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Special Issue Reprint

Latest Review Papers in Molecular Biology 2024

Edited by
Madhav Bhatia

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Guest Editor

Madhav Bhatia

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About the Editor

Madhav Bhatia

Madhav Bhatia is a world-renowned scientist known for his seminal research on inflammation. He has researched hydrogen sulphide and substance P as mediators of inflammation and novel therapeutic targets for inflammatory diseases, making significant contributions to show the mechanism through which they act. In recent years, Professor Bhatia has also been working on novel markers for the diagnosis and prognosis of inflammatory and infectious diseases. He has authored more than 200 publications which have been cited more than 22,000 times; his "h" index is 76, and he has been recognised as being among the top 2% of scientists in the world in the area of Immunology. He has also been acknowledged as a Highly Ranked Scholar (among the top 0.05% of scholars globally) by ScholarGPS, and as one of the top scientists in the discipline of Biology and Biochemistry by Research.com.

Professor Madhav Bhatia began his career at the All India Institute of Medical Sciences in India, after which he worked at the Institut de Génétique et de Biologie Moléculaire et Cellulaire (France), Harvard Medical School (USA), University of Liverpool (UK) and the National University of Singapore (Singapore). He was then recruited by the University of Otago, New Zealand. Therefore, he has worked and lived in six different countries, developing a vast experience in interacting with people from various nations representing diverse cultures.

Professor Madhav Bhatia plays a major role in research supervision and leadership, and has supervised more than 50 doctoral students, post-doctoral fellows, and research scientists, many of whom are now independent investigators in their own right.

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Editorial

Molecular Biology: Challenges and Opportunities

Madhav Bhatia

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"Latest Review Papers in Molecular Biology 2024", a Special Issue of *Current Issues in Molecular Biology (CIMB)* that I have had the privilege of editing, called for articles on the challenges and opportunities in the field of molecular biology. Following an excellent response from researchers around the world, this Special Issue now boasts 29 published articles, which have all helped enhance our understanding of different aspects of molecular biology. The articles published in this Special Issue have already been viewed a total of 75,862 times at the time of writing of this Editorial (22 January 2025), and the number continues to rise daily.

All the articles in this Special Issue are of exceptionally high standards, and it has been my pleasure to read them. I would, therefore, encourage the readers of *CIMB* to read all the articles in this Special Issue, and I am sure you will enjoy reading them as I have. I would, however, like to highlight a few seminal review articles published in the Special Issue, which have dealt with complex ideas in an easy-to-read manner.

The first article [1] describes the use of collagen in cosmetic products. Collagen, a structural protein, is a frequently employed ingredient in several cosmetic products [2,3]. This review presented the types of collagen proteins, the factors affecting their structure, and their unusual role in the functions of the human body. The authors also summarize the type of cosmetic products where collagen is used, including cosmetics administered via injection, orally, as well as topically (creams, ointments, serums, cosmetic masks, etc.). This article elegantly deals with a complex subject, spanning from the basic chemistry of collagen to its practical applications in cosmetic products. In light of the increasing interest in this rapidly developing area, in the short time since it has been published, this article has already been viewed a total of 11,115 times.

The second article [4] is of outmost relevance to the present times, and it tackles the issue of micro- and nano-plastics and their impact on gut health. Micro- and nano-plastics are tiny particles derived from plastics, representing a significant health risk [5,6]. This review described recent research on the toxicological impact of micro- and nano-plastics on the gut, as observed in human cell lines and mammalian models. Available data suggest that these particles may result in adverse consequences, including epithelial toxicity, immune toxicity, and the disruption of the gut microbiota. This article concludes with directions for future research in this area, given the current limitations to our understanding of the subject. To date, this article has been viewed 4775 times.

The third article [7] describes the pharmacological effects of capsaicin. Capsaicin is the pungent compound found in chili peppers and, therefore, plays a significant role in the cuisine of several cultures around the world. Capsaicin acts through the TRPV1 (transient receptor potential cation channel subfamily V member 1) receptors. TRPV1 has been shown to play a key role in systemic inflammatory conditions such as sepsis [8–10]. The local application of capsaicin, however, produces an anti-inflammatory effect. Additionally, capsaicin has been reported to have anti-cancer and anti-microbial effects, with a long

history of use in traditional medicine systems for several centuries. Though a major limitation in the therapeutic applications of capsaicin remains, which is related to its toxicity, especially in sensitive tissues. The research reviewed in this article suggests the potential of capsaicin in monotherapy or adjunct therapy for various pathologies. Further research is required in order to investigate capsaicin's therapeutic properties that extend its usage beyond food. This article has been viewed 4064 times so far.

The fourth article [11] describes the application of multi-omics in biological systems. Biological systems are quite complex, and traditional approaches have limitations due to our incomplete understanding of the interactions within these systems. Every biological process may involve thousands of molecules, which traditional approaches are not capable of investigating. Biology-based multi-omics system approaches provide us with a global picture of complex biological systems, using approaches such as genomics, transcriptomics, proteomics, glycomics, glycoproteomics, metabolomics, and lipidomics analyses. Multi-omics approaches have thus far been used in the diagnosis for Alzheimer's disease [12], the identification of osteoporosis biomarkers [13], and the diagnosis and therapy prostate cancer [14]. Investigating biological systems from a holistic viewpoint using multi-omics approaches, therefore, promises to significantly enhance our understanding of the diagnosis, treatment, and prevention of human diseases. This article describes the current state of knowledge and provides potential directions for the future of this very important and rapidly developing subject. This article has been viewed 4192 times so far.

Finally, I would like to highlight a review article that summarizes our current understanding of the positive effects of physical activity on insulin signaling [15]. Physical activity plays a vital role in maintaining metabolic health, particularly in addressing insulin resistance and the related disorders such as type 2 diabetes mellitus [16]. Research has shown that a strong association between physical activity levels and insulin sensitivity exists [17]. In this article, the author has described insulin signaling pathways, focusing on insulin signaling in skeletal muscles, the liver, and white adipose tissues. The article proceeds to elucidate the relationship between physical inactivity and islet cell insufficiency, which leads to the exacerbation of insulin resistance through various pathways including endoplasmic reticulum stress, mitochondrial dysfunction, oxidative stress, and inflammation. Physical activity, on the other hand, preserves and restores islet function, thus enhancing peripheral insulin sensitivity. Based on the literature on the molecular biology of insulin signaling, the author recommends that individuals be encouraged to adopt active lifestyles and engage in regular exercise. This may help them prevent and manage insulin resistance and related metabolic disorders, ultimately promoting overall health and well-being. Similarly to the other articles highlighted in this Editorial, this article deals with a complex subject but explains it clearly and comprehensibly. So far, this article has been viewed 4520 times.

In conclusion, this Special Issue represents a collection of review papers from a wide range of scientific fields. I am confident that the articles published herein will form the basis for more ground-breaking research in times to come. Encouraged by the response to this Special Issue in 2024—from the authors as well as readers—we are launching another Special Issue of *CIMB*, "Latest Review Papers in Molecular Biology 2025" (https://www.mdpi.com/journal/cimb/special_issues/5CV2UG4RK8, accessed on 22 January 2025), and I cordially invite you to contribute review articles pertaining to the challenges and opportunities in the discipline of molecular biology.

Conflicts of Interest: The author declares no conflict of interest.

References

1. Jadach, B.; Mielcarek, Z.; Osmałek, T. Use of Collagen in Cosmetic Products. *Curr. Issues Mol. Biol.* **2024**, *46*, 2043–2070. [CrossRef] [PubMed]
2. Prajaputra, V.; Isnaini, N.; Maryam, S.; Ernawati, E.; Deliana, F.; Haridhi, H.A.; Fadli, N.; Karina, S.; Agustina, S.; Nurfadillah, N.; et al. Exploring marine collagen: Sustainable sourcing, extraction methods, and cosmetic applications. *S. Afr. J. Chem. Eng.* **2024**, *47*, 197–211. [CrossRef]
3. Al-Atif, H. Collagen Supplements for Aging and Wrinkles: A Paradigm Shift in the Fields of Dermatology and Cosmetics. *Dermatol. Pract. Concept.* **2022**, *12*, 2022018–2022019. [CrossRef] [PubMed]
4. Covello, C.; Di Vincenzo, F.; Cammarota, G.; Pizzoferrato, M. Micro(nano)plastics and Their Potential Impact on Human Gut Health: A Narrative Review. *Curr. Issues Mol. Biol.* **2024**, *46*, 2658–2677. [CrossRef] [PubMed]
5. Marcelino, R.C.; Cardoso, R.M.; Domingues, E.L.B.C.; Gonçalves, R.V.; Lima, G.D.A.; Novaes, R.D. The Emerging Risk of Microplastics and Nanoplastics on the Microstructure and Function of Reproductive Organs in Mammals: A Systematic Review of Preclinical Evidence. *Life Sci.* **2022**, *295*, 120404. [CrossRef] [PubMed]
6. Lim, X. Microplastics Are Everywhere—But Are They Harmful? *Nature* **2021**, *593*, 22–25. [CrossRef] [PubMed]
7. Petran, E.M.; Periferakis, A.; Troumpata, L.; Periferakis, A.-T.; Scheau, A.-E.; Badarau, I.A.; Periferakis, K.; Caruntu, A.; Savulescu-Fiedler, I.; Sima, R.-M.; et al. Capsaicin: Emerging Pharmacological and Therapeutic Insights. *Curr. Issues Mol. Biol.* **2024**, *46*, 7895–7943. [CrossRef] [PubMed]
8. Ang, S.F.; Moochhala, S.M.; Bhatia, M. Hydrogen sulfide promotes transient receptor potential vanilloid 1-mediated neurogenic inflammation in polymicrobial sepsis. *Crit. Care Med.* **2010**, *38*, 619–628. [CrossRef] [PubMed]
9. Ang, S.F.; Moochhala, S.M.; MacAry, P.A.; Bhatia, M. Hydrogen sulfide and neurogenic inflammation in polymicrobial sepsis: Involvement of substance P and ERK-NF-κB signaling. *PLoS ONE* **2011**, *6*, e24535. [CrossRef] [PubMed]
10. Ang, S.F.; Sio, S.W.; Moochhala, S.M.; MacAry, P.A.; Bhatia, M. Hydrogen sulfide upregulates cyclooxygenase-2 and prostaglandin E metabolite in sepsis-evoked acute lung injury via transient receptor potential vanilloid type 1 channel activation. *J. Immunol.* **2011**, *187*, 4778–4787. [CrossRef]
11. Gutierrez Reyes, C.D.; Alejo-Jacuinde, G.; Perez Sanchez, B.; Chavez Reyes, J.; Onigbinde, S.; Mogut, D.; Hernández-Jasso, I.; Calderón-Vallejo, D.; Quintanar, J.L.; Mechref, Y. Multi Omics Applications in Biological Systems. *Curr. Issues Mol. Biol.* **2024**, *46*, 5777–5793. [CrossRef]
12. Horgusluoglu, E.; Neff, R.; Song, W.-M.; Wang, M.; Wang, Q.; Arnold, M.; Krumsiek, J.; Galindo-Prieto, B.; Ming, C.; Nho, K.; et al. Integrative metabolomics-genomics approach reveals key metabolic pathways and regulators of Alzheimer's disease. *Alzheimer's Dement.* **2022**, *18*, 1260–1278. [CrossRef]
13. Qiu, C.; Yu, F.; Su, K.; Zhao, Q.; Zhang, L.; Xu, C.; Hu, W.; Wang, Z.; Zhao, L.; Tian, Q.; et al. Multi-omics Data Integration for Identifying Osteoporosis Biomarkers and Their Biological Interaction and Causal Mechanisms. *iScience* **2020**, *23*, 100847. [CrossRef] [PubMed]
14. Wang, Z.; Li, Y.; Zhao, W.; Jiang, S.; Huang, Y.; Hou, J.; Zhang, X.; Zhai, Z.; Yang, C.; Wang, J.; et al. Integrative multi-omics and drug–response characterization of patient-derived prostate cancer primary cells. *Signal Transduct. Target. Ther.* **2023**, *8*, 175. [CrossRef]
15. Małkowska, P. Positive Effects of Physical Activity on Insulin Signaling. *Curr. Issues Mol. Biol.* **2024**, *46*, 5467–5487. [CrossRef] [PubMed]
16. Eaton, S.B.; Eaton, S.B. Physical Inactivity, Obesity, and Type 2 Diabetes: An Evolutionary Perspective. *Res. Q. Exerc. Sport* **2017**, *88*, 1–8. [CrossRef]
17. Sampath Kumar, A.; Maiya, A.G.; Shastry, B.A.; Vaishali, K.; Ravishankar, N.; Hazari, A.; Gundmi, S.; Jadhav, R. Exercise and Insulin Resistance in Type 2 Diabetes Mellitus: A Systematic Review and Meta-Analysis. *Ann. Phys. Rehabil. Med.* **2019**, *62*, 98–103. [CrossRef] [PubMed]

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Review

Sodium Dodecyl Sulfate Analogs as a Potential Molecular Biology Reagent

Tsutomu Arakawa [1,*], Takako Niikura [2], Yoshiko Kita [1] and Teruo Akuta [3]

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Abstract: In this study, we review the properties of three anionic detergents, sodium dodecyl sulfate (SDS), Sarkosyl, and sodium lauroylglutamate (SLG), as they play a critical role in molecular biology research. SDS is widely used in electrophoresis and cell lysis for proteomics. Sarkosyl and, more frequently, SDS are used for the characterization of neuropathological protein fibrils and the solubilization of proteins. Many amyloid fibrils are resistant to SDS or Sarkosyl to different degrees and, thus, can be readily isolated from detergent-sensitive proteins. SLG is milder than the above two detergents and has been used in the solubilization and refolding of proteins isolated from inclusion bodies. Here, we show that both Sarkosyl and SLG have been used for protein refolding, that the effects of SLG on the native protein structure are weaker for SLG, and that SLG readily dissociates from the native proteins. We propose that SLG may be effective in cell lysis for functional proteomics due to no or weaker binding of SLG to the native proteins.

Keywords: SDS; Sarkosyl; sodium lauroyl glutamate; cell lysis; refolding; neuropathological

1. Introduction

Detergents are one of the most widely used reagents in molecular biology research [1–3]. Their use includes but is not limited to cell lysis, electrophoresis, protein refolding, and the suppression of macromolecular surface adsorption or aggregation, i.e., a manipulation of macromolecular solutions [4]. Sodium dodecyl sulfate (SDS, see Figure 1 for chemical structure) is perhaps the most highly used reagent because of its use in SDS-polyacrylamide gel electrophoresis (SDS-PAGE). There are many advantages to the use of SDS in SDS-PAGE. SDS disrupts almost all the non-covalent molecular interactions and thereby allows the separation of individual components and the determination of their molecular weights [5]. SDS is effective at disrupting molecular interactions, including the lipid–lipid interactions that make cell membranes, and hence in releasing cytoplasmic and nuclear components [6]. This ability to disrupt molecular interactions is due to the strong micellar binding of SDS to the proteins, which provides the following advantage of using SDS in SDS-PAGE [7]. The SDS–protein complex in the gel can be readily electrophoresed to membranes in Western blotting technology [8,9]. However, such effects of SDS on disrupting molecular interactions means it can inherently denature macromolecules and disrupt their complexes, thereby destroying their functions.

There are two SDS analogs that are frequently used in protein or molecular biology research. As shown in Figure 1, they are sodium lauroly glutamate (SLG) and sodium lauroyl sarcosince (Sarkosyl). These are identical to SDS in their aliphatic portion and differ from SDS in the charged group. SLG has been used for cosmetic and cleaning applications [10]. Sarkosyl and SDS are consistently used for separating soluble and insoluble neuropathological fibrillar proteins, including tau, which is one of the microtubule-associating proteins

(MAPs) [11–13]. We examine the physical properties of these detergents and their mechanism in affecting protein structure, folding, and molecular interactions.

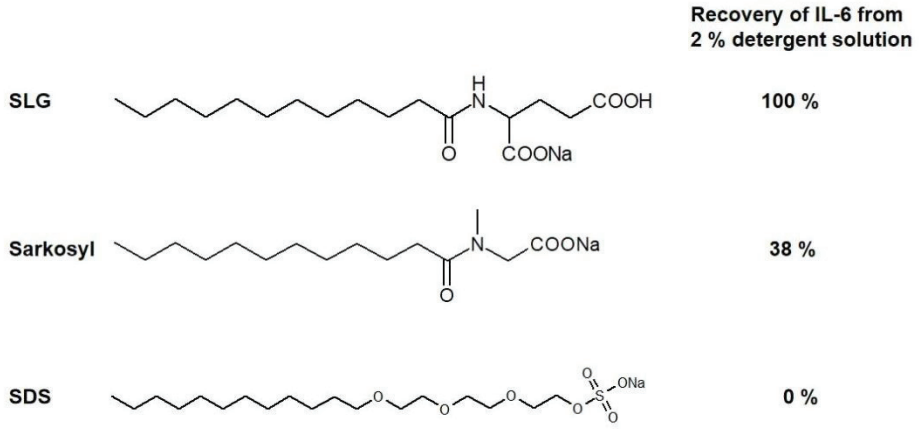


Figure 1. The structures of sodium lauroyl glutamate (SLG), Sarkosyl, and SDS. The recovery of interleukin-6 (L-6) from 2% detergent solutions is given in the last column. Native IL-6 was exposed to 0–2% detergent solutions and subjected to Superdex 75 gel filtration. The recovery of IL-6 was estimated from the absorbance of the protein eluted in the native IL-6 elution position.

2. Properties of SDS, Sarkosyl and SLG

Table 1 summarizes the properties of sodium dodecyl sulfate (SDS), sodium lauroyl sarcosine (Sarkosyl), and sodium lauroyl glutamate (SLG). Sarkosyl is unique in that it has a small micellar size compared to SDS and SLG, clearly forming a micellar structure with an aggregation number of ~80, as schematically depicted in Figure 2, in which red circles, yellow ellipsoids, and green squares depicted the different head groups of SDS, Sarkosyl, and SLG [13,14]. Sarkosyl is reported to have an aggregation number of two and, hence, does not appear to form a true micelle, although how this aggregation number is determined is not described [4]. There is no explanation about the stability of dimeric Sarkosyl: it may be worth mentioning that sodium caprylate with eight carbon aliphatic chains has a high monomeric concentration of 300 mM before forming micelles, meaning that the non-polar aliphatic chain can be stable in water. However, whether Sarkosyl forms a true micelle or not is questionable, and the critical micelle concentration (CMC) was reported to be 9.5–15 mM. Whether this corresponds to a CMC or a dissociation constant of the dimeric Sarkosyl (aggregation number 2) to a monomer may depend on how the CMC is determined. If it is determined from the surface tension measurement, then it simply refers to the equilibrium concentration of Sarkosyl between the air–water interface and the bulk solution. In fact, one of the CMC measurements is based on the air–water or solid–water interface adsorption of Sarkosyl [15] but not the actual equilibrium between micelles and free Sarkosyl in the bulk. The same authors claim that Sarkosyl is non-denaturing to proteins without showing data or references and may bind to proteins as assembled states, such as in the air–water interface.

Table 1. Properties of SDS, Sarkosyl, and sodium lauroyl glutamate (SLG).

Detergent	CMC	Aggregation Number	MW
SDS	8.2 mM in water 1.4 mM in 0.1 M NaCl	~80 in water	288.4
Sarkosyl	14.57 mM	2	293.4
SLG	10.6 mM	~80	351.4

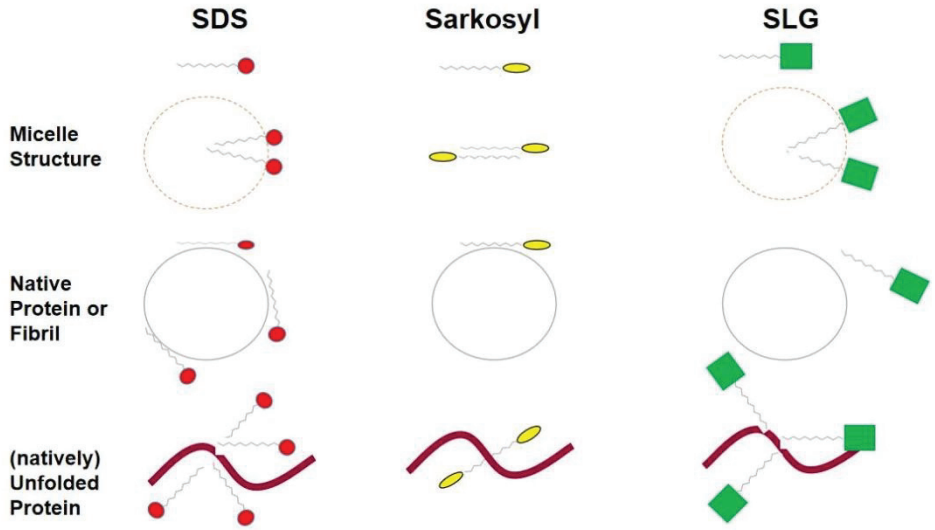


Figure 2. Schematic illustration of the micellar structure and binding mode of SDS, Sarkosyl, and SLG. Red circles (SDS), yellow ellipsoid (Sarkosyl), and green squares (SLG). Top row, the structure of the micelle. Middle row, the binding mode to native proteins (or fibrils). Bottom row, the binding mode to (intrinsically) native proteins. In these illustrations, the size of each structure does not reflect differences in the sizes of micelles and proteins.

The cleaning properties of SLG have been studied because of its cosmetic and cleansing applications [16]. When compared to Sarkosyl, SLG was slightly weaker in suppressing the surface tension of water, although both detergents are strong surface tension suppressors [17]. SLG is much stronger at stabilizing the foams than Sarkosyl despite their similarity in decreasing the surface tension of water. Interestingly, SLG is extremely weak in cleaning the greasy stains from surfaces below CMC, suggesting its weakness in hydrophobic binding as a monomeric detergent. Nevertheless, SLG can interact with hydrophobic aliphatic chains, as it enhances the solubility of alkyl-gallates, particularly for those with longer alkyl chains [18].

3. Effects on Native Proteins

The effects of Sarkosyl, SDS, and SLG were examined on the structure of native, folded interleukin-6 (IL-6) [19]. The protein was exposed to 0–2% detergent solutions and loaded onto a Superdex-75 gel filtration column run without the detergent. If the detergents dissociate from (or do not bind to) the protein without altering the protein structure, it shows native elution on the column. The peak area corresponding to the native protein was determined by the absorbance measurement. The recovery of native IL-6 from the 2% detergent solution is shown in Table 1. When diluted from 2% SLG upon gel filtration chromatography, the recovery was 100%. On the contrary, sodium lauroyl ether sulfate, used instead of SDS, resulted in 0% recovery. It is noted that sodium lauroyl ether sulfate was used here as an alternative to SDS, as the use of SDS in this experiment would give stronger denaturing effects on the protein and damage the column due to the strong (irreversible) binding of the SDS-denatured protein to the column. The recovery from 2% Sarkosyl was 38%, intermediate between lauroyl ether sulfate and SLG, indicating that the binding ability may be between lauroyl ether sulfate (and likely SDS) and lauroyl glutamate (SLG). These results for Sarkosyl and, likely, for SDS suggest binding to the native proteins, as shown in Figure 2.

An extensive analysis of the effects of SLG on native proteins was also carried out [20]. Circular dichroism (CD) was used to monitor the structural changes in IL-6 in the absence and presence of SLG. The CD spectrum of BSA in 2% SLG showed a distinct near UV CD spectrum with characteristic peaks and shoulders present in the native BSA but clearly with different intensities, indicating that 2% SLG alters the tertiary structure of BSA without

completely unfolding the structure. This, in turn, suggests that BSA can assume a native-like tertiary structure fold even in the presence of 2% SLG. The CD spectra of BSA in the presence of 0.1% SLG, generated whether diluted from 2% SLG or added to the native BSA, were essentially identical to the spectrum of native BSA, clearly indicating that BSA has a native fold in the presence of 0.1% SLG or regains the native structure when diluted from 2% SLG, as depicted in Figure 2 (middle row). Different from the above results with BSA, IL-6 showed a greater loss of the tertiary structure in the presence of 2% SLG, as determined by near UV CD analysis, indicating that the effects of SLG on the folded structure depend on the proteins. When SLG was removed, IL-6 completely regained the tertiary structure, as analyzed by CD, and consistent with the results of BSA. The tertiary structure of IL-6 was monitored using CD and fluorescence spectroscopy as a function of the SLG concentration, indicating that the structure change occurs around 0.2% SLG, and the structure is native below 0.1% SLG and unfolded above 0.5% SLG. Native gel electrophoresis indicated that SLG fully dissociates from BSA when contained only in the loading sample at 0.1–2%. However, IL-6 showed a doublet band, one of which is native, when 0.5% SLG is present in the loading sample, indicating that SLG at this concentration may still be bound to the non-native band of the doublet. Above 0.5% SLG, the native band was not observed, indicating that the dissociation of the bound SLG was not fast enough to generate the native IL-6 during the electrophoresis run time. To our knowledge, there appear to be no studies on the interaction of these detergents side-by-side with other proteins. Both IL-6 and BSA are small globular proteins, suggesting that it is of great interest to study larger proteins such as antibodies and protein complexes.

4. Effects on Protein Refolding

SDS is well known to bind to unfolded proteins, which is depicted in Figure 2 (bottom row). Sarkosyl has been shown to be non-denatured [21]. It was used to solubilize actin-binding proteins expressed as inclusion bodies in *E. coli* [22]. Sarkosyl was found to be able to extract active proteins, including the green fluorescent protein (GFP), tumor necrosis factor (TNF), and lymphotoxin from *E. coli* inclusion bodies [23]. Sarkosyl has been used to solubilize and refold proteins expressed recombinantly in bacterial cells [24,25]. A rather low concentration, 0.3–0.4%, of Sarkosyl was used to solubilize the polymerase sigma factor, which yielded a recovery of the folded protein greater than 50% [25]. Refolding was performed by the 10-fold dilution of the Sarkosyl concentration followed by dialysis. After dialysis, 0.01–0.02% Sarkosyl was still found to be trapped with the protein, most likely bound by the protein and, hence, un-dialyzable. It is speculated that Sarkosyl is bound by the unfolded sigma factor during solubilization, refolding during dialysis, and staying bound, though to a limited extent, but perhaps strongly, even after extensive dialysis. Sarkosyl was used to solubilize the granulocyte colony-stimulating factor (G-CSF) [26]. Native G-SCF has two disulfide bonds. When expressed in *E. coli*, G-CSF forms inclusion bodies. They were solubilized equally well using 6 M of guanidine hydrochloride or 2% Sarkosyl. Reverse-phase chromatography analysis showed a similar profile between these solubilizing solutions. G-CSF solubilized by these reagents was eluted between the fully reduced, unfolded structure and the native structure. Successful oxidation and refolding were achieved in the presence of 2% Sarkosyl with $CuSO_4$. In this study, the final products were analyzed using reverse phase chromatography, in which free (unbound-to protein) Sarkosyl was retained by the reverse phase column and eluted before the native G-CSF. It is evident that the strong binding of Sarkosyl by the reverse phase resin can completely be removed from the refolded G-CSF. It is not clear from this study how well Sarkosyl can be removed from the protein if a simpler process, such as diafiltration, gel filtration, and ion exchange chromatography, is used. A similar result was obtained in refolding a chymeric receptor binding domain (RBD) of SARS-CoV-2 fused to a DNA-binding CRM197 protein (i.e., CRM197-RBD fusion protein) [27]. The bacterial expression resulted in inclusion bodies, from which the chymeric protein was solubilized by 1% Sarkosyl. The Sarkosyl

was removed using a detergent removal resin, Amberlite XAD-4, leading to the formation of the functional structures of both the DNA binding protein and RBD.

The detailed protocol of application of Sarkosyl for the solubilization and purification of recombinant proteins from bacterial *E. coli* cells has been described, clearly showing its usefulness in protein refolding [13,28]. An interesting observation was made with glycerol kinase [29]. When expressed in *E. coli*, the glycerol kinase formed inclusion bodies. A cationic detergent CTAB (cetyltrimethylammonium bromide) at 0.5% solubilized the kinase without activity. SDS at 1% did the same but also without activity. Sarkosyl at 1% resulted in solubilization and an active enzyme. However, it was active only in the presence of 1% Sarkosyl, as its removal resulted in inactivation, perhaps due to the aggregation of the folded enzyme after removing Sarkosyl. This suggests that the bound Sarkosyl helps the kinase become soluble in an aqueous solution. As an Fc scaffold model, an attempt was made to express and characterize an antibody CH2-CH3 construct [30]. When the construct was expressed in *E. coli*, it was expressed in inclusion bodies. Sarkosyl (1.5%) was successfully used to solubilize the construct, and refolding into a native dimer was performed by dialysis. However, the recovery was rather low, which was ascribed to the persistent binding of the Sarkosyl, which hampered either the refolding process, chromatographic purification, or both. It is, thus, likely that Sarkosyl binds to the unfolded proteins, as depicted in Figure 2 (bottom row), and to the native proteins, though to a limited extent, as also shown in Figure 2.

SLG was used to solubilize or refold several proteins, including somatotropin. Native somatotropins contain two disulfide bonds. The solubilization and refolding of somatotropins are reported in [31]. As an example, bovine somatotropin was solubilized in 2% SLG at an alkaline pH and refolded in the presence of oxidizing reagents via 2-fold dilution. A simple diafiltration was successfully used to remove SLG. SLG was also used to refold various proteins [32]. When expressed in *E. coli* cells, IL-6 was reduced and unfolded in insoluble inclusion bodies. It was solubilized by 2% SLG in the presence of disulfide exchange reagents. As described in the previous section, the CD analysis of native IL-6 showed that its structure is altered by SLG above 0.1%, clearly indicating that it can bind to the protein even below its CMC (~10 mM, see Table 1). After solubilization and incubation with 2% SLG, the solution was diluted 20-fold to 0.1% with 10 mM of the phosphate buffer at pH 7.0 and room temperature. The native, refolded IL-6 was identified with a high yield via ion exchange chromatography, indicating that dilution and ion exchange purification are sufficient to remove the bound detergent. Below 0.1%, SLG dissociates from the protein and leads to the formation of a native structure. It is interesting that Sarkosyl gave a low yield under the conditions used above. The use of arginine above 0.4 M in the phosphate refolding buffer (used for 20-fold dilution) resulted in greater refolding yield, suggesting that arginine assists the refolding and dissociation of SLG. The same solubilization and refolding technology was successfully applied to transglutaminase and the single-chain variable domain or Fab domain of antibodies [19,32,33].

The effects of temperature on the refolding and dissociation of SLG were examined using two single-chain antibodies (scFv) that also formed inclusion bodies [33]. Inclusion bodies containing these scFv were solubilized in 2.5% SLG and diluted with a phosphate buffer containing a redox agent at different SLG concentrations. The refolding and dissociation of SLG were assessed by gel filtration using the Superdex-75 column. Refolding was independent of whether SLG was in micellar or monomeric forms, as the refolding yield was high below and above the CMC. Incubation at an elevated temperature, e.g., 45 °C, resulted in higher recovery, suggesting that higher temperature accelerated refolding. SLG at 2% was also used to refold an scFv-corestreptavidin fusion protein. The dilution of 2% SLG by 100-fold to 0.02% resulted in the refolding of corestreptavidin into a monomeric structure. The complete removal of SLG by dialysis led to the formation of tetrameric scFv-corestrepavidin fusion, which, in turn, suggested that a small amount of SLG was bound at 0.02%, most likely to the corestreptavidin moiety, preventing the formation of

the tetramer structure. These results clearly indicate that SLG does bind to the unfolded proteins, as shown in Figure 2.

5. Amyloid Fractionation by Sarkosyl or SDS

Tau is a member of "microtubule binding proteins (MAPs)", which bind to the surface of assembled tubulin subunits, i.e., microtubules, with a constellation of positive charges distributed through the tau structure [34,35]. Tau refers to natively (intrinsically) unfolded basic proteins with isoelectric points above nine and, hence, are positively charged at a physiological pH. The tau binds to the microtubules electrostatically and stabilizes the assembled structures. The phosphorylation of tau lowers net-positive charges and weakens the electrostatic interactions with the microtubules, making them less water-soluble. Neurofibrillary tangles are one of the major culprits in Alzheimer's disease and are formed by intra-cellular aggregates of abnormally hyperphosphorylated tau, a disease called "tauopathies". Tau aggregates have been characterized based on their solubility (or insolubility) in 1% Sarkosyl. The homogenization of brains in a buffer containing high salt (0.8 M NaCl) and 10% sucrose leads to the dissociation of tau from microtubules and helps the elimination of myelin and associated lipids [36]. The centrifugation of the suspension leads to the supernatant containing various forms of tau, to which the addition of 1% Sarkosyl with a reducing agent leads to the fractionation of the tau fibrils as Sarkosyl-insoluble aggregates [37]. It appears that the binding of Sarkosyl retains the "native"-like structures and hence antigenic epitopes of tau, which are lost when SDS is used instead of Sarkosyl and lead to the formation of insoluble aggregates that can be obtained via high-speed centrifugation [36]. One issue that is not clear is how 1% Sarkosyl binds to tau and generates (or retains) insoluble tau [38]. The 1% Sarkosyl was used not only in identifying and characterizing tauopathy-related insoluble tau fibrils but also the pathogenic fibrils derived from synucleinopathy α-synuclein and proteinopathy TDP-43. Thus, the solubility or, more directly, the insolubility of neuropathological fibrils by 1% Sarkosyl, or, as described below, 1–2% SDS, appears to be the hallmark of a technique for protein aggregates in neuron diseases, including Alzheimer's, Parkinson's and multiple system atrophy disease.

Cryoelectron microscopy analysis showed no major effects of the Sarkosyl treatment on the structure of Aß and tau fibrils [39], although Sarkosyl-untreated Aß, i.e., the ultra-centrifugation supernatant of Tris-buffered saline extracts of Alzheimer's disease brain, showed shorter fibrils that clumped less, suggesting that Sarkosyl may enhance fibril association [40]. Unfortunately, no data are available on the effects of SLG on fibrils. Based on its mild detergent properties, it would be of great interest to examine its ability to bind to fibrillar aggregates and other proteins and separate the fibrils from SLG-sensitive proteins and complexes.

SDS has also been used to fractionate amyloid fibrils, taking advantage of the SDS-resistant properties of amyloid fibrils. Prion amyloids were separated by treating cell lysates with 2% SDS at 37 °C, which enabled proteins other than the amyloids to solubilize; however, care must be exercised not to overheat to avoid the disaggregation of the amyloid structures. Instead of 2% SDS, 1% Sarkosyl may be used [41]. The use of Sarkosyl was found to detect a wider range of amyloids than SDS due to Sarkosyl's weaker detergent capability and the susceptibility of amyloids to SDS, although Sarkosyl may retain non-amyloid complexes. Polyglutamine (polyQ) fibrillar aggregates can be readily fractionated by SDS treatment, as polyQ is SDS-resistant [42]. As noted above, there may be a disadvantage to using a weak Sarkosyl detergent, which can retain certain non-amyloidogenic complexes, resulting in contaminating proteins in amyloid fractions. A different combination of temperature and SDS concentration, e.g., 1% SDS and room temperature, can also be applied to retain and purify amyloid aggregates [43]. Utilizing the detergent-resistance properties, previously unidentified amyloid-like structures have been discovered in different organisms, e.g., such eukaryotes as yeast and plant seeds [44–47]. Amyloid-like proteins were obtained from pea seeds as 1% SDS-resistant proteins and identified as vicilin.

6. Cell Lysis

Many in vitro cell biology experiments require cell lysis, as depicted in Figure 3, which releases soluble and membrane proteins and may or may not preserve the native integrity of cellular components. One such procedure is called decellularization, which is often performed on tissues for xenografts. For example, corneal transplantation is the only option to cure corneal opacities using decellularized corneas and is performed with different decellularized protocols [48]. Decellularization removes xenogenic cells and antigens, which can cause rejection, for tissue transplantation. SDS, in combination with nucleases, has been used in traditional decellularization, as this detergent can remove nuclear and cytoplasmic components from various tissues, including the dermis, kidney, and lung [49]. However, SDS can damage biologically essential components that constitute the extracellular matrix (ECM). Thus, SDS was compared with Sarkosyl, Triton X-100, or SLG in combination with nucleases for the decellularization of porcine corneas that may be applied to human patients [50]. The results are clear that SLG was better than the other three detergents in preserving the transparency of the cornea and removing residual cellular nuclei and other components from the porcine cornea. In addition, SLG resulted in the regular alignment of corneal stromal fibrils, while another three detergents resulted in the disordered alignment and retention of residual nuclei. These results indicate that SLG is superior in removing xenogenic cells and antigens and preserving the transparency and ultrastructure of corneal stroma.

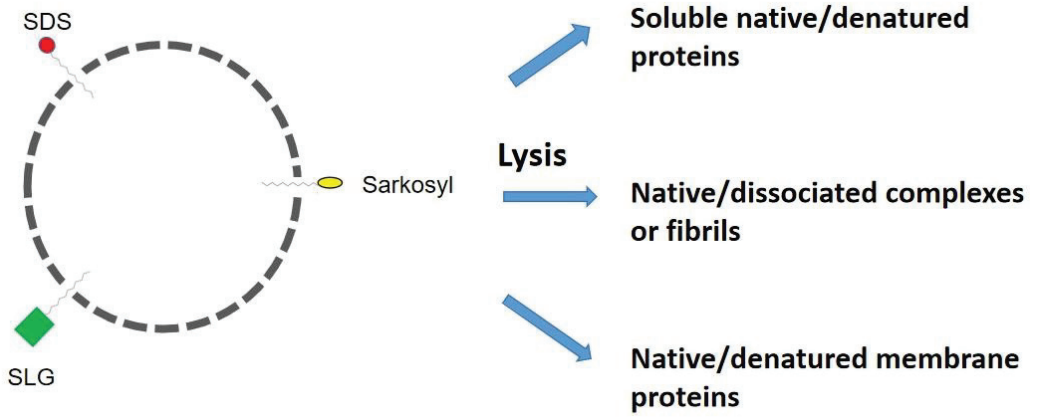


Figure 3. Schematic illustration of a detergent binding to membranes and its effects on proteins released from lysed cells. SDS, Sarkosyl, and SLG are shown to penetrate cell membranes through their aliphatic chains. Their head groups are in the bulk solvent, as indicated by the red circle (SDS), yellow ellipsoid (Sarkosyl), and green square (SLG). These detergents are expected to affect soluble, complex, or fibrillar membrane proteins differently.

SDS has been extensively used to lyse cells under denaturing conditions for proteomic analysis, namely, cell lysis above the CMC, followed by the removal of SDS via protein precipitation, washing steps, and protease digestion before mass analysis [51]. While this is highly effective in cell lysis and proteomic analysis, the extracted proteins from the cells are inherently denatured and, hence, may not be suitable for functional proteomics. However, there is an advantage of this denaturing/dissociating ability of SDS for the isolation of SDS-resistant fibrils, as described below and depicted in Figure 3. An even harsher form of cell lysis is to use SDS in combination with heating, which guarantees complete cell lysis and protein denaturation for immunoprecipitation using antibodies that react only with linear peptide sequences [52].

Sarkosyl at 1% has been used to aid with cell lysis, thereby removing nucleic acids from purified proteins [53]. They also showed that a higher Sarkosyl concentration, even 10%, is effective at solubilizing multiple proteins from inclusion bodies, conferring folding into the native structure and speculating the mechanism of solubilization via the encapsulation of native-like proteins trapped in inclusion bodies, assuming that these inclusion bodies

are made of native or native-like structures that are made insoluble due to a high protein concentration or the presence of nucleic acids, lipids or other contaminants. Sarkosyl at 2.5% was used to increase the permeability of *E. coli* cell membranes [54].

The subcellular fractionation of *Shewanella oneidensis* proteins was successfully obtained with 0.5% Sarkosyl [55]. After mechanical cell lysis, the subcellular complex of the lipid–protein complex was solubilized by Sarkosyl for proteomic analysis via mass spectrometry. Identification of bacterial Rhodococcus strains was performed using a lysis protocol made of Sarkosyl and lysozyme, combined with freeze–thaw, followed by PCR analysis [56]. The lysis of *E. coli* cells and solubilization of proteins by Sarkosyl was carried out for the expression of the ecdysteroid receptor (EcR) and ultraspiracle (USP) together. The purification of these proteins resulted in the expression of the functional heterodimer and homodimers of both EcR and USP [57]. The combination of Sarkosyl-based cell lysis and a different *E. coli* strain resulted in the expression of soluble actin, which otherwise formed inclusion bodies [58].

Although these detergents may be equally effective in cell lysis, they are different in their effects on proteins released from lysed cells. How these detergents affect the soluble, complex, and membrane proteins, as shown in Figure 3, depends on the detergent concentration and such solution conditions as pH and temperature. For protein purification, SDS is most unfavorable at obtaining native soluble proteins and native molecular complexes from the cytoplasm, nucleus, and membrane proteins. The use of Sarkosyl or SLG may require adequate procedures for the purification of native proteins, as they add negative charges when bound to the proteins and may dissociate native complexes, although their dissociating capacity is weaker than SDS. The extracellular domain structure of the membrane proteins is most likely destroyed by SDS. To isolate and purify membrane proteins, however, it is essential to use detergents to stabilize their membrane-spanning domains. The detergents most frequently used are non-ionic (e.g., Triton X-100) or zwitterionic (e.g., CHAPS) detergents or mild ionic detergents (e.g., sodium cholate), which are milder than Sarkosyl, SLG, and SDS [59,60]. Depending on the subsequent experiments, the detergent used to lyse the cells and extract membrane proteins may need to be removed or replaced with another detergent, meaning that the ability of the detergent to dissociate from the solubilized proteins plays a key role in the purification and characterization of proteins.

7. Mechanism

Here, we do our best to interpret the effects of these three anionic detergents. One of the most critical questions is the effects of SDS, Sarkosyl, and sodium lauroyl glutamate (SLG) on membranes and macromolecules during cell lysis, decellularization, and purification. These three detergents may be expected to penetrate the cell membrane equally well because of their identical aliphatic chains (see Figure 3). However, they are different in their effects on proteins released from lysed cells. Among the three detergents, SDS is strongest in its ability to bind to and denature proteins. Why is SDS stronger than the other two detergents? They have an identical aliphatic chain and differ mainly at the charged group, as is clear from the different molecular weights (see Table 1) depicted in Figure 2 (different sizes of the head groups). One of the mechanisms that is caused by the different charged groups may be the uniqueness of the sulfate group in SDS. Sulfate salts, e.g., sodium, ammonium, and even guanidinium sulfate, are known as strong salting-out salts [61–63]. Although pure speculation, the sulfate group in SDS may salt out itself (SDS) either intra-molecularly or inter-molecularly at a local high SDS concentration when bound by proteins. Namely, hydrophobic interactions between the aliphatic chain of SDS and the non-polar surface of the protein molecules are enhanced by their own sulfate group. By the same token, it is expected that these head groups are different in size and hydration and play a role in determining their differences. SLG has the largest head group, which may destabilize its binding as a cluster due to steric hindrance. The sulfate group in SDS is perhaps the most hydrated and, hence, the most effective in stabilizing SDS-bound structures. Namely, SDS not only masks the hydrophobic surface of the proteins but also makes the bound structure

more hydrated, stabilizing the denatured and dissociated structures. There may be other factors involved in the different strengths of these detergents, which require more studies.

The next question is their effects on denatured proteins. These three detergents are used to solubilize proteins from inclusion bodies. One of the mechanisms for their solubilization of proteins from inclusion bodies is the dispersion or dissociation of contaminants that are trapped in inclusion bodies and possibly bound by the proteins. Lipids and nucleic acids may be effectively dispersed and dissociated from the proteins by the detergents. For example, Sarkosyl helps dissociate and remove lipopolysaccharides (LPS) from Gram-negative bacteria such as *E. coli* and *Chlamydia* [64]. At elevated temperatures, LPS undergoes thermal transition, making its binding weaker, leading to its replacement by Sarkosyl. The binding of Sarkosyl then prevents the protein from aggregation [65]. The dispersion of lipids by the detergents can be explained by the binding of the aliphatic groups of the detergents. Above the CMC of the detergents, lipids may form mixed micelles with the detergents, although Sarkosyl does not appear to form micelles by itself, even above the CMC. However, Sarkosyl may be able to bind to the lipids through its aliphatic chains and increase the solubility of the lipids. Nucleic acids may be bound by the detergents through aromatic/hydrophobic interactions with the nucleobases [66]. However, it is unlikely that dispersing contaminants from inclusion bodies is sufficient to make the denatured proteins soluble. These denatured proteins in the inclusion bodies aggregate to a different extent depending on the proteins expressed in inclusion bodies.

SDS has been shown to bind to denatured proteins as stable SDS–protein complexes. As shown in Figure 2, SDS binds to an (unfolded) polypeptide as micellar forms, masking its non-polar surface and, more importantly, adding negative charges and, possibly, hydration to the polypeptide. SLG, which also forms micelles (see Table 1), most likely binds to proteins in a similar manner but less stably than SDS micelles due to the bulky head group of SLG. Sarkosyl may also bind to proteins similarly, but perhaps not in a micellar manner. It is also possible that Sarkosyl binds to proteins molecularly, also masking non-polar surfaces and adding negative charges. Refolding from the detergent-solubilized proteins can be effectively undertaken both for SLG and Sarkosyl, which may be ascribed to unstable detergent binding to the proteins.

An attempt was made to use Sarkosyl in place of SDS in gel electrophoresis [67,68]. Two purified recombinant proteins, one with an isoelectric point of 4.64 and molecular weight of ~17,000 and another with an electric point of 8.0 and a molecular weight of ~30,000, were subjected to Sarkosyl-PAGE, containing 0.05% Sarkosyl, which is below the CMC. Unfortunately, the running pH was not reported in that paper [54], although the pH of the loading sample was 6.8. These proteins showed the electrophoretic mobility of the monomer expected from their molecular weight, most likely due to Sarkosyl binding proportionally increasing with the molecular size of the proteins. These proteins showed a doublet band due to dimerization, as expected from their self-association properties. NMR experiments indicated the retention of the native fold for several proteins and the perturbation of surface residues. These results clearly indicate the binding of Sarkosyl on the native proteins during electrophoresis and the NMR experiment. A successful Sarkosyl solubilization (even 10%) followed by dilution refolding has also been shown for the maltose-binding protein expressed as a fusion protein to glutathione S-transferase (GST) in inclusion bodies [69]. It was shown, however, that reducing the Sarkosyl concentration alone does not restore the native structure of the GST; instead, a combination of dilution with the addition of Triton X-100 and CHAPS resulted in the renaturation of the GST portion. This was ascribed to the binding of Sarkosyl to the GST, which disabled binding to the glutathione column. Those bound to Sarkosyl can then be removed from the glutathione binding site via the addition of milder detergents (Triton X-100 and CHAPS). Thus, Sarkosyl appears to persistently bind to proteins even after refolding, as described earlier.

The most frequently employed solubilization methods are the use of urea and guanidine hydrochloride, which not only fully denature the proteins but also lead to effective solubilization by cleaving inter-molecular and intra-molecular hydrogen bonding and

hydrophobic interactions. Their binding to the proteins is generally weak compared to the detergents. The binding of these detergents occurs in the mM concentration range, while urea and guanidine hydrochloride require an M concentration range. After refolding, they can be readily dialyzed out. Which of the detergents or the denaturants is more effective in refolding proteins depends on the proteins. However, it seems clear that the detergent process is more cost-effective.

Detergents are frequently used for chemical cell lysis procedures. Since SDS, Sarkosyl, and SLG all have the same aliphatic chain, they may be equally effective in penetrating cell membranes, as shown in Figure 3, and disrupting the membrane structures. SDS has been used as a means of denaturing cell lysis, as this detergent most likely denatures the proteins extracted from the cells. Namely, excessive SDS binds to the proteins and denatures them. However, it may be effective at solubilizing membrane proteins but can still denature their extracellular domains. Sarkosyl may similarly disrupt the membrane structures and release cellular proteins. Although it may bind to the proteins, the extracted proteins are most likely functional. There is no report on the use of SLG for cell lysis, but one of us (Tsutomu Arakawa) tested SLG and observed functional proteins when extracted from the cells using SLG. It would be of great interest to try this detergent for cell lysis because of its weak protein-binding properties and non-denaturing nature.

The last question is how Sarkosyl or SDS separates these insoluble neuropathogenic fibrils. Taking tau as an example, this protein is natively unfolded and bound to microtubules, as described earlier. It undergoes post-translational modifications, including phosphorylation, which, in turn, leads to conformational changes and a series of oligomerization, eventually leading to the formation of filaments and fibrils [70]. The native proteins and their oligomers are actually more likely to be less folded than the late-stage filamentous and fibrillar structures and may present binding sites for Sarkosyl or SDS. The early-stage and late-stage oligomers were both characterized as granules using atomic force microscopy, dynamic light scattering, and CD spectroscopy [71]. The early-stage oligomer granules are Sarkosyl-soluble, smaller in size, and have extended structures, while late-stage oligomer granules (closer in structure to fibrils) are Sarkosyl (or SDS)-insoluble and β-sheet structures. Thus, the late-stage samples may contain various forms of tau or other neuropathogenic proteins. The binding of Sarkosyl to these less-folded species may make them more soluble and facilitate their separation from less-soluble (Sarkosyl-insoluble) forms. In this sense, it also presents binding sites for SDS. However, SDS can cause structural changes in fibrils that are different from Sarkosyl, which may preserve the fibril structures more effectively. SLG may also be effective in keeping the fibrillar structure intact, although its binding to the natively unfolded structure is not known. It should be noted, nevertheless, that SLG can bind to the denatured proteins expressed in inclusion bodies. The binding of Sarkosyl to natively unfolded proteins may make them more soluble and, thereby, facilitate their separation from Sarkosyl-insoluble fibrils, which may bind Sarkosyl to a limited extent and hence may not be solubilized and converted to the natively unfolded state.

8. Conclusions

Here, we review the properties and effects of three anionic detergents, SDS, Sarkosyl, and Sodium lauroyl glutamate (SLG), which have been used in molecular biology research. SDS is effective in cell lysis, the solubilization of proteins, and the purification of SDS-resistant fibrils, but inherently denatures proteins and disrupts native complexes. Sarkosyl binds to unfolded proteins and, most likely, to native proteins, though to a limited extent, and can be used for protein solubilization and the separation of soluble proteins from Sarkosyl-insoluble neuropathological protein fibrils. SLG is mostly mild as a detergent and can be used to refold proteins, although its use as a molecular biology research reagent has been limited. We hope that this review promotes research on its use in cell lysis, the protein refolding of inclusion bodies, and the characterization of protein fibrils.

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References

1. Izumida, K.; Hara, Y.; Furukawa, Y.; Ishida, K.; Tabata, K.; Morita, E. Purification of hepatitis C virus core protein in non-denaturing condition. *J. Virol. Mthods* **2023**, *323*, 114852. [CrossRef] [PubMed]
2. Krishnan, S.; Sasi, S.; Kodakkattumannil, P.; Senaani, S.; Lekshmi, G.; KOttackal, M.; Amiri, K.M.A. Cationic and anionic detergent buffers in sequence yield high-quality genomic DNA from diverse plant species. *Anal. Biochem.* **2024**, *1*, 115372. [CrossRef] [PubMed]
3. Niebling, S.; Burastero, O.; Garcia-Alai, M. Biophysical characterization of membrane proteins. *Methods Mol. Biol.* **2023**, *2652*, 215–230. [CrossRef] [PubMed]
4. Linke, D. Detergents: An overview. *Methods Enzymol.* **2009**, *463*, 603–617. [CrossRef] [PubMed]
5. Miyake, J.; Ochiai-Yanagi, S.; Kasumi, T.; Takagi, T. Isolation of a membrane protein from R rubrun chromatophores and its abnormal behavior in SDS-polyacrylamide gel electrophoresis due to a high binding capacity for SDS. *J. Biochem.* **1978**, *83*, 1679–1686. [CrossRef]
6. Neset, L.; Takayidza, G.; Berven, F.S.; Hernandez-Valladares, M. Comparing effciency of lysis buffer solutions and sample preparation methods for liquid chromatography-mass spectrometry analysis of human cells and plasma. *Molecules* **2022**, *27*, 3390. [CrossRef]
7. Kubo, K.; Isemura, T.; Takagi, T. Electrophoretic behavior of micellar and monomeric sodium dodecyl sulfate in polyacrylamide gel electrophoresis with reference to those of SDS-protein complexes. *Anal. Biochem.* **1979**, *92*, 243–247. [CrossRef]
8. Sakuma, C.; Nakagawa, M.; Tomioka, Y.; Maruyama, T.; Entzminger, K.; Fleming, J.K.; Shibata, T.; Kurosawa, Y.; Okumura, C.J.; Arakawa, T. Western blotting of native proteins from agarose gels. *Biotechniques* **2022**, *72*, 207–218. [CrossRef]
9. Akuta, T.; Maruyama, T.; Sakuma, C.; Nakagawa, M.; Tomioka, Y.; Entzminger, K.; Fleming, J.K.; Sato, R.; Shibata, T.; Kurosawa, Y. A new method to characterize conformation-specific antibody by a combination of agarose native gel electrophoresis and contact blotting. *Antibodies* **2022**, *11*, 36. [CrossRef]
10. Dam, D.V.; Valkenburg, F.; Kolen, K.V.; Pintelon, I.; Timmermans, J.-P.; Deyn, P.P.D. Behavioral and neuropathological phenotyping of the Tau58.2 and Tau58/4 transgenic mouse models for FTDP-17. *Life* **2023**, *13*, 2088. [CrossRef]
11. Matsuki, H.; Mandai, S.; Shiwaku, H.; Koide, T.; Takahashi, N.; Yanagi, T.; Inaba, S.; Ida, S.; Fujiki, T.; Mori, Y.; et al. Chronic kidney disease causes blood-brain barrier breakdown via urea-activated matrix metalloproteinase-2 and insolubility of tau protein. *Aginig* **2023**, *25*, 15. [CrossRef]
12. Tomé, S.O.; Tsaka, G.; Ronisz, A.; Ospitalieri, S.; Gawor, K.; Gomes, L.A.; Otto, M.; von Arnim, C.A.F.; Damme, P.V.; Bosch, L.V.D. TDP-43 pathology is associated with increased tau buerdens and seeding. *Mol. Neurodegener.* **2023**, *18*, 71. [CrossRef] [PubMed]
13. Chisnall, B.; Johnson, C.; Kulaberoglu, Y. Insoluble protein purification with Sarkosyl: Facts and precautions. *Methods Mol. Biol.* **2014**, *1091*, 179–186. [CrossRef] [PubMed]
14. Morrow, B.H.; Koening, P.H.; Shen, J.K. Self-assembly and bilayer-micelle transition of fatty acids studied by replica-exchange constant molecular dynamics. *Langmiur* **2013**, *29*, 14823–14830. [CrossRef] [PubMed]
15. Bordes, R.; Tropsch, J.; Holmberg, K. Role of an amide bond for self-assembly of surfactants. *Langmuir* **2010**, *26*, 3077–3083. [CrossRef] [PubMed]
16. Xia, J.; Nnanna, I.A.; Sakamoto, K. Amino acid surfactants: Chemistry, synthesis, and properties. In *Protein-Based Surfactants. Synthesis, Physicochemical Properties, and Applications*; Nnanna, I.A., Xia, J., Eds.; Marcel Dekker: New York, NY, USA, 2001; pp. 75–122. Available online: https://books.google.co.jp/books?hl=ja&lr=lang_ja%7Clang_en&id=CWK1DwAAQBAJ&oi=fnd&pg=PA75&ots=fEoUpD7MJE&sig=XcfIl8ys3o410mGlCPLT26tMoYc&redir_esc=y#v=onepage&q&f=false (accessed on 4 March 2014).
17. Arakawa, T.; Kita, Y.; Ejima, D. Refolding technology for scFv using a new detergent, N-lauroyl-L-glutamate and arginine. *Antibodies* **2012**, *1*, 215–238. [CrossRef]
18. Ariki, R.; Hirano, A.; Arakawa, T.; Shiraki, K. Drug solubilization effect of lauroyl-L-glutamate. *J. Biochem.* **2012**, *151*, 27–33. [CrossRef]
19. Yumioka, R.; Shimada, M.; Takino, K.; Ejima, D. Evaluation Method for Detergents. Japan Patent JP 2006292686, 26 October 2006.
20. Kudou, M.; Yumioka, R.; Ejima, D.; Arakawa, T.; Tsumoto, K. A novel protein refolding system using lauroyl-L-glutamate as a solubilizing detergent and arginine as a folding assisting agent. *Protein Expr. Purif.* **2011**, *75*, 46–54. [CrossRef]

21. Carratalá, J.V.; Atienza-Garriga, J.; López-Laguna, H.; Vázquez, E.; Villaverde, A.; Sánchez, J.M.; Ferrer-Miralles, N. Enhanced recombinant protein capture, purity and yield from crude bacterial cell extracts by N-Lauroylsarcosine-assisted affinity chromatography. *Microb. Cell Fact.* **2023**, *22*, 81. [CrossRef]
22. Chung, J.M.; Lee, S.; Jung, H.S. Effective non-denaturing purification method for improving the solubility of recombinant actin-binding proteins produced by bacterial expression. *Protein Expr. Purif.* **2017**, *133*, 193–198. [CrossRef]
23. Peternel, S.; Grdadolnik, J.; Garberc-Porekar, V.; Komel, R. Engineering inclusion bodies for non denaturing extraction of functional proteins. *Microb. Cell Fact.* **2008**, *7*, 34. [CrossRef]
24. Gentry, D.R.; Burgess, R.R. Overproduction and purification of the ω subunit of *Escherichia coli* RNA polymerase. *Protein Expr. Purifi.* **1990**, *1*, 81. [CrossRef]
25. Burgess, R.R. Purification of overproduced *Escherichia coli* RNA polymerase sigma factors by solubilizing and refolding from Sarkosyl. *Methods Enzymol.* **1996**, *273*, 145–149. [CrossRef]
26. Lu, H.S.; Clogston, C.L.; Narhi, L.O.; Merewether, L.A.; Pearl, W.R.; Boone, T.S. Folding and oxidation of recombinant human granulocyte colony stimulating factor produced in *Escherichia coli*. *J. Biol. Chem.* **1992**, *267*, 8770–8777. [CrossRef]
27. Maltoni, G.; Scutteri, L.; Mensitieri, F.; Piaz, F.D.; Hochkoeppler, A. High-yield production in *Escherichia coli* and convenient purification of a candidate vaccine against SARS-CoV-2. *Biotechnol. Lett.* **2022**, *44*, 1313–1322. [CrossRef]
28. Padhiar, A.A.; Chanda, W.; Joseph, T.P.; Guo, X.; Liu, M.; Sha, L.; Batool, S.; Gao, Y.; Zhang, W.; Huang, M. Comparative study to develop a single method for retrieving wide class of recombinant proteins from classical inclusion bodies. *Appl. Microbiol. Biotechnol.* **2018**, *102*, 2363–2377. [CrossRef] [PubMed]
29. Rani, R.M.; Syngkli, S.; Nongkhlaw, J.; Das, B. Expression and characterization of human glycerol kinase: The role of solubilizing agents and molecular chaperones. *Biosci. Rep.* **2023**, *43*, BSR20222258. [CrossRef]
30. Ishikawa, S.; Ishikawa, H.; Sato, A. Improved refolding of a human IgG Fc (CH2-CH3) scaffold from its inclusion body in *E. coli* by alkaline solubilization. *Biol. Pharm. Bull.* **2022**, *45*, 284–291. [CrossRef]
31. Storrs, S.B.; Tou, J.S.; Ballinger, J.M. Method for Solubilization and Renaturation of Somatotropins. U.S. Patent US6410694B, 15 December 1999.
32. Yumioka, R.; Ejima, D. Protein Refolding Method. Japan Patent WO 2009136568A1, 12 November 2009.
33. Kudou, M.; Ejima, D.; Sato, H.; Yumioka, R.; Arakawa, T.; Tsumoto, K. Refolding single-chain antibody (scFv) using lauroyl-L-glutamate as a solubilizing detergent and arginine as a refolding additive. *Protein Expr. Purif.* **2011**, *77*, 68–74. [CrossRef]
34. Melki, R.; Carlier, M.F.; Pantaloni, D.; Timasheff, S.N. Cold depolymerization of microtubules to double rings: Geometric stabilization of assemblies. *Biochemistry* **1989**, *28*, 9143–9152. [CrossRef]
35. Julien, C.; Bretteville, A.; Planel, E. Biochemical isolation of insoluble tau in transgenic mouse models of tauopathies. *Methods Mol. Biol.* **2012**, *849*, 473–491. [CrossRef] [PubMed]
36. Greenberg, S.G.; Davies, P. A preparation of Alzheimer paired helical filaments that displays distinct tau proteins by polyacrylamide gel electrophoresis. *Proc. Natl. Acad. Sci. USA* **1990**, *87*, 5827–5831. [CrossRef] [PubMed]
37. Kondo, J.; Honda, T.; Mori, H.; Hamada, Y.; Miura, R.; Ogawa, M.; Ihara, Y. The carboxyl third of tau is tightly bound to paired helical filaments. *Neuron* **1988**, *1*, 827–834. [CrossRef]
38. Tarutani, A.; Adachi, T.; Akatsu, H.; Hashizume, Y.; Hasegawa, K.; Saito, Y.; Robinson, A.C.; Mann, D.M.A.; Yoshida, M.; Maruyama, S. Ultrastrucrural and biochemical classification of pathogenic tau, α-synuclein and TDP-43. *Acta Neuropathol.* **2022**, *143*, 613–640. [CrossRef] [PubMed]
39. Fitzpatrick, A.W.P.; Falcon, B.; He, S.; Murzin, A.G.; Murshudov, G.; Garringer, H.J.; Crowther, R.A.; Ghetti, B.; Goedert, M.; Scheres, S.H.W. Cryo-EM structures of tau filaments frin Alzheimer's disease. *Nature* **2017**, *547*, 185–190. [CrossRef] [PubMed]
40. Stern, A.M.; Yang, Y.; Jin, S.; Yamashita, K.; Meunier, A.L.; Liu, W.; Cai, Y.; Ericsson, M.; Liu, L.; Goedert, M. Abundant Aß fibrils in ultracentrifugal supernatants of aqueous extracts from Alzheimer's disease brains. *Neuron* **2012**, *111*, 2012–2020. [CrossRef] [PubMed]
41. Kryndushkin, D.; Pripuzova, N.; Shewmaker, F.P. Isolation and analysis of prion and amyloid aggregates. *Cold Spring Harb. Protoc.* **2017**, *2*, 118–125. [CrossRef]
42. Nizhnikov, A.A.; Alexandrov, A.I.; Ryzhova, T.A.; Mitkevich, O.V.; Dergalev, A.A.; Ter-Avanesyan, M.D.; Galkin, A.P. Proteomic screening for amyloid proteins. *PLoS ONE* **2014**, *9*, e116002. [CrossRef]
43. Belashova, T.A.; Valina, A.A.; Sysoev, E.L.; Velizhanina, M.E.; Zelensky, A.A.; Galkin, A.P. Search and identification of amyloid proteins. *Methods Protoc.* **2023**, *6*, 16. [CrossRef]
44. Ryzhova, T.A.; Sopova, J.V.; Zadorsky, S.P.; Siniukova, V.A.; Sergeeva, A.V.; Galkina, S.A.; Nizhnikov, A.A.; Shenfeld, A.A.; Volkov, K.V.; Galkin, A.P. Screening for amyloid proteins in the yeast proteome. *Curr. Genet.* **2018**, *64*, 469–478. [CrossRef]
45. Sergeeva, A.V.; Galkin, A.P. Functional amyloids of eukaryotes: Criteria, classification, and biological significance. *Curr. Genet.* **2020**, *66*, 849–866. [CrossRef] [PubMed]
46. Sopova, J.V.; Koshel, E.I.; Belashova, T.A.; Zadorsky, S.P.; Sergeeva, A.V.; Siniukova, V.A.; Shenfeld, A.A.; Velizhanina, M.E.; Volkov, K.V.; Nizhnikov, A.A. RNA-binding protein FXR1 is presented in rat brain in amyloid form. *Sci. Rep.* **2019**, *9*, 18983. [CrossRef] [PubMed]
47. Antonets, K.S.; Belousov, M.V.; Sulatskaya, A.I.; Belousova, M.E.; Kosolapova, A.O.; Sulatsky, M.I.; Andreeva, E.A.; Zykin, P.A.; Malovichko, Y.V.; Shtark, O.Y. Accumulation of storage proteins in plant seeds is mediated by amyloid formation. *PLoS Biol.* **2020**, *18*, e3000564. [CrossRef] [PubMed]

48. Isidan, A.; Liu, S.; Li, P.; Lashmet, M.; Smith, L.J.; Hara, H.; Cooper, D.K.C.; Ekser, B. Decellularization methods for developing porcine corneal xenografts and future perspectives. *Xenotransplantation* **2019**, *26*, e12564. [CrossRef] [PubMed]
49. da Palma, R.K.; Fratini, P.; Schiavo Matias, G.S.; Cereta, A.D.; Guimarães, L.L.; Anunciação, A.R.A.; de Oliveira, L.V.F.; Farre, R.; Miglino, M.A. Equine lung decellularization: A potential approach for in vitro modeling the role of the extracellular matrix in asthma. *J. Tissue Eng.* **2018**, *9*, 2041731418810164. [CrossRef] [PubMed]
50. Dong, M.; Zhao, L.; Wang, F.; Hu, X.; Li, H.; Liu, T.; Zhou, Q.; Shi, W. Rapid porcine corneal decellurization through the use of sodium N-lauroyl glutamate and supernuclease. *J. Tissue Eng.* **2019**, *10*, 2041731419875876. [CrossRef]
51. Chen, Y.; Gin, J.W.; Wang, Y.; de Raad, M.; Tan, S.; Hillson, N.J.; Northen, T.R.; Adams, P.D.; Petzold, J. Alkaline-SDS cell lysis of microbes with acetone protein precipitation for proteomic sample preparation in 96-well plate format. *PLoS ONE* **2023**, *18*, e0288102. [CrossRef]
52. DeCaprio, J.; Kohl, T.O. Denaturing lysis of cells for immunoprecipitation. *Cold Spring Harb. Protoc.* **2020**, *2020*, 098616. [CrossRef]
53. Massiah, M.A.; Wright, K.M.; Du, H. Obtaining soluble folded proteins from inclusion bodies using Sarkosyl, Triton X-100, and CHAPS: Application to LB and M9 minimal media. Curr. *Proto. Protein Sci.* **2016**, *84*, 6–13. [CrossRef]
54. Wang, H.; Gill, C.O.; Yang, X. Use of sodium lauroyl sarcosinate (Sarkosyl) in viable real-time PCR for enumeration of *Escherichia coli*. *J. Microbiol. Methods* **2014**, *98*, 89–93. [CrossRef]
55. Brown, R.N.; Romine, M.F.; Schepmoes, A.A.; Smith, R.D.; Lipton, M.S. Mapping the subcellular proteome of *Shewanella oneidensis* MR-1 using Sarkosyl-based fractionation and LC-MS/MS protein identification. *J. Proteome Res.* **2010**, *9*, 4454–4463. [CrossRef] [PubMed]
56. Bell, K.S.; Kuyukina, M.S.; Heidbrink, S.; Philp, J.C.; Aw, D.W.; Ivshina, I.B.; Christofi, N. Identification and environmental detection of Rhodococcus species by 1S rDNA-targeted PCR. *J. Appl. Microbiol.* **1999**, *87*, 472–480. [CrossRef] [PubMed]
57. Elke, C.; Vögtli, M.; Rauch, P.; Spindler-Barth, M.; Lezzi, M. Expression of EcR and USP in *Escherichi coli*: Purification and functional studies. *Arch. Insect Biochem. Physiol.* **1997**, *35*, 59–69. [CrossRef]
58. Frankel, S.; Sohn, R.; Leinwand, L. The use of salkosyl in generating soluble protein after bacterial expression. *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 1192–1196. [CrossRef] [PubMed]
59. Lin, S.-H.; Guidotti, G. Purification of membrane proteins. *Methods Enzymol.* **2009**, *463*, 619–629. [CrossRef] [PubMed]
60. Smith, S.M. Strategies for the purification of membrane proteins. *Methods Mol. Biol.* **2017**, *1485*, 389–400. [CrossRef] [PubMed]
61. Hofmeister, F. Zur Lehre von der wirkung der salze. *Arch. Expt. Pathol. Pharmakol.* **1888**, *24*, 247–260. [CrossRef]
62. Arakawa, T.; Timasheff, S.N. Mechanism of protein salting in and salting out by divalent cation salts: Balance between hydration and salt binding. *Biochemistry* **1984**, *23*, 5912–5923. [CrossRef]
63. Arakawa, T.; Timasheff, S.N. Protein stabilization and destabilization by guanidinium salts. *Biochemistry* **1984**, *23*, 5924–5929. [CrossRef]
64. Petyaev, I.M.; Zigangirova, N.A.; Tsibezov, V.V.; Ross, A.; Bashmakov, Y.K. Monoclonal antibodies against lipopolysaccharide of Chlamydia trachomatis with cross reactivity to human ApoB. *Hybridoma* **2011**, *30*, 131–136. [CrossRef]
65. Impeller, D. Hemoglobin Binding Protein from Actinobacillus pleuropneumoniae: A novel Method for Extraction and Isolation. 2007. Available online: https://escholarship.mcgill.ca/concern/thesis/z029p754m (accessed on 1 November 2023).
66. Nandy, A.; Shekhar, S.; Paul, B.K.; Mukherjee, S. Exploring the nucleobase-specific hydrophobic interaction of cryptolepine hydrate with RNA and its subsequenct sequestration. *Langmuir* **2021**, *37*, 11176–11187. [CrossRef] [PubMed]
67. Reichel, C. SARKOSYL-PAGE: A new electrophoretic method for the separation and immunological detection of PEGylated proteins. *Methods Mol. Biol.* **2012**, *869*, 65–79. [CrossRef] [PubMed]
68. Huang, L.; Kuo, X.; Zheng, W.; Xiao, X.; Li, C.; Liu, M.; Jiang, L. 05SAR-PAGE: Separation of protein dimerization and modification using a gel with 0.5% Sarkosyl. *Anal. Chim. Acta* **2020**, *1101*, 193–198. [CrossRef]
69. Tao, H.; Liu, W.; Simmons, B.N.; Harris, H.K.; Cox, T.C.; Massiah, M.A. Purifying natively folded proteins from inclusion bodies using sarkosyl, Triton X-100, and CHAPS. *BioTechniques* **2010**, *48*, 61–64. [CrossRef] [PubMed]
70. Hu, J.; Sha, W.; Yuan, S.; Wu, J.; Huang, Y. Aggregation, transmission, and toxicity of the microtubule-associated protein tau. A complex comprehension. *Int. J. Mol. Sci.* **2023**, *24*, 15023. [CrossRef]
71. Maeda, S.; Sahara, N.; Saito, Y.; Murayama, M.; Yoshiike, Y.; Kim, Y.; Miyasaka, T.; Murayama, S.; Ikai, A.; Takashima, A. Granular tau oligomers as intermediates of tau filaments. *Biochemistry* **2007**, *46*, 3856–3861. [CrossRef]

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Review

A Narrative Review of Preclinical In Vitro Studies Investigating microRNAs in Myocarditis

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Abstract: According to the World Health Organization's statement, myocarditis is an inflammatory myocardium disease. Although an endometrial biopsy remains the diagnostic gold standard, it is an invasive procedure, and thus, cardiac magnetic resonance imaging has become more widely used and is called a non-invasive diagnostic gold standard. Myocarditis treatment is challenging, with primarily symptomatic therapies. An increasing number of studies are searching for novel diagnostic biomarkers and potential therapeutic targets. Microribonucleic acids (miRNAs) are small, non-coding RNA molecules that decrease gene expression by inhibiting the translation or promoting the degradation of complementary mRNAs. Their role in different fields of medicine has been recently extensively studied. This review discusses all relevant preclinical in vitro studies regarding microRNAs in myocarditis. We searched the PubMed database, and after excluding unsuitable studies and clinical and preclinical in vivo trials, we included and discussed 22 preclinical in vitro studies in this narrative review. Several microRNAs presented altered levels in myocarditis patients in comparison to healthy controls. Moreover, microRNAs influenced inflammation, cell apoptosis, and viral replication. Finally, microRNAs were also found to determine the level of myocardial damage. Further studies may show the vital role of microRNAs as novel therapeutic agents or diagnostic/prognostic biomarkers in myocarditis management.

Keywords: in vitro studies; inflammation; microRNAs; myocarditis; preclinical studies

1. Introduction

1.1. Myocarditis—Basic Information

The World Health Organization statement defines myocarditis as an inflammatory disease of the myocardium, diagnosed using histological, immunological, or immunohistochemical criteria [1]. According to the European Society of Cardiology's statement, endomyocardial biopsy remains the gold standard for a definite diagnosis [2]; however, cardiac magnetic resonance imaging is increasingly recommended as the preferable non-invasive approach [3]. Various etiologies are listed among causes that may lead to developing myocarditis, headed by infections, mainly viral, and autoimmune conditions [4]. Notably, the most commonly affected group, in contrast to other well-known cardiac diseases, such as myocardial infarction or heart failure, are young adults, with a slight dominance of men [5]. Noteworthily, myocarditis was indicated as one of the leading causes of sudden cardiac death and dilated cardiomyopathy [6]. However, it is hard to estimate the prevalence

worldwide due to the diverse clinical presentation. According to the reports, it varies between 0.12% and 12% [5].

Although much progress has been made in recent years in myocarditis diagnosis [7,8] with the development of novel non-invasive diagnostic tools [7], there remains a lack of treatment-focused studies [9]. Therefore, therapy remains mainly supportive and symptomatic, focusing on hemodynamic stabilization [10]. The long-term prognosis depends primarily on the cause of the disease [3,9]. Establishing a targeted therapy would allow us to provide patients with optimal management and lead to better long-term outcomes.

1.2. microRNAs—Small Regulators of Gene Expression

Microribonucleic acids (microRNAs, miRNAs, and miRs) are small, non-coding RNA molecules that regulate gene expression [11]. They consist of about 20–25 nucleotides [12] and fulfil their molecular role through binding to the complementary sequences of messenger RNAs (mRNAs), thus promoting mRNA degradation or inhibiting translation. In turn, protein synthesis is blocked [13]. MicroRNAs can be found and measured in different body fluids, such as blood, urine, or cerebrospinal fluid.

Briefly, miRNA biosynthesis starts in the nucleus and continues in the cytoplasm, where Drosha and Dicer, two RNase III proteins, process premature molecules [14,15]. In the final step, the guide strand remains and represents mature miRNA, while a passenger strand is discarded [16]. This is the canonical way and results in the formation of the 5′ miRNA involved in regulating gene expression. Most miRNAs are produced likewise; nevertheless, the different pathways independent of Drosha and Dicer are also known [17]. In turn, the 3′ miRNA can also be formed to bind with complementary mRNAs. This review includes information about the miRNA's end whenever the authors clearly state it.

MiRNAs have been thoroughly studied in recent years. Their putative role in multiple diseases in diverse fields of medicine was described, including cardiology [18,19], oncology [20,21], neurology [22], and autoimmune diseases [23]. Researchers often investigated the involvement of miRNAs in the pathogenesis of the disease, trying to find cause-and-effect relationships. Nevertheless, in some cases, they solely explored miRNAs as potential disease biomarkers without searching for an underlying cause of uncovered miRNA deregulation.

1.3. Suggested Value of MicroRNAs in Myocarditis

MiRNAs are altered in several cardiological conditions, including myocarditis. A recent study showed that not only circulating but also exosome-derived miRNAs may play a vital role in the mechanisms of myocarditis [24]. Regarding usually uncertain diagnoses [22] and mainly symptomatic treatment without targeted therapy options [25], microRNAs are considered a chance to facilitate myocarditis management. Several clinical and preclinical studies analyzed miRNAs' potential role in myocarditis, drawing promising conclusions. We aimed to discuss all of these studies comprehensively.

In previous reviews, we have already summarized clinical trials and in vivo preclinical trials [26,27]. In this review, the last one from the series about microRNAs in myocarditis, we aimed to analyze preclinical in vitro studies thoroughly. Apart from the diagnostic utilities of microRNAs, we also aimed to focus on the possibilities that miRNAs bring to myocarditis therapy.

2. Methodology

The role of miRNAs in myocarditis has been investigated in multiple studies. Therefore, only preclinical in vitro trials have been included in this narrative review to narrow the list of suitable research items and allow for a more accurate and solid discussion. Commentaries, letters to the editors, case reports, reviews, and clinical and preclinical in vivo studies were excluded. Articles written in languages other than English were not included.

We searched the PubMed database with the following query: "(miRNA OR microRNA) AND (myocarditis)". After removing inappropriate studies by title, type, or abstract, we

were left with 73 articles regarding the role of miRNAs in myocarditis. Excluding all of the papers other than those of original preclinical in vitro studies yielded 22 papers to be included in this review (Figure 1).

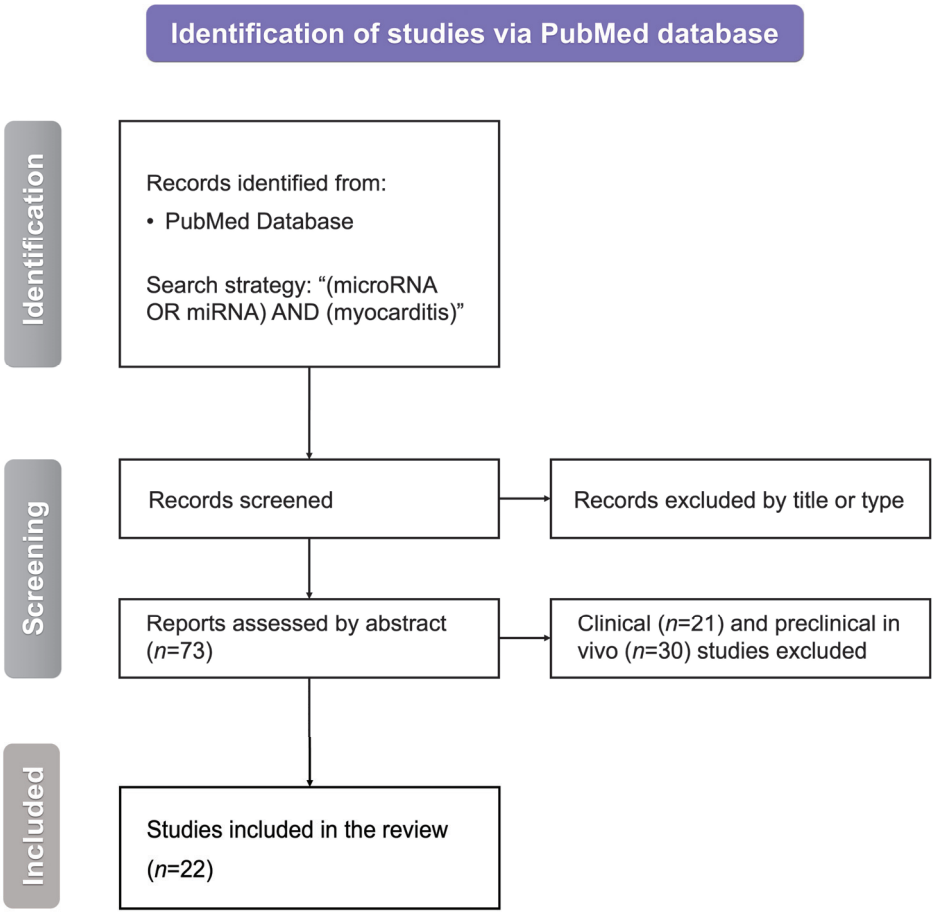


Figure 1. The flowchart for the selection process. miRNA—microRNA, *n*—the number of studies, and RNA—ribonucleic acid.

All included studies were divided into five paragraphs: (i) microRNA alterations in myocarditis, (ii) microRNA influence on myocardial inflammation, (iii) viral replication dependence on microRNAs, (iv) microRNA impact on cell apoptosis, and (v) in vitro models presenting the role of microRNAs in cardiac dysfunction.

3. MicroRNAs in an In Vitro Model of Myocarditis

3.1. microRNA Alterations in Myocarditis

Some in vitro studies presented clearly that miRNAs are altered significantly in the myocarditis model. Yao et al. [28] conducted a study investigating HeLa cells infected with CVB3 (Coxsackievirus B3) and compared them to non-infected cells. It was shown that the miR-107 level was elevated in the study group in comparison to the control group. The higher expression of miR-107 was related to CVB3 infection and associated with viral replication. Liu et al. [29] carried out a study consisting of an in vivo and in vitro part. In this review, we focused on the latter. It was shown that CVB3-infected HeLa cells demonstrated higher levels of miR-324-3p than non-infected cells. Lin et al. [30] studied CVB3-infected HL-1 cells. It was demonstrated that these cells presented increased levels of miR-19b compared to mock-infected HL-1 cells. All studies mentioned in this paragraph, with some additional information, are summarized in Table 1.

Table 1. A summary of studies focusing on microRNA alterations in myocarditis.

Ref.	Year	Population	Comparison	miRNA	Outcome	Methodology
Yao et al. [28]	2020	CVB3-infected HeLa cells	normal HeLa cells	miR-107	↑ miR-107 in CVB3-infected HeLa cells	miRs by PCR
Liu et al. [29]	2022	CVB3-infected HeLa cells	normal HeLa cells	miR-324-3p	↑ miR-324-3p in CVB3-infected HeLa cells	miRs by qPCR
Lin et al. [30]	2016	CVB3-infected HL-1 cells	mock-infected HL-1 cells	miR-19b	↑ miR-19b in CVB3-infected HL-1 cells	miRs by microarray analysis and qPCR

↑—increased, CVB3—Coxsackievirus B3, miR—microRNA, qPCR—quantitative polymerase chain reaction, ref.—reference, and RNA—ribonucleic acid.

3.2. microRNA Influence on Myocardial Inflammation

Several studies focused on miRNA impact on cardiac inflammation, the pathogenic base of myocarditis. Pan et al. [31] analyzed human cardiomyocytes that had been exposed to lipopolysaccharide (LPS) to induce myocarditis. Researchers measured the levels of interleukin 1β (IL-1β) and interleukin 6 (IL-6) to assess pyroptosis and cardiac inflammation. The levels of inflammatory factors were reduced in cells treated with miR-223-3p encapsulated in extracellular vesicles compared to the control group without any intervention. Zhu et al. [32], similar to the previous researchers, studied LPS-induced cardiomyocytes in which *SOX2* overlapping transcript (SOX2OT), which substantially contributes to heart damage, was silenced. An miR-215-5p inhibitor was transfected into the cells to abolish the suppressive role of SOX2OT-knockdown on IL-6 and tumor necrosis factor α (TNF-α) production. This intervention restored the elevated levels of these inflammatory factors in cells primarily caused by induction with LPS. After all, miR-215-5p was shown to play an anti-inflammatory role. Another study in which human cardiomyocytes were treated with LPS to imitate viral myocarditis was performed by Wang et al. [33]. Due to this intervention, the level of miR-16 was profoundly downregulated. These cells were then transfected with miR-16 mimic and compared to (i) normal cells, (ii) LPS-induced cells, and (iii) LPS-induced cells treated with an miR-16 inhibitor. Cardiomyocytes treated with LPS presented considerably higher levels of inflammatory factors—IL-6, interleukin 8 (IL-8), and TNF-α. However, it was repressed by miR-16 mimic administration.

Fei et al. [34] showed that the miR-146a level was elevated in CVB3-infected HeLa cells in comparison to non-infected ones. Moreover, it was proved that adding an miR-146a mimic exerted an anti-inflammatory effect on CVB3-infected cells. The group treated with this miR mimic showed decreased levels of inflammatory cytokines (IL-6 and TNF-α) compared to cells treated with the miR-146a antagonist. Toll-like receptor 3 (detecting viral RNA) was identified as a main target for miR-146a. Fan et al. [35] also conducted research using the HeLa cells model. It was shown that CVB3-infected cells presented elevated levels of miR-181d and miR-30a compared to control cells. Treating infected cells with miR-181d or miR-30a mimics caused an increase in the level of IL-6, while transfecting cells with inhibitors of these miRNAs caused a decrease in IL-6 levels. Chen et al. [36] compared the levels of inflammatory cytokines in CVB3-infected HeLa cells treated with either a mimic or an antagonist of miR-214. Transfection with miR-214 mimic was shown to increase the levels of TNF-α and IL-6. MiR-214 inhibition might be a possible strategy in myocarditis treatment.

A slightly different study was conducted by Chen et al. [37]. Apart from the in vivo part of their study, the researchers also isolated B-cells from the hearts of mice with myocarditis. Wild B-cells with normal miR-98 expression were compared to miR-98-deficient ones. LPS alone and LPS with TNF-α were added to both cultures. It was demonstrated that in LPS-treated cultures, the level of IL-10 was increased. The presence of TNF-α abolished this effect but only in wild B-cells. In B-cells presenting reduced levels of miR-98, the presence of TNF-α did not impact the IL-10 increase caused by LPS. Overall, miR-98 was shown to play a protective role in myocarditis. As concluded by the authors, miR-98 might be considered a potential therapeutic target in the treatment of myocarditis. All

studies mentioned in this paragraph, with some additional information, are summarized in Table 2.

Table 2. A summary of studies regarding the influence of microRNAs on inflammation.

Ref.	Year	Population	Comparison	miRNA	Outcome	Methodology
Pan et al. [31]	2022	LPS-induced HCM treated with EVs with miR-223p	LPS-induced HCM (without intervention)	miR-223-3p	Cardiac inflammation and pyroptosis restriction caused by miR-223-3p.	IL-1β and IL-6 by ELISA
Zhu et al. [32]	2022	SOX2OT-silenced LPS-induced HCM + miR-215-5p inhibitor	SOX2OT-silenced LPS-induced HCM	miR-215-5p	↑ IL-6 and TNF-α (IFs) in HCM with miR-215-5p inhibitor.	IFs by WB
Wang et al. [33]	2020	miR-16 mimic-treated LPS-induced HCM	normal HCM, LPS-induced HCM, or miR-16 inhibitor-treated LPS-induced-HCM	miR-16	↓ miR-16 in LPS cells compared to others; ↓ IL-6, IL-8, and TNF-α (IFs) in LPS-cells treated with miR-16 compared to others.	miRs by qPCR IFs by ELISA
Fei et al. [34]	2020	miR-146a mimic-treated CVB3-infected HeLa cells	normal HeLa cells or miR-146a antagonist-treated CVB3-infected HeLa cells	miR-146a	↑ miR-146a in infected cells compared to others; ↓ IL-6 and TNF-α (Ifs) in cells treated with miR-146a mimic.	miRs by qPCR; Ifs by ELISA
Fan et al. [35]	2019	miR-181d or miR-30a inhibitors or mimic-treated CVB3-infected HeLa cells	normal HeLa cells, CVB3-infected HeLa cells, or miR-NC CVB3-infected HeLa cells	miR-181d miR-30a	↑ miR-181d and miR-30a in infected HeLa cells compared to normal cells; ↑ IL-6 after injecting miR mimic; ↓ IL-6 after injecting miR inhibitor.	miRs by qPCR; IL-6 by ELISA
Chen et al. [36]	2015	miR-214 mimic-treated CVB3-infected HeLa cells	miR214 antagonist-treated CVB3-infected HeLa cells	miR-214	↑ TNF-α and IL-6 (IFs) in miR-214 mimic-treated HeLa cells.	IFs by ELISA
Chen et al. [37]	2017	LPS and LPS/TNF-α-treated miR-98-deficient B-cells	LPS and LPS/TNF-α-treated wild B-cells	miR-98	↑ IL-10 in LPS-treated wild B-cells (but not in LPS/TNF-α-treated wild B-cells); ↑ IL-10 in LPS and LPS/TNF-α-treated miR-98-deficient B-cells.	IL-10 by qPCR

↑—increased, ↓—decreased, CVB3—Coxsackievirus B3, ELISA—enzyme-linked immunosorbent assay, HCM—human cardiomyocytes, Ifs—inflammatory factors, IL—interleukin, LPS—lipopolysaccharide, miR—microRNA, NC—negative control, qPCR—quantitative polymerase chain reaction, SOX2OT—SOX2 overlapping transcript, ref.—reference, RNA—ribonucleic acid, TNF—tumor necrosis factor, and WB—Western blotting.

3.3. Viral Replication Dependence on microRNAs

Germano et al. [38] studied the role of miR-590-5p in promoting viral infection. It was presented that cardiomyocytes exposed to CVB had significantly higher levels of miR-590-5p than normal cells. However, the injection of antagomiR-590-5p was shown to decrease viral load and replication. Another microRNA promoting viral replication was explored by Ye et al. [39], who also conducted a study on CVB3-infected HeLa cells. To analyze the role of miR-126 in viral myocarditis, the cells were treated with a mimic, an inhibitor, or a negative control of miR-126. Administration of an miR-126 mimic caused an increase in viral replication. Accordingly, the injection of an miR-126 inhibitor decreased the viral load. Overall, it was shown that miR-126 promoted CVB3 myocarditis.

Similarly, He et al. [40] conducted a study with HeLa cells infected with CVB3. It appeared that the viral replication was suppressed after miR-21 injection as compared to non-mammal miR. Liu et al. [29] modified CVB3-infected HeLa cells to overexpress miR-343-3p or to knockdown this miR expression. Cells overexpressing miR-343-3p had lower levels of viral capsid protein 1, a marker of viral replication, than miRNA-silenced ones. He et al. [41] engineered CVB3 to contain target sequences for miRNAs important in suppressing viral myocarditis (miR-133 and miR-206). Afterward, these modified viruses were injected into HeLa cells. As a result, viral replication was lowered, and cell viability was increased compared to the controls treated with either CVB3 containing negative control miRNA target sequences or non-modified CVB3. Considering the above, CVB3 containing target sequences for miR-133 or miR-206 is a promising candidate for CVB3 vaccines. Finally, Corsten et al. [42] investigated neonatal rat cardiomyocytes infected with

CVB3. The cells were then treated with an inhibitor, a mimic, and a scrambled control of the miR-221/-222 cluster. After inhibitor addition, the viral replication was elevated. Consistently, transfection with the mimic of the miR-221/-222 cluster caused a decrease in viral load. All studies mentioned in this paragraph, with some additional information, are summarized in Table 3.

Table 3. A summary of studies showing the effect of administrating microRNA mimics or antagonists on viral replication.

Ref.	Year	Population	Comparison	miRNA	Outcome	Methodology
Germano et al. [38]	2019	antagomiR-590-5p-treated CVB-infected CM	normal CM miR-590-5p mimic-treated CVB-infected CM.	miR-590-5p	↑ miR-590-5p in infected CM compared to normal CM; ↓ viral load and ↓ VP1 in antagomiR-590-5p-treated CVB-infected CM compared to other infected cells.	miRs by qPCR; viral load by plaque assay; VP1 by WB.
Ye et al. [39]	2013	miR-126 mimic-treated CVB3-infected HeLa cells	miR-126 inhibitor-treated CVB3-infected HeLa cells; miR-NC-treated CVB3-infected HeLa cells.	miR-126	↑ miR-126 after CVB3 infection VP1 and viral replication; ↑ miR-126 mimic treated and ↓ miR-126 inhibitor-treated cells compared to NC.	miRs by qPCR; VP-1 by WB; replication by plaque assay.
He et al. [40]	2019	miR-21-treated CVB3-infected HeLa cells	non-mammal-miR-treated CVB3-infected HeLa cells.	miR-21	↓ viral load in miR-21-treated CVB3-infected cells compared to others.	Viral load by plaque assay.
Liu et al. [29]	2022	miR-324-3p-overexpressing CVB3-infected HeLa cells	miR-324-3p-silenced CVB3-infected HeLa cells.	miR-324-3p	↓ VP1 in miR-324-3p-overexpressing cells.	VP1 by WB.
He et al. [41]	2015	miR-133 or -206-Ts-engineered CVB3-infected HeLa cells	non-mammal-miR-Ts-engineered CVB3-infected HeLa cells; CVB3-infected HeLa cells.	miR-133 miR-206	↓ viral replication and ↑ cell viability in miR-133 or -206-Ts-engineered CVB3-infected cells.	Viral replication by plaque assay; cell viability by MTS assay.
Corsten et al. [42]	2015	miR-221/-222-inhibited CVB3-infected neonatal rat CMs	miR-221/-222 mimic-treated CVB3-infected neonatal rat CMs; miR-NC-treated CVB3-infected neonatal rat CMs.	miR-221/-222	↑ viral load in miR-221/-222-inhibited CM compared to other VMC CM.	Viral load by qPCR.

↑—increased, ↓—decreased, CM—cardiomyocytes, CVB3—Coxsackievirus B3, miR—microRNA, miR-Ts—miRNA target sequences, MTS—3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, NC—negative control, qPCR—quantitative polymerase chain reaction, ref.—reference, RNA—ribonucleic acid, VMC—viral myocarditis, VP1—viral capsid protein 1, and WB—Western blotting.

3.4. microRNA Impact on Cell Apoptosis

Several studies explored not only the influence on inflammatory response or viral load but also how microRNAs affect apoptosis. Zhang et al. [43] analyzed human cardiomyocytes overexpressing miR-8055 and compared them to the miR-8055-silenced cardiomyocytes. Cells with miR-8055 overexpression presented decreased levels of inflammatory factors, myocardial injury biomarkers, and apoptotic cell ratio. Similarly, Xiang et al. [44] showed that H9c2 cells exposed to LPS and subsequently treated with an miR-27a mimic had ameliorated cell viability; thus, the apoptotic cell ratio was decreased (control cells were injected with an miR-27a inhibitor). Overall, it was proved that miR-27a was protective in LPS-damaged H9c2 cells. Li et al. [45] explored the influence of an miR-203 inhibitor and mimic on H9c2 cells exposed to LPS. The downregulation of miR-203 promoted cell survival, lowered apoptotic rate, and decreased levels of inflammatory cytokines. On the other hand, miR-203 overexpression exacerbated cell apoptosis and contributed to an increased inflammatory response. As suggested by the authors, miR-203 plays its role by inhibiting the expression of a nuclear factor interleukine-3 (NFIL3), a survival mediator in the heart.

Tong et al. [46] studied the role of miR-15 in CVB3-infected H9c2 cells. The miR-15 inhibitor was added to these cells and compared to three other groups: (i) normal H9c2 cells,

(ii) CVB3-infected H9c2 cells, and (iii) CVB3-infected cells treated with the negative control miR. It was demonstrated that the group receiving the miR-15 inhibitor presented lower levels of inflammatory factors—IL-1β, IL-6, and interleukin 18 (IL-18). Moreover, miR-15 inhibition promoted the viability of CVB3-infected cells. Xia et al. [47] also conducted the in vitro part of their study on CVB3-infected H9c2 cells. They demonstrated that both miR-217 and miR-543 inhibitors caused a reduction in cell apoptosis and mitigation of inflammatory response (measured as IL-1β and IL-6 levels). Li et al. [48] presented that CVB3-infected H9c2 cells showed restrained expression levels of miR-16 compared to non-infected cells. Furthermore, the levels of inflammatory factors (IL-6 and TNF-α) and cell apoptosis decreased in the viral myocarditis cell model after transfection with an miR-16 mimic.

Zhang et al. [49] performed the preclinical part of their study on human cardiomyocytes. They showed that treating these cells with an miR-98 mimic caused a decrease in the expression of *FAS* and *FASL* genes. Furthermore, the apoptotic cell ratio was decreased in cells overexpressing miR-98. All studies discussed in this paragraph, with some additional information, are summarized in Table 4.

Table 4. A summary of studies presenting the influence of microRNAs on apoptosis.

Ref.	Year	Population	Comparison	miRNA	Outcome	Methodology
Zhang et al. [43]	2021	miR-8055-overexpressing LPS-treated HCM	miR-8055-silenced LPS-treated HCM.	miR-8055	↓ IL-1β, IL-6, and TNFα (IFs); ↓ cTnT, CKMB, BNP, and apoptotic rate in miR-8055-overexpressing cells.	IFs, cTnT, CKMB, and BNP by ELISA; apoptotic cells by FC.
Xiang et al. [44]	2019	miR-27 mimic-treated LPS-H9c2 cells	miR-27 inhibitor-treated LPS-H9c2 cells.	miR-27	↓ cell apoptosis in miR-27 mimic-treated cells.	Cell apoptosis by MTT assay and FC.
Li et al. [45]	2019	miR-203 inhibitor-treated LPS-H9c2 cells	miR-203 mimic-treated LPS-H9c2 cells; miR NC-treated LPS-cells.	miR-203	↓ IL-6, IL-8 (IFs), and cell apoptosis after miR-203 inhibition.	Cell apoptosis and IFs by ELISA.
Tong et al. [46]	2020	miR-15 inhibitor-treated CVB3-infected H9c2 cells	normal H9c2 cells; CVB3-infected H9c2 cells; miR NC-treated CVB3-infected cells.	miR-15	↑ miR-15 in CVB3-infected cells compared to normal cells; ↓ cell apoptosis and ↓ IL-1β, IL-6, and IL-18 (Ifs) in cells treated with miR-15-inhibitor compared to other CVB3-infected cells.	miRs by qPCR; cell apoptosis by FC; Ifs by ELISA.
Xia et al. [47]	2020	miR-217 inhibitor- or miR-543 inhibitor-treated CVB3-infected H9c2 cells	normal H9c2 cells; miR NC-treated CVB3-infected H9c2 cells.	miR-217 miR-543	↑ miR-217 and miR-543 in CVB3-infected H9c2 cells compared to normal cells; ↓ cell apoptosis, ↓ IL-1β, and IL-6 (Ifs) caused by miR inhibitor.	miRs by qPCR; cell apoptosis by FC; Ifs by ELISA.
Li et al. [48]	2020	miR-16 mimic-treated LPS-induced H9c2 cells	normal H9c2 cells; NC-treated LPS-induced-H9c2 cells.	miR-16	↓ miR-16 in LPS-induced cells than in normal cells; ↓ cell apoptosis, IL-6, and TNF-α (IFs) in LPS-induced cells treated with miR-16 compared to other LPS-induced cells.	miRs by qPCR; cell apoptosis by FC; IFs by WB.
Zhang et al. [49]	2016	miR-98 mimic-treated HCM	miR-98 inhibitor-treated HCM; miR NC-treated HCM.	miR-98	↓ *FAS* and *FASL* mRNA and ↓ apoptotic cells in HCM treated with miR-98 mimic compared to others.	miRs, *FAS*, and *FASL* by qPCR; apoptotic cells by FC.

↑—increased, ↓—decreased, BNP—brain natriuretic peptide, CKMB—creatine phosphokinase MB, cTnT—cardiac troponin T, CVB3—Coxsackievirus B3, ELISA—enzyme-linked immunosorbent assay, FC—flow cytometry, HCM—human cardiomyocytes, Ifs—inflammatory factors, IL—interleukin, LPS—lipopolysaccharide, miR—microRNA, MTT—3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, NC—negative control, qPCR—quantitative polymerase chain reaction, ref.—reference, RNA—ribonucleic acid, TNF—tumor necrosis factor, and WB—Western blotting

3.5. In Vitro Models Presenting the Role of microRNAs in Cardiac Dysfunction

Xu et al. [50] investigated cardiomyocytes derived from neonatal rats, aiming to assess the influence of overexpressed miR-1 on the level of connexin 43 (Cx43)—an important transmembrane protein. It was observed that the upregulation of miR-1 decreased Cx43 expression. The decrease in Cx43 may lead to interference with cardiac function in my-

ocarditis. In the previously mentioned study [30], researchers transfected human-induced pluripotent stem cell-derived cardiomyocytes (hiPSCs-CMs) with the miR-19b mimic since it was shown to be upregulated in the VMC model. Irregular beats and decreased beating rates were observed as a result of this intervention. Gap junction protein α1, responsible for the electrical synchrony of cardiomyocytes, was indicated as a target for miR-19b. Thus, the dysregulation of miR-19b might explain arrhythmia occurrence in viral myocarditis patients. Both studies mentioned in this paragraph, with additional information, are summarized in Table 5.

Table 5. A summary of studies showing the role of microRNAs in cardiac dysfunction.

Ref.	Year	Population	Comparison	miRNA	Outcome	Methodology
Xu et al. [50]	2012	miR-1-treated neonatal rat CM	miR-1-inhibited neonatal rat CM; miR NC-treated neonatal rat CM.	miR-1	↓ Cx43 protein in miR-1-treated CM compared to other CM	Cx43 by WB
Lin et al. [30]	2016	miR-19b mimic-treated hi-PSCs-CMs	NC-treated hi-PSCs-CMs.	miR-19b	Irregular beating patterns and decreased beating rate of CM treated with miR-19b mimic	Beating assessed in timeslot

↓—decreased, CM—cardiomyocytes, Cx—connexin, hi-PSCs-CMs—human-induced pluripotent stem cell-derived cardiomyocytes, miR—microRNA, NC—negative control, ref.—reference, and WB—Western blotting.

4. Conclusions and Future Perspectives

MiRNAs are being increasingly investigated in many diseases in various fields of medicine, thus creating novel opportunities for their better management. Amongst them, myocarditis has also been thoroughly researched. Here, we have presented different perspectives from preclinical in vitro trials on the value of miRNAs in myocarditis. MiRNAs influence mechanisms in myocarditis, such as inflammation, apoptosis, and viral replication. Moreover, particular miRNAs are observed to have different levels in the groups of patients and healthy controls. It is crucial not only for myocarditis diagnostic options but also for targeted therapies using either mimics or antagonists of miRNAs (Figure 2).

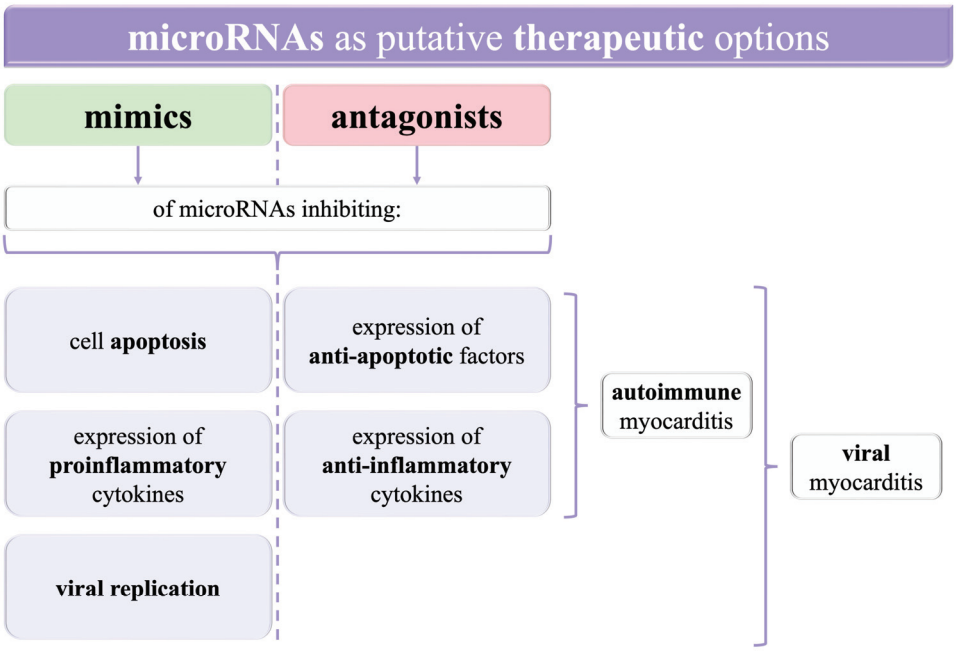


Figure 2. A graphical summary of the opportunities to use microRNAs as potential targets for therapeutic agents. RNA—ribonucleic acid.

Since microRNAs have been shown to influence the levels of diverse proteins, such as inflammatory factors, further investigations into myocarditis should bring new insights into the molecular bases of the disease. Since the treatment remains mainly symptomatic in both viral and autoimmune myocarditis, with attempts to administer antiviral drugs in the former and immunosuppressives in the latter, a better understanding of the pathogenesis may increase the role of targeted therapy. For instance, mimics of miRNAs decreasing the expression of proinflammatory cytokines, as well as antagonists of miRNAs exerting an anti-inflammatory effect, can presumably alleviate inflammation directly in the myocardium. This could enable the avoidance of harmful systemic adverse reactions of immunosuppressive drugs.

Some miRNAs may be crucial in terms of viral replication. Administrating inhibitors of those miRNAs that were shown to increase viral load might be a potential treatment for viral myocarditis. Similarly, inducing overexpression of microRNAs which are related to decreased viral replication is another putative therapy option. Finally, medications based on microRNAs may be used to reduce cell apoptosis and therefore halt the deterioration of the myocardium and prevent impaired heart function after recovery from myocarditis.

Few in vitro studies focused on the correlation between miRNAs and cardiac function; nevertheless, they cannot be missed. Myocarditis, in some cases, leads to permanent impairment of cardiac function, for instance, cardiomyopathies, especially dilated cardiomyopathy, or potentially life-threatening arrhythmias. Therefore, finding a valuable prognostic biomarker would help assess the patient's prognosis and identify those patients who need a more comprehensive approach. As discussed, arrhythmia occurrence in viral myocarditis patients may be partially explained by the dysregulation of miR-19b, and thus, it emerges as another potential therapeutic target.

In conclusion, miRNAs can also be useful as prognostic biomarkers or in targeted therapy, in addition to their role in myocarditis diagnosis. However, more studies exploring this issue are needed. Investigations of the effects of mimics or antagonists of particular microRNAs in myocarditis models may bring us closer to better management of myocarditis with more specific approaches.

5. Limitations

First, we searched only the PubMed database, which might have led to omitting some studies in the field. Nevertheless, it should not cause any statistical or reasoning bias since we performed solely a literature overview in a narrative style. The lack of statistical analysis of discussed data is another limitation. The narrative nature of our review can also explain this. Due to the same reasons, we evaded the risk of bias analysis.

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References

1. Richardson, P.; McKenna, W.; Bristow, M.; Maisch, B.; Mautner, B.; O'Connell, J.; Olsen, E.; Thiene, G.; Goodwin, J.; Gyarfas, I.; et al. Report of the 1995 World Health Organization/International Society and Federation of Cardiology Task Force on the Definition and Classification of cardiomyopathies. *Circulation* **1996**, *93*, 841–842. [CrossRef] [PubMed]
2. Caforio, A.L.; Pankuweit, S.; Arbustini, E.; Basso, C.; Gimeno-Blanes, J.; Felix, S.B.; Fu, M.; Heliö, T.; Heymans, S.; Jahns, R.; et al. Current state of knowledge on aetiology, diagnosis, management, and therapy of myocarditis: A position statement of the European Society of Cardiology Working Group on Myocardial and Pericardial Diseases. *Eur. Heart J.* **2013**, *34*, 2636–2648. [CrossRef] [PubMed]
3. Sagar, S.; Liu, P.P.; Cooper, L.T., Jr. Myocarditis. *Lancet* **2012**, *379*, 738–747. [CrossRef]
4. Lampejo, T.; Durkin, S.M.; Bhatt, N.; Guttmann, O. Acute myocarditis: Aetiology, diagnosis and management. *Clin. Med.* **2021**, *21*, e505–e510. [CrossRef]

5. Blauwet, L.A.; Cooper, L.T. Myocarditis. *Prog. Cardiovasc. Dis.* **2010**, *52*, 274–288. [CrossRef] [PubMed]
6. Ali-Ahmed, F.; Dalgaard, F.; Al-Khatib, S.M. Sudden cardiac death in patients with myocarditis: Evaluation, risk stratification, and management. *Am. Heart J.* **2020**, *220*, 29–40. [CrossRef]
7. Jeserich, M.; Konstantinides, S.; Pavlik, G.; Bode, C.; Geibel, A. Non-invasive imaging in the diagnosis of acute viral myocarditis. *Clin. Res. Cardiol.* **2009**, *98*, 753–763. [CrossRef]
8. Piccirillo, F.; Nusca, A.; Di Sciascio, G. Incidence, diagnosis, and prognosis of myocarditis: Does gender matter? *Pol. Arch. Intern. Med.* **2022**, *132*, 16246. [CrossRef]
9. Ammirati, E.; Frigerio, M.; Adler, E.D.; Basso, C.; Birnie, D.H.; Brambatti, M.; Friedrich, M.G.; Klingel, K.; Lehtonen, J.; Moslehi, J.J.; et al. Management of Acute Myocarditis and Chronic Inflammatory Cardiomyopathy: An Expert Consensus Document. *Circ. Heart Fail.* **2020**, *13*, e007405. [CrossRef]
10. Pettit, M.A.; Koyfman, A.; Foran, M. Myocarditis. *Pediatr. Emerg. Care* **2014**, *30*, 832–838. [CrossRef]
11. Ho, P.T.B.; Clark, I.M.; Le, L.T.T. MicroRNA-Based Diagnosis and Therapy. *Int. J. Mol. Sci.* **2022**, *23*, 7167. [CrossRef] [PubMed]
12. Kara, G.; Arun, B.; Calin, G.A.; Ozpolat, B. miRacle of microRNA-Driven Cancer Nanotherapeutics. *Cancers* **2022**, *14*, 3818. [CrossRef]
13. Catalanotto, C.; Cogoni, C.; Zardo, G. MicroRNA in Control of Gene Expression: An Overview of Nuclear Functions. *Int. J. Mol. Sci.* **2016**, *17*, 1712. [CrossRef]
14. Ha, M.; Kim, V.N. Regulation of microRNA biogenesis. *Nat. Rev. Mol. Cell Biol.* **2014**, *15*, 509–524. [CrossRef]
15. Mustafin, R.N.; Khusnutdinova, E. Perspective for Studying the Relationship of miRNAs with Transposable Elements. *Curr. Issues Mol. Biol.* **2023**, *45*, 3122–3145. [CrossRef] [PubMed]
16. Lin, S.; Gregory, R.I. MicroRNA biogenesis pathways in cancer. *Nat. Rev. Cancer* **2015**, *15*, 321–333. [CrossRef]
17. Hammond, S.M. An overview of microRNAs. *Adv. Drug Deliv. Rev.* **2015**, *87*, 3–14. [CrossRef] [PubMed]
18. Krauze, A.; Procyk, G.; Gąsecka, A.; Garstka-Pacak, I.; Wrzosek, M. The Role of MicroRNAs in Aortic Stenosis-Lessons from Recent Clinical Research Studies. *Int. J. Mol. Sci.* **2023**, *24*, 3095. [CrossRef]
19. Procyk, G.; Klimczak-Tomaniak, D.; Sygitowicz, G.; Tomaniak, M. Circulating and Platelet MicroRNAs in Cardiovascular Risk Assessment and Antiplatelet Therapy Monitoring. *J. Clin. Med.* **2022**, *11*, 1763. [CrossRef]
20. Hussen, B.M.; Hidayat, H.J.; Salihi, A.; Sabir, D.K.; Taheri, M.; Ghafouri-Fard, S. MicroRNA: A signature for cancer progression. *Biomed. Pharmacother.* **2021**, *138*, 111528. [CrossRef]
21. Dziechciowska, I.; Dąbrowska, M.; Mizielska, A.; Pyra, N.; Lisiak, N.; Kopczyński, P.; Jankowska-Wajda, M.; Rubiś, B. miRNA Expression Profiling in Human Breast Cancer Diagnostics and Therapy. *Curr. Issues Mol. Biol.* **2023**, *45*, 9500–9525. [CrossRef]
22. Grodzka, O.; Słyk, S.; Domitrz, I. The Role of MicroRNA in Migraine: A Systemic Literature Review. *Cell Mol. Neurobiol.* **2023**, *43*, 3315–3327. [CrossRef] [PubMed]
23. Mirzaei, R.; Zamani, F.; Hajibaba, M.; Rasouli-Saravani, A.; Noroozbeygi, M.; Gorgani, M.; Hosseini-Fard, S.R.; Jalalifar, S.; Ajdarkosh, H.; Abedi, S.H.; et al. The pathogenic, therapeutic and diagnostic role of exosomal microRNA in the autoimmune diseases. *J. Neuroimmunol.* **2021**, *358*, 577640. [CrossRef]
24. Wu, Q.; Huang, C.; Chen, R.; Li, D.; Zhang, G.; Yu, H.; Li, Y.; Song, B.; Zhang, N.; Li, B.; et al. Transcriptional and functional analysis of plasma exosomal microRNAs in acute viral myocarditis. *Genomics* **2024**, *116*, 110775. [CrossRef]
25. Kyaw, T.; Drummond, G.; Bobik, A.; Peter, K. Myocarditis: Causes, mechanisms, and evolving therapies. *Expert. Opin. Ther. Targets* **2023**, *27*, 225–238. [CrossRef] [PubMed]
26. Grodzka, O.; Procyk, G.; Gąsecka, A. The Role of MicroRNAs in Myocarditis-What Can We Learn from Clinical Trials? *Int. J. Mol. Sci.* **2022**, *23*, 6022. [CrossRef]
27. Procyk, G.; Grodzka, O.; Procyk, M.; Gąsecka, A.; Głuszek, K.; Wrzosek, M. MicroRNAs in Myocarditis-Review of the Preclinical In Vivo Trials. *Biomedicines* **2023**, *11*, 2723. [CrossRef] [PubMed]
28. Yao, M.; Xu, C.; Shen, H.; Liu, T.; Wang, X.; Shao, C.; Shao, S. The regulatory role of miR-107 in Coxsackie B3 virus replication. *Aging* **2020**, *12*, 14467–14479. [CrossRef]
29. Liu, T.; Tong, J.; Shao, C.; Qu, J.; Wang, H.; Shi, Y.; Lin, Y.; Liu, Y.; Shao, S.; Shen, H. MicroRNA-324-3p Plays A Protective Role Against Coxsackievirus B3-Induced Viral Myocarditis. *Virol. Sin.* **2021**, *36*, 1585–1599. [CrossRef]
30. Lin, J.; Xue, A.; Li, L.; Li, B.; Li, Y.; Shen, Y.; Sun, N.; Chen, R.; Xu, H.; Zhao, Z. MicroRNA-19b Downregulates Gap Junction Protein Alpha1 and Synergizes with MicroRNA-1 in Viral Myocarditis. *Int. J. Mol. Sci.* **2016**, *17*, 741. [CrossRef]
31. Pan, L.; Yan, B.; Zhang, J.; Zhao, P.; Jing, Y.; Yu, J.; Hui, J.; Lu, Q. Mesenchymal stem cells-derived extracellular vesicles-shuttled microRNA-223-3p suppress lipopolysaccharide-induced cardiac inflammation, pyroptosis, and dysfunction. *Int. Immunopharmacol.* **2022**, *110*, 108910. [CrossRef]
32. Zhu, W.; Peng, F.; Cui, X.; Li, J.; Sun, C. LncRNA SOX2OT facilitates LPS-induced inflammatory injury by regulating intercellular adhesion molecule 1 (ICAM1) via sponging miR-215-5p. *Clin. Immunol.* **2022**, *238*, 109006. [CrossRef]
33. Wang, L.; Zhang, Y.; Zhu, G.; Ma, Y.; Zuo, H.; Tian, X. miR-16 exhibits protective function in LPS-treated cardiomyocytes by targeting DOCK2 to repress cell apoptosis and exert anti-inflammatory effect. *Cell Biol. Int.* **2020**, *44*, 1760–1768. [CrossRef] [PubMed]
34. Fei, Y.; Chaulagain, A.; Wang, T.; Chen, Y.; Liu, J.; Yi, M.; Wang, Y.; Huang, Y.; Lin, L.; Chen, S.; et al. MiR-146a down-regulates inflammatory response by targeting TLR3 and TRAF6 in Coxsackievirus B infection. *RNA* **2020**, *26*, 91–100. [CrossRef] [PubMed]

35. Fan, K.L.; Li, M.F.; Cui, F.; Feng, F.; Kong, L.; Zhang, F.H.; Hao, H.; Yin, M.X.; Liu, Y. Altered exosomal miR-181d and miR-30a related to the pathogenesis of CVB3 induced myocarditis by targeting SOCS3. *Eur. Rev. Med. Pharmacol. Sci.* **2019**, *23*, 2208–2215. [CrossRef] [PubMed]
36. Chen, Z.G.; Liu, H.; Zhang, J.B.; Zhang, S.L.; Zhao, L.H.; Liang, W.Q. Upregulated microRNA-214 enhances cardiac injury by targeting ITCH during coxsackievirus infection. *Mol. Med. Rep.* **2015**, *12*, 1258–1264. [CrossRef]
37. Chen, X.; Dong, S.; Zhang, N.; Chen, L.; Li, M.G.; Yang, P.C.; Song, J. MicroRNA-98 plays a critical role in experimental myocarditis. *Int. J. Cardiol.* **2017**, *229*, 75–81. [CrossRef]
38. Germano, J.F.; Sawaged, S.; Saadaeijahromi, H.; Andres, A.M.; Feuer, R.; Gottlieb, R.A.; Sin, J. Coxsackievirus B infection induces the extracellular release of miR-590-5p, a proviral microRNA. *Virology* **2019**, *529*, 169–176. [CrossRef]
39. Ye, X.; Hemida, M.G.; Qiu, Y.; Hanson, P.J.; Zhang, H.M.; Yang, D. MiR-126 promotes coxsackievirus replication by mediating cross-talk of ERK1/2 and Wnt/β-catenin signal pathways. *Cell Mol. Life Sci.* **2013**, *70*, 4631–4644. [CrossRef]
40. He, F.; Xiao, Z.; Yao, H.; Li, S.; Feng, M.; Wang, W.; Liu, Z.; Liu, Z.; Wu, J. The protective role of microRNA-21 against coxsackievirus B3 infection through targeting the MAP2K3/P38 MAPK signaling pathway. *J. Transl. Med.* **2019**, *17*, 335. [CrossRef]
41. He, F.; Yao, H.; Wang, J.; Xiao, Z.; Xin, L.; Liu, Z.; Ma, X.; Sun, J.; Jin, Q.; Liu, Z. Coxsackievirus B3 engineered to contain microRNA targets for muscle-specific microRNAs displays attenuated cardiotropic virulence in mice. *J. Virol.* **2015**, *89*, 908–916. [CrossRef]
42. Corsten, M.F.; Heggermont, W.; Papageorgiou, A.P.; Deckx, S.; Tijsma, A.; Verhesen, W.; van Leeuwen, R.; Carai, P.; Thibaut, H.J.; Custers, K.; et al. The microRNA-221/-222 cluster balances the antiviral and inflammatory response in viral myocarditis. *Eur. Heart J.* **2015**, *36*, 2909–2919. [CrossRef]
43. Zhang, L.; Han, B.; Liu, H.; Wang, J.; Feng, X.; Sun, W.; Cai, D.; Jia, H.; Jiang, D. Circular RNA circACSL1 aggravated myocardial inflammation and myocardial injury by sponging miR-8055 and regulating MAPK14 expression. *Cell Death Dis.* **2021**, *12*, 487. [CrossRef]
44. Xiang, J.F.; Yu, J.C.; Zhu, J.Y. Up-regulation of miR-27 extenuates lipopolysaccharide-induced injury in H9c2 cells via modulating ICAM1 expression. *Genes Genom.* **2019**, *41*, 1467–1474. [CrossRef]
45. Li, Y.; Liu, X.; Du, A.; Zhu, X.; Yu, B. miR-203 accelerates apoptosis and inflammation induced by LPS via targeting NFIL3 in cardiomyocytes. *J. Cell Biochem.* **2019**, *120*, 6605–6613. [CrossRef] [PubMed]
46. Tong, R.; Jia, T.; Shi, R.; Yan, F. Inhibition of microRNA-15 protects H9c2 cells against CVB3-induced myocardial injury by targeting NLRX1 to regulate the NLRP3 inflammasome. *Cell Mol. Biol. Lett.* **2020**, *25*, 6. [CrossRef] [PubMed]
47. Xia, K.; Zhang, Y.; Sun, D. miR-217 and miR-543 downregulation mitigates inflammatory response and myocardial injury in children with viral myocarditis by regulating the SIRT1/AMPK/NF-κB signaling pathway. *Int. J. Mol. Med.* **2020**, *45*, 634–646. [CrossRef]
48. Li, Q.Q.; Xi, J.; Li, B.Q.; Li, N. MiR-16, as a potential NF-κB-related miRNA, exerts anti-inflammatory effects on LPS-induced myocarditis via mediating CD40 expression: A preliminary study. *J. Biochem. Mol. Toxicol.* **2020**, *34*, e22426. [CrossRef]
49. Zhang, B.Y.; Zhao, Z.; Jin, Z. Expression of miR-98 in myocarditis and its influence on transcription of the FAS/FASL gene pair. *Genet. Mol. Res.* **2016**, *15*, mr.15027627. [CrossRef]
50. Xu, H.F.; Ding, Y.J.; Shen, Y.W.; Xue, A.M.; Xu, H.M.; Luo, C.L.; Li, B.X.; Liu, Y.L.; Zhao, Z.Q. MicroRNA- 1 represses Cx43 expression in viral myocarditis. *Mol. Cell Biochem.* **2012**, *362*, 141–148. [CrossRef] [PubMed]

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Review

Subjective Cognitive Decline and Genetic Propensity for Dementia beyond Apolipoprotein ε_4: A Systematic Review

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Abstract: Subjective cognitive decline (SCD) has been described as a probable early stage of dementia, as it has consistently appeared to precede the onset of objective cognitive impairment. SCD is related to many risk factors, including genetic predisposition for dementia. The Apolipoprotein (APOE) ε4 allele, which has been thoroughly studied, seems to explain genetic risk for SCD only partially. Therefore, we aimed to summarize existing data regarding genetic factors related to SCD, beyond APOE ε4, in order to improve our current understanding of SCD. We conducted a PRISMA systematic search in PubMed/MEDLINE and Embase databases using the keywords "subjective cognitive decline" and "genetic predisposition" with specific inclusion and exclusion criteria. From the 270 articles identified, 16 were finally included for the qualitative analysis. Family history of Alzheimer's disease (AD) in regard to SCD was explored in eight studies, with conflicting results. Other genes implicated in SCD, beyond APOE ε4, were investigated in six studies, which were not strong enough to provide clear conclusions. Very few data have been published regarding the association of polygenic risk for AD and SCD. Thus, many more genes related to AD must be studied, with polygenic risk scores appearing to be really promising for future investigation.

Keywords: subjective cognitive decline; cognitive complaints; early dementia stages; genetic propensity; polygenic risk

1. Introduction

Nowadays, awareness of brain health and symptoms of dementia in the general population is growing. Therefore, an increasing number of cognitively unimpaired individuals seek medical help for their cognitive function [1]. A new medical term, subjective cognitive decline (SCD), was conceived in 2014 in order to describe an individual's perception of their own cognitive impairment [2]. At the same time, the SCD initiative (SCD-I), an international working group, established standardized criteria for SCD [2], consisting of a self-experienced persistent decline in cognition, unrelated to an acute event, in comparison to a previously normal cognitive status, along with normal performance on standardized cognitive tests.

Existing data suggest that SCD may precede the onset of objective memory impairment [3], acting as an early dementia stage [4]. Specifically, longitudinal studies have shown that individuals reporting SCD had an increased incidence of dementia [5,6]. Moreover, cognitively normal (CN) individuals with SCD have been related to abnormal Alzheimer's disease (AD) biomarkers, such as low cerebrospinal fluid amyloid-beta [7,8], as well as low hippocampal and medial temporal cortex volume [9,10]. Individuals with SCD have also been associated with peripheral blood biomarkers [11] of AD, such as the peripheral blood transcriptome, which might be considered a pre-disease biomarker.

However, it remains uncertain which factors affect the progression from SCD to dementia. In fact, SCD has been related to many different underlying causes, apart from

preclinical dementia, including psychiatric symptoms or disorders [12], medication effects [13], different personality traits [14], poor physical functioning and alcohol abuse [15]. Additionally, the context (community-based or clinical setting) [16] as well as the way SCD is assessed (via a single question or structured questionnaire) [17] have been shown to affect its prevalence. Thus, individuals with SCD appear to be a highly heterogeneous population. Given that, to date, there is no single gold standard instrument which can differentiate individuals with SCD from those without SCD in the clinical setting; SCD is not considered a diagnostic entity in the Diagnostic and Statistical Manual of Mental Disorders [18]. What is of utmost importance, though, is to identify those individuals belonging in the predementia spectrum in whom SCD may yield additional information for future objective cognitive decline [19].

In an effort to determine the factors increasing the risk of an individual with SCD to develop objective cognitive impairment, the SCD-I working group has proposed the SCD-plus criteria [2]. The specific criteria focused on memory rather than other cognitive domains. Moreover, confirmation of perceived cognitive decline by an informant was included, in accordance with previous studies showing that partner reports of memory impairment were more consistent in estimating objective impairment and predicting longitudinal decline [20,21]. Interestingly, Apolipoprotein (APOE) ε4 carriership, which is most commonly associated with late onset AD [22], was also included in the SCD-plus criteria, enhancing the hypothesis of an association between SCD and genetic predisposition for dementia. Thus, genetic risk factors for AD might be helpful in distinguishing individuals with SCD with a higher risk for dementia.

To date, the vast majority of studies regarding genetic predisposition for dementia in individuals with SCD have compared APOE ε4 carriers with non-carriers. The APOE ε4 allele has been associated with cognitive decline [23], abnormal levels of amyloid and tau proteins [24], cortical atrophy [25], disruption of the cerebral white matter in neuroimaging [26,27] and functional brain network changes [28] in individuals with SCD. A previously published systematic review summarized existing data regarding the contribution of APOE ε4 in SCD [29], including 36 articles published between 2001 and 2018, and concluded that there is not enough evidence to suggest that the specific allele predisposes individuals to developing SCD. Recent research work has indicated that APOE ε4 allele is not the only genetic factor implicated in objective cognitive decline among individuals with SCD [30]. Moreover, in the FACEHBI study [31], the APOE genotype has been shown to explain the variance of cerebral amyloid levels only partially in individuals with SCD. Those findings have aroused interest in the possible involvement of other genetic factors for dementia, beyond APOE ε4, in SCD.

The genetic profile of individuals with SCD, beyond APOE ε4, is relatively unexplored. Family history (FH) of AD has been related to increased worrying about dementia symptoms [32], as well as increased risk of developing dementia [33]. Thus, family studies investigating siblings or individuals with positive family history (FH) of AD in relation to SCD have been conducted. Furthermore, other genes beyond the APOE ε4 allele have appeared to contribute to SCD, including presenilin-1 (PSEN1) gene mutations [34,35] as well as period circadian clock 2 (PER_2) gene polymorphisms [36]. Nevertheless, only a small proportion of relevant genes has been studied. Additionally, the polygenic risk for AD has recently emerged, with research concerning the polygenic risk for SCD being relatively limited.

Therefore, our aim in undertaking the current systematic review was to summarize existing knowledge concerning the association of genetic propensity for dementia, beyond APOE ε4, and SCD, as well as to detect whether such an association might be used in order to ameliorate screening for future decline and identify effective interventions for dementia risk reduction.

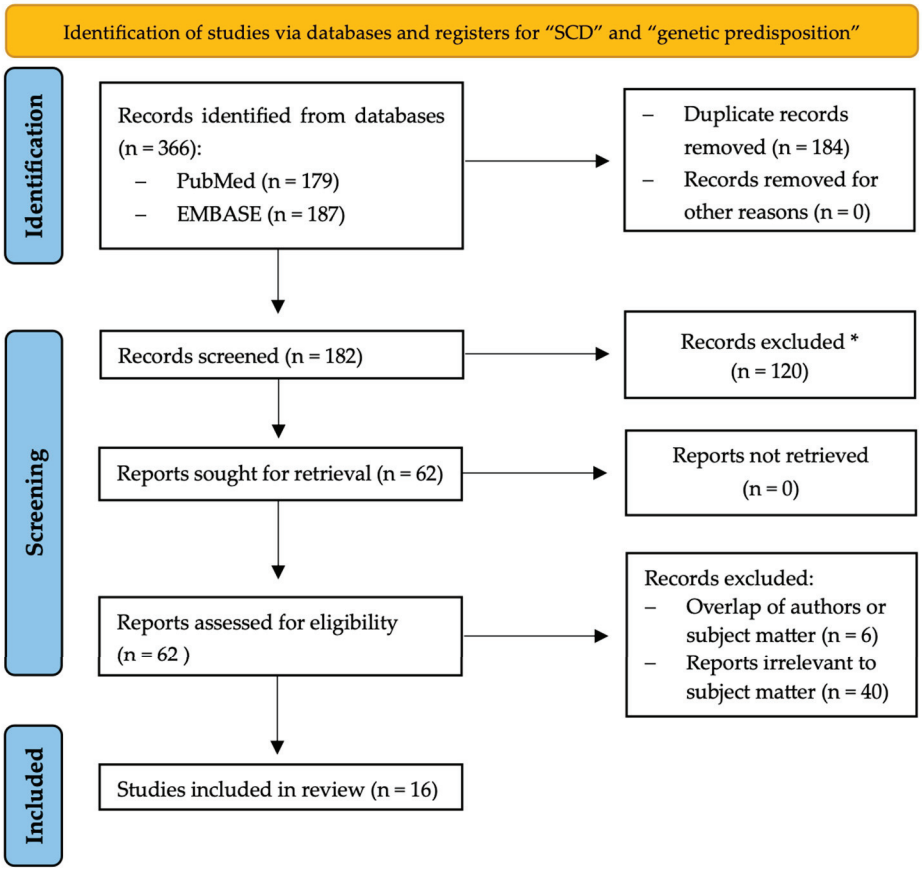
2. Methods

2.1. Systematic Review Search Strategy

The systematic review was conducted in accordance with the Preferred Reporting Items for Systematic Review and Meta-analysis Protocols (PRISMA) statement [37]. The PRISMA 2020 checklist can be found in Table S2 of the Supplementary Materials, along with the PRISMA 2020 checklist for abstracts (Table S3). The protocol of the systematic review was registered in PROSPERO (ID: CRD42024507607).

PubMed/MEDLINE and EMBASE, two electronic bibliographic databases, were searched from inception until 15 December 2023. The search term "subjective cognitive decline" or "subjective cognitive complaints" or "subjective memory complaints" was used in combination with the terms "genetic predisposition" or "genetic risk" or "genetic factors" or "polygenic risk". Overall, 366 records were identified. The exact search strings of the systematic review search strategy can be found in the Supplementary Materials.

The different phases of the systematic review performed are described in Figure 1.



* No automation tools were used. Exclusion was performed only by humans.

Figure 1. PRISMA flow diagram of systematic review.

2.2. Screening and Eligibility Strategy

The screening and eligibility phase was blindly conducted by two individual researchers (S.N.S. and M.R.). All titles and abstracts retrieved from the search were independently examined by the researchers on the same day to promote reliability. After removing 184 duplicate records, the first screening of the remaining 182 reports resulted in 62 full articles. Inclusion criteria were the following: full-text original peer-reviewed research articles (abstracts of poster presentations available in the bibliographic databases were not included), study population including a proportion of individuals with subjective

cognitive complaints (SCC) or subjective memory complaints (SMC) and English language. We mainly selected publications from the past ten years but did not exclude commonly referenced and highly regarded older publications. In total, 120 records were excluded based on the exclusion criteria presented in detail in Table S1 of the Supplementary Materials.

The 62 full-text articles identified as relevant during the first screening stage were assessed for eligibility. In particular, studies were evaluated for potential overlap of authors and subject matter, leading to exclusion of 6 more reports. As this specific review concerns the relationship between SCD and genetic predisposition for dementia beyond APOE ε4, articles examining only the APOE ε4 allele were excluded as irrelevant to subject matter (40 reports), leading to 16 studies which were finally included. In case of disagreement during the eligibility assessment, another investigator (NS) reviewed the full text in question and gave a final approval.

3. Results

In total, 16 studies were selected for the final stage and qualitative synthesis to achieve the objectives of this systematical review. The results were stratified into "family studies" including studies concerning family history (FH) of AD as well as studies in twin individuals, "other genes beyond APOE ε4", and "polygenic risk". In all studies, SCD was assessed in the context of reported SMC via single questions or structured questionnaires.

3.1. Family Studies

In total, eight family studies were included [38–45], either relating FH of AD to SCD (five studies) or examining SCD heritability in twin individuals (three studies). Family studies are shown in Table 1.

Table 1. Family Studies in subjects with SMC.

Study	Population	Family History	SMC [1] Estimation	Main Results
Nicholas et al., 2017 [38] Longitudinal	N = 1545, United States CN [2] Mean age: 53.6	1118 individuals with parental FH [3] of AD [4]	MFQ: Memory Functioning Questionnaire	SMC (baseline or longitudinal) were not associated with FH
Heun et al., 2003 [39] Case-Control	N = 550, Germany CN, MCI [5] or dementia Mean age: 67.9 ± 10.2	238 individuals with parental FH of AD, 59 spouses	In-person interview with two questions	SMC were not associated with FH of AD
Heser et al., 2023 [40] Longitudinal	N = 1240, Germany Dementia-free Mean age: 79.6	422 individuals with parental FH of AD	In-person interview with two questions	SMC were associated with FH of AD (p = 0.008). FH was related to increased dementia risk (HR [6]: 1.56, p = 0.002) in SMC participants
Tsai et al., 2006 [41] Case-Control	N = 1499, United States and Germany, CN Mean age: 69.6 ± 10.5	1203 individuals with parental FH of AD, 296 spouses	In-person interview with a single question	SMC were greater in participants with FH of AD (OR [7]: 1.9, 95% CI [8]: 1.3, 3.0)
Haussmann et al., 2018 [42] Case-control	N = 75, Germany CN (40) or MCI (35) Mean age: 68.1 ± 7.1	21 individuals with parental FH of AD	7-item Likert scale (1 = severe to 7 = none)	SMC were greater in participants with FH of AD in the CN group (p = 0.019)
Selwood et al., 2017 [43] Longitudinal	N = 612, Australia Dementia-free Mean age: 71.3 ± 5.7	Twin individuals 338 monozygotic 274 dizygotic twins	Telephone interview with two questions	SMC were low to moderately heritable (h^2 [9] = 0.33, CI: 0.15, 0.49)

Table 1. *Cont.*

Study	Population	Family History	SMC [1] Estimation	Main Results
Caracciolo et al., 2012 [45] Longitudinal	N = 11,926, Sweden Dementia-free Aged $\geq$ 65	Twin individuals	Telephone interview with specific questions	SMC were not related to genetic background
Bell et al., 2023 [44] Longitudinal	N = 1555, United States Male adults Mean age: 37.8 $\pm$ 2.5	Twin individuals 872 monozygotic 570 dizygotic twins	In-person interview with a single question	SMC were not related to FH of AD

[1] Subjective memory complaints, [2] cognitively normal, [3] family history, [4] Alzheimer's disease, [5] mild cognitive impairment, [6] hazard ratio, [7] odds ratio, [8] confidence interval, [9] heritability.

FH studies have produced conflicting results regarding SMC. On the one hand, Nicholas et al. [38] did not find an association between FH and baseline or longitudinal SMC in 1545 CN subjects, among whom 72.4% reported a parental FH of AD. Similarly, in the case–control study of Heun et al. [39] including 550 individuals (238 first-degree relatives, 59 spouses of AD patients and 253 controls), SMC were not associated with FH of AD, as no difference was found between relatives, spouses and controls. In contrast, Heser et al. [40] found that SMC were associated with FH of AD (p = 0.008) in a prospective multicenter study including 1240 individuals without dementia, among whom 422 had a parental FH of AD. Additionally, FH of AD was associated with subsequent risk of all-type dementia in participants with SMC [hazard ratio (HR): 1.56, 95% confidence intervals (CI): 1.18–2.07, p = 0.002)]. These findings are in accordance with the case–control study of Tsai et al. [41], which showed that first-degree relatives were more likely to report SMC than spouses of AD patients [odds ratio (OR): 1.9, 95% CI: 1.3–3.0]. Moreover, another case–control study conducted by Haussmann et al. [42] investigating 40 CN and 35 mild cognitive impairment (MCI) individuals, showed that SMC were greater in participants with FH of AD only in the CN group (p = 0.019).

In all twin studies included in this systematic review, SMC were not related to genetic background. In particular, a longitudinal study conducted by Selwood et al. [43] included 338 monozygotic and 274 dizygotic twin pairs without dementia, revealing that SMC were low to moderately heritable, as just one third (33%) of the SMC variance was explained by genetic factors, while environmental factors were responsible for 67% of the SMC variance. Similar results were obtained by Bell et al. [44] who did not find an association between SMC and parental FH of AD in 1.555 twin male individuals studied prospectively, showing that the heritability for SMC ranged from 26% to 34% in males aged 38–67. Last but not least, Caracciolo et al. [45] assessed 11.926 twin individuals from the large Swedish cohort study for SMC via a telephone interview, concluding that SMC occurrence was not related to genetic background.

3.2. Other Genes beyond APOE ε4

Overall, six studies [34–36,46–48] regarding other genes, beyond APOE ε4, were included in this specific review, as presented in Table 2.

Table 2. Studies concerning other genes beyond APOE ε4.

Study	Population	Genes	SMC [1] Estimation	Main Results
Norton et al. [34], 2017 Cross-Sectional	N = 52, Colombia CN [2], 26 carriers Mean age: 36.4 $\pm$ 7.1	PSEN-1 280A mutation	Self-reported and partner-based with Memory Complaint Scale	Only self-reported SMC were higher in carriers (p = 0.019).

Table 2. *Cont.*

Study	Population	Genes	SMC [1] Estimation	Main Results
Laws et al. [35], 2002 Case-Control	N = 124, Australia 58 SMC individuals Mean age: 62.0 ± 1.8	PSEN-1 Glu318Gly mutation	Cambridge Cognition Examination (CAMCOG)	Only 4 SMC subjects (6.8%) had the mutation. No difference in SMC.
Bessi et al. [36], 2021 Longitudinal	N = 68, Italy 41 SCD [3], 27 MCI [4] Mean age: 65.2 ± 7.2	CLOCK $_{T3111C}$ PER2 $_{C111G}$	Self-reported with a single question	Only 2 SCD subjects progressed to AD (all were PER2 G carriers, $p = 0.003$).
Mazzeo et al. [46], 2017 Longitudinal	N = 101, Italy 70 SCD, 31 MCI Mean age: 61.3 ± 7.9	KIBRA T	Memory Assessment Clinics-Questionnaire (MAC-Q)	CT or TT carriers had 2.273 higher odds of MCI than CC carriers ($p = 0.049$)
Youn et al. [47], 2017 Case-Control	N = 689 Republic of Korea 344 SMC individuals Mean age: 70.0	APOE ε7	Seoul Neuro-Psychological Screening Battery (SNSB)	Only SMC subjects had APOE ε7 variants (heterozygotes ε3/ε7)
Watfa et al. [48], 2010 Cross-Sectional	N = 369, France SMC, hypertensive Mean age: 70.0 ± 6.0	50 vascular-related genes	Cognitive Difficulties Scale of McNair	No association of any of the polymorphisms with cognitive decline

[1] Subjective memory complaints, [2] cognitively normal, [3] subjective cognitive decline, [4] mild cognitive impairment.

Two studies investigated different PSEN-1 mutations. The E280A mutation was studied in 52 individuals who all had a parent who was carrier of the specific mutation. Among participants, 26 were carriers themselves, while 26 were non carriers [34]. In that study, self-reported SMC were higher in carriers of the mutation ($p = 0.019$), while partner-based SMC did not differ between carriers and non-carriers. Furthermore, the PSEN-1 Glu318Gly mutation was investigated in a case–control study of Australian individuals, including participants with SMC [35]. The specific mutation was found only in four individuals in the SMC group, while no-one among controls had the mutation. Although Glu318Gly carriers had lower objective cognitive scores, SMC did not differ between carriers and non-carriers.

Circadian CLOCK and PER genes polymorphisms, implicated in a sleep–wake cycle, were investigated by Bessi et al. [36] in a longitudinal study. CLOCK T3111C polymorphism was detected in 51.2% of cases with SMC, while PER2 C111G polymorphism was found in 19.5% of cases with SMC. During follow-up, only two subjects with SMC progressed to AD (all were PER2 G carriers), while none of the SMC non-carriers converted to AD ($p = 0.003$).

The KIBRA (kidney and brain expressed protein) T allele, mainly expressed in memory-related brain regions, was investigated in a prospective study conducted by Mazzeo et al. [46] with a long follow-up period. Among 70 individuals with SCD, 16 developed MCI at follow-up [11 were carriers of one or two T alleles (CT/TT) and 5 were not (CC)]. Carriers of the T allele (CT or TT) had 2.273 times higher odds of MCI development than CC carriers ($p = 0.049$) at follow-up.

The APOE ε7 allele, a mutant form of APOE ε3, was investigated in a case–control study by Youn et al. [47], in which ε3/ε7 heterozygotes were found only in the SMC group of individuals. Lastly, Watfa et al. [48] examined the contribution of 50 different gene polymorphisms associated with vascular alterations in dementia-free participants with hypertension and SMC at the time of investigation. No significant associations were found for the genes investigated.

3.3. Polygenic Risk and SCD

Data regarding the association of polygenic risk for AD and SCD are very limited as only three relevant studies have been published [44,49,50], shown in Table 3.

Table 3. Polygenic risk scores in individuals with SCD.

Study	Population	Polygenic Risk	SMC [1] Estimation	Main Results
Ebenau et al. [49], 2021 Cross-Sectional	N = 829, The Netherlands All SCD [2] individuals Mean age: 59.6 ± 8.8	PRS [3] including 39 genetic loci related to AD [4]	Amsterdam Instrumental Activities of Daily Living questionnaire	PRS was associated with amyloid positivity (OR: 1.5)
Sathyan et al. [50], 2019 Case-Control	N = 4915, United States 1928 SMC individuals Mean age: 74.8 ± 6.7	PRS including 19 genetic loci related to AD	In-person interview: 2 questions	PRS for AD was not associated with SMC (p = 0.407).
Bell et al. [44], 2023 Longitudinal	N = 1555, United States Male adults Mean age: 37.8 ± 2.5		In-person interview: single question	PRS for AD was not associated with SMC

[1] Subjective memory complaints, [2] subjective cognitive decline, [3] polygenic risk score, [4] Alzheimer's disease.

A study conducted by Ebenau et al. [49] examined the effect of a polygenic risk score (PRS) predicting AD in a population of cognitively normal subjects with SCD from the Amsterdam Dementia Cohort. The specific PRS was calculated based on 39 genetic variants previously shown to be related to AD [51–53], excluding APOE, and was associated with amyloid positivity, according to the ATN classification (OR: 1.5, 95% CI 1.2–2.0), in subjects with SCD. In addition, a higher PRS increased AD risk (HR: 1.7, 95% CI 1.1–2.8), while individuals with a higher PRS had a lower risk of non-AD dementia (HR: 0.5, 95% CI 0.3–0.9). Nevertheless, after adjustment for APOE, the relationship between the PRS and progression to AD was not significant anymore.

Another study conducted by Sathyan et al. [50] examined the polygenic inheritance of SCD in the context of motoric cognitive risk syndrome, a predementia syndrome characterized by the presence of SMC and slow gait. In that case, the existence of SMC in older individuals was not associated with the PRS related to AD, including 19 genetic loci from previous GWAS studies [54]. However, increased neuroticism PRS was associated with SMC (OR: 1.10, p = 0.007). The same PRS for AD was also used in the aforementioned twin study conducted by Bell et al. [44], in which it was not associated with SMC in a statistically significant manner.

4. Discussion

In our systematic review, we sought to summarize existing evidence on AD genetic factors beyond APOE ε4 in regard to SCD. We found that family studies, both FH and twin studies, have produced contradictory results regarding the association of interest. Moreover, studies of specific gene alleles, apart from PSEN1, including KIBRA T, PER2 and APOE ε7 cannot establish a direct association with SCD. Last but not least, the polygenic risk for AD, in the context of PRSs, has not yet been thoroughly studied to provide clear conclusions.

4.1. Family Studies

To begin with, FH of AD was studied using first-degree relatives of patients with AD as a composite risk factor for dementia, as shown previously [55]. FH studies have yielded conflicting results regarding the association of FH of AD and SMC. On the one hand, the longitudinal study of Nicholas et al. [38] as well as the case–control study of Heun et al. [39] showed that SMC were not associated with FH of AD. On the other hand, Tsai et al. [41] as well as Haussman et al. [42] found that SMC were related to FH of AD in all participants and in the CN group, respectively. The most recent multicenter study of Heser et al. [40] showed a strong association between SMC and FH of AD (p = 0.008), as well as increased all-type dementia risk (p = 0.002). In that study, the authors considered a negative participant's answer valid regarding FH only if the respective maternal age or age of death was at least 75 years (or paternal age at least 70 years), resulting in a very high

missing data rating for FH (approximately 60%). Additionally, the mean age of participants was approximately 80 years old; hence, the results may not apply to younger cohorts.

Selection bias is the most important limitation of FH studies, as some participants were excluded due to absence of living relatives [40], while self-selection may have resulted in a healthier sample with fewer memory complaints than the general population [41]. In any case, the generalizability of results is limited. Moreover, SCD estimation in most family studies was made using simple questions, either via in-person or telephone interviews, except for one study [38], in which a structured SMC questionnaire was used. Thus, the SMC assessment raises questions concerning the reliability of the results, as simple questions are likely to be vague and, therefore, hide useful information, leading to an increased risk of misclassification. Last but not least, an important concern regarding FH studies, compared to single genetic variants such as APOE ε4, is the possible contribution of the psychological distress caused by the knowledge of being at increased risk of SCD. There is an ongoing debate about whether disclosing genetic dementia risk information has negative psychological consequences on relatives of patients with AD [56].

All twin studies [43–45] concluded that there was no association between SCD and genetic background. However, questions arise regarding the generalizability of these results in different populations, as the ethnic background of participants was limited, and all studies included community-dwelling populations, while one study included only male individuals [44]. The twin study design itself offers an advantage, allowing researchers to differentiate between the contributions of genetic and environmental factors [43]; thus, the conduction of multi-ethnic, larger twin studies in the future is essential.

4.2. Other Genes beyond APOE ε4

As far as other alleles beyond APOE ε4 are concerned, only PSEN1 280A mutation carriers were cross-sectionally related to SCD [34]. PSEN1 280A mutation, which is recognized as the most common cause of familial early-onset AD with complete penetrance, meaning that eventually all carriers will develop AD until their fifth decade of age, appears to be associated with SCD as well. Prospectively, KIBRA T allele [46], which is involved in cell migration and synaptogenesis, as well as PER2 G carriership [36], which is involved in sleep–wake cycle alterations and neurodegeneration, were related to increased longitudinal cognitive decline in subjects with SCD; however, the number of events was relatively small. Among the rest of the gene variants studied [35,47], neither the APOE ε7 allele nor the PSEN-1 Glu318Gly mutation were directly associated with SCD and longitudinal cognitive decline, even though these were found only in the SMC groups. The 50 genes related to vascular damage [48] were not associated with cognitive decline in subjects with SMC receiving anti-hypertensive treatment, similarly to inflammatory genes studied in the past [57].

The aforementioned studies offered indications rather than conclusions concerning the genetic profile of individuals with SMC, presenting remarkable limitations, such as the generalizability of the findings due to the specific features and characteristics of the population samples and the statistical power being limited by the relatively short sample sizes. Additionally, each study used a different questionnaire to evaluate SMC, enhancing the heterogeneity of participants' answers. A recent study aimed to link questionnaire data from international aging studies and was able to differentiate items that made the greatest contribution to measurement precision, opening new perspectives of developing new self-perceived cognitive functioning questionnaires [58]. In any case, many more genes need to be investigated, such as the bridging integrator 1 (BIN_1), clusterin (CLU), and phosphatidylinositol-binding clathrin assembly protein (PICALM), which have been previously shown to be associated with increased risk for late onset AD [54] and also predict objective verbal memory ($p < 0.05$) [59].

4.3. Polygenic Risk

To date, polygenic risk in subjects with SCD has not been studied in depth. Only in one study [49] higher PRS for AD was associated with increased amyloid positivity as well as progression to AD in a population with SCD (an association which was no longer significant after adjusting for APOE), while the other two studies [44,50] which used a different PRS for AD did not show an association. Another study, not included in our results due to the unavailability of the full text, examined the application of a PRS algorithm (genoSCORE), including over 100.000 genetic variations related to AD, in subjects with SCD and MCI in a London Memory Clinic [60], with promising results, as individuals with SCD who were most likely to decline cognitively towards AD had higher PRS values. Taking the above-mentioned results [44,49,50] into consideration, it appears that a variety of different genes is implicated in SCD, just like AD [61]; thus, the PRS method can be helpful in future studies in revealing the genetic underpinnings of SCD.

4.4. Limitations of Our Study

This systematic review is not without limitations. First of all, data regarding SCD and genetic predisposition for dementia, beyond APOE ε4, are heterogeneous. Study populations were heterogeneous in terms of age, cognitive status (including CN, MCI or dementia-free participants) and ethnicity. Additionally, SCD was not uniformly defined in included studies. SCD assessments were performed based on different questionnaires, which may in turn lead to differences in outcomes. As we mentioned before, efforts have already been made in order to combine existing questionnaires into one reliable and standardized questionnaire concerning self-perceived cognitive functioning [58]. The systematic review strategy as well as adequate blinding of researchers have mitigated selection bias. However, selection bias might anyway exist (might be attributed to several exclusion criteria such as year of publication or language of the text). Nevertheless, the overall outcomes of this review provide a comprehensive overview of existing knowledge.

5. Conclusions

Individuals with SCD appear to be a genetically heterogeneous population. The APOE genotype represents only a small proportion of the genetic propensity for SCD. The contribution of FH of AD to SCD remains controversial, as conducted studies present remarkable limitations in terms of selection bias, SMC assessment and probable psychological effects of FH; thus, many more twin studies should be performed. Apart from the PSEN1 280A mutation, studies concerning other candidate genetic variants such as KIBRA T, PER2 and APOE ε7 offer only indications of a possible association with SCD; therefore, many more genes associated with AD must be investigated in relation to SMC. In the near future, the application of the PRS method, which has already shown promising results, has the potential to contribute towards management planning and might be used in individualized risk profiling for longitudinal cognitive decline in subjects with SCD.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cimb46030129/s1, Table S1: Systematic Review inclusion and exclusion Criteria; Table S2: PRISMA 2020 Systematic Review Checklist; Table S3: PRISMA 2020 checklist for abstracts.

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References

1. Jessen, F.; Amariglio, R.E.; Buckley, R.F.; van der Flier, W.M.; Han, Y.; Molinuevo, J.L.; Rabin, L.; Rentz, D.M.; Rodriguez-Gomez, O.; Saykin, A.J.; et al. The characterisation of subjective cognitive decline. *Lancet Neurol.* **2020**, *19*, 271–278. [CrossRef]
2. Jessen, F.; Amariglio, R.E.; van Boxtel, M.; Breteler, M.; Ceccaldi, M.; Chételat, G.; Dubois, B.; Dufouil, C.; Ellis, K.A.; van der Flier, W.M.; et al. A conceptual framework for research on subjective cognitive decline in preclinical Alzheimer's disease. *Alzheimer's Dement.* **2014**, *10*, 844–852. [CrossRef]
3. Reisberg, B.; Prichep, L.; Mosconi, L.; John, E.R.; Glodzik-Sobanska, L.; Boksay, I.; Monteiro, I.; Torossian, C.; Vedvyas, A.; Ashraf, N.; et al. The pre-mild cognitive impairment, subjective cognitive impairment stage of Alzheimer's disease. *Alzheimer's Dement.* **2008**, *4*, S98–S108. [CrossRef]
4. Choe, Y.M.; Byun, M.S.; Lee, J.H.; Sohn, B.K.; Lee, D.Y.; Kim, J.W. Subjective memory complaint as a useful tool for the early detection of Alzheimer's disease. *Neuropsychiatr. Dis. Treat.* **2018**, *14*, 2451–2460. [CrossRef] [PubMed]
5. Pike, K.E.; Cavuoto, M.G.; Li, L.; Wright, B.J.; Kinsella, G.J. Subjective Cognitive Decline: Level of Risk for Future Dementia and Mild Cognitive Impairment, a Meta-Analysis of Longitudinal Studies. *Neuropsychol. Rev.* **2022**, *32*, 703–735. [CrossRef] [PubMed]
6. Reisberg, B.; Shulman, M.B.; Torossian, C.; Leng, L.; Zhu, W. Outcome over seven years of healthy adults with and without subjective cognitive impairment. *Alzheimer's Dement.* **2010**, *6*, 11–24. [CrossRef] [PubMed]
7. Janssen, O.; Jansen, W.J.; Vos, S.J.B.; Boada, M.; Parnetti, L.; Gabryelewicz, T.; Fladby, T.; Molinuevo, J.L.; Villeneuve, S.; Hort, J.; et al. Characteristics of subjective cognitive decline associated with amyloid positivity. *Alzheimer's Dement.* **2022**, *18*, 1832–1845. [CrossRef] [PubMed]
8. van Harten, A.C.; Visser, P.J.; Pijnenburg, Y.A.; Teunissen, C.E.; Blankenstein, M.A.; Scheltens, P.; van der Flier, W.M. Cerebrospinal fluid Aβ42 is the best predictor of clinical progression in patients with subjective complaints. *Alzheimer's Dement.* **2013**, *9*, 481–487. [CrossRef] [PubMed]
9. Rogne, S.; Vangberg, T.; Eldevik, P.; Wikran, G.; Mathiesen, E.B.; Schirmer, H. Magnetic Resonance Volumetry: Prediction of Subjective Memory Complaints and Mild Cognitive Impairment, and Associations with Genetic and Cardiovascular Risk Factors. *Dement. Geriatr. Cogn. Disord. Extra* **2016**, *6*, 529–540. [CrossRef] [PubMed]
10. Verfaillie, S.C.; Tijms, B.; Versteeg, A.; Benedictus, M.R.; Bouwman, F.H.; Scheltens, P.; Barkhof, F.; Vrenken, H.; van der Flier, W.M. Thinner temporal and parietal cortex is related to incident clinical progression to dementia in patients with subjective cognitive decline. *Alzheimer's Dement.* **2016**, *5*, 43–52. [CrossRef]
11. Song, L.; Chen, J.; Lo, C.Z.; Guo, Q.; ZIB Consortium; Feng, J.; Zhao, X.M. Impaired type I interferon signaling activity implicated in the peripheral blood transcriptome of preclinical Alzheimer's disease. *EBioMedicine* **2022**, *82*, 104175. [CrossRef] [PubMed]
12. Zlatar, Z.Z.; Muniz, M.; Galasko, D.; Salmon, D.P. Subjective Cognitive Decline Correlates with Depression Symptoms and Not with Concurrent Objective Cognition in a Clinic-Based Sample of Older Adults. *J. Gerontol. B Psychol. Sci. Soc. Sci.* **2018**, *73*, 1198–1202. [CrossRef] [PubMed]
13. Quon, R.J.; Mazanec, M.T.; Schmidt, S.S.; Andrew, A.S.; Roth, R.M.; MacKenzie, T.A.; Sajatovic, M.; Spruill, T.; Jobst, B.C. Antiepileptic drug effects on subjective and objective cognition. *Epilepsy Behav.* **2020**, *104*, 106906. [CrossRef]
14. Comijs, H.C.; Deeg, D.J.; Dik, M.G.; Twisk, J.W.; Jonker, C. Memory complaints; the association with psycho-affective and health problems and the role of personality characteristics. A 6-year follow-up study. *J. Affect. Disord.* **2002**, *72*, 157–165. [CrossRef]
15. Mewton, L.; Sachdev, P.; Anderson, T.; Sunderland, M.; Andrews, G. Demographic, clinical, and lifestyle correlates of subjective memory complaints in the Australian population. *Am. J. Geriatr. Psychiatry* **2014**, *22*, 1222–1232. [CrossRef]
16. Numbers, K.; Crawford, J.D.; Kochan, N.A.; Draper, B.; Sachdev, P.S.; Brodaty, H. Participant and informant memory-specific cognitive complaints predict future decline and incident dementia: Findings from the Sydney Memory and Ageing Study. *PLoS ONE* **2020**, *15*, e0232961. [CrossRef]
17. Tandetnik, C.; Farrell, M.T.; Cary, M.S.; Cines, S.; Emrani, S.; Karlawish, J.; Cosentino, S. Ascertaining Subjective Cognitive Decline: A Comparison of Approaches and Evidence for Using an Age-Anchored Reference Group. *J. Alzheimer's Dis.* **2015**, *48*, S43–S55. [CrossRef]
18. Mitchell, A.J.; Beaumont, H.; Ferguson, D.; Yadegarfar, M.; Stubbs, B. Risk of dementia and mild cognitive impairment in older people with subjective memory complaints: Meta-analysis. *Acta Psychiatr. Scand.* **2014**, *130*, 439–451. [CrossRef]
19. Molinuevo, J.L.; Rabin, L.A.; Amariglio, R.; Buckley, R.; Dubois, B.; Ellis, K.A.; Ewers, M.; Hampel, H.; Klöppel, S.; Rami, L.; et al. Implementation of subjective cognitive decline criteria in research studies. *Alzheimer's Dement.* **2017**, *13*, 296–311. [CrossRef] [PubMed]
20. Slot, R.E.R.; Sikkes, S.A.M.; Berkhof, J.; Brodaty, H.; Buckley, R.; Cavedo, E.; Dardiotis, E.; Guillo-Benarous, F.; Hampel, H.; Kochan, N.A.; et al. Subjective cognitive decline and rates of incident Alzheimer's disease and non-Alzheimer's disease dementia. *Alzheimer's Dement.* **2019**, *15*, 465–476. [CrossRef] [PubMed]
21. Zuroff, L.; Wisse, L.E.; Glenn, T.; Xie, S.X.; Nasrallah, I.M.; Habes, M.; Dubroff, J.; de Flores, R.; Xie, L.; Yushkevich, P.; et al. Self- and Partner-Reported Subjective Memory Complaints: Association with Objective Cognitive Impairment and Risk of Decline. *J. Alzheimer's Dis. Rep.* **2022**, *6*, 411–430. [CrossRef]
22. Slot, R.E.R.; Verfaillie, S.C.J.; Overbeek, J.M.; Timmers, T.; Wesselman, L.M.P.; Teunissen, C.E.; Dols, A.; Bouwman, F.H.; Prins, N.D.; Barkhof, F.; et al. Subjective Cognitive Impairment Cohort (SCIENCe): Study design and first results. *Alzheimer's Res. Ther.* **2018**, *10*, 76. [CrossRef]

23. Vlachos, G.S.; Cosentino, S.; Kosmidis, M.H.; Anastasiou, C.A.; Yannakoulia, M.; Dardiotis, E.; Hadjigeorgiou, G.; Sakka, P.; Ntanasi, E.; Scarmeas, N. Prevalence and determinants of subjective cognitive decline in a representative Greek elderly population. *Int. J. Geriatr. Psychiatry* **2019**, *34*, 846–854. [CrossRef]
24. Risacher, S.L.; Kim, S.; Nho, K.; Foroud, T.; Shen, L.; Petersen, R.C.; Jack, C.R.; Beckett, L.A.; Aisen, P.S.; Koeppe, R.A.; et al. Alzheimer's Disease Neuroimaging Initiative (ADNI). APOE effect on Alzheimer's disease biomarkers in older adults with significant memory concern. *Alzheimer's Dement.* **2015**, *11*, 1417–1429. [CrossRef]
25. Stewart, R.; Godin, O.; Crivello, F.; Maillard, P.; Mazoyer, B.; Tzourio, C.; Dufouil, C. Longitudinal neuroimaging correlates of subjective memory impairment: 4-year prospective community study. *Br. J. Psychiatry* **2011**, *198*, 199–205. [CrossRef] [PubMed]
26. Jung, Y.; Viviano, R.P.; Van Rooden, S.; Van Der Grond, J.; Rombouts, S.A.R.B.; Damoiseaux, J.S. White matter hyperintensities and apolipoprotein e affect the association between mean arterial pressure and objective and subjective cognitive functioning in older adults. *J. Alzheimer's Dis.* **2021**, *84*, 1337–1350. [CrossRef] [PubMed]
27. Lee, Y.M.; Ha, J.K.; Park, J.M.; Lee, B.D.; Moon, E.; Chung, Y.I.; Kim, J.H.; Kim, H.J.; Mun, C.W.; Kim, T.H.; et al. Impact of Apolipoprotein E4 Polymorphism on the Gray Matter Volume and the White Matter Integrity in Subjective Memory Impairment without White Matter Hyperintensities: Voxel-Based Morphometry and Tract-Based Spatial Statistics Study under 3-Tesla MRI. *J. Neuroimaging* **2016**, *26*, 144–149. [CrossRef]
28. Deng, S.; Sun, L.; Chen, W.; Liu, X.; Chen, S. Effect of APOEε4 on functional brain network in patients with subjective cognitive decline: A resting state functional MRI study. *Int. J. Gen. Med.* **2021**, *14*, 9761–9771. [CrossRef]
29. Ali, J.I.; Smart, C.M.; Gawryluk, J.R. Subjective Cognitive Decline and APOE ε4: A Systematic Review. *J. Alzheimer's Dis.* **2018**, *65*, 303–320. [CrossRef] [PubMed]
30. Munro, C.E.; Boyle, R.; Chen, X.; Coughlan, G.; Gonzalez, C.; Jutten, R.J.; Martinez, J.; Orlovsky, I.; Robinson, T.; Weizenbaum, E.; et al. Recent contributions to the field of subjective cognitive decline in aging: A literature review. *Alzheimer's Dement.* **2023**, *15*, e12475. [CrossRef]
31. Moreno-Grau, S.; Rodríguez-Gómez, O.; Sanabria, Á.; Pérez-Cordón, A.; Sánchez-Ruiz, D.; Abdelnour, C.; Valero, S.; Hernández, I.; Rosende-Roca, M.; Mauleón, A.; et al. Exploring APOE genotype effects on Alzheimer's disease risk and amyloid β burden in individuals with subjective cognitive decline: The FundacioACE Healthy Brain Initiative (FACEHBI) study baseline results. *Alzheimer's Dement.* **2018**, *14*, 634–643. [CrossRef] [PubMed]
32. Cutler, S.J. Worries about getting Alzheimer's: Who's concerned? *Am. J. Alzheimer's Dis. Other Dement.* **2015**, *30*, 591–598. [CrossRef] [PubMed]
33. van Duijn, C.M.; Clayton, D.; Chandra, V.; Fratiglioni, L.; Graves, A.B.; Heyman, A.; Jorm, A.F.; Kokmen, E.; Kondo, K.; EURODEM Risk Factors Research Group; et al. Familial aggregation of Alzheimer's disease and related disorders: A collaborative re-analysis of case-control studies. *Int. J. Epidemiol.* **1991**, *20*, S13–S20. [CrossRef] [PubMed]
34. Norton, D.J.; Amariglio, R.; Protas, H.; Chen, K.; Aguirre-Acevedo, D.C.; Pulsifer, B.; Castrillon, G.; Tirado, V.; Munoz, C.; Tariot, P.; et al. Subjective memory complaints in preclinical autosomal dominant Alzheimer disease. *Neurology* **2017**, *89*, 1464–1470. [CrossRef] [PubMed]
35. Laws, S.M.; Clarnette, R.M.; Taddei, K.; Martins, G.; Paton, A.; Almeida, O.P.; Förstl, H.; Martins, R.N. Association between the presenilin-1 mutation Glu318Gly and complaints of memory impairment. *Neurobiol. Aging* **2002**, *23*, 55–58. [CrossRef] [PubMed]
36. Bessi, V.; Giacomucci, G.; Mazzeo, S.; Bagnoli, S.; Padiglioni, S.; Balestrini, J.; Tomaiuolo, G.; Piaceri, I.; Carraro, M.; Bracco, L.; et al. PER2 C111G polymorphism, cognitive reserve and cognition in subjective cognitive decline and mild cognitive impairment: A 10-year follow-up study. *Eur. J. Neurol.* **2021**, *28*, 56–65. [CrossRef]
37. Page, M.J.; McKenzie, J.E.; Bossuyt, P.M.; Boutron, I.; Hoffmann, T.C.; Mulrow, C.D.; Shamseer, L.; Tetzlaff, J.M.; Akl, E.A.; Brennan, S.E.; et al. The PRISMA 2020 statement: An updated guideline for reporting systematic reviews. *Int. J. Surg.* **2021**, *88*, 105906. [CrossRef]
38. Nicholas, C.R.; Dowling, N.M.; Racine, A.M.; Clark, L.R.; Berman, S.E.; Koscik, R.L.; Asthana, S.; Hermann, B.; Sager, M.A.; Johnson, S.C. Longitudinal Assessment of Self- and Informant-Subjective Cognitive Complaints in a Sample of Healthy Late-Middle Aged Adults Enriched with a Family History of Alzheimer's Disease. *J. Int. Neuropsychol. Soc.* **2017**, *23*, 617–626. [CrossRef]
39. Heun, R.; Kockler, M.; Ptok, U. Subjective memory complaints of family members of patients with Alzheimer's disease and depression. *Dement. Geriatr. Cogn. Disord.* **2003**, *16*, 78–83. [CrossRef]
40. Heser, K.; Kleineidam, L.; Wagner, M.; Luppa, M.; Löbner, M.; Wiese, B.; Oey, A.; König, H.H.; Brettschneider, C.; van der Leeden, C.; et al. Family History of Dementia in Old-Age Participants with Subjective Memory Complaints Predicts Own Risk for Dementia in a Longitudinal Multi-Center Cohort Study. *J. Alzheimer's Dis.* **2023**, *96*, 579–589. [CrossRef]
41. Tsai, D.H.; Green, R.C.; Benke, K.S.; Silliman, R.A.; Farrer, L.A. Predictors of subjective memory complaint in cognitively normal relatives of patients with Alzheimer's disease. *J. Neuropsychiatry Clin. Neurosci.* **2006**, *18*, 384–388. [CrossRef] [PubMed]
42. Haussmann, R.; Ganske, S.; Gruschwitz, A.; Werner, A.; Osterrath, A.; Lange, J.; Buthut, M.; Donix, K.L.; Linn, J.; Donix, M. Family History of Alzheimer's Disease and Subjective Memory Performance. *Am. J. Alzheimer's Dis. Other Dement.* **2018**, *33*, 458–462. [CrossRef] [PubMed]
43. Selwood, A.E.; Catts, V.S.; Numbers, K.; Lee, T.; Thalamuthu, A.; Wright, M.J.; Sachdev, P.S. The Heritability of Subjective Cognitive Complaints in Older Australian Twins. *J. Alzheimer's Dis.* **2023**, *92*, 1015–1026. [CrossRef] [PubMed]

44. Bell, T.R.; Beck, A.; Gillespie, N.A.; Reynolds, C.A.; Elman, J.A.; Williams, M.E.; Gustavson, D.E.; Lyons, M.J.; Neale, M.C.; Kremen, W.S.; et al. A Traitlike Dimension of Subjective Memory Concern Over 30 Years Among Adult Male Twins. *JAMA Psychiatry* **2023**, *80*, 718–727. [CrossRef] [PubMed]
45. Caracciolo, B.; Gatz, M.; Xu, W.; Pedersen, N.L.; Fratiglioni, L. Differential distribution of subjective and objective cognitive impairment in the population: A nation-wide twin-study. *J. Alzheimer's Dis.* **2012**, *29*, 393–403. [CrossRef] [PubMed]
46. Mazzeo, S.; Bessi, V.; Padiglioni, S.; Bagnoli, S.; Bracco, L.; Sorbi, S.; Nacmias, B. KIBRA T allele influences memory performance and progression of cognitive decline: A 7-year follow-up study in subjective cognitive decline and mild cognitive impairment. *Neurol. Sci.* **2019**, *40*, 1559–1566. [CrossRef] [PubMed]
47. Youn, Y.C.; Lim, Y.K.; Han, S.H.; Giau, V.V.; Lee, M.K.; Park, K.Y.; Kim, S.; Bagyinszky, E.; An, S.S.A.; Kim, H.R. Apolipoprotein ε7 allele in memory complaints: Insights through protein structure prediction. *Clin. Interv. Aging* **2017**, *12*, 1095–1102. [CrossRef] [PubMed]
48. Watfa, G.; Marteau, J.B.; Rossignol, P.; Kearney-Schwartz, A.; Fay, R.; Bracard, S.; Felblinger, J.; Boivin, J.M.; Lacolley, P.; Visvikis-Siest, S.; et al. Association study of gene polymorphisms involved in vascular alterations in elderly hypertensives with subjective memory complaints. *Dement. Geriatr. Cogn. Disord.* **2010**, *30*, 440–448. [CrossRef]
49. Ebenau, J.L.; van der Lee, S.J.; Hulsman, M.; Tesi, N.; Jansen, I.E.; Verberk, I.M.W.; van Leeuwenstijn, M.; Teunissen, C.E.; Barkhof, F.; Prins, N.D.; et al. Risk of dementia in *APOE* ε4 carriers is mitigated by a polygenic risk score. *Alzheimer's Dement.* **2021**, *13*, e12229. [CrossRef]
50. Sathyan, S.; Wang, T.; Ayers, E.; Verghese, J. Genetic basis of motoric cognitive risk syndrome in the Health and Retirement Study. *Neurology* **2019**, *92*, e1427–e1434. [CrossRef]
51. Kunkle, B.W.; Grenier-Boley, B.; Sims, R.; Bis, J.C.; Damotte, V.; Naj, A.C.; Boland, A.; Vronskaya, M.; van der Lee, S.J.; Amlie-Wolf, A.; et al. Genetic meta-analysis of diagnosed Alzheimer's disease identifies new risk loci and implicates Aβ, tau, immunity and lipid processing. *Nat. Genet.* **2019**, *51*, 414–430. [CrossRef]
52. Sims, R.; van der Lee, S.J.; Naj, A.C.; Bellenguez, C.; Badarinarayan, N.; Jakobsdottir, J.; Kunkle, B.W.; Boland, A.; Raybould, R.; Bis, J.C.; et al. Rare coding variants in PLCG2, ABI3, and TREM2 implicate microglial-mediated innate immunity in Alzheimer's disease. *Nat. Genet.* **2017**, *49*, 1373–1384. [CrossRef] [PubMed]
53. de Rojas, I.; Moreno-Grau, S.; Tesi, N.; Grenier-Boley, B.; Andrade, V.; Jansen, I.E.; Pedersen, N.L.; Stringa, N.; Zettergren, A.; Hernández, I.; et al. Common variants in Alzheimer's disease and risk stratification by polygenic risk scores. *J. Nat. Commun.* **2021**, *12*, 3417. [CrossRef] [PubMed]
54. Lambert, J.C.; Ibrahim-Verbaas, C.A.; Harold, D.; Naj, A.C.; Sims, R.; Bellenguez, C.; DeStafano, A.L.; Bis, J.C.; Beecham, G.W.; Grenier-Boley, B.; et al. Meta-analysis of 74,046 individuals identifies 11 new susceptibility loci for Alzheimer's disease. *Nat. Genet.* **2013**, *45*, 1452–1458. [CrossRef] [PubMed]
55. Donix, M.; Small, G.W.; Bookheimer, S.Y. Family history and APOE-4 genetic risk in Alzheimer's disease. *Neuropsychol. Rev.* **2012**, *22*, 298–309. [CrossRef] [PubMed]
56. Haussmann, R.; Donix, M. Disclosing Pleiotropic Effects During Genetic Risk Assessment for Alzheimer Disease. *Ann. Intern. Med.* **2016**, *165*, 673. [CrossRef]
57. Lau, S.; Bates, K.A.; Sohrabi, H.R.; Rodrigues, M.; Martins, G.; Dhaliwal, S.S.; Taddei, K.; Laws, S.M.; Martins, I.J.; Mastaglia, F.L.; et al. Functional effects of genetic polymorphism in inflammatory genes in subjective memory complainers. *Neurobiol. Aging* **2012**, *33*, 1054–1056. [CrossRef] [PubMed]
58. Rabin, L.A.; Sikkes, S.A.M.; Tommet, D.; Jones, R.N.; Crane, P.K.; Elbulok-Charcape, M.M.; Dubbelman, M.A.; Koscik, R.; Amariglio, R.E.; Buckley, R.F.; et al. Linking self-perceived cognitive functioning questionnaires using item response theory: The subjective cognitive decline initiative. *Neuropsychology* **2023**, *37*, 463–499. [CrossRef]
59. Tyndall, A.V.; Longman, R.S.; Sajobi, T.T.; Parboosingh, J.S.; Drogos, L.L.; Davenport, M.H.; Eskes, G.A.; Hogan, D.B.; Hill, M.D.; Poulin, M.J. Genetic Risk, Vascular Function, and Subjective Cognitive Complaints Predict Objective Cognitive Function in Healthy Older Adults: Results from the Brain in Motion Study. *Front. Integr. Neurosci.* **2020**, *14*, 571683. [CrossRef]
60. Sauer, J.; Ballard, C.; Gibson, A.; Daunt, P.; Pither, R. Clinical experience with polygenic risk score in subjects with early cognitive concerns. *Alzheimer's Dement.* **2021**, *17*, e053509. [CrossRef]
61. Baker, E.; Escott-Price, V. Polygenic Risk Scores in Alzheimer's Disease: Current Applications and Future Directions. *Front. Digit. Health* **2020**, *2*, 14. [CrossRef] [PubMed]

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Review

Use of Collagen in Cosmetic Products

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Abstract: Collagen (CLG) belongs to the family of fibrillar proteins and is composed of left-handed α polypeptide chains, which, twisting around themselves and their axis, form a right-handed superhelix. In the chemical structure, it contains mainly proline, hydroxyproline, glycine, and hydroxylysine. It occurs naturally in the dermis in the form of fibers that provide the skin with proper density and elasticity. The review aimed to present the types of collagen protein, factors affecting its structure and its unusual role in the functioning of the human body. Also, an overview of cosmetic products containing collagen or its derivatives, the characteristics of the formulas of these products, and the effects of their use were presented. Throughout the market, there are many cosmetic and cosmeceutical products containing CLG. They are in the form of fillers administered as injections, belonging to the group of the oldest tissue fillers; products administered orally and for topical use, such as creams, gels, serums, or cosmetic masks. Analyzed studies have shown that the use of products with collagen or its peptides improves the general condition of the skin and delays the aging process by reducing the depth of wrinkles, improving hydration (in the case of oral preparations), reducing transepithelial water loss (TEWL), as well as improving skin density and elasticity. In addition, oral application of bioactive CLG peptides has shown a positive effect on the nails, reducing the frequency of their breakage.

Keywords: collagen; skin; cosmetics; cosmeceutical products

1. Introduction

The cosmetics industry is one of the best-developing industries in recent years [1,2]. The cosmetics market offers countless products in various formulas so that everyone can find a cosmetic that suits their needs. Consumer awareness of the composition of cosmetic products is also regularly increasing, to which companies producing cosmetics and cosmeceuticals respond by offering a wide range of active ingredients that act on specific skin properties [1]. The main purpose of the cosmetic is to improve the appearance. The term cosmeceutical describes a cosmetic with a healing effect due to the supposedly higher content of active ingredients. In the eyes of the law, it is subject to the same legal regulations as a cosmetic product [2,3]. One of the most frequently used ingredients in cosmetics is collagen (CLG) [4,5]. It is a structural protein composed of amino acids that create collagen fibers, characterized by exceptional strength and high elasticity [6,7]. This protein is composed of three left-handed α polypeptides that wind around themselves and their axis to form a right-handed superhelix [8]. Its structure varies depending on its functions and place of occurrence. CLG is one of the most important proteins in the human body because it is responsible for maintaining the appropriate structure of tissues and organs and constitutes as much as one-third of the total body protein mass [7]. It occurs, among the main organs in the body that provide appropriate elasticity and strength [8–11]. It is also an essential building block of the skin; without CLG, it would not be able to perform its functions properly. CLG proteins in the dermis are constantly being rebuilt, and

their fibers are particularly resistant to stretching and tearing [12–14]. It owes its success in the cosmetics industry to skin biocompatibility, as it is a natural skin component [5,15]. With age, the production of natural collagen decreases, which is why producers of cosmetics and cosmeceuticals offer products that allow for replenishment of this ingredient both from the inside and outside [5,16]. The cosmetics industry is constantly developing and looking for new, innovative solutions, so sources of obtaining collagen for cosmetic products have become the subject of many studies. Currently, the best tested and used CLG in cosmetics is of marine origin [3,6,16–19]. Among the CLG products used for cosmetic and aesthetic purposes, one can distinguish collagen fillers, which belong to the group of the oldest tissue fillers; products with CLG or its hydrolysates for topical use on the skin; and oral products [3,19,20]. Each of these forms is intended to somehow replenish the loss of this protein in the skin. Taking into account the fact that CLG is a large-molecule protein and is unable to cross the epidermal barrier, its hydrolysates, i.e., smaller peptide fragments with a lower molecular weight, are used in topical products [5,21,22].

This work aims to show the unusual structure of collagen protein, its irreplaceable role in the skin, but also in the entire body, which makes it a very interesting ingredient of cosmetic products with various mechanisms of action. This paper presents the types of cosmetics containing CLG or its derivatives, in what form it occurs, and how it affects the properties of the skin, as well as the effects of its action confirmed by research.

2. Characteristics of Collagen

2.1. Structure and Biosynthesis

Collagens are family of fibrillar proteins that dominate the extracellular matrix of most connective tissues in mammals [23,24]. They are the basic proteins responsible for the structure and biochemical properties of connective tissue, and in the case of skin, they constitute approximately 70% of its dry weight. These are natural polymers constituting 1/3 of the total mass of proteins in the human body, mainly performing building functions [7]. They are characterized by a unique form that varies depending on their function and place of occurrence [23,25]. CLG commonly found in organisms is type I. Its presence, e.g., in bone tissue, provides elasticity and strength. Collagens are a fibrous components of the skin, tendons, ligaments, cartilage, and blood vessel walls. They are also present in bones and teeth and internal organs such as the heart, lungs, and liver [7,23].

In terms of structure, collagens are among the most complex natural polymers [8,12–14,26,27]. More than 20 amino acids may be involved in the construction of these protein molecules. Regardless of the type of CLG, the most abundant amino acids are proline (Pro), hydroxyproline (Pro-OH), glycine (Gly), and hydroxylysine (Lys-OH). The unique structure of CLG proteins consists of three left-handed polypeptide α chains, the so-called procollagen. They are wrapped around each other and have a common axis and thus form a right-handed conformation of a superhelix called tropocollagen [8,12,23].

The superhelix is held together by hydrogen bonds, electrostatic interactions, and van der Waals forces [8]. In addition to three-helical domains, non-helical fragments, i.e., telopeptides, are also involved in the structure of collagens (Figure 1a). They occur at the ends of CLG macromolecules or are built into superhelix structures. The presence of a triple helix and its content ranges from 96% in type I CLG to less than 10% in type XII CLG [8,23]. The fibers of most types of collagen form delicate networks, except types I and II, whose fibers are thick and resistant to stretching. Natural collagen superhelices are resistant to proteases such as pepsin, trypsin, and chymotrypsin [14,23,27,28].

The only enzymes that can degrade these macromolecules are called collagenases; they are extracellular matrix metalloproteinases [29]. Each chain contains about 1000 amino acids. A characteristic feature of the amino acid composition of CLG polypeptides is the equimolar amount of acidic amino acids, i.e., glutamic acid (Glu) and aspartic acid (Asp), and basic amino acids, i.e., lysine (Lys) and arginine (Arg) [29]. The most frequently repeated sequence in the collagen polypeptide chain (Figure 1b) is Gly-X-Y-, of which Gly constitutes the third amino acid residue, and X and Y are the amino acid residues:

Pro (approx. 28%) and Pro-OH (approx. 38%) [8,12,29]. The number of kinks occurring in the collagen structure, resulting from disruptions in the regularly repeating Gly-X-Y sequence, determines its elasticity. Better aggregation possibilities are provided by greater flexibility, e.g., in membrane structures. The unique structure of the triple helix and strong bonds between amino acids make collagen fibers maintain their flexibility and are resistant to stretching. Collagen proteins are encoded by many genes, which is why they are structurally and functionally heterogeneous [29]. Thanks to this, organisms protect themselves against the loss of these important macromolecules. Also, changes occurring during post-translational modification are important in maintaining the heterogeneity of collagen proteins, which are constantly synthesized and degraded in the extracellular space [12]. This means that the vast majority of CLG fibers are made of more than one type of CLG [12,14]. Before CLG appears in the extracellular space, it undergoes some modifications like hydroxylation, glycosylation, and the process of creating triplet procollagen particles. The resulting procollagen is expelled outside the cell. Then, using peptidases, telopeptides are removed and tropocollagen molecules are formed [12,14]. The next stage of CLG biosynthesis is the formation of crosslinks between tropocollagen molecules. Finally, spontaneous association occurs into microfibrils, and then into fibrils and mature collagen fibers. Type I CLG is the most common type, found in the human body [9]. It constitutes 85–90% of the organic content of bone connective tissue, skin, or other organs. It is primarily responsible for the tensile strength of tissues and bone stiffness [13,14]. It can be found in bones, which is a product of the same genes as skin type I CLG. Both of these collagens differ significantly from each other, which is the result of post-translational modifications [8,12]. Many factors can stimulate or inhibit collagen biosynthesis. This occurs at various stages of gene expression. TGF-β (transforming growth factor beta), which is one of the most important stimulating factors, influences the production of type I CLG in fibroblasts as a result of inducing the activity of the promoter of the gene encoding this protein [30]. In addition to TGF-β, the following also have a stimulating effect on the synthesis: FGF (fibroblast growth factor), EGF (epidermal growth factor), and various isoforms of PDGF (platelet-derived growth factor). The opposite effect, i.e., inhibition of CLG biosynthesis, is demonstrated by, among others, interferon α and TNF (tumor necrosis factor) [30].

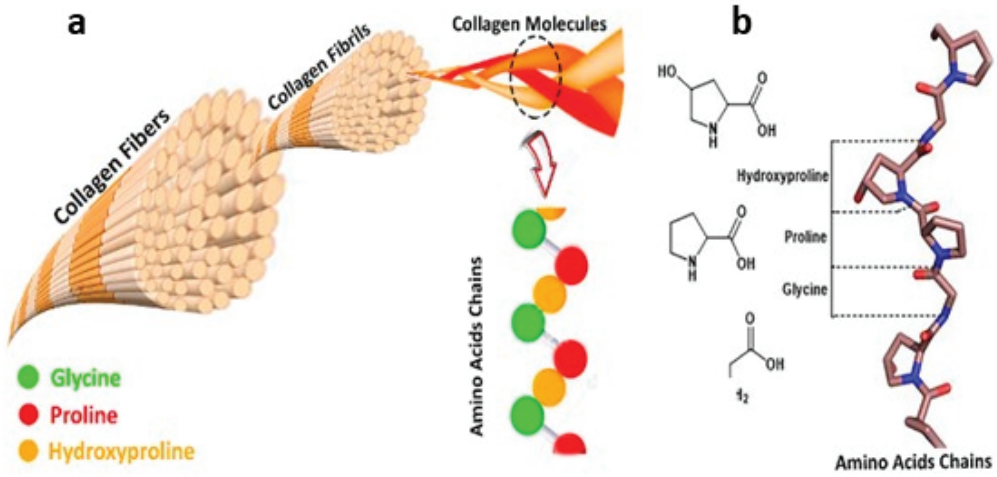


Figure 1. (**a**) Structure of collagen fibers; (**b**) main amino acids in collagen structure (reprinted from [28]).

2.2. Types of Collagen

A characteristic feature of the structure of all collagen proteins is the formation of the so-called rope, i.e., a right-handed superhelix, which is formed by winding three left-handed, single polypeptide chains around its own axis [8]. Each type of CLG has a different initiation stage in the biosynthesis process. Common steps include intracellular reactions

and triple helix formation, post-translational modifications, enzymatic glycosylation and hydroxylation of lysine, proteolytic cleavage of procollagen, and natural crosslinking [8,15]. So far, 30 different forms of collagen, which perform various functions and have different structures (Table 1), have been detected in human tissues [8]. Moreover, they differ in their location and content in the tissues (Table 1).

Table 1. Types of fibrillar collagens and their basic functions and occurrence in the body [23,25,31].

Type	Structure	Localization	Function
I	Triple superhelix composed of two identical α_1 and one α_2 chain; a form consisting of three α_1 chains (homotrimeric) is also found	Bones, tendons, ligaments, skin, cornea	Tensile strength of tissues and bone stiffness
II	Three identical $\alpha_1[III]_3$ chains—homotrimer; similar properties and size to type I, but higher content of hydroxylysine and glucose and galactose residues	The dominant component of vitreous tissue (approx. 80%), corneal epithelium, and cartilage	One of the main components of ECM (extracellular matrix), maintains chondrocyte functions, induces cell adhesion and proliferation
III	Three identical $\alpha_1[III]_3$ chains	Element of the dermis, liver and lung tissues, spleen and blood vessels	Gives elasticity to tissues
V	Three different chains—heterotrimers	Bones, skin, placenta, cornea	Initiation of collagen fibrillogenesis
XI	Three different chains—heterotrimers	Cartilage, intervertebral discs	Initiation of collagen fibrillogenesis
XXIV	The fragment of the Gly-Xaa-Yaa repeated sequence is relatively short, constitutes approximal	Bones, cornea	A marker of osteoblast differentiation and bone formation in mice
XXVII	Forms non-striated, thread-like structures, unlike other members of the subfamily	Cartilage	Deposited mainly in skeletal tissues at the junction of cartilage and bone

The polymorphism of CLG may result from differences in the expression of genes encoding enzymes that are involved in the biosynthesis of these proteins [8,9,12,32]. Another hypothesis is that the diversity of these proteins is caused by changes occurring in post-translational modifications [29]. The description of the subunit composition facilitates the nomenclature based on numbering in Roman numerals (I–XXIX) [23,25,31]. Arabic numerals indicate the polypeptide chains they are composed of (α1–α6). The family of collagen proteins is divided into two main groups: fibrillar collagens and non-fibrillar collagens [12,14,27]. In mammals, they are encoded by 11 genes and were discovered as the first among the CLG protein family. A common feature of this group is a long central triple helix. The fibrillar molecule has a diameter of 1.5 nm and a length of 300 nm. Table 1 presents the group of fibrillar collagens, taking into account the functions, locations, and characteristics of individual types of CLG [12,14,27]. The characteristic transverse striations of CLG fibers visible under an electron microscope are caused by the aggregation of macromolecules. The vast majority of them are heterotypical. This means that they are made of more than one type of collagen (Table 2). This phenomenon is confirmed by the presence of CLG fibers in the bones and cornea, composed mainly of type I and V collagens [12,14,27].

Table 2. Types of non-fibrillar collagens and their location in the body [12,14,27].

Collagen	Type	Localization
Basement membrane	IV	Basement membranes, capillaries
Forming microfibers	XVI	Bones, vessels, skin, cornea, cartilage
	XXVIII	Cells of the nervous system
	XXIX	Skin
Anchoring	VII	Mucosa, skin, bladder, umbilical cord, amnion
Forming hexagonal lattice systems	VIII	Mucosa, skin, bladder, umbilical cord, amnion
	X	Cartilage
FACITs type	IX	Cornea, vitreous body, cartilage
	XII	Cartilage, tendons, skin
	XIV	Vessels, eye, nerves, tendons, bones, skin, cartilage
	XVI	Heart, smooth muscles, skin, kidney
	XIX	Band of basement membranes in skeletal muscles, skin, kidneys, liver, placenta, spleen, prostate
	XX	Corneal epithelium
	XXI	Stomach, kidneys, vessels, heart, placenta, skeletal muscles
	XXII	Tissue connections
	XXVI	Testicles, ovaries
MACITs type	XII	Skeletal muscles, heart, eye, skin, endothelial cells
	XVII	Skin
	XXIII	Metastatic carcinogenic cells
	XXV	Eye, brain, heart, testicles
MULTIPLEXINs type	X	Capillaries, ovaries, heart, testicles, skin, placenta, kidneys
	XVIII	Kidney, lungs, liver

However, the structure of the skin is characterized by the coexistence of types I and III, and in the cartilage, there are combinations of types II, III, IX, and XI. The composition of the fibrillar collagen group constitutes approximately 90% of all CLG proteins found in animal organisms. Fibrillar CLG types are I, II, III, V, XI, XXIV, and XXVI [8,12–14]. Non-fibrillar CLGs do not form typical fibrils. Among this group of collagen proteins, we can distinguish basement membrane, forming microfibers, anchoring, forming hexagonal network systems, the FACITs type, containing transmembrane domains—MACITs and collagens of the MULTIPLEXINs type. The affiliation of individual types of CLG with the above groups and their location in the body [14] are presented in Table 2.

2.3. Physicochemical Characteristic of Collagen

The molecular weight of collagen is approximately 300,000 Da, the diameter is approximately 14–15 Å (Angstrom), and the length is 2800 Å [8]. Collagen has high water-binding capacity, which makes it a good ingredient for texturizing, thickening, and creating gels [8,14]. It has properties related to its behavior on the surface: emulsion formation, foam formation, stabilization, adhesion, cohesion, film-forming properties, and a protective function for the colloid (Figure 2). Additionally, CLG is a good surfactant. It can penetrate lipid-free structures. It has good biocompatibility and low immunogenicity [6]. The proper-

ties of CLG depend on the age of the body. With age, solubility tends to decrease due to the greater crosslinking of collagen in older animals [20].

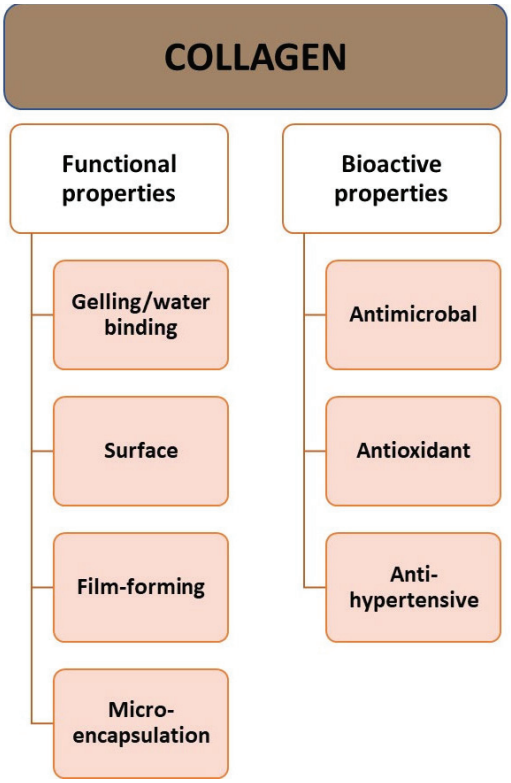


Figure 2. Properties of collagen.

Aging processes lead to crosslinking of the protein structure and affect its mechanical properties [15,20,33]. Mature CLG has a highly crosslinked structure and is usually insoluble in water. Water-soluble and acid-soluble CLG can only be obtained from young tissues. Age-related differences in CLG solubility have been examined based on susceptibility to pepsin digestion. Research has established that the solubility of collagen in acetic acid decreases rapidly as it matures [15]. Like other proteins, it is denatured under the influence of certain factors. If collagen is denatured by heat, the weak bonds (hydrogen, dipole–dipole, ionic bonds, or van der Waals forces) are broken, while the covalent bonds remain intact. The temperature at which thermal denaturation of CLG occurs (the so-called melting point) depends on the water content, pH of the environment, and the degree of crosslinking. The triple helix unwinds, and the chains separate. The denatured mass of tangled chains cools and absorbs all the water from the surroundings. This denatured collagen is called gelatin. Gelatin itself is a mixture of water-soluble proteins, mainly derived from collagen. It usually binds more water than CLG because it is partially degraded and more active groups are exposed to interactions with water through hydrogen bonds. Due to their structural role and compatibility with the body, collagen and gelatin are commonly used biomaterials in medicine, the pharmaceutical and cosmetics industries, with gelatin being a much cheaper material [34,35]. Collagen is a highly crosslinked material, usually insoluble in water and oils, so in the case of cosmetic preparations, it is usually hydrolyzed into smaller peptides [15,20].

2.4. The Role of Collagen in the Body

Collagen proteins perform various functions in the body (Figure 3). The most important of them are maintaining structural integrity, and being responsible for the processes of cell adhesion, differentiation, growth, survival, and regeneration [12,30,36]. CLG is found

in various tissues of the body (Tables 1 and 2), and its structure varies depending on its location and function.

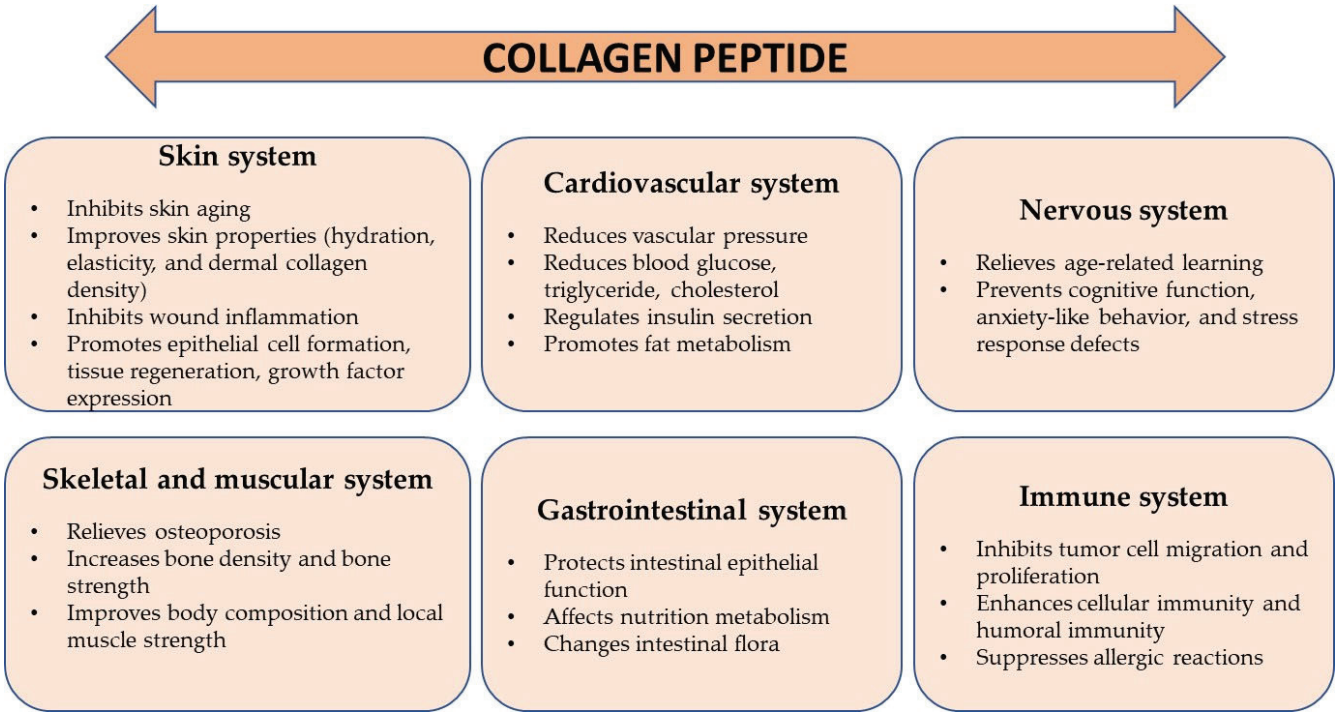


Figure 3. Main role of collagen in the body.

The basic task of collagen is to connect cells, which is why it is a building block of most organs, especially skin, bones, teeth, cartilage, blood vessels, and the cornea of the eye [28]. At the same time, it protects internal organs such as the kidneys, stomach, and liver, creating a flexible scaffolding around them. It also takes part in regenerative processes and ensures proper hydration of the skin thanks to its ability to bind water [23,25,37]. In the immune system, it prevents the entry of pathogenic microorganisms and toxic substances [30]. It ensures the continuity of cell renewal processes in the skin and maintains the appropriate level of hydration, which affects its elasticity, appearance, and condition [33,38,39]. It accelerates wound healing, creates scars, and promotes the reconstruction of connective tissue [40,41]. Collagen increases the absorption of minerals and increases bone density. It stimulates the activity of cartilage cells and supports protective processes within cartilage tissue, providing cartilage with the appropriate shape and resistance to stretching [38]. It is responsible for the production of synovial fluid and the condition of cartilage. Moreover, it reduces the activity of enzymes responsible for causing inflammation and rheumatic pain [30,42]. CLG fibers can also be carriers of some drugs, including interferon. It provides essential amino acids that nourish hair bulbs and ensure their proper growth [34]. Its proteins also play an important role in the functioning of the circulatory system because they are a component of blood vessel walls. The functioning of the circulatory system directly depends on the composition and structure of the vessel walls [6,7]. Collagen, as a fibrillar support protein, interacts with the structures of the extracellular matrix of vessel walls, which gives them appropriate elasticity and mechanical strength. CLG reorganization can impair the effective function of veins and arteries in transporting blood. It also significantly influences the development of circulatory system diseases [23,25]. Collagens are the largest group of proteins that make up the blood vessels of the human body; types I and III constitute approximately 90% of all CLG in the vessels. They are the main supporting proteins of vessels—both healthy and damaged ones. However, among the non-fibrillar collagens that support the functioning of vessels, we can distinguish type VI CLG, which forms microfibers, and collagens that combine with fibrillar CLG—types

XII and XIV [13,26]. In the basement membrane of the inner layer of the vessel, there is also a network of CLG molecules of type IV, anchoring of type VII, and CLG of types XV, XVIII, and XIX, which act as connectors between cells and the basement membrane. Due to the high stiffness of their fibers, their main mechanical role is to limit the expansion of blood vessels under the influence of the pressure of blood flowing through them [25–27]. The number of bonds between collagen molecules affects the quality of the mechanical functions of the vessel walls, as well as the organization of crosslinked collagen fibers within the tissue and their interaction with other matrix components [14,26].

2.5. Effects of Collagen Deficiency in the Body

In a healthy, young body, collagen is regularly replaced—about 3 kg per year—and systematically rebuilt, and over time, the ability to regenerate CLG fibers disappears. The level in the body begins to decrease from the age of 25, its sharp decline occurs after the age of 50, and after the age of 60, this protein ceases to be synthesized by the body. However, the reduction in endogenous collagen biosynthesis is not limited to adulthood [8,14,27]. A decrease (Figure 4) in its concentration in the body can also be observed in young people, which is genetically and hormonally determined. This process is also favored by stress and other external factors, and the CLG biosynthesis process is also disrupted during menopause.

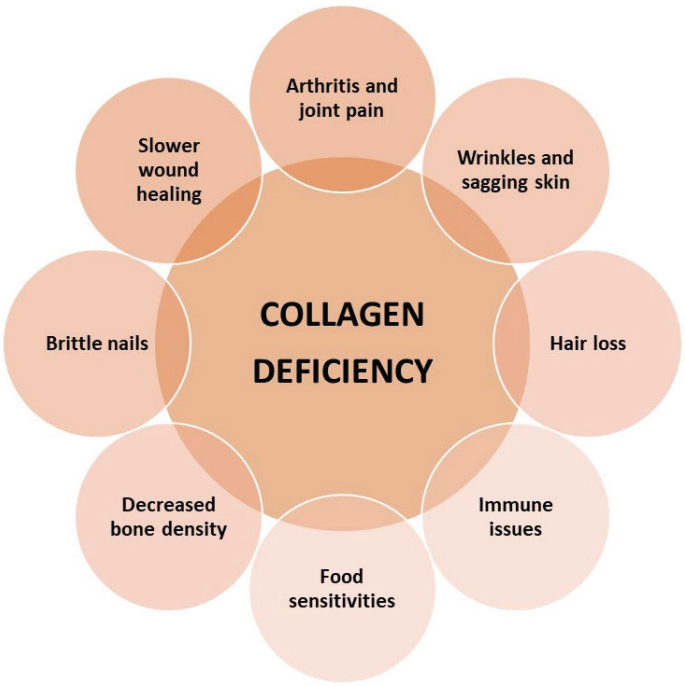


Figure 4. The main effects of collagen deficiency in the body.

Then, the level of estrogens decreases, which affects the synthesis of this protein and changes its properties. CLG fibers become less elastic and thinner [7,16,23,27,36,43]. Changes in the structure can occur under the influence of various external factors, such as mechanical loads, hormonal changes, or diseases [8,32,44,45]. Increased physical activity and practicing extreme sports contribute to the increased destruction of collagen fibers and disturbances in their synthesis. Accelerated fiber breakdown is also caused by excessive exposure to the sun, too high and low temperatures, and some compounds contained in cosmetics [10,46]. These changes may result (Figure 4) in problems with movement, spine and joint pain. With age, the concentration of vitamins A, C, and E, and minerals (copper), which are responsible for the natural renewal of collagen, decreases. Disturbances in the synthesis of CLG protein and its transformation in bone tissue may cause bone decalcification, brittleness, and susceptibility to fractures. The most noticeable symptom is

the loss of skin firmness and general deterioration of its condition. Hair becomes brittle and falls out excessively, which may lead to premature baldness [17,37,47,48].

3. The Importance of Collagen in the Skin

The main role in the structure of the dermis is played by collagen fibers; they constitute 80% of the connective tissue, 72% of the dry matter of the skin, and 30% of all proteins that make up the body. In addition, they can be found in the underlying connective tissue, cartilage, tendons, and muscles. They exist as network structures by crossing and intertwining with each other (Figure 5) [45]. Despite their slight extensibility, they are characterized by significant tearing strength. CLG fibers cut the gel of the proteoglycan matrix and thus provide it with tensile strength. They are constantly being rebuilt—new CLG is created in place of damaged, old protein [33,49]. However, it can be destroyed, e.g., by UV rays (as a result of photocrosslinking) [46,50], sugar molecules (as a result of glycation) [44,51,52], and free radicals (released as a result of diseases, smoking, stress, or practicing high-level sports) [33,43,49,53,54]. There is a natural mechanism in the skin that regulates the production of collagen.

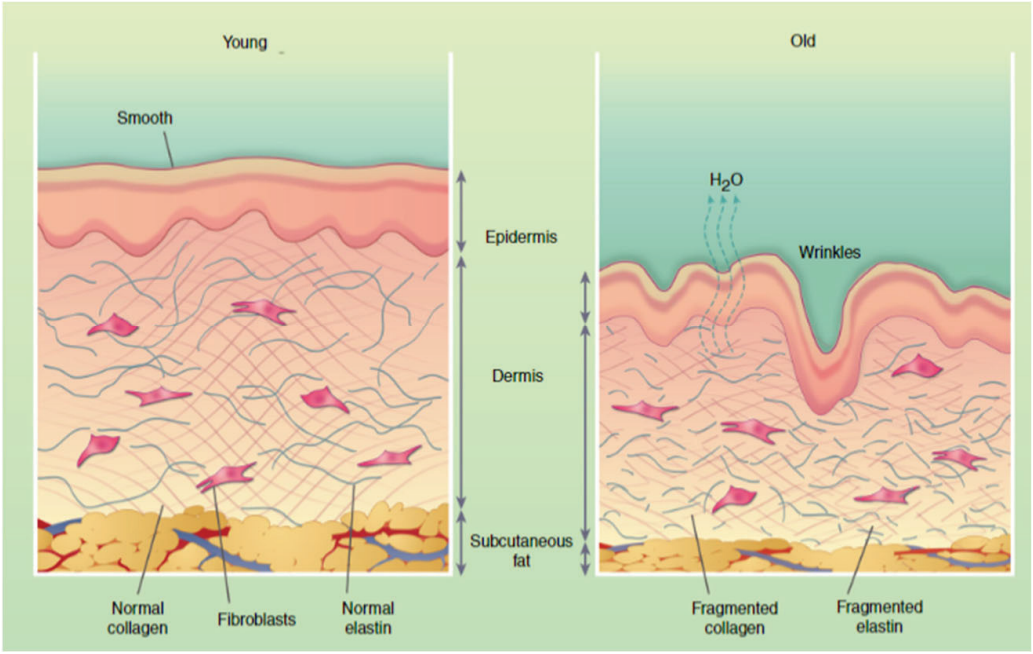


Figure 5. Comparison young and aged skin (reprinted with permeation from [45]).

Enzymes—metalloproteinases—play an important role in this process, e.g., a collagen molecule damaged by UV rays is cut into pieces by MMP 1 (matrix metalloproteinase I) [11,55]. Such fragments created from cut peptides (e.g., tripeptides) become feedback for fibroblasts, which will be able to produce new, properly constructed collagen. Cosmetic procedures are largely based on this knowledge, the mechanism of which is to destroy old CLG fibers, then produce signal peptides from them, and then stimulate fibroblasts to produce new CLG [53]. Between the epidermis and the dermis, there is a basement membrane, which is a thin layer of intercellular substance on which epithelial cells rest. Cells are connected to the membrane through hemidesmosomes and focal attachments [43,53,54]. The epidermis is placed on the basement membrane to provide proper support and proper proliferation of its cells. This also allows for it to adhere properly to the dermis. Moreover, the basement membrane participates in the exchange of nutrients between blood vessels and avascular epithelium and in the differentiation of epithelial cells [45]. The basement membrane consists of i. lamina lucidum, which lies below the epithelial cells; it contains characteristic glycoproteins such as fibronectin, laminin, and entactin, which ensure adhesion, i.e., adhesion of the basement membrane to epithelial cells and connective tissue

elements; ii. lamina densa, made of type IV collagen, which creates a multilayer network that scaffolds the basement membrane; iv. reticular lamina, composed of reticulin fibers consisting of type III collagen [13,27,45]. The building block of anchoring fibers that are involved in attaching the basement membrane to the connective tissue substrate is type VII collagen. The lamina lucidum and the lamina densa form the basal lamina, which, apart from being present in the epithelia, also covers fat and muscle cells. Collagen proteins are characterized by high resistance to physical, chemical, and, above all, mechanical factors [34,36,47,56]. Thanks to this, CLG can bond structural elements at the cellular level. By bonding individual tissue elements together, it ensures their complete integrity. It also fulfills this function at higher levels of cellular organization, e.g., in the skin—it ensures the integrity of the dermis, consisting of connective tissue, with the epidermis, which is an epithelial tissue [45]. Because CLG fibers form an ordered spatial network, other elements of the extracellular matrix can attach to them via receptors. When a mechanical stimulus is activated, the network deforms and then returns to its initial state when the factor ceases to be active [45]. Thanks to this ability to deform the collagen network, the elements attached to the fibers or located close to them will not be damaged under pressure and can continue to perform their functions properly [23]. Another important feature of CLG is the ability to bind water. For protein fibers to retain their properties and structure, adequate hydration is necessary [56]. The skin is then appropriately tense and elastic and can act as a protective barrier and be resistant to mechanical factors [23]. Since most biochemical reactions take place in an aqueous environment, high water content is essential for the proper functioning of the epidermis and dermis. In dry skin, a decrease in metabolism is observed. Research suggests that collagen molecules also play a role in the process of cell proliferation, thanks to their ability to bind cytokines, and the stimulation of cell division in the skin is extremely important, especially in the process of regeneration of skin defects [30,37,48]. According to research, amino acids derived from the breakdown of fish collagen (glycine and hydroxyproline) [3,19], which was applied to the skin, have a possible impact on the amount of cytokines produced by fibroblasts and keratinocytes. Transdermal fish CLG stimulates the synthesis and secretion of the following cytokines: FGF and TGF [6]. TGF-β in skin fibroblasts is an important regulator of CLG homeostasis, stimulates procollagens I and III, and also reduces MMP-1 transcription [17].

3.1. The Influence of External Factors on Collagen Fibers

Collagen constantly changes its structure as a result of external factors (Figure 6) [9]. Collagen structures are damaged by factors such as UV radiation, hormonal changes, mechanical loads, vascular changes, and inflammation [15,35,37].

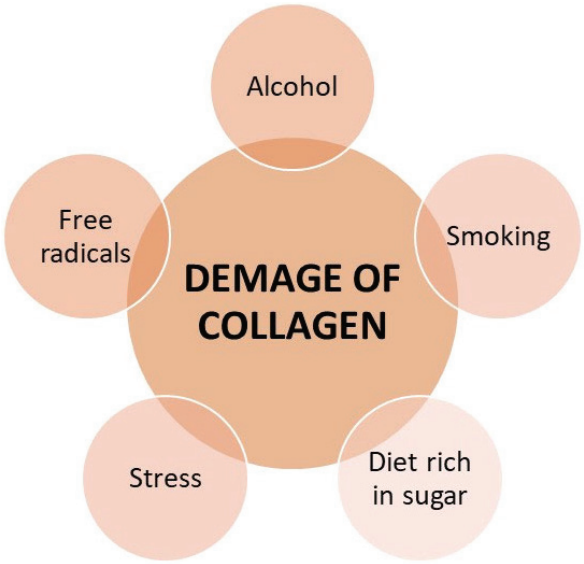


Figure 6. Factors influencing the structure of collagen.

Damaged collagen is cut by collagenases (matrix metalloproteinases) so that it can be replaced by new, properly crosslinked collagen. Among the factors that damage collagen proteins, we can also distinguish free radicals generated under the influence of stress [36], drinking alcohol or smoking cigarettes, and an incorrect diet rich in sugar causing glycation of collagen and low in, e.g., flavonoids, i.e., anti-inflammatory factors. Toxic compounds may be factors that block collagen synthesis, e.g., heavy metals such as mercury, lead or cadmium [8,14,34,36,47,53,54,57].

3.1.1. Collagen Glycation

As a result of the glycation process, the so-called AGE (advanced glycation end-product)—i.e., products of advanced glycation—compounds have the ability to create crosslinks in proteins [44,51,52,58]. Glycation (Figure 7) is a non-enzymatic reaction between reducing sugars and proteins, lipids, or nucleic acids. Enzymatic glycosylation of proteins in the body is a deliberate process and occurs in a specific place, while glycation, i.e., non-enzymatic glycosylation, is spontaneous [44,58]. Its severity depends on the content of simple sugars in the body, e.g., glucose. AGEs, both directly and indirectly, cause the destruction of many cellular structures, including by inducing oxidative stress [59,60].

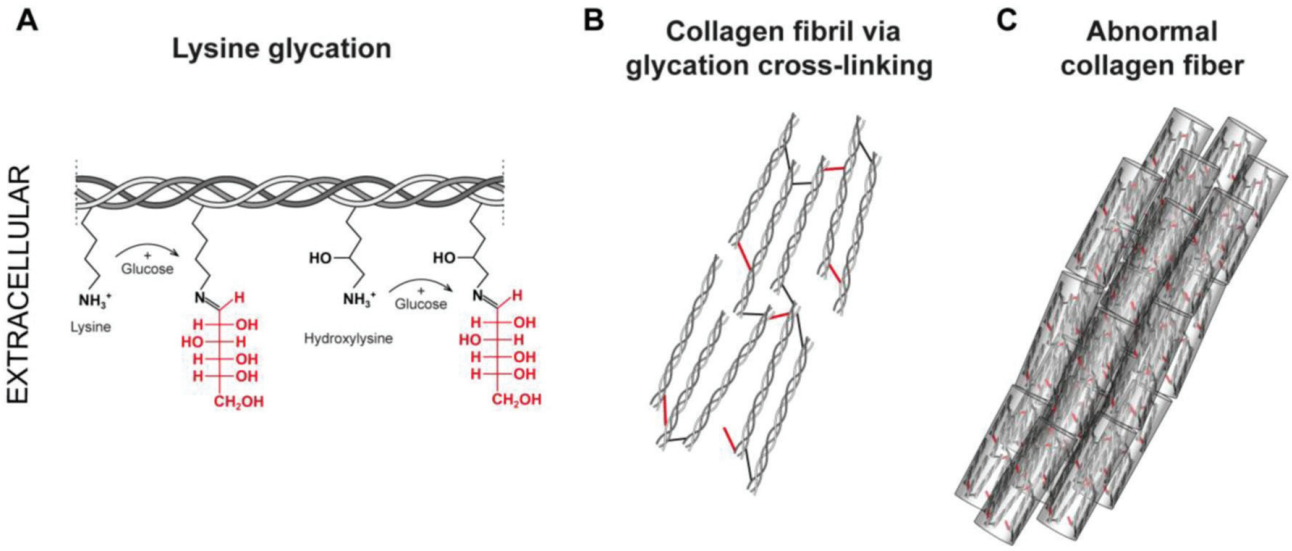


Figure 7. Steps of glycation of collagen fibers, (**A**) Lysine glycation (red font—reduced glucose attached to lysine molecule and forming a Schiff base); (**B**) Collagen fibril via glycation cross-linking (red lines—bonds formed during the glycation process); (**C**) Abnormal collagen fiber (red lines—bonds formed between fibers) (reprinted from [58]).

Protein glycation involves the reaction of reducing sugars (glucose, fructose, glucose-6-phosphate) with the amino groups of proteins and takes place in stages. Initially, the free amino group (mainly arginine or lysine) interacts with the carbonyl groups of sugars [44]. As a result of this action, a Schiff's base is formed, which in the next stage is rearranged to enaminol. [44,51,52]. The next stage, i.e., the transformation of the resulting product into ketolamine, is irreversible. Enaminols and ketolamines are early products of glycation, while final glycation products are formed as a result of the Maillard reaction. As for collagen molecules, asparagine, polyproline, or glutamine take part in the formation of the Schiff's base. They react with the carbonyl group of sugar—usually the aldehyde group of glucose and galactose or the ketone group of fructose [51]. In living organisms, the glycation process occurs slowly, over many weeks, because a small part of glucose exists in a form containing a free aldehyde group (open form), which could react with the amino groups of proteins [52]. Therefore, this process mainly involves proteins with a long half-life, such as collagen. They then become resistant to proteolysis, which accelerates their aging process [36]. As a result of the glycation of type IV collagen, which is responsible for

the structure of the basement membrane, Its turnover process is significantly slowed down. However, glycation of type I collagen and proteoglycan causes stiffening of their fibers and a reduction in their elasticity [31,44]. The AGE formation process is catalyzed by copper and zinc and inhibited by, e.g., ascorbate (reducing agent). Glycation of proteins is promoted by, among others, insulin resistance, hyperglycemia, as well as low levels of antioxidant factors such as vitamin E, vitamin A, and selenium. The final glycation products may not only be of endogenous origin—but their source may also be food products in which AGEs are formed as a result of heat treatment [44,51,52].

3.1.2. The Effect of UV Radiation on Collagen Fibers

Ultraviolet radiation is characterized by high biological activity. Radiation absorbed by a given substance may cause changes in its physicochemical properties, e.g., ionization or heating [46]. Moreover, it may cause the initiation of photochemical reactions such as oxidation, reduction, decomposition, or polymerization [33,36,53,54]. Ultraviolet radiation leads to the thickening of the stratum corneum (SC) as a result of increased cell division in its creative layer [10,46]. The thicker the SC, the more radiation it can absorb. Direct contact of the skin with UV rays does not benefit the tissues, the main structures exposed are proteins [55]. About 50% of UVA radiation reaches the dermis [46,50]. However, before damage occurs, the body activates various defense mechanisms. By stimulating individual membrane receptors, information about the radiation reaching the skin is transmitted to the cell nucleus, where the transcription of various genes is activated. Genes encoding metalloproteinases are activated, which attack collagen, hyaluronic acid, and elastin molecules and form the stroma of the dermis [49]. The c-Jun transcription factor is also activated, which is intended to inhibit CLG synthesis until the cell repairs any damage. The human body can lose up to 20% of normal skin collagen as a result of careless exposure to the sun in one day [11,55].

UVB radiation stimulates the expression of genes responsible for the apoptosis of fibroblasts, melanocytes, and keratinocytes [53]. In tissue culture studies, fibroblasts and keratinocytes collected from tanned skin have a shorter lifespan compared to cells from untanned areas. As a result of radiation, the process of elastosis also takes place, in which amorphous, thickened elastin fibers move toward the upper layers of the dermis, where they replace collagen fibers [46]. UV rays cause photocrosslinking and photodegradation of collagen. As a result of photocrosslinking, abnormal connections are created between fibers, which changes their mechanical properties [11,16,46,50,55]. Free radicals produced under the influence of radiation, which are capable of further reactions, also have a harmful effect (Figure 8) [49]. As a result of irradiation of the skin surface, the energy of UV rays is easily absorbed by cellular chromophores such as NADH2, NADPH, riboflavin, tryptophan, and trans-urocanic acid [49].

The stimulation of chromophores occurs with the participation of molecular oxygen, which results in the formation of oxidation products and oxygen radicals, including the highly reactive hydroxyl radical HO* [10,46]. Free radicals stimulate the synthesis of collagenases that maintain CLG damaged by photocrosslinking [10,50]. As a result of the reaction of free radicals to CLG fibers, tissue stiffness and density increase, and skin pigmentations and wrinkles form [11,50,55]. However, prolonged exposure to UV radiation on the skin may lead to the development of rheumatic diseases, inhibition of wound healing processes, and the development of cancer [46,50]. In studies on the effect of UV radiation on collagen isolated from fish scales, conformational changes in the CLG molecule and water loss were observed [59,60].

Initially, absorbed UV rays cause the loosening of the protein structure and then its degradation. Under the influence of radiation, hydrogen bonds that stabilize the triple helix are broken, which leads to changes in conformation and the transition of collagen molecules from a helical structure to a coil structure. Photodegradation results from the disruption of peptide bonds as well as the formation of new products: phenylalanine and tyrosine [33,60]. Moreover, as a result of radiation exposure, an initial increase and then a

rapid decrease in the optical activity and viscosity of CLG were observed. In the study of mouse skin collagen, crosslinking of type I and IV was observed as a result of radiation with a wavelength of 290–320 nm [11]. Type IV collagen produced 3,4-dihydroxyphenylalanine (DOPA), which is involved in skin aging, while the analysis of the amino acid composition showed that the amount of aromatic amino acids decreases under the influence of UV rays [47]. Taking into account previous research on the photostability of collagen, it can be concluded that UV radiation is harmful not only to the tissues in which protein occurs but also to preparations containing this ingredient, e.g., cosmetic products. To sum up, UV radiation causes a deficit in collagen as a result of shifting its homeostasis from production to degradation [11,46,53,55,59–61].

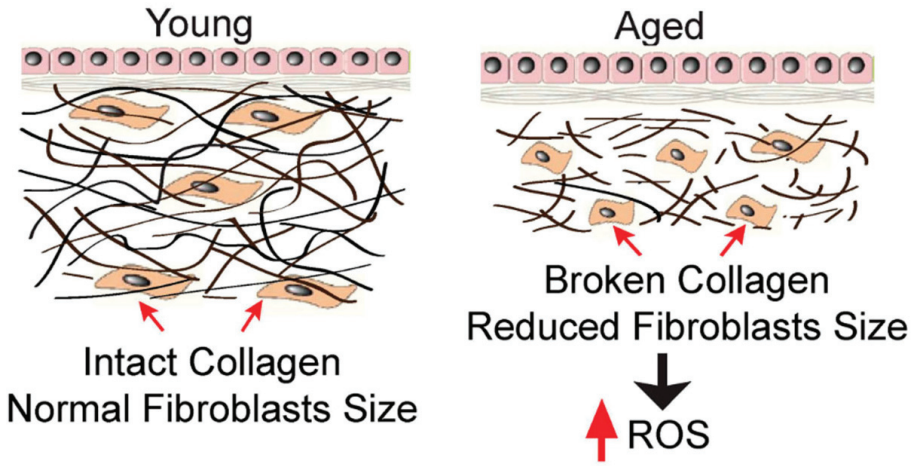


Figure 8. Comparison of fibroblasts and collagen fibrils in young and aged skin (reprinted from [49]). Reduced fibroblasts size stimulates intracellular ROS generation. In the young human skin dermis, intact collagen fibrils interact with cells to maintain normal cell spreading and size (**left**). In contrast, in the aged human skin dermis (**right**), broken collagen fibrils are unable to support normal cell spreading, and this causes reduced cell size. One of the prominent features of the collapsed cells is the increase in intracellular ROS generation.

4. Characteristics of Cosmetic Products Containing Collagen

CLG is obtained from many sources; the main one is beef, especially from cow bones and skin. Due to the occurrence of cow diseases that threaten human life, such as BSE (bovine spongiform encephalopathy), TSE (transmissible spongiform encephalopathies), FMD (fibromuscular dysplasia), and mad cow disease in particular, scientists are looking for an alternative, safer source of collagen [28,40,62–67]. One of the main disadvantages of bovine CLG is that approximately 3% of the total human population is allergic to it, making it difficult to use. Beef Achilles' tendon is used to obtain type I CLG. Type IV is obtained from the placental villi, while type II is obtained from the nose or articular cartilage. Cattle are used at various stages of their development. The dermis of the fetus is used to strengthen tendons and improve wound healing, while the dermis of newborn cattle is used to treat hernias and is also used in plastic and reconstructive surgery [29,40,41]. The pericardium of adult cattle has been used in the treatment of hernias and muscle strengthening [29]. Porcine collagen is widely used for industrial purposes; pig bones and skin are used. Pork CLG is very similar to that of humans, but it does not cause as many allergic reactions. As with bovine CLG, there is a risk of zoonotic diseases. The dermis of adult pigs and the mucosa of the small intestine are used to strengthen tendons, treat hernias, support wound healing, and in plastic and reconstructive surgery [12,13,29]. Currently, the safest source of collagen is considered to be marine [3,6,18,19,28,67].

Extracting animal collagen is complex, expensive, and time consuming. Due to concerns about the occurrence of defense reactions and the occurrence of diseases among land animals, marine sources began to be investigated (Figure 9) [62]. Marine CLG has many advantages over that received from land animals: mainly, lack of diseases, environmental

friendliness, better absorption due to lower body temperature (than terrestrial animals), greater absorption due to low molecular weight, minimal presence of biological contaminants and toxic substances (practically negligible), lower immunogenicity, and metabolic compatibility [29,62,63].

Marine sources include the use of fish, starfish, jellyfish, sponges, sea urchins, octopus, squid, cuttlefish, anemones, and shrimp [6,18]. Freshwater and saltwater fish are used to obtain fish collagen, specifically their skin, bones, fins, and scales. These fish parts are considered waste during fish processing, so using them to obtain CLG reduces environmental pollution [66–68]. Type I CLG is obtained mainly from the skins of Atlantic cod, silver carp, Japanese sea bass, chub mackerel, and sole and from the bones of carp, yellow sea bream, and Japanese sea bass [29]. Other animal sources include chicken, kangaroo tails, rat tail tendons, duck legs, horse tendons, alligator skin and bones, sheep skin, frog skin, and sometimes even human skin [62]. Recombinant human collagen is used due to its lower immunogenicity compared to other sources [63,69]. The pericardium of adult horses is used to strengthen tendons, treat and heal wounds, and treat hernias. Collagen types I and II are isolated, among others, from horse skins and their joint cartilage. Types I, II, III, and V are obtained from chicken necks, with type I being dominant [70]. Chicken feet are also a rich source of CLG. Type I is also obtained from the oviducts of bullfrogs [29].

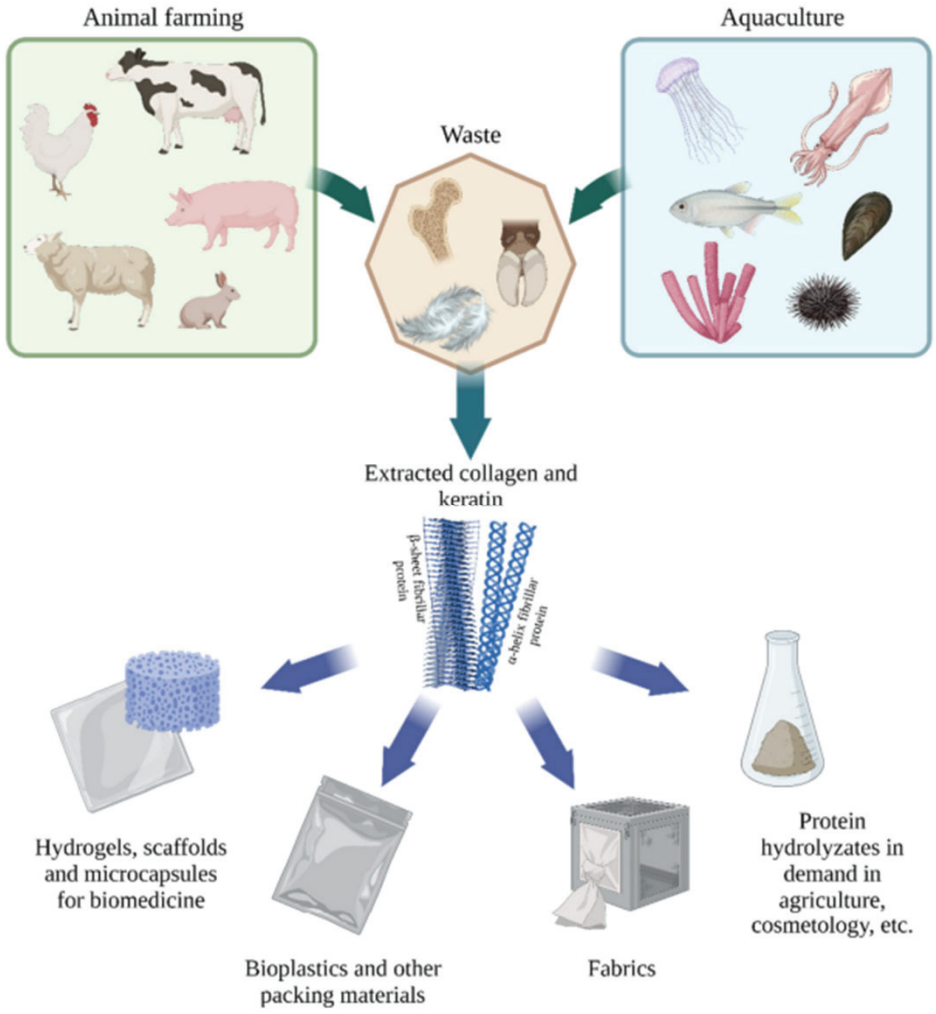


Figure 9. Sources and application of collagen (Reprinted from [62]).

4.1. Sources of Collagen Used in Cosmetics

Since collagen acts as a natural humectant and has moisturizing properties, it is one of the basic ingredients of many cosmetic products [69,71,72]. The cosmetics industry is

constantly developing and looking for innovative, effective products, which makes collagen sources the subject of many studies. CLG used in cosmetics comes from many sources: from bovine through pork to marine [1,4,73]. However, as previously mentioned, pork and bovine collagen is associated with the risk of zoonotic diseases [15,19,20].

The research contributed to understanding the structural and physicochemical features of CLG of marine origin, which constitutes a significant disjunction with animal sources. CLG derived from jellyfish, more specifically from the species *R. pulmo*, has similar biological activity to human collagen type I [6,7,18,19]. Studies have shown that certain human receptors can identify collagen derived from jellyfish. It follows that there is a similar response regarding cell adhesion, proliferation, and migration to jellyfish CLG [15]. The protein amount in jellyfish is approximately 60%. Test results showed that the amino acid level was similar to that of vertebrate CLG, but much lower hydroxyproline content was observed. This indicates relatively low denaturation temperatures, ranging from 26 to 29.9 °C [15,20]. Another source of CLG for cosmetic purposes is cuttlefish skin [28]. The Sepia lycidas species contains between 2 and 35% of collagen in freeze-dried dry matter. However, Argentine squid skin contains approximately 35.6% CLG. Among marine sources, fish are the most valuable source of CLG. The main reason is that 75% of a fish's weight is collagen. Fish skin is mainly used to obtain type I CLG [28]. Type II is found in cartilage. According to research, the highest collagen content was observed in silver carp, brown toads, cod, and tilapia [4,15,18,28,66–68].

As mentioned, marine collagen, depending on its source, can be divided into two categories—isolated from invertebrate or vertebrate animals [28,61,65,67,68,74]. Type IV collagen received from sponges is used in cosmetics for dry skin and type I CLG from salmon skin has moisturizing properties in cosmetics. For the extracted peptide to be suitable for use in cosmetics, it must be properly isolated and purified. Due to the presence of strong crosslinks in the triple helix structure of CLG, it is poorly soluble in cold water. Although solubility is improved by heating, chemical treatment of the animal tissue is necessary, so dilute acids and bases are used to break the links. The acid most often used to extract CLG from tissues is dilute acetic acid, as well as lactic and citric acid [6]. As a result of research on acid extraction of CLG, it was observed that CLG isolated from salmon skin was more soluble than that isolated from cod skin [3]. However, CLG also has strong intermolecular covalent bonds in the telopeptide regions, which cannot be cleaved by acetic acid alone—in this case, enzymes are also used, e.g., pepsin, trypsin, papain, collagenase [6]. Thanks to their use, the solubility of collagen and the efficiency of its extraction increase. The most commonly used enzyme is pepsin [6,65]. Many cosmetic preparations contain CLG (Figure 10) as a main component due to its benefits of being a natural humectant and having moisturizing properties. Preparations based on marine collagen are promising alternatives due to the continuous development and search for innovative and effective products. They will differ in composition and properties depending on their origin [1,56,57,65,67,74]. High molecular weight proteins such as CLG are unable to be absorbed by the stratum corneum, so they remain on the surface, preventing water loss—they maintain proper hydration of the skin and protect against microorganisms in the case of injured tissue.

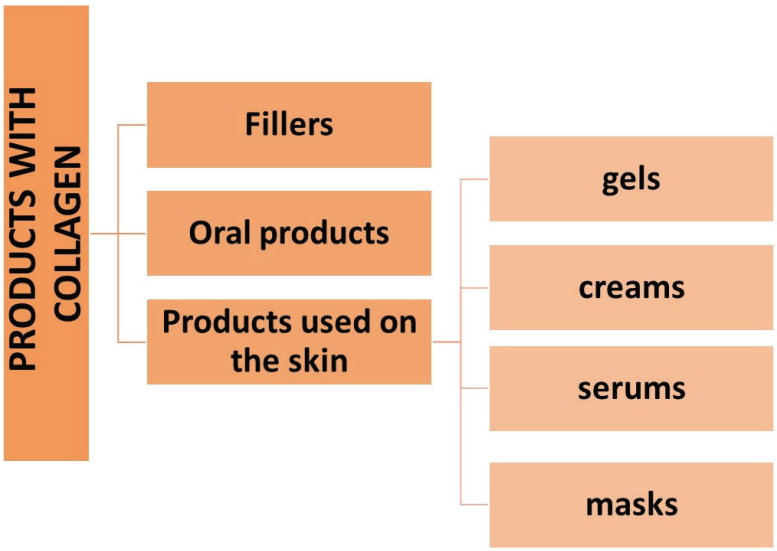


Figure 10. Types of products with collagen.

4.2. Collagen Fillers

The use of collagen fillers is an increasingly developing method in minimally invasive aesthetic medicine and involves leveling the skin surface, i.e., "smoothing" wrinkles and places with, for example, atrophic scars [75,76]. They are also used to shape lips or face contours. Fillers are substances that can fill soft tissue defects permanently or temporarily and are injected intradermally [4,16,72]. We can distinguish synthetic and natural fillers, which are obtained from animal tissues (xenogeneic), human tissues (allogeneic), and our tissues (autogenic) [15,16,20,75,76]. Collagen fillers belong to the group of the oldest tissue fillers; they are divided into non-absorbable (permanent), and absorbable (temporary). Their feature is full biodegradability; they are also relatively cheap and easy to produce, non-carcinogenic, non-teratogenic, and non-toxic, and provide repeatable results, which is why they are gaining popularity [15,16]. Their significant disadvantage is the occurrence of side effects of varying intensity, depending on the preparation used, such as erythema, swelling, itching, infections, or discoloration [72,75]. As a result of the search for collagen fillers with lower immunogenicity, preparations such as Cosmoderm® and Cosmoplast® (INAMED Aesthetics, Santa Barbara, CA, USA) were introduced to the market. Both of these products are obtained by culturing human fibroblasts and contain mainly type I collagen (as much as 93%) and type III CLG (7%), as well as lidocaine [72,77]. They belong to allogeneic fillers and are obtained from the tissues of deceased people after they have been previously tested for the presence of teratogenic and infectious factors. Cosmoderm® specializes in the correction of more superficial wrinkles, while Cosmoplast® is used for deeper wrinkles, the nose, and lifting the corners of the mouth, as well as for correcting the contours of the lips [76]. When using these preparations, allergy tests are not required, and the effects last from 3 to 7 months (Cosmoderm® 3–4 months, Cosmoplast® 3–7 months) [72]. One other collagen preparation is Isolagen® (FibroCell Science, Exton, PA, USA), which is obtained from the patient's skin to create a suspension of live fibroblast cultures and extracellular matrix [75]. The effect of this preparation appears with a delay because it is related to stimulating the remodeling of existing tissues and the synthesis of new components of the extracellular matrix. After the period necessary to perform cell culture (about 6 weeks), it is administered by injection, and it is recommended to perform an allergy test beforehand [72]. A representative of the xenogeneic filler is Artefill® (Artes Medica, San Diego, CA, USA) [75], containing bovine collagen, which is also the filler with the longest-lasting effect. Bovine CLG biodegrades and is replaced by the body's collagen after 1–3 months. Collagen fillers are also used to fill the lips, and the collagen concentration in such preparations is 3.5–6.5% [15]. To sum up, xenogeneic fillers have a wide range

of applications in medicine, e.g., in surgical sutures, but the factor limiting their use is immunogenicity [75,76]. However, autogenous and allogeneic fillers are characterized by high therapy costs, and if the products are not thoroughly cleaned, there is a risk of transmitting pathogenic prions or viruses to the patient [72].

4.3. Oral Products with Collagen and Its Hydrolysates and Their Effects

A typical CLG hydrolysate consists of peptides of various lengths depending on the source and is characterized by a special amino acid composition [17]. So far, oral preparations with collagen or its hydrolysates were mainly intended for people struggling with connective tissue diseases, mainly cartilage and joint changes [38]. However, there are more and more dietary supplements with collagen appearing on the market, the effect of which is to improve the appearance of the skin and delay its aging. Such preparations with aesthetic indications have been popular for a long time, e.g., in Japan, where they constitute a significant category of nutraceuticals, while in Europe they appeared later [72]. Hydrolyzed CLG works in the dermis [33] in two ways (Figure 11): first, free amino acids provide the building blocks for the formation of collagen and elastin fibers, and second, collagen oligopeptides act as ligands—they bind to receptors on the fibroblast membrane and stimulate the production of new CLG, elastin, and hyaluronic acid [33,63]. In the literature, there are descriptions of studies on the impact of oral preparations with CLG on the body [38,42,78] in which an increase in collagen synthesis by fibroblasts (thanks to the amino acids contained in CLG) increased proliferation of skin cells and increased CLG synthesis due to the presence of hydroxyproline. Taking collagen hydrolysates resulted in an increase in skin hydration [22,72].

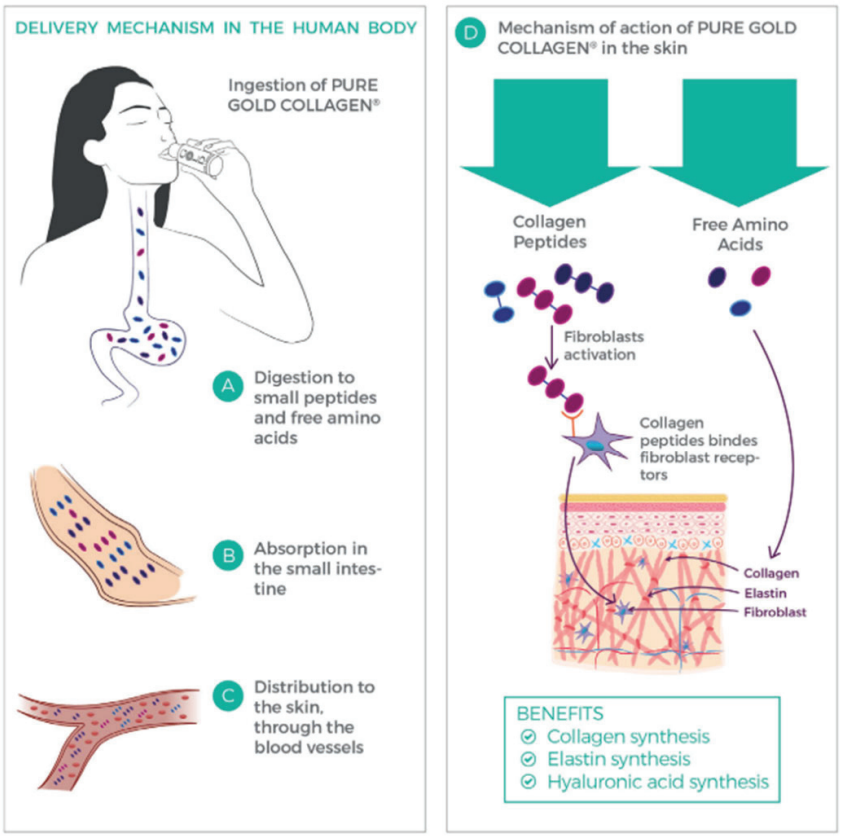


Figure 11. Delivery mechanism of collagen in human body (**A**–**C**); effect of action of collagen in the body (**D**) (reprinted from [33]).

Numerous studies have shown that as a result of consuming hydrolyzed collagen, peptides are produced in the bloodstream (after oral consumption, CLG hydrolysate is degraded to dipeptides and tripeptides [15]). After this, they have chemotactic properties for skin fibroblasts, supporting their reconstruction processes [20]. Other studies have shown that oral consumption of a preparation containing bioactive CLG peptide (BCP) by a group of women aged 45–65 years resulted in a significant reduction in, among others, wrinkle depth—a difference of 7.2% compared to the placebo group after eight weeks of use, this difference increased to 20.1%, and four weeks after taking the last dose, the wrinkle depth reduction effect remained at the level of 11.5% [79]. After eight weeks of BCP use, the content of tropocollagen type I was as much as 65% higher than in the placebo group, which could have contributed to greater synthesis of natural CLG and, consequently, to increased skin elasticity and better hydration [15]. In the case of hydrolyzed fish collagen, which has lower stability compared to animal one due to a smaller amount of hydroxyproline and a larger amount of amino acids such as serine, methionine, and threonine, liposomes have been used to increase the bioactivity of CLG [20]. Studies conducted on rats have shown that daily supplementation with bovine CLG hydrolysates has a positive effect on extracellular matrix proteins—the amount of type I and IV CLG in skin samples increased [78], while number of enzymes responsible for its degradation decreased, including type II collagenase. Based on such studies, it can be concluded that supplementation with collagen hydrolysates may delay the formation of changes in the extracellular matrix that occur with age, and the mitigating effect of these preparations on oxidative stress has also been proven [72,77,80]. When supplementing with oral products with CLG, it is worth ensuring the appropriate level of vitamin C, because it plays an important role in the synthesis of collagen fibers [33]. There are a lot of websites that recommend dietary supplements in sachets with CLG. Usually, they contain 5000 mg of collagen with a molecular weight of 3000 Da in one sachet, which, according to the manufacturer's description, is characterized by high absorption and provides protection against oxidative stress [79]. The CLG in the preparation is of marine origin, more specifically from cod fish, and is intended to restore the skin's density, elasticity, and hydration. According to the opinions of customers who used such products regularly for a month, the level of skin hydration and its density improves [33,38].

As previously mentioned, oral supplementation with collagen preparations is currently very popular. Table 3 summarizes information presented in various publications about research on these effects.

An example of a study prepared to be conducted on the effect of collagen supplementation on skin hydration, elasticity, smoothness, and density was proposed by Bolke and co-workers [17]. The study aimed to investigate the effect of the drinking nutraceutical ELASTEN® (QUIRIS Healthcare, Gütersloh, Germany) on aging and skin health. The ampoules were supposed to provide a mixture of 2.5 g of CLG peptides, acerola extract, zinc, biotin, vitamin C, and a native vitamin E complex. The study was conducted on a group of 72 healthy women aged 35 and over who took a supplement or a placebo for 12 weeks [17]. Then, the skin properties were assessed using a corneometer (assessment of skin hydration), a cutometer (assessment of skin elasticity), a silicone skin replica with in vivo optical measurements with a 3D phase shift (assessment of skin roughness), and a skin ultrasound (assessment of skin density) [17]. The tested ELASTEN® collagen complex contained short-chain oligopeptides containing between 5 and 26 amino acids, which largely overlap with the amino acid sequences found in collagen proteins of human skin. They are obtained as a result of the enzymatic hydrolysis of natural CLG, and after consumption, they are further metabolized in the digestive tract to bioactive di- and tripeptides. Then, they reach the bloodstream and the skin, where they accumulate to form a CLG biomatrix [17].

Table 3. The impact of collagen supplementation on individual systems and organs of the human body [17,38,47,63,81,82].

Place of Action	Effect of Supplementation
Immune system	Inhibition of inflammatory processes resulting from autoimmunity.
Hormone system	Amino acids included in collagen may be involved in the production of hormones and neurotransmitters, e.g., GABA.
Cartilage and skeletal system	Increased production of collagen in cartilage—improved resistance to mechanical loads, reduction in joint pain and joint inflammation, stimulation of the production of synovial fluid.
Skin	Bioactive short peptides (dipeptide glycine—hydroxyproline) send signals to fibroblasts, stimulating the collagen production process, reducing wrinkles, improving the smoothness, tension, and elasticity of the skin, reducing cellulite and stretch marks by improving the elasticity and cohesion of the skin.
Hair, nails	Preventing graying and hair loss and brittle nails.
Liver	Assistance in the removal of toxic substances and alcohol metabolites.
Muscle	Increase in muscle mass, increased energy efficiency of the body.
Circulatory system	Amino acids contained in collagen, such as proline and lysine, prevent the accumulation of fats in the walls of arteries—protection of the circulatory system.
Digestive system	The amino acids proline and glycine seal the walls of the small intestine; collagen helps absorb water in the intestines.

Studies have shown (Figure 12) that age-dependent reductions in CLG synthesis can be reversed by oral administration of specific bioactive CLG peptides. Objective dermatological measurements such as corneometry and cutometry confirmed that oral use of collagen peptides along with other ingredients significantly improves skin hydration, density, elasticity, and roughness after three months of use [17]. In addition, study participants, in their subjective assessments, concluded that the appearance of their skin had improved significantly. The CLG supplement did not cause any side effects, was well tolerated, and was safe to use. Since CLG peptides were taken orally, the effects reached deeper layers of the skin, permanently improving its physiology and appearance [17,33]. The subject of the next study was to compare the consequences of using oral collagen with the use of topical collagen preparations in reducing or delaying skin aging. Evidence from peer-reviewed studies suggests that both oral and topical CLG preparations improve skin hydration and elasticity, and skin hydration improves when administered orally. Additionally, CLG has been proven to reduce wrinkles and skin roughness, and existing research has not shown any side effects from the use of CLG supplements [38,42,83]. Among the publications was a study conducted in Japan in which collagen peptides were administered orally to patients with aging, wrinkled skin [83]. The study involved women over 40 years of age who used 10 g of collagen for 56 consecutive days or a placebo. The authors observed a statistical difference in skin hydration during the entire experiment compared to the placebo group. A device using bioelectrical impedance analysis was used to test skin hydration—the skin hydration level is determined based on the time needed for the current to flow through the skin [83]. The same study enrolled French women over 40 years of age and followed a collagen treatment protocol for three consecutive months [83]. At the end of the study, the authors found a noticeable increase in hydration in the collagen group compared to the placebo group.

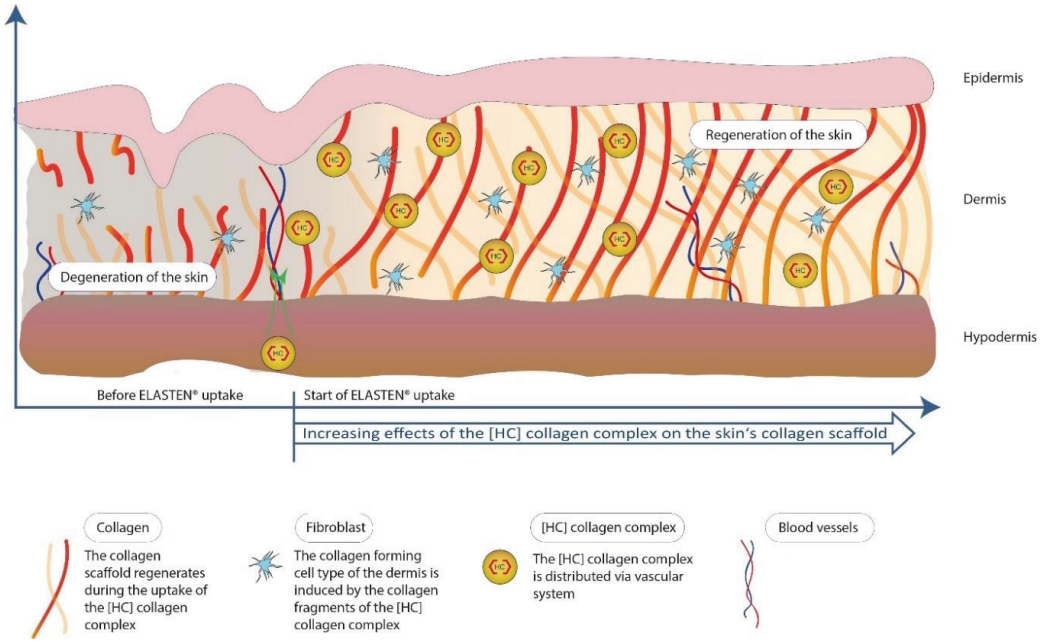


Figure 12. Effects of the use of ELASTEN® product (reprinted from [17]).

In another study, women were divided into four groups differing in the dose of collagen they took [81]. Participants were followed up for 60 days, and there was a statistical increase in skin elasticity according to the administered doses compared to the placebo group. Moreover, improvement in skin elasticity among older women was noticed relatively earlier—after just one month [5]. Brittle nail syndrome is a common problem among women, and is characterized by a rough, flaky, jagged surface of the nail. The aim of the study was to check whether daily oral supplementation with collagen peptides alleviates the symptoms of brittle nails and improves their growth rate. The trial involved 25 participants who took 2.5 g of specific collagen peptides (BCP, VERISOL® Gelita, Eberbach, Germany) once a day for 24 weeks. After this period, there was a 4-week break in therapy. The rate of nail growth, as well as the frequency of cracking and overall clinical improvement were assessed by the doctor during and after the therapy. The results of the study showed that treatment with bioactive collagen peptides promoted nail growth by 12% and also reduced the frequency of nail breakage by 42%. Additionally, 64% of study participants reported global clinical improvement in brittle nails and 88% experienced improvement four weeks after treatment. The conclusion of the study is that daily oral consumption of bioactive collagen peptides contributes to improving the rate of nail growth and a noticeable decrease in the frequency of nail breakage [21].

4.4. Products with Collagen for Use on the Skin and Mucous Membrane

Collagen found in cosmetics applied to the skin (Figure 13) is not able to replenish its losses in the skin; its role is to retain and absorb water in the outer layer of the skin, i.e., the epidermis [5]. It will also influence the appropriate level of skin hydration. Cosmetic preparations with natural CLG (native collagen, tropocollagen) penetrate the epidermis, where they actively bind water [16,56]; they can also penetrate deeper layers—the dermis—combining there with other water-insoluble proteins, creating a hydrophilic layer on the skin surface, successively retaining moisture in the skin's intercellular spaces [3,71]. CLG is a common ingredient in hydrogel forms, which include widely used beauty masks. Such products are supposed to restore skin elasticity, and have anti-aging properties [39,57]. It can also be used as a thickening agent, but the high price of native CLG limits this choice, and instead gelatin, a denatured form of CLG, is used, which is a much cheaper alternative [15,20]. During the maturation process of collagen, the number of crosslinks in its molecule increases, making it less and less soluble in water and in acidic pH. For

this reason, cosmetic preparations mainly use hydrolyzed peptides, which contain short polypeptides, and small peptides that dissolve well in water. Such hydrophilic molecules are easier to introduce into cosmetic formulations, and thanks to their smaller molecules, they can penetrate deeper layers of the skin and ensure its regeneration [20,33,72]. Based on research on hydrolyzed collagen from marine fish, it was shown that in the deep layers of the skin, the moisturizing properties of hydrolyzed CLG are very high and can have a very good effect on the appearance of the skin [28,56,65,67,74]. Hydrolyzed CLG is also widely known in cosmetics as an ingredient with antioxidant properties and as a natural humectant [39]. Its wide range of application possibilities is due to its good compatibility with human tissues and complete biodegradability. It has been shown that local application and supplementation of hydrolyzed collagen can improve the appearance and properties of the skin by participating in various mechanisms [64] and can also accelerate the wound healing process [40]—in a study in which CLG gel was used in a skin treatment ointment in research group A, higher hydroxyproline content was observed than in the control group that did not use the gel [40,67]. Gel is a form of preparation very often used in dermatological treatment and cosmetics due to its ease of application and non-greasy nature. It is characterized by high water content (95–99%). Its ingredients are water-soluble or form micelles. It also has the ability to create a film, thanks to which it has the ability to maintain active ingredients on the skin surface [69,70,72,84].

Probably, applying collagen gel to the wound stimulates the healthy cells adjacent to it to initiate the healing process [15]. Also important in cosmetic formulas are the film-forming properties of CLG, which are used to create a film on the skin whose task is to reduce TEWL (transepidermal water loss) and protect the skin and hair against damage by surfactants [69,85]. Such properties may be enhanced by binding CLG to biopolymers and/or other polymeric molecules, e.g., polyvinylpyrrolidone (PVP). As a result of the reaction of this compound with CLG, hydrogen bonds are formed. The film-forming properties of collagen can also be modified by substances such as chitosan, silk fibroin, hyaluronic acid, elastin, or keratin [15,20,33,71]. It is important that CLG is introduced into cosmetic preparations after cooling the preparation, at the end of the emulsification process, to avoid the process of protein denaturation. The only completely safe material used in cosmetics, and medicine, is the so-called atelocollagen, i.e., a derivative of type I CLG without telopeptides [67,69]. Research on CLG obtained from the jellyfish "umbrella" has shown that it may have an impact on inhibiting the photoaging process of the skin. Animals were exposed to UVA and UVB rays after being injected with collagen or its hydrolysate in various doses. It was observed that hydrolysate applied topically to the skin protected natural collagen against degradation and also had a protective effect on elastin [61]. Ineffectiveness of topical CLG skin care products may occur due to the inability to penetrate the stratum corneum, which prevents the product from reaching the fibroblasts in the dermis [1,35,40,48]. Therefore, when it comes to collagen products, the chosen route and how they will be used is important [4,34,39,47,56,57,63,73,79]. Much of the research on CLG used for cosmetic applications is carried out by the cosmetics industry, but their results are patented, so there are not many publications in the public domain regarding the uses of collagen.

The other forms of cosmetic products with collagen are creams, serums, or masks. Creams are complex preparations composed of lipophilic and hydrophilic phases. They have the character of an emulsion, i.e., a system consisting of at least two immiscible liquids, one of which is dispersed in the other, creating o/w (oil in water), w/o (water in oil) emulsions or multiple emulsions, e.g., w/o/w. Creams differ in terms of emulsion type, composition, and application [2,5,73]. There can be distinguished creams with a light emulsion formula intended for day use, night creams with more dense and compact consistency, as well as creams in the form of a delicate cream used under the eyes, which are intended for very sensitive skin [71].



Figure 13. Application of collagen in the cosmetic products.

Collagen has been used in creams for decades, so it can be argued that it improves the structure of the skin. However, CLG molecules are too large to penetrate the epidermal barrier when applied to the skin surface. Due to the poor penetration of CLG, cosmetic products use partially hydrolyzed collagen, i.e., amino acids or peptides that would be able to penetrate the skin, but the properties of hydrolyzed CLG differ from those of native one [86]. The larger the peptide (more than 6–7 amino acids), the less likely it is to penetrate the deeper layers of the skin [22]. As a result of CLG hydrolysis, hydrogen bonds are dissociated and the triple helix structure of native CLG changes into a random coil form. Polypeptide chains are also broken, resulting in the formation of a large number of peptides. However, such resulting fragments are unable to reassemble to build CLG in the layers of the skin. Peptides and amino acids have beneficial biological functions such as water retention and cell proliferation, but they are different from those found in CLG itself [86]. In a study in which micronized marine CLG was used in a cream, it was found that it was able to penetrate the stratum corneum. The depth of penetration in human skin was measured using a non-invasive optical device that uses a laser and camera to detect light phase changes that follow the presence of collagen molecules in the deep layers of the skin [86]. In this study, the number of collagen fibers was reduced to facilitate their penetration into the deeper layers of the skin. As a result of the passage through the stratum corneum, the skin's elasticity, texture, and mechanical properties improved. Moreover, after applying the tested collagen cream, the stratum corneum seemed to be better connected to the stratum lucidum/granular layer [86]. Another study focused on the immediate and long-term clinical effects of a topical CLG hydrolysate formulation showed a significant reduction in wrinkles within minutes of the first application, and after three months of use, continued improvement was due to the action of peptides that have been shown to prevent matrix damage, extracellularly induced by metalloproteinases [22].

One other example of a product with collagen is serum. It is a cosmetic preparation in concentrated form, contains higher doses of active substances, and is supposed to act stronger and faster than cream. It can be in the form of an emulsion, gel, milk, or oil and has very light consistency, which facilitates the penetration of active ingredients into the skin. Applying the cream over the serum will prevent it from evaporating. A frequently used ingredient in cheeses is collagen, which improves the elasticity and tension of the skin, as well as hyaluronic acid, plant extracts, and vitamins A, C, and E. In order for the active substances to fulfill their role in the skin, cheeses (and creams) are equipped with carrier systems, i.e., microscopic fat bodies capable of crossing the epidermal barrier [71].

On the cosmetic market there are also masks, which are preparations in the form of a cream or paste, the main role of which is to provide a high dose of nutritional and regenerative ingredients. They are applied to the cleansed skin of the face, neck, or cleavage

and washed off with water or removed completely by removing the elastic layer [34]. There are also masks used for professional treatments in beauty salons, as well as masks for individual use at home. The most frequently used masks are regenerating and nourishing ones, e.g., algae masks, moisturizing or lifting masks aimed at smoothing the skin, reducing wrinkles, and improving skin tension, thereby improving the face oval and minimizing signs of fatigue. Natural collagen hydrolysate is the main ingredient found in the formulas of CLG masks, while CLG flakes soaked in nourishing and moisturizing substances are used to improve the absorption of active substances on facial skin [33,69,71]. CLG masks are also used in anti-aging and moisturizing treatments for people struggling with atopic dermatitis, where they appear as dry flakes made of freeze-dried ingredients applied to the skin, activated by soaking in individual active ingredients. They are supposed to have a lifting effect and retain moisture in the skin [87].

As for the number of studies assessing the effect of local use of collagen products, it is much smaller compared to the studies conducted on oral CLG supplements [5]. A study conducted in 2015 showed [88] that approximately three-quarters of treated women experienced a reduction in wrinkles and a significant improvement in skin density and elasticity after seven days of applying a product containing CLG. A similar study was conducted in Germany and South Africa on 480 patients with wrinkles, sagging skin, scars, and stretch marks, who were administered CLG in transdermal form after preparing the skin with the necessary vitamins and creams for at least a month. The results of the study indicated an improvement in the patients' skin by 60–80% compared to the condition before treatment [88]. Moreover, scientists conducted a histological examination on a subgroup of patients, which showed a significant increase in the amount of CLG and elastin, as well as thickening of the epidermis, mainly the spinous layer, after the first year after the procedure [56,89]. The anti-wrinkle effect of triple peptide (3%) was confirmed by clinicians in the study. The results suggested that their topical application significantly improves the condition of sun-damaged skin (decreases in the number of wrinkles) at the end of the first month of use compared to the placebo group [89]. Previous research shows that both oral and topical use of collagen products reduces or delays skin aging, and there is no evidence that one of these forms is better than the other [5]. One of the critical parameters that accelerate skin aging is the glycation process, especially in cases exposed to environmental factors such as ultraviolet radiation [11,44,50]. Previous research has shown that collagen tripeptide hydrolysate has anti-inflammatory and anti-aging properties, but the exact mechanism of its action is not yet fully known [89]. A 4-week study on the effects of topical application of CLG tripeptide on facial skin was conducted in 22 Asian women with visible wrinkles around the eyes. The age range was from 30 to 54 years. The ampoule used contained hydrolyzed fish skin extract, which is a form of CLG hydrolysate obtained from the skin of Pangasius hypophthalmus, containing 4% Gly-Pro-Hyp with tripeptide content exceeding 25%. The results showed a significant improvement in skin density and elasticity and reduced wrinkles. In addition, a reduction in the accumulation of advanced glycation end products (AGEs) in the skin was demonstrated in the fourth week of the study, without any adverse effects [31]. In vitro studies have shown a preventive effect of local CLG tripeptide on the accumulation of AGEs, on the production of denatured collagen, and on the occurrence of reactive oxygen species in skin fibroblasts. The topical application of collagen tripeptide also resulted in a reduction in the induction of metalloproteinases in the matrix while increasing the level of type I collagen. These study results suggest that the use of topical CLG tripeptide may improve the clinical phenotypes of aging by inhibiting oxidative stress and the glycation process [89]. Other studies have shown that oral and topical use of CLG tripeptide in people with atopic dermatitis results in reduced itching and improved skin hydration due to its anti-inflammatory properties [87,89].

5. Side Effects of Overused of Collagen Products

The above examples and discussion showed the positive action of collagen; generally, this peptide is very well tolerated and no adverse effects of oral and topical use have been

observed [5]. However, this does not mean that the use of CLG is absolutely safe. The first effect is allergic reaction, which can be observed in some people. This is the simplest side effect [30]. For example, some people may have a shellfish allergy and could even experience anaphylaxis if they take marine collagen supplements; also, other sources of CLG may cause some allergy problems. In addition, bovine-origin CLG carries a risk of transmitting illnesses such as bovine BSE [30]. Studies have not reported any side effects such as vomiting, diarrhea, nausea, or constipation in treatment or control groups [5]. In his review, Al-Atif reported a wide range of trial studies that found no adverse effects of collagen until they observed their participants [5].

6. Conclusions

Collagen has various functions in the body. It maintains the appropriate elasticity of blood vessels, provides mechanical strength to cartilage and bone tissue, is present in the cornea of the eye, and, of course, in the skin, to which it provides appropriate density and elasticity. With age, the production of natural CLG in the skin decreases. Various external factors have a similar effect, such as UV radiation causing photocrosslinking of this protein. The regulation of CLG production involves enzymes called metalloproteinases, whose task is to cut damaged CLG proteins so that new ones can be formed in their place. The most important types of CLG found in the skin are type I and type III. It is an ingredient in various cosmetic products—gels, creams, beauty masks, and serums that accelerate wound healing—and is also a component of oral supplements designed to improve the appearance of skin from its deeper layers. When used in the form of topical products, it is unable to penetrate the epidermal barrier due to its high molecular weight, which means it does not reach fibroblasts in the dermis. For this reason, partially hydrolyzed CLG is used, but its properties will differ from those of native CLG. This protein, after application, remains on the skin surface, preventing water loss, making the skin better hydrated, and, consequently, improving its color, brightness, and overall appearance. It also has a protective role against microorganisms in the case of injured skin. Thanks to this, it has been used in cosmetics for atopic skin. In order for collagen to be included in a cosmetic product, it must be properly extracted and purified. In order to break the strong crosslinks in its molecule, dilute acids and bases are used. Oral supplements used to improve the appearance of the skin and delay its aging process are becoming more and more popular. The site of their action is the dermis, where free amino acids provide building blocks for the synthesis of collagen and elastin fibers, while CLG oligopeptides stimulate fibroblasts to produce new collagen, elastin, and hyaluronic acid. If we want to temporarily improve the appearance of the skin or reduce wrinkles, we should use collagen fillers that have the ability to fill soft tissue defects permanently or temporarily. Numerous studies confirm the effects of CLG preparations as a result of their use: the depth of wrinkles is reduced, the density and elasticity of the skin improves, transepidermal water loss (TEWL) is reduced, and when using oral supplements, it also provides hydration. CLG peptides also prevent damage to the extracellular matrix caused by metalloproteinases. Oral preparations with CLG peptides have an especially positive effect on nails; a significant reduction in nail brittleness during and after using a preparation was observed. The presented work certainly does not exhaust such an extensive topic as the use of collagen protein in cosmetics. It is only an attempt to demonstrate what a huge role this protein plays in the processes taking place in the body, and that the largest organ of the body, which is the skin, would not be able to function properly without its presence. The cosmetic market offers a wide range of collagen products, which was confirmed in this work, but it is important how we use them (topically, orally, or by injection), and this depends on the needs of the skin.

To end, it is worth mentioning potential avenues for future research. These could include exploring novel formulations, investigating the long-term effects of collagen use, or delving into the molecular mechanisms underlying collagen's impact on skin health.

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References

1. Usman, R.; Bharadvaja, N. Nutricosmetics: Role in health, nutrition, and cosmetics. *Proc. Indian Natl. Sci. Acad.* **2023**, *89*, 584–599. [CrossRef]
2. Martin, I.K.; Glaser, D.A. Cosmeceuticals: The New Medicine of Beauty 'Cosmeceuticals' blur the line between drug and cosmetic, doctor and aesthetician, and patient and consumer. *Sci. Med.* **2011**, *108*, 60.
3. Alves, A.L.; Marques, A.L.P.; Martins, E.; Silva, T.H.; Reis, R.L. Cosmetic Potential of Marine Fish Skin Collagen. *Cosmetics* **2017**, *4*, 39. [CrossRef]
4. Prajaputra, V.; Isnaini, N.; Maryam, S.; Ernawati, E.; Deliana, F.; Haridhi, H.A.; Fadli, N.; Karina, S.; Agustina, S.; Nurfadillah, N.; et al. Exploring marine collagen: Sustainable sourcing, extraction methods, and cosmetic applications. *S. Afr. J. Chem. Eng.* **2024**, *47*, 197–211. [CrossRef]
5. Al-Atif, H. Collagen Supplements for Aging and Wrinkles: A Paradigm Shift in the Fields of Dermatology and Cosmetics. *Dermatol. Pr. Concept.* **2022**, *12*, 2022018–2022019. [CrossRef]
6. Felician, F.F.; Xia, C.; Qi, W.; Xu, H. Collagen from Marine Biological Sources and Medical Applications. *Chem. Biodivers.* **2018**, *15*, e1700557. [CrossRef]
7. Alcaide-Ruggiero, L.; Molina-Hernández, V.; Granados, M.M.; Domínguez, J.M. Main and minor types of collagens in the articular cartilage: The role of collagens in repair tissue evaluation in chondral defects. *Int. J. Mol. Sci.* **2021**, *22*, 13329. [CrossRef]
8. Engel, J.; Bächinger, H.P. Structure, stability and folding of the collagen triple helix. *Top. Curr. Chem.* **2005**, *247*, 7–33. [CrossRef]
9. Gordon, M.K.; Hahn, R.A. Collagens. *Cell Tissue Res.* **2010**, *339*, 247–257. [CrossRef]
10. Rittié, L.; Fisher, G.J. Natural and Sun-Induced Aging of Human Skin. *Cold Spring Harb. Perspect. Med.* **2015**, *5*, a015370. [CrossRef]
11. Jariashvili, K.; Madhan, B.; Brodsky, B.; Kuchava, A.; Namicheishvili, L.; Metreveli, N. UV Damage of Collagen: Insights from Model Collagen Peptides. *Biopolymers* **2011**, *97*, 189–198. [CrossRef]
12. Owczarzy, A.; Kurasiński, R.; Kulig, K.; Rogóż, W.; Szkudlarek, A.; Maciążek-Jurczyk, M. Collagen—Structure, properties and application. *Eng. Biomater.* **2020**, *23*, 17–23. [CrossRef]
13. Ottani, V.; Raspanti, M.; Ruggeri, A. Collagen structure and functional implications. *Micron* **2001**, *32*, 251–260. [CrossRef]
14. Shoulders, M.D.; Raines, R.T. Collagen Structure and Stability. *Annu. Rev. Biochem.* **2009**, *78*, 929–958. [CrossRef]
15. Sionkowska, A.; Skrzyński, S.; Śmiechowski, K.; Kołodziejczak, A. The review of versatile application of collagen. *Polym. Adv. Technol.* **2017**, *28*, 4–9. [CrossRef]
16. Samad, N.; Sikarwar, A. Collagen: New Dimension in Cosmetic and Healthcare. *Int. J. Biochem. Res. Rev.* **2016**, *14*, 1–8. [CrossRef]
17. Bolke, L.; Schlippe, G.; Gerß, J.; Voss, W. A Collagen Supplement Improves Skin Hydration, Elasticity, Roughness, and Density: Results of a Randomized, Placebo-Controlled, Blind Study. *Nutrients* **2019**, *11*, 2494. [CrossRef]
18. Gr up SM Marine Collagen: Extraction and Applications. Available online: www.smgebooks.com (accessed on 4 January 2024).
19. Venkatesan, J.; Anil, S.; Kim, S.-K.; Shim, M.S. Marine Fish Proteins and Peptides for Cosmeceuticals: A Review. *Mar. Drugs* **2017**, *15*, 143. [CrossRef]
20. Sionkowska, A.; Adamiak, K.; Musiał, K.; Gadomska, M. Collagen Based Materials in Cosmetic Applications: A Review. *Materials* **2020**, *13*, 4217. [CrossRef]
21. Hexsel, D.; Zague, V.; Schunck, M.; Bsc, C.S.; Camozzato, F.O.; Oesser, S. Oral supplementation with specific bioactive collagen peptides improves nail growth and reduces symptoms of brittle nails. *J. Cosmet. Dermatol.* **2017**, *16*, 520–526. [CrossRef]
22. Aguirre-Cruz, G.; León-López, A.; Cruz-Gómez, V.; Jiménez-Alvarado, R.; Aguirre-Álvarez, G. Collagen Hydrolysates for Skin Protection: Oral Administration and Topical Formulation. *Antioxidants* **2020**, *9*, 181. [CrossRef]
23. Karsdal, M.A. *Biochemistry of Collagens, Laminins and Elastin: Structure, Function and Biomarkers*; Elsevier: Amsterdam, The Netherlands, 2023.
24. Matinong, A.M.E.; Chisti, Y.; Pickering, K.L.; Haverkamp, R.G. Collagen Extraction from Animal Skin. *Biology* **2022**, *11*, 905. [CrossRef]

25. Lindholm, M.; Manon-Jensen, T.; Karsdal, M.A. Chapter 14—Type XIV collagen. In *Biochemistry of Collagens, Laminins and Elastin*, 2nd ed.; Academic Press: Cambridge, MA, USA, 2019; pp. 121–125. Available online: https://www.sciencedirect.com/science/article/pii/B9780128170687000148 (accessed on 4 January 2024).
26. Orgel, J.P.R.O.; Antonio, J.D.S.; Antipova, O. Molecular and structural mapping of collagen fibril interactions. *Connect. Tissue Res.* **2011**, *52*, 2–17. [CrossRef] [PubMed]
27. Okuyama, K. Revisiting the Molecular Structure of Collagen. *Connect. Tissue Res.* **2008**, *49*, 299–310. [CrossRef]
28. Jafari, H.; Lista, A.; Siekapen, M.M.; Ghaffari-Bohlouli, P.; Nie, L.; Alimoradi, H.; Shavandi, A. Fish Collagen: Extraction, Characterization, and Applications for Biomaterials Engineering. *Polymers* **2020**, *12*, 2230. [CrossRef]
29. Silvipriya, K.S.; Kumar, K.K.; Bhat, A.R.; Kumar, B.D.; John, A.; Lakshmanan, P. Collagen: Animal sources and biomedical application. *J. Appl. Pharm. Sci.* **2015**, *5*, 123–127. [CrossRef]
30. Wang, H. A Review of the Effects of Collagen Treatment in Clinical Studies. *Polymers* **2021**, *13*, 3868. [CrossRef]
31. Collier, T.A.; Nash, A.; Birch, H.L.; De Leeuw, N.H. Effect on the mechanical properties of type I collagen of intra-molecular lysine-arginine derived advanced glycation end-product cross-linking. *J. Biomech.* **2017**, *67*, 55–61. [CrossRef]
32. Chang, S.W.; Buehler, M.J. Molecular biomechanics of collagen molecules. *Mater. Today* **2014**, *17*, 70–76. [CrossRef]
33. Reilly, D.M.; Lozano, J. Skin collagen through the lifestages: Importance for skin health and beauty. *Plast. Aesthetic Res.* **2021**, *8*, 231801211. [CrossRef]
34. Sibilla, S.; Godfrey, M.; Brewer, S.; Budh-Raja, A.; Genovese, L. An overview of the beneficial effects of hydrolysed collagen as a nutraceutical on skin properties: Scientific background and clinical studies. *Open Nutraceuticals J.* **2015**, *8*, 29–42. [CrossRef]
35. Li, G.Y.; Fukunaga, S.; Takenouchi, K.; Nakamura, F. Comparative study of the physiological properties of collagen, gelatin and collagen hydrolysate as cosmetic materials. *Int. J. Cosmet. Sci.* **2005**, *27*, 101–106. [CrossRef] [PubMed]
36. Calleja-Agius, J.; Brincat, M.; Borg, M. Skin connective tissue and ageing. *Best Pr. Res. Clin. Obs. Gynaecol.* **2013**, *27*, 727–740. [CrossRef]
37. Shekhter, A.B.; Fayzullin, A.L.; Vukolova, M.N.; Rudenko, T.G.; Osipycheva, V.D.; Litvitsky, P.F. Medical Applications of Collagen and Collagen-Based Materials. *Curr. Med. Chem.* **2017**, *26*, 506–516. [CrossRef]
38. Asserin, J.; Lati, E.; Shioya, T.; Prawitt, J. The effect of oral collagen peptide supplementation on skin moisture and the dermal collagen network: Eevidence from an ex vivo model and randomized, placebo-controlled clinical trials. *J. Cosmet. Dermatol.* **2015**, *14*, 291–301. [CrossRef]
39. Inoue, N.; Sugihara, F.; Wang, X. Ingestion of bioactive collagen hydrolysates enhance facial skin moisture and elasticity and reduce facial ageing signs in a randomised double-blind placebo-controlled clinical study. *J. Sci. Food Agric.* **2016**, *96*, 4077–4081. [CrossRef]
40. Davison-Kotler, E.; Marshall, W.S.; García-Gareta, E. Sources of collagen for biomaterials in skin wound healing. *Bioengineering* **2019**, *6*, 56. [CrossRef]
41. Dong, C.; Lv, Y. Application of collagen scaffold in tissue engineering: Recent advances and new perspectives. *Polymers* **2016**, *8*, 42. [CrossRef]
42. Shimizu, J.; Asami, N.; Kataoka, A.; Sugihara, F.; Inoue, N.; Kimira, Y.; Wada, M.; Mano, H. Oral collagen-derived dipeptides, prolyl-hydroxyproline and hydroxyprolyl-glycine, ameliorate skin barrier dysfunction and alter gene expression profiles in the skin. *Biochem. Biophys. Res. Commun.* **2015**, *456*, 626–630. [CrossRef]
43. Chung, J.H.; Seo, J.Y.; Choi, H.R.; Lee, M.K.; Youn, C.S.; Rhie, G.-E.; Cho, K.H.; Kim, K.H.; Park, K.C.; Eun, H.C. Modulation of skin collagen metabolism in aged and photoaged human skin in vivo. *J. Investig. Dermatol.* **2001**, *117*, 1218–1224. [CrossRef] [PubMed]
44. Bansode, S.; Bashtanova, U.; Li, R.; Clark, J.; Müller, K.H.; Puszkarska, A.; Goldberga, I.; Chetwood, H.H.; Reid, D.G.; Colwell, L.J.; et al. Glycation changes molecular organization and charge distribution in type I collagen fibrils. *Sci. Rep.* **2020**, *10*, 1–13. [CrossRef]
45. Chambers, E.S.; Vukmanovic-Stejic, M. Skin barrier immunity and ageing. *Immunology* **2020**, *160*, 116–125. [CrossRef]
46. Mohania, D.; Chandel, S.; Kumar, P.; Verma, V.; Digvijay, K.; Tripathi, D.; Choudhury, K.; Mitten, S.K.; Shah, D. Ultraviolet radiations: Skin defense-damage mechanism. *Adv. Exp. Med. Biol.* **2017**, *996*, 71–87. [CrossRef]
47. De Luca, C.; Mikhal'Chik, E.V.; Suprun, M.V.; Papacharalambous, M.; Truhanov, A.I.; Korkina, L.G. Skin antiageing and systemic Redox effects of supplementation with marine collagen peptides and plant-derived antioxidants: A single-blind case-control clinical study. *Oxid. Med. Cell Longev.* **2016**, *2016*, 4389410. [CrossRef]
48. Cherim, M.; Mustafa, A.; Cadar, E.; Lupaşcu, N.; Paris, S.; Sirbu, R. Collagen Sources and Areas of Use. *Eur. J. Med. Nat. Sci.* **2019**, *2*, 8–13. [CrossRef]
49. Tu, Y.; Quan, T. Oxidative Stress and Human Skin Connective Tissue Aging. *Cosmetics* **2016**, *3*, 28. [CrossRef]
50. Gromkowska-Kępka, K.J.; Puścion-Jakubik, A.; Markiewicz-Żukowska, R.; Socha, K. The impact of ultraviolet radiation on skin photoaging—Review of in vitro studies. *J. Cosmet. Dermatol.* **2021**, *20*, 3427–3431. [CrossRef] [PubMed]
51. Yamauchi, M.; Sricholpech, M.; Terajima, M.; Tomer, K.B.; Perdivara, I. Glycosylation of Type I Collagen. *Methods Mol. Biol.* **2019**, *1934*, 127–144. [CrossRef]
52. Hennet, T. Collagen glycosylation. *Curr. Opin. Struct. Biol.* **2019**, *56*, 131–138. [CrossRef] [PubMed]
53. Tanaka, M.; Koyama, Y.I.; Nomura, Y. Effects of collagen peptide ingestion on UV-B-induced skin damage. *Biosci. Biotechnol. Biochem.* **2009**, *73*, 930–932. [CrossRef] [PubMed]

54. Oba, C.; Ohara, H.; Morifuji, M.; Ito, K.; Ichikawa, S.; Kawahata, K.; Koga, J. Collagen hydrolysate intake improves the loss of epidermal barrier function and skin elasticity induced by UVB irradiation in hairless mice. *Photodermatol. Photoimmunol. Photomed.* **2013**, *29*, 204–211. [CrossRef] [PubMed]
55. Silveira, J.E.P.S.; Pedroso, D.M.M. UV light and skin aging. *Rev. Environ. Health* **2014**, *29*, 243–254. [CrossRef]
56. Choi, S.Y.; Ko, E.J.; Lee, Y.H.; Kim, B.G.; Shin, H.J.; Seo, D.B.; Lee, S.J.; Kim, B.J.; Kim, M.N. Effects of collagen tripeptide supplement on skin properties: A prospective, randomized, controlled study. *J. Cosmet. Laser Ther.* **2014**, *16*, 132–137. [CrossRef]
57. Evans, M.; Lewis, E.D.; Zakaria, N.; Pelipyagina, T.; Guthrie, N. A randomized, triple-blind, placebo-controlled, parallel study to evaluate the efficacy of a freshwater marine collagen on skin wrinkles and elasticity. *J. Cosmet. Dermatol.* **2021**, *20*, 825–834. [CrossRef]
58. Añazco, C.; Riedelsberger, J.; Vega-Montoto, L.; Rojas, A. Exploring the Interplay between Polyphenols and Lysyl Oxidase Enzymes for Maintaining Extracellular Matrix Homeostasis. *Int. J. Mol. Sci.* **2023**, *24*, 10985. [CrossRef]
59. Sionkowska, A.; Lewandowska, K.; Adamiak, K. The Influence of UV Light on Rheological Properties of Collagen Extracted from Silver Carp Skin. *Materials* **2020**, *13*, 4453. [CrossRef]
60. Adamiak, K.; Sionkowska, A. The influence of UV irradiation on fish skin collagen films in the presence of xanthohumol and propanediol. *Spectrochim. Acta Part A Mol. Biomol. Spectrosc.* **2022**, *282*, 121652. [CrossRef]
61. Song, H.H.; Hong, K.-B.; Kim, S.; Kim, B.-Y.; Shik, S.H.; Suh, H.J.; Ahn, Y. Effects of fish collagen on hairless mice skin photoaging induced by ultraviolet irradiation via regulation of the TGF-β signaling pathway Anti-photoaging effect of fish collagen in UVB-induced hairless mice. *J. Funct. Foods* **2023**, *105*, 105554. [CrossRef]
62. Timorshina, S.; Popova, E.; Osmolovskiy, A. Sustainable Applications of Animal Waste Proteins. *Polymers* **2022**, *14*, 1601. [CrossRef]
63. León-López, A.; Morales-Peñaloza, A.; Martínez-Juárez, V.M.; Vargas-Torres, A.; Zeugolis, D.I.; Aguirre-Álvarez, G. Hydrolyzed Collagen—Sources and Applications. *Molecules* **2019**, *24*, 4031. [CrossRef]
64. Feng, B.; Yang, H.; Zhu, M.; Li, J.; Chang, H.-M.; Leung, P.C.K.; Guo, J.; Zhang, Y. Collagen-based biomaterials in organoid technology for reproductive medicine: Composition, characteristics, and applications. *Collagen Leather* **2023**, *5*, 1–26. [CrossRef]
65. Coppola, D.; Oliviero, M.; Vitale, G.A.; Lauritano, C.; D'ambra, I.; Iannace, S.; de Pascale, D. Marine Collagen from Alternative and Sustainable Sources: Extraction, Processing and Applications. *Mar. Drugs* **2020**, *18*, 214. [CrossRef] [PubMed]
66. Sotelo, C.G.; Comesaña, M.B.; Ariza, P.R.; Pérez-Martín, R.I. Characterization of Collagen from Different Discarded Fish Species of the West Coast of the Iberian Peninsula. *J. Aquat. Food Prod. Technol.* **2016**, *25*, 388–399. [CrossRef]
67. Silva, T.H.; Moreira-Silva, J.; Marques, A.L.P.; Domingues, A.; Bayon, Y.; Reis, R.L. Marine origin collagens and its potential applications. *Mar. Drugs* **2014**, *12*, 5881–5901. [CrossRef] [PubMed]
68. Mahboob, S. Isolation and characterization of collagen from fish waste material- skin, scales and fins of *Catla catla* and *Cirrhinus mrigala*. *J. Food Sci. Technol.* **2015**, *52*, 4296–4305. [CrossRef]
69. Rodríguez, M.I.A.; Barroso, L.G.R.; Sánchez, M.L. Collagen: A review on its sources and potential cosmetic applications. *J. Cosmet. Dermatol.* **2018**, *17*, 20–26. [CrossRef]
70. Prokopová, A.; Pavlačková, J.; Mokrejš, P.; Gál, R.; Marrocchi, A.; Marconi, O. Collagen Hydrolysate Prepared from Chicken By-Product as a Functional Polymer in Cosmetic Formulation. *Molecules* **2021**, *26*, 2021. [CrossRef] [PubMed]
71. Bernat, M.; Matysek-Nawrocka, M.; Cioczek, W.; Społecznych, W.N.; Medycznych, N.; Nauk, W.S. Składniki aktywne w kosmetykach przeciwstarzeniowych Active agents in anti-aging cosmetics. *Kosmetol. Estet.* **2016**, *5*, 575–579.
72. Żelaszczyk, D.; Waszkielewicz, A.; Marona, H. Kolagen—Struktura oraz zastosowanie w kosmetologii i medycynie estetycznej. *Estetologia Med. Kosmetol.* **2012**, *2*, 14–20. [CrossRef]
73. Prokopowicz, M.; Różycki, K.M. News, and Undefined 2017. Innovation in Cosmetics. Available online: https://bibliotekanauki.pl/articles/1179767.pdf (accessed on 4 January 2024).
74. Hou, H.; Li, B.; Zhang, Z.; Xue, C.; Yu, G.; Wang, J.; Bao, Y.; Bu, L.; Sun, J.; Peng, Z.; et al. Moisture absorption and retention properties, and activity in alleviating skin photodamage of collagen polypeptide from marine fish skin. *Food Chem.* **2012**, *135*, 1432–1439. [CrossRef]
75. Cohen, S.R.; Berner, C.F.; Busso, M.; Gleason, M.C.; Hamilton, D.; Holmes, R.E.; Romano, J.J.; Rullan, P.P.; Thaler, M.P.; Ubogy, Z.; et al. ArteFill: A long-lasting injectable wrinkle filler material—Summary of the U.S. Food and Drug Administration trials and a progress report on 4- to 5-year outcomes. *Plast. Reconstr. Surg.* **2006**, *118*, 64S–76S. [CrossRef] [PubMed]
76. Bauman, L. CosmoDerm/CosmoPlast (Human Bioengineered Collagen) for the Aging Face. *Facial Plast. Surg.* **2004**, *20*, 125–128. [CrossRef]
77. Baumann, L. Collagen-Containing Fillers: Alone and in Combination. *Clin. Plast. Surg.* **2006**, *33*, 587–596. [CrossRef]
78. Watanabe-Kamiyama, M.; Shimizu, M.; Kamiyama, S.; Taguchi, Y.; Sone, H.; Morimatsu, F.; Shirakawa, H.; Furukawa, Y.; Komai, M. Absorption and effectiveness of orally administered low molecular weight collagen hydrolysate in rats. *J. Agric. Food Chem.* **2010**, *58*, 835–841. [CrossRef] [PubMed]
79. Yoon, H.S.; Cho, H.H.; Cho, S.; Lee, S.R.; Shin, M.H.; Chung, J.H. Supplementating with dietary astaxanthin combined with collagen hydrolysate improves facial elasticity and decreases matrix metalloproteinase-1 and -12 expression: A comparative study with placebo. *J. Med. Food* **2014**, *17*, 810–816. [CrossRef] [PubMed]
80. Baumann, L.; Kaufman, J.; Saghari, S. Collagen fillers. *Dermatol. Ther.* **2006**, *19*, 134–140. [CrossRef]

81. Proksch, E.; Segger, D.; Degwert, J.; Schunck, M.; Zague, V.; Oesser, S. Oral supplementation of specific collagen peptides has beneficial effects on human skin physiology: A double-blind, placebo-controlled study. *Ski. Pharmacol. Physiol.* **2013**, *27*, 47–55. [CrossRef]
82. Proksch, E.; Schunck, M.; Zague, V.; Segger, D.; Degwert, J.; Oesser, S. Oral intake of specific bioactive collagen peptides reduces skin wrinkles and increases dermal matrix synthesis. *Skin Pharmacol. Physiol.* **2014**, *27*, 113–119. [CrossRef]
83. Addor, F.A.S.; Vieira, J.C.; Melo, C.S.A. Improvement of dermal parameters in aged skin after oral use of a nutrient supplement. *Clin. Cosmet. Investig. Dermatol.* **2018**, *11*, 195–201. [CrossRef]
84. Ciszek, A. Variability of skin pH after the use of different collagen gels. *J. Cosmet. Dermatol.* **2017**, *16*, 531–536. [CrossRef]
85. Luebberding, S.; Krueger, N.; Kerscher, M. Skin physiology in men and women: In vivo evaluation of 300 people including TEWL, SC hydration, sebum content and skin surface pH. *Int. J. Cosmet. Sci.* **2013**, *35*, 477–483. [CrossRef] [PubMed]
86. Lubart, R.; Yariv, I.; Fixler, D.; Lipovsky, A. A Novel Facial Cream Based on Skin-penetrable Fibrillar Collagen Microparticles. *J. Clin. Aesthet. Dermatol.* **2022**, *15*, 59–64. [PubMed]
87. Lehmann, K.M. Skin care of atopic dermatitis in a beauty salon. *Aesthetic Cosmetol. Med.* **2022**, *11*, 87–90. [CrossRef]
88. Aust, M.C.; Fernandes, D.; Kolokythas, P.; Kaplan, H.M.; Vogt, P.M. Percutaneous collagen induction therapy: An alternative treatment for scars, wrinkles, and skin laxity. *Plast. Reconstr. Surg.* **2008**, *121*, 1421–1429. [CrossRef] [PubMed]
89. Lee, Y.I.; Lee, S.G.; Jung, I.; Suk, J.; Lee, M.-H.; Kim, D.-U.; Lee, J.H. Effect of a Topical Collagen Tripeptide on Antiaging and Inhibition of Glycation of the Skin: A Pilot Study. *Int. J. Mol. Sci.* **2022**, *23*, 1101. [CrossRef]

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Review

Micro(nano)plastics and Their Potential Impact on Human Gut Health: A Narrative Review

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Abstract: Microplastics and nanoplastics (MNPs) are becoming an increasingly severe global problem due to their widespread distribution and complex impact on living organisms. Apart from their environmental impact, the effects of MNPs on living organisms have also continued to attract attention. The harmful impact of MNPs has been extensively documented in marine invertebrates and larger marine vertebrates like fish. However, the research on the toxicity of these particles on mammals is still limited, and their possible effects on humans are poorly understood. Considering that MNPs are commonly found in food or food packaging, humans are primarily exposed to them through ingestion. It would be valuable to investigate the potential harmful effects of these particles on gut health. This review focuses on recent research exploring the toxicological impacts of micro- and nanoplastics on the gut, as observed in human cell lines and mammalian models. Available data from various studies indicate that the accumulation of MNPs in mammalian models and human cells may result in adverse consequences, in terms of epithelial toxicity, immune toxicity, and the disruption of the gut microbiota. The paper also discusses the current research limitations and prospects in this field, aiming to provide a scientific basis and reference for further studies on the toxic mechanisms of micro- and nanoplastics.

Keywords: digestive system; gut microbiota; human cells; intestinal toxicity; microplastics; mouse models; nanoplastics; plastic; polymers

1. Introduction

Microplastics (MPs) are tiny particles derived from plastics, or synthetic or semisynthetic polymers produced from hydrocarbon or biomass materials. Most plastics are petroleum-derived polymers that consist of "molecules of high relative molecular mass, whose structure essentially comprises multiple repetitions of derived units, from molecules of low relative molecular mass" [1]. These polymers, like polypropylene (PP), polyethylene (PE), polyvinyl chloride (PVC), polyethylene terephthalate (PET), and polystyrene (PS), are non-biodegradable [2].

Plastic is a widely used material in industrial applications and its production has been consistently increasing over the years. In 2016, annual plastic production reached 300 million tons. However, if this trend continues, it is estimated that approximately 25 million tons of plastic waste will be produced by 2050 [3–7]. Plastic is a key component in a diverse range of industrial and consumer products, including cosmetics, detergents, paints, synthetic fertilizers, and pesticides, among others. Unfortunately, MPs have been detected in various food products, such as processed foods, beer, seafood, and sugar-sweetened beverages [8,9].

This widespread plastic contamination, which has been attributed to the limited recycling efforts and the absence of regulatory frameworks, has had a substantial impact

on aquatic, terrestrial, and atmospheric environments. The issue of plastic pollution has become a pressing concern as it is now present in almost all water bodies, including oceans, seas, rivers, and lakes, thereby posing a significant threat to biodiversity and public health [10].

Plastic waste, once discarded in nature, is exposed to different factors, including physical (e.g., ultraviolet radiation and temperature), chemical (e.g., salinity, pH, and corrosive agents), and biological (e.g., bacteria, microalgae, and plankton). These factors decompose plastic waste into particles of different sizes and ecological impact. Three main classification groups are commonly used to describe plastic waste based on its particle size: macro- (>25 mm), meso- (between 5 and 25 mm), and microplastics (MPs < 5 mm). Additionally, the intentional production and further degradation of microplastics can generate smaller waste particles, known as nanoplastics (NPs < 1 μm) [11].

Microplastics are categorized by their origin. "Primary" microplastics are intentionally created at the microscale, while "secondary" microplastics come from the fragmentation of larger plastics [12].

Microplastic pollution is widespread in soil environments, including agricultural soils, greenhouses, and coastal, industrial, and floodplain soils. This type of pollution is a result of the inappropriate management and unsustainable use of plastic waste and agricultural processes [13,14]. Microplastic pollution is also a significant issue in aquatic environments, such as the marine environment, where plastic debris can be found on the sea floor, surface, and shoreline [15]. It has been estimated that 80% of the plastic pollution in oceans and seas comes from land [16]. Microplastics have also been detected in freshwater, including lakes, rivers, and groundwater. These particles mainly come from urban pollution, shipping, fishing, tourism, oil and gas platforms, wastewater treatment plants, discharged personal care products, textiles, and packaging [17]. Furthermore, microplastics have been found in the atmospheric fallout in both megacities and sparsely populated areas [6,18–21], and suspended atmospheric microplastics have also been repeatedly detected in indoor air [22,23].

The wide distribution of microplastics and nanoplastics (MNPs) promotes contamination by different animal species, especially by integumentary exposure, inhalation, and ingestion [24–26]. Specifically, for mammals and humans, the inhalation of nanomaterials and ingestion of contaminated water, sea salt, and seafood are the main routes of exposure to these plastic particles [27].

Although previously considered safe and inert materials, the negative biological impact of the contamination of microplastics and nanoplastics has been demonstrated recently [26,28,29]. As a result, the smallest particles (such as 10 μm and 2.5 μm) can penetrate organs like lungs and intestines, as well as cells like enterocytes and macrophages. These particles are recognized as foreign elements that stimulate immune response and oxidative stress [29,30]. Due to their difficulty in being cleared biologically, particles can accumulate and result in chronic inflammation, potentially leading to the development of tumors [29,31,32]. In addition, microplastics and nanoplastics pose a high toxicological risk, as they contain hazardous additives like plasticizers, flame retardants, stabilizers, dyes, antistatic agents, lubricants, sliding agents, curing agents, foaming agents, and biocides [33]. It is worth noting that microplastics have the potential to adopt a fibrous form, which is commonly referred to as "microplastic fibers" [34]. The contamination of environments with microplastic fibers is probably as much as, or even more than, that caused by microplastic particles [35,36]. Because of their elongated shape, microplastic fibers have a higher potential for bioaccumulation and can cause direct harm to organisms or lead to adverse effects [37].

It is currently not feasible to conduct clinical studies that analyze the health risks of MNPs in humans due to ethical concerns. As a result, we do not have a clear understanding of the health impact of MNPs on humans. We are unaware of the extent to which humans can absorb and accumulate MNPs, and the pharmacokinetic and pharmacodynamic mechanisms associated with them [38].

However, it is still a major concern that MNPs could have toxicological effects on the entire intestinal system, especially through ingestion, which remains one of the main exposure mechanisms of these particles.

This paper aims to present an objective overview of the potential impacts that these polymers could have on the intestinal system by highlighting the toxicological effects related to MNPs for in vivo mammalian and in vitro human cell studies found in the literature. This review focuses specifically on the toxicological effects of MNPs on the gut and the complex immunological system related to it, examining the various interrelationships that these particles have with the gut microbiota. These studies cannot provide clinical data. However, they can lay an important foundation for future research by providing an overview of these issues.

2. Main Pathogenetic Mechanisms of MNP-Induced Cell Toxicity

Experimental models have revealed that the mechanisms of membrane damage, oxidative stress, immune response, and genotoxicity contribute to the toxicity of MNPs.

Among them, the cytotoxicity of MNPs was mainly attributed to membrane damage and oxidative stress [39]. Particles can damage the plasma membrane, which is often observed with cationic particles [40,41]. Polyethylene nanoparticles have been found to penetrate the hydrophobic milieu of the bilayer of the plasma membrane and cause structural changes [42]. Endocytosed particles can permeabilize the endosomal and lysosomal membrane and interact with intracellular organelles [43,44].

Reactive oxygen species (ROS) can be generated during plastic polymerization and particle processing and, upon interaction with the bioenvironment, cause cellular stress [45]. On the other hand, the direct or indirect impairment of DNA through the translocation of particles or ROS into the nucleus and damage to the DNA replication or repair mechanism may contribute to the genotoxicity of particles [46,47].

In mammalian cells, MNPs can cause nuclear membrane disruption, oxidative stress, the release of damage-associated molecular patterns, and the downstream activation of inflammatory and apoptotic and necrotic pathways [44,48].

The absorption of micro- or nanoplastics can lead to the loss of integrity of plasma, endosomal, and nuclear membranes, causing pore formation in membranes and the subsequent generation of ROS from mitochondria. Elevated levels of intracellular ROS can cause mitochondrial damage due to increased mitochondrial Ca2+, concomitant mitochondrial membrane depolarization, the release of pro-apoptotic factors from mitochondria, the reduction of ATP, the release of damage-associated molecular patterns (DAMPs) from mitochondria or other organelles, resulting in the production of pro-inflammatory cytokines, and, finally, the activation of cell death pathways, leading to apoptosis or necrosis [38].

3. MNPs and the Intestinal System

The main source of exposure to microplastic and nanoplastic particles is through the ingestion of food or water that is contaminated with these particles. Plastics inevitably find their way into the food chain and carry contaminants that can affect intestinal homeostasis. Studies have found the presence of microplastics and nanoplastics in many types of foods, including fruits, vegetables, marine products, livestock (such as chickens), and drinking water [49–53]. Other foods such as sugar, honey, beer, cow's milk, and sea salt have also been found to contain microplastics [9,52,54–56]. These particles have even been found in the gastrointestinal contents of more than 220 different marine species, such as *mussels*, *oysters*, *clams*, and *common shrimp*, as well as in various seafood products [57–59].

The most commonly detected polymers in food and drinking water are polyethylene (PE), polypropylene (PP), polystyrene (PS), polyvinyl chloride (PVC), and polyethylene terephthalate (PET). Polyamide (PA), acrylic, or acrylic-related compounds, polyesters, and polymethyl methacrylate (PMMA) are also detected, but less frequently [60].

It is uncertain whether the ingestion of MNPs poses a significant risk to the intestinal system, given the conflicting data on human exposure and the biodistribution of these

particles. It has been observed that human adults can potentially ingest up to 458,000 microplastic particles per annum through tap water and 3,569,000 microplastic particles per annum through bottled water [61]. However, there exists a considerable variation in the estimates of human exposure to microplastics due to differences in the type of plastic and experimental methodologies employed in various studies [9]. In a recent study, Schawabl et al. endeavored to estimate human contamination by measuring the amount of microplastics in the feces of eight healthy volunteers. The study established an average of 20 microplastic particles per 10 g of feces, ranging in size from 50 to 500 μm, and belonging to nine types of plastics, with PP and PET being the most prevalent [62,63].

The distribution of micro- and nanoplastics after ingestion is not well-understood. Due to the stability of plastic materials, enzymatic or chemical degradation is challenging, especially since mammalian intestines lack specialized enzymes for plastic degradation. This means that plastic particles are not significantly degraded during digestion. Larger microplastics (>150 μm) remain attached to the intestinal mucus layer, directly contacting the apical part of intestinal epithelial cells. Smaller particles (<150 μm), however, can cross the intestinal mucus layer. The uptake of micro- and nanoplastics depends on their size and occurs through various mechanisms, including transcytosis through microfold cells, endocytosis through enterocytes, persorption (which is the passage through crevices at the end of the villus, following the loss of enterocytes), and paracellular uptake [64–67].

When micro- and nanoplastics are ingested, most of them are excreted through feces [68,69], while a small portion stays in the intestine for several days [68]. In the gut, MNPs can cause damage and inflammation by entering the bloodstream, spreading to other tissues, and persisting for prolonged periods [70]. The bioavailability of MNPs after oral intake depends on intestinal translocation. In a study of three intestinal cell models [71], it was observed that the size and surface chemistry of the particles influenced translocation, with 50 nm nanoparticles having a higher translocation rate than 100 nm NPs. The translocation of MNPs is influenced by various factors, including the characteristics of plastic particles and animal behavior and development [72].

Despite the low rate of intestinal absorption, exposure to significant amounts of micro- and nanoparticles could lead to systemic toxicity, as their small size allows them to penetrate deep into organs. Specifically, a study by Walczak et al. investigating the impact of in vitro gastrointestinal digestion on the protein crown of PS-NPs revealed that, after digestion, translocation was 4 times higher for positively charged NPs and 80 and 1.7 times higher for two different types of negatively charged NPs. In vitro digestion also reduced the presence of higher-molecular-weight proteins, shifting the protein content of the corona toward lower-molecular-weight proteins [73].

Comprehensive studies of the 55 most widely used polymer types developed a model for ranking the hazard of each polymer, according to the monomer chemicals that formed the polymer. The most hazardous polymers were those produced from carcinogenic, mutagenic, or both monomers. Hazard classification data were mainly taken from Annex VI of the EU Classification, Labelling and Packaging (CLP) regulation which is based on the UN Globally Harmonized System (GHS). However, while this approach determined a high ranking for polyurethanes, epoxy resins, and polyvinylchloride and styrene polymers, no hazard classification was available for many of the listed substances, such as suspected endocrine disruptors, due to the lack of safety data [67,74].

3.1. Toxicity of Micro-/Nanoplastics in the Intestine

The effects of MNPs on the intestinal system and gut microbiota in mammals and humans, and the associated mechanisms, are still not fully understood. Figure 1 summarizes the main postulated toxicological effects of MNPs on the intestinal system.

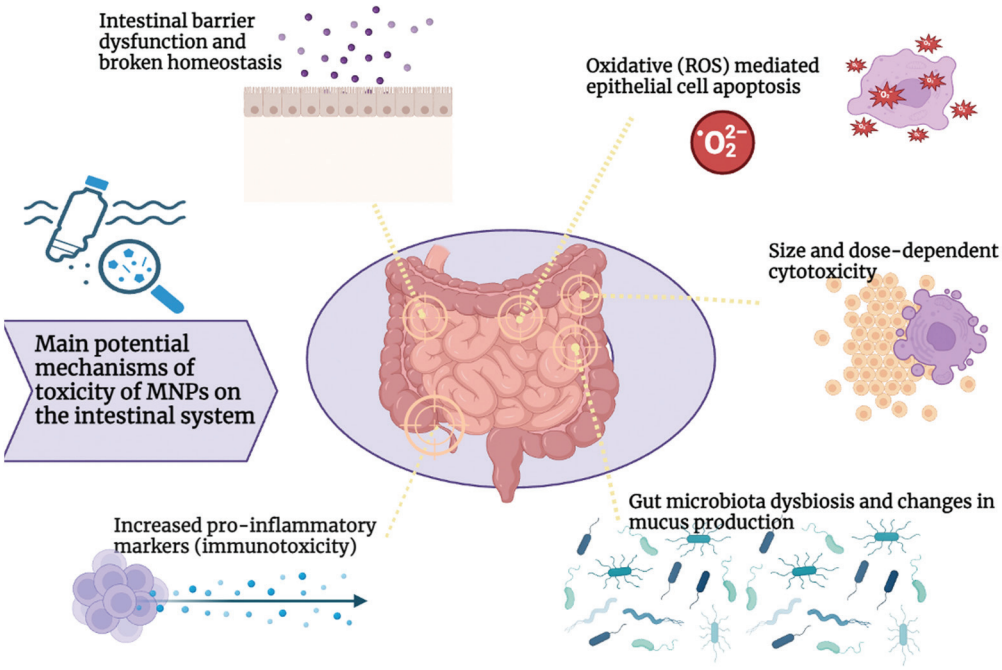


Figure 1. Main potential mechanisms of MNP toxicity on the intestinal system.

Studies have shown that microparticles have a harmful impact on the intestines of invertebrates and vertebrates like fish. For instance, research conducted on *Caenorhabditis elegans*, *Artemia parthenogenetica* zooplankton larvae, and *Eisenia fetida* earthworm has revealed that intestinal oxidative damage is a significant mechanism in microplastic toxicity. Moreover, exposure to microparticles was found to be associated with the progression of cellular deformations and enterocyte decomposition [75–77]. Further evidence comes from studies that involved oral exposure to microplastics in aquatic vertebrates such as *rainbow trout*, *juvenile intertidal fish Girella laevifrons*, *juvenile large yellow croaker Larimichthys crocea*, or *Oryzias melastigma*. Overall, these studies have found that PS microbeads and nanoparticles cause a decrease in digestive enzyme activity (lipase, trypsin, and lysozyme) [78,79], and induce goblet cell enlargement and increased mucus secretion [78,80], the secretion of proinflammatory cytokines like Tumor Necrosis Factor α (TNF α), Interferon γ (IFN γ), and Interleukin-6 (IL-6) [78], leukocyte infiltration, hyperemia, and the loss of villi and crypt cells [81]. Interestingly, intestinal levels of oxidative stress enzymes were found to be modified in opposite ways between nano- and microparticles [80].

The toxic effects of microplastics on the gut have been assessed in several aquatic species, pointing out inflammation, genotoxicity, and oxidative stress responses [82]. Several studies have been conducted to investigate the effects of PS on the intestines of zebrafish. Exposure to PS beads resulted in an increase in the secretion of proinflammatory cytokines such as Interleukin-1α (IL1α), Interleukin-1β (IL1β), and IFN γ. This exposure also enhanced the activity of enzymes that respond to excessive oxidative stress. It was observed that exposure to PS was associated with reduced levels of antioxidant enzyme diamine oxidase and of D-lactate, which could indicate increased intestinal permeability [83]. Furthermore, a single-cell analysis revealed a dysfunction of intestinal cell populations, a decrease in the detoxification/antioxidant capacity of enterocytes, and a decrease in the cell chemotaxis of secretory cells.

It appears that the impact of microplastics on the intestinal epithelium depends not only on the size of the particles but also on their shape. In fact, when exposed to microplastic fibers, the volume of mucus in the intestine of zebrafish declined sharply. Additionally, both microplastic fibers and fragments led to a decrease in intestinal D-lactate, caused inflammation in the intestine, and increased the activity of superoxide dismutase [84]. Exposure to PVC induced a histological alteration in the intestine of

European sea bass *Dicentrarchus labrax* L. [85], increasing the globet cell number, villus thickness, and expression of intestinal nuclear factor E2-related factor 2 (Nrf2). On the other side, exposure to irregularly shaped high-density PE and PS particles determined an epithelial detachment, increase in the neutrophil count, and decrease in the globet cell count in the intestine of zebrafish [86].

Notwithstanding the available evidence, the data from in vitro and in vivo studies in mammalian models are comparatively restricted and conflicting (Table 1).

Table 1. Major studies investigating the potential effects of MNPs on the intestinal system in human cell lines and mammalian models.

Molecules	Species	Design	Size/Exposure	Effects	Study
Nanoplastics	Human	Human colon adenocarcinoma Caco-2 cell; in vitro design	PS particles between 50 and 200 nm at a concentration of 250 mg/mL for 10 to 120 min	Absence of cellular toxicity.	Abdelkhaliq 2018 [87]
Nanoplastics	Human	Human colon adenocarcinoma Caco-2 cell; in vitro design	100 nm PE terephthalate particles at a concentration between 1 and 30 mg/mL for an incubation time of 24 h	No evidence of increased inflammatory factors.	Magrì 2018 [88]
Nanoplastics, microplastics	Human	Human Caco-2 and HT29-MTX-E12 cells; in vitro design	50 nm and 0.5 μm COOH-modified PS particles, concentration (0.01 μg/mL–100 μg/mL) for an incubation time of 24 h	Absence of cellular toxicity.	Hesler 2019 [89]
Microplastics	Human	Human Caco-2 cells and gut microbiota; in vitro design	PE microplastics between 30 and 140 μm tested at various concentrations for 48 h	Significant reduction in Caco-2 cell viability, only for high concentrations (1000 mg/L).	Huang 2021 [90]
Microplastics	Human, mice	- Human colon adenocarcinoma Caco-2 cell; in vitro design - Male reporter gene mice; in vivo design	- 4 μm and 10 μm PS particles, variable concentration for an incubation time of 48 h - Mixture of 1 μm to 10 μm PS microplastics at a volume of 10 mL/kg and a total of one dose for 3 weeks	- Reduction in cell vitality for high concentrations (1×10^8 particles/mL); no effect on cell polarization. - Absence of histologically detectable lesions and inflammatory responses.	Stock 2019 [91]
Microplastics	Human	HRT-18 and CMT-93 epithelial human cell lines; in vitro design	PS microparticles of 4.8–5.8 μm for a concentration of 1 mg/mL and a time between 6 and 48 h	Significant cytotoxicity in both cell lines. Oxidative stress activity was increased only in CMT-93 cells.	Mattioda 2023 [92]
Microplastics	Mice	IRC mice divided into control and exposed group; in vivo design	PS microparticles of 5 μm for a concentration of 100 and 1000 μg/L for six weeks	Reduced mucus production and damage to the intestinal barrier. Decreased Actinobacteria content and altered microbial alpha diversity. At the genus level, a total of 15 types of bacteria changed significantly.	Jin 2019 [93]

Table 1. *Cont.*

Molecules	Species	Design	Size/Exposure	Effects	Study
Microplastics	Mice	Male mice exposed to two different MP sizes; in vivo design	Oral exposure to 1000 μg/L of 0.5 and 50 μm PS-MP for five weeks	Decreased mucus secretion in the intestine in both sizes of treated groups. Decreased relative abundance of Firmicutes and α-Proteobacteria in the feces. Significant changes in the richness and diversity of the caecal intestinal microbiota.	Lu 2018 [94]
Microplastics	Mice	SPF grade C57BL/6 male mice were divided into four groups; in vivo design	Exposure to different amounts of PE microplastics between 10 and 150 μm (6, 60, and 600 μg/day for 5 consecutive weeks)	Induction of histologic inflammation in the colon and duodenum (a higher expression of TLR4, AP-1, and IRF5). Changes of IL1α and granulocyte colony-stimulating factor (G-CSF) in the blood, decrease in the count of regulatory T-lymphocytes, and an increase in the proportion of Th17 cells in the spleen. Increased number of intestinal microbial species, bacterial abundance, and diversity of flora. Significant increase in *Staphylococcus* abundance along with a significant decrease in *Parabacteroides* abundance.	Li 2020 [95]
Microplastics, phthalate esters	Mice	Male mice (Mus musculus CD-1) divided into 12 groups and exposed to MPs and MPs contaminated with phthalate esters; in vivo design	Virgin PE spheres of size between 45 and 53 μm and concentration of 0.2 g/L (about 1.5×10^5 particles/L) for 30 days of exposure	Disruption of intestinal permeability. Increased abundance of phylum Actinobacteria and genera *Lactobacillus*, *Adlercreutzia*, *Butyricimonas*, and *Parabacteroides*.	Deng 2020 [96]
Microplastics, nanoplastics	Mice	6-week-old C57BL/6 J mice; in vivo design	Combined exposure to PS-NP and PS-MP (50 nm, 500, and 5000 nm, respectively, at a concentration of 20 mL/kg body weight for 28 days)	Gut barrier dysfunction by apoptosis of epithelial cells through ROS production.	Liang 2021 [97]
Microplastics, nanoplastics	Human, mice	- CCD18-Co cells from normal human colon fibroblasts, human colon organoids; in vitro design - Seven-week-old male C57BL/6 mice; in vivo design	- Exposure to 50- and 100 nm MNP particles at varying concentrations for 48 h of incubation - 50 nm MNPs at the concentration at which the highest toxicity was found in colonic organoids, for 7 days	Concentrations of 5 mg/mL induced > 20% decrease in colonic organoid viability and increased expression of genes related to inflammation, apoptosis, and immunity. 50 nm MNPs accumulate in various mouse organs, including colon, liver, pancreas, and testes after 7 days of exposure.	Park 2023 [98]

Table 1. *Cont.*

Molecules	Species	Design	Size/Exposure	Effects	Study
Microplastics	Human	3D in vitro intestinal model comprising human intestinal epithelial cell lines Caco-2 and HT29-MTX-E12	Exposure to 50–500 μm MP at the concentration of 823.5–1380.0 μg/cm^2 for 24 h	No induction of cytotoxicity, nor pro-inflammatory response.	Lehner 2020 [99]
Microplastics	Human, mice/sheep	Murine and sheep blood and immune cells; human-derived cell lines; in vitro design	Polypropylene MPs (50–500 μm) at various concentrations	Induction of proinflammatory cytokines in a size- and concentration-dependent manner.	Hwang 2019 [100]
Microplastics	Mice	Six-week-old male and female ICR mice; in vivo design	40−48 μm PE-MPs (0.125, 0.5, 2 mg/day/mouse) by gavage to mice (10 mice/sex/dose) for 90 days	Increase in the number of blood neutrophils and immunoglobulin IgA levels, alteration of spleen lymphocytes.	Park 2020 [101]
Nanoplastics	Mice	Mice with chronic colitis; in vivo design	100 nm polystyrene nanospheres (PS-NPs, at concentrations of 1 mg/kg, 5 mg/kg, and 25 mg/kg) for 28 consecutive days	Increase in oxidative stress and intestinal inflammation by activating the MAPK signaling pathway.	Ma 2023 [102]
Microplastics	Mice	Male and female ICR mice; in vivo design	PS-MPs (0.5 μm and 5 μm) at a concentration of 100 μg/L and 1000 μg/L, from day 1 of gestation to the day of birth	Abundance of Actinobacteria increased, while that of Proteobacteria and Firmicutes remained unchanged.	Luo 2019 [103]
Microplastics	Mice	Seven-week-old male C57BL/6J mice; in vivo design	Oral exposure of 5 μm MPs (0.1 mg/day) for 33 days	Increased relative abundance of Proteobacteria. Decrease in *Bacteroides* and *Marvinbryantia* and increase in *Bifidobacterium*.	Jiang 2021 [104]
Microplastics, nanoplastics	Mice	C57/B6 mice (male, 8 weeks old); in vivo design	PS M/NPLs, and carboxyl-modified (PS-COOH) and aminomodified (PS-NH2) PS M/NPLs (70 nm, 5 μm in diameter), at a concentration between 2 mg and 0.2 mg/kg, for 28 days	Increased relative abundance of Proteobacteria. Increase of Verrucomicrobia at a high concentration. Reduced several short-chain fatty acid (SCFA)-producing genera.	Qiao 2021 [105]
Microplastics	Mice	4-week-old female mice (KM mice); in vivo design	PET-MPs (2 μm to 631 μm) at a concentration of 500 mg/kg for 28 days	Decreased abundances of Bacteroidetes and increased abundance of Firmicutes. Increased abundance of *Lactobacillus* and decreased abundance of *Parabacteroides*.	Liu 2022 [106]

MP: microplastic; NP: nanoplastic; MNPs: micro- and nanoplastics; PS: polystyrene; PE: polyethylene; PET: polyethylene terephthalate.

In 2018, Abdelkhaliq et al. showed no cytotoxicity of polystyrene (PS) particles (50 nm and 200 nm) on Caco-2 cells at a concentration of 250 mg/mL for 10 to 120 min of exposure [87]. Accordingly, with 1 to 30 mg/mL, laser-ablated, approximately 100 nm PET particles, no impact on Caco-2 cell viability and no inflammation was measured up to 24 h of incubation [88]. Similarly, Hesler et al., in 2019, showed the absence of toxicity at a

concentration below 100 mg/mL PS particles (between 40–52 nm and 457–477 nm) after 24 h of incubation [89]. A significant decrease of Caco-2 cell viability was only measured at very high concentrations of 4–10 μm PS particles (1×10^8 particles per mL) after 48 h of incubation. Furthermore, in investigating the effect of PS particles on the macrophage cell line THP-1, no effect on cell polarization was detected after particle exposure [91]. According to a recent study, when HRT-18 and CMT-93 epithelial cell lines were exposed to PS microparticles (with a diameter of 4.8–5.8 μm, a concentration of 1 mg/mL, and a time between 6 and 48 h), it resulted in a significant increase in cytotoxicity in both cell lines. However, only CMT-93 cells showed an increase in oxidative stress activity [92]. Moreover, after being tested at various concentrations for 48 h, polyethylene (PE) microplastics between 30 and 140 μm caused a significant reduction in Caco-2 cell viability at high concentrations (1000 mg/L) [90].

Notably, a recent comparative systematic analysis monitored the influence of small microplastics, of size 50–100 nm, on human colon cells and human colon organoids, and in vivo in a mouse model. According to the authors, the viability of colon organoids decreased by over 20% when exposed to concentrations of 5 mg/mL of MPs. This exposure also led to an increase in the expression of genes linked to inflammation, apoptosis, and immunity. Additionally, in vivo data from a murine model indicated that 50 nm MPs accumulated in several mouse organs, including the colon, after 7 days of exposure [98].

Several studies in mice exposed to PS microspheres have shown a transcriptional decrease in major genes related to mucin expression, such as mucin 1 (Muc1) and Klf4 [93,94], and to ion transport, such as cystic fibrosis transmembrane conductance regulator (Cftr), Na-K-2Cl cotransporter 1 (Nkcc1), Na+/H+ exchanger 3 (Nhe3), anoctamin 1 (Ano1), and solute carrier family 26 member 6 (Slc26a6) [93]. In a research study conducted on mouse models, it was found that exposure to a mixture of microplastics ranging from 1 μm to 10 μm in size, at a volume of 10 mL/kg, and for a total of one dose for three weeks, did not lead to any evidence of intestinal inflammation [91].

Accordingly, in a recent study, mice were fed with 5 μm pristine and fluorescent polystyrene MP for 6 weeks [93]. The results revealed that PS-MPs were observed in the intestine of mice, and reduced the intestinal mucus secretion, thus causing damage to the intestinal barrier function. Similarly, male mice exposed to polystyrene MP from 0.5 and 50 μm at 1000 μg/L for 5 weeks exhibited decreased intestinal mucus secretion following oral exposure [94]. On the other hand, when mice were exposed to different amounts of polyethylene microplastics, it led to histological inflammation in their colon and duodenum. Specifically, exposure to PE-MP (10–150 μm) at various concentrations (2, 20, and 200 μg/g for 5 weeks) resulted in the increased secretion of proinflammatory cytokines and higher levels of toll-like receptor 4 (TLR4), c-Jun, and interferon regulatory factor 5 [95].

Virgin PE spheres with a size between 45 and 53 μm and a concentration of 0.2 g/L (1.5×10^5 particles/L) after 30 days of exposure have been found to cause impaired intestinal permeability in mouse models [96]. Another study on mammals confirms that exposure to MNPs may cause adverse effects on the intestinal system. When exposed to PS-NPs and PS-MPs (50 nm, 500 nm, and 5000 nm at a concentration of 20 mL/kg body weight for 28 days), there was a combined exposure that caused intestinal barrier dysfunction by the apoptosis of epithelial cells through ROS production in the mouse model [97]

In terms of toxicity, a mention must be made of the ability of MPs to transport pollutants and plasticizers. In this review, considering the focus of this paper; we will only refer to pollutants and plasticizers having the greatest potential to harm the gut system.

Chemical compounds called plasticizers can expose humans through occupational exposure, product use, or transfer from plastic packaging [33,107]. Exposure to these compounds can occur through ingestion, inhalation, and skin contact [108]. Among the various plasticizers, phthalates (PAEs) are known to be harmful to human health according to several studies. This group of chemicals is a major concern as they have been identified as endocrine-metabolic disruptors, which can affect the reproductive system based on available evidence from human epidemiological studies [109]. Numerous reports

have found high levels of phthalate contamination in drinking water and various foods, including meat, oil, fats, dairy products, and even infant formula [110–112]. This suggests that these substances can easily enter the food chain, and ingestion may be the primary route of exposure [113,114]. Research has shown that the ingestion of various PAEs can lead to different health problems, such as reproductive, hepatic, cardiac, and neurodevelopmental disorders [115–118].

Although little research exists on how phthalates directly affect the intestinal system, these harmful substances are commonly found in contaminated food and water, making it highly likely that they negatively impact the gastrointestinal tract and gut microbiota.

Exposure of female CD-1 mice to phthalates at doses ranging from 0.2 to 200 mg/kg for 10–14 days caused colonic damage and inflammation. This was due to the dysregulation of the tight junction gene (Zo-3), cell cycle regulatory gene (Ccnb1), and cytokine levels (sICAM-1 and TNF-α) [119]. Additionally, Xiong et al. (2020) and Fu et al. (2021) observed elevated serum lipopolysaccharides (LPS) levels in mice exposed to PAEs, indicating epithelial barrier disruption and intestinal permeability [117,120]. Similarly, Deng et al. (2020) also reported reduced serum diaminoxidase (DAO) activity in CD-1 mice exposed to PAEs, which is an important indicator of impaired intestinal function [96]. Lastly, recent evidence suggests that the gut microbiota, due to its complex interaction with the intestinal epithelium and barrier, may play a significant role in influencing the local and systemic toxicity of these molecules [121].

It is widely acknowledged that PAEs and other plasticizers, including Bisphenol A, have the potential to negatively impact human health. In light of this, the European Food Safety Authority (EFSA) has recently advised lowering the acceptable daily intake (TDI) of such substances to safer levels [122,123].

Micro- and nanoplastics can also act as vectors for toxic heavy metals or other pollutants that can be released [124] into the environment and lead to health risks [38].

One example is chromium (Cr), which has a greater potential to adsorb on microplastics than other heavy metals. Microplastics can carry 19–7970 ng of Cr per g of microplastics [124]. When Cr (IV) enters the body, it causes DNA damage in various tissues at high acute doses or with chronic oral exposure [125]. To study the effect of the ingestion of adsorbed Cr on microplastics, ~150 μ PE, PP, PVC, and PS-MP contaminated with Cr at concentrations commensurate with water Cr-MP levels were prepared [124]. Using an in vitro method to model the entire digestive system, the researchers found that Cr (IV) availability was high for PLA in the stomach, small intestine, and large intestine. However, the risk quotients for adults and children calculated from the bioavailability did not raise concerns about the carcinogenicity.

3.2. MNP Gut Immunological Impact

The immune system present in the intestine is continuously exposed to external antigens, which are derived from food and non-pathogenic micro-organisms that need to be tolerated immunologically. However, the intestinal immune system also needs to be prepared to respond to pathogenic micro-organisms and external toxins. This balance is maintained by the equilibrium between pro- and anti-inflammatory stimuli, which involves innate lymphocytes, myeloid cells, and T- and B-lymphocytes residing in the lamina propria of the gut epithelium and draining in the mesenteric lymph nodes [126].

After being exposed to MNPs, immune cells trigger a significant modulation at the transcriptional level, affecting enzyme levels and cytokine release. Several studies, both on invertebrates and vertebrates, revealed an immune-toxic effect caused by nano- and microplastics on the intestinal immune system.

Exposure to PS nanoparticles has been found to cause higher hemocyte counts in *Daphnia magna*, while also decreasing the total antioxidant capacity and increasing DNA damage in mussels [127,128]. Amino-modified PS nanoparticles, on the other hand, have been shown to induce hemocyte changes in mussels, depending on the duration of exposure [129,130]. Additionally, exposure to PS microbeads or nanoparticles has been found to

increase the production of oxygen reactive and nitrogen species, result in higher hemocyte mortality, and modify several enzymes related to the immune system, such as acid phosphatase, alkaline phosphatase, lysozyme, and phenoloxidase, depending on the duration and dose of exposure [131–135]. Studies have also shown that PS nanoparticles cause more damage than PS microparticles [134,135].

Studies on vertebrates have revealed some interesting findings. Exposure to PS nanoparticles led to a dose-dependent increase in myeloperoxidase activity and the release of neutrophil extracellular traps in fathead minnows *Pimephales promelas*. Similarly, polycarbonate microplastics dose-dependently disrupted neutrophil functions [136]. Exposure to PE microparticles in carp impaired the activity of the complement system and immunity-related enzymes [3]. Furthermore, in zebrafish, exposure to PE and PS particles reduced the liver transcript levels of two immune genes, leukotriene B4 receptor (ltb4r) and interferon-induced transmembrane protein (ifitm1) [86]. Furthermore, microplastics in the gastrointestinal tract have been found to upregulate the expression of T-cell receptors β and δ (TCRβ and TCRδ) and IgM in the spleen of *Scyliorhinus canicula* [137].

Lehner et al. (2020) developed a 3D in vitro intestinal model comprising human intestinal epithelial cell lines Caco-2 and HT29-MTX-E12 to study the effects of ingested MPs such as the 50-MP polymer of 500 μm representing tire wear and polyolefins at the concentration of 823.5–1380.0 μg/cm^2. Although the results showed some changes in the levels of inflammatory cytokines (IL-8, TNFα, and IL-1β) and barrier integrity, these changes were not significant [99]. In contrast, other forms of MPs, polypropylene MPs (50–500 μm), have been shown to induce immune responses by triggering the production of proinflammatory cytokines such as IL-6 and TNF α in a size- and concentration-dependent manner [100].

A study on mice models has shown that exposure to PE microparticles can cause changes in the levels of certain proteins such as IL1α and granulocyte colony-stimulating factor (G-CSF) in the blood, a decrease in the count of regulatory T-lymphocytes, and an increase in the proportion of Th17 cells in the spleen [95]. In this study, it was found that high concentrations (600 μg/day) of PE-MPs (10–150 μm) caused inflammatory reactions by increasing the expression of Toll-like receptor 4 (TLR4), Activator Protein 1 (AP-1), and Interferon regulatory factor 5 (IRF5). The exposure to MP also led to a significant increase in the serum level of IL-1α and a decrease in Th17 and Treg cells in CD4+ T cells [95]. Additionally, PE microplastic exposure (40–48 μm per dosing volume of 200 μL/day for 90 days) can lead to an increase in the number of blood neutrophils and immunoglobulin IgA levels in female mice and an alteration of spleen lymphocytes in both dams and offspring [101].

While there is evidence of the effects of MNPs on the immune system, most studies have focused solely on the innate immune response, and the impact of MNPs on the adaptive immune response remains unclear.

A recent study [138] found that there is a connection between microplastics (MPs) in feces and inflammatory bowel disease (IBD). The study discovered that the fecal concentration of MPs in IBD patients was significantly higher (41.8 items/g dm) than in healthy individuals (28.0 items/g dm), including 15 different types of MPs. Among the MPs found, polyethylene terephthalate (22.3–34.0%) and polyamide (8.9–12.4%) were the most dominant types. The researchers observed that the primary shapes of the detected MPs were sheets and fibers [138]. Additionally, the study showed that there is a positive correlation between the concentration of MPs and the activity level of IBD, suggesting that MP exposure may be related to the disease process, or that IBD could promote the retention of MPs.

Indeed, further recent evidence in mouse models confirms these suspicions. It was observed that PS-NPs aggravate inflammation and intestinal injury in mice with chronic colitis [102]. Specifically, mice subjected to sodium dextran sulfate (DSS) exposures were subsequently fed via gastric tube with water containing 100 nm polystyrene nanospheres (PS-NPs, at concentrations of 1 mg/kg, 5 mg/kg, and 25 mg/kg) for 28 consecutive days.

The results showed that PS-NPs exacerbated intestinal inflammation by activating the MAPK signaling pathway and also aggravated inflammation and oxidative stress in mice with chronic colitis.

These findings show that the intestinal immune system is altered by exposure to microplastics; however, further studies, especially in species more closely related to humans, are warranted.

3.3. MNP Effects on Gut Microbiota

The human gut is home to numerous communities of micro-organisms, collectively referred to as the "gut microbiota". This microbiota comprises over 250 species of viruses, fungi, bacteria, and archaea, and is a dynamic system that changes over the course of a human's life. The relationship between the gut microbiota and the host is mutually beneficial, as the former plays a crucial role in several physiological and pathological pathways of human life [139]. Human gut microbiota is primarily composed of five bacterial phyla: Firmicutes (60% to 80%), Bacteroidetes (20% to 40%), Verrucomicrobia, Actinobacteria, and a lesser extent of Proteobacteria; and one Archea phyla, the Euryarchaeota [139]. The gut microbiota is a crucial component of the gut ecosystem that plays a vital role in human health. It helps in the formation and maturation of immunity, acts as a barrier against pathogens, facilitates the absorption of nutrients and drugs, and regulates metabolic intake [140]. When there is an imbalance in the gut microbiota, it can lead to various gastrointestinal and extraintestinal disorders [140]. As a result, several therapeutic approaches, such as fecal microbiota transplantation [141], are increasingly being investigated for the treatment of microbiome-based disorders.

There is a lack of data concerning the effects of MNPs on the gut microbiota in humans. However, studies conducted on mammals have shown that both short- and long-term exposure to MNPs can cause modifications in microbial communities. Dysbiosis, or an imbalance in the gut microbiota, is a common finding in murine mole studies, with reduced alpha- and beta-diversity, and a loss of resilience. This can lead to frequent outbreaks of pathogens and metabolic disorders, both locally and systemically [93,95]. Particularly, at the phylum level, exposure to PS particles caused changes in the abundance of Bacteroidetes, Firmicutes, Actinobacteria, and Proteobacteria. At the genus level, variations in the abundance of *Staphylococcus*, *Clostridium*, and *Bacteroides* were observed when compared to animals that were not exposed to PS particles. In addition, up to 15 types of bacteria were affected by exposure to MPs, in particular, *Bifidobacterium*, *Prevotella*, *Veillonella*, Actinobacteria, and *Ruminococcus*.

Discrepancies were found regarding the abundance of Proteobacteria. In 2019, Lu et al. reported a decrease or increase in its abundance, while Jin et al. discovered a relative reduction in the abundance of Proteobacteria after PS microparticle exposure [93,94].

Conversely, in the same year, Luo et al. (2019) discovered that the Actinobacteria abundance increased while the abundance of Proteobacteria and Firmicutes remained unchanged in mice that were exposed to PS-MPs (0.5 μm and 5 μm) at a concentration of 100 μg/L and 1000 μg/L during their gestation and lactation period [103].

On the other hand, in 2021, Jiang et al. (2021) noted, following a 33-day period of the ingestion of 0.1 mg/kg MPs (5 μm), a shift in the relative abundance of bacterial taxa in mice models [104]. Specifically, there was a significant increase in the presence of Proteobacteria, while *Bacteroides* and *Marvinbryantia* exhibited a marked decrease. Additionally, *Bifidobacterium* also exhibited an increase. Qiao et al. confirmed mice exposed to PS-MNPs (70 nm, 5 μm in diameter) at a concentration between 2 mg and 0.2 mg kg^{-1} for 28 days experienced an increase in the relative abundance of Proteobacteria and Verrucomicrobia, while the major short-chain fatty acid (SCFA)-producing genera decreased in abundance [105].

In a study conducted by Liu et al. in 2022 [106], female mice were exposed to polyethylene terephthalate (PET) microplastics (ranging from 2 μm to 631 μm) at a concentration of 500 mg/kg for a period of 28 days. The study reported a decrease in the abundance of Bac-

teroidetes and an increase in the abundance of Firmicutes, which was accompanied by an increase in the abundance of *Lactobacillus* and a decrease in the abundance of *Parabacteroides*.

Two different studies focusing on PE microplastic exposure in mice, respectively, found an increase in the abundance of Firmicutes and Melainabacteria phyla and *Staphylococcus* genera, with a decrease in the abundance of Bacteroidetes phylum and *Parabacteroides* genera [95], and an increase in the abundance of the Actinobacteria phylum and *Lactobacillus*, *Adlercreutzia*, *Butyricimonas*, and *Parabacteroides* genera [96].

It is important to note that exposure to MNPs has been shown to reduce the abundance of bacteria that are known to promote tight junction functions. This reduction may have additional indirect toxic effects due to the dysbiosis of the gut microbiota [105].

Although plastic particles are inert to biodegradation due to their hydrophobic nature, high molecular weight, and long polymer chain, some micro-organisms ingest these polymers and convert them into environmentally friendly carbon compounds [142–144].

Polymer biodegradation is a process that occurs due to micro-organisms present in three domains of life, namely, Bacteria, Archaea, and Eukarya. Among the different kingdoms, fungi and bacteria are the most vital players in biodegradation processes in natural environments. The effectiveness of micro-organisms in degrading a specific type of plastic depends on the environmental conditions and the plastic typologies [145,146]. *Arthrobacter*, *Bacillus*, *Micrococcus*, *Pseudomonas*, *Corynebacterium*, *Streptomyces*, and *Nocardia* are the most commonly studied bacteria for their ability to degrade various types of plastics [147–149]. Besides free-living micro-organisms in the environment, the gut microbiota is an important driver of MNPs degradation, with most of the attention focused on insects and their larvae [150]. Indeed, several studies showed that MPs biodegradation does not occur after antibiotic treatment in mealworms, thus suggesting a crucial role played by the gut microbiota [151].

However, little is known about the microbial degradation capacity in mammals, probably due to the lack of appropriate high-resolution analytical methods to quantify small MPs and NPs and chemical intermediates in animal and human feces. Similarly, research regarding the microbial degradation of MPs and the human gut microbiota is still scarce; however, numerous plastic-degrading bacteria described in insects or larvae are part of the core of human gut microbiota, particularly, several potentially pathogenic Proteobacteria, such as *Enterobacteriaceae*, *Enterococcaceae*, *Listeria*, *Pseudomonas*, and *Klebsiella*, but also *Lactococcus* [152,153].

4. Conclusions

Annually, the global production of plastic waste amounts to millions of tons, a considerable quantity which disintegrates and accumulates in the form of minute particles that pollute and disseminate throughout terrestrial environments. Ingestion is a prevalent means of exposure of animals and humans to micro- and nanoplastics that can accumulate in the intestinal system to a degree and in a manner that remain incompletely understood.

Studies conducted in vitro on human cell lines have shown conflicting results regarding the toxicity of MNPs on the intestinal system. The discrepancies could be due to the different dosages of particles used in each study. Additionally, the various treatment periods and particle concentrations employed could also contribute to the conflicting nature of the findings. Furthermore, the studies cited only assess the short-term effects of MNPs on different endpoints, while possible long-term effects remain unexplored.

In contrast, studies conducted on mammals suggest that MNPs may have adverse effects in terms of intestinal cells toxicity, immunotoxicity, and dysbiosis. Nonetheless, the use of various study designs generates a degree of unclearness, and the absence of a definitive classification system for plastic waste based on parameters such as size, shape, and physical and chemical properties further complicates the issue. Additionally, the toxicological studies cited in this context do not account for the impact of realistic environmental exposure, nor do they consider the possible interactions between plastics and other pollutants.

Thus, we understand how these studies are not yet robust enough to determine their intestinal toxicity on mammals and humans with any degree of certainty. To gain a better understanding of the impact of MNPs ingestion on human gut health, it is essential to introduce validated and shared analytical methods. These arrangements will allow animal and cell studies to understand toxicological effects and will allow reference values to be generated to assess dietary intake and help stratify dietary risk. Observational and biomarker-based studies, on the other hand, will be able to help us unravel the real adverse effects of these particles on human gut health.

In conclusion, further studies and analytical methodologies are needed to characterize the real toxicological effects of MPNs on the intestinal human system and the precise role of the gut microbiota as a potential key player in this context.

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References

1. Hodge, P.; Hellwich, K.-H.; Hiorns, R.C.; Jones, R.G.; Kahovec, J.; Luscombe, C.K.; Purbrick, M.D.; Wilks, E.S. A Concise Guide to Polymer Nomenclature for Authors of Papers and Reports in Polymer Science and Technology (IUPAC Technical Report). *Pure Appl. Chem.* **2020**, *92*, 797–813. [CrossRef]
2. Singh Jadaun, J.; Bansal, S.; Sonthalia, A.; Rai, A.K.; Singh, S.P. Biodegradation of Plastics for Sustainable Environment. *Bioresour. Technol.* **2022**, *347*, 126697. [CrossRef]
3. Banaee, M.; Soltanian, S.; Sureda, A.; Gholamhosseini, A.; Haghi, B.N.; Akhlaghi, M.; Derikvandy, A. Evaluation of Single and Combined Effects of Cadmium and Micro-Plastic Particles on Biochemical and Immunological Parameters of Common Carp (Cyprinus Carpio). *Chemosphere* **2019**, *236*, 124335. [CrossRef]
4. Rhodes, C.J. Plastic Pollution and Potential Solutions. *Sci. Prog.* **2018**, *101*, 207–260. [CrossRef] [PubMed]
5. Shahul Hamid, F.; Bhatti, M.S.; Anuar, N.; Anuar, N.; Mohan, P.; Periathamby, A. Worldwide Distribution and Abundance of Microplastic: How Dire Is the Situation? *Waste Manag. Res. J. A Sustain. Circ. Econ.* **2018**, *36*, 873–897. [CrossRef]
6. Wright, S.L.; Ulke, J.; Font, A.; Chan, K.L.A.; Kelly, F.J. Atmospheric Microplastic Deposition in an Urban Environment and an Evaluation of Transport. *Environ. Int.* **2020**, *136*, 105411. [CrossRef]
7. Alimba, C.G.; Faggio, C. Microplastics in the Marine Environment: Current Trends in Environmental Pollution and Mechanisms of Toxicological Profile. *Environ. Toxicol. Pharmacol.* **2019**, *68*, 61–74. [CrossRef]
8. Naegelen, I. Mikroplastik Als Gesundheitsgefahr Für Den Menschen. *Biol. Unserer Zeit* **2019**, *49*, 241–242. [CrossRef]
9. Toussaint, B.; Raffael, B.; Angers-Loustau, A.; Gilliland, D.; Kestens, V.; Petrillo, M.; Rio-Echevarria, I.M.; Van den Eede, G. Review of Micro- and Nanoplastic Contamination in the Food Chain. *Food Addit. Contam. Part A* **2019**, *36*, 639–673. [CrossRef]
10. Lambert, S.; Wagner, M. *Microplastics Are Contaminants of Emerging Concern in Freshwater Environments: An Overview*; Springer International Publishing: Berlin/Heidelberg, Germany, 2018; pp. 1–23.
11. Marcelino, R.C.; Cardoso, R.M.; Domingues, E.L.B.C.; Gonçalves, R.V.; Lima, G.D.A.; Novaes, R.D. The Emerging Risk of Microplastics and Nanoplastics on the Microstructure and Function of Reproductive Organs in Mammals: A Systematic Review of Preclinical Evidence. *Life Sci.* **2022**, *295*, 120404. [CrossRef]
12. Osman, A.I.; Hosny, M.; Eltaweil, A.S.; Omar, S.; Elgarahy, A.M.; Farghali, M.; Yap, P.-S.; Wu, Y.-S.; Nagandran, S.; Batumalaie, K.; et al. Microplastic Sources, Formation, Toxicity and Remediation: A Review. *Environ. Chem. Lett.* **2023**, *21*, 2129–2169. [CrossRef]
13. Wang, J.; Liu, X.; Li, Y.; Powell, T.; Wang, X.; Wang, G.; Zhang, P. Microplastics as Contaminants in the Soil Environment: A Mini-Review. *Sci. Total Environ.* **2019**, *691*, 848–857. [CrossRef]
14. Xu, B.; Liu, F.; Cryder, Z.; Huang, D.; Lu, Z.; He, Y.; Wang, H.; Lu, Z.; Brookes, P.C.; Tang, C.; et al. Microplastics in the Soil Environment: Occurrence, Risks, Interactions and Fate—A Review. *Crit. Rev. Environ. Sci. Technol.* **2020**, *50*, 2175–2222. [CrossRef]

15. Eriksen, M.; Lebreton, L.C.M.; Carson, H.S.; Thiel, M.; Moore, C.J.; Borerro, J.C.; Galgani, F.; Ryan, P.G.; Reisser, J. Plastic Pollution in the World's Oceans: More than 5 Trillion Plastic Pieces Weighing over 250,000 Tons Afloat at Sea. *PLoS ONE* **2014**, *9*, e111913. [CrossRef] [PubMed]
16. Schwarz, A.E.; Ligthart, T.N.; Boukris, E.; van Harmelen, T. Sources, Transport, and Accumulation of Different Types of Plastic Litter in Aquatic Environments: A Review Study. *Mar. Pollut. Bull.* **2019**, *143*, 92–100. [CrossRef]
17. Bouwmeester, H.; Hollman, P.C.H.; Peters, R.J.B. Potential Health Impact of Environmentally Released Micro- and Nanoplastics in the Human Food Production Chain: Experiences from Nanotoxicology. *Environ. Sci. Technol.* **2015**, *49*, 8932–8947. [CrossRef]
18. Prata, J.C. Airborne Microplastics: Consequences to Human Health? *Environ. Pollut.* **2018**, *234*, 115–126. [CrossRef]
19. Chen, G.; Feng, Q.; Wang, J. Mini-Review of Microplastics in the Atmosphere and Their Risks to Humans. *Sci. Total Environ.* **2020**, *703*, 135504. [CrossRef]
20. Liu, K.; Wang, X.; Fang, T.; Xu, P.; Zhu, L.; Li, D. Source and Potential Risk Assessment of Suspended Atmospheric Microplastics in Shanghai. *Sci. Total Environ.* **2019**, *675*, 462–471. [CrossRef]
21. Allen, S.; Allen, D.; Phoenix, V.R.; Le Roux, G.; Durántez Jiménez, P.; Simonneau, A.; Binet, S.; Galop, D. Atmospheric Transport and Deposition of Microplastics in a Remote Mountain Catchment. *Nat. Geosci.* **2019**, *12*, 339–344. [CrossRef]
22. Zhang, Q.; Zhao, Y.; Du, F.; Cai, H.; Wang, G.; Shi, H. Microplastic Fallout in Different Indoor Environments. *Environ. Sci. Technol.* **2020**, *54*, 6530–6539. [CrossRef] [PubMed]
23. Vianello, A.; Jensen, R.L.; Liu, L.; Vollertsen, J. Simulating Human Exposure to Indoor Airborne Microplastics Using a Breathing Thermal Manikin. *Sci. Rep.* **2019**, *9*, 8670. [CrossRef] [PubMed]
24. Kosuth, M.; Mason, S.A.; Wattenberg, E.V. Anthropogenic Contamination of Tap Water, Beer, and Sea Salt. *PLoS ONE* **2018**, *13*, e0194970. [CrossRef] [PubMed]
25. Gasperi, J.; Wright, S.L.; Dris, R.; Collard, F.; Mandin, C.; Guerrouache, M.; Langlois, V.; Kelly, F.J.; Tassin, B. Microplastics in Air: Are We Breathing It in? *Curr. Opin. Environ. Sci. Health* **2018**, *1*, 1–5. [CrossRef]
26. Enyoh, C.E.; Shafea, L.; Verla, A.W.; Verla, E.N.; Qingyue, W.; Chowdhury, T.; Paredes, M. Microplastics Exposure Routes and Toxicity Studies to Ecosystems: An Overview. *Environ. Anal. Health Toxicol.* **2020**, *35*, e2020004. [CrossRef]
27. Yee, M.S.-L.; Hii, L.-W.; Looi, C.K.; Lim, W.-M.; Wong, S.-F.; Kok, Y.-Y.; Tan, B.-K.; Wong, C.-Y.; Leong, C.-O. Impact of Microplastics and Nanoplastics on Human Health. *Nanomaterials* **2021**, *11*, 496. [CrossRef]
28. Amereh, F.; Babaei, M.; Eslami, A.; Fazelipour, S.; Rafiee, M. The Emerging Risk of Exposure to Nano(Micro)Plastics on Endocrine Disturbance and Reproductive Toxicity: From a Hypothetical Scenario to a Global Public Health Challenge. *Environ. Pollut.* **2020**, *261*, 114158. [CrossRef]
29. Lim, X. Microplastics Are Everywhere—But Are They Harmful? *Nature* **2021**, *593*, 22–25. [CrossRef]
30. Wagner, S.; Reemtsma, T. Things We Know and Don't Know about Nanoplastic in the Environment. *Nat. Nanotechnol.* **2019**, *14*, 300–301. [CrossRef]
31. Guerrera, M.C.; Aragona, M.; Porcino, C.; Fazio, F.; Laurà, R.; Levanti, M.; Montalbano, G.; Germanà, G.; Abbate, F.; Germanà, A. Micro and Nano Plastics Distribution in Fish as Model Organisms: Histopathology, Blood Response and Bioaccumulation in Different Organs. *Appl. Sci.* **2021**, *11*, 5768. [CrossRef]
32. Sökmen, T.Ö.; Sulukan, E.; Türkoğlu, M.; Baran, A.; Özkaraca, M.; Ceyhun, S.B. Polystyrene Nanoplastics (20 Nm) Are Able to Bioaccumulate and Cause Oxidative DNA Damages in the Brain Tissue of Zebrafish Embryo (Danio Rerio). *Neurotoxicology* **2020**, *77*, 51–59. [CrossRef]
33. Hahladakis, J.N.; Velis, C.A.; Weber, R.; Iacovidou, E.; Purnell, P. An Overview of Chemical Additives Present in Plastics: Migration, Release, Fate and Environmental Impact during Their Use, Disposal and Recycling. *J. Hazard. Mater.* **2018**, *344*, 179–199. [CrossRef]
34. Barrows, A.P.W.; Cathey, S.E.; Petersen, C.W. Marine Environment Microfiber Contamination: Global Patterns and the Diversity of Microparticle Origins. *Environ. Pollut.* **2018**, *237*, 275–284. [CrossRef]
35. Gago, J.; Carretero, O.; Filgueiras, A.V.; Viñas, L. Synthetic Microfibers in the Marine Environment: A Review on Their Occurrence in Seawater and Sediments. *Mar. Pollut. Bull.* **2018**, *127*, 365–376. [CrossRef]
36. Woods, M.N.; Stack, M.E.; Fields, D.M.; Shaw, S.D.; Matrai, P.A. Microplastic Fiber Uptake, Ingestion, and Egestion Rates in the Blue Mussel (Mytilus Edulis). *Mar. Pollut. Bull.* **2018**, *137*, 638–645. [CrossRef]
37. Rebelein, A.; Int-Veen, I.; Kammann, U.; Scharsack, J.P. Microplastic Fibers—Underestimated Threat to Aquatic Organisms? *Sci. Total Environ.* **2021**, *777*, 146045. [CrossRef]
38. Banerjee, A.; Shelver, W.L. Micro- and Nanoplastic Induced Cellular Toxicity in Mammals: A Review. *Sci. Total Environ.* **2021**, *755*, 142518. [CrossRef]
39. Bhattacharjee, S.; Ershov, D.; Islam, M.A.; Kämpfer, A.M.; Maslowska, K.A.; van der Gucht, J.; Alink, G.M.; Marcelis, A.T.M.; Zuilhof, H.; Rietjens, I.M.C.M. Role of Membrane Disturbance and Oxidative Stress in the Mode of Action Underlying the Toxicity of Differently Charged Polystyrene Nanoparticles. *RSC Adv.* **2014**, *4*, 19321–19330. [CrossRef]
40. Feng, L.-J.; Li, J.-W.; Xu, E.G.; Sun, X.-D.; Zhu, F.-P.; Ding, Z.; Tian, H.; Dong, S.-S.; Xia, P.-F.; Yuan, X.-Z. Short-Term Exposure to Positively Charged Polystyrene Nanoparticles Causes Oxidative Stress and Membrane Destruction in Cyanobacteria. *Environ. Sci. Nano* **2019**, *6*, 3072–3079. [CrossRef]
41. Fröhlich, E. The Role of Surface Charge in Cellular Uptake and Cytotoxicity of Medical Nanoparticles. *Int. J. Nanomed.* **2012**, 5577–5591. [CrossRef]

42. Hollóczki, O.; Gehrke, S. Can Nanoplastics Alter Cell Membranes? *ChemPhysChem* **2020**, *21*, 9–12. [CrossRef] [PubMed]
43. Wang, F.; Salvati, A.; Boya, P. Lysosome-Dependent Cell Death and Deregulated Autophagy Induced by Amine-Modified Polystyrene Nanoparticles. *Open Biol.* **2018**, *8*, 170271. [CrossRef] [PubMed]
44. Yong, C.; Valiyaveettil, S.; Tang, B. Toxicity of Microplastics and Nanoplastics in Mammalian Systems. *Int. J. Environ. Res. Public. Health* **2020**, *17*, 1509. [CrossRef]
45. Rubio, L.; Marcos, R.; Hernández, A. Potential Adverse Health Effects of Ingested Micro- and Nanoplastics on Humans. Lessons Learned from in Vivo and in Vitro Mammalian Models. *J. Toxicol. Environ. Health Part B* **2020**, *23*, 51–68. [CrossRef]
46. Zheng, T.; Yuan, D.; Liu, C. Molecular Toxicity of Nanoplastics Involving in Oxidative Stress and Desoxyribonucleic Acid Damage. *J. Mol. Recognit.* **2019**, *32*, e2804. [CrossRef]
47. Liu, X.; Tian, X.; Xu, X.; Lu, J. Design of a Phosphinate-Based Bioluminescent Probe for Superoxide Radical Anion Imaging in Living Cells. *Luminescence* **2018**, *33*, 1101–1106. [CrossRef]
48. Hwang, J.; Choi, D.; Han, S.; Jung, S.Y.; Choi, J.; Hong, J. Potential Toxicity of Polystyrene Microplastic Particles. *Sci. Rep.* **2020**, *10*, 7391. [CrossRef]
49. Auta, H.S.; Emenike, C.U.; Fauziah, S.H. Distribution and Importance of Microplastics in the Marine Environment: A Review of the Sources, Fate, Effects, and Potential Solutions. *Environ. Int.* **2017**, *102*, 165–176. [CrossRef]
50. Horton, A.A.; Walton, A.; Spurgeon, D.J.; Lahive, E.; Svendsen, C. Microplastics in Freshwater and Terrestrial Environments: Evaluating the Current Understanding to Identify the Knowledge Gaps and Future Research Priorities. *Sci. Total Environ.* **2017**, *586*, 127–141. [CrossRef]
51. Cox, K.D.; Covernton, G.A.; Davies, H.L.; Dower, J.F.; Juanes, F.; Dudas, S.E. Human Consumption of Microplastics. *Environ. Sci. Technol.* **2019**, *53*, 7068–7074. [CrossRef]
52. Kutralam-Muniasamy, G.; Pérez-Guevara, F.; Elizalde-Martínez, I.; Shruti, V.C. Branded Milks—Are They Immune from Microplastics Contamination? *Sci. Total Environ.* **2020**, *714*, 136823. [CrossRef] [PubMed]
53. Paul, M.B.; Stock, V.; Cara-Carmona, J.; Lisicki, E.; Shopova, S.; Fessard, V.; Braeuning, A.; Sieg, H.; Böhmert, L. Micro- and Nanoplastics—Current State of Knowledge with the Focus on Oral Uptake and Toxicity. *Nanoscale Adv.* **2020**, *2*, 4350–4367. [CrossRef] [PubMed]
54. Liebezeit, G.; Liebezeit, E. Non-Pollen Particulates in Honey and Sugar. *Food Addit. Contam. Part A* **2013**, *30*, 2136–2140. [CrossRef] [PubMed]
55. Liebezeit, G.; Liebezeit, E. Synthetic Particles as Contaminants in German Beers. *Food Addit. Contam. Part A* **2014**, *31*, 1574–1578. [CrossRef]
56. Yang, D.; Shi, H.; Li, L.; Li, J.; Jabeen, K.; Kolandhasamy, P. Microplastic Pollution in Table Salts from China. *Environ. Sci. Technol.* **2015**, *49*, 13622–13627. [CrossRef]
57. Bai, Z.; Wang, N.; Wang, M. Effects of Microplastics on Marine Copepods. *Ecotoxicol. Environ. Saf.* **2021**, *217*, 112243. [CrossRef]
58. Hasan, J.; Islam, S.M.M.; Alam, M.S.; Johnson, D.; Belton, B.; Hossain, M.A.R.; Shahjahan, M. Presence of Microplastics in Two Common Dried Marine Fish Species from Bangladesh. *Mar. Pollut. Bull.* **2022**, *176*, 113430. [CrossRef]
59. Ahmadi, A.; Moore, F.; Keshavarzi, B.; Soltani, N.; Sorooshian, A. Potentially Toxic Elements and Microplastics in Muscle Tissues of Different Marine Species from the Persian Gulf: Levels, Associated Risks, and Trophic Transfer. *Mar. Pollut. Bull.* **2022**, *175*, 113283. [CrossRef]
60. Novotna, K.; Cermakova, L.; Pivokonska, L.; Cajthaml, T.; Pivokonsky, M. Microplastics in Drinking Water Treatment—Current Knowledge and Research Needs. *Sci. Total Environ.* **2019**, *667*, 730–740. [CrossRef]
61. Danopoulos, E.; Twiddy, M.; Rotchell, J.M. Microplastic Contamination of Drinking Water: A Systematic Review. *PLoS ONE* **2020**, *15*, e0236838. [CrossRef]
62. Schwabl, P.; Köppel, S.; Königshofer, P.; Bucsics, T.; Trauner, M.; Reiberger, T.; Liebmann, B. Detection of Various Microplastics in Human Stool. *Ann. Intern. Med.* **2019**, *171*, 453–457. [CrossRef] [PubMed]
63. Van Raamsdonk, L.W.D.; van der Zande, M.; Koelmans, A.A.; Hoogenboom, R.L.A.P.; Peters, R.J.B.; Groot, M.J.; Peijnenburg, A.A.C.M.; Weesepoel, Y.J.A. Current Insights into Monitoring, Bioaccumulation, and Potential Health Effects of Microplastics Present in the Food Chain. *Foods* **2020**, *9*, 72. [CrossRef] [PubMed]
64. Powell, J.J.; Faria, N.; Thomas-McKay, E.; Pele, L.C. Origin and Fate of Dietary Nanoparticles and Microparticles in the Gastrointestinal Tract. *J. Autoimmun.* **2010**, *34*, J226–J233. [CrossRef] [PubMed]
65. Coméra, C.; Cartier, C.; Gaultier, E.; Catrice, O.; Panouille, Q.; El Hamdi, S.; Tirez, K.; Nelissen, I.; Théodorou, V.; Houdeau, E. Jejunal Villus Absorption and Paracellular Tight Junction Permeability Are Major Routes for Early Intestinal Uptake of Food-Grade TiO2 Particles: An in Vivo and Ex Vivo Study in Mice. *Part. Fibre Toxicol.* **2020**, *17*, 26. [CrossRef]
66. Smith, M.; Thomas, N.; Jenkins, P.; Miller, N.; Cremaschi, D.; Porta, C. Selective Transport of Microparticles across Peyer's Patch Follicle-associated M Cells from Mice and Rats. *Exp. Physiol.* **1995**, *80*, 735–743. [CrossRef]
67. Galloway, T.S. Micro- and Nano-Plastics and Human Health. In *Marine Anthropogenic Litter*; Springer International Publishing: Cham, Switzerland, 2015; pp. 343–366.
68. Dawson, A.L.; Kawaguchi, S.; King, C.K.; Townsend, K.A.; King, R.; Huston, W.M.; Bengtson Nash, S.M. Turning Microplastics into Nanoplastics through Digestive Fragmentation by Antarctic Krill. *Nat. Commun.* **2018**, *9*, 1001. [CrossRef]
69. Ma, C.; Li, L.; Chen, Q.; Lee, J.-S.; Gong, J.; Shi, H. Application of Internal Persistent Fluorescent Fibers in Tracking Microplastics in Vivo Processes in Aquatic Organisms. *J. Hazard. Mater.* **2021**, *401*, 123336. [CrossRef]

70. Volkheimer, G. Hematogenous Dissemination of Ingested Polyvinyl Chloride Particles. *Ann. N. Y. Acad. Sci.* **1975**, *246*, 164–171. [CrossRef]
71. Walczak, A.P.; Kramer, E.; Hendriksen, P.J.M.; Tromp, P.; Helsper, J.P.F.G.; van der Zande, M.; Rietjens, I.M.C.M.; Bouwmeester, H. Translocation of Differently Sized and Charged Polystyrene Nanoparticles in in Vitro Intestinal Cell Models of Increasing Complexity. *Nanotoxicology* **2015**, *9*, 453–461. [CrossRef]
72. Hu, L.; Zhou, Y.; Wang, Y.; Zhang, D.; Pan, X. Transfer of Micro(Nano)Plastics in Animals: A Mini-Review and Future Research Recommendation. *J. Hazard. Mater. Adv.* **2022**, *7*, 100101. [CrossRef]
73. Walczak, A.P.; Kramer, E.; Hendriksen, P.J.M.; Helsdingen, R.; van der Zande, M.; Rietjens, I.M.C.M.; Bouwmeester, H. In Vitro Gastrointestinal Digestion Increases the Translocation of Polystyrene Nanoparticles in an in Vitro Intestinal Co-Culture Model. *Nanotoxicology* **2015**, *9*, 886–894. [CrossRef]
74. Lithner, D.; Larsson, Å.; Dave, G. Environmental and Health Hazard Ranking and Assessment of Plastic Polymers Based on Chemical Composition. *Sci. Total Environ.* **2011**, *409*, 3309–3324. [CrossRef]
75. Lei, L.; Wu, S.; Lu, S.; Liu, M.; Song, Y.; Fu, Z.; Shi, H.; Raley-Susman, K.M.; He, D. Microplastic Particles Cause Intestinal Damage and Other Adverse Effects in Zebrafish Danio Rerio and Nematode Caenorhabditis Elegans. *Sci. Total Environ.* **2018**, *619–620*, 1–8. [CrossRef]
76. Wang, Y.; Mao, Z.; Zhang, M.; Ding, G.; Sun, J.; Du, M.; Liu, Q.; Cong, Y.; Jin, F.; Zhang, W.; et al. The Uptake and Elimination of Polystyrene Microplastics by the Brine Shrimp, Artemia Parthenogenetica, and Its Impact on Its Feeding Behavior and Intestinal Histology. *Chemosphere* **2019**, *234*, 123–131. [CrossRef]
77. Jiang, X.; Chang, Y.; Zhang, T.; Qiao, Y.; Klobučar, G.; Li, M. Toxicological Effects of Polystyrene Microplastics on Earthworm (Eisenia Fetida). *Environ. Pollut.* **2020**, *259*, 113896. [CrossRef] [PubMed]
78. Huang, J.-N.; Wen, B.; Zhu, J.-G.; Zhang, Y.-S.; Gao, J.-Z.; Chen, Z.-Z. Exposure to Microplastics Impairs Digestive Performance, Stimulates Immune Response and Induces Microbiota Dysbiosis in the Gut of Juvenile Guppy (Poecilia Reticulata). *Sci. Total Environ.* **2020**, *733*, 138929. [CrossRef]
79. Gu, H.; Wang, S.; Wang, X.; Yu, X.; Hu, M.; Huang, W.; Wang, Y. Nanoplastics Impair the Intestinal Health of the Juvenile Large Yellow Croaker Larimichthys Crocea. *J. Hazard. Mater.* **2020**, *397*, 122773. [CrossRef] [PubMed]
80. Kang, H.-M.; Byeon, E.; Jeong, H.; Kim, M.-S.; Chen, Q.; Lee, J.-S. Different Effects of Nano- and Microplastics on Oxidative Status and Gut Microbiota in the Marine Medaka Oryzias Melastigma. *J. Hazard. Mater.* **2021**, *405*, 124207. [CrossRef]
81. Ahrendt, C.; Perez-Venegas, D.J.; Urbina, M.; Gonzalez, C.; Echeveste, P.; Aldana, M.; Pulgar, J.; Galbán-Malagón, C. Microplastic Ingestion Cause Intestinal Lesions in the Intertidal Fish Girella Laevifrons. *Mar. Pollut. Bull.* **2020**, *151*, 110795. [CrossRef] [PubMed]
82. De Sá, L.C.; Oliveira, M.; Ribeiro, F.; Rocha, T.L.; Futter, M.N. Studies of the Effects of Microplastics on Aquatic Organisms: What Do We Know and Where Should We Focus Our Efforts in the Future? *Sci. Total Environ.* **2018**, *645*, 1029–1039. [CrossRef]
83. Qiao, R.; Sheng, C.; Lu, Y.; Zhang, Y.; Ren, H.; Lemos, B. Microplastics Induce Intestinal Inflammation, Oxidative Stress, and Disorders of Metabolome and Microbiome in Zebrafish. *Sci. Total Environ.* **2019**, *662*, 246–253. [CrossRef]
84. Qiao, R.; Deng, Y.; Zhang, S.; Wolosker, M.B.; Zhu, Q.; Ren, H.; Zhang, Y. Accumulation of Different Shapes of Microplastics Initiates Intestinal Injury and Gut Microbiota Dysbiosis in the Gut of Zebrafish. *Chemosphere* **2019**, *236*, 124334. [CrossRef]
85. Pedà, C.; Caccamo, L.; Fossi, M.C.; Gai, F.; Andaloro, F.; Genovese, L.; Perdichizzi, A.; Romeo, T.; Maricchiolo, G. Intestinal Alterations in European Sea Bass Dicentrarchus Labrax (Linnaeus, 1758) Exposed to Microplastics: Preliminary Results. *Environ. Pollut.* **2016**, *212*, 251–256. [CrossRef]
86. Limonta, G.; Mancia, A.; Benkhalqui, A.; Bertolucci, C.; Abelli, L.; Fossi, M.C.; Panti, C. Microplastics Induce Transcriptional Changes, Immune Response and Behavioral Alterations in Adult Zebrafish. *Sci. Rep.* **2019**, *9*, 15775. [CrossRef]
87. Abdelkhaliq, A.; van der Zande, M.; Punt, A.; Helsdingen, R.; Boeren, S.; Vervoort, J.J.M.; Rietjens, I.M.C.M.; Bouwmeester, H. Impact of Nanoparticle Surface Functionalization on the Protein Corona and Cellular Adhesion, Uptake and Transport. *J. Nanobiotechnology* **2018**, *16*, 70. [CrossRef]
88. Magrì, D.; Sánchez-Moreno, P.; Caputo, G.; Gatto, F.; Veronesi, M.; Bardi, G.; Catelani, T.; Guarnieri, D.; Athanassiou, A.; Pompa, P.P.; et al. Laser Ablation as a Versatile Tool To Mimic Polyethylene Terephthalate Nanoplastic Pollutants: Characterization and Toxicology Assessment. *ACS Nano* **2018**, *12*, 7690–7700. [CrossRef]
89. Hesler, M.; Aengenheister, L.; Ellinger, B.; Drexel, R.; Straskraba, S.; Jost, C.; Wagner, S.; Meier, F.; von Briesen, H.; Büchel, C.; et al. Multi-Endpoint Toxicological Assessment of Polystyrene Nano- and Microparticles in Different Biological Models in Vitro. *Toxicol. Vitr.* **2019**, *61*, 104610. [CrossRef]
90. Huang, W.; Yin, H.; Yang, Y.; Jin, L.; Lu, G.; Dang, Z. Influence of the Co-Exposure of Microplastics and Tetrabromobisphenol A on Human Gut: Simulation in Vitro with Human Cell Caco-2 and Gut Microbiota. *Sci. Total Environ.* **2021**, *778*, 146264. [CrossRef]
91. Stock, V.; Böhmert, L.; Lisicki, E.; Block, R.; Cara-Carmona, J.; Pack, L.K.; Selb, R.; Lichtenstein, D.; Voss, L.; Henderson, C.J.; et al. Uptake and Effects of Orally Ingested Polystyrene Microplastic Particles in Vitro and in Vivo. *Arch. Toxicol.* **2019**, *93*, 1817–1833. [CrossRef] [PubMed]
92. Mattioda, V.; Benedetti, V.; Tessarolo, C.; Oberto, F.; Favole, A.; Gallo, M.; Martelli, W.; Crescio, M.I.; Berio, E.; Masoero, L.; et al. Pro-Inflammatory and Cytotoxic Effects of Polystyrene Microplastics on Human and Murine Intestinal Cell Lines. *Biomolecules* **2023**, *13*, 140. [CrossRef] [PubMed]

93. Jin, Y.; Lu, L.; Tu, W.; Luo, T.; Fu, Z. Impacts of Polystyrene Microplastic on the Gut Barrier, Microbiota and Metabolism of Mice. *Sci. Total Environ.* **2019**, *649*, 308–317. [CrossRef] [PubMed]
94. Lu, L.; Wan, Z.; Luo, T.; Fu, Z.; Jin, Y. Polystyrene Microplastics Induce Gut Microbiota Dysbiosis and Hepatic Lipid Metabolism Disorder in Mice. *Sci. Total Environ.* **2018**, *631–632*, 449–458. [CrossRef]
95. Li, B.; Ding, Y.; Cheng, X.; Sheng, D.; Xu, Z.; Rong, Q.; Wu, Y.; Zhao, H.; Ji, X.; Zhang, Y. Polyethylene Microplastics Affect the Distribution of Gut Microbiota and Inflammation Development in Mice. *Chemosphere* **2020**, *244*, 125492. [CrossRef]
96. Deng, Y.; Yan, Z.; Shen, R.; Wang, M.; Huang, Y.; Ren, H.; Zhang, Y.; Lemos, B. Microplastics Release Phthalate Esters and Cause Aggravated Adverse Effects in the Mouse Gut. *Environ. Int.* **2020**, *143*, 105916. [CrossRef]
97. Liang, B.; Zhong, Y.; Huang, Y.; Lin, X.; Liu, J.; Lin, L.; Hu, M.; Jiang, J.; Dai, M.; Wang, B.; et al. Underestimated Health Risks: Polystyrene Micro- and Nanoplastics Jointly Induce Intestinal Barrier Dysfunction by ROS-Mediated Epithelial Cell Apoptosis. *Part. Fibre Toxicol.* **2021**, *18*, 20. [CrossRef] [PubMed]
98. Park, S.B.; Jung, W.H.; Choi, K.J.; Koh, B.; Kim, K.Y. A Comparative Systematic Analysis of The Influence of Microplastics on Colon Cells, Mouse and Colon Organoids. *Tissue Eng. Regen. Med.* **2023**, *20*, 49–58. [CrossRef]
99. Lehner, R.; Wohlleben, W.; Septiadi, D.; Landsiedel, R.; Petri-Fink, A.; Rothen-Rutishauser, B. A Novel 3D Intestine Barrier Model to Study the Immune Response upon Exposure to Microplastics. *Arch. Toxicol.* **2020**, *94*, 2463–2479. [CrossRef] [PubMed]
100. Hwang, J.; Choi, D.; Han, S.; Choi, J.; Hong, J. An Assessment of the Toxicity of Polypropylene Microplastics in Human Derived Cells. *Sci. Total Environ.* **2019**, *684*, 657–669. [CrossRef]
101. Park, E.-J.; Han, J.-S.; Park, E.-J.; Seong, E.; Lee, G.-H.; Kim, D.-W.; Son, H.-Y.; Han, H.-Y.; Lee, B.-S. Repeated-Oral Dose Toxicity of Polyethylene Microplastics and the Possible Implications on Reproduction and Development of the next Generation. *Toxicol. Lett.* **2020**, *324*, 75–85. [CrossRef]
102. Ma, J.; Wan, Y.; Song, L.; Wang, L.; Wang, H.; Li, Y.; Huang, D. Polystyrene Nanobeads Exacerbate Chronic Colitis in Mice Involving in Oxidative Stress and Hepatic Lipid Metabolism. *Part. Fibre Toxicol.* **2023**, *20*, 49. [CrossRef]
103. Luo, T.; Wang, C.; Pan, Z.; Jin, C.; Fu, Z.; Jin, Y. Maternal Polystyrene Microplastic Exposure during Gestation and Lactation Altered Metabolic Homeostasis in the Dams and Their F1 and F2 Offspring. *Environ. Sci. Technol.* **2019**, *53*, 10978–10992. [CrossRef]
104. Jiang, P.; Yuan, G.; Jiang, B.; Zhang, J.; Wang, Y.; Lv, H.; Zhang, Z.; Wu, J.; Wu, Q.; Li, L. Effects of Microplastics (MPs) and Tributyltin (TBT) Alone and in Combination on Bile Acids and Gut Microbiota Crosstalk in Mice. *Ecotoxicol. Environ. Saf.* **2021**, *220*, 112345. [CrossRef] [PubMed]
105. Qiao, J.; Chen, R.; Wang, M.; Bai, R.; Cui, X.; Liu, Y.; Wu, C.; Chen, C. Perturbation of Gut Microbiota Plays an Important Role in Micro/Nanoplastics-Induced Gut Barrier Dysfunction. *Nanoscale* **2021**, *13*, 8806–8816. [CrossRef] [PubMed]
106. Liu, J.; Lv, M.; Sun, A.; Ding, J.; Wang, Y.; Chang, X.; Chen, L. Exposure to Microplastics Reduces the Bioaccumulation of Sulfamethoxazole but Enhances Its Effects on Gut Microbiota and the Antibiotic Resistome of Mice. *Chemosphere* **2022**, *294*, 133810. [CrossRef]
107. Montano, D. Chemical and Biological Work-Related Risks across Occupations in Europe: A Review. *J. Occup. Med. Toxicol.* **2014**, *9*, 28. [CrossRef] [PubMed]
108. Lyche, L. Chapter 48—Phthalates. In *Reproductive and Developmental Toxicology*; Gupta, R.C., Ed.; Academic Press: Cambridge, MA, USA, 2011; pp. 637–655. ISBN 9780123820327. [CrossRef]
109. Eales, J.; Bethel, A.; Galloway, T.; Hopkinson, P.; Morrissey, K.; Short, R.E.; Garside, R. Human Health Impacts of Exposure to Phthalate Plasticizers: An Overview of Reviews. *Environ. Int.* **2022**, *158*, 106903. [CrossRef]
110. Cirillo, T.; Latini, G.; Castaldi, M.A.; Dipaola, L.; Fasano, E.; Esposito, F.; Scognamiglio, G.; Francesco, F.D.; Cobellis, L. Exposure to Di-2-Ethylhexyl Phthalate, Di-N-Butyl Phthalate and Bisphenol A through Infant Formulas. *J. Agric. Food Chem.* **2015**, *63*, 3303–3310. [CrossRef]
111. Kıralan, S.S.; Toptancı, İ.; Öncül Abacıgil, T.; Ramadan, M.F. Phthalates Levels in Olive Oils and Olive Pomace Oils Marketed in Turkey. *Food Addit. Contam. Part A* **2020**, *37*, 1332–1338. [CrossRef]
112. Serrano, S.E.; Braun, J.; Trasande, L.; Dills, R.; Sathyanarayana, S. Phthalates and Diet: A Review of the Food Monitoring and Epidemiology Data. *Environ. Health* **2014**, *13*, 43. [CrossRef]
113. Zhang, Y.-J.; Guo, J.-L.; Xue, J.; Bai, C.-L.; Guo, Y. Phthalate Metabolites: Characterization, Toxicities, Global Distribution, and Exposure Assessment. *Environ. Pollut.* **2021**, *291*, 118106. [CrossRef]
114. Polinski, K.J.; Dabelea, D.; Hamman, R.F.; Adgate, J.L.; Calafat, A.M.; Ye, X.; Starling, A.P. Distribution and Predictors of Urinary Concentrations of Phthalate Metabolites and Phenols among Pregnant Women in the Healthy Start Study. *Environ. Res.* **2018**, *162*, 308–317. [CrossRef] [PubMed]
115. Wang, G.; Chen, Q.; Tian, P.; Wang, L.; Li, X.; Lee, Y.; Zhao, J.; Zhang, H.; Chen, W. Gut Microbiota Dysbiosis Might Be Responsible to Different Toxicity Caused by Di-(2-Ethylhexyl) Phthalate Exposure in Murine Rodents. *Environ. Pollut.* **2020**, *261*, 114164. [CrossRef]
116. Yu, Z.; Shi, Z.; Zheng, Z.; Han, J.; Yang, W.; Lu, R.; Lin, W.; Zheng, Y.; Nie, D.; Chen, G. DEHP Induce Cholesterol Imbalance via Disturbing Bile Acid Metabolism by Altering the Composition of Gut Microbiota in Rats. *Chemosphere* **2021**, *263*, 127959. [CrossRef]

117. Fu, X.; Han, H.; Li, Y.; Xu, B.; Dai, W.; Zhang, Y.; Zhou, F.; Ma, H.; Pei, X. Di-(2-ethylhexyl) Phthalate Exposure Induces Female Reproductive Toxicity and Alters the Intestinal Microbiota Community Structure and Fecal Metabolite Profile in Mice. *Environ. Toxicol.* **2021**, *36*, 1226–1242. [CrossRef]
118. Lei, M.; Menon, R.; Manteiga, S.; Alden, N.; Hunt, C.; Alaniz, R.C.; Lee, K.; Jayaraman, A. Environmental Chemical Diethylhexyl Phthalate Alters Intestinal Microbiota Community Structure and Metabolite Profile in Mice. *mSystems* **2019**, *4*, 10–1128. [CrossRef]
119. Chiu, K.; Bashir, S.T.; Nowak, R.A.; Mei, W.; Flaws, J.A. Subacute Exposure to Di-Isononyl Phthalate Alters the Morphology, Endocrine Function, and Immune System in the Colon of Adult Female Mice. *Sci. Rep.* **2020**, *10*, 18788. [CrossRef]
120. Xiong, Z.E.; Zeng, Y.; Zhou, J.; Shu, R.; Xie, X.; Fu, Z. Exposure to Dibutyl Phthalate Impairs Lipid Metabolism and Causes Inflammation via Disturbing Microbiota-Related Gut—Liver Axis. *Acta Biochim. Biophys. Sin.* **2020**, *52*, 1382–1393. [CrossRef] [PubMed]
121. Goyal, S.P.; Saravanan, C. An Insight into the Critical Role of Gut Microbiota in Triggering the Phthalate-Induced Toxicity and Its Mitigation Using Probiotics. *Sci. Total Environ.* **2023**, *904*, 166889. [CrossRef]
122. Dekant, W. Grouping of Phthalates for Risk Characterization of Human Exposures. *Toxicol. Lett.* **2020**, *330*, 1–6. [CrossRef]
123. Zoeller, R.T.; Birnbaum, L.S.; Collins, T.J.; Heindel, J.; Hunt, P.A.; Iguchi, T.; Kortenkamp, A.; Myers, J.P.; Vom Saal, F.S.; Sonnenschein, C.; et al. European Medicines Agency Conflicts With the European Food Safety Authority (EFSA) on Bisphenol A Regulation. *J. Endocr. Soc.* **2023**, *7*, bvad107. [CrossRef]
124. Liao, Y.; Yang, J. Microplastic Serves as a Potential Vector for Cr in an In-Vitro Human Digestive Model. *Sci. Total Environ.* **2020**, *703*, 134805. [CrossRef] [PubMed]
125. Nickens, K.P.; Patierno, S.R.; Ceryak, S. Chromium Genotoxicity: A Double-Edged Sword. *Chem. Biol. Interact.* **2010**, *188*, 276–288. [CrossRef]
126. Ahmad, R.; Sorrell, M.F.; Batra, S.K.; Dhawan, P.; Singh, A.B. Gut Permeability and Mucosal Inflammation: Bad, Good or Context Dependent. *Mucosal Immunol.* **2017**, *10*, 307–317. [CrossRef] [PubMed]
127. Sadler, D.E.; Brunner, F.S.; Plaistow, S.J. Temperature and Clone-Dependent Effects of Microplastics on Immunity and Life History in Daphnia Magna. *Environ. Pollut.* **2019**, *255*, 113178. [CrossRef]
128. Brandts, I.; Teles, M.; Gonçalves, A.P.; Barreto, A.; Franco-Martinez, L.; Tvarijonaviciute, A.; Martins, M.A.; Soares, A.M.V.M.; Tort, L.; Oliveira, M. Effects of Nanoplastics on Mytilus Galloprovincialis after Individual and Combined Exposure with Carbamazepine. *Sci. Total Environ.* **2018**, *643*, 775–784. [CrossRef]
129. Auguste, M.; Balbi, T.; Ciacci, C.; Canonico, B.; Papa, S.; Borello, A.; Vezzulli, L.; Canesi, L. Shift in Immune Parameters After Repeated Exposure to Nanoplastics in the Marine Bivalve Mytilus. *Front. Immunol.* **2020**, *11*, 503705. [CrossRef]
130. Auguste, M.; Lasa, A.; Balbi, T.; Pallavicini, A.; Vezzulli, L.; Canesi, L. Impact of Nanoplastics on Hemolymph Immune Parameters and Microbiota Composition in Mytilus Galloprovincialis. *Mar. Environ. Res.* **2020**, *159*, 105017. [CrossRef]
131. Paul-Pont, I.; Lacroix, C.; González Fernández, C.; Hégaret, H.; Lambert, C.; Le Goïc, N.; Frère, L.; Cassone, A.-L.; Sussarellu, R.; Fabioux, C.; et al. Exposure of Marine Mussels Mytilus Spp. to Polystyrene Microplastics: Toxicity and Influence on Fluoranthene Bioaccumulation. *Environ. Pollut.* **2016**, *216*, 724–737. [CrossRef]
132. Murano, C.; Agnisola, C.; Caramiello, D.; Castellano, I.; Casotti, R.; Corsi, I.; Palumbo, A. How Sea Urchins Face Microplastics: Uptake, Tissue Distribution and Immune System Response. *Environ. Pollut.* **2020**, *264*, 114685. [CrossRef]
133. Liu, Z.; Yu, P.; Cai, M.; Wu, D.; Zhang, M.; Chen, M.; Zhao, Y. Effects of Microplastics on the Innate Immunity and Intestinal Microflora of Juvenile Eriocheir Sinensis. *Sci. Total Environ.* **2019**, *685*, 836–846. [CrossRef]
134. Shi, W.; Han, Y.; Sun, S.; Tang, Y.; Zhou, W.; Du, X.; Liu, G. Immunotoxicities of Microplastics and Sertraline, Alone and in Combination, to a Bivalve Species: Size-Dependent Interaction and Potential Toxication Mechanism. *J. Hazard. Mater.* **2020**, *396*, 122603. [CrossRef]
135. Tang, Y.; Rong, J.; Guan, X.; Zha, S.; Shi, W.; Han, Y.; Du, X.; Wu, F.; Huang, W.; Liu, G. Immunotoxicity of Microplastics and Two Persistent Organic Pollutants Alone or in Combination to a Bivalve Species. *Environ. Pollut.* **2020**, *258*, 113845. [CrossRef]
136. Greven, A.; Merk, T.; Karagöz, F.; Mohr, K.; Klapper, M.; Jovanović, B.; Palić, D. Polycarbonate and Polystyrene Nanoplastic Particles Act as Stressors to the Innate Immune System of Fathead Minnow (*Pimephales promelas*). *Environ. Toxicol. Chem.* **2016**, *35*, 3093–3100. [CrossRef] [PubMed]
137. Mancia, A.; Chenet, T.; Bono, G.; Geraci, M.L.; Vaccaro, C.; Munari, C.; Mistri, M.; Cavazzini, A.; Pasti, L. Adverse Effects of Plastic Ingestion on the Mediterranean Small-Spotted Catshark (Scyliorhinus Canicula). *Mar. Environ. Res.* **2020**, *155*, 104876. [CrossRef] [PubMed]
138. Yan, Z.; Liu, Y.; Zhang, T.; Zhang, F.; Ren, H.; Zhang, Y. Analysis of Microplastics in Human Feces Reveals a Correlation between Fecal Microplastics and Inflammatory Bowel Disease Status. *Environ. Sci. Technol.* **2022**, *56*, 414–421. [CrossRef] [PubMed]
139. Donaldson, G.P.; Lee, S.M.; Mazmanian, S.K. Gut Biogeography of the Bacterial Microbiota. *Nat. Rev. Microbiol.* **2016**, *14*, 20–32. [CrossRef] [PubMed]
140. Fusco, W.; Lorenzo, M.B.; Cintoni, M.; Porcari, S.; Rinninella, E.; Kaitsas, F.; Lener, E.; Mele, M.C.; Gasbarrini, A.; Collado, M.C.; et al. Short-Chain Fatty-Acid-Producing Bacteria: Key Components of the Human Gut Microbiota. *Nutrients* **2023**, *15*, 2211. [CrossRef] [PubMed]
141. Cammarota, G.; Ianiro, G.; Tilg, H.; Rajilić-Stojanović, M.; Kump, P.; Satokari, R.; Sokol, H.; Arkkila, P.; Pintus, C.; Hart, A.; et al. European Consensus Conference on Faecal Microbiota Transplantation in Clinical Practice. *Gut* **2017**, *66*, 569–580. [CrossRef]

142. Urbanek, A.K.; Rymowicz, W.; Mirończuk, A.M. Degradation of Plastics and Plastic-Degrading Bacteria in Cold Marine Habitats. *Appl. Microbiol. Biotechnol.* **2018**, *102*, 7669–7678. [CrossRef]
143. Yeom, S.-J.; Le, T.-K.; Yun, C.-H. P450-Driven Plastic-Degrading Synthetic Bacteria. *Trends Biotechnol.* **2022**, *40*, 166–179. [CrossRef]
144. Gambarini, V.; Pantos, O.; Kingsbury, J.M.; Weaver, L.; Handley, K.M.; Lear, G. Phylogenetic Distribution of Plastic-Degrading Microorganisms. *mSystems* **2021**, *6*, 10–1128. [CrossRef]
145. García-Depraect, O.; Bordel, S.; Lebrero, R.; Santos-Beneit, F.; Börner, R.A.; Börner, T.; Muñoz, R. Inspired by Nature: Microbial Production, Degradation and Valorization of Biodegradable Bioplastics for Life-Cycle-Engineered Products. *Biotechnol. Adv.* **2021**, *53*, 107772. [CrossRef]
146. Maity, W.; Maity, S.; Bera, S.; Roy, A. Emerging Roles of PETase and MHETase in the Biodegradation of Plastic Wastes. *Appl. Biochem. Biotechnol.* **2021**, *193*, 2699–2716. [CrossRef]
147. Amobonye, A.; Bhagwat, P.; Singh, S.; Pillai, S. Plastic Biodegradation: Frontline Microbes and Their Enzymes. *Sci. Total Environ.* **2021**, *759*, 143536. [CrossRef]
148. Jacquin, J.; Cheng, J.; Odobel, C.; Pandin, C.; Conan, P.; Pujo-Pay, M.; Barbe, V.; Meistertzheim, A.-L.; Ghiglione, J.-F. Microbial Ecotoxicology of Marine Plastic Debris: A Review on Colonization and Biodegradation by the "Plastisphere". *Front. Microbiol.* **2019**, *10*, 424560. [CrossRef]
149. Lear, G.; Kingsbury, J.M.; Franchini, S.; Gambarini, V.; Maday, S.D.M.; Wallbank, J.A.; Weaver, L.; Pantos, O. Plastics and the Microbiome: Impacts and Solutions. *Environ. Microbiome* **2021**, *16*, 2. [CrossRef]
150. Zhang, J.; Gao, D.; Li, Q.; Zhao, Y.; Li, L.; Lin, H.; Bi, Q.; Zhao, Y. Biodegradation of Polyethylene Microplastic Particles by the Fungus Aspergillus Flavus from the Guts of Wax Moth Galleria Mellonella. *Sci. Total Environ.* **2020**, *704*, 135931. [CrossRef]
151. Yang, Y.; Yang, J.; Wu, W.-M.; Zhao, J.; Song, Y.; Gao, L.; Yang, R.; Jiang, L. Biodegradation and Mineralization of Polystyrene by Plastic-Eating Mealworms: Part 2. Role of Gut Microorganisms. *Environ. Sci. Technol.* **2015**, *49*, 12087–12093. [CrossRef] [PubMed]
152. Plaza Oñate, F.; Le Chatelier, E.; Almeida, M.; Cervino, A.C.L.; Gauthier, F.; Magoulès, F.; Ehrlich, S.D.; Pichaud, M. MSPminer: Abundance-Based Reconstitution of Microbial Pan-Genomes from Shotgun Metagenomic Data. *Bioinformatics* **2019**, *35*, 1544–1552. [CrossRef]
153. Ruan, W.; Engevik, M.A.; Spinler, J.K.; Versalovic, J. Healthy Human Gastrointestinal Microbiome: Composition and Function After a Decade of Exploration. *Dig. Dis. Sci.* **2020**, *65*, 695–705. [CrossRef] [PubMed]

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Review

Revisiting Neuroblastoma: Nrf2, NF-κB and Phox2B as a Promising Network in Neuroblastoma

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Abstract: Neuroblastoma is the most common solid extracranial tumor during childhood; it displays extraordinary heterogeneous clinical courses, from spontaneous regression to poor outcome in high-risk patients due to aggressive growth, metastasizing, and treatment resistance. Therefore, the identification and detailed analysis of promising tumorigenic molecular mechanisms are inevitable. This review highlights the abnormal regulation of NF-κB, Nrf2, and Phox2B as well as their interactions among each other in neuroblastoma. NF-κB and Nrf2 play a key role in antioxidant responses, anti-inflammatory regulation and tumor chemoresistance. Recent studies revealed a regulation of NF-κB by means of the Nrf2/antioxidant response element (ARE) system. On the other hand, Phox2B contributes to the differentiation of immature sympathetic nervous system stem cells: this transcription factor regulates the expression of *RET*, thereby facilitating cell survival and proliferation. As observed in other tumors, we presume striking interactions between NF-κB, Nrf2, and Phox2B, which might constitute an important crosstalk triangle, whose decompensation may trigger a more aggressive phenotype. Consequently, these transcription factors could be a promising target for novel therapeutic approaches and hence, further investigation on their regulation in neuroblastoma shall be reinforced.

Keywords: neuroblastoma; signaling network; genetic alterations; Nrf2; NF-κB; Phox2B

1. Introduction

Neuroblastoma derives from sympathetic nervous system stem cells and accounts for the most common solid extracranial tumor during childhood, accounting for 5.5% of all pediatric malignancies. Hence, 11 out of 1,000,000 children and adolescents between 0 and 17 years are diagnosed with this disease [1,2]. Different parameters have been used to classify patients with neuroblastoma into subgroups by their risk of death: age at diagnosis, metastatic spread, degree of tumor differentiation as well as histological features, mitosis-index, *MYCN* oncogene amplification, 1p/17q/11q status, DNA ploidy and LDH and ferritin levels [3,4].

For example, patients with high-risk disease (stage 4) are older than 18 months (median presentation age) and present metastases; their cancers show *MYCN* amplification, 1p deletion, 17q gain, 11q LOH, diploidy, as well as high LDH and ferritin levels—otherwise they are grouped to non-high-risk disease (stage 1–3) [5].

Children with stage 4 neuroblastoma, accounting for approximately 60%, have one of the worst prognoses in pediatric oncology [4]. Despite significant therapeutic improvements including high-dose chemotherapy, surgical resection, radiotherapy, immunotherapy, and myeloablative therapy with autologous stem cell transplantation, the 5-year overall survival rate remains below 50% in these patients [6–9].

Cancer development and progression are multifactorial processes in which several elements (both intrinsic and extra-tumoral) interact with one another. Accordingly, abnormal regulation of genetic factors, such as NF-κB, *MYCN*, *ALK*, and Nrf2, has been constantly

and similarly observed in various cancer types [10]. Although neuroblastoma characterizes for a wide range of different chromosomal abnormalities and gene mutations [5], to date no common neuroblastoma-specific genomic alteration, LOH, or genetic translocation has been uniformly linked to all aggressive neuroblastoma phenotypes [11].

A *MYCN* amplification is displayed by approximately 18–38% of tumors and linked with the most aggressive disease course. Its alteration is associated with segmental loss of the distal short arm of chromosome 1 (1p). On the other hand, 11q deletion also correlates with poor prognosis, but shows a negative correlation with *MYCN* amplification [1,5].

Other factors, in turn, are altered in a tumor-specific manner and may consequently qualify as tumor biomarkers (e.g., GD2 and Phox2B for neuroblastoma) [12]. Despite the hitherto conducted efforts to characterize neuroblastoma, a proper interpretation of the genetic and epigenetic features of neuroblastoma is still missing.

The goal of this scope review is to shed light onto overlooked signaling pathways possibly involved in the carcinogenesis of neuroblastoma, with emphasis on Nrf2, NF-κB, and Phox2B. Additionally, we aim to uncover their interaction and contextualize it in neuroblastoma. We presume to portray and then partially fill the current knowledge gap regarding these functional networks. This review shall motivate for novel research approaches and deeper analyses ensuring better comprehension of these factors' role in the context of neuroblastoma, with a focus on their possible striking role in the development of aggressive tumor traits.

2. Nrf2 Signaling Pathway

2.1. Nrf2 Molecular and Functional Biology

The nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor, belonging to a family of basic leucine zipper (bZIP) proteins, a member of the cap'n'collar (CNC) subfamily that in humans is encoded by the *NFE2L2* gene [13,14]. Nrf2 has seven functional domains (Nrf2-ECH homology, Neh1–7), which are either implicated in the regulation of Nrf2 stability or necessary for its transcriptional activity. It is primarily involved in response to oxidative stress, in fact regulating the expression of hundreds of cytoprotective genes which contain antioxidant response elements (ARE) in their promoters, such as *HO-1* [15,16].

As depicted in Figure 1, different Nrf2-regulation pathways have been described. Traditionally the ETGE and DLG motifs of the N-terminal domain (Neh2) of Nrf2 bind two molecules of E3 ligase adaptor Kelch-like ECH-associated protein 1 (Keap1) under homeostatic conditions. This interaction activates the Cullin3 (Cul3) E3 ubiquitin ligase complex-mediated ubiquitination of Nrf2, which leads to its degradation by 26S proteasome [17,18]. In cases of oxidative damage, i.e., through cell exposure to exogenous chemicals, the cytoplasmatic concentration of ROS and electrophiles increases [19]. The resulting modification of cysteine residues of Keap1 reduces the affinity between Keap1 and Nrf2, leading to complex dissociation and the subsequent translocation of Nrf2 into the nucleus [18]. The phosphorylation of Nrf2 appears to be a further important event in the release of Nrf2 from Keap1 [20]. Another model displays a Keap1-independent regulation of Nrf2: under physiological conditions its Neh6 domain binds Beta-transducin repeats-containing protein (β-TrCP), enabling Nrf2 ubiquitination by Skp1-Cul1-Rbx1/Roc1 ubiquitin ligase complex and ultimately its proteasomal degradation [19]. The stability of this complex is jeopardized by phosphorylation of Nrf2, which is in turn initialized by elevated oxidative stress levels [21]. An additional regulation of Nrf2 is via phosphatidylinositol 3-kinase (PI3K) and Akt, which leads to Nrf2 activation by phosphorylation of its phosphatidylinositol at the D-3 position of the inositol ring [22].

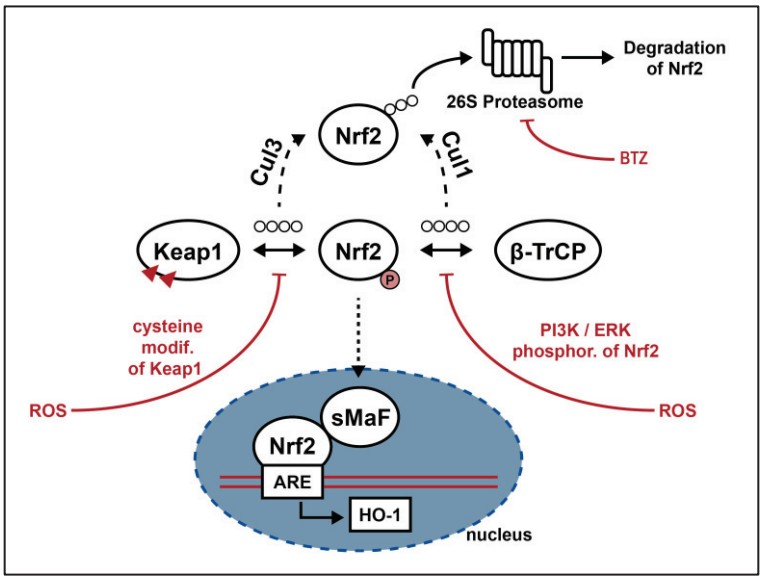


Figure 1. Regulatory axis of Nrf2: in basal conditions the interaction between Nrf2 and either Keap1 or β-TrcP leads to Nrf2 ubiquitination and proteasomal degradation. In cases of cell exposure to oxidative stress, cysteine modifications on Keap1 decrease its affinity to Nrf2, which translocate into the nucleus and, together with its cofactor sMaf, binds cytoprotective genes containing ARE in their promoters. PI3K/ERK-mediated phosphorylation of Nrf2 prevents its β-TrcP-driven ubiquitination, thus enabling its translocation into the nucleus too [16,18]. BTZ is a selective and reversible 26S proteasome inhibitor, able to improve Nrf2 activity by reducing its proteasomal degradation [16]. (↔ interaction; ⇢ translocation; ↳ transcription; ⊣ inhibition).

Furthermore, the inhibition of proteasome activity can increase cytoplasmatic level of Nrf2 and indirectly its activity [16]. Recent studies have identified numerous proteins with motifs that are very similar to that of ETGE in Nrf2; these proteins might be able to compete with Nrf2 for Keap1 binding, thus stabilizing Nrf2 [14].

Once Nrf2 is in the nucleus, the bZip motif in the Neh1 domain of Nrf2 heterodimerizes with small musculoaponeurotic fibrosarcoma proteins (sMafs) and binds to ARE, initiating the transcription of downstream target genes. The resulting expressed proteins are involved in the response against stress and inflammation as well as in maintaining redox homeostasis. They can be classified into three major groups: phase I and phase II drug-metabolizing enzymes as well as phase III drug transporters [18].

Various studies outlined how the stimulus-induced activation of Nrf2 might be able to suppress carcinogenesis, at least in its early phase. Indeed, Nrf2-deficient mice have been shown to easily develop drug toxicity as well as a larger number of different tumors after exposure to exogenous chemicals/carcinogens compared to wild-type mice [18,23]. On the contrary, constitutive Nrf2 activation or its overactivation promotes carcinogenesis in plural ways: metabolic reprogramming, cell-cycle regulation [24], healthy mitochondrial maintenance [18,25], induction of angiogenesis [26], suppression of cell apoptosis [27], reduction of tumor-associated chronic inflammation, maintenance of the self-renewal potential of cancer stem cells, and contribution to chemo- and radio-resistance [16].

2.2. Nrf2 in Neuroblastoma

Although the oncogenic role of Nrf2 has been repeatedly highlighted, there is only fragmentary knowledge about its involvement in the carcinogenesis and development of neuroblastoma, mostly based on single experiments on neuroblastoma-derived cell lines.

In neuroblastoma cells the activation of TRPM2, an ion channel permeable to Ca^{2+}, K^{+}, and Na^{2+}, might induce the cAMP response element-binding protein(CREB)-mediated expression of Nrf2, thus promoting oxidative stress regulation and cell survival [28].

In the work of Kaufman et al. (2020), human IMR-32 neuroblastoma cells were exposed to high dopamine concentrations to induce oxidative stress. In the presence of sufficient zinc in the cell culture medium, this cell line responded by upregulating heme oxygenase 1 (HO-1), a downstream target of Nrf2 [29].

In highly chemo-resistant neuroblastoma cells HTLA-230, treatment with bortezomib (BTZ), the first selective and reversible 26S proteasome inhibitor, led to Nrf2 activation, thus reducing the expected improvement in cell chemosensitivity. BTZ usually increases the apoptotic rate of tumoral cells due to the enhanced response to chemotherapy. In the aforementioned neuroblastoma cells, the Nrf2-mediated upregulation of HO-1 and ultimately the increment of total glutathione (GSH) levels after BTZ exposure, prevented cell damage [16].

In the study of Wang et al. (2008), stable overexpression or induced upregulation of Nrf2 in SH-SY5Y neuroblastoma cells was reliably linked to enhanced resistance to cisplatin, etoposide and doxorubicin, which are utilized in the treatment regimen for high-risk neuroblastoma [30,31]. Chemicals which selectively inhibit the Nrf2 signal pathway might be used as adjuvants to chemotherapy in order to potentiate the efficacy of well-established treatment protocols [32,33]. So far, the only known selective Nrf2-inhibitor is brusatol, a quassinoid derived from the plant *Brucea sumatrana*, which was shown to sensitize cancer cells and xenografts to cheloid agents. Clinical application was limited by systemic toxicity in early phase clinical studies. Furthermore, its regulation on the Nrf2 pathway was never tested in neuroblastoma cells or samples [34,35].

De Miranda Ramos et al. (2016) used the cell line SH-SY5Y as a retinoic acid (RA)-induced differentiation model. RA acts as a morphogen, controlling tissue growth and differentiation through genomic and non-genomic actions. RA-treatment of SH-SY5Y cells induced Nrf2 activation via thiol-dependent signaling and a rapid non-genomic activation of PI3K/Akt and ERK1/2, leading to cell survival by augmented resistance to RA cytotoxicity [36]. The activation of the PI3K/AKT/Nrf2 pathway and increasing Nrf2-induced expression of HO-1 after ROS impairment in human neuroblastoma cells SH-SY5Y has also been reported in other studies [37,38]. Additionally, combined treatment with all-trans retinoic acid (ATRA) and 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO) on human IMR32 neuroblastoma cells and mouse neuroblasts Neuro2a induced differentiation and neuron outgrowth; this was accompanied by a decreased expression of *MYCN* in the case of IMR32 cells. CDDO belongs to the compound group of triterpenoids and their (semi)synthetic derivatives, which characterize for their anti-inflammatory and cytotoxic activity as well-known Nrf2 activators [39,40]. Another group member, soloxone methyl (SM), was used to treat the aforementioned murine Neuro2a neuroblastoma cells by Odarenko and colleagues, after cell stimulation with the carcinogenic leptine. Interestingly, their results showed that leptin-associated phosphorylation of ERK1/2 was suppressed by SM [41].

Alternatively, epigenetic therapeutic strategies may be considered. Although there is no direct proof of chromatin structure alterations in either *NFE2L2* or *KEAP-1* in neuroblastoma, there are a few studies supporting potential epigenetic modifications of both genes. Accordingly, Zhao et al. showed an increase of Nrf2 expression after treating murine neuroblastoma cells with sulforaphane (SFN), possibly mediated by decreased DNA demethylation of the *NFE2L2* promoter [42]. Moreover, other groups demonstrated a correlation between promoter methylation, histone acetylation status of *NFE2L2*/*KEAP-1* and the biological function of several tumor cells. Alike, the regulation of histone deacetyltransferases (HDACs) and DNA methyltransferases (DNMTs) were shown to modulate the expression and activity of Nrf2 and Keap-1 in prostate cancer, lung cancer and colorectal carcinoma [42–44]. In contrast to Zhao's findings, in these tumor entities the hypermethylation on the *NFE2L2* promoter suppresses its transcription and leads to a subsequent inhibition of cancer progression. On the other hand, some cases of malignant glioma are characterized by an increase of *KEAP-1* promoter methylation which leads to an upregulation of *KEAP-1* transcription and a consequent higher degradation of Nrf2. In such cases,

the therapeutic application of compounds such as Fumonisin B may induce cell death, as shown in hepatocellular carcinoma-derived cells. In this case, treatment of the HepG2 cell line with the aforementioned mycotoxin led to hypermethylation of the *KEAP-1*-promoter and to hypomethylation of *NFE2L2*-promoters. Consequently, decreased Keap-1 expression was observed, whereas Nrf2 was up-regulated [45].

3. *NF-kB* Signaling Pathway

3.1. NF-kB Molecular and Functional Biology

NF-κB (nuclear factor 'kappa-light-chain-enhancer' of activated B-cells) is a family of pleiotropic transcription factors expressed in all mammalian cells. This group comprises NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB, and c-Rel. All of them share the Rel-homologue domain, but only the p65, c-Rel, and RelB proteins contain a transactivation domain (TAD), responsible for DNA binding. The functional biology of NF-κB differs based on which members of this superfamily form homo- or heterodimers [46,47].

Signal-induced activation of NF-κB occurs either through the canonical or the non-canonical pathway [47].

In the canonical pathway (Figure 2), inactive dimers are anchored in the cytosol associated with one of the ankyrin-containing NF-κB inhibitors, the IκBs: IκBα, IκBβ, and IκBε [46]. Exposure to extracellular signals activates, for example, interleukin receptors, and TNF receptors type I as well as T- and B-cell receptors. Subsequent activation of the IκB kinase (IKK) complex, represented by IKK alpha, IKK beta, and IKK gamma/NEMO, leads to phosphorylation of IκBα, which undergoes ubiquitination and proteasomal degradation, freeing the NF-κB dimer p50/p65 [46,48]. Dimer translocation into the cell nucleus and adjunctive post-translational modifications are the final steps which permit DNA binding and the transcription of genes related to inflammation, cell survival, and proliferation (e.g., induction of IL-6, IL-8, Bcl-2, Fas, HO-1, BDNF and NGF) [28].

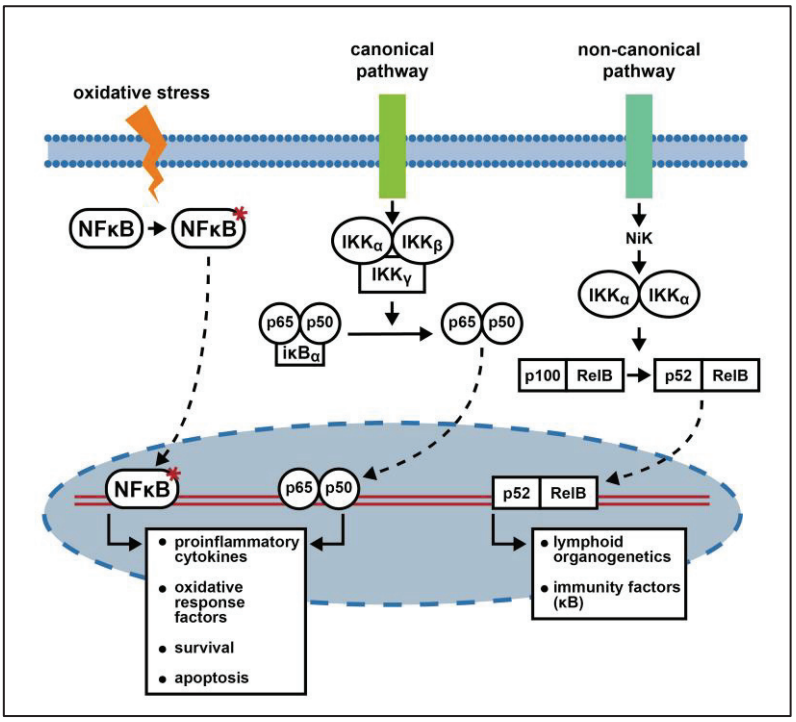


Figure 2. The regulatory axis of NF-κB: NF-κB activation can be mediated by three different pathways, depending on the activated membrane receptors. Each pathway leads to formation of a specific hetero- or homodimer, which in turn promotes the transcription of a distinct pattern of downstream targets, thus mediating pleomorphic effects [46,47]. (→ activation; ⇢ translocation; ↳ transcription; * phosphorylation).

Interestingly, *IκBα* belongs to the downstream target genes of the canonical NF-κB activation cascade, thereby ensuring a negative control loop [47,49].

On the other hand, lymphotoxin B and B cell-activating factor (BAFF) are two of the few known factors able to induce the non-canonical pathway. In this pathway, NF-κB heterodimers contain p100, which is an inactive precursor of p52 [50]. Downstream, NIK (NF-κB-inducing kinase) is activated by phosphorylating the IKK-α dimer. Consequently, the NF-κB factor p100 is processed, turning into p52, allowing the heterodimer p52/RelB to translocate into the nucleus and activate the transcription of genes involved in immune cell development, such as clusters of differentiation 40 (CD40) and major histocompatibility complex type I (MHC I) [47,51,52].

Other alternative pathways responsible for NF-κB activation involve an oxidative stress response and rely on IKK-independent phosphorylation of IκBα [49]—(Figure 2).

As mentioned above, NF-κB has been identified as a pivotal mediator of inflammation and responsible for multiple innate and adaptative immune functions [53]. It also plays a crucial role in cell survival mechanisms: in tumors, the activation of NF-κB usually leads to impaired cell apoptosis and to the activation of genes regulating cell proliferation (cyclin D1/D2/D3). In most cancers, the activation of NF-κB is enhanced upon increased pathway stimulation, due to higher levels of TNFα and IL-1 in the tumor microenvironment [54].

Through analysis of in silico data from publicly available expression arrays, Medeiros and her team were able to depict increased NF-κB expression as a common denominator of pediatric brain tumors. Furthermore, NF-κB levels have been shown to be higher after chemo- or radiotherapeutic toxic aggression [55]. In fact, it has been reported that NF-κB also induces drug resistance in cancer cells, which is a major reason for therapy failure. Bentires-Aly and colleagues (2003) observed how NF-κB inhibition in HCT15 colon cancer cells led to an increased apoptosis rate after cell treatment with daunomycin, whose uptake was significantly increased [56].

3.2. NF-κB in Neuroblastoma

To date, neither chromosome rearrangements nor mutations are described for NF-κB in the context of neuroblastoma. In this tumor entity, NF-κB alterations are shown to be restricted to protein expression impairment, mostly induced. As for Nrf2, studies on NF-κB in neuroblastoma and its role as a possible therapeutic target are mostly based on in vitro assays.

Nevertheless, its activity is linked to metastatic spread and regulation of the tumor microenvironment. TNFα-mediated activation of the NF-κB signaling pathway leads to upregulation of CX chemokine receptor-4 (CXCR4) expression in neuroblastoma samples compared to ganglioneuroma tissue specimens. CXCR4 fosters tumor metastasis, since it enables the homing in of cancer cells to organs expressing its ligand CXCL12, such as liver and bone marrow. Analogous results were observed in SH-SY5Y cells treated with TNFα, which displayed overexpression of both NF-κB and CXCR4. Contrarily, inhibition of NF-κB activity led to a reduced cell migration capacity [36].

As already mentioned, a further key role assigned to NF-κB is the regulation of apoptosis. Programmed cell death is a very complex and multifactorial process which is usually compromised in cancer; the role of NF-κB in apoptosis is equally complex. Both apoptosis suppression and induction have been reported for this transcription factor. Bian and colleagues showed that doxorubicin-induced apoptotic cell death in N-type neuroblastoma cells was mediated by NF-κB activation [56].

In SH-SY5Y cells, for instance, treatment with acetaminophen (AAP) resulted in the generation of ROS and the subsequent translocation of the NF-κB subunit p65 into the nucleus. This, in turn, led to the production of IL-1β with concomitant enhanced apoptosis [56–58]. Karacay et al. showed an NF-κB-mediated anti-apoptotic effect in several neuroblastoma cell lines. Transfection of these cancer cells with the *TRAIL* (TNF-related apoptosis-inducing ligand) gene led to apoptosis. However, the cell death rate was increased in those cells in which inhibition of the NF-κB signaling pathway occurred

simultaneously. Strikingly, TRAIL is described as an inducer of NF-κB and thus it may be assumed that this ligand possibly act as a double-edged sword [59].

The ability of NF-κB to regulate apoptosis, together with the transcriptional regulation of immune-related genes, lend neuroblastoma cells the advantage of evading the immune system and developing treatment resistance. Transcriptional inhibition of NF-κB downregulates MHC Class I expression in human neuroblastoma cells [57,60]. To the downstream genes of NF-κB also belongs *MYCN:* N-myc is suppressed after treating neuroblastoma cells with NF-κB inhibitor withanolide [61].

Another known NF-κB inhibitor is dimethylamino parthenolide (DMAPT), which induces the expression of two methyltransferases, i.e., SETD2 and NSD1; the latter is epigenetically silenced in neuroblastoma and functions therefore as a tumor suppressor [62].

4. Phox2B Signaling Pathway

4.1. Phox2B Molecular and Functional Biology

Paired-like homeobox 2b gene (*PHOX2B*) is located on chromosome 4p12; it consists of 3 exons and encodes for a protein of 314 amino acids, i.e., a transcription factor which is a fundamental regulator of the initial differentiation and development of neural crest cells. Phox2B is expressed in sympathetic, parasympathetic and enteric ganglia of the developing peripheral autonomic nervous system. Up to date, very little is known about the regulation of Phox2B. Bone morphogenetic protein (BMP) signaling released by the dorsal aorta induces the neural crest cells to express high levels of Phox2B, and an interplay between microRNA(miR)-204, *MYCN* and *PHOX2B* has been described [63]. Whereas *MYCN* positively controls *PHOX2B* transcriptional expression, miR-204 targets *PHOX2B* leading to its downregulation [64,65]. *PHOX2B*, on the other hand, regulates the Delta–Notch pathway leading cells towards differentiation. Upstream, the helix–loop–helix transcription factor MASH1, a pro-neural factor promoting neuronal differentiation, and several genes such as *PHOX2A*, *ALK* and *RET* show activity in enteric neuron precursors [63]. At the same time, Phox2A acts as a transcriptional regulator of Phox2B [66]. Interestingly, the NF-κB pathway is involved in *PHOX2B* transcriptional regulation: the inhibition of NF-κB causes the accumulation of Nestin, Sox2, and glial fibrillary acidic protein with the consequent maintenance of neural stem cells in an undifferentiated state [67].

4.2. Phox2B in Neuroblastoma

Mutations in *PHOX2B* lead to neurodevelopmental disorders and have been linked to several congenital human diseases, including congenital central hypoventilation syndrome and Hirschsprung's disease [68–71]. Additionally, in the last decade *PHOX2B* has moved to the main genetic landscape of neuroblastoma [69,72], becoming a reliable diagnostic marker in tissue and cytology specimens [12,73], and a specific and sensitive biomarker for minimal residual disease in neuroblastoma [74]. Indeed, a heterogenic pool of mutations in the PHOX2B gene have been observed in familial and sporadic forms of neuroblastoma, but in most neuroblastoma cells *PHOX2B* is highly expressed rather than downregulated [75]. Neuroblastoma exclusive *PHOX2B* mutations are missense mutations occurring in exon 1, frameshift mutations arising in exon 1 and 3 and unbalanced gene expressions [76]. Syndromic neuroblastoma is caused by mutations in the homeodomain and in exon 3. Some missense mutations lead to a decrease of Phox2B-mediated genes transcription [77]. This is also the case of frameshift mutations which either lead to elongated proteins or truncated Phox2B proteins [78]. *PHOX2B* mutations do impair inhibition of MSX1 (Msh Homeobox 1), thus resulting in upregulation of the Delta–Notch pathway, *NOTCH3* overexpression and activation, and aberrant transcriptional regulation of the anaplastic lymphoma kinase (*ALK*) gene by directly binding its promoter [72,79,80]. Among the many transcription factors involved at different neuroblastoma differentiation stages, *PHOX2B* and *MYCN* with their transcriptional targets *ALK* and *LIN28B*, and the tumor suppressor miRNAs let-7, miR-34, and miR-204 are mainly involved [65,81]. Ke et al. presented that *MYCN* expression promotes the proliferation of *PHOX2B*-positive neuronal progenitors, thus

forming hyperplastic lesions in sympathetic ganglia, and surmised this for the initiation and progression of neuroblastoma development [82]. Reiff et al. provided the first demonstration that *PHOX2B* mutations give rise to gain-of-function protein variants that stimulate the proliferation of potential tumor founder cells of the sympathoadrenergic lineage. Thus, they speculated that tumorigenesis may rely on the acquisition of novel, proliferation-stimulating properties, and that impaired differentiation [83]. However, *PHOX2B* also has tumor suppressor properties in the final metastatic phase: knockdown of *PHOX2B* in human neuroblastoma micrometastasis increased the metastatic potential of the cells, which were orthotopically inoculated in nude mice [84].

Based on the fact that *PHOX2B* transcription is regulated by the tumor microenvironment [84,85], Di Zanni et al. considered downregulation of *PHOX2B* expression by treating neuroblastoma cells with pharmacological compounds. Incubation of cell lines with mycophenolate mofetil and chloroquine, which negatively act on *PHOX2B* promoters, led to decreased cell proliferation and increased apoptosis and cell differentiation [67].

Similarly, de Pontual et al. showed that the differential expression of *PHOX2B* in several human neuroblastoma cell lines and tumor samples partially relied on the methylation-mediated silencing of its promoter. Among all 13 studied cell lines, SK-N-BE, GIMEN, and SK-N-SH expressed no Phox2B, and only SK-N-BE and GIMEN were able to re-express it after treatment with 5′-aza, a methyltransferase inhibitor [86].

5. Potential Nrf2—NF-κB—Phox2B Crosstalk in Neuroblastoma

Inflammation, as a response against tissue homeostasis impairment, is involved in a plethora of pathologic processes [13]. After signal recognition, Toll-like receptors (TLRs) mediate the activation of specific immune cascades leading to NF-κB dissociation from IκB. Subsequently, NF-κB is able to translocate into the nucleus, where it acts as a transcription factor, eventually upregulating the secretion of pro-inflammatory cytokines (e.g., IL-1β, IL-6 and, TNF-α) [69]. Consequent recruitment of immune cells at an injury site results in increased ROS and reactive nitrogen species (RNS). Since Nrf2-signaling plays a key role in redox homeostasis by reducing intracellular ROS levels, it might counteract NF-κB effects under determined conditions. A further regulatory mechanism is represented by Nrf2-mediated overexpression of phase II enzymes which block proteasomal degradation of IκB-α, necessary for NF-κB translocation into the nucleus [70]. An additional attractive crosstalk point between the two cascades has been found in CREB binding protein (CBP), which is a transcription co-activator of NF-κB, able to acetylate histones for chromatin remodeling as well as non-histone proteins [71]; in the case of Nrf2, this is a mandatory step for Neh1-domain-mediated DNA-binding. Similar to NF-κB, CBP acts as a transcriptional co-activator of Nrf2, binding its Neh4 and Neh5 domains, nevertheless with a lower affinity than to p65. Thus, both transcription factors compete for CBP accessibility: when p65 is overexpressed the amount of free CBP for Nrf2 is reduced and, hence, Nrf2-mediated gene transcription decreases, as depicted in Figure 3 [13].

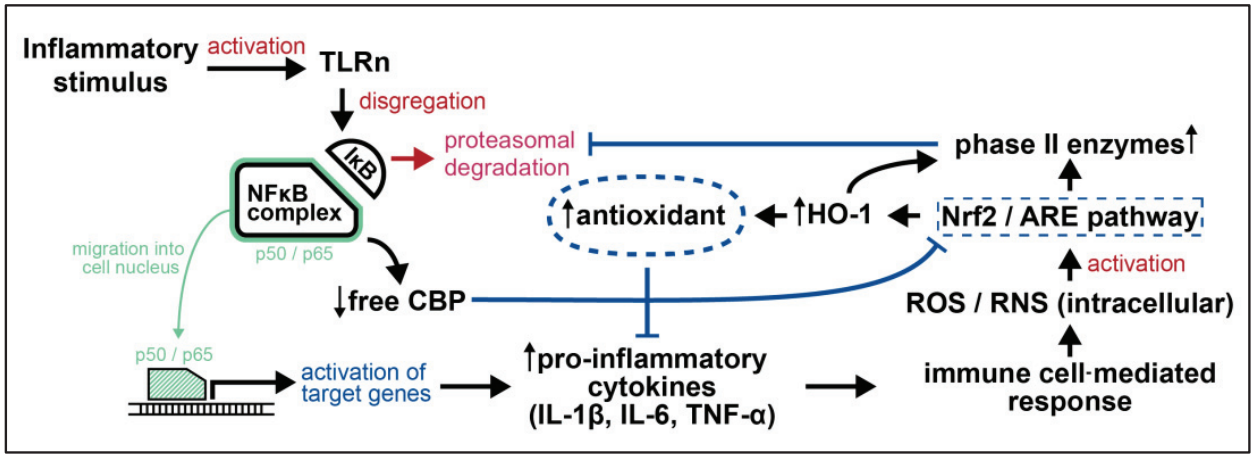


Figure 3. Mutual interaction between Nrf2 and NF-κB pathways in a proinflammatory state: CBP is a crucial intersection between the two axes. CBP is able to acetylate histone and promote chromatin remodeling, which is mandatory for NF-κB to start its translational activity. At the same time CBP-mediated acetylation of Nrf2 is necessary to allow DNA-binding. Nrf2 and NF-κB compete for the same co-activator. The NF-κB member p65 shows higher affinity for CBP than Nrf2: in the case of overexpression and/or overactivation of p65, the level of free CBP decreases, thereby downregulating Nrf2 transcriptional activity [19,87,88]. (→ activation; ↳ transcription; ⊣ inhibition; ↑ up-regulation; ↓ down-regulation).

To the best of our knowledge this multifaceted network has not been systematically investigated in neuroblastoma yet. A limited number of studies utilized SH-SY5Y human neuroblastoma cells to test the neuroprotective effect of multiple compounds, such as glucomoringin isothiocyanate (GMG-ITC), Klotho, Tavarua Deoxyriboside A, and Jasplakinolide [89–91]. The common denominator among these substances is their ability to inhibit the NF-κB signaling pathway while activating the Nrf2/ARE system. This crosstalk might be even more byzantine, considering the possible linkage between the aforementioned cascades and the paired-like homeobox 2B (*PHOX2B*) pathway via anaplastic lymphoma kinase (ALK).

As already underlined, various research works on SH-SY5Y cells suggested a rapid non-genomic activation of the Nrf2 axis mediated by PI3K/Akt and ERK1/2 [36–38]. Activation of ALK stimulates downstream signaling through both the PI3K/Akt and the MAPK/ERK cascade. Therefore, it may be presumed that an overactivation or overexpression of ALK indirectly leads to a reinforced activity of Nrf2 [92]. ALK-activating mutations have been detected in most familial and in 10% of sporadic neuroblastoma cases [93]. Concordantly, Heukamp et al. were able to induce neuroblastoma in transgenic mice through targeted expression of the most aggressive variant, ALKF1174. Chromosomal aberrations in this model, e.g., 17q gain and *MYCN* amplification, resembled those in human high-risk neuroblastoma [94]. Additionally, in the work of Lopez-Delisle et al. neuroblastoma patients with mutation-driven ALK activation showed poorer outcome than patients with non-mutated ALK [95]. An additional regulating element of *ALK* is Phox2B, since *ALK* is the major known target gene of Phox2B transcriptional activity [67].

In patients with neuroblastoma, different heterozygous missense and nonsense germline mutations of *PHOX2B* have been detected [69,76]. Its pathogenic role is traditionally associated with gene overexpression, which can in turn increase ALK expression and activity [67]. ALK upregulation might also be associated to a co-amplification with *MYCN*, either due to their proximity on 2p23, or as a direct transcriptional target of N-Myc [93]. The latter represents one of the most important hallmarks of high-risk neuroblastoma. The overmentioned PI3K was shown to stabilize N-Myc in mouse models for pediatric neural cancers [96]; PI3K overactivation has been reported in both neuroblastoma samples and cell lines, although its mutations are quite rare. Guo et al. have been able to correlate

high expression of ALK subunit p110α with worse treatment outcomes of neuroblastoma patients by analyzing gene expression data from primary neuroblastoma samples [97]. PI3K is usually recruited to the cell membrane by transmembrane receptor tyrosine kinases (RTKs) after binding with specific growth factors. A further and crucial step is the activation of Akt. Opel et al. first provided evidence that Akt activation correlates with poor outcome by analyzing 116 primary neuroblastoma samples [98]. More recent studies on multiple neuroblastoma cell lines demonstrated how inhibitors of Akt (e.g., Hu7691 [99]) or PI3K (e.g., BI-D1870 [100]) induced suppression of cell growth. Moreover, it shall not be forgotten that Akt is able to regulate activation of other transcription factors, e.g., NF-κB [101]. It is noteworthy that NF-κB signaling is required for neural stem cells' early differentiation, as it is involved in *PHOX2B* transcriptional regulation. In fact, inhibition of NF-κB in transgenic mice maintains neural stem cells in an undifferentiated state [102]. This transcription factor also controls the transcription of *RET*, another RTK such as PI3K. RET is indispensable for physiologic development of sympathetic neurons [103] and its inactivating mutations and polymorphisms are a major cause of Hirschsprung's disease, defined by colonic aganglionosis [104]. In addition, RET interacts with ALK and TRK, likewise members of the RTK family [103]. Recently it has been more precisely defined that RET hyperactivation is driven by ALK in neuroblastoma [95]. In SH-SY5Y neuroblastoma cells this regulation seems to be mediated through the PI3K/AKT pathway, which in turn can be activated by RET. Indeed, the RET-induced activation of RAS/ERK and PI3K/Akt would lead to hyperfunction of both Nrf2 and NF-κB pathways, which seem to primarily inhibit each other, shedding light on the complexity of the crosstalk between Phox2B and both Nrf2 and NF-κB cascades [105].

According to the proposed interactions, it may be possible to presume a shared therapeutic pattern in neuroblastoma in which a few compounds regulate at least two of the hereby described transcription factors. Some groups have shown the effects of phytocompounds, such as sulforaphane and luteolin, on the methylation status of *NFE2L2/KEAP-1* promoters, thereby influencing cell survival and tumor progression in different cancer entities [43,106]. Luteolin regulates not only the Nrf2 axis via epigenetic modulation, but also inhibits NF-κB, thereby blocking the degradation of IκBα [43]. The use of 5´-aza may also be considered in neuroblastoma treatment due to its regulatory effect on the activity of both *KEAP-1* and *PHOX2B* promoters [86].

In order to reinforce the importance of analyzing the NF-κB—Nrf2—Phox2B network we used the well-known core data source STRING, which shows active and predicted protein–protein interactions based on a given query. These interactions include both physical and functional associations and rely on sources like laboratory experiments data, automated text mining and previous knowledge in datasets [107].

Introducing Phox2B, NF-κB (family members), Nrf2 (encoded as *NFE2L2*), Keap1, CREB/CREB binding protein (CBP), Akt/ERK, PI3K, ALK and *MYCN* in the STRING search tool and filtering for interactions with a high minimum-required interaction score (>0.750—high confidence), the STRING algorithm system output the scheme depicted in Figure 4. STRING also showed differentiated interaction confidence scores, based on each of the aforementioned sources. In the case of RelA and Nrf2, for instance, a direct interaction at the experimental level accounted for a score of 0.322. As described above, both transcription factors share —at least—the two binding proteins Keap1 and CBP (with respective purely experimental confidence scores of 0.370/0.991 for RelA and of 0.999/0.625 for Nrf2). Interestingly, NF-κB (RelA/p65) and Nrf2 are involved in both metabolic and in stress pathways.

Subsequently, CREB/CBP, Nrf2 and RelA converge in AKT1 which, in turn, affiliates with *MYCN* with a combined score of 0.798. At this point, *MYCN*, ALK, RET and Phox2B built a complex network involved in different types of cancer, mostly involving the nervous system, also in regard to NF-κB (RelA/p65).

By screening for common GO pathways (Gene Ontology—see Supplementary Material), NF-κB (RelA/p65), Nrf2, and Phox2B join in the regulation of metabolic processes.

Furthermore, Nrf2 and Phox2B share a role in the regulation of neuron projection development, whereas RelA/p65 and Nrf2 are functionally close in the regulation of the immunological and inflammatory response, especially to reactive oxygen species (Figure 4).

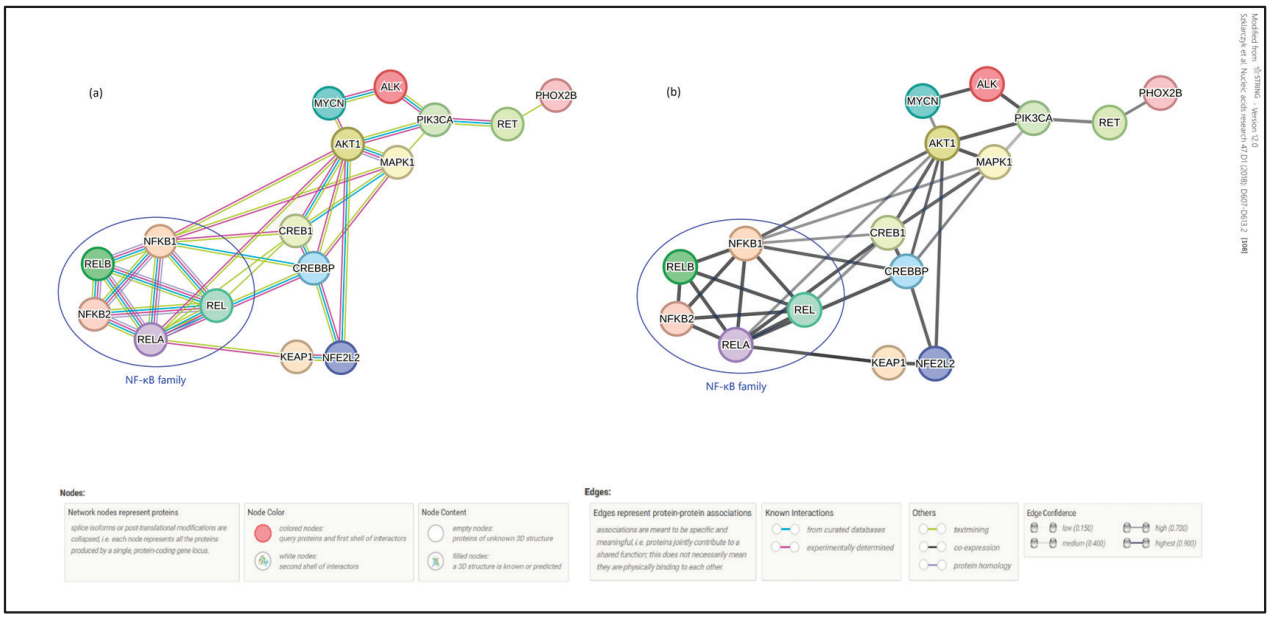


Figure 4. STRING-derived protein–protein interaction network: inserting in the STRING research tool the main proteins and genes highlighted in the review and filtering for interactions with a high minimum-required interaction score (>0.750—high confidence), the STRING algorithm system outputs a network in which all elements are linked. In (**a**) different levels of interaction are shown; in (**b**) the line thickness and grey color intensity indicate the strength of data support [108].

6. Conclusions

This review outlines the molecular and functional biology of the three transcription factors Nrf2, NF-κB and Phox2B, insinuating the role of their interaction in the development, progression and aggressive behaviour of neuroblastoma.

Unlike Phox2B, which is well described in the context of neuroblastoma, it is not possible to clearly infer the potential actions of Nrf2 and NF-κB in the molecular machinery of this cancer type. Based on recent studies and extrapolating the regulation patterns from other tumor entities, hereby we have intended to depict a structural and functional model in which Nrf2, NF-κB and Phox2B may compose a transcription factor network. The regulation of it may be finely orchestrated by a wide range of stimuli interacting with several receptors.

Considering neuroblastoma as the result of a dysregulated network rather than the sum of multiple genetic alterations, we encourage the screening and analysis of neuroblastoma in a more comprehensive way, allowing better patient stratification and therefore optimized identification of novel therapeutic targets.

Starting from the analysis we performed with STRING, the development of a highly stringent computational model for the hereby presented networks might be a decisive next step, as shown by Lombardo et al. for PD-L1 expression in neuroblastoma [108].

In summary, a structured description of understudied signaling pathways in neuroblastoma might exert a better understanding of tumor progression and drug resistance in aggressive neuroblastoma phenotypes and, thus, facilitate the individuation of novel therapeutic targets.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cimb46040200/s1, File S1: Gene ontology dataset of the STRING-derived protein–protein interaction network displayed in Figure 4.

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References

1. Matthay, K.K.; Maris, J.M.; Schleiermacher, G.; Nakagawara, A.; Mackall, C.L.; Diller, L.; Weiss, W.A. Neuroblastoma. *Nat. Rev. Dis. Primers* **2016**, *2*, 16078. [CrossRef] [PubMed]
2. Erdmann, F.; Kaatsch, P.; Grabow, D.; Spix, C. *'German Childhood Cancer Registry—Annual Report 2019 (1980–2018)*; Institute of Medical Biostatistics, Epidemiology and Informatics (IMBEI) at the University Medical Center of the Johannes Gutenberg University Mainz: Mainz, Germany, 2020.
3. Shimada, H.; Chatten, J.; Newton, W.A.; Sachs, N.; Hamoudi, A.B.; Chiba, T.; Marsden, H.B.; Misugi, K. Histopathologic Prognostic Factors in Neuroblastic Tumors: Definition of Subtypes of Ganglioneuroblastoma and an Age-Linked Classification of Neuroblastomas. *JNCI J. Natl. Cancer Inst.* **1984**, *73*, 405–416. [CrossRef]
4. Zafar, A.; Wang, W.; Liu, G.; Wang, X.; Xian, W.; McKeon, F.; Foster, J.; Zhou, J.; Zhang, R. Molecular targeting therapies for neuroblastoma: Progress and challenges. *Med. Res. Rev.* **2020**, *41*, 961–1021. [CrossRef]
5. Aygun, N. Biological and Genetic Features of Neuroblastoma and Their Clinical Importance. *Curr. Pediatr. Rev.* **2018**, *14*, 73–90. [CrossRef] [PubMed]
6. Berthold, F.; Spix, C.; Kaatsch, P.; Lampert, F. Incidence, Survival, and Treatment of Localized and Metastatic Neuroblastoma in Germany 1979–2015. *Pediatr. Drugs* **2017**, *19*, 577–593. [CrossRef]
7. Park, J.R.; Bagatell, R.; London, W.B.; Maris, J.M.; Cohn, S.L.; Mattay, K.M.; Hogarty, M.; on behalf of the COG Neuroblastoma Committee. Children's Oncology Group's 2013 blueprint for research: Neuroblastoma. *Pediatr. Blood Cancer* **2013**, *60*, 985–993. [CrossRef]
8. Pinto, N.R.; Applebaum, M.A.; Volchenboum, S.L.; Matthay, K.K.; London, W.B.; Ambros, P.F.; Nakagawara, A.; Berthold, F.; Schleiermacher, G.; Park, J.R.; et al. Advances in Risk Classification and Treatment Strategies for Neuroblastoma. *J. Clin. Oncol.* **2015**, *33*, 3008–3017. [CrossRef]
9. Berlanga, P.; Cañete, A.; Castel, V. Advances in emerging drugs for the treatment of neuroblastoma. *Expert Opin. Emerg. Drugs* **2016**, *22*, 63–75. [CrossRef]
10. Megison, M.L.; Gillory, L.A.; Beierle, E.A. Cell Survival Signaling in Neuroblastoma. *Anti-Cancer Agents Med. Chem.* **2013**, *13*, 563–575. [CrossRef]
11. Louis, C.U.; Shohet, J.M. Neuroblastoma: Molecular pathogenesis and therapy. *Annu. Rev. Med.* **2015**, *66*, 49–63. [CrossRef]
12. Ma, Y.; Feng, J.; Zhao, J.; Ding, D.; Tian, F.; Chen, L.; Zheng, J.; Xiao, X. PHOX2B as a Reliable Marker for Neuroblastoma in Tissue and Cytology Specimens. *J. Neuropathol. Exp. Neurol.* **2021**, *80*, 1108–1116. [CrossRef]
13. Kobayashi, M.; Yamamoto, M. Molecular Mechanisms Activating the Nrf2-Keap1 Pathway of Antioxidant Gene Regulation. *Antioxidants Redox Signal.* **2005**, *7*, 385–394. [CrossRef]
14. Krajka-Kuźniak, V.; Paluszczak, J.; Baer-Dubowska, W. The Nrf2-ARE signaling pathway: An update on its regulation and possible role in cancer prevention and treatment. *Pharmacol. Rep.* **2017**, *69*, 393–402. [CrossRef]
15. Ahmed, S.M.U.; Luo, L.; Namani, A.; Wang, X.J.; Tang, X. Nrf2 signaling pathway: Pivotal roles in inflammation. *Biochim. Biophys. Acta Mol. Basis Dis.* **2017**, *1863*, 585–597. [CrossRef]
16. Furfaro, A.L.; Piras, S.; Domenicotti, C.; Fenoglio, D.; De Luigi, A.; Salmona, M.; Moretta, L.; Marinari, U.M.; Pronzato, M.A.; Traverso, N.; et al. Role of Nrf2, HO-1 and GSH in Neuroblastoma Cell Resistance to Bortezomib. *PLoS ONE* **2016**, *11*, e0152465. [CrossRef]
17. Sekhar, K.R.; Rachakonda, G.; Freeman, M.L. Cysteine-based regulation of the CUL3 adaptor protein Keap1. *Toxicol. Appl. Pharmacol.* **2010**, *244*, 21–26. [CrossRef]
18. Wu, S.; Lu, H.; Bai, Y. Nrf2 in cancers: A double-edged sword. *Cancer Med.* **2019**, *8*, 2252–2267. [CrossRef]
19. Saha, S.; Buttari, B.; Panieri, E.; Profumo, E.; Saso, L. An Overview of Nrf2 Signaling Pathway and Its Role in Inflammation. *Molecules* **2020**, *25*, 5474. [CrossRef]

20. Huang, Y.; Li, W.; Su, Z.-Y.; Kong, A.-N.T. The complexity of the Nrf2 pathway: Beyond the antioxidant response. *J. Nutr. Biochem.* **2015**, *26*, 1401–1413. [CrossRef] [PubMed]
21. Rada, P.; Rojo, A.I.; Chowdhry, S.; McMahon, M.; Hayes, J.D.; Cuadrado, A. SCF/β-TrCP Promotes Glycogen Synthase Kinase 3-Dependent Degradation of the Nrf2 Transcription Factor in a Keap1-Independent Manner. *Mol. Cell. Biol.* **2011**, *31*, 1121–1133. [CrossRef] [PubMed]
22. Wang, L.; Chen, Y.; Sternberg, P.; Cai, J. Essential Roles of the PI3 Kinase/Akt Pathway in Regulating Nrf2-Dependent Anti-oxidant Functions in the RPE. *Invest. Ophthalmol. Vis. Sci.* **2008**, *49*, 1671–1678. [CrossRef] [PubMed]
23. Ramos-Gomez, M.; Kwak, M.-K.; Dolan, P.M.; Itoh, K.; Yamamoto, M.; Talalay, P.; Kensler, T.W. Sensitivity to carcinogenesis is increased and chemoprotective efficacy of enzyme inducers is lost in *nrf2* transcription factor-deficient mice. *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 3410–3415. [CrossRef] [PubMed]
24. Reddy, N.M.; Kleeberger, S.R.; Bream, J.H.; Fallon, P.G.; Kensler, T.W.; Yamamoto, M.; Reddy, S.P. Genetic disruption of the Nrf2 compromises cell-cycle progression by impairing GSH-induced redox signaling. *Oncogene* **2008**, *27*, 5821–5832. [CrossRef] [PubMed]
25. Holmström, K.M.; Baird, L.; Zhang, Y.; Hargreaves, I.; Chalasani, A.; Land, J.M.; Stanyer, L.; Yamamoto, M.; Dinkova-Kostova, A.T.; Abramov, A.Y. Nrf2 Impacts Cellular Bioenergetics by Controlling Substrate Availability for Mitochondrial Respiration. *Biol. Open* **2013**, *2*, 761–770. [CrossRef] [PubMed]
26. Shibuya, A.; Onda, K.; Kawahara, H.; Uchiyama, Y.; Nakayama, H.; Omi, T.; Nagaoka, M.; Matsui, H.; Hirano, T. Sofalcone, a gastric mucosa protective agent, increases vascular endothelial growth factor via the Nrf2-heme-oxygenase-1 dependent pathway in gastric epithelial cells. *Biochem. Biophys. Res. Commun.* **2010**, *398*, 581–584. [CrossRef]
27. Niture, S.K.; Jaiswal, A.K. Nrf2 Protein Up-regulates Antiapoptotic Protein Bcl-2 and Prevents Cellular Apoptosis. *J. Biol. Chem.* **2012**, *287*, 9873–9886. [CrossRef] [PubMed]
28. Miller, B.A. TRPM2 in Cancer. *Cell Calcium* **2019**, *80*, 8–17. [CrossRef] [PubMed]
29. Kaufman, Z.; Salvador, G.; Liu, X.; Oteiza, P. Zinc and the modulation of Nrf2 in human neuroblastoma cells. *Free. Radic. Biol. Med.* **2020**, *155*, 1–9. [CrossRef] [PubMed]
30. Kim, J.-Y.; Surh, Y.-J. The Role of Nrf2 in Cellular Innate Immune Response to Inflammatory Injury. *Toxicol. Res.* **2009**, *25*, 159–173. [CrossRef]
31. High-Risk Neuroblastoma Standard Clinical Practice Recommendations. Available online: https://siope.eu/media/documents/escp-high-risk-neuroblastoma-standard-clinical-practice-recommendations.pdf (accessed on 1 January 2024).
32. Taguchi, K.; Motohashi, H.; Yamamoto, M. Molecular mechanisms of the Keap1-Nrf2 pathway in stress response and cancer evolution. *Genes Cells* **2011**, *16*, 123–140. [CrossRef]
33. Wang, X.-J.; Sun, Z.; Villeneuve, N.F.; Zhang, S.; Zhao, F.; Li, Y.; Chen, W.; Yi, X.; Zheng, W.; Wondrak, G.T.; et al. Nrf2 enhances resistance of cancer cells to chemotherapeutic drugs, the dark side of Nrf2. *Carcinogenesis* **2008**, *29*, 1235–1243. [CrossRef] [PubMed]
34. Cai, S.J.; Liu, Y.; Han, S.; Yang, C. Brusatol, an NRF2 inhibitor for future cancer therapeutic. *Cell Biosci.* **2019**, *9*, 1–3. [CrossRef]
35. Ren, D.; Villeneuve, N.F.; Jiang, T.; Wu, T.; Lau, A.; Toppin, H.A.; Zhang, D.D. Brusatol enhances the efficacy of chemotherapy by inhibiting the Nrf2-mediated defense mechanism. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 1433–1438. [CrossRef] [PubMed]
36. Ramos, V.d.M.; Gasparotto, J.; Figueiró, F.; Dias, A.d.F.; Rostirolla, D.C.; Somensi, N.; da Rosa, H.T.; Grun, L.K.; Barbé-Tuana, F.M.; Gelain, D.P.; et al. Retinoic acid downregulates thiol antioxidant defences and homologous recombination while promotes A549 cells sensitization to cisplatin. *Cell. Signal.* **2019**, *62*, 109356. [CrossRef] [PubMed]
37. He, D.; Fu, S.; Zhou, A.; Su, Y.; Gao, X.; Zhang, Y.; Huang, B.; Du, J.; Liu, D. Camptothecin Regulates Microglia Polarization and Exerts Neuroprotective Effects via Activating AKT/Nrf2/HO-1 and Inhibiting NF-κB Pathways In Vivo and In Vitro. *Front. Immunol.* **2021**, *12*, 619761. [CrossRef] [PubMed]
38. Yang, R.; Ma, S.; Zhuo, R.; Xu, L.; Jia, S.; Yang, P.; Yao, Y.; Cao, H.; Ma, L.; Pan, J.; et al. Suppression of endoplasmic reticulum stress-dependent autophagy enhances cynaropicrin-induced apoptosis via attenuation of the P62/Keap1/Nrf2 pathways in neuroblastoma. *Front. Pharmacol.* **2022**, *13*, 977622. [CrossRef] [PubMed]
39. Chaudhari, N.; Ravanan, P. Bardoxolone methyl induces neuritogenesis in Neuro2a cells. *Pharmacol. Rep.* **2018**, *70*, 730–736. [CrossRef] [PubMed]
40. Chaudhari, N.; Talwar, P.; D'Hellencourt, C.L.; Ravanan, P. CDDO and ATRA Instigate Differentiation of IMR32 Human Neuroblastoma Cells. *Front. Mol. Neurosci.* **2017**, *10*, 310. [CrossRef]
41. Odarenko, K.V.; Salomatina, O.V.; Chernikov, I.V.; Salakhutdinov, N.F.; Zenkova, M.A.; Markov, A.V. Soloxolone Methyl Reduces the Stimulatory Effect of Leptin on the Aggressive Phenotype of Murine Neuro2a Neuroblastoma Cells via the MAPK/ERK1/2 Pathway. *Pharmaceuticals* **2023**, *16*, 1369. [CrossRef] [PubMed]
42. Zhao, F.; Zhang, J.; Chang, N. Epigenetic modification of Nrf2 by sulforaphane increases the antioxidative and anti-inflammatory capacity in a cellular model of Alzheimer's disease. *Eur. J. Pharmacol.* **2018**, *824*, 1–10. [CrossRef]
43. Kang, K.A.; Piao, M.J.; Hyun, Y.J.; Zhen, A.X.; Cho, S.J.; Ahn, M.J.; Yi, J.M.; Hyun, J.W. Luteolin promotes apoptotic cell death via upregulation of Nrf2 expression by DNA demethylase and the interaction of Nrf2 with p53 in human colon cancer cells. *Exp. Mol. Med.* **2019**, *51*, 1–14. [CrossRef] [PubMed]

44. Wang, C.; Shu, L.; Zhang, C.; Li, W.; Wu, R.; Guo, Y.; Yang, Y.; Kong, A. Histone Methyltransferase Setd7 Regulates Nrf2 Signaling Pathway by Phenethyl Isothiocyanate and Ursolic Acid in Human Prostate Cancer Cells. *Mol. Nutr. Food Res.* **2018**, *62*, e1700840. [CrossRef] [PubMed]
45. Arumugam, T.; Ghazi, T.; Chuturgoon, A.A. Fumonisin B1 alters global m6A RNA methylation and epigenetically regulates Keap1-Nrf2 signaling in human hepatoma (HepG2) cells. *Arch. Toxicol.* **2021**, *95*, 1367–1378. [CrossRef] [PubMed]
46. Huang, T.T.; Kudo, N.; Yoshida, M.; Miyamoto, S. A nuclear export signal in the N-terminal regulatory domain of IκBα controls cytoplasmic localization of inactive NF-κB/IκBα complexes. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 1014–1019. [CrossRef] [PubMed]
47. Solan, N.J.; Miyoshi, H.; Carmona, E.M.; Bren, G.D.; Paya, C.V. RelB Cellular Regulation and Transcriptional Activity Are Regulated by p100. *J. Biol. Chem.* **2002**, *277*, 1405–1418. [CrossRef] [PubMed]
48. Jardin, F. NFkB Pathway and Hodgkin Lymphoma. *Biomedicines* **2022**, *10*, 2153. [CrossRef] [PubMed]
49. KEGG PATHWAY: NF-Kappa B Signaling Pathway—Reference Pathway'. Available online: https://www.kegg.jp/pathway/map=map04064&keyword=nfkb (accessed on 8 January 2024).
50. Savinova, O.V.; Hoffmann, A.; Ghosh, G. The Nfkb1 and Nfkb2 Proteins p105 and p100 Function as the Core of High-Molecular-Weight Heterogeneous Complexes. *Mol. Cell* **2009**, *34*, 591–602. [CrossRef] [PubMed]
51. Sun, S.-C. The non-canonical NF-κB pathway in immunity and inflammation. *Nat. Rev. Immunol.* **2017**, *17*, 545–558. [CrossRef]
52. Kayagaki, N.; Yan, M.; Seshasayee, D.; Wang, H.; Lee, W.; French, D.M.; Grewal, I.S.; Cochran, A.G.; Gordon, N.C.; Yin, J.; et al. BAFF/BLyS Receptor 3 Binds the B Cell Survival Factor BAFF Ligand through a Discrete Surface Loop and Promotes Processing of NF-κB2. *Immunity* **2002**, *17*, 515–524. [CrossRef]
53. Fagiani, F.; Catanzaro, M.; Buoso, E.; Basagni, F.; Di Marino, D.; Raniolo, S.; Amadio, M.; Frost, E.H.; Corsini, E.; Racchi, M.; et al. Targeting Cytokine Release Through the Differential Modulation of Nrf2 and NF-κB Pathways by Electrophilic/Non-Electrophilic Compounds. *Front. Pharmacol.* **2020**, *11*, 1256. [CrossRef]
54. Zhang, T.; Ma, C.; Zhang, Z.; Zhang, H.; Hu, H. NF-κB signaling in inflammation and cancer. *Medcomm* **2021**, *2*, 618–653. [CrossRef] [PubMed]
55. Medeiros, M.; Candido, M.F.; Valera, E.T.; Brassesco, M.S. The multifaceted NF-kB: Are there still prospects of its inhibition for clinical intervention in pediatric central nervous system tumors? *Cell. Mol. Life Sci.* **2021**, *78*, 6161–6200. [CrossRef] [PubMed]
56. Bian, X.; McAllister-Lucas, L.M.; Shao, F.; Schumacher, K.R.; Feng, Z.; Porter, A.G.; Castle, V.P.; Opipari, A.W. NF-κB Activation Mediates Doxorubicin-induced Cell Death in N-type Neuroblastoma Cells. *J. Biol. Chem.* **2001**, *276*, 48921–48929. [CrossRef] [PubMed]
57. Brandetti, E.; Focaccetti, C.; Pezzolo, A.; Ognibene, M.; Folgiero, V.; Cotugno, N.; Benvenuto, M.; Palma, P.; Manzari, V.; Rossi, P.; et al. Enhancement of Neuroblastoma NK-Cell-Mediated Lysis through NF-kB p65 Subunit-Induced Expression of FAS and PVR, the Loss of Which Is Associated with Poor Patient Outcome. *Cancers* **2021**, *13*, 4368. [CrossRef] [PubMed]
58. Posadas, I.; Santos, P.; Ceña, V. Acetaminophen Induces Human Neuroblastoma Cell Death through NFKB Activation. *PLoS ONE* **2012**, *7*, e50160. [CrossRef] [PubMed]
59. Karacay, B.; Sanlioglu, S.; Griffith, T.S.; Sandler, A.; Bonthius, D.J. Inhibition of the NF-κB pathway enhances TRAIL-mediated apoptosis in neuroblastoma cells. *Cancer Gene Ther.* **2004**, *11*, 681–690. [CrossRef] [PubMed]
60. Lorenzi, S.; Forloni, M.; Cifaldi, L.; Antonucci, C.; Citti, A.; Boldrini, R.; Pezzullo, M.; Castellano, A.; Russo, V.; van der Bruggen, P.; et al. IRF1 and NF-kB Restore MHC Class I-Restricted Tumor Antigen Processing and Presentation to Cytotoxic T Cells in Aggressive Neuroblastoma. *PLoS ONE* **2012**, *7*, e46928. [CrossRef] [PubMed]
61. Forloni, M.; Albini, S.; Limongi, M.Z.; Cifaldi, L.; Boldrini, R.; Nicotra, M.R.; Giannini, G.; Natali, P.G.; Giacomini, P.; Fruci, D. NF-κB, and not MYCN, Regulates MHC Class I and Endoplasmic Reticulum Aminopeptidases in Human Neuroblastoma Cells. *Cancer Res.* **2010**, *70*, 916–924. [CrossRef] [PubMed]
62. Nakshatri, H.; Appaiah, H.N.; Anjanappa, M.; Gilley, D.; Tanaka, H.; Badve, S.; Crooks, P.A.; Mathews, W.; Sweeney, C.; Bhat-Nakshatri, P. NF-κB-dependent and -independent epigenetic modulation using the novel anti-cancer agent DMAPT. *Cell Death Dis.* **2015**, *6*, e1608. [CrossRef]
63. Pattyn, A.; Morin, X.; Cremer, H.; Goridis, C.; Brunet, J.-F. The homeobox gene Phox2b is essential for the development of autonomic neural crest derivatives. *Nature* **1999**, *399*, 366–370. [CrossRef]
64. Ooi, C.Y.; Carter, D.R.; Liu, B.; Mayoh, C.; Beckers, A.; Lalwani, A.; Nagy, Z.; De Brouwer, S.; Decaesteker, B.; Hung, T.-T.; et al. Network Modeling of microRNA–mRNA Interactions in Neuroblastoma Tumorigenesis Identifies miR-204 as a Direct Inhibitor of MYCN. *Cancer Res.* **2018**, *78*, 3122–3134. [CrossRef] [PubMed]
65. Perri, P.; Ponzoni, M.; Corrias, M.V.; Ceccherini, I.; Candiani, S.; Bachetti, T. A Focus on Regulatory Networks Linking MicroRNAs, Transcription Factors and Target Genes in Neuroblastoma. *Cancers* **2021**, *13*, 5528. [CrossRef] [PubMed]
66. Flora, A.; Lucchetti, H.; Benfante, R.; Goridis, C.; Clementi, F.; Fornasari, D. SP Proteins and PHOX2B Regulate the Expression of the Human*PHOX2a*Gene. *J. Neurosci.* **2001**, *21*, 7037–7045. [CrossRef] [PubMed]
67. Di Zanni, E.; Bianchi, G.; Ravazzolo, R.; Raffaghello, L.; Ceccherini, I.; Bachetti, T. Targeting of*PHOX2B*expression allows the identification of drugs effective in counteracting neuroblastoma cell growth. *Oncotarget* **2017**, *8*, 72133–72146. [CrossRef] [PubMed]
68. Amiel, J.; Laudier, B.; Attié-Bitach, T.; Trang, H.; de Pontual, L.; Gener, B.; Trochet, D.; Etchevers, H.; Ray, P.; Simonneau, M.; et al. Polyalanine expansion and frameshift mutations of the paired-like homeobox gene PHOX2B in congenital central hypoventilation syndrome. *Nat. Genet.* **2003**, *33*, 459–461. [CrossRef] [PubMed]

69. Mosse, Y.P.; Laudenslager, M.; Khazi, D.; Carlisle, A.J.; Winter, C.L.; Rappaport, E.; Maris, J.M. Germline PHOX2B Mutation in Hereditary Neuroblastoma. *Am. J. Hum. Genet.* **2004**, *75*, 727–730. [CrossRef]
70. Zhao, J.; Zhu, Y.; Xie, X.; Yao, Y.; Zhang, J.; Zhang, R.; Huang, L.; Cheng, J.; Xia, H.; He, J.; et al. Pleiotropic effect of common PHOX2B variants in Hirschsprung disease and neuroblastoma. *Aging* **2019**, *11*, 1252–1261. [CrossRef]
71. Di Lascio, S.; Benfante, R.; Cardani, S.; Fornasari, D. Research Advances on Therapeutic Approaches to Congenital Central Hypoventilation Syndrome (CCHS). *Front. Neurosci.* **2021**, *14*, 615666. [CrossRef] [PubMed]
72. van Limpt, V.; Chan, A.; Schramm, A.; Eggert, A.; Versteeg, R. Phox2B mutations and the Delta–Notch pathway in neuroblastoma. *Cancer Lett.* **2005**, *228*, 59–63. [CrossRef]
73. Hung, Y.P.; Lee, J.P.; Bellizzi, A.M.; Hornick, J.L. PHOX2B reliably distinguishes neuroblastoma among small round blue cell tumours. *Histopathology* **2017**, *71*, 786–794. [CrossRef]
74. Stutterheim, J.; Gerritsen, A.; Zappeij-Kannegieter, L.; Kleijn, I.; Dee, R.; Hooft, L.; van Noesel, M.M.; Bierings, M.; Berthold, F.; Versteeg, R.; et al. *PHOX2B* Is a Novel and Specific Marker for Minimal Residual Disease Testing in Neuroblastoma. *J. Clin. Oncol.* **2008**, *26*, 5443–5449. [CrossRef] [PubMed]
75. Longo, L.; Borghini, S.; Schena, F.; Parodi, S.; Albino, D.; Bachetti, T.; Da Prato, L.; Truini, M.; Gambini, C.; Tonini, G.P.; et al. PHOX2A and PHOX2B genes are highly co-expressed in human neuroblastoma. *Int. J. Oncol.* **1992**, *33*, 985–991. [CrossRef]
76. Trochet, D.; Bourdeaut, F.; Janoueix-Lerosey, I.; Deville, A.; de Pontual, L.; Schleiermacher, G.; Coze, C.; Philip, N.; Frébourg, T.; Munnich, A.; et al. Germline Mutations of the Paired–Like Homeobox 2B (PHOX2B) Gene in Neuroblastoma. *Am. J. Hum. Genet.* **2004**, *74*, 761–764. [CrossRef] [PubMed]
77. Di Lascio, S.; Bachetti, T.; Saba, E.; Ceccherini, I.; Benfante, R.; Fornasari, D. Transcriptional dysregulation and impairment of PHOX2B auto-regulatory mechanism induced by polyalanine expansion mutations associated with congenital central hypoventilation syndrome. *Neurobiol. Dis.* **2012**, *50*, 187–200. [CrossRef] [PubMed]
78. Di Lascio, S.; Benfante, R.; Di Zanni, E.; Cardani, S.; Adamo, A.; Fornasari, D.; Ceccherini, I.; Bachetti, T. Structural and functional differences in*PHOX2B*frameshift mutations underlie isolated or syndromic congenital central hypoventilation syndrome. *Hum. Mutat.* **2017**, *39*, 219–236. [CrossRef] [PubMed]
79. Revet, I.; Huizenga, G.; Chan, A.; Koster, J.; Volckmann, R.; van Sluis, P.; Øra, I.; Versteeg, R.; Geerts, D. The MSX1 homeobox transcription factor is a downstream target of PHOX2B and activates the Delta–Notch pathway in neuroblastoma. *Exp. Cell Res.* **2008**, *314*, 707–719. [CrossRef] [PubMed]
80. Chen, Y.; Takita, J.; Choi, Y.L.; Kato, M.; Ohira, M.; Sanada, M.; Wang, L.; Soda, M.; Kikuchi, A.; Igarashi, T.; et al. Oncogenic mutations of ALK kinase in neuroblastoma. *Nature* **2008**, *455*, 971–974. [CrossRef] [PubMed]
81. Pugh, T.J.; Morozova, O.; Attiyeh, E.F.; Asgharzadeh, S.; Wei, J.S.; Auclair, D.; Carter, S.L.; Cibulskis, K.; Hanna, M.; Kiezun, A.; et al. The genetic landscape of high-risk neuroblastoma. *Nat. Genet.* **2013**, *45*, 279–284. [CrossRef] [PubMed]
82. Ke, X.-X.; Zhang, D.; Zhao, H.; Hu, R.; Dong, Z.; Yang, R.; Zhu, S.; Xia, Q.; Ding, H.-F.; Cui, H. Phox2B correlates with MYCN and is a prognostic marker for neuroblastoma development. *Oncol. Lett.* **2015**, *9*, 2507–2514. [CrossRef] [PubMed]
83. Reiff, T.; Tsarovina, K.; Majdazari, A.; Schmidt, M.; del Pino, I.; Rohrer, H. Neuroblastoma Phox2b Variants Stimulate Proliferation and Dedifferentiation of Immature Sympathetic Neurons. *J. Neurosci.* **2010**, *30*, 905–915. [CrossRef]
84. Naftali, O.; Maman, S.; Meshel, T.; Sagi-Assif, O.; Ginat, R.; Witz, I.P. PHOX2B is a suppressor of neuroblastoma metastasis. *Oncotarget* **2016**, *7*, 10627–10637. [CrossRef] [PubMed]
85. Maman, S.; Edry-Botzer, L.; Sagi-Assif, O.; Meshel, T.; Yuan, W.; Lu, W.; Witz, I.P. The metastatic microenvironment: Lung-derived factors control the viability of neuroblastoma lung metastasis. *Int. J. Cancer* **2013**, *133*, 2296–2306. [CrossRef] [PubMed]
86. de Pontual, L.; Trochet, D.; Bourdeaut, F.; Thomas, S.; Etchevers, H.; Chompret, A.; Minard, V.; Valteau, D.; Brugieres, L.; Munnich, A.; et al. Methylation-associated PHOX2B gene silencing is a rare event in human neuroblastoma. *Eur. J. Cancer* **2007**, *43*, 2366–2372. [CrossRef] [PubMed]
87. Iyer, N.G.; Özdag, H.; Caldas, C. p300/CBP and cancer. *Oncogene* **2004**, *23*, 4225–4231. [CrossRef] [PubMed]
88. Yerra, V.G.; Negi, G.; Sharma, S.S.; Kumar, A. Potential therapeutic effects of the simultaneous targeting of the Nrf2 and NF-κB pathways in diabetic neuropathy. *Redox Biol.* **2013**, *1*, 394–397. [CrossRef] [PubMed]
89. Sedighi, M.; Baluchnejadmojarad, T.; Afshin-Majd, S.; Amiri, M.; Aminzade, M.; Roghani, M. Anti-aging Klotho Protects SH-SY5Y Cells Against Amyloid β1–42 Neurotoxicity: Involvement of Wnt1/pCREB/Nrf2/HO-1 Signaling. *J. Mol. Neurosci.* **2020**, *71*, 19–27. [CrossRef] [PubMed]
90. Alvariño, R.; Alonso, E.; Tabudravu, J.N.; Pérez-Fuentes, N.; Alfonso, A.; Botana, L.M. Tavarua Deoxyriboside A and Jasplakinolide as Potential Neuroprotective Agents: Effects on Cellular Models of Oxidative Stress and Neuroinflammation. *ACS Chem. Neurosci.* **2020**, *12*, 150–162. [CrossRef] [PubMed]
91. Jaafaru, M.S.; Nordin, N.; Rosli, R.; Shaari, K.; Bako, H.Y.; Saad, N.; Noor, N.M.; Razis, A.F.A. Neuroprotective effects of glucomoringin-isothiocyanate against H2O2-Induced cytotoxicity in neuroblastoma (SH-SY5Y) cells. *NeuroToxicology* **2019**, *75*, 89–104. [CrossRef] [PubMed]
92. Holla, V.R.; Elamin, Y.Y.; Bailey, A.M.; Johnson, A.M.; Litzenburger, B.C.; Khotskaya, Y.B.; Sanchez, N.S.; Zeng, J.; Shufean, A.; Shaw, K.R.; et al. ALK: A tyrosine kinase target for cancer therapy. *Mol. Case Stud.* **2017**, *3*, a001115. [CrossRef] [PubMed]
93. Nakagawara, A.; Li, Y.; Izumi, H.; Muramori, K.; Inada, H.; Nishi, M. Neuroblastoma. *Jpn. J. Clin. Oncol.* **2018**, *48*, 214–241. [CrossRef]

94. Heukamp, L.C.; Thor, T.; Schramm, A.; De Preter, K.; Kumps, C.; De Wilde, B.; Odersky, A.; Peifer, M.; Lindner, S.; Spruessel, A.; et al. Targeted Expression of Mutated ALK Induces Neuroblastoma in Transgenic Mice. *Sci. Transl. Med.* **2012**, *4*, 141ra91. [CrossRef]
95. Lopez-Delisle, L.; Pierre-Eugène, C.; Louis-Brennetot, C.; Surdez, D.; Raynal, V.; Baulande, S.; Boeva, V.; Grossetête-Lalami, S.; Combaret, V.; Peuchmaur, M.; et al. Activated ALK signals through the ERK–ETV5–RET pathway to drive neuroblastoma oncogenesis. *Oncogene* **2018**, *37*, 1417–1429. [CrossRef] [PubMed]
96. Cage, T.A.; Chanthery, Y.; Chesler, L.; Grimmer, M.; Knight, Z.; Shokat, K.; Weiss, W.A.; Gustafson, W.C. Downregulation of MYCN through PI3K Inhibition in Mouse Models of Pediatric Neural Cancer. *Front. Oncol.* **2015**, *5*, 111. [CrossRef]
97. Guo, Y.; Guo, D.; Zhang, S.; Zhang, Y.; He, X.; Jiang, X.; Chan, A.M.-L.; Zou, L.; Sun, J.; Zhao, H. Inhibition of PI3 kinase isoform p110α suppresses neuroblastoma growth and induces the reduction of Anaplastic Lymphoma Kinase. *Cell Biosci.* **2022**, *12*, 1–15. [CrossRef]
98. Opel, D.; Poremba, C.; Simon, T.; Debatin, K.-M.; Fulda, S. Activation of Akt Predicts Poor Outcome in Neuroblastoma. *Cancer Res.* **2007**, *67*, 735–745. [CrossRef]
99. Heiss, A.; Ammer, H.; Eisinger, D.A. δ-Opioid receptor-stimulated Akt signaling in neuroblastoma×glioma (NG108-15) hybrid cells involves receptor tyrosine kinase-mediated PI3K activation. *Exp. Cell Res.* **2009**, *315*, 2115–2125. [CrossRef] [PubMed]
100. Jin, L.; Mi, T.; Wu, X.; Wang, Z.; Zhang, Z.; Liu, J.; Wang, Z.; Wang, J.; Li, M.; Ren, C.; et al. BI-D1870 Induces Mitotic Dysfunction and Apoptosis in Neuroblastoma by Regulating the PI3K-Akt-mTORC1 Signal Axis. *Cancers* **2023**, *15*, 2023. [CrossRef]
101. Fulda, S. Tumor resistance to apoptosis. *Int. J. Cancer* **2008**, *124*, 511–515. [CrossRef] [PubMed]
102. Di Zanni, E.; Fornasari, D.; Ravazzolo, R.; Ceccherini, I.; Bachetti, T. Identification of novel pathways and molecules able to down-regulate PHOX2B gene expression by in vitro drug screening approaches in neuroblastoma cells. *Exp. Cell Res.* **2015**, *336*, 43–57. [CrossRef]
103. Tetri, L.H.; Kolla, V.; Golden, R.L.; Iyer, R.; Croucher, J.L.; Choi, J.; Macfarland, S.P.; Naraparaju, K.; Guan, P.; Nguyen, F.; et al. RET receptor expression and interaction with TRK receptors in neuroblastomas. *Oncol. Rep.* **2020**, *44*, 263–272. [CrossRef]
104. Müller, C.M.; Haase, M.G.; Kemnitz, I.; Fitze, G. Genetic mosaicism of a frameshift mutation in the RET gene in a family with Hirschsprung disease. *Gene* **2014**, *541*, 51–54. [CrossRef]
105. Lambertz, I.; Kumps, C.; Claeys, S.; Lindner, S.; Beckers, A.; Janssens, E.; Carter, D.R.; Cazes, A.; Cheung, B.B.; De Mariano, M.; et al. Upregulation of MAPK Negative Feedback Regulators and RET in Mutant ALK Neuroblastoma: Implications for Targeted Treatment. *Clin. Cancer Res.* **2015**, *21*, 3327–3339. [CrossRef] [PubMed]
106. Zhang, C.; Su, Z.-Y.; Khor, T.O.; Shu, L.; Kong, A.-N.T. Sulforaphane enhances Nrf2 expression in prostate cancer TRAMP C1 cells through epigenetic regulation. *Biochem. Pharmacol.* **2013**, *85*, 1398–1404. [CrossRef]
107. Szklarczyk, D.; Kirsch, R.; Koutrouli, M.; Nastou, K.; Mehryary, F.; Hachilif, R.; Gable, A.L.; Fang, T.; Doncheva, N.T.; Pyysalo, S.; et al. The STRING database in 2023: Protein–protein association networks and functional enrichment analyses for any sequenced genome of interest. *Nucleic Acids Res.* **2022**, *51*, D638–D646. [CrossRef]
108. Lombardo, S.D.; Presti, M.; Mangano, K.; Petralia, M.C.; Basile, M.S.; Libra, M.; Candido, S.; Fagone, P.; Mazzon, E.; Nicoletti, F.; et al. Prediction of PD-L1 Expression in Neuroblastoma via Computational Modeling. *Brain Sci.* **2019**, *9*, 221. [CrossRef]

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Review

Sudden Cardiac Death in the Young: State-of-the-Art Review in Molecular Autopsy

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Abstract: Sudden cardiac death (SCD) is defined as unexpected death due to a cardiac cause that occurs rapidly. Despite the identification of prevention strategies, SCD remains a serious public health problem worldwide, accounting for 15–20% of all deaths, and is therefore a challenge for modern medicine, especially when it affects young people. Sudden cardiac death in young people affects the population aged $\leq$ 35 years, including athletes and non-athletes, and it is due to various hereditary and non-hereditary causes. After an autopsy, if the cause remains unknown, it is called sudden unexplained death, often attributable to genetic causes. In these cases, molecular autopsy—post-mortem genetic testing—is essential to facilitate diagnostic and therapeutic pathways and/or the monitoring of family members of the cases. This review aims to elaborate on cardiac disorders marked by genetic mutations, necessitating the post-mortem genetic investigation of the deceased for an accurate diagnosis in order to facilitate informed genetic counseling and to implement preventive strategies for family members of the cases.

Keywords: sudden cardiac death; young; causes; molecular autopsy; molecular medicine

1. Introduction

Sudden death (SD) is defined as "a death occurs within one hour of the onset of symptoms, within 24 h of the last alive visit in the absence of witnesses, or for those resuscitated after cardiac arrest who die during hospitalization" [1]. SD is often the first clinical manifestation of a disease in apparently healthy subjects defined as asymptomatic, and an autopsy is the only instrument to define the cause of death [2].

Sudden cardiac death (SCD) is defined as "a natural and unexpected fatal event that occurs within one hour of the onset of symptoms, in an apparently healthy subject or in one in whom the illness was not so serious as to suggest a sudden event outcome" [3]. According to other authors, SCD is defined as "an unexpected and premature death caused by a cardiac condition in a person with known or unknown heart disease" [4].

Despite the identification of prevention strategies, death from cardiac causes is still the leading cause of death in the world; in particular, SCD represents a serious international public health problem and therefore a real challenge for modern medicine, especially when it affects young people.

Sudden cardiac death in the young (SCDY) is defined as "SCD that affects a population aged $\leq$ 35 years" [4–7], not athletes and athletes [8,9], with multiple hereditary/genetic or non-hereditary/genetic causes.

When the cause of death remains unknown despite an autopsy being performed, it is called sudden unexplained death (SUD), often caused by cardiomyopathies and cardiac channelopathies. In these cases, a post-mortem genetic study such as molecular autopsy (MA) is necessary.

In 2008 (Figure 1), the European Association of Cardiovascular Pathology (AECVP) published the "Guidelines for autopsy investigation of sudden cardiac death" with the

aim of standardizing the autopsy procedure in cases of SCD [10]. In 2017 (Figure 1), given the greater understanding of cardiovascular genetics, the introduction of new techniques, and the experience gained in the field, the following version was updated and published: "Guidelines for autopsy investigation of sudden cardiac death: 2017 update from the Association for European Cardiovascular Pathology" [2].

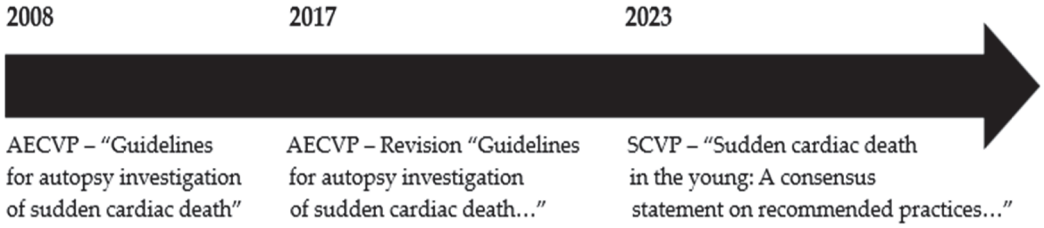


Figure 1. Temporal evolution of the guidelines and recommendations in SCD.

In 2023 (Figure 1), the Society for Cardiovascular Pathology (SCVP) published the recommendations "Sudden cardiac death in the young: a consensus statement on recommended practices for cardiac examination by pathologists from the Society for Cardiovascular Pathology" [4], underlining the importance of determining a genetic disease in a proband post-mortem to start a life-saving diagnostic–therapeutic and/or monitoring process for family members.

2. Sudden Cardiac Death in the Young

2.1. Epidemiology

SCD is a major public health challenge, contributing to approximately 15–20% of all mortality cases due to natural causes [4].

Each year, approximately 350,000 cases of sudden and unexpected deaths are recorded in Europe, while the United States records a range between 300,000 and 400,000 cases [11]. Previous investigations in the United States indicate an incidence ranging from 250,000 to 400,000, equivalent to an overall rate of 1–2 per 1000 inhabitants per year [12]. The European Society of Cardiology (ESC) reports incidence rates of between 36 and 128 deaths per 100,000 individuals per year, while other reviews cite figures ranging from 50 to 100 per 100,000 inhabitants [13].

The incidence of SCD among individuals aged $\leq$ 35 years deserves particular attention and depends on the age considered. Indeed, in individuals aged < 30 years, the overall risk of MCI is approximately 1–2.8 per 100,000 individuals [14,15], with rates of 19% among children aged between 1 and 13 years and 30% among adolescents aged 14–21 years [16]. Alarmingly, in 33% of cases of sudden death, the cause of death remains unknown despite autopsy examination [17,18].

In Italy, the frequency of SCD and SCDY is based on ISTAT data due to the absence of Regional Registers and the National Register, with the exception of the Veneto region [3]. According to ISTAT data, SCD is responsible for approximately 50,000 deaths per year, while SCDY affects 1000 individuals every year. In particular, in the Veneto region, the cumulative incidence is equal to 1 per 100,000 per year, with rates of 0.9 per 100,000 per year among non-athletes and 2.3 per 100,000 per year among athletes [3].

2.2. Causes Hereditary Heart Diseases

The pathophysiological mechanisms underlying SCD can stem from mechanical origins or alterations in the conduction system. The causes of SCD are multifaceted and vary across different age groups.

In the elderly population, chronic structural diseases are predominant, with coronary artery disease accounting for 75% of SCD cases followed by cardiomyopathies, including dilated cardiomyopathy, hypertrophic cardiomyopathy, and arrhythmogenic right ventricular cardiomyopathy, which contribute to 15% of cases. Valvular heart diseases

represent 5% of cases, while genetic and acquired cardiac abnormalities and hereditary arrhythmic syndromes, such as long QT syndrome, Brugada syndrome, catecholaminergic polymorphic ventricular tachycardia, and early repolarization syndrome, constitute 3% and 2% of cases, respectively [6,19].

In individuals aged $\leq$ 35 years, inherited heart diseases are more prevalent. Hypertrophic cardiomyopathy accounts for 36% of cases, while arrhythmogenic right ventricular cardiomyopathy contributes to 20% [6,19]. Congenital heart disease is more frequently observed in neonates (less than 1 year of age).

Moreover, SCD can be categorized based on the certainty of the cause–effect relationship, namely, certain, highly probable, and uncertain; in such instances, a comprehensive assessment encompassing clinical history, circumstances of death, and ancillary tests such as toxicological, metabolic, and molecular studies become imperative [2].

Lastly, there exist cardiac conditions where delineating between physiological changes, secondary alterations, and pathological manifestations presents challenges, thus defining diagnostic gray areas [2,20].

2.3. Cardiomyopathy

Cardiomyopathies are a heterogeneous group of diseases characterized by the intrinsic pathology of the heart muscle associated with electrical and/or mechanical dysfunction and are among the most frequent causes of heart transplantation.

These conditions are classified based on morphology in hypertrophic cardiomyopathy, dilated cardiomyopathy, restrictive cardiomyopathy, arrhythmogenic cardiomyopathy, and non-compact cardiomyopathy and are classified by causes in primary and secondary cardiomyopathy or familial (genetic) and non-familial (non-genetic) cardiomyopathy.

Moreover, other classifications have been developed by the World Health Organization (WHO) in 1995, the American Heart Association (AHA) in 2006, the European Society of Cardiology (ESC) in 2008, and the phenotype–genotype–cause classification by the MOGE(S) in 2013 by the World Heart Federation (WHF).

Hypertrophic and arrhythmogenic cardiomyopathies are the most frequent cause of sudden cardiac death associated with sport, especially in young individuals.

2.3.1. Hypertrophic Cardiomyopathy (HCM)

Hypertrophic cardiomyopathy (HCM) is a cardiac condition characterized by significant primary hypertrophy of the left ventricle, with a wall thickness of 15 mm or greater. This hypertrophy leads to various functional impairments, including left ventricular outflow tract obstruction, diastolic dysfunction, mitral regurgitation, and myocardial ischemia.

While HCM can occur as a compensatory response or secondary to conditions such as hypertension or aortic stenosis, it typically presents with symptoms such as fatigue, dyspnea, chest pain, palpitations, and syncope. In some cases, it can lead to SCD due to malignant ventricular arrhythmias like rapid ventricular tachycardia and ventricular fibrillation [21].

The prevalence of HCM in the general population is approximately 0.2% [22], making it a relatively rare condition. However, it is the second most common cause of cardiomyopathy in the pediatric population, with a prevalence of 3 cases per 100,000 live births. The incidence of SCD in individuals with HCM ranges from 1% to 7.2%, with peak presentations occurring before 1 year of age and in the age group of 8–17 years [23]. According to some studies, it is the leading cause of SCD, while several studies have reported that it accounts for 2–36% of SCD cases [9].

Familial HCM often exhibits autosomal dominant inheritance, and more than 50 different pathogenic variants have been identified. Approximately 20–30% of cases are caused by pathogenic variants in genes encoding cardiac sarcomeric contractile proteins, including *MYH7*, *MYL2*, *MYL3*, *MYBPC3*, *TNNT2*, *TNNI3*, and *TPM1*. Pathogenic vari-

ants in *MYBPC3* and *MYH7* are particularly common, accounting for over 70% of cases (Figure 2) [22].

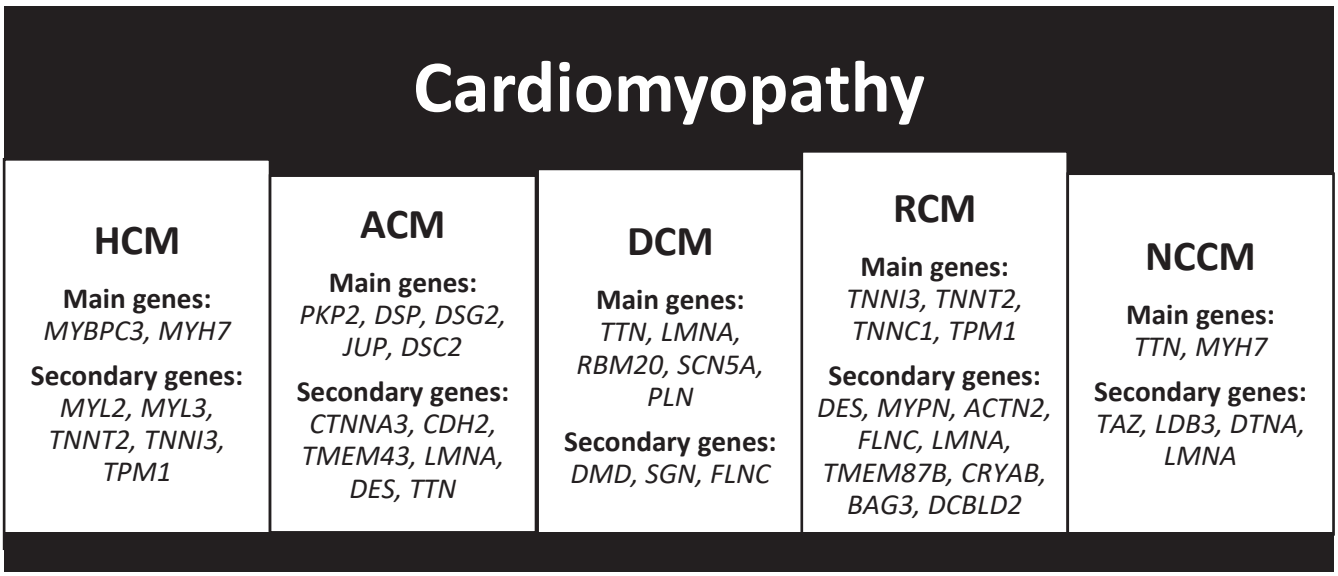


Figure 2. Main and secondary genes responsible for cardiomyopathies.

In addition to familial cases, other causes of HCM include mitochondrial gene pathogenic variants transmitted through the maternal line, referred to as mitochondrial hypertrophic cardiomyopathy [24], as well as inherited metabolic diseases, neuromuscular disorders, and RASopathies [23].

At autopsy, characteristic findings include a heart weighing over 500 g, predominant left ventricular hypertrophy, spiral myocardium, areas of whitish scars, and subaortic endocardial fibrosis plaque. Depending on the location of hypertrophy, HCM can be classified into four forms: hypertrophy of the anterior portion of the septum (type I), predominant septal hypertrophy (type II), hypertrophy of the interventricular septum and anterior wall (type III), and hypertrophy of all ventricular walls and the apex (type IV).

Histologically, HCM is characterized by hypertrophy of cardiomyocytes with hyperchromatic nuclei, the disorganization of cardiomyocytes and myofibrils, increased interstitial cellularity, the thickening of intramyocardial coronary arteries with disorganization of the media, intimal fibrosis, interstitial or replacement fibrosis, and focal myocarditis.

2.3.2. Arrhythmogenic Cardiomyopathy (ACM)

Arrhythmogenic cardiomyopathy (ACM), formerly known as arrhythmogenic right ventricular dysplasia/cardiomyopathy (ARVD), is a cardiac disorder characterized by the progressive fibrotic or fibroadipose replacement of the myocardium, leading to ventricular arrhythmias and sudden cardiac death, particularly among young athletes.

ACM has a prevalence ranging from 1:2000 to 1:5000, with a higher occurrence in males and clinical symptoms typically emerging between the second and fourth decades of life [25]. Among young athletes, the incidence of sudden cardiac death varies from 3% to 20% in some studies [25], while in individuals under the age of 65, it ranges from 5% to 10% [26].

In most cases, ACM is a genetic disorder with autosomal dominant inheritance, variable expressivity, and incomplete penetrance, resulting in diverse clinical presentations. The clinical spectrum includes progressive heart failure and ventricular arrhythmias such as ventricular tachycardia and ventricular fibrillation, often leading to sudden cardiac arrest as the initial manifestation.

Pathogenic variants in genes encoding structural proteins of cardiac intercellular junctions, particularly desmosomes, account for 30–50% of cases [27]. Specifically, the

five genes encoding desmosomal proteins, in order of frequency, are plakophilin (*PKP2*) in 10–45%, desmoplakin (*DSP*) in 10–15%, desmoglein (*DSG2*) in 10%, and plakoglobin (*JUP*) and desmocolin (*DSC2*) in 1–2% [25]. Non-desmosomal genes involved in ACM pathogenesis include alpha-T-catenin (*CTNNA3*), N-cadherin (*CDH2*), transmembrane protein 43 (*TMEM43*), lamin A/C (*LMNA*), desmin (*DES*), and titin (*TTN*), particularly in left or biventricular forms (Figure 2) [25].

In a minority of cases, ACM follows an autosomal recessive pattern, as observed in Naxos disease and Carvajal-Huerta syndrome, caused by homozygous mutations in *JUP* and certain *DSP* mutations, respectively [27].

The diagnosis of ACM is based on the Padua criteria [28], which consist of major and minor criteria varying according to the three phenotypic variants: classic or right dominant (ARVC), biventricular, and left dominant (ALVC). Notably, the diagnosis of ALVC without clinically demonstrable right ventricular anomalies necessitates the confirmation of a genetic mutation.

At autopsy, the heart typically exhibits increased weight, with a fibrotic or fibroadipose replacement of the myocardium, predominantly affecting the sub-epicardial ventricular region, either in the right, left, or biventricular distribution, depending on the phenotypic variants. Ventricular dilation with aneurysm formation, especially in the right ventricle, may also be observed.

Histologically, findings include fibro-adipose replacement, morphological alterations of cardiomyocytes with apoptosis and cytoplasmic vacuolization, and focal inflammatory infiltration.

2.3.3. Dilated Cardiomyopathy (DCM)

Dilated cardiomyopathy (DCM) is characterized by the dilation of the left and/or right ventricle, causing a progressive contractile dysfunction with a decrease in the cardiac ejection volume with each beat.

The residual volume produces an increase in the diameter and pressure of the ventricle; consequently, the compensatory response is hypertrophy and the progressive increase in the length of the sarcomeres to improve contractility. As a result, the heart gradually enlarges to a spherical shape.

DCM can be due to genetic and non-genetic causes including coronary artery disease, valvular disease, hypertension, myocarditis, alcoholism, drugs, and chemotherapy, and it can be associated with systemic diseases such as muscular dystrophy.

The prevalence of DCM is 1:250, of which 30–50% are familial cases [29] and approximately 40% of cases have an identifiable genetic cause [30], and DCM is responsible for 1–3% of SCD cases in athletes [9].

In most cases, DCM is autosomal dominant with variable expressivity and incomplete penetrance, but also specific forms of autosomal recessive, X-linked, mitochondrial inheritance, de novo mutations, polygenic disorders, and over 60 genes have been identified by genome sequencing.

In order of frequency, the protein coding genes are titin (*TTN*) in 20–25%, lamin A/C (*LMNA*) in 8%, RNA-binding protein 20 (*RBM20*) in 1–5%, type V cardiac voltage-dependent Na channel (*SCN5A*) in 2–3%, and phospholamban (*PLN*) (Figure 2) [30,31].

Furthermore, various genes encoding cardiac cytoskeletal proteins such as dystrophin (*DMD*) for Duchenne muscular dystrophy, sarcoglycan (*SGC*) for sarcolemmal instability muscular dystrophy, and filamin C (*FLNC*) are associated with arrhythmias and SCD (Figure 2) [31].

Genetic DCM is suspected in cases of family history with two first-degree relatives who have had idiopathic DCM or juvenile sudden cardiac death [31].

At autopsy, the heart weighs > 500 g and is globoid in shape, with the dilation of all cardiac chambers with the thinning of the walls and flattening of the trabeculae, as well as in areas of mural fibrosis and thrombosis.

The histology is characterized by degenerative changes and the variable size of the cardiomyocytes and with interstitial or replacement fibrosis.

2.3.4. Restrictive Cardiomyopathy (RCM)

Restrictive cardiomyopathy (RCM) is a rare cardiac muscle disorder characterized by a marked reduction in compliance, leading to disproportionately increased telediastolic ventricular pressure and impaired ventricular filling, often resulting in SCD in children.

RCM can manifest as either a primary familial or acquired condition or as a secondary complication of systemic diseases linked to congenital metabolic errors, infiltrative disorders, or skeletal myopathies [32]. The precise prevalence of RCM remains unknown, although its estimated incidence ranges from 0.03 to 0.04 per 100,000 children [32], with an average age of onset between 6 and 11 years [33]. Due to the rarity of primary RCM, the genetic underpinnings are not well understood. Conversely, secondary forms, such as amyloidosis linked to mutations in the transthyretin (*TTR*) gene or syndromic disorders like Alström syndrome or Myhre syndrome [34], are better characterized.

In most instances, RCM demonstrates autosomal dominant transmission, de novo mutations, or autosomal recessive inheritance patterns. Pathogenic variants have been identified in 19 genes encoding sarcomeric, cytoskeletal, or Z-disc proteins. Predominantly, pathogenic variants are concentrated within ten genes encoding sarcomere proteins, including cardiac troponins (*TNNI3*, *TNNT2*, and *TNNC1*) and alpha-tropomyosin (*TPM1*), with *TNNI3* being the most frequently implicated (Figure 2) [34]. Additionally, pathogenic variants have been identified in genes encoding non-sarcomeric proteins such as desmin (*DES*), myopalladin (*MYPN*), alpha-actinin-2 (*ACTN2*), filamin-C (*FLNC*), lamin A/C (*LMNA*), transmembrane protein 87B (*TMEM87B*), alphaB-crystallin (*CRYAB*), Bcl2-associated athanogene 3 (*BAG3*), discoidin, CUB, and LCCL domain-containing protein 2 (*DCBLD2*) (Figure 2) [34].

During autopsy, the heart typically appears normal or slightly diminished in size, with dilated atria and thickened, rigid walls exhibiting areas of fibrosis. Occasionally, mural thrombi may be present as a consequence of stasis.

Histologically, the findings may vary depending on the underlying causes but commonly include features indicative of cardiomyocyte degeneration and hypertrophy, areas of cellular disarray and interstitial fibrosis, the thickening of intramyocardial vessel walls, and focal inflammatory cell infiltration.

2.3.5. Non-Compaction Cardiomyopathy (NCCM)

Non-compaction cardiomyopathy (NCCM), also referred to as left ventricular non-compaction (LVNC) or hypertrabeculation, is distinguished by excessive trabeculation and profound intertrabecular recesses, presenting with a spectrum of clinical manifestations ranging from asymptomatic to heart failure, thromboembolism, arrhythmias, and SCD. It can involve the right, left, or both ventricles and may manifest as an isolated condition or in conjunction with other cardiomyopathies and severe congenital heart disease.

This condition is rare, and its prevalence in the general population remains unknown; however, some studies have reported an estimated prevalence ranging from 0.014% to 1.3% based on echocardiographic findings. Familial cases account for approximately 20–40% of occurrences, rendering NCCM the third most common cardiomyopathy in pediatric patients [35].

NCCM primarily exhibits autosomal dominant transmission, although autosomal recessive and X-linked inheritance patterns have also been documented. Pathogenic variants in genes encoding sarcomeric, cytoskeletal, and nuclear membrane proteins disrupt embryonic myocardial compaction, leading to NCCM.

The most prevalent genetic pathogenic variants involve sarcomeric proteins, such as *TTN* (including *ACTC* and *SNTA1*) and *MYH7* (including *SCN5A* and *NKX2-5*), as well as cytoskeletal protein mutations like *TAZ* (associated with Barth syndrome, including *TNN13*

and *P121L*), *LDB3* mutations (including *TNNT2* and *PRDM16*), *DTNA* (including *TNNT2* and *TPM1*), and *LMNA* (including *MYBPC3* and *HCN4*) (Figure 2) [35,36].

Macroscopically, NCCM is typified by an abundance of non-compacted myocardium relative to normal myocardium (with a ratio > 2) at the apical and mid-third levels, featuring polypoid or anastomotic trabeculae.

Histologically, deep intertrabecular recesses extending from the endocardium to the epicardium are observed, with depths exceeding 50% of the thickness of the left ventricular wall.

2.4. Cardiac Channelopathies

Cardiac channelopathies are a group of inherited heart diseases caused by abnormalities in the genes that code for cardiac regulatory proteins, sodium channels, potassium channels, calcium channels, and adrenergic receptors.

These anomalies affect the cardiac action potential or intracellular calcium homeostasis, with electrical instability and a greater predisposition to malignant arrhythmias.

The most frequent channelopathies responsible for SCDY are long QT syndrome (LQTS), Brugada syndrome (BrS), short QT syndrome (SQTS), catecholaminergic polymorphic ventricular tachycardia (CPVT), and early repolarization syndrome (ERS).

They predominantly have an autosomal dominant transmission with incomplete penetrance and variable expressivity, so the clinical manifestation varies from asymptomatic to malignant arrhythmias.

Mutations in sodium channels such as *SCN5A*, *SCN4B*, and *SCN5A* alter the phase of depolarization and manifest as LQTS and BrS. Mutations in potassium channels such as *KCNQ1*, *KCNH2*, *KCNE1*, *KCNE2*, *KCNJ2*, and *KCNJ5* alter the repolarization phase and manifest as LQTS and SQTS. Pathogenic variants affecting components of voltage-gated calcium channels such as *CACNA1C* and calmodulin and its subtypes such as *CALM1*, *CALM2*, *CALM3*, and caveolin-3 can cause arrhythmias, LQTS, and Timothy syndrome [37].

They are characterized by the absence of structural heart disease, and the first manifestation can be SCD; in these cases, the only way to reach a diagnosis is via a molecular autopsy which is essential to the direct genetic counseling of family members and identifying carriers of genetic pathogenic variants.

Genetic screening can often give negative results in 20% of patients with LQTS and BrS, in 80% with SQTS, and in 40% with CPVT [37].

2.4.1. Long QT Syndrome (LQTS)

Long QT syndrome (LQTS) is a cardiac channelopathy characterized by delayed myocardial repolarization, causing prolonged QT intervals on the electrocardiogram (ECG) and clinically manifesting with ventricular tachyarrhythmias such as torsades de pointes (TdP) with an increased risk of MCIG.

LQTS can be congenital or acquired and are associated with pharmacological or electrolyte imbalance.

The prevalence ranges approximately from 1 in 2000 individuals to 1 in 2500 individuals [38], and patients with positive genetics but asymptomatic have a 36% probability of a non-fatal cardiac event and a risk of MCI of 13%, approximately 50% of survivors have a probability of recurrence of ventricular fibrillation [39], and it is a major cause of SCD from ventricular arrhythmias in young people and athletes [40].

Moreover, 17 genes have been identified as responsible of LQTS, with 3 major genes such as *KCNQ1* (LQT1), *KCNH2* (LQT2), and *SCN5A* (LQT3) responsible for 75% of cases and 14 minor genes such as *AKAP9*, *CACNA1C*, *CALM1*, *CALM2*, *CALM3*, *CAV3*, *KCNE1*, *KCNE2*, *KCNJ2*, *KCNJ5*, *SCN4B*, *SCN5A*, *SNTA1*, and *TRDN* responsible for less than 5% of cases (Figure 3) [41].

Moreover, there are three atypical LQTS or multisystem syndromic disorders including Ankyrin-B syndrome gene *ANK2* (LQT4), Anderson–Tawil syndrome (ATS) gene *KCNJ2* (LQT7), and Timothy syndrome (TS) gene *CACNA1C* (LQT8) [42].

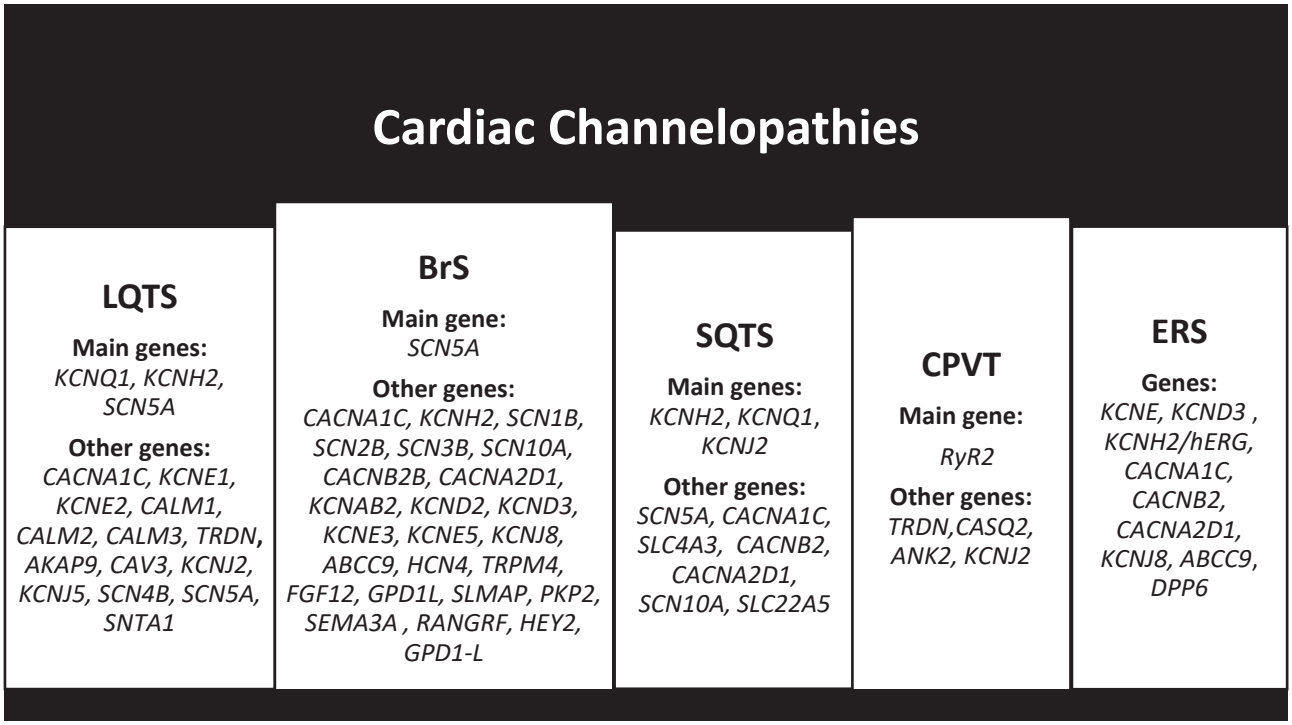


Figure 3. Main and other genes associated with cardiac channelopathies.

2.4.2. Brugada Syndrome (BrS)

Brugada syndrome (BrS) is an autosomal dominant hereditary cardiac channelopathy characterized by anomalies in repolarization, depolarization, and current–load correspondence, with accentuated J wave and coved-type ST segment elevation in the right precordial leads V1–V3 on an ECG, responsible for tachycardia polymorphic ventricular fibrillation and ventricular fibrillation and with an increased risk of MCIG.

Three types of ECGs have been described: type 1 ECG is characterized by an ST segment elevation of $\geq$2 mm in V1–V3, followed by negative T waves, type 2 ECG has >2 mm of ST segment elevation and a saddle-shaped ST segment, and type 3 has the morphology of type 1 or type 2, but with <2 mm of ST segment elevation [43].

It is responsible for 4% of all SCD cases and almost 20% of SCD in individuals with structurally normal hearts [44], and in fact, 80% of patients are asymptomatic until MI occurs [45].

The global prevalence of BrS is estimated to be around 0.05%, and in Southeast Asia, it represents the main cause of natural death in males under the age of 50 [46] with a prevalence 14 times higher than that worldwide.

It is generally asymptomatic, whereas the symptoms usually occur following triggering events such as feverish episodes, large meals, or during sleep. Syncope is the most frequent symptom, and other clinical manifestations are sudden infant death syndrome (SIDS) and sudden and unexpected nocturnal death syndrome (SUNDS).

BrS is associated with over 300 *SCN5A* pathogenic variants with incomplete penetrance and variable expression, representing 11–28% of cases, and pathogenic variants in other genes have been detected such as *SCN1B*, *SCN2B*, *SCN3B*, *SCN10A*, *CACNA1C*, *CACNB2B*, *CACNA2D1*, *KCNAB2*, *KCND2*, *KCND3*, *KCNE3*, *KCNE5*, *KCNJ8*, *KCNH2*, *ABCC9*, *HCN4*, *TRPM4*, *FGF12*, *GPD1L*, *SLMAP*, *PKP2*, *SEMA3A*, *RANGRF*, *HEY2*, and *GPD1-L* (Figure 3) [46].

2.4.3. Short QT Syndrome (SQTS)

Short QT syndrome (SQTS) is a rare autosomal dominant inherited cardiac channelopathy characterized by a shortened QTc interval (<340 ms), and it is associated with tachyarrhythmias and an increased risk of SCDY.

The most frequent clinical presentation of SQTS is SCD (30% of cases), and it is often the first clinical manifestation of the disease [47], especially in the first year of life and among individuals aged between 20 and 40 years. It is difficult to accurately estimate the prevalence of SQTS due to the rarity of occurrence and the diagnosis of this pathological entity [48].

SQTS is caused by mutations in potassium and calcium channel genes, and six genes responsible for the different subtypes have been identified which explain only 25% of cases. Specifically, the SQT1, SQT2, and SQT3 subtypes are caused by gain-of-function mutations in the *KCNH2*, *KCNQ1*, and *KCNJ2* genes, respectively. Conversely, the SQT4, SQT5, and SQT6 subtypes are caused by loss-of-function mutations in the *CACNA1C*, *CACNB2*, and *CACNA2D1* genes, respectively [37]. Other mutations are in the *SCN5A*, *SLC4A3* and *SCN10A* genes, respectively, the SQT7, SQT8, and SQT9 subtypes, and finally mutations in the *SLC22A5* gene (Figure 3) [49].

2.4.4. Catecholaminergic Polymorphic Ventricular Tachycardia (CPVT)

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is a hereditary cardiac channelopathy caused by mutations in ion channels characterized by the presence of polymorphic VT and bidirectional VT, triggered by physical or emotional stress and manifesting with malignant arrhythmias and MCIG.

It has an incidence of approximately 1 in 5000/10,000 individuals, with a mortality rate of up to 30–35% within 30 years of age in untreated patients [50] and up to 13% in patients receiving therapy, and 30% have a family history of MCI before the age of 40 [49].

The most frequent symptom is syncope, and its presentation varies from two years to 21 years of age, with a poor prognosis of up to 50% mortality in those who are 20 years of age [50].

CPVT has been classified based on the gene pathogenic variant into five subtypes: CPVT1, which involves the *RyR2* gene with autosomal dominant transmission, is responsible for approximately 60% of cases; CPVT3, which involves the *TRDN* gene with autosomal recessive transmission, manifests around the age of 10 age; CPVT2 involving the *CASQ2* gene with autosomal recessive transmission manifests around seven years of age; and CPVT4 involving the *ANK2* gene and CPVT5 involving the *KCNJ2* gene are autosomal dominant and manifest at 4 and 2.5 years of age and with a frequency of <1% (Figure 3) [50,51].

2.4.5. Early Repolarization Syndrome (ERS)

Early repolarization syndrome (ERS) is an inherited channelopathy, and it is a variant of J wave syndromes like SB with which it shares multiple similarities. The syndrome is defined as the occurrence of an early repolarization (ERP) pattern in individuals who have been successfully resuscitated from a confirmed case of unexplained VF or polymorphic VT [52], with an increased risk of malignant arrhythmias and SCD.

It has a prevalence in the general population between 1.3% and 13.3%, with higher prevalence in physically active individuals up to 33.9%, and in men [48,53], and it is responsible for 7.3% of SCD [48].

ERS is classified into three different subtypes based on the spatial localization of the early repolarization (ER) pattern in the lateral (type 1), infero-lateral (type 2), or right infero-lateral and precordial (type 3) leads [37].

It is caused by missense mutations in ion channels, specifically pathogenic variants in the *KCNE*, *KCND3*, and *KCNH2/hERG* genes for the VGKC channel; pathogenic variants in the *CACNA1C*, *CACNB2*, and *CACNA2D1* genes for the VGCC channel; and pathogenic variants in the *KCNJ8*, *ABCC9*, and *DPP6* genes for the Okay channel (Figure 3) [37,48].

3. Molecular Autopsy (MA)

The term "autopsy" originates from Greek roots, combining "Auto" (αὐτό), meaning "self" or "from oneself," and "Opsis" (ὄψις), meaning "vision" or "observation." Thus, it translates to "to observe from oneself" or "to see from oneself".

Autopsy can be categorized into two main types: a hospital autopsy and a forensic autopsy. A hospital autopsy involves the examination of a deceased individual by a pathologist to determine the cause of death and to validate or revise diagnoses made during the person's lifetime on behalf of the health authority. On the other hand, a forensic autopsy is conducted by a forensic pathologist to establish the time, cause, means, and manner of death, especially in cases where foul play is suspected, on behalf of the judicial system.

Autopsy is the only tool to definitively determine the cause of death post-mortem, and according to some studies, in 5–10% of cases, the cause remains unknown, termed SUD [54,55]. Often, in these instances, the cause of SCDY is a malignant hereditary arrhythmogenic disease (MHAD) [54,55].

MHAD can be caused by cardiomyopathies characterized by a structurally altered heart, pathogenic variants in genes encoding contractile sarcomeric proteins, and pathogenic variants in genes encoding ion channels (sodium, chloride, and potassium).

MHAD predominantly exhibits autosomal dominant inheritance, with incomplete penetrance, variable expressivity, genetic overlap, and pleiotropy, leading to variable clinical manifestations and consequently challenges in genetic diagnosis and risk stratification, especially in asymptomatic carriers [56,57].

Furthermore, SCD has been associated with oligogenic models of heart disease, where rare potentially pathogenic variants may synergistically contribute to the risk of SD, and an over-representation of rare variants in cardiac genes has been identified in young deceased individuals [58,59].

Moreover, more common variants might act as modulatory factors to rare variants causing a more or less severe phenotype, and variants in non-coding regions (intronic regions and UTRs) or regions encoding microRNAs can modulate or play a key role [60].

MHAD predispose individuals to alterations in electrical conduction, leading to malignant arrhythmias and SCD. Notably, nearly 30% of SCD cases in the young population remain without a conclusive cause of death after a comprehensive autopsy examination [56]. In these instances, MA, which is a post-mortem genetic analysis, becomes crucial to enable diagnoses in the decedent and guide the family towards genetic counseling.

A post-mortem genetic analysis is conducted on 5–10 milliliters of blood preserved with ethylenediaminetetraacetic acid (EDTA) or on tissues either frozen or fixed in formalin and embedded in paraffin (FFPE) collected during the autopsy [4]. This analysis identifies a genetic mutation in up to 25% of cases and, according to other studies, in 10–20% of SCDY cases [55].

Cardiac channelopathies are the most common cause of SUD in the pediatric and young population (Table 1).

LQTS, depending on the genetic mutation, is associated with physical exercise, swimming, and sleep; in 85% of cases, the genetic analysis of all genes identifies the cause of the disease, with 80% carrying the pathogenic variant in three principal genes, *KCNQ1*, *KCNH2*, and *SCN5A*, which are recommended by current guidelines [52,61].

BrS should be suspected in young individuals who died suddenly at night; genetic analysis identifies causative gene mutations in 35% of cases, with 30% of patients carrying the pathogenic variant in *SCN5A*, the only one recommended by current guidelines to be analyzed [52,61].

SQTS should be suspected in a newborn who died suddenly, being considered the main cause of death in the first year of life (sudden infant death syndrome—SIDS); genetic analysis identifies pathogenic variants primarily in three genes, *KCNQ1*, *KCNJ2*, and *KCNH2*, in 45% of cases, as recommended by current guidelines [52,61].

Table 1. Malignant hereditary arrhythmogenic diseases—cardiac channelopathies and recommended gene mutation for diagnosis.

Cardiac Channelopathies	Recommended Gene Mutations	Suspected
LQTS	*KCNQ1*, *KCNH2*, *SCN5A* (80%)	Young died suddenly during physical exercise or swimming or sleep
BrS	*SCN5A* (30%)	Young died suddenly at night
SQTS	*KCNQ1*, *KCNJ2*, *KCNH2* (45%)	Newborn died suddenly
CPVT	*RyR2* (55%)	Young died suddenly during adrenergic stress.

CPVT should be suspected in young patients who died suddenly during adrenergic stress. It is one of the leading causes of SCD, especially in adolescents and children before the age of 10. Genetic analysis identifies mutations in 65% of cases, with 55% due to genetic alterations in the *RyR2* gene, as recommended by current guidelines [52,61].

Cardiomyopathies are the most frequent cause of SUD in the population aged < 40 years (Table 2).

Table 2. Malignant hereditary arrhythmogenic diseases—cardiomyopathies.

Cardiomyopathies	Most Frequent Gene Mutations
HCM	*MYBPC3* (40–45%), *MYH7* (15–25%)
ACM	*PKP2* (10–45%), *DSP* (10–15%), *DSG2* (10%), *JUP* and *DSC2* (1–2%)
DCM	*TTN* (20–25%), *LMNA* (8%), *RBM20* (1–5%), *SCN5A* (2–3%)

Even when no structural abnormalities are identified post autopsy, it is imperative to conduct a molecular autopsy of the associated genes. In cases where a pathogenic variant is identified in the associated genes, further investigations are needed to conclude whether the arrhythmia occurred before the structural alteration. Indeed, a concealed cardiomyopathy may occur whereby the arrhythmia manifests before the structural alteration [62,63]. Furthermore, detailed diagnostics are needed to exclude other conditions with overlapping phenotypes.

The genes most frequently mutated in HCM are *MYBPC3* and *MYH7*, accounting for 70% of cases, in particular *MYBPC3* for 40–45% and *MYH7* for 15–25% [22,55].

The most commonly mutated genes in ACM are *PKP2* in 10–45% of cases, *DSP* in 10–15%, *DSG2* in 10%, and *JUP* and *DSC2* in 1–2% [25].

The genes most frequently mutated in DCM are *TTN* in 20–25% of cases, *LMNA* in 8%, *RBM20* in 1–5%, and *SCN5A* in 2–3% [30,31].

Despite MA being recommended in cases of SUD suspected to be caused by MHAD [4], it is not included in the autopsy protocols of both hospital and forensic settings in most countries. Recent data show that only 37% of SUD cases with a suspected arrhythmic cause undergo post-mortem genetic testing [64,65].

Furthermore, due to the increasing number of rare variants that remain ambiguous—defined variants of unknown significance (VUS) [66–68]—a significant portion of SUD cases concludes without definitive answers after molecular autopsy. In order to reduce the frequency of ambiguous variants, it has been reported that rare variants associated with hereditary arrhythmogenic syndromes should be reanalyzed and reclassified within five years [68–70] if already classified according to the American College of

Medical Genetics and Genomics and the Association for Molecular Pathology recommendations [71].

Therefore, it would be beneficial to develop guidelines or recommendations focused on the interpretation of genetic variants in the context of autopsy procedures.

4. Conclusions and Future Prospects

The identification of a genetic–molecular alteration underlying a malignant hereditary arrhythmic syndrome is essential to guide genetic counseling and to implement personalized preventive strategies such as the monitoring and/or therapy of the patients' family members in order to reduce sudden cardiac death.

The challenge for modern medicine is to ensure that molecular autopsy becomes routine in autopsy practice to ensure the correct genetic interpretation of identified variants and their clinically useful translation into asymptomatic patients. Therefore, for the appropriate management of these cardiac syndromes, it is essential to enable the formation and collaboration of a multidisciplinary team composed of a cardiovascular pathologist, a forensic pathologist, a geneticist, a cardiologist, a pediatrician, and a general practitioner.

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References

1. De Gaspari, M.; Rizzo, S.; Thiene, G.; Basso, C. Causes of sudden death. *Eur. Heart J. Suppl.* **2023**, *25* (Suppl. B), B16–B20. [CrossRef] [PubMed]
2. Basso, C.; Aguilera, B.; Banner, J.; Cohle, S.; d'Amati, G.; de Gouveia, R.H.; di Gioia, C.; Fabre, A.; Gallagher, P.J.; Leone, O.; et al. Guidelines for autopsy investigation of sudden cardiac death: 2017 update from the Association for European Cardiovascular Pathology. *Virchows. Arch.* **2017**, *471*, 691–705. [CrossRef] [PubMed]
3. Thiene, G. Autopsy and sudden death. *Eur. Heart J. Suppl.* **2023**, *25* (Suppl. C), C118–C129. [CrossRef] [PubMed]
4. Kelly, K.L.; Lin, P.T.; Basso, C.; Bois, M.; Buja, L.M.; Cohle, S.D.; D'Amati, G.; Duncanson, E.; Fallon, J.T.; Firchau, D.; et al. Sudden cardiac death in the young: A consensus statement on recommended practices for cardiac examination by pathologists from the Society for Cardiovascular Pathology. *Cardiovasc. Pathol.* **2023**, *63*, 107497. [CrossRef] [PubMed]
5. Abbas, R.; Abbas, A.; Khan, T.K.; Sharjeel, S.; Amanullah, K.; Irshad, Y. Sudden Cardiac Death in Young Individuals: A Current Review of Evaluation, Screening and Prevention. *J. Clin. Med. Res.* **2023**, *15*, 1–9. [CrossRef] [PubMed]
6. Patel, M.A.; Malhotra, A.; Mpondo, F.H.M.; Gupta, V.; Jain, R.; Gupta, S.; Jain, R. Sudden cardiac death in the adolescent population: A narrative review. *Egypt. J. Intern. Med.* **2023**, *35*, 36. [CrossRef]
7. DeWitt, E.; Etheridge, S.P. Sudden Cardiac Death in Adolescents: Allowing the Dead to Speak. *J. Am. Coll. Cardiol.* **2023**, *81*, 1018–1020. [CrossRef]
8. D'Ascenzi, F.; Valentini, F.; Pistoresi, S.; Frascaro, F.; Piu, P.; Cavigli, L.; Valente, S.; Focardi, M.; Cameli, M.; Bonifazi, M.; et al. Causes of sudden cardiac death in young athletes and non-athletes: Systematic review and meta-analysis: Sudden cardiac death in the young. *Trends. Cardiovasc. Med.* **2022**, *32*, 299–308. [CrossRef]
9. Finocchiaro, G.; Westaby, J.; Sheppard, M.N.; Papadakis, M.; Sharma, S. Sudden Cardiac Death in Young Athletes: JACC State-of-the-Art Review. *J. Am. Coll. Cardiol.* **2024**, *83*, 350–370. [CrossRef]
10. Basso, C.; Fornes, P.; Gallagher, P.J.; de Gouveia, R.H.; Sheppard, M.; Thiene, G.; van der Wal, A.; on behalf of the Association for European Cardiovascular Pathology. Guidelines for autopsy investigation of sudden cardiac death. *Virchows. Arch.* **2008**, *452*, 11–18. [CrossRef]
11. Markwerth, P.; Bajanowski, T.; Tzimas, I.; Dettmeyer, R. Sudden cardiac death-update. *Int. J. Legal. Med.* **2021**, *135*, 483–495. [CrossRef] [PubMed]
12. Hayashi, M.; Shimizu, W.; Albert, C.M. The spectrum of epidemiology underlying sudden cardiac death. *Circ. Res.* **2015**, *116*, 1887–1906. [CrossRef] [PubMed]
13. Banner, J.; Basso, C.; Tolkien, Z.; Kholova, I.; Michaud, K.; Gallagher, P.J. Autopsy examination in sudden cardiac death: A current perspective on behalf of the Association for European Cardiovascular Pathology. *Virchows Arch.* **2021**, *478*, 687–693. [CrossRef] [PubMed]
14. Isbister, J.; Semsarian, C. Sudden cardiac death: An update. *Intern. Med. J.* **2019**, *49*, 826–833. [CrossRef] [PubMed]

15. Corrado, D.; Basso, C.; Thiene, G. Sudden cardiac death in young people with apparently normal heart. *Cardiovasc. Res.* **2001**, *50*, 399–408. [CrossRef] [PubMed]
16. Risgaard, B. Sudden cardiac death: A nationwide cohort study among the young. *Dan. Med. J.* **2016**, *63*, B5321. [PubMed]
17. Virmani, R.; Burke, A.P.; Farb, A. Sudden cardiac death. *Cardiovasc. Pathol.* **2001**, *10*, 211–218. [CrossRef] [PubMed]
18. Bagnall, R.D.; Weintraub, R.G.; Ingles, J.; Duflou, J.; Yeates, L.; Lam, L.; Davis, A.M.; Thompson, T.; Connell, V.; Wallace, J.; et al. A Prospective Study of Sudden Cardiac Death among Children and Young Adults. *N. Engl. J. Med.* **2016**, *374*, 2441–2452. [CrossRef]
19. Srinivasan, N.T.; Schilling, R.J. Sudden Cardiac Death and Arrhythmias. *Arrhythm. Electrophysiol. Rev.* **2018**, *7*, 111–117. [CrossRef]
20. Papadakis, M.; Raju, H.; Behr, E.R.; De Noronha, S.V.; Spath, N.; Kouloubinis, A.; Sheppard, M.N.; Sharma, S. Sudden cardiac death with autopsy findings of uncertain significance: Potential for erroneous interpretation. *Circ. Arrhythm. Electrophysiol.* **2013**, *6*, 588–596. [CrossRef]
21. Siontis, K.C.; Ommen, S.R.; Geske, J.B. Art and science of risk stratification of sudden cardiac death in hypertrophic cardiomyopathy: Current state, unknowns, and future directions. *Prog. Cardiovasc. Dis.* **2023**, *80*, 25–31. [CrossRef] [PubMed]
22. Basit, H.; Brito, D.; Sharma, S. Hypertrophic Cardiomyopathy. In *StatPearls [Internet]*; StatPearls Publishing: Treasure Island, FL, USA, 2024.
23. Thakkar, K.; Karajgi, A.R.; Kallamvalappil, A.M.; Avanthika, C.; Jhaveri, S.; Shandilya, A.; Anusheel; Al-Masri, R. Sudden cardiac death in childhood hypertrophic cardiomyopathy. *Dis. Mon.* **2023**, *69*, 101548. [CrossRef] [PubMed]
24. Zhuang, Q.; Guo, F.; Fu, L.; Dong, Y.; Xie, S.; Ding, X.; Hu, S.; Zhou, X.D.; Jiang, Y.; Zhou, H.; et al. 1-Deoxynojirimycin promotes cardiac function and rescues mitochondrial cristae in mitochondrial hypertrophic cardiomyopathy. *J. Clin. Investig.* **2023**, *133*, e164660. [CrossRef]
25. De la Guía-Galipienso, F.; Ugedo-Alzaga, K.; Grazioli, G.; Quesada-Ocete, F.J.; Feliu-Rey, E.; Perez, M.V.; Quesada-Dorador, A.; Sanchis-Gomar, F. Arrhythmogenic Cardiomyopathy and Athletes: A Dangerous Relationship. *Curr. Probl. Cardiol.* **2023**, *48*, 101799. [CrossRef] [PubMed]
26. Alblaihed, L.; Kositz, C.; Brady, W.J.; Al-Salamah, T.; Mattu, A. Diagnosis and management of arrhythmogenic right ventricular cardiomyopathy. *Am. J. Emerg. Med.* **2023**, *65*, 146–153. [CrossRef] [PubMed]
27. Chua, C.J.; Morrissette-McAlmon, J.; Tung, L.; Boheler, K.R. Understanding Arrhythmogenic Cardiomyopathy: Advances through the Use of Human Pluripotent Stem Cell Models. *Genes* **2023**, *14*, 1864. [CrossRef] [PubMed]
28. Corrado, D.; Marra, M.P.; Zorzi, A.; Beffagna, G.; Cipriani, A.; De Lazzari, M.; Migliore, F.; Pilichou, K.; Rampazzo, A.; Rigato, I.; et al. Diagnosis of arrhythmogenic cardiomyopathy: The Padua criteria. *Int. J. Cardiol.* **2020**, *319*, 106–114. [CrossRef] [PubMed]
29. Hustings, N.; Van Ballaer, V.; Barrios, L. Cardiac MRI after Sudden Cardiac Arrest in a Young Woman Prompts Diagnosis of Familial Dilated Cardiomyopathy. *J. Belg. Soc. Radiol.* **2023**, *107*, 21. [CrossRef] [PubMed]
30. McNally, E.M.; Mestroni, L. Dilated Cardiomyopathy: Genetic Determinants and Mechanisms. *Circ. Res.* **2017**, *121*, 731–748. [CrossRef]
31. Eldemire, R.; Mestroni, L.; Taylor, M.R.G. Genetics of Dilated Cardiomyopathy. *Annu. Rev. Med.* **2024**, *75*, 417–426. [CrossRef]
32. Tsatsopoulou, A.; Protonotarios, I.; Xylouri, Z.; Papagiannis, I.; Anastasakis, A.; Germanakis, I.; Patrianakos, A.; Nyktari, E.; Gavras, C.; Papadopoulos, G.; et al. Cardiomyopathies in children: An overview. *Hellenic. J. Cardiol.* **2023**, *72*, 43–56. [CrossRef] [PubMed]
33. Ditaranto, R.; Caponetti, A.G.; Ferrara, V.; Parisi, V.; Minnucci, M.; Chiti, C.; Baldassarre, R.; Di Nicola, F.; Bonetti, S.; Hasan, T.; et al. Pediatric Restrictive Cardiomyopathies. *Front. Pediatr.* **2022**, *9*, 745365. [CrossRef]
34. Brodehl, A.; Gerull, B. Genetic Insights into Primary Restrictive Cardiomyopathy. *J. Clin. Med.* **2022**, *11*, 2094. [CrossRef] [PubMed]
35. Srivastava, S.; Yavari, M.; Al-Abcha, A.; Banga, S.; Abela, G. Ventricular non-compaction review. *Heart Fail. Rev.* **2022**, *27*, 1063–1076. [CrossRef] [PubMed]
36. Richard, P.; Ader, F.; Roux, M.; Donal, E.; Eicher, J.; Aoutil, N.; Huttin, O.; Selton-Suty, C.; Coisne, D.; Jondeau, G.; et al. Targeted panel sequencing in adult patients with left ventricular non-compaction reveals a large genetic heterogeneity. *Clin. Genet.* **2019**, *95*, 356–367. [CrossRef]
37. Sebastian, S.A.; Panthangi, V.; Sethi, Y.; Padda, I.; Khan, U.; Affas, Z.R.; Mareddy, C.; Dolack, L.; Johal, G. Precision Medicine and Cardiac Channelopathies: Human iPSCs Take the Lead. *Curr. Probl. Cardiol.* **2023**, *48*, 101990. [CrossRef] [PubMed]
38. Huang, S.; Chen, J.; Song, M.; Yu, Y.; Geng, J.; Lin, D.; Yang, J.; Wu, J.; Li, K.; Yu, Y.; et al. Whole-exome sequencing and electrophysiological study reveal a novel loss-of-function mutation of KCNA10 in epinephrine provoked long QT syndrome with familial history of sudden cardiac death. *Leg. Med.* **2023**, *62*, 102245. [CrossRef]
39. Pappone, C.; Ciconte, G.; Anastasia, L.; Gaita, F.; Grant, E.; Micaglio, E.; Locati, E.T.; Calovic, Z.; Vicedomini, G.; Santinelli, V. Right ventricular epicardial arrhythmogenic substrate in long-QT syndrome patients at risk of sudden death. *Europace* **2023**, *25*, 948–955. [CrossRef]
40. Mahendran, S.; Gupta, I.; Davis, J.; Davis, A.J.; Orchard, J.W.; Orchard, J.J. Comparison of methods for correcting QT interval in athletes and young people: A systematic review. *Clin. Cardiol.* **2023**, *46*, 1106–1115. [CrossRef]
41. Adler, A.; Novelli, V.; Amin, A.S.; Abiusi, E.; Care, M.; Nannenberg, E.A.; Feilotter, H.; Amenta, S.; Mazza, D.; Bikker, H.; et al. An International, Multicentered, Evidence-Based Reappraisal of Genes Reported to Cause Congenital Long QT Syndrome. *Circulation* **2020**, *141*, 418–428. [CrossRef]

42. Bauer, R.; Timothy, K.W.; Golden, A. Update on the Molecular Genetics of Timothy Syndrome. *Front. Pediatr.* **2021**, *9*, 668546. [CrossRef] [PubMed]
43. Li, K.H.C.; Lee, S.; Yin, C.; Liu, T.; Ngarmukos, T.; Conte, G.; Yan, G.X.; Sy, R.W.; Letsas, K.P.; Tse, G.; et al. Brugada syndrome: A comprehensive review of pathophysiological mechanisms and risk stratification strategies. *Int. J. Cardiol. Heart Vasc.* **2020**, *26*, 100468. [CrossRef] [PubMed]
44. Popa, I.P.; Șerban, D.N.; Mărănducă, M.A.; Șerban, I.L.; Tamba, B.I.; Tudorancea, I. Brugada Syndrome: From Molecular Mechanisms and Genetics to Risk Stratification. *Int. J. Mol. Sci.* **2023**, *24*, 3328. [CrossRef] [PubMed]
45. Gaita, F.; Cerrato, N.; Giustetto, C.; Martino, A.; Bergamasco, L.; Millesimo, M.; Barbonaglia, L.; Carvalho, P.; Caponi, D.; Saglietto, A.; et al. Asymptomatic Patients with Brugada ECG Pattern: Long-Term Prognosis From a Large Prospective Study. *Circulation* **2023**, *148*, 1543–1555. [CrossRef] [PubMed]
46. Moras, E.; Gandhi, K.; Narasimhan, B.; Brugada, R.; Brugada, J.; Brugada, P.; Krittanawong, C. Genetic and Molecular Mechanisms in Brugada Syndrome. *Cells* **2023**, *12*, 1791. [CrossRef] [PubMed]
47. Pasero, E.; Gaita, F.; Randazzo, V.; Meynet, P.; Cannata, S.; Maury, P.; Giustetto, C. Artificial Intelligence ECG Analysis in Patients with Short QT Syndrome to Predict Life-Threatening Arrhythmic Events. *Sensors* **2023**, *23*, 8900. [CrossRef]
48. Badura, K.; Buławska, D.; Dąbek, B.; Witkowska, A.; Lisińska, W.; Radzioch, E.; Skwira, S.; Młynarska, E.; Rysz, J.; Franczyk, B. Primary Electrical Heart Disease-Principles of Pathophysiology and Genetics. *Int. J. Mol. Sci.* **2024**, *25*, 1826. [CrossRef] [PubMed]
49. Hancox, J.C.; Du, C.Y.; Butler, A.; Zhang, Y.; Dempsey, C.E.; Harmer, S.C.; Zhang, H. Pro-arrhythmic effects of gain-of-function potassium channel mutations in the short QT syndrome. *Philos. Trans. R Soc. Lond. B Biol. Sci.* **2023**, *378*, 20220165. [CrossRef] [PubMed]
50. Henriquez, E.; A Hernandez, E.; Mundla, S.R.; Wankhade, D.H.; Saad, M.; Ketha, S.S.; Martinez, G.C.; Ahmed, F.S.; Hussain, M.S.; Hernandez, E.A.; et al. Catecholaminergic Polymorphic Ventricular Tachycardia and Gene Therapy: A Comprehensive Review of the Literature. *Cureus* **2023**, *15*, e47974. [CrossRef]
51. Jurisic, S.; Medeiros-Domingo, A.; Berger, F.; Balmer, C.; Brunckhorst, C.; Ruschitzka, F.; Saguner, A.M.; Duru, F. Catecholaminergic Polymorphic Ventricular Tachycardia: Multiple Clinical Presentations of a Genetically Determined Disease. *J. Clin. Med.* **2023**, *13*, 47. [CrossRef]
52. Zeppenfeld, K.; Tfelt-Hansen, J.; De Riva, M.; Winkel, B.G.; Behr, E.R.; Blom, N.A.; Charron, P.; Corrado, D.; Dagres, N.; de Chillou, C.; et al. 2022 ESC Guidelines for the management of patients with ventricular arrhythmias and the prevention of sudden cardiac death. *Eur. Heart J.* **2022**, *43*, 3997–4126.
53. Ji, H.Y.; Hu, N.; Liu, R.; Zhou, H.R.; Gao, W.L.; Quan, X.Q. Worldwide prevalence of early repolarization pattern in general population and physically active individuals: A meta-analysis. *Medicine* **2021**, *100*, e25978. [CrossRef] [PubMed]
54. Campuzano, O.; Sarquella-Brugada, G. Molecular autopsy in sudden cardiac death. *Glob. Cardiol. Sci. Pract.* **2023**, *2023*, e202308. [CrossRef] [PubMed]
55. Martínez-Barrios, E.; Grassi, S.; Brión, M.; Toro, R.; Cesar, S.; Cruzalegui, J.; Coll, M.; Alcalde, M.; Brugada, R.; Greco, A.; et al. Molecular autopsy: Twenty years of post-mortem diagnosis in sudden cardiac death. *Front. Med.* **2023**, *10*, 1118585. [CrossRef] [PubMed]
56. Martínez-Barrios, E.; Cesar, S.; Cruzalegui, J.; Hernandez, C.; Arbelo, E.; Fiol, V.; Brugada, J.; Brugada, R.; Campuzano, O.; Sarquella-Brugada, G. Clinical Genetics of Inherited Arrhythmogenic Disease in the Pediatric Population. *Biomedicines* **2022**, *10*, 106. [CrossRef] [PubMed]
57. Coll, M.; Pérez-Serra, A.; Mates, J.; Del Olmo, B.; Puigmulé, M.; Fernandez-Falgueras, A.; Iglesias, A.; Picó, F.; Lopez, L.; Brugada, R.; et al. Incomplete Penetrance and Variable Expressivity: Hallmarks in Channelopathies Associated with Sudden Cardiac Death. *Biology* **2017**, *7*, 3. [CrossRef] [PubMed]
58. Webster, G.; Puckelwartz, M.J.; Pesce, L.L.; Dellefave-Castillo, L.M.; Vanoye, C.G.; Potet, F.; Page, P.; Kearns, S.D.; Pottinger, T.; White, S.; et al. Genomic Autopsy of Sudden Deaths in Young Individuals. *JAMA Cardiol.* **2021**, *6*, 1247–1256. [CrossRef] [PubMed]
59. Goudal, A.; Karakachoff, M.; Lindenbaum, P.; Baron, E.; Bonnaud, S.; Kyndt, F.; Arnaud, M.; Minois, D.; Bourcereau, E.; Thollet, A.; et al. Burden of rare variants in arrhythmogenic cardiomyopathy with right dominant form-associated genes provides new insights for molecular diagnosis and clinical management. *Hum. Mutat.* **2022**, *43*, 1333–1342. [CrossRef] [PubMed]
60. Pérez-Agustín, A.; Pinsach-Abuin, M.L.; Pagans, S. Role of Non-Coding Variants in Brugada Syndrome. *Int. J. Mol. Sci.* **2020**, *21*, 8556. [CrossRef]
61. Mont Girbau, J.L.; Wilde, A.A.; Semsarian, C.; Márquez, M.F.; Deneke, T.; Kaufman, E.S. European Heart Rhythm Association (EHRA)/Heart Rhythm Society (HRS)/Asia Pacific Heart Rhythm Society (APHRS)/Latin American Heart Rhythm Society (LAHRS) Expert Consensus Statement on the state of genetic testing for cardiac diseases. *Europace* **2022**, *24*, 1307–1367.
62. Isbister, J.C.; Nowak, N.; Yeates, L.; Singer, E.S.; Sy, R.W.; Ingles, J.; Raju, H.; Bagnall, R.D.; Semsarian, C. Concealed Cardiomyopathy in Autopsy-Inconclusive Cases of Sudden Cardiac Death and Implications for Families. *J. Am. Coll. Cardiol.* **2022**, *80*, 2057–2068. [CrossRef] [PubMed]
63. Karra, R.; Nafissi, N.A. Expanding the Molecular Autopsy to Uncover Occult Cardiomyopathy: Concealed to Revealed. *J. Am. Coll. Cardiol.* **2022**, *80*, 2069–2071. [CrossRef] [PubMed]

64. Stiles, M.K.; Wilde, A.A.; Abrams, D.J.; Ackerman, M.J.; Albert, C.M.; Behr, E.R.; Chugh, S.S.; Cornel, M.C.; Gardner, K.; Ingles, J.; et al. 2020 APHRS/HRS expert consensus statement on the investigation of decedents with sudden unexplained death and patients with sudden cardiac arrest, and of their families. *Heart Rhythm* **2021**, *18*, e1–e50. [CrossRef]
65. Behr, E.R.; Scrocco, C.; Wilde, A.A.M.; Marijon, E.; Crotti, L.; E Iliodromitis, K.; A Remme, C.; Kosiuk, J.; Rudaka, I.; Brugada, G.S.; et al. Investigation on Sudden Unexpected Death in the Young (SUDY) in Europe: Results of the European Heart Rhythm Association Survey. *Europace* **2022**, *24*, 331–339. [CrossRef] [PubMed]
66. Semsarian, C.; Ingles, J.; Wilde, A.A. Sudden cardiac death in the young: The molecular autopsy and a practical approach to surviving relatives. *Eur. Heart J.* **2015**, *36*, 1290–1296. [CrossRef] [PubMed]
67. Coll, M.; Fernandez-Falgueras, A.; Iglesias, A.; del Olmo, B.; Nogue-Navarro, L.; Simon, A.; Serra, A.P.; Puigmule, M.; Lopez, L.; Pico, F.; et al. Unpredicted Aberrant Splicing Products Identified in Postmortem Sudden Cardiac Death Samples. *Int. J. Mol. Sci.* **2022**, *23*, 12640. [CrossRef] [PubMed]
68. Martinez-Barrios, E.; Sarquella-Brugada, G.; Perez-Serra, A.; Fernandez-Falgueras, A.; Cesar, S.; Alcalde, M.; Coll, M.; Puigmulé, M.; Iglesias, A.; Ferrer-Costa, C.; et al. Reevaluation of ambiguous genetic variants in sudden unexplained deaths of a young cohort. *Int. J. Legal. Med.* **2023**, *137*, 345–351. [CrossRef] [PubMed]
69. Campuzano, O.; Sarquella-Brugada, G.; Fernandez-Falgueras, A.; Coll, M.; Iglesias, A.; Ferrer-Costa, C.; Cesar, S.; Arbelo, E.; García-Álvarez, A.; Jordà, P.; et al. Reanalysis and reclassification of rare genetic variants associated with inherited arrhythmogenic syndromes. *EBioMedicine* **2020**, *54*, 102732. [CrossRef]
70. Sarquella-Brugada, G.; Fernandez-Falgueras, A.; Cesar, S.; Arbelo, E.; Coll, M.; Perez-Serra, A.; Puigmulé, M.; Iglesias, A.; Alcalde, M.; Vallverdú-Prats, M.; et al. Clinical impact of rare variants associated with inherited channelopathies: A 5-year update. *Hum. Genet.* **2022**, *141*, 1579–1589. [CrossRef]
71. Richards, S.; Aziz, N.; Bale, S.; Bick, D.; Das, S.; Gastier-Foster, J.; Grody, W.W.; Hegde, M.; Lyon, E.; Spector, E.; et al. Standards and guidelines for the interpretation of sequence variants: A joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet. Med.* **2015**, *17*, 405–424. [CrossRef]

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Review

Soil and Mineral Nutrients in Plant Health: A Prospective Study of Iron and Phosphorus in the Growth and Development of Plants

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Abstract: Plants being sessile are exposed to different environmental challenges and consequent stresses associated with them. With the prerequisite of minerals for growth and development, they coordinate their mobilization from the soil through their roots. Phosphorus (P) and iron (Fe) are macro- and micronutrient; P serves as an important component of biological macromolecules, besides driving major cellular processes, including photosynthesis and respiration, and Fe performs the function as a cofactor for enzymes of vital metabolic pathways. These minerals help in maintaining plant vigor via alterations in the pH, nutrient content, release of exudates at the root surface, changing dynamics of root microbial population, and modulation of the activity of redox enzymes. Despite this, their low solubility and relative immobilization in soil make them inaccessible for utilization by plants. Moreover, plants have evolved distinct mechanisms to cope with these stresses and coregulate the levels of minerals (Fe, P, etc.) toward the maintenance of homeostasis. The present study aims at examining the uptake mechanisms of Fe and P, and their translocation, storage, and role in executing different cellular processes in plants. It also summarizes the toxicological aspects of these minerals in terms of their effects on germination, nutrient uptake, plant–water relationship, and overall yield. Considered as an important and indispensable component of sustainable agriculture, a separate section covers the current knowledge on the cross-talk between Fe and P and integrates complete and balanced information of their effect on plant hormone levels.

Keywords: soil; iron; phosphorus; plant growth; productivity; sustainable agriculture

1. Introduction

Agriculture contributes immensely to the growth, development, and economic prosperity of the nation. Considered as the backbone of a nation's economy, it plays an active role in the reduction in poverty, rural and social development, and food security [1,2]. As the agriculture sector is challenged by different stresses, like drought, salinity, etc., their influences on plant growth and development escalate the problem of food security and agricultural sustainability [3]. Under such circumstances, plants greatly rely on the acquisition of soil nutrients (both macro- and micronutrients) to increase their survival and their productivity is often supplemented by the use of agrochemicals in agricultural practices. Iron (Fe; a micronutrient) and phosphorus (P; a macronutrient) are part of the essential mineral nutrients for the survival of plants [4]. Owing to their relative immobilization and low solubility in soils, their inaccessibility to plants makes them deficient in these

essential mineral nutrients with a serious impact on the metabolic machinery of the plant cell. Given the United Nations' sustainability goal of achieving zero hunger, deficiencies of both Fe and P create serious complications within the plant system that severely impact the yield and quality of the plants, and as such raise concerns at a global scale regarding the availability of food to feed the population. To overcome this problem, the global population has widely become dependent on the use of fertilizers to meet soil requirements in order to achieve agricultural sustainability through increases in crop production and quality. With serious ecological and economic impacts, the use of chemical fertilizers that adversely affect plant health put forth the need to have crops with improved Fe and P requirements toward reducing the use of fertilizers in agricultural practices. Additionally, the growth of plants and their fitness for survival under mineral-deficiency conditions stress the need to have genotypes with efficient mineral nutrient usage to fit the low input of these nutrients in agricultural soils. Keeping this in view, there is a need to have an understanding of the interaction of these mineral elements among themselves, their relation to other mineral nutrients, and, more importantly, the requirement of information that pertains to different signaling pathways operating in response to nutrients in plants. In such a context, the present study aims at summarizing the existing knowledge about these mineral nutrients, the importance of nutrient transporters, the role of siderophores and phosphate-solubilizing bacteria in mineral uptake, cross-talk between them, if any, and information on the signaling pathways that regulate levels of the mineral nutrients, preferably Fe and P, in plants.

2. Iron in Plant Health

Iron (Fe), a key micronutrient essential for cellular processes (respiration, photosynthesis, etc.), also functions as a cofactor for the enzymes of vital metabolic pathways, like the ETC (electron transport chain) (oxidoreductases and oxygenases), in the respiration process of plants [5–9]. Fe occupies a key position related to its role: (1) in the synthesis of chlorophyll during photosynthesis [9,10]; (2) as a cofactor of iron–sulfur and heme proteins for the synthesis of phenylpropanoids, oxylipins, and other primary and secondary metabolites, besides being associated with the synthesis of hormones such as gibberellins and brassinosteroids [11–13]; and (3) as part of enzymes in nutrient assimilation, such as nitrite and nitrate reductase [14,15]. Plants generally rely on soil Fe content to fulfill their needs as an important component of proteins in chlorophyll synthesis and other metabolic activities. However, only a small portion of it is bioavailable (ferrous form, Fe^{2+}) and the rest is present as insoluble matter (ferric form, Fe^{3+}), which plants are unable to utilize for various biochemical activities, and as such face Fe deficiency [16,17]. Plants suffer from both deficient (growth on neutral to basic soils), observed mostly in upland regions, and toxic levels of Fe, in soil from lowland agricultural practices where a submerged condition favors the reduction in Fe^{3+}-Fe^{2+} (increased solubility) that increases its uptake by plants [18,19]. Under fluctuating environmental conditions, a precise homeostatic mechanism operates in plants that balances nutrient uptake by avoiding surplus storage catalyzing the generation of reactive oxygen species (ROS; preferably hydroxyl radical via the Fenton reaction) [20,21]. As such, optimized Fe uptake is regulated via a transcriptional switch that induces its uptake in response to low internal levels or represses it on account of sufficient Fe stores in plants.

2.1. Iron Uptake, Translocation, and Storage in Plants

Though abundant in soils, plants have evolved different strategies for maintaining a strict balance of iron by controlling its absorption at the root surface, translocation to aerial parts, compartmentalization for storage, and limiting their remobilization to cope with its deficiency (Figure 1). Plants, being sessile, employ two common strategies: reduction-based (strategy I: non-graminaceous plants, e.g., *Arabidopsis thaliana*) and chelation-based (strategy II: graminaceous plants, e.g., rice) that fine-tunes the mobilization of iron for

efficient acquisition at root surfaces [22,23]. The basic components and regulations for the two strategies are discussed below.

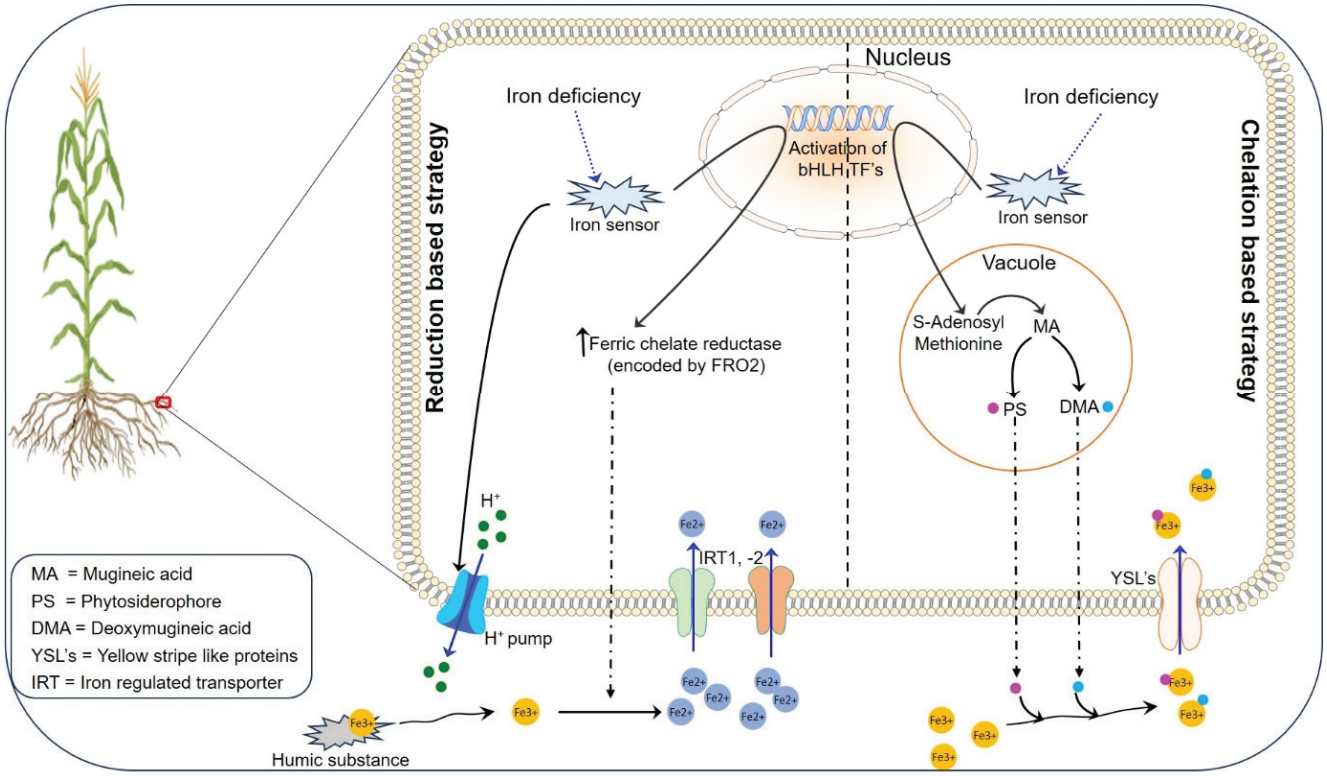


Figure 1. Strategies employed for the uptake of iron at the root surface.

2.1.1. Reduction-Based Strategy for Iron Uptake

Strategy I is predominantly utilized by non-graminaceous monocots and dicots, and is based on iron reduction to enhance its uptake under limited conditions. The approach involves the concerted action of different types of plasma membrane proteins of the root epidermis. These proteins are expressed in response to low Fe levels to cope with poorly available soil iron, Fe^{3+} (exhibiting limited solubility and being more prone to precipitation), in the surrounding environment [24]. For this strategy, plants undergo acidification by pumping protons (H^+) via ATPase AHA2 at the rhizosphere causing the release of Fe^{3+} bound to humic substances and a subsequent reduction to Fe^{2+} by enzyme ferric chelate reductase [10] encoded by FRO2 for its uptake by the IRT (iron-regulated transporter), two genes, IRT1 and IRT2, in *Arabidopsis thaliana* [25,26].

Initially, P-type ATPases play a pivotal role by releasing protons into the rhizosphere, leading to a decrease in soil pH. This acidification enhances the solubility of Fe, with additional contributions from the secretion of solubilizing agents like carboxylates and phenolates. Subsequently, FERRIC-REDUCTION OXIDASES, activated under acidic conditions [27], facilitate the conversion of Fe^{3+} to Fe^{2+}. Finally, ZINC-REGULATED TRANSPORTER/IRON-REGULATED TRANSPORTER-LIKE PROTEIN (ZIP) transporters facilitate the uptake of Fe^{2+} into plant roots. In Arabidopsis, H^+-ATPase 2 (AHA2), FERRIC-REDUCTION OXIDASES 2 (FRO2), and IRON-REGULATED TRANSPORTER 1 (IRT1) encode these three proteins, respectively [24]. IRT1 functions as a high-affinity transporter of Fe, playing a crucial role in both its uptake and distribution from roots to shoots [26,28]. Additionally, NATURAL-RESISTANCE-ASSOCIATED MACROPHAGE PROTEIN 1 (NRAMP1) contributes to the uptake of Fe as a low-affinity Fe^{2+} transporter [29,30]. Recently, a study has unveiled that a complex is formed by FRO2, IRT1, and AHA2 to create a microenvironment with an optimal pH and Fe^{2+} concentration in the rhizosphere. This complex formation

helps optimize Fe uptake by preventing the reoxidation of Fe^{2+} into Fe^{3+}, a process that may occur due to the presence of oxygen in most soils [31].

The reduction-based strategy was believed to be exclusive to non-graminaceous monocots and dicots. However, subsequent research has demonstrated that grasses also employ this strategy for iron (Fe) uptake. Notably, *Oryza sativa* (rice) utilizes the reduction-based approach by absorbing Fe^{2+} through the activity of OsIRT1, OsNRAMP1, OsIRT2, and OsNRAMP5 [8,32]. In response to the deficiency of Fe, the expressions of OsNRAMP1, OsIRT1, and OsIRT2 are induced, indicating their role in plant Fe nutrition under the conditions of low Fe availability [8]. In contrast, OsNRAMP5, which maintains constitutive Fe^{2+} uptake, does not influence Fe deficiency [32].

Dicotyledonous plants, such as tomatoes, cucumbers, and soybeans, predominantly employ strategy I in response to Fe deficiency, though their specific reactions vary. In the case of cucumber and tomato, Fe-deficient conditions lead to an enlargement of root tips and coarseness near the tips, accompanied by increased root hairs, features characteristic of metastatic cells [33]. These cells actively release a substantial H^+ concentration, thereby significantly enhancing the root's Fe^{3+} reduction capability, ultimately improving Fe availability in the rhizosphere. Conversely, soybeans primarily address insoluble Fe^{3+} through the thickening and swelling of root tips, coupled with the accumulation of numerous phenolic substances in the cortex and epidermis [34]. Consequently, certain plants exhibit diverse mechanisms when responding to Fe-deficiency stress. Furthermore, Fe deficiency induces the biosynthesis and signal transduction of ethylene, with ethylene, in turn, playing a role in enhancing Fe transport and distribution in rice [35].

2.1.2. Chelation-Based Strategy for Iron Uptake

Strategy II, prevalent among graminaceous plants, involves Fe^{3+} that undergoes a complex formation with mugineic acid (MA) synthesized from L-methionine with the involvement of nicotinamide synthase (NAS) (catalyzing production of non-proteinaceous amino acid nicotianamine, (NA)), nicotianamine aminotransferase (NAAT), deoxymugineic acid synthase (DMAS) that catalyzes the production of a phytosiderophore (PS), and deoxymugineic acid (DMA), which undergoes hydroxylation to release MA at the root surface [36]. The ferric chelate complex (Fe^{3+}-PS) formed in the exterior milieu is transported inside the roots by specific transporters of the yellow stripe-like (YSL) protein family, with its first report in maize, i.e., YS1 [37,38]. To date, 9 variants of MA and 18 YSLs have been reported [8,10]. Though the synthesis of NA is reported as a metal chelator from several plants, only grasses were found undergoing its conversion to PS [22]. As per the reports, OsYSL15 is the major transporter of Fe^{3+}-PS in rice, while all other members of the YSL protein family in both graminaceous and non-graminaceous plants are involved in the transportation of metal-Na complexes [39,40]. The efflux of PS DMA into the exterior milieu was recently found to occur through a major facilitator superfamily (MFS) transporter, TOM1 [41]. Additionally, two other MFS member transporters, ENA1 and ENA2, were found to be associated with the transport of NA in plants [41]. AtZIF1 (an analog of ENA1) in *Arabidopsis thaliana* with a zinc-sensitive phenotype and localized on the vacuolar membrane initially thought to be a Zn transporter was recently found to be associated with the accumulation of NA in vacuoles [42]. It is now believed that ENA1 plays a role in metal detoxification via the transportation of NA to the vacuole.

An analogy for rice plants is the occurrence of the Fe^{2+} transporter OsIRT1 that attributes it with the property to take up Fe^{2+} similar to strategy I [43]. However, unlike strategy I, the induction of H^+-ATPase and ferric chelate reductase was not observed under iron-deficiency conditions. This simply reflects an adaptation to flooded rice where a surplus of Fe^{2+} is present due to reduced oxygen levels [44]. The depleted oxygen levels in soil cause microorganisms to use Fe^{3+} as an alternate electron acceptor, leading to the conversion of Fe^{3+} to Fe^{2+}, which is transported into plant roots through the Fe^{2+} transporter, OsIRT1 [45]. The genetic components involved in iron metabolism in rice, particularly *OsIRT1* and *OsIRT2* genes, share similarities with the well-documented strategy I plants.

It has been previously reported that ferric chelate reductase activity is induced in iron-deficient conditions, facilitating the reductions in Fe(III) to Fe(II) in growth medium [43]. Connolly et al. [46] observed a decline in the expression of IRT1 and FRO2 mRNA levels as iron concentrations increased from deficient to sufficient levels. Pereira et al. [47] demonstrated the upregulation of the functional gene *OsFRO2* under stringent iron conditions. *OsFRDL1* represents another set of genes accountable for iron efflux transport in rice. Yokosho et al. [48] findings revealed that *OsFRDL1* plays a vital role in iron distribution from the nodes–panicles. Knocking out this gene results in the accumulation of iron in the nodal region deprived of a proper distribution to other plant parts.

2.1.3. Iron Translocation and Storage

During absorption at the root's surface, Fe undergoes a translocation from the roots to aerial parts having a high iron demand. The transport of Fe inside the shoots takes place through the xylem in a passive mode, until it is unloaded by a specific transporter, and through the phloem in an active mode via the involvement of a specific transport system [49,50]. Inside the plants, the immobilization of Fe takes place as part of its binding to different substrates, such as citric acid, owing to the low solubility in the Fe^{3+} state and being harmful in the Fe^{2+} state [51,52]. Several studies have reported the involvement of different citrate transporters, such as OsFRDL1 (rice) and FRD3 (Arabidopsis), restricted to the xylem-encircling plasma membrane of the root pericycle, for their role of transporting citrate to the xylem [53,54]. Similar to citrate, NA and 2′-deoxymugenic acid are not only involved in the transport of iron through the xylem, but have been reported to be involved in the transport of Fe through the phloem [55,56]. Additionally, YS1 transporters often localized to plasma membranes have been reported to facilitate Fe uptake at the rhizospheric plane and in translocating Fe in the plant body [37,57]. OsYSL15 is regarded as an Fe^{3+}-DMA transporter, playing a role in the uptake of iron at the rhizosphere, besides being involved in the translocation of iron following their expression in the shoots and developing seeds [40]. Similarly, OsYSL16 and OsYSL18 are regarded as Fe^{3+}-DMA transporters that are involved in transporting Fe to the reproductive organs and allocating it via vascular bundles [58]. In Arabidopsis, vacuole iron transporter-1 (VIT-1) transports Fe into the vacuole [59]. OsVIT-1 and OsVIT-2 (orthologs of VIT-1 in rice) were involved in the translocation of Fe along with zinc (Zn) from leaves to seeds [60]. Natural-resistance-associated microphage proteins (NRAMPs) are recognized as being involved in immobilizing Fe from the vacuoles to cytoplasm for utilization in different plant processes [61]. NRAMP3 and -4 (in Arabidopsis) and OsNRAMP1, -2, and -3 (in rice) are involved in immobilizing iron from the vacuole for its utilization by plants [62,63].

3. Phosphorus in Plant Health

Phosphorus (P) (macronutrient) serves as an important structural and functional component of biological molecules like nucleic acids, proteins, ATP, NADPH, vitamins, etc., besides playing an important role in the regulation of signaling cascades occurring in the plant system [64]. It serves as an important component for regulating plant growth, utilized in cellular process, including respiration and photosynthesis, and plays a critical role in the metabolism of carbohydrates and nitrogen compounds [65,66]. Additionally, phosphorus has a significant influence on stress tolerance and a plant's dependence on mycorrhizal fungi for phosphorus absorption, which is necessary for optimum crop development, yield, and quality [67]. Phosphorus deficiency is among the main factors limiting crop development in tropical and subtropical areas. This deficiency of phosphorus nutrients is caused by extreme temperatures and prolonged rainfall, along with the fixation of phosphorus by aluminum and iron oxides in soil [68]. In the world's arable land, about 30–40% is affected by phosphorus deficiency [69]. The phosphorus requirement of crops is fulfilled through the use of fertilizers in different agricultural practices. However, ~80% of phosphorus applied as a fertilizer is inaccessible for the majority of crops as inorganic phosphorus rapidly undergoes a complex formation with Ca^{2+} in alkaline soil and with

aluminum and iron oxides and hydroxides in acidic soil [70]. As the majority of phosphorus in soil remains inaccessible to plants, it worsens the condition by influencing nutrient availability for crop development. Plants have adapted to changes (biochemical, metabolic, and morphological) in phosphorus availability by increasing the root/shoot ratio, lateral root development, and producing longer and denser root hairs for a larger root surface area, thereby improving its acquisition in response to P limitation [68].

Phosphorus Uptake, Translocation, and Storage in Plants

Inorganic phosphate (P) is a major source of phosphorus for plants. The concentration of free P ions available for utilization by plants is relatively low (0.01 mmol/L or even less) as the majority of it is present in a complex with metal ions [70]. Though abundant in soil, it is often challenging for plants to absorb it; thus plants have evolved a complex signaling network against fluctuating external phosphate levels and have come up with a selective process of a distinct uptake (transporter) system that improves its availability and internalization for use by the plants [71]. The majority of transporters operating for the uptake of P from soil into the roots includes proton-coupled phosphate transporters (PHTs), SPX domain containing proteins, and sulphate transporter (SULTR)-like phosphorus distributor transporters (SPDTs) (Figure 2).

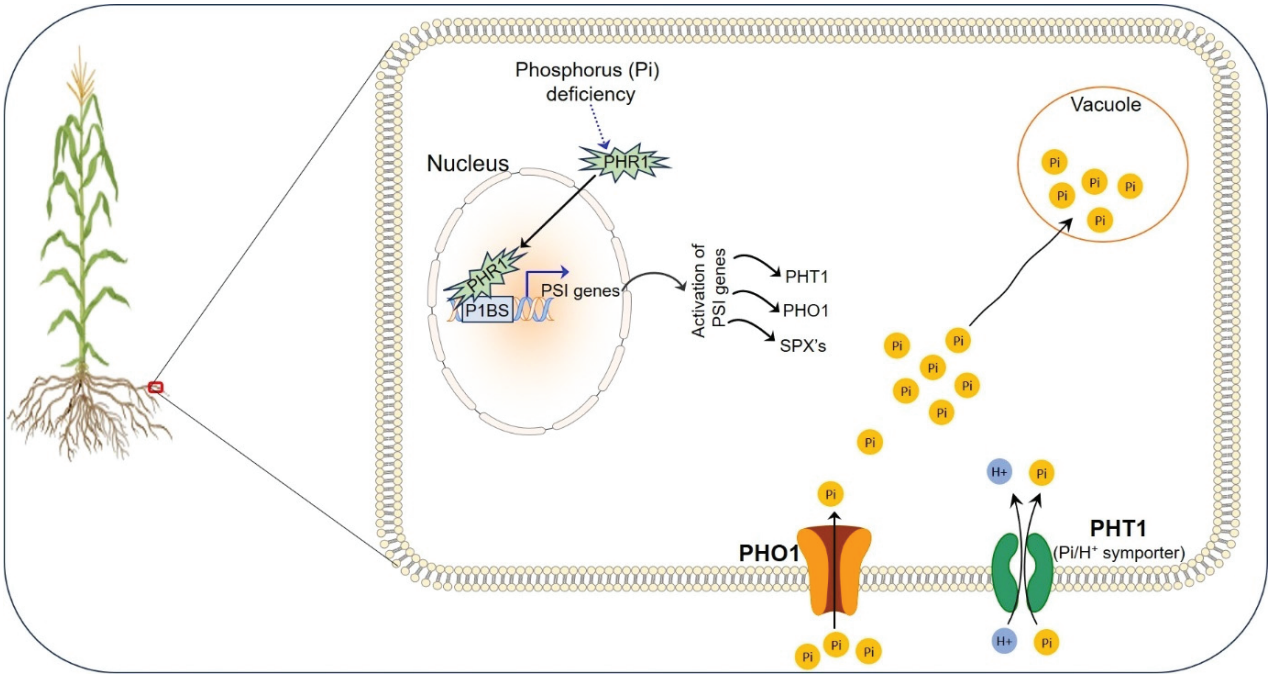


Figure 2. Phosphorus acquisition at the root surface of plants.

PHTs, based on subcellular localization, are classified into PHT1 (preferable plasma membrane), PHT2 (localized to chloroplasts), PHT3 (localized to mitochondria), PHT4 (chloroplast, Golgi, and non-green plastids), and PHT5 (localized to vacuoles) [72–74]. PHT1 transporters employ the P/H^+ symporter for transporting P from the soil into the roots and its translocation between cells and tissues. Their number varies across different plant species: Arabidopsis has 9 [75,76], 15 in soybean [77,78], 13 each in rice and maize [72,73,79,80], and tomato having 8 [81]. P uptake via PHT1 relies on the co-transport of 2-4 protons per P [82,83]. PHT2/3/4 facilitates the intracellular transport of P into and out of different sub-cellular organs playing a role in stress response and energy metabolism [74]. These transporters undergo complex formations with each other and, with PHT1 (homomeric as well as heteromeric), play a role in maintaining the homeostasis of phosphate [84]. The vacuole, acting as a depository for the storage of surplus phosphate (~90%), facilitates its mobilization as per the need via PHT5 [73,85]. SPX-domain containing proteins are

sub-divided into SPX-major facility superfamily (SPX-MFS), SPX-really interesting new gene (RING), and SPX-EXS (ERD1/XPR1/SYG1) [86], with only SPX-MFS and SPX-EXS reported for their involvement in the transport of phosphate [87].

A potential phosphate transporter, PHOSPHATE 1 (PHO1, a member of SPX-EXS) localized to Golgi is involved in the efflux of cellular P for its loading into the xylem [88]. The Arabidopsis genome contains 10 PHO1 homologs, while in rice, only three have been reported [89,90]. Other members of the SULTR family (SULTR3; 3 and -3;4) are present in rice grains for the accumulation of phosphate [91,92]. Root cells absorb P, loaded into the xylem by the symplastic or apoplastic pathway, transported to the shoot by xylem flow, and dispersed by the phloem across various shoot tissues [64]. Subcellular localization among different organs following transport (root-to-shoot translocation) through the xylem is believed to be mediated by PHO1 [93]. The majority of P entering the roots is absorbed by the symplasm in the outermost cell layers or the root hairs (RHs). In addition to direct absorption using different transporters, an indirect mode facilitated by fungi also contributes to the uptake of phosphate in plants [94,95]. P is released into plant cells by the AMF from pools of the P, which are inaccessible to plant roots [96]. P absorption, translocation, and remobilization are all carried out by P transporters. Current investigations show that their activity and abundance are highly controlled [97,98].

4. Roles of Iron and Phosphorus in Plants

In the production of nucleic acids and membranes, phosphorus and iron play a significant role. Fe is therefore necessary for a variety of biological processes, since it is an important component of several essential enzymes, including cytochromes of the ETC (electron transport chain) [99]. In macromolecules, like RNA and DNA, phosphorus connects ribonucleosides. Inorganic phosphate (P) or its organic derivates provide energy to several fundamental biological activities, including photosynthesis and respiration. P plays a critical function in energy transfer processes as a component of nucleotides, such as ATP [99]. Phosphate esters are also used in many different metabolic processes as energy carriers. A vital feature of cellular membranes, phospholipids include the element phosphorus. The transfer and removal of phosphate by enzymes (phosphorylation and dephosphorylation) has a significant impact on how proteins function. Proteins can signal one another through phosphorylation, including a protein's interactions [99].

In many agricultural plants, iron deficiency is a frequent nutritional disease that leads to low yields and worse nutritional quality. Iron is typically present in aerobic soils in the Fe^{3+} state, mostly as a component of oxyhydroxide polymers with very little solubility. This type frequently falls short of a plant's requirements [7]. Young leaves that have interveinal chlorosis and stunted root development display visible signs of low iron feeding in higher plants. Iron is necessary to preserve the structure and functionality of chloroplasts and is involved in the production of chlorophyll. In certain conditions, like when the soil is too alkaline (has a high pH), plants face a challenge of absorbing iron because it is mainly in the form of Fe^{3+} chelates in the soil. This difficulty in obtaining enough iron makes it hard for plants in high-pH soils to make and keep chlorophyll, which causes the leaves to become yellow, poor growth, and decreased production [7].

4.1. Photosynthesis

Photosynthesis takes place in the chloroplasts, which need a variety of proteins that are encoded in the nuclear genome [100]. While chloroplast to nucleus communication (retrograde regulation) is carefully orchestrated in plants, its molecular mechanisms are little known. Moreover, for chloroplasts to function at their best level, appropriate nutrient accumulation, like iron (Fe), is necessary [101,102]. Fe is present in all electron transfer complexes, including PSI, PSII, ferredoxins, and the cytochrome b6f complex, and is necessary for the synthesis of cofactors such as heme and Fe-S clusters [6,103]. Fe-deficient conditions cause plants to exhibit chlorotic symptoms [104] and have impaired photosynthesis [101,102]. Despite abundant Fe levels, chlorotic leaves may also form in

high-phosphorus (P) environments [105], casting doubt on the relationship between Fe concentration and chlorophyll synthesis. According to a study, rice plants growing in environments with a combined Fe and P shortage (FeP) do not display a chlorotic phenotype [106]. The impact of an iron deficit on chlorophyll synthesis and photosystem activity is influenced by the availability of phosphorus [107].

According to Monteiro and Winterbourn, excessive levels of iron stimulated the production of active free ROS (reactive oxygen species), which may have ultimately oxidized the chlorophyll and thus resulted in a drop in chlorophyll concentration [108]. Chlorophyll and carotenoid contents were shown to be lower with higher iron concentrations, according to Arunachala et al. [109]. The regularity of pigment production is the cause of this behavior, and carotenoids are essential metabolites that quench singlet oxygen to protect cellular and subcellular systems from the harmful effects of ROS [110].

4.2. Enzyme Activity

According to Asad and Rafique, iron is crucial for plants since it is needed for biological systems and because it is a crucial part of several enzymes, which are important for the biochemical and physiological functions of plants [111]. It takes part in a variety of electron transfer activities and functions as a cofactor of important enzymes of plant hormones [112]. In the metabolism of carbohydrates, the enzyme amylase is involved. According to Hofner, the fact that iron forms complexes with carbohydrates like maltose in plants is the reason why amylase activity is reduced as iron concentrations rise [113]. Together, glutamine synthetase and glutamate synthetase work to convert NH_4^+ into amino acids. α-ketoglutarate and glutamine are converted into two molecules of glutamate by the action of glutamate synthase. It is generally accepted that this process is how plants primarily assimilate nitrogen [114]. Because there was less activity, there was also reduced amino acid synthesis, which prevented the plants from assimilating nitrogen. According to Hemalatha and Venkatesan, aspartate amino transferase, amylase, invertase, and glutamate synthase in tea plants were all adversely impacted by excessive iron concentrations [115]. Also, they noted that pigment amounts, like chlorophyll and carotenes, decreased when the iron content dropped. The localized creation of an Fe-polyphenol complex has an impact on polyphenol concentration, which is crucial to tea quality. In graminaceous plants, Bashir et al. investigated the enzyme activity and expression patterns of GR (glutathione reductase) in both iron conditions (iron-deficient and -sufficient) by using two clones of cDNA for cytosolic GR (HvGR2) and chloroplastic GR (HvGR1) [116]. The projected polypeptide lengths for HvGR1 and HvGR2 were 550 and 497 amino acids, respectively, based on their nucleotide sequences. In graminaceous plants, they discovered that GR1 and GR2 genomes were significantly preserved. Both proteins had in vitro GR activity, however HvGR1 had a threefold-higher specific activity than HvGR2. During iron deprivation, HvGR1, HvGR2, and TaGR2 were increased [117]. Furthermore, TaGR1 and HvGR1 were mostly expressed in the shoots, whereas TaGR2 and HvGR2 were mainly seen in the roots. In response to iron deprivation, the activity of GR increased in the shoot tissues of barley, maize, and rice, as well as in the roots of wheat, maize, and barley. The researchers also discovered that rice, wheat, and barley did not present post-transcriptional regulations of GR. The expression of OsGR1 was mostly seen in the roots and calli, rather than the leaves [118]. In graminaceous plants, iron shortage as well as internal iron homeostasis may be influenced by GR.

4.3. Metabolism

Iron is a vital micronutrient for nearly all living organisms, owing to its indispensable involvement in fundamental metabolic processes, including DNA synthesis, respiration, and photosynthesis. Additionally, it serves as a cofactor for numerous enzymes, thus activating various metabolic pathways essential for cellular function [7]. Vigani et al. documented that mitochondria serve as the primary sites of energy production in root cells and are notably impacted by deficiencies in iron. Their research highlighted the remarkable

functional adaptability exhibited by mitochondria, facilitating the preservation of cellular viability under such conditions [119]. Within mitochondria, a substantial proportion of proteins are metalloproteins, necessitating iron for their proper enzymatic function [120]. Phosphorus (P) plays a pivotal role in plant metabolism by facilitating the synthesis of adenosine triphosphate (ATP), nicotinamide adenine dinucleotide phosphate hydrogen (NADPH), nucleic acids, and phospholipids. These compounds are essential for various aspects of plant development, productivity, signal transduction, and photosynthesis [121,122]. Phosphorus has five key functions in the metabolism of green plants, according to several biochemical investigations on plants conducted over the past fifteen years. 1. Phosphorus may have a role in nitrate reduction in the roots, according to Eckerson [123]. 2. Phosphorus plays a role in the production of starch in the leaves and other green portions of plants. 3. Respiratory functions and phosphorus are somewhat related. 4. Nuclear division is aided by phosphorus. 5. Other researchers believe that phosphorus, which is a component of lipoids and is found in the cytoplasm, helps to facilitate selective absorption by altering the solubility of cytoplasmic membranes or by controlling surface charges [124]. Reduced phosphorus (P) availability has been observed to negatively impact gas-exchange parameters in plants, including the net photosynthetic rate, transpiration rate, and stomatal conductance. Additionally, it has been noted to elevate the intercellular concentration of CO_2 [125].

5. Deficiencies of Iron and Phosphorus in Plants

Phosphorus has gained widely accepted status as part of membrane components, lipids, and in the structural organization of nucleic acids. Playing a vital role in maintaining the structural integrity of macromolecules, its role has also been observed in the processes of respiration and photosynthesis. As per the existing literature, phosphorus (P) deficiency has been demonstrated to exert a significant impact on plants' physiological parameters. Specifically, it leads to a substantial reduction in the dry weight (DW) of both shoot and root tissues in lettuce and tomato, along with a decrease in leaf numbers observed in lettuce, Chinese milk vetch, alfalfa, tomato, and marigold [126]. Across various plants, such as sunflower, rice, and maize, P deficiency induces a noteworthy decline in both the net photosynthesis rate and PSII reaction center's efficiency for energy capture [127]. This deleterious effect on the photosynthesis rate due to P deficiency has also been documented in diverse crops, including sugar beet [128], soybean, tobacco, *Zizania latifolia*, oat, sheep grass, barley, and tea [129–131]. Consequently, the insufficiency of phosphorus compromises the grain yield or overall crop production [125,127]. Moreover, short-term phosphorus deprivation not only leads to a reduction in phosphorus concentration, but also results in the diminished content of total chlorophyll and carotenoids in tomato seedlings [132].

Phosphorus (P) deficiency is known to perturb metabolic processes and carbohydrate translocation, including organic acids and soluble sugars [133,134]. The response to P deficiency often involves an augmented accumulation of carbohydrates, particularly sucrose, in the leaves of various species of plants [135,136]. This accumulation is attributed to reduced sink demand and constrained leaf expansion under conditions of P starvation [137]. Notably, the secretion of organic acids represents a pivotal low-P response in plants, facilitating the dissolution of soil P through acidification and complexation. This mechanism contributes to diverse levels of low-P tolerance in crops [138,139]. Some species, such as tea [140], white lupin [141], barley, soybean [138], and Chinese fir [139] have been observed to exhibit an induced exudation of organic acids under low-P conditions. Moreover, investigations suggest that the internal metabolism of organic acids may also play a role in low-P responses, exemplified by significantly elevated concentrations of citric, malic, and succinic acids in the roots of low-P alfalfa [142]. The deficiency of P as compared to other abiotic stresses inevitably triggers an escalation in the generation of ROS as byproducts of photosynthesis. A feedback inhibition mechanism, orchestrated by the accumulation of sugars, results in the diminished utilization of the electron transport chain [143].

The deficiency of phosphorus in plants proceeds with a change in the pigmentation (dark-green coloration) of the leaves and stunted plant growth. Phosphorus deficiency leads to the excessive production of anthocyanins that change the coloration of leaves to slightly purple (not correlated with leaf chlorosis). Its deficiency causes a delay in the maturation process of plants and in inducing symptoms of slender-like stem, as observed for the deficiency of nitrogen and the development of small spots of dead tissue (often referred to as necrotic spots). Considering its essentiality for plant growth and development, its requirement is directly correlated with the enhancement in the yield of different crops [144,145]. Its deficiency is generally addressed through the application of phosphate-based fertilizers that complement soil nutrients and make it available for use by plants [146].

Approximately one-third of globally cultivated land consists of calcareous soils, and these soils are a major contributor to iron (Fe) deficiency, which is prevalent in about 40% of all soil types [147]. The manifestation of Fe deficiency gives rise to diverse metabolic irregularities, leading to anomalies in chloroplast structure and morphology, diminished chlorophyll levels, reduced photosynthetic rates, and compromised respiratory functions in plants. These adverse effects collectively result in a substantial reduction in both the yield and quality of crops [148,149]. Consequently, iron deficiency poses a widespread challenge impacting agricultural productivity. In plants, iron is required for the assembly and maintenance of chlorophyl structure. Having such important functions, its deficiency often causes the chlorosis of the leaves. The symptoms of iron deficiency progress from intravenous chlorosis to the chlorosis of whole leaves (for prolonged deficiency) that turns the color of the leaves to white. The formation of insoluble iron decreases its mobility that increases its precipitation in older leaves. Iron undergoes a complex formation with the iron-binding protein, phytoferritin, as observed in the leaves and other plant parts [150].

6. Bacteria-Mediated Uptake of Fe and P

Bacteria mediates the uptake of mineral nutrients, especially Fe and P, through the production of siderophore (for Fe) and solubilization of P reserves for uptake by the plant system.

6.1. Siderophores in Fe Uptake

Several studies have demonstrated the efficacy of siderophore-producing rhizobacteria in promoting plant growth. Examples of such bacteria include *Bacillus licheniformis*, *B. subtilis*, *B. coagulans*, *B. circulans*, *Pseudomonas fluorescens*, *P. koreensis* [151], *Bacillus cereus*, *Pseudoalteromonas tetraodonis*, *Micrococcus aloeverae*, *Psychrobacter pocilloporae*, *P. weihenstephanensis* [152], *P. aeruginosa* [153,154], *Bacillus,* and *Rhodococcus*, and Enterobacter genera [155] and Pseudomonas sp [156]. Additionally, *Pantoae cypripedii* [157], *Pantoae dispersa* [158], *Bacillus megaterium* [159], and *Kosakonia radicincitans* [160] have also been identified as contributors to plant growth promotion through siderophore production.

Siderophores play a vital role in numerous physiological processes in plants, including respiration, bioremediation, photosynthesis [161,162], promoting the growth of plants, and aiding in the phytoremediation of heavy metals [151,163]. These compounds, produced through both non-ribosomal peptide bonds and multi-dentate iron-chelating compounds, demonstrate the ability to dissolve and bind various compounds (organic and inorganic) in soil [164,165]. Siderophore-producing bacteria primarily release proteins (like permeases and ATPases), which are iron-binding, facilitating the chelation of Fe^{3+} (ferric iron) and subsequent transport of Fe^{3+} ions in Gram-positive bacteria through the cell membrane [166]. In contrast, the transport of Fe^{3+} (ferric iron) in Gram-negative bacteria employs a sophisticated mechanism involving various enzymes, outer membrane receptors, periplasmic binding proteins, and cytoplasmic membrane proteins for ferric iron (Fe^{3+}) transportation, ultimately making Fe^{3+} available to plants [167].

Plant growth stimulation and health protection are two functions of siderophores. Using radio-labeled ferric siderophores as a plant's only source of iron has shown the

advantages of microbial siderophores [156]. Further evidence for the siderophores' importance in plant nutrition comes from the lack of indications of an iron shortage, such as chlorosis, and the relatively high iron content in the roots of plants grown in non-sterile soils as opposed to plants produced in sterile environments [168]. In plants that were not infected with the siderophore-producing Pseudomonas strain GRP3 and cultivated in environments with a limited iron supply, the injected plants displayed less chlorotic signs and more chlorophyll [169]. When nickel is introduced to *A. thaliana*, the plants were inoculated with two mutants of the *Pseudomonas putida* ARB86 strain—one overproducing siderophores, and the other lacking siderophore synthesis—the symptoms caused by the metal were relieved to the same degree in plants inoculated with the wild type and both mutants, indicating that siderophore independence is involved, in this case, of alleviating Ni toxicity [170]. Similarly, two strains of bacteria that produce siderophores decreased the amount of zinc that *Salix caprea* (willow) absorbed, indicating that siderophores of bacteria may bind to heavy metals in the soil and prevent plants from taking up those metals. However, improvements in zinc and copper absorption in willow infected with a strain of Streptomyces incapable of producing siderophores emphasize the significance of other physiological characteristics for heavy metals [171].

6.2. Phosphate Solubilization

Phosphate-solubilizing bacteria (PSB) play a pivotal role in fostering a symbiotic relationship with plants by contributing to the efficient utilization of phosphorus, an essential nutrient for plant growth [172]. Phosphorus is often present in soil in insoluble forms, primarily as phosphate minerals or organic compounds, making it challenging for plants to access [173]. In response to this challenge, PSB has evolved mechanisms to solubilize these otherwise inaccessible phosphates, rendering them in a form that plants can readily absorb (Table 1).

Table 1. Phosphate-solubilizing microorganisms, production of organic acids, and their ecological niches.

Phosphate-Solubilizing Microorganisms	Predominantly Produced Acids	Ecological Niche	References
Escherichia freundii	Lactic acid	Soil	[174,175]
Penicillium sp., *Aspergillus niger*	Citric acid, succinic acid, oxalic acid, glycolic acid, gluconic acid, lactic acid	Soil	[176,177]
Bacillus subtilus, *Bacillus megaterium*, *Pseudomonas* sp.	Lactic acid, malic acid	Soil rizoshpere	[177]
Arthrobacter sp., *Bacillus* sp. *Bacillus firmus* B-7650	Citric acid, lactic acid	Cowpea and wheat rhizospheres	[178]
Chaetomium nigricolor, *Penicillium* sp., *Aspergillus* sp.	Oxalic acid, succinic acid, citric acid, 2-ketogluconic acid	Lateritic soil	[179,180]
A. foetidus, *A. japonicus*	Citric acid, tartaric acid, succinic acid, oxalic acid, gluconic acid	Indian rock phosphate	[175]
P. radicum	Gluconic acid	Wheat rhizosphere	[181]
Enterobacter agglomerans	Citric acid, oxalic acid	Wheat rhizosphere	[182]
Enterobacter aerogenes, *E. asburiae*, *E. taylorae*, *Penibacillus macerans*, *Vibrio proteolyticus*, *Kluyvera cryocrescens*, *Xanthobacter agilis*, *Pseudomonas aeromonassens*, *Bacillus amyloliquefaciens*, *B. atrophaeus*, *B. licheniformis*	Acetic acid, itaconic acid, isobutyric acid, isovaleric acid, lactic acid	Mangrove	[177,183]
Penicillium rugulosum	Gluconic acid, citric acid	Venezuelan phosphate rocks	[184]
Enterobacter intermedius	2-ketogluconic acid	Grass rhizosphere	[185]
Penicillium canescens, *Aspergillus flavus*, *A. niger*	Citric acid, gluconic acid, oxalic acid, succinic acid	Wheat grains	[176,186]

Table 1. *Cont.*

Phosphate-Solubilizing Microorganisms	Predominantly Produced Acids	Ecological Niche	References
Pseudomonas fluorescens	Tartaric acid, citric acid, malic acid, gluconic acid	Oil palm rhizosphere	[182,187]
Aspergillus niger	Gluconic acid, oxalic acid	Tropical and subtropical soils	[188]
P. trivialis	Lactic acid, formic acid	Rhizosphere (*Hippophae rhamnoides*) (Trans-Himalayas, cold Howl and Spiti deserts)	[177]
Actinomadura oligospora; B. pumilus var.2; *B. subtilis var.2*; *Citrobacter* sp.	Propionic acid, gluconic acid, isovaleric acid, caproic acid, heptonic acid, isocaproic acid, formic acid, valeric acid, succinic acid, oxalic acid, oxaloacetic acid, malonic acid	Giant cardon cactus (*P. pringlei*)	[189,190]
B. pumilus CHOO8A; B. fusiformis	Succinic acid, citric acid, gluconic acid, oxalic acid, 2-ketogluconic acid, lactic acid, malic acid, formic acid	*Opuntia cholla*	[177]
Bacillus sp. *SENDO 6*	Gluconic acid, isovaleric acid, propionic acid, lactic acid, formic acid, succinic acid	*P. pringlei*	[177]
Bacillus megaterium M1PCa, *Enterobacter sakazakii M2PFe*, *Pseudomonas putida M5TSA*	Acetic acid, formic acid, gluconic acid, lactic acid, oxalic acid, propionic acid, succinic acid	*Mammillaria fraileana* cactus	[173,191]

One of the key functions of PSB involves the secretion of enzymes, such as phosphatases, which break down complex organic and inorganic phosphates into simpler, soluble forms [192]. This solubilization process occurs in the rhizosphere, the region surrounding plant roots, where phosphate-solubilizing bacteria are often found [193]. By converting insoluble phosphates into accessible forms, these bacteria aid in the enhancement of phosphorus availability for plants [194]. The intimate association between phosphate-solubilizing bacteria and plant roots is crucial for the success of this relationship [195]. PSB can colonize the rhizosphere and adhere to root surfaces. This colonization facilitates direct interactions between the bacteria and plant roots, enabling a more efficient exchange of nutrients [172]. As a result, plants can absorb solubilized phosphorus more effectively, supporting critical physiological processes, like energy transfer, nucleic acid synthesis, and root development [196]. Furthermore, the impact of phosphate-solubilizing bacteria extends beyond nutrient solubilization. Some strains of these bacteria exhibit plant growth-promoting traits, producing substances like phytohormones and organic acids. In plants, these compounds contribute not only to enhanced nutrient absorption, but also improved stress tolerance [197]. The presence of phosphate-solubilizing bacteria can positively influence the overall health and resilience of plants, particularly under conditions of nutrient stress. In the broader context of soil health, the activities of phosphate-solubilizing bacteria contribute to nutrient cycling. By solubilizing phosphates, these bacteria facilitate the recycling of phosphorus within the soil ecosystem, benefiting a diverse range of plants. This nutrient cycling is fundamental for maintaining soil fertility over the long term [198]. As a practical application of this symbiotic relationship, biofertilizers containing phosphate-solubilizing bacteria have been developed. Farmers can use these biofertilizers to enhance the fertility of soil and promote the growth of plants in an environmentally sustainable manner [199].

7. Toxicity Effects of Excess Fe and P on Plants

The inherent redox properties of Fe enable it to serve as both an electron donor and acceptor in cellular environments. In the presence of unsequestered iron within the cell, it can catalyze the transformation of hydrogen peroxide into free radicals through Fenton chemistry. These free radicals, possessing unpaired electrons, exhibit high reactivity and can induce oxidative damage to diverse cellular components. Cumulative oxidative stress inflicted by free radicals may culminate in cellular demise [200]. Enzymes involved in the transport of electrons need iron as a key component in redox reactions (e.g., Fe-S proteins and cytochromes). Moreover, Fe is a component of proteins (non-heme iron) that are essential for respiration, photosynthesis, and N_2 fixation [201]. One-third of the world's arable land is affected by iron restriction, which is a significant issue for agriculture. Several

photosynthetic components, such as ferredoxin (Fd), the Fe-S protein, involved in crucial oxidoreductive processes of chloroplasts, suffer from iron deficiency, contributing to this loss [202]. During the electron transfer process, it is reversibly oxidized from Fe^{2+} to Fe^{3+} in this function. Interveinal chlorosis, a symptom of iron insufficiency related to Mg deficiency, is one of its defining signs [203]. Iron deficiency typically causes young leaves to exhibit interveinal chlorotic signs as well as poor root development, and when severe, the shortage results in stasis, growth retardation, and mortality [204]. Phosphorus toxicity in plants is a significant concern that arises when there is an excess of phosphorus available in the soil or when plants are subjected to high levels of phosphorus fertilization. This condition can have detrimental effects on plant health and productivity. Phosphorus toxicity often manifests through visible symptoms on plant leaves [205]. These symptoms can include necrotic spots or patches on the leaves, particularly along the leaf margins. In severe cases, the leaves may become entirely necrotic and die off. Additionally, plants may exhibit reduced growth, including the stunted growth of both the shoots and roots. Different plant species exhibit varying degrees of tolerance to phosphorus toxicity. Some species are highly sensitive and may show severe symptoms, even with relatively low levels of phosphorus, while others are more tolerant and can withstand higher concentrations of phosphorus without significant damage [206].

7.1. Germination and Plant Growth

Iron (Fe) toxicity poses a significant threat to seed germination. In *Vigna radiata* (green gram), Verma and Pandey, highlighted that germination peaked at a 100 μM Fe supply, but a decline was observed between 200 and 300 μM Fe applications [207]. Similarly, an increase in Fe toxicity led to a reduction in seed germination in wheat and cowpea [208,209]. Consistent findings were reported by El Rasafi et al., [210] in common bean (*Phaselous vulgaris*) and wheat (*Triticum aestivum*), reinforcing the negative impact of elevated Fe levels on seed germination. Additionally, Nozoe et al. [211] noted an inhibition of the germination of rice weed (*Echinochloa oryzicola* cv. Hoshino yume) and cockspur grass (*Echinochloa crus*-galli) with the application of 100 mg L^{-1} of Fe^{2+}. Their examinations of rice and weed tolerance suggested the potential use of Fe for weed control fertilization amendments. Furthermore, Rodrigues Filho et al. found that an increased Fe^{2+} content in the substrate significantly reduced radicle formation [212]. A dose of Fe^{2+} (50 mg/L) resulted in the increased peroxidation of lipid, particularly in the radicle zone, causing growth stunt and decreased dry weight production [213]. Inhibited radical growth has severe consequences for various processes related to development, including the loss of radicle hairs and volume reduction [19]. Phosphorus toxicity proceeds with oxidation damage to the macromolecular structure. Lipid peroxidation, which is the oxidative degradation of lipids, may occur due to the accumulation of reactive oxygen species (ROS), further damaging plant tissues [205]. The mechanisms underlying phosphorus toxicity involve disruptions in various physiological processes in the plant. Moreover, high levels of P can inhibit the activity of certain antioxidant enzymes, such as Cu/Zn-type superoxide dismutase, which are essential for scavenging ROS and protecting plant cells from oxidative damage [205]. One significant consequence of phosphorus excess is the accumulation of phytic acid in plant cells. Phytic acid is a phosphorus-rich compound that forms when plants store excess phosphorus in the form of phytate.

7.2. Plant–Water Relation and Yield

The translocation of Fe occurs along the xylem transpiration stream [214]. Hence, closing the stoma might be viewed as an avoidance strategy to reduce its absorption and translocation. Many investigations have found that elevated Fe toxicity results in reduced stomatal conductivity [215,216]. In plants (tolerant and sensitive), this diminishes stomatal conductance as soon as Fe is applied [217]. Yield decrease and the severity of Fe have some relationships. This connection is influenced by both the cropping scheme and the season. Sahrawat (2010) estimates that typical yield losses brought on by Fe toxicity vary from

12% to 35% [218]. Rice yield loss occurs above a critical toxicity of Fe around 500 mg/kg (dry weight of leaves) [219,220]. Rice production loss is closely related to the soil solution Fe^{2+} concentration and cultivar tolerance level [221]. A maximum yield loss was noted at a 500 μM Fe supply [207]. In the condition of Fe toxicity, the formation of both reproductive and vegetative tillers was reduced. Becker and Asch discovered spikelet sterility, maturity, and delayed flowering in the range of 20–25 days; they also saw no blooming of sensitive cultivars [214]. Fe poisoning at early reproductive and late vegetative stages resulted in less panicles per hill [222]. The critical threshold of phosphorus concentration associated with a decline in plant yield varies among species. This threshold is influenced by factors such as the plant's nutrient requirements, growth habits, and environmental conditions. Reported critical phosphorus concentrations range from 0.26% to 1.00%, depending on the specific plant species and growing conditions [223,224]. Excessive levels of inorganic phosphorus, particularly phosphate ions (Ps), can interfere with photosynthesis. This interference may occur through several pathways, including a reduction in the activation of Rubisco. The accumulation of phytic acid can have several negative effects on plant metabolism. It can interfere with the availability of essential metals within the plant, such as iron and zinc, by forming insoluble complexes with these metals. This, in turn, can impair various metabolic processes and reduce the overall health and productivity of the plant.

7.3. Nutrient Uptake

As per various studies, a heightened iron (Fe) concentration in the soil causes in chemical interactions with Fe^{2+}, resulting in the diminished uptake of essential nutrients [225]. Researchers have made a distinction between "pseudo-Fe toxicity" and "real Fe toxicity," the latter being induced by elevated Fe^{2+} levels, impeding the absorption of some other crucial nutrients. The phenomenon is evident in rice plants, where an increased concentration of Fe^{2+} in the soil leads to reductions in calcium (Ca), potassium (K), manganese (Mn), phosphorus (P), and magnesium (Mg) contents in the shoots [226]. This occurs due to the increased content of metal in the root, stem, and lamina. Olaleye and Ogunkunle, argue that Fe poisoning amplifies the nutritional demands for phosphorus (P) and potassium (K) to alleviate stress reactions [227]. Additionally, plants receiving 10 mg L^{-1} of Fe experienced an increase in iron (Fe) content and reduction in potassium (K) content in both the shoots and roots [228]. Soils deficient in potassium (K) often exhibit high or hazardous levels of iron (Fe) [225]. Furthermore, in zinc-deficient soils, an excess of iron (Fe) not only leads to a deficiency in calcium (Ca), magnesium (Mg), phosphorus (P), and potassium (K), but also exacerbates the antagonistic relationship between iron (Fe) and zinc (Zn) [229]. This intricate interplay emphasizes the far-reaching consequences of iron toxicity on nutrient dynamics in the soil–plant system. The effects of phosphorus toxicity can be influenced by the availability of other nutrients in the soil [205]. High levels of nitrogen or potassium, for example, may help alleviate the toxic effects of excess phosphorus to some extent by promoting plant growth and mitigating nutrient imbalances [230]. Compared to Fe, excessive phosphorus can interfere with the uptake of other essential nutrients, such as iron, zinc, and manganese, leading to nutrient deficiencies [4]. High phosphorus levels can inhibit root growth and development, reducing the plant's ability to absorb water and nutrients from the soil [231]. A high phosphorus concentration can disrupt various metabolic pathways in the plant, affecting processes such as photosynthesis, respiration, and hormone regulation [126].

8. Iron–Phosphorus Homeostasis in Plants

It has long been known that plants may interact with macronutrients and micronutrients, and this interaction is somewhat understood. The relationships between Zn, P, and iron (Fe) homeostasis at molecular and physiological levels are therefore highlighted in this article. Crop species have shown evidence of the connection between the homeostasis of two nutrients. In plants, the relationship of P and Fe homeostasis is largely recognized.

During Fe-deficit conditions, P acquisition in both the shoots and roots is encouraged, while in contrast, in the plants, P deficiency greatly enhances Fe availability [103,232,233].

Recent plant research has unveiled intricate interactions between macronutrients and micronutrients, particularly highlighting the complex tripartite relationship between Fe and P homeostasis in Arabidopsis [234]. The interplay of P and Fe shortages governs iron transport in rice [106]. The tripartite interaction between Fe and P homeostasis is highlighted in this study as a factor in increased plant survival and fitness.

Molecular and physiological relationships have been established between Fe and P nutrition in well-studied model systems in rice and Arabidopsis [235,236]. However, limited knowledge exists regarding these interactions in mycorrhizal plants. Studies on edible crop species in mycorrhizal plants have indicated a negative association between Fe and P absorption [237,238]. Mycorrhizal plants tend to acquire more Fe under a low-P supply, while host plants acquire less Fe under P circumstances during arbuscular mycorrhizal (AM) symbiosis. Notably, the Fe content in straw significantly increased during AM symbiosis with a low supply of P, indicating that mycorrhized rice reduces the amount of Fe transferred to the shoots at a high P level [238]. Similar detrimental effects on Fe concentration were observed in maize treated with P fertilizers [239]. Consequently, in mycorrhizal plants, the accumulation of Fe is adversely affected by the application of high P to soil [240]. While these studies shed light on the impact of a high level of P on Fe absorption during AM symbiosis and transport in maize and rice, the reciprocal effect on P nutrient uptake by Fe treatments in mycorrhizal plants remains an underexplored area.

8.1. Fe-P Cross-Talk

Studies on the nutrient status of plants have revealed the strong physio-biochemical interaction of Fe and P occurring in plants [241]. On the rhizospeheric plane, the formation of the Fe-P complex renders Fe and P unavailable for uptake by the roots for its utilization in different cellular processes in the plant. For plants growing in limited-Fe and surplus-P conditions, a special uptake system for Fe is activated, while, in vice versa conditions, the accumulation of Fe accompanied by aluminum has been reported [103,233,235]. Other mechanisms of plant tolerance to P deficiency include an increase in the solubility of P for uptake at the root surface following the release of organic acids via transporters of citrate and malate [242]. Having a high affinity for Ca^{2+}, Al^{3+}, and Fe salts, organic acids easily replace P from insoluble precipitate for acquisition by the roots [243]. The interaction of Fe with P progresses with a reduction in its translocation to the aerial parts of the plants [244,245]. A similar pattern has been observed on the leaf surface, where chlorosis mediated by a high level of P occurs, irrespective of the presence of significant amounts of Fe [105]. Inside the seeds, the storage of Fe occurs in the vacuoles following the formation of the inositol hexakisphosphate-Fe complex [246]. This all shows that P acts as an Fe chelator in plants that affects its availability during changes in P homeostasis; therefore, there are hints of similar types of interactions between Fe and P that maintain their optimum levels in the shoots as well as seeds.

In arabidopsis, the availability of Fe plays a vital role in changing root architecture following the induction by a deficiency of P [235]. The reduction in the level of P results in the inhibition of root elongation that is restored under Fe-deficient conditions without any interference in P availability. Contrary to this, an increase in Fe accumulation was reported in the shoots and roots of Arabidopsis following growth under P-starved conditions [235,247]. In plants, a higher Fe concentration observed under P-deficient conditions is attributed to the expression of the Fe-responsive genes [232].

In phosphate-deficient plants, a transcriptomic analysis revealed that the expression of genes associated with the regulation of excess iron is enhanced, while a decrease in gene expression associated with iron deficiency was observed [247,248]. The expression of the gene *AtFER1* (encode ferritin protein) that regulates iron storage was induced in plants with a P deficiency. However, this regulation of the expression of *AtFER1* was found to be independent of the requirement of cis-acting element iron-dependent regulatory

sequences (IDRSs) found in the promoter region [249]. Irrespective of iron availability, the expression of the proteins associated with iron storage is mediated by two members of the MYB transcription factor family (PHL1 and PHR1) through binding with the cis-regulatory element, located in the promoter region (P1BS) of the *AtFER1* gene [249]. In response to excessive Fe, the upregulation of the expression of *AtFER1* remains unchanged in single (phr1) as well as in double mutants (phl1 and phr1) [249]. The deficiency of phosphorus (P) was observed by inhibiting the expressions of *FRO3, -6, IRT1, -2*, and *NAS1* genes in wild-type plants. Conversely, in phr1 × phl1 double mutants, these genes were found to be upregulated [103,233,250]. These results underscore the vital role of PHL1 and PHR1 in preserving the homeostatic interaction between phosphorus (P) and iron (Fe) in plant systems.

The core transcription factors PHR1 and PHL1 control P transporters' expression (PHO1 and PHT1) under P-deficiency conditions via signaling across the PHR1-miR399-PHO2 signaling cascade. P deficiency promotes Fe accumulation at the root surface via modulation signaling across the STOP1 cascade (PDR2-LPR1-STOP1-ALMT1) [251,252]. Transcription factors PHR1 and PHL1also help in maintaining Fe homeostasis via regulating the expression of Fe transporters (NAS3, FER1, and YLS8). Under conditions of Fe sufficiency, IDRS (cis-acting element) plays an important role in maintaining Fe homeostasis via upregulation in the expression of ferritin genes (FER2, FER3, and FER4) with the simultaneous inactivation of FIT to inhibit the expression of genes related to Fe uptake (IRT1 and FRO2) [253]. Following the interaction of bHLH IVc and bHLH121, a deficiency of Fe was found to exert a positive regulatory effect on bHLH Ib and FIT expressions [254]. The interaction of FIT and bHLH Ib in turn induces the expression of genes (IRT1 and FRO2) and P transporter genes, such as PHO1, necessary for P homeostasis [26,253]. Taken together, proteins such as PHL1, PHR1, PHO1; PHO1;1, H1, STOP1, FER1, and MPK6 extend help in integrating the signals for Fe and P as part of their cross-talk in plants.

8.2. Hormones concerning Fe and P in Plants

Phytohormones, being small endogenous molecules, play a crucial role in eliciting effective defense responses against stresses (biotic and abiotic) in plants. Beyond their involvement in defense signaling, these phytohormones serve as growth regulators, displaying various physiological and development processes. Phytohormones, including auxins (AUXs), gibberellins (GAs), cytokinins (CKs), ethylene (ET), brassinosteroids (BRs), salicylic acid (SA), abscisic acid (ABA), and jasmonic acid (JA), respond to stress through antagonistic and synergistic actions, commonly referred to as signaling cross-talk. This intricate coordination enables plants to respond adaptively to developmental and environmental cues. While phytohormones are well-established for their roles in both development and defense, specific hormones, like auxins, brassinosteroids, cytokinins (CKs), and gibberellic acid (GA), are particularly associated with the growth and development of plants [255–257]. Despite their primary impact on growth, these phytohormones also play a regulatory role in modulating plant defense responses [258–260]. In the context of systemic resistance pathways, phytohormones like jasmonic acid (JA), ethylene (ET) [261], salicylic acid (SA) [262], and abscissic acid (ABA) are notably involved [263]. When phosphorus (P) or iron (Fe) are deficient, auxin and ethylene have been linked to the development of roots [264–267]

In Arabidopsis, an increase in auxin synthesis is induced by a deficiency in iron, leading to elevated expressions of *FIT* and *FRO2* genes. Furthermore, the transcription of these genes is stimulated by the application of exogenous auxins [268]. An increase in ethylene production was observed in cucumber (*Cucumis sativus* L.), peas (*Pisum sativum* L.), and tomato (*Lycopersicon esculentum* Mill.), when there was a deficiency in iron [269]. Treating Arabidopsis and tomato with ethylene precursor ACC (1-aminocyclopropane-1-carboxylate) resulted in the heightened expression of *FRO*, *FIT*, and *IRT* genes. Conversely, in plants grown under Fe-deficient conditions, ethylene inhibitors' application led to the repression of these gene expressions [270]. Further investigations have shown that iron deficiency triggers an increased expression of genes involved in ethylene signaling (AtCTR1,

AtEIN2, AtEIN3, AtETR1, AtEIL1, and AtEIL3) and ethylene synthesis (*AtACS4*, *AtACS6*, *AtACS9*, *AtSAM1*, *AtSAM2*, *AtACO1*, and *AtACO2*) [271]. Jasmonic acid is a negative regulator of iron-deficiency response [272]. Arabidopsis subjected to the conditions of iron-deficiency and methyl jasmonate treatment (100 μM) exhibited a reduced expression of genes (*AtIRT1*, *AtFRO2*, and *AtFIT*). Similarly, when JA (20 μM) was applied to *Medicago truncatula*, the expressions (*MtFIT, MtFRO3*, *MtbHLH38,* and *MtbHLH39*) of iron-deficiency response genes were reduced, which in turn caused a decrease in the chlorophyll content [273]. In contrast, the application of salicylic acid (SA) to Arabidopsis increased the expression of genes (*AtbHLH38* and *AtbHLH39*), pivotal for establishing the response to iron deficiency [274]. Nevertheless, in *M. truncatula* exposed to 100 μM of SA, the gene expression related to iron deficiency decreased [273]. Interestingly, the content of chlorophyll increased by the treatment of peanut plants (*Arachis hypogaea*) with SA under both iron-sufficient and -deficient conditions [275], presenting a contrasting result compared to *M. truncatula* [273].

The expansion of the absorptive surface area in response to P and Fe deficiencies is achieved through the increased length and frequency of root hairs in Arabidopsis. These extra root hairs frequently appear across a tangential wall of underlying cortical cells, which is a location typically inhabited by non-hair cells under normal circumstances. Through phenotypic investigations of hormone-related Arabidopsis mutants and the application of chemicals that interfere with transport, or perception, hormone production, the role of ethylene and auxin in the growth of the root's epidermal cell under P and Fe deprivation was inferred. The application of the auxin analog (2,4-D) or ethylene precursor (1-aminocyclopropane-1-carboxylic acid) notably increased root hair density, resembling the phenotype of ethylene-overproducing mutants. Further investigations revealed that the application of hormone antagonists and hormone insensitivity inhibited the initiation of extra root hairs induced by the deficiency of Fe. However, these measures did not counteract the formation of additional hairs in response to P deprivation. A model is proposed, outlining potential pathways for alterations in root epidermal cell patterning induced by environmental stress.

Ethylene (ET) and nitric oxide (NO), with other signaling substances, have been implicated in the development of most morphological responses to Fe deficiency. Pioneering studies by Romera's group delved into the investigation of ET/NO interactions in this regulatory process [269]. ET and NO also contribute by regulating the cell wall composition and the dynamics of endodermal cell suberization [271,276]. Under Fe-deficient conditions, a naturally occurring polyamine (putrescine) is synthesized at an increased rate, which causes an NO burst that serves as a positive regulator for Fe solubilization that is attached to cell walls. Notably, the advantages of Fe utilization for the cell wall of putrescine is compromised in mutants of NO synthesis, including the *noa1* mutant (NO-associated 1) and *nia1nia2* mutants (nitrate reductases 1 and 2) [277]. Furthermore, this positive effect was absent in the Xyloglucan endotransglucosylase/hydrolase 31 mutant (*xth31*), the hemicellulose production mutant [277], and the reduced putrescine (Put) content mutant *adc2* [278]. Lei et al. demonstrated that genes PT1 and PT2-encoding P transporters are expressed less regularly in *etr1* and *ein2-5* (ET-insensitive Arabidopsis mutants), whereas the constitutive ET signaling mutant *hps2* displays enhanced responses to P deficiency [279]. ET has also been implicated in the regulation of *PHT1;5* [280,281]. In plants like *Medicago falcata*, the evidence suggests the involvement of ethylene (ET) in regulating phosphorus (P)-deficiency responses [282]. The expression of P transporter-encoding (*MfPT1* and *MfPT5*) genes as well as the acid phosphatase-encoding (*MfPAP1*) gene was strongly suppressed by ET synthesis inhibitors, including cobalt (Co^{2+}) and aminoethoxyvinyl glycine (AVG). On the other hand, applying the ACC to plants increased the expression of these genes, especially in plants that were cultivated in settings where P was adequate [282].

Cytokinins play a crucial role in the regulation of phosphorus (P)- and iron (Fe)-deficiency responses [283]. Their involvement in nutritional signaling responses was initially recognized in the early 1980s when studies demonstrated a decrease in cytokinin

levels under conditions of P and nitrogen (N) deficiencies [284]. In the context of P deficiency, the exogenous application of cytokinins has been shown to repress the expression of P-acquisition genes [285]. More recently, a similar repressive effect of cytokinins was observed for the expression of Fe-acquisition genes, including *IRT1*, *FRO2*, and *FIT* [286]. These findings underscore the presence of shared components in the responses to both Fe and P deficiencies, highlighting the significance of cytokinins alongside ethylene in these regulatory processes.

9. Conclusions and Future Perspectives

Plants, being sessile, are exposed to variable stresses, including soil nutrient availability, exposure to metals, etc., which exerts significant pressure on the survival and overall development of plants. Influenced by root architecture, plants are easily exposed to nutrient stress, such as the presence of a reduced amount of minerals, such as Fe, P, etc., influencing the growth and development parameters in their life cycle. The imbalance of mineral nutrients is rectified by increasing the use of inorganic and organic fertilizers that amend the availability of nutrients for uptake by plants. The use of fertilizers requires high energy, a large capital base, and a significant amount of effort, and mainly focuses on increasing productivity with little or no concern for damage to the ecosystem. As mineral nutrients play a pivotal role in the nutrition of plants, unraveling the intricacies of the transporters and pathways associated therein would help in understanding the significance of mineral nutrient cross-talk for efficient nutrient management. It highlights the need to unlock the avenues for crop improvement by undertaking integrative studies toward developing stress-resilient crop varieties. Although the imbalance of nutrients, specifically minerals, are adjusted by increasing the biomass of organs involved in acquiring scarce resources, they often regulate these imbalances through the development of effective strategies toward maintaining nutrient homeostasis. To this end, stable strategies and methodological procedures are needed to be undertaken, not only to minimize environmental challenges, but to increase plant productivity and profitability that would boost the agriculture-based economy.

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References

1. Diao, X.; Silver, J.; Takeshima, H. *Agricultural Mechanization and Agricultural Transformation*; International Food Policy Research Institute: Washington, DC, USA, 2016; Volume 1527.
2. Vanlauwe, B.; Wendt, J.; Giller, K.; Corbeels, M.; Gerard, B.; Nolte, C. A fourth principle is required to define Conservation Agriculture in sub-Saharan Africa: The appropriate use of fertilizer to enhance crop productivity. *Field Crops Res.* **2014**, *155*, 10–13. [CrossRef]
3. Bhat, M.A.; Kumar, V.; Bhat, M.A.; Wani, I.A.; Dar, F.L.; Farooq, I.; Bhatti, F.; Koser, R.; Rahman, S.; Jan, A.T. Mechanistic Insights of the Interaction of Plant Growth-Promoting Rhizobacteria (PGPR) with Plant Roots Toward Enhancing Plant Productivity by Alleviating Salinity Stress. *Front. Microbiol.* **2020**, *11*, 1952. [CrossRef] [PubMed]
4. Bhatla, S.C.; ALal, M.; Kathpalia, R.; Bhatla, S.C. Plant mineral nutrition. In *Plant Physiology, Development and Metabolism*; Springer: Singapore, 2018; pp. 37–81.

5. Hänsch, R.; Mendel, R.R. Physiological functions of mineral micronutrients (Cu, Zn, Mn, Fe, Ni, Mo, B, Cl). *Curr. Opin. Plant Biol.* **2009**, *12*, 259–266. [CrossRef] [PubMed]
6. Balk, J.; Pilon, M. Ancient and essential: The assembly of iron–sulfur clusters in plants. *Trends Plant Sci.* **2011**, *16*, 218–226. [CrossRef] [PubMed]
7. Rout, G.R.; Sahoo, S. Role of iron in plant growth and metabolism. *Rev. Agric. Sci.* **2015**, *3*, 1–24. [CrossRef]
8. Kobayashi, T.; Itai, R.N.; Nishizawa, N.K. Iron deficiency responses in rice roots. *Rice* **2014**, *7*, 1–11. [CrossRef] [PubMed]
9. Kobayashi, T.; Nozoye, T.; Nishizawa, N.K. Iron transport and its regulation in plants. *Free Radic. Biol. Med.* **2019**, *133*, 11–20. [CrossRef] [PubMed]
10. Kar, S.; Panda, S.K. Iron homeostasis in rice: Deficit and excess. *Proc. Natl. Acad. Sci. India Sect. B Biol. Sci.* **2020**, *90*, 227–235. [CrossRef]
11. Helliwell, C.A.; Poole, A.; Peacock, W.J.; Dennis, E.S. Arabidopsis *ent*-Kaurene Oxidase Catalyzes Three Steps of Gibberellin Biosynthesis. *Plant Physiol.* **1999**, *119*, 507–510. [CrossRef]
12. Kim, T.-W.; Hwang, J.-Y.; Kim, Y.-S.; Joo, S.-H.; Chang, S.C.; Lee, J.S.; Takatsuto, S.; Kim, S.-K. Arabidopsis CYP85A2, a Cytochrome P450, Mediates the Baeyer-Villiger Oxidation of Castasterone to Brassinolide in Brassinosteroid Biosynthesis. *Plant Cell* **2005**, *17*, 2397–2412. [CrossRef]
13. Fraser, C.M.; Chapple, C. The phenylpropanoid pathway in Arabidopsis. *Arab. Book/Am. Soc. Plant Biol.* **2011**, *9*, e0152. [CrossRef]
14. Murphy, M.J.; Siegel, L.M.; Tove, S.R.; Kamin, H. Siroheme: A New Prosthetic Group Participating in Six-Electron Reduction Reactions Catalyzed by Both Sulfite and Nitrite Reductases. *Proc. Natl. Acad. Sci. USA* **1974**, *71*, 612–616. [CrossRef]
15. Lancaster, J.; Vega, J.; Kamin, H.; Orme-Johnson, N.; Orme-Johnson, W.; Krueger, R.; Siegel, L. Identification of the iron-sulfur center of spinach ferredoxin-nitrite reductase as a tetranuclear center, and preliminary EPR studies of mechanism. *J. Biol. Chem.* **1979**, *254*, 1268–1272. [CrossRef] [PubMed]
16. Tanaka, A.; Loe, R.; Navasero, S.A. Some mechanisms involved in the development of iron toxicity symptoms in the rice plant. *Soil Sci. Plant Nutr.* **1966**, *12*, 32–38. [CrossRef]
17. Yamauchi, M. Rice bronzing in Nigeria caused by nutrient imbalances and its control by potassium sulfate application. *Plant Soil* **1989**, *117*, 275–286. [CrossRef]
18. Asch, F.; Becker, M.; Kpongor, D.S. A quick and efficient screen for resistance to iron toxicity in lowland rice. *J. Plant Nutr. Soil Sci.* **2005**, *168*, 764–773. [CrossRef]
19. Dorlodot, S.D.; Lutts, S.; Bertin, P. Effects of ferrous iron toxicity on the growth and mineral composition of an interspecific rice. *J. Plant Nutr.* **2005**, *28*, 1–20. [CrossRef]
20. Grotz, N.; Guerinot, M.L. Molecular aspects of Cu, Fe and Zn homeostasis in plants. *Biochim. Biophys. Acta BBA—Mol. Cell Res.* **2006**, *1763*, 595–608. [CrossRef]
21. Jan, A.T.; Azam, M.; Siddiqui, K.; Ali, A.; Choi, I.; Haq, Q.M.R. Heavy metals and human health: Mechanistic insight into toxicity and counter defense system of antioxidants. *Int. J. Mol. Sci.* **2015**, *16*, 29592–29630. [CrossRef]
22. Hindt, M.N.; Guerinot, M.L. Getting a sense for signals: Regulation of the plant iron deficiency response. *Biochim. Biophys. Acta (BBA)—Mol. Cell Res.* **2012**, *1823*, 1521–1530. [CrossRef]
23. Grillet, L.; Ouerdane, L.; Flis, P.; Hoang, M.T.T.; Isaure, M.-P.; Lobinski, R.; Curie, C.; Mari, S. Ascorbate Efflux as a New Strategy for Iron Reduction and Transport in Plants. *J. Biol. Chem.* **2014**, *289*, 2515–2525. [CrossRef]
24. Gao, F.; Dubos, C. Transcriptional integration of plant responses to iron availability. *J. Exp. Bot.* **2021**, *72*, 2056–2070. [CrossRef] [PubMed]
25. Varotto, C.; Maiwald, D.; Pesaresi, P.; Jahns, P.; Salamini, F.; Leister, D. The metal ion transporter IRT1 is necessary for iron homeostasis and efficient photosynthesis in *Arabidopsis thaliana*. *Plant J.* **2002**, *31*, 589–599. [CrossRef]
26. Vert, G.; Grotz, N.; Dédaldéchamp, F.; Gaymard, F.; Guerinot, M.L.; Briat, J.-F.; Curie, C. IRT1, an Arabidopsis Transporter Essential for Iron Uptake from the Soil and for Plant Growth. *Plant Cell* **2002**, *14*, 1223–1233. [CrossRef]
27. Li, M.; Watanabe, S.; Gao, F.; Dubos, C. Iron Nutrition in Plants: Towards a New Paradigm? *Plants* **2023**, *12*, 384. [CrossRef] [PubMed]
28. Quintana, J.; Bernal, M.; Scholle, M.; Holländer-Czytko, H.; Nguyen, N.T.; Piotrowski, M.; Krämer, U. Root-to-shoot iron partitioning in Arabidopsis requires IRON-REGULATED TRANSPORTER1 (IRT1) protein but not its iron (II) transport function. *Plant J.* **2022**, *109*, 992–1013. [CrossRef]
29. Castaings, L.; Caquot, A.; Loubet, S.; Curie, C. The high-affinity metal Transporters NRAMP1 and IRT1 Team up to Take up Iron under Sufficient Metal Provision. *Sci. Rep.* **2016**, *6*, 37222. [CrossRef] [PubMed]
30. Tergemina, E.; Elfarargi, A.F.; Flis, P.; Fulgione, A.; Göktay, M.; Neto, C.; Scholle, M.; Flood, P.J.; Xerri, S.-A.; Zicola, J.; et al. A two-step adaptive walk rewires nutrient transport in a challenging edaphic environment. *Sci. Adv.* **2022**, *8*, eabm9385. [CrossRef]
31. Martin-Barranco, A.; Spielmann, J.; Dubeaux, G.; Vert, G.; Zelazny, E. Dynamic Control of the High-Affinity Iron Uptake Complex in Root Epidermal Cells. *Plant Physiol.* **2020**, *184*, 1236–1250. [CrossRef]
32. Ishimaru, Y.; Bashir, K.; Nakanishi, H.; Nishizawa, N.K. The role of rice phenolics efflux transporter in solubilizing apoplasmic iron. *Plant Signal. Behav.* **2011**, *6*, 1624–1626. [CrossRef]
33. Tripathi, D.K.; Singh, S.; Gaur, S.; Singh, S.; Yadav, V.; Liu, S.; Singh, V.P.; Sharma, S.; Srivastava, P.; Prasad, S.M.; et al. Acquisition and Homeostasis of Iron in Higher Plants and Their Probable Role in Abiotic Stress Tolerance. *Front. Environ. Sci.* **2018**, *5*, 86. [CrossRef]

34. Wang, Y.; Kang, Y.; Zhong, M.; Zhang, L.; Chai, X.; Jiang, X.; Yang, X. Effects of Iron Deficiency Stress on Plant Growth and Quality in Flowering Chinese Cabbage and Its Adaptive Response. *Agronomy* **2022**, *12*, 875. [CrossRef]
35. Rai, S.; Singh, P.K.; Mankotia, S.; Swain, J.; Satbhai, S.B. Iron homeostasis in plants and its crosstalk with copper, zinc, and manganese. *Plant Stress* **2021**, *1*, 100008. [CrossRef]
36. Kobayashi, T.; Nishizawa, N.K. Iron Uptake, Translocation, and Regulation in Higher Plants. *Annu. Rev. Plant Biol.* **2012**, *63*, 131–152. [CrossRef]
37. Curie, C.; Panaviene, Z.; Loulergue, C.; Dellaporta, S.L.; Briat, J.-F.; Walker, E.L. Maize yellow stripe1 encodes a membrane protein directly involved in Fe(III) uptake. *Nature* **2001**, *409*, 346–349. [CrossRef]
38. Curie, C.; Cassin, G.; Couch, D.; Divol, F.; Higuchi, K.; Le Jean, M.; Misson, J.; Schikora, A.; Czernic, P.; Mari, S. Metal movement within the plant: Contribution of nicotianamine and yellow stripe 1-like transporters. *Ann. Bot.* **2009**, *103*, 1–11. [CrossRef]
39. Lee, S.; Chiecko, J.C.; Kim, S.A.; Walker, E.L.; Lee, Y.; Guerinot, M.L.; An, G. Disruption of OsYSL15 leads to iron inefficiency in rice plants. *Plant Physiol.* **2009**, *150*, 786–800. [CrossRef]
40. Inoue, H.; Kobayashi, T.; Nozoye, T.; Takahashi, M.; Kakei, Y.; Suzuki, K.; Nakazono, M.; Nakanishi, H.; Mori, S.; Nishizawa, N.K. Rice OsYSL15 Is an Iron-regulated Iron(III)-Deoxymugineic Acid Transporter Expressed in the Roots and Is Essential for Iron Uptake in Early Growth of the Seedlings. *J. Biol. Chem.* **2009**, *284*, 3470–3479. [CrossRef]
41. Nozoye, T.; Nagasaka, S.; Kobayashi, T.; Takahashi, M.; Sato, Y.; Sato, Y.; Uozumi, N.; Nakanishi, H.; Nishizawa, N.K. Phytosiderophore Efflux Transporters Are Crucial for Iron Acquisition in Graminaceous Plants. *J. Biol. Chem.* **2011**, *286*, 5446–5454. [CrossRef]
42. Haydon, M.J.; Kawachi, M.; Wirtz, M.; Hillmer, S.; Hell, R.; Krämer, U. Vacuolar Nicotianamine Has Critical and Distinct Roles under Iron Deficiency and for Zinc Sequestration in *Arabidopsis*. *Plant Cell* **2012**, *24*, 724–737. [CrossRef]
43. Ishimaru, Y.; Suzuki, M.; Tsukamoto, T.; Suzuki, K.; Nakazono, M.; Kobayashi, T.; Wada, Y.; Watanabe, S.; Matsuhashi, S.; Takahashi, M.; et al. Rice plants take up iron as an Fe^{3+}-phytosiderophore and as Fe^{2+}. *Plant J.* **2006**, *45*, 335–346. [CrossRef] [PubMed]
44. Cheng, L.; Wang, F.; Shou, H.; Huang, F.; Zheng, L.; He, F.; Li, J.; Zhao, F.-J.; Ueno, D.; Ma, J.F.; et al. Mutation in Nicotianamine Aminotransferase Stimulated the Fe(II) Acquisition System and Led to Iron Accumulation in Rice. *Plant Physiol.* **2007**, *145*, 1647–1657. [CrossRef] [PubMed]
45. Shabala, S. Physiological and cellular aspects of phytotoxicity tolerance in plants: The role of membrane transporters and implications for crop breeding for water logging tolerance. *New Phytol.* **2011**, *190*, 289–298. [CrossRef] [PubMed]
46. Connolly, E.L.; Campbell, N.H.; Grotz, N.; Prichard, C.L.; Guerinot, M.L. Overexpression of the FRO2 ferric chelate reductase confers tolerance to growth on low iron and uncovers posttranscriptional control. *Plant Physiol.* **2003**, *133*, 1102–1110. [CrossRef] [PubMed]
47. Pereira, M.P.; Santos, C.; Gomes, A.; Vasconcelos, M.W. Cultivar variability of iron uptake mechanisms in rice (*Oryza sativa* L.). *Plant Physiol. Biochem.* **2014**, *85*, 21–30. [CrossRef]
48. Yokosho, K.; Yamaji, N.; Feng Ma, J. OsFRDL1 expressed in nodes is required for distribution of iron into grains in rice. *J. Exp. Bot.* **2016**, *67*, 5485–5494. [CrossRef] [PubMed]
49. Mori, S. Iron transport in graminaceous plants. *Met. Ions Biol. Syst.* **1998**, *35*, 215–238.
50. Tsukamoto, T.; Nakanishi, H.; Uchida, H.; Watanabe, S.; Matsuhashi, S.; Mori, S.; Nishizawa, N.K. ^{52}Fe Translocation in Barley as Monitored by a Positron-Emitting Tracer Imaging System (PETIS): Evidence for the Direct Translocation of Fe from Roots to Young Leaves via Phloem. *Plant Cell Physiol.* **2009**, *50*, 48–57. [CrossRef]
51. Takahashi, M.; Terada, Y.; Nakai, I.; Nakanishi, H.; Yoshimura, E.; Mori, S.; Nishizawa, N.K. Role of Nicotianamine in the Intracellular Delivery of Metals and Plant Reproductive Development. *Plant Cell* **2003**, *15*, 1263–1280. [CrossRef]
52. Hell, R.; Stephan, U.W. Iron uptake, trafficking and homeostasis in plants. *Planta* **2003**, *216*, 541–551. [CrossRef]
53. Inoue, H.; Mizuno, D.; Takahashi, M.; Nakanishi, H.; Mori, S.; Nishizawa, N.K. A rice FRD3-like (*OsFRDL1*) gene is expressed in the cells involved in long-distance transport. *Soil Sci. Plant Nutr.* **2004**, *50*, 1133–1140. [CrossRef]
54. Durrett, T.P.; Gassmann, W.; Rogers, E.E. The FRD3-Mediated Efflux of Citrate into the Root Vasculature Is Necessary for Efficient Iron Translocation. *Plant Physiol.* **2007**, *144*, 197–205. [CrossRef] [PubMed]
55. Rellán-Álvarez, R.; Giner-Martínez-Sierra, J.; Orduna, J.; Orera, I.; Rodríguez-Castrillón, J.; García-Alonso, J.I.; Abadía, J.; Álvarez-Fernández, A. Identification of a Tri-Iron(III), Tri-Citrate Complex in the Xylem Sap of Iron-Deficient Tomato Resupplied with Iron: New Insights into Plant Iron Long-Distance Transport. *Plant Cell Physiol.* **2010**, *51*, 91–102. [CrossRef] [PubMed]
56. Nishiyama, R.; Kato, M.; Nagata, S.; Yanagisawa, S.; Yoneyama, T. Identification of Zn–nicotianamine and Fe–2′-deoxymugineic acid in the phloem sap from rice plants (*Oryza sativa* L.). *Plant Cell Physiol.* **2012**, *53*, 381–390. [CrossRef] [PubMed]
57. Ueno, D.; Yamaji, N.; Ma, J.F. Further characterization of ferric--phytosiderophore transporters ZmYS1 and HvYS1 in maize and barley. *J. Exp. Bot.* **2009**, *60*, 3513–3520. [CrossRef]
58. Aoyama, T.; Kobayashi, T.; Takahashi, M.; Nagasaka, S.; Usuda, K.; Kakei, Y.; Ishimaru, Y.; Nakanishi, H.; Mori, S.; Nishizawa, N.K. OsYSL18 is a rice iron(III)–deoxymugineic acid transporter specifically expressed in reproductive organs and phloem of lamina joints. *Plant Mol. Biol.* **2009**, *70*, 681–692. [CrossRef] [PubMed]
59. Kim, S.A.; Punshon, T.; Lanzirotti, A.; Li, L.; Alonso, J.M.; Ecker, J.R.; Kaplan, J.; Guerinot, M.L. Localization of Iron in *Arabidopsis* Seed Requires the Vacuolar Membrane Transporter VIT1. *Science* **2006**, *314*, 1295–1298. [CrossRef] [PubMed]

60. Zhang, Y.; Xu, Y.; Yi, H.; Gong, J. Vacuolar membrane transporters OsVIT1 and OsVIT2 modulate iron translocation between flag leaves and seeds in rice. *Plant J.* **2012**, *72*, 400–410. [CrossRef] [PubMed]
61. Darbani, B.; Briat, J.-F.; Holm, P.B.; Husted, S.; Noeparvar, S.; Borg, S. Dissecting plant iron homeostasis under short and long-term iron fluctuations. *Biotechnol. Adv.* **2013**, *31*, 1292–1307. [CrossRef]
62. Belouchi, A.; Kwan, T.; Gros, P. Cloning and characterization of the OsNramp family from *Oryza sativa*, a new family of membrane proteins possibly implicated in the transport of metal ions. *Plant Mol. Biol.* **1997**, *33*, 1085–1092. [CrossRef]
63. Lanquar, V.; Ramos, M.S.; Lelièvre, F.; Barbier-Brygoo, H.; Krieger-Liszkay, A.; Krämer, U.; Thomine, S. Export of Vacuolar Manganese by AtNRAMP3 and AtNRAMP4 Is Required for Optimal Photosynthesis and Growth under Manganese Deficiency. *Plant Physiol.* **2010**, *152*, 1986–1999. [CrossRef] [PubMed]
64. Poirier, Y.; Bucher, M. Phosphate transport and homeostasis in Arabidopsis. *Arab. Book/Am. Soc. Plant Biol.* **2002**, *1*, e0024. [CrossRef] [PubMed]
65. Marschner, H. Functions of mineral nutrients, macronutrients. In *Mineral Nutrition of Higher Plants*; Academic Press: London, UK, 1997.
66. Kaur, G.; Shukla, V.; Meena, V.; Kumar, A.; Tyagi, D.; Singh, J.; Kandoth, P.K.; Mantri, S.; Rouached, H.; Pandey, A.K. Physiological and molecular responses to combinatorial iron and phosphate deficiencies in hexaploid wheat seedlings. *Genomics* **2021**, *113*, 3935–3950. [CrossRef] [PubMed]
67. Graham, J.H.; Syvertsen, J.P. Host determinants of mycorrhizal dependency of citrus rootstock seedlings. *New Phytol.* **1985**, *101*, 667–676. [CrossRef]
68. López-Arredondo, D.L.; Leyva-González, M.A.; González-Morales, S.I.; López-Bucio, J.; Herrera-Estrella, L. Phosphate Nutrition: Improving Low-Phosphate Tolerance in Crops. *Annu. Rev. Plant Biol.* **2014**, *65*, 95–123. [CrossRef] [PubMed]
69. Von Uexküll, H.R.; Mutert, E. Global extent, development and economic impact of acid soils. *Plant Soil* **1995**, *171*, 1–15. [CrossRef]
70. Raghothama, K.G. Phosphate acquisition. *Annu. Rev. Plant Biol.* **1999**, *50*, 665–693. [CrossRef] [PubMed]
71. Puga, M.I.; Rojas-Triana, M.; de Lorenzo, L.; Leyva, A.; Rubio, V.; Paz-Ares, J. Novel signals in the regulation of Pi starvation responses in plants: Facts and promises. *Curr. Opin. Plant Biol.* **2017**, *39*, 40–49. [CrossRef]
72. Liu, F.; Xu, Y.; Jiang, H.; Jiang, C.; Du, Y.; Gong, C.; Wang, W.; Zhu, S.; Han, G.; Cheng, B. Systematic identification, evolution and expression analysis of the *Zea mays* PHT1 gene family reveals several new members involved in root colonization by arbuscular mycorrhizal fungi. *Int. J. Mol. Sci.* **2016**, *17*, 930. [CrossRef]
73. Liu, T.-Y.; Huang, T.-K.; Yang, S.-Y.; Hong, Y.-T.; Huang, S.-M.; Wang, F.-N.; Chiang, S.-F.; Tsai, S.-Y.; Lu, W.-C.; Chiou, T.-J. Identification of plant vacuolar transporters mediating phosphate storage. *Nat. Commun.* **2016**, *7*, 11095. [CrossRef]
74. Versaw, W.K.; Garcia, L.R. Intracellular transport and compartmentation of phosphate in plants. *Curr. Opin. Plant Biol.* **2017**, *39*, 25–30. [CrossRef] [PubMed]
75. Mudge, S.R.; Rae, A.L.; Diatloff, E.; Smith, F.W. Expression analysis suggests novel roles for members of the Pht1 family of phosphate transporters in *Arabidopsis*. *Plant J.* **2002**, *31*, 341–353. [CrossRef]
76. Shin, H.; Shin, H.S.; Dewbre, G.R.; Harrison, M.J. Phosphate transport in Arabidopsis: Pht1;1 and Pht1;4 play a major role in phosphate acquisition from both low- and high-phosphate environments. *Plant J.* **2004**, *39*, 629–642. [CrossRef] [PubMed]
77. Qin, L.; Guo, Y.; Chen, L.; Liang, R.; Gu, M.; Xu, G.; Zhao, J.; Walk, T.; Liao, H. Functional characterization of 14 Pht1 family genes in yeast and their expressions in response to nutrient starvation in soybean. *PLoS ONE* **2012**, *7*, e47726. [CrossRef] [PubMed]
78. Fan, C.; Wang, X.; Hu, R.; Wang, Y.; Xiao, C.; Jiang, Y.; Zhang, X.; Zheng, C.; Fu, Y.-F. The pattern of Phosphate transporter 1 genes evolutionary divergence in *Glycine max* L. *BMC Plant Biol.* **2013**, *13*, 48. [CrossRef] [PubMed]
79. Liu, F.; Chang, X.-J.; Ye, Y.; Xie, W.-B.; Wu, P.; Lian, X.-M. Comprehensive Sequence and Whole-Life-Cycle Expression Profile Analysis of the Phosphate Transporter Gene Family in Rice. *Mol. Plant* **2011**, *4*, 1105–1122. [CrossRef] [PubMed]
80. Wang, X.; Wang, H.-F.; Chen, Y.; Sun, M.-M.; Wang, Y.; Chen, Y.-F. The Transcription Factor NIGT1.2 Modulates Both Phosphate Uptake and Nitrate Influx during Phosphate Starvation in Arabidopsis and Maize. *Plant Cell* **2020**, *32*, 3519–3534. [CrossRef] [PubMed]
81. Chen, A.; Chen, X.; Wang, H.; Liao, D.; Gu, M.; Qu, H.; Sun, S.; Xu, G. Genome-wide investigation and expression analysis suggest diverse roles and genetic redundancy of Pht1 family genes in response to Pi deficiency in tomato. *BMC Plant Biol.* **2014**, *14*, 61. [CrossRef] [PubMed]
82. Ullrich-Eberius, C.I.; Novacky, A.; van Bel, A.J.E. Phosphate uptake inLemna gibba G1: Energetics and kinetics. *Planta* **1984**, *161*, 46–52. [CrossRef] [PubMed]
83. Sakano, K. Proton/Phosphate Stoichiometry in Uptake of Inorganic Phosphate by Cultured Cells of *Catharanthus roseus* (L.) G. Don. *Plant Physiol.* **1990**, *93*, 479–483. [CrossRef]
84. Karlsson, P.M.; Herdean, A.; Adolfsson, L.; Beebo, A.; Nziengui, H.; Irigoyen, S.; Ünnep, R.; Zsiros, O.; Nagy, G.; Garab, G.; et al. The Arabidopsis thylakoid transporter PHT4;1 influences phosphate availability for ATP synthesis and plant growth. *Plant J.* **2015**, *84*, 99–110. [CrossRef] [PubMed]
85. Liu, J.; Yang, L.; Luan, M.; Wang, Y.; Zhang, C.; Zhang, B.; Shi, J.; Zhao, F.-G.; Lan, W.; Luan, S. A vacuolar phosphate transporter essential for phosphate homeostasis in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA* **2015**, *112*, E6571–E6578. [CrossRef] [PubMed]
86. Secco, D.; Wang, C.; Arpat, B.A.; Wang, Z.; Poirier, Y.; Tyerman, S.D.; Wu, P.; Shou, H.; Whelan, J. The emerging importance of the SPX domain-containing proteins in phosphate homeostasis. *New Phytol.* **2012**, *193*, 842–851. [CrossRef] [PubMed]

87. Wang, L.; Jia, X.; Zhang, Y.; Xu, L.; Menand, B.; Zhao, H.; Zeng, H.; Dolan, L.; Zhu, Y.; Yi, K. Loss of two families of SPX domain-containing proteins required for vacuolar polyphosphate accumulation coincides with the transition to phosphate storage in green plants. *Mol. Plant* **2021**, *14*, 838–846. [CrossRef] [PubMed]
88. Wang, Z.; Kuo, H.-F.; Chiou, T.-J. Intracellular phosphate sensing and regulation of phosphate transport systems in plants. *Plant Physiol.* **2021**, *187*, 2043–2055. [CrossRef] [PubMed]
89. Secco, D.; Baumann, A.; Poirier, Y. Characterization of the Rice *PHO1* Gene Family Reveals a Key Role for *OsPHO1;2* in Phosphate Homeostasis and the Evolution of a Distinct Clade in Dicotyledons. *Plant Physiol.* **2010**, *152*, 1693–1704. [CrossRef]
90. Wang, Y.; Ribot, C.; Rezzonico, E.; Poirier, Y. Structure and Expression Profile of the Arabidopsis *PHO1* Gene Family Indicates a Broad Role in Inorganic Phosphate Homeostasis. *Plant Physiol.* **2004**, *135*, 400–411. [CrossRef] [PubMed]
91. Zhao, H.; Frank, T.; Tan, Y.; Zhou, C.; Jabnoune, M.; Arpat, A.B.; Cui, H.; Huang, J.; He, Z.; Poirier, Y.; et al. Disruption of *OsSULTR3;3* reduces phytate and phosphorus concentrations and alters the metabolite profile in rice grains. *New Phytol.* **2016**, *211*, 926–939. [CrossRef]
92. Yamaji, N.; Takemoto, Y.; Miyaji, T.; Mitani-Ueno, N.; Yoshida, K.T.; Ma, J.F. Reducing phosphorus accumulation in rice grains with an impaired transporter in the node. *Nature* **2017**, *541*, 92–95. [CrossRef]
93. Arpat, A.B.; Magliano, P.; Wege, S.; Rouached, H.; Stefanovic, A.; Poirier, Y. Functional expression of PHO1 to the Golgi and *trans*-Golgi network and its role in export of inorganic phosphate. *Plant J.* **2012**, *71*, 479–491. [CrossRef]
94. Rausch, C.; Bucher, M. Molecular mechanisms of phosphate transport in plants. *Planta* **2002**, *216*, 23–37. [CrossRef] [PubMed]
95. Javot, H.; Pumplin, N.; Harrison, M.J. Phosphate in the arbuscular mycorrhizal symbiosis: Transport properties and regulatory roles. *Plant Cell Environ.* **2007**, *30*, 310–322. [CrossRef] [PubMed]
96. Chen, J.; Wang, Y.; Wang, F.; Yang, J.; Gao, M.; Li, C.; Liu, Y.; Liu, Y.; Yamaji, N.; Ma, J.F.; et al. The Rice CK2 Kinase Regulates Trafficking of Phosphate Transporters in Response to Phosphate Levels. *Plant Cell* **2015**, *27*, 711–723. [CrossRef] [PubMed]
97. Wang, F.; Deng, M.; Xu, J.; Zhu, X.; Mao, C. Molecular mechanisms of phosphate transport and signaling in higher plants. *Semin. Cell Dev. Biol.* **2018**, *74*, 114–122. [CrossRef] [PubMed]
98. Yang, Z.; Yang, J.; Wang, Y.; Wang, F.; Mao, W.; He, Q.; Xu, J.; Wu, Z.; Mao, C. PROTEIN PHOSPHATASE95 Regulates Phosphate Homeostasis by Affecting Phosphate Transporter Trafficking in Rice. *Plant Cell* **2020**, *32*, 740–757. [CrossRef] [PubMed]
99. Raghothama, K.G. Phosphorus and plant nutrition: An overview. *Phosphorus Agric. Environ.* **2005**, *46*, 353–378.
100. Myouga, F.; Akiyama, K.; Motohashi, R.; Kuromori, T.; Ito, T.; Iizumi, H.; Ryusui, R.; Sakurai, T.; Shinozaki, K. The Chloroplast Function Database: A large-scale collection of Arabidopsis Ds/Spm-or T-DNA-tagged homozygous lines for nuclear-encoded chloroplast proteins, and their systematic phenotype analysis. *Plant J.* **2010**, *61*, 529–542. [CrossRef] [PubMed]
101. Chen, Y.; Barak, P. Iron nutrition of plants in calcareous soils. *Adv. Agron.* **1982**, *35*, 217–240.
102. Carstensen, A.; Herdean, A.; Schmidt, S.B.; Sharma, A.; Spetea, C.; Pribil, M.; Husted, S. The Impacts of Phosphorus Deficiency on the Photosynthetic Electron Transport Chain. *Plant Physiol.* **2018**, *177*, 271–284. [CrossRef]
103. Briat, J.-F.; Rouached, H.; Tissot, N.; Gaymard, F.; Dubos, C. Integration of P, S, Fe, and Zn nutrition signals in *Arabidopsis thaliana*: Potential involvement of PHOSPHATE STARVATION RESPONSE 1 (PHR1). *Front. Plant Sci.* **2015**, *6*, 290. [CrossRef]
104. Marschner, H. *Mineral Nutrition of Higher Plants*, 2nd ed.; Institute of Plant Nutrition University of Hohenheim: Stuttgart, Germany, 1995.
105. Dekock, P.C.; Hall, A.; Inkson, R.H.E. Active Iron in Plant Leaves. *Ann. Bot.* **1979**, *43*, 737–740. [CrossRef]
106. Saenchai, C.; Bouain, N.; Kisko, M.; Prom-U-Thai, C.; Doumas, P.; Rouached, H. The Involvement of OsPHO1;1 in the Regulation of Iron Transport Through Integration of Phosphate and Zinc Deficiency Signaling. *Front. Plant Sci.* **2016**, *7*, 396. [CrossRef] [PubMed]
107. Nam, H.-I.; Shahzad, Z.; Dorone, Y.; Clowez, S.; Zhao, K.; Bouain, N.; Lay-Pruitt, K.S.; Cho, H.; Rhee, S.Y.; Rouached, H. Interdependent iron and phosphorus availability controls photosynthesis through retrograde signaling. *Nat. Commun.* **2021**, *12*, 7211. [CrossRef] [PubMed]
108. Monteiro, H.P.; Winterbourn, C.C. The superoxide-dependent transfer of iron from ferritin to transferrin and lactoferrin. *Biochem. J.* **1988**, *256*, 923–928. [CrossRef] [PubMed]
109. Arunachala, R.; Paulkumar, K.; Ranjitsin, A.; Annadurai, G. Environmental Assessment due to Air Pollution near Iron Smelting Industry. *J. Environ. Sci. Technol.* **2009**, *2*, 179–186. [CrossRef]
110. Wilkinson, R.E.; Ohki, K. Influence of Manganese Deficiency and Toxicity on Isoprenoid Syntheses. *Plant Physiol.* **1988**, *87*, 841–846. [CrossRef]
111. Asad, A.; Rafique, R. Effect of Zinc, Copper, Iron, Manganese and Boron on the Yield and Yield Components of Wheat Crop in Tehsil Peshawar. *Pak. J. Biol. Sci.* **2000**, *3*, 1615–1620. [CrossRef]
112. Kerkeb, L.; Connolly, E.L. Iron transport and metabolism in plants. In *Genetic Engineering: Principles and Methods*; Springer: Boston, MA, USA, 2006; pp. 119–140.
113. Hofner, W. Iron and manganese compounds in the Blutungssaft of Helianthus annuus. *Physiol. Plant* **1970**, *23*, 673–677. [CrossRef]
114. Temple, S.J.; Vance, C.P.; Gantt, J.S. Glutamate synthase and nitrogen assimilation. *Trends Plant Sci.* **1998**, *3*, 51–56. [CrossRef]
115. Hemalatha, K.; Venkatesan, S. Impact of Iron Toxicity on Certain Enzymes and Biochemical Parameters of Tea. *Asian J. Biochem.* **2011**, *6*, 384–394. [CrossRef]

116. Bashir, K.; Nagasaka, S.; Itai, R.N.; Kobayashi, T.; Takahashi, M.; Nakanishi, H.; Mori, S.; Nishizawa, N.K. Expression and enzyme activity of glutathione reductase is upregulated by Fe-deficiency in graminaceous plants. *Plant Mol. Biol.* **2007**, *65*, 277–284. [CrossRef]
117. Romero-Puertas, M.C.; Corpas, F.J.; Sandalio, L.M.; Leterrier, M.; Rodríguez-Serrano, M.; Del Río, L.A.; Palma, J.M. Glutathione reductase from pea leaves: Response to abiotic stress and characterization of the peroxisomal isozyme. *New Phytol.* **2006**, *170*, 43–52. [CrossRef] [PubMed]
118. Kaminaka, H.; Morita, S.; Nakajima, M.; Masumura, T.; Tanaka, K. Gene cloning and expression of cytosolic glutathione reductase in rice (*Oryza sativa* L.). *Plant Cell Physiol.* **1998**, *39*, 1269–1280. [CrossRef] [PubMed]
119. Vigani, G.; Maffi, D.; Zocchi, G. Iron availability affects the function of mitochondria in cucumber roots. *New Phytol.* **2009**, *182*, 127–136. [CrossRef] [PubMed]
120. Bertini, I.; Rosato, A. From Genes to Metalloproteins: A Bioinformatic Approach. *Eur. J. Inorg. Chem.* **2007**, *2007*, 2546–2555. [CrossRef]
121. Hammond, J.P.; White, P.J. Sucrose transport in the phloem: Integrating root responses to phosphorus starvation. *J. Exp. Bot.* **2008**, *59*, 93–109. [CrossRef] [PubMed]
122. Lu, H.; Wang, F.; Wang, Y.; Lin, R.; Wang, Z.; Mao, C. Molecular mechanisms and genetic improvement of low-phosphorus tolerance in rice. *Plant Cell Environ.* **2023**, *46*, 1104–1119. [CrossRef] [PubMed]
123. Eckerson, S.H. Influence of phosphorus deficiency on the metabolism of tomato plants. *Am. J. Bot.* **1929**, *16*, 852.
124. Levene, P.A.; Rolf, I.P. Structure and significance of the phosphatides. *Physiol. Rev.* **1921**, *1*, 327–393. [CrossRef]
125. Kayoumu, M.; Iqbal, A.; Muhammad, N.; Li, X.; Li, L.; Wang, X.; Gui, H.; Qi, Q.; Ruan, S.; Guo, R.; et al. Phosphorus Availability Affects the Photosynthesis and Antioxidant System of Contrasting Low-P-Tolerant Cotton Genotypes. *Antioxidants* **2023**, *12*, 466. [CrossRef]
126. Khan, F.; Siddique, A.B.; Shabala, S.; Zhou, M.; Zhao, C. Phosphorus Plays Key Roles in Regulating Plants' Physiological Responses to Abiotic Stresses. *Plants* **2023**, *12*, 2861. [CrossRef] [PubMed]
127. Zhang, W.; Chen, X.-X.; Liu, Y.-M.; Liu, D.-Y.; Du, Y.-F.; Chen, X.-P.; Zou, C.-Q. The role of phosphorus supply in maximizing the leaf area, photosynthetic rate, coordinated to grain yield of summer maize. *Field Crops Res.* **2018**, *219*, 113–119. [CrossRef]
128. Meng, X.; Chen, W.-W.; Wang, Y.-Y.; Huang, Z.-R.; Ye, X.; Chen, L.-S.; Yang, L.-T. Effects of phosphorus deficiency on the absorption of mineral nutrients, photosynthetic system performance and antioxidant metabolism in Citrus grandis. *PLoS ONE* **2021**, *16*, e0246944. [CrossRef] [PubMed]
129. Li, L.; Yang, H.; Peng, L.; Ren, W.; Gong, J.; Liu, P.; Wu, X.; Huang, F. Comparative Study Reveals Insights of Sheep grass (*Leymus chinensis*) Coping with Phosphate-Deprived Stress Condition. *Front. Plant Sci.* **2019**, *10*, 170. [CrossRef] [PubMed]
130. Seleiman, M.F.; Alotaibi, M.A.; Alhammad, B.A.; Alharbi, B.M.; Refay, Y.; Badawy, S.A. Effects of ZnO Nanoparticles and Biochar of Rice Straw and Cow Manure on Characteristics of Contaminated Soil and Sunflower Productivity, Oil Quality, and Heavy Metals Uptake. *Agronomy* **2020**, *10*, 790. [CrossRef]
131. Geng, G.; Wang, G.; Stevanato, P.; Lv, C.; Wang, Q.; Yu, L.; Wang, Y. Physiological and Proteomic Analysis of Different Molecular Mechanisms of Sugar Beet Response to Acidic and Alkaline pH Environment. *Front. Plant Sci.* **2021**, *12*, 682799. [CrossRef] [PubMed]
132. Satheesh, V.; Zhang, J.; Li, J.; You, Q.; Zhao, P.; Wang, P.; Lei, M. High transcriptome plasticity drives phosphate starvation responses in tomato. *Stress Biol.* **2022**, *2*, 18. [CrossRef] [PubMed]
133. Zheng, L.; Karim, M.R.; Hu, Y.G.; Shen, R.; Lan, P. Greater morphological and primary metabolic adaptations in roots contribute to phosphate-deficiency tolerance in the bread wheat cultivar Kenong199. *BMC Plant Biol.* **2021**, *21*, 381. [CrossRef] [PubMed]
134. Zhang, J.; Sun, W.; Zhao, H.; Liang, Q.; Zhang, G.; Cai, S. Integrated Transcriptome and Metabolome Analysis Reveals the Divergent Evolution of Low-P Tolerance in Cultivated and Tibetan Wild Barley. *Environ. Exp. Bot.* **2024**, *219*, 105641. [CrossRef]
135. Soumya, P.R.; Sharma, S.; Meena, M.K.; Pandey, R. Response of diverse bread wheat genotypes in terms of root architectural traits at seedling stage in response to low phosphorus stress. *Plant Physiol. Rep.* **2021**, *26*, 152–161. [CrossRef]
136. Naz, M.; Hussain, S.; Ashraf, I.; Farooq, M. Exogenous application of proline and phosphorus help improving maize performance under salt stress. *J. Plant Nutr.* **2023**, *46*, 2342–2350. [CrossRef]
137. Glanz-Idan, N.; Wolf, S. Upregulation of photosynthesis in mineral nutrition-deficient tomato plants by reduced source-to-sink ratio. *Plant Signal. Behav.* **2020**, *15*, 1712543. [CrossRef] [PubMed]
138. Nadira, U.A.; Ahmed, I.M.; Zeng, J.; Bibi, N.; Cai, S.; Wu, F.; Zhang, G. The changes in physiological and biochemical traits of Tibetan wild and cultivated barley in response to low phosphorus stress. *Soil Sci. Plant Nutr.* **2014**, *60*, 832–842. [CrossRef]
139. Zou, X.; Wei, D.; Wu, P.; Zhang, Y.; Hu, Y.; Chen, S.; Ma, X. Strategies of organic acid production and exudation in response to low-phosphorus stress in Chinese fir genotypes differing in phosphorus-use efficiencies. *Trees* **2018**, *32*, 897–912. [CrossRef]
140. Lin, Z.-H.; Chen, L.-S.; Chen, R.-B.; Zhang, F.-Z.; Jiang, H.-X.; Tang, N. CO_2 assimilation, ribulose-1,5-bisphosphate carboxylase/oxygenase, carbohydrates and photosynthetic electron transport probed by the JIP-test, of tea leaves in response to phosphorus supply. *BMC Plant Biol.* **2009**, *9*, 43. [CrossRef] [PubMed]
141. Xu, W.; Zhang, Q.; Yuan, W.; Xu, F.; Aslam, M.M.; Miao, R.; Li, Y.; Wang, Q.; Li, X.; Zhang, X.; et al. The genome evolution and low-phosphorus adaptation in white lupin. *Nat. Commun.* **2020**, *11*, 1069. [CrossRef]
142. Li, Z.; Xu, H.; Li, Y.; Wan, X.; Ma, Z.; Cao, J.; Li, Z.; He, F.; Wang, Y.; Wan, L.; et al. Analysis of physiological and miRNA responses to Pi deficiency in alfalfa (*Medicago sativa* L.). *Plant Mol. Biol.* **2018**, *96*, 473–492. [CrossRef] [PubMed]

143. Yang, L.-T.; Zhou, Y.-F.; Wang, Y.-Y.; Wu, Y.-M.; Ye, X.; Guo, J.-X.; Chen, L.-S. Magnesium Deficiency Induced Global Transcriptome Change in *Citrus sinensis* Leaves Revealed by RNA-Seq. *Int. J. Mol. Sci.* **2019**, *20*, 3129. [CrossRef] [PubMed]
144. Tisdale, S.L.; Nelson, W.L. Soil Fertility and Fertilizers. *Soil Sci.* **1966**, *101*, 346. [CrossRef]
145. Denison, R.F.; Kiers, E.T. 11 Sustainable crop nutrition: Constraints and opportunities. In *Plant Nutritional Genomics*; Wiley-Blackwell: Oxford, UK, 2009; p. 242.
146. White, P.J.; Brown, P.H. Plant nutrition for sustainable development and global health. *Ann. Bot.* **2010**, *105*, 1073–1080. [CrossRef]
147. Kobayashi, T.; Maeda, K.; Suzuki, Y.; Nishizawa, N.K. Simultaneous Enhancement of iron Deficiency Tolerance and Iron Accumulation in Rice by Combining the Knockdown of OsHRZ Ubiquitin Ligases with the Introduction of Engineered Ferric-chelate Reductase. *Rice* **2022**, *15*, 54. [CrossRef] [PubMed]
148. Dey, S.; Chowardhara, B.; Regon, P.; Kar, S.; Saha, B.; Panda, S.K. Iron deficiency in blackgram (*Vigna mungo* L.): Redox status and antioxidant activity. *Plant Biosyst.-Int. J. Deal. All Asp. Plant Biol.* **2022**, *156*, 411–426.
149. Riaz, N.; Guerinot, M.L. All together now: Regulation of the iron deficiency response. *J. Exp. Bot.* **2021**, *72*, 2045–2055. [CrossRef] [PubMed]
150. Oh, S.H.; Cho, S.W.; Kwon, T.H.; Yang, M.S. Purification and characterization of phytoferritin. *BMB Rep.* **1996**, *29*, 540–544.
151. Ghazy, N.; El-Nahrawy, S. Siderophore production by *Bacillus subtilis* MF497446 and *Pseudomonas koreensis* MG209738 and their efficacy in controlling *Cephalosporium maydis* in maize plant. *Arch. Microbiol.* **2021**, *203*, 1195–1209. [CrossRef]
152. Sinha, A.K.; Venkateswaran, B.P.; Tripathy, S.C.; Sarkar, A.; Prabhakaran, S. Effects of growth conditions on siderophore producing bacteria and siderophore production from Indian Ocean sector of Southern Ocean. *J. Basic Microbiol.* **2019**, *59*, 412–424. [CrossRef] [PubMed]
153. Subramanium, N.; Sundaram, L. Siderophore producing Pseudomonas spp. isolated from rhizospheric soil and enhancing iron content in *Arachis hypogaea* L. plant. *J. Agric. Technol* **2020**, *16*, 429–442.
154. Singh, P.; Singh, R.K.; Guo, D.-J.; Sharma, A.; Singh, R.N.; Li, D.-P.; Malviya, M.K.; Song, X.-P.; Lakshmanan, P.; Yang, L.-T.; et al. Whole Genome Analysis of Sugarcane Root-Associated Endophyte *Pseudomonas aeruginosa* B18-A Plant Growth-Promoting Bacterium with Antagonistic Potential Against *Sporisorium scitamineum*. *Front. Microbiol.* **2021**, *12*, 628376. [CrossRef]
155. Sah, S.; Singh, R. Siderophore: Structural and functional characterization-a comprehensive review. *Agriculture* **2015**, *6*, 97–114. [CrossRef]
156. Singh, P.; Singh, R.K.; Zhou, Y.; Wang, J.; Jiang, Y.; Shen, N.; Wang, Y.; Yang, L.; Jiang, M. Unlocking the strength of plant growth promoting *Pseudomonas* in improving crop productivity in normal and challenging environments: A review. *J. Plant Interact.* **2022**, *17*, 220–238. [CrossRef]
157. Singh, R.K.; Singh, P.; Guo, D.-J.; Sharma, A.; Li, D.-P.; Li, X.; Verma, K.K.; Malviya, M.K.; Song, X.-P.; Lakshmanan, P.; et al. Root-Derived Endophytic Diazotrophic Bacteria *Pantoea cypripedii* AF1 and *Kosakonia arachidis* EF1 Promote Nitrogen Assimilation and Growth in Sugarcane. *Front. Microbiol.* **2021**, *12*, 774707. [CrossRef] [PubMed]
158. Singh, P.; Singh, R.K.; Li, H.-B.; Guo, D.-J.; Sharma, A.; Lakshmanan, P.; Malviya, M.K.; Song, X.-P.; Solanki, M.K.; Verma, K.K.; et al. Diazotrophic Bacteria *Pantoea dispersa* and *Enterobacter asburiae* Promote Sugarcane Growth by Inducing Nitrogen Uptake and Defense-Related Gene Expression. *Front. Microbiol.* **2021**, *11*, 600417. [CrossRef]
159. Singh, R.K.; Singh, P.; Li, H.B.; Song, Q.Q.; Guo, D.J.; Solanki, M.K.; Verma, K.K.; Malviya, M.K.; Song, X.P.; Lakshmanan, P.; et al. Diversity of nitrogen-fixing rhizobacteria associated with sugarcane, a comprehensive study of plant-microbe interactions for growth enhancement in *Saccharum* spp. *BMC Plant Biol.* **2020**, *20*, 220. [CrossRef] [PubMed]
160. Singh, R.K.; Singh, P.; Li, H.-B.; Guo, D.-J.; Song, Q.-Q.; Yang, L.-T.; Malviya, M.K.; Song, X.-P.; Li, Y.-R. Plant-PGPR interaction study of plant growth-promoting diazotrophs *Kosakonia radicincitans* BA1 and *Stenotrophomonas maltophilia* COA2 to enhance growth and stress-related gene expression in *Saccharum* spp. *J. Plant Interact.* **2020**, *15*, 427–445. [CrossRef]
161. Aznar, A.; Dellagi, A. New insights into the role of siderophores as triggers of plant immunity: What can we learn from animals? *J. Exp. Bot.* **2015**, *66*, 3001–3010. [CrossRef] [PubMed]
162. Saha, M.; Sarkar, S.; Sarkar, B.; Sharma, B.K.; Bhattacharjee, S.; Tribedi, P. Microbial siderophores and their potential applications: A review. *Environ. Sci. Pollut. Res.* **2016**, *23*, 3984–3999. [CrossRef] [PubMed]
163. Ustiatik, R.; Nuraini, Y.; Suharjono, S.; Handayanto, E. Siderophore Production of the Hg-Resistant Endophytic Bacteria Isolated from Local Grass in the Hg-Contaminated Soil. *J. Ecol. Eng.* **2021**, *22*, 129–138. [CrossRef] [PubMed]
164. Hu, Q.P.; Xu, J.U. A simple double layered chrome azurol S agar (SD CASA) plate assay to optimize the production of siderophores by a potential biocontrol agent Bacillus. *Afr. J. Microbiol. Res.* **2011**, *5*, 4321–4327.
165. Singh, R.; Pandey, D.K.; Kumar, A. PGPR isolates from the rhizosphere of vegetable crop *Momordica charantia*: Characterization and application as biofertilizer. *Int. J. Curr. Microbiol. Appl Sci.* **2017**, *6*, 1789–1802.
166. Ahmed, E.; Holmstrom, S.J.M. Siderophores in environmental research: Roles and applications. *Microb. Biotechnol.* **2014**, *7*, 196–208. [CrossRef]
167. Schütze, E.; Ahmed, E.; Voit, A.; Klose, M.; Greyer, M.; Svatoš, A.; Merten, D.; Roth, M.; Holmström, S.J.M.; Kothe, E. Siderophore production by streptomycetes—Stability and alteration of ferrihydroxamates in heavy metal-contaminated soil. *Environ. Sci. Pollut. Res.* **2015**, *22*, 19376–19383. [CrossRef] [PubMed]
168. Lurthy, T.; Cantat, C.; Jeudy, C.; Declerck, P.; Gallardo, K.; Barraud, C.; Leroy, F.; Ourry, A.; Lemanceau, P.; Salon, C.; et al. Impact of Bacterial Siderophores on Iron Status and Ionome in Pea. *Front. Plant Sci.* **2020**, *11*, 730. [CrossRef] [PubMed]

169. Zhang, Y.; Ren, J.; Wang, W.; Chen, B.; Li, E.; Chen, S. Siderophore and indolic acid production by *Paenibacillus triticisoli* BJ-18 and their plant growth-promoting and antimicrobe abilities. *PeerJ* **2020**, *8*, e9403. [CrossRef] [PubMed]
170. Grosse, C.; Brandt, N.; Van Antwerpen, P.; Wintjens, R.; Matthijs, S. Two new siderophores produced by *Pseudomonas* sp. NCIMB 10586: The anti-oomycete non-ribosomal peptide synthetase-dependent mupirochelin and the NRPS-independent triabactin. *Front. Microbiol.* **2023**, *14*, 1143861. [CrossRef] [PubMed]
171. Saharan, B.S.; Chaudhary, T.; Mandal, B.S.; Kumar, D.; Kumar, R.; Sadh, P.K.; Duhan, J.S. Microbe-Plant Interactions Targeting Metal Stress: New Dimensions for Bioremediation Applications. *J. Xenobiotics* **2023**, *13*, 252–269. [CrossRef] [PubMed]
172. Cheng, Y.; Narayanan, M.; Shi, X.; Chen, X.; Li, Z.; Ma, Y. Phosphate-solubilizing bacteria: Their agroecological function and optimistic application for enhancing agro-productivity. *Sci. Total Environ.* **2023**, *901*, 166468. [CrossRef] [PubMed]
173. da Silva, L.I.; Pereira, M.C.; de Carvalho, A.M.X.; Buttrós, V.H.; Pasqual, M.; Dória, J. Phosphorus-Solubilizing Microorganisms: A Key to Sustainable Agriculture. *Agriculture* **2023**, *13*, 462. [CrossRef]
174. Kouas, S.; Labidi, N.; Debez, A.; Abdelly, C. Effect of P on nodule formation and N fixation in bean. *Agron. Sustain. Dev.* **2005**, *25*, 389–393. [CrossRef]
175. Rajguru, B.R.; Bhatt, V.D. Review on Mechanism of Mineral Phosphate Solubilization in Fast-Growing Rhizobia Based on Sugar Utilization. *Plant Breed. Biotechnol.* **2022**, *10*, 203–211. [CrossRef]
176. Kalayu, G. Phosphate Solubilizing Microorganisms: Promising Approach as Biofertilizers. *Int. J. Agron.* **2019**, *2019*, 4917256. [CrossRef]
177. Timofeeva, A.; Galyamova, M.; Sedykh, S. Prospects for Using Phosphate-Solubilizing Microorganisms as Natural Fertilizers in Agriculture. *Plants* **2022**, *11*, 2119. [CrossRef] [PubMed]
178. Sharma, S.B.; Sayyed, R.Z.; Trivedi, M.H.; Gobi, T.A. Phosphate solubilizing microbes: Sustainable approach for managing phosphorus deficiency in agricultural soils. *Springer Plus* **2013**, *2*, 587. [CrossRef] [PubMed]
179. Yu, C.; Wang, Y.; Cao, H.; Zhao, Y.; Li, Z.; Wang, H.; Chen, M.; Tang, Q. Simultaneous Determination of 13 Organic Acids in Liquid Culture Media of Edible Fungi Using High-Performance Liquid Chromatography. *BioMed. Res. Int.* **2020**, *2020*, 2817979. [CrossRef] [PubMed]
180. Djuuna, I.A.F.; Prabawardani, S.; Massora, M. Population Distribution of Phosphate-solubilizing Microorganisms in Agricultural Soil. *Microbes Environ.* **2022**, *37*, ME21041. [CrossRef] [PubMed]
181. Guzmán-Guzmán, P.; Santoyo, G. Action mechanisms, biodiversity, and omics approaches in biocontrol and plant growth-promoting Pseudomonas: An updated review. *Biocontrol. Sci. Technol.* **2022**, *32*, 527–550. [CrossRef]
182. Devi, R.; Kaur, T.; Kour, D.; Yadav, A.; Yadav, A.N.; Suman, A.; Ahluwalia, A.S.; Saxena, A.K. Minerals solubilizing and mobilizing microbiomes: A sustainable approach for managing minerals' deficiency in agricultural soil. *J. Appl. Microbiol.* **2022**, *133*, 1245–1272. [CrossRef] [PubMed]
183. Adnan, M.; Fahad, S.; Saleem, M.H.; Ali, B.; Mussart, M.; Ullah, R.; Jr, A.; Arif, M.; Ahmad, M.; Shah, W.A.; et al. Comparative efficacy of phosphorous supplements with phosphate solubilizing bacteria for optimizing wheat yield in calcareous soils. *Sci. Rep.* **2022**, *12*, 11997. [CrossRef] [PubMed]
184. Liu, X.; Han, R.; Cao, Y.; Turner, B.L.; Ma, L.Q. Enhancing Phytate Availability in Soils and Phytate-P Acquisition by Plants: A Review. *Environ. Sci. Technol.* **2022**, *56*, 9196–9219. [CrossRef]
185. de Freitas Duarte, N.; Paiva, C.A.O.; Pagano, M.C.; Correa, E.J.A. Phosphate solubilization by microorganisms. In *New and Future Developments in Microbial Biotechnology and Bioengineering*; Elsevier: Amsterdam, The Netherlands, 2022; pp. 257–282.
186. Ramamoorthy, P.; Karthikeyan, M.; Nirubana, V. Role of Phosphate Solubilizing Microorganisms in Agriculture. *Agric. Mirror Future India* **2020**, *1*, 104.
187. Mandal, P.; Tiru, Z. Soil application of plant growth promoting fungi for sustainable agriculture in the new decade. In *Plant Stress: Challenges and Management in the New Decade*; Springer Int. Publishing: Cham, Switzerland, 2022; pp. 321–330.
188. Zhao, S.; Zheng, B.-W.; Wang, Y.-C.; He, F.; Wang, L.-J.; Lin, X.; Luo, X.-M.; Feng, J.-X. Environmentally-friendly biorecovery of manganese from electrolytic manganese residue using a novel *Penicillium oxalicum* strain Z6-5-1: Kinetics and mechanism. *J. Hazard. Mater.* **2023**, *446*, 130662. [CrossRef]
189. Aberathna, A.A.A.U.; Satharasinghe, D.A.; Jayasooriya, A.P.; Jinadasa, R.N.; Manopriya, S.; Jayaweera, B.P.A.; Fernando, C.A.N.; Weerathilake, W.A.D.V.; Prathapasinghe, G.A.; Liyanage, J.A.; et al. Increasing the Bioavailability of Phosphate by Using Microorganisms. *Int. J. Agron.* **2022**, *2022*, 4305501. [CrossRef]
190. Aberathna, A.A.U.; Satharasinghe, D.A.; Jayasooriya, A.P.; Jinadasa, H.R.N.; Manopriya, S.; Jayaweera, B.A.; Fernando, C.A.N.; Weerathilake, W.A.D.V.; Prathapasinghe, G.A.; Liyanage, J.A.; et al. Managing Soil and Plant Nutrients: Role of Microbial Phosphate Solubilisation. In *Phosphorus in Soils and Plants*; IntechOpen: London, UK, 2023.
191. Yadav, A.N. Phosphate-Solubilizing Microorganisms for Agricultural Sustainability. *J. Appl. Biol. Biotechnol.* **2022**, *10*, 1–6. [CrossRef]
192. Tian, J.; Ge, F.; Zhang, D.; Deng, S.; Liu, X. Roles of Phosphate Solubilizing Microorganisms from Managing Soil Phosphorus Deficiency to Mediating Biogeochemical P Cycle. *Biology* **2021**, *10*, 158. [CrossRef] [PubMed]
193. Rawat, P.; Das, S.; Shankhdhar, D.; Shankhdhar, S.C. Phosphate-Solubilizing Microorganisms: Mechanism and Their Role in Phosphate Solubilization and Uptake. *J. Soil Sci. Plant Nutr.* **2021**, *21*, 49–68. [CrossRef]
194. Elhaissoufi, W.; Ghoulam, C.; Barakat, A.; Zeroual, Y.; Bargaz, A. Phosphate bacterial solubilization: A key rhizosphere driving force enabling higher P use efficiency and crop productivity. *J. Adv. Res.* **2022**, *38*, 13–28. [CrossRef]

195. Saeed, Q.; Xiukang, W.; Haider, F.U.; Kučerik, J.; Mumtaz, M.Z.; Holatko, J.; Naseem, M.; Kintl, A.; Ejaz, M.; Naveed, M.; et al. Rhizosphere Bacteria in Plant Growth Promotion, Biocontrol, and Bioremediation of Contaminated Sites: A Comprehensive Review of Effects and Mechanisms. *Int. J. Mol. Sci.* **2021**, *22*, 10529. [CrossRef] [PubMed]
196. Elhaissoufi, W.; Khourchi, S.; Ibnyasser, A.; Ghoulam, C.; Rchiad, Z.; Zeroual, Y.; Lyamlouli, K.; Bargaz, A. Phosphate Solubilizing Rhizobacteria Could Have a Stronger Influence on Wheat Root Traits and Aboveground Physiology Than Rhizosphere P Solubilization. *Front. Plant Sci.* **2020**, *11*, 979. [CrossRef] [PubMed]
197. Alori, E.T.; Glick, B.R.; Babalola, O.O. Microbial Phosphorus Solubilization and Its Potential for Use in Sustainable Agriculture. *Front. Microbiol.* **2017**, *8*, 971. [CrossRef]
198. Bargaz, A.; Elhaissoufi, W.; Khourchi, S.; Benmrid, B.; Borden, K.A.; Rchiad, Z. Benefits of phosphate solubilizing bacteria on belowground crop performance for improved crop acquisition of phosphorus. *Microbiol. Res.* **2021**, *252*, 126842. [CrossRef]
199. Kumar, S.; Diksha; Sindhu, S.S.; Kumar, R. Biofertilizers: An ecofriendly technology for nutrient recycling and environmental sustainability. *Curr. Res. Microb. Sci.* **2021**, *3*, 100094. [CrossRef]
200. Crichton, R.R.; Wilmet, S.; Legssyer, R.; Ward, R.J. Molecular and cellular mechanisms of iron homeostasis and toxicity in mammalian cells. *J. Inorg. Biochem.* **2002**, *91*, 9–18. [CrossRef] [PubMed]
201. Taiz, L.; Zeiger, E. *Plant Physiology*; The Benyamin. Cummings: Redwood City, CA, USA, 1991.
202. Tognetti, V.B.; Zurbriggen, M.D.; Morandi, E.N.; Fillat, M.F.; Valle, E.M.; Hajirezaei, M.-R.; Carrillo, N. Enhanced plant tolerance to iron starvation by functional substitution of chloroplast ferredoxin with a bacterial flavodoxin. *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 11495–11500. [CrossRef] [PubMed]
203. Bienfait, H.F.; Van der Mark, F. Phytoferritin and its role in iron metabolism. In *Metals and Micronutrients. Uptake and Utilization by Plants*; Academic Press: Cambridge, MA, USA, 1983; pp. 111–123.
204. Kobayashi, T.; Nakayama, Y.; Itai, R.N.; Nakanishi, H.; Yoshihara, T.; Mori, S.; Nishizawa, N.K. Identification of novel cis-acting elements, IDE1 and IDE2, of the barley IDS2 gene promoter conferring iron-deficiency-inducible, root-specific expression in heterogeneous tobacco plants. *Plant J.* **2003**, *36*, 780–793. [CrossRef] [PubMed]
205. Takagi, D.; Miyagi, A.; Tazoe, Y.; Suganami, M.; Kawai-Yamada, M.; Ueda, A.; Suzuki, Y.; Noguchi, K.; Hirotsu, N.; Makino, A. Phosphorus toxicity disrupts Rubisco activation and reactive oxygen species defence systems by phytic acid accumulation in leaves. *Plant Cell Environ.* **2020**, *43*, 2033–2053. [CrossRef] [PubMed]
206. Gikaara, D.M.; Johnston, M.E.; Edwards, D.G. Phosphorus management of Australian native plants. In *V International Symposium on New Floricultural Crops*; International Society for Horticultural Science: Leuven, Belgium, 2003; Volume 683, pp. 133–140.
207. Verma, L.; Pandey, N. The effect of Fe toxicity on seed germination and early seedling growth of green gram (*Vigna radiata* L. Wilczek). *Int. J. Sci. Res.* **2017**, *6*, 1427–1430.
208. Reis, S.; Pavia, I.; Carvalho, A.; Moutinho-Pereira, J.; Correia, C.; LimaBrito, J. Seed priming with Fe and zinc in bread wheat: Effects in germination, mitosis and grain yield. *Protoplasma* **2018**, *255*, 1179–1194. [CrossRef] [PubMed]
209. Ifie, J.E.; Ifie-Etumah, S.O.; Ikhajiagbe, B. Physiological and biochemical responses of selected cowpea (*Vigna unguiculata* (L.) Walp.) accessions to Fe toxicity. *Acta Agric. Slov.* **2020**, *115*, 25–38. [CrossRef]
210. El Rasafi, T.; Nouri, M.; Bouda, S.; Haddioui, A. The Effect of Cd, Zn and Fe on Seed Germination and Early Seedling Growth of Wheat and Bean. *Ekologia* **2016**, *35*, 213–223. [CrossRef]
211. Nozoe, T.; Agbisit, R.; Fukuta, Y.; Rodriguez, R.; Yanagihara, S. Characteristics of Fe tolerant rice lines developed at IRRI under field conditions. *Jpn. Agric. Res. Q.* **2008**, *42*, 187–192. [CrossRef]
212. Rodrigues Filho, J.; Corte, V.B.; Perin, I.T.; dos Santos, C.R.; da Silva, R.W. Effects of Fe toxicity on germination and initial growth of *Carica papaya* L. *Sci. Plena* **2020**, *16*, 101201. [CrossRef]
213. Peña-Olmos, J.E.; Casierra-Posada, F.; Olmos-Cubides, M.A. The effect of high Fe doses (Fe^{2+}) on the growth of broccoli plants (*Brassica oleracea* var. italica). *Agron. Colomb.* **2014**, *32*, 22–28. [CrossRef]
214. Becker, M.; Asch, F. Iron toxicity in rice—Conditions and management concepts. *J. Plant Nutr. Soil Sci.* **2005**, *168*, 558–573. [CrossRef]
215. dos Santos, M.S.; Sanglard, L.M.; Barbosa, M.L.; Namorato, F.A.; de Melo, D.C.; Franco, W.C.; Pérez-Molina, J.P.; Martins, S.C.V.; DaMatta, F.M. Silicon nutrition mitigates the negative impacts of Fe toxicity on rice photosynthesis and grain yield. *Ecotoxicol. Environ. Saf.* **2020**, *189*, 110008. [CrossRef] [PubMed]
216. Tadaiesky, L.B.A.; da Silva, B.R.S.; Batista, B.L.; Lobato, A.K.D.S. Brassinosteroids trigger tolerance to iron toxicity in rice. *Physiol. Plant.* **2020**, *171*, 371–387. [CrossRef] [PubMed]
217. Onyango, D.A.; Entila, F.; Dida, M.M.; Ismail, A.M.; Drame, K.N. Mechanistic understanding of Fe toxicity tolerance in contrasting rice varieties from Africa: 1. Morpho-physiological and biochemical responses. *Funct. Plant Biol.* **2019**, *46*, 93–105. [CrossRef] [PubMed]
218. Sahrawat, K.L. Reducing Fe toxicity in lowland rice with tolerant genotypes and plant nutrition. *Plant Stress* **2010**, *4*, 70–75.
219. Mengel, K.; Kirkby, E. *Principles of Plant Nutrition*; International Potash Institute: Bern, Switzerland, 1987; pp. 687–695.
220. Marschner, H. *Marschner's Mineral Nutrition of Higher Plants*; Academic Press: Amsterdam, The Netherlands, 2011; pp. 7–44.
221. Audebert, A. Iron toxicity in rice: Environmental conditions and symptoms. In *Iron Toxicity in Rice-Based Systems in West Africa*; Audebert, A., Narteh, L.T., Kiepe, P., Millar, D., Beks, B., Eds.; WARDA [Africa Rice Center]: Cotonou, Benin, 2006; pp. 18–33.
222. Olaleye, A.; Ogunkunle, A.; Singh, B.; Akinbola, G.; Tabi, F.; Fayinminu, O.; Iji, M.E. Ratios of nutrients in lowland rice grown on two Fe toxic soils in Nigeria. *J. Plant Nutr.* **2009**, *32*, 1336–1352. [CrossRef]

223. Jones, J.B., Jr. Phosphorus toxicity in tomato plants: When and how does it occur? *Commun. Soil Sci. Plant Anal.* **1998**, *29*, 1779–1784. [CrossRef]
224. Dixit, R.K.; Thomas, M.B.; Farooqui, U.M.; Patra, A.K.; Spurway, M.I. Phosphorus response and toxicity in *Acacia* spp. *Indian For.* **1999**, *125*, 770–774.
225. Sahrawat, K.L. Iron Toxicity in Wetland Rice and the Role of Other Nutrients. *J. Plant Nutr.* **2005**, *27*, 1471–1504. [CrossRef]
226. Majerus, V.; Bertin, P.; Lutts, S. Effects of iron toxicity on osmotic potential, osmolytes and polyamines concentrations in the African rice (*Oryza glaberrima* Steud.). *Plant Sci.* **2007**, *173*, 96–105. [CrossRef]
227. Olaleye, A.; Ogunkunle, A. Management of two potentially Fe toxic benchmark wetlands using integrated approach for rice production in Nigeria. *J. Agron. Crop Sci.* **2008**, *194*, 237–243. [CrossRef]
228. Mehraban, P.; Zadeh, A.A.; Sadeghipour, H.R. Iron toxicity in rice (*Oryza sativa* L.), under different potassium nutrition. *Asian J. Plant Sci.* **2008**, *7*, 251–259. [CrossRef]
229. Küpper, H.; Andresen, E. Mechanisms of metal toxicity in plants. *Metallomics* **2016**, *8*, 269–285. [CrossRef] [PubMed]
230. Nichols, D.G.; Beardsell, D.V. Interactions of calcium, nitrogen and potassium with phosphorus on the symptoms of toxicity in Grevillea cv. 'Poorinda Firebird'. *Plant Soil* **1981**, *61*, 437–445. [CrossRef]
231. Fageria, N.K.; Moreira, A. The role of mineral nutrition on root growth of crop plants. In *Advances in Agronomy*; Elsevier: Amsterdam, The Netherlands, 2011; Volume 110, pp. 251–331.
232. Zheng, L.; Huang, F.; Narsai, R.; Wu, J.; Giraud, E.; He, F.; Cheng, L.; Wang, F.; Wu, P.; Whelan, J.; et al. Physiological and Transcriptome Analysis of Iron and Phosphorus Interaction in Rice Seedlings. *Plant Physiol.* **2009**, *151*, 262–274. [CrossRef] [PubMed]
233. Briat, J.-F.; Dubos, C.; Gaymard, F. Iron nutrition, biomass production, and plant product quality. *Trends Plant Sci.* **2015**, *20*, 33–40. [CrossRef] [PubMed]
234. Rai, V.; Sanagala, R.; Sinilal, B.; Yadav, S.; Sarkar, A.K.; Dantu, P.K.; Jain, A. Iron Availability Affects Phosphate Deficiency-Mediated Responses, and Evidence of Cross-Talk with Auxin and Zinc in Arabidopsis. *Plant Cell Physiol.* **2015**, *56*, 1107–1123. [CrossRef] [PubMed]
235. Ward, J.T.; Lahner, B.; Yakubova, E.; Salt, D.E.; Raghothama, K.G. The Effect of Iron on the Primary Root Elongation of Arabidopsis during Phosphate Deficiency. *Plant Physiol.* **2008**, *147*, 1181–1191. [CrossRef]
236. Jain, A.; Sinilal, B.; Dhandapani, G.; Meagher, R.B.; Sahi, S.V. Effects of Deficiency and Excess of Zinc on Morphophysiological Traits and Spatiotemporal Regulation of Zinc-Responsive Genes Reveal Incidence of Cross Talk between Micro- and Macronutrients. *Environ. Sci. Technol.* **2013**, *47*, 5327–5335. [CrossRef]
237. Ferrol, N.; Tamayo, E.; Vargas, P. The heavy metal paradox in arbuscular mycorrhizas: From mechanisms to biotechnological applications. *J. Exp. Bot.* **2016**, *67*, 6253–6265. [CrossRef] [PubMed]
238. Hoseinzade, H.; Ardakani, M.R.; Shahdi, A.; Rahmani, H.A.; Noormohammadi, G.; Miransari, M. Rice (*Oryza sativa* L.) nutrient management using mycorrhizal fungi and endophytic *Herbaspirillum seropedicae*. *J. Integr. Agric.* **2016**, *15*, 1385–1394. [CrossRef]
239. Nafady, N.A.; Elgharably, A. Mycorrhizal symbiosis and phosphorus fertilization effects on *Zea mays* growth and heavy metals uptake. *Int. J. Phytoremediation* **2018**, *20*, 869–875. [CrossRef] [PubMed]
240. Azcón, R.; Ambrosano, E.; Charest, C. Nutrient acquisition in mycorrhizal lettuce plants under different phosphorus and nitrogen concentration. *Plant Sci.* **2003**, *165*, 1137–1145. [CrossRef]
241. Hirsch, J.; Marin, E.; Floriani, M.; Chiarenza, S.; Richaud, P.; Nussaume, L.; Thibaud, M. Phosphate deficiency promotes modification of iron distribution in Arabidopsis plants. *Biochimie* **2006**, *88*, 1767–1771. [CrossRef] [PubMed]
242. Delhaize, E.; Ma, J.F.; Ryan, P.R. Transcriptional regulation of aluminium tolerance genes. *Trends Plant Sci.* **2012**, *17*, 341–348. [CrossRef] [PubMed]
243. de Bang, T.C.; Husted, S.; Laursen, K.H.; Persson, D.P.; Schjoerring, J.K. The molecular–physiological functions of mineral macronutrients and their consequences for deficiency symptoms in plants. *New Phytol.* **2021**, *229*, 2446–2469. [CrossRef] [PubMed]
244. Cumbus, I.P.; Hornsey, D.J.; Robinson, L.W. The influence of phosphorus, zinc and manganese on absorption and translocation of iron in watercress. *Plant Soil* **1977**, *48*, 651–660. [CrossRef]
245. Mathan, K.K.; Amberger, A. Influence of iron on the uptake of phosphorus by maize. *Plant Soil* **1977**, *46*, 413–422. [CrossRef]
246. Lanquar, V.; Lelièvre, F.; Bolte, S.; Hamès, C.; Alcon, C.; Neumann, D.; Vansuyt, G.; Curie, C.; Schröder, A.; Krämer, U.; et al. Mobilization of vacuolar iron by AtNRAMP3 and AtNRAMP4 is essential for seed germination on low iron. *EMBO J.* **2005**, *24*, 4041–4051. [CrossRef]
247. Misson, J.; Raghothama, K.G.; Jain, A.; Jouhet, J.; Block, M.A.; Bligny, R.; Ortet, P.; Creff, A.; Somerville, S.; Rolland, N.; et al. A genome-wide transcriptional analysis using *Arabidopsis thaliana* Affymetrix gene chips determined plant responses to phosphate deprivation. *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 11934–11939. [CrossRef] [PubMed]
248. Thibaud, M.-C.; Arrighi, J.-F.; Bayle, V.; Chiarenza, S.; Creff, A.; Bustos, R.; Paz-Ares, J.; Poirier, Y.; Nussaume, L. Dissection of local and systemic transcriptional responses to phosphate starvation in Arabidopsis. *Plant J.* **2010**, *64*, 775–789. [CrossRef] [PubMed]
249. Bournier, M.; Tissot, N.; Mari, S.; Boucherez, J.; Lacombe, E.; Briat, J.-F.; Gaymard, F. Arabidopsis Ferritin 1 (AtFer1) Gene Regulation by the Phosphate Starvation Response 1 (AtPHR1) Transcription Factor Reveals a Direct Molecular Link between Iron and Phosphate Homeostasis. *J. Biol. Chem.* **2013**, *288*, 22670–22680. [CrossRef] [PubMed]

250. Bustos, R.; Castrillo, G.; Linhares, F.; Puga, M.I.; Rubio, V.; Pérez-Pérez, J.; Solano, R.; Leyva, A.; Paz-Ares, J. A Central Regulatory System Largely Controls Transcriptional Activation and Repression Responses to Phosphate Starvation in Arabidopsis. *PLoS Genet.* **2010**, *6*, e1001102. [CrossRef] [PubMed]
251. Godon, C.; Mercier, C.; Wang, X.; David, P.; Richaud, P.; Nussaume, L.; Liu, D.; Desnos, T. Under phosphate starvation conditions, Fe and Al trigger accumulation of the transcription factor STOP1 in the nucleus of Arabidopsis root cells. *Plant J.* **2019**, *99*, 937–949. [CrossRef] [PubMed]
252. Hanikenne, M.; Esteves, S.M.; Fanara, S.; Rouached, H. Coordinated homeostasis of essential mineral nutrients: A focus on iron. *J. Exp. Bot.* **2021**, *72*, 2136–2153. [CrossRef] [PubMed]
253. Chen, S.-Y.; Gu, T.-Y.; Qi, Z.-A.; Yan, J.; Fang, Z.-J.; Lu, Y.-T.; Li, H.; Gong, J.-M. Two NPF transporters mediate iron long-distance transport and homeostasis in Arabidopsis. *Plant Commun.* **2021**, *2*, 100244. [CrossRef] [PubMed]
254. Cai, Y.; Li, Y.; Liang, G. FIT and bHLH Ib transcription factors modulate iron and copper crosstalk in Arabidopsis. *Plant Cell Environ.* **2021**, *44*, 1679–1691. [CrossRef] [PubMed]
255. Eyster, H.C. Auxin action. *Science* **1943**, *97*, 358–359. [CrossRef]
256. Phinney, B.O. Growth Response of single-gene dwarf mutants in maize to gibberellic acid. *Proc. Natl. Acad. Sci. USA* **1956**, *42*, 185–189. [CrossRef]
257. Braun, P.; Wild, A. The Influence of Brassinosteroid on Growth and Parameters of Photosynthesis of Wheat and Mustard Plants. *J. Plant Physiol.* **1984**, *116*, 189–196. [CrossRef] [PubMed]
258. Nakashita, H.; Yasuda, M.; Nitta, T.; Asami, T.; Fujioka, S.; Arai, Y.; Sekimata, K.; Takatsuto, S.; Yamaguchi, I.; Yoshida, S. Brassinosteroid functions in a broad range of disease resistance in tobacco and rice. *Plant J.* **2003**, *33*, 887–898. [CrossRef] [PubMed]
259. Walters, D.R.; McRoberts, N. Plants and biotrophs: A pivotal role for cytokinins? *Trends Plant Sci.* **2006**, *11*, 581–586. [CrossRef] [PubMed]
260. Kazan, K.; Manners, J.M. Linking development to defense: Auxin in plant–pathogen interactions. *Trends Plant Sci.* **2009**, *14*, 373–382. [CrossRef] [PubMed]
261. Penninckx, I.A.; Eggermont, K.; Terras, F.R.; Thomma, B.P.; De Samblanx, G.W.; Buchala, A.; Métraux, J.P.; Manners, J.M.; Broekaert, W.F. Pathogen-induced systemic activation of a plant defensin gene in Arabidopsis follows a salicylic acid-independent pathway. *Plant Cell* **1996**, *8*, 2309–2323. [PubMed]
262. Gaffney, T.; Friedrich, L.; Vernooij, B.; Negrotto, D.; Nye, G.; Uknes, S.; Ward, E.; Kessmann, H.; Ryals, J. Requirement of Salicylic Acid for the Induction of Systemic Acquired Resistance. *Science* **1993**, *261*, 754–756. [CrossRef] [PubMed]
263. Ton, J.; Flors, V.; Mauch-Mani, B. The multifaceted role of ABA in disease resistance. *Trends Plant Sci.* **2009**, *14*, 310–317. [CrossRef] [PubMed]
264. Landsberg, E.C. Fe stress induced transfer cell formation: Regulated by auxin. *Plant Physiol.* **1981**, *67*, 563.
265. Schmidt, W.; Tittel, J.; Schikora, A. Role of Hormones in the Induction of Iron Deficiency Responses in Arabidopsis Roots. *Plant Physiol.* **2000**, *122*, 1109–1118. [CrossRef]
266. Landsberg, E.C. Hormonal regulation of iron-stress response in sunflower roots: A morphological and cytological investigation. *Protoplasma* **1996**, *194*, 69–80. [CrossRef]
267. Bates, T.R.; Lynch, J.P. Stimulation of root hair elongation in *Arabidopsis thaliana* by low phosphorus availability. *Plant Cell Environ.* **1996**, *19*, 529–538. [CrossRef]
268. Chen, W.W.; Yang, J.L.; Qin, C.; Jin, C.W.; Mo, J.H.; Ye, T.; Zheng, S.J. Nitric Oxide Acts Downstream of Auxin to Trigger Root Ferric-Chelate Reductase Activity in Response to Iron Deficiency in Arabidopsis. *Plant Physiol.* **2010**, *154*, 810–819. [CrossRef]
269. Romera, F.J.; Alcantara, E.; DE LA Guardia, M.D. Ethylene production by Fe-deficient roots and its involvement in the regulation of Fe-deficiency stress responses by strategy I plants. *Ann. Bot.* **1999**, *83*, 51–55. [CrossRef]
270. Lucena, C.; Waters, B.M.; Romera, F.J.; García, M.J.; Morales, M.; Alcántara, E.; Pérez-Vicente, R. Ethylene could influence ferric reductase, iron transporter, and H+-ATPase gene expression by affecting FER (or FER-like) gene activity. *J. Exp. Bot.* **2006**, *57*, 4145–4154. [CrossRef] [PubMed]
271. García, M.J.; Lucena, C.; Romera, F.J.; Alcántara, E.; Pérez-Vicente, R. Ethylene and nitric oxide involvement in the up-regulation of key genes related to iron acquisition and homeostasis in Arabidopsis. *J. Exp. Bot.* **2010**, *61*, 3885–3899. [CrossRef] [PubMed]
272. Maurer, F.; Müller, S.; Bauer, P. Suppression of Fe deficiency gene expression by jasmonate. *Plant Physiol. Biochem.* **2011**, *49*, 530–536. [CrossRef]
273. Montejano-Ramírez, V.; García-Pineda, E.; Valencia-Cantero, E. Bacterial compound N, N-dimethylhexadecylamine modulates expression of iron deficiency and defense response genes in *Medicago truncatula* independently of the jasmonic acid pathway. *Plants* **2020**, *9*, 624. [CrossRef]
274. Kang, H.G.; Foley, R.C.; Oñate-Sánchez, L.; Lin, C.; Singh, K.B. Target genes for OBP3, a Dof transcription factor, include novel basic helix-loop-helix domain proteins inducible by salicylic acid. *Plant J.* **2003**, *35*, 362–372. [CrossRef] [PubMed]
275. Kong, J.; Dong, Y.; Xu, L.; Liu, S.; Bai, X. Effects of Exogenous Salicylic Acid on Alleviating Chlorosis Induced by Iron Deficiency in Peanut Seedlings (*Arachis hypogaea* L.). *J. Plant Growth Regul.* **2014**, *33*, 715–729. [CrossRef]
276. García, M.J.; Suárez, V.; Romera, F.J.; Alcántara, E.; Pérez-Vicente, R. A new model involving ethylene, nitric oxide and Fe to explain the regulation of Fe-acquisition genes in Strategy I plants. *Plant Physiol. Biochem.* **2011**, *49*, 537–544. [CrossRef]

277. Zhu, X.F.; Wang, B.; Song, W.F.; Zheng, S.J.; Shen, R.F. Putrescine Alleviates Iron Deficiency via NO-Dependent Reutilization of Root Cell-Wall Fe in Arabidopsis. *Plant Physiol.* **2016**, *170*, 558–567. [CrossRef] [PubMed]
278. Curie, C.; Mari, S. New routes for plant iron mining. *New Phytol.* **2017**, *214*, 521–525. [CrossRef] [PubMed]
279. Lei, M.; Zhu, C.; Liu, Y.; Karthikeyan, A.S.; Bressan, R.A.; Raghothama, K.G.; Liu, D. Ethylene signalling is involved in regulation of phosphate starvation-induced gene expression and production of acid phosphatases and anthocyanin in *Arabidopsis*. *New Phytol.* **2011**, *189*, 1084–1095. [CrossRef] [PubMed]
280. Nagarajan, V.K.; Smith, A.P. Ethylene's role in phosphate starvation signaling: More than just a root growth regulator. *Plant Cell Physiol.* **2012**, *53*, 277–286. [CrossRef] [PubMed]
281. Nagarajan, V.K.; Jain, A.; Poling, M.D.; Lewis, A.J.; Raghothama, K.G.; Smith, A.P. Arabidopsis Pht1;5 Mobilizes Phosphate between Source and Sink Organs and Influences the Interaction between Phosphate Homeostasis and Ethylene Signaling. *Plant Physiol.* **2011**, *156*, 1149–1163. [CrossRef] [PubMed]
282. Li, Y.-S.; Gao, Y.; Tian, Q.-Y.; Shi, F.-L.; Li, L.-H.; Zhang, W.-H. Stimulation of root acid phosphatase by phosphorus deficiency is regulated by ethylene in *Medicago falcata*. *Environ. Exp. Bot.* **2011**, *71*, 114–120. [CrossRef]
283. García, M.J.; Romera, F.J.; Lucena, C.; Alcántara, E.; Pérez-Vicente, R. Ethylene and the Regulation of Physiological and Morphological Responses to Nutrient Deficiencies. *Plant Physiol.* **2015**, *169*, 51–60. [CrossRef] [PubMed]
284. Horgan, J.M.; Wareing, P.F. Cytokinins and the growth responses of seedlings of *Betula pendula* Roth. and *Acer pseudoplatanus* L. to nitrogen and phosphorus deficiency. *J. Exp. Bot.* **1980**, *31*, 525–532. [CrossRef]
285. Martín, A.C.; Del Pozo, J.C.; Iglesias, J.; Rubio, V.; Solano, R.; De La Peña, A.; Leyva, A.; Paz-Ares, J. Influence of cytokinins on the expression of phosphate starvation responsive genes in *Arabidopsis*. *Plant J.* **2000**, *24*, 559–567. [CrossRef]
286. Séguéla, M.; Briat, J.; Vert, G.; Curie, C. Cytokinins negatively regulate the root iron uptake machinery in Arabidopsis through a growth-dependent pathway. *Plant J.* **2008**, *55*, 289–300. [CrossRef]

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Review

Single-Cell Sequencing Technology in Ruminant Livestock: Challenges and Opportunities

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Abstract: Advancements in single-cell sequencing have transformed the genomics field by allowing researchers to delve into the intricate cellular heterogeneity within tissues at greater resolution. While single-cell omics are more widely applied in model organisms and humans, their use in livestock species is just beginning. Studies in cattle, sheep, and goats have already leveraged single-cell and single-nuclei RNA-seq as well as single-cell and single-nuclei ATAC-seq to delineate cellular diversity in tissues, track changes in cell populations and gene expression over developmental stages, and characterize immune cell populations important for disease resistance and resilience. Although challenges exist for the use of this technology in ruminant livestock, such as the precise annotation of unique cell populations and spatial resolution of cells within a tissue, there is vast potential to enhance our understanding of the cellular and molecular mechanisms underpinning traits essential for healthy and productive livestock. This review intends to highlight the insights gained from published single-cell omics studies in cattle, sheep, and goats, particularly those with publicly accessible data. Further, this manuscript will discuss the challenges and opportunities of this technology in ruminant livestock and how it may contribute to enhanced profitability and sustainability of animal agriculture in the future.

Keywords: single-cell RNA-seq; single-cell ATAC-seq; cattle; sheep; goat

1. Introduction

Single-cell sequencing technologies have revolutionized the field of genomics, enabling researchers to explore the cellular heterogeneity within tissues at greater resolution [1–3]. Among these technologies, single-cell and single-nuclei RNA sequencing (sc/snRNA-seq) and single-cell and single-nuclei assays for transposase-accessible chromatin sequencing (sc/snATAC-seq) have emerged as powerful tools for dissecting the transcriptomic and chromatin landscapes of individual cells [3–5]. While these techniques have been more extensively applied across studies in model organisms and humans, their adoption in livestock species is just beginning. Several studies in cattle, sheep, and goats have utilized sc/snRNA-seq and sc/snATAC-seq to characterize cellular heterogeneity within tissues, track cell population and gene expression changes across developmental time, and profile immune cell populations essential for disease resistance and resilience (Tables 1–3) [6–39]. There is immense potential to further utilize these technologies to advance the understanding of cellular and molecular mechanisms governing traits essential for more productive and sustainable livestock while supporting the welfare of these species.

Ruminant livestock including cattle, sheep, and goats play a critical role in global food production; and as the global population is poised to reach 9.7 billion by 2050, the demand for animal products is also increasing [40,41]. Ruminants provide a unique opportunity to convert human-inedible plant material to nutrient-rich foods [40,41]. In order to accomplish the goal of feeding a growing population with fewer animals while using less land and water, ruminant livestock must undergo intense selection aimed at improving traits such as

meat quality, milk production, disease resistance, and reproduction [40,41]. Understanding the genetic and biological factors that contribute to these traits at single-cell resolution can provide valuable insights into the genetic and epigenetic factors that drive phenotypic variability [42,43]. Furthermore, the application of single-cell genomics in livestock research has the potential to accelerate the pace of genetic improvement by facilitating the identification of cell-type-specific gene expression, along with gene regulatory elements and networks associated with various traits important to livestock production [6–39].

Table 1. Single-cell and single-nuclei RNA and ATAC sequencing studies published with publicly available data for cattle.

Tissue(s) or Cell Type(s)	Isolation Type	Data Type	Database and Accession *	Reference
Abomasum, ileum, liver, mammary gland, omasum, PBMC *, rectum, reticulum, rumen, salivary gland	Cell	RNA	GEO GSE176512	[6]
Adipose	Nuclei	RNA	GEO GSE211707	[7]
Embryo (8- and 16-cell stage)	Cell	RNA	GEO GSE99210	[8]
Embryo (peri-implantation)	Cell	RNA	GEO GSE234335	[9]
Embryo (SCNT)	Cell	RNA	SRA PRJNA727165	[10]
Embryo (trophectoderm)	Cell	RNA	GEO GSE200216	[11]
Fetal Gonads	Cell	RNA	GEO GSE162952	[12]
Milk Somatic Cells	Cell	RNA	ENA PRJEB73560	[13]
Muscle	Cell	RNA	GEO GSE205347	[14]
Muscle	Cell	RNA, ATAC	GSA CRA006626	[15]
PBMC *	Cell	RNA, ATAC	GEO GSE225962	[16]
PBMC *	Cell	RNA, ATAC	GEO GSE166473	[17]
Placenta (developing)	Cell	RNA	GEO GSE234524	[18]
Placenta (mature)	Nuclei	RNA	GEO GSE214407	[19]
Primary Mammary Epithelial Cells	Cell	RNA	FAANG PRJEB41576	[20]
Ruminal epithelial cells	Cell	RNA	GEO GSE166473	[21]
Satellite cells	Cell	RNA	GEO GSE184128	[22]
Satellite cells	Cell	RNA	GEO GSE211428	[23]

* Abbreviations: PBMC (peripheral blood mononuclear cells), GEO (Gene Expression Omnibus), SRA (Sequence Read Archive), ENA (European Nucleotide Archive), GSA (Genome Sequence Archive), FAANG (Functional Annotation of Animal Genomes).

Despite the potential benefits, the application of sc/snRNA-seq and sc/snATAC-seq in ruminant livestock species presents challenges. Standardized computational analysis pipelines that are species-agnostic are essential, and the discovery and annotation of cell populations can be difficult in non-model organisms and for tissues such as the rumen, which are unique to these species [44–46]. Further limitations of single-cell sequencing include the lack of spatial resolution of cells within a tissue structure and the cost of library preparation [44–46]. However, ongoing efforts to improve sequencing protocols, genomic tools and resources, and computational pipelines in livestock are poised to overcome these barriers and pave the way for further use of single-cell sequencing technology in agriculturally important species [47–50]. This review aims to highlight the findings of published sc/snRNA-seq and sc/snATAC-seq studies in cattle, sheep, and goats that have publicly available data and discuss their impact on the field's understanding of cellular

and molecular mechanisms governing important traits within ruminant livestock. This manuscript will also explore the emerging trends and future directions in the field and how these will contribute to the continued scientific advancement in animal agriculture.

Table 2. Single-cell and single-nuclei RNA and ATAC sequencing studies published with publicly available data for sheep.

Tissue(s) or Cell Type(s)	Isolation Type	Data Type	Database and Accession *	Reference
Conceptus	Cell	RNA	NCBI PRJNA987334	[24]
Embryo	Cell	RNA	GEO GSE185233	[25]
Ovary	Cell	RNA	GEO GSE233801	[26]
Rumen	Cell	RNA	GSA CRA007511	[27]
Rumen	Cell	RNA	NCBI PRJNA919098	[28]
Skin	Cell	RNA	GEO GSE186204	[29]
Testes	Cell	RNA	GEO GSE184343	[30]
Testes	Cell	RNA	GSA CRA005236	[31]

* Abbreviations: NCBI (National Center for Biotechnology Information), GEO (Gene Expression Omnibus), GSA (Genome Sequence Archive).

Table 3. Single-cell and single-nuclei RNA and ATAC sequencing studies published with publicly available data for goats.

Tissue(s) or Cell Type(s)	Isolation Type	Data Type	Database and Accession *	Reference
Fetal Gonad	Cell	RNA, ATAC	GSA CRA006304, CRA006365	[32]
Lung	Nuclei	RNA	GEO GSE183300	[33]
Ovarian Follicle	Cell	RNA	GEO GSE135688	[34]
Ovary	Cell	RNA	NCBI PRJNA1010653	[35]
Rumen	Cell	RNA	NCBI PRJNA919098	[28]
Skin	Cell	RNA	GEO GSE144351	[36]
Skin	Cell	RNA	GEO GSE182474	[37]
Skin	Cell	RNA	GEO GSE141284	[38]
Testes	Cell	RNA	GEO GSE234407	[39]

* Abbreviations: NCBI (National Center for Biotechnology Information), GEO (Gene Expression Omnibus), GSA (Genome Sequence Archive).

2. Brief Overview of Single-Cell and Single-Nuclei mRNA and ATAC Sequencing

Single-cell sequencing, including sc/snRNA-seq and sc/snATAC-seq, is a powerful tool that provides insights into cellular heterogeneity, gene expression profiles across cell types, and regulatory networks. The most commonly used platform for single-cell sequencing in ruminant livestock is currently 10× Genomics Chromium [6–39]. The 10× Genomics Chromium workflow for sc/snRNA-seq broadly entails cell or nuclei isolation, encapsulation of cells or nuclei in oil droplets with barcoded beads using microfluidics, cell and/or nuclei lysis, and reverse transcription of mRNA while incorporating unique barcodes, adapter ligation, and sequencing (Figure 1) [51].

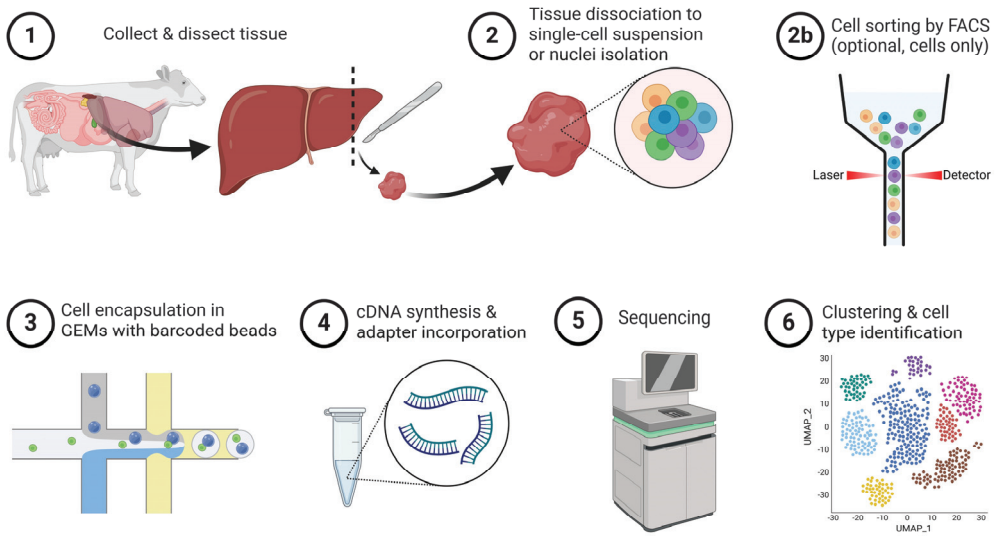


Figure 1. Schematic of single-cell and single-nuclei RNA sequencing workflow (created with BioRender). Abbreviations: FACS (fluorescence-activated cell sorting) and GEMs (gel bead-in emulsions).

2.1. Cell Isolation, Library Preparation, and Sequencing

Cells are first dissociated from whole tissue and can be sorted using fluorescence-activated cell sorting (FACS) to enrich for specific cell types or subset only live cells to be carried forward into sc/snRNA-seq or sc/snATAC-seq library preparation and sequencing [52]. Tissue can also be dissected and snap frozen for nuclei isolation at a later time, which can be advantageous for tissues that can be damaged by enzymatic digestion, are difficult to dissociate into a single-cell suspension such as striated muscle or adipose tissues [53–55] or are multinucleated and could be mistakenly filtered out in downstream analyses [19]. Cells or nuclei are then encapsulated in an oil droplet with a uniquely barcoded bead (GEMs; gel in bead emulsions) [51]. For sc/snRNA-seq, cells or nuclei are lysed, and mRNA is reverse-transcribed into complementary DNA (cDNA) while incorporating a unique molecular identifier (UMI) [51]. Sequencing adapters are then incorporated, and libraries are sequenced on either a short- or long-read platform (most commonly short-read) [51]. The sc/snATAC-seq workflow is similar to sc/snRNA-seq and begins with tissue dissociation to single-cell suspension or nuclei isolation from snap-frozen tissue [56–58]. Encapsulation with barcoded beads in oil (GEMs) is followed by cell/nuclei lysis and the addition of the Tn5 transposase to fragment-accessible chromatin regions [58]. Sequencing can be performed on many different platforms, but short-read platforms are most commonly used [51]. Multiomic profiling of snRNA-seq and snATAC-seq is also becoming more popular, which provides insight into both the gene expression and chromatin landscape within the same nucleus [59].

2.2. Bioinformatic Analyses

There are several tools available for bioinformatic analyses of sc/snRNA-seq and sn/scATAC-seq data. If using a 10× Genomics platform, CellRanger is generally the first step in mapping raw FASTQs to a reference genome and generating count matrices of gene expression across individually barcoded cells [60]. This step will allow the use of any reference genome and is not limited to model organisms. Decontamination of ambient RNA can be incorporated into the bioinformatic workflow and can prove beneficial for snRNA-seq or snATAC-seq datasets, as they can contain a greater amount of ambient RNA carryover from outside the nucleus [60–62]. Doublet removal, filtering, and clustering workflows are available through a number of different computational frameworks including Bioconductor [63], Seurat [64], and Scanpy [65] for sc/snRNA-seq and sc/snATAC-seq [60]. After clustering and cell type identification, cell trajectories [66–68], RNA velocity [69,70],

and cell–cell communication [71,72] can be inferred to gain additional insight into cell population dynamics within the sampled tissue. Additional processing for sc/snATAC-seq datasets can be performed for quality control and doublet removal with specific consideration for sc/snATAC-seq datasets [73,74], followed by peak calling and cell population clustering with packages such as SnapATAC [75], ArchR [76], and Signac [77]. Other informative analyses with sc/snATAC-seq data include identifying differentially accessible regions across cell types, enrichment of transcription factor binding motifs within these regions, and construction of gene regulatory networks [78–80]. Within these bioinformatic tools, reference genomes and annotations must be customized to livestock genomes. The tools listed here allow for the creation of custom genomes and annotation, but whether a tool or pipeline allows for custom genomes and annotation is a major consideration for livestock researchers.

2.3. Data Interpretation

Performing sc/snRNA-seq and sc/snATAC-seq results in large datasets that can define cellular heterogeneity within complex tissues, model cell differentiation and RNA velocity across developmental time, identify differentially expressed genes and differentially accessible chromatin regions across cell populations, and many other applications. Acquiring both RNA and ATAC datasets provides an opportunity to integrate and putatively link gene expression changes to differentially accessible chromatin regions, likely housing regulatory elements, and construct gene regulatory networks with transcription factor binding sites [78–80]. However, open chromatin data only define the accessibility of putative regulatory regions and do not specify which type of regulatory element (i.e., promoter or enhancer) resides within that region (histone modification data will indicate. Further, putative regulatory elements for specific genes can only be identified in a linear context and interpreted in *cis*, as there is no three-dimensional genome information provided by these datasets.

In summary, the library preparation and bioinformatic analysis workflows for sc/snRNA-seq and sc/snATAC-seq include tissue dissociation or nuclei isolation, individual barcoding of cells or nuclei, sequencing, unsupervised clustering of cells and population identification, and further bioinformatic analyses to elucidate trajectories and regulatory networks. There are many different platforms and tools to accomplish library preparation and data analysis goals, and standardizing quality control metrics and data processing will become even more important as this technology is more widely adopted in livestock research. Data from these technologies provide insight into cellular heterogeneity and differentially expressed genes across cell populations of complex tissues among other applications; however, they have limitations in precisely identifying the type of regulatory element associated with gene expression changes.

3. Current Uses of Single-Cell Sequencing in Ruminant Livestock Research

As with studies in model organisms and humans, single-cell sequencing can reveal the underlying heterogeneity of tissues or cells and identify cell-specific regulatory elements and biomarkers in ruminant livestock tissues (Tables 1–3, Figure 2). It can also track cell populations and gene expression across developmental time and provide precise insights into the expression and chromatin accessibility of immune cell populations essential for resistance and resilience to disease. Research in ruminant livestock has made significant advancements to date, and scientists will likely continue to employ this technology to discover novel associations with economically important traits.

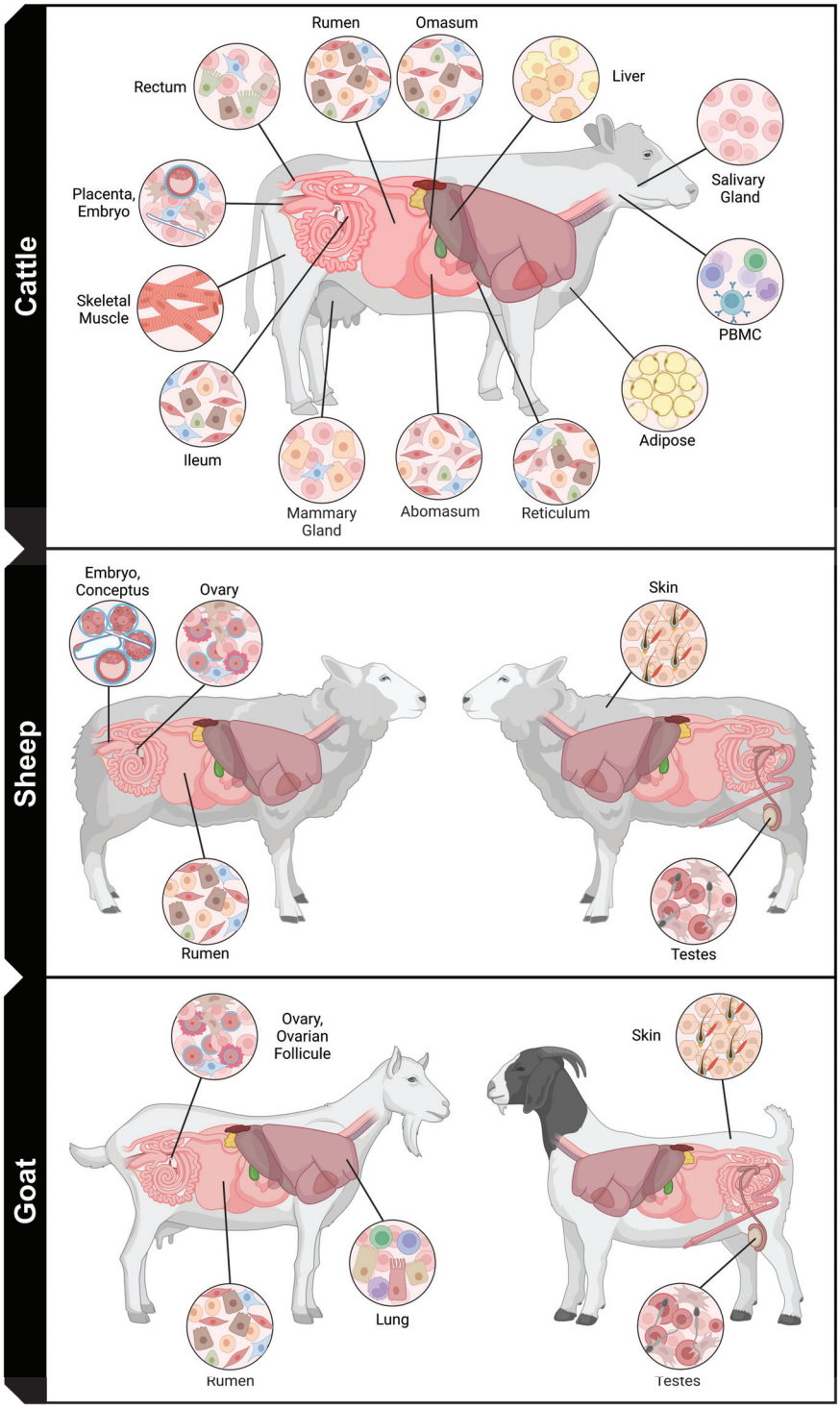


Figure 2. Schematic of tissues sampled for single-cell gene expression and chromatin accessibility assays in cattle, sheep, and goats in published studies with publicly available datasets (created with BioRender). Abbreviations: PBMC (peripheral blood mononuclear cells).

3.1. Cellular Heterogeneity within Tissues Important for Food and Fiber Production

The enhanced resolution provided by single-cell sequencing has been instrumental in identifying rare cell populations, such as stem cells, immune cells, and specialized tissue-specific cells, which play critical roles in development, growth, and disease resistance in livestock. One study identified 55 cell types across the abomasum, ileum, liver, mammary gland, omasum, rectum, reticulum, rumen, salivary gland, and isolated PBMCs in cattle [6].

All of these tissues in ruminant livestock play a critical role in converting human-inedible plant material into nutrient-rich milk and meat products [6]. This study constructed a single-cell atlas of these tissues in dairy cattle, which provided insight into the cellular heterogeneity and complexity within each tissue, and identified key cell types including epithelial and immune cells involved in milk production [6]. Because milk production is a critical aspect of the dairy industry, another study in dairy cattle performed scRNA-seq of cells isolated from fresh milk at mid-lactation to characterize cell types and gene expression [13]. This study identified many different cell types isolated from milk, including immune cells (T-cells, neutrophils, B-cells, monocytes, macrophages, and others) as well as luminal epithelial and progenitor cells [13]. An additional study performed scRNA-seq from a similar isolation of somatic cells from milk as well as primary bovine mammary epithelial cells in culture [20]. Both immune and epithelial cell types were identified from scRNA-seq from milk, while cell populations from the original tissue source (ductal region of the udder) were identified from cultured primary mammary epithelial cells [20]. These studies lay the groundwork for further examining cell types critical for lactation in dairy cattle.

In addition, transcriptomically distinct cell populations of the rumen, a tissue that is critical for digestion and feed efficiency and is unique to ruminants, were identified using scRNA-seq in sheep [27,28] and goats [28] as well as cultured primary epithelial cells in cattle [21]. In cattle, six cell populations were identified from cultured primary ruminal epithelial cells [21]. Cell lineages were then modeled across developmental time, and regulatory networks were constructed to better understand the unique gene expression profiles of these cells and their roles in rumen development in function [21]. A study in sheep examined changes in rumen cell populations and gene expression before, during, and after the development of papillae by sampling lambs during prenatal and early postnatal development [27]. Cell populations identified from this study included basal cells and keratinocytes, and marker genes for papillary growth and development in the rumen were characterized [27]. These results provide critical information about cellular dynamics during the establishment of rumen papillae, which are critical for digestion in sheep and other ruminants [27]. A comparative study of rumen maturation in sheep and goats identified cell populations at different developmental time points [28]. This study characterized cellular communication and transcription factor networks critical for rumen development, and correlated changes in cell populations across development to changes in the rumen microbiome [28]. Overall, studies examining cell populations in the rumen across development and maturation, including potential interactions with the microbial populations, provide valuable insights into biological processes essential for rumen development and ultimately the capacity to convert human inedible feedstuffs into nutrient-rich meat and milk products for consumption.

Other agriculturally important traits in ruminants such as growth and fat deposition were investigated in cattle by performing scRNA-seq and scATAC-seq in skeletal muscle [14,15], scRNA-seq of cultured bovine satellite cells [22,23], and snRNA-seq of adipose tissue [7], which uncovered previously unrealized cellular heterogeneity. Both scRNA-seq and scATAC-seq were employed in the skeletal muscle of cattle at fetal, postnatal, and adult stages of development [15]. This study identified several transcriptionally distinct cell types within skeletal muscle along with putative regulatory elements in open chromatin regions, cell–cell communication pathways, and transcription factor regulatory networks [15]. Another study used scRNA-seq in skeletal muscles of Wagyu, Brahman, and Wagyu/Brahman crosses to examine the cell types potentially involved with the deposition of intramuscular fat and connective tissue [14]. By using these specific breeds, the study was able to identify unique cell types predicted to be involved with enhanced intramuscular fat deposition (Wagyu) and intramuscular connective tissue deposition (Brahman), which both influence meat quality [15]. Further, single-cell RNA-seq of bovine satellite cells in culture isolated from a 2-week-old calf revealed different cell populations expressing markers of adipose progenitor cells that contribute to intramuscular fat deposition [22]. Another study reca-

pitulated these findings from bovine satellite cells in culture and further characterized fibro-adipogenic precursor cells using scRNA-seq [23]. These studies are critical first steps in understanding underlying cellular heterogeneity in skeletal muscle and how different cell types contribute to desirable traits such as increased growth or greater meat quality in ruminants.

Wool and hair quality are traits important to sheep and goat production across the world. The skin and hair follicle cell populations were characterized in sheep [29] as well as cashmere goats [36–38]. In sheep, differences in wool curvature due to hair follicle cell types were compared using scRNA-seq [29]. This study revealed differences in gene expression signatures of dermal papilla cells between curly and non-curly wool that may regulate curvature [29]. Several studies in cashmere goats identified heterogeneity of dermal papillae from different follicle types during fetal development [36], between goats producing course and fine cashmere [37], and during different times of the year corresponding to specific hair growth periods [38]. These studies provided insight into hair follicle development across different developmental time points, hair characteristics, and stages of the growth cycle, which are all important for the production of quality fiber in sheep and goats as well as other ruminants.

3.2. Gene Expression Dynamics during Reproduction and Development at Single-Cell Resolution

Reproductive efficiency in livestock is essential for profitable and sustainable farms. Previous work characterized the development of gonads at the fetal stage in cattle [12] and goats [32] with scRNA-seq and scATAC-seq, respectively, and compared these cell populations with other mammalian species. Cell populations of mature female gonads including the ovary in sheep [26] and goats [35] as well as the ovarian follicle in goats [34] were also identified with scRNA-seq. Heterogeneity of testes was identified with scRNA-seq in sheep [30,31] and goats [39]. Overall, these studies provide insights into gonadal and germ cell development in ruminants, which facilitate successful reproduction.

Single-cell sequencing has also proven useful for detangling dynamic gene expression within specific cell populations during embryonic and placental development in ruminants. Cellular heterogeneity was uncovered during early embryonic development at the 8- and 16-cell stage in cattle, which coincides with embryonic genome activation [8]. Cell populations and gene expression at single-cell resolution were also investigated during embryonic and conceptus development in sheep, providing additional information on biological processes contributing to pregnancy establishment in ruminants [24,25]. In vitro, scRNA-seq profiled gene expression across cell types in bovine trophectoderm outgrowth of embryos in culture [11] and bovine embryos that had undergone somatic cell nuclear transfer [10]. Further, cell population and gene expression dynamics of bovine embryos pre- and post-implantation, as well as the developing and mature placenta, provided insight into important processes governing implantation and placentation in cattle, which is critical for successful pregnancy establishment and maintenance [9,18,19]. Successful pregnancy establishment and maintenance are critical for the sustainability of ruminant livestock production.

3.3. Immune Cell Gene Expression and Chromatin Profiling

Disease resistance and resilience are very important for healthy animals and sustainable livestock production. scRNA-seq and scATAC-seq were performed on peripheral blood mononuclear cells (PBMC) in cattle, including cellular responses to lipopolysaccharide (LPS) challenge [16,17]. Further, snRNA-seq was utilized with lung tissue from 11 non-model species including goats, which revealed conserved cell populations and gene expression across mammals [33]. Future studies using single-cell sequencing technologies in immune cells and tissues, including their responses to disease challenges, have vast potential for identifying molecular mechanisms governing disease resistance and resilience in ruminant livestock species.

The field of single-cell genomics is rapidly evolving, and new datasets are continually being generated. Public data repositories such as the National Center for Biotechnology Information (NCBI), the Gene Expression Omnibus (GEO), the European Nucleotide Archive (ENA), and the Functional Annotation of Animal Genomes (FAANG) Data Portal are all currently being used to deposit raw data. A list of publicly available sc/snRNA-seq and sc/snATAC-seq datasets for cattle (Table 1), sheep (Table 2), and goats (Table 3) is provided. As these technologies continue to evolve and become more accessible, they hold tremendous promise for transforming our understanding of genetics, breeding, and disease resistance in ruminant livestock species and beyond. Ultimately, this will contribute to sustainable and efficient livestock production systems.

4. Challenges and Limitations of Single-Cell Technologies in Ruminant Livestock Research

Despite the transformative impact of sc/snRNA-seq and sc/snATAC-seq on livestock genomics research, these technologies are not without limitations. Understanding these challenges is crucial for the accurate interpretation and application of single-cell data in biological contexts. Technical variability, bioinformatic challenges in non-model species, cost, spatial resolution of cell populations, and consistency of data and metadata submitted to public repositories all pose challenges. However, initiatives within the genetics and bioinformatics community are poised to address many of these challenges.

4.1. Technical and Bioinformatic Challenges

One of the technical challenges associated with sc/snRNA-seq and sc/snATAC-seq is the presence of technical variability and dropout events during library preparation and sequencing, which can lead to the incomplete detection of gene expression or accessible chromatin regions [44,81,82]. Dropout events occur when the expression level of a gene or accessibility of a region falls below the detection threshold, leading to false negatives and potential biases in downstream analyses [81]. Livestock species often exhibit high levels of genetic diversity and complex transcriptomic and chromatin profiles, which can exacerbate the impact of technical variability and dropout events, making it difficult to accurately quantify gene expression levels and identify differentially expressed genes [45]. This could influence the reliability and reproducibility of single-cell sequencing data, providing further challenges in interpreting downstream results and comparing findings across studies. As library preparation, sequencing, and analysis algorithms continue to improve, these challenges will likely be mitigated.

Many different tools and pipelines exist for the analysis of sc/snRNA-seq and sc/snATAC-seq datasets, with more being developed and released regularly. This speaks to the rapid advancement of the field; however, there is a lack of standardized pipelines for quality control, filtering, and clustering, especially for non-model organisms. This can prove challenging for comparing and reproducing results across studies [60,83]. Using a variety of computational languages to analyze data can pose challenges for researchers if this is not their area of expertise. The analysis of sc/snRNA-seq and sc/snATAC-seq data also presents bioinformatics challenges in non-model species, including ruminant livestock. Many analysis tools built for model organisms are expanding to become species-agnostic, which provides substantial opportunities for livestock researchers to utilize these with annotation from their own genomes. The quality and utility of ruminant livestock genomes and annotation are advancing rapidly driven by community efforts, including pangenome construction [47–50], complete telomere-to-telomere assemblies [84], and sophisticated annotation pipelines and resources [85]. These efforts will enhance the ability to precisely differentiate cell clusters based on gene expression, providing even greater insights into cellular heterogeneity and gene expression patterns within tissues important for the understanding of economically important traits in ruminant livestock.

4.2. Cell Cluster Identification and Spatial Resolution

Cell cluster identification and naming can be a challenging aspect of single-cell data analyses. This step in the analysis is critical, as it leads to many novel discoveries; however, it can prove to be the most difficult in non-model species, especially in tissues such as the placenta, which are highly specialized structurally across mammals [18,19]. Currently, cell populations in non-model organisms such as livestock are frequently identified with the top differentially expressed genes for each cluster, using databases such as the Human Protein Atlas [86] and PanglaoDB [87], or automated cluster identification using databases built from humans and mouse cell clusters [88,89]. Often, cell types are classified as "unknown" if no definitive answer can be reached. One potential new way to combat these obstacles is to use generative pre-trained transformers (GPT) to identify single cell clusters, which could shift the annotation process from manual to semi- or fully automated [90]. Using GPT was deemed to be competent in naming clusters and identifying marker genes with a 70% match rate in most tissues in a recent study [90]. As artificial intelligence technology continues to evolve, it could be incorporated into single-cell omics pipelines to identify cell clusters.

Another limitation of sc/snRNA-seq and sc/snATAC-seq is the lack of spatial resolution, which impedes the precise characterization of cell–cell interactions in spatial context and organization within tissues and organs [91]. Cell populations identified from single-cell sequencing have been localized in a spatial context using techniques such as RNA in-situ hybridization and immunofluorescence [19,92]. In livestock species, where tissue architecture and cellular microenvironments play crucial roles in development, growth, and disease resistance, the inability to capture spatial information can limit the biological insights gained from single-cell sequencing data. Emerging spatial transcriptomics and spatial genomics technologies offer promising solutions to this challenge by enabling the profiling of gene expression within intact tissue sections [91]. These technologies are rapidly improving in robustness and resolution and are becoming more widely utilized in non-model organisms [93,94]. Spatial omics technologies have vast potential for ruminant livestock research by providing spatial resolution and insights into cell–cell interaction and communications in tissues important for production such as the rumen.

4.3. Reproducibility, Collaboration, and Knowledge Sharing

The availability and re-use of single-cell genomics datasets are important for answering important biological questions in ruminant livestock research. The accessibility of high-quality, well-annotated datasets accelerates new discoveries and enhances the reproducibility and robustness of the initial single-cell atlases [83,95,96]. Data availability and re-use enable researchers to build upon existing knowledge and leverage the collective insights gained from multiple studies, thereby accelerating scientific discoveries and facilitating the identification of novel biomarkers, regulatory elements, and gene networks associated with economically important traits. By accessing and utilizing existing datasets, researchers can validate and extend previous findings, generate new hypotheses, and explore biological questions more efficiently and cost-effectively [47,83,95]. Further, the availability of single-cell omics datasets across different species, breeds, and experimental conditions facilitates comparative analyses, enabling researchers to identify conserved and species-specific gene expression profiles and regulatory networks associated with complex traits and biological processes in livestock and across species [97–99].

In summary, single-cell sequencing technologies can provide valuable insights into important biological questions in livestock species; however, these technologies are not without challenges or limitations. Single-cell library preparation and sequencing can be quite costly and more resource-intensive than traditional bulk sequencing methods; however, the increase in throughput and the decrease in cost per sample for library preparation and sequencing as this technology evolves will enable greater adoption and accessibility of single-cell sequencing in ruminant livestock research [47,100]. Continued collaborative efforts to develop better tools and standardized pipelines for sample preparation, analyses,

and data submissions to public databases are essential for realizing the full potential of single-cell omics technologies in ruminant livestock [48,96,101].

5. The Future of Single-Cell Sequencing in Ruminant Livestock Research

Despite the current limitations, the rapid advancements in single-cell genomic technologies offer promising opportunities for research in ruminant livestock genetics and physiology, encompassing economically important phenotypes spanning from reproduction and growth to disease resistance. This in turn has tremendous potential for addressing key challenges and driving innovation in the agricultural sector, contributing to sustainable and profitable animal agriculture globally.

Continued development of single-cell gene expression atlases across complex, heterogenous tissues and cell populations in livestock across developmental time will provide greater insights into cell-type-specific expression dynamics. One very promising use of single-cell omics sequencing in livestock research is the comprehensive profiling of immune cell populations and their interactions within the host [16,17,102]. Understanding the dynamics of immune cell heterogeneity and function is crucial for developing strategies to improve disease resistance in livestock. Single-cell omics enables precise characterization of immune cell subsets, including T cells, B cells, macrophages, and dendritic cells, and the identification of cell-type-specific gene expression signatures and regulatory networks associated with immune response and pathogen resistance [16,17,102]. Future research leveraging single-cell omics could focus on elucidating molecular mechanisms underlying host–pathogen interactions, vaccine efficacy, and immune system development in livestock. Another promising application of single-cell technologies is further examining the interaction between host and microbes in terms of the microbiome by expanding on previous work associating cell populations of the rumen with the microbial population composition in sheep and goats [28].

The development of single-cell isoform sequencing and epigenetic assays, along with spatial transcriptomics, also hold promising applications in ruminant livestock research. Single-cell isoform sequencing enables precise identification of alternate isoform expression across cell types within a tissue [103]. Single-cell implementations of CUT&TAG [104], chromatin structure [105,106], and methylation [107,108] also provide opportunities to improve the functional annotation of different cell types within complex tissues [47] when integrated with sc/snRNA-seq and sc/snATAC-seq. Spatial transcriptomics also offers exciting opportunities to localize the spatial context of gene expression and better characterize cell–cell communication [93,94]. Future studies could leverage multiple single-cell omics sequencing technologies to elucidate regulatory networks, contributing to complex traits such as growth, milk production, meat quality, and reproduction in livestock. This will facilitate the development of more precise breeding strategies for livestock operations to increase the productivity and sustainability of animal agriculture.

6. Conclusions

Ultimately, incorporating single-cell omics technologies into livestock research holds the potential to facilitate new discoveries to accelerate the pace of genetic improvement, enhance disease resistance, support the welfare of these species, and improve the overall profitability and sustainability of livestock production. Ruminant livestock researchers have already implemented sc/snRNA-seq and sc/snATAC-seq across multiple tissues to uncover cellular heterogeneity, track gene expression dynamics across developmental time, and profile immune cells. Although several limitations to this technology exist, including the lack of spatial resolution, cost, and the need for standardized bioinformatics and data deposit guidelines, the collaborative livestock genomics community is poised to help overcome these challenges [47,109]. The future of single-cell omics technology in livestock research is promising, with many opportunities for advancing understanding of the molecular mechanisms governing complex traits to optimize breeding strategies, make

advancements in animal welfare, and drive innovation in sustainable and efficient livestock production systems to meet the growing global demand for high-quality animal products.

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References

1. Sun, S.; Shen, X.; Li, Y.; Wang, S.; Li, R.; Zhang, H.; Shen, G.; Guo, B.; Wei, J.; Xu, J.; et al. Single-cell RNA sequencing provides a high-resolution roadmap for understanding the multicellular compartmentation of specialized metabolism. *Nat. Plants* **2023**, *9*, 179–190. [CrossRef]
2. Ke, M.; Elshenawy, B.; Sheldon, H.; Arora, A.; Buffa, F.M. Single cell RNA-sequencing: A powerful yet still challenging technology to study cellular heterogeneity. *Bioessays* **2022**, *44*, e2200084. [CrossRef] [PubMed]
3. Lim, J.; Park, C.; Kim, M.; Kim, H.; Kim, J.; Lee, D.S. Advances in single-cell omics and multiomics for high-resolution molecular profiling. *Exp. Mol. Med.* **2024**, *56*, 515–526. [CrossRef]
4. Ogbeide, S.; Giannese, F.; Mincarelli, L.; Macaulay, I.C. Into the multiverse: Advances in single-cell multiomic profiling. *Trends Genet.* **2022**, *38*, 831–843. [CrossRef]
5. Baysoy, A.; Bai, Z.; Satija, R.; Fan, R. The technological landscape and applications of single-cell multi-omics. *Nat. Rev. Mol. Cell Biol.* **2023**, *24*, 695–713. [CrossRef]
6. Wu, J.J.; Zhu, S.; Gu, F.; Valencak, T.G.; Liu, J.X.; Sun, H.Z. Cross-tissue single-cell transcriptomic landscape reveals the key cell subtypes and their potential roles in the nutrient absorption and metabolism in dairy cattle. *J. Adv. Res.* **2022**, *37*, 1–18. [CrossRef]
7. Michelotti, T.C.; Kisby, B.R.; Flores, L.S.; Tegeler, A.P.; Fokar, M.; Crasto, C.; Menarim, B.C.; Loux, S.C.; Strieder-Barboza, C. Single-nuclei analysis reveals depot-specific transcriptional heterogeneity and depot-specific cell types in adipose tissue of dairy cows. *Front. Cell Dev. Biol.* **2022**, *10*, 1025240. [CrossRef]
8. Lavagi, I.; Krebs, S.; Simmet, K.; Beck, A.; Zakhartchenko, V.; Wolf, E.; Blum, H. Single-cell RNA sequencing reveals developmental heterogeneity of blastomeres during major genome activation in bovine embryos. *Sci. Rep.* **2018**, *8*, 4071. [CrossRef] [PubMed]
9. Scatolin, G.N.; Ming, H.; Wang, Y.; Iyyappan, R.; Gutierrez-Castillo, E.; Zhu, L.; Sagheer, M.; Song, C.; Bondioli, K.; Jiang, Z. Single-cell transcriptional landscapes of bovine peri-implantation development. *iScience* **2024**, *27*, 109605. [CrossRef] [PubMed]
10. Zhao, L.; Long, C.; Zhao, G.; Su, J.; Ren, J.; Sun, W.; Wang, Z.; Zhang, J.; Liu, M.; Hao, C.; et al. Reprogramming barriers in bovine cells nuclear transfer revealed by single-cell RNA-seq analysis. *J. Cell Mol. Med.* **2022**, *26*, 4792–4804. [CrossRef]
11. Ortega, M.S.; Rizo, J.A.; Drum, J.N.; O'Neil, E.V.; Pohler, K.G.; Kerns, K.; Schmelze, A.; Green, J.; Spencer, T.E. Development of an Improved in vitro Model of Bovine Trophectoderm Differentiation. *Front. Anim. Sci.* **2022**, *3*, 898808. [CrossRef]
12. Soto, D.A.; Ross, P.J. Similarities between bovine and human germline development revealed by single-cell RNA sequencing. *Reproduction* **2021**, *161*, 239–253. [CrossRef] [PubMed]
13. Zorc, M.; Dolinar, M.; Dovč, P. A Single-Cell Transcriptome of Bovine Milk Somatic Cells. *Genes* **2024**, *15*, 349. [CrossRef] [PubMed]
14. Wang, L.; Gao, P.; Li, C.; Liu, Q.; Yao, Z.; Li, Y.; Zhang, X.; Sun, J.; Simintiras, C.; Welborn, M.; et al. A single-cell atlas of bovine skeletal muscle reveals mechanisms regulating intramuscular adipogenesis and fibrogenesis. *J. Cachexia Sarcopenia Muscle* **2023**, *14*, 2152–2167. [CrossRef] [PubMed]
15. Cai, C.; Wan, P.; Wang, H.; Cai, X.; Wang, J.; Chai, Z.; Zhang, M.; Yang, N.; Wu, Z.; Zhu, J.; et al. Transcriptional and open chromatin analysis of bovine skeletal muscle development by single-cell sequencing. *Cell Prolif.* **2023**, *56*, e13430. [CrossRef]
16. Wang, X.; Gao, Y.; Li, C.J.; Fang, L.; Liu, G.E.; Zhao, X.; Zhang, Y.; Cai, G.; Xue, G.; Liu, Y.; et al. The single-cell transcriptome and chromatin accessibility datasets of peripheral blood mononuclear cells in Chinese holstein cattle. *BMC Genom. Data* **2023**, *24*, 39. [CrossRef] [PubMed]
17. Gao, Y.; Li, J.; Cai, G.; Wang, Y.; Yang, W.; Li, Y.; Zhao, X.; Li, R.; Tuo, W.; Baldwin, R.L.; et al. Single-cell transcriptomic and chromatin accessibility analyses of dairy cattle peripheral blood mononuclear cells and their responses to lipopolysaccharide. *BMC Genom.* **2022**, *23*, 338. [CrossRef]
18. Davenport, K.M.; O'Neil, E.V.; Ortega, M.S.; Patterson, A.; Kelleher, A.M.; Warren, W.C.; Spencer, T.E. Single-cell insights into development of the bovine placenta. *Biol. Reprod.* **2024**, *110*, 169–184. [CrossRef] [PubMed]

19. Davenport, K.M.; Ortega, M.S.; Liu, H.; O'Neil, E.V.; Kelleher, A.M.; Warren, W.C.; Spencer, T.E. Single-nuclei RNA sequencing (snRNA-seq) uncovers trophoblast cell types and lineages in the mature bovine placenta. *Proc. Natl. Acad. Sci. USA* **2023**, *120*, e2221526120. [CrossRef]
20. Becker, D.; Weikard, R.; Hadlich, F.; Kühn, C. Single-cell RNA sequencing of freshly isolated bovine milk cells and cultured primary mammary epithelial cells. *Sci. Data* **2021**, *8*, 177. [CrossRef]
21. Gao, Y.; Fang, L.; Baldwin, R.L.; Connor, E.E.; Cole, J.B.; Van Tassell, C.P.; Ma, L.; Li, C.J.; Liu, G.E. Single-cell transcriptomic analyses of dairy cattle ruminal epithelial cells during weaning. *Genomics* **2021**, *113*, 2045–2055. [CrossRef] [PubMed]
22. Lyu, P.; Qi, Y.; Tu, Z.J.; Jiang, H. Single-cell RNA Sequencing Reveals Heterogeneity of Cultured Bovine Satellite Cells. *Front. Genet.* **2021**, *12*, 742077. [CrossRef] [PubMed]
23. Messmer, T.; Dohmen, R.G.J.; Schaeken, L.; Melzener, L.; Hueber, R.; Godec, M.; Didoss, C.; Post, M.J.; Flack, J.E. Single-cell analysis of bovine muscle-derived cell types for cultured meat production. *Front. Nutr.* **2023**, *10*, 1212196. [CrossRef] [PubMed]
24. Jia, G.X.; Ma, W.J.; Wu, Z.B.; Li, S.; Zhang, X.Q.; He, Z.; Wu, S.X.; Tao, H.P.; Fang, Y.; Song, Y.W.; et al. Single-cell transcriptomic characterization of sheep conceptus elongation and implantation. *Cell Rep.* **2023**, *42*, 112860. [CrossRef] [PubMed]
25. He, T.; Guo, W.; Yang, G.; Su, H.; Dou, A.; Chen, L.; Ma, T.; Su, J.; Liu, M.; Su, B.; et al. A Single-Cell Atlas of an Early Mongolian Sheep Embryo. *Vet. Sci.* **2023**, *10*, 543. [CrossRef] [PubMed]
26. Ge, T.; Wen, Y.; Li, B.; Huang, X.; Jiang, S.; Zhang, E. Single-cell sequencing reveals the reproductive variations between primiparous and multiparous Hu ewes. *J. Anim. Sci. Biotechnol.* **2023**, *14*, 144. [CrossRef] [PubMed]
27. Yuan, Y.; Sun, D.M.; Qin, T.; Mao, S.Y.; Zhu, W.Y.; Yin, Y.Y.; Huang, J.; Heller, R.; Li, Z.P.; Liu, J.H.; et al. Single-cell transcriptomic landscape of the sheep rumen provides insights into physiological programming development and adaptation of digestive strategies. *Zool. Res.* **2022**, *43*, 634–647. [CrossRef] [PubMed]
28. Deng, J.; Liu, Y.J.; Wei, W.T.; Huang, Q.X.; Zhao, L.P.; Luo, L.Y.; Zhu, Q.; Zhang, L.; Chen, Y.; Ren, Y.L.; et al. Single-cell transcriptome and metagenome profiling reveals the genetic basis of rumen functions and convergent developmental patterns in ruminants. *Genome Res.* **2023**, *33*, 1690–1707. [CrossRef] [PubMed]
29. Wang, S.; Wu, T.; Sun, J.; Li, Y.; Yuan, Z.; Sun, W. Single-Cell Transcriptomics Reveals the Molecular Anatomy of Sheep Hair Follicle Heterogeneity and Wool Curvature. *Front. Cell Dev. Biol.* **2021**, *9*, 800157. [CrossRef]
30. Wu, Y.; Guo, T.; Li, J.; Niu, C.; Sun, W.; Zhu, S.; Zhao, H.; Qiao, G.; Han, M.; He, X.; et al. The Transcriptional Cell Atlas of Testis Development in Sheep at Pre-Sexual Maturity. *Curr. Issues Mol. Biol.* **2022**, *44*, 483–497. [CrossRef]
31. Tian, Y.; Sun, P.; Liu, W.X.; Shan, L.Y.; Hu, Y.T.; Fan, H.T.; Shen, W.; Liu, Y.B.; Zhou, Y.; Zhang, T. Single-cell RNA sequencing of the Mongolia sheep testis reveals a conserved and divergent transcriptome landscape of mammalian spermatogenesis. *FASEB J.* **2022**, *36*, e22348. [CrossRef] [PubMed]
32. Chen, M.; Long, X.; Hao, F.; Kang, J.; Wang, N.; Wang, Y.; Wang, M.; Gao, Y.; Zhou, M.; Duo, L.; et al. Integration of single-cell transcriptome and chromatin accessibility of early gonads development among goats, pigs, macaques, and humans. *Cell Rep.* **2022**, *41*, 111587. [CrossRef] [PubMed]
33. Chen, D.; Sun, J.; Zhu, J.; Ding, X.; Lan, T.; Wang, X.; Wu, W.; Ou, Z.; Zhu, L.; Ding, P.; et al. Single cell atlas for 11 non-model mammals, reptiles and birds. *Nat. Commun.* **2021**, *12*, 7083. [CrossRef]
34. Li, Z.; Wang, J.; Zhao, Y.; Ma, D.; Zhao, M.; Li, N.; Men, Y.; Zhang, Y.; Chu, H.; Lei, C.; et al. scRNA-seq of ovarian follicle granulosa cells from different fertility goats reveals distinct expression patterns. *Reprod. Domest. Anim.* **2021**, *56*, 801–811. [CrossRef] [PubMed]
35. Xu, D.; Song, S.; Wang, F.; Li, Y.; Li, Z.; Yao, H.; Zhao, Y.; Zhao, Z. Single-cell transcriptomic atlas of goat ovarian aging. *J. Anim. Sci. Biotechnol.* **2023**, *14*, 151. [CrossRef]
36. Ge, W.; Zhang, W.; Zhang, Y.; Zheng, Y.; Li, F.; Wang, S.; Liu, J.; Tan, S.; Yan, Z.; Wang, L.; et al. A Single-cell Transcriptome Atlas of Cashmere Goat Hair Follicle Morphogenesis. *Genom. Proteom. Bioinform.* **2021**, *19*, 437–451. [CrossRef]
37. Wang, Z.; Wang, Y.; Hui, T.; Chen, R.; Xu, Y.; Zhang, Y.; Tian, H.; Wang, W.; Cong, Y.; Guo, S.; et al. Single-Cell Sequencing Reveals Differential Cell Types in Skin Tissues of Liaoning Cashmere Goats and Key Genes Related Potentially to the Fineness of Cashmere Fiber. *Front. Genet.* **2021**, *12*, 726670. [CrossRef]
38. Yang, F.; Li, R.; Zhao, C.; Che, T.; Guo, J.; Xie, Y.; Wang, Z.; Li, J.; Liu, Z. Single-cell sequencing reveals the new existence form of dermal papilla cells in the hair follicle regeneration of cashmere goats. *Genomics* **2022**, *114*, 110316. [CrossRef]
39. Chen, M.; Wang, N.; Yang, H.; Liu, D.; Gao, Y.; Duo, L.; Cui, X.; Hao, F.; Ye, J.; Gao, F.; et al. Single-cell transcriptome analysis of the germ cells and somatic cells during mitotic quiescence stage in goats. *FASEB J.* **2023**, *37*, e23244. [CrossRef]
40. Sargison, N.D. The critical importance of planned small ruminant livestock health and production in addressing global challenges surrounding food production and poverty alleviation. *N. Z. Vet. J.* **2020**, *68*, 136–144. [CrossRef]
41. Davis, T.C.; White, R.R. Breeding animals to feed people: The many roles of animal reproduction in ensuring global food security. *Theriogenology* **2020**, *150*, 27–33. [CrossRef] [PubMed]
42. Cuomo, A.S.E.; Nathan, A.; Raychaudhuri, S.; MacArthur, D.G.; Powell, J.E. Single-cell genomics meets human genetics. *Nat. Rev. Genet.* **2023**, *24*, 535–549. [CrossRef] [PubMed]
43. Vandereyken, K.; Sifrim, A.; Thienpont, B.; Voet, T. Methods and applications for single-cell and spatial multi-omics. *Nat. Rev. Genet.* **2023**, *24*, 494–515. [CrossRef] [PubMed]
44. Chen, G.; Ning, B.; Shi, T. Single-Cell RNA-Seq Technologies and Related Computational Data Analysis. *Front. Genet.* **2019**, *10*, 317. [CrossRef] [PubMed]

45. Alfieri, J.M.; Wang, G.; Jonika, M.M.; Gill, C.A.; Blackmon, H.; Athrey, G.N. A Primer for Single-Cell Sequencing in Non-Model Organisms. *Genes* **2022**, *13*, 380. [CrossRef] [PubMed]
46. Conte, M.I.; Fuentes-Trillo, A.; Domínguez Conde, C. Opportunities and tradeoffs in single-cell transcriptomic technologies. *Trends Genet.* **2024**, *40*, 83–93. [CrossRef] [PubMed]
47. Clark, E.L.; Archibald, A.L.; Daetwyler, H.D.; Groenen, M.A.M.; Harrison, P.W.; Houston, R.D.; Kühn, C.; Lien, S.; Macqueen, D.J.; Reecy, J.M.; et al. From FAANG to fork: Application of highly annotated genomes to improve farmed animal production. *Genome Biol.* **2020**, *21*, 285. [CrossRef] [PubMed]
48. Harrison, P.W.; Sokolov, A.; Nayak, A.; Fan, J.; Zerbino, D.; Cochrane, G.; Flicek, P. The FAANG Data Portal: Global, Open-Access, "FAIR", and Richly Validated Genotype to Phenotype Data for High-Quality Functional Annotation of Animal Genomes. *Front. Genet.* **2021**, *12*, 639238. [CrossRef] [PubMed]
49. Smith, T.P.L.; Bickhart, D.M.; Boichard, D.; Chamberlain, A.J.; Djikeng, A.; Jiang, Y.; Low, W.Y.; Pausch, H.; Demyda-Peyrás, S.; Prendergast, J.; et al. The Bovine Pangenome Consortium: Democratizing production and accessibility of genome assemblies for global cattle breeds and other bovine species. *Genome Biol.* **2023**, *24*, 139. [CrossRef]
50. Woolley, S.A.; Salavati, M.; Clark, E.L. Recent advances in the genomic resources for sheep. *Mamm. Genome* **2023**, *34*, 545–558. [CrossRef]
51. Jovic, D.; Liang, X.; Zeng, H.; Lin, L.; Xu, F.; Luo, Y. Single-cell RNA sequencing technologies and applications: A brief overview. *Clin. Transl. Med.* **2022**, *12*, e694. [CrossRef]
52. Yao, C.; Bora, S.A.; Chen, P.; Goodridge, H.S.; Gharib, S.A. Sample processing and single cell RNA-sequencing of peripheral blood immune cells from COVID-19 patients. *STAR Protoc.* **2021**, *2*, 100582. [CrossRef] [PubMed]
53. Petrany, M.J.; Swoboda, C.O.; Sun, C.; Chetal, K.; Chen, X.; Weirauch, M.T.; Salomonis, N.; Millay, D.P. Single-nucleus RNA-seq identifies transcriptional heterogeneity in multinucleated skeletal myofibers. *Nat. Commun.* **2020**, *11*, 6374. [CrossRef]
54. Sun, W.; Dong, H.; Balaz, M.; Slyper, M.; Drokhlyansky, E.; Colleluori, G.; Giordano, A.; Kovanicova, Z.; Stefanicka, P.; Balazova, L.; et al. snRNA-seq reveals a subpopulation of adipocytes that regulates thermogenesis. *Nature* **2020**, *587*, 98–102. [CrossRef] [PubMed]
55. Kim, N.; Kang, H.; Jo, A.; Yoo, S.A.; Lee, H.O. Perspectives on single-nucleus RNA sequencing in different cell types and tissues. *J. Pathol. Transl. Med.* **2023**, *57*, 52–59. [CrossRef]
56. Cusanovich, D.A.; Daza, R.; Adey, A.; Pliner, H.A.; Christiansen, L.; Gunderson, K.L.; Steemers, F.J.; Trapnell, C.; Shendure, J. Multiplex single cell profiling of chromatin accessibility by combinatorial cellular indexing. *Science* **2015**, *348*, 910–914. [CrossRef] [PubMed]
57. Buenrostro, J.D.; Wu, B.; Chang, H.Y.; Greenleaf, W.J. ATAC-seq: A Method for Assaying Chromatin Accessibility Genome-Wide. *Curr. Protoc. Mol. Biol.* **2015**, *109*, 21–29. [CrossRef]
58. Shi, Z.; Das, S.; Morabito, S.; Miyoshi, E.; Swarup, V. Protocol for single-nucleus ATAC sequencing and bioinformatic analysis in frozen human brain tissue. *STAR Protoc.* **2022**, *3*, 101491. [CrossRef] [PubMed]
59. Sandoval, L.; Mohammed Ismail, W.; Mazzone, A.; Dumbrava, M.; Fernandez, J.; Munankarmy, A.; Lasho, T.; Binder, M.; Simon, V.; Kim, K.H.; et al. Characterization and Optimization of Multiomic Single-Cell Epigenomic Profiling. *Genes* **2023**, *14*, 1245. [CrossRef]
60. Heumos, L.; Schaar, A.C.; Lance, C.; Litinetskaya, A.; Drost, F.; Zappia, L.; Lücken, M.D.; Strobl, D.C.; Henao, J.; Curion, F.; et al. Best practices for single-cell analysis across modalities. *Nat. Rev. Genet.* **2023**, *24*, 550–572. [CrossRef]
61. Yang, S.; Corbett, S.E.; Koga, Y.; Wang, Z.; Johnson, W.E.; Yajima, M.; Campbell, J.D. Decontamination of ambient RNA in single-cell RNA-seq with DecontX. *Genome Biol.* **2020**, *21*, 57. [CrossRef] [PubMed]
62. Fleming, S.J.; Chaffin, M.D.; Arduini, A.; Akkad, A.D.; Banks, E.; Marioni, J.C.; Philippakis, A.A.; Ellinor, P.T.; Babadi, M. Unsupervised removal of systematic background noise from droplet-based single-cell experiments using CellBender. *Nat. Methods* **2023**, *20*, 1323–1335. [CrossRef] [PubMed]
63. Amezquita, R.A.; Lun, A.T.L.; Becht, E.; Carey, V.J.; Carpp, L.N.; Geistlinger, L.; Marini, F.; Rue-Albrecht, K.; Risso, D.; Soneson, C.; et al. Orchestrating single-cell analysis with Bioconductor. *Nat. Methods* **2020**, *17*, 137–145. [CrossRef] [PubMed]
64. Hao, Y.; Hao, S.; Andersen-Nissen, E.; Mauck, W.M.; Zheng, S.; Butler, A.; Lee, M.J.; Wilk, A.J.; Darby, C.; Zager, M.; et al. Integrated analysis of multimodal single-cell data. *Cell* **2021**, *184*, 3573–3587.e29. [CrossRef] [PubMed]
65. Wolf, F.A.; Angerer, P.; Theis, F.J. SCANPY: Large-scale single-cell gene expression data analysis. *Genome Biol.* **2018**, *19*, 15. [CrossRef] [PubMed]
66. Street, K.; Risso, D.; Fletcher, R.B.; Das, D.; Ngai, J.; Yosef, N.; Purdom, E.; Dudoit, S. Slingshot: Cell lineage and pseudotime inference for single-cell transcriptomics. *BMC Genom.* **2018**, *19*, 477. [CrossRef] [PubMed]
67. Cao, J.; Spielmann, M.; Qiu, X.; Huang, X.; Ibrahim, D.M.; Hill, A.J.; Zhang, F.; Mundlos, S.; Christiansen, L.; Steemers, F.J.; et al. The single-cell transcriptional landscape of mammalian organogenesis. *Nature* **2019**, *566*, 496–502. [CrossRef] [PubMed]
68. Van den Berge, K.; Roux de Bézieux, H.; Street, K.; Saelens, W.; Cannoodt, R.; Saeys, Y.; Dudoit, S.; Clement, L. Trajectory-based differential expression analysis for single-cell sequencing data. *Nat. Commun.* **2020**, *11*, 1201. [CrossRef] [PubMed]
69. La Manno, G.; Soldatov, R.; Zeisel, A.; Braun, E.; Hochgerner, H.; Petukhov, V.; Lidschreiber, K.; Kastriti, M.E.; Lönnerberg, P.; Furlan, A.; et al. RNA velocity of single cells. *Nature* **2018**, *560*, 494–498. [CrossRef]
70. Zheng, S.C.; Stein-O'Brien, G.; Boukas, L.; Goff, L.A.; Hansen, K.D. Pumping the brakes on RNA velocity by understanding and interpreting RNA velocity estimates. *Genome Biol.* **2023**, *24*, 246. [CrossRef]

71. Jin, S.; Guerrero-Juarez, C.F.; Zhang, L.; Chang, I.; Ramos, R.; Kuan, C.H.; Myung, P.; Plikus, M.V.; Nie, Q. Inference and analysis of cell-cell communication using CellChat. *Nat. Commun.* **2021**, *12*, 1088. [CrossRef]
72. Wang, S.; Zheng, H.; Choi, J.S.; Lee, J.K.; Li, X.; Hu, H. A systematic evaluation of the computational tools for ligand-receptor-based cell-cell interaction inference. *Brief. Funct. Genom.* **2022**, *21*, 339–356. [CrossRef] [PubMed]
73. Ou, J.; Liu, H.; Yu, J.; Kelliher, M.A.; Castilla, L.H.; Lawson, N.D.; Zhu, L.J. ATACseqQC: A Bioconductor package for post-alignment quality assessment of ATAC-seq data. *BMC Genom.* **2018**, *19*, 169. [CrossRef] [PubMed]
74. Germain, P.L.; Lun, A.; Garcia Meixide, C.; Macnair, W.; Robinson, M.D. Doublet identification in single-cell sequencing data using. *F1000Research* **2021**, *10*, 979. [CrossRef] [PubMed]
75. Chen, H.; Lareau, C.; Andreani, T.; Vinyard, M.E.; Garcia, S.P.; Clement, K.; Andrade-Navarro, M.A.; Buenrostro, J.D.; Pinello, L. Assessment of computational methods for the analysis of single-cell ATAC-seq data. *Genome Biol.* **2019**, *20*, 241. [CrossRef] [PubMed]
76. Granja, J.M.; Corces, M.R.; Pierce, S.E.; Bagdatli, S.T.; Choudhry, H.; Chang, H.Y.; Greenleaf, W.J. ArchR is a scalable software package for integrative single-cell chromatin accessibility analysis. *Nat. Genet.* **2021**, *53*, 403–411. [CrossRef] [PubMed]
77. Stuart, T.; Srivastava, A.; Madad, S.; Lareau, C.A.; Satija, R. Single-cell chromatin state analysis with Signac. *Nat. Methods* **2021**, *18*, 1333–1341. [CrossRef] [PubMed]
78. Liu, L.; Liu, C.; Quintero, A.; Wu, L.; Yuan, Y.; Wang, M.; Cheng, M.; Leng, L.; Xu, L.; Dong, G.; et al. Deconvolution of single-cell multi-omics layers reveals regulatory heterogeneity. *Nat. Commun.* **2019**, *10*, 470. [CrossRef] [PubMed]
79. Kartha, V.K.; Duarte, F.M.; Hu, Y.; Ma, S.; Chew, J.G.; Lareau, C.A.; Earl, A.; Burkett, Z.D.; Kohlway, A.S.; Lebofsky, R.; et al. Functional inference of gene regulation using single-cell multi-omics. *Cell Genom.* **2022**, *2*, 100166. [CrossRef]
80. Fleck, J.S.; Jansen, S.M.J.; Wollny, D.; Zenk, F.; Seimiya, M.; Jain, A.; Okamoto, R.; Santel, M.; He, Z.; Camp, J.G.; et al. Inferring and perturbing cell fate regulomes in human brain organoids. *Nature* **2023**, *621*, 365–372. [CrossRef]
81. Haque, A.; Engel, J.; Teichmann, S.A.; Lonnberg, T. A practical guide to single-cell RNA-sequencing for biomedical research and clinical applications. *Genom. Med.* **2017**, *9*, 75. [CrossRef] [PubMed]
82. Hicks, S.C.; Townes, F.W.; Teng, M.; Irizarry, R.A. Missing data and technical variability in single-cell RNA-sequencing experiments. *Biostatistics* **2018**, *19*, 562–578. [CrossRef] [PubMed]
83. Skinnider, M.A.; Squair, J.W.; Courtine, G. Enabling reproducible re-analysis of single-cell data. *Genome Biol.* **2021**, *22*, 215. [CrossRef]
84. Nurk, S.; Koren, S.; Rhie, A.; Rautiainen, M.; Bzikadze, A.V.; Mikheenko, A.; Vollger, M.R.; Altemose, N.; Uralsky, L.; Gershman, A.; et al. The complete sequence of a human genome. *Science* **2022**, *376*, 44–53. [CrossRef]
85. Triant, D.A.; Walsh, A.T.; Hartley, G.A.; Petry, B.; Stegemiller, M.R.; Nelson, B.M.; McKendrick, M.M.; Fuller, E.P.; Cockett, N.E.; Koltes, J.E.; et al. AgAnimalGenomes: Browsers for viewing and manually annotating farm animal genomes. *Mamm. Genome* **2023**, *34*, 418–436. [CrossRef] [PubMed]
86. Thul, P.J.; Lindskog, C. The human protein atlas: A spatial map of the human proteome. *Protein Sci.* **2018**, *27*, 233–244. [CrossRef] [PubMed]
87. Franzén, O.; Gan, L.M.; Björkegren, J.L.M. PanglaoDB: A web server for exploration of mouse and human single-cell RNA sequencing data. *Database* **2019**, *2019*, baz046. [CrossRef] [PubMed]
88. Ianevski, A.; Giri, A.K.; Aittokallio, T. Fully-automated and ultra-fast cell-type identification using specific marker combinations from single-cell transcriptomic data. *Nat. Commun.* **2022**, *13*, 1246. [CrossRef]
89. Galdos, F.X.; Xu, S.; Goodyer, W.R.; Duan, L.; Huang, Y.V.; Lee, S.; Zhu, H.; Lee, C.; Wei, N.; Lee, D.; et al. devCellPy is a machine learning-enabled pipeline for automated annotation of complex multilayered single-cell transcriptomic data. *Nat. Commun.* **2022**, *13*, 5271. [CrossRef]
90. Hou, W.; Ji, Z. Assessing GPT-4 for cell type annotation in single-cell RNA-seq analysis. *Nat. Methods* 2024. [CrossRef]
91. Adema, K.; Schon, M.A.; Nodine, M.D.; Kohlen, W. Lost in space: What single-cell RNA sequencing cannot tell you. *Trends Plant Sci.* 2024, *in press*. [CrossRef]
92. Spencer, T.E.; Lowke, M.T.; Davenport, K.M.; Kelleher, A.M. Single-cell insights into epithelial morphogenesis in the neonatal mouse uterus. *Proc. Natl. Acad. Sci. USA* **2023**, *120*, e2316410120. [CrossRef]
93. Marx, V. Publisher Correction: Method of the Year: Spatially resolved transcriptomics. *Nat. Methods* **2021**, *18*, 219. [CrossRef]
94. Williams, C.G.; Lee, H.J.; Asatsuma, T.; Vento-Tormo, R.; Haque, A. An introduction to spatial transcriptomics for biomedical research. *Genome Med.* **2022**, *14*, 68. [CrossRef]
95. Phan, Q.M.; Driskell, I.M.; Driskell, R.R. The Three Rs of Single-Cell RNA Sequencing: Reuse, Refine, and Resource. *J. Investig. Dermatol.* **2021**, *141*, 1627–1629. [CrossRef]
96. Grones, C.; Eekhout, T.; Shi, D.; Neumann, M.; Berg, L.S.; Ke, Y.; Shahan, R.; Cox, K.L.; Gomez-Cano, F.; Nelissen, H.; et al. Best practices for the execution, analysis, and data storage of plant single-cell/nucleus transcriptomics. *Plant Cell* **2024**, *36*, 812–828. [CrossRef] [PubMed]
97. Shafer, M.E.R. Cross-Species Analysis of Single-Cell Transcriptomic Data. *Front. Cell Dev. Biol.* **2019**, *7*, 175. [CrossRef] [PubMed]
98. Song, Y.; Miao, Z.; Brazma, A.; Papatheodorou, I. Benchmarking strategies for cross-species integration of single-cell RNA sequencing data. *Nat. Commun.* **2023**, *14*, 6495. [CrossRef] [PubMed]
99. Jiao, A.; Zhang, C.; Wang, X.; Sun, L.; Liu, H.; Su, Y.; Lei, L.; Li, W.; Ding, R.; Ding, C.; et al. Single-cell sequencing reveals the evolution of immune molecules across multiple vertebrate species. *J. Adv. Res.* **2024**, *55*, 73–87. [CrossRef]

100. Tang, X.; Huang, Y.; Lei, J.; Luo, H.; Zhu, X. The single-cell sequencing: New developments and medical applications. *Cell Biosci.* **2019**, *9*, 53. [CrossRef]
101. Fahlgren, N.; Kapoor, M.; Yordanova, G.; Papatheodorou, I.; Waese, J.; Cole, B.; Harrison, P.; Ware, D.; Tickle, T.; Paten, B.; et al. Toward a data infrastructure for the Plant Cell Atlas. *Plant Physiol.* **2023**, *191*, 35–46. [CrossRef] [PubMed]
102. Herrera-Uribe, J.; Wiarda, J.E.; Sivasankaran, S.K.; Daharsh, L.; Liu, H.; Byrne, K.A.; Smith, T.P.L.; Lunney, J.K.; Loving, C.L.; Tuggle, C.K. Reference Transcriptomes of Porcine Peripheral Immune Cells Created Through Bulk and Single-Cell RNA Sequencing. *Front. Genet.* **2021**, *12*, 689406. [CrossRef] [PubMed]
103. Shi, Z.X.; Chen, Z.C.; Zhong, J.Y.; Hu, K.H.; Zheng, Y.F.; Chen, Y.; Xie, S.Q.; Bo, X.C.; Luo, F.; Tang, C.; et al. High-throughput and high-accuracy single-cell RNA isoform analysis using PacBio circular consensus sequencing. *Nat. Commun.* **2023**, *14*, 2631. [CrossRef] [PubMed]
104. Bartosovic, M.; Kabbe, M.; Castelo-Branco, G. Single-cell CUT&Tag profiles histone modifications and transcription factors in complex tissues. *Nat. Biotechnol.* **2021**, *39*, 825–835. [CrossRef] [PubMed]
105. Zhang, R.; Zhou, T.; Ma, J. Multiscale and integrative single-cell Hi-C analysis with Higashi. *Nat. Biotechnol.* **2022**, *40*, 254–261. [CrossRef] [PubMed]
106. Qu, J.; Sun, J.; Zhao, C.; Liu, X.; Zhang, X.; Jiang, S.; Wei, C.; Yu, H.; Zeng, X.; Fan, L.; et al. Simultaneous profiling of chromatin architecture and transcription in single cells. *Nat. Struct. Mol. Biol.* **2023**, *30*, 1393–1402. [CrossRef] [PubMed]
107. Nichols, R.V.; O'Connell, B.L.; Mulqueen, R.M.; Thomas, J.; Woodfin, A.R.; Acharya, S.; Mandel, G.; Pokholok, D.; Steemers, F.J.; Adey, A.C. High-throughput robust single-cell DNA methylation profiling with sciMETv2. *Nat. Commun.* **2022**, *13*, 7627. [CrossRef] [PubMed]
108. Liu, H.; Zeng, Q.; Zhou, J.; Bartlett, A.; Wang, B.A.; Berube, P.; Tian, W.; Kenworthy, M.; Altshul, J.; Nery, J.R.; et al. Single-cell DNA methylome and 3D multi-omic atlas of the adult mouse brain. *Nature* **2023**, *624*, 366–377. [CrossRef]
109. Tuggle, C.K.; Clarke, J.L.; Murdoch, B.M.; Lyons, E.; Scott, N.M.; Beneš, B.; Campbell, J.D.; Chung, H.; Daigle, C.L.; Das Choudhury, S.; et al. Current challenges and future of agricultural genomes to phenomes in the USA. *Genome Biol.* **2024**, *25*, 8. [CrossRef]

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Review

Positive Effects of Physical Activity on Insulin Signaling

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Abstract: Physical activity is integral to metabolic health, particularly in addressing insulin resistance and related disorders such as type 2 diabetes mellitus (T2DM). Studies consistently demonstrate a strong association between physical activity levels and insulin sensitivity. Regular exercise interventions were shown to significantly improve glycemic control, highlighting exercise as a recommended therapeutic strategy for reducing insulin resistance. Physical inactivity is closely linked to islet cell insufficiency, exacerbating insulin resistance through various pathways including ER stress, mitochondrial dysfunction, oxidative stress, and inflammation. Conversely, physical training and exercise preserve and restore islet function, enhancing peripheral insulin sensitivity. Exercise interventions stimulate β-cell proliferation through increased circulating levels of growth factors, further emphasizing its role in maintaining pancreatic health and glucose metabolism. Furthermore, sedentary lifestyles contribute to elevated oxidative stress levels and ceramide production, impairing insulin signaling and glucose metabolism. Regular exercise induces anti-inflammatory responses, enhances antioxidant defenses, and promotes mitochondrial function, thereby improving insulin sensitivity and metabolic efficiency. Encouraging individuals to adopt active lifestyles and engage in regular exercise is crucial for preventing and managing insulin resistance and related metabolic disorders, ultimately promoting overall health and well-being.

Keywords: physical activity; insulin sensitivity; metabolic health; inflammation; insulin signaling; exercise interventions; type 2 diabetes; metabolic syndrome

1. Introduction

Insulin, an endocrine peptide hormone, interacts with receptors on cell membranes in specific cells to coordinate a comprehensive anabolic reaction to the presence of nutrients [1]. When glucose levels in the bloodstream rise to a certain point after eating, insulin is released by pancreatic β-cells. Typically, insulin facilitates the uptake of carbohydrates in vital locations for storage and utilization, like adipose tissue and skeletal muscle, where they are converted into lipids alongside proteins [2]. While insulin receptors are found in many somatic cell types, their role in maintaining glucose levels is mainly associated with their direct impact on the skeletal muscle, liver, and white adipocytes. These tissues have specific roles in metabolic balance, necessitating insulin's tissue-specific signaling pathways. For instance, in skeletal muscle, insulin enhances glucose uptake and storage by boosting glucose transport and glycogen synthesis. In the liver, insulin activates glycogen synthesis, increases lipogenic gene expression, and reduces gluconeogenic gene expression. In white adipose tissue (WAT), insulin suppresses lipolysis while promoting glucose transport and lipogenesis. Despite these varied effects, the initial components of insulin signal transduction are quite similar across insulin-sensitive cells. However, the diverse physiological responses to insulin in different cell types mainly arise from distinct downstream effectors [1].

Decreased responsiveness of tissues to insulin results in insulin resistance (IR), a multifaceted pathological state marked by diminished sensitivity. This condition impairs the ability to suppress glucose production and enhance peripheral glucose disposal, often

leading to elevated insulin levels to stabilize blood sugar levels [3]. IR is typified by compromised insulin-mediated blood glucose regulation, hindered glucose utilization, abnormal lipid accumulation, and heightened lipid breakdown in adipocytes. This constellation of symptoms is often referred to as insulin resistance syndrome or metabolic syndrome. IR is associated with conditions such as obesity, type 2 diabetes mellitus (T2DM) and its complications, non-alcoholic fatty liver disease (NAFLD), cancer, cardiovascular disease, and various other metabolic disorders [4]. The condition is considered a key precursor to the development of T2DM, as prolonged insulin resistance can lead to pancreatic β cell dysfunction and eventual failure to compensate for insulin resistance with increased insulin secretion [5,6]. Beyond its role in glucose metabolism, insulin resistance also affects lipid and protein metabolism. In adipose tissue, insulin resistance promotes lipolysis (breakdown of fats), leading to increased release of free fatty acids into the bloodstream, which contributes to dyslipidemia and ectopic fat deposition in non-adipose tissues [7]. Moreover, insulin resistance is associated with systemic inflammation and oxidative stress, further exacerbating metabolic dysfunction and increasing the risk of cardiovascular complications [8].

Given its profound implications for metabolic health, understanding and addressing insulin resistance are of paramount importance in preventing and managing metabolic disorders. Lifestyle modifications, including regular physical activity, healthy dietary habits, weight management, and stress reduction, play a crucial role in improving insulin sensitivity and mitigating the adverse effects of insulin resistance on metabolic health [9–11].

Physical activity plays a pivotal role in modulating the biomarkers associated with insulin resistance by exerting beneficial effects on various physiological pathways involved in glucose and lipid metabolism (Figure 1). Regular physical activity enhances insulin sensitivity and improves glycemic control through multiple mechanisms, thereby mitigating the development and progression of insulin resistance-related disorders such as T2DM, obesity, and cardiovascular disease. As most studies indicate, insulin resistance is associated with excessive lipid accumulation in skeletal muscle and the liver, which is also linked to adipose tissue [12]. For this reason, this review will focus on the insulin signaling pathway in skeletal muscle, liver and adipose tissue.

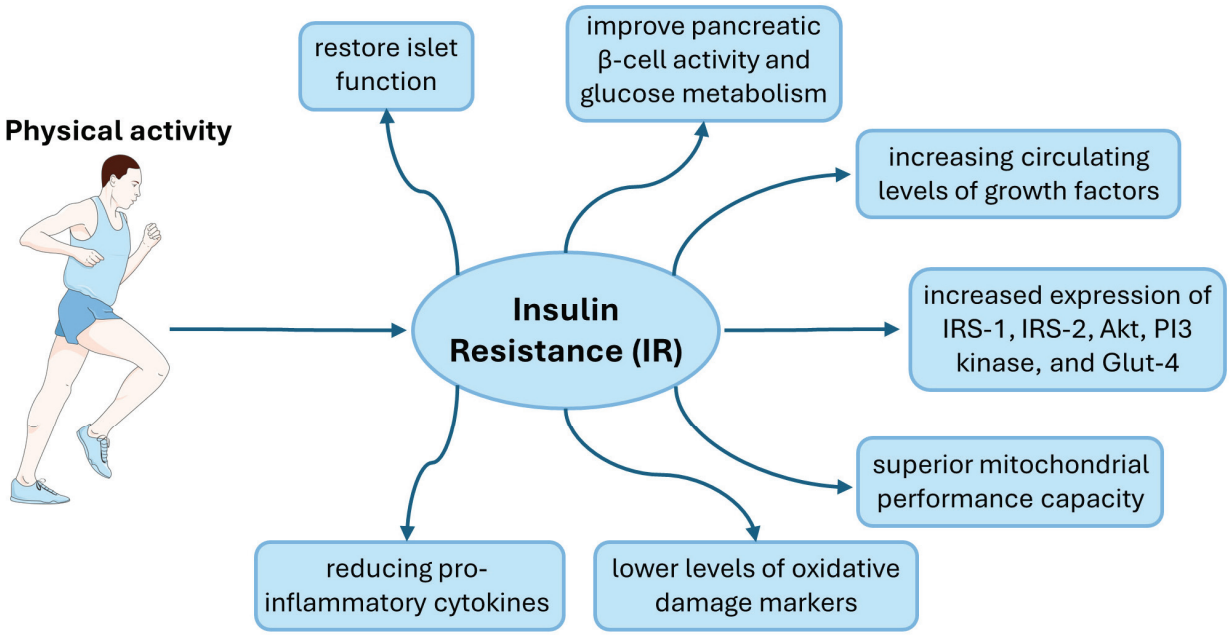


Figure 1. Graphic representation of the impact of physical activity in people with insulin resistance. This figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license.

2. Insulin Signaling Pathway

Insulin exerts its various physiological effects by binding to the insulin receptor (INSR) situated on the cell membrane of target cells. The INSR is a complex receptor composed of two extracellular α subunits, responsible for insulin binding, and two membrane-spanning β subunits, each containing a tyrosine kinase domain [13]. Among the two isoforms of INSR, isoform B is more specific for insulin and is predominantly expressed in mature liver, muscle, and WAT, mediating the majority of insulin's metabolic effects. In contrast, isoform A, prevalent during fetal development due to its high affinity for IGF-2, differs by the exclusion of exon 11 [14]. The INSR possesses two insulin binding sites but exhibits negative cooperativity, whereby insulin binding at one site decreases the affinity for insulin binding at the other site. Thus, evidence suggests that one insulin molecule activates one INSR under physiological conditions [15]. Upon insulin binding, the β-subunit undergoes a conformational change, leading to autophosphorylation of tyrosine residues and recruitment of various adaptor proteins, such as insulin receptor substrate (IRS), growth factor receptor-bound protein-2 (GRB-2), GRB-10, SHC-transforming protein (SHC), and SH2B adapter protein-2 (SH2B-2) [16].

Insulin's impact on glucose and lipid metabolism primarily occurs through a series of molecular events initiated by the insulin receptor tyrosine kinase (IRTK). This process involves the phosphorylation of insulin receptor substrate (IRS), which subsequently attracts phosphatidylinositol-3-OH kinase (PI3K). PI3K then triggers the conversion of phosphatidylinositol-4,5-bisphosphate (PIP2) into phosphatidylinositol-3,4,5-trisphosphate (PIP3). Akt, once recruited to the cell membrane by PIP3, becomes activated through phosphorylation by both 3-phosphoinositide-dependent kinase-1 (PDK1) and mechanistic target of rapamycin complex 2 (mTORC2) [17]. This activated Akt proceeds to phosphorylate a variety of downstream substrates in metabolic tissues such as skeletal muscle, liver, and adipose tissue (Figure 2). These phosphorylation events initiate insulin-induced mechanisms that promote the retention of nutrients within these tissues.

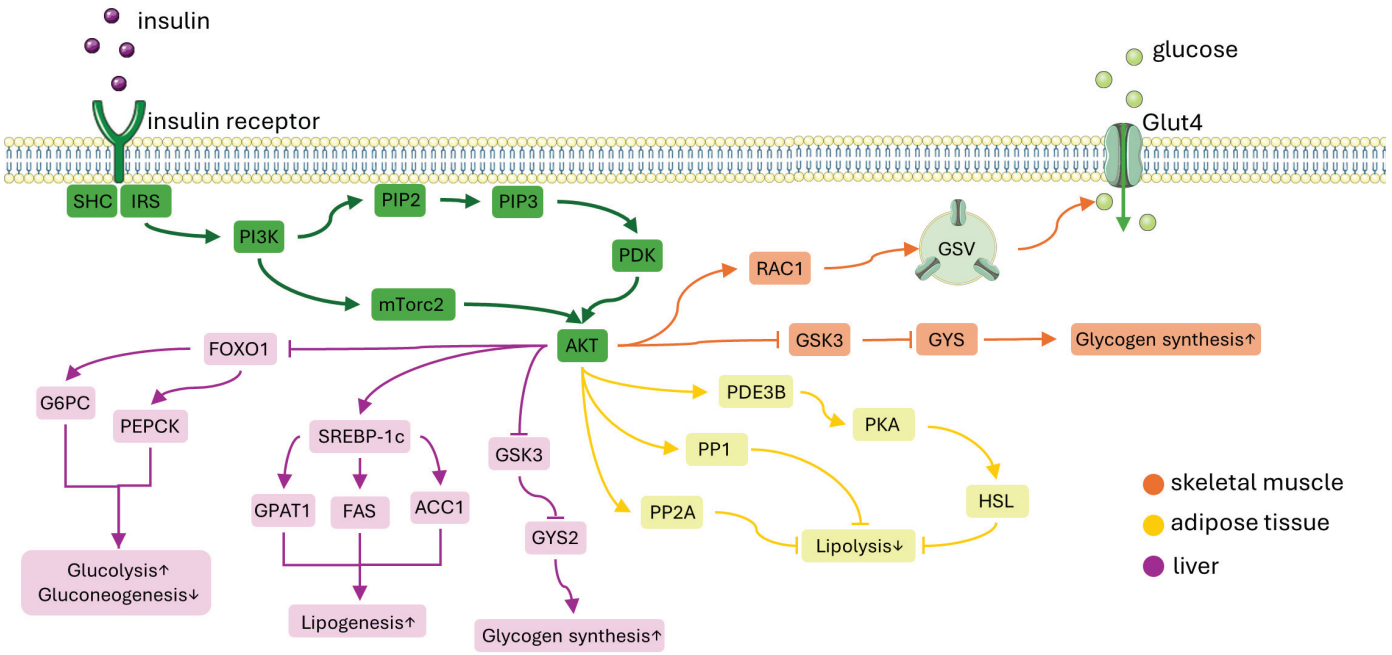


Figure 2. A simplified diagram of the insulin signaling pathway. Insulin attaches to the insulin receptor which activates IRS-1. This activation leads to the recruitment of PI3K and subsequently activates Akt. ORANGE PATH: In skeletal muscle, Akt facilitates glucose uptake by moving GSVs to the plasma membrane. This process involves the activation of the GTP-bound form of RAC1. Additionally,

insulin promotes glycogen synthesis by inhibiting GSK3, which activates GYS, and by inactivating glycogen phosphorylase through the dephosphorylation of phosphorylase kinase. YELLOW PATH: In adipose tissue, insulin inhibits lipolysis by decreasing the availability of gluconeogenic substrates. This suppression is thought to be mediated by PDE3B, PP1, and PP2A. PURPLE PATH: In the liver, Akt reduces gluconeogenesis by inhibiting the expression of gluconeogenic genes mediated by FOXO1. Insulin enhances hepatic glycogen synthesis by regulating GYS2 and GSK3. Moreover, insulin promotes lipogenesis by up-regulating SREBP-1c. Abbreviations: IRS-1, insulin receptor substrate-1; PI3K, phosphatidylinositol-3-OH kinase; GSVs, glucose transporter type 4 (GLUT4) storage vesicles; RAC1, Ras-related C3 botulinum toxin substrate 1; GYS, glycogen synthease; PDE3B, phosphodiesterase 3B; PP1, protein phosphatase 1; PP2A, protein phosphatase-2A; FOXO1, forkhead box O1; GSK3, glycogen synthase kinase 3; SREBP-1c, sterol regulatory element-binding protein 1c; PIP2, phosphatidylinositol-4,5-bisphosphate; PIP3, phosphatidylinositol-3,4,5-trisphosphate; mTORC1, mechanistic target of rapamycin complex 1; G6PC, glucose-6-phosphatase; PEPCK, phosphoenolpyruvate carboxykinase 1; GPAT1, glycerol-3-phosphate acyltransferase 1; ACC, acetyl-CoA carboxylase; FAS, fatty acid synthase. This figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license.

2.1. Insulin Signaling Pathway in Skeletal Muscle

Skeletal muscle is a highly energetically demanding tissue, requiring substantial resources to function efficiently. The energy stored within muscle cells is predominantly reserved for future needs. However, there is an exception with the three-carbon molecules, such as lactate and alanine. Lactate is formed by the reduction of pyruvate by NADH in a reaction catalyzed by lactate dehydrogenase as the end product of anaerobic glycolysis. Alanine can be formed from pyruvate and glutamate catalyzed by alanine aminotransferase. These molecules are released from the skeletal muscle and are transported to the liver, serving as substrates for further metabolic processes [1]. In skeletal muscle, insulin signaling plays a crucial role in facilitating glucose uptake and promoting net glycogen synthesis. Upon insulin activation, there is a coordinated process that enhances glucose transport activity through the translocation and fusion of glucose transporter type 4 (GLUT4) storage vesicles (GSVs) to the plasma membrane within skeletal muscle. This process is mediated by the inactivation of AS160 (GTPase-activating protein [GAP] AKT substrate of 160 kDa, also known as TBC1D4) by Akt, which, in turn, activates small Rab GTPase proteins that control vesicle trafficking [18]. Additionally, insulin-induced Akt activation promotes the GTP-bound form of Ras-related C3 botulinum toxin substrate 1 (RAC1), facilitating GLUT4 translocation by inducing the reorganization of cortical actin [19–21]. RAC1, a protein involved in cellular signaling, has direct targets such as p21-associated kinase (PAK). Insulin enhances the activation of RAC1, promoting its GTP-bound form, which in turn stimulates the phosphorylation of PAK by alleviating PAK autoinhibition [19,20]. When RAC1 is specifically knocked out in muscle cells, there is a significant impairment in insulin-stimulated glucose uptake, even though AKT activation remains intact [22]. Conversely, when constitutively active RAC1 is overexpressed in muscle cells, it induces GLUT4 translocation, facilitating glucose uptake, even in the absence of insulin stimulation [23].

Furthermore, insulin regulates net glycogen synthesis in skeletal muscle by suppressing glycogenolysis and promoting glycogen synthesis. This regulation occurs through several mechanisms. Firstly, insulin signaling enhances the activity of glycogen synthase (GYS) by phosphorylating glycogen synthase kinase 3 (GSK3) via Akt activation. Secondly, insulin activates protein phosphatase 1 (PP1), promoting the dephosphorylation of GYS, thus further enhancing glycogen synthesis [24,25]. PP1 phosphatase activity serves multiple targets in various cellular pathways. However, its specificity towards glycogen synthase is mediated by four glycogen-targeted PP1 regulatory subunits [25]. These regulatory subunits contain binding domains for PP1, GYS, and glycogen, functioning as metabolic scaffolds [26]. Among these regulatory subunits, G_M exhibits the highest expression in skeletal muscle. Mice lacking G_M expression demonstrate reduced muscle glycogen stores [27–29]. Insulin promotes the recruitment of PP1 to glycogen molecules

and enhances PP1 activity towards GYS. However, the precise molecular mechanisms underlying this action remain incompletely understood [26]. Conventionally, the combination of inactive glycogen synthase kinase 3 (GSK3) and active PP1 promotes the formation of active, dephosphorylated muscle glycogen synthase, thereby facilitating glycogen synthesis [30]. Additionally, insulin regulates glycogen phosphorylase activity by promoting the dephosphorylation of phosphorylase kinase. Collectively, these insulin-mediated processes promote glucose uptake and glycogen synthesis, contributing to the efficient storage of glucose within skeletal muscle cells [31].

2.2. Insulin Signaling Pathway in WAT

The primary physiological role of insulin in white adipose tissue is to inhibit lipolysis, which consequently suppresses hepatic glucose production (HGP) by reducing gluconeogenic substrates [32]. In vivo, white adipocytes exhibit high sensitivity to insulin [1]. Insulin's ability to regulate plasma levels of non-esterified fatty acids (NEFAs) is essential for maintaining normal blood glucose levels. Inhibition of lipolysis, a process by which triglycerides are broken down into NEFAs and glycerol, represents a significant physiological function of insulin in WAT [33,34]. The precise mechanism behind insulin-induced suppression of lipolysis is not entirely elucidated, but evidence suggests that phosphodiesterase 3B (PDE3B) plays a role through decreased activity of cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) [35]. PKA plays a crucial role in the regulation of WAT lipolysis by phosphorylating two key proteins: hormone-sensitive lipase (HSL) and perilipin (PLIN) [36]. HSL undergoes phosphorylation at three serine residues located at the COOH end, resulting in its translocation from the cytosol to the surface of lipid droplets [37,38]. The significance of HSL in the hormonal control of WAT lipolysis was underscored by the discovery of frame shift mutations in the human HSL gene. Individuals homozygous for this mutation lack HSL expression and exhibit severely impaired lipolysis regulation [39]. Perilipins, which encompass five isoforms with tissue-specific functions, are abundantly expressed [40]. PLIN1, predominantly found in white adipocytes, is phosphorylated by PKA at multiple serine residues [40,41]. While the precise functions of PLIN phosphorylation in lipolytic regulation are not fully elucidated, they are believed to involve several major mechanisms. Firstly, phosphorylation of PLIN reduces its affinity for the adipose triglyceride lipase (ATGL) cofactor CGI-58, facilitating CGI-58 binding to ATGL and thereby enhancing ATGL activity by approximately 20-fold [41]. Secondly, PLIN phosphorylation is crucial for the complete activation of HSL on the surface of lipid droplets [38]. Thirdly, PLIN phosphorylation was demonstrated to increase the surface area-to-volume ratio of lipid droplets by promoting the budding of lipid microbubbles [42]. PDE3B serves to degrade cAMP, thereby dampening prolipolytic PKA signaling towards HSL and PLIN. In Pde3b-deficient adipocytes, stimulated lipolysis remains unimpeded by insulin suppression, and Pde3b knockout mice exhibit impaired suppression of plasma NEFA levels during glucose tolerance tests [35].

Furthermore, protein phosphatase-1 (PP1) and protein phosphatase-2A (PP2A) seem to facilitate PI3K-dependent insulin-induced suppression of lipolysis by dephosphorylating lipolytic regulatory proteins [43,44]. PP2A emerges as the primary mediator responsible for the dephosphorylation of HSL, even in scenarios where PKA activity is undetectable [45]. Conversely, PP1 is identified as the primary perilipin phosphatase in adipocytes. In response to insulin, both the phosphorylation and activity of PP1 regulatory subunits are observed to increase [43].

Although insulin facilitates glucose transport by triggering phosphorylation of targets involved in vesicle tethering, docking, and fusion, its impact on whole-body glucose disposal is relatively modest [46]. Insulin also stimulates lipogenesis in white adipose tissue by activating sterol regulatory element-binding protein 1c (SREBP-1c), promoting the translocation of glucose or fatty acid transport proteins (FATPs), encouraging fatty acid esterification, and fostering adipogenesis through the transcription factor peroxisome proliferator-activated receptor-γ (PPARγ) [47,48].

2.3. Insulin Signaling Pathway in Liver

Insulin, produced by the pancreatic endocrine gland, is released into the portal vein. As a result, the liver is exposed to higher concentrations of insulin, typically two to three times greater than those found in the general circulation [49]. Insulin plays a crucial role in promoting the synthesis of all major classes of metabolic macromolecules, including glycogen, lipids, and proteins. Additionally, insulin rapidly and effectively reduces hepatic glucose production. This reduction in HGP is particularly significant because increased fasting concentrations of HGP and its insensitivity to insulin are characteristic features of type 2 diabetes. Therefore, measuring the suppression of HGP by insulin is a widely recognized physiological indicator of liver insulin sensitivity [1,49]. In the liver, insulin initiates a signaling cascade by activating INSR, which subsequently phosphorylates insulin receptor substrate 1 (IRS1) and IRS2. The different genetic disorders affecting IRS1 and IRS2 expression in the liver have not definitively outlined the distinct functions of any specific isoform [1]. This leads to the activation of Akt2, a pivotal regulator of hepatic glucose metabolism. Akt2 exerts several effects that collectively reduce HGP, promote glycogen synthesis, and enhance lipogenesis [50].

The primary function of insulin signaling in the liver is to decrease HGP by inhibiting gluconeogenesis. This occurs through Akt-induced phosphorylation of forkhead box O1 (FOXO1), which results in the exclusion of FOXO1 from the nucleus. As a consequence, FOXO1 is unable to activate the transcription of gluconeogenic genes, such as glucose-6-phosphatase (G6PC) and phosphoenolpyruvate carboxylase (PEPCK) [50,51]. Active FOXO1 additionally associates with the co-repressor SIN3A to diminish the expression of glucokinase, thereby enhancing the preference for glucose export [52]. Additionally, insulin suppresses hepatic gluconeogenesis by inhibiting adipocyte lipolysis, which reduces the availability of gluconeogenic substrates in the liver [33].

In addition to inhibiting gluconeogenesis, insulin promotes hepatic glycogen synthesis by regulating glycogen synthase (especially GYS2 in the liver) and glycogen phosphorylase. This regulation is mediated through glycogen synthase kinase 3 (GSK3) and PP1, similar to the mechanisms observed in skeletal muscle [53].

Furthermore, insulin activates lipid synthesis (lipogenesis) in the liver by up-regulating SREBP-1c, a master transcriptional regulator of hepatic de novo lipogenesis. Subsequently, SREBP-1c enhances the transcription of several lipogenic genes, including acetyl-CoA carboxylase 1 (ACC1), fatty acid synthase (FAS), and glycerol-3-phosphate acyltransferase 1 (GPAT1). This results in increased synthesis of fatty acids and triglycerides, contributing to lipid storage and energy metabolism in the liver [54,55]. Liver-specific overexpression of SREBP-1c alone is adequate to induce hepatic steatosis [56]. Insulin primarily influences SREBP-1c by enhancing its transcription, but it also facilitates SREBP-1c cleavage and its subsequent translocation into the nucleus, which are the conventional mechanisms of SREBP activation [54,57]. These actions of insulin can be hindered by inhibiting PI3K, AKT, or mTORC1, indicating that these kinases serve as upstream regulators of SREBP-1c [58].

3. Mechanisms of Insulin Resistance

Insulin resistance (IR) is a complex metabolic disorder characterized by impaired cellular responses to insulin, primarily affecting skeletal muscle, liver, and adipose tissue. Several underlying molecular and cellular mechanisms contribute to the development and progression of insulin resistance (Figure 3).

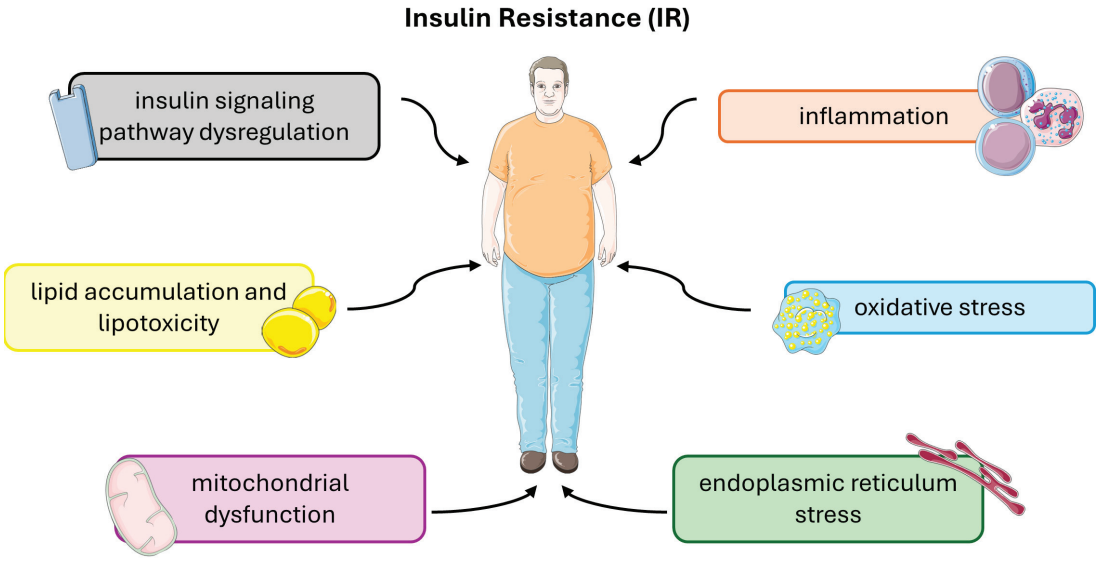


Figure 3. Molecular and cellular mechanisms that contribute to the development and progression of insulin resistance. This figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license.

3.1. Insulin Signaling Pathway Dysregulation

Insulin receptors (INSRs), functioning as tyrosine kinases, have a specific affinity for insulin and are pivotal in regulating insulin-dependent glucose homeostasis and cellular growth [59,60]. Impaired binding of INSR primarily refers to reduced affinity or numbers of receptors on the cell membrane, or structural abnormalities affecting insulin's interaction with the receptor [61]. The insulin receptor substrate protein serves as a critical node in the insulin signaling network and is closely linked to the development of insulin resistance. Molecularly, crosstalk between downstream nucleotide-binding oligomerization domain (NOD) 1 and the insulin receptor pathway can hinder insulin signaling by impeding the action of insulin receptor substrates [62].

Recent research indicates that insulin-stimulated kinases play a role in feedback phosphorylation of serine/threonine residues in IRS, contributing to the desensitization of proximal insulin signaling, which is significant in insulin resistance development [63]. For instance, a double-stranded RNA-dependent protein kinase (PKR) was found to elevate inhibitory IRS1 phosphorylation and IRS2 expression in liver and muscle cells, thereby modulating the insulin signaling pathway. PKR, facilitated by other protein kinases like JNK and IKK, heightens IRS1 phosphorylation at Ser312 and inhibits its tyrosine phosphorylation [64,65]. IκB kinase β (IKK-β) is a key mediator of inflammatory signaling pathways, particularly the NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) pathway. In conditions of chronic inflammation, such as obesity, IKKβ is activated by pro-inflammatory cytokines like TNF-α (tumor necrosis factor-alpha) and IL-6 (interleukin-6). Activated IKKβ phosphorylates IRS proteins on serine residues, leading to their degradation and impairing insulin signaling. Additionally, IKKβ activation promotes the expression of inflammatory genes that further exacerbate insulin resistance [66]. JNK (c-Jun N-terminal Kinase) is a stress-activated protein kinase that is activated by various stressors, including inflammatory cytokines, oxidative stress, and lipid intermediates [67]. Once activated, JNK phosphorylates IRS proteins on serine residues, interfering with their ability to transmit insulin signals [68]. JNK activation also promotes the expression of pro-inflammatory genes and induces apoptotic pathways [69], contributing to insulin resistance and pancreatic β-cells dysfunction.

Although the exact location of the defect within the insulin signaling pathway remains uncertain, numerous key components were identified. These components encompass proximal elements such as insulin receptors, insulin receptor substrates, PI3K, and AKT/PKB,

as well as distal elements representing various components downstream of AKT/PKB, including TBC1D4, GSK3, and PDE3B. Insulin resistance stems from malfunctions in one or more of these signaling components [70].

3.2. Inflammation and Oxidative Stress

Chronic low-grade inflammation and oxidative stress are associated with insulin resistance. Chronic low-grade inflammation, characterized by elevated levels of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β, is a hallmark of obesity and insulin resistance [71,72]. Adipose tissue, particularly visceral adipose tissue, serves as a major source of pro-inflammatory cytokines in obesity [73]. These cytokines activate inflammatory signaling pathways, such as the NF-κB pathway, in target tissues including skeletal muscle, liver, and adipose tissue. Activation of NF-κB leads to increased expression of inflammatory genes, including those encoding cytokines, chemokines, and adhesion molecules [74]. Inflammatory signaling pathways can directly interfere with insulin signaling by promoting serine phosphorylation of IRS proteins, impairing their ability to transmit insulin signals, as described earlier. Additionally, inflammation contributes to insulin resistance by promoting adipose tissue dysfunction, ectopic lipid accumulation, and systemic insulin resistance.

Oxidative stress arises from an imbalance between the production of reactive oxygen species (ROS) and the ability of antioxidant defense mechanisms to neutralize them [75]. ROS, including superoxide radicals, hydrogen peroxide, and hydroxyl radicals, are generated by various cellular processes, including mitochondrial respiration, NADPH oxidase activity, and enzymatic reactions [76]. In conditions of excess nutrient intake, mitochondrial dysfunction and increased flux through metabolic pathways, such as the electron transport chain and the pentose phosphate pathway, can lead to increased ROS production. Elevated ROS levels can damage cellular macromolecules, including lipids, proteins, and DNA, leading to cellular dysfunction and death [77]. ROS can also directly impair insulin signaling by inhibiting insulin receptor autophosphorylation and activating serine kinases such as JNK and IKKβ, which phosphorylate IRS proteins and interfere with insulin signaling [78]. Additionally, ROS promote the production of pro-inflammatory cytokines and chemokines, exacerbating inflammation and insulin resistance [79].

3.3. Lipid Accumulation and Lipotoxicity

Lipid accumulation and lipotoxicity are key factors contributing to the development of insulin resistance and metabolic dysfunction. Lipids, including triglycerides, cholesterol, and free fatty acids (FFAs), play essential roles as energy substrates and signaling molecules in cellular metabolism [80]. However, excessive lipid accumulation, particularly in non-adipose tissues such as skeletal muscle, liver, and pancreatic β-cells, can lead to cellular dysfunction and insulin resistance [81]. Lipid accumulation and lipotoxicity contribute to insulin resistance in several ways.

Ectopic lipid accumulation refers to the deposition of lipids, such as triglycerides and fatty acids, in tissues where they are not typically stored, such as skeletal muscle, liver, and pancreatic β-cells [82]. While adipose tissue serves as the primary site for lipid storage, excess lipid uptake or impaired lipid metabolism can lead to lipid spillover into non-adipose tissues, contributing to various metabolic dysfunctions [83]. Skeletal muscle is a major site for glucose uptake and utilization, making it crucial for maintaining glucose homeostasis [84]. In conditions of positive energy balance or impaired lipid metabolism, skeletal muscle can accumulate excess lipids, particularly intramuscular triglycerides (IMTGs) and fatty acid metabolites [85]. Ectopic lipid accumulation in skeletal muscle is strongly associated with insulin resistance, as it disrupts insulin signaling pathways and impairs glucose uptake and utilization. Lipid metabolites, such as diacylglycerol (DAG) and ceramides, can activate serine kinases, including protein kinase C (PKC) isoforms and JNK, leading to the phosphorylation of IRS and inhibition of insulin signaling [4,86,87]. The liver plays a central role in lipid metabolism, including fatty acid uptake, synthesis, oxidation, and export. Excessive dietary fat intake, increased lipolysis from adipose tissue,

or impaired hepatic lipid metabolism can lead to hepatic lipid accumulation, a condition known as non-alcoholic fatty liver disease (NAFLD) [88]. Hepatic lipid accumulation is strongly associated with insulin resistance and type 2 diabetes, as it disrupts hepatic insulin signaling and promotes gluconeogenesis, leading to increased hepatic glucose production [89]. Additionally, the accumulation of lipid intermediates, such as ceramides and DAG, can impair insulin signaling and promote hepatic inflammation and fibrosis [90]. Ectopic lipid accumulation in pancreatic β-cells has emerged as a potential contributor to β-cell dysfunction and insulin resistance. Excess lipid exposure can impair β-cell function by inducing lipotoxicity, oxidative stress, and ER stress, leading to impaired insulin secretion and glucose intolerance. Lipid metabolites, such as ceramides, were shown to inhibit insulin signaling pathways and promote β-cell apoptosis, contributing to the progression of type 2 diabetes [2]. Dysfunction of adipose tissue, characterized by adipocyte hypertrophy, inflammation, and altered adipokine secretion, can promote ectopic lipid accumulation in non-adipose tissues [74]. Enlarged adipocytes are less insulin-sensitive and exhibit increased lipolysis, releasing more fatty acids into circulation [91]. These excess fatty acids can then be taken up by non-adipose tissues, exacerbating ectopic lipid accumulation and insulin resistance.

3.4. Mitochondrial Dysfunction

Mitochondria serve as the cellular power stations, playing vital roles in fundamental cellular functions such as generating ATP, regulating intracellular calcium levels, producing and detoxifying reactive oxygen species, modulating apoptotic cell death, and activating caspase proteases [92]. Their dysfunction refers to impaired function of the mitochondria, the organelles responsible for cellular energy production through oxidative phosphorylation (OXPHOS) and various metabolic processes. Emerging evidence suggests that mitochondrial dysfunction plays a crucial role in the development of insulin resistance and metabolic disorders [93].

Mitochondrial dysfunction can lead to impaired oxidative phosphorylation, resulting in decreased ATP production and energy deficits in cells. Reduced ATP levels can impair cellular processes that require energy, including insulin-stimulated glucose uptake and metabolism. Skeletal muscle, which is a major site of glucose disposal, is particularly sensitive to mitochondrial dysfunction. Impaired mitochondrial function in skeletal muscle can lead to decreased mitochondrial ATP production, reduced glucose oxidation, and accumulation of lipid intermediates, contributing to insulin resistance [94].

Mitochondria are a major source of reactive oxygen species (ROS), which are natural byproducts of mitochondrial respiration. Under conditions of mitochondrial dysfunction, excessive ROS production can occur, leading to oxidative stress and damage to cellular macromolecules, including lipids, proteins, and DNA. ROS can also impair insulin signaling pathways by directly modifying IRS and inhibiting insulin-stimulated glucose uptake. Moreover, ROS-mediated oxidative stress can activate stress-sensitive kinases, such as JNK and IKK, which can phosphorylate IRS proteins on serine residues and interfere with insulin signaling [77,78].

Mitochondria undergo dynamic processes of fusion, fission, and mitophagy to maintain mitochondrial function and integrity [95,96]. Dysregulation of mitochondrial dynamics and impaired mitophagy can lead to the accumulation of dysfunctional mitochondria and mitochondrial DNA (mtDNA) damage. Accumulation of damaged mitochondria can further exacerbate oxidative stress and impair cellular function, contributing to insulin resistance. Additionally, impaired mitochondrial quality control mechanisms were implicated in the pathogenesis of metabolic disorders such as type 2 diabetes and obesity [92].

Mitochondrial dysfunction can also disrupt lipid metabolism, leading to ectopic lipid accumulation and lipotoxicity in insulin-sensitive tissues. Impaired mitochondrial β-oxidation of fatty acids can lead to increased accumulation of lipid intermediates, such as diacylglycerol (DAG) and ceramides, which can impair insulin signaling pathways and contribute to insulin resistance. Moreover, dysfunctional mitochondria may exhibit altered

substrate utilization and inefficient energy metabolism, further exacerbating metabolic dysfunction and insulin resistance [94].

4. Physical Activity and Insulin Resistance

Engaging in physical exercise encompasses activities that are systematically planned, structured, and undertaken to enhance or sustain physical fitness and overall health [97]. The advantages of physical exercise are extensive, impacting both physical and mental well-being [98]. Regular participation in physical exercise significantly enhances cardiovascular health, lowering the risk of heart disease, stroke, and hypertension. Additionally, it boosts bone density, thereby diminishing the likelihood of osteoporosis and fractures. Maintaining an active lifestyle aids in weight management, enhances insulin sensitivity, and decreases the risk of type 2 diabetes [99–102].

Physical exercise comes in many forms, including aerobic activities, strength training, and flexibility exercises. Aerobic activities, such as running, biking, and swimming, are highly effective for enhancing cardiovascular health and burning calories. Strength training, which includes exercises like weight lifting and resistance band routines, builds muscle strength and preserves bone density. Flexibility exercises, like yoga and stretching, enhance mobility and reduce injury risk. Exercise intensity and duration can differ greatly from person to person, making it challenging to assess the overall effectiveness of exercise programs.

Epidemiological studies have consistently demonstrated a strong association between physical activity levels and insulin sensitivity, highlighting the crucial role of exercise in metabolic health [103]. Regular exercise is widely recognized for its beneficial effects on metabolic health, particularly in addressing obesity and enhancing insulin sensitivity. A meta-analysis was conducted to evaluate the efficacy of structured exercise intervention programs for insulin resistance in T2DM. The findings underscore the effectiveness of regular exercise in improving glycemic control, thus warranting its recommendation as a therapeutic strategy for reducing IR, supported by a moderate level of evidence [104]. Studies have shown that physical activity with high energy expenditure and higher intensity has greater benefits for increasing insulin sensitivity. This includes high-intensity interval training (HIIT) [105].

Currently, the homeostasis model assessment of insulin resistance index, or HOMA-IR, is most commonly used to diagnose insulin resistance. It involves assessing the disruption of the homeostatic relationship between plasma insulin and glucose concentrations [106]. It was shown that during aerobic exercise, the intensity of which is gradually increased, a reduction in abdominal fat and subcutaneous fat is observed after 6 months. These changes are also believed to cause a 16% decrease in the HOMA-IR index [107]. However, a similar study using exercise training in which participants reduced visceral fat area after 12 weeks showed no effect on the HOMA-IR index [108]. However, it should be taken into account that this study lasted twice as long despite being conducted on a similar group. In addition, the authors emphasize that the HOMA-IR index does not take into account small changes within the body that can significantly affect changes in insulin sensitivity [109].

Strong evidence suggests that physical inactivity is closely linked to islet cell insufficiency. Adopting a sedentary lifestyle can exacerbate insulin resistance by imposing a greater workload on islets and diminishing their efficiency. This phenomenon occurs through various pathways, including ER stress, mitochondrial dysfunction, oxidative stress, and inflammation, ultimately promoting β-cell apoptosis and death [110]. Conversely, engaging in physical training and exercise serves to preserve and even restore islet function, thereby enhancing peripheral insulin sensitivity [111]. Furthermore, exercise can stimulate β-cell proliferation by increasing circulating levels of growth factors such as growth hormone, insulin-like growth factor 1 (IGF-1), and glucagon-like peptide 1 (GLP-1) [112]. Inactive individuals were found to exhibit lower β-cell sufficiency compared to trained individuals. Long periods of inactivity lead to increased fasting plasma glucose levels, while moderate-to-high-intensity exercise can reverse these changes, improving islet function

and glucose metabolism [113]. Sedentary individuals also show reduced insulin sensitivity and β-cell sufficiency in response to carbohydrate intake [111]. Moreover, individuals with lower physical activity levels, including T2DM patients, exhibit compromised islet function and irregular glucose metabolism compared to those engaged in aerobic exercise [114]. Short-term exercise interventions were shown to improve pancreatic β-cell activity and glucose metabolism [115]. Additionally, studies in animal models have demonstrated that inactive obese diabetic rats have lower pancreatic β-cell function compared to exercise groups [116,117]. These findings collectively emphasize the importance of physical activity in preserving and enhancing islet function while underscoring the detrimental effects of physical inactivity on β-cell health.

Physical inactivity was linked to disruptions in the expression, translocation, and function of genes and proteins involved in glucose homeostasis, while aerobic exercise was shown to stimulate these genes [10,118–120]. GLUT4, crucial for insulin-stimulated glucose uptake, is particularly affected. Research indicates that physical inactivity can lead to decreased *GLUT4* expression in skeletal muscles, contributing to insulin resistance. Studies using the hyperinsulinemic–euglycemic clamp method have demonstrated that prolonged bed rest significantly down-regulated genes related to insulin signaling, including *GLUT4*, *HK2* (hexokinase 2), *RRAD* (Ras-related glycolysis inhibitor and calcium channel regulator), and *TXNIP*, thereby reducing insulin sensitivity in skeletal muscles [119]. Conversely, evidence suggests that physical activity increases the expression of *IRS1*, *IRS2*, *Akt*, *PI3 kinase*, and *GLUT4* in animal models [121]. Similarly, studies in humans have shown that short-term bed rest interventions result in downregulation of GLUT4, HK2, GS, and Akt proteins, leading to decreased insulin sensitivity in skeletal muscles [118]. These findings underscore the importance of regular physical activity in maintaining proper gene expression and insulin sensitivity in skeletal muscles.

As was pointed out, inflammation may play a role in insulin resistance. However, in the context of skeletal muscle, no macrophages, lymphocytes or granulocytes were observed in myocytes in images obtained by electron microscopy [122]. For this reason, inflammation in skeletal muscle is thought to be caused by the presence of immune cells in perimuscular adipose tissue [123]. It is the inflammation of adipose tissue that is increasingly recognized as an activating factor in the development of IR. However, despite the clear contribution of adipose tissue to the development of the disorder, there are still few reports on the effect of physical activity on it [12]. There are many studies confirming the effect of physical activity on plasma levels of specific pro- and anti-inflammatory cytokines [124]. Studies indicate that a sedentary lifestyle can lead to increased inflammatory markers. Replacing sedentary activities with physical activity resulted in a reduction in circulating cytokines and an improvement in insulin sensitivity among obese adults [125]. Additional evidence suggested that even short periods of physical inactivity in healthy individuals could trigger inflammatory responses and increase the risk of insulin resistance and type 2 diabetes mellitus [126]. Regular physical exercise is consistently linked to alterations in inflammatory cytokine profiles, favoring a shift towards anti-inflammatory responses. Notably, exercise was shown to elevate levels of anti-inflammatory cytokines while reducing pro-inflammatory ones, such as TNF-α and IL-1β, which are implicated in the pathogenesis of diabetes [127,128]. Furthermore, studies have demonstrated that exercise interventions, particularly in combination with dietary modifications, lead to reductions in circulating levels of inflammatory markers like IL-6, IL-18, and CRP (C-reactive protein), coupled with increased levels of adiponectin, a hormone with potent anti-inflammatory and insulin-sensitizing effects [129,130]. Additionally, physically active individuals tend to exhibit lower levels of leptin, a hormone associated with inflammation [131]. Importantly, exercise exerts anti-inflammatory effects by improving endothelial function and reducing peripheral markers of endothelial dysfunction, including soluble intracellular and vascular adhesion molecules, as well as granulocyte-macrophage colony-stimulating factor [132]. Given the undeniable contribution of adipose tissue to the development of IR, adipokines, which are signaling molecules produced and released by adipose tissue, also deserve

special attention. Studies have shown that prolonged exercise affects the expression of leptin and adiponectin in subcutaneous adipose tissue in people at risk of developing T2DM. Compared to a control group containing healthy subjects, the expression of these adipokines normalized [133].

There is compelling evidence suggesting that physical inactivity contributes to increased oxidative stress levels [134]. Studies have indicated that individuals with sedentary lifestyles exhibit higher levels of oxidative damage markers, such as elevated malondialdehyde (MDA) and reduced total antioxidant capacity (TAC) in plasma, compared to their physically active counterparts [135]. Additionally, research has shown that inactive older individuals have diminished activity of key antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), along with higher plasma MDA levels [136]. Interestingly, acute bouts of exercise were observed to transiently elevate reactive oxygen and nitrogen species (RONS) levels; however, this phenomenon serves as a stimulus for the up-regulation of endogenous antioxidant defenses [137]. Indeed, studies in untrained animals have demonstrated increased oxidant levels following acute exercise [138,139], yet long-term exercise training counteracts this effect by promoting the expression of antioxidant enzymes, consequently reducing free radical production. Further evidence from animal studies has revealed that endurance training leads to elevated levels of antioxidants and antioxidant enzymes in skeletal and cardiac muscles, thereby providing protection against oxidative stress [140–142].

There is compelling evidence suggesting that physical inactivity contributes to increased ceramide production [143]. Studies have shown that prolonged periods of inactivity lead to impaired insulin sensitivity by disrupting the trafficking and metabolism of lipids, including ceramides, in both cellular and plasma compartments. For instance, research has demonstrated that just two months of bed rest can result in the accumulation of saturated fats and sphingosine in muscle cells, ultimately impairing insulin sensitivity [144]. Similarly, findings from animal studies indicate that 14 days of inactivity can elevate ceramide levels, disrupt insulin signaling in skeletal muscle, and worsen glucose tolerance [145]. Moreover, direct evidence from studies on obese volunteers has linked increased muscle ceramide levels during physical inactivity to insulin resistance. Conversely, acute exercise was shown to reduce sphingolipid synthesis during recovery periods, leading to improved insulin sensitivity in trained individuals [146]. However, conflicting reports on the short-term effects of inactivity on ceramide levels and glucose homeostasis suggest that ceramides may require more prolonged exposure to exert pathogenic effects and disrupt insulin signaling [147].

Considerable evidence indicates that physical inactivity and sedentary behaviors have adverse effects on mitochondrial function [148,149]. Trained individuals typically demonstrate superior mitochondrial performance capacity compared to their sedentary counterparts [50]. For example, prolonged periods of inactivity were shown to impair mitochondrial respiratory function in skeletal muscles, leading to decreased mitochondrial oxidative capacity due to oxidative damage in immobilized tissues [149]. Furthermore, inactive subjects tend to exhibit lower mitochondrial oxidative capacity compared to their physically active counterparts. Active older adults, in particular, often display improved mitochondrial capacity, highlighting mitochondria as a critical therapeutic target for sedentary-related complications and insulin resistance [148]. Clinical evidence has also linked physical inactivity-induced insulin resistance to significant changes in mitochondrial genes involved in glucose metabolism [119]. A link between obesity and mitochondrial dysfunction was also observed. Expression of the mitochondrial gene *ESR1* in adipocytes, which is responsible for encoding estrogen receptor α (ERα), may be dependent on obesity [150]. Studies in animal models have shown that deletion of ERα from adipocytes increases their size and contributes to obesity [151]. However, long-term physical activity improves ERα function and increases the number of mitochondria in adipose tissue which then reduces insulin resistance [152].

A summary of the effect of physical activities on insulin resistance is shown in Table 1.

Table 1. Effect of physical activities on insulin resistance.

Mechanism	Research Group	Physical Activity	Impact of Physical Activity	References
Islet cells	12 type 2 diabetic male patients	12-week programme on ergometer cycle (5 sessions/week)	preserve and even restore islet function, thereby enhancing peripheral insulin sensitivity	[111]
	40 type 2 diabetic patients	accumulated million steps for 1 year	even short-term exercise interventions improve pancreatic β-cell activity and glucose metabolism	[113]
Genes and proteins	female Wistar rats	two 3 h exercise bouts, separated by one 45 min rest period	increased expression of IRS-1, IRS-2, Akt, PI3 kinase, and Glut-4	[121]
Inflammation	General data from review articles. A detailed summary was presented in a previous paper [124]		increased levels of anti-inflammatory cytokines and reduced pro-inflammatory ones	[124,127,128]
	100 healthy volunteers (48 men, and 52 women)	lifestyle evaluation	decreased level of leptin	[131]
Oxidative Stress	75 type 2 diabetic patients	based on Global Physical Activity Questionnaire	lower levels of oxidative damage markers, such as MDA and higher TAC in plasma	[135]
	87 older males	engage in any physical activity every day for 12 months	higher activity of key antioxidant enzymes like SOD, CAT and GPx	[136]
	344 male rats	10 weeks of exercise training	endurance training leads to elevated levels of antioxidants and antioxidant enzymes in skeletal and cardiac muscles	[140–142]
Lipid Accumulation and Lipotoxicity	14 obese sedentary individuals, 15 type 2 diabetic patients and 15 endurance-trained athletes	ergometer cycle for 1.5 h	acute exercise reduces sphingolipid synthesis during recovery periods, leading to improved insulin sensitivity in trained individuals	[146]
Mitochondrial Dysfunction	26 sedentary (<1 exercise session/week) men	12-week intensive exercise intervention	increased number of mitochondria in adipose tissue	[152]

It is also important in this review to highlight gender differences that may affect insulin resistance. A woman's body is hormonally and biologically different from a man's, which also contributes to a different response to physical activity. One such difference is the presence of the female sex hormone, estrogen. It was suggested that estrogen protects against the development of insulin resistance through two mechanisms: modulating the metabolic processes involved in energy balance and down-regulating and/or repressing inflammation [153]. For this reason, men are thought to be more susceptible to metabolic syndromes. However, the situation is different for post-menopausal women, as with decreasing estrogen levels, their risk of developing IR increases. Studies have also proven that menopause is a factor that increases the risk of weight gain and impairs glucose tolerance [154,155]. On the other hand, studies have proven that the incidence of metabolic syndrome and diabetes is closely related to testosterone and changes in abdominal body composition [156]. It was proven that low testosterone levels are strongly correlated

with the incidence of insulin resistance and type 2 diabetes [157]. Also common is high visceral obesity with low testosterone levels. It was suggested that changes in abdominal body composition are a secondary consequence of metabolic syndrome [158]. In a study examining gender differences in the effect of type 2 diabetes on exercise performance, a decrease in peak oxygen consumption was observed in diabetic men and women compared to controls. However, it should be noted that this abnormality was significantly greater in the female group (24%) than in the male group (16%) [159]. Another study comparing the effects of physical activity on IR in both men and women with type 2 diabetes involved a 12-week training program consisting of aerobic and resistance training three days a week. The men showed increased improvements in peak oxygen consumption and stroke volume index compared to the women's group. However, only women significantly improved heart rate recovery (after 1 min, 48%), insulin resistance (25%), insulin C-peptide (27%) and right ventricular systolic function (21%) [160]. Another study looked at the relationship between participation in strength training and insulin resistance. This was a cross-sectional study using data from the National Health and Nutrition Examination Survey. The study found no significant association between strength training and insulin resistance in women. However, higher HOMA-IR was observed in men in the absence of strength training compared to men who reported moderate to high levels of strength training. From the data, it was also estimated that men who did not strength train had a 2.42 times higher risk of insulin resistance compared to men who exercised at a moderate level and a 2.5 times higher risk compared to men who strength trained intensively [161].

5. Conclusions

Studies consistently highlight the association between physical activity levels and insulin sensitivity, underscoring exercise's pivotal role in metabolic health. Regular exercise is widely acknowledged for its positive effects on metabolic parameters, particularly in addressing obesity and enhancing insulin sensitivity. A meta-analysis evaluating structured exercise intervention programs for insulin resistance in T2DM reinforced the effectiveness of regular exercise in improving glycemic control, substantiating its recommendation as a therapeutic approach for reducing insulin resistance. One of the key pathways through which exercise exerts its beneficial effects is by modulating ceramide production. Ceramides, lipid molecules associated with insulin resistance, are influenced by physical activity levels. Regular exercise was shown to reduce ceramide levels, thereby improving cellular lipid metabolism and insulin sensitivity.

Mitochondrial dysfunction is another target of physical activity interventions aimed at improving insulin sensitivity. Sedentary behaviors are associated with impaired mitochondrial function, leading to decreased oxidative capacity and tissue damage. In contrast, regular exercise promotes mitochondrial biogenesis and enhances mitochondrial function, thereby enhancing cellular energy production and metabolic efficiency. By improving mitochondrial function, exercise helps alleviate oxidative stress and mitigate insulin resistance.

Furthermore, physical activity has anti-inflammatory effects that can counteract the chronic low-grade inflammation observed in individuals with insulin resistance. Sedentary lifestyles are associated with elevated levels of inflammatory cytokines, which contribute to the development of metabolic disorders. Regular exercise was shown to reduce systemic inflammation by decreasing circulating levels of pro-inflammatory cytokines and increasing anti-inflammatory cytokines. By modulating inflammatory pathways, exercise helps improve insulin sensitivity and metabolic health.

In addition to its anti-inflammatory effects, physical activity positively influences pancreatic beta cell function and insulin secretion. Sedentary behaviors increase the workload on pancreatic islets, leading to impaired insulin secretion and reduced efficiency in glucose metabolism. Conversely, regular exercise preserves and enhances β-cell function, promoting insulin secretion and glucose homeostasis. Aerobic exercise, resistance training, and high-intensity interval training have all been shown to improve beta cell function and insulin sensitivity.

Overall, physical activity has multifaceted effects on insulin resistance, targeting various mechanisms involved in metabolic dysfunction. By reducing ceramide production, improving mitochondrial function, and modulating inflammatory pathways, exercise helps enhance insulin sensitivity and promote metabolic health. Additionally, exercise promotes β-cell function and insulin secretion, further contributing to glucose homeostasis. Furthermore, physical inactivity disrupts gene and protein expression involved in glucose homeostasis, leading to decreased insulin sensitivity. In contrast, aerobic exercise up-regulates key genes related to insulin signaling, such as *GLUT4*, promoting insulin-stimulated glucose uptake and improving insulin sensitivity in skeletal muscles. Physical inactivity also contributes to elevated oxidative stress levels, characterized by increased oxidative damage markers and diminished antioxidant enzyme activity. In contrast, regular exercise enhances antioxidant defenses, reducing free radical production and mitigating oxidative stress-induced insulin resistance.

Encouraging individuals to adopt active lifestyles and engage in regular exercise is essential for preventing and managing insulin resistance and related metabolic disorders. Public health initiatives aimed at promoting physical activity can have significant benefits for improving metabolic health and reducing the burden of insulin resistance and type 2 diabetes. By addressing the underlying mechanisms of insulin resistance, physical activity interventions offer promising strategies for improving overall health and well-being.

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References

1. Petersen, M.C.; Shulman, G.I. Mechanisms of Insulin Action and Insulin Resistance. *Physiol. Rev.* **2018**, *98*, 2133–2223. [CrossRef] [PubMed]
2. Lee, S.-H.; Park, S.-Y.; Choi, C.S. Insulin Resistance: From Mechanisms to Therapeutic Strategies. *Diabetes Metab. J.* **2022**, *46*, 15–37. [CrossRef] [PubMed]
3. Bugianesi, E.; McCullough, A.J.; Marchesini, G. Insulin Resistance: A Metabolic Pathway to Chronic Liver Disease. *Hepatology* **2005**, *42*, 987–1000. [CrossRef] [PubMed]
4. Zhao, X.; An, X.; Yang, C.; Sun, W.; Ji, H.; Lian, F. The Crucial Role and Mechanism of Insulin Resistance in Metabolic Disease. *Front. Endocrinol.* **2023**, *14*, 1149239. [CrossRef]
5. Kasuga, M. Insulin Resistance and Pancreatic Beta Cell Failure. *J. Clin. Investig.* **2006**, *116*, 1756–1760. [CrossRef]
6. Kahn, S.E. The Relative Contributions of Insulin Resistance and Beta-Cell Dysfunction to the Pathophysiology of Type 2 Diabetes. *Diabetologia* **2003**, *46*, 3–19. [CrossRef] [PubMed]
7. Morigny, P.; Houssier, M.; Mouisel, E.; Langin, D. Adipocyte Lipolysis and Insulin Resistance. *Biochimie* **2016**, *125*, 259–266. [CrossRef] [PubMed]
8. Kosmas, C.E.; Bousvarou, M.D.; Kostara, C.E.; Papakonstantinou, E.J.; Salamou, E.; Guzman, E. Insulin Resistance and Cardiovascular Disease. *J. Int. Med. Res.* **2023**, *51*, 03000605231164548. [CrossRef] [PubMed]
9. Sung, K.-C.; Lee, M.Y.; Kim, Y.-H.; Huh, J.-H.; Kim, J.-Y.; Wild, S.H.; Byrne, C.D. Obesity and Incidence of Diabetes: Effect of Absence of Metabolic Syndrome, Insulin Resistance, Inflammation and Fatty Liver. *Atherosclerosis* **2018**, *275*, 50–57. [CrossRef]
10. Yaribeygi, H.; Atkin, S.L.; Simental-Mendía, L.E.; Sahebkar, A. Molecular Mechanisms by Which Aerobic Exercise Induces Insulin Sensitivity. *J. Cell Physiol.* **2019**, *234*, 12385–12392. [CrossRef]
11. Yaribeygi, H.; Maleki, M.; Sathyapalan, T.; Jamialahmadi, T.; Sahebkar, A. Pathophysiology of Physical Inactivity-Dependent Insulin Resistance: A Theoretical Mechanistic Review Emphasizing Clinical Evidence. *J. Diabetes Res.* **2021**, *2021*, 7796727. [CrossRef] [PubMed]
12. Lee-Ødegård, S.; Olsen, T.; Norheim, F.; Drevon, C.A.; Birkeland, K.I. Potential Mechanisms for How Long-Term Physical Activity May Reduce Insulin Resistance. *Metabolites* **2022**, *12*, 208. [CrossRef]
13. Hubbard, S.R. The Insulin Receptor: Both a Prototypical and Atypical Receptor Tyrosine Kinase. *Cold Spring Harb. Perspect. Biol.* **2013**, *5*, a008946. [CrossRef]
14. Belfiore, A.; Malaguarnera, R.; Vella, V.; Lawrence, M.C.; Sciacca, L.; Frasca, F.; Morrione, A.; Vigneri, R. Insulin Receptor Isoforms in Physiology and Disease: An Updated View. *Endocr. Rev.* **2017**, *38*, 379–431. [CrossRef] [PubMed]

15. De Meyts, P. The Insulin Receptor: A Prototype for Dimeric, Allosteric Membrane Receptors? *Trends Biochem. Sci.* **2008**, *33*, 376–384. [CrossRef]
16. Youngren, J.F. Regulation of Insulin Receptor Function. *Cell. Mol. Life Sci.* **2007**, *64*, 873–891. [CrossRef]
17. Khalid, M.; Alkaabi, J.; Khan, M.A.B.; Adem, A. Insulin Signal Transduction Perturbations in Insulin Resistance. *Int. J. Mol. Sci.* **2021**, *22*, 8590. [CrossRef] [PubMed]
18. Leto, D.; Saltiel, A.R. Regulation of Glucose Transport by Insulin: Traffic Control of GLUT4. *Nat. Rev. Mol. Cell Biol.* **2012**, *13*, 383–396. [CrossRef]
19. Chiu, T.T.; Jensen, T.E.; Sylow, L.; Richter, E.A.; Klip, A. Rac1 Signalling towards GLUT4/Glucose Uptake in Skeletal Muscle. *Cell. Signal.* **2011**, *23*, 1546–1554. [CrossRef]
20. Sylow, L.; Kleinert, M.; Pehmøller, C.; Prats, C.; Chiu, T.T.; Klip, A.; Richter, E.A.; Jensen, T.E. Akt and Rac1 Signaling Are Jointly Required for Insulin-Stimulated Glucose Uptake in Skeletal Muscle and Downregulated in Insulin Resistance. *Cell. Signal.* **2014**, *26*, 323–331. [CrossRef]
21. Belman, J.P.; Habtemichael, E.N.; Bogan, J.S. A Proteolytic Pathway That Controls Glucose Uptake in Fat and Muscle. *Rev. Endocr. Metab. Disord.* **2014**, *15*, 55–66. [CrossRef] [PubMed]
22. Sylow, L.; Jensen, T.E.; Kleinert, M.; Højlund, K.; Kiens, B.; Wojtaszewski, J.; Prats, C.; Schjerling, P.; Richter, E.A. Rac1 Signaling Is Required for Insulin-Stimulated Glucose Uptake and Is Dysregulated in Insulin-Resistant Murine and Human Skeletal Muscle. *Diabetes* **2013**, *62*, 1865–1875. [CrossRef]
23. Ueda, S.; Kitazawa, S.; Ishida, K.; Nishikawa, Y.; Matsui, M.; Matsumoto, H.; Aoki, T.; Nozaki, S.; Takeda, T.; Tamori, Y.; et al. Crucial Role of the Small GTPase Rac1 in Insulin-Stimulated Translocation of Glucose Transporter 4 to the Mouse Skeletal Muscle Sarcolemma. *FASEB J.* **2010**, *24*, 2254–2261. [CrossRef] [PubMed]
24. Cross, D.A.; Alessi, D.R.; Cohen, P.; Andjelkovich, M.; Hemmings, B.A. Inhibition of Glycogen Synthase Kinase-3 by Insulin Mediated by Protein Kinase B. *Nature* **1995**, *378*, 785–789. [CrossRef] [PubMed]
25. Newgard, C.B.; Brady, M.J.; O'Doherty, R.M.; Saltiel, A.R. Organizing Glucose Disposal: Emerging Roles of the Glycogen Targeting Subunits of Protein Phosphatase-1. *Diabetes* **2000**, *49*, 1967–1977. [CrossRef] [PubMed]
26. Jurczak, M.J.; Danos, A.M.; Rehrmann, V.R.; Brady, M.J. The Role of Protein Translocation in the Regulation of Glycogen Metabolism. *J. Cell Biochem.* **2008**, *104*, 435–443. [CrossRef] [PubMed]
27. Brady, M.J.; Saltiel, A.R. The Role of Protein Phosphatase-1 in Insulin Action. *Recent Prog. Horm. Res.* **2001**, *56*, 157–173. [CrossRef] [PubMed]
28. Delibegovic, M.; Armstrong, C.G.; Dobbie, L.; Watt, P.W.; Smith, A.J.H.; Cohen, P.T.W. Disruption of the Striated Muscle Glycogen Targeting Subunit PPP1R3A of Protein Phosphatase 1 Leads to Increased Weight Gain, Fat Deposition, and Development of Insulin Resistance. *Diabetes* **2003**, *52*, 596–604. [CrossRef] [PubMed]
29. Suzuki, Y.; Lanner, C.; Kim, J.H.; Vilardo, P.G.; Zhang, H.; Yang, J.; Cooper, L.D.; Steele, M.; Kennedy, A.; Bock, C.B.; et al. Insulin Control of Glycogen Metabolism in Knockout Mice Lacking the Muscle-Specific Protein Phosphatase PP1G/RGL. *Mol. Cell. Biol.* **2001**, *21*, 2683–2694. [CrossRef]
30. Cohen, P.; Frame, S. The Renaissance of GSK3. *Nat. Rev. Mol. Cell Biol.* **2001**, *2*, 769–776. [CrossRef]
31. Agius, L. Role of Glycogen Phosphorylase in Liver Glycogen Metabolism. *Mol. Asp. Med.* **2015**, *46*, 34–45. [CrossRef] [PubMed]
32. Rebrin, K.; Steil, G.M.; Mittelman, S.D.; Bergman, R.N. Causal Linkage between Insulin Suppression of Lipolysis and Suppression of Liver Glucose Output in Dogs. *J. Clin. Investig.* **1996**, *98*, 741–749. [CrossRef] [PubMed]
33. Perry, R.J.; Camporez, J.-P.G.; Kursawe, R.; Titchenell, P.M.; Zhang, D.; Perry, C.J.; Jurczak, M.J.; Abudukadier, A.; Han, M.S.; Zhang, X.-M.; et al. Hepatic Acetyl CoA Links Adipose Tissue Inflammation to Hepatic Insulin Resistance and Type 2 Diabetes. *Cell* **2015**, *160*, 745–758. [CrossRef] [PubMed]
34. Reaven, G.M. Banting Lecture 1988. Role of Insulin Resistance in Human Disease. *Diabetes* **1988**, *37*, 1595–1607. [CrossRef]
35. Choi, Y.H.; Park, S.; Hockman, S.; Zmuda-Trzebiatowska, E.; Svennelid, F.; Haluzik, M.; Gavrilova, O.; Ahmad, F.; Pepin, L.; Napolitano, M.; et al. Alterations in Regulation of Energy Homeostasis in Cyclic Nucleotide Phosphodiesterase 3B–Null Mice. *J. Clin. Investig.* **2006**, *116*, 3240–3251. [CrossRef]
36. Jaworski, K.; Sarkadi-Nagy, E.; Duncan, R.E.; Ahmadian, M.; Sul, H.S. Regulation of Triglyceride Metabolism. IV. Hormonal Regulation of Lipolysis in Adipose Tissue. *Am. J. Physiol. Gastrointest. Liver Physiol.* **2007**, *293*, G1–G4. [CrossRef] [PubMed]
37. Holm, C. Molecular Mechanisms Regulating Hormone-Sensitive Lipase and Lipolysis. *Biochem. Soc. Trans.* **2003**, *31*, 1120–1124. [CrossRef]
38. Sztalryd, C.; Xu, G.; Dorward, H.; Tansey, J.T.; Contreras, J.A.; Kimmel, A.R.; Londos, C. Perilipin A Is Essential for the Translocation of Hormone-Sensitive Lipase during Lipolytic Activation. *J. Cell Biol.* **2003**, *161*, 1093–1103. [CrossRef] [PubMed]
39. Albert, J.S.; Yerges-Armstrong, L.M.; Horenstein, R.B.; Pollin, T.I.; Sreenivasan, U.T.; Chai, S.; Blaner, W.S.; Snitker, S.; O'Connell, J.R.; Gong, D.-W.; et al. Null Mutation in Hormone-Sensitive Lipase Gene and Risk of Type 2 Diabetes. *N. Engl. J. Med.* **2014**, *370*, 2307. [CrossRef]
40. Brasaemle, D.L. Thematic Review Series: Adipocyte Biology. The Perilipin Family of Structural Lipid Droplet Proteins: Stabilization of Lipid Droplets and Control of Lipolysis. *J. Lipid Res.* **2007**, *48*, 2547–2559. [CrossRef]
41. Zechner, R. FAT FLUX: Enzymes, Regulators, and Pathophysiology of Intracellular Lipolysis. *EMBO Mol. Med.* **2015**, *7*, 359–362. [CrossRef]

42. Marcinkiewicz, A.; Gauthier, D.; Garcia, A.; Brasaemle, D.L. The Phosphorylation of Serine 492 of Perilipin a Directs Lipid Droplet Fragmentation and Dispersion. *J. Biol. Chem.* **2006**, *281*, 11901–11909. [CrossRef] [PubMed]
43. Begum, N. Stimulation of Protein Phosphatase-1 Activity by Insulin in Rat Adipocytes. Evaluation of the Role of Mitogen-Activated Protein Kinase Pathway. *J. Biol. Chem.* **1995**, *270*, 709–714. [CrossRef] [PubMed]
44. Resjö, S.; Göransson, O.; Härndahl, L.; Zolnierowicz, S.; Manganiello, V.; Degerman, E. Protein Phosphatase 2A Is the Main Phosphatase Involved in the Regulation of Protein Kinase B in Rat Adipocytes. *Cell. Signal.* **2002**, *14*, 231–238. [CrossRef]
45. Strålfors, P.; Honnor, R.C. Insulin-Induced Dephosphorylation of Hormone-Sensitive Lipase. Correlation with Lipolysis and cAMP-Dependent Protein Kinase Activity. *Eur. J. Biochem.* **1989**, *182*, 379–385. [CrossRef]
46. Virtanen, K.A.; Lönnroth, P.; Parkkola, R.; Peltoniemi, P.; Asola, M.; Viljanen, T.; Tolvanen, T.; Knuuti, J.; Rönnemaa, T.; Huupponen, R.; et al. Glucose Uptake and Perfusion in Subcutaneous and Visceral Adipose Tissue during Insulin Stimulation in Nonobese and Obese Humans. *J. Clin. Endocrinol. Metab.* **2002**, *87*, 3902–3910. [CrossRef]
47. Kersten, S. Mechanisms of Nutritional and Hormonal Regulation of Lipogenesis. *EMBO Rep.* **2001**, *2*, 282–286. [CrossRef]
48. Rieusset, J.; Andreelli, F.; Auboeuf, D.; Roques, M.; Vallier, P.; Riou, J.P.; Auwerx, J.; Laville, M.; Vidal, H. Insulin Acutely Regulates the Expression of the Peroxisome Proliferator-Activated Receptor-Gamma in Human Adipocytes. *Diabetes* **1999**, *48*, 699–705. [CrossRef] [PubMed]
49. Cherrington, A.D.; Edgerton, D.; Sindelar, D.K. The Direct and Indirect Effects of Insulin on Hepatic Glucose Production In Vivo. *Diabetologia* **1998**, *41*, 987–996. [CrossRef]
50. Dong, X.C.; Copps, K.D.; Guo, S.; Li, Y.; Kollipara, R.; DePinho, R.A.; White, M.F. Inactivation of Hepatic Foxo1 by Insulin Signaling Is Required for Adaptive Nutrient Homeostasis and Endocrine Growth Regulation. *Cell Metab.* **2008**, *8*, 65–76. [CrossRef]
51. Tzivion, G.; Dobson, M.; Ramakrishnan, G. FoxO Transcription Factors; Regulation by AKT and 14-3-3 Proteins. *Biochim. Biophys. Acta* **2011**, *1813*, 1938–1945. [CrossRef] [PubMed]
52. Langlet, F.; Haeusler, R.A.; Lindén, D.; Ericson, E.; Norris, T.; Johansson, A.; Cook, J.R.; Aizawa, K.; Wang, L.; Buettner, C.; et al. Selective Inhibition of FOXO1 Activator/Repressor Balance Modulates Hepatic Glucose Handling. *Cell* **2017**, *171*, 824–835.e18. [CrossRef] [PubMed]
53. Ros, S.; García-Rocha, M.; Domínguez, J.; Ferrer, J.C.; Guinovart, J.J. Control of Liver Glycogen Synthase Activity and Intracellular Distribution by Phosphorylation. *J. Biol. Chem.* **2009**, *284*, 6370–6378. [CrossRef] [PubMed]
54. Horton, J.D.; Goldstein, J.L.; Brown, M.S. SREBPs: Activators of the Complete Program of Cholesterol and Fatty Acid Synthesis in the Liver. *J. Clin. Investig.* **2002**, *109*, 1125–1131. [CrossRef] [PubMed]
55. Krycer, J.R.; Sharpe, L.J.; Luu, W.; Brown, A.J. The Akt-SREBP Nexus: Cell Signaling Meets Lipid Metabolism. *Trends Endocrinol. Metab.* **2010**, *21*, 268–276. [CrossRef]
56. Jelenik, T.; Kaul, K.; Séquaris, G.; Flögel, U.; Phielix, E.; Kotzka, J.; Knebel, B.; Fahlbusch, P.; Hörbelt, T.; Lehr, S.; et al. Mechanisms of Insulin Resistance in Primary and Secondary Nonalcoholic Fatty Liver. *Diabetes* **2017**, *66*, 2241–2253. [CrossRef] [PubMed]
57. Eberlé, D.; Hegarty, B.; Bossard, P.; Ferré, P.; Foufelle, F. SREBP Transcription Factors: Master Regulators of Lipid Homeostasis. *Biochimie* **2004**, *86*, 839–848. [CrossRef] [PubMed]
58. Li, S.; Brown, M.S.; Goldstein, J.L. Bifurcation of Insulin Signaling Pathway in Rat Liver: mTORC1 Required for Stimulation of Lipogenesis, but Not Inhibition of Gluconeogenesis. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 3441–3446. [CrossRef] [PubMed]
59. Horita, S.; Nakamura, M.; Suzuki, M.; Satoh, N.; Suzuki, A.; Seki, G. Selective Insulin Resistance in the Kidney. *Biomed Res. Int.* **2016**, *2016*, 5825170. [CrossRef] [PubMed]
60. Ashraf, A.; Palakkott, A.; Ayoub, M.A. Anti-Insulin Receptor Antibodies in the Pathology and Therapy of Diabetes Mellitus. *Curr. Diabetes Rev.* **2021**, *17*, 198–206. [CrossRef]
61. Hall, C.; Yu, H.; Choi, E. Insulin Receptor Endocytosis in the Pathophysiology of Insulin Resistance. *Exp. Mol. Med.* **2020**, *52*, 911–920. [CrossRef] [PubMed]
62. Rivers, S.L.; Klip, A.; Giacca, A. NOD1: An Interface Between Innate Immunity and Insulin Resistance. *Endocrinology* **2019**, *160*, 1021–1030. [CrossRef]
63. Copps, K.D.; White, M.F. Regulation of Insulin Sensitivity by Serine/Threonine Phosphorylation of Insulin Receptor Substrate Proteins IRS1 and IRS2. *Diabetologia* **2012**, *55*, 2565–2582. [CrossRef]
64. Carvalho-Filho, M.A.; Carvalho, B.M.; Oliveira, A.G.; Guadagnini, D.; Ueno, M.; Dias, M.M.; Tsukumo, D.M.; Hirabara, S.M.; Reis, L.F.; Curi, R.; et al. Double-Stranded RNA-Activated Protein Kinase Is a Key Modulator of Insulin Sensitivity in Physiological Conditions and in Obesity in Mice. *Endocrinology* **2012**, *153*, 5261–5274. [CrossRef] [PubMed]
65. Hage Hassan, R.; Pacheco de Sousa, A.C.; Mahfouz, R.; Hainault, I.; Blachnio-Zabielska, A.; Bourron, O.; Koskas, F.; Górski, J.; Ferré, P.; Foufelle, F.; et al. Sustained Action of Ceramide on the Insulin Signaling Pathway in Muscle Cells: IMPLICATION OF THE DOUBLE-STRANDED RNA-ACTIVATED PROTEIN KINASE. *J. Biol. Chem.* **2016**, *291*, 3019–3029. [CrossRef] [PubMed]
66. Arkan, M.C.; Hevener, A.L.; Greten, F.R.; Maeda, S.; Li, Z.-W.; Long, J.M.; Wynshaw-Boris, A.; Poli, G.; Olefsky, J.; Karin, M. IKK-β Links Inflammation to Obesity-Induced Insulin Resistance. *Nat. Med.* **2005**, *11*, 191–198. [CrossRef]
67. Yung, J.H.M.; Giacca, A. Role of C-Jun N-Terminal Kinase (JNK) in Obesity and Type 2 Diabetes. *Cells* **2020**, *9*, 706. [CrossRef]
68. Hilder, T.L.; Tou, J.C.L.; Grindeland, R.E.; Wade, C.E.; Graves, L.M. Phosphorylation of Insulin Receptor Substrate-1 Serine 307 Correlates with JNK Activity in Atrophic Skeletal Muscle. *FEBS Lett.* **2003**, *553*, 63–67. [CrossRef]
69. Yan, H.; He, L.; Lv, D.; Yang, J.; Yuan, Z. The Role of the Dysregulated JNK Signaling Pathway in the Pathogenesis of Human Diseases and Its Potential Therapeutic Strategies: A Comprehensive Review. *Biomolecules* **2024**, *14*, 243. [CrossRef]

70. James, D.E.; Stöckli, J.; Birnbaum, M.J. The Aetiology and Molecular Landscape of Insulin Resistance. *Nat. Rev. Mol. Cell Biol.* **2021**, *22*, 751–771. [CrossRef]
71. Saltiel, A.R.; Kahn, C.R. Insulin Signalling and the Regulation of Glucose and Lipid Metabolism. *Nature* **2001**, *414*, 799–806. [CrossRef] [PubMed]
72. Schenk, S.; Saberi, M.; Olefsky, J.M. Insulin Sensitivity: Modulation by Nutrients and Inflammation. *J. Clin. Investig.* **2008**, *118*, 2992–3002. [CrossRef] [PubMed]
73. Kolb, H. Obese Visceral Fat Tissue Inflammation: From Protective to Detrimental? *BMC Med.* **2022**, *20*, 494. [CrossRef] [PubMed]
74. Kawai, T.; Autieri, M.V.; Scalia, R. Adipose Tissue Inflammation and Metabolic Dysfunction in Obesity. *Am. J. Physiol. Cell Physiol.* **2021**, *320*, C375–C391. [CrossRef] [PubMed]
75. Pizzino, G.; Irrera, N.; Cucinotta, M.; Pallio, G.; Mannino, F.; Arcoraci, V.; Squadrito, F.; Altavilla, D.; Bitto, A. Oxidative Stress: Harms and Benefits for Human Health. *Oxidative Med. Cell. Longev.* **2017**, *2017*, 8416763. [CrossRef] [PubMed]
76. de Almeida, A.J.P.O.; de Oliveira, J.C.P.L.; da Silva Pontes, L.V.; de Souza Júnior, J.F.; Gonçalves, T.A.F.; Dantas, S.H.; de Almeida Feitosa, M.S.; Silva, A.O.; de Medeiros, I.A. ROS: Basic Concepts, Sources, Cellular Signaling, and Its Implications in Aging Pathways. *Oxidative Med. Cell. Longev.* **2022**, *2022*, 1225578. [CrossRef] [PubMed]
77. Juan, C.A.; Pérez de la Lastra, J.M.; Plou, F.J.; Pérez-Lebeña, E. The Chemistry of Reactive Oxygen Species (ROS) Revisited: Outlining Their Role in Biological Macromolecules (DNA, Lipids and Proteins) and Induced Pathologies. *Int. J. Mol. Sci.* **2021**, *22*, 4642. [CrossRef]
78. Besse-Patin, A.; Estall, J.L. An Intimate Relationship between ROS and Insulin Signalling: Implications for Antioxidant Treatment of Fatty Liver Disease. *Int. J. Cell Biol.* **2014**, *2014*, 519153. [CrossRef] [PubMed]
79. Masenga, S.K.; Kabwe, L.S.; Chakulya, M.; Kirabo, A. Mechanisms of Oxidative Stress in Metabolic Syndrome. *Int. J. Mol. Sci.* **2023**, *24*, 7898. [CrossRef]
80. Yoon, H.; Shaw, J.L.; Haigis, M.C.; Greka, A. Lipid Metabolism in Sickness and in Health: Emerging Regulators of Lipotoxicity. *Mol. Cell* **2021**, *81*, 3708–3730. [CrossRef]
81. Wen, Y.; Chen, C.; Kong, X.; Xia, Z.; Kong, W.; Si, K.; Han, P.; Vivian Liu, W.; Li, X. Pancreatic Fat Infiltration, β-Cell Function and Insulin Resistance: A Study of the Young Patients with Obesity. *Diabetes Res. Clin. Pract.* **2022**, *187*, 109860. [CrossRef] [PubMed]
82. Snel, M.; Jonker, J.T.; Schoones, J.; Lamb, H.; de Roos, A.; Pijl, H.; Smit, J.W.A.; Meinders, A.E.; Jazet, I.M. Ectopic Fat and Insulin Resistance: Pathophysiology and Effect of Diet and Lifestyle Interventions. *Int. J. Endocrinol.* **2012**, *2012*, 983814. [CrossRef] [PubMed]
83. Yang, A.; Mottillo, E.P. Adipocyte Lipolysis: From Molecular Mechanisms of Regulation to Disease and Therapeutics. *Biochem. J.* **2020**, *477*, 985–1008. [CrossRef] [PubMed]
84. Merz, K.E.; Thurmond, D.C. Role of Skeletal Muscle in Insulin Resistance and Glucose Uptake. *Compr. Physiol.* **2020**, *10*, 785–809. [CrossRef] [PubMed]
85. Park, S.S.; Seo, Y.-K. Excess Accumulation of Lipid Impairs Insulin Sensitivity in Skeletal Muscle. *Int. J. Mol. Sci.* **2020**, *21*, 1949. [CrossRef] [PubMed]
86. Perreault, L.; Newsom, S.A.; Strauss, A.; Kerege, A.; Kahn, D.E.; Harrison, K.A.; Snell-Bergeon, J.K.; Nemkov, T.; D'Alessandro, A.; Jackman, M.R.; et al. Intracellular Localization of Diacylglycerols and Sphingolipids Influences Insulin Sensitivity and Mitochondrial Function in Human Skeletal Muscle. *JCI Insight* **2018**, *3*, e96805. [CrossRef] [PubMed]
87. Schmitz-Peiffer, C.; Biden, T.J. Protein Kinase C Function in Muscle, Liver, and Beta-Cells and Its Therapeutic Implications for Type 2 Diabetes. *Diabetes* **2008**, *57*, 1774–1783. [CrossRef] [PubMed]
88. Geng, Y.; Faber, K.N.; de Meijer, V.E.; Blokzijl, H.; Moshage, H. How Does Hepatic Lipid Accumulation Lead to Lipotoxicity in Non-Alcoholic Fatty Liver Disease? *Hepatol. Int.* **2021**, *15*, 21–35. [CrossRef] [PubMed]
89. London, A.; Lundsgaard, A.-M.; Kiens, B.; Bojsen-Møller, K.N. The Role of Hepatic Fat Accumulation in Glucose and Insulin Homeostasis—Dysregulation by the Liver. *J. Clin. Med.* **2021**, *10*, 390. [CrossRef]
90. Lipke, K.; Kubis-Kubiak, A.; Piwowar, A. Molecular Mechanism of Lipotoxicity as an Interesting Aspect in the Development of Pathological States—Current View of Knowledge. *Cells* **2022**, *11*, 844. [CrossRef]
91. Ahmed, B.; Sultana, R.; Greene, M.W. Adipose Tissue and Insulin Resistance in Obese. *Biomed. Pharmacother.* **2021**, *137*, 111315. [CrossRef]
92. Bhatti, J.S.; Bhatti, G.K.; Reddy, P.H. Mitochondrial Dysfunction and Oxidative Stress in Metabolic Disorders—A Step towards Mitochondria Based Therapeutic Strategies. *Biochim. Biophys. Acta (BBA)-Mol. Basis Dis.* **2017**, *1863*, 1066–1077. [CrossRef]
93. Chistiakov, D.A.; Sobenin, I.A.; Revin, V.V.; Orekhov, A.N.; Bobryshev, Y.V. Mitochondrial Aging and Age-Related Dysfunction of Mitochondria. *Biomed Res. Int.* **2014**, *2014*, 238463. [CrossRef]
94. Sergi, D.; Naumovski, N.; Heilbronn, L.K.; Abeywardena, M.; O'Callaghan, N.; Lionetti, L.; Luscombe-Marsh, N. Mitochondrial (Dys)Function and Insulin Resistance: From Pathophysiological Molecular Mechanisms to the Impact of Diet. *Front. Physiol.* **2019**, *10*, 532. [CrossRef]
95. Hales, K.G. The Machinery of Mitochondrial Fusion, Division, and Distribution, and Emerging Connections to Apoptosis. *Mitochondrion* **2004**, *4*, 285–308. [CrossRef]
96. Youle, R.J.; van der Bliek, A.M. Mitochondrial Fission, Fusion, and Stress. *Science* **2012**, *337*, 1062–1065. [CrossRef]
97. Caspersen, C.J.; Powell, K.E.; Christenson, G.M. Physical Activity, Exercise, and Physical Fitness: Definitions and Distinctions for Health-Related Research. *Public Health Rep.* **1985**, *100*, 126–131.

98. Schuch, F.B.; Vancampfort, D. Physical Activity, Exercise, and Mental Disorders: It Is Time to Move On. *Trends Psychiatry Psychother.* **2021**, *43*, 177–184. [CrossRef]
99. Nystoriak, M.A.; Bhatnagar, A. Cardiovascular Effects and Benefits of Exercise. *Front. Cardiovasc. Med.* **2018**, *5*, 135. [CrossRef]
100. Benedetti, M.G.; Furlini, G.; Zati, A.; Letizia Mauro, G. The Effectiveness of Physical Exercise on Bone Density in Osteoporotic Patients. *Biomed Res. Int.* **2018**, *2018*, 4840531. [CrossRef]
101. Pinckard, K.; Baskin, K.K.; Stanford, K.I. Effects of Exercise to Improve Cardiovascular Health. *Front. Cardiovasc. Med.* **2019**, *6*, 69. [CrossRef]
102. Conn, V.S.; Koopman, R.J.; Ruppar, T.M.; Phillips, L.J.; Mehr, D.R.; Hafdahl, A.R. Insulin Sensitivity Following Exercise Interventions: Systematic Review and Meta-Analysis of Outcomes Among Healthy Adults. *J. Prim. Care Community Health* **2014**, *5*, 211–222. [CrossRef]
103. Eaton, S.B.; Eaton, S.B. Physical Inactivity, Obesity, and Type 2 Diabetes: An Evolutionary Perspective. *Res. Q. Exerc. Sport* **2017**, *88*, 1–8. [CrossRef]
104. Sampath Kumar, A.; Maiya, A.G.; Shastry, B.A.; Vaishali, K.; Ravishankar, N.; Hazari, A.; Gundmi, S.; Jadhav, R. Exercise and Insulin Resistance in Type 2 Diabetes Mellitus: A Systematic Review and Meta-Analysis. *Ann. Phys. Rehabil. Med.* **2019**, *62*, 98–103. [CrossRef]
105. Bird, S.R.; Hawley, J.A. Update on the Effects of Physical Activity on Insulin Sensitivity in Humans. *BMJ Open Sport Exerc. Med.* **2017**, *2*, e000143. [CrossRef]
106. Barseem, N.F.; Helwa, M.A. Homeostatic Model Assessment of Insulin Resistance as a Predictor of Metabolic Syndrome: Consequences of Obesity in Children and Adolescents. *Egypt. Pediatr. Assoc. Gaz.* **2015**, *63*, 19–24. [CrossRef]
107. Jenkins, N.T.; Hagberg, J.M. Aerobic Training Effects on Glucose Tolerance in Prediabetic and Normoglycemic Humans. *Med. Sci. Sports Exerc.* **2011**, *43*, 2231–2240. [CrossRef]
108. Hays, N.P.; Starling, R.D.; Sullivan, D.H.; Fluckey, J.D.; Coker, R.H.; Evans, W.J. Comparison of Insulin Sensitivity Assessment Indices with Euglycemic-Hyperinsulinemic Clamp Data after a Dietary and Exercise Intervention in Older Adults. *Metabolism* **2006**, *55*, 525–532. [CrossRef]
109. Keshel, T.E.; Coker, R.H. Exercise Training and Insulin Resistance: A Current Review. *J. Obes. Weight Loss Ther.* **2015**, *5*, S5-003. [CrossRef]
110. Hudish, L.I.; Reusch, J.E.B.; Sussel, L. β Cell Dysfunction during Progression of Metabolic Syndrome to Type 2 Diabetes. *J. Clin. Investig.* **2019**, *129*, 4001–4008. [CrossRef]
111. Dela, F.; von Linstow, M.E.; Mikines, K.J.; Galbo, H. Physical Training May Enhance Beta-Cell Function in Type 2 Diabetes. *Am. J. Physiol. Endocrinol. Metab.* **2004**, *287*, E1024–E1031. [CrossRef]
112. Narendran, P.; Solomon, T.P.; Kennedy, A.; Chimen, M.; Andrews, R.C. The Time Has Come to Test the Beta Cell Preserving Effects of Exercise in Patients with New Onset Type 1 Diabetes. *Diabetologia* **2015**, *58*, 10–18. [CrossRef]
113. Slentz, C.A.; Tanner, C.J.; Bateman, L.A.; Durheim, M.T.; Huffman, K.M.; Houmard, J.A.; Kraus, W.E. Effects of Exercise Training Intensity on Pancreatic β-Cell Function. *Diabetes Care* **2009**, *32*, 1807–1811. [CrossRef]
114. Lee, S.F.; Pei, D.; Chi, M.J.; Jeng, C. An Investigation and Comparison of the Effectiveness of Different Exercise Programmes in Improving Glucose Metabolism and Pancreatic β Cell Function of Type 2 Diabetes Patients. *Int. J. Clin. Pract.* **2015**, *69*, 1159–1170. [CrossRef]
115. Bloem, C.J.; Chang, A.M. Short-Term Exercise Improves β-Cell Function and Insulin Resistance in Older People with Impaired Glucose Tolerance. *J. Clin. Endocrinol. Metab.* **2008**, *93*, 387–392. [CrossRef]
116. Gomes, R.M.; Tófolo, L.P.; Rinaldi, W.; Scomparin, D.X.; Grassiolli, S.; Barella, L.F.; de Oliveira, J.C.; Branco, R.C.S.; Agostinho, A.R.; da Silva Ribeiro, T.A.; et al. Moderate Exercise Restores Pancreatic Beta-Cell Function and Autonomic Nervous System Activity in Obese Rats Induced by High-Fat Diet. *Cell Physiol. Biochem.* **2013**, *32*, 310–321. [CrossRef]
117. Delghingaro-Augusto, V.; Décary, S.; Peyot, M.-L.; Latour, M.G.; Lamontagne, J.; Paradis-Isler, N.; Lacharité-Lemieux, M.; Akakpo, H.; Birot, O.; Nolan, C.J.; et al. Voluntary Running Exercise Prevents β-Cell Failure in Susceptible Islets of the Zucker Diabetic Fatty Rat. *Am. J. Physiol. Endocrinol. Metab.* **2012**, *302*, E254–E264. [CrossRef]
118. Biensø, R.S.; Ringholm, S.; Kiilerich, K.; Aachmann-Andersen, N.-J.; Krogh-Madsen, R.; Guerra, B.; Plomgaard, P.; van Hall, G.; Treebak, J.T.; Saltin, B.; et al. GLUT4 and Glycogen Synthase Are Key Players in Bed Rest–Induced Insulin Resistance. *Diabetes* **2012**, *61*, 1090–1099. [CrossRef]
119. Alibegovic, A.C.; Sonne, M.P.; Højbjerre, L.; Bork-Jensen, J.; Jacobsen, S.; Nilsson, E.; Faerch, K.; Hiscock, N.; Mortensen, B.; Friedrichsen, M.; et al. Insulin Resistance Induced by Physical Inactivity Is Associated with Multiple Transcriptional Changes in Skeletal Muscle in Young Men. *Am. J. Physiol. Endocrinol. Metab.* **2010**, *299*, E752–E763. [CrossRef]
120. Hawley, J.A. Exercise as a Therapeutic Intervention for the Prevention and Treatment of Insulin Resistance. *Diabetes Metab. Res. Rev.* **2004**, *20*, 383–393. [CrossRef]
121. Chibalin, A.V.; Yu, M.; Ryder, J.W.; Song, X.M.; Galuska, D.; Krook, A.; Wallberg-Henriksson, H.; Zierath, J.R. Exercise-Induced Changes in Expression and Activity of Proteins Involved in Insulin Signal Transduction in Skeletal Muscle: Differential Effects on Insulin-Receptor Substrates 1 and 2. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 38–43. [CrossRef] [PubMed]
122. Li, Y.; Lee, S.; Langleite, T.; Norheim, F.; Pourteymour, S.; Jensen, J.; Stadheim, H.K.; Storås, T.H.; Davanger, S.; Gulseth, H.L.; et al. Subsarcolemmal Lipid Droplet Responses to a Combined Endurance and Strength Exercise Intervention. *Physiol. Rep.* **2014**, *2*, e12187. [CrossRef] [PubMed]

123. Roden, M.; Shulman, G.I. The Integrative Biology of Type 2 Diabetes. *Nature* **2019**, *576*, 51–60. [CrossRef]
124. Małkowska, P.; Sawczuk, M. Cytokines as Biomarkers for Evaluating Physical Exercise in Trained and Non-Trained Individuals: A Narrative Review. *Int. J. Mol. Sci.* **2023**, *24*, 11156. [CrossRef] [PubMed]
125. Phillips, C.M.; Dillon, C.B.; Perry, I.J. Does Replacing Sedentary Behaviour with Light or Moderate to Vigorous Physical Activity Modulate Inflammatory Status in Adults? *Int. J. Behav. Nutr. Phys. Act.* **2017**, *14*, 138. [CrossRef] [PubMed]
126. Højbjerre, L.; Sonne, M.P.; Alibegovic, A.C.; Nielsen, N.B.; Dela, F.; Vaag, A.; Bruun, J.M.; Stallknecht, B. Impact of Physical Inactivity on Adipose Tissue Low-Grade Inflammation in First-Degree Relatives of Type 2 Diabetic Patients. *Diabetes Care* **2011**, *34*, 2265–2272. [CrossRef] [PubMed]
127. Febbraio, M.A.; Pedersen, B.K. Muscle-Derived Interleukin-6: Mechanisms for Activation and Possible Biological Roles. *FASEB J.* **2002**, *16*, 1335–1347. [CrossRef] [PubMed]
128. Suzuki, K.; Nakaji, S.; Yamada, M.; Totsuka, M.; Sato, K.; Sugawara, K. Systemic Inflammatory Response to Exhaustive Exercise. Cytokine Kinetics. *Exerc. Immunol. Rev.* **2002**, *8*, 6–48. [PubMed]
129. Esposito, K.; Pontillo, A.; Di Palo, C.; Giugliano, G.; Masella, M.; Marfella, R.; Giugliano, D. Effect of Weight Loss and Lifestyle Changes on Vascular Inflammatory Markers in Obese Women: A Randomized Trial. *JAMA* **2003**, *289*, 1799–1804. [CrossRef]
130. Stefan, N.; Stumvoll, M. Adiponectin—Its Role in Metabolism and Beyond. *Horm. Metab. Res.* **2002**, *34*, 469–474. [CrossRef]
131. Shamsuzzaman, A.S.M.; Winnicki, M.; Wolk, R.; Svatikova, A.; Phillips, B.G.; Davison, D.E.; Berger, P.B.; Somers, V.K. Independent Association between Plasma Leptin and C-Reactive Protein in Healthy Humans. *Circulation* **2004**, *109*, 2181–2185. [CrossRef]
132. Adamopoulos, S.; Parissis, J.; Kroupis, C.; Georgiadis, M.; Karatzas, D.; Karavolias, G.; Koniavitou, K.; Coats, A.J.; Kremastinos, D.T. Physical Training Reduces Peripheral Markers of Inflammation in Patients with Chronic Heart Failure. *Eur. Heart J.* **2001**, *22*, 791–797. [CrossRef]
133. Lee, S.; Norheim, F.; Langleite, T.M.; Gulseth, H.L.; Birkeland, K.I.; Drevon, C.A. Effects of Long-Term Exercise on Plasma Adipokine Levels and Inflammation-Related Gene Expression in Subcutaneous Adipose Tissue in Sedentary Dysglycaemic, Overweight Men and Sedentary Normoglycaemic Men of Healthy Weight. *Diabetologia* **2019**, *62*, 1048–1064. [CrossRef]
134. Bilet, L.; Phielix, E.; van de Weijer, T.; Gemmink, A.; Bosma, M.; Moonen-Kornips, E.; Jorgensen, J.A.; Schaart, G.; Zhang, D.; Meijer, K.; et al. One-Leg Inactivity Induces a Reduction in Mitochondrial Oxidative Capacity, Intramyocellular Lipid Accumulation and Reduced Insulin Signalling upon Lipid Infusion: A Human Study with Unilateral Limb Suspension. *Diabetologia* **2020**, *63*, 1211–1222. [CrossRef]
135. Alghadir, A.H.; Gabr, S.A.; Anwer, S.; Al-Eisa, E. Fatigue and Oxidative Stress Response to Physical Activity in Type 2 Diabetic Patients. *Int. J. Diabetes Dev. Ctries.* **2016**, *36*, 59–64. [CrossRef]
136. Kozakiewicz, M.; Rowiński, R.; Kornatowski, M.; Dąbrowski, A.; Kędziora-Kornatowska, K.; Strachecka, A. Relation of Moderate Physical Activity to Blood Markers of Oxidative Stress and Antioxidant Defense in the Elderly. *Oxid. Med. Cell Longev.* **2019**, *2019*, 5123628. [CrossRef]
137. Powers, S.K.; Ji, L.L.; Leeuwenburgh, C. Exercise Training-Induced Alterations in Skeletal Muscle Antioxidant Capacity: A Brief Review. *Med. Sci. Sports Exerc.* **1999**, *31*, 987–997. [CrossRef]
138. Leeuwenburgh, C.; Hansen, P.A.; Holloszy, J.O.; Heinecke, J.W. Hydroxyl Radical Generation during Exercise Increases Mitochondrial Protein Oxidation and Levels of Urinary Dityrosine. *Free Radic. Biol. Med.* **1999**, *27*, 186–192. [CrossRef]
139. Bejma, J.; Ji, L.L. Aging and Acute Exercise Enhance Free Radical Generation in Rat Skeletal Muscle. *J. Appl. Physiol.* **1999**, *87*, 465–470. [CrossRef]
140. Leeuwenburgh, C.; Fiebig, R.; Chandwaney, R.; Ji, L.L. Aging and Exercise Training in Skeletal Muscle: Responses of Glutathione and Antioxidant Enzyme Systems. *Am. J. Physiol.* **1994**, *267*, R439–R445. [CrossRef]
141. Leeuwenburgh, C.; Hollander, J.; Leichtweis, S.; Griffiths, M.; Gore, M.; Ji, L.L. Adaptations of Glutathione Antioxidant System to Endurance Training Are Tissue and Muscle Fiber Specific. *Am. J. Physiol.* **1997**, *272*, R363–R369. [CrossRef] [PubMed]
142. Powers, S.K.; Criswell, D.; Lawler, J.; Martin, D.; Lieu, F.K.; Ji, L.L.; Herb, R.A. Rigorous Exercise Training Increases Superoxide Dismutase Activity in Ventricular Myocardium. *Am. J. Physiol.* **1993**, *265*, H2094–H2098. [CrossRef]
143. Reidy, P.T.; Mahmassani, Z.S.; McKenzie, A.I.; Petrocelli, J.J.; Summers, S.A.; Drummond, M.J. Influence of Exercise Training on Skeletal Muscle Insulin Resistance in Aging: Spotlight on Muscle Ceramides. *Int. J. Mol. Sci.* **2020**, *21*, 1514. [CrossRef]
144. Bergouignan, A.; Trudel, G.; Simon, C.; Chopard, A.; Schoeller, D.A.; Momken, I.; Votruba, S.B.; Desage, M.; Burdge, G.C.; Gauquelin-Koch, G.; et al. Physical Inactivity Differentially Alters Dietary Oleate and Palmitate Trafficking. *Diabetes* **2009**, *58*, 367–376. [CrossRef]
145. Kwon, O.S.; Tanner, R.E.; Barrows, K.M.; Runtsch, M.; Symons, J.D.; Jalili, T.; Bikman, B.T.; McClain, D.A.; O'Connell, R.M.; Drummond, M.J. MyD88 Regulates Physical Inactivity-Induced Skeletal Muscle Inflammation, Ceramide Biosynthesis Signaling, and Glucose Intolerance. *Am. J. Physiol. Endocrinol. Metab.* **2015**, *309*, E11–E21. [CrossRef]
146. Bergman, B.C.; Brozinick, J.T.; Strauss, A.; Bacon, S.; Kerege, A.; Bui, H.H.; Sanders, P.; Siddall, P.; Wei, T.; Thomas, M.K.; et al. Muscle Sphingolipids during Rest and Exercise: A C18:0 Signature for Insulin Resistance in Humans. *Diabetologia* **2016**, *59*, 785–798. [CrossRef]
147. Reidy, P.T.; McKenzie, A.I.; Mahmassani, Z.; Morrow, V.R.; Yonemura, N.M.; Hopkins, P.N.; Marcus, R.L.; Rondina, M.T.; Lin, Y.K.; Drummond, M.J. Skeletal Muscle Ceramides and Relationship with Insulin Sensitivity after 2 Weeks of Simulated Sedentary Behaviour and Recovery in Healthy Older Adults. *J. Physiol.* **2018**, *596*, 5217–5236. [CrossRef]

148. Distefano, G.; Standley, R.A.; Zhang, X.; Carnero, E.A.; Yi, F.; Cornnell, H.H.; Coen, P.M. Physical Activity Unveils the Relationship between Mitochondrial Energetics, Muscle Quality, and Physical Function in Older Adults. *J. Cachexia Sarcopenia Muscle* **2018**, *9*, 279–294. [CrossRef]
149. Figueiredo, P.A.; Powers, S.K.; Ferreira, R.M.; Amado, F.; Appell, H.J.; Duarte, J.A. Impact of Lifelong Sedentary Behavior on Mitochondrial Function of Mice Skeletal Muscle. *J. Gerontol. A Biol. Sci. Med. Sci.* **2009**, *64*, 927–939. [CrossRef]
150. Heinonen, S.; Muniandy, M.; Buzkova, J.; Mardinoglu, A.; Rodríguez, A.; Frühbeck, G.; Hakkarainen, A.; Lundbom, J.; Lundbom, N.; Kaprio, J.; et al. Mitochondria-Related Transcriptional Signature Is Downregulated in Adipocytes in Obesity: A Study of Young Healthy MZ Twins. *Diabetologia* **2017**, *60*, 169–181. [CrossRef]
151. Drew, B.G.; Hamidi, H.; Zhou, Z.; Villanueva, C.J.; Krum, S.A.; Calkin, A.C.; Parks, B.W.; Ribas, V.; Kalajian, N.Y.; Phun, J.; et al. Estrogen Receptor (ER)α-Regulated Lipocalin 2 Expression in Adipose Tissue Links Obesity with Breast Cancer Progression*. *J. Biol. Chem.* **2015**, *290*, 5566–5581. [CrossRef]
152. Zhou, Z.; Moore, T.M.; Drew, B.G.; Ribas, V.; Wanagat, J.; Civelek, M.; Segawa, M.; Wolf, D.M.; Norheim, F.; Seldin, M.M.; et al. Estrogen Receptor α Controls Metabolism in White and Brown Adipocytes by Regulating Polg1 and Mitochondrial Remodeling. *Sci. Transl. Med.* **2020**, *12*, eaax8096. [CrossRef]
153. De Paoli, M.; Zakharia, A.; Werstuck, G.H. The Role of Estrogen in Insulin Resistance: A Review of Clinical and Preclinical Data. *Am. J. Pathol.* **2021**, *191*, 1490–1498. [CrossRef]
154. Pu, D.; Tan, R.; Yu, Q.; Wu, J. Metabolic Syndrome in Menopause and Associated Factors: A Meta-Analysis. *Climacteric* **2017**, *20*, 583–591. [CrossRef]
155. Christakis, M.K.; Hasan, H.; De Souza, L.R.; Shirreff, L. The Effect of Menopause on Metabolic Syndrome: Cross-Sectional Results from the Canadian Longitudinal Study on Aging. *Menopause* **2020**, *27*, 999. [CrossRef]
156. Zarotsky, V.; Huang, M.-Y.; Carman, W.; Morgentaler, A.; Singhal, P.K.; Coffin, D.; Jones, T.H. Systematic Literature Review of the Risk Factors, Comorbidities, and Consequences of Hypogonadism in Men. *Andrology* **2014**, *2*, 819–834. [CrossRef]
157. Caliber, M.; Saad, F. Testosterone Therapy for Prevention and Reversal of Type 2 Diabetes in Men with Low Testosterone. *Curr. Opin. Pharmacol.* **2021**, *58*, 83–89. [CrossRef]
158. Han, S.; Jeon, Y.-J.; Lee, T.Y.; Park, G.-M.; Park, S.; Kim, S.C. Testosterone Is Associated with Abdominal Body Composition Derived from Computed Tomography: A Large Cross Sectional Study. *Sci. Rep.* **2022**, *12*, 22528. [CrossRef]
159. Regensteiner, J.G.; Bauer, T.A.; Huebschmann, A.G.; Herlache, L.; Weinberger, H.D.; Wolfel, E.E.; Reusch, J.E.B. Sex Differences in the Effects of Type 2 Diabetes on Exercise Performance. *Med. Sci. Sports Exerc.* **2015**, *47*, 58–65. [CrossRef]
160. Rehman, D. *Sex Differences in Exercise Response in Type 2 Diabetes*; Norwegian University of Science and Technology: Trondheim, Norway, 2019.
161. Niemann, M.J.; Tucker, L.A.; Bailey, B.W.; Davidson, L.E. Strength Training and Insulin Resistance: The Mediating Role of Body Composition. *J. Diabetes Res.* **2020**, *2020*, 7694825. [CrossRef]

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Review

Multi Omics Applications in Biological Systems

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Abstract: Traditional methodologies often fall short in addressing the complexity of biological systems. In this regard, system biology omics have brought invaluable tools for conducting comprehensive analysis. Current sequencing capabilities have revolutionized genetics and genomics studies, as well as the characterization of transcriptional profiling and dynamics of several species and sample types. Biological systems experience complex biochemical processes involving thousands of molecules. These processes occur at different levels that can be studied using mass spectrometry-based (MS-based) analysis, enabling high-throughput proteomics, glycoproteomics, glycomics, metabolomics, and lipidomics analysis. Here, we present the most up-to-date techniques utilized in the completion of omics analysis. Additionally, we include some interesting examples of the applicability of multi omics to a variety of biological systems.

Keywords: systems biology; omics; genomics; transcriptomics; proteomics; glycoproteomics; glycomics; metabolomics; lipidomics; foodomics

1. Introduction

Omics is an interdisciplinary research field focused on understanding the interactions within biological systems from a holistic perspective, considering the entire system rather than individual parts [1,2]. This field emphasizes the use of high-throughput technologies and large-scale data generated at the molecular level. Computational tools are then employed to integrate and analyze the data, enabling an understanding of biological processes. The "omics" concept encloses the subfields of genomics [3], transcriptomics [4], proteomics [5], glycoproteomics [6], glycomics [7], metabolomics [8], lipidomics [9], and several others, providing a comprehensive analysis of the corresponding biomolecules.

In recent years, omics science has emerged as a pivotal area of research in understanding cellular and molecular systems, significantly enhancing our comprehension of human health, development, diagnosis, prognosis, and aging [10]. Omics technologies offer detailed insights by cataloging variations linked with different stages of diseases or a variety of biological processes, thereby serving as a critical tool for monitoring disease progression and developing biomarkers. Stand-alone omics approaches offer a restricted point of view of the studied system, offering insights into specific molecules and their

potential significance in metabolic pathways. Additionally, these approaches lack the necessary information for a comprehensive understanding of dynamic biological processes. In this regard, multi omics plays a central role in unraveling the relationships between different biomolecules and their interactions by simultaneously examining different single omics approaches (Figure 1), to provide a holistic view of the biological system [11]. This allows for the validation of individual findings and reduces the risk of false positives. As a fundamental component of system biology, multi omics allows researchers to explore biological systems as interconnected networks. However, the integration of omics data is challenging due to factors such as large data sets, data heterogeneity, sample sizes, and the need for advanced statistical methods [1]. Multi omics approaches could shed light on the fundamental causes of diseases, their functional repercussions, and pertinent interactions [12,13]. Together, omics technologies offer a comprehensive view of the biological status and changes occurring within an organism, especially in the context of disease. The integration of data from these diverse sources allows researchers to construct a more comprehensive model of disease mechanisms. This integrated approach helps identify potential diagnostic markers and therapeutic targets and enhances our understanding of the complex network of biological pathways involved in disease etiology and progression. By enabling a systems-level analysis, omics sciences facilitate the identification of key regulatory nodes and pathways that could be targeted for intervention, paving the way for personalized medicine and improved healthcare outcomes [14–16].

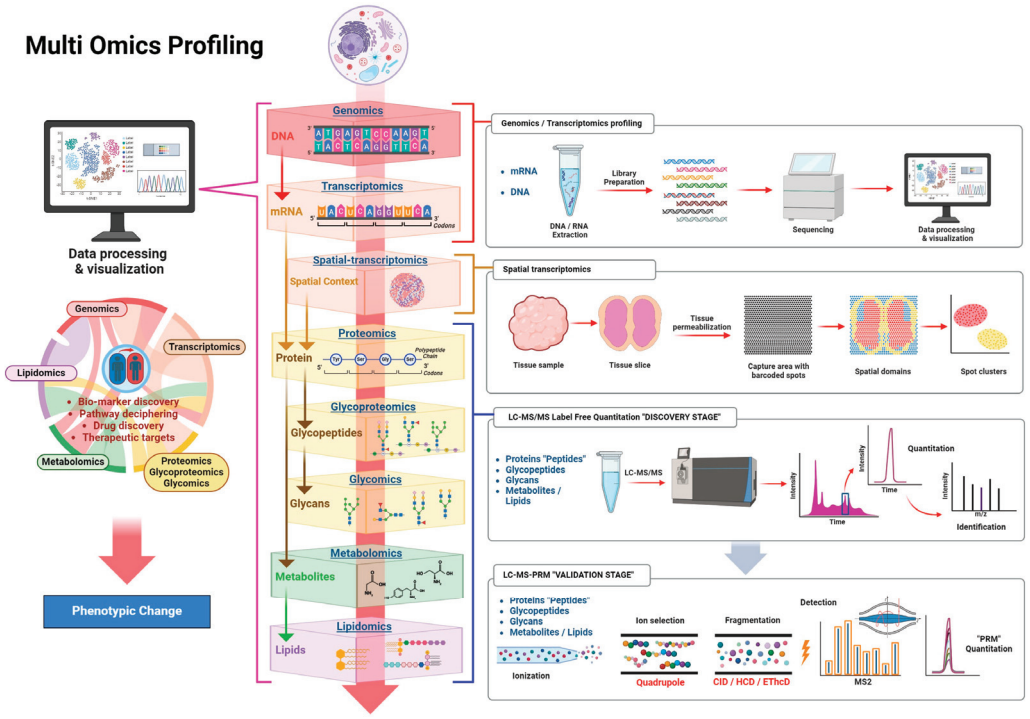


Figure 1. Multi omics. Inside the eukaryotic nucleus, spatial conformations of genomic material and interactions participate in regulating gene expression. Different RNAs are accumulated, and some splice variants are produced in response to internal and external stimuli. Transcripts can be rapidly degraded or preserved and can sustain high or low translation rates. Post translational modifications provide different protein functions that can modify ligand affinity, protein interactions, catalytic activity, etc. The cellular regulatory processes favor synthesis, degradation, modification, and interaction of a multitude of molecules. The right section of the figure shows high-throughput sequencing methods and technologies to measure gene expression in situ, and the common LC-MS/MS label-free quantitation "discovery stage" followed by the targeted "validation stage".

This review provides a comprehensive and up-to-date description of the most widely used omics techniques and describes their applicability for understanding biological sys-

tems. Additionally, our review focuses on holistic approaches that have demonstrated the benefits of multi omics applications.

2. Genomics and Transcriptomics

A few decades ago, scientists studied single genes at a time. Current technological innovations allow fast and cost-effective sequencing of entire genomes [17]. This tremendous change in sequencing capability is reflected in the first human genome, which was produced by an international consortium that cost billions of dollars; today, the sequencing of individual genomes can be completed for a reasonable price in a relatively short time [18]. Such technological progress encourages research efforts focused on genomic studies rather than single genes. Genomics encompasses a wide range of approaches to study and understand organisms' DNA. This includes the analysis of genome structure and composition (genes as well as non-coding regions), their functions and interaction with each other, variation, evolution, and more. The significant improvement in the throughput and accuracy of sequencing capabilities has had an important impact on the identification of novel coding and regulatory regions [19], as well as on our understanding of the spatial organization of the DNA within a cell nucleus [20]. Genomics has also revolutionized the pharmaceutical industry by allowing the identification of protein targets and accelerating drug discovery [21–23]. The field has seen rapid expansion, with models and methods developed for analyzing genome rearrangements [24], gene duplication, phylogenetic networks [25], and gene clustering [26].

While genomics provides an overview of all the genetic information of organisms, transcriptomics refers to the study of gene expression patterns of the entire set of RNA molecules in an individual [27]. High-throughput sequencing technologies (e.g., Illumina) allow quantifying the number of times a given sequence is present in a sample. In global transcriptomics analyses, RNA is isolated from tissue samples and further prepared to enrich messenger RNAs (mRNAs), small RNAs (sRNAs), long non-coding RNAs (lncRNA), etc. The resulting data represent a snapshot of the enriched RNA abundance from the bulk of cells within the sample. These analyses provide an average representation of gene expression across a population of cells [28]. Although transcriptomics offers an important source of information, it does not tell the whole story about transcripts. It does not consider the rate at which transcripts are produced or degraded, nor how much of the transcripts are translated into proteins. Additional techniques, such as polysome profiling, allow researchers to estimate the translation rate of a transcript by measuring the number of ribosomes attached to individual mRNAs [29,30], and ribosome profiling can tell the ribosome footprint by isolating the section of the transcript that is bound to the ribosome [31,32]. More sophisticated library preparations involving reversible inhibition of transcription elongation combined with tagging nascent RNA can also provide information about the specific transcription rates of mRNAs and their degradation, as well as providing clues about the splicing process [33–35]. More recently, long-read sequencing techniques, such as PacBio Iso-Seq or Oxford Nanopore direct RNA-seq, allow the sequencing of complete transcripts to detect multiple isoforms from the same gene and their dynamics between distinct treatments [36].

Multicellular organisms show a great diversity in cellular composition. For instance, even though a human being originates from a single diploid cell as tissue develops, different cells fall into a heterogeneity of cellular fates influenced by internal and external factors [37,38]. Moreover, once fully developed, cells from the same tissues undergo distinct tasks. Currently, platforms such as Fluidigm C1, Clontech iCell8, BD Rhapsody, and 10× Genomics Chromium allow the isolation of individual cells or nuclei to prepare single-cell RNA-seq (scRNA-seq) libraries [39]. Every platform has its strengths and limitations. A recent study compared the droplet-based 10× Chromium and the microwell-based BD Rhapsody [40]. The results of this study indicate that the microwell-based platform exhibits better mRNA capture efficiency, but it can be under-represented or even lose larger cell types compared to the droplet-based system. The scRNA-seq technology has been

applied to understand the dynamic activities of transcription regulation over time and the trajectories of cell states at the individual cell level, for instance, in response to neuronal activation [41]. Moreover, generating multiple single-cell data has allowed scientists to produce reference single-cell atlases to compare cellular conditions across various research investigations [42]. Although scRNA-seq has significantly increased our understanding of the complex cellular heterogeneity of tissues and organs, it has some disadvantages, such as the effect of cell dissociation and the loss of positional information [43]. The spatial context of the cells has a significant role in developmental biology, cellular communication, and disease study, among others. Emerging technologies to determine cell gene expression preserving in situ spatial locations within a tissue are known as spatial transcriptomics (ST). These technologies can be broadly divided into imaging-based and sequencing-based methods [44]. Imaging-based technologies rely on fluorescence in situ hybridization of probes that target specific genes. Conversely, sequencing-based technologies depend on spatial barcodes on an array to capture transcripts unbiasedly. Recent reviews have discussed currently available ST technologies' characteristics, applications, advantages, and limitations [45,46]. Commercially available ST technologies for whole transcriptome profiling include the 10× Visium, which resolves several to dozens of cells [47]. New methods such as Stereo-seq can provide single-cell resolution, and it has been successfully used to study the spatiotemporal transcriptomic dynamics during organogenesis [48].

3. Proteomics, Glycoproteomics, and Glycomics

Proteomics and its associated glycoproteomics and glycomics techniques are dynamic research areas working to elucidate the associations between protein structure and function [49–51]. Protein abundance and post-translational modifications are the fundamental parameters utilized to evaluate the dynamic behavior of proteins. The exponential growth of these omics was possible due to their integration with mass spectrometry-based (MS-based) analysis, which enormously contributed to the creation of large and reliable data sets from a variety of sample types such as plants [52], animal models, human bodily fluids, tissues, etc. [53–56].

In MS-based proteomics, the proteins are identified using two common approaches: bottom up and top down. In the bottom-up approach, the intact proteins are digested into peptides prior to introduction to the mass spectrometer, where they are detected and fragmented to facilitate identification [57]. In top-down proteomics, the proteins are ionized and fragmented in the intact form in the mass spectrometer [58]. This approach allows an important characterization of protein isoforms and provides a general picture of the protein's post-translational modifications, such as glycosylation, acetylation, and phosphorylation [59,60]. The protein purification stages are crucial for the obtention of sensitive and reproducible results. In the past decade, proteomics analysis was commonly performed on bodily fluids such as serum, plasma, and cerebrospinal fluid (CSF) and different types of tissues [61–63]. These investigations mainly searched for abundance changes in the proteome of disease samples compared to healthy controls. At present, proteomics analysis has extended to more complicated samples such as extra vesicular exosomes (EVs) [64], nucleosomes [65], or single-cell approaches [66]. For instance, Van den Ackerveken et al. [65] developed a fast and robust enrichment method able to isolate nucleosomes from plasma. Further top-down proteomics analysis of colorectal cancer (CRC) samples showed alterations in the histone PTMs after alterations of epigenetic modification [65,67]. The approach was able to identify and quantify 13 histone PTMs with significant changes in abundance between the CRC and the control samples. Recent advancements in MS instruments, such as the development of the Orbitrap Astral (Thermo Fisher Sci.), allowed researchers in the field to address single-cell proteomics using label-free quantitative (LFQ) analysis. Ye et al. [68] developed a lossless LFQ single-cell proteomics (SCP) method used for the identification of over 40,000 peptides and 5000 proteins in Hela cells. The approach integrated sample preparation utilizing the cellenONE, the proteoCHIP EVO96, and the direct transference of the sample to Evotip disposal trap

columns. The samples were subsequently analyzed using the Evosep One LC, with whisper flow gradients linked to a narrow-window data independent acquisition (DIA) system.

Glycoproteomics and glycomics MS-based analysis is dedicated to the comprehensive study of carbohydrate moieties present in cells and organisms. These branches of omics science delve into understanding the structure, function, and dynamics of glycans attached to proteins and lipids across different biological systems and their role in health and disease. By analyzing these structures, glycomics and glycoproteomics analysis provides insights into cellular communication [69,70], immune response [71], pathogen interaction [72,73], and disease progression [74], making it a crucial field in biomedical research and therapeutic development. Advanced technologies such as high-performance liquid chromatography–mass spectrometry (LC-MS) are employed to decipher the complex and diverse structures of glycans and the protein *O*- and *N*-site heterogeneity. Derivatization techniques are essential due to the low ionization efficiency and poor stability of native glycan and glycopeptide structures that hinder their accurate quantitation and detection by MS systems [75–77]. Through derivatization, researchers can achieve more accurate glycan and glycopeptide profiling, facilitating a deeper understanding of glycan functions in biological processes and disease mechanisms. Common glycan derivatization techniques include hydrazide chemistry [78], carbamate derivatization [79], chemical labeling by reductive amination [80], and permethylation [81]. In the case of glycopeptides, the glycan moiety can be derivatized through selective reductive amination reactions directed to the sialic acid [76,77]. The glycopeptides can also be derivatized, targeting the peptide *N*-terminus using tandem mass tags (TMTs) [82]. Alterations in glycan expression are linked to various diseases, including cancer [83–85], neurodegenerative diseases [86–88], autoimmune disorders [89,90], and infectious diseases [73,91,92]. These changes can impact cell signaling [93], immune recognition [94], and molecular stability [95], leading to altered cellular behaviors and disease progression. For example, in cancer, altered glycosylation can promote tumor growth, facilitate metastasis, and enable evasion of immune surveillance [96]. In autoimmune diseases, aberrant glycosylation can lead to improper immune responses against self-antigens [90]. Understanding these changes is crucial for developing diagnostic markers and therapeutic targets. Integrating glycomics and proteomics analyses enhances our understanding of disease mechanisms by providing a comprehensive view of the glycome and its interaction with proteins [97–99]. Mechref and co-workers [53] have shown that integrating proteomics and glycomics analysis can shed light on the glycoprotein source of glycans and provide mechanistic insight into the clinicopathological changes observed in different disease conditions [53]. This strategy was applied to samples derived from healthy and mild cognitive impairment (MCI) patients. The results pointed to the isomeric *N*-glycans $GlcNAc_5$,Hex_6,$Neu5Ac_3$ and $GlcNAc_4$,Hex_5,Fuc,Neu5Ac as biomarker candidates to differentiate healthy and MCI patients. Moreover, the proteomics analysis showed a correlation of the identified glycoproteins with neuroinflammation, an important process in the progression of brain-related diseases. The same research group also utilized proteomics-glycomics approaches to describe the protein glycosylation differences in 11 SARS-CoV2 variants. A significantly large abundance of sialofucosylated *N*-glycans was observed in the variants of concern when compared to the variants of interest. The proteomics analyses were used for the validation of variant amino acid sequences [92].

4. Metabolomics and Lipidomics

The metabolome includes all the small-molecule metabolite parts of a biological sample, including structural functions, stimulatory and inhibitory effects, fuel storage molecules, and others [100]. Lipids are structurally diverse molecules that perform multiple important functions within the cell. They are characterized by their hydrophobic nature and fundamental components of the cellular membranes [101]. Either the metabolome or the lipidome can be studied at the single-cell level or in a more complex matrix such as a bodily fluid or an organ tissue [100,102,103]. The analyses of these biological systems using

high-throughput targeted or untargeted metabolomics or lipidomics MS-based analysis provide an invaluable opportunity to study and quantify differential abundance [104].

Recently, a few techniques based on single-cell metabolomics (SCM) have been developed, including Patch clamp-based nano ESI-MS, CE-MS-based SCMS, MALDI-MSI-based SCMS, LALDI-MSI-based SCMS, SIMS-MSI-based SCMS, and mass cytometry-based SCMS [105]. Metabolites are crucial components of biochemical pathways and cellular functions; therefore, quantitative metabolomics has become a powerful tool in many research fields, including those investigating cancer and neurodegenerative disorders. In this sense, there is a challenge to explore and identify metabolic hallmarks that can track disease development and progression [106]. Cancer cells can reprogram their metabolic pathways to adapt tumor microenvironments and survive pharmacological treatments [107]. This phenomenon, known as metabolic plasticity, results in a heterogenous tumor with different metabolic phenotypes that are hard to characterize and consequently difficult to treat [108]. In this regard, SCM has been applied in the identification of sub-types of circulating tumor cells with distinct metastatic potential [109], and provided useful information for drug therapy [110,111]. In addition, SCM has been used to study the dynamic cancer cell–cell interaction during the drug resistance process [112] and the potential role of microbiota in the development and progression of different cancer types [113]. As metabolites directly reflect the cell behavior, the integration of SCM with other single-cell omics could suggest mechanisms underlying tumor development and drug resistance. Metabolomics have also been used to investigate neurodegenerative disorders [114]. Despite the expanding prevalence of SCM, scarce evidence of studying neurodegenerative disorders at the single-cell level has been reported. Metabolomics was applied to a preclinical model of Alzheimer's disease (AD); seven AD-related neurotransmitters and sixteen biomarkers were identified by SCM in PC12 cells [115]. It is only a matter of time before SCM will be employed on neurodegenerative disorders to explore the individual role of each neuron and glia cell in the progress of neurodegeneration.

Other most affordable LC–MS/MS strategies employed for metabolomics analysis of biological samples have served to identify potential biomarkers in human serum from patients with diabetes [116], schizophrenia [117], ischemic stroke [118], and other health conditions [119–121]. Similar technique also have been used to assess food quality [122–126] and the toxic effects of xenobiotics [127,128]. In order to enhance accuracy, precision, and throughput, common LC-MS techniques have employed ^{13}C or ^{18}O isotopic approaches [129–132]. MALDI imagining is an MS-based technique widely used for the characterization of tissues derived from several health conditions facilitating their diagnosis and therapy [133–137].

Lipids possess significant nutritional value due to their high caloric density and their capacity to transport essential substances that the human body cannot produce independently. Lipids are also involved in intra- and extracellular signaling processes [138]. Lipidomics focuses on the study and characterization of cellular and bodily fluid lipids, along with their interactions and functions within the body. This field is a crucial tool in healthcare for identifying biomarkers useful in diagnosing and developing therapies for various diseases [139,140]. For example, the nervous system contains a large amount of lipids. It has been suggested that neurological disorders may be associated with lipid homeostasis, among other factors [103,141]. It has also been described that there are changes in the lipidomic profiles of bioactive lipids in plasma and CSF, as well as in specific anatomical regions of the brain associated with the pathological characteristics of AD and Parkinson's disease [142]. Significant alterations in plasma lipids have also been described in rats and mice with spinal cord and sciatic nerve injury, which indicates that lipid metabolism could be related to the recovery and/or damage processes following nerve injury [143,144]. The application of lipidomics to disorders associated with metabolic syndrome has been widely described. Lipidomics plays a key role in risk prediction studies and therapeutic monitoring of diseases related to metabolic syndrome, given the close association of lipids with these diseases [145]. Furthermore, this discipline has been

valuable for determining population profiles, conducting research on pathogenesis, and for identifying biomarkers and monitoring therapeutic responses [146]. It has been described that the lipidome is altered in many neoplastic diseases. The main applications of lipidomics in these types of disorders have focused on the detection and classification of neoplastic cells or tissues, on differentiation between neoplastic and normal environments, in the evaluation of cancer treatments, and for the discovery of new tumor biomarkers [147].

5. Multi Omics Integration

The study of organisms as complex systems requires the integration of distinct approaches (Figure 1). For instance, although genomic and transcriptomic analyses offer powerful tools in our understanding of molecular processes, downstream gaps cannot be filled without integrating other approaches. None of the above methods provide information about what happens to a gene's translated product or the effect of metabolite accumulation. Consideration of these questions highlights the relevance of multi omics analysis, as they provide a more complete and holistic landscape of cell molecular interactions. Table 1 summarizes additional omics approaches, focusing on the multi-integration of molecular data sets.

Table 1. Complemental integrative omics applications.

Omics Spotlight	Omics Integration	Applications	References
Diagnosis and prognosis	G+T+P+M	Alzheimer's disease diagnosis	[148]
	T+Me+scT	Evaluation of Covid-19 prognosis	[149]
	G+T+P+pP	Hepatocellular carcinoma classification	[150]
	G+T	Identification of prognostic biomarkers for gastric cancer	[151]
	P+M+L	Search for pathway alterations in Alzheimer's disease	[152,153]
	G+T+M	Identification of metabolite biomarkers for predicting radiation resistance	[154]
	T+P+E	Epigenetic alterations associated with Alzheimer's disease	[155]
	G+T+P	Gastrointestinal microbiome and diabetes mellitus (type 1)	[156]
	G+P+M	Liver disease pathogenesis	[157]
	T+P+M	Effect of heavy-metal exposure in neurodevelopment	[158]
	sT+sM	Spatial resolution approaches to study brain injuries	[159]
	sT+sM	Multimodal spatial approach used in Parkinson's disease	[160]
	scT+E	Identification of genomic variants associated with eye diseases	[161]
Biomarker	T+M+L	Progression of lung fibrosis	[162]
	G+T+P	Metabolic mapping of Alzheimer's disease	[163]
	G+T+M+Me	Identification of osteoporosis biomarkers	[164]
	M+L	Predictive biomarkers for COVID-19 severity	[165]
	G+T+P+pP	Exploration for Hepatocellular carcinoma biomarkers	[150]
	P+Gly	Identification of mild cognitive impairment biomarkers	[53]
	T+P	Temporal lobe epilepsy biomarkers	[166]
	T+P	Emphysema biomarkers	[167]
	T+P	Idiopathic pulmonary fibrosis	[168]
Drug targets and therapeutics	G+T+P	Alzheimer's drug discovery	[169]
	G+T+P	Prostate cancer diagnosis and therapies	[170]
	P+Gly	Glycosylation profile of the S1 protein of eleven SARS-CoV-2 variants	[92]
	G+T	Drug response profiling of childhood acute lymphoblastic leukemia cell lines	[171]
	G+T	Prioritization of therapeutic targets for dyslipidemia	[172]

Abbreviations: G, genomics; T, transcriptomics; P, proteomics; M, metabolomics; L, lipidomics; Gly, glycomics; sc, single cell; p, phospho; Me, methylomics; E, epigenomics; and s, spatial.

Omics technologies are high-throughput molecular assays that produce large data sets of information. Public repositories facilitate the accessibility of this information to the scientific community. Some examples are the following: for high-throughput sequencing SRA [173]; for functional genomics and DNA sequence information NCBI GEO [174], GenBank [175]; for spatial transcriptomics CROST [176]; for proteomics and glycoproteomics

PRIDE [177], Proteome Xchange [178]; for glycomics GlycoPOST [179]; for metabolomics and lipidomics MetaboLights [180], HMDB [181]; and many others for multi omics as listed in the references [182–184]. However, single omics relay in the application of common statistics mainly focusing on the differentiation of the studied groups, for example, principal component analysis (PCA) plots; heat maps; receiver operating characteristic (ROC) curves; dot plots; statistical tests such as Anova, t-test, Mann–Whitney U Test, and several others in accordance with the data size and distribution; and the appropriated false discovery rate (FDR) corrections such as Benjamini-Hochberg, Bonferroni, etc. [185–187]. Regarding the multi omics integration, the application of computational approaches is necessary due to the data complexity. Ingenuity pathway analysis (IPA) is an extensive database from QIAGEN Co. able to integrate transcriptomics, proteomics, and metabolomics data in diverse biological pathways [188]. A similar free alternative is iPathwayGuide developed from ADVAITA Co. [189]; many other software developments and its applications in diverse multi omics investigations are described in the following references: IOAT [190], multi omics of primary immunodeficiencies [191], Omics TIDE [192], IBAG [193], cancer omics [194], and TiMEG [195].

Foodomics

Foodomics represents the integration of advanced omics technologies in food and nutrition studies, aimed at enhancing consumer welfare, health, and understanding [196]. This interdisciplinary field merges food chemistry, biological sciences, and data analysis techniques to comprehend food and its constituents at a molecular level, with the overarching goal of improving food quality, safety, and nutrition by exploring the intricate interactions between food components and the human body. In foodomics research, a primary focus lies in understanding the interplay between food constituents and biological systems, striving to identify biomarkers that objectively measure food intake and shed light on its physiological effects [197]. Foodomics methodologies find application in various studies aimed at linking dietary patterns with health outcomes. Research outcomes can utilize metabolomics techniques to identify specific compounds in diets and analyze their impact on health. For instance, ongoing studies apply metabolome analysis to unveil food-specific compounds (FSCs) that can bridge dietary patterns, such as the Mediterranean-style (MED) diet, with overall health outcomes. Drawing from a controlled feeding MED intervention, the study validates the identification of FSCs from eight distinct foods, detectable in biospecimens post-consumption [198]. Another example is the use of nuclear magnetic resonance (NMR) to reveal alterations of protein and carbohydrate metabolisms in different diets [199]. This study provides new insights into the effects of a healthy diet on glycemia, reduction of inflammation, and weight loss among obese individuals, as well as alteration of the gut microbiota metabolism.

Dilmore et al. [200] evidenced that the modified Mediterranean ketogenic diet (MMKD) could offer potential benefits in combating memory decline associated with AD. The study implemented MMKD dietary interventions for participants with mild cognitive impairment (MCI) to assess their cognitive function. The study utilized shotgun metagenomics to analyze the gut microbiota composition of participants, providing insights into how these dietary interventions influenced the microbiome. Additionally, untargeted metabolomics was employed to analyze metabolite profiles in samples collected at various intervals during the dietary interventions, to identify changes in metabolites related to cognitive function and gut health. However, the untargeted metabolomics platform has limitations in characterizing lipid metabolism and mitochondrial function compared to targeted platforms. Through metagenomic and metabolomic analyses, potential biomarkers associated with cognitive function and gut health were identified. For example, changes in the levels of GABA-producing microbes and GABA-regulating bacteria were observed in individuals with MCI following the MMKD, suggesting a link between gut microbiota composition and cognitive status. Overall, the study integrated a multi omics approach to investigate

the relationship between dietary interventions, gut microbiota composition, metabolite profiles, and cognitive function in individuals at risk for AD.

Foodomics represents a dynamic and interdisciplinary field that holds promise for enhancing our understanding of food and its effects on health and well-being. This field has implications for food industry practices, public health policies, and personalized nutrition strategies, especially with the rise of alternative sources of nutrients.

6. Concluding Remarks

Omics sciences stand at the forefront of biomedical research, offering unprecedented insights into the molecular underpinnings of diseases. Their application in disease study promises to revolutionize the diagnosis, treatment, and prevention of human diseases, highlighting their indispensable role in advancing medical science and improving patient care. In this review, we have discussed the current technological innovations that allow fast and cost-effective sequencing of entire genomes. Additionally, we revised a set of novel and accurate transcriptomics techniques focused on showing how the transcript patterns are affected by the development or disease progression. The development of modern MS techniques has allowed the acquisition of high-throughput data sets in the proteomics, metabolomics, and lipidomics fields. Additionally, we described examples of recent studies using single cells or derived from diverse bodily fluids, as well as plant materials. This review offers an extensive and up-to-date overview of the most widely used omics techniques, detailing the relevance of the omics field in understanding different types of biological systems using a holistic view.

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References

1. Karahalil, B. Overview of Systems Biology and Omics Technologies. *Curr. Med. Chem.* **2016**, *23*, 4221–4230. [CrossRef] [PubMed]
2. Westerhoff, H.V.; Palsson, B.O. The evolution of molecular biology into systems biology. *Nat. Biotechnol.* **2004**, *22*, 1249–1252. [CrossRef] [PubMed]
3. Munung, N.S.; Ambele, M.A.; Moela, P. Advancing global equity in cancer genomics—Challenges and opportunities in Sub-Saharan Africa. *Curr. Opin. Genet. Dev.* **2021**, *66*, 20–24. [CrossRef] [PubMed]
4. Heidecker, B.; Hare, J.M. The use of transcriptomic biomarkers for personalized medicine. *Heart Fail. Rev.* **2007**, *12*, 1–11. [CrossRef]
5. He, T. Implementation of Proteomics in Clinical Trials. *Proteom. Clin. Appl.* **2019**, *13*, e1800198. [CrossRef] [PubMed]
6. Mechref, Y.; Peng, W.; Gautam, S.; Ahmadi, P.; Lin, Y.; Zhu, J.; Zhang, J.; Liu, S.; Singal, A.G.; Parikh, N.D.; et al. Mass spectrometry based biomarkers for early detection of HCC using a glycoproteomic approach. *Adv. Cancer Res.* **2023**, *157*, 23–56. [CrossRef]
7. Peng, W.; Zhao, J.; Dong, X.; Banazadeh, A.; Huang, Y.; Hussien, A.; Mechref, Y. Clinical application of quantitative glycomics. *Expert Rev. Proteom.* **2018**, *15*, 1007–1031. [CrossRef]
8. Dona, A.C.; Coffey, S.; Figtree, G. Translational and emerging clinical applications of metabolomics in cardiovascular disease diagnosis and treatment. *Eur. J. Prev. Cardiol.* **2016**, *23*, 1578–1589. [CrossRef]
9. Astarita, G.; Stocchero, M.; Paglia, G. Unbiased Lipidomics and Metabolomics of Human Brain Samples. *Methods Mol. Biol.* **2018**, *1750*, 255–269. [CrossRef]
10. Aizat, W.M.; Ismail, I.; Noor, N.M. Recent Development in Omics Studies. *Adv. Exp. Med. Biol.* **2018**, *1102*, 1–9. [CrossRef]
11. Hasin, Y.; Seldin, M.; Lusis, A. Multi-omics approaches to disease. *Genome Biol.* **2017**, *18*, 83. [CrossRef] [PubMed]

12. Pinu, F.R.; Beale, D.J.; Paten, A.M.; Kouremenos, K.; Swarup, S.; Schirra, H.J.; Wishart, D. Systems Biology and Multi-Omics Integration: Viewpoints from the Metabolomics Research Community. *Metabolites* **2019**, *9*, 76. [CrossRef]
13. Dihazi, H.; Asif, A.R.; Beißbarth, T.; Bohrer, R.; Feussner, K.; Feussner, I.; Jahn, O.; Lenz, C.; Majcherczyk, A.; Schmidt, B.; et al. Integrative omics—From data to biology. *Expert Rev. Proteom.* **2018**, *15*, 463–466. [CrossRef] [PubMed]
14. Raufaste-Cazavieille, V.; Santiago, R.; Droit, A. Multi-omics analysis: Paving the path toward achieving precision medicine in cancer treatment and immuno-oncology. *Front. Mol. Biosci.* **2022**, *9*, 962743. [CrossRef] [PubMed]
15. Sen, P.; Orešič, M. Integrating Omics Data in Genome-Scale Metabolic Modeling: A Methodological Perspective for Precision Medicine. *Metabolites* **2023**, *13*, 855. [CrossRef]
16. Misra, B.B.; Langefeld, C.D.; Olivier, M.; Cox, L.A. Integrated Omics: Tools, Advances, and Future Approaches. *J. Mol. Endocrinol.* **2018**, *62*, R21–R45. [CrossRef]
17. Katara, A.; Chand, S.; Chaudhary, H.; Chaudhry, V.; Chandra, H.; Dubey, R.C. Evolution and applications of Next Generation Sequencing and its intricate relations with chromatographic and spectrometric techniques in modern day sciences. *J. Chromatogr. Open* **2024**, *5*, 100121. [CrossRef]
18. McGuire, A.L.; Gabriel, S.; Tishkoff, S.A.; Wonkam, A.; Chakravarti, A.; Furlong, E.E.M.; Treutlein, B.; Meissner, A.; Chang, H.Y.; López-Bigas, N.; et al. The road ahead in genetics and genomics. *Nat. Rev. Genet.* **2020**, *21*, 581–596. [CrossRef]
19. Cano-Gamez, E.; Trynka, G. From GWAS to Function: Using Functional Genomics to Identify the Mechanisms Underlying Complex Diseases. *Front. Genet.* **2020**, *11*, 505357. [CrossRef]
20. Jerkovic', I.; Cavalli, G. Understanding 3D genome organization by multidisciplinary methods. *Nat. Rev. Mol. Cell Biol.* **2021**, *22*, 511–528. [CrossRef]
21. Schneider, M.; Radoux, C.J.; Hercules, A.; Ochoa, D.; Dunham, I.; Zalmas, L.-P.; Hessler, G.; Ruf, S.; Shanmugasundaram, V.; Hann, M.M.; et al. The PROTACtable genome. *Nat. Rev. Drug Discov.* **2021**, *20*, 789–797. [CrossRef] [PubMed]
22. Zhang, L.; Song, J.; Kong, L.; Yuan, T.; Li, W.; Zhang, W.; Hou, B.; Lu, Y.; Du, G. The strategies and techniques of drug discovery from natural products. *Pharmacol. Ther.* **2020**, *216*, 107686. [CrossRef] [PubMed]
23. Kiriiri, G.K.; Njogu, P.M.; Mwangi, A.N. Exploring different approaches to improve the success of drug discovery and development projects: A review. *Future J. Pharm. Sci.* **2020**, *6*, 27. [CrossRef]
24. Goel, M.; Sun, H.; Jiao, W.-B.; Schneeberger, K. SyRI: Finding genomic rearrangements and local sequence differences from whole-genome assemblies. *Genome Biol.* **2019**, *20*, 277. [CrossRef]
25. Blair, C.; Ané, C. Phylogenetic Trees and Networks Can Serve as Powerful and Complementary Approaches for Analysis of Genomic Data. *Syst. Biol.* **2019**, *69*, 593–601. [CrossRef] [PubMed]
26. Gilchrist, C.L.M.; Booth, T.J.; van Wersch, B.; van Grieken, L.; Medema, M.H.; Chooi, Y.-H. Cblaster: A remote search tool for rapid identification and visualization of homologous gene clusters. *Bioinform. Adv.* **2021**, *1*, vbab016. [CrossRef]
27. Vishwanath, P.P.; Bidaramali, V.; Lata, S.; Yadav, R.K. Transcriptomics: Illuminating the molecular landscape of vegetable crops: A review. *J. Plant Biochem. Biotechnol.* **2024**. [CrossRef]
28. Lee, J.-Y. The Principles and Applications of High-Throughput Sequencing Technologies. *Dev. Reprod.* **2023**, *27*, 9–24. [CrossRef]
29. Nguyen, H.L.; Duviau, M.-P.; Cocaign-Bousquet, M.; Nouaille, S.; Girbal, L. Multiplexing polysome profiling experiments to study translation in *Escherichia coli*. *PLoS ONE* **2019**, *14*, e0212297. [CrossRef]
30. Pringle, E.S.; McCormick, C.; Cheng, Z. Polysome Profiling Analysis of mRNA and Associated Proteins Engaged in Translation. *Curr. Protoc. Mol. Biol.* **2019**, *125*, e79. [CrossRef]
31. Brar, G.A.; Weissman, J.S. Ribosome profiling reveals the what, when, where and how of protein synthesis. *Nat. Rev. Mol. Cell Biol.* **2015**, *16*, 651–664. [CrossRef] [PubMed]
32. Liang, S.; Bellato, H.M.; Lorent, J.; Lupinacci, F.C.S.; Oertlin, C.; van Hoef, V.; Andrade, V.P.; Roffé, M.; Masvidal, L.; Hajj, G.N.M.; et al. Polysome-profiling in small tissue samples. *Nucleic Acids Res.* **2018**, *46*, e3. [CrossRef] [PubMed]
33. Fuchs, G.; Voichek, Y.; Rabani, M.; Benjamin, S.; Gilad, S.; Amit, I.; Oren, M. Simultaneous measurement of genome-wide transcription elongation speeds and rates of RNA polymerase II transition into active elongation with 4sUDRB-seq. *Nat. Protoc.* **2015**, *10*, 605–618. [CrossRef] [PubMed]
34. Fuchs, G.; Voichek, Y.; Benjamin, S.; Gilad, S.; Amit, I.; Oren, M. 4sUDRB-seq: Measuring genomewide transcriptional elongation rates and initiation frequencies within cells. *Genome Biol.* **2014**, *15*, R69. [CrossRef] [PubMed]
35. Zhang, X.-O.; Fu, Y.; Mou, H.; Xue, W.; Weng, Z. The temporal landscape of recursive splicing during Pol II transcription elongation in human cells. *PLoS Genet.* **2018**, *14*, e1007579. [CrossRef] [PubMed]
36. Zhao, L.; Zhang, H.; Kohnen, M.V.; Prasad, K.V.S.K.; Gu, L.; Reddy, A.S.N. Analysis of Transcriptome and Epitranscriptome in Plants Using PacBio Iso-Seq and Nanopore-Based Direct RNA Sequencing. *Front. Genet.* **2019**, *10*, 430951. [CrossRef] [PubMed]
37. Song, Y.; Xu, X.; Wang, W.; Tian, T.; Zhu, Z.; Yang, C. Single cell transcriptomics: Moving towards multi-omics. *Analyst* **2019**, *144*, 3172–3189. [CrossRef] [PubMed]
38. Adil, A.; Kumar, V.; Jan, A.T.; Asger, M. Single-Cell Transcriptomics: Current Methods and Challenges in Data Acquisition and Analysis. *Front. Neurosci.* **2021**, *15*, 591122. [CrossRef] [PubMed]
39. Wen, L.; Li, G.; Huang, T.; Geng, W.; Pei, H.; Yang, J.; Zhu, M.; Zhang, P.; Hou, R.; Tian, G.; et al. Single-cell technologies: From research to application. *Innovation* **2022**, *3*, 100342. [CrossRef]

40. Salcher, S.; Heidegger, I.; Untergasser, G.; Fotakis, G.; Scheiber, A.; Martowicz, A.; Noureen, A.; Krogsdam, A.; Schatz, C.; Schäfer, G.; et al. Comparative analysis of 10X Chromium vs. BD Rhapsody whole transcriptome single-cell sequencing technologies in complex human tissues. *Heliyon* **2024**, *10*, e28358. [CrossRef]
41. Qiu, Q.; Hu, P.; Qiu, X.; Govek, K.W.; Cámara, P.G.; Wu, H. Massively parallel and time-resolved RNA sequencing in single cells with scNT-seq. *Nat. Methods* **2020**, *17*, 991–1001. [CrossRef]
42. Andreatta, M.; Corria-Osorio, J.; Müller, S.; Cubas, R.; Coukos, G.; Carmona, S.J. Interpretation of T cell states from single-cell transcriptomics data using reference atlases. *Nat. Commun.* **2021**, *12*, 2965. [CrossRef]
43. Adema, K.; Schon, M.A.; Nodine, M.D.; Kohlen, W. Lost in space: What single-cell RNA sequencing cannot tell you. *Trends Plant Sci.* **2024**. [CrossRef]
44. Wang, Y.; Liu, B.; Zhao, G.; Lee, Y.; Buzdin, A.; Mu, X.; Zhao, J.; Chen, H.; Li, X. Spatial transcriptomics: Technologies, applications and experimental considerations. *Genomics* **2023**, *115*, 110671. [CrossRef]
45. Chen, T.-Y.; You, L.; Hardillo, J.A.U.; Chien, M.-P. Spatial Transcriptomic Technologies. *Cells* **2023**, *12*, 2042. [CrossRef]
46. Piñeiro, A.J.; Houser, A.E.; Ji, A.L. Research Techniques Made Simple: Spatial Transcriptomics. *J. Investig. Dermatol.* **2022**, *142*, 993–1001.e1001. [CrossRef]
47. Shi, W.; Zhang, J.; Huang, S.; Fan, Q.; Cao, J.; Zeng, J.; Wu, L.; Yang, C. Next-Generation Sequencing-Based Spatial Transcriptomics: A Perspective from Barcoding Chemistry. *JACS Au* **2024**, *4*, 1723–1743. [CrossRef]
48. Chen, A.; Liao, S.; Cheng, M.; Ma, K.; Wu, L.; Lai, Y.; Qiu, X.; Yang, J.; Xu, J.; Hao, S.; et al. Spatiotemporal transcriptomic atlas of mouse organogenesis using DNA nanoball-patterned arrays. *Cell* **2022**, *185*, 1777–1792.e1721. [CrossRef]
49. Al-Amrani, S.; Al-Jabri, Z.; Al-Zaabi, A.; Alshekaili, J.; Al-Khabori, M. Proteomics: Concepts and applications in human medicine. *World J. Biol. Chem.* **2021**, *12*, 57–69. [CrossRef]
50. Gutierrez Reyes, C.D.; Jiang, P.; Donohoo, K.; Atashi, M.; Mechref, Y.S. Glycomics and glycoproteomics: Approaches to address isomeric separation of glycans and glycopeptides. *J. Sep. Sci.* **2021**, *44*, 403–425. [CrossRef]
51. Peng, W.; Gutierrez Reyes, C.D.; Gautam, S.; Yu, A.; Cho, B.G.; Goli, M.; Donohoo, K.; Mondello, S.; Kobeissy, F.; Mechref, Y. MS-based glycomics and glycoproteomics methods enabling isomeric characterization. *Mass. Spectrom. Rev.* **2023**, *42*, 577–616. [CrossRef] [PubMed]
52. Alejo-Jacuinde, G.; Nájera-González, H.-R.; Chávez Montes, R.A.; Gutierrez Reyes, C.D.; Barragán-Rosillo, A.C.; Perez Sanchez, B.; Mechref, Y.; López-Arredondo, D.; Yong-Villalobos, L.; Herrera-Estrella, L. Multi-omic analyses reveal the unique properties of chia (*Salvia hispanica*) seed metabolism. *Commun. Biol.* **2023**, *6*, 820. [CrossRef]
53. Reyes, C.D.G.; Hakim, M.A.; Atashi, M.; Goli, M.; Gautam, S.; Wang, J.; Bennett, A.I.; Zhu, J.; Lubman, D.M.; Mechref, Y. LC-MS/MS Isomeric Profiling of N-Glycans Derived from Low-Abundant Serum Glycoproteins in Mild Cognitive Impairment Patients. *Biomolecules* **2022**, *12*, 1657. [CrossRef] [PubMed]
54. Cho, B.G.; Gutierrez Reyes, C.D.; Mechref, Y. N-Glycomics of Cerebrospinal Fluid: Method Comparison. *Molecules* **2021**, *26*, 1712. [CrossRef]
55. Gonçalves, E.; Poulos, R.C.; Cai, Z.; Barthorpe, S.; Manda, S.S.; Lucas, N.; Beck, A.; Bucio-Noble, D.; Dausmann, M.; Hall, C.; et al. Pan-cancer proteomic map of 949 human cell lines. *Cancer Cell* **2022**, *40*, 835–849.e838. [CrossRef]
56. Rontogianni, S.; Synadaki, E.; Li, B.; Liefaard, M.C.; Lips, E.H.; Wesseling, J.; Wu, W.; Altelaar, M. Proteomic profiling of extracellular vesicles allows for human breast cancer subtyping. *Commun. Biol.* **2019**, *2*, 325. [CrossRef]
57. Dupree, E.J.; Jayathirtha, M.; Yorkey, H.; Mihasan, M.; Petre, B.A.; Darie, C.C. A Critical Review of Bottom-Up Proteomics: The Good, the Bad, and the Future of this Field. *Proteomes* **2020**, *8*, 14. [CrossRef]
58. Toby, T.K.; Fornelli, L.; Srzentić, K.; DeHart, C.J.; Levitsky, J.; Friedewald, J.; Kelleher, N.L. A comprehensive pipeline for translational top-down proteomics from a single blood draw. *Nat. Protoc.* **2019**, *14*, 119–152. [CrossRef] [PubMed]
59. Chen, L.; Kashina, A. Post-translational Modifications of the Protein Termini. *Front. Cell Dev. Biol.* **2021**, *9*, 719590. [CrossRef]
60. Lee, J.M.; Hammarén, H.M.; Savitski, M.M.; Baek, S.H. Control of protein stability by post-translational modifications. *Nat. Commun.* **2023**, *14*, 201. [CrossRef]
61. Johnson, E.C.B.; Bian, S.; Haque, R.U.; Carter, E.K.; Watson, C.M.; Gordon, B.A.; Ping, L.; Duong, D.M.; Epstein, M.P.; McDade, E.; et al. Cerebrospinal fluid proteomics define the natural history of autosomal dominant Alzheimer's disease. *Nat. Med.* **2023**, *29*, 1979–1988. [CrossRef] [PubMed]
62. Palstrøm, N.B.; Matthiesen, R.; Rasmussen, L.M.; Beck, H.C. Recent Developments in Clinical Plasma Proteomics-Applied to Cardiovascular Research. *Biomedicines* **2022**, *10*, 162. [CrossRef] [PubMed]
63. Ma, M.; Huo, S.; Zhang, M.; Qian, S.; Zhu, X.; Pu, J.; Rasam, S.; Xue, C.; Shen, S.; An, B.; et al. In-depth mapping of protein localizations in whole tissue by micro-scaffold assisted spatial proteomics (MASP). *Nat. Commun.* **2022**, *13*, 7736. [CrossRef] [PubMed]
64. Kangas, P.; Nyman, T.A.; Metsähonkala, L.; Burns, C.; Tempest, R.; Williams, T.; Karttunen, J.; Jokinen, T.S. Towards optimised extracellular vesicle proteomics from cerebrospinal fluid. *Sci. Rep.* **2023**, *13*, 9564. [CrossRef] [PubMed]
65. Van den Ackerveken, P.; Lobbens, A.; Turatsinze, J.-V.; Solis-Mezarino, V.; Völker-Albert, M.; Imhof, A.; Herzog, M. A novel proteomics approach to epigenetic profiling of circulating nucleosomes. *Sci. Rep.* **2021**, *11*, 7256. [CrossRef] [PubMed]
66. Petrosius, V.; Schoof, E.M. Recent advances in the field of single-cell proteomics. *Transl. Oncol.* **2023**, *27*, 101556. [CrossRef]
67. Liu, R.; Wu, J.; Guo, H.; Yao, W.; Li, S.; Lu, Y.; Jia, Y.; Liang, X.; Tang, J.; Zhang, H. Post-translational modifications of histones: Mechanisms, biological functions, and therapeutic targets. *MedComm* **2023**, *4*, e292. [CrossRef] [PubMed]

68. Ye, Z.; Sabatier, P.; Hoeven, L.V.D.; Phlairaharn, T.; Hartlmayr, D.; Izaguirre, F.; Seth, A.; Joshi, H.J.; Bekker-Jensen, D.B.; Bache, N.; et al. High-throughput and scalable single cell proteomics identifies over 5000 proteins per cell. *bioRxiv* **2023**. [CrossRef]
69. Chandler, K.B.; Leon, D.R.; Kuang, J.; Meyer, R.D.; Rahimi, N.; Costello, C.E. N-Glycosylation regulates ligand-dependent activation and signaling of vascular endothelial growth factor receptor 2 (VEGFR2). *J. Biol. Chem.* **2019**, *294*, 13117–13130. [CrossRef]
70. Perkey, E.; Maurice De Sousa, D.; Carrington, L.; Chung, J.; Dils, A.; Granadier, D.; Koch, U.; Radtke, F.; Ludewig, B.; Blazar, B.R.; et al. GCNT1-Mediated O-Glycosylation of the Sialomucin CD43 Is a Sensitive Indicator of Notch Signaling in Activated T Cells. *J. Immunol.* **2020**, *204*, 1674–1688. [CrossRef]
71. Van Coillie, J.; Schulz, M.A.; Bentlage, A.E.H.; de Haan, N.; Ye, Z.; Geerdes, D.M.; van Esch, W.J.E.; Hafkenscheid, L.; Miller, R.L.; Narimatsu, Y.; et al. Role of N-Glycosylation in FcγRIIIa interaction with IgG. *Front. Immunol.* **2022**, *13*, 987151. [CrossRef]
72. Ozdilek, A.; Paschall, A.V.; Dookwah, M.; Tiemeyer, M.; Avci, F.Y. Host protein glycosylation in nucleic acid vaccines as a potential hurdle in vaccine design for nonviral pathogens. *Proc. Natl. Acad. Sci. USA* **2020**, *117*, 1280–1282. [CrossRef]
73. Onigbinde, S.; Reyes, C.D.G.; Fowowe, M.; Daramola, O.; Atashi, M.; Bennett, A.I.; Mechref, Y. Variations in O-Glycosylation Patterns Influence Viral Pathogenicity, Infectivity, and Transmissibility in SARS-CoV-2 Variants. *Biomolecules* **2023**, *13*, 1467. [CrossRef]
74. Munkley, J.; Mills, I.G.; Elliott, D.J. The role of glycans in the development and progression of prostate cancer. *Nat. Rev. Urol.* **2016**, *13*, 324–333. [CrossRef]
75. Zhou, S.; Veillon, L.; Dong, X.; Huang, Y.; Mechref, Y. Direct comparison of derivatization strategies for LC-MS/MS analysis of N-glycans. *Analyst* **2017**, *142*, 4446–4455. [CrossRef]
76. Jiang, P.; Huang, Y.; Gutierrez Reyes, C.D.; Zhong, J.; Mechref, Y. Isomeric Separation of α2,3/α2,6-Linked 2-Aminobenzamide (2AB)-Labeled Sialoglycopeptides by C18-LC-MS/MS. *Anal. Chem.* **2023**, *95*, 18388–18397. [CrossRef]
77. Zhong, J.; Huang, Y.; Mechref, Y. Derivatization of Sialylated Glycopeptides (DOSG) Enabling Site-Specific Isomeric Profiling Using LC-MS/MS. *Anal. Chem.* **2021**, *93*, 5763–5772. [CrossRef]
78. Jiang, K.; Aloor, A.; Qu, J.; Xiao, C.; Wu, Z.; Ma, C.; Zhang, L.; Wang, P.G. Rapid and sensitive MALDI MS analysis of oligosaccharides by using 2-hydrazinopyrimidine as a derivative reagent and co-matrix. *Anal. Bioanal. Chem.* **2017**, *409*, 421–429. [CrossRef]
79. Wu, Y.; Sha, Q.; Du, J.; Wang, C.; Zhang, L.; Liu, B.F.; Lin, Y.; Liu, X. Determination of N-glycans by high performance liquid chromatography using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate as the glycosylamine labeling reagent. *J. Chromatogr. A* **2018**, *1535*, 114–122. [CrossRef]
80. Ruhaak, L.R.; Zauner, G.; Huhn, C.; Bruggink, C.; Deelder, A.M.; Wuhrer, M. Glycan labeling strategies and their use in identification and quantification. *Anal. Bioanal. Chem.* **2010**, *397*, 3457–3481. [CrossRef]
81. Mechref, Y.; Kang, P.; Novotny, M.V. Solid-phase permethylation for glycomic analysis. *Methods Mol. Biol.* **2009**, *534*, 53–64. [CrossRef] [PubMed]
82. Saraswat, M.; Mangalaparthi, K.K.; Garapati, K.; Pandey, A. TMT-Based Multiplexed Quantitation of N-Glycopeptides Reveals Glycoproteome Remodeling Induced by Oncogenic Mutations. *ACS Omega* **2022**, *7*, 11023–11032. [CrossRef] [PubMed]
83. Dube, D.H.; Bertozzi, C.R. Glycans in cancer and inflammation—Potential for therapeutics and diagnostics. *Nat. Rev. Drug Discov.* **2005**, *4*, 477–488. [CrossRef] [PubMed]
84. Taniguchi, N.; Kizuka, Y. Glycans and cancer: Role of N-glycans in cancer biomarker, progression and metastasis, and therapeutics. *Adv. Cancer Res.* **2015**, *126*, 11–51. [CrossRef] [PubMed]
85. Gutierrez Reyes, C.D.; Huang, Y.; Atashi, M.; Zhang, J.; Zhu, J.; Liu, S.; Parikh, N.D.; Singal, A.G.; Dai, J.; Lubman, D.M.; et al. PRM-MS Quantitative Analysis of Isomeric N-Glycopeptides Derived from Human Serum Haptoglobin of Patients with Cirrhosis and Hepatocellular Carcinoma. *Metabolites* **2021**, *11*, 563. [CrossRef] [PubMed]
86. Veillon, L.; Huang, Y.; Peng, W.; Dong, X.; Cho, B.G.; Mechref, Y. Characterization of isomeric glycan structures by LC-MS/MS. *Electrophoresis* **2017**, *38*, 2100–2114. [CrossRef]
87. Kizuka, Y.; Kitazume, S.; Taniguchi, N. N-glycan and Alzheimer's disease. *Biochim. Biophys. Acta Gen. Subj.* **2017**, *1861*, 2447–2454. [CrossRef]
88. Tena, J.; Tang, X.; Zhou, Q.; Harvey, D.; Barajas-Mendoza, M.; Jin, L.W.; Maezawa, I.; Zivkovic, A.M.; Lebrilla, C.B. Glycosylation alterations in serum of Alzheimer's disease patients show widespread changes in N-glycosylation of proteins related to immune function, inflammation, and lipoprotein metabolism. *Alzheimers Dement.* **2022**, *14*, e12309. [CrossRef] [PubMed]
89. Pinho, S.S.; Alves, I.; Gaifem, J.; Rabinovich, G.A. Immune regulatory networks coordinated by glycans and glycan-binding proteins in autoimmunity and infection. *Cell Mol. Immunol.* **2023**, *20*, 1101–1113. [CrossRef]
90. Chien, M.W.; Fu, S.H.; Hsu, C.Y.; Liu, Y.W.; Sytwu, H.K. The Modulatory Roles of N-glycans in T-Cell-Mediated Autoimmune Diseases. *Int. J. Mol. Sci.* **2018**, *19*, 780. [CrossRef]
91. Unione, L.; Gimeno, A.; Valverde, P.; Calloni, I.; Coelho, H.; Mirabella, S.; Poveda, A.; Arda, A.; Jimenez-Barbero, J. Glycans in Infectious Diseases. A Molecular Recognition Perspective. *Curr. Med. Chem.* **2017**, *24*, 4057–4080. [CrossRef] [PubMed]
92. Reyes, C.D.G.; Onigbinde, S.; Sanni, A.; Bennett, A.I.; Jiang, P.; Daramola, O.; Ahmadi, P.; Fowowe, M.; Atashi, M.; Sandilya, V.; et al. N-Glycome Profile of the Spike Protein S1: Systemic and Comparative Analysis from Eleven Variants of SARS-CoV-2. *Biomolecules* **2023**, *13*, 1421. [CrossRef]

93. Freitas, D.; Campos, D.; Gomes, J.; Pinto, F.; Macedo, J.A.; Matos, R.; Mereiter, S.; Pinto, M.T.; Polónia, A.; Gartner, F.; et al. O-glycans truncation modulates gastric cancer cell signaling and transcription leading to a more aggressive phenotype. *EBioMedicine* **2019**, *40*, 349–362. [CrossRef] [PubMed]
94. Amon, R.; Reuven, E.M.; Leviatan Ben-Arye, S.; Padler-Karavani, V. Glycans in immune recognition and response. *Carbohydr. Res.* **2014**, *389*, 115–122. [CrossRef] [PubMed]
95. Liwosz, A.; Lei, T.; Kukuruzinska, M.A. N-glycosylation affects the molecular organization and stability of E-cadherin junctions. *J. Biol. Chem.* **2006**, *281*, 23138–23149. [CrossRef] [PubMed]
96. Rodrigues, J.G.; Balmaña, M.; Macedo, J.A.; Poças, J.; Fernandes, Â.; de-Freitas-Junior, J.C.M.; Pinho, S.S.; Gomes, J.; Magalhães, A.; Gomes, C.; et al. Glycosylation in cancer: Selected roles in tumour progression, immune modulation and metastasis. *Cell Immunol.* **2018**, *333*, 46–57. [CrossRef] [PubMed]
97. Zhang, Q.; Ma, C.; Chin, L.S.; Li, L. Integrative glycoproteomics reveals protein N-glycosylation aberrations and glycoproteomic network alterations in Alzheimer's disease. *Sci. Adv.* **2020**, *6*, eabc5802. [CrossRef] [PubMed]
98. Bennun, S.V.; Hizal, D.B.; Heffner, K.; Can, O.; Zhang, H.; Betenbaugh, M.J. Systems Glycobiology: Integrating Glycogenomics, Glycoproteomics, Glycomics, and Other 'Omics Data Sets to Characterize Cellular Glycosylation Processes. *J. Mol. Biol.* **2016**, *428*, 3337–3352. [CrossRef] [PubMed]
99. Peixoto, A.; Relvas-Santos, M.; Azevedo, R.; Santos, L.L.; Ferreira, J.A. Protein Glycosylation and Tumor Microenvironment Alterations Driving Cancer Hallmarks. *Front. Oncol.* **2019**, *9*, 380. [CrossRef]
100. Guo, S.; Zhang, C.; Le, A. The limitless applications of single-cell metabolomics. *Curr. Opin. Biotechnol.* **2021**, *71*, 115–122. [CrossRef]
101. Blanco, A.; Blanco, G. Chapter 5—Lipids. In *Medical Biochemistry*, 2nd ed.; Blanco, A., Blanco, G., Eds.; Academic Press: Cambridge, MA, USA, 2022; pp. 105–129.
102. Li, Z.; Cheng, S.; Lin, Q.; Cao, W.; Yang, J.; Zhang, M.; Shen, A.; Zhang, W.; Xia, Y.; Ma, X.; et al. Single-cell lipidomics with high structural specificity by mass spectrometry. *Nat. Commun.* **2021**, *12*, 2869. [CrossRef]
103. Yang, K.; Han, X. Lipidomics: Techniques, Applications, and Outcomes Related to Biomedical Sciences. *Trends Biochem. Sci.* **2016**, *41*, 954–969. [CrossRef] [PubMed]
104. Hoang, G.; Udupa, S.; Le, A. Chapter Six—Application of metabolomics technologies toward cancer prognosis and therapy. In *International Review of Cell and Molecular Biology*; Montrose, D.C., Galluzzi, L., Eds.; Academic Press: Cambridge, MA, USA, 2019; Volume 347, pp. 191–223.
105. Wang, Z.; Zhu, H.; Xiong, W. Advances in mass spectrometry-based multi-scale metabolomic methodologies and their applications in biological and clinical investigations. *Sci. Bull.* **2023**, *68*, 2268–2284. [CrossRef] [PubMed]
106. Zhang, Z.; Bao, C.; Jiang, L.; Wang, S.; Wang, K.; Lu, C.; Fang, H. When cancer drug resistance meets metabolomics (bulk, single-cell and/or spatial): Progress, potential, and perspective. *Front. Oncol.* **2022**, *12*, 1054233. [CrossRef] [PubMed]
107. Hanahan, D. Hallmarks of Cancer: New Dimensions. *Cancer Discov.* **2022**, *12*, 31–46. [CrossRef] [PubMed]
108. Bhat, G.R.; Sethi, I.; Sadida, H.Q.; Rah, B.; Mir, R.; Algehainy, N.; Albalawi, I.A.; Masoodi, T.; Subbaraj, G.K.; Jamal, F.; et al. Cancer cell plasticity: From cellular, molecular, and genetic mechanisms to tumor heterogeneity and drug resistance. *Cancer Metastasis Rev.* **2024**, *43*, 197–228. [CrossRef] [PubMed]
109. Zhang, W.; Xu, F.; Yao, J.; Mao, C.; Zhu, M.; Qian, M.; Hu, J.; Zhong, H.; Zhou, J.; Shi, X.; et al. Single-cell metabolic fingerprints discover a cluster of circulating tumor cells with distinct metastatic potential. *Nat. Commun.* **2023**, *14*, 2485. [CrossRef]
110. Zhu, G.; Zhang, W.; Zhao, Y.; Chen, T.; Yuan, H.; Liu, Y.; Guo, G.; Liu, Z.; Wang, X. Single-Cell Metabolomics-Based Strategy for Studying the Mechanisms of Drug Action. *Anal. Chem.* **2023**, *95*, 4712–4720. [CrossRef]
111. Zeng, Q.; Mousa, M.; Nadukkandy, A.S.; Franssens, L.; Alnaqbi, H.; Alshamsi, F.Y.; Safar, H.A.; Carmeliet, P. Understanding tumour endothelial cell heterogeneity and function from single-cell omics. *Nat. Rev. Cancer* **2023**, *23*, 544–564. [CrossRef]
112. Chen, X.; Peng, Z.; Yang, Z. Metabolomics studies of cell–cell interactions using single cell mass spectrometry combined with fluorescence microscopy. *Chem. Sci.* **2022**, *13*, 6687–6695. [CrossRef]
113. León-Letelier, R.A.; Dou, R.; Vykoukal, J.; Yip-Schneider, M.T.; Maitra, A.; Irajizad, E.; Wu, R.; Dennison, J.B.; Do, K.-A.; Zhang, J.; et al. Contributions of the Microbiome-Derived Metabolome for Risk Assessment and Prognostication of Pancreatic Cancer. *Clin. Chem.* **2024**, *70*, 102–115. [CrossRef] [PubMed]
114. Zahra, W.; Rai, S.N.; Birla, H.; Singh, S.S.; Dilnashin, H.; Rathore, A.S.; Singh, S.P. The Global Economic Impact of Neurodegenerative Diseases: Opportunities and Challenges. In *Bioeconomy for Sustainable Development*; Keswani, C., Ed.; Springer: Singapore, 2020; pp. 333–345.
115. Zheng, Y.; Liu, Z.; Xing, J.; Zheng, Z.; Pi, Z.; Song, F.; Liu, S. In situ analysis of single cell and biological samples with rGO-Cu functional probe ESI-MS spectrometry. *Talanta* **2020**, *211*, 120751. [CrossRef] [PubMed]
116. Bao, L.; Wu, B.; Yang, C.; Xu, X.; Shi, Z.; Jiang, D. Targeted Metabolomics Analysis of Serum Amino Acids in T2DM Patients. *Diabetes Metab. Syndr. Obes.* **2024**, *17*, 203–212. [CrossRef] [PubMed]
117. Yan, H.; Li, G.; Zhang, X.; Zhang, C.; Li, M.; Qiu, Y.; Sun, W.; Dong, Y.; Li, S.; Li, J. Targeted metabolomics-based understanding of the sleep disturbances in drug-naïve patients with schizophrenia. *BMC Psychiatry* **2024**, *24*, 355. [CrossRef] [PubMed]
118. Lee, E.-J.; Kim, D.J.; Kang, D.-W.; Yang, W.; Jeong, H.-Y.; Kim, J.-M.; Ko, S.-B.; Lee, S.-H.; Yoon, B.-W.; Cho, J.-Y.; et al. Targeted Metabolomic Biomarkers for Stroke Subtyping. *Transl. Stroke Res.* **2024**, *15*, 422–432. [CrossRef] [PubMed]

119. Wang, S.; He, T.; Wang, H. Non-targeted metabolomics study for discovery of hepatocellular carcinoma serum diagnostic biomarker. *J. Pharm. Biomed. Anal.* **2024**, *239*, 115869. [CrossRef]
120. Sun, S.; Chen, M.; Zhang, T.; Wang, Y.; Shen, W.; Zhang, T.; Liu, J.; Lan, H.; Zhao, J.; Lin, F.; et al. Identification of Key Factors in Cartilage Tissue During the Progression of Osteoarthritis Using a Non-targeted Metabolomics Strategy. *Phenomics* **2024**. [CrossRef]
121. Chen, W.; Guo, W.; Li, Y.; Chen, M. Integrative analysis of metabolomics and transcriptomics to uncover biomarkers in sepsis. *Sci. Rep.* **2024**, *14*, 9676. [CrossRef] [PubMed]
122. Wang, S.; Liu, G.; Xie, C.; Zhou, Y.; Yang, R.; Wu, J.; Xu, J.; Tu, K. Metabolomics Analysis of Different Quinoa Cultivars Based on UPLC-ZenoTOF-MS/MS and Investigation into Their Antioxidant Characteristics. *Plants* **2024**, *13*, 240. [CrossRef]
123. Windarsih, A.; Arifah, M.F.; Utami, I.D.; Suratno; Rohman, A. Detection of goat milk adulteration in horse milk using LC-HRMS-based non-targeted metabolomics and chemometrics. *Chem. Pap.* **2024**, *78*, 809–821. [CrossRef]
124. Tian, Y.; Wang, W.; Jiang, W.; Zhang, G.; He, J.; Dong, S.; Zhou, Y.; Yang, W.; Tang, Q.; Yu, Y.; et al. Non-targeted metabolomics provides insights into the distinct amino acid and lipid metabolism in liver tissues of rainbow trout (*Oncorhynchus mykiss*) cultured in seawater at different temperatures. *Aquaculture* **2024**, *579*, 740188. [CrossRef]
125. Yang, S.; Pathak, S.; Tang, H.; Zhang, D.; Chen, Y.; Ntezimana, B.; Ni, D.; Yu, Z. Non-Targeted Metabolomics Reveals the Effects of Different Rolling Methods on Black Tea Quality. *Foods* **2024**, *13*, 325. [CrossRef] [PubMed]
126. Feng, X.; Ma, R.; Wang, Y.; Tong, L.; Wen, W.; Mu, T.; Tian, J.; Yu, B.; Gu, Y.; Zhang, J. Non-targeted metabolomics identifies biomarkers in milk with high and low milk fat percentage. *Food Res. Int.* **2024**, *179*, 113989. [CrossRef] [PubMed]
127. Mo, L.; Fang, L.; Yao, W.; Nie, J.; Dai, J.; Liang, Y.; Qin, L. LC-QTOF/MS-based non-targeted metabolomics to explore the toxic effects of di(2-ethylhexyl) phthalate (DEHP) on *Brassica chinensis* L. *Sci. Total Environ.* **2024**, *918*, 170817. [CrossRef] [PubMed]
128. Song, Z.; Wu, H.; Fang, X.; Feng, X.; Zhou, L. The cardiovascular toxicity of polystyrene microplastics in rats: Based on untargeted metabolomics analysis. *Front. Pharmacol.* **2024**, *15*, 1336369. [CrossRef]
129. Xiao, X.; Zhou, Y.; Li, X.; Jin, J.; Durham, J.; Ye, Z.; Wang, Y.; Hennig, B.; Deng, P. 13C-Stable isotope resolved metabolomics uncovers dynamic biochemical landscape of gut microbiome-host organ communications in mice. *Microbiome* **2024**, *12*, 90. [CrossRef] [PubMed]
130. Wen, S.; Tu, X.; Zang, Q.; Zhu, Y.; Li, L.; Zhang, R.; Abliz, Z. Liquid chromatography–mass spectrometry-based metabolomics and fluxomics reveals the metabolic alterations in glioma U87MG multicellular tumor spheroids versus two-dimensional cell cultures. *Rapid Commun. Mass. Spectrom.* **2024**, *38*, e9670. [CrossRef]
131. Peper, C.J.; Kilgore, M.D.; Jiang, Y.; Xiu, Y.; Xia, W.; Wang, Y.; Shi, M.; Zhou, D.; Dumont, A.S.; Wang, X.; et al. Tracing the path of disruption: 13C isotope applications in traumatic brain injury-induced metabolic dysfunction. *CNS Neurosci. Ther.* **2024**, *30*, e14693. [CrossRef]
132. Osipenko, S.; Bashilov, A.; Vishnevskaya, A.; Rumiantseva, L.; Levashova, A.; Kovalenko, A.; Tupertsev, B.; Kireev, A.; Nikolaev, E.; Kostyukevich, Y. Investigating the Metabolism of Plants Germinated in Heavy Water, D_2O, and $H_{218}O$-Enriched Media Using High-Resolution Mass Spectrometry. *Int. J. Mol. Sci.* **2023**, *24*, 15396. [CrossRef]
133. Brorsen, L.F.; McKenzie, J.S.; Tullin, M.F.; Bendtsen, K.M.S.; Pinto, F.E.; Jensen, H.E.; Haedersdal, M.; Takats, Z.; Janfelt, C.; Lerche, C.M. Cutaneous squamous cell carcinoma characterized by MALDI mass spectrometry imaging in combination with machine learning. *Sci. Rep.* **2024**, *14*, 11091. [CrossRef]
134. Bag, S.; Oetjen, J.; Shaikh, S.; Chaudhary, A.; Arun, P.; Mukherjee, G. Impact of spatial metabolomics on immune-microenvironment in oral cancer prognosis: A clinical report. *Mol. Cell. Biochem.* **2024**, *479*, 41–49. [CrossRef] [PubMed]
135. Vermeulen, I.; Rodriguez-Alvarez, N.; François, L.; Viot, D.; Poosti, F.; Aronica, E.; Dedeurwaerdere, S.; Barton, P.; Cillero-Pastor, B.; Heeren, R.M.A. Spatial omics reveals molecular changes in focal cortical dysplasia type II. *Neurobiol. Dis.* **2024**, *195*, 106491. [CrossRef] [PubMed]
136. Wang, J.; Sun, N.; Kunzke, T.; Shen, J.; Zens, P.; Prade, V.M.; Feuchtinger, A.; Berezowska, S.; Walch, A. Spatial metabolomics identifies distinct tumor-specific and stroma-specific subtypes in patients with lung squamous cell carcinoma. *Npj Precis. Oncol.* **2023**, *7*, 114. [CrossRef] [PubMed]
137. Conroy, L.R.; Clarke, H.A.; Allison, D.B.; Valenca, S.S.; Sun, Q.; Hawkinson, T.R.; Young, L.E.A.; Ferreira, J.E.; Hammonds, A.V.; Dunne, J.B.; et al. Spatial metabolomics reveals glycogen as an actionable target for pulmonary fibrosis. *Nat. Commun.* **2023**, *14*, 2759. [CrossRef] [PubMed]
138. Züllig, T.; Trötzmüller, M.; Köfeler, H.C. Lipidomics from sample preparation to data analysis: A primer. *Anal. Bioanal. Chem.* **2020**, *412*, 2191–2209. [CrossRef]
139. Kenwood, B.M.; Merrill, A.H. Lipidomics. In *Encyclopedia of Cell Biology*; Bradshaw, R.A., Stahl, P.D., Eds.; Academic Press: Waltham, MA, USA, 2016; pp. 147–159.
140. Zhao, Y.-Y.; Cheng, X.-l.; Lin, R.-C. Chapter One—Lipidomics Applications for Discovering Biomarkers of Diseases in Clinical Chemistry. In *International Review of Cell and Molecular Biology*; Jeon, K.W., Ed.; Academic Press: Cambridge, MA, USA, 2014; Volume 313, pp. 1–26.
141. Costa, A.C.; Riça, L.B.; van de Bilt, M.; Zandonadi, F.S.; Gattaz, W.F.; Talib, L.L.; Sussulini, A. Application of Lipidomics in Psychiatry: Plasma-Based Potential Biomarkers in Schizophrenia and Bipolar Disorder. *Metabolites* **2023**, *13*, 600. [CrossRef]
142. Chiurchiù, V.; Tiberi, M.; Matteocci, A.; Fazio, F.; Siffeti, H.; Saracini, S.; Mercuri, N.B.; Sancesario, G. Lipidomics of Bioactive Lipids in Alzheimer's and Parkinson's Diseases: Where Are We? *Int. J. Mol. Sci.* **2022**, *23*, 6235. [CrossRef]

143. Graham, Z.A.; Siedlik, J.A.; Toro, C.A.; Harlow, L.; Cardozo, C.P. Boldine Alters Serum Lipidomic Signatures after Acute Spinal Cord Transection in Male Mice. *Int. J. Environ. Res. Public Health* **2023**, *20*, 6591. [CrossRef]
144. Senko, D.; Gorovaya, A.; Stekolshchikova, E.; Anikanov, N.; Fedianin, A.; Baltin, M.; Efimova, O.; Petrova, D.; Baltina, T.; Lebedev, M.A.; et al. Time-Dependent Effect of Sciatic Nerve Injury on Rat Plasma Lipidome. *Int. J. Mol. Sci.* **2022**, *23*, 15544. [CrossRef]
145. Meikle, P.J.; Wong, G.; Barlow, C.K.; Kingwell, B.A. Lipidomics: Potential role in risk prediction and therapeutic monitoring for diabetes and cardiovascular disease. *Pharmacol. Ther.* **2014**, *143*, 12–23. [CrossRef]
146. Rasmiena, A.A.; Ng, T.W.; Meikle, P.J. Metabolomics and ischaemic heart disease. *Clin. Sci.* **2012**, *124*, 289–306. [CrossRef] [PubMed]
147. Perrotti, F.; Rosa, C.; Cicalini, I.; Sacchetta, P.; Del Boccio, P.; Genovesi, D.; Pieragostino, D. Advances in Lipidomics for Cancer Biomarkers Discovery. *Int. J. Mol. Sci.* **2016**, *17*, 1992. [CrossRef] [PubMed]
148. Horgusluoglu, E.; Neff, R.; Song, W.-M.; Wang, M.; Wang, Q.; Arnold, M.; Krumsiek, J.; Galindo-Prieto, B.; Ming, C.; Nho, K.; et al. Integrative metabolomics-genomics approach reveals key metabolic pathways and regulators of Alzheimer's disease. *Alzheimer's Dement.* **2022**, *18*, 1260–1278. [CrossRef] [PubMed]
149. Bernardes, J.P.; Mishra, N.; Tran, F.; Bahmer, T.; Best, L.; Blase, J.I.; Bordoni, D.; Franzenburg, J.; Geisen, U.; Josephs-Spaulding, J.; et al. Longitudinal Multi-omics Analyses Identify Responses of Megakaryocytes, Erythroid Cells, and Plasmablasts as Hallmarks of Severe COVID-19. *Immunity* **2020**, *53*, 1296–1314.e1299. [CrossRef] [PubMed]
150. Xing, X.; Hu, E.; Ouyang, J.; Zhong, X.; Wang, F.; Liu, K.; Cai, L.; Zhou, Y.; Wang, Y.; Chen, G.; et al. Integrated omics landscape of hepatocellular carcinoma suggests proteomic subtypes for precision therapy. *Cell Rep. Med.* **2023**, *4*, 101315. [CrossRef] [PubMed]
151. Liu, N.; Wu, Y.; Cheng, W.; Wu, Y.; Wang, L.; Zhuang, L. Identification of novel prognostic biomarkers by integrating multi-omics data in gastric cancer. *BMC Cancer* **2021**, *21*, 460. [CrossRef] [PubMed]
152. Clark, C.; Dayon, L.; Masoodi, M.; Bowman, G.L.; Popp, J. An integrative multi-omics approach reveals new central nervous system pathway alterations in Alzheimer's disease. *Alzheimer's Res. Ther.* **2021**, *13*, 71. [CrossRef] [PubMed]
153. Strefeler, A.; Jan, M.; Quadroni, M.; Teav, T.; Rosenberg, N.; Chatton, J.-Y.; Guex, N.; Gallart-Ayala, H.; Ivanisevic, J. Molecular insights into sex-specific metabolic alterations in Alzheimer's mouse brain using multi-omics approach. *Alzheimer's Res. Ther.* **2023**, *15*, 8. [CrossRef] [PubMed]
154. Lewis, J.E.; Kemp, M.L. Integration of machine learning and genome-scale metabolic modeling identifies multi-omics biomarkers for radiation resistance. *Nat. Commun.* **2021**, *12*, 2700. [CrossRef]
155. Nativio, R.; Lan, Y.; Donahue, G.; Sidoli, S.; Berson, A.; Srinivasan, A.R.; Shcherbakova, O.; Amlie-Wolf, A.; Nie, J.; Cui, X.; et al. An integrated multi-omics approach identifies epigenetic alterations associated with Alzheimer's disease. *Nat. Genet.* **2020**, *52*, 1024–1035. [CrossRef]
156. Heintz-Buschart, A.; May, P.; Laczny, C.C.; Lebrun, L.A.; Bellora, C.; Krishna, A.; Wampach, L.; Schneider, J.G.; Hogan, A.; de Beaufort, C.; et al. Integrated multi-omics of the human gut microbiome in a case study of familial type 1 diabetes. *Nat. Microbiol.* **2016**, *2*, 16180. [CrossRef] [PubMed]
157. Lupberger, J.; Croonenborghs, T.; Roca Suarez, A.A.; Van Renne, N.; Jühling, F.; Oudot, M.A.; Virzì, A.; Bandiera, S.; Jamey, C.; Meszaros, G.; et al. Combined Analysis of Metabolomes, Proteomes, and Transcriptomes of Hepatitis C Virus–Infected Cells and Liver to Identify Pathways Associated With Disease Development. *Gastroenterology* **2019**, *157*, 537–551.e539. [CrossRef]
158. Hudson, K.M.; Shiver, E.; Yu, J.; Mehta, S.; Jima, D.D.; Kane, M.A.; Patisaul, H.B.; Cowley, M. Transcriptomic, proteomic, and metabolomic analyses identify candidate pathways linking maternal cadmium exposure to altered neurodevelopment and behavior. *Sci. Rep.* **2021**, *11*, 16302. [CrossRef] [PubMed]
159. Zheng, P.; Zhang, N.; Ren, D.; Yu, C.; Zhao, B.; Zhang, Y. Integrated spatial transcriptome and metabolism study reveals metabolic heterogeneity in human injured brain. *Cell Rep. Med.* **2023**, *4*, 101057. [CrossRef]
160. Vicari, M.; Mirzazadeh, R.; Nilsson, A.; Shariatgorji, R.; Bjärterot, P.; Larsson, L.; Lee, H.; Nilsson, M.; Foyer, J.; Ekvall, M.; et al. Spatial multimodal analysis of transcriptomes and metabolomes in tissues. *Nat. Biotechnol.* **2023**. [CrossRef] [PubMed]
161. Lopez Soriano, V.; Dueñas Rey, A.; Mukherjee, R.; Inglehearn, C.F.; Coppieters, F.; Bauwens, M.; Willaert, A.; De Baere, E.; Genomics England Research, C. Multi-omics analysis in human retina uncovers ultraconserved cis-regulatory elements at rare eye disease loci. *Nat. Commun.* **2024**, *15*, 1600. [CrossRef]
162. Pattaroni, C.; Begka, C.; Cardwell, B.; Jaffar, J.; Macowan, M.; Harris, N.L.; Westall, G.P.; Marsland, B.J. Multi-omics integration reveals a nonlinear signature that precedes progression of lung fibrosis. *Clin. Transl. Immunol.* **2024**, *13*, e1485. [CrossRef]
163. Garcia-Segura, M.E.; Durainayagam, B.R.; Liggi, S.; Graça, G.; Jimenez, B.; Dehghan, A.; Tzoulaki, I.; Karaman, I.; Elliott, P.; Griffin, J.L. Pathway-based integration of multi-omics data reveals lipidomics alterations validated in an Alzheimer's disease mouse model and risk loci carriers. *J. Neurochem.* **2023**, *164*, 57–76. [CrossRef]
164. Qiu, C.; Yu, F.; Su, K.; Zhao, Q.; Zhang, L.; Xu, C.; Hu, W.; Wang, Z.; Zhao, L.; Tian, Q.; et al. Multi-omics Data Integration for Identifying Osteoporosis Biomarkers and Their Biological Interaction and Causal Mechanisms. *iScience* **2020**, *23*, 100847. [CrossRef]
165. Byeon, S.K.; Madugundu, A.K.; Garapati, K.; Ramarajan, M.G.; Saraswat, M.; Kumar, M.P.; Hughes, T.; Shah, R.; Patnaik, M.M.; Chia, N.; et al. Development of a multiomics model for identification of predictive biomarkers for COVID-19 severity: A retrospective cohort study. *Lancet Digit. Health* **2022**, *4*, e632–e645. [CrossRef]

166. Huang, C.; You, Z.; He, Y.; Li, J.; Liu, Y.; Peng, C.; Liu, Z.; Liu, X.; Sun, J. Combined transcriptomics and proteomics forecast analysis for potential biomarker in the acute phase of temporal lobe epilepsy. *Front. Neurosci.* **2023**, *17*, 1145805. [CrossRef] [PubMed]
167. Suryadevara, R.; Gregory, A.; Lu, R.; Xu, Z.; Masoomi, A.; Lutz, S.M.; Berman, S.; Yun, J.H.; Saferali, A.; Ryu, M.H.; et al. Blood-based Transcriptomic and Proteomic Biomarkers of Emphysema. *Am. J. Respir. Crit. Care Med.* **2024**, *209*, 273–287. [CrossRef] [PubMed]
168. Sivakumar, P.; Ammar, R.; Thompson, J.R.; Luo, Y.; Streltsov, D.; Porteous, M.; McCoubrey, C.; Cantu, E.; Beers, M.F.; Jarai, G.; et al. Integrated plasma proteomics and lung transcriptomics reveal novel biomarkers in idiopathic pulmonary fibrosis. *Respir. Res.* **2021**, *22*, 273. [CrossRef] [PubMed]
169. Zhou, Y.; Fang, J.; Bekris, L.M.; Kim, Y.H.; Pieper, A.A.; Leverenz, J.B.; Cummings, J.; Cheng, F. AlzGPS: A genome-wide positioning systems platform to catalyze multi-omics for Alzheimer's drug discovery. *Alzheimer's Res. Ther.* **2021**, *13*, 24. [CrossRef] [PubMed]
170. Wang, Z.; Li, Y.; Zhao, W.; Jiang, S.; Huang, Y.; Hou, J.; Zhang, X.; Zhai, Z.; Yang, C.; Wang, J.; et al. Integrative multi-omics and drug–response characterization of patient-derived prostate cancer primary cells. *Signal Transduct. Target. Ther.* **2023**, *8*, 175. [CrossRef] [PubMed]
171. Leo, I.R.; Aswad, L.; Stahl, M.; Kunold, E.; Post, F.; Erkers, T.; Struyf, N.; Mermelekas, G.; Joshi, R.N.; Gracia-Villacampa, E.; et al. Integrative multi-omics and drug response profiling of childhood acute lymphoblastic leukemia cell lines. *Nat. Commun.* **2022**, *13*, 1691. [CrossRef] [PubMed]
172. Kim, M.S.; Song, M.; Kim, B.; Shim, I.; Kim, D.S.; Natarajan, P.; Do, R.; Won, H.-H. Prioritization of therapeutic targets for dyslipidemia using integrative multi-omics and multi-trait analysis. *Cell Rep. Med.* **2023**, *4*, 101112. [CrossRef] [PubMed]
173. Leinonen, R.; Sugawara, H.; Shumway, M.; International Nucleotide Sequence Database Collaboration. The Sequence Read Archive. *Nucleic Acids Res.* **2010**, *39*, D19–D21. [CrossRef]
174. Barrett, T.; Wilhite, S.E.; Ledoux, P.; Evangelista, C.; Kim, I.F.; Tomashevsky, M.; Marshall, K.A.; Phillippy, K.H.; Sherman, P.M.; Holko, M.; et al. NCBI GEO: Archive for functional genomics data sets—Update. *Nucleic Acids Res.* **2012**, *41*, D991–D995. [CrossRef]
175. Clark, K.; Karsch-Mizrachi, I.; Lipman, D.J.; Ostell, J.; Sayers, E.W. GenBank. *Nucleic Acids Res.* **2015**, *44*, D67–D72. [CrossRef]
176. Wang, G.; Wu, S.; Xiong, Z.; Qu, H.; Fang, X.; Bao, Y. CROST: A comprehensive repository of spatial transcriptomics. *Nucleic Acids Res.* **2023**, *52*, D882–D890. [CrossRef] [PubMed]
177. Vizcaíno, J.A.; Côté, R.; Reisinger, F.; Foster, J.M.; Mueller, M.; Rameseder, J.; Hermjakob, H.; Martens, L. A guide to the Proteomics Identifications Database proteomics data repository. *Proteomics* **2009**, *9*, 4276–4283. [CrossRef] [PubMed]
178. Deutsch, E.W.; Bandeira, N.; Sharma, V.; Perez-Riverol, Y.; Carver, J.J.; Kundu, D.J.; García-Seisdedos, D.; Jarnuczak, A.F.; Hewapathirana, S.; Pullman, B.S.; et al. The ProteomeXchange consortium in 2020: Enabling 'big data' approaches in proteomics. *Nucleic Acids Res* **2020**, *48*, D1145–D1152. [CrossRef] [PubMed]
179. Watanabe, Y.; Aoki-Kinoshita, K.F.; Ishihama, Y.; Okuda, S. GlycoPOST realizes FAIR principles for glycomics mass spectrometry data. *Nucleic Acids Res.* **2020**, *49*, D1523–D1528. [CrossRef] [PubMed]
180. Haug, K.; Salek, R.M.; Conesa, P.; Hastings, J.; de Matos, P.; Rijnbeek, M.; Mahendraker, T.; Williams, M.; Neumann, S.; Rocca-Serra, P.; et al. MetaboLights—An open-access general-purpose repository for metabolomics studies and associated meta-data. *Nucleic Acids Res.* **2013**, *41*, D781–D786. [CrossRef] [PubMed]
181. Wishart, D.S.; Tzur, D.; Knox, C.; Eisner, R.; Guo, A.C.; Young, N.; Cheng, D.; Jewell, K.; Arndt, D.; Sawhney, S.; et al. HMDB: The Human Metabolome Database. *Nucleic Acids Res.* **2007**, *35*, D521–D526. [CrossRef] [PubMed]
182. Conesa, A.; Beck, S. Making multi-omics data accessible to researchers. *Sci. Data* **2019**, *6*, 251. [CrossRef] [PubMed]
183. Guo, X.; Chen, F.; Gao, F.; Li, L.; Liu, K.; You, L.; Hua, C.; Yang, F.; Liu, W.; Peng, C.; et al. CNSA: A data repository for archiving omics data. *Database* **2020**, *2020*, baaa055. [CrossRef] [PubMed]
184. Athieniti, E.; Spyrou, G.M. A guide to multi-omics data collection and integration for translational medicine. *Comput. Struct. Biotechnol. J.* **2023**, *21*, 134–149. [CrossRef]
185. Yamada, R.; Okada, D.; Wang, J.; Basak, T.; Koyama, S. Interpretation of omics data analyses. *J. Hum. Genet.* **2021**, *66*, 93–102. [CrossRef]
186. Dunkler, D.; Sánchez-Cabo, F.; Heinze, G. Statistical analysis principles for Omics data. *Methods Mol. Biol.* **2011**, *719*, 113–131. [CrossRef] [PubMed]
187. Rouam, S. False Discovery Rate (FDR). In *Encyclopedia of Systems Biology*; Dubitzky, W., Wolkenhauer, O., Cho, K.-H., Yokota, H., Eds.; Springer: New York, NY, USA, 2013; pp. 731–732.
188. Krämer, A.; Green, J.; Pollard, J., Jr.; Tugendreich, S. Causal analysis approaches in Ingenuity Pathway Analysis. *Bioinformatics* **2014**, *30*, 523–530. [CrossRef] [PubMed]
189. Ahsan, S.; Drăghici, S. Identifying Significantly Impacted Pathways and Putative Mechanisms with iPathwayGuide. *Curr. Protoc. Bioinform.* **2017**, *57*, 7.15.11–17.15.30. [CrossRef]
190. Wu, L.; Liu, F.; Cai, H. IOAT: An interactive tool for statistical analysis of omics data and clinical data. *BMC Bioinform.* **2021**, *22*, 326. [CrossRef]
191. Buy, M.; Digan, W.; Chen, X.; Husson, J.; Ménager, M.; Rieux-Laucat, F.; Garcelon, N. A Multi-Omics Common Data Model for Primary Immunodeficiencies. *Stud. Health Technol. Inform.* **2022**, *290*, 56–60. [CrossRef]

192. Harbig, T.A.; Fratte, J.; Krone, M.; Nieselt, K. OmicsTIDE: Interactive exploration of trends in multi-omics data. *Bioinform. Adv.* **2023**, *3*, vbac093. [CrossRef]
193. Fang, Z.; Ma, T.; Tang, G.; Zhu, L.; Yan, Q.; Wang, T.; Celedón, J.C.; Chen, W.; Tseng, G.C. Bayesian integrative model for multi-omics data with missingness. *Bioinformatics* **2018**, *34*, 3801–3808. [CrossRef]
194. Ma, C.; Wu, M.; Ma, S. Analysis of cancer omics data: A selective review of statistical techniques. *Brief. Bioinform.* **2022**, *23*, bbab585. [CrossRef] [PubMed]
195. Das, S.; Mukhopadhyay, I. TiMEG: An integrative statistical method for partially missing multi-omics data. *Sci. Rep.* **2021**, *11*, 24077. [CrossRef] [PubMed]
196. Cifuentes, A. Food analysis and Foodomics. *J. Chromatogr. A* **2009**, *1216*, 7109. [CrossRef]
197. Picó, C.; Serra, F.; Rodríguez, A.M.; Keijer, J.; Palou, A. Biomarkers of Nutrition and Health: New Tools for New Approaches. *Nutrients* **2019**, *11*, 1092. [CrossRef] [PubMed]
198. Hill, E.B.; Tang, M.; Long, J.M.; Kemp, J.F.; Westcott, J.L.; Hendricks, A.E.; Reisdorph, N.A.; Campbell, W.W.; Krebs, N.F.; Schaefer, C.; et al. mini-MED: Study protocol for a randomized, multi-intervention, semi-controlled feeding trial of a Mediterranean-amplified vs. habitual Western dietary pattern for the evaluation of food-specific compounds and cardiometabolic health. *Trials* **2024**, *25*, 101. [CrossRef] [PubMed]
199. Trimigno, A.; Khakimov, B.; Savorani, F.; Poulsen, S.K.; Astrup, A.; Dragsted, L.O.; Engelsen, S.B. Human urine 1H NMR metabolomics reveals alterations of protein and carbohydrate metabolism when comparing habitual Average Danish diet vs. healthy New Nordic diet. *Nutrition* **2020**, *79–80*, 110867. [CrossRef] [PubMed]
200. Dilmore, A.H.; Martino, C.; Neth, B.J.; West, K.A.; Zemlin, J.; Rahman, G.; Panitchpakdi, M.; Meehan, M.J.; Weldon, K.C.; Blach, C.; et al. Effects of a ketogenic and low-fat diet on the human metabolome, microbiome, and foodome in adults at risk for Alzheimer's disease. *Alzheimer's Dement.* **2023**, *19*, 4805–4816. [CrossRef]

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Review

Cadmium Stress Signaling Pathways in Plants: Molecular Responses and Mechanisms

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Abstract: Heavy metal (HM) pollution, specifically cadmium (Cd) contamination, is a worldwide concern for its consequences for plant health and ecosystem stability. This review sheds light on the intricate mechanisms underlying Cd toxicity in plants and the various strategies employed by these organisms to mitigate its adverse effects. From molecular responses to physiological adaptations, plants have evolved sophisticated defense mechanisms to counteract Cd stress. We highlighted the role of phytochelatins (PC_n) in plant detoxification, which chelate and sequester Cd ions to prevent their accumulation and minimize toxicity. Additionally, we explored the involvement of glutathione (GSH) in mitigating oxidative damage caused by Cd exposure and discussed the regulatory mechanisms governing GSH biosynthesis. We highlighted the role of transporter proteins, such as ATP-binding cassette transporters (ABCs) and heavy metal ATPases (HMAs), in mediating the uptake, sequestration, and detoxification of Cd in plants. Overall, this work offered valuable insights into the physiological, molecular, and biochemical mechanisms underlying plant responses to Cd stress, providing a basis for strategies to alleviate the unfavorable effects of HM pollution on plant health and ecosystem resilience.

Keywords: pollution; heavy metal; cadmium; phytochelatins; glutathione; transporter

1. Introduction: Environmental Heavy Metal Contamination

Heavy metals (HMs) are elements characterized by an atomic number greater than 20, an atomic density exceeding 5 g/cm^3, and metallic properties. Transition metals, like cadmium (Cd), mercury (Hg), and chromium (Cr), elements in the bottom left section of the periodic table like lead (Pb) and tin (Sn), and certain metalloids like arsenic (As) are essentially what are referred to as HMs. The latter can be broadly classified into two categories: essential and non-essential HMs. The first ones are necessary for living organisms to perform fundamental processes such as growth, metabolism, and organ development. Therefore, HMs, such as iron (Fe), zinc (Zn), copper (Cu), and cobalt (Co) function as cofactors that support redox and protein structure processes, making them crucial for plant growth and development. The non-essential ones are dangerous because they can bind to a variety of functional groups in biomolecules, including amino, carboxylic acid, and sulfur-containing groups [1]. Moreover, they compete with the plant for nutrient uptake; they are dangerous even at low concentrations as they can result in macronutrient deficiencies. Increased HM buildup results in acute toxicity, growth inhibition, and ultimately plant death [2]. In contrast to organic pollutants, HMs are non-biodegradable and tend to accumulate in living organisms [3]. The pervasive problem of HM pollution in the environment is a significant risk to public health worldwide. Reports indicate that 20 million hectares of China's farmland, constituting roughly 20% of the nation's total agricultural area, are affected by HM pollution, notably Cd, Pb, As, and Hg. Within this affected area, 14,000 hectares are particularly afflicted by Cd contamination [1,4].

Groundwater, agricultural products, and crops alike will soon suffer the far-reaching consequences of the presence of Cd in soil [5]. Pollution of the environment by HMs can result from both natural processes and human activities. Among the latter, the primary artificial causes of soil contamination include farming practices, urban sprawl, industrial activities, mining operations [6], and the expansion of transport infrastructure [7]. Industrial operations contribute to ecological pollution since HMs and their numerous chemical combinations are frequently employed as catalysts in chemical reactions. Transportation-wise, the emission of gases from tires, brakes, and gasoline fuel have been shown to be the main contributors of Pb and Cd emissions to the air due to increased traffic [8]. Concerning the mining activity, although there are no mines present nearby, agricultural goods cultivated in roadside fields have higher levels of HM contamination than crops grown in other parts [9]. Moreover, the mine environment is further polluted by soil-borne metal particles carried by wind or mineral transportation [10]. The soil pollution of HMs in close proximity to mines is also significantly influenced by climate [10,11]. The expanding trash mounds have a negative effect on the surrounding areas of mining and could potentially transform some agricultural regions into wasteland [12]. Besides, one of the primary causes of ecological contamination in these areas is the water utilized in the mining process. People living close to mines suffer from a variety of autoimmune and non-autoimmune disorders that can be fatal due to toxic HMs in the surrounding soil [12]. Furthermore, human-induced soil modifications, such as the standard agricultural methods used in different areas, cause crops to take in HMs. The most common causes of liquid-induced HM environmental pollution are the application of phosphate fertilizers and the irrigation of agricultural lands with dirty water [13].

One trace element that is not necessary for biological systems yet is present in large quantities in the environment is Cd. Due to its exceptional toxicity to all biological systems, Cd is an HM that is frequently released into the environment by both natural and man-made sources [14]. For these reasons, it is considered a major metal pollutant [15,16]. Cd and its compounds are soluble, making them more mobile than other metals in environmental matrices. They also have high bioavailability, which makes them easily absorbable by plants. Once inside the plant, Cd can interfere with important physiological processes [17,18]. Given that Cd is highly toxic, this poses a significant risk to human health, leading to cancer and extensive environmental harm [19]. According to numerous studies, dumps often contain Cd levels exceeding the permissible limit in soil [20–23]. With a global mean of 0.36 mg/kg, Cd usually occurs in soils at amounts ranging from 0.01 to 1 mg/kg [16] and can be found in soil water at up to 5 μg/L [24] and groundwater at up to 1 μg/L [25]. Increases in Cd concentrations in soils and groundwater, which are crucial for preserving a wholesome food supply and clean drinking water, can come from both natural and man-made sources. The WHO Guidelines for Drinking-Water Quality recommend a maximum value of 3 μg/L for Cd. The potential for Cd accumulation in plants to transfer to humans via the food chain, causing serious harm, is nowadays a considerable concern [26,27]. Due to competition with calcium (Ca) and other nutrients, chronic Cd poisoning, also known as itai-itai disease and initially identified in Japan in the early 20th century, results in renal tubular dysfunction, osteomalacia, and osteoporosis [28–30]. According to Khan et al. 2017 [30], Cd exposure is also linked to problems with glucose metabolism, lung and breast cancer, myocardial infarction, and heart failure.

2. Effects of Cd in Plants

Crops, agricultural goods, and groundwater are significantly affected by Cd [5], a trace element that is not necessary for plant growth, although it does accumulate in all sections of plants after absorption [2,31]. The first signs of Cd toxic effects on plants include browning of the roots, a decrease in the number of lateral roots, and the death of the tips of the roots due to inhibition of root growth. Furthermore, the accumulation of Cd could result in stem development retardation, terminal leaf chlorosis, and leaf curl [32,33]. By substituting the sulfhydryl groups of proteins and enzymes, Cd replaces the essential elements of enzyme

active sites needed in biological reactions. It also modifies macromolecule conformation, causes protein denaturation, and deteriorates cell membranes. The plant defense system reacts to the entry of Cd into plant cells by accumulating proline [34–36], variations in the synthesis and levels of phytohormones [37–39], and the initiation of several signaling pathways, including the Ca-calmodulin pathway as well as the activation of various antioxidant enzymes [40,41].

Cd significantly increases the production of reactive oxygen species (ROS) after binding with proteins and enzyme complexes, which leads to oxidative stress [42,43]. Reactive nitrogen species (RNS) and ROS are byproducts of regular cellular metabolism and are known to play two distinct roles in biological systems [44]. High ROS levels cause the cell to arrest and eventually become necrotic or apoptotic, whereas low ROS levels accelerate the cell cycle's progression [45]. The toxicity due to elevated ROS concentrations reflects the oxidation of proteins, DNA, and membrane lipids, as well as the breakdown of polyphenols, catecholamines, and thiols. Previous reports highlight ROS dual function in plant defense systems as a signaling molecule and as a source of toxicity [46,47]. According to multiple studies [48,49] these molecules are produced by the Fenton or Haber-Weiss reaction, respiratory burst oxidase homologs (also known as NADPH oxidases), and the inhibition of antioxidative enzymes, such as oxidases and peroxidases. The cell has evolved a sophisticated antioxidant system that may shield biological molecules from potentially hazardous, purely physiological, species-producing oxidative stress through both enzymatic and non-enzymatic methods. Proteins like catalase (CAT), glutathione peroxidase (GPx), ascorbic acid (Vitamin C), ρ-tocopherol (Vitamin E), glutathione (GSH), carotenoids, flavonoids, and other antioxidant agents are part of the enzymatic defense system [50] (Figure 1).

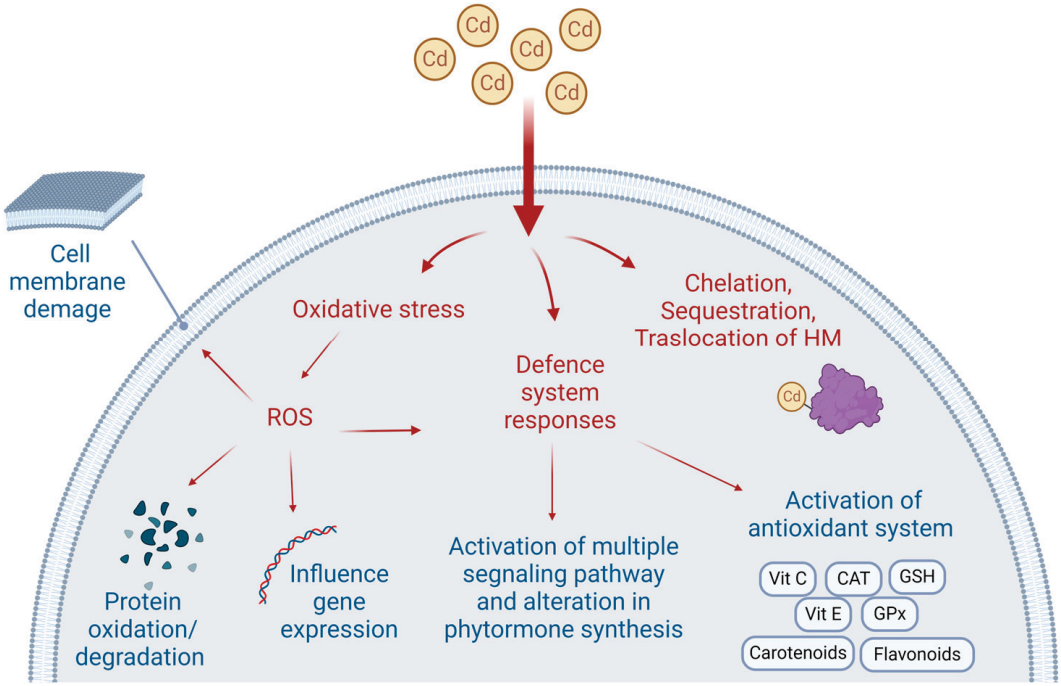


Figure 1. Toxic effects of Cd in plants. Induction of oxidative stress leads to ROS generation, resulting in cell membrane damage, protein oxidation, and degradation, with effects on gene expression. Cellular responses to Cd entry include activation of the defense system as well as chelation, sequestration, and translocation of the HM.

3. Plant Responses to Cd Stress and Detoxification via PC_n Production

Different plant species are affected by nutrient variance under Cd stress [15,51,52]. Certain molecular alterations control Cd mitigation, tolerance, and adaptation, including the overexpression of specific genes. It has been observed that natural resistance

and macrophage proteins (NRAMPs), iron-regulated transporter-like proteins (IRTs), zinc-regulated transporter-like proteins (ZIPs), heavy metal ATPases (HMAs), and metal tolerance or transporter proteins (MTPs) play roles in the uptake, translocation, and sequestration of Cd within plants [53]. Jia et al. 2019 [54] revealed that a high concentration of Cd (6.5 μM Cd) increases the expression of genes (*SlQUA1* and *SlPME1*) associated with pectin synthesis in the cell walls of tomatoes, leading to an increase in pectin methyl esterase activity and ion binding sites, which counteracts Cd stress. Additionally, Song, Feng, et al. 2017 [55] demonstrated that overexpression of *AtFC1* in *A. thaliana* enhances Cd accumulation and tolerance under Cd exposure. Conversely, genes like *HvIRT1* and *HvNramp5* in barley [56] downregulate Cd uptake, while lower expression of *HMA-A*, *HMA-B*, *IRT1*, and *ZIP1* in tobacco leads to reduced Cd accumulation [57]. Genes such as *ZIP2*, *ZIP3*, *IRT1*, *HMA2*, and *HMA4* and *CAX4*, *HMA3*, *MRP7*, *MTP3*, and *COPT5* are overexpressed in Pak choi (*B. rapa*) grown in sand artificially contaminated with 10 mg Cd Kg^{-1} [58]. Other studies on the response to Cd stress have been conducted in rape, *A. thaliana*, maize, rice, and other crops [59,60]. *NcNramp1* was shown to be crucial for the process of plant absorption and accumulation of the ionic form Cd^{2+} when transcriptome technology was used to examine *N. caerulescens* under Cd stress in various genotypes [61].

As a result of the significant toxic impact on plant growth and development, plants have evolved a variety of tolerance and detoxification strategies to control the uptake, transportation, and accumulation of Cd and lessen its toxicity [62]. The plant root hairs, along with the cuticle and epidermis, produce protective tissue to keep Cd out [63]. They also release organic acids, including citric, malic, and lactic acids, which mix with Cd to form compounds that prevent Cd from passing through membranes. In order to reduce the quantity of Cd that migrates from the soil into the root, they also modify the pH and EH of the rhizosphere [64]. Furthermore, to reduce the toxicity of Cd to cell activity, Cd that does enter cells will be enriched in vacuoles. This enrichment occurs due to the ability of polysaccharides and proteins in the cell wall to bond with the metal and prevent it from entering the cell [65].

Plants, particularly the hyperaccumulator ones, react in different ways to the toxicity of HMs, including Cd. HM chelation utilizing peptide ligands called phytochelatins (PC_n) is one of the novel defense mechanisms [66]. These peptides are primarily composed of (c-Glu-Cys)n-Gly, with ($2 < n < 11$), although other PC_n structures such as (c-Glu-Cys)n-Glu, (c-Glu-Cys)n-Ser, and (c-Glu-Cys)n-c-Ala have also been identified [67,68]. PC_n are synthesized from GSH by the activation of the enzyme phytochelatin synthase (PCS), a γ-EC dipeptidyl (trans)peptidase that is constitutively expressed in the plant cytosol [69–71]. Synthesized PC_n can form a complex with Cd, referred to as a chelator-Cd complex. Intracellular Cd promotes PCS activity, and the subsequent metal-induced production of PC_n allows intracellular Cd detoxification [72]. Indeed, the PC_n complex aids in transporting Cd from the cytosol into the vacuole, where it becomes inactive [73,74] (Figure 2).

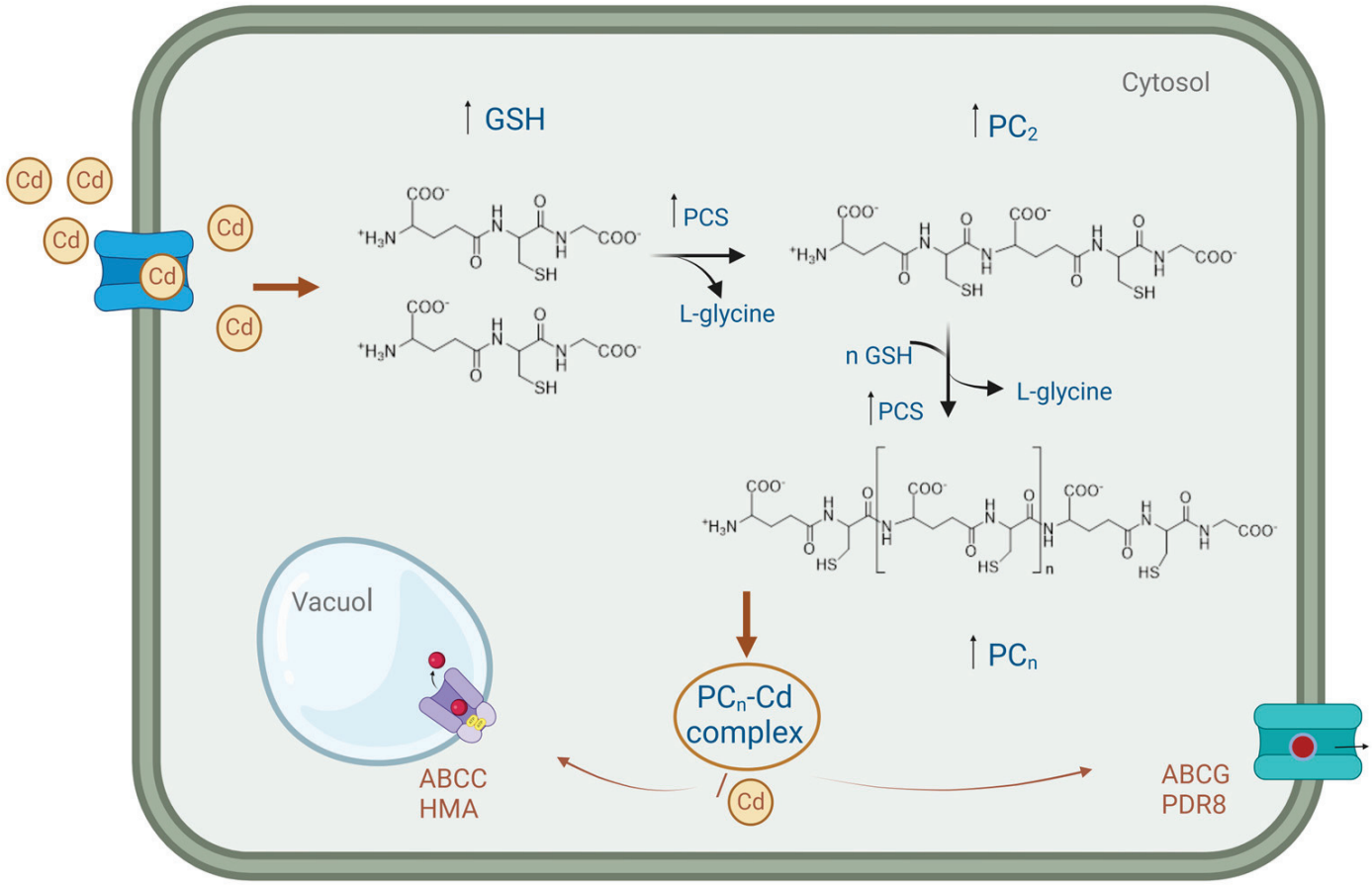


Figure 2. The Cd-PC_n complex. Cd influx into cells induces overexpression of PCS and involves glutathione in PC_2 formation, resulting in the synthesis of other PC_n. This production of PC_n leads to cadmium chelation, and the complex is transported to the vacuole for detoxification or removed from the cell via membrane transporters and efflux pumps.

A study reports the exogenous presence of GSH to reduce Cd translocation to shoots and enhance Cd accumulation in *B. campestris* roots treated with 20 mM of Cd [40]. According to the authors, exogenous GSH promotes the synthesis of PC_n by increasing Cd chelation and facilitating its translocation into the root vacuoles. In fact, recent studies [72,75–77] confirm that exposure to Cd raises GSH levels in addition to activating the constitutively expressed enzyme PCS in *M. polymorpha* (*MpPCS*), which significantly increases the amount of PC_n generated overall. Particularly, a detailed gene expression analysis confirmed the constitutive transcription of *MpPCS*; indeed, Cd is able to induce the activity, but not the gene expression, of the enzyme [72]. As a result, PC_n synthesis is essential for plant detoxification and tolerance to high Cd concentrations. Another notable instance is seen in rice plants, where PC_n increases by 23% and 47%, respectively, in response to 1 and 2 mM Cd [78]. Similarly, *B. auriculata* treated with 125 mM of Cd showed similar outcomes [70]. In response to metallic micronutrients including Fe, Zn, Cu, and the hazardous element Cd, PC_n activation has recently been found in tracheophytes (mostly in angiosperms), as well as bryophytes and charophytes [79–81]. Therefore, *M. polymorpha* may be a great model organism to study the cellular and molecular mechanisms involved in Cd detoxification. Studies examining how metal detoxifying mechanisms evolved from aquatic to terrestrial environments can greatly benefit from the study of bryophytes. Not to mention, these plants do not form strong hydrophobic barriers, have a very high surface/volume ratio, an elevated cation exchange capacity, and are hence more susceptible to unrestricted, seemingly uncontrolled metal absorption [18,82]. Consequently, bryophytes have been employed as a crucial biological monitoring system for metal environmental

pollution because of their widespread distribution [82–84]. Additionally, a study conducted by Bellini et al. 2020 [85] examined the medium acidification of this liverwort and its thiol-peptide synthesis (GSH, PC_n) following a 72 h exposure to Cd. The loss of selective permeability, a well-known property of biological membranes, can lead to swelling or shrinking of cell compartments due to HM-induced membrane damage or energy loss. Moreover, changes in chloroplast ultrastructure have been linked to HM concentrations in studies conducted both in the field and in vitro on bryophytes [83,86]. *L. riparium* has been explored to determine whether this moss could be suitable for biomonitoring in natural field conditions. Recent studies showed that Cd exposure severely affected the amount of photosynthesis, PC_n, GSH, and nitrogen metabolism in *L. riparium* plants. Nitrogen (N_2) and Cd metabolism are known to be closely correlated [87–90]. N_2 is typically associated with the abiotic stress response because it competes with essential reductants required for antioxidant responses, particularly in the presence of metals and metalloids [89,91]. High N_2 levels promote the uptake of Fe, Zn, Cu, Ca, Hg, and other cations in certain plants [90]. Instead, the activity of glutamate dehydrogenase, another important enzyme involved in abiotic stress, is reduced because it serves as a substitute route for assimilating N and detoxifies excess NH_4 that is produced under stressful circumstances [91,92]. On the other hand, several major crops, such as rice, beans, peas, and corn, exhibited reduced glutathione synthase (GS) activity in response to HM uptake [93,94]. Research conducted by Maresca et al., 2022 [95] demonstrated that *L. riparium* has a natural tendency to increase the occurrence of GS2 proteins. Cd exposure causes the metal to be chelated in the roots and transferred to the shoots via GSH and PC_n [90]. Maintaining this chelation mechanism, which controls the amount of GSH and non-protein thiols, as well as the expression of *PCS* and GSH synthase genes, requires a sufficient absorption of N_2 [90].

4. Role of GSH in Mitigating Cd-Induced Abiotic Stress

Due to the implication of GSH in mitigating HM stress, which is linked to its involvement in PC_n synthesis, it is relevant to deepen our understanding of the GSH response during abiotic stress. Two ATP-dependent enzymes, glutamate-cysteine ligase (GSH1, previously known as γ-glutamylcysteine synthetase) and GSH synthase (GSH2), sequentially synthesize GSH from its three amino acid building blocks [96]. The first enzyme, GSH1, conjugates Cys and Glu to produce the dipeptide γ-glutamylcysteine. The second step is catalyzed by GSH2, which joins Gly to γ-glutamylcysteine to generate GSH. Growth and viability are restored when cytosol-specific GSH2 is added to the seedling-lethal GSH2 mutant strains. Therefore, for normal plant growth, cytosolic production of GSH alone is sufficient [97]. Although both *GSH1* and *GSH2* are relatively widely expressed in plant tissues, biotic stressors such as methyl jasmonate and high light [98], as well as abiotic stresses like HMs [99,100], but not H_2O_2 or oxidative agents [99], increase transcriptional levels of both *GSH1* and *GSH2*. Its homodimerization is therefore thought to increase enzyme stability by shielding sensitive regions [101]. Since the chloroplast is home to GSH1, the redox state of this organelle plays a crucial role in controlling the synthesis of GSH. Due to the inherent instability of oxygenic photosynthesis, chloroplasts are highly reactive to unfavorable environmental conditions, which subsequently provides quick responses to abiotic and biotic challenges [102]. The recognized function of GSH in maintaining intracellular redox homeostasis largely depends on the redox control of GSH biosynthesizing enzymes. Indeed, oxidative stress-induced metabolic circumstances create a demand for GSH and the production of its oxidated form, GSSG, while also promoting GSH synthesis through the post-translational activation of GSH1. Consequently, elevated GSH levels establish a reducing environment, triggering a regulatory feedback loop to slow down GSH synthesis and decrease GSH1 activity. The activity of the sulfate assimilation pathway also influences GSH synthesis [103]. Cys serves as the initial organic product of sulfur assimilation in plants, and thus, its availability plays a crucial role in determining GSH production [103,104]. Consequently, several regulatory mechanisms influence the GSH biosynthesis process. Various environmental conditions and stresses impact the coarse con-

trol of *GSH1* and *GSH2* expression. Additionally, precursor availability affects the system's activity, and a redox mechanism that considers feedback information finely regulates GSH1 activity. Currently, it remains uncertain whether a redox-coupling mechanism is necessary for the transformation of GSH1 from its oxidized to reduced form. The redox state of GSH, along with its cellular concentration, plays a critical role in mediating and finely regulating metabolism and stress responses. The flavoenzyme NADPH-dependent GSH reductase (GR) efficiently regenerates GSSG to GSH and permits recurrent redox cycling. In order to sustain fundamental functions in the cytosol, mitochondria, and chloroplast stroma in the light, GR contributes to the maintenance of a negative EGSH (i.e., high GSH/GSSG). Gill et al. 2013 [105] conducted a thorough literature analysis about *GR* genes and found evidence that a variety of abiotic stressors, including exposure to HMs, including Cd, salt, drought, UV radiation, and cold temperatures, boost GR activity in many plant species. Moreover, several stress-tolerant plants exhibit significant GR activity [106]. Because of its effective ROS scavenging capacity, transgenic techniques to control GR activity in plants also demonstrate that increased GR activity significantly improves tolerance to oxidative stress brought on by a range of abiotic stimuli [105]. Two nuclear genes, *GR1* (*At3g24170*) and *GR2* (*At3g54660*), which both code for differently localized isoforms, are responsible for encoding GRs in *A. thaliana*. *GR1* is more highly expressed in roots, while *GR2* expression is more pronounced in photosynthetic tissues [98]. *GR1* is localized in the cytoplasm, nucleus, and peroxisomes [107–110] while *GR2* targets mitochondria as well as chloroplasts [108].

5. Transporters Involved in Cd Remediation

There are various forms of Cd in soil, but many of them are not absorbed by plants [111]. For Cd to be absorbed, it must be available for uptake, which depends on the plant species, the physicochemical conditions of the soil, and the speciation of the metals [112,113].

A number of technologies have been developed to lower soil Cd levels. Phytoremediation, used by plants to absorb Cd and store it in easily harvested aerial sections of the plant, is one of the approaches [114]. The choice of appropriate plants that can grow in Cd-polluted soils and accumulate Cd in aerial parts is crucial to the effectiveness of phytoremediation. Over the past ten years, Cd hyperaccumulating plants have received attention [114]. However, only a few plant species that can withstand high amounts of Cd have been identified, including *A. halleri*, *N. caerulescens*, and *S. alfredii.*

Meighan et al., 2011 [115] found that herbaceous Cd hyperaccumulators had limited relevance for phytoremediation in Cd-polluted fields due to their slow development and low biomass output. The use of *Populus* species has been suggested as an alternative for phytoremediation because these woody species can be managed in short rotation coppicing plantations and are known for their fast growth and deep rooting systems [116–119].

Metal uptake is significantly influenced by root features such as surface area, size, and shape [113,120–124]. Furthermore, plants with thin, hairy roots have demonstrated high absorption and accumulation of metals [125]. In most cases, bivalent forms of trace elements are absorbed [126]. The same transporters that carry Ca, Fe, Mg, Cu, and Zn must carry Cd across root cell walls [15]. By passive transport (diffusion), Cd can move from the soil to plant roots through the cell walls [120,127]. Furthermore, studies have shown that Cd uses nonspecific membrane transport proteins (IRTs and ZIPs) and metal pumping ATPases to facilitate active transport across the plasma membrane of root cells [128,129]. Different transporters are used by plants to sequester metal ions inside organelles, whether in a free ionic state or as chelates. Plants can store extra metals in vacuoles when they are in neutral chelated forms. Long-distance transportation also makes use of chelated metal forms. To lessen the harmful effects of metals, metal chelators can be proteins that bind to metals or other compounds, such as amino acids, their derivatives, or organic acids [130].

These metal chelators also exhibit antioxidant properties. By detoxifying metal ions, specific protein metal chelators like glutaredoxins (GRXs), metallothionins (MTs), and PC_n are essential in reducing the effects of metal stress-mediated oxidative damage [130]. Thereby, according to Kumar et al., 2020 [131], the overexpression of glutaredoxin *Ca-*

GRX in chickpeas decreases the absorption of metal ions and boosts the ROS scavenging mechanism, thus mitigating metal stress.

Transporters serve dual roles as receptors and transporters, known as transceptors, within the plant's sensory apparatus aimed at preserving metal homeostasis [132–135]. Transceptors have the capability to discern the availability of metal ions in soil and their scarcity within the cytosol, thereby orchestrating the regulation of metal ion uptake through low- or high-affinity transporters. This intricate process leads to a substantial decrease in metal ions, effectively maintaining metal homeostasis within plant cells through the expulsion and exclusion mechanisms facilitated by transporters.

Among these transporters, Cd, Co, Fe, Mn, and Zn divalent ions are transported by the IRT1. Within root cells, IRT1 acts as a transceptor within sensing and signaling mechanisms, prioritizing the sensing of Fe above other ions. Additionally, the IRT1 regulatory domain senses additional ions such as Co, Mn, and Zn, thereby inhibiting IRT1 function [132]. In a different study, CBL-interacting protein kinase 23 (CIPK23) phosphorylates the metal-bound IRT1, leading to ubiquitination by the IRT1 degradation factor 1 (IDF1) protein. This ubiquitination targets IRT1 for breakdown in the vacuole, effectively halting further metal absorption [136].

Wu et al., 2020 [137] discovered in their intriguing study on *A. thaliana* that the use of hydrogen-rich water accelerates the uptake of Cd. Elevated levels of Cd result in hydrogen production, which in turn triggers a cytosolic influx of Ca. Moreover, the activation of respiratory burst oxidase homolog protein D (RBOHD) by Ca elevation results in the production of ROS, primarily H_2O_2. In the end, the induced-H_2O_2 can inhibit IRT1 transcripts, which will lessen the absorption of more Cd [137].

Another instance in which Cd stress is mitigated is shown in apples, where the kinase salt overly sensitive 2-like1 (SOS2L1) phosphorylates the malate transporter aluminum-activated malate transporter 14 (ALMT14), enhancing its functionality [49]. Otherwise, a rice natural resistance-associated macrophage protein 1 (OsNRAMP1) plasma-membrane-localized Fe transporter that is involved in absorbing Cd is overexpressed, increasing the amount of Cd stored in leaves. Additionally, it was found that the expression of *OsNRAMP1* is correlated with the amount of Cd found across different rice cultivars. Compared to the hyperaccumulating cultivar Sasanishiki, it was expressed up to two times greater in the Cd hyperaccumulating rice cultivar Habataki [138]. According to Lang et al., 2011 [139], *B. juncea* exhibits Cd tolerance through the involvement of two cation efflux transporters, BjCET3 and BjCET4, whose overexpression results in increased Cd accumulation in the shoot without lowering plant biomass.

Otherwise, in *A. thaliana*, the gene *AtNRAMP6* confers Cd tolerance by locating at the Golgi apparatus. By transferring the Fe contained in Golgi vesicles into the cytosol during Fe depletion, *AtNRAMP6* overexpression plays a critical role in preserving intracellular Fe equilibrium [140]. Consequently, by compartmentalizing and redistributing metals to specific organelles, including vacuoles, the endoplasmic reticulum, chloroplasts, and mitochondria, Golgi apparatus-localized metal transporters are necessary to maintain metal homeostasis of Fe, Cd, and Mn.

Furthermore, plants utilize energy-dependent metal transport systems to expel or remove metals, primarily relying on HM ATPases (HMAs) and ABC (ATP-binding cassette) transporters. Typically, these transporters expel metals from root cells into the surrounding air or other sections of the plant, where they can accumulate harmlessly. The role of ABC transporters in the translocation and redistribution of metals has been extensively studied. Fu et al. 2019 [141] demonstrated that Cd treatment in rice promoted the expression of the G-type ABC transporter ABCG36. A loss-of-function mutant exhibited a higher Cd concentration in its roots, suggesting that ABCG36 facilitates Cd translocation from roots to shoots. An interesting study revealed that the *P. tomentosa ABCG36* gene can be overexpressed to improve Cd tolerance in *A. thaliana* plants, suggesting that this transporter serves as a Cd extrusion pump [142]. In accordance with a different study, the *A. thaliana* ABC transporter pleiotropic drug resistance 8 (AtPDR8) acts as a plasma membrane-

localized Cd efflux pump, pushing Cd out of the cytosol and onto the cell's exterior, thus providing HM stress tolerance [143]. In certain plants, only C-type ABC transporters (ABCCs) localize on tonoplasts and contribute to metal tolerance. According to Brunetti et al. 2015 [144] and Song et al. 2010 [145], tonoplast-localized ABCCs in *A. thaliana*, such as AtABCC1, AtABCC2, and AtABCC3, facilitate the transport of PC_n and its complexes with As, Cd, Mn, and Zn into the vacuole, thus enhancing metal tolerance.

A recent study compared wild-type (WT) *P. alba* clone Villafranca plants with transgenic plants overexpressing aquaporin (*aqua1*) in a hydroponic treatment with 10μM Cd. *PaHMA2*, *PaNRAMP1.3*, *PaNRAMP2*, *PaNRAMP3.1*, *PaNRAMP3.2*, *PaABCC9*, and *PaABCC13* gene expressions were evaluated, revealing that the majority of the genes analyzed exhibited a prompt transcriptional response in WT plants at 1 day post-treatment and adaptation at 60 days. Conversely, *aqua1* plants showed a weaker transcriptional response along with a higher Cd content in the medial leaves. In conclusion, *aqua1* overexpression in poplar enhanced Cd translocation, indicating that *aqua1* plants are less sensitive to Cd. Given the significance of *PaNRAMP3* in Cd compartmentalization, a different transcription of this gene in the WT line may account for this distinct response. Nevertheless, even after two months of treatment, Cd administration did not cause observable toxicity symptoms in WT and *aqua1* plants, demonstrating the remarkable resistance of this poplar species to Cd [146].

As part of a redistribution strategy, the HMA family of P1B-ATPases also serves as metal pumps, transporting metals into the xylem, phloem, and apoplast for transfer to other plant components, including shoots and grains. Members of the HMA family are essential for the mobilization of metal ions in a variety of plants, including Cu, Cd, and Zn. HvHMA1 is essential for scavenging Cu and Zn from the chloroplast to the cytosol in barley, facilitating their transport to areas of need [147]. To prevent their intracellular accumulation, HMA2 in *A. thaliana* expels excess cytosolic Zn, Cd, and other ions into the apoplast [148]. In response to Zn deficiency in the shoot, HMA2 and metal transporter protein 2 (MTP2) are stimulated to redistribute Zn from the root to the shoot [149]. Additionally, when cultivated in environments with high concentrations of metals, the HMA2 mutant exhibits raised intracellular Cd and Zn levels.

AtHMA3, localized in tonoplasts, plays a role in storing Cd, Zn, Co, and Pb. According to Morel et al. 2009 [150], overexpression of *AtHMA3* enhances resistance to metal stress and boosts Cd storage capacity. Furthermore, research by Sasaki et al. 2014 [151] observed that vacuolar sequestration of Cd in rice roots contributes to its accumulation. In a comparable way, *GmHMA3* in soybeans is essential for Zn and Cd tolerance. Moreover, a study on rice showed how it has the capability to diminish the quantity of Cd present in its grains by adjusting the Zn-to-Cd ratio via the mutation of OsHMA2 [152].

Additionally, the endomembrane system includes the endoplasmic reticulum (ER), housing a spacious lumen utilized for the storage and utilization of metals for various purposes [153]. Within the ER membrane, there exist broad-specificity transporters for Cd, Cu, and Zn, which are occasionally observed to also localize on the plasma membrane. The endomembrane system's continuity as an element of the secretory system may be the cause of this shared location. For example, OsZIP1 in rice is situated at both the ER and plasma membrane, primarily in roots where Cd, Cu, and zinc levels are elevated. All the different aforementioned transporters are summarized in Table 1.

Table 1. Main features of transporters implicated in Cd tolerance.

Subfamily	Name	Subcellular Localization	Substrate
IRT	IRT1	Plasma membrane	Fe, Co, Mn, Zn, Cd
SOS	SOS2L1 *	Plasma membrane	Malate and malic acid
ALMT	ALMT14 *	Plasma membrane	Malate and malic acid

Table 1. *Cont.*

Subfamily	Name	Subcellular Localization	Substrate
CET	BjCET3 BjCET4	Plasma membrane	Zn, Co, Ni, Cd
RAMP	OsNRAMP1 AtNRAMP6	Plasma membrane Golgi	Fe, Cd Fe, Mn, Cd
ABCG (PDR)	PtABCG36 AtPDR8	Plasma membrane	Pb, Cd
ABCC	AtABCC1 AtABCC2 AtABCC3	Tonoplast	PC_n and its complexes with As, Mn, Zn, Cd
HMA	HvHMA1	Chloroplast	Zn, Cd
	PaHMA2 AtHMA2	Plasma membrane	Zn, Cd
	AtHMA3	Tonoplast	Zn, Co, Pb, Cd
ZIP	OsZIP1	Endoplasmic reticulum and plasma membrane	Zn, Cu, Cd

* Not directly involved in Cd transport. Cd presence enhances their functionality, increasing Cd tolerance.

6. Conclusions

HMs pose significant risks to plant health, causing toxicity, growth inhibition, and oxidative stress. Essential metals are necessary but can be harmful in excess, while non-essential metals like Cd disrupt critical functions. Plants employ various defense mechanisms, including the synthesis of PC_n from GSH via PCS, which chelates Cd and facilitates its detoxification by sequestering it into vacuoles. Due to the implication of GSH in mitigating Cd stress, this review highlights the GSH role in response to abiotic stress.

Moreover, plant cells employ a variety of transporters to preserve metal homeostasis. Different transporters are used by plants to sequester metal ions inside organelles, whether in a free ionic state or as chelates. Transporters belonging to the IRT, NRAMP, and HMA subfamily play crucial roles in Cd absorption, translocation, and sequestration across various plant species; ATPases and ABC transporters facilitate the removal of metals from root cells, ensuring metal stress tolerance. Furthermore, the endomembrane system, particularly the ER, houses transporters for Cd, Cu, and Zn, contributing to metal storage and utilization within plant cells.

The limitations of this study include detailing only a few of the methods plants use for detoxification. A more comprehensive study would explore further mechanisms that could act additively or synergistically. It could distinguish mechanisms of avoidance, where the plant limits the entry of metals, such as the release of radical exudates or wall lignification, and mechanisms for damage repair [14,154], in addition to those that have been mentioned in greater depth.

However, this review highlights the importance of understanding the sources and pathways of Cd contamination in soil, emphasizing the need for comprehensive strategies to mitigate HM pollution. By elucidating the physiological, molecular, and biochemical mechanisms underlying plant responses to Cd stress, this work provides a basis for the development of effective strategies to safeguard plant health and ensure ecosystem resilience in the face of HM pollution.

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References

1. Jiang, Y.; Jiang, S.; Li, Z.; Yan, X.; Qin, Z.; Huang, R. Field Scale Remediation of Cd and Pb Contaminated Paddy Soil Using Three Mulberry (*Morus alba* L.) Cultivars. *Ecol. Eng.* **2019**, *129*, 38–44. [CrossRef]
2. Bhattacharya, S. Protective Role of the Essential Trace Elements in the Obviation of Cadmium Toxicity: Glimpses of Mechanisms. *Biol. Trace Elem. Res.* **2022**, *200*, 2239–2246. [CrossRef]
3. Yadav, M.; Gupta, R.; Sharma, R.K. Chapter 14-Green and Sustainable Pathways for Wastewater Purification. In *Advances in Water Purification Techniques*; Ahuja, S.B.T.-A., Ed.; Elsevier: Amsterdam, The Netherlands, 2019; pp. 355–383, ISBN 978-0-12-814790-0.
4. Lin, X.; Li, S.; Wei, Z.; Chen, Y.; Hei, L.; Wu, Q.-T. Indirect Application of Sludge for Recycling in Agriculture to Minimize Heavy Metal Contamination of Soil. *Resour. Conserv. Recycl.* **2021**, *166*, 105358. [CrossRef]
5. Qin, X.; Xia, Y.; Hu, C.; Yu, M.; Shabala, S.; Wu, S.; Tan, Q.; Xu, S.; Sun, X. Ionomics Analysis Provides New Insights into the Co-Enrichment of Cadmium and Zinc in Wheat Grains. *Ecotoxicol. Environ. Saf.* **2021**, *223*, 112623. [CrossRef]
6. Gu, Y.-G.; Li, Q.; Fang, J.; He, B.; Fu, H.; Tong, Z. Identification of Heavy Metal Sources in the Reclaimed Farmland Soils of the Pearl River Estuary in China Using a Multivariate Geostatistical Approach. *Ecotoxicol. Environ. Saf.* **2014**, *105*, 7–12. [CrossRef]
7. Ministry of Ecology and Environment of the People's Republic of China. *2021 China Ecology and Environment Statement*; Ministry of Ecology and Environment of the People's Republic of China: Beijing, China, 2022.
8. Kim, E.; Hopke, P.; Edgerton, E. Improving Source Identification of Atlanta Aerosol Using Temperature Resolved Carbon Fractions in Positive Matrix Factorization. *Atmos. Environ.* **2004**, *38*, 3349–3362. [CrossRef]
9. Fei, X.; Lou, Z.; Xiao, R.; Ren, Z.; Lv, X. Source Analysis and Source-Oriented Risk Assessment of Heavy Metal Pollution in Agricultural Soils of Different Cultivated Land Qualities. *J. Clean. Prod.* **2022**, *341*, 130942. [CrossRef]
10. Xiao, R.; Ali, A.; Wang, P.; Li, R.; Tian, X.; Zhang, Z. Comparison of the Feasibility of Different Washing Solutions for Combined Soil Washing and Phytoremediation for the Detoxification of Cadmium (Cd) and Zinc (Zn) in Contaminated Soil. *Chemosphere* **2019**, *230*, 510–518. [CrossRef] [PubMed]
11. Ekrami, E.; Pouresmaieli, M.; Hashemiyoon, E.; Noorbakhsh, N.; Mahmoudifard, M. Nanotechnology: A Sustainable Solution for Heavy Metals Remediation. *Environ. Nanotechnol. Monit. Manag.* **2022**, *18*, 100718. [CrossRef]
12. Chen, L.; Beiyuan, J.; Hu, W.; Zhang, Z.; Duan, C.; Cui, Q.; Zhu, X.; He, H.; Huang, X. Phytoremediation of Potentially Toxic Elements (PTEs) Contaminated Soils Using Alfalfa (*Medicago sativa* L.): A Comprehensive Review. *Chemosphere* **2022**, *293*, 133577. [CrossRef]
13. Chaoua, S.; Boussaa, S.; Gharmali, A.; Boumezzough, A. Impact of Irrigation with Wastewater on Accumulation of Heavy Metals in Soil and Crops in the Region of Marrakech in Morocco. *J. Saudi Soc. Agric. Sci.* **2018**, *18*, 429–436. [CrossRef]
14. Sanita' di Toppi, L.; Gabbrielli, R. Response to Cadmium in Higher Plants. *Environ. Exp. Bot.* **1999**, *41*, 105–130. [CrossRef]
15. Ismael, M.A.; Elyamine, A.M.; Moussa, M.G.; Cai, M.; Zhao, X.; Hu, C. Cadmium in Plants: Uptake, Toxicity, and Its Interactions with Selenium Fertilizers. *Metallomics* **2019**, *11*, 255–277. [CrossRef] [PubMed]
16. Kubier, A.; Wilkin, R.T.; Pichler, T. Cadmium in Soils and Groundwater: A Review. *Appl. Geochem. J. Int. Assoc. Geochemistry Cosmochem.* **2019**, *108*, 104388. [CrossRef] [PubMed]
17. Clemens, S.; Aarts, M.; Thomine, S.; Verbruggen, N. Plant Science: The Key to Preventing Slow Cadmium Poisoning. *Trends Plant Sci.* **2012**, *18*, 92. [CrossRef] [PubMed]
18. Stankovic, J.; Sabovljevic, A.; Sabovljevic, M. Bryophytes and Heavy Metals: A Review. *Acta Bot. Croat.* **2018**, *77*, 109–118. [CrossRef]
19. Zhao, H.; Li, P.; He, X. Remediation of Cadmium Contaminated Soil by Modified Gangue Material: Characterization, Performance and Mechanisms. *Chemosphere* **2021**, *290*, 133347. [CrossRef]
20. Alimba, C.G.; Gandhi, D.; Sivanesan, S.; Bhanarkar, M.D.; Naoghare, P.K.; Bakare, A.A.; Krishnamurthi, K. Chemical Characterization of Simulated Landfill Soil Leachates from Nigeria and India and Their Cytotoxicity and DNA Damage Inductions on Three Human Cell Lines. *Chemosphere* **2016**, *164*, 469–479. [CrossRef]
21. Wang, R.; Lin, K.; Chen, H.; Qi, Z.; Liu, B.; Cao, F.; Chen, H.; Wu, F. Metabolome Analysis Revealed the Mechanism of Exogenous Glutathione to Alleviate Cadmium Stress in Maize (*Zea mays* L.) Seedlings. *Plants* **2021**, *10*, 105. [CrossRef]
22. Wang, Y.; Hu, Y.; Xue, C.; Khan, A.; Zheng, X.; Cai, L. Risk Assessment of Lead and Cadmium Leaching from Solidified/Stabilized MSWI Fly Ash under Long-Term Landfill Simulation Test. *Sci. Total Environ.* **2022**, *816*, 151555. [CrossRef]
23. Hölzle, I.; Somani, M.; Ramana, G.V.; Datta, M. Heavy Metals in Soil-like Material from Landfills–Resource or Contaminants? *J. Clean. Prod.* **2022**, *369*, 133136. [CrossRef]
24. Alloway, B. *Heavy Metals in Soils: Trace Metals and Metalloids in Soils and Their Bioavailability*; Springer: Berlin/Heidelberg, Germany, 2013; ISBN 978-94-007-4469-1.
25. Naseem, S.; Hamza; Nawaz-Ul-Huda, S.; Erum, B.; ul-Haq, Q. Geochemistry of Cd in Groundwater of Winder, Balochistan and Suspected Health Problems. *Environ. Earth Sci.* **2013**, *71*, 1683–1690. [CrossRef]

26. Alengebawy, A.; Abdelkhalek, S.; Qureshi, S.; Wang, M.-Q. Heavy Metals and Pesticides Toxicity in Agricultural Soil and Plants: Ecological Risks and Human Health Implications. *Toxics* **2021**, *9*, 42. [CrossRef]
27. Rehman, A.; Nazir, S.; Irshad, R.; Tahir, K.; Rehman, K.; Islam, R.; Wahab, Z. Toxicity of Heavy Metals in Plants and Animals and Their Uptake by Magnetic Iron Oxide Nanoparticles. *J. Mol. Liq.* **2020**, *321*, 114455. [CrossRef]
28. Aoshima, K. Itai-Itai Disease: Renal Tubular Osteomalacia Induced by Environmental Exposure to Cadmium—Historical Review and Perspectives. *Soil Sci. Plant Nutr.* **2016**, *62*, 319–326. [CrossRef]
29. Arain, M.B.; Kazi, T.G.; Baig, J.A.; Afridi, H.I.; Sarajuddin; Brehman, K.D.; Panhwar, H.; Arain, S.S. Co-Exposure of Arsenic and Cadmium through Drinking Water and Tobacco Smoking: Risk Assessment on Kidney Dysfunction. *Environ. Sci. Pollut. Res. Int.* **2015**, *22*, 350–357. [CrossRef] [PubMed]
30. Khan, M.A.; Khan, S.; Khan, A.; Alam, M. Soil Contamination with Cadmium, Consequences and Remediation Using Organic Amendments. *Sci. Total Environ.* **2017**, *601–602*, 1591–1605. [CrossRef] [PubMed]
31. Meysam Hoseini, S.; Zargari, F. Cadmium in Plants: A Review. *Int. J. Farming Allied Sci.* **2013**, *2*, 579–581.
32. Li, Y.; Sun, M.; He, W.; Wang, H.; Pan, H.; Yang, Q.; Lou, Y.; Zhuge, Y. Effect of Phosphorus Supplementation on Growth, Nutrient Uptake, Physiological Responses, and Cadmium Absorption by Tall Fescue (*Festuca arundinacea* Schreb.) Exposed to Cadmium. *Ecotoxicol. Environ. Saf.* **2021**, *213*, 112021. [CrossRef]
33. Meng, Y.; Jing, H.; Huang, J.; Shen, R.; Zhu, X. The Role of Nitric Oxide Signaling in Plant Responses to Cadmium Stress. *Int. J. Mol. Sci.* **2022**, *23*, 6901. [CrossRef]
34. Rady, M.; Elrys, A.; Aboelmaati, M.; Desoky, E.-S. Interplaying Roles of Silicon and Proline Effectively Improve Salt and Cadmium Stress Tolerance in Phaseolus Vulgaris Plant. *Plant Physiol. Biochem.* **2019**, *139*, 558–568. [CrossRef] [PubMed]
35. Semida, W.; Hemida, K.; Rady, M. Sequenced Ascorbate-Proline-Glutathione Seed Treatment Elevates Cadmium Tolerance in Cucumber Transplants. *Ecotoxicol. Environ. Saf.* **2018**, *154*, 171–179. [CrossRef] [PubMed]
36. Zouari, M.; Ben Ahmed, C.; Zorrig, W.; Elloumi, N.; Rabhi, M.; Delmail, D.; Ben Rouina, B.; Labrousse, P.; Ben Abdallah, F. Exogenous Proline Mediates Alleviation of Cadmium Stress by Promoting Photosynthetic Activity, Water Status and Antioxidative Enzymes Activities of Young Date Palm (*Phoenix dactylifera* L.). *Ecotoxicol. Environ. Saf.* **2016**, *128*, 100–108. [CrossRef] [PubMed]
37. Shahid, M.; Javed, M.T.; Mushtaq, A.; Akram, M.S.; Mahmood, F.; Ahmed, T.; Noman, M.; Azeem, M. Microbe-Mediated Mitigation of Cadmium Toxicity in Plants. In *Cadmium Toxicity and Tolerance in Plants*; Elsevier: Amsterdam, The Netherlands, 2019; pp. 427–449, ISBN 9780128148648.
38. Sidhu, G.P.S.; Bali, A.; Kumar, V.; Bhardwaj, R. Mitigating Cadmium Toxicity in Plants by Phytohormones. In *Cadmium Toxicity and Tolerance in Plants: From Physiology to Remediation*; Hasanuzzaman, M., Prasad, M.N.V., Fujita, M., Eds.; Elsevier: London, UK, 2019; pp. 375–396, ISBN 978-0-12-814864-8.
39. Hameed, A.; Rasool, S.; Azooz, M.M.; Hossain, M.A.; Ahanger, M.A.; Ahmad, P. Chapter 24-Heavy Metal Stress: Plant Responses and Signaling. In *Plant Metal Interaction*; Ahmad, P., Ed.; Elsevier: Amsterdam, The Netherlands, 2016; pp. 557–583, ISBN 978-0-12-803158-2.
40. Huang, Y.; Zhu, Z.; Wu, X.; Liu, Z.; Zou, J.; Chen, Y.; Su, N.; Cui, J. Lower Cadmium Accumulation and Higher Antioxidative Capacity in Edible Parts of *Brassica campestris* L. Seedlings Applied with Glutathione under Cadmium Toxicity. *Environ. Sci. Pollut. Res.* **2019**, *26*, 13235–13245. [CrossRef] [PubMed]
41. Meng, Y.; Zhang, L.; Wang, L.; Zhou, C.; Shangguan, Y.; Yang, Y. Antioxidative Enzymes Activity and Thiol Metabolism in Three Leafy Vegetables under Cd Stress. *Ecotoxicol. Environ. Saf.* **2019**, *173*, 214–224. [CrossRef] [PubMed]
42. Shahid, M.; Dumat, C.; Khalid, S.; Schreck, E.; Xiong, T.; Niazi, N.K. Foliar Heavy Metal Uptake, Toxicity and Detoxification in Plants: A Comparison of Foliar and Root Metal Uptake. *J. Hazard. Mater.* **2017**, *325*, 36–58. [CrossRef] [PubMed]
43. Faizan, M.; Alam, P.; Rajput, V.; Faraz, A.; Afzal, S.; Ahmed, S.; Yu, F.-Y.; Minkina, T.; Hayat, S. Nanoparticle Mediated Plant Tolerance to Heavy Metal Stress: What We Know? *Sustainability* **2023**, *15*, 1446. [CrossRef]
44. Valko, M.; Rhodes, C.; Izakovic, M.; Mazur, M. Valko M, Rhodes CJ, Moncol J, Izakovic M, Mazur MFree Radicals, Metals and Antioxidants in Oxidative Stress-Induced Cancer. *Chem. Biol. Interact.* **2006**, *160*, 1–40. [CrossRef]
45. Boonstra, J.; Post, J. Molecular Events Associated with Reactive Oxygen Species and Cell Cycle Progression in Mammalian Cells. *Gene* **2004**, *337*, 1–13. [CrossRef]
46. Kumar, A.; Majeti, P. Plant-Lead Interactions: Transport, Toxicity, Tolerance, and Detoxification Mechanisms. *Ecotoxicol. Environ. Saf.* **2018**, *166*, 401–418. [CrossRef]
47. Liu, Y.; Liu, L.; Qi, J.; Dang, P.; Xia, T. Cadmium Activates ZmMPK3-1 and ZmMPK6-1 via Induction of Reactive Oxygen Species in Maize Roots. *Biochem. Biophys. Res. Commun.* **2019**, *516*, 747–752. [CrossRef]
48. Berni, R.; Luyckx, M.; Xu, X.; Legay, S.; Sergeant, K.; Hausman, J.-F.; Lutts, S.; Cai, G.; Guerriero, G. Reactive Oxygen Species and Heavy Metal Stress in Plants: Impact on the Cell Wall and Secondary Metabolism. *Environ. Exp. Bot.* **2019**, *161*, 98–106. [CrossRef]
49. Ma, Q.-J.; Sun, M.-H.; Lu, J.; Hu, D.-G.; Kang, H.; You, C.-X.; Hao, Y.-J. Phosphorylation of a Malate Transporter Promotes Malate Excretion and Reduces Cadmium Uptake in Apple. *J. Exp. Bot.* **2020**, *71*, 3437–3449. [CrossRef]
50. Sharma, S.K.; Singh, D.; Pandey, H.; Jatav, R.B.; Singh, V.; Pandey, D. *An Overview of Roles of Enzymatic and Nonenzymatic Antioxidants in Plant BT-Antioxidant Defense in Plants: Molecular Basis of Regulation*; Aftab, T., Hakeem, K.R., Eds.; Springer Nature Singapore: Singapore, 2022; pp. 1–13, ISBN 978-981-16-7981-0.
51. Metwally, A.; Safronova, V.I.; Belimov, A.A.; Dietz, K.-J. Genotypic Variation of the Response to Cadmium Toxicity in *Pisum sativum* L. *J. Exp. Bot.* **2005**, *56*, 167–178. [CrossRef] [PubMed]

52. Nada, E.; Ferjani, B.A.; Ali, R.; Bechir, B.R.; Imed, M.; Makki, B. Cadmium-Induced Growth Inhibition and Alteration of Biochemical Parameters in Almond Seedlings Grown in Solution Culture. *Acta Physiol. Plant.* **2007**, *29*, 57–62. [CrossRef]
53. Fan, W.; Liu, C.; Cao, B.; Qin, M.; Long, D.; Xiang, Z.; Zhao, A. Genome-Wide Identification and Characterization of Four Gene Families Putatively Involved in Cadmium Uptake, Translocation and Sequestration in Mulberry. *Front. Plant Sci.* **2018**, *9*, 879. [CrossRef] [PubMed]
54. Jia, H.; Wang, X.; Wei, T.; Zhou, R.; Muhammad, H.; Hua, L.; Ren, X.; Guo, J.; Ding, Y. Accumulation and Fixation of Cd by Tomato Cell Wall Pectin under Cd Stress. *Environ. Exp. Bot.* **2019**, *167*, 103829. [CrossRef]
55. Song, J.; Feng, S.J.; Chen, J.; Zhao, W.T.; Yang, Z.M. A Cadmium Stress-Responsive Gene AtFC1 Confers Plant Tolerance to Cadmium Toxicity. *BMC Plant Biol.* **2017**, *17*, 187. [CrossRef]
56. Astolfi, S.; Ortolani, M.R.; Catarcione, G.; Paolacci, A.R.; Cesco, S.; Pinton, R.; Ciaffi, M. Cadmium Exposure Affects Iron Acquisition in Barley (*Hordeum vulgare*) Seedlings. *Physiol. Plant.* **2014**, *152*, 646–659. [CrossRef]
57. Bahmani, R.; Modareszadeh, M.; Kim, D.; Hwang, S. Overexpression of Tobacco UBQ2 Increases Cd Tolerance by Decreasing Cd Accumulation and Oxidative Stress in Tobacco and Arabidopsis. *Environ. Exp. Bot.* **2019**, *166*, 103805. [CrossRef]
58. Yu, R.; Li, D.; Du, X.; Xia, S.; Liu, C.; Shi, G. Comparative Transcriptome Analysis Reveals Key Cadmium Transport-Related Genes in Roots of Two Pak Choi (*Brassica rapa* L. ssp. *chinensis*) Cultivars. *BMC Genom.* **2017**, *18*, 587. [CrossRef] [PubMed]
59. Rui, H.; Zhang, X.; Shinwari, K.; Zheng, L.; Shen, Z.G. Comparative Transcriptomic Analysis of Two *Vicia sativa* L. Varieties with Contrasting Responses to Cadmium Stress Reveals the Important Role of Metal Transporters in Cadmium Tolerance. *Plant Soil* **2018**, *423*, 241–255. [CrossRef]
60. Shu, X.; Zhang, K.; Zhang, Q.; Wang, W. Ecophysiological Responses of *Jatropha curcas* L. Seedlings to Simulated Acid Rain under Different Soil Types. *Ecotoxicol. Environ. Saf.* **2019**, *185*, 109705. [CrossRef] [PubMed]
61. Milner, M.; Mitani-Ueno, N.; Yamaji, N.; Yokosho, K.; Craft, E.; Fei, Z.; Ebbs, S.; Zambrano, M.; Kochian, L. Root and Shoot Transcriptome Analysis of Two Ecotypes of Noccaea Caerulescens Uncovers the Role of NcNramp1 in Cd Hyperaccumulation. *Plant J.* **2014**, *78*, 398–410. [CrossRef] [PubMed]
62. Zhang, J.; Zhang, C.; Huang, S.; Chang, L.; Li, J.; Tang, H.; Dey, S.; Biswas, A.; Du, D.; Li, D.; et al. Key Cannabis Salt-Responsive Genes and Pathways Revealed by Comparative Transcriptome and Physiological Analyses of Contrasting Varieties. *Agronomy* **2021**, *11*, 2338. [CrossRef]
63. Feng, Z.; Bartholomew, E.S.; Liu, Z.; Cui, Y.; Dong, Y.; Li, S.; Wu, H.; Ren, H.; Liu, X. Glandular Trichomes: New Focus on Horticultural Crops. *Hortic. Res.* **2021**, *8*, 158. [CrossRef] [PubMed]
64. Chen, H.; Li, Y.; Ma, X.; Guo, L.; He, Y.; Ren, Z.; Kuang, Z.; Zhang, X.; Zhang, Z. Analysis of Potential Strategies for Cadmium Stress Tolerance Revealed by Transcriptome Analysis of Upland Cotton. *Sci. Rep.* **2019**, *9*, 86. [CrossRef] [PubMed]
65. Wei, W.; Peng, H.; Xie, Y.; Wang, X.; Huang, R.; Chen, H.; Ji, X. The Role of Silicon in Cadmium Alleviation by Rice Root Cell Wall Retention and Vacuole Compartmentalization under Different Durations of Cd Exposure. *Ecotoxicol. Environ. Saf.* **2021**, *226*, 112810. [CrossRef] [PubMed]
66. Ghuge, S.A.; Nikalje, G.C.; Kadam, U.S.; Suprasanna, P.; Hong, J.C. Comprehensive Mechanisms of Heavy Metal Toxicity in Plants, Detoxification, and Remediation. *J. Hazard. Mater.* **2023**, *450*, 131039. [CrossRef]
67. Ahmad, J.; Ali, A.A.; Baig, M.A.; Iqbal, M.; Haq, I.; Irfan Qureshi, M. Chapter 8-Role of Phytochelatins in Cadmium Stress Tolerance in Plants. In *Cadmium Toxicity and Tolerance in Plants*; Hasanuzzaman, M., Prasad, M.N.V., Fujita, M., Eds.; Academic Press: Cambridge, MA, USA, 2019; pp. 185–212, ISBN 978-0-12-814864-8.
68. Hendrix, S.; Schröder, P.; Keunen, E.; Huber, C.; Cuypers, A. Chapter Six-Molecular and Cellular Aspects of Contaminant Toxicity in Plants: The Importance of Sulphur and Associated Signalling Pathways. In *Phytoremediation*; Cuypers, A., Vangronsveld, J., Eds.; Academic Press: Cambridge, MA, USA, 2017; Volume 83, pp. 223–276, ISBN 0065-2296.
69. Al Mahmud, J.; Hasanuzzaman, M.; Nahar, K.; Bhuyan, M.H.M.B.; Fujita, M. Insights into Citric Acid-Induced Cadmium Tolerance and Phytoremediation in *Brassica juncea* L.: Coordinated Functions of Metal Chelation, Antioxidant Defense and Glyoxalase Systems. *Ecotoxicol. Environ. Saf.* **2018**, *147*, 990–1001. [CrossRef]
70. Peco, J.D.; Campos, J.A.; Romero-Puertas, M.C.; Olmedilla, A.; Higueras, P.; Sandalio, L.M. Characterization of Mechanisms Involved in Tolerance and Accumulation of Cd in *Biscutella auriculata* L. *Ecotoxicol. Environ. Saf.* **2020**, *201*, 110784. [CrossRef] [PubMed]
71. Clemens, S.; Peršoh, D. Multi-Tasking Phytochelatin Synthases. *Plant Sci.* **2009**, *177*, 266–271. [CrossRef]
72. Bellini, E.; Bandoni, E.; Giardini, S.; Sorce, C.; Spanò, C.; Bottega, S.; Fontanini, D.; Kola, A.; Valensin, D.; Bertolini, A.; et al. Glutathione and Phytochelatins Jointly Allow Intracellular and Extracellular Detoxification of Cadmium in the Liverwort Marchantia Polymorpha. *Environ. Exp. Bot.* **2023**, *209*, 105303. [CrossRef]
73. Huang, L.; Li, W.C.; Tam, N.F.Y.; Ye, Z. Effects of Root Morphology and Anatomy on Cadmium Uptake and Translocation in Rice (*Oryza sativa* L.). *J. Environ. Sci.* **2019**, *75*, 296–306. [CrossRef] [PubMed]
74. Zhao, F.-J.; Wang, P. Arsenic and Cadmium Accumulation in Rice and Mitigation Strategies. *Plant Soil* **2020**, *446*, 1–21. [CrossRef]
75. Bellini, E.; Varotto, C.; Borsò, M.; Rugnini, L.; Bruno, L.; Di Toppi, L.S. Eukaryotic and Prokaryotic Phytochelatin Synthases Differ Less in Functional Terms than Previously Thought: A Comparative Analysis of Marchantia Polymorpha and Geitlerinema Sp. PCC 7407. *Plants* **2020**, *9*, 914. [CrossRef] [PubMed]
76. Li, M.; Barbaro, E.; Bellini, E.; Saba, A.; di Toppi, L.S.; Varotto, C. Ancestral Function of the Phytochelatin Synthase C-Terminal Domain in Inhibition of Heavy Metal-Mediated Enzyme Overactivation. *J. Exp. Bot.* **2020**, *71*, 6655–6669. [CrossRef] [PubMed]

77. Li, M.; Leso, M.; Buti, M.; Bellini, E.; Bertoldi, D.; Saba, A.; Larcher, R.; Sanità di Toppi, L.; Varotto, C. Phytochelatin Synthase De-Regulation in Marchantia Polymorpha Indicates Cadmium Detoxification as Its Primary Ancestral Function in Land Plants and Provides a Novel Visual Bioindicator for Detection of This Metal. *J. Hazard. Mater.* **2022**, *440*, 129844. [CrossRef]
78. Bhuyan, M.H.M.B.; Parvin, K.; Mohsin, S.M.; Mahmud, J.A.; Hasanuzzaman, M.; Fujita, M. Modulation of Cadmium Tolerance in Rice: Insight into Vanillic Acid-Induced Upregulation of Antioxidant Defense and Glyoxalase Systems. *Plants* **2020**, *9*, 188. [CrossRef]
79. Degola, F.; De Benedictis, M.; Petraglia, A.; Massimi, A.; Fattorini, L.; Sorbo, S.; Basile, A.; Sanita' di Toppi, L. A Cd/Fe/Zn-Responsive Phytochelatin Synthase Is Constitutively Present in the Ancient Liverwort *Lunularia cruciata* (L.) Dumort. *Plant Cell Physiol.* **2014**, *55*, 1884–1891. [CrossRef]
80. Petraglia, A.; De Benedictis, M.; Degola, F.; Pastore, G.; Calcagno, M.; Ruotolo, R.; Mengoni, A.; Sanita' di Toppi, L. The Capability to Synthesize Phytochelatins and the Presence of Constitutive and Functional Phytochelatin Synthases Are Ancestral (Plesiomorphic) Characters for Basal Land Plants. *J. Exp. Bot.* **2014**, *65*, 1153–1163. [CrossRef] [PubMed]
81. Fontanini, D.; Andreucci, A.; Castiglione, M.; Basile, A.; Sorbo, S.; Petraglia, A.; Degola, F.; Bellini, E.; Bruno, L.; Varotto, C.; et al. The Phytochelatin Synthase from Nitella Mucronata (Charophyta) Plays a Role in the Homeostatic Control of Iron(II)/(III). *Plant Physiol. Biochem.* **2018**, *127*, 88–96. [CrossRef] [PubMed]
82. Bellini, E.; Betti, C.; Sanità di Toppi, L. Responses to Cadmium in Early-Diverging Streptophytes (Charophytes and Bryophytes): Current Views and Potential Applications. *Plants* **2021**, *10*, 770. [CrossRef] [PubMed]
83. Maresca, V.; Sorbo, S.; Loppi, S.; Funaro, F.; Del Prete, D.; Basile, A. Biological Effects from Environmental Pollution by Toxic Metals in the "Land of Fires" (Italy) Assessed Using the Biomonitor Species *Lunularia cruciata* L. (Dum). *Environ. Pollut.* **2020**, *265*, 115000. [CrossRef] [PubMed]
84. Fasani, E.; Li, M.; Varotto, C.; Furini, A.; DalCorso, G. Metal Detoxification in Land Plants: From Bryophytes to Vascular Plants. STATE of the Art and Opportunities. *Plants* **2022**, *11*, 237. [CrossRef] [PubMed]
85. Bellini, E.; Maresca, V.; Betti, C.; Castiglione, M.R.; Fontanini, D.; Capocchi, A.; Sorce, C.; Borsò, M.; Bruno, L.; Sorbo, S.; et al. The Moss *Leptodictyum riparium* Counteracts Severe Cadmium Stress by Activation of Glutathione Transferase and Phytochelatin Synthase, but Slightly by Phytochelatins. *Int. J. Mol. Sci.* **2020**, *21*, 1583. [CrossRef] [PubMed]
86. Esposito, S.; Loppi, S.; Monaci, F.; Paoli, L.; Vannini, A.; Sorbo, S.; Maresca, V.; Fusaro, L.; Karam, E.; Lentini, M.; et al. In-Field and in-Vitro Study of the Moss *Leptodictyum riparium* as Bioindicator of Toxic Metal Pollution in the Aquatic Environment: Ultrastructural Damage, Oxidative Stress and HSP70 Induction. *PLoS ONE* **2018**, *13*, e0195717. [CrossRef] [PubMed]
87. Landi, S.; Esposito, S. Nitrate Uptake Affects Cell Wall Synthesis and Modeling. *Front. Plant Sci.* **2017**, *8*, 1376. [CrossRef] [PubMed]
88. Singh, S.; Prasad, S.M. Effects of 28-Homobrassinoloid on Key Physiological Attributes of Solanum Lycopersicum Seedlings under Cadmium Stress: Photosynthesis and Nitrogen Metabolism. *Plant Growth Regul.* **2017**, *82*, 161–173. [CrossRef]
89. Lentini, M.; De Lillo, A.; Paradisone, V.; Liberti, D.; Landi, S.; Esposito, S. Early Responses to Cadmium Exposure in Barley Plants: Effects on Biometric and Physiological Parameters. *Acta Physiol. Plant.* **2018**, *40*, 178. [CrossRef]
90. Yang, Y.; Xiong, J.; Tao, L.; Cao, Z.; Tang, W.; Zhang, J.; Yu, X.; Fu, G.; Zhang, X.; Lu, Y. Regulatory Mechanisms of Nitrogen (N) on Cadmium (Cd) Uptake and Accumulation in Plants: A Review. *Sci. Total Environ.* **2020**, *708*, 135186. [CrossRef]
91. Ben Azaiez, F.E.; Ayadi, S.; Capasso, G.; Landi, S.; Paradisone, V.; Jallouli, S.; Hammami, Z.; Chamekh, Z.; Zouari, I.; Trifa, Y.; et al. Salt Stress Induces Differentiated Nitrogen Uptake and Antioxidant Responses in Two Contrasting Barley Landraces from MENA Region. *Agronomy* **2020**, *10*, 1426. [CrossRef]
92. Jallouli, S.; Ayadi, S.; Landi, S.; Capasso, G.; Santini, G.; Chamekh, Z.; Zouari, I.; Ben Azaiez, F.E.; Trifa, Y.; Esposito, S. Physiological and Molecular Osmotic Stress Responses in Three Durum Wheat (*Triticum Turgidum* ssp *Durum*) Genotypes. *Agronomy* **2019**, *9*, 550. [CrossRef]
93. Lee, H.J.; Abdula, S.E.; Jang, D.W.; Park, S.-H.; Yoon, U.-H.; Jung, Y.J.; Kang, K.K.; Nou, I.S.; Cho, Y.-G. Overexpression of the Glutamine Synthetase Gene Modulates Oxidative Stress Response in Rice after Exposure to Cadmium Stress. *Plant Cell Rep.* **2013**, *32*, 1521–1529. [CrossRef] [PubMed]
94. Saini, S.; Kaur, N.; Pati, P.K. Phytohormones: Key Players in the Modulation of Heavy Metal Stress Tolerance in Plants. *Ecotoxicol. Environ. Saf.* **2021**, *223*, 112578. [CrossRef]
95. Maresca, V.; Bellini, E.; Landi, S.; Capasso, G.; Cianciullo, P.; Carraturo, F.; Pirintsos, S.; Sorbo, S.; Sanità di Toppi, L.; Esposito, S.; et al. Biological Responses to Heavy Metal Stress in the Moss *Leptodictyum riparium* (Hedw.) Warnst. *Ecotoxicol. Environ. Saf.* **2022**, *229*, 113078. [CrossRef]
96. Meister, A. Glutathione Metabolism. *Methods Enzymol.* **1995**, *251*, 3–7. [CrossRef] [PubMed]
97. Pasternak, M.; Lim, B.; Wirtz, M.; Hell, R.; Cobbett, C.S.; Meyer, A.J. Restricting Glutathione Biosynthesis to the Cytosol Is Sufficient for Normal Plant Development. *Plant J.* **2008**, *53*, 999–1012. [CrossRef] [PubMed]
98. Belin, C.; Bashandy, T.; Cela, J.; Delorme-Hinoux, V.; Riondet, C.; Reichheld, J.P. A Comprehensive Study of Thiol Reduction Gene Expression under Stress Conditions in *Arabidopsis thaliana*. *Plant. Cell Environ.* **2015**, *38*, 299–314. [CrossRef]
99. Xiang, C.; Oliver, D.J. Glutathione Metabolic Genes Coordinately Respond to Heavy Metals and Jasmonic Acid in Arabidopsis. *Plant Cell* **1998**, *10*, 1539–1550. [CrossRef]

100. Wachter, A.; Wolf, S.; Steininger, H.; Bogs, J.; Rausch, T. Differential Targeting of GSH1 and GSH2 Is Achieved by Multiple Transcription Initiation: Implications for the Compartmentation of Glutathione Biosynthesis in the Brassicaceae. *Plant J.* **2005**, *41*, 15–30. [CrossRef] [PubMed]
101. Yang, Y.; Lenherr, E.D.; Gromes, R.; Wang, S.; Wirtz, M.; Hell, R.; Peskan-Berghöfer, T.; Scheffzek, K.; Rausch, T. Plant Glutathione Biosynthesis Revisited: Redox-Mediated Activation of Glutamylcysteine Ligase Does Not Require Homo-Dimerization. *Biochem. J.* **2019**, *476*, 1191–1203. [CrossRef] [PubMed]
102. Watson, S.J.; Sowden, R.G.; Jarvis, P. Abiotic Stress-Induced Chloroplast Proteome Remodelling: A Mechanistic Overview. *J. Exp. Bot.* **2018**, *69*, 2773–2781. [CrossRef] [PubMed]
103. Kopriva, S.; Rennenberg, H. Control of Sulphate Assimilation and Glutathione Synthesis: Interaction with N and C Metabolism. *J. Exp. Bot.* **2004**, *55*, 1831–1842. [CrossRef] [PubMed]
104. Strohm, M.; Jouanin, L.; Kunert, K.J.; Pruvost, C.; Polle, A.; Foyer, C.H.; Rennenberg, H. Regulation of Glutathione Synthesis in Leaves of Transgenic Poplar (*Populus tremula* X *P. alba*) Overexpressing Glutathione Synthetase. *Plant J.* **1995**, *7*, 141–145. [CrossRef]
105. Gill, S.S.; Anjum, N.A.; Hasanuzzaman, M.; Gill, R.; Trivedi, D.K.; Ahmad, I.; Pereira, E.; Tuteja, N. Glutathione and Glutathione Reductase: A Boon in Disguise for Plant Abiotic Stress Defense Operations. *Plant Physiol. Biochem.* **2013**, *70*, 204–212. [CrossRef] [PubMed]
106. Sairam, R.K.; Saxena, D.C. Oxidative Stress and Antioxidants in Wheat Genotypes: Possible Mechanism of Water Stress Tolerance. *J. Agron. Crop Sci.* **2000**, *184*, 55–61. [CrossRef]
107. Romero-Puertas, M.C.; Corpas, F.J.; Sandalio, L.M.; Leterrier, M.; Rodríguez-Serrano, M.; Del Río, L.A.; Palma, J.M. Glutathione Reductase from Pea Leaves: Response to Abiotic Stress and Characterization of the Peroxisomal Isozyme. *New Phytol.* **2006**, *170*, 43–52. [CrossRef] [PubMed]
108. Marty, L.; Siala, W.; Schwarzländer, M.; Fricker, M.D.; Wirtz, M.; Sweetlove, L.J.; Meyer, Y.; Meyer, A.J.; Reichheld, J.-P.; Hell, R. The NADPH-Dependent Thioredoxin System Constitutes a Functional Backup for Cytosolic Glutathione Reductase in Arabidopsis. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 9109–9114. [CrossRef]
109. Kataya, A.R.A.; Reumann, S. Arabidopsis Glutathione Reductase 1 Is Dually Targeted to Peroxisomes and the Cytosol. *Plant Signal. Behav.* **2010**, *5*, 171–175. [CrossRef]
110. Delorme-Hinoux, V.; Bangash, S.A.K.; Meyer, A.J.; Reichheld, J.-P. Nuclear Thiol Redox Systems in Plants. *Plant Sci.* **2016**, *243*, 84–95. [CrossRef] [PubMed]
111. Liu, L.; Li, W.; Song, W.; Guo, M. Remediation Techniques for Heavy Metal-Contaminated Soils: Principles and Applicability. *Sci. Total Environ.* **2018**, *633*, 206–219. [CrossRef]
112. Rizwan, M.; Ali, S.; Zia ur Rehman, M.; Rinklebe, J.; Tsang, D.C.W.; Bashir, A.; Maqbool, A.; Tack, F.M.G.; Ok, Y.S. Cadmium Phytoremediation Potential of Brassica Crop Species: A Review. *Sci. Total Environ.* **2018**, *631–632*, 1175–1191. [CrossRef] [PubMed]
113. Luo, Z.-B.; He, J.; Polle, A.; Rennenberg, H. Heavy Metal Accumulation and Signal Transduction in Herbaceous and Woody Plants: Paving the Way for Enhancing Phytoremediation Efficiency. *Biotechnol. Adv.* **2016**, *34*, 1131–1148. [CrossRef] [PubMed]
114. Krämer, U. Metal Hyperaccumulation in Plants. *Annu. Rev. Plant Biol.* **2010**, *61*, 517–534. [CrossRef] [PubMed]
115. Meighan, M.M.; Fenus, T.; Karey, E.; MacNeil, J. The Impact of EDTA on the Rate of Accumulation and Root/Shoot Partitioning of Cadmium in Mature Dwarf Sunflowers. *Chemosphere* **2011**, *83*, 1539–1545. [CrossRef] [PubMed]
116. Di Lonardo, S.; Capuana, M.; Arnetoli, M.; Gabbrielli, R.; Gonnelli, C. Exploring the Metal Phytoremediation Potential of Three *Populus alba* L. Clones Using an in Vitro Screening. *Environ. Sci. Pollut. Res.* **2011**, *18*, 82–90. [CrossRef] [PubMed]
117. Marmiroli, M.; Visioli, G.; Maestri, E.; Marmiroli, N. Correlating SNP Genotype with the Phenotypic Response to Exposure to Cadmium in *Populus* spp. *Environ. Sci. Technol.* **2011**, *45*, 4497–4505. [CrossRef] [PubMed]
118. Pietrini, F.; Zacchini, M.; Iori, V.; Pietrosanti, L.; Bianconi, D.; Massacci, A. Screening of Poplar Clones for Cadmium Phytoremediation Using Photosynthesis, Biomass and Cadmium Content Analyses. *Int. J. Phytoremediation* **2010**, *12*, 105–120. [CrossRef]
119. Wu, F.; Yang, W.; Zhang, J.; Zhou, L. Cadmium Accumulation and Growth Responses of a Poplar (*Populus deltoids* x *Populus nigra*) in Cadmium Contaminated Purple Soil and Alluvial Soil. *J. Hazard. Mater.* **2010**, *177*, 268–273. [CrossRef]
120. Redjala, T.; Zelko, I.; Sterckeman, T.; Legué, V.; Lux, A. Relationship between Root Structure and Root Cadmium Uptake in Maize. *Environ. Exp. Bot.* **2011**, *71*, 241–248. [CrossRef]
121. Thakur, S.; Singh, L.; Wahid, Z.; Siddiqui, M.; Atnaw, S.; Md Din, M.F. Plant-Driven Removal of Heavy Metals from Soil: Uptake, Translocation, Tolerance Mechanism, Challenges, and Future Perspectives. *Environ. Monit. Assess.* **2016**, *188*, 206. [CrossRef] [PubMed]
122. Kumar Yadav, K.; Gupta, N.; Kumar, A.; Reece, L.M.; Singh, N.; Rezania, S.; Ahmad Khan, S. Mechanistic Understanding and Holistic Approach of Phytoremediation: A Review on Application and Future Prospects. *Ecol. Eng.* **2018**, *120*, 274–298. [CrossRef]
123. Laghlimi, M.; Baghdad, B.; EL Hadi, H.; Bouabdli, A. Phytoremediation Mechanisms of Heavy Metal Contaminated Soils: A Review. *Open J. Ecol.* **2015**, *5*, 375–388. [CrossRef]
124. Fahr, M.; Laplaze, L.; Bendaou, N.; Hocher, V.; EL Mzibri, M.; Bogusz, D.; Smouni, A. Effect of Lead on Root Growth. *Front. Plant Sci.* **2013**, *4*, 175. [CrossRef] [PubMed]
125. Gupta, N.; Yadav, K.K.; Kumar, V.; Kumar, S.; Chadd, R.P.; Kumar, A. Trace Elements in Soil-Vegetables Interface: Translocation, Bioaccumulation, Toxicity and Amelioration–A Review. *Sci. Total Environ.* **2019**, *651*, 2927–2942. [CrossRef] [PubMed]

126. Gupta, N.; Ram, H.; Kumar, B. Mechanism of Zinc Absorption in Plants: Uptake, Transport, Translocation and Accumulation. *Rev. Environ. Sci. Bio/Technol.* **2016**, *15*, 89–109. [CrossRef]
127. Kim, R.-Y.; Yoon, J.-K.; Kim, T.-S.; Yang, J.; Owens, G.; Kim, K.-R. Bioavailability of Heavy Metals in Soils: Definitions and Practical Implementation—A Critical Review. *Environ. Geochem. Health* **2015**, *37*, 1041–1061. [CrossRef] [PubMed]
128. Sebastian, A.; Prasad, M.N. V Exogenous Citrate and Malate Alleviate Cadmium Stress in Oryza Sativa L.: Probing Role of Cadmium Localization and Iron Nutrition. *Ecotoxicol. Environ. Saf.* **2018**, *166*, 215–222. [CrossRef] [PubMed]
129. Wu, Z.; Zhao, X.; Sun, X.; Tan, Q.; Tang, Y.; Nie, Z.; Hu, C. Xylem Transport and Gene Expression Play Decisive Roles in Cadmium Accumulation in Shoots of Two Oilseed Rape Cultivars (*Brassica napus*). *Chemosphere* **2015**, *119*, 1217–1223. [CrossRef]
130. Hall, J.L. Cellular Mechanisms for Heavy Metal Detoxification and Tolerance. *J. Exp. Bot.* **2002**, *53*, 1–11. [CrossRef]
131. Kumar, A.; Dubey, A.; Kumar, V.; Ansari, M.; Narayan, S.M.; Kumar, S.; Meenakshi; Pandey, V.; Shirke, P.; Pande, V.; et al. Over-Expression of Chickpea Glutaredoxin (CaGrx) Provides Tolerance to Heavy Metals by Reducing Metal Accumulation and Improved Physiological and Antioxidant Defence System. *Ecotoxicol. Environ. Saf.* **2020**, *192*, 110252. [CrossRef] [PubMed]
132. Cointry, V.; Vert, G. The Bifunctional Transporter-receptor IRT1 at the Heart of Metal Sensing and Signaling. *New Phytol.* **2019**, *223*, 1173–1178. [CrossRef] [PubMed]
133. Narayan, O.; Verma, N.; Jogawat, A.; Dua, M.; Johri, A. Role of Sulphate Transporter (PiSulT) of Endophytic Fungus Serendipita Indica in Plant Growth and Development. *bioRxiv* **2020**. [CrossRef]
134. Narayan, O.; Verma, N.; Jogawat, A.; Dua, M.; Johri, A. Sulfur Transfer from the Endophytic Fungus Serendipita Indica Improves Maize Growth and Requires the Sulfate Transporter SiSulT. *Plant Cell* **2021**, *33*, 1268–1285. [CrossRef] [PubMed]
135. Yadav, B.; Jogawat, A.; Lal, S.K.; Lakra, N.; Mehta, S.; Shabek, N.; Narayan, O. Plant Mineral Transport Systems and the Potential for Crop Improvement. *Planta* **2021**, *253*, 45. [CrossRef] [PubMed]
136. Dubeaux, G.; Julie, N.; Zelazny, E.; Vert, G. Metal Sensing by the IRT1 Transporter-Receptor Orchestrates Its Own Degradation and Plant Metal Nutrition. *Mol. Cell* **2018**, *69*, 953–964.e5. [CrossRef] [PubMed]
137. Wu, Q.; Huang, L.; Su, N.; Shabala, L.; Wang, H.; Huang, X.; Wen, R.; Yu, M.; Cui, J.; Shabala, S. Calcium-Dependent Hydrogen Peroxide Mediates Hydrogen-Rich Water-Reduced Cadmium Uptake in Plant Roots. *Plant Physiol.* **2020**, *183*, 1331–1344. [CrossRef] [PubMed]
138. Takahashi, R.; Ishimaru, Y.; Senoura, T.; Shimo, H.; Ishikawa, S.; Arao, T.; Nakanishi, H.; Nishizawa, N. The OsNRAMP1 Iron Transporter Is Involved in Cd Accumulation in Rice. *J. Exp. Bot.* **2011**, *62*, 4843–4850. [CrossRef]
139. Lang, M.; Hao, M.; Fan, Q.; Wang, W.; Mo, S.; Zhao, W.; Zhou, J. Functional Characterization of BjCET3 and BjCET4, Two New Cation-Efflux Transporters from *Brassica juncea* L. *J. Exp. Bot.* **2011**, *62*, 4467–4480. [CrossRef]
140. Li, J.; Wang, Y.; Zheng, L.; Li, Y.; Zhou, X.; Li, J.; Gu, D.; Xu, E.; Lu, Y.; Chen, X.; et al. The Intracellular Transporter AtNRAMP6 Is Involved in Fe Homeostasis in Arabidopsis. *Front. Plant Sci.* **2019**, *10*, 1124. [CrossRef] [PubMed]
141. Fu, S.; Lu, Y.; Zhang, X.; Yang, G.; Chao, D.; Wang, Z.; Shi, M.; Chen, J.; Chao, D.-Y.; Li, R.; et al. The ABC Transporter ABCG36 Is Required for Cadmium Tolerance in Rice. *J. Exp. Bot.* **2019**, *70*, 5909–5918. [CrossRef] [PubMed]
142. Wang, H.; Liu, Y.; Peng, Z.; Li, J.; Huang, W.; Liu, Y.; Wang, X.; Xie, S.; Sun, L.; Han, E.; et al. Ectopic Expression of Poplar ABC Transporter PtoABCG36 Confers Cd Tolerance in *Arabidopsis thaliana*. *Int. J. Mol. Sci.* **2019**, *20*, 3293. [CrossRef] [PubMed]
143. Kim, D.Y.; Bovet, L.; Maeshima, M.; Martinoia, E.; Lee, Y. The ABC Transporter AtPDR8 Is a Cadmium Extrusion Pump Conferring Heavy Metal Resistance. *Plant J.* **2007**, *50*, 207–218. [CrossRef] [PubMed]
144. Brunetti, P.; Zanella, L.; De Paolis, A.; Di Litta, D.; Cecchetti, V.; Falasca, G.; Barbieri, M.; Altamura, M.M.; Costantino, P.; Cardarelli, M. Cadmium-Inducible Expression of the ABC-Type Transporter AtABCC3 Increases Phytochelatin-Mediated Cadmium Tolerance in Arabidopsis. *J. Exp. Bot.* **2015**, *66*, 3815–3829. [CrossRef]
145. Song, W.-Y.; Park, J.; Mendoza Cozatl, D.; Grotemeyer, M.; Shim, D.; Hörtensteiner, S.; Geisler, M.; Weder, B.; Rea, P.; Rentsch, D.; et al. Arsenic Tolerance in Arabidopsis Is Mediated by Two ABCC-Type Phytochelatin Transporters. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 21187–21192. [CrossRef] [PubMed]
146. Neri, A.; Traversari, S.; Andreucci, A.; Francini, A.; Sebastiani, L. The Role of Aquaporin Overexpression in the Modulation of Transcription of Heavy Metal Transporters under Cadmium Treatment in Poplar. *Plants* **2021**, *10*, 54. [CrossRef] [PubMed]
147. Mikkelsen, M.; Pedas, P.; Stokholm, M.; Vincze, E.; Mills, R.; Borg, S.; Møller, A.; Schjoerring, J.; Williams, L.; Baekgaard, L.; et al. Barley HvHMA1 Is a Heavy Metal Pump Involved in Mobilizing Organellar Zn and Cu and Plays a Role in Metal Loading into Grains. *PLoS ONE* **2012**, *7*, e49027. [CrossRef]
148. Eren, E.; Argüello, J. Arabidopsis HMA2, a Divalent Heavy Metal-Transporting PIB-Type ATPase, Is Involved in Cytoplasmic Zn2+ Homeostasis. *Plant Physiol.* **2004**, *136*, 3712–3723. [CrossRef]
149. Sinclair, S.; Senger, T.; Talke, I.; Cobbett, C.; Haydon, M.; Kraemer, U. Systemic Upregulation of MTP2- and HMA2-Mediated Zn Partitioning to the Shoot Supplements Local Zn Deficiency Responses of Arabidopsis. *Plant Cell* **2018**, *30*, 2463–2479. [CrossRef]
150. Morel-Rouhier, M.; Crouzet, J.; Gravot, A.; Auroy, P.; Leonhardt, N.; Vavasseur, A.; Richaud, P. AtHMA3, a P1B-ATPase Allowing Cd/Zn/Co/Pb Vacuolar Storage in Arabidopsis. *Plant Physiol.* **2008**, *149*, 894–904. [CrossRef] [PubMed]
151. Sasaki, A.; Yamaji, N. Overexpression of OsHMA3 Enhances Cd Tolerance and Expression of Zn Transporter Genes in Rice. *J. Exp. Bot.* **2014**, *65*, 6013–6021. [CrossRef] [PubMed]
152. Satoh-Nagasawa, N.; Mori, M.; Nakazawa, N.; Kawamoto, T.; Nagato, Y.; Sakurai, K.; Takahashi, H.; Watanabe, A.; Akagi, H. Mutations in Rice (*Oryza sativa*) Heavy Metal ATPase 2 (OsHMA2) Restrict the Translocation of Zinc and Cadmium. *Plant Cell Physiol.* **2012**, *53*, 213–224. [CrossRef] [PubMed]

153. De caroli, M.; Furini, A.; Dalcorso, G.; Rojas, M.; Di Sansebastiano, G. Endomembrane Reorganization Induced by Heavy Metals. *Plants* **2020**, *9*, 482. [CrossRef]
154. Sanita' di Toppi, L.; Bruno, L.; Bruni, R.; Ligrone, R.; Ferrarese, A.; Paoli, L.; Lingua, G. *Interazioni Piante-Ambiente*; PICCIN: Padova, Italy, 2018; ISBN 978-88-299-2870-5.

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Review

Molecular Biomarkers of Canine Reproductive Functions

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Abstract: The aim of the current study is to review potential molecular biomarker substances selected so far as useful for assessing the quality of dog semen. Proteins, lipids, carbohydrates, and ions can serve as molecular biomarkers of reproductive functions (BRFs) for evaluating male reproductive health and identifying potential risk factors for infertility or reproductive disorders. Evaluation of BRF levels in semen samples or reproductive tissues may provide insights into the underlying causes of infertility, such as impaired sperm function, abnormal sperm–egg interaction, or dysfunction of the male reproductive tract. Molecular biomarker proteins may be divided into two groups: proteins that are well-studied, such as A-kinase anchoring proteins (AKAPs), albumins (ALBs), alkaline phosphatase (ALPL), clusterin (CLU), canine prostate-specific esterase (CPSE), cysteine-rich secretory protein 2 (CRISP2), lactotransferrin (LTF), metalloproteinases (MMPs), and osteopontin (OPN) and proteins that are not well-studied. Non-protein markers include lipid-based substances (fatty acids, phosphatidylcholine), carbohydrates (glycosaminoglycans), and ions (zinc, calcium). Assessing the levels of BRFs in semen samples may provide valuable information for breeding management and reproductive assessments in dogs. This review systematizes current knowledge that could serve as a starting point for developing practical tests with the use of biomarkers of canine reproductive functions and their predictive value for assisted reproductive technique outcomes and semen preservation.

Keywords: canine; semen; biomarkers; proteins; lipids; carbohydrates

1. Introduction

Different reproductive biotechniques nowadays play a significant role in dog breeding. These biotechniques offer numerous benefits and advancements that enhance breeding programs and address various challenges faced by breeders. Fertility prediction and control in dogs using molecular biomarkers of reproductive functions (BRFs) is a key aspect of responsible breeding, health management, and population control. According to the Merriam-Webster Dictionary [1] definition, a biomarker is any biological component that can function as an indicator of a physiological pathway. Biomarkers are by definition objective, quantifiable rates of biological processes [2]. The development of simple molecular tests for advanced epididymal and ejaculated semen quality evaluation would be highly beneficial. Advanced molecular tests can more accurately assess the quality of semen samples (fresh and preserved), including sperm concentration, motility, morphology, and viability, leading to higher success rates in artificial insemination and natural breeding [3]. Additionally, knowing the exact quality of dog semen allows breeders to use it more effectively, ensuring that only high-quality doses are used for breeding, increasing the likelihood of conception. Additionally, modern semen evaluation can prevent costly unsuccessful breeding attempts and reduce the financial burden associated with poor-quality semen. Breeders can command higher prices for stud services if they can demonstrate that their sires produce high-quality ejaculates, backed by modern testing. Better semen quality evaluation improves the success rates of semen cryopreservation, ensuring that stored samples remain viable and effective for future use. High-quality semen is critical

for successful in vitro fertilization (IVF) and subsequent embryo transfer, leading to better outcomes in reproductive technologies. With detailed information on quality of male used for reproduction, breeders can create more precise and effective breeding plans tailored to the specific needs and conditions of their breeding program. Modern semen evaluation with the use of molecular biomarkers contributes to a better understanding of canine reproduction and fertility, leading to further advancements in veterinary reproductive medicine. Implementing new technologies requires expanding knowledge and further research on canine semen storage techniques and procedures for handling semen. This is important for using valuable male reproductors, rare breeds of dogs and endangered canine species.

Thanks to access to increasingly modern research equipment, knowledge related to canine reproduction is developing in parallel to the expansion of areas of knowledge regarding human reproduction. The increasing demands of dog breeders and the changing living environment of these animals accompanying humans force a change in the approach of owners, veterinarians, and scientists to issues related to the reproduction of these animals. Modern technologies facilitate both detailed examination of the health of the reproductive organs and measurement of the quality of semen obtained from the male. Today, breeders expect accurate information on the health of their most valuable males because the success of their breeding and its profitability depends on it. Research has been ongoing for years to identify biomarkers of semen quality that could be easily linked to key parameters for the fertilization process and the specificity of the insemination dose. The most common substances contained in semen for this purpose include proteins, lipids, carbohydrates, and ions. All of these components are tightly interlinked, and their effective cooperation provides cellular signals required for sufficient sperm functional features. Relationships are sought between the level of a given substance in the animal's reproductive organ fluids, sperm, blood, and selected semen quality parameters. Most often, the relationship between sperm motility, the condition of sperm plasma membranes, and DNA integrity is taken into account.

In canines, proteins from epididymal fluid or seminal plasma may coat sperm plasma membranes via phospholipid binding, exerting protective effects [4]. Additionally, these proteins, when bound, may also act as different ion carrier proteins or proteins with enzymatic functions [5,6]. Their role may be to alter ion concentrations in seminal fluid and spermatozoa, acting as decapacitation factors or protease inhibitors, as well as serine proteases affecting the activity of other proteins [6]. Moreover, these proteins might influence various aspects of sperm functions, for example, sperm membrane permeability and sperm motility, and they may modulate fertilization-associated events [7]. Lipids are involved in a wide range of reproductive events, from spermatogenesis and maturation to fertilization. As major compartments of sperm membranes, lipids maintain their integrity, control fluidity, and provide functional membrane microstructural domains and signaling molecules. Usually, different types of lipids do not function separately; rather, they act together in complex signaling pathways [8]. The maintenance of proper sperm function is also dependent on the balance between carbohydrates and ions, working intracellularly and extracellularly. Therefore, when searching for BRFs of dogs, it is necessary to take not only an individual approach but also a holistic, comprehensive approach to the analyzed molecules. Due to the growing interest in dog breeding over the years, as well as the dog being a model of the male reproductive system [9,10], it seems advisable to conduct a comprehensive examination of dog semen, including high-throughput OMICs (proteomics, lipidomics, metabolomics) and molecular biology tools, considering different variables such as age, breed, and pathological and environment conditions.

The aim of the current study is to review potential molecular biomarker substances selected so far as useful for assessing the quality of dog semen.

2. Protein Markers

Protein profiling of a reproductive tissue or a sperm cell, defined as proteomics, provides new possibilities and promising potential in dog reproduction research [11]. The

detection and identification of protein biochemical properties, which influence their functional features and result in reproductive success, is important. Identification of proteins influencing sperm motility or sperm membrane integrity may be used in biomedical strategies for semen assessment, infertility treatment, and semen conservation improvement [12]. Some proteins that are abundant in semen or reproductive tissue were recognized many years ago in canines. Their functions in reproduction are well known. However, certain proteins have been newly discovered in canine semen and tissue and are not yet well-known; however, they possess a potential that needs to be recognized more widely. In this article, the characteristics of both types of proteins are provided, and their potential as markers in dog breeding is explored.

2.1. Well-Studied/Highly Abundant Proteins

Some proteins have been well studied and are recognized as being correlated with canine semen quality parameters.

A-Kinase Anchoring Proteins (AKAPs) share the property of binding to the regulatory subunit of protein kinase A (PKA) [13]. ProAKAP4 (Pro A-Kinase Anchoring Protein 4) is expressed as a precursor of the AKAP4 (A-Kinase An-choring Protein 4) [14]. ProAKAP4 and AKAP4 are involved in a signaling cascade including adenosine monophosphate cyclic, protein kinase A, tyrosine kinase, and phosphatase [15]. These proteins are actively phosphorylated on their tyrosine and serin residues during capacitation, which allows us to consider them as biomarkers of the capacitation status [16,17]. ProAKAP4 is produced during the round spermatid stage and is incorporated into the fibrous sheath of the developing sperm flagellum [18]. ProAKAP4 is cleaved during the condensed spermatid stage, resulting in its conversion into active AKAP4 [13,18]. It has been described as a functional marker of sperm in mammals [19,20]. AKAP4 is associated with good-quality spermatogenesis and is involved in the regulation of sperm motility [14,21]. Le Couazer and Bencharif (2021) [14] showed, for the first time, the localization of proAKAP4 and AKAP4 in the fibrous sheath of the dog's spermatozoid and their expression in the spermatic part of the dog's ejaculate (not in the urethral or prostatic fractions) [22]. Additionally, in humans, the absence or weak expression of proAKAP4 and AKAP4 was associated with poor sperm motility [23]. There are kits (Dog 4MID® kits, 4BioDx, Lille, France) available on the market allowing for objective validation of semen quality by quantifying the proAKAP4 in dog semen. The translation of fundamental discoveries around proAKAP4 and AKAP4 semen quality markers into a practical tool (such as 4MID® kits) is of great importance in breeding and veterinary practice [20]. However, further detailed research is needed on the expression of proAKAP4 and AKAP4 in canine semen and its association with canine reproductive functions.

Albumins (ALBs) are proteins with a molecular mass of about 65 kDa [24]. In addition to being the main component of blood serum, they are also known to be an important component of the seminal plasma of the canine [25] epididymal spermatozoa [26] and epididymal fluid [27]. A semi-quantitative analysis of ALBs in canine epididymal fluid showed its higher abundance in the good sperm motility group [27]. In the reproductive system, they are mainly secreted by the testes, epididymides, and prostate [24]. The addition of heterogenous bovine serum albumin (BSA) to the ejaculated canine spermatozoa preserves sperm viability and function [28] and improves sperm capacitation and acrosome reaction [29]. Studies of homologous ALBs (found in the seminal plasma) have shown that they are implicated in fertilization-associated events, such as sperm capacitation [30] and zona pellucida penetration [31]. The seminal plasma ALB content is highly correlated with the motility parameters of animal spermatozoa [32]. The exact mechanism of action of ALBs is not known. It has been postulated that they may act via two mechanisms. One is via their antioxidant properties and the other is by binding bivalent cations to regulate their number in the reproductive fluids [5,33]. Even a simple mechanism of binding seminal plasma or epididymal fluid ALBs to the plasma membrane of ejaculated or epididymal spermatozoa plays a protective role in sperm motility [27,32,34]. Due to their antioxidant

properties, ALBs can absorb lipid peroxides from reproductive fluids, which contribute to their protective effect on the structures involved in sperm motility and sperm membrane integrity [25]. It has also been demonstrated that ALBs possess a strong ability to bind zinc ions, thus reducing their concentrations in the spermatozoa and enhancing the motility of the cells [32].

Alkaline phosphatase (ALPL) is a dephosphorylation enzyme present at very high concentrations in canine reproductive tracts [35]. In dogs, most of the ALPL is produced in the epididymis, but it is also found in seminal plasma [36,37]. It also plays a role in the transport of sugars and other organic molecules across biological membranes [38]. Its activity has been demonstrated in cytoplasmic droplets, which raises the hypothesis that ALPL catalyzes dephosphorylation and transport of phosphate groups between the epididymal sperm and epididymal fluid. ALPL might be a potential biomarker for prostate and testicular cancer [39]. This enzyme inhibitor can reduce the growth and invasion of cancer cells [39]. Measurement of the enzymatic activity of ALPL in the canine seminal plasma has been used for the diagnosis of incomplete ejaculation or azoospermia in dogs [38], and a reduced concentration of ALPL in the seminal plasma suggests bilateral obstruction of the vas deferens or epididymis [40].

Clusterin (CLU) is a common SP protein in many species [41–43]. CLU is an extracellular chaperone secreted in high amounts by epididymides and testes [44,45]. Its presence in the canine epididymal sperm and fluid has been established [26,27]. CLU might be adsorbed from the epididymal fluid and cover epididymal sperm plasma membranes, positively influencing sperm motility [27]. However, it may also be an indicator of low semen quality because secretion of this protein is enhanced in case of cellular damage or heat shock [42,46,47]. It is overexpressed in several human cancers, such as prostate cancer [48]. Morphologically defective sperm extensively bind CLU to its plasma membrane [49]. This protein has a role in inhibiting cell apoptosis, mediation of female tolerance to seminal antigens, and sperm maturation processes [50]. It also participates in membrane remodeling and DNA reparation [48]. CLU participates in sperm maturation by affecting lipid transport and membrane remodeling [48].

Canine prostate-specific esterase (CPSE) accounts for more than 90% of the proteins secreted by the prostate and about 30% of the canine SP proteins. The enzyme has a molecular weight of 29 kDa and it can be dissociated into two subunits with molecular masses of 12–14 kDa and 15 kDa [51]. The CPSE identified in canine seminal plasma is a multifunctional protein due to its zinc-binding and phosphorylcholine-binding properties [4,6]. The enzyme has been detected in the post-acrosomal region and sperm tail of ejaculated spermatozoa of dogs [51]. It can bind to phosphorylcholine of the sperm plasma membrane and coat ejaculated spermatozoa, which could be implicated in sperm fertilization-related events. It is noteworthy that CPSE is utilized as a marker of glandular secretion and serves as a promising diagnostic tool for non-neoplastic canine prostatic disorders [40]. Interestingly, CPSE is an enzyme homologous to human prostate-specific antigen (PSA), which is a direct indicator of the development of prostate cancer. Both CPSE and PSA are members of the kallikrein family, a group of serine proteases [52]. This family also includes the horse prostate kallikrein (HPK), which, like human PSA, has the ability to bind zinc and mercury ions [52]. Findings by our team confirmed that CPSE can bind zinc ions, a property previously demonstrated only by Isaacs and Coffey (1984) [51]. This underscores the role of zinc ions in the proteases' activity in dog semen.

Members of the cysteine-rich secretory protein (CRISP) family have been found in spermatids [53,54] as well as in the acrosome and tail of ejaculated sperm [55–58]. CRISP expression is high in the testis, and it is present at the junction between germ and Sertoli cells within the seminiferous epithelium [55–58]. CRISP2 specifically regulates calcium flow through ryanodine receptors [59,60], is implicated in cell–cell adhesion, and is capable of binding to steroids [61,62]. A decrease in CRISP2 content in sperm is associated with infertility in humans [53,54] and horses [63]. It was found that the content of this protein in the canine epididymal spermatozoa was low in very young and senile dogs [26].

Lactotransferrin (LTF) is an iron-binding protein that regulates the availability and catalytic activity of iron [64]. In canines, LTF was found in seminal plasma, and further studies showed that it originates in the epididymis [4,27], similar to other animal species e.g., mice, boars, and stallions [65]. It was also found to be a component of canine epididymal sperm [26,34]. LTF binds to phosphorylcholine-containing phospholipids of the sperm plasma membrane in dogs [4]. LTF has antibiotic properties in the reproductive system, conveyed by its ability to sequester iron and prevent the harmful effects of pathogens on spermatozoa [64,65]. Additionally, LTF can bind lipopolysaccharides, heparin, glycosaminoglycans, DNA, and ions like Mn^{3+}, Co^{3+}, Cu^{2+}, and Zn^{2+} [65]. The exact function of LTF in the canine reproductive system is not known, but since it is present in high amounts, it must be important for canine reproductive functions. The addition of LTF to a cryoprotective extender can significantly improve the function of frozen ram sperm [66].

Metalloproteinases (MMPs) are mostly expressed in the epididymis, where they contribute to the modification of the sperm membrane and the regulation of sperm maturation and storage [67]. MMPs aid in the processing of proteins on the sperm surface, which is crucial for sperm function and fertilization [68]. MMP-2, MMP-9, proMMP-2, and proMMP-9 were identified in canine seminal plasma [69]. Saengsoi et al., (2011) [69] suggested that higher activation of proMMP-2, proMMP-9, and MMP-9 may be caused by an abnormal spermatogenesis process, whereas MMP-2 may benefit sperm motility and viability. Studies have shown associations between MMP amounts in seminal plasma and parameters such as sperm concentration, motility, and morphology [70]. Zinc-binding abilities have also been demonstrated for MMPs [70]. The levels of MMPs and their tissue inhibitors are correlated with sperm motility and sperm DNA fragmentation in men [71,72]. Alterations in MMP expression or activity may indicate abnormalities in sperm function [71,72]. MMPs have been proposed as predictive biomarkers for the success of assisted reproductive techniques (ART), such as IVF and intracytoplasmic sperm injection (ICSI) [73]. Assessing MMP amounts in semen or reproductive tissues may help predict fertilization outcomes and the likelihood of successful embryo implantation.

Osteopontin (OPN) is expressed in the male reproductive tract, including the testes and epididymis [74]. It has been implicated in various aspects of sperm function, such as sperm maturation, motility, and capacitation, which are essential for successful fertilization. Lower OPN concentrations have been linked to poorer sperm motility and morphology [75]. OPN levels in seminal plasma vary depending on factors such as breed, age, and reproductive health status [76]. OPN has been identified in the seminal plasma of dogs, indicating its secretion by the male reproductive tract and its presence in the ejaculate [75–77]. Erikson et al., (2007) [78] suggested that OPN localized to the post-acrosomal region on sperm membranes may participate in bovine fertilization by interacting with egg integrins. OPN may also regulate sperm adhesion to the female reproductive tract, their migration towards the egg, and sperm–oocyte interaction [79,80]. OPN has been shown to play a major role in tumorigenesis, tumor invasion, and metastasis, with reported associations with breast, prostate, and ovarian cancer [81].

The prostaglandin-H2 D-isomerase, also known as prostaglandin-D2-synthase (PTGDS) or lipocalin-type prostaglandin-D-synthase, is an enzyme that converts the cyclooxygenase product of prostaglandin H2 (PGH2) to prostaglandin D2 PGD2 [82]. It is an enzyme that binds small non-substrate lipophilic molecules such as retinoids [83]. It also acts as a scavenger of harmful hydrophobic molecules [83]. Moreover, lipocalins bind to specific cell-surface receptors and may form macromolecular complexes [84]. PTGDS also affects plasma membrane permeability, resulting in changes in the input of ions from the outside, which may be correlated to the regulation of sperm motility and access to Ca^{2+} ions by the sperm cell [83,85,86]. PTGDS has been found in the epididymal fluid and seminal plasma of rams [86] and cats [87]. A positive correlation between PTGDS content in human sperm and cell progressive motility has been shown [88]. PTGDS is interconnected with several essential proteins involved in sperm metabolism, such as CRISP2 and lipocalin cytosolic FA-bd domain-containing protein (LCNL1). However, the exact function of PTGDS in sperm metabolism is unknown. A pro-

teomic study revealed that PTGDS is present in greater abundance in dog epididymal fluid surrounding sperm showing good motility [27].

Well-studied molecular biomarker proteins that coat canine sperm and change its surface properties, biochemistry, and metabolism are shown in Figure 1.

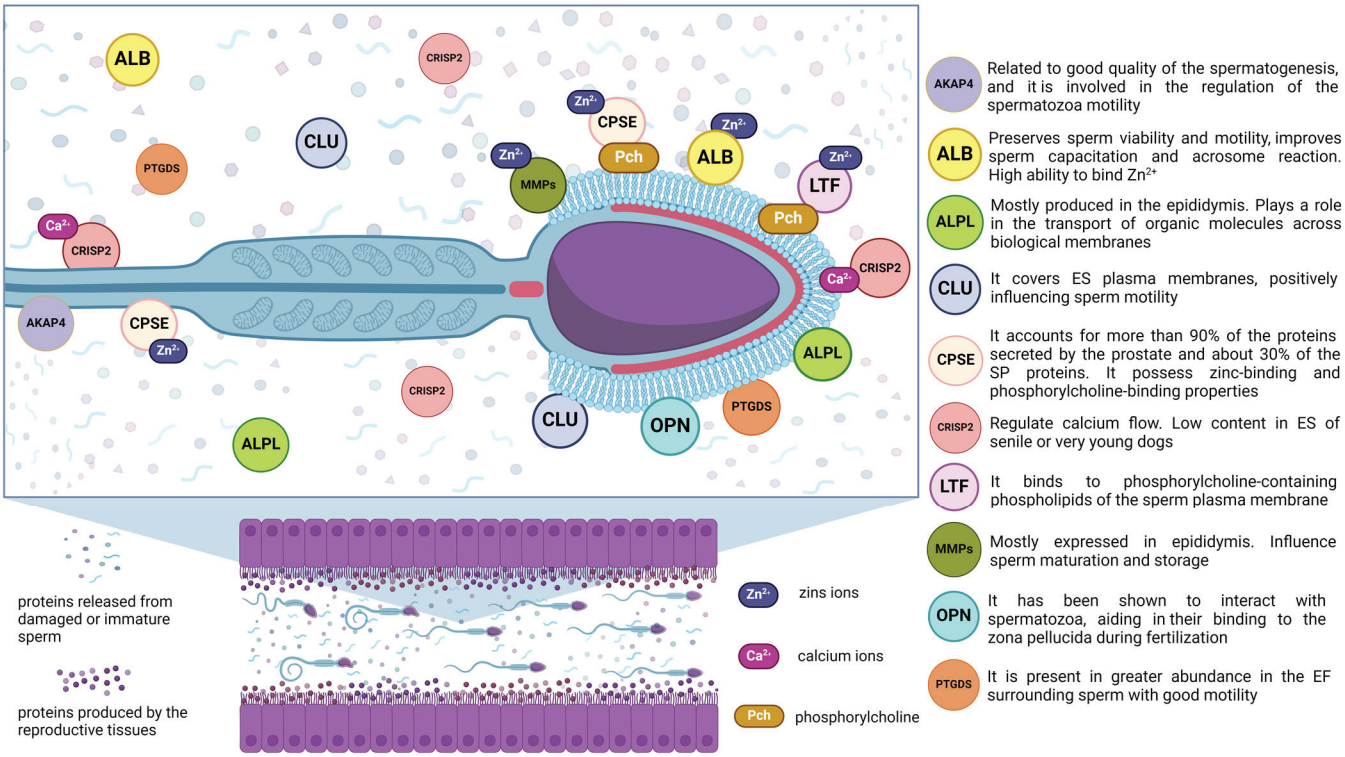


Figure 1. Well-studied/highly abundant molecular biomarker proteins that coat canine sperm and change its surface properties, biochemistry, and metabolism. AKAP4—A-kinase anchoring protein; ALB—albumin; ALPL—alkaline phosphatase; CLU—clusterin; CPSE—canine prostate-specific esterase; CRISP2—cysteine-rich secretory protein 2; LTF—lactotransferrin; MMPs—metalloproteinases; OPN—osteopontin; PTGDS—prostaglandin-H2 D-isomerase; SP—seminal plasma; EF—epididymal fluid; ES—epididymal spermatozoa. Created with BioRender.com.

2.2. Poorly Studied/Newly Recognized/Low-Abundance Proteins

Canine reproductive tissues, fluids, and sperm contain newly recognized proteins that could serve as potential protein markers for semen quality.

Acrosin binding protein (ACBP) is a calcium-binding protein detected in the semen of dogs (Bernese Mountain) [29]. The ACBP is a binding protein for both the precursor and intermediate forms of serine protease acrosin, a protein that is specifically localized in the acrosomes of germ and sperm cells [89]. ACBP improves sperm capacitation, acrosome reaction, and semen quality [29]. ACBP can be used as a molecular marker for pachytene spermatocytes, and for round, elongating, and elongated spermatids. ACBP can be used to monitor either normal spermatogenesis in the testicular tissues, or germ cell development in vitro [90]. ACBP may be a good marker for predicting boar sperm freezing capacity [91].

Actin binding protein (ACTB) is a structural protein that builds the cytoskeleton and is present in the flagellar and acrosomal membranes of spermatozoa. It is also responsible for cell volume changes [92,93]. Based on its location, its role was proposed to be in sperm capacitation and motility [92–94]. ACTB was previously found in the canine epididymal spermatozoa, and its expression was correlated with the dog's age [26]. Additionally, it was found in canine epididymal fluid, and it is highly abundant in semen with good sperm motility [27]. The exact mechanism of ACTB's influence on sperm motility needs to be evaluated in the future.

Abnormal spindle-like microcephaly-associated protein homolog (ASPM) is expressed in a variety of embryonic and adult tissues and is upregulated in cancer [95]. It has been found in canine epididymal spermatozoa [26]. ASPM misfunction affects chromosome segregation, which leads to reduced ability of fetal stem cells to produce neurons [95]. ASPM plays a role in sperm flagellar function [96].

Caspase recruitment domain containing protein 6 (CARD6) is found in the canine epididymal fluid [27]. Its function is to regulate cell proliferation, immune response, and cell apoptosis [97]. CARD6 may be involved in microtubular transport mechanisms, and proteins that interact with it may be targeted to the microtubule-organizing center, resulting in either their inactivation or translocation [98].

Hyaluronoglucosaminidase (CEMIP), also named KIAA1199, is a unique protein found in the epididymal spermatozoa of young dogs [26]. It is expressed in the human testis, and its biological role in cancer biology has been studied in humans [99]. It has not been described in other species, including dogs. This protein causes significant changes in cell morphology and actin cytoskeletal dynamics. It exerts its effects through regulation of the canonical Wnt and P38/MAPK signaling pathways [100] and the expression of CEMIP may be regulated depending on cell mortality rather than cell age [99].

Epididymal sperm-binding protein 1 (ELSPBP1) was first described in humans and dogs as a sperm-binding protein of epididymal origin that binds to the spermatozoa during their transit through the epididymis [101,102]. Since then, orthologs have been identified in horses [103], pigs [104,105], and bovines [102]. More recently, ELSPBP1 was shown to negatively correlate with bull fertility [106] and was associated with the dead sperm population [107]. It has been found in epididymal spermatozoa, and its presence is age-dependent [26].

The function of a family with sequence similarity 135 member A (FAM 135A) is the regulation of cellular proliferation, cell differentiation, development, and growth control [108]. Expression of the protein was observed in the testis, epididymis, prostate, and seminal vesicles in humans [109]. This protein has also been found to be a unique protein in the epididymal spermatozoa of older dogs [26].

N-acetylgalactosaminyltransferase-like proteins (GALNTL) were found in male reproductive organs in rodents and cattle [110–113]. In canines, it has been shown that polypeptide N-acetylgalactosaminyltransferase 6 (GALNT6) is important for epididymal sperm motility [27] and participates in mucin-type-O-glycan biosynthesis [26]. We can point out more reports about GALNTL5. In mice, it is localized in the head–tail coupling apparatus of cauda epididymal spermatozoa [112], and its expression is positively correlated with sperm motility [114].

Lipocalin cytosolic FA-bd domain-containing protein (LCNL1) is a member of the lipocalin family. These proteins transport or store small molecules, such as vitamins, hormones, and secondary metabolites [115]. LCN family proteins are important for sperm maturation, and they are expressed in different regions of the epididymis [116,117]. LCNL1, present in canine epididymal fluid, is important for sperm motility, although the exact mechanism is unknown [27].

Cystatin domain-containing protein (LOC607874) may serve as potential biomarker of canine aging [26]. A record for *Canis lupus familiaris* LOC607874 mRNA is found in the National Center for Biotechnology Information (NCBI). The cystatin-related epididymal-specific (*CRES*) gene is almost entirely restricted to the epididymis and much less is expressed in the testis, mainly in spermatids [118]. A *CRES* gene, which shares similarities with well-known protein inhibitors called cystatins, was found in the mouse epididymis [119]. This protein is unique to the epididymal spermatozoa of older dogs [26] and its presence in the epididymal fluid has been associated with poor sperm motility [27].

Niemann-Pick type C2 protein (NPC2), intracellular cholesterol transporter 2, also called epididymal secretory protein E1, is found in the canine epididymal spermatozoa [26]. In canines, epididymal secretory protein E1 (CE1/NPC2) is encoded by genes similar to those in humans [120]. The CE1 protein is a highly abundant, conserved, secretory

protein [121] and its mRNA is found in large amounts in the epididymal duct epithelium, while the protein is found in the duct lumen [122]. Recently, *CE1* has been identified as a gene that is important in the etiology of Niemann-Pick type C disease [123]. This protein is involved in cholesterol efflux from lysosomes [123]. In human ejaculated spermatozoa, a human sperm antigen (HE2), human homolog of CE1, was found in the acrosome and equatorial region of the cells [124]. NPC2 epididymal sperm-membrane-coating ability has been suggested [34] because similar results were shown by Araujo et al. [29,125], who found NPC2 to be an ejaculated sperm component.

Olfactomedin 4 (OLFM4) is an olfactomedin domain-containing glycoprotein [126]. OLFM-1, -2, -3, -4 are known to regulate cellular growth, differentiation, and pathological processes [127]. The absence of *OLFM4* gene expression is associated with the progression of human prostate cancer [128]. OLFM4 is found in human spermatozoa [129]. The OLFM-4 precursor is found in human epididymosomes [130]. The role of OLFM4 in sperm physiology is unknown. This protein was found to be expressed in the epididymal fluid of dogs and was correlated with good sperm motility [27].

Pleckstrin homolog, MyTH4, and FERM domain-containing H1 (PLEKHH1) is a protein constituent of the cytoskeleton with a role in intracellular signaling [131]. It binds phosphatidylinositol lipids within biological membranes and plays a role in recruiting proteins to membranes. PLEKHH1 expression is found in human testis, epididymis, seminal vesicles, and prostate [132]. This protein has also been found in the canine epididymal spermatozoa [26]; however, its function has not yet been established.

The tubulin (TUBB) family proteins are associated with structural cell organization and flagella movement [29,133–135]. These proteins have also been identified in the sperm membrane of Morada Nova rams [136] and mice [137]. In humans, tubulins are associated with low sperm motility [138,139]. A study by Araujo et al., (2022) [29] indicated a high abundance of tubulin alpha-3E chain in Maremmano-Abruzzese Sheepdogs and tubulin alpha-3 chain in Bernese Mountain Dogs [29]. Tubulins in seminal plasma are linked to damaged sperm in the ejaculate, possibly due to sample handling [140].

Poorly studied/newly recognized/low-abundance molecular biomarker proteins in canines, which represent less studied proteins that change the biochemistry and metabolism of sperm, are shown in Figure 2.

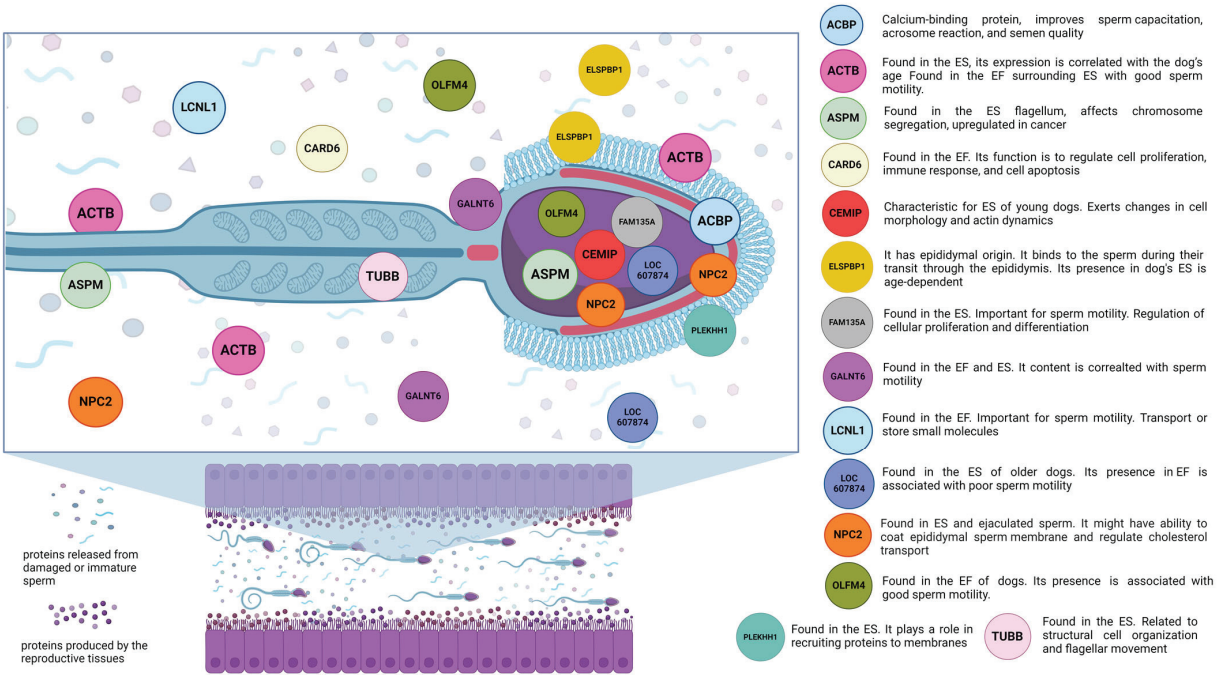


Figure 2. Poorly studied/newly recognized/low-abundance molecular biomarker proteins in canines that change sperm biochemistry and metabolism. ACBP—Acrosin binding protein; ACTB—Actin binding protein;

ASPM—Abnormal spindle-like microcephaly-associated protein homolog; CARD6—Caspase recruitment domain containing protein 6; CEMIP—Hyaluronoglucosaminidase; ELSPBP1—Epididymal sperm-binding protein 1; FAM135—A family with sequence similarity 135 member A; GALNT6—Polypeptide N-acetylgalactosaminyltransferase 6; LCNL1—Lipocalin cytosolic FA-bd domain-containing protein; LOC607874—Cystatin domain-containing protein; NPC2—Niemann-Pick type C2 protein; OLFM4—Olfactomedin 4; PLEKHH1—Pleckstrin homolog, MyTH4, and FERM domain-containing H1; TUBB—Tubulin; EF—epididymal fluid; ES—epididymal spermatozoa. Created with BioRender.com.

2.3. Antioxidant Enzymes

Glutathione peroxidase (GPX) is a well-known, highly abundant protein in the canine epididymis [141]. GPX5 is highly expressed in the epididymis and is then secreted into the lumen. Its role is to protect sperm from lipid peroxidation [141]. GPX is found in, among others, bull, boar, and dog seminal plasma [142–144] and in boar epididymal spermatozoa [144]. This protein also participates in important metabolic pathways in feline seminal plasma [87]. The above-mentioned protein works in a SOD/CAT system. Antioxidant enzyme activities of mammalian spermatozoa comprise those provided by superoxide dismutase (SOD) and GPX. SOD has been shown to play a key role in the intracellular scavenging of superoxide anions [145]. The superoxide anion generated by the inner mitochondrial membrane is dis-mutated to hydrogen peroxide by the Mn-SOD present in the mitochondrial matrix and by the Cu/Zn-SOD present in the cytosol. In turn, hydrogen peroxide is eliminated by either GPX or catalase to avoid cell damage. Because it has been shown that sperm from most mammalian species lack catalase activity, sperm mostly rely on GPX activity to eliminate intracellularly generated hydrogen peroxide [146]. It has been reported that both SOD and GPX play a central role in protecting mammalian sperm against oxygen-radical-induced damage leading to motility loss [146]. SOD could be a valid marker for resistance to cryodamage because the time observed for complete loss of motility due to lipoperoxidative damage to sperm under experimental conditions is strongly correlated with the SOD activity of the sperm sample [147]. A study of dogs confirmed the presence of endogenous antioxidants in the seminal plasma of pre-spermatic, spermatic, and post-spermatic fractions, with SOD representing the major enzymatic antioxidant in all dog ejaculate fractions, whereas GPX activity was present in the sperm-rich and post-spermatic fractions, while CAT activity was deficient [148]. Another study [149], however, demonstrated CAT activity in dog ejaculates and showed that the addition of SOD and CAT in the dilution extender of canine semen improved sperm quality. The addition of a GPX and SOD combination to dog semen can protect sperm viability and DNA integrity [150]. The addition of SOD, CAT, and GPX in the extender allows the preservation of semen quality in cold storage for both fertile and hypofertile dogs [151].

Antioxidant enzymes functioning as molecular biomarkers that participate in sperm biochemistry and metabolism in canines are shown in Figure 3.

Table 1 contains a summary of the most promising protein molecular biomarkers of canine reproductive functions.

Table 1. Summary of the most promising protein molecular biomarkers of canine reproductive functions described in the review. T—testis; EP — epididymis; EF—epididymal fluid; ES—epididymal spermatozoa; S—ejaculated spermatozoa; SP—seminal plasma; P—prostate; BS—blood serum, CD—cytoplasmic droplets.

Biomarker	Origin of Biomarker	Influence on Reproductive Functions	Implementation in Clinical/Breeding Practice	References
Well-studied proteins/highly abundant proteins				
AKAP4/ProAKAP4 (A-kinase anchoring protein)	T, S	- regulation of spermatogenesis - regulation of sperm capacitation - regulation of sperm motility	- biomarkers of capacitation status - associated with good-quality of spermatogenesis - involved in the regulation of spermatozoa motility - translation of semen quality markers into a practical tool (such as 4MID® kits, 4BioDx, Lille, France)	[14,16,17,21]

Table 1. *Cont.*

Biomarker	Origin of Biomarker	Influence on Reproductive Functions	Implementation in Clinical/Breeding Practice	References
ALBs (Albumins)	BS, T, EF, ES, SP, P	- preserves sperm viability - improves sperm capacitation and acrosome reaction - involved in zona pellucida penetration - regulates sperm motility - antioxidant properties	- The addition of heterogenous bovine serum albumin to the ejaculated canine spermatozoa preserves sperm viability and function -improves sperm capacitation and acrosome reaction	[28,29]
ALPL (Alkaline phosphatase)	CD, BS, EP, SP	- dephosphorylation enzyme - transport organic molecules across biological membranes	- potential biomarker for prostate and testicular cancer - ALPL inhibitors can reduce the growth and invasion of cancer cells—measurement of ALPL enzymatic activity in the canine SP has been used in the diagnosis of incomplete ejaculation or azoospermia in dogs - a lower concentration of ALPL in the SP suggests bilateral obstruction of the vas deferens or epididymis	[37,39,40]
CLU (Clusterin)	T, ES, EF, SP, P	- positively influences sperm motility - inhibiting cell apoptosis - mediation of female tolerance to seminal antigens - sperm maturation processes	- an indicator of low semen quality because secretion of this protein is enhanced in case of cellular damage or heat shock	[42,46,47]
CPSE (Canine prostate-specific esterase)	S, SP, P	- multifunctional protein due to its zinc-binding and phosphorylcholine-binding properties - coat ejaculated spermatozoa, which could be implicated in sperm fertilization-related events	- marker of glandular secretion and serves as a promising diagnostic tool for non-neoplastic canine prostatic disorders	[4,6,40]
CRISP2 (Cysteine-rich secretory protein)	T, ES, S	- specifically regulates calcium flow through ryanodine receptors - is implicated in cell–cell adhesion and is capable of steroid binding	- decrease in CRISP2 content in sperm is associated with infertility in humans and horses - its content is low in the ES of very young and senile dogs	[26,53,54,59–63]
LTF (Lactotransferrin)	EP, SP, ES	- regulates the availability and catalytic activity of iron - has antibiotic properties in the reproductive system - able to bind lipopolysaccharides, heparin, glycosaminoglycans, and DNA, and ions like Mn^{3+}, Co^{3+}, Cu^{2+}, and Zn^{2+}	- its addition to a cryoprotective extender can significantly improve the function of frozen ram sperm	[64–66]
MMPs (Metalloproteinases)	E, SP, S	- contribute to the modification of the sperm membrane and the regulation of sperm maturation and storage - higher activation of proMMP-2, proMMP-9, and MMP-9 may be caused by an abnormal spermatogenesis process, whereas MMP-2 may benefit sperm motility and viability	- associations between MMP amounts in SP and parameters such as sperm concentration, motility, and morphology - the levels of MMPs and their tissue inhibitors are correlated with sperm motility and sperm DNA fragmentation in men - alterations in MMP expression or activity may indicate abnormalities in sperm function - MMPs have been proposed as predictive biomarkers for the success of assisted reproductive techniques (ART) such as in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI)	[67,70–73]
OPN (Osteopontin)	T, EP	- implicated in various aspects of sperm function such as sperm maturation, motility, and capacitation, which are essential for successful fertilization - OPN localized to the post-acrosomal region on sperm membranes may participate in bovine fertilization by interacting with egg integrins	- lower OPN concentrations are linked to poorer sperm motility and morphology - it plays a major role in tumorigenesis, tumor invasion, and metastasis in prostate cancer	[75,76,78]
PTGDS (Prostaglandin-H2 D-isomerase)	EF, SP	- an enzyme that binds small non-substrate lipophilic molecules such as retinoids	- has a positive effect on human sperm's progressive motility - PTGDS is present in greater abundance in the dog EF surrounding sperm showing good motility	[27,83,88]
		Poorly studied/newly recognized/low-abundance proteins		
ACBP (Acrosin binding protein)	S	- improves sperm capacitation, acrosome reaction, and semen quality	- molecular marker for monitoring spermatogenesis in testicular tissues or germ cell development in vitro - marker for predicting boar sperm freezing capacity	[29,90,91]
ACTB (Actin binding protein)	ES, EF	- sperm capacitation and motility	- potential marker of canine ES aging - found in canine EF (highly abundant in EF of dogs with good sperm motility)	[26,27,92–94]
ELSPBP1 (Epididymal sperm-binding protein 1)	EP, ES	- binds to spermatozoa during their transit through the epididymis	- negatively correlated with bull fertility - is associated with the dead sperm population - is found in the epididymal spermatozoa, and its presence is age-dependent	[26,106,107]
LOC607874 (Cystatin domain-containing protein)	T, EP, ES, EF	- sperm motility	- potential biomarker of aging - its presence in EF has been associated with poor sperm motility	[26,27,118]
NPC2 (Niemann-Pick type C2 protein)	EP, S	- intracellular cholesterol transporter - gene is important in the etiology of Niemann-Pick type C disease - involved in cholesterol efflux from lysosomes	- higher content of NPC2 in poor-freezability semen leads to higher efflux of cholesterol - potential freezability marker of semen	[26,123,152]
OLFM4 (Olfactomedin 4)	S, EF, Epididymosomes	- regulates cellular growth, differentiation, and pathological processes	- the absence of its gene expression is associated with the progression of human prostate cancer - found in the EF of dogs, and is correlated with good sperm motility	[27,28,128]
TUBB (Tubulin)	SP, S	- associated with structural cell organization and flagella movement	- presence in SP may be correlated to sample handling	[29]
		Antioxidant enzymes		
GPX5 (Glutathione peroxidase 5)	EP, SP, ES	- protects sperm from lipid peroxidation - works in the SOD/CAT system	- marker for resistance to cryodamage - the combination of GPX5 and SOD as an additive in the dilution extender of canine semen protects sperm viability and DNA integrity - addition of SOD, CAT, and GPX to the extender allows the preservation of semen quality in cold storage for both fertile and hypofertile dogs	[141,147,150, 151]
SOD (Superoxide dismutase)	EP, SP, ES	- protects sperm from lipid peroxidation - works in the SOD/CAT system	as above	as above
CAT (Catalase)	SP	- enzymatic decomposition of hydrogen peroxide into water and oxygen - works in the SOD/CAT system	- addition of SOD and CAT to the dilution extender of canine semen improves sperm quality - addition of CAT to the extender allows the preservation of semen quality in cold storage for both fertile and hypofertile dogs	[119,149]

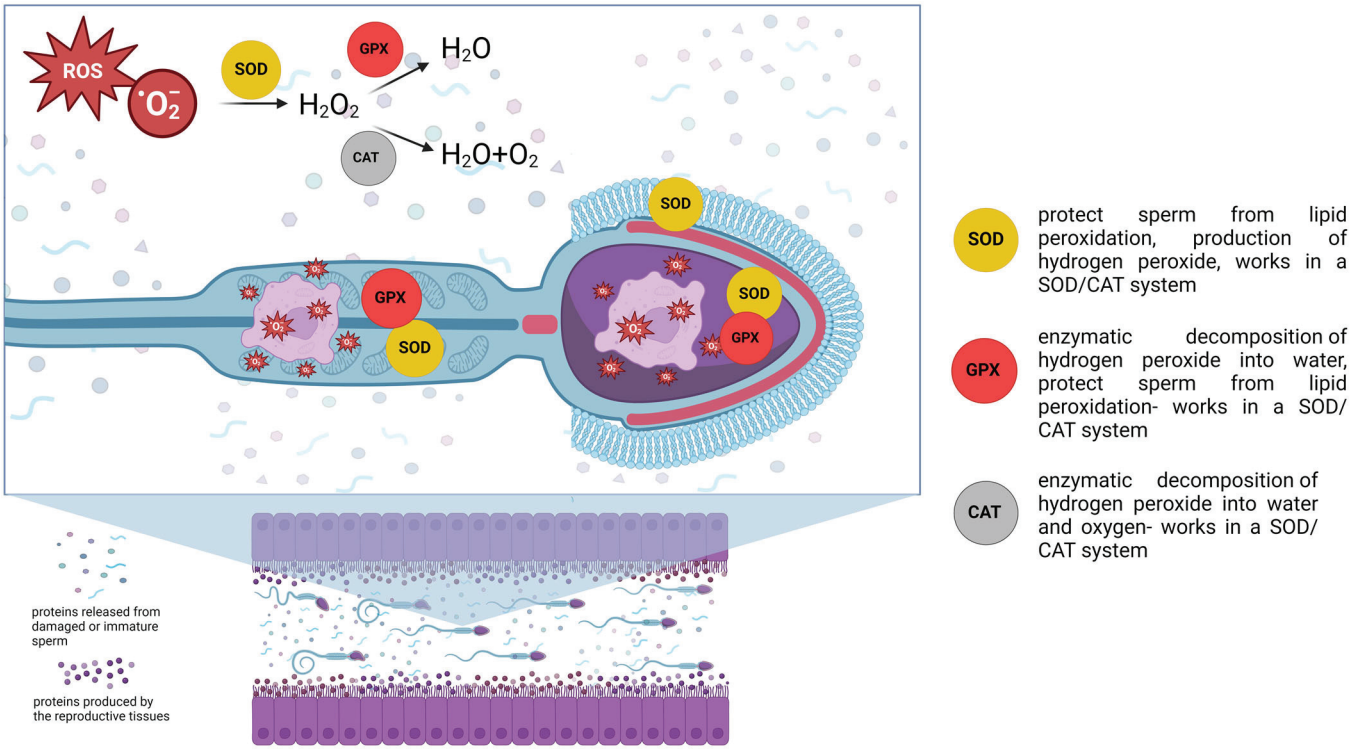


Figure 3. Antioxidant enzymes that function as molecular biomarkers and participate in sperm biochemistry and metabolism in canines. SOD—superoxide dismutase; GPX—glutathione peroxidase, CAT—catalase, ROS—reactive oxygen species.

3. Non-Protein Markers

3.1. Lipids

Lipids are essential building compartments of cells, including most of all their plasma membranes, the endoplasmic reticulum, the nuclear membranes, endosomes, and lysosomes [153]. Lipids form highly specialized sperm plasma membranes. Phospholipids, fatty acids, glycolipids, and neutral lipids (such as cholesterol and diacylglycerols) are among the lipids found in plasma membranes [8,154]. Changes in lipid composition within membranes generally correspond to their physiological needs [8]. The sperm plasma membrane's lipid profile varies during sperm transfer along the epididymal tract [155]. This facilitates the preservation of cellular stability and integrity, which are crucial for preventing sperm damage, as well as the progressive acquisition of functional competence by sperm, such as motility and the ability to fertilize [156]. Lipid reorganization of the sperm plasma membrane during sperm maturation occurs due to the region-specific environment surrounding sperm. Therefore, remodeling of the sperm plasma membrane's lipid profile is influenced by substances present in the epididymal fluid [157–159]. In relation to the above findings, the arrangement of fatty acids in the sperm membrane appears to be a significant factor that could potentially elucidate the quality of sperm, including their cryotolerance [160–162]. Only a few studies have been conducted on canine semen's lipid composition and its impact on semen quality.

3.1.1. Fatty Acids

Angrimani et al., (2017) [159] identified differences between fatty acid profiles of epididymal spermatozoa and epididymal fluid derived from different regions of canine epididymis (caput, corpus, cauda). The authors study also showed that the fatty acids content correlate with sperm motility parameters and sperm plasma membrane integrity. Díaz et al., (2014) [163] identified canine seminal plasma fatty acids. Lucio et al., (2017) [164]

conducted an analysis of the lipid profile of dog-ejaculated sperm, which the authors used to identify lipid markers of sperm motility.

Results obtained by Angrimani et al., (2017) [159] indicate a progressive increase in the dog sperm motility and plasmatic membrane integrity from caput to cauda epididymis. The same pattern was also observed for the concentrations of fatty acids (saturated, monounsaturated, and polyunsaturated).

Saturated Fatty Acids

According to Angrimani et al., (2017) [159], epididymal sperm had a higher concentration of saturated fatty acids (SFAs) compared to the epididymal fluid. Among fatty acids in canine seminal plasma, SFAs were observed to be the most abundant [163]. Caprylic and stearic fatty acids are the most abundant SFAs found in cauda epididymal sperm, as identified in a study by Angrimani et al., (2017) [159], while palmitic and stearic fatty acids are the most abundant in canine seminal plasma, according to Díaz et al., (2014) [163]. Caprylic fatty acid plays a dual role in the sperm membrane by exhibiting both bactericidal properties and protective functions, as per a study by [165]. Additionally, caprylic fatty acid was found to be effective in maintaining the viability of rooster sperm during in vitro storage when used as an extender [166]. It has been shown that stearic fatty acid regulates sperm function and provides the energy necessary for sperm motility and metabolism [167]. However, stearic fatty acid was also identified in human spermatozoa and seminal plasma and was negatively correlated with sperm motility [168]. High levels of stearic fatty acid were observed in lipid profiles of asthenozoospermic spermatozoa [169–171]. In humans, a positive correlation was observed between palmitic fatty acid and both sperm concentration and total sperm count, suggesting its potential significance in sperm production [172].

Monounsaturated Fatty Acids

For monounsaturated fatty acids (MUFAs), pentadecenoic fatty acid concentration was higher in the dog cauda epididymal sperm and fluid compared to epididymal caput and corpus, and the concentration increased from caput to cauda epididymal regions [159]. Pentadecenoic fatty acid has also been identified in canine seminal plasma, albeit in small amounts [163]; however, knowledge about its influence on epididymal sperm maturation and further semen quality is lacking. Oleic fatty acid is a characteristic component of the seminal plasma in both canines [163] and humans [168,173]. This fatty acid, along with stearic fatty acid, is associated with sperm metabolism and has a positive effect on both the motility and viability of boar spermatozoa [174].

Polyunsaturated Fatty Acids

Among polyunsaturated fatty acids (PUFAs), docosahexaenoic acid (DHA) is present in the dog cauda epididymal spermatozoa [159]. Cauda epididymal fluid also contains higher DHA levels than the remaining regions of the epididymis, although it was lower than in the dog cauda epididymal sperm [159]. The contents of DHA in mammalian sperm differ among species and range from low percentages in rats and rabbits (1%) to almost half of the total content of PUFAs in human sperm [160,175–177]. Waterhouse et al., (2006) [178] suggested that, when freeze-thawed, boar sperm with higher levels of DHA in their membranes were more cryotolerant, which was attributed to the higher membrane fluidity. Docosahexaenoic acid adds unique fluidity to the sperm plasma membrane [179], thus affecting its integrity, which is essential for sperm motility and acrosome reaction [179,180]. Moreover, DHA positively influences testicular testosterone secretion and enhances sperm antioxidant capacity [181]. A positive correlation between DHA in the sperm plasma membrane and sperm motility in boars was demonstrated [182], and a low content of this fatty acid was observed in human asthenospermic semen [160]. Based on the above findings, Angrimani et al., (2017) [159] proposed that increasing DHA concentration in the epididymal sperm and fluid is important for the final steps of epididymal maturation in dogs due to its direct involvement in fertilization.

It should be noted that the highest concentration of individual fatty acids occurred both in the sperm and fluids derived from the cauda epididymis—the place where sperm achieve the highest maturity level. Changes in the structure of the sperm plasma membrane's lipids occur during sperm transfer through the epididymis. These changes are associated with the binding of fatty acids as well as other components, such as proteins, from the epididymal fluid [44,155]. This suggests that during epididymal maturation, the sperm takes up essential lipids, which may be vital for acquiring functional features required for its mission in the female reproductive tract. The highest concentration of fatty acids in the dog cauda epididymal sperm may contribute to the enhanced stability of the cell membrane [159]. Sperm cells are highly susceptible to oxidative stress due to the concentration of PUFAs found within the plasma membranes [183]. Unsaturated fatty acids add elasticity and fluidity to the plasma membrane, which are crucial for the proper development of sperm functional features such as motility [184]. However, their presence stimulates free radical activity and, thus, lipid peroxidation, which are the main causes of membrane disintegration [185]. A higher concentration of SFAs may reduce sperm damage during storage at low temperatures [176].

3.1.2. Phospholipids

Among the group of phospholipids, very early reports have shown that the dog spermatozoa contain large amounts of phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, ethanolamine plasmalogen, and choline plasmalogen [186]. Phosphatidylcholine and phosphatidylethanolamine were identified in the motile spermatozoa and less frequently in the spermatozoa of asthenozoospermic individuals [164]. The reduction in phosphatidylcholine and phosphatidylethanolamine content in the sperm of asthenozoospermic individuals stems from the extensive degradation and impairment of PUFAs caused by oxidation induced by free radicals [170,187]. Canine sperm motility is indicated by the lipid markers phosphatidylcholine and phosphatidylethanolamine, according to the above-mentioned reports. Interestingly, phosphatidylethanolamine in the sperm membranes has the capacity to bind to lipocalin 2 in the female reproductive tract. In turn, lipocalin 2 can stimulate the rearrangement of lipid rafts and the efflux of cholesterol [188].

Plasmalogens

Among the group of lipids, plasmalogens are of great interest based on their protective functions against oxidative damage in sperm. Shan et al., (2021) [8], in their thorough review, explained the mechanism of plasmalogen antioxidant properties. Moreover, in canines, plasmenyl phosphatidylcholine (40:5) and plasmanyl phosphatidylcholine (40:4) were reported as lipid sperm motility markers [164] due to their increased content in motile sperm and cells with the lowest percentage of acrosomal membrane damage. These findings might provide evidence that the presence of plasmalogens in the motile sperm may be part of a protective mechanism protecting the sperm plasma membrane against lipid peroxidation and preventing damage induced by reactive oxygen species (ROS) [164].

Sphingomyelins

Sphingomyelins are part of biological membranes [189], including the sperm plasma membrane [190,191], and function as bioactive lipids [189]. Sphingomyelin has an affinity for cholesterol [192]. Interestingly, this combination may generate low-density lipid rafts in the plasma membrane, thereby modifying the lateral arrangement of proteins [193]. An early study by Darin-Bennet et al., (1974) [186] showed that dog spermatozoa contain large amounts of sphingomyelin; however, the latest reports on this matter do not indicate the significance of sphingomyelin in dog's reproductive functions.

Phosphorylcholine

The coating on the sperm plasma membrane is formed by phosphorylcholine moieties of phospholipids [194]. Protein complexes with the involvement of phosphorylcholine

moieties also form the coating of the dog sperm plasma membrane during ejaculation and protect the spermatozoa against the adverse conditions in the female reproductive tract, as well as against cold shock during semen freezing [4,195]. These findings suggested that the attachment of phospholipids containing phosphorylcholine is an important mechanism of protein coating on the dog sperm plasma membrane [4,195]. Mogielnicka-Brzozowska et al., (2017) [4] have shown that the phosphorylcholine-binding proteins (PchBPs) in the canine seminal plasma are high molecular weight aggregates. Mass spectrometric analysis of canine seminal plasma PchBPs identified five different types of proteins: LTF, ALB, PTGDS, CPSE, and lipocalin-like 1 protein isoform.

3.1.3. Cholesterol

Cholesterol is an important structural component maintaining the stability and fluidity of the cell membrane [196]. Cellular efflux of cholesterol is required to maintain homeostasis and is an essential step during the capacitation of spermatozoa [197]. Cholesterol is the precursor of steroid hormones, such as testosterone, produced by the Leydig cells, and plays an important role in normal spermatogenesis [198]. Cholesterol concentration in spermatozoa was shown to be associated with cryotolerance, motility, and morphology of sperm cells. Therefore, cholesterol in sperm cells provides protection against sperm damage induced by freeze/thaw processes [199]. Canine sperm membranes are not highly sensitive to cold damage due to their relatively high cholesterol:phospholipid ratio and the PUFA content of the membrane [200]. Schäfer-Somi and Palme (2016) [25] showed that in dogs, higher concentrations of cholesterol are found in the seminal plasma of ejaculates with good freezability than in the seminal plasma of ejaculates with bad freezability. The removal of seminal plasma from good-quality ejaculates decreased post-thaw motility, increased the percentage of morphologically abnormal sperm, and increased post-thaw DNA damage [201]. The movement of cholesterol from the sperm plasma membrane involves specific molecules with a high affinity for cholesterol, for example, NPC2, which is closely associated with the lipid structure of the cell membrane [202,203]. It was found that the NPC2 protein contains a functional cholesterol-binding site through which it exerts its high affinity for cholesterol and binds to the sperm membrane [204]. In humans, NPC2 is associated with cholesterol efflux from the sperm membrane during epididymal sperm maturation [205]. Its role in the removal of cholesterol from the lipid rafts in bull spermatozoa was suggested [206]. In mice, NPC2 is associated with the maintenance of cholesterol content in the sperm membrane [203]. These findings are consistent with results showing that higher content of NPC2 in poor-freezability semen leads to higher efflux of cholesterol [152]. This phenomenon affects the fluidity of the sperm plasma membrane [207] and increases the influx of calcium ions, which leads to impaired membrane integrity [207,208]. Based on NPC2's cholesterol-binding ability, a possible protective mechanism of NPC2 in reducing boar sperm membrane lipid loss during cryopreservation was proposed [152]. NPC2 was specified as a potential freezability marker of semen [152]. These investigations show that cholesterol content in semen might be a potential marker of canine semen resistance to cold shock [25]. Moreover, the positive correlation between seminal plasma cholesterol and semen quality in humans suggests that the cholesterol profile of seminal plasma can be used as an indicator of semen quality [209].

Lipids exhibit significant complexity and dynamism, undergoing continuous alterations in response to physiological and environmental factors [210]. Lipidomics is a field of research on the complete lipid profile (lipidome) within a cell, tissue, or organism. It also provides a quantitative analysis of the identified lipids [211]. Lipidomics can be used to study signal processing, lipid metabolism, molecular mechanisms, and biomarker discovery [210]. Despite the growing popularity and development of lipidomic tools, a lack of knowledge remains. The lipid composition of canine semen has also not been fully explored. This study would shed new light on understanding the impact of various lipids on reproductive processes in dogs. Combined with other molecular biology methods, lipidomics can be used to highlight the lipid markers of male fertility [183]. The identi-

fied biomarkers can be subjected to further research and used to determine, in detail, the molecular basis of male fertility in the future.

3.2. Carbohydrates

The effect of carbohydrates on the reproductive functions of dogs has not been described in detail. The available literature is dominated primarily by reports on the addition of sugars (glucose, fructose) to semen extenders [212] or basal medium [213]. Sugars have long been incorporated into semen diluents as exogenous energy substrates, osmotic constituents, and cryoprotective agents [214]. The carbohydrate composition of dog semen (sperm and seminal fluids) and the impact of individual sugar components on semen quality need to be investigated to fill the existing lack of current knowledge. Research based on modern, accurate measurement methods may allow the identification of new sugar markers of dogs' reproductive functions.

Heparin

Heparin belongs to the class of glycosaminoglycans [215]. Early studies showed that glycosaminoglycans are involved in events preceding the acrosome reaction [216,217] and stimulate acrosome reaction in sperm in vitro [218–221]. Interestingly, it has been speculated that sperm motility in the bitch reproductive tract is maintained by the glycosaminoglycans present in the uterine fluid, and the glycosaminoglycans in the oviductal and uterine fluids of estrous bitches are associated with in vivo capacitation of the dog sperm [222]. The role of heparin in reproductive processes can be considered in three ways: (a) heparin as an addition to the sperm capacitation medium; (b) heparin as a ligand bound to proteins present in the fluids of the reproductive system; and (c) heparin-binding proteins (HBPs) as a semen additive.

Heparin as an additive in the in vitro medium is effective in stimulating metabolic enzymes, prolonging sperm motility, and successfully inducing hyperactivation even in the sperm of asthenozoospermic dogs [223]. Additionally, Risopatrón et al., (2005) [224] showed the positive effect of the addition of heparin to the capacitating medium on the acrosome reaction of the canine sperm. The presence of heparin in an in vitro medium induces acceleration in the fertilizing ability of the bull epididymal sperm [225].

The ability of sperm to bind heparin is attributed to the presence of seminal plasma proteins. These proteins attach to the sperm surface (especially to lipids containing the phosphoryl-choline group) after ejaculation, which increases the number of heparin-binding sites on the cell [226–228]. The sperm is then allowed to respond to heparin [227]. Heparin binding to bovine seminal plasma proteins induces the efflux of cholesterol and phospholipids from sperm, leading to capacitation [229,230]. The binding of heparin to receptors localized in the proximal region of the sperm head results in alterations in the structure of the plasma membrane covering that specific area [216,231]. In consequence, this mechanism influences the modulation of the acrosomal reaction by zona pellucida glycoproteins [228]. Due to this, heparin is known as a potent enhancer of capacitation [218]. De Souza et al., (2006) [232] described HBPs of the canine seminal plasma. They showed that approximately 50% of the canine seminal plasma proteins are bound to heparin. Of the dog seminal plasma HBPs, CPSE is predominant [232], similar to the group of ZnBPs [6]. Moreover, there is a high homology between the CPSE and the PSA from the seminal plasma, and PSA has been identified as a heparin-binding protein. PSA is the protein marker for human prostate cancer [233–235]. Moreover, high concentrations of OPN were identified in the dog seminal plasma and recognized as HBP [77]. The functions of CPSE and OPN in canine reproduction are described above. The capacity of some proteins to bind several different ligands should be taken into account. It has been shown that boar seminal plasma ZnBPs have the ability to also bind heparin, which suggests that these proteins participate in events associated with the regulation of capacitation and acrosome reactions in boar sperm [7]. Seminal plasma HBPs play an essential role in the fertilization process, and any variations in the structures, content, and functions of these proteins may

be associated with infertility [236]. Treatment of bull semen with seminal plasma HBPs improved sperm motility, viability, acrosome integrity, in vitro capacitation, and acrosome reaction by reducing the oxidative stress induced by cold shock and, consequently, damage to the sperm membrane [237].

This clearly indicates a positive effect of heparin and HBPs on dog semen quality and its participation in the fertilization process, suggesting that heparin and its binding proteins are distinguished as having marker potential.

3.3. Ions

The maintenance of proper sperm function is dependent on the balance of intracellular and extracellular ions and proper ion channel functioning. Sperm cells react rapidly to environmental fluctuations and the regulation of ion balance across their membrane has been shown to be of critical importance for sperm functional features such as motility and fertilization capacity [238]. Ion channels contribute significantly to modulating membrane potential through the maintenance of intracellular pH and osmotic balance [239]. Sperm motility acquisition, capacitation, acrosome reaction, hyperactivation, and transport in the female reproductive tract are ion channel-dependent processes, and the intracellular milieu is regulated by the activity of these ion channels [240]. The control of sperm membrane potential is essential for all these events, and it is regulated by complex signaling pathways that involve the activation of many ion transporters and ion channels present in the sperm membrane [241,242]. Thus, ion channel dysfunctions, interference with ion homeostasis, and sperm membrane potential alterations have important consequences for fertilization [238].

3.3.1. Zinc Ions

Zinc is an essential biological component that plays significant roles in several processes, including signaling, enzymatic activities, and regulation of sexual maturation, ensuring the stability of membrane lipid bilayers and the quaternary structure of the chromatin, and mitochondrial oxidative stress [243–246]. Zinc ions regulate a number of events that are crucial for fertilization, such as normal testicular development, spermatogenesis, and sperm function [247]. Any alterations in zinc can cause abnormalities in the functioning of the organism and have been associated with several diseases in dogs (see review [248]).

It has been shown that most of the zinc in canine seminal plasma originates from the prostate [249]. Large amounts of free zinc in seminal plasma inhibit sperm motility, while reduced free zinc improves their motility [250,251]. Zinc released by the prostate into seminal plasma during ejaculation can be utilized in two different manners. It can be incorporated into sperm cells and thus perform a protective role towards sperm chromatin and membrane stabilization and sperm motility [252–254]. Free zinc ions can also bind to different protein ligands that are implicated in fertilization processes in the female reproductive tract [195,255–257], reducing the zinc fraction available for sperm cells [258]. Free zinc availability in semen is regulated by high molecular proteins, which bind this element and reduce its amount in seminal plasma [259]. The authors' recent studies have shown that high molecular weight protein complexes of canine seminal plasma possess the ability to bind zinc ions, and this has a positive effect on the motility of spermatozoa stored at low temperatures [7].

Zinc–protein complexes were isolated from seminal plasma in canines and characterized for the first time by Mogielnicka-Brzozowska et al., (2012) [258]. This study showed that canine zinc-binding proteins (ZnBPs) occurred in their native state as high molecular aggregates. Mogielnicka-Brzozowska et al., (2015) [6] also showed that under denaturing and reducing conditions, these macromolecules disaggregate. Spectrometric analysis revealed that seminal plasma zinc ions play both structural and regulatory roles in the activity of CPSE, which is crucial for maintaining the normal function of prostate and sperm cells. Prostasomes have also been studied in the context of zinc. Prostasomes are small lipid membrane-confined extracellular vesicles in the mammalian reproductive tract fluids [260]

with the ability to bind zinc ions [261–263]. Canine prostasome proteins have a very high affinity for zinc ions, which suggests the important role of the vesicles in the metabolism of zinc in the canine semen [263]. It was shown that the addition of prostasomes to dog ejaculates had a positive effect on sperm quality (sperm plasma membrane and acrosome integrity) during storage at low temperatures [263].

There are some works systematizing knowledge on the role of zinc in male reproduction [248,264–267]. However, zinc ions found in dog semen should be further investigated for their potential as markers of semen quality.

3.3.2. Calcium Ions

Ca^{2+} levels are low in freshly ejaculated cells; however, during capacitation, Ca^{2+} levels increase due to the influx of extracellular calcium ions over the sperm plasma membrane after the opening of voltage-gated calcium channels [268]. Calcium ionophore is a substance that enables the transport of Ca^{2+} ions across the spermatozoa membrane by forming stable complexes with divalent cations [200]. Szász et al., (2000) [269] suggested that Ca^{2+}-ionophore-induced membrane changes in dog sperm cells are valuable parameters for predicting the suitability of dog ejaculates for cryopreservation. Calcium ions regulate several reproductive processes and events that are crucial for proper fertilization: spermatogenesis, testosterone secretion, sperm capacitation, hyperactivation, motility, and acrosome reaction (in review [270]). Despite reports indicating the important role of calcium ions in the regulation of reproductive events in humans, knowledge of the function of calcium in the canine reproductive tract is very limited. With the current state of knowledge, it is difficult to define calcium as a BRF in dogs. However, according to studies conducted in humans, calcium ions should not be underestimated, and further research is needed to explain the molecular basis of their action as a potential BRF in dogs.

Lipids, carbohydrates, and ions and their binding proteins that are involved in canine sperm functions are shown in Figure 4.

Table 2 contains a summary of the most promising lipid, carbohydrate, and ion molecular biomarkers of canine reproductive functions.

Table 2. Summary of the most promising non-protein molecular biomarkers of canine reproductive functions described in the review. EF—epididymal fluid; ES—epididymal spermatozoa; S—ejaculated spermatozoa; SP—seminal plasma; P—prostate.

Biomarker	Origin of Biomarker	Influence on Reproductive Functions	Implementation in Clinical/Breeding Practice	References
Lipids				
Saturated fatty acids (SFAs)				
Caprylic fatty acid	ES	- bactericidal properties - sperm membrane protection	- fatty acids supplementation for gamete manipulation techniques such as in vitro sperm maturation for immature canine spermatozoa - contraception, such as the use of inhibitors of the enzymes that are responsible for fatty acid biosynthesis, promoting lipid disruption of sperm - formulation of diluents and extenders, and improving cryopreservation protocols	[159,165,176]
Stearic fatty acid	ES, SP, S	- provides the energy for sperm motility and metabolism	as above	[159,167–171,176]
Palmitic fatty acid	SP	- positive effect on sperm production	as above	[159,172,176]
Monounsaturated fatty acids (MUFAs)				
Oleic fatty acid	SP	- regulation of sperm metabolism - positive effect on sperm motility and viability	- formulation of better diluents and extenders, and improving cryopreservation protocols	[159,174,176]
Polyunsaturated fatty acids (PUFAs)				
Docosahexaenoic acid (DHA)	ES, EF	- important for epididymal maturation - provides fluidity to the sperm plasma membrane affecting its integrity - positive effect on sperm cryotolerance	- fatty acids supplementation for gamete manipulation techniques such as in vitro sperm maturation for immature canine spermatozoa - contraception, such as the use of inhibitors of the enzymes that are responsible for fatty acid biosynthesis, promoting lipid disruption of sperm - formulation of better diluents and extenders, and improving cryopreservation protocols	[159,176,179,182]
Phospholipids				
Phosphatidylcholine Phosphatidylethanolamine	S	- sperm motility	- identification of asthenozoospermia	[164,170]

Table 2. *Cont.*

Biomarker	Origin of Biomarker	Influence on Reproductive Functions	Implementation in Clinical/Breeding Practice	References
		Plasmalogens		
Plasmenyl phosphatidylcholine (40:5) Plasmanyl phosphatidylcholine (40:4)	S	- sperm motility - antioxidant properties	- formulation of diluents and extenders, and improving cryopreservation protocols	[8,164]
		Neutral lipids		
Cholesterol	SP	- maintaining the stability and fluidity of the cell membrane - protection against sperm damage induced by freeze/thaw processes	- potential marker of canine semen crytolerance - higher concentrations of cholesterol in the SP of ejaculates with good freezability - indicator of semen quality	[25,199,201,209]
		Carbohydrates		
Heparin	oviductal and uterine fluids	- preceding and stimulation of sperm acrosome reaction in vitro - binding to SP proteins and performing different functions	- as an additive in the in vitro/capacitating medium: stimulates metabolic enzymes, prolongs sperm motility, and induces hyperactivation (even in the sperm of asthenozoospermic dogs), and has a positive effect on the acrosome reaction	[218–225]
		Ions		
Zinc	SP, P	- sperm motility regulation - binding to SP proteins and performing different functions	- high levels of free zinc in SP inhibit sperm motility, while reduced free zinc levels improve sperm motility	[250,251,258]

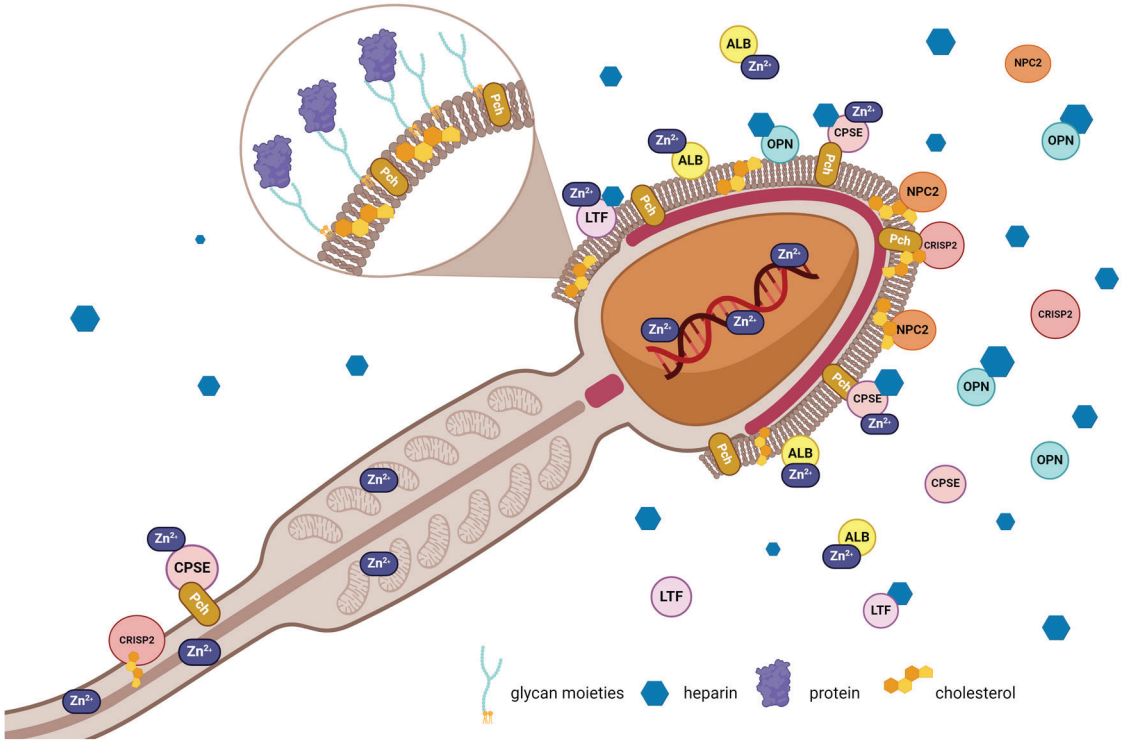


Figure 4. Lipids, carbohydrates, and ions and their binding proteins that are involved in canine sperm functions. ALB—albumin; CPSE—canine prostate-specific esterase; CRISP2—cysteine-rich secretory protein 2; LTF—lactotransferrin; NPC2—Niemann-Pick type C2 protein; OPN—osteopontin; Pch—phosphorylcholine; Zn^{2+}—zinc ions. Created with BioRender.com.

4. Differences in Biomarkers between Breeds

Purebred domestic dogs were created for backcrossing and inbreeding to determine desirable phenotypic or behavioral characteristics. These alterations may influence semen quality and impair biotechnologies of reproduction results [29]. Many canine breeds dealing with genetically specific diseases directly correlated with inbreeding in these breeds [271]. Some breeds may be more affected by reproductive impairments such as decreased semen quality or compromised fertility rates [272,273]. Araujo et al., (2022) [29] showed the influence of dog breed on the proteome of the spermatozoa and seminal plasma (for Golden Retriever, Great Dane, Bernese Mountain dog, and Maremmano-Abruzzese sheepdog). ALB was useful for the separation of the breeds based on spermatozoa proteins. Maremmano-Abruzzese Sheepdog had an increased abundance of ALB in the spermatozoa, and the Bernese Mountain dog had a low abundance of this protein. Tubulin alpha-3E

chain, ACRBP, and tubulin alpha-3 chain were considered relevant in dogs' seminal plasma. High abundances of the tubulin alpha-3E chain and the tubulin alpha-3 chain were found in the Maremmano-Abruzzese sheepdog and the Bernese Mountain dog, respectively. ACBP protein was found in higher abundance in the Bernese Mountain dog. According to the practical expertise of Araujo et al., (2022) [29], the Bernese Mountain dogs have low semen quality, which may be hypothesized as a pattern characteristic of this breed. The variations found between distinct canine breeds suggest that some cellular and molecular phenotypes associated with semen quality, intra-species cellular modulation, and motility could be breed-specific, similar to molecular markers of reproductive functions. Hallberg et al., (2024) [274] investigated the correlation between biomarkers for testicular cell function in the Bernese Mountain dog. Based on the markers discussed in our study, ALPL and CPSE levels vary between dogs based on semen quality and fertility. We found studies on the influence of breed and individual variation on the quality of frozen canine semen (Beagle, Schnauzer, Boxer, and Doberman) [275]. Based on the in vitro evaluation of the semen of different dog breeds, the quality of fresh semen does not show significant variation across the breeds studied; however, the integrity and viability of the sperm membrane and longevity of the thawed semen varies significantly both between individuals and between breeds. The canine spermatozoa cryotolerance varies based on the individual, and this variation may present some genetically inherited components that manifest as a characteristic of the seminal freezing of the breeds. These findings suggest that further studies on semen proteins that may be potential BRFs and functional investigations of breed-specific proteins in purebred dogs need to be performed to improve reproduction biotechnologies.

5. Characteristic Features of Molecular Biomarkers of Reproductive Processes

Most BRFs in dogs have certain unique characteristics. They are produced in the testicles, epididymis, or prostate, or are derived from blood and secreted into the reproductive organ fluids. These biomarkers interact with the sperm plasma membrane with the participation of proteins, lipids, carbohydrates, or ions. This interaction changes the functional characteristics of sperm. BRFs can be an integral part of sperm's structure or may only be temporarily attached and detached from it, influencing sperm physiology. These biomarkers may also have functions associated with antioxidant properties. The levels of BRFs in semen or fluids surrounding sperm can vary depending on the physiological characteristics of the cells or reproductive organs. The content of molecular biomarkers directly or indirectly affects the condition of reproductive tissues or semen quality parameters, which in turn affects reproductive success. The levels of biomarkers can be measured accurately and reproducibly.

6. Conclusions

This review systematizes current knowledge that could serve as a starting point for developing practical tests for the use of biomarkers of canine reproductive functions and their predictive value for assisted reproductive technique outcomes and semen preservation. The literature review allowed for the systematization of the features of a typical molecular marker of reproductive processes in dogs. From the entire pool of molecular markers of reproductive functions in dogs, it is possible to distinguish a pool of markers that can be used with great probability in the future to improve reproductive processes in dogs, including semen conservation. Their functions include improving sperm capacitation, the acrosomal reaction, and sperm motility. They may be potential biomarkers of prostate and testicular cancer. There are markers whose expressions were correlated with the dog's age. The addition of these biomarkers to the canine spermatozoa before or after preservation techniques preserves sperm viability and function, preventing cold shock consequences. Some described substances may be predictive biomarkers for the success of ART, such as IVF and ICSI. However, each type of marker must be treated individually in relation to these matters and this requires further in-depth research.

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References

1. Merriam-Webster Dictionary. Available online: https://www.merriam-webster.com/ (accessed on 6 June 2022).
2. Strimbu, K.; Tavel, J.A. What are biomarkers? *Curr. Opin. HIV AIDS* **2010**, *5*, 463–466. [CrossRef]
3. Tanga, B.M.; Qamar, A.Y.; Raza, S.; Bang, S.; Fang, X.; Yoon, K.; Cho, J. Semen evaluation: Methodological advancements in sperm quality-specific fertility assessment—A review. *Anim. Biosci.* **2021**, *34*, 1253–1270. [CrossRef]
4. Mogielnicka-Brzozowska, M.; Słowińska, M.; Fraser, L.; Wysocki, P.; Strzeżek, R.; Wasilewska, K.; Kordan, W. Identification and characterization of non-phosphorylcholine-binding and phosphorylcholine-binding proteins of canine seminal plasma. *Ann. Anim. Sci.* **2017**, *17*, 771–786. [CrossRef]
5. Lu, J.; Stewart, A.J.; Sadler, P.J.; Pinheiro, T.J.; Blindauer, C.A. Albumin as a zinc carrier: Properties of its high-affinity zinc-binding site. *Biochem. Soc. Trans.* **2008**, *36*, 1317–1321. [CrossRef]
6. Mogielnicka-Brzozowska, M.; Kowalska, N.; Fraser, L.; Kordan, W. Proteomic Characterization of Zinc-Binding Proteins of Canine Seminal Plasma. *Reprod. Domest. Anim.* **2015**, *50*, 1017–1021. [CrossRef]
7. Mogielnicka-Brzozowska, M.; Strzezek, J.; Dziekonska, A.; Kordan, W. The ability of zinc-binding proteins from boar seminal plasma to bind heparin and phosphorylcholine. *Anim. Sci. Pap. Rep.* **2014**, *32*, 327–336.
8. Shan, S.; Xu, F.; Hirschfeld, M.; Brenig, B. Sperm Lipid Markers of Male Fertility in Mammals. *Int. J. Mol. Sci.* **2021**, *22*, 8767. [CrossRef]
9. Peña, F.J.; Núñez-Martínez, I.; Morán, J.M. Semen technologies in dog breeding: An update. *Reprod. Domest. Anim.* **2006**, *41*, 21–29. [CrossRef]
10. Wright, S.J. Spotlight on Reproduction in Domestic Dogs as a Model for Human Reproduction. In *Animal Models and Human Reproduction*; Constantinescu, G., Schatten, H., Eds.; John Wiley & Sons, Inc.: Hoboken, NJ, USA, 2017; pp. 247–358.
11. Panner Selvam, M.K.; Finelli, R.; Agarwal, A.; Henkel, R. Proteomics and metabolomics—Current and future perspectives in clinical andrology. *Andrologia* **2021**, *53*, e13711. [CrossRef]
12. Chakraborty, S.; Saha, S. Understanding sperm motility mechanisms and the implication of sperm surface molecules in promoting motility. *Middle East Fertil. Soc. J.* **2022**, *27*, 4. [CrossRef]
13. Luconi, M.; Cantini, G.; Baldi, E.; Forti, G. Role of a-kinase anchoring proteins (AKAPs) in reproduction. *Front. Biosci.* **2011**, *16*, 1315–1330. [CrossRef]
14. Le Couazer, D.; Bencharif, D. Are AKAP4 and proAKAP4 Found in Canine Semen? Preliminary Results. *Asp. Nanotechnol.* **2021**, *3*, 70–75. [CrossRef]
15. Marquez, B.; Suarez, S.S. Different signalling pathways in bovine sperm regulate capacitation and hyperactivation. *Biol. Reprod.* **2004**, *70*, 1626–1633. [CrossRef]
16. Ficarro, S.; Chertihin, O.; Westbrook, V.A.; White, F.; Jayes, F.; Kalab, P.; Marto, J.A.; Shabanowitz, J.; Herr, J.C.; Hunt, D.F.; et al. Phosphoproteome analysis of capacitated human sperm. *J. Biol. Chem.* **2003**, *278*, 11579–11589. [CrossRef]
17. Jagan Mohanarao, G.; Atreja, S.K. Identification of capacitation associated tyrosine phosphoproteins in buffalo (Bubalus bubalis) and cattle spermatozoa. *Anim. Reprod. Sci.* **2011**, *123*, 40–47. [CrossRef]
18. Malo, C.; Carracedo, S.; Delehedde, M.; Sergeant, N.; Skidmore, J.A. Identification of proAKAP4 concentration variations in dromedary sperm and their correlation with monthly semen parameters. *Reprod. Fertil.* **2021**, *2*, 268–279. [CrossRef]
19. Delehedde, M.; Blommaert, D.; Jouy, N.; Scabello, J.; Miersman, H.; Franck, T.; Serteyn, D.; Mitchell, V.; Sergeant, N. Concentration of ProAKAP4 as a pertinent read-out of sperm quality in mammals. *Anim. Reprod. Sci.* **2018**, *194*, e24. [CrossRef]
20. Sergeant, N.; Briand-Amirat, L.; Bencharif, D.; Delehedde, M. The sperm specific protein ProAKAP4 as an innovative marker to evaluate sperm quality and fertility. *J. Dairy Vet. Sci.* **2019**, *11*, 111–119. [CrossRef]
21. Dordas-Perpinyà, M.; Yanez-Ortiz, I.; Sergeant, N.; Mevel, V.; Bruyas, J.F.; Catalán, J.; Delehedde, M.; Briand-Amirat, L.; Miró, J. ProAKAP4 Concentration Is Related to Sperm Motility and Motile Sperm Subpopulations in Frozen-Thawed Horse Semen. *Animals* **2022**, *12*, 3417. [CrossRef]
22. Le Couazer, D.; Delehedde, M.; Ruelle, I.; Sergeant, N.; Michaud, S.; Briand, L.; Bencharif, D. ProAKAP4 as a valuable marker to assess sperm quality in dogs. *Rep. Dom. Anim.* **2019**, *54*, 91–92.
23. Moretti, E.; Scapigliati, G.; Pascarelli, N.A.; Baccetti, B.; Collodel, G. Localization of AKAP4 and tubulin proteins in sperm with reduced motility. *Asian J. Androl.* **2007**, *9*, 641–649. [CrossRef]
24. Elzanaty, S.; Malm, J. Effects of ejaculation-to-analysis delay on levels of markers of epididymal and accessory sex gland function and sperm motility. *J. Androl.* **2007**, *28*, 847–852. [CrossRef]

25. Schäfer-Somi, S.; Palme, N. Seminal Plasma Characteristics and Expression of ATP-binding Cassette Transporter A1 (ABCA1) in Canine Spermatozoa from Ejaculates with Good and Bad Freezability. *Reprod. Domest. Anim.* **2016**, *51*, 232–239. [CrossRef]
26. Zmudzinska, A.; Wisniewski, J.; Mlynarz, P.; Olejnik, B.; Mogielnicka-Brzozowska, M. Age-dependent Variations in Functional Quality and Proteomic Characteristics of Canine (*Canis lupus* familiaris) Epididymal Spermatozoa. *Int. J. Mol. Sci.* **2022**, *23*, 9143. [CrossRef]
27. Cichowska, A.W.; Wisniewski, J.; Bromke, M.A.; Olejnik, B.; Mogielnicka-Brzozowska, M. Proteome Profiling of Canine Epididymal Fluid: In Search of Protein Markers of Epididymal Sperm Motility. *Int. J. Mol. Sci.* **2023**, *24*, 14790. [CrossRef]
28. Risopatrón, J.; Catalán, S.; Miska, W.; Schill, W.B.; Sánchez, R. Effect of albumin and polyvinyl alcohol on the vitality, motility and acrosomal integrity of canine spermatozoa incubated in vitro. *Reprod. Domest. Anim.* **2002**, *37*, 347–351. [CrossRef]
29. Araujo, M.S.; de Oliveira Henriques Paulo, O.L.D.; Scott, C.; Paranzini, C.S.; Codognoto, V.M.; de Paula Freitas Dell'Aqua, C.; Papa, F.O.; de Souza, F.F. Insights into the influence of canine breed on proteomics of the spermatozoa and seminal plasma. *J. Proteom.* **2022**, *257*, 104508. [CrossRef]
30. Macıas-Garcıa, B.; González-Fernández, L.; Loux, S.C.; Rocha, A.M.; Guimarães, T.; Peña, F.J.; Varner, D.D.; Hinrichs, K. Effect of calcium, bicarbonate, and albumin on capacitation-related events in equine sperm. *Reproduction* **2015**, *149*, 87–99. [CrossRef]
31. Arroteia, K.F.; Barbieri, M.F.; Souza, G.H.; Tanaka, H.; Eberlin, M.N.; Hyslop, S.; Alvares, L.E.; Pereira, L.A. Albumin is synthesized in epididymis and aggregates in a high molecular mass glycoprotein complex involved in sperm-egg fertilization. *PLoS ONE* **2014**, *9*, e103566. [CrossRef]
32. Mogielnicka-Brzozowska, M.; Fraser, L.; Dziekońska, A.; Gackowska, K.; Sobiewska, M.; Kuzborska, A.; Majewska, A.M.; Filipowicz, K.; Kordan, W. Identification of proteoforms of albumin and kallikrein in stallion seminal plasma and their correlations with sperm motility. *Pol. J. Vet. Sci.* **2019**, *22*, 227–235. [CrossRef]
33. Roche, M.; Rondeau, P.; Singh, N.R.; Tarnus, E.; Bourdon, E. The antioxidant properties of serum albumin. *FEBS Lett.* **2008**, *582*, 1783–1787. [CrossRef] [PubMed]
34. Zmudzinska, A.; Bromke, M.A.; Strzezek, R.; Zielinska, M.; Olejnik, B.; Mogielnicka-Brzozowska, M. Proteomic Analysis of Intracellular and Membrane-Associated Fractions of Canine (*Canis lupus familiaris*) Epididymal Spermatozoa and Sperm Structure Separation. *Animals* **2022**, *12*, 772. [CrossRef] [PubMed]
35. Johnston, S.D. Performing a complete canine semen evaluation in a small animal hospital. *Vet. Clin. N. Am. Small Anim. Pract.* **1991**, *21*, 545–551. [CrossRef] [PubMed]
36. Frenette, G.; Dubé, J.Y.; Tremblay, R.R. Origin of alkaline phosphatase of canine semen. *Arch. Androl.* **1986**, *16*, 235–241. [CrossRef] [PubMed]
37. Schäfer-Somi, S.; Fröhlich, T.; Schwendenwein, I. Measurement of alkaline phosphatase in canine seminal plasma—An update. *Reprod. Dom. Anim.* **2013**, *48*, e10–e12. [CrossRef] [PubMed]
38. Adams, C.S. Localization of alkaline phosphatase and NADH diaphorase in the principal cells of the guinea pig epididymis. *Acta Anat.* **1983**, *116*, 146–151. [CrossRef] [PubMed]
39. Jiang, T.; Zeng, Q.; He, J. Do alkaline phosphatases have great potential in the diagnosis, prognosis, and treatment of tumors? *Transl. Cancer Res.* **2023**, *12*, 2932–2945. [CrossRef] [PubMed]
40. Gobello, C.; Castex, G.; Corrada, Y. Serum and seminal markers in the diagnosis of disorders of the genital tract of the dog: A mini-review. *Theriogenology* **2002**, *57*, 1285–1291. [CrossRef] [PubMed]
41. Santos, E.A.A.; Sousa, P.C.; Martins, J.A.M.; Moreira, R.A.; Monteiro-Moreira, A.C.O.; Moreno, F.B.M.B.; Oliveira, M.F.; Moura, A.A.; Silva, A.R. Protein profile of the seminal plasma of collared peccaries (*Pecari tajacu* Linnaeus, 1758). *Reproduction* **2014**, *147*, 753–764. [CrossRef]
42. Boe-Hansen, G.B.; Rego, J.P.A.; Crisp, J.M.; Moura, A.A.; Nouwens, A.S.; Li, Y.; Venus, B.; Burns, B.M.; McGowan, M.R. Seminal plasma proteins and their relationship with percentage of morphologically normal sperm in 2-year-old Brahman (*Bos indicus*) bulls. *Anim. Reprod. Sci.* **2015**, *162*, 20–30. [CrossRef]
43. Waheed, M.M.; Ghoneim, I.M.; Alhaider, A.K. Seminal plasma and serum fertility biomarkers in dromedary camels (*Camelus dromedarius*). *Theriogenology* **2015**, *83*, 650–654. [CrossRef]
44. Fouchécourt, S.; Métayer, S.; Locatelli, A.; Dacheux, F.; Dacheux, J.L. Stallion epididymal fluid proteome: Qualitative and quantitative characterization; secretion and dynamic changes of major proteins. *Biol. Reprod.* **2000**, *62*, 1790–1803. [CrossRef]
45. Dacheux, J.L.; Belleannée, C.; Jones, R.; Labas, V.; Belghazi, M.; Guyonnet, B.; Druart, X.; Gatti, J.L.; Dacheux, F. Mammalian epididymal proteome. *Mol. Cell Endocrinol.* **2009**, *306*, 45–50. [CrossRef]
46. Carver, J.A.; Rekas, A.; Thorn, D.C.; Wilson, M.R. Small heat-shock proteins and clusterin: Intra- and extracellular molecular chaperones with a common mechanism of action and function? *IUBMB Life* **2003**, *55*, 661–668. [CrossRef]
47. Souza, C.E.A.; Rego, J.P.A.; Lobo, C.H.; Oliveira, J.T.A.; Nogueira, F.C.S.; Domont, G.B.; Fioramonte, M.; Gozzo, F.C.; Moreno, F.B.; Monteiro-Moreira, A.C.; et al. Proteomic analysis of the reproductive tract fluids from tropically-adapted Santa Ines rams. *J. Proteom.* **2012**, *75*, 4436–4456. [CrossRef]
48. Zhang, H.; Kim, J.K.; Edwards, C.A.; Xu, Z.; Taichman, R.; Wang, C.Y. Clusterin inhibits apoptosis by interacting with activated Bax. *Nat. Cell Biol.* **2005**, *7*, 909–915. [CrossRef] [PubMed]
49. Ibrahim, N.M.; Foster, D.N.; Crabo, B.G. Localization of clusterin on freeze-preserved bull spermatozoa before and after glass wool-sephadex filtration. *J. Androl.* **2001**, *22*, 891–902. [CrossRef]

50. Naponelli, V.; Bettuzzi, S. Clusterin. In *The Complement FactsBook*, 2nd ed.; Barnum, S., Schein, T., Eds.; Academic Press: Cambridge, MA, USA, 2018; pp. 341–349.
51. Isaacs, W.B.; Coffey, D.S. The predominant protein of canine seminal plasma is an enzyme. *J. Biol. Chem.* **1984**, *259*, 11520–11526. [CrossRef] [PubMed]
52. Carvalho, A.L.; Sanz, L.; Barettino, D.; Romero, A.; Calvete, J.J.; Romão, M.J. Crystal structure of a prostate kallikrein isolated from stallion seminal plasma: A homologue of human PSA. *J. Mol. Biol.* **2002**, *322*, 325–337. [CrossRef] [PubMed]
53. Jamsai, D.; Reilly, A.; Smith, S.J.; Gibbs, G.M.; Baker, H.W.G.; McLachlan, R.I.; de Kretser, D.M.; O'Bryan, M.K. Polymorphisms in the human cysteine-rich secretory protein 2 (CRISP2) gene in Australian men. *Hum. Reprod.* **2008**, *23*, 2151–2159. [CrossRef]
54. Zhou, J.H.; Zhou, Q.Z.; Lyu, X.M.; Zhu, T.; Chen, Z.J.; Chen, M.K.; Xia, H.; Wang, C.Y.; Qi, T.; Li, X.; et al. The expression of cysteine-rich secretory protein 2 (CRISP2) and its specific regulator miR-27b in the spermatozoa of patients with asthenozoospermia. *Biol. Reprod.* **2015**, *92*, 28. [CrossRef]
55. Foster, J.A.; Gerton, G.L. Autoantigen 1 of the guinea pig sperm acrosome is the homologue of mouse Tpx-1 and human TPX1 and is a member of the cysteine-rich secretory protein (CRISP) family. *Mol. Reprod. Dev.* **1996**, *44*, 221–229. [CrossRef]
56. O'Bryan, M.K.; Loveland, K.L.; Herszfeld, D.; McFarlane, J.R.; Hearn, M.T.; de Kretser, D.M. Identification of a rat testis-specific gene encoding a potential rat outer dense fibre protein. *Mol. Reprod. Dev.* **1998**, *50*, 313–322. [CrossRef]
57. Reddy, T.; Gibbs, G.M.; Merriner, D.J.; Kerr, J.B.; O'Bryan, M.K. Cysteine-rich secretory proteins are not exclusively expressed in the male reproductive tract. *Dev. Dyn.* **2008**, *237*, 3313–3323. [CrossRef] [PubMed]
58. Lim, S.; Kierzek, M.; O'Connor, A.E.; Brenker, C.; Merriner, D.J.; Okuda, H.; Volpert, M.; Gaikwad, A.; Bianco, D.; Potter, D.; et al. CRISP2 Is a Regulator of Multiple Aspects of Sperm Function and Male Fertility. *Endocrinology* **2019**, *160*, 915–924. [CrossRef] [PubMed]
59. Guo, M.; Teng, M.; Niu, L.; Liu, Q.; Huang, Q.; Hao, Q. Crystal structure of the cysteine-rich secretory protein stecrisp reveals that the cysteine-rich domain has a K^+ channel inhibitor-like fold. *J. Biol. Chem.* **2005**, *280*, 12405–12412. [CrossRef] [PubMed]
60. Gibbs, G.M.; Scanlon, M.J.; Swarbrick, J.; Curtis, S.; Gallant, E.; Dulhunty, A.F.; O'Bryan, M.K. The cysteine-rich secretory protein domain of Tpx-1 is related to ion channel toxins and regulates ryanodine receptor Ca^{2+} signaling. *J. Biol. Chem.* **2006**, *281*, 4156–4163. [CrossRef] [PubMed]
61. Maeda, T.; Sakashita, M.; Ohba, Y.; Nakanishi, Y. Molecular cloning of the rat Tpx-1 responsible for the interaction between spermatogenic and Sertoli cells. *Biochem. Biophys. Res. Commun.* **1998**, *248*, 140–146. [CrossRef] [PubMed]
62. Choudhary, V.; Schneiter, R. Pathogen-related yeast (PRY) proteins and members of the CAP superfamily are secreted sterolbinding proteins. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 16882–16887. [CrossRef]
63. Gottschalk, M.; Metzger, J.; Martinsson, G.; Sieme, H.; Distl, O. Genome-wide association study for semen quality traits in German Warmblood stallions. *Anim. Reprod. Sci.* **2016**, *171*, 81–86. [CrossRef]
64. Kiso, W.K.; Selvaraj, V.; Nagashima, J.; Asano, A.; Brown, J.L.; Schmitt, D.L.; Leszyk, J.; Travis, A.J.; Pukazhenthi, B.S. Lactotransferrin in Asian elephant (*Elephas maximus*) seminal plasma correlates with semen quality. *PLoS ONE* **2013**, *8*, e71033. [CrossRef]
65. Adlerova, L.; Bartoskova, A.; Faldyna, M. Lactoferrin: A review. *Vet. Med.* **2008**, *53*, 457–468. [CrossRef]
66. Su, J.; Wang, C.; Song, Y.; Yang, Y.; Cao, G. Effect of lactoferrin on ram sperm motility after cryopreservation. *Anim. Biosci.* **2022**, *35*, 1351–1359. [CrossRef] [PubMed]
67. Kim, S.-H.; Song, Y.-S.; Hwang, S.-Y.; Min, K.-S.; Yoon, J.-T. Effects of hormones on the expression of matrix metalloproteinases and their inhibitors in bovine spermatozoa. *Asian-Australas. J. Anim. Sci.* **2013**, *26*, 334–342. [CrossRef] [PubMed]
68. Cornwall, G.A. New insights into epididymal biology and function. *Hum. Reprod. Update* **2009**, *15*, 213–227. [CrossRef] [PubMed]
69. Saengsoi, W.; Shia, W.Y.; Shyu, C.L.; Wu, J.T.; Warinrak, C.; Lee, W.M.; Cheng, F.P. Detection of matrix metalloproteinase (MMP)-2 and MMP-9 in canine seminal plasma. *Anim. Reprod. Sci.* **2011**, *127*, 114–119. [CrossRef] [PubMed]
70. Tentes, I.; Asimakopoulos, B.; Mourvati, E.; Diedrich, K.; Al-Hasani, S.; Nikolettos, N. Matrix metalloproteinase (MMP)-2 and MMP-9 in seminal plasma. *J. Assist. Reprod. Genet.* **2007**, *24*, 278–281. [CrossRef] [PubMed]
71. Baumgart, E.; Lenk, S.V.; Loening, S.A.; Jung, K. Quantitative differences in matrix metalloproteinase (MMP)-2, but not in MMP-9, tissue inhibitor of metalloproteinase (TIMP)-1 or TIMP-2, in seminal plasma of normozoospermic and azoospermic patients. *Hum. Reprod.* **2002**, *17*, 2919–2923. [CrossRef] [PubMed]
72. Belardin, L.B.; Antoniassi, M.P.; Camargo, M.; Intasqui, P.; Fraietta, R.; Bertolla, R.P. Semen levels of matrix metalloproteinase (MMP) and tissue inhibitor of metallorproteinases (TIMP) protein families members in men with high and low sperm DNA fragmentation. *Sci. Rep.* **2019**, *9*, 903. [CrossRef]
73. Benkhalifa, M.; Zidi, W.; Bahri, H.; Mahjoub, S.; Boudhraa, K.; Sanhaji, H.; Khorsi-Cauet, H.; Feki, M.; Benkhalifa, M.; Allal-Elasmi, M. Circulating MMP-7 and VEGF as potential predictive biomarkers for recurrent implantation failures. *Zygote* **2021**, *29*, 365–371. [CrossRef]
74. Luedtke, C.C.; McKee, M.D.; Cyr, D.G.; Gregory, M.; Kaartinen, M.T.; Mui, J.; Hermo, L. Osteopontin expression and regulation in the testis, efferent ducts, and epididymis of rats during postnatal development through to adulthood. *Biol. Reprod.* **2002**, *66*, 1437–1448. [CrossRef] [PubMed]
75. Tekin, K.; Kurtdede, E.; Salmanoğlu, B.; Uysal, O.; Stelletta, C. Osteopontin Concentration in Prostates Fractions: A Novel Marker of Sperm Quality in Dogs. *Vet. Sci.* **2023**, *10*, 646. [CrossRef] [PubMed]
76. Abedin, S.N.; Leela, V.; Devendran, P.; Suganya, G.; Rangasamy, S.; Loganathasamy, K. Seminal Plasma Osteopontin: A Marker for Potential Fertility in Dogs. *Indian J. Anim. Res.* **2021**, *55*, 758–762. [CrossRef]

77. Souza, F.F.; Chirinéa, V.H.; Martins, M.I.; Lopes, M.D. Osteopontin in seminal plasma and sperm membrane of dogs. *Reprod. Domest. Anim.* **2009**, *44*, 283–286. [CrossRef] [PubMed]
78. Erikson, D.W.; Way, A.L.; Chapman, D.A.; Killian, G.J. Detection of osteopontin on Holstein bull spermatozoa, in cauda epididymal fluid and testis homogenates, and its potential role in bovine fertilization. *Reproduction* **2007**, *133*, 909–917. [CrossRef] [PubMed]
79. Dai, J.; Peng, L.; Fan, K.; Wang, H.; Wei, R.; Ji, G.; Cai, J.; Lu, B.; Li, B.; Zhang, D.; et al. Osteopontin induces angiogenesis through activation of PI3K/AKT and ERK1/2 in endothelial cells. *Oncogene* **2009**, *28*, 3412–3422. [CrossRef] [PubMed]
80. Moura, A.A. Seminal plasma proteins and fertility indexes in the bull: The case for osteopontin. *Anim. Reprod.* **2005**, *2*, 3–10.
81. Furger, K.A.; Menon, R.K.; Tuck, A.B.; Bramwell, V.H.; Chambers, A.F. The functional and clinical roles of osteopontin in cancer and metastasis. *Curr. Mol. Med.* **2001**, *1*, 621–632. [CrossRef] [PubMed]
82. Candenas, L.; Chianese, R. Exosome composition and seminal plasma proteome: A promising source of biomarkers of male infertility. *Int. J. Mol. Sci.* **2020**, *21*, 7022. [CrossRef]
83. Zhou, Y.; Shaw, N.; Li, Y.; Zhao, Y.; Zhang, R.; Liu, Z.-J. Structure-function analysis of human l-prostaglandin D synthase bound with fatty acid molecules. *FASEB J.* **2010**, *24*, 4668–4677. [CrossRef]
84. Flower, D.R. Beyond the superfamily: The lipocalin receptors. *Biochim. Biophys. Acta* **2000**, *1482*, 327–336. [CrossRef] [PubMed]
85. Luvoni, G.C.; Morselli, M.G. Canine epididymal spermatozoa: A hidden treasure with great potential. *Reprod. Domest. Anim.* **2016**, *52*, 197–201. [CrossRef] [PubMed]
86. van Tilburg, M.; Sousa, S.; Lobo, M.D.P.; Monteiro-Azevedo, A.C.O.M.; Azevedo, R.A.; Araújo, A.A.; Moura, A.A. Mapping the major proteome of reproductive fluids and sperm membranes of rams: From the cauda epididymis to ejaculation. *Theriogenology* **2021**, *159*, 98–107. [CrossRef] [PubMed]
87. Mogielnicka-Brzozowska, M.; Prochowska, S.; Niżański, W.; Bromke, M.A.; Wiśniewski, J.; Olejnik, B.; Kuzborska, A.; Fraser, L.; Młynarz, P.; Kordan, W. Proteome of cat semen obtained after urethral catheterization. *Theriogenology* **2020**, *141*, 68–81. [CrossRef] [PubMed]
88. Chen, D.-Y.; Zhu, M.-Y.; Cui, Y.-D.; Huang, T.-H. Relationship between contents of lipocalin-type prostaglandin D synthase on the surface of infertility sperm and in seminal plasma. *Biochemistry* **2007**, *72*, 215–218. [CrossRef] [PubMed]
89. Baba, T.; Niida, Y.; Michikawa, Y.; Kashiwabara, S.; Kodaira, K.; Takenaka, M.; Kohno, N.; Gerton, G.L.; Arai, Y. An acrosomal protein, sp32, in mammalian sperm is a binding protein specific for two proacrosins and an acrosin intermediate. *J. Biol. Chem.* **1994**, *269*, 10133–10140. [CrossRef] [PubMed]
90. Kim, J.T.; Jung, H.J.; Song, H.; Yoon, M.J. Acrosin-binding protein (ACRBP) in the testes of stallions. *Anim. Reprod. Sci.* **2015**, *163*, 179–186. [CrossRef]
91. Vilagran, I.; Castillo, J.; Bonet, S.; Sancho, S.; Yeste, M.; Estanyol, J.M.; Oliva, R. Acrosin-binding protein (ACRBP) and triosephosphate isomerase (TPI) are good markers to predict boar sperm freezing capacity. *Theriogenology* **2013**, *80*, 443–450. [CrossRef] [PubMed]
92. Pedersen, S.F.; Hoffmann, E.K.; Mills, J.W. The cytoskeleton and cell volume regulation. *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* **2001**, *130*, 385–399. [CrossRef]
93. Naresh, S. Effect of cooling (4 °C) and cryopreservation on cytoskeleton actin and protein tyrosine phosphorylation in buffalo spermatozoa. *Cryobiology* **2016**, *72*, 7–13. [CrossRef]
94. Cabello-Agüeros, J.F.; Hernández-González, E.O.; Mújica, A. The role of F-actin cytoskeleton-associated gelsolin in the guinea pig capacitation and acrosome reaction. *Cell Motil. Cytoskelet.* **2003**, *56*, 94–108. [CrossRef] [PubMed]
95. Kouprina, N.; Pavlicek, A.; Collins, N.K.; Nakano, M.; Noskov, V.N.; Ohzeki, J.-I.; Mochida, G.H.; Risinger, J.I.; Goldsmith, P.; Gunsior, M.; et al. The microcephaly ASPM gene is expressed in proliferating tissues and encodes for a mitotic spindle protein. *Hum. Mol. Genet.* **2005**, *14*, 2155–2165. [CrossRef] [PubMed]
96. Lüers, G.H.; Michels, M.; Schwaab, U.; Franz, T. Murine calmodulin-binding protein 1 (Calmbp1): Tissue-specific expression during development and in adult tissues. *Mech. Dev.* **2002**, *118*, 229–232. [CrossRef] [PubMed]
97. Bouchier-Hayes, L.; Martin, S.J. CARD games in apoptosis and immunity. *EMBO Rep.* **2002**, *3*, 616–621. [CrossRef] [PubMed]
98. Dufner, A.; Pownall, S.; Mak, T.W. Caspase recruitment domain protein 6 is a microtubule-interacting protein that positively modulates NF-κB activation. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 988–993. [CrossRef] [PubMed]
99. Michishita, E.; Garcés, G.; Barrett, J.C.; Horikawa, I. Upregulation of the KIAA1199 gene is associated with cellular mortality. *Cancer Lett.* **2006**, *239*, 71–77. [CrossRef] [PubMed]
100. Chen, L.; Shi, K.; Andersen, T.L.; Qiu, W.; Kassem, M. KIAA1199 is a secreted molecule that enhances osteoblastic stem cell migration and recruitment. *Cell Death Dis.* **2019**, *10*, 126. [CrossRef]
101. Saalmann, A.; Münz, S.; Ellerbrock, K.; Ivell, R.; Kirchhoff, C. Novel sperm-binding proteins of epididymal origin contain four fibronectin type II-modules. *Mol. Reprod. Dev.* **2001**, *58*, 88–100. [CrossRef]
102. Sahin, E.; Petrunkina, A.M.; Ekhlasi-Hundrieser, M.; Hettel, C.; Waberski, D.; Harrison, R.A.P.; Töpfer-Petersen, E. Fibronectin type II-module proteins in the bovine genital tract and their putative role in cell volume control during sperm maturation. *Reprod. Fertil. Dev.* **2009**, *21*, 479–488. [CrossRef]
103. Ekhlasi-Hundrieser, M.; Schäfer, B.; Kirchhoff, C.; Hess, O.; Bellair, S.; Müller, P.; Töpfer-Petersen, E. Structural and molecular characterization of equine sperm-binding fibronectin-II module proteins. *Mol. Reprod. Dev.* **2005**, *70*, 45–57. [CrossRef]
104. Schäfer, B.; von Horsten, H.H.; Dacheux, J.L.; Holtz, W.; Kirchhoff, C. Cloning and characterization of boar epididymal secretory proteins by homology to the human. *Reprod. Domest. Anim.* **2003**, *38*, 111–118. [CrossRef] [PubMed]

105. Ekhlasi-Hundrieser, M.; Schäfer, B.; Philipp, U.; Kuiper, H.; Leeb, T.; Mehta, M.; Kirchhoff, C.; Töpfer-Petersen, E. Sperm-binding fibronectin type II-module proteins are genetically linked and functionally related. *Gene* **2007**, *392*, 253–265. [CrossRef] [PubMed]
106. D'Amours, O.; Frenette, G.; Fortier, M.; Leclerc, P.; Sullivan, R. Proteomic comparison of detergent-extracted sperm proteins from bulls with different fertility indexes. *Reproduction* **2010**, *139*, 545–556. [CrossRef] [PubMed]
107. D'Amours, O.; Bordeleau, L.-J.; Frenette, G.; Blondin, P.; Leclerc, P.; Sullivan, R. Binder of sperm 1 and epididymal sperm binding protein 1 are associated with different bull sperm subpopulations. *Reproduction* **2012**, *143*, 759–771. [CrossRef] [PubMed]
108. Yao, H.L.; Liu, M.; Wang, W.J.; Wang, X.L.; Song, J.; Song, Q.Q.; Han, J. Construction of miRNA-target networks using microRNA profiles of CVB3-infected HeLa cells. *Sci. Rep.* **2019**, *9*, 17876. [CrossRef] [PubMed]
109. The Human Protein Atlas FAM135A. Available online: https://www.proteinatlas.org/ENSG00000082269-FAM135A/tissue (accessed on 20 June 2022).
110. Kierszenbaum, A.L.; Rivkin, E.; Tres, L.L. Acroplaxome, an F-actin-keratin-containing plate, anchors the acrosome to the nucleus during shaping of the spermatid head. *Mol. Biol. Cell* **2003**, *14*, 4628–4640. [CrossRef] [PubMed]
111. Raman, J.; Guan, Y.; Perrine, C.L.; Gerken, T.A.; Tabak, L.A. UDP-N-acetyl-α-D galactosamine:polypeptide N-acetylgalactosaminy ltransferases: Completion of the family tree. *Glycobiology* **2012**, *22*, 768–777. [CrossRef] [PubMed]
112. Takasaki, N.; Tachibana, K.; Ogasawara, S.; Matsuzaki, H.; Hagiuda, J.; Ishikawa, H.; Mochida, K.; Inoue, K.; Ogonuki, N.; Ogura, A.; et al. A heterozygous mutation of GALNTL5 affects male infertility with impairment of sperm motility. *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 1120–1125. [CrossRef] [PubMed]
113. Li, H.; Jiang, Q.; Ju, Z.H.; Huang, J.M.; Wang, X.G.; Yang, C.H.; Sun, Y.; Yang, G.W.; Zhong, J.F.; Wang, C.F. A novel splice variant of the bovine GALNTL5 gene identified in Chinese Holstein bull testis tissue and its mRNA expression. *Genet. Mol. Res.* **2016**, *15*, 15027723. [CrossRef]
114. Yao, X.; Yang, H.; Zhang, Y.; Ren, C.; Nie, H.; Fan, Y.; Zhou, W.; Wang, S.; Feng, X.; Wang, F. Characterization of GALNTL5 gene sequence and expression in ovine testes and sperm. *Theriogenology* **2017**, *95*, 54–61. [CrossRef]
115. Salier, J.P. Chromosomal location, exon/intron organization and evolution of lipocalin genes. *Biochim. Biophys. Acta* **2000**, *1482*, 25–34. [CrossRef] [PubMed]
116. Hamil, K.G.; Liu, Q.; Sivashanmugam, P.; Anbalagan, M.; Yenugu, S.; Soundararajan, R.; Grossman, G.; Rao, A.J.; Birse, C.E.; Ruben, S.M.; et al. LCN6, a novel human epididymal lipocalin. *Reprod. Biol. Endocrinol.* **2003**, *1*, 112. [CrossRef] [PubMed]
117. Thimon, V.; Koukoui, O.; Calvo, E.; Sullivan, R. Region-specific gene expression profiling along the human epididymis. *Mol. Hum. Reprod.* **2007**, *13*, 691–704. [CrossRef] [PubMed]
118. Sutton, H.G.; Fusco, A.; Cornwall, G.A. Cystatin-related epididymal spermatogenic protein colocalizes with luteinizing hormone-β protein in mouse anterior pituitary gonadotropes. *Endocrinology* **1999**, *140*, 2721–2732. [CrossRef] [PubMed]
119. Cornwall, G.A.; Orgebin-Crist, M.C.; Hann, S.R. The CRES gene: A unique testis-regulated gene related to the cystatin family is highly restricted in its expression to the proximal region of the mouse epididymis. *Mol. Endocrinol.* **1992**, *6*, 1653–1664. [CrossRef] [PubMed]
120. Kirchhoff, C. The dog as model to study human epididymal function at a molecular level. *Mol. Hum. Reprod.* **2002**, *8*, 695–701. [CrossRef] [PubMed]
121. Froman, D.P.; Amann, R.P.; Riek, P.M.; Olar, T.T. Acrosin activity of canine spermatozoa as an index of cellular damage. *J. Reprod. Fert.* **1984**, *70*, 301–308. [CrossRef] [PubMed]
122. Pera, I.; Ivell, R.; Kirchhoff, C. Regional variation of gene expression in the dog epididymis as revealed by in-situ transcript hybridization. *Int. J. Androl.* **1994**, *17*, 324–330. [CrossRef] [PubMed]
123. Naureckiene, S.; Sleat, D.E.; Lackland, H.; Fensom, A.; Vanier, M.T.; Wattiaux, R.; Jadot, M.; Lobel, P. Identification of HE1 as the second gene of Niemann-Pick C disease. *Science* **2000**, *290*, 2298–2301. [CrossRef]
124. Osterhoff, C.; Kirchhoff, C.; Krull, N.; Ivell, R. Molecular cloning and characterization of a novel human sperm antigen (HE2) specifically expressed in the proximal epididymis. *Biol. Reprod.* **1994**, *50*, 516–525. [CrossRef]
125. Araujo, M.S.; Oliveira Henriques Paulo, O.L.D.; Paranzini, C.S.; Scott, C.; Codognoto, V.M.; de Paula Freitas Dell'Aqua, C.; Papa, F.O.; de Souza, F.F. Proteomic data of seminal plasma and spermatozoa of four purebred dogs. *Data Brief* **2020**, *30*, 105498. [CrossRef] [PubMed]
126. Liu, W.; Rodgers, G.P. Olfactomedin 4 expression and functions in innate immunity, inflammation, and cancer. *Cancer Metastasis Rev.* **2016**, *35*, 201–212. [CrossRef] [PubMed]
127. Kodithuwakku, S.P.; Pang, R.T.K.; Ng, E.H.Y.; Cheung, A.N.Y.; Horne, A.W.; Ho, P.-C.; Yeung, W.S.B.; Lee, K.-F. Wnt activation downregulates Olfactomedin-1 (Olfm-1) in Fallopian tubal epithelial cells: A microenvironment predisposed to tubal ectopic pregnancy. *Lab. Investig.* **2012**, *92*, 256–264. [CrossRef] [PubMed]
128. Li, H.; Liu, W.; Chen, W.; Zhu, J.; Deng, C.X.; Rodgers, G.P. Olfactomedin 4 deficiency promotes prostate neoplastic progression and is associated with upregulation of the hedgehog-signaling pathway. *Sci. Rep.* **2015**, *5*, 16974. [CrossRef] [PubMed]
129. Sharma, R.; Agarwal, A.; Mohanty, G.; Hamada, A.J.; Gopalan, B.; Willard, B.; Yadav, S.; du Plessis, S. Proteomic analysis of human spermatozoa proteins with oxidative stress. *Reprod. Biol. Endocrinol.* **2013**, *11*, 48. [CrossRef] [PubMed]
130. Thimon, V.; Frenette, G.; Saez, F.; Thabet, M.; Sullivan, R. Protein composition of human epididymosomes collected during surgical vasectomy reversal: A proteomic and genomic approach. *Hum. Reprod.* **2008**, *23*, 1698–1707. [CrossRef] [PubMed]
131. Pawson, T. Protein modules and signalling networks. *Nature* **1995**, *373*, 573–580. [CrossRef]

132. The Human Protein Atlas PLEKHH1. Available online: https://www.proteinatlas.org/ENSG00000054690-PLEKHH1/tissue (accessed on 20 June 2022).
133. Wolff, J. Plasma membrane tubulin. *Biochim. Biophys. Acta Biomembr.* **2009**, *1788*, 1415–1433. [CrossRef]
134. Mendonça, G.A.; Filho, R.M.; Souza, E.T.; Gaggini, T.S.; Silva-Mendonça, M.C.A.; Antunes, R.C.; Beletti, M.E. Isolation and identification of proteins from swine sperm chromatin and nuclear matrix. *Anim. Reprod.* **2017**, *14*, 418–428. [CrossRef]
135. Kierszenbaum, A.L.; Rivkin, E.; Tres, L.L. Cytoskeletal track selection during cargo transport in spermatids is relevant to male fertility. *Spermatogenesis* **2011**, *1*, 221–230. [CrossRef]
136. van Tilburg, M.F.; Rodrigues, M.A.M.; Moreira, R.A.; Moreno, F.B.; Monteiro-Moreira, A.C.O.; Cândido, M.J.D.; Moura, A.A. Membrane-associated proteins of ejaculated sperm from Morada Nova rams. *Theriogenology* **2013**, *79*, 1247–1261. [CrossRef] [PubMed]
137. Li, H.Y.; He, Y.X.; Zhang, H.; Liu, Y.Y.; Miao, G.Y.; Zhao, Q.Y. Carbon ion irradiation induces reduction of β-tubulin in sperm of pubertal mice. *Biomed. Environ. Sci.* **2014**, *27*, 130–133. [CrossRef] [PubMed]
138. Peknicova, J.; Pexidrova, M.; Kubatova, A.; Koubek, P.; Tepla, O.; Sulimenko, T.; Draber, P. Expression of beta-tubulin epitope in human sperm with pathological spermiogram. *Fertil. Steril.* **2007**, *88*, 1120–1128. [CrossRef]
139. Shen, S.; Wang, J.; Liang, J.; He, D. Comparative proteomic study between human normal motility sperm and idiopathic asthenozoospermia. *World J. Urol.* **2013**, *31*, 1395–1401. [CrossRef]
140. Kelly, V.C.; Kuy, S.; Palmer, D.J.; Xu, Z.; Davis, S.R.; Cooper, G.J. Characterization of bovine seminal plasma by proteomics. *Proteomics* **2006**, *6*, 5826–5833. [CrossRef] [PubMed]
141. Angrimani, D.S.R.; Losano, J.D.A.; Lucio, C.F.; Veiga, G.A.L.; Pereda, M.C.; Nichi, M.; Vannucchi, C.I. Role of residual cytoplasm on oxidative status during sperm maturation in dogs. *Anim. Reprod. Sci.* **2014**, *151*, 256–261. [CrossRef] [PubMed]
142. Neagu, V.R.; Macıas-Garcıa, B.; Rodríguez, A.M.; Ortega-Ferrusola, C.; Bolaños, J.M.G.; Fernández, L.G.; Tapia, J.A.; Peña, F.J. Determination of glutation peroxidase and superoxide dismutase activities in canine seminal plasma and its relation with sperm quality and lipid peroxidation post thaw. *Theriogenology* **2011**, *75*, 10–16. [CrossRef]
143. Waheed, M.M.; Gouda, E.M.; Khalifa, T.A.A. Impact of seminal plasma superoxide dismutase and glutathione peroxidase on cryopreserved buffalo spermatozoa. *Anim. Reprod. Sci.* **2013**, *142*, 126–130. [CrossRef] [PubMed]
144. Barranco, I.; Tvarijonaviciute, A.; Perez-Patiño, C.; Vicente-Carrillo, A.; Parrilla, I.; Ceron, J.J.; Martinez, E.A.; Rodriguez-Martinez, H.; Roca, J. Glutathione Peroxidase 5 Is Expressed by the Entire Pig Male Genital Tract and Once in the Seminal Plasma Contributes to Sperm Survival and In Vivo Fertility. *PLoS ONE* **2016**, *11*, e0162958:1–e0162958:17. [CrossRef]
145. Alvarez, J.G.; Storey, B.T. Evidence for increased lipid peroxidative damage and loss of superoxide dismutase activity as a mode of sublethal cryodamage to human sperm during cryopreservation. *J. Androl.* **1992**, *13*, 232–241. [CrossRef]
146. Alvarez, J.G.; Storey, B.T. Role of glutathione peroxidase in protecting mammalian spermatozoa from loss of motility caused by spontaneous lipid peroxidation. *Gamete Res.* **1989**, *23*, 77–90. [CrossRef]
147. Buffone, M.G.; Calamera, J.C.; Brugo-Olmedo, S.; De Vincentiis, S.; Calamera, M.M.; Storey, B.T.; Doncel, G.F.; Alvarez, J.G. Superoxide dismutase content in sperm correlates with motility recovery after thawing of cryopreserved human spermatozoa. *Fertil. Steril.* **2012**, *97*, 293–298. [CrossRef] [PubMed]
148. Strzezek, R.; Koziorowska-Gilun, M.; Kowalówka, M.; Strzezek, J. Characteristics of antioxidant system in dog semen. *Pol. J. Vet. Sci.* **2009**, *12*, 55–60. [PubMed]
149. Kawakami, E.; Takemura, A.; Sakuma, M.; Takano, M.; Hirano, T.; Hori, T.; Tsutsui, T. Superoxide dismutase and catalase activities in the seminal plasma of normozoospermic and asthenozoospermic Beagles. *J. Vet. Med. Sci.* **2007**, *69*, 133–136. [CrossRef]
150. Chatdarong, K.; Chaivechakarn, A.; Thuwanut, P.; Ponglowhapan, S. Effects of cold storage prior to freezing on superoxide dismutase, glutathione peroxidase activities, level of total reactive oxygen species and sperm quality in dogs. *Reprod. Domest. Anim.* **2012**, *47*, 274–277. [CrossRef]
151. Prete, C.D.; Ciani, F.; Tafuri, S.; Pasolini, M.P.; Valle, G.D.; Palumbo, V.; Abbondante, L.; Calamo, A.; Barbato, V.; Gualtieri, R.; et al. Effect of superoxide dismutase, catalase, and glutathione peroxidase supplementation in the extender on chilled semen of fertile and hypofertile dogs. *J. Vet. Sci.* **2018**, *19*, 667–675. [CrossRef] [PubMed]
152. Valencia, J.; Yeste, M.; Quintero-Moreno, A.; Niño-Cardenas, C.D.P.; Henao, F.J. Relative content of Niemann-Pick C2 protein (NPC2) in seminal plasma, but not that of spermadhesin AQN-1, is related to boar sperm cryotolerance. *Theriogenology* **2020**, *145*, 181–189. [CrossRef]
153. Muro, E.; Atilla-Gokcumen, G.E.; Eggert, U.S. Lipids in cell biology: How can we understand them better? *Mol. Biol. Cell* **2014**, *25*, 1819–1823. [CrossRef]
154. Vance, J.E.; Vance, D.E. *Biochemistry of Lipids, Lipoproteins and Membranes*, 5th ed.; Elsevier Science: Amsterdam, The Netherlands, 2008; p. 624.
155. Christova, Y.; James, P.; Mackie, A.; Cooper, T.G.; Jones, R. Molecular diffusion in sperm plasma membranes during epididymal maturation. *Mol. Cell Endocrinol.* **2004**, *216*, 41–46. [CrossRef]
156. Parks, J.E.; Hammerstedt, R.H. Development changes occurring in the lipids of ram epididymal spermatozoa plasma membrane. *Biol. Reprod.* **1985**, *32*, 653–668. [CrossRef]
157. Lenzi, A.; Picardo, M.; Gandini, L.; Dondero, F. Lipids of the sperm plasma membrane: From polyunsaturated fatty acids considered as markers of sperm function to possible scavenger therapy. *Hum. Reprod. Update* **1996**, *2*, 246–256. [CrossRef]
158. Belleannée, C.; Belghazi, M.; Labas, V.; Teixeira-Gomes, A.P.; Gatti, J.L.; Dacheux, J.L.; Dacheux, F. Purification and identification of sperm surface proteins and changes during epididymal maturation. *Proteomics* **2011**, *11*, 1952–1964. [CrossRef] [PubMed]

159. Angrimani, D.S.R.; Nichi, M.; Losano, J.D.A.; Lucio, C.F.; Lima Veiga, G.A.; Franco, M.V.M.J.; Vannucchi, C.I. Fatty acid content in epididymal fluid and spermatozoa during sperm maturation in dogs. *J. Anim. Sci. Biotechnol.* **2017**, *8*, 18. [CrossRef]
160. Aksoy, Y.; Aksoy, H.; Altinkaynak, K.; Aydin, H.R.; Ozkan, A. Sperm fatty acid composition in subfertile men. *Prostaglandins Leukot. Essent. Fat. Acids* **2006**, *75*, 75–79. [CrossRef] [PubMed]
161. Macıas-Garcıa, B.; González-Fernández, L.; Ortega-Ferrusola, C.; Salazar-Sandoval, C.; Rodríguez, A.M.; Martinez, H.R.; Tapia, J.A.; Morcuende, D.; Peña, F.J. Membrane lipids of the stallion spermatozoon in relation to sperm quality and susceptibility to lipid peroxidation. *Reprod. Domest. Anim.* **2011**, *46*, 141–148. [CrossRef]
162. Kim, N.; Nakamura, H.; Masaki, H.; Kumasawa, K.; Hirano, K.-I.; Kimura, T. Effect of lipid metabolism on male fertility. *Biochem. Biophys. Res. Commun.* **2017**, *485*, 686–692. [CrossRef] [PubMed]
163. Díaz, R.; Inostroza, K.; Risopatrón, J.; Sanchez, R.; Sepúlveda, N. Identification of fatty acids in canine seminal plasma. *Andrologia* **2014**, *46*, 194–197. [CrossRef]
164. Lucio, C.F.; Brito, M.M.; Angrimani, D.S.R.; Belaz, K.R.A.; Morais, D.; Zampieri, D.; Losano, J.D.A.; Assumpção, M.E.O.A.; Nichi, M.; Eberlin, M.N.; et al. Lipid composition of the canine sperm plasma membrane as markers of sperm motility. *Reprod. Domest. Anim.* **2017**, *52*, 208–213. [CrossRef]
165. Lin, Y.Q.; Li, J.Y.; Wang, H.Y.; Liu, J.; Zhang, C.L.; Wang, W.T.; Liu, J.; Li, N.; Jin, S.H. Cloning and identification of a novel sperm binding protein, HEL-75, with antibacterial activity and expressed in the human epididymis. *Hum. Reprod.* **2008**, *23*, 2086–2094. [CrossRef]
166. Liu, G.; Donoghue, A.; Venkitanarayanan, K.; Donoghue, D. Caprylic acid maintains rooster sperm viability during in vitro storage. *Poult. Sci.* **2008**, *87*, 16.
167. Ortega-Ferrusola, C.; González Fernández, L.; Morrell, J.M.; Salazar Sandoval, C.; Macıas-Garcıa, B.; Rodríguez-Martinez, H.; Tapia, J.A.; Peña, F.J. Lipid peroxidation, assessed with BODIPY-C11, increases after cryopreservation of stallion spermatozoa, is stallion-dependent and is related to apoptotic-like changes. *Reproduction* **2009**, *138*, 55–63. [CrossRef] [PubMed]
168. Zerbinati, C.; Caponecchia, L.; Rago, R.; Leoncini, E.; Bottaccioli, A.G.; Ciacciarelli, M.; Pacelli, A.; Salacone, P.; Sebastianelli, A.; Pastore, A.; et al. Fatty acids profiling reveals potential candidate markers of semen quality. *Andrology* **2016**, *4*, 1094–1101. [CrossRef]
169. Zalata, A.A.; Christophe, A.B.; Depuydt, C.E.; Schoonjans, F.; Comhaire, F.H. The fatty acid composition of phospholipids of spermatozoa from infertile patients. *Mol. Hum. Reprod.* **1998**, *4*, 111–118. [CrossRef] [PubMed]
170. Tavilani, H.; Doosti, M.; Nourmohammadi, I.; Mahjub, H.; Vaisiraygani, A.; Salimi, S.; Hosseinipanah, S.M. Lipid composition of spermatozoa in normozoospermic and asthenozoospermic males. *Prostaglandins Leukot. Essent. Fat. Acids* **2007**, *77*, 45–50. [CrossRef] [PubMed]
171. Falsig, A.-M.L.; Gleerup, C.S.; Knudsen, U.B. The influence of omega-3 fatty acids on semen quality markers: A systematic PRISMA review. *Andrology* **2019**, *7*, 794–803. [CrossRef] [PubMed]
172. Andersen, J.M.; Rønning, P.O.; Herning, H.; Bekken, S.D.; Haugen, T.B.; Witczak, O. Fatty acid composition of spermatozoa is associated with BMI and with semen quality. *Andrology* **2016**, *4*, 857–865. [CrossRef] [PubMed]
173. Cooray, A.; Kim, J.H.; Chae, M.R.; Lee, S.; Lee, K.P. Perspectives on Potential Fatty Acid Modulations of Motility Associated Human Sperm Ion Channels. *Int. J. Mol. Sci.* **2022**, *23*, 3718. [CrossRef] [PubMed]
174. Hossain, M.S.; Tareq, K.M.A.; Hammano, K.-I.; Tsujii, H. Effect of fatty acids on boar sperm motility, viability and acrosome reaction. *Reprod. Med. Biol.* **2007**, *6*, 235–239. [CrossRef] [PubMed]
175. Tavilani, H.; Doosti, M.; Abdi, K.; Vaisiraygani, A.; Joshaghani, H.R. Decreased polyunsaturated and increased saturated fatty acid concentration in spermatozoa from asthenozoospermic males as compared with normozoospermic males. *Andrologia* **2006**, *38*, 173–178. [CrossRef]
176. Martínez-Soto, J.C.; Landeras, J.; Gadea, J. Spermatozoa and seminal plasma fatty acids as predictors of cryopreservation success. *Andrology* **2013**, *1*, 365–375. [CrossRef]
177. delBarco-Trillo, J.; Roldan, E.R.S. Effects of metabolic rate and sperm competition on the fatty-acid composition of mammalian sperm. *J. Evol. Biol.* **2014**, *27*, 55–62. [CrossRef] [PubMed]
178. Waterhouse, K.E.; Hofmo, P.O.; Tverdal, A.; Miller, R.R., Jr. Within and between breed differences in freezing tolerance and plasma membrane fatty acid composition of boar sperm. *Reproduction* **2006**, *131*, 887–894. [CrossRef] [PubMed]
179. Varesi, S.; Vernocchi, V.; Faustini, M.; Luvoni, G.C. Morphological and acrosomal changes of canine spermatozoa during epididymal transit. *Acta Vet. Scand.* **2013**, *55*, 17. [CrossRef] [PubMed]
180. Tanghe, S.; De Smet, S. Does sow reproduction and piglet performance benefit from the addition of n-3 polyunsaturated fatty acids to the maternal diet? *Vet. J.* **2013**, *197*, 560–569. [CrossRef] [PubMed]
181. Martínez-Soto, J.C.; Domingo, J.C.; Cordobilla, B.; Nicolás, M.; Fernández, L.; Albero, P.; Gadea, J.; Landeras, J. Dietary supplementation with docosahexaenoic acid (DHA) improves seminal antioxidant status and decreases sperm DNA fragmentation. *Syst. Biol. Reprod. Med.* **2016**, *62*, 387–395. [CrossRef]
182. Cerolini, S.; Maldjian, A.; Surai, P.; Noble, R. Viability, susceptibility to peroxidation and fatty acid composition of boar semen during liquid storage. *Anim. Reprod. Sci.* **2000**, *58*, 99–111. [CrossRef] [PubMed]
183. Evans, H.C.; Dinh, T.T.N.; Hardcastle, M.L.; Gilmore, A.A.; Ugur, M.R.; Hitit, M.; Jousan, F.D.; Nicodemus, M.C.; Memili, E. Advancing Semen Evaluation Using Lipidomics. *Front. Vet. Sci.* **2021**, *8*, 601794. [CrossRef]
184. Aboagla, E.M.-E.; Terada, T. Trehalose-enhanced fluidity of the goat sperm membrane and its protection during freezing. *Biol. Reprod.* **2003**, *69*, 1245–1250. [CrossRef]

185. Tapia, J.A.; Macıas-Garcıa, B.; Miro-Moran, A.; Ortega-Ferrusola, C.; Salido, G.M.; Peña, F.J.; Aparicio, I.M. The membrane of the mammalian spermatozoa: Much more than an inert envelope. *Reprod. Domest. Anim.* **2012**, *47*, 65–75. [CrossRef]
186. Darin-Bennett, A.; Poulos, A.; White, I.G. The phospholipids and phospholipid-bound fatty acids and aldehydes of dog and fowl spermatozoa. *J. Reprod. Fertil.* **1974**, *41*, 471–474. [CrossRef]
187. Conquer, J.A.; Martin, J.B.; Tummon, I.; Watson, L.; Tekpetey, F. Fatty acid analysis of blood serum, seminal plasma, and spermatozoa of normozoospermic vs. asthenozoospermic males. *Lipids* **1999**, *34*, 793–799. [CrossRef] [PubMed]
188. Watanabe, H.; Takeo, T.; Tojo, H.; Sakoh, K.; Berger, T.; Nakagata, N.; Mak, T.W.; Kondoh, G. Lipocalin 2 binds to membrane phosphatidylethanolamine to induce lipid raft movement in a PKA-dependent manner and modulates sperm maturation. *Development* **2014**, *141*, 2157–2164. [CrossRef] [PubMed]
189. Adada, M.; Luberto, C.; Canals, D. Inhibitors of the sphingomyelin cycle: Sphingomyelin synthases and sphingomyelinases. *Chem. Phys. Lipids* **2016**, *197*, 45–59. [CrossRef] [PubMed]
190. Nikolopoulou, M.; Soucek, D.A.; Vary, J.C. Changes in the lipid content of boar sperm membranes during epididymal maturation. *Biochim. Biophys. Acta* **1985**, *815*, 486–498. [CrossRef] [PubMed]
191. Parks, J.E.; Arion, J.W.; Foote, R.H. Lipids of plasma membrane and outer acrosomal membranes from bovine spermatozoa. *Biol. Reprod.* **1987**, *37*, 1249–1258. [CrossRef] [PubMed]
192. Cross, N.L. Sphingomyelin modulates capacitation of human sperm in vitro. *Biol. Reprod.* **2000**, *63*, 1129–1134. [CrossRef] [PubMed]
193. Simons, K.; Ikonen, E. Functional rafts in cell membranes. *Nature* **1997**, *387*, 569–572. [CrossRef]
194. Leblond, E.; Desnoyers, L.; Manjunath, P. Phosphorylcholine-binding proteins from the seminal fluids of different species share antigenic determinants with the major proteins of bovine seminal plasma. *Mol. Reprod. Dev.* **1993**, *34*, 443–449. [CrossRef]
195. Mogielnicka-Brzozowska, M.; Kordan, W. Characteristics of selected seminal plasma proteins and their application in the improvement of the reproductive processes in mammals. *Pol. J. Vet. Sci.* **2011**, *14*, 489–499. [CrossRef]
196. Sèdes, L.; Thirouard, L.; Maqdasy, S.; Garcia, M.; Caira, F.; Lobaccaro, J.-M.A.; Beaudoin, C.; Volle, D.H. Cholesterol: A Gatekeeper of Male Fertility? *Front. Endocrinol.* **2018**, *9*, 369. [CrossRef]
197. Schäfer-Somi, S.; Budik, S. The ABCA1 blocking agent probucol decreases capacitation in ejaculated dog spermatozoa. *Acta Vet. Scand.* **2020**, *62*, 2. [CrossRef] [PubMed]
198. Payne, A.H.; Youngblood, G.L. Regulation of expression of steroidogenic enzymes in Leydig cells. *Biol. Reprod.* **1995**, *52*, 217–225. [CrossRef] [PubMed]
199. Dearing, C.G.; Lindsay, K.S. Corrected cholesterol, a novel marker for predicting semen post-thaw quality: A pilot study. *Hum. Fertil.* **2017**, *22*, 94–103. [CrossRef] [PubMed]
200. Schäfer-Somi, S.; Colombo, M.; Luvoni, G.C. Canine Spermatozoa—Predictability of Cryotolerance. *Animals* **2022**, *12*, 733. [CrossRef] [PubMed]
201. Koderle, M.; Aurich, C.; Schäfer-Somi, S. The influence of cryopreservation and seminal plasma on the chromatin structure of dog spermatozoa. *Theriogenology* **2009**, *72*, 1215–1220. [CrossRef] [PubMed]
202. Manaskova-Postlerova, P.; Davidova, N.; Jonakova, V. Biochemical and binding characteristics of boar epididymal fluid proteins. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **2011**, *879*, 100–106. [CrossRef] [PubMed]
203. Busso, D.; Onate-Alvarado, M.J.; Balboa, E.; Castro, J.; Lizama, C.; Morales, G.; Vargas, S.; Härtel, S.; Moreno, R.D.; Zanlungo, S. Spermatozoa from mice deficient in Niemann-Pick disease type C2 (NPC2) protein have defective cholesterol content and reduced in vitro fertilizing ability. *Reprod. Fertil. Dev.* **2014**, *26*, 609–621. [CrossRef] [PubMed]
204. Okamura, N.; Kiuchi, S.; Tamba, M.; Kashima, T.; Hiramoto, S.; Baba, T.; Dacheux, F.; Dacheux, J.L.; Sugita, Y.; Jin, Y.Z. A porcine homolog of the major secretory protein of human epididymis, HE1, specifically binds cholesterol. *Biochim. Biophys. Acta* **1999**, *1438*, 377–387. [CrossRef] [PubMed]
205. Légaré, C.; Thabet, M.; Gatti, J.-L.; Sullivan, R. HE1/NPC2 status in human reproductive tract and ejaculated spermatozoa: Consequence of vasectomy. *Mol. Hum. Reprod.* **2006**, *12*, 461–468. [CrossRef]
206. Girouard, J.; Frenette, G.; Sullivan, R. Seminal plasma proteins regulate the association of lipids and proteins within detergent-resistant membrane domains of bovine spermatozoa. *Biol. Reprod.* **2008**, *78*, 921–931. [CrossRef]
207. Drobnis, E.Z.; Crowe, L.M.; Berger, T.; Anchordoguy, T.J.; Overstreet, J.W.; Crowe, J.H. Cold shock damage is due to lipid phase transitions in cell membranes: A demonstration using sperm as a model. *J. Exp. Zool.* **1993**, *265*, 432–437. [CrossRef] [PubMed]
208. Yeste, M. Recent advances in boar sperm cryopreservation: State of the art and current perspectives. *Reprod. Domest. Anim.* **2015**, *50*, 71–79. [CrossRef] [PubMed]
209. de Neergaard, R.; Nielsen, J.E.; Jørgensen, A.; Toft, B.G.; Goetze, J.P.; Jørgensen, N. Positive association between cholesterol in human seminal plasma and sperm counts: Results from a cross-sectional cohort study and immunohistochemical investigations. *Andrology* **2018**, *6*, 817–828. [CrossRef]
210. Yang, K.; Han, X. Lipidomics: Techniques, Applications, and Outcomes Related to Biomedical Sciences. *Trends Biochem. Sci.* **2016**, *41*, 954–969. [CrossRef]
211. Lam, S.M.; Wang, Z.; Li, B.; Shui, G. High-coverage lipidomics for functional lipid and pathway analyses. *Anal. Chim. Acta* **2021**, *1147*, 199–210. [CrossRef]
212. Ponglowhapan, S.; Essén-Gustavsson, B.; Linde Forsberg, C. Influence of glucose and fructose in the extender during long-term storage of chilled canine semen. *Theriogenology* **2004**, *62*, 1498–1517. [CrossRef]

213. Rigau, T.; Farré, M.; Ballester, J.; Mogas, T.; Peña, A.; Rodríguez-Gil, J.E. Effects of glucose and fructose on motility patterns of dog spermatozoa from fresh ejaculates. *Theriogenology* **2001**, *56*, 801–815. [CrossRef] [PubMed]
214. Martínez-Barbitta, M.; Rivera Salinas, C. Evaluation of Chilled Dog Semen Extended with Sperm Activator. *Front. Vet. Sci.* **2022**, *8*, 764750. [CrossRef]
215. Fleet, T.L.; Varner, D.D.; Blanchard, T.L.; Vogelsang, M.M.; Thompson, J.A. Use of Heparin to Accelerate Capacitation of Equine Spermatozoa In Vivo. *Biol. Reprod.* **1995**, *52*, 713–718. [CrossRef]
216. Parrish, J.J.; Susko-Parrish, J.; Winer, M.A.; First, N.L. Capacitation of bovine sperm by heparin. *Biol. Reprod.* **1988**, *38*, 1171–1180. [CrossRef]
217. Florman, H.M.; First, N.L. Regulation of acrosomal exocytosis I. Sperm capacitation is required for the induction of acrosome reaction by the bovine zona pellucida in vitro. *Dev. Biol.* **1988**, *128*, 453–463. [CrossRef]
218. Handrow, R.R.; Lenz, R.W.; Ax, R.L. Structural comparisons among glycosaminoglycans to promote acrosome reactions in bovine spermatozoa. *Biochem. Biophys. Res. Commun.* **1982**, *107*, 1326–1332. [CrossRef]
219. Lenz, R.W.; Bellin, M.E.; Ax, R.L. Rabbit spermatozoa undergo acrosome reaction in the presence of glycosaminoglycans. *Gamete Res.* **1983**, *8*, 11–19. [CrossRef]
220. Reyes, T.; Carranco, A.; Hernández, O.; Rosado, A.; Merchant, H.; Delgado, N.M. Glycosamineglycan sulfate as acrosomal reaction-inducing factor of follicular fluid. *Arch. Androl.* **1984**, *12*, 203–209. [CrossRef]
221. Miller, D.J.; Hunter, A.G. Effect of osmolality and glycosaminoglycans on motility, capacitation, acrosome reaction and in vitro fertilization of bovine ejaculated sperm. *J. Dairy Sci.* **1986**, *69*, 2915–2924. [CrossRef]
222. Kawakami, E.; Arai, T.; Oishi, I.; Hori, T.; Tsutsui, T. Induction of dog sperm capacitation by glycosaminoglycans and glycosaminoglycan amounts of oviductal and uterine fluids in bitches. *J. Vet. Med. Sci.* **2000**, *62*, 65–68. [CrossRef] [PubMed]
223. Kawakami, E.; Arai, T.; Nakamura, U. Effects of medium containing heparin and theophylline on capacitation and metabolic enzyme activities of ejaculated spermatozoa from dogs with asthenozoospermia. *Anim. Reprod. Sci.* **1999**, *54*, 251–259. [CrossRef] [PubMed]
224. Risopatrón, J.; Catalán, S.; Sepúlveda, N.G.; Sanchez, R. Effect of different concentrations of heparin on canine sperm capacitation in vitro. *Rev. Cient. Fac. Cien. Vet. Univ. Zulia* **2005**, *15*, 528–535.
225. Cunha, A.T.M.; Carvalho, J.O.; Guimarães, A.L.S.; Leme, L.O.; Caixeta, F.M.; Viana, J.H.M.; Dode, M.A.N. Bovine epididymal spermatozoa treatment for in vitro fertilization: Heparin accelerates fertilization and enables a reduction in coincubation time. *PLoS ONE* **2019**, *14*, e0209692. [CrossRef]
226. Miller, D.J.; First, N.L.; Ax, R.L. Isolation and characterization of seminal fluid proteins that bind heparin. *Adv. Exp. Med. Biol.* **1987**, *219*, 597–601. [CrossRef]
227. Nass, S.J.; Miller, D.J.; Winer, M.A.; Ax, R.L. Male accessory sex glands produce heparin-binding proteins that bind to cauda epididymal spermatozoa and are testosterone dependent. *Mol. Reprod. Dev.* **1990**, *25*, 237–246. [CrossRef] [PubMed]
228. Manjunath, P.; Chandonnet, L.; Leblond, E.; Desnoyers, L. Major proteins of bovine seminal vesicles bind to spermatozoa. *Biol. Reprod.* **1994**, *50*, 27–37. [CrossRef] [PubMed]
229. Manjunath, P.; Thérien, I. Role of seminal plasma phospholipids-binding proteins in sperm membrane lipid modification that occurs during capacitation. *J. Reprod. Immunol.* **2002**, *53*, 109–119. [CrossRef] [PubMed]
230. Parrish, J.J. Bovine in vitro fertilization: In vitro oocyte maturation and sperm capacitation with heparin. *Theriogenology* **2014**, *81*, 67–73. [CrossRef] [PubMed]
231. Miller, A.; Ax, R.L. Carbohydrates and fertilization in animals. *Mol. Reprod. Dev.* **1990**, *26*, 184–198. [CrossRef] [PubMed]
232. de Souza, F.F.; Martins, M.I.M.; dos Santos Fernandes, C.E.; Ribolla, P.E.; Lopes, M.D. Herapin-binding proteins of canine seminal plasma. *Theriogenology* **2006**, *66*, 1606–1609. [CrossRef] [PubMed]
233. Dube, J.Y.; Lazure, C.; Tremblay, R.R. Dog prostate arginine esterase is related to human prostate specific antigen. *Clin. Investig. Med.* **1986**, *9*, 51–54. [PubMed]
234. Clements, J.A. The glandular kallikrein family of enzymes: Tissue-specific expression and hormonal regulation. *Endocr. Rev.* **1989**, *10*, 393–419. [CrossRef] [PubMed]
235. España, F.; Sandaz-Cuenca, J.; Vera, C.D.; Estelles, A.; Gilabert, J. A quantitative ELISA for the measurement of complexes of prostate-specific antigen with protein C inhibitor when using a purified standard. *J. Lab. Clin. Med.* **1993**, *122*, 711–719. [CrossRef]
236. Kumar, V.; Hassan, M.I.; Tomar, A.K.; Kashav, T.; Nautiyal, J.; Singh, S.; Singh, T.P.; Yadav, S. Proteomic analysis of heparin-binding proteins from human seminal plasma: A step towards identification of molecular markers of male fertility. *J. Biosci.* **2009**, *34*, 899–908. [CrossRef]
237. Patel, M.; Gandotra, V.K.; Cheema, R.S.; Bansal, A.K.; Kumar, A. Seminal Plasma Heparin Binding Proteins Improve Semen Quality by Reducing Oxidative Stress during Cryopreservation of Cattle Bull Semen. *Asian Australas. J. Anim. Sci.* **2016**, *29*, 1247–1255. [CrossRef] [PubMed]
238. Pinto, F.M.; Odriozola, A.; Candenas, L.; Subirán, N. The Role of Sperm Membrane Potential and Ion Channels in Regulating Sperm Function. *Int. J. Mol. Sci.* **2023**, *24*, 6995. [CrossRef] [PubMed]
239. Benko, F.; Urminská, D.; Ďuračka, M.; Tvrdá, E. Signaling Roleplay between Ion Channels during Mammalian Sperm Capacitation. *Biomedicines* **2023**, *11*, 2519. [CrossRef] [PubMed]

240. Swain, D.K.; Sharma, P.; Shah, N.; Sethi, M.; Mahajan, A.; Gupta, S.; Mishra, A.K.; Yadav, S. Introduction to the pathways involved in the activation and regulation of sperm motility: A review of the relevance of ion channels. *Anim. Reprod. Sci.* **2022**, *246*, 107052. [CrossRef] [PubMed]
241. Lishko, P.V.; Kirichok, Y.; Ren, D.; Navarro, B.; Chung, J.; Clapham, D.E. The Control of Male Fertility by Spermatozoan Ion Channels. *Annu. Rev. Physiol.* **2012**, *74*, 453–475. [CrossRef] [PubMed]
242. Puga Molina, L.C.; Luque, G.M.; Balestrini, P.A.; Marín-Briggiler, C.I.; Romarowski, A.; Buffone, M.G. Molecular Basis of Human Sperm Capacitation. *Front. Cell Dev. Biol.* **2018**, *6*, 72. [CrossRef] [PubMed]
243. Aonuma, S.; Okabe, M.; Kawaguchi, M.; Kishi, Y. Zinc effects on mouse spermatozoa and in vitro fertilization. *J. Reprod. Fertil.* **1981**, *63*, 463–466. [CrossRef]
244. Björndahl, L.; Kvist, U. Influence of seminal vesicular fluid on the zinc content of human sperm chromatin. *Int. J. Androl.* **1990**, *13*, 232–237. [CrossRef]
245. Lee, S.R. Critical Role of Zinc as Either an Antioxidant or a Prooxidant in Cellular Systems. *Oxid. Med. Cell. Longev.* **2018**, *2018*, 9156285. [CrossRef]
246. Levaot, N.; Hershfinkel, M. How cellular Zn^{2+} signaling drives physiological functions. *Cell Calcium* **2018**, *75*, 53–63. [CrossRef]
247. Henkel, R.; Bittner, J.; Weber, R.; Huther, F.; Miska, W. Relevance of zinc in human spermatozoa flagella and its relation to motility. *Fertil. Steril.* **1999**, *71*, 1138–1143. [CrossRef] [PubMed]
248. Pereira, A.M.; Maia, M.R.G.; Fonseca, A.J.M.; Cabrita, A.R.J. Zinc in Dog Nutrition, Health and Disease: A Review. *Animals* **2021**, *11*, 978. [CrossRef] [PubMed]
249. Johnson, L.; Wikstrom, S.; Nylander, G. The vehicle for zinc in the prostatic secretion of dogs. *Scand. J. Urol. Nephrol.* **1969**, *3*, 9–11. [CrossRef] [PubMed]
250. Riffo, M.; Leiva, S.; Astudillo, J. Effect of zinc on human sperm motility and the acrosome reaction. *Int. J. Androl.* **1992**, *15*, 229–237. [CrossRef] [PubMed]
251. Sorensen, M.B.; Stoltenberg, M.; Danscher, G.; Ernst, E. Chelation of intracellular zinc ions affects human sperm cell motility. *Mol. Hum. Reprod.* **1999**, *5*, 338–341. [CrossRef] [PubMed]
252. Björndahl, L.; Kjellberg, S.; Roomans, G.M.; Kvist, U. The human sperm nucleus takes up zinc at ejaculation. *Int. J. Androl.* **1986**, *9*, 77–80. [CrossRef]
253. Foresta, C.; Garolla, A.; Cosci, I.; Menegazzo, M.; Ferigo, M.; Gandin, V.; De Toni, L. Role of zinc trafficking in male fertility: From germ to sperm. *Hum. Reprod.* **2014**, *29*, 1134–1145. [CrossRef]
254. Samanta, L.; Parida, R.; Dias, T.R.; Agarwal, A. The enigmatic seminal plasma: A proteomics insight from ejaculation to fertilization. *Reprod. Biol. Endocrinol.* **2018**, *16*, 41. [CrossRef] [PubMed]
255. Arver, S. Zinc and zinc ligands in human seminal plasma. III. The principal low molecular weight zinc ligands in prostatic secretion and seminal plasma. *Acta Physiol. Scand.* **1982**, *116*, 67–73. [CrossRef]
256. Hołody, D.; Strzezek, J. Heparin- and Zn^{2+}-binding proteins from boar seminal plasma. *Acta Biochim. Pol.* **1999**, *46*, 935–939. [CrossRef]
257. Siciliano, L.; De Stefano, C.; Petroni, M.F.; Vivacqua, A.; Rago, V.; Carpino, A. Prostatic origin of a zinc binding high molecular weight protein complex in human seminal plasma. *Mol. Hum. Reprod.* **2000**, *6*, 215–218. [CrossRef] [PubMed]
258. Mogielnicka-Brzozowska, M.; Fraser, L.; Czarzasta, J.; Kordan, W. Isolation and characterization of zinc-binding proteins of canine seminal plasma. *Pol. J. Vet. Sci.* **2012**, *15*, 493–498. [CrossRef] [PubMed]
259. Caprino, A.; Siciliano, L.; Petroni, M.F.; De Stefano, C.; Aquila, S.; Andó, S. Low seminal zinc bound to high molecular weight proteins in asthenozoospermic patients: Evidence of increased sperm zinc content in oligoasthenozoospermic patients. *Hum. Reprod.* **1998**, *13*, 111–114. [CrossRef] [PubMed]
260. Ghaoui, R.E.-H.; Thomson, P.C.; Evans, G.; Maxwell, W.M.C. Characterization and localization of membrane vesicles in ejaculate fractions from the ram, boar and stallion. *Reprod. Domest. Anim.* **2004**, *39*, 173–180. [CrossRef] [PubMed]
261. Vivacqua, A.; Siciliano, L.; Sabato, M.; Palma, A.; Caprino, A. Prostasomes as zinc ligands in human seminal plasma. *Int. J. Androl.* **2004**, *27*, 27–31. [CrossRef] [PubMed]
262. Burden, H.P.; Holmes, C.H.; Persad, R.; Whittington, K. Prostasomes—Their effects on human male reproduction and fertility. *Hum. Reprod. Update* **2006**, *12*, 283–292. [CrossRef] [PubMed]
263. Mogielnicka-Brzozowska, M.; Strzeżek, R.; Wasilewska, K.; Kordan, W. Prostasomes of canine seminal plasma—Zinc-binding ability and effects on motility characteristics and plasma membrane integrity of spermatozoa. *Reprod. Domest. Anim.* **2015**, *50*, 484–491. [CrossRef] [PubMed]
264. Fallah, A.; Mohammad-Hasani, A.; Colagar, A.H. Zinc is an Essential Element for Male Fertility: A Review of Zn Roles in Men's Health, Germination, Sperm Quality, and Fertilization. *J. Reprod. Infertil.* **2018**, *19*, 69–81. [PubMed]
265. Kerns, K.; Zigo, M.; Sutovsky, P. Zinc: A Necessary Ion for Mammalian Sperm Fertilization Competency. *Int. J. Mol. Sci.* **2018**, *19*, 4097. [CrossRef]
266. Vickram, S.; Rohini, K.; Srinivasan, S.; Nancy Veenakumari, D.; Archana, K.; Anbarasu, K.; Jeyanthi, P.; Thanigaivel, S.; Gulothungan, G.; Rajendiran, N.; et al. Role of Zinc (Zn) in Human Reproduction: A Journey from Initial Spermatogenesis to Childbirth. *Int. J. Mol. Sci.* **2021**, *22*, 2188. [CrossRef]
267. Kageyama, A.; Terakawa, J.; Ito, J.; Kashiwazaki, N. Roles of zinc signaling in mammalian reproduction. *Met. Res.* **2022**, *2*, rev-64–rev-73. [CrossRef]

268. Florman, H.M. Sequential focal and global elevations of sperm intracellular Ca^{2+} are initiated by the zona pellucida during acrosomal exocytosis. *Dev. Biol.* **1994**, *165*, 152–164. [CrossRef] [PubMed]
269. Szász, F.; Sirivaidyapong, S.; Cheng, F.P.; Voorhout, W.F.; Marks, A.; Colenbrander, B.; Solti, L.; Gadella, B.M. Detection of calcium ionophore induced membrane changes in dog sperm as a simple method to predict the cryopreservability of dog semen. *Mol. Reprod. Dev.* **2000**, *55*, 289–298. [CrossRef]
270. Naz, T.; Chakraborty, S.; Saha, S. Role of fatty acids and calcium in male reproduction. *Reprod. Dev. Med.* **2022**, *6*, 57–64. [CrossRef]
271. Patterson, D.F. Understanding and controlling inherited diseases in dog and cat. *Tidjschr Diergeneeskd* **1993**, *118*, 23–27.
272. Axnér, E.; Rasmus, L.S.; Melangen, T. Factors affecting reproductive performance in the Swedish Bernese mountain dog. *Acta Vet. Scand.* **2022**, *64*, 28. [CrossRef] [PubMed]
273. Dahlbom, M.; Andersson, M.; Juga, J.; Alanko, M. Fertility parameters in male Irish wolfounds: A two-year follow-up study. *J. Small Anim. Pract.* **1997**, *38*, 547–550. [CrossRef] [PubMed]
274. Hallberg, I.; Olsson, H.; Lau, A.; Wallander, S.; Snell, A.; Bergman, D.; Holst, B.S. Endocrine and dog factors associated with semen quality. *Sci. Rep.* **2024**, *14*, 718. [CrossRef]
275. Mascarenhas, R.M.; de Paula Tarcízio Antonio, R. Influence of Breed and Individual Variation in the Quality of Frozen Canine Semen. *J. Vet. Sci. Technol.* **2018**, *9*, 1. [CrossRef]

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Review

The Omics Revolution in Understanding Chicken Reproduction: A Comprehensive Review

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Simple Summary: This review article discusses the impact of omics technologies on the understanding of chicken reproduction. Omics technologies such as genomics, transcriptomics, proteomics, and metabolomics have provided valuable insights into the molecular mechanisms underlying chicken reproduction. These technologies have allowed researchers to identify the key genes, proteins, and metabolites involved in reproductive processes, as well as their interactions. This article highlights the importance of omics approaches in advancing our understanding of chicken reproduction and their potential applications in improving reproductive efficiency and health in poultry production. Overall, this article emphasizes the transformative impact of omics technologies in unraveling the complexities of chicken reproduction.

Abstract: Omics approaches have significantly contributed to our understanding of several aspects of chicken reproduction. This review paper gives an overview of the use of omics technologies such as genomics, transcriptomics, proteomics, and metabolomics to elucidate the mechanisms of chicken reproduction. Genomics has transformed the study of chicken reproduction by allowing the examination of the full genetic makeup of chickens, resulting in the discovery of genes associated with reproductive features and disorders. Transcriptomics has provided insights into the gene expression patterns and regulatory mechanisms involved in reproductive processes, allowing for a better knowledge of developmental stages and hormone regulation. Furthermore, proteomics has made it easier to identify and quantify the proteins involved in reproductive physiology to better understand the molecular mechanisms driving fertility, embryonic development, and egg quality. Metabolomics has emerged as a useful technique for understanding the metabolic pathways and biomarkers linked to reproductive performance, providing vital insights for enhancing breeding tactics and reproductive health. The integration of omics data has resulted in the identification of critical molecular pathways and biomarkers linked with chicken reproductive features, providing the opportunity for targeted genetic selection and improved reproductive management approaches. Furthermore, omics technologies have helped to create biomarkers for fertility and embryonic viability, providing the poultry sector with tools for effective breeding and reproductive health management. Finally, omics technologies have greatly improved our understanding of chicken reproduction by revealing the molecular complexities that underpin reproductive processes.

Keywords: omics; proteomics; transcriptomics; metabolomics; chicken; reproduction

1. Introduction

Genomics, transcriptomics, proteomics, and metabolomics are all important approaches for better understanding chicken reproduction [1–3] (Figure 1). Genomic research has helped discover genes linked to reproductive features like fertility and egg

production. Transcriptomics is used to understand the gene expression patterns at various phases of reproduction [4]. Proteomics research has provided insight into the proteins involved in reproductive processes, whereas metabolomics has assisted in discovering the metabolic pathways critical for fertility [5]. The integration of omics data has provided complete insights into the molecular mechanisms underpinning chicken reproduction, allowing for improved breeding methods and reproductive success in poultry [6].

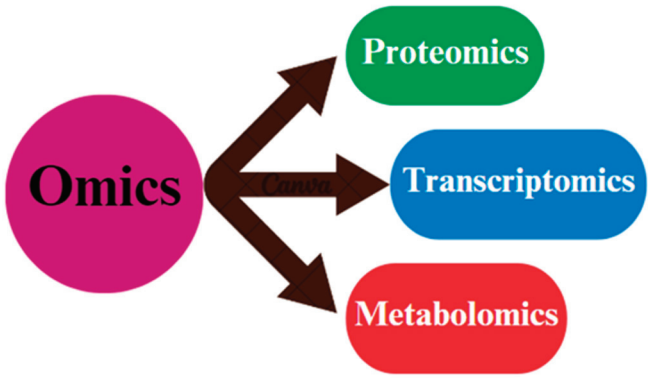


Figure 1. A schematic illusion diagram of the omics techniques (proteomics, transcriptomics, and metabolomics) that are commonly applied in biological samples.

Genomic research has helped to identify the genetic variants related to desirable reproductive features in chickens, such as egg quality, fertility, and broiler performance [7]. By studying the full genome, we can identify the genes associated with specific reproductive features, allowing for selective breeding to boost productivity and efficiency [8]. Single-cell sequencing methods have been used to evaluate gene expression patterns at the single-cell level, providing unparalleled resolution for investigating the variety of cell populations involved in chicken reproduction [9]. This method has revealed previously undiscovered cell types, regulatory networks, and dynamic changes throughout follicle development, embryogenesis, and gametogenesis.

Transcriptomic analysis enables the investigation of gene expression patterns across several stages of poultry reproduction. By analyzing the mRNA levels in reproductive organs or cells, we can identify the essential genes involved in follicle development, ovulation, sperm generation, and other reproductive processes [10]. This knowledge aids in understanding the regulatory networks that govern fertility and reproductive performance. Proteomics research has enhanced our understanding of the proteins involved in chicken reproduction and the intricate protein interactions that govern reproductive activities via the study of the entire set of proteins found in reproductive organs or cells [3,11]. Proteomic techniques have found biomarkers associated with fertility, egg quality, and sperm function, allowing for the development of diagnostic tools and breeding strategies [12].

Metabolomic analysis is concerned with investigating the entire range of small molecules (metabolites) found in biological samples and revealing metabolic pathways crucial for fertility, embryonic development, and reproductive health in chickens by characterizing the metabolites linked with reproductive activities. Metabolomics has revealed information on nutrient use, energy metabolism, and biomarkers in poultry reproduction [13,14].

Epigenomic research, concentrating on DNA methylation, histone modifications, and noncoding RNAs, has revealed the epigenetic regulation of gene expression in chicken reproductive organs [15,16]. We can learn about how environmental factors, stressors, and nutritional aspects affect reproductive outcomes in chickens by looking at epigenetic changes. Epigenomics has provided vital information about the heredity and plasticity of reproductive characteristics [17]. Metagenomic investigation of the chicken reproductive microbiome has arisen as a new area of study, providing insight into the relationships between host genetics, microbiota composition, and reproductive health. We can investigate

the role of the microbiome in regulating fertility, disease resistance, and immunological responses in chickens by identifying the microbial populations in the reproductive tract [18]. Metagenomics has implications for developing techniques to modify the microbiome for better reproductive health [19].

Deciphering the intricate regulatory networks in chicken reproduction has been possible through the integration of several omics datasets, including those from transcriptomics, proteomics, metabolomics, and genomics [20]. Through the integration of several omics datasets, we can reveal the associations between genotype and phenotype, pinpoint pivotal biomarkers, and clarify the functional pathways that contribute to reproductive functioning [21,22]. A systems biology viewpoint on chicken reproduction is offered by multiomics integration, which provides a thorough grasp of the molecular pathways involved. Large-scale omics data in chicken reproductive studies may now be efficiently analyzed and interpreted because of developments in machine learning techniques and bioinformatics tools [23]. The identification of novel genetic markers, regulatory elements, and functional annotations pertinent to poultry reproduction has been expedited by bioinformatic pipelines for processing omics data, genome assembly, pathway analysis, and predictive modeling [24]. Additionally, phenotype–genotype correlations and predictions of complex features in chickens have been modeled using artificial intelligence techniques [25]. One of the most significant advantages of omics techniques is their ability to generate large volumes of biological data [26]. Integrating genomic, transcriptomic, proteomic, and metabolomic data provides a comprehensive picture of the molecular mechanisms that control chicken reproduction [2]. By merging multiomics datasets, we can find critical genetic regulators, signaling pathways, and metabolic networks that control reproductive features, providing a comprehensive picture of poultry genetics and reproduction. In conclusion, omics technologies have altered poultry genetics and reproductive research by enabling the investigation of the complicated molecular mechanisms that underpin these processes. The application of genomics, transcriptomics, proteomics, and metabolomics has improved our understanding of chicken reproduction, resulting in advances in breeding methods, disease management, and overall poultry production efficiency. Overall, recent advances in omics technology have improved our understanding of chicken reproduction by giving deep molecular insights, revealing novel biological insights, and hastening the discovery of genetic and regulatory variables that influence reproductive features in poultry [27].

1.1. Role of Proteomics in Chicken Reproduction

Proteomics is the large-scale analysis of proteins and has emerged as a strong tool in biology, providing useful insights into a wide range of biological processes, including chicken reproduction [28]. The function of proteomics in chicken reproduction is an intriguing and difficult field of study that has the potential to transform our understanding of the mechanisms underpinning fertility, development, and the regulation of reproductive processes in these birds [29]. Proteomics, which can detect and quantify thousands of proteins at the same time, provides a complete perspective of the molecular alterations occurring in the chicken reproductive system [30] (Figure 2). One of the key applications of omics research in chicken reproduction is the identification of biomarkers that can be used as indicators of fertility or egg quality. Proteomics, which involves the study of the complete set of proteins in a cell, tissue, or organism, has particularly been instrumental in identifying protein biomarkers associated with chicken reproduction.

Proteomics has made major contributions to our understanding of chicken reproduction, particularly in the identification of the essential proteins involved in ovarian development and function [31]. The ovary is essential in female reproduction because it produces and releases eggs for fertilization [32]. Proteomic research on the chicken ovary has uncovered important proteins involved in follicular development, ovulation, and hormonal regulation, providing insight into the complex interplay of the molecular events that drive these processes [21,22,33]. Furthermore, proteomics has been useful in understanding the role of proteins in sperm production and function in male chickens.

Sperm generation, maturation, and fertilization capacity are all important aspects of male fertility, and proteomic studies have discovered crucial proteins associated with these processes, providing vital insights into the molecular mechanisms underpinning successful rooster reproduction [34,35]. By analyzing the proteome of chicken reproductive tissues, researchers can identify specific proteins that are differentially expressed in fertile and infertile individuals, or in eggs of varying quality. These protein biomarkers can then be used to develop diagnostic tools that can accurately assess the fertility of breeding chickens or the quality of eggs produced. For example, a study may identify a specific protein that is abundantly expressed in the reproductive tract of fertile hens but is absent or expressed at lower levels in infertile hens. This protein biomarker can then be used in a diagnostic test to assess the fertility of individual hens or to predict the likelihood of successful fertilization.

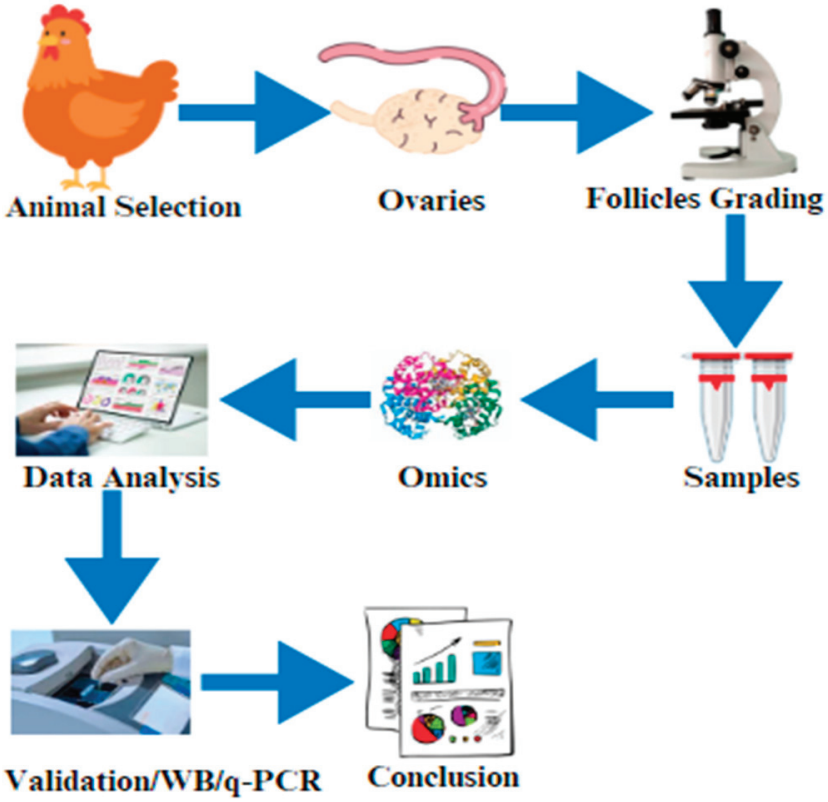


Figure 2. The graphical abstract of this current review article indicates the selection of the animals and the isolation of the ovaries. After that, ovary/follicle samples are prepared for omics (proteomics, transcriptomics, or metabolomics), data are validated by using Western blotting (WB)/real-time q-PCR, and the results are finally compiled.

In addition to studying reproductive organs, proteomics has been used to analyze seminal plasma, the fluid that surrounds and nourishes the sperm in the male reproductive tract [35]. Seminal plasma is a complex mixture of proteins that play important roles in sperm survival, motility, and fertilization [36]. Proteomic investigations of chicken seminal plasma have identified proteins that may act as indicators for male fertility and could be used to build novel reproductive technologies [37,38]. Furthermore, proteomics has been frequently utilized to explore how environmental factors such as nutrition, stress, and disease affect chicken reproduction [39]. Profiling the proteome of reproductive tissues under various conditions can identify the protein markers associated with poor reproductive performance, providing valuable insights into how external factors influence fertility and reproductive success in chickens [40]. The field of assisted reproductive technologies (ARTs), including artificial insemination and embryo modification, has also been affected by the application of proteomics in chicken reproduction [41]. The characterization of

the proteome profiles of gametes, embryos, and reproductive organs can improve the success rates of assisted reproduction in chickens, refine ART procedures, and increase the effectiveness of fertility therapies [41]. Similarly, proteomics can also be used to identify the biomarkers associated with egg quality, such as proteins that are involved in the formation of the eggshell or proteins that are indicative of the nutritional quality of the egg. By developing diagnostic tools that utilize these protein biomarkers, farmers can assess the quality of the eggs produced by their chickens and make informed decisions on breeding strategies or nutritional interventions to improve egg quality.

Overall, proteomics has revolutionized our understanding of the molecular processes driving fertility, development, and reproductive function in these birds, which has fundamentally changed our understanding of chicken reproduction [42]. Proteome studies have cleared the path for cutting-edge research and useful applications in chicken breeding and production by revealing the complex interactions between the proteins involved in ovarian and sperm development, seminal plasma composition, and the effect of environmental factors on reproductive performance [43]. To summarize, the use of proteomics in chicken reproduction is a fast-emerging field with significant potential for improving our understanding of avian fertility and reproductive health [2].

1.1.1. Role of Proteomics in Egg Production

Follicular formation in chickens is a complex biological phenomenon that is carefully controlled by a succession of molecular events [44,45]. The proteomic analysis enables the identification and quantification of the proteins involved in this process, revealing important insights into the underlying mechanisms [46]. By performing proteomic analysis on chicken samples, researchers can gain a better understanding of the molecular mechanisms involved in various physiological and pathological processes. This can help with identifying the key proteins that play a role in specific functions, such as growth and development, immune response, and disease progression. We can acquire a better grasp of the parameters influencing egg production by examining the protein content of chicken follicles at various developmental stages [47]. The capacity of proteomic analysis to find proteins that might act as indicators for reproductive success is one of the main benefits of using proteomics in poultry research [42]. Proteomic can also be used to identify particular proteins linked to higher egg productivity by comparing the protein profiles of hens that produce eggs well and those that do not. Proteomic studies can also provide insights into how different environmental conditions affect the development of chicken follicles and the quality of their eggs to find potential stress biomarkers and create plans to lessen their effects on egg production by examining the changes in follicles' protein composition in response to various stressors, such as heat or nutrition [48]. Furthermore, proteomic analysis can help with identifying potential biomarkers for certain conditions or diseases, as well as with evaluating the effectiveness of treatments or interventions. Overall, proteomic analysis is a powerful tool that can provide important insights into the complex biological processes in chickens and other organisms.

Proteomics can be utilized not just to comprehend the molecular underpinnings of egg productivity but also to identify and track the presence of pollutants or diseases in chicken products [49], uncover proteins linked to threats to food safety, and create strategies for identifying and eliminating dangerous materials from the food supply chain by examining the protein profiles of eggs [50]. As such, the use of proteomics in poultry research could fundamentally alter our knowledge of the development of chicken follicles and egg output [51]. Proteomic analysis offers a comprehensive perspective on the protein landscape within chicken follicles [29], which offers new options for enhancing the reproductive efficiency in poultry farming and providing significant insights into the molecular mechanisms driving egg production [6]. In the context of enhancing reproductive efficiency and driving egg production in chickens, proteomics can be used to identify key proteins and pathways involved in processes such as ovulation, fertilization, and egg formation. By conducting proteomic analysis on reproductive tissues, fluids, or cells of chickens, researchers can

gain insights into the molecular mechanisms underlying these processes. This information can then be used to develop strategies to enhance reproductive efficiency and increase egg production in chickens. For example, proteomic analysis may reveal the proteins that are crucial for oocyte maturation, sperm function, or hormone regulation. By targeting these proteins with specific interventions, such as nutritional supplements or gene editing techniques, researchers may be able to improve the reproductive performance of chickens.

1.1.2. Proteomic Techniques Used in Chickens

Proteomics is an essential tool for comprehending the molecular mechanisms underpinning a variety of physiological activities in chickens, including health, illness resistance, and growth performance [29]. Two-dimensional gel electrophoresis is a technique used to separate proteins based on their isoelectric point and molecular weight. It provides high resolution but can be time consuming and have low reproducibility. Liquid chromatography–tandem mass spectrometry (LC-MS) is a powerful tool used for protein identification and quantification that has high sensitivity and accuracy. It can be used in conjunction with 2-D gel electrophoresis to overcome its limitations. Two-dimensional gel electrophoresis (2-DE) is one of the most popular proteomic methods used in chicken research [52,53]. By dividing proteins according to their molecular weight and isoelectric point (pI), this technique offers a thorough understanding of the chicken proteome. Through the process of comparing the protein profiles between distinct situations, such as ill versus healthy birds or treated versus untreated groups, the proteins that exhibit differential expression can be discerned, potentially having an impact on the phenotypes that are seen [53,54].

Matrix-assisted laser desorption/ionization (MALDI) is a mass spectrometry technique that is often used for protein profiling and identification. It has a fast analysis speed and high sensitivity, but it may not be as effective as LC-MS in terms of data quality and proteome coverage. MALDI makes it possible to identify and measure proteins in complicated biological samples [55]. Liquid chromatography–mass spectrometry (LC-MS) and matrix-assisted laser desorption/ionization (MALDI) are two popular forms of mass spectrometry (MS) [56]. Because of the excellent sensitivity and specificity of these methods, we can even identify proteins with low abundances and post-translational modifications [57].

Apart from 2-DE and MS, protein microarray technology has become an important tool in the field of chicken proteomics [58] (Figure 3). Protein microarray technology is a high-throughput method for studying protein interactions and functions. It allows for the simultaneous analysis of thousands of proteins but may have limited sensitivity and specificity compared to mass-spectrometry-based methods. Protein interactions with DNA, other proteins, and small molecules can be studied in high-throughput using protein microarrays, enabling systematic analysis of the roles and interactions of thousands of chicken proteins by organizing them on a stable substrate [58]. Furthermore, the field of chicken research has seen a rise in the use of shotgun proteomics, which breaks down proteins into peptides and analyzes them using mass spectrometry. Shotgun proteomics is a mass-spectrometry-based approach that involves digesting proteins into peptides and analyzing them in a high-throughput manner. It provides comprehensive proteome coverage but may have limitations in terms of quantifying proteins and identifying low-abundance proteins. This method is especially helpful for large-scale research that attempts to thoroughly characterize the chicken proteome since it makes a high number of proteins identifiable in a single experiment [59]. Recent developments in targeted proteomic methods, such as parallel reaction monitoring (PRM) and selective reaction monitoring (SRM), have made it possible to measure particular proteins very precisely and sensitively [60,61]. They offer high sensitivity and specificity but are more labor-intensive compared to shotgun proteomics. These methods yield important information, particularly when examining important regulatory proteins or biomarkers in chickens [59,62].

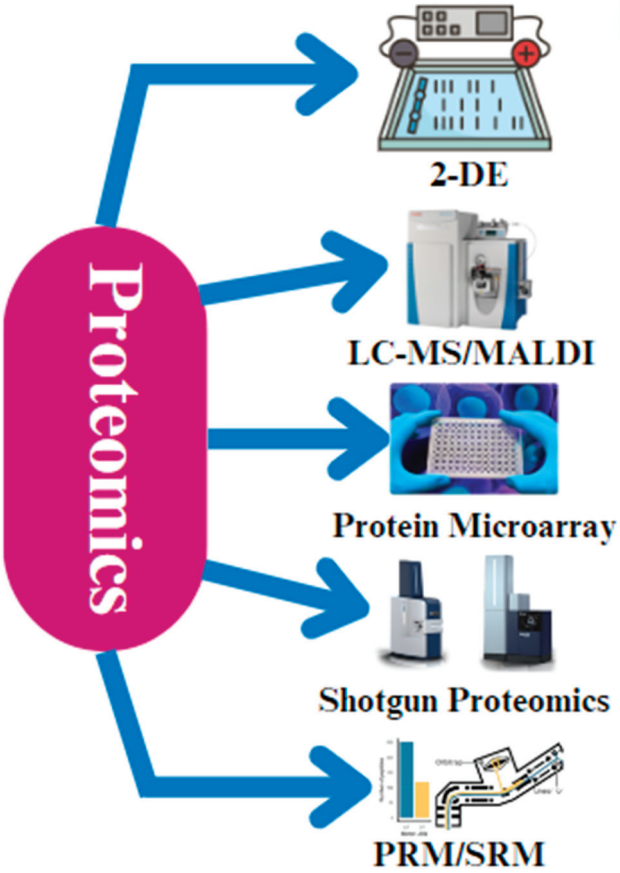


Figure 3. The different techniques that are applied during proteomic analysis. The most common techniques that are used are 2-D gel electrophoresis, liquid chromatography–mass spectrometry (LC-MS), matrix-assisted laser desorption/ionization (MALDI), protein microarray technology, shotgun proteomics, parallel reaction monitoring (PRM), and selective reaction monitoring (SRM).

1.2. Role of Transcriptomics in Chicken Reproduction

Transcriptomics, or the study of an organism's entire set of RNA transcripts, is an important technique used for understanding numerous biological processes, including chicken reproduction [63]. Transcriptomics analysis can provide valuable insights into the molecular mechanisms driving fertility, embryo development, and overall reproductive success by studying the gene expression profiles of chickens during critical reproductive stages [64].

Transcriptomics is important in investigating chicken reproduction because it can uncover the critical genes and pathways involved in reproductive activities and identify genes that are up- or downregulated during critical events such as ovulation, fertilization, and embryonic development by comparing gene expression levels across distinct reproductive states [65]. Such studies might be useful in understanding the molecular basis of reproductive failure and creating fertility-enhancing techniques in chicken breeding programs [66]. Transcriptomics can also shed light on the effect of external factors such as nutrition, stress, and environment on chicken reproductive health to discover the biological mechanisms by which these factors influence reproductive outcomes by examining changes in gene expression in response to them [67].

Transcriptomic studies in chicken reproduction have also helped to identify molecular markers linked to key reproductive features and to uncover genetic markers that can be employed in selective breeding by comparing gene expression patterns to phenotypic features such as egg production, hatchability, and sperm quality [68,69]. Furthermore, transcriptomics is critical for understanding epigenetic control in chicken reproduction. Epigenetic changes, such as DNA methylation and histone modifications, can affect gene

expression patterns without changing the underlying DNA sequence [70]. Transcriptomic analysis enables us to understand how these epigenetic mechanisms control gene expression during critical reproductive events and how they might be targeted to improve chicken reproductive outcomes [71].

Transcriptomic investigations can assist veterinarians and breeders in early diagnosis and the implementation of focused treatment methods by identifying the biomarkers linked to reproductive diseases or inadequate fertility [72]. Transcriptomic research, by deciphering the complex gene expression profiles associated with critical reproductive processes, provides the path for the identification of genetic markers, breeding program optimization, and improved reproductive outcomes in poultry production [13,73].

Techniques Used for Chicken Transcriptomics

Various methodologies are employed in transcriptomics to examine the patterns of gene expression in animal reproduction [74] (Figure 4). These methodologies enable us to discern the genes that are expressed differently, as well as the signaling pathways and regulatory networks that play a role in reproductive processes [74]. The common techniques used in transcriptomics for animal reproduction include RNA sequencing (RNA-Seq), microarray analysis [75], quantitative real-time polymerase chain reaction (qRT-PCR) [76], and in situ hybridization [77]. RNA sequencing, also known as RNA-Seq, is a very efficient method that enables the sequencing and measuring of the abundance of all RNA molecules present in a given sample. RNA-Seq is a technique that can be used to gather data on gene expression levels, identify alternative splicing processes, and uncover new transcripts [78]. Through the comparison of transcriptomes from various reproductive organs or developmental stages, we can discern the genes that exhibit unique expression in reproductive cells and tissues [79]. RNA-Seq techniques are used in chicken reproduction research to study the gene expression patterns in reproductive tissues and unravel the molecular mechanisms underlying various reproductive processes. In particular, the genes RAC1, MRE11A, MAP7, and SOX5 have been identified as playing important roles in chicken reproduction based on RNA-seq studies, which uncovered differentially expressed genes in the ovaries, testes, and reproductive tracts of male and female chickens at distinct developmental stages [80].

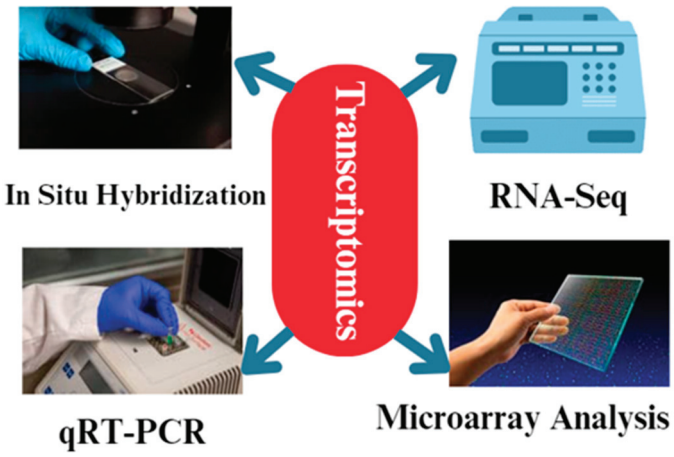


Figure 4. The different methodologies used during transcriptomics analysis. The most common techniques are qRT-PCR and RNA-Seq. In addition, microarray analysis and in situ hybridization techniques are used for transcriptomics analysis.

Microarray analysis is a commonly employed method in transcriptomics that enables the evaluation of the expression levels of several genes simultaneously in a single experiment [81]. Microarrays are composed of many DNA probes that bind to RNA molecules in a sample, yielding data on the levels of gene expression [82]. Microarray analysis was

employed to examine the gene expression patterns in reproductive tissues, including the ovary, testis, and uterus [83]. It was also utilized to identify the genes that exhibit differential expression during reproductive processes [84]. Microarray analysis is a prominent transcriptomics approach [74]. Microarrays have enabled the concurrent evaluation of the expression levels of thousands of genes in a sample and the uncovering of the essential genes and pathways involved in chicken reproduction by comparing gene expression profiles across reproductive tissues or healthy and sick hens [85,86].

In addition to RNA-Seq and microarray analysis, quantitative real-time polymerase chain reaction (qRT-PCR) is a popular technique in transcriptomics [87]. With qRT-PCR, we can estimate the expression levels of individual genes with high sensitivity and accuracy [88]. This technique is frequently used to validate the results of RNA-Seq or microarray research, as well as to investigate the dynamics of gene expression at various phases of chicken reproduction [89]. Quantitative real-time polymerase chain reaction (qRT-PCR) is a very accurate and precise method frequently employed to confirm the gene expression data acquired from RNA-Seq or microarray research [90]. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) enables the calculation of the gene expression levels in a sample and confirms the differential expression of certain genes of interest. qRT-PCR is commonly employed to confirm the candidate genes identified in transcriptome investigations and to assess the expression patterns of genes implicated in reproductive functions [91].

Currently, single-cell RNA sequencing (scRNA-seq) has been developed as a strong transcriptomics method [92]. This technique enables the study of gene expression patterns at the individual cell level, resulting in a more complete understanding of the heterogeneity within complex tissues such as the chicken reproductive system. By analyzing the transcriptomes of individual germ cells, follicle cells, and embryonic cells, we can discover the regulatory networks and cell-to-cell communication routes involved in chicken reproduction [93]. Overall, transcriptomics has transformed our understanding of the molecular pathways that drive chicken reproduction and can be used to understand the complexity of gene expression in chickens by using modern techniques such as RNA sequencing, microarray analysis, qRT-PCR, and single-cell RNA sequencing [94]. These findings not only have expanded our understanding of avian reproduction but also have implications for increasing chicken production and fertility management tactics in the poultry business.

In situ hybridization is a method used to observe the precise location of particular RNA molecules within cells and tissues [95]. In situ hybridization employs tagged RNA probes that bind to the complementary RNA molecules in a tissue segment, enabling observation of the expression patterns of particular genes [96]. In situ hybridization has been utilized to investigate the expression patterns of the genes implicated in reproductive processes such as folliculogenesis, spermatogenesis, and embryo implantation [97].

1.3. Role of Metabolomics in Chicken Reproduction

Metabolomics is an advanced scientific method that is used to thoroughly analyze the small-molecule metabolites present in biological systems [98]. Through the analysis of metabolite profiles, useful knowledge of the metabolic pathways, biochemical processes, and physiological functions that support different biological functions can be described [99]. Metabolomics is a valuable technique in the study of chicken reproduction since it allows for the investigation of the metabolic changes that take place during several reproductive phases, including follicular development, ovulation, fertilization, and embryogenesis [100].

Metabolomics has been used to investigate the relationship between chicken reproduction efficiency and metabolic changes, yielding important insights into the factors that affect reproductive outcomes [24] and detecting distinct metabolic indicators linked to fertility, fecundity, and hatchability in chickens by examining the metabolite profiles of various reproductive organs, fluids, and biofluids [101]. Metabolomics is essential for comprehending the influence of many environmental conditions, genetic factors, and dietary interventions on the efficiency of chicken reproduction [102]. Through the comparison of

metabolite profiles in hens exposed to various environments, we can detect the metabolic signatures linked to enhanced reproductive success [103] and can be used to monitor the reproductive health of chickens, including hormone levels and fertility. This can help identify and address any potential issues that may affect breeding success.

Moreover, metabolomics enables the detection of possible biomarkers associated with fertility-related illnesses and reproductive diseases in chickens [1,104]. Through the examination of metabolic disruptions linked to illnesses including ovarian dysfunction, reproductive tract infections, and hormonal abnormalities, diagnostic instruments can be developed to identify and track these problems at an early stage [105]. By taking a proactive approach, timely interventions can be implemented to manage reproductive health issues in hens. This helps to minimize economic losses and promote animal welfare. Furthermore, it can be used to analyze the nutritional content of chicken feed and optimize the diet for optimal growth and reproductive performance. This can help improve the overall productivity of the chicken population.

Techniques Applied in Metabolomics

Gas chromatography–mass spectrometry (GC-MS) is a commonly employed method in metabolomics that enables the isolation and identification of metabolites by analyzing their mass and chemical characteristics [106]. It involves separating and analyzing compounds based on their volatility and mass-to-charge ratio, so is ideal for analyzing small volatile molecules to identify compounds that are thermo-stable and can be vaporized without decomposition. GC-MS can be employed to examine the metabolic profiles of reproductive tissues, such as the ovary and oviduct [107]. Through the comparison of metabolite profiles throughout several reproductive phases, scientists can pinpoint the crucial metabolites linked to activities such as ovulation and fertilization [108] (Figure 5).

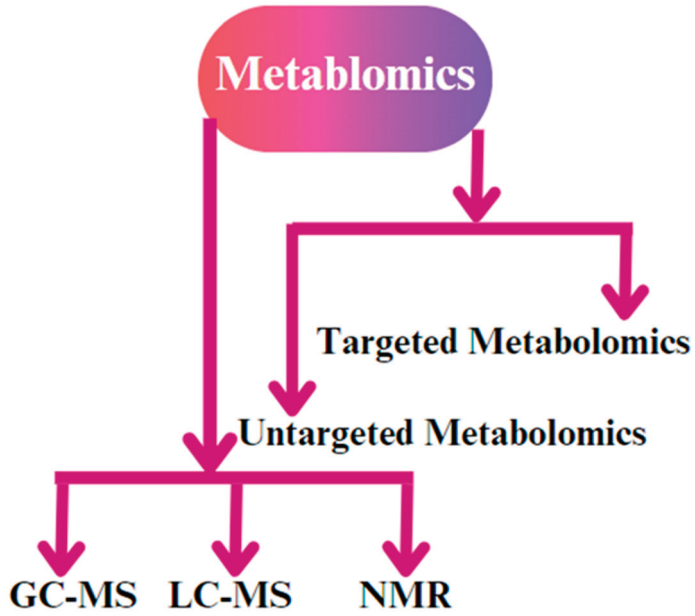


Figure 5. The different types of metabolomics techniques used for biological sample analysis. The most common methodologies that are applied are gas chromatography–mass spectrometry (GC-MS), liquid chromatography–mass spectrometry (LC-MS), and nuclear magnetic resonance (NMR) spectroscopy. In addition, untargeted metabolomic and targeted metabolomic techniques are used for biological replicates.

Liquid chromatography–mass spectrometry (LC-MS) is a highly effective technique used in metabolomics to accurately detect and measure the concentration of metabolites in intricate biological samples [109]. It involves separating and analyzing compounds based on their polarity and mass-to-charge ratio, so is ideal for analyzing the polar compounds

and large molecules for analyzing compounds that are not volatile or thermo-stable. LC-MS is an essential tool in the examination of chicken reproduction as it enables the analysis of the metabolic alterations that take place throughout embryonic development [110]. Through the analysis of the metabolites in embryonic tissue samples at various stages of development, scientists can reveal the essential metabolic pathways that are crucial for appropriate growth and differentiation [111].

Nuclear magnetic resonance (NMR) spectroscopy is a noninvasive method that offers comprehensive insights into the chemical composition of metabolites [112]. It involves analyzing the chemical structure of compounds based on the interactions between nuclei and magnetic fields and provides information on the chemical environment and the connectivity of atoms in a molecule. NMR spectroscopy is a useful tool for examining the metabolic alterations in reproductive fluids, including blood plasma and follicular fluid [113]. Through the examination of the NMR spectra of these fluids, scientists can detect biomarkers linked to reproductive health and fertility [114].

Untargeted metabolomics is a comprehensive method that seeks to identify a wide range of metabolites in a biological sample without any prior information about their chemical identification [115], and it can be used to discover novel biomarkers or metabolic pathways related to chicken reproduction. In the study of chicken reproduction, untargeted metabolomics has provided a broad overview of the metabolic pathways involved in key reproductive processes [14,116]. In contrast to untargeted metabolomics, targeted metabolomics focuses on the quantification of specific metabolites or metabolic pathways of interest [117]. Targeted metabolomics is a useful technique in chicken reproduction research for quantifying the amounts of crucial metabolites involved in reproductive processes, including hormone synthesis and embryonic growth. Researchers might enhance their comprehension of the metabolic changes that take place during crucial stages of chicken reproduction by focusing on particular metabolites [118].

In summary, metabolomics approaches have greatly transformed the investigation of chicken reproduction by offering a thorough examination of the metabolic alterations that take place throughout crucial reproductive processes. Gas chromatography–mass spectrometry and nuclear magnetic resonance spectroscopy are two techniques that offer distinct advantages in the realm of metabolomics research.

1.4. Genomics/Epigenomics and Chicken Reproduction

Genomics is the scientific discipline that investigates the complete genetic material of an organism, encompassing its DNA sequence [119]. Genomics has facilitated the identification of crucial genes and genetic markers linked to qualities such as fertility, hatchability, and egg output in the field of chicken reproduction [120]. Through the examination of the chicken genome, we can identify specific genetic changes that have an impact on reproductive performance [121].

A notable progress in genomics is the utilization of genome-wide association studies (GWAS) to chart the genetic markers associated with reproductive characteristics in chickens [122,123]. GWAS have revealed genes and genomic areas that are crucial in reproductive functions, such as hormone control, follicle growth, and sperm quality [122,124]. Genomic data can be utilized by breeders to make well-informed choices regarding mating pairs, thereby maximizing genetic variety and enhancing the overall reproductive efficacy of chicken populations [125].

Epigenomics is the field of study that specifically examines the modifications in gene expression that are not a result of changes in the DNA sequence [126]. Epigenomics is essential in controlling gene expression during important stages of development and reproduction in chickens [127]. Epigenetic alterations, such as DNA methylation and histone modification, can influence reproductive processes in chickens [15,128]. Epigenetic modifications in the genes associated with sex determination and embryo development can impact the rates of fertility and hatchability, as an example [129]. Studying the epigenetic mechanisms involved in chicken reproduction has the potential to generate novel

approaches for enhancing reproductive efficiency [130]. Epigenomics can impact gene expression without modifying the DNA sequence, which is one of its main advantages. Epigenetic control enables the modulation of gene activity in hens' reproductive processes in response to environmental cues, facilitating precise adjustments [131].

Integration of Genomics/Epigenomics via Omics

Omics techniques, such as genomics and epigenomics, allow for the comprehensive analysis of the chicken genome and epigenome [132]. These techniques involve high-throughput sequencing and bioinformatics analysis and generate large-scale datasets that can provide a detailed picture of the genetic and epigenetic factors influencing chicken reproduction [133]. By integrating these omics approaches, we can identify the key genes and pathways that are involved in reproductive processes and gain a better understanding of the complex interactions between genetics and the environment [134].

One of the main advantages of integrating genomics and epigenomics using omics in chicken reproduction is the ability to identify novel biomarkers for reproductive traits [15]. By analyzing the genetic and epigenetic signatures associated with desirable reproductive traits, researchers can develop molecular markers that can be used for breeding selection [135]. This can help poultry breeders to more effectively select for desirable traits, such as increased egg production and fertility, leading to improved breeding programs and overall productivity.

Additionally, the integration of genomics and epigenomics in chicken reproduction can provide valuable insights into the molecular mechanisms underlying reproductive disorders and diseases [15]. By studying the genetic and epigenetic factors associated with reproductive disorders, researchers can identify potential therapeutic targets and develop targeted interventions to improve reproductive health in chickens [136]. In conclusion, the integration of genomics and epigenomics through omics techniques in chicken reproduction holds great promise for advancing our understanding of the molecular mechanisms underlying important reproductive processes in chickens [137].

2. Overall Conclusions

The incorporation of omics in investigating chicken reproduction has greatly enhanced our comprehension of the molecular pathways that regulate reproductive efficiency, utilizing the genomes, transcriptomics, metabolomics, and proteomics data integration to pinpoint the crucial genes, pathways, and biomarkers linked to fertility, egg production, and other reproductive features. Omics techniques can be utilized to formulate precise breeding tactics, enhance reproductive results, and optimize the overall efficiency of chicken farming. Overall, omics techniques have demonstrated their effectiveness in improving chicken reproductive efficiency. We can utilize the knowledge acquired from genomic, transcriptomic, metabolomic, and proteomic investigations to make well-informed choices that enhance breeding programs and reproductive performance in chicken populations. By integrating data from these different omics approaches, researchers can create a more comprehensive and detailed picture of the molecular mechanisms that underlie chicken reproduction. This holistic approach can help identify potential biomarkers for reproductive health and fertility, as well as inform breeding strategies to improve reproductive performance in chicken populations. This knowledge holds great potential for improving the efficiency and sustainability of poultry farming in the future.

3. Recommendations

Omics technologies have the potential to aid researchers and breeders in comprehending the genetic foundation of reproductive features, detecting biomarkers for fertility and egg production, and refining breeding tactics to improve overall reproductive efficiency in chickens. An important suggestion for the use of omics techniques in chicken reproduction efficiency is the use of genetic information to enhance breeding strategies. Genomic technologies, such as next-generation sequencing and genotyping arrays, have greatly enhanced

our capacity to detect the genetic changes linked to significant reproductive characteristics in chickens. Through the implementation of genome-wide association studies (GWAS) and genomic selection, breeders can identify specific genes and genomic regions that have an impact on fertility, hatchability, and other reproductive features. Researchers can obtain useful insights into the genetic and molecular pathways that control reproductive qualities in chickens by utilizing genomics, transcriptomics, proteomics, and metabolomics.

4. Future Prospectives

Omics techniques, such as genomics, transcriptomics, proteomics, and metabolomics, have fundamentally transformed our approach to studying biological systems. These technologies have the potential to significantly improve our comprehension of the fundamental mechanisms that control fertility, hatchability, and overall reproductive efficiency in poultry, specifically in the realm of chicken reproduction. Through the implementation of genomic studies on diverse chicken breeds exhibiting differing levels of reproductive efficiency, we can precisely identify the genetic variants that are associated with specific attributes, including egg production, embryo development, and sperm quality. Through transcriptomic analysis, we can examine gene expression profiles in fertile and infertile chickens and pinpoint crucial genes and pathways linked to successful reproduction. Proteomics, which investigates the complete collection of proteins generated by an organism, can be beneficial in comprehending the mechanisms that contribute to the effectiveness of chicken reproduction. Through the examination of the protein makeup of reproductive organs such as the ovary, oviduct, and sperm, we can pinpoint crucial proteins that play a role in significant reproductive functions such as egg production, fertilization, and embryo growth.

Metabolomics, a field that examines tiny molecules like metabolites and hormones, might offer vital insights into the metabolic pathways that regulate reproductive activities in chickens. Through the examination of the metabolic profiles of chickens that are capable of reproducing and those that are not, we can detect crucial metabolites that are linked to the effectiveness of reproduction. Epigenetic modifications have been shown to play a crucial role in reproductive processes. Omics techniques, such as epigenomics, can be used to study the epigenetic changes that occur during different stages of the reproductive cycle in chickens.

Omics techniques can be used to develop personalized reproductive management strategies for chickens. By understanding the genetic and molecular profiles of individual birds, it is possible to tailor management practices to optimize reproductive performance. This can lead to improved efficiency and sustainability in the poultry industry. Omics techniques can be used to identify biomarkers of reproductive diseases and disorders in chickens. This can aid in the early diagnosis and treatment of these conditions, leading to improved reproductive health and productivity in poultry flocks. The use of omics techniques, such as gene editing, can allow for the manipulation of specific genes involved in reproductive processes, which can lead to the development of chickens with improved reproductive performance and disease resistance. In order to improve disease resistance and reproductive performance in chickens, omics techniques, such as gene editing, can be utilized to specifically target and manipulate genes involved in reproductive processes. One way gene editing can be used is through the CRISPR/Cas9 system, which allows for the precise editing of genes by introducing specific mutations or deletions. By targeting the genes involved in immune response or reproductive processes, it is possible to enhance disease resistance and reproductive performance in chickens. For example, the genes involved in immune response can be edited to increase the chicken's ability to fight off pathogens and improve overall health. Additionally, the genes involved in reproductive processes, such as fertility and egg production, can be targeted to enhance reproductive performance in chickens. By using omics techniques like gene editing, researchers can selectively manipulate specific genes to improve disease resistance and reproductive performance in chickens. This can lead to healthier and more productive poultry populations, ultimately benefiting both producers and consumers.

By integrating omics data with bioinformatics, researchers can analyze and interpret large datasets to identify the key genes, pathways, and biological processes involved in chicken reproduction. Bioinformatics tools and algorithms help in the identification of the biomarkers, gene regulatory networks, and signaling pathways that are critical for reproductive processes. Systems biology takes the integration of omics data a step further by incorporating computational modeling and simulation to understand the complex interactions and dynamics of biological systems. By modeling the regulatory networks and pathways involved in chicken reproduction, researchers can gain insights into the underlying mechanisms and predict the outcomes of genetic or environmental perturbations. Overall, the integration of omics with bioinformatics and systems biology has revolutionized our understanding of chicken reproduction by providing a comprehensive view of the molecular mechanisms underlying this critical biological process. This interdisciplinary approach has the potential to drive future advancements in poultry breeding and production, ultimately leading to improved efficiency and sustainability in the poultry industry.

The role of omics techniques in chicken reproduction is constantly evolving and expanding. These techniques have already provided valuable insights into the genetic and molecular mechanisms involved in reproductive processes. With further advancements in technology and research, omics techniques have the potential to revolutionize the way we manage and improve reproductive performance in chickens. They can also aid in the development of novel treatments for reproductive disorders, leading to improved productivity and sustainability in the poultry industry. Therefore, it is essential to continue investing in research and development in this field to fully realize the potential of omics techniques in chicken reproduction.

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References

1. Long, J.A. The 'omics' revolution: Use of genomic, transcriptomic, proteomic and metabolomic tools to predict male reproductive traits that impact fertility in livestock and poultry. *Anim. Reprod. Sci.* **2020**, *220*, 106354. [CrossRef] [PubMed]
2. Suravajhala, P.; LKogelman, J.; Kadarmideen, H.N. Multi-omic data integration and analysis using systems genomics approaches: Methods and applications in animal production, health and welfare. *Genet. Sel. Evol.* **2016**, *48*, 38. [CrossRef] [PubMed]
3. Cogburn, L.; Porter, T.E.; Duclos, M.J.; Simon, J.; Burgess, S.C.; Zhu, J.J.; Cheng, H.H.; Dodgson, J.B.; Burnside, J. Functional genomics of the chicken—A model organism. *Poult. Sci.* **2007**, *86*, 2059–2094. [CrossRef]
4. Ma, J.; Li, R.; Wang, H.; Li, D.; Wang, X.; Zhang, Y.; Zhen, W.; Duan, H.; Yan, G.; Li, Y. Transcriptomics analyses reveal wheat responses to drought stress during reproductive stages under field conditions. *Front. Plant Sci.* **2017**, *8*, 592. [CrossRef]
5. Selvam, M.K.P.; Baskaran, S.; Agarwal, A. Proteomics of reproduction: Prospects and perspectives. *Adv. Clin. Chem.* **2019**, *92*, 217–243. [CrossRef]
6. Maru, D.; Kumar, A. Applications of Omics Technologies in Livestock Production, Improvement and Sustainability. In *Sustainable Agriculture Reviews: Animal Biotechnology for Livestock Production 4*; Springer: Cham, Switzerland, 2024; pp. 1–54. [CrossRef]
7. Zhao, X.; Nie, C.; Zhang, J.; Li, X.; Zhu, T.; Guan, Z.; Chen, Y.; Wang, L.; Lv, X.Z.; Yang, W.; et al. Identification of candidate genomic regions for chicken egg number traits based on genome-wide association study. *BMC Genom.* **2021**, *22*, 610. [CrossRef] [PubMed]
8. Hayes, B.J.; Lewin, H.A.; Goddard, M.E. The future of livestock breeding: Genomic selection for efficiency, reduced emissions intensity, and adaptation. *Trends Genet.* **2013**, *29*, 206–214. [CrossRef]
9. Liu, Y.; Liang, S.; Wang, B.; Zhao, J.; Zi, X.; Yan, S.; Dou, T.; Jia, J.; Wang, K.; Ge, C. Advances in single-cell sequencing technology and its application in poultry science. *Genes* **2022**, *13*, 2211. [CrossRef]
10. Atikuzzaman, M.; Alvarez-Rodriguez, M.; Vicente-Carrillo, A.; Johnsson, M.; Wright, D.; Rodriguez-Martinez, H. Conserved gene expression in sperm reservoirs between birds and mammals in response to mating. *BMC Genom.* **2017**, *18*, 98. [CrossRef]
11. Karr, T.L. Reproductive proteomics comes of age. *Mol. Cell. Proteom.* **2019**, *18*, S1–S5. [CrossRef]
12. Krzastek, S.C.; Smith, R.P.; Kovac, J.R. Future diagnostics in male infertility: Genomics, epigenetics, metabolomics and proteomics. *Transl. Androl. Urol.* **2020**, *9* (Suppl. S2), S195. [CrossRef] [PubMed]

13. Huang, X.; Zhang, H.; Cao, H.; Zhou, W.; Xiang, X.; Yin, Z. Transcriptomics and metabolomics analysis of the ovaries of high and low egg production chickens. *Animals* **2022**, *12*, 2010. [CrossRef] [PubMed]
14. Zhao, J.; Pan, H.; Zhao, W.; Li, W.; Li, H.; Tian, Z.; Meng, D.; Teng, Y.; Li, X.; He, Y.; et al. Untargeted Metabolomics Revealed Potential Biomarkers of Small Yellow Follicles of Chickens during Sexual Maturation. *Metabolites* **2023**, *13*, 176. [CrossRef] [PubMed]
15. Wang, M.; EIbeagha-Awemu, M. Impacts of epigenetic processes on the health and productivity of livestock. *Front. Genet.* **2021**, *11*, 613636. [CrossRef] [PubMed]
16. Kumar, H.; Chaudhary, A.; Singh, A.; Sukhija, N.; Panwar, A.; Saravanan, K.; Bhaladhare, A.; Kaisa, K.; Panigrahi, M. A review on epigenetics: Manifestations, modifications, methods & challenges. *J. Entomol. Zool. Stud.* **2020**, *8*, 780–786.
17. Duncan, E.J.; Gluckman, P.D.; Dearden, P.K. Epigenetics, plasticity, and evolution: How do we link epigenetic change to phenotype? *J. Exp. Zool. Part B Mol. Dev. Evol.* **2014**, *322*, 208–220. [CrossRef] [PubMed]
18. Ashonibare, V.J.; Akorede, B.A.; Ashonibare, P.J.; Akhigbe, T.M.; Akhigbe, R.E. Gut microbiota-gonadal axis: The impact of gut microbiota on reproductive functions. *Front. Immunol.* **2024**, *15*, 1346035. [CrossRef] [PubMed]
19. Aruwa, C.E.; Pillay, C.; Nyaga, M.M.; Sabiu, S. Poultry gut health–microbiome functions, environmental impacts, microbiome engineering and advancements in characterization technologies. *J. Anim. Sci. Biotechnol.* **2021**, *12*, 119. [CrossRef] [PubMed]
20. Shahzad, Q.; Pu, L.; Wadood, A.A.; Waqas, M.; Xie, L.; Pareek, C.S.; Xu, H.; Liang, X.; Lu, Y. Proteomics analysis reveals that Warburg effect along with modification in lipid metabolism improves in vitro embryo development under low oxygen. *Int. J. Mol. Sci.* **2020**, *21*, 1996. [CrossRef]
21. Wadood, A.A.; Wang, J.; Pu, L.; Shahzad, Q.; Waqas, M.; Liu, X.; Xie, L.; Yu, L.; Chen, D.; Akhtar, R.W.; et al. Proteomic analysis identifies potential markers for chicken primary follicle development. *Animals* **2021**, *11*, 1108. [CrossRef]
22. Wadood, A.A.; Pu, L.; Shahzad, Q.; Waqas, M.; Yu, L.; Liao, Y.; Rehman, S.U.; Chen, D.; Huang, Z.; Lu, Y.; et al. Proteomic analysis identifies potential markers in small white and small yellow follicle development in chickens. *Reprod. Fertil. Dev.* **2022**, *34*, 516–525. [CrossRef] [PubMed]
23. Nayeri, S.; Sargolzaei, M.; Tulpan, D. A review of traditional and machine learning methods applied to animal breeding. *Anim. Health Res. Rev.* **2019**, *20*, 31–46. [CrossRef] [PubMed]
24. Urgessa, O.E.; Woldesemayat, A.A. OMICs approaches and technologies for understanding low-high feed efficiency traits in chicken: Implication to breeding. *Anim. Biotechnol.* **2023**, *34*, 4147–4166. [CrossRef] [PubMed]
25. Polewko-Klim, A.; Lesiński, W.; Golińska, A.K.; Mnich, K.; Siwek, M.; Rudnicki, W.R. Sensitivity analysis based on the random forest machine learning algorithm identifies candidate genes for regulation of innate and adaptive immune response of chicken. *Poult. Sci.* **2020**, *99*, 6341–6354. [CrossRef]
26. Golestan Hashemi, F.S.; Razi Ismail, M.; Rafii Yusop, M.; Golestan Hashemi, M.S.; Nadimi Shahraki, M.H.; Rastegari, H.; Miah, G.; Aslani, F. Intelligent mining of large-scale bio-data: Bioinformatics applications. *Biotechnol. Biotechnol. Equip.* **2018**, *32*, 10–29. [CrossRef]
27. Bornelöv, S.; Seroussi, E.; Yosefi, S.; Benjamini, S.; Miyara, S.; Ruzal, M.; Grabherr, M.; Rafati, N.; Molin, A.-M.; Pendavis, K.; et al. Comparative omics and feeding manipulations in chicken indicate a shift of the endocrine role of visceral fat towards reproduction. *BMC Genom.* **2018**, *19*, 295. [CrossRef] [PubMed]
28. Almeida, A.M.d.; Bassols, A.; Bendixen, E.; Bhide, M.; Ceciliani, F.; Cristobal, S.; Eckersall, P.; Hollung, K.; Lisacek, F.; Mazzucchelli, G.; et al. Animal board invited review: Advances in proteomics for animal and food sciences. *Animal* **2015**, *9*, 1–17. [CrossRef] [PubMed]
29. Ferver, A.; Burgess, S.C.; Scanes, C.G.; Dridi, S. Avian proteomics. In *Sturkie's Avian Physiology*; Elsevier: Amsterdam, The Netherlands, 2022; pp. 29–47. [CrossRef]
30. Kunec, D.; Burgess, S.C. Avian proteomics. In *Sturkie's Avian Physiology*; Elsevier: Amsterdam, The Netherlands, 2015; pp. 25–37. [CrossRef]
31. Cui, Z.; Ning, Z.; Deng, X.; Du, X.; Amevor, F.K.; Liu, L.; Kang, X.; Tian, Y.; Wang, Y.; Li, D.; et al. Integrated proteomic and metabolomic analyses of chicken ovary revealed the crucial role of lipoprotein lipase on lipid metabolism and steroidogenesis during sexual maturity. *Front. Physiol.* **2022**, *13*, 885030. [CrossRef] [PubMed]
32. Chen, Q.; Wang, Y.; Liu, Z.; Guo, X.; Sun, Y.; Kang, L.; Jiang, Y. Transcriptomic and proteomic analyses of ovarian follicles reveal the role of VLDLR in chicken follicle selection. *BMC Genom.* **2020**, *21*, 486. [CrossRef]
33. Brégeon, M.; Tomas, D.; Bernay, B.; Zatylny-Gaudin, C.; Georgeault, S.; Labas, V.; Réhault-Godbert, S.; Guyot, N. Multifaceted roles of the egg perivitelline layer in avian reproduction: Functional insights from the proteomes of chicken egg inner and outer sublayers. *J. Proteom.* **2022**, *258*, 104489. [CrossRef]
34. Labas, V.; Grasseau, I.; Cahier, K.; Gargaros, A.; Harichaux, G.; Teixeira-Gomes, A.-P.; Alves, S.; Bourin, M.; Gérard, N.; Blesbois, E. Qualitative and quantitative peptidomic and proteomic approaches to phenotyping chicken semen. *J. Proteom.* **2015**, *112*, 313–335. [CrossRef] [PubMed]
35. Słowińska, M.; Nynca, J.; Arnold, G.J.; Fröhlich, T.; Jankowski, J.; Kozłowski, K.; Mostek, A.; Ciereszko, A. Proteomic identification of turkey (*Meleagris gallopavo*) seminal plasma proteins. *Poult. Sci.* **2017**, *96*, 3422–3435. [CrossRef] [PubMed]
36. Rodríguez-Martínez, H.; Kvist, U.; Ernerudh, J.; Sanz, L.; Calvete, J.J. Seminal plasma proteins: What role do they play? *Am. J. Reprod. Immunol.* **2011**, *66*, 11–22. [CrossRef] [PubMed]

37. Sun, Y.; Li, Y.; Shi, L.; Xue, F.; Xu, H.; Meng, Q.; Liu, Y.; Zong, Y.; Ma, H.; Chen, J. Differential proteomics highlights specific testicular proteins associated with chicken sperm motility and fertility potential. *Ann. Agric. Sci.* **2023**, *68*, 36–47. [CrossRef]
38. Luo, X.; Guo, Y.; Huang, Y.; Cheng, M.; Wu, X.; Gong, Y. Characterization and proteomics of chicken seminal plasma extracellular vesicles. *Reprod. Domest. Anim.* **2022**, *57*, 98–110. [CrossRef] [PubMed]
39. Marco-Ramell, A.; de Almeida, A.M.; Cristobal, S.; Rodrigues, P.; Roncada, P.; Bassols, A. Proteomics and the search for welfare and stress biomarkers in animal production in the one-health context. *Mol. Biosyst.* **2016**, *12*, 2024–2035. [CrossRef] [PubMed]
40. Borziak, K.; Álvarez-Fernández, A.; Karr, T.L.; Pizzari, T.; Dorus, S. The Seminal fluid proteome of the polyandrous Red junglefowl offers insights into the molecular basis of fertility, reproductive ageing and domestication. *Sci. Rep.* **2016**, *6*, 35864. [CrossRef] [PubMed]
41. Pereira, R.M.L.N.; Marques, C.C.; Pimenta, J.; Barbas, J.P.; Baptista, M.C.; Diniz, P.; Torres, A.; Lopes-da-Costa, L. Assisted reproductive technologies (ART) directed to germplasm preservation. In *Advances in Animal Health, Medicine and Production: A Research Portrait of the Centre for Interdisciplinary Research in Animal Health (CIISA), University of Lisbon, Portugal*; Springer: Berlin/Heidelberg, Germany, 2020. [CrossRef]
42. Wright, P.; Noirel, J.; Ow, S.-Y.; Fazeli, A. A review of current proteomics technologies with a survey on their widespread use in reproductive biology investigations. *Theriogenology* **2012**, *77*, 738–765.e52. [CrossRef]
43. Zampiga, M.; Flees, J.; Meluzzi, A.; Dridi, S.; Sirri, F. Application of omics technologies for a deeper insight into quali-quantitative production traits in broiler chickens: A review. *Anim. Sci. Biotechnol.* **2018**, *9*, 61. [CrossRef]
44. Hrabia, A. Reproduction in the female. In *Sturkie's Avian Physiology*; Elsevier: Amsterdam, The Netherlands, 2022; pp. 941–986. [CrossRef]
45. Jeong, W.; Lim, W.; Ahn, S.E.; Lim, C.-H.; Lee, J.-Y.; Bae, S.-M.; Kim, J.; Bazer, F.W.; Song, G. Recrudescence mechanisms and gene expression profile of the reproductive tracts from chickens during the molting period. *PLoS ONE* **2013**, *8*, e76784. [CrossRef]
46. Zhang, Y.; Zheng, X.; Zhang, Y.; Zhang, H.; Zhang, X.; Zhang, H. Comparative transcriptomic and proteomic analyses provide insights into functional genes for hypoxic adaptation in embryos of Tibetan chickens. *Sci. Rep.* **2020**, *10*, 11213. [CrossRef] [PubMed]
47. Du, Y.; Liu, L.; He, Y.; Dou, T.; Jia, J.; Ge, C. Endocrine and genetic factors affecting egg laying performance in chickens: A review. *Br. Poult. Sci.* **2020**, *61*, 538–549. [CrossRef] [PubMed]
48. Wang, Y.; Wang, J.; Shi, Y.; Ye, H.; Luo, W.; Geng, F. Quantitative proteomic analyses during formation of chicken egg yolk. *Food Chem.* **2022**, *374*, 131828. [CrossRef]
49. Katsarou, E.I.; Billinis, C.; Galamatis, D.; Fthenakis, G.C.; Tsangaris, G.T.; Katsafadou, A.I. Applied proteomics in 'one health'. *Proteomes* **2021**, *9*, 31. [CrossRef] [PubMed]
50. Piras, C.; Roncada, P.; Rodrigues, P.M.; Bonizzi, L.; Soggiu, A. Proteomics in food: Quality, safety, microbes, and allergens. *Proteomics* **2016**, *16*, 799–815. [CrossRef] [PubMed]
51. Meng, Y.; Qiu, N.; Guyonnet, V.; Mine, Y. Omics as a window to unravel the dynamic changes of egg components during chicken embryonic development. *J. Agric. Food Chem.* **2021**, *69*, 12947–12955. [CrossRef] [PubMed]
52. Alessandroni, L.; Sagratini, G.; Gagaoua, M. Proteomics and bioinformatics analyses based on two-dimensional electrophoresis and LC-MS/MS for the primary characterization of protein changes in chicken breast meat from divergent farming systems: Organic versus antibiotic-free. *Food Chem. Mol. Sci.* **2024**, *8*, 100194. [CrossRef] [PubMed]
53. Wu, Y.; Zhou, J.; Zhang, X.; Zheng, X.; Jiang, X.; Shi, L.; Yin, W.; Wang, J. Optimized sample preparation for two-dimensional gel electrophoresis of soluble proteins from chicken bursa of Fabricius. *Proteome Sci.* **2009**, *7*, 38. [CrossRef] [PubMed]
54. Hu, S.; Qiu, N.; Liu, Y.; Zhao, H.; Gao, D.; Song, R.; Ma, M. Identification and comparative proteomic study of quail and duck egg white protein using 2-dimensional gel electrophoresis and matrix-assisted laser desorption/ionization time-of-flight tandem mass spectrometry analysis. *Poult. Sci.* **2016**, *95*, 1137–1144. [CrossRef]
55. Soares, R.; Franco, C.; Pires, E.; Ventosa, M.; Palhinhas, R.; Koci, K.; de Almeida, A.M.; Coelho, A.V. Mass spectrometry and animal science: Protein identification strategies and particularities of farm animal species. *J. Proteom.* **2012**, *75*, 4190–4206. [CrossRef]
56. Murray, K.K. Coupling matrix-assisted laser desorption/ionization to liquid separations. *Mass Spectrom. Rev.* **1997**, *16*, 283–299. [CrossRef]
57. Stachniuk, A.; Sumara, A.; Montowska, M.; Fornal, E. Liquid chromatography–mass spectrometry bottom-up proteomic methods in animal species analysis of processed meat for food authentication and the detection of adulterations. *Mass Spectrom. Rev.* **2021**, *40*, 3–30. [CrossRef] [PubMed]
58. Templin, M.F.; Stoll, D.; Schwenk, J.M.; Pötz, O.; Kramer, S.; Joos, T.O. Protein microarrays: Promising tools for proteomic research. *Proteomics* **2003**, *3*, 2155–2166. [CrossRef] [PubMed]
59. Fournier, M.L.; Gilmore, J.M.; Martin-Brown, S.A.; Washburn, M.P. Multidimensional separations-based shotgun proteomics. *Chem. Rev.* **2007**, *107*, 3654–3686. [CrossRef] [PubMed]
60. Ronsein, G.E.; Pamir, N.; von Haller, P.D.; Kim, D.S.; Oda, M.N.; Jarvik, G.P.; Vaisar, T.; Heinecke, J.W. Parallel reaction monitoring (PRM) and selected reaction monitoring (SRM) exhibit comparable linearity, dynamic range and precision for targeted quantitative HDL proteomics. *J. Proteom.* **2015**, *113*, 388–399. [CrossRef] [PubMed]
61. Peterson, A.C.; Russell, J.D.; Bailey, D.J.; Westphall, M.S.; Coon, J.J. Parallel reaction monitoring for high resolution and high mass accuracy quantitative, targeted proteomics. *Mol. Cell. Proteom.* **2012**, *11*, 1475–1488. [CrossRef] [PubMed]

62. Samperi, R.; Capriotti, A.L.; Cavaliere, C.; Colapicchioni, V.; Chiozzi, R.Z.; Laganà, A. Food proteins and peptides. In *Comprehensive Analytical Chemistry*; Elsevier: Amsterdam, The Netherlands, 2015; pp. 309–357. [CrossRef]
63. Jax, E.; Wink, M.; Kraus, R.H. Avian transcriptomics: Opportunities and challenges. *J. Ornithol.* **2018**, *159*, 599–629. [CrossRef]
64. Tao, Z.; Song, W.; Zhu, C.; Xu, W.; Liu, H.; Zhang, S.; Li, H. Comparative transcriptomic analysis of high and low egg-producing duck ovaries. *Poult. Sci.* **2017**, *96*, 4378–4388. [CrossRef] [PubMed]
65. Yin, Z.; Lian, L.; Zhu, F.; Zhang, Z.-H.; Hincke, M.; Yang, N.; Hou, Z.-C. The transcriptome landscapes of ovary and three oviduct segments during chicken (*Gallus gallus*) egg formation. *Genomics* **2020**, *112*, 243–251. [CrossRef]
66. Gao, G.; Zhang, H.; Ni, J.; Zhao, X.; Zhang, K.; Wang, J.; Kong, X.; Wang, Q. Insights into genetic diversity and phenotypic variations in domestic geese through comprehensive population and pan-genome analysis. *J. Anim. Sci. Biotechnol.* **2023**, *14*, 150. [CrossRef]
67. Elfwing, M.; Nätt, D.; Goerlich-Jansson, V.C.; Persson, M.; Hjelm, J.; Jensen, P. Early stress causes sex-specific, life-long changes in behaviour, levels of gonadal hormones, and gene expression in chickens. *PLoS ONE* **2015**, *10*, e0125808. [CrossRef] [PubMed]
68. Shen, X.; Bai, X.; Xu, J.; Zhou, M.; Xu, H.; Nie, Q.; Lu, X.; Zhang, X. Transcriptome sequencing reveals genetic mechanisms underlying the transition between the laying and brooding phases and gene expression changes associated with divergent reproductive phenotypes in chickens. *Mol. Biol. Rep.* **2016**, *43*, 977–989. [CrossRef] [PubMed]
69. Sun, T.; Xiao, C.; Deng, J.; Yang, Z.; Zou, L.; Du, W.; Li, S.; Huo, X.; Zeng, L.; Yang, X. Transcriptome analysis reveals key genes and pathways associated with egg production in Nandan-Yao domestic chicken. *Comp. Biochem. Physiol. Part D Genom. Proteom.* **2021**, *40*, 100889. [CrossRef]
70. Cedar, H.; Bergman, Y. Linking DNA methylation and histone modification: Patterns and paradigms. *Nat. Rev. Genet.* **2009**, *10*, 295–304. [CrossRef] [PubMed]
71. Chen, X.; Sun, X.; Chimbaka, I.M.; Qin, N.; Xu, X.; Liswaniso, S.; Xu, R.; Gonzalez, J.M. Transcriptome analysis of ovarian follicles reveals potential pivotal genes associated with increased and decreased rates of chicken egg production. *Front. Genet.* **2021**, *12*, 622751. [CrossRef] [PubMed]
72. Ugur, M.R.; Guerreiro, D.D.; Moura, A.A.; Memili, E. Identification of biomarkers for bull fertility using functional genomics. *Anim. Reprod.* **2022**, *19*, e20220004. [CrossRef] [PubMed]
73. Shao, D.; Hu, Y.; Wang, Q.; Tong, H.; Shi, S. Transcriptome sequencing reveals genetic mechanisms of reproduction performance stimulated by dietary daidzein in laying breeder hens. *Theriogenology* **2020**, *142*, 120–130. [CrossRef] [PubMed]
74. Lowe, R.; Shirley, N.; Bleackley, M.; Dolan, S.; Shafee, T. Transcriptomics technologies. *PLoS Comput. Biol.* **2017**, *13*, e1005457. [CrossRef] [PubMed]
75. Vijayalakshmy, K.; Kumar, D.; Virmani, M.; Jacob, N.; Kumar, P. Sperm Transcriptomics: An emerging technique to assess male fertility. *Int. J. Curr. Microbiol. Appl. Sci.* **2018**, *7*, 1188–1200. [CrossRef]
76. Magar, N.D.; Shah, P.; Harish, K.; Bosamia, T.C.; Barbadikar, K.M.; Shukla, Y.M.; Phule, A.; Zala, H.N.; Madhav, M.S.; Mangrauthia, S.K.; et al. Gene expression and transcriptome sequencing: Basics, analysis, advances. In *Gene Expression*; IntechOpen: London, UK, 2022. [CrossRef]
77. Bytov, M.V.; Zubareva, V.D.; Volskaya, S.V.; Khatsko, S.L.; Shkuratova, I.A.; Sokolova, O.V. Current State of In Situ Gene Expression Studies in Animal Tissues. *Genetika* **2024**, *60*, 3–15. [CrossRef]
78. Guo, W.; Tzioutziou, N.A.; Stephen, G.; Milne, I.; Calixto, C.P.; Waugh, R.; Brown, J.W.; Zhang, R. 3D RNA-seq: A powerful and flexible tool for rapid and accurate differential expression and alternative splicing analysis of RNA-seq data for biologists. *RNA Biol.* **2021**, *18*, 1574–1587. [CrossRef] [PubMed]
79. Luk, A.C.-S.; Chan, W.-Y.; Rennert, O.M.; Lee, T.-L. Long noncoding RNAs in spermatogenesis: Insights from recent high-throughput transcriptome studies. *Reproduction* **2014**, *147*, R131–R141. [CrossRef]
80. Ayers, K.L.; Lambeth, L.S.; Davidson, N.M.; Sinclair, A.H.; Oshlack, A.; Smith, C.A. Identification of candidate gonadal sex differentiation genes in the chicken embryo using RNA-seq. *BMC Genom.* **2015**, *16*, 704. [CrossRef] [PubMed]
81. Conway, T.; Schoolnik, G.K. Microarray expression profiling: Capturing a genome-wide portrait of the transcriptome. *Mol. Microbiol.* **2003**, *47*, 879–889. [CrossRef]
82. Stoughton, R.B. Applications of DNA microarrays in biology. *Annu. Rev. Biochem.* **2005**, *74*, 53–82. [CrossRef]
83. Bethin, K.E.; Nagai, Y.; Sladek, R.; Asada, M.; Sadovsky, Y.; Hudson, T.J.; Muglia, L.J. Microarray analysis of uterine gene expression in mouse and human pregnancy. *Mol. Endocrinol.* **2003**, *17*, 1454–1469. [CrossRef]
84. Hennebold, J.D. Characterization of the ovarian transcriptome through the use of differential analysis of gene expression methodologies. *Hum. Reprod. Updat.* **2004**, *10*, 227–239. [CrossRef]
85. Zhang, T.; Chen, Y.; Wen, J.; Jia, Y.; Wang, L.; Lv, X.; Yang, W.; Qu, C.; Li, H.; Wang, H.; et al. Transcriptomic analysis of laying hens revealed the role of aging-related genes during forced molting. *Genes* **2021**, *12*, 1767. [CrossRef] [PubMed]
86. Röll, S.; Härtle, S.; Lütteke, T.; Kaspers, B.; Härtle, S. Tissue and time specific expression pattern of interferon regulated genes in the chicken. *BMC Genom.* **2017**, *18*, 264. [CrossRef]
87. Ma, R.; Xu, S.; Zhao, Y.; Xia, B.; Wang, R. Selection and validation of appropriate reference genes for quantitative real-time PCR analysis of gene expression in Lycoris aurea. *Front. Plant Sci.* **2016**, *7*, 183134. [CrossRef]
88. Jozefczuk, J.; Adjaye, J. Quantitative real-time PCR-based analysis of gene expression. In *Methods in Enzymology*; Elsevier: Amsterdam, The Netherlands, 2011; pp. 99–109. [CrossRef]

89. Yi, G.; Yuan, J.; Bi, H.; Yan, W.; Yang, N.; Qu, L. In-depth duodenal transcriptome survey in chickens with divergent feed efficiency using RNA-Seq. *PLoS ONE* **2015**, *10*, e0136765. [CrossRef] [PubMed]
90. Noel, D.; Malerba, G.; Ferrarini, A.; Delledonne, M. Evaluation of microarray sensitivity and specificity in gene expression differential analysis by RNA-seq and quantitative RT-PCR. *J. Multidiscip. Res.* **2014**, *2*, 5–9.
91. Wu, Y.; Zhao, X.; Chen, L.; Wang, J.; Duan, Y.; Li, H.; Lu, L. Transcriptomic analyses of the hypothalamic-pituitary-gonadal axis identify candidate genes related to egg production in Xinjiang Yili geese. *Animals* **2020**, *10*, 90. [CrossRef] [PubMed]
92. Jovic, D.; Liang, X.; Zeng, H.; Lin, L.; Xu, F.; Luo, Y. Single-cell RNA sequencing technologies and applications: A brief overview. *Clin. Transl. Med.* **2022**, *12*, e694. [CrossRef] [PubMed]
93. Jung, K.M.; Seo, M.; Han, J.Y. Comparative single-cell transcriptomic analysis reveals differences in signaling pathways in gonadal primordial germ cells between chicken (*Gallus gallus*) and zebra finch (*Taeniopygia guttata*). *FASEB J.* **2023**, *37*, e22706. [CrossRef] [PubMed]
94. Alessio, E.; Bonadio, R.S.; Buson, L.; Chemello, F.; Cagnin, S. A single cell but many different transcripts: A journey into the world of long non-coding RNAs. *Int. J. Mol. Sci.* **2020**, *21*, 302. [CrossRef]
95. Kalhor, K.; Chen, C.-J.; Lee, H.S.; Cai, M.; Nafisi, M.; Que, R.; Palmer, C.R.; Yuan, Y.; Zhang, Y.; Li, X.; et al. Mapping human tissues with highly multiplexed RNA in situ hybridization. *Nat. Commun.* **2024**, *15*, 2511. [CrossRef] [PubMed]
96. Veselinyová, D.; Mašlanková, J.; Kalinová, K.; Mičková, H.; Mareková, M.; Rabajdová, M. Selected in situ hybridization methods: Principles and application. *Molecules* **2021**, *26*, 3874. [CrossRef]
97. Shang, E.; Salazar, G.; E Crowley, T.; Wang, X.; A Lopez, R.; Wang, X.; Wolgemuth, D.J. Identification of unique, differentiation stage-specific patterns of expression of the bromodomain-containing genes Brd2, Brd3, Brd4, and Brdt in the mouse testis. *Gene Expr. Patterns* **2004**, *4*, 513–519. [CrossRef]
98. Muthubharathi, B.C.; Gowripriya, T.; Balamurugan, K. Metabolomics: Small molecules that matter more. *Mol. Omics* **2021**, *17*, 210–229. [CrossRef]
99. Fiehn, O. Combining genomics, metabolome analysis, and biochemical modelling to understand metabolic networks. *Comp. Funct. Genom.* **2001**, *2*, 155–168. [CrossRef] [PubMed]
100. Xiang, X.; Huang, X.; Wang, J.; Zhang, H.; Zhou, W.; Xu, C.; Huang, Y.; Tan, Y.; Yin, Z. Transcriptomic and metabolomic analyses of the ovaries of Taihe black-bone silky fowls at the peak egg-laying and nesting period. *Front. Genet.* **2023**, *14*, 1222087. [CrossRef] [PubMed]
101. Miretti, S.; Lecchi, C.; Ceciliani, F.; Baratta, M. MicroRNAs as biomarkers for animal health and welfare in livestock. *Front. Vet. Sci.* **2020**, *7*, 578193. [CrossRef]
102. Tonks, A.A. Exploring the Effects of Management Strategies on the Gut Microbiome and Metabolome of Growing Broiler Chickens: An Integrated Metagenomic and Metabolomic Approach. Ph.D. Thesis, University of Reading, Reading, UK, 2019. [CrossRef]
103. Mon, K.K.Z.; Zhu, Y.; Chanthavixay, G.; Kern, C.; Zhou, H. Integrative analysis of gut microbiome and metabolites revealed novel mechanisms of intestinal Salmonella carriage in chicken. *Sci. Rep.* **2020**, *10*, 4809. [CrossRef] [PubMed]
104. Mills, K.M. Identification of Protein and Lipid Biomarkers of Infertility in Young Boars and Prepubertal Gilts. Ph.D. Thesis, Purdue University, West Lafayette, IN, USA, 2021. Available online: https://docs.lib.purdue.edu/dissertations/AAI30505196/ (accessed on 1 August 2021).
105. Qi, X.; Yun, C.; Pang, Y.; Qiao, J. The impact of the gut microbiota on the reproductive and metabolic endocrine system. *Gut Microbes* **2021**, *13*, 1894070. [CrossRef] [PubMed]
106. Garcia, A.; Barbas, C. Gas chromatography-mass spectrometry (GC-MS)-based metabolomics. In *Metabolic Profiling: Methods and Protocols*; Humana Press: Totowa, NJ, USA, 2011; pp. 191–204. [CrossRef]
107. Milojevic, V. Investigating Fatty Acid Metabolism in Mammals, with Specific Emphasis on the Female Reproductive Tract. Ph.D. Thesis, ETH, Zurich, Switzerland, 2019.
108. Malekinejad, H.; Rezabakhsh, A. Hormones in dairy foods and their impact on public health-a narrative review article. *Iran. J. Public Health* **2015**, *44*, 742. [PubMed Central]
109. Ivanisevic, J.; Want, E.J. From samples to insights into metabolism: Uncovering biologically relevant information in LC-HRMS metabolomics data. *Metabolites* **2019**, *9*, 308. [CrossRef]
110. Peng, M.L.; Li, S.N.; He, Q.Q.; Zhao, J.L.; Li, L.L.; Ma, H.T. Based serum metabolomics analysis reveals simultaneous interconnecting changes during chicken embryonic development. *J. Anim. Physiol. Anim. Nutr.* **2018**, *102*, 1210–1219. [CrossRef] [PubMed]
111. Li, J.; Zhang, Z.; Wei, Y.; Zhu, P.; Yin, T.; Wan, Q. Metabonomic analysis of follicular fluid in patients with diminished ovarian reserve. *Front. Endocrinol.* **2023**, *14*, 1132621. [CrossRef]
112. Zahra, Z.S.A. Nuclear Magnetic Resonance (NMR): Principle, Applications, Types, and Uses in Metabolite Identification and Medical Biotechnology. *Curr. Clin. Med. Edu.* **2024**, *2*, 33–48.
113. Moore, R.E.; Kirwan, J.; Doherty, M.K.; Whitfield, P.D. Biomarker discovery in animal health and disease: The application of post-genomic technologies. *Biomark. Insights* **2007**, *2*, 185–196. [CrossRef] [PubMed]
114. Deepinder, F.; Chowdary, H.T.; Agarwal, A. Role of metabolomic analysis of biomarkers in the management of male infertility. *Expert Rev. Mol. Diagn.* **2007**, *7*, 351–358. [CrossRef] [PubMed]
115. Schrimpe-Rutledge, A.C.; Codreanu, S.G.; Sherrod, S.D.; McLean, J.A. Untargeted metabolomics strategies—Challenges and emerging directions. *J. Am. Soc. Mass. Spectrom.* **2016**, *27*, 1897–1905. [CrossRef] [PubMed]

116. Shi, K.; Zhao, Q.; Shao, M.; Duan, Y.; Li, D.; Lu, Y.; Tang, Y.; Feng, C. Untargeted metabolomics reveals the effect of selective breeding on the quality of chicken meat. *Metabolites* **2022**, *12*, 367. [CrossRef] [PubMed]
117. Roberts, L.D.; Souza, A.L.; Gerszten, R.E.; Clish, C.B. Targeted metabolomics. *Curr. Protoc. Mol. Biol.* **2012**, *98*, 30.2.1–30.2.24. [CrossRef] [PubMed]
118. Hanlon, C.; Ramachandran, R.; Zuidhof, M.J.; Bédécarrats, G.Y. Should I lay or should I grow: Photoperiodic versus metabolic cues in chickens. *Front. Physiol.* **2020**, *11*, 707. [CrossRef] [PubMed]
119. Barnes, B.; Dupré, J. *Genomes and What to Make of Them*; University of Chicago Press: Chicago, IL, USA, 2009. Available online: https://academic.oup.com/chicago-scholarship-online/book/22203 (accessed on 13 June 2024).
120. Makanjuola, B.O.; Abdalla, E.A.; Baes, C.F.; Wood, B.J. Selection for Reproductive Efficiency in Turkeys and Broiler Chickens: Egg Production, Hatchability and Fertility. 2021. Available online: https://www.cabidigitallibrary.org/doi/full/10.5555/20210433721 (accessed on 13 June 2024).
121. Rubin, C.-J.; Zody, M.C.; Eriksson, J.; Meadows, J.R.S.; Sherwood, E.; Webster, M.T.; Jiang, L.; Ingman, M.; Sharpe, T.; Ka, S.; et al. Whole-genome resequencing reveals loci under selection during chicken domestication. *Nature* **2010**, *464*, 587–591. [CrossRef] [PubMed]
122. Shen, M.; Qu, L.; Ma, M.; Dou, T.; Lu, J.; Guo, J.; Hu, Y.; Yi, G.; Yuan, J.; Sun, C.; et al. Genome-wide association studies for comb traits in chickens. *PLoS ONE* **2016**, *11*, e0159081. [CrossRef]
123. Zhang, H.; Zhang, H.; Wang, Z.; Wang, Z.; Wang, S.; Wang, S.; Li, H.; Li, H. Progress of genome wide association study in domestic animals. *J. Anim. Sci. Biotechnol.* **2012**, *3*, 26. [CrossRef]
124. Gao, G.; Gao, D.; Zhao, X.; Xu, S.; Zhang, K.; Wu, R.; Yin, C.; Li, J.; Xie, Y.; Hu, S.; et al. Genome-wide association study-based identification of SNPs and haplotypes associated with goose reproductive performance and egg quality. *Front. Genet.* **2021**, *12*, 602583. [CrossRef]
125. Stock, K.; Reents, R. Genomic selection: Status in different species and challenges for breeding. *Reprod. Domest. Anim.* **2013**, *48*, 2–10. [CrossRef]
126. Gibney, E.; Nolan, C. Epigenetics and gene expression. *Heredity* **2010**, *105*, 4–13. [CrossRef]
127. Piferrer, F. Epigenetics of sex determination and gonadogenesis. *Dev. Dyn.* **2013**, *242*, 360–370. [CrossRef]
128. Li, C.; Guo, S.; Zhang, M.; Gao, J.; Guo, Y. DNA methylation and histone modification patterns during the late embryonic and early postnatal development of chickens. *Poult. Sci.* **2015**, *94*, 706–721. [CrossRef]
129. Ellison, A.; López, C.M.R.; Moran, P.; Breen, J.; Swain, M.; Megias, M.; Hegarty, M.; Wilkinson, M.; Pawluk, R.; Consuegra, S.; et al. Epigenetic regulation of sex ratios may explain natural variation in self-fertilization rates. *Proc. R. Soc. B Biol. Sci.* **2015**, *282*, 20151900. [CrossRef]
130. Bednarczyk, M.; Dunislawska, A.; Stadnicka, K.; Grochowska, E. Chicken embryo as a model in epigenetic research. *Poult. Sci.* **2021**, *100*, 101164. [CrossRef]
131. Jensen, P. Behaviour epigenetics–the connection between environment, stress and welfare. *Appl. Anim. Behav. Sci.* **2014**, *157*, 1–7. [CrossRef]
132. Nyholm, L.; Koziol, A.; Marcos, S.; Botnen, A.B.; Aizpurua, O.; Gopalakrishnan, S.; Limborg, M.T.; Gilbert, M.P.; Alberdi, A. Holo-omics: Integrated host-microbiota multi-omics for basic and applied biological research. *iScience* **2020**, *23*, 101414. [CrossRef]
133. Arora, I.; Tollefsbol, T.O. Computational methods and next-generation sequencing approaches to analyze epigenetics data: Profiling of methods and applications. *Methods* **2021**, *187*, 92–103. [CrossRef]
134. Stamou, M.; Cox, K.; Crowley, W.F., Jr. Discovering genes essential to the hypothalamic regulation of human reproduction using a human disease model: Adjusting to life in the "-omics" era. *Endocr. Rev.* **2015**, *36*, 603–621. [CrossRef]
135. Ibeagha-Awemu, E.M.; Zhao, X. Epigenetic marks: Regulators of livestock phenotypes and conceivable sources of missing variation in livestock improvement programs. *Front. Genet.* **2015**, *6*, 139313. [CrossRef] [PubMed]
136. Colon-Diaz, M.; Jaramillo, A.J.; Soto, E.Y.; Elosegui, P.M. Reproductive disease epigenetics. In *Medical Epigenetics*; Elsevier: Amsterdam, The Netherlands, 2021; pp. 309–346. [CrossRef]
137. Fu, W.; Wang, R.; Xu, N.; Wang, J.; Li, R.; Nanaei, H.A.; Nie, Q.; Zhao, X.; Han, J.; Yang, N.; et al. Galbase: A comprehensive repository for integrating chicken multi-omics data. *BMC Genom.* **2022**, *23*, 364. [CrossRef] [PubMed]

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Review

The Latest Look at PDT and Immune Checkpoints

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Abstract: Photodynamic therapy (PDT) can not only directly eliminate cancer cells, but can also stimulate antitumor immune responses. It also affects the expression of immune checkpoints. The purpose of this review is to collect, analyze, and summarize recent news about PDT and immune checkpoints, along with their inhibitors, and to identify future research directions that may enhance the effectiveness of this approach. A search for research articles published between January 2023 and March 2024 was conducted in PubMed/MEDLINE. Eligibility criteria were as follows: (1) papers describing PDT and immune checkpoints, (2) only original research papers, (3) only papers describing new reports in the field of PDT and immune checkpoints, and (4) both in vitro and in vivo papers. Exclusion criteria included (1) papers written in a language other than Polish or English, (2) review papers, and (3) papers published before January 2023. 24 papers describing new data on PDT and immune checkpoints have been published since January 2023. These included information on the effects of PDT on immune checkpoints, and attempts to associate PDT with ICI and with other molecules to modulate immune checkpoints, improve the immunosuppressive environment of the tumor, and resolve PDT-related problems. They also focused on the development of new nanoparticles that can improve the delivery of photosensitizers and drugs selectively to the tumor. The effect of PDT on the level of immune checkpoints and the associated activity of the immune system has not been fully elucidated further, and reports in this area are divergent, indicating the complexity of the interaction between PDT and the immune system. PDT-based strategies have been shown to have a beneficial effect on the delivery of ICI to the tumor. The utility of PDT in enhancing the induction of the antitumor response by participating in the triggering of immunogenic cell death, the exposure of tumor antigens, and the release of various alarm signals that together promote the activation of dendritic cells and other components of the immune system has also been demonstrated, with the result that PDT can enhance the antitumor immune response induced by ICI therapy. PDT also enables multifaceted regulation of the tumor's immunosuppressive environment, as a result of which ICI therapy has the potential to achieve better antitumor efficacy. The current review has presented evidence of PDT's ability to modulate the level of immune checkpoints and the effectiveness of the association of PDT with ICIs and other molecules in inducing an effective immune response against cancer cells. However, these studies are at an early stage and many more observations need to be made to confirm their efficacy. The new research directions indicated may contribute to the development of further strategies.

Keywords: PDT; immune checkpoints; effectiveness; cancer cell

1. Introduction

Cancer is one of the common diseases with a high mortality rate worldwide and is the most important obstacle to improving the overall life expectancy of the population in the 21st century [1]. New therapeutic options are constantly being sought to improve this

situation. In this regard, photodynamic therapy (PDT) has attracted increasing attention in recent years due to its potential in cancer treatment [2–4]. Although PDT results vary depending on the type of cancer, minimal tissue toxicity, low systemic effects, high efficacy, and minimal side effects make it a valuable therapeutic option [5]. PDT has been clinically approved for the treatment of head and neck cancer, esophageal cancer, pancreatic cancer, prostate cancer, and squamous cell carcinoma [6]. PDT uses three main agents: a photosensitizer (PS), light, and oxygen [7,8]. Absorption of light by the photosensitizer molecule causes it to go from a ground state to a short-lived excited singlet state. An intersystem transition then occurs to the more stable excited triplet state. From this triplet state, the photosensitizer molecules can return to the ground state through a type I or type II photodynamic reaction. In a type I reaction, the activated photosensitizer transfers an electron to the substrate to form various reactive oxygen species. In a type II reaction, energy is directly transferred to molecular oxygen in the ground state, resulting in the formation of highly reactive singlet oxygen [9]. It is widely believed that singlet oxygen is the main agent responsible for the photodynamic destruction of cells and tissues. Most of the PSs currently in use work primarily through a type II mechanism [10]. Reactive oxygen species kill tumor cells, destroy tumor blood vessels, and lead to tumor regression through cell death mechanisms [11]. Depending on the subcellular localization of the photosensitizer, cell membrane damage, or disruption of mitochondria, endoplasmic reticulum or lysosomes can occur. This in turn triggers stress reactions, leading to cell necrosis, apoptosis, or autophagy [12]. Photosensitizer photoactivation promotes the detachment of endothelial tissues from the basement membrane of blood vessels. This creates thrombogenic areas characterized by the aggregation of thrombocytes, production of vasoactive molecules, and increased permeability and vasoconstriction [13]. This leads to cancer cell death due to a lack of oxygen and nutrition, thus inhibiting cancer growth [14]. PDT also stimulates the immune response. Destruction of cancer cell organelles and membranes activates phospholipases and cyclooxygenases, leading to the release of inflammatory mediators that attract leukocytes that fight cancer cells [15]. However, the antitumor effects induced by PDT are influenced by several factors, including PS localization in cells, PS concentration, light fluence rate, oxygen concentration, and integrity of immune function [16]. Therefore, PDT still has many limitations, such as metastatic tumors, insufficient light delivery, and a lack of adequate oxygen [17]. As a result, PDT is limited to treating thin and superficial tumors. Recently, however, emerging nanotechnology has contributed to advances in many areas, including PDT, improving its ability to penetrate deep tumor tissues and produce the desired therapeutic effects [10,18]. In addition, photodynamic therapy can not only eliminate cancer cells, but can also stimulate antitumor immune responses [19]. Results have shown that PDT at a high dose of light, as a therapeutic dose, induced early immune activation followed by late immunosuppression, which was mediated by activated TGF-β1 regulation [20]. Also, photogenerated cytotoxic molecules localized in tumor tissue can lead to immunogenic cell death (ICD), which releases damage-related molecular patterns and tumor-specific antigens [21]. The field of cancer photodynamic therapy, like oncology research in general, is showing increasing interest in cancer immunology and the immunological effects of cancer treatment [22]. Immunotherapy has made tremendous clinical progress in recent years [23]. However, it is only effective in a fraction of cancer patients due to low response rates and serious side effects. Findings indicate that the challenges of immunotherapy in the clinic can be solved by inducing immunogenic cell death, among others. ICD is currently considered one of the most promising ways to achieve complete eradication of cancer cells [24]. It is induced to release threat-associated molecular patterns (DAMPs) and tumor-associated antigens to facilitate dendritic cell (DC) maturation and infiltration of cytotoxic T lymphocytes (CTLs). This process can reverse the immunosuppressive tumor microenvironment (TME) and significantly improve therapeutic efficacy [25,26]. Consequently, immune checkpoint blockade is now one of the most important types of antitumor immunotherapy, which aims to restore the function of effector immune cells, most commonly T cells, by administering antibodies that block

inhibitory molecules [27]. It has been shown that the efficacy of PDT and immunotherapy in preventing cancer metastasis and recurrence can be significantly improved primarily through combined strategies [28,29]. Synergistic enhancement of ICD may be particularly important in this area [30] (Figure 1).

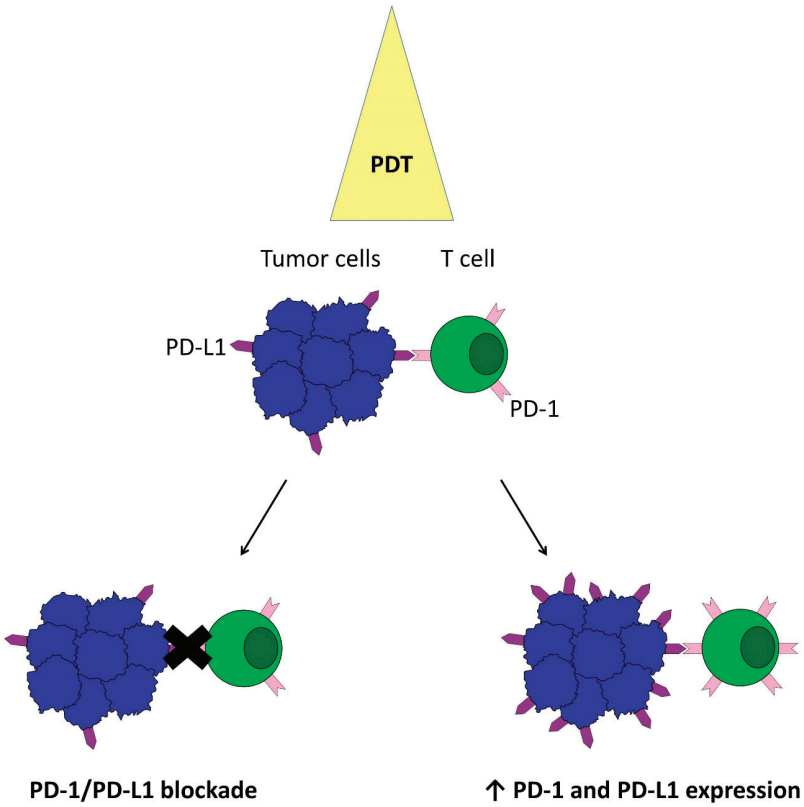


Figure 1. Effects of photodynamic therapy on the PD1/PD-L1 axis. (1) PDT with PS chlorin e6 through an unexplained mechanism has the ability to block the PD-1/PD-L1 axis and increase CD8+ T-cell activity, but without inducing an antitumor immune response [31–35]; (2) ALA-PDT can increase PD-1 expression on lymphocytes, while Redaporfin-PDT can increase PD-L1 expression on tumor cells [36,37]. The impact of these changes on the effectiveness of therapy is described further in the text.

2. Methods

A literature search that focused on papers describing photodynamic therapy and immune checkpoints in the treatment of malignant tumors was conducted using the PubMed/MEDLINE database. The following terms were searched: "PDT AND immunotherapy", "PDT and immune checkpoint inhibitors", and "PDT and immune checkpoint" (Figure 2). A total of 812 papers were identifed. Only research papers, both in vivo and in vitro, were eligible. To describe recent developments, articles written before January 2023 were excluded. Only articles written in English or Polish were eligible. The final number of papers selected was 24. The inclusion and exclusion criteria are shown in Table 1.

Table 1. Inclusion and exclusion criteria.

Inclusion
Papers describing PDT and immunological checkpoints were included
Only original research papers were included
Only papers describing new strategies or reports in the field of PDT and immune checkpoints were included

Table 1. *Cont.*

Both in vitro and in vivo papers were included
Exclusion
Review papers were excluded
Articles in a language other than English or Polish were excluded
Papers written before 2023 were excluded

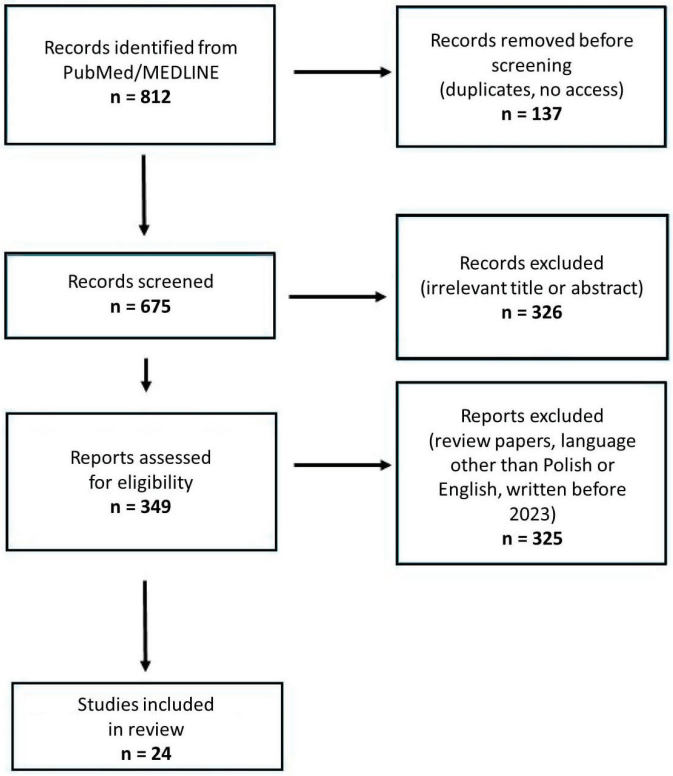


Figure 2. PRISMA flow diagram of the studies included.

3. Results and Discussion

3.1. PDT versus Immunological Checkpoints

Programmed cell death protein 1 (PD-1) and programmed death-ligand (PD-L1) are typical immune checkpoints that transmit inhibitory signals to silence host immunity [31,32]. The up-regulation of PD-L1 in tumor cells has been shown to be generally associated with tumor progression, proliferation, and invasion; anti-apoptotic signaling; and T-cell inhibitory activity through PD-1 engagement [33,34]. Reducing their negative effects is particularly desirable in cancer therapy. A number of recent studies describe the potential of PDT to improve the efficacy of the immune response by affecting immune checkpoints. Work by Gurung et al. demonstrates that PDT with PS chlorin e6 through an unexplained mechanism has the ability to block the PD-1/PD-L1 axis and increase CD8+ T-cell activity. However, they observed that despite the increased infiltration of these cells in some of the mice tested, they were depleted and an antitumor immune response was not induced [35]. On the other hand, Lobo et al. and Anand et al. show that in some tumor cells, PDT increased CTLA-4 and PD-L1 and PD-1 expression on lymphocytes, as schematically shown in Figure 1 [36,37]. However, this may have a potentially beneficial effect on immune checkpoint inhibitors (ICI) therapy, as pre-existing T cells in the tumor are a prerequisite for response to anti-PD-1 therapy, and the lack of reactive PD-L1 expression may indicate a poor response to PD-1 blockade therapy due to impaired T cells infiltrating

the tumor [38]. Moreover, it has been found that PDT can stimulate cells in some tumors to exhibit high levels of CD80 molecules on their surfaces. Overexpression of CD80 enhances tumor immunogenicity [36]. The formation of a cis-PD-L1-CD80 duplex, by binding CD80 to PD-L1, attenuates PD-L1-PD-1 binding and abrogates PD-1 function [39]. Removing this PD-1 restriction has been shown to have an immunosuppressive effect and may be effective in treating autoimmune disease [40]. The cis-PD-L1-CD80 duplex also inhibits CTLA-4 inhibitory receptors binding to CD80 in cancer cells [36]. Delcanale et al. point out that there does not appear to be a correlation between PDT efficacy and PD-L1 expression levels, indicating that mechanisms other than simple receptor density determine treatment outcome [40]. In conclusion, reports on PDT's ability to modulate PD-1 and PD-L1 levels lead to conflicting conclusions, and the mechanism is not completely elucidated.

3.2. Association of PDT with Immune Checkpoint Blockers

Checkpoint-blocking immunotherapy has recently been successful in the clinic, but elicits only limited rates of systemic antitumor response for most cancers due to insufficient activation of the host immune system [41,42]. Many reasons for this phenomenon have been identified [34,43–45]. Photodynamic therapy-based combination therapies have gained much attention in various cancers due to their potent therapeutic effects [46]. These include induction of immunogenic cell death, production of damage-related molecular patterns and tumor-associated antigens, and reprogramming of the tumor microenvironment [47]. All of these processes promote the activation of antigen-presenting cells and promote T-cell infiltration, so combining ICI treatment with PDT can address the reasons for the ineffectiveness of ICI therapy alone and enhance the antitumor immune response leading to effective immunotherapy, as we describe below [42,48,49].

3.2.1. Abnormal Tumor Vascularization

Increased angiogenesis in TME, the resulting abnormal vascularization, and high interstitial pressure within the tumor can impair the penetration of checkpoint inhibitors [50]. Moreover, endothelial cells may express PD-L1, which may further impair T-cell function in the tumor microenvironment [50,51]. In recent years, PDT has been found to have the ability to affect the biochemical and physical properties of TME [52]. It has been shown that by damaging endothelial components, PDT can significantly increase vascular permeability (Figure 3). However, these same effects can also lead to platelet aggregation, coagulation, and reduced blood flow in the tumor area [53]. In their novel work, Bhandari et al. doubled the delivery of α-PD-L1 antibodies in AT-84 mouse head and neck tumors using a single protocol of subtherapeutic photodynamic therapy regimens utilizing a liposomal benzoporphyrin derivative. Moreover, this improves the immune response by inducing immunogenic cell death and a 3–11 fold increase in tumor cell exposure to damage-related molecular patterns [52]. Zheng et al. developed a new nanoparticle (Combo-NP) consisting of a biodegradable pseudo-conjugate of the fluorescent polymer NIR II with disulfide bonds in its main chain and the vascular inhibitor lenvatinib [54]. Lenvatinib is a multi-kinase inhibitor that targets VEGFR1/2/3, FGFR, PDGFRB, KIT, and RET [55]. In a number of studies, lenvatinib in combination with PD-1/PD-L1 signaling inhibitors showed more potent antitumor activity than either drug alone [56]. Clinically, lenvatinib in combination with pembrolizumab has also shown promise, such as in the treatment of endometrial cancer [56,57]. Photodynamic therapy with Combo-NP led to remodeling and normalization of the tumor vasculature, thus enhancing the efficacy of PDT while promoting increased lymphocyte infiltration in the tumor microenvironment. Moreover, it also induced immunogenic cell death. In addition, in combination with anti-PD-L1, it showed a significant inhibitory effect on tumor metastasis [54].

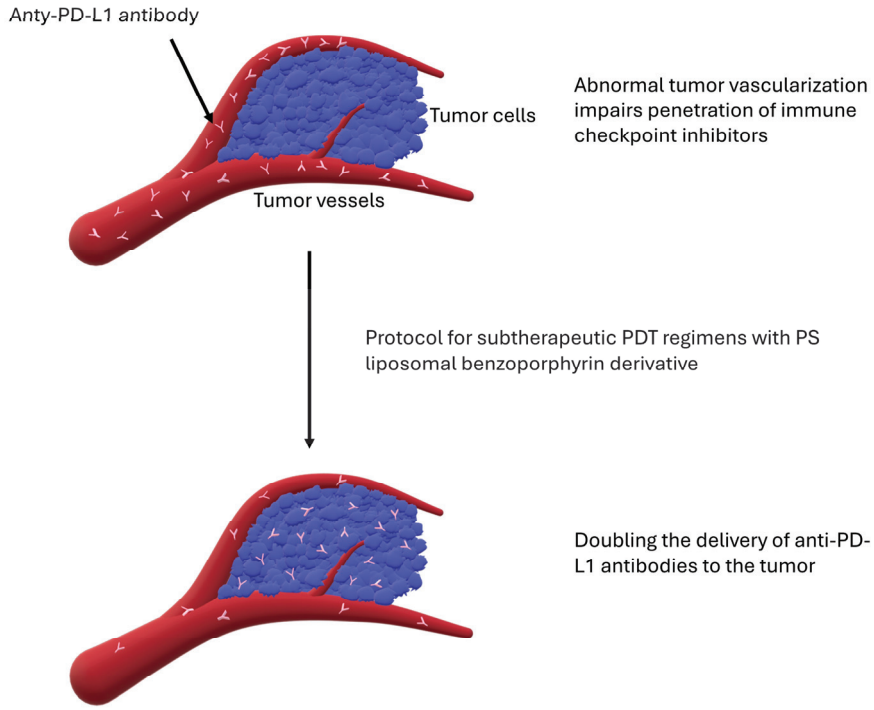


Figure 3. Abnormal tumor vascularization impairs the penetration of immune checkpoint inhibitors. It can be doubled by a regimen of subtherapeutic PDT with PS liposomal benzoporphyrin derivative [52].

3.2.2. Immunosuppressive Cells

The immunosuppressive effect of the tumor microenvironment is also characterized by the presence of cells with a suppressor function against functional immune cells, such as Tregs, myeloid-derived suppressor cells, and tumor-associated macrophages (TAMs) [38,45]. These may be responsible for the failure of immune checkpoint blocker therapy [58,59]. Recent studies have shown that human TAMs also express PD-1, which increases with increasing disease stage, and exhibit an M2-like surface profile. PD-1 expression on PD-1+TAM is associated with decreased macrophage phagocytosis and increased tumor cell invasion, which can lead to poor cancer prognosis [43]. Moreover, it has been found that tumor-associated macrophages can be a major source of PD-L1 [60]. Recruitment of M2 macrophages thus appears to lead to resistance to immunotherapy by affecting tumor angiogenesis, invasion, metastasis, and immunosuppression [34,61]. In the context of reducing the negative impact of M2 macrophages on ICI efficacy, the work of Sun et al. is interesting. They designed extracellular vesicles (EV) expressing Siglec-10 previously obtained from 4T1 breast cancer cells, additionally combined with a photosensitizer-loaded lipid nanosystem [62]. Siglec-10, a member of the sialic acid-binding immunoglobulin superfamily, has been identified as an immune checkpoint that is expressed on macrophages in various types of cancer [63,64]. The interaction of this molecule with CD24 on the surface of cancer cells can activate SHP-1 and/or SHP-2 phosphatases associated with tyrosine inhibitory motifs (ITIMs), which prevents TLR-mediated inflammation and cell engulfment by macrophages [65,66]. By combining Siglec-10 from nanoparticles with CD24 of cancer cells, blocking Siglec-10 attachment of macrophages was achieved increasing their anti-inflammatory activity. The generation of ROS by the photosensitizer during PDT can convert immunosuppressive M2 macrophages into M1 macrophages [62]. The EVs contained all tumor antigens, thus directly activating dendritic cells and a specific immune response [62]. In summary, this approach not only activated the antitumor immune response, but also reduced the negative impact of the immunosuppressive tumor environment on the effectiveness of the immune response. A new strategy for effective

immunotherapy against "cold" tumors was developed by Wang et al. The molecule they created, with activity similar to a number of enzymes, including superoxide dismutase, catalase, peroxidase, and glutathione oxidase, solved a number of problems associated with tumor immunosuppression. It promoted M2 to M1 macrophage polarization, alleviated hypoxia in TME, generated ROS, and depleted glutathione in TME to expose necrotic cell fragments and reverse immunosuppressive TME by inducing dendritic cell maturation and infiltration of cytotoxic T cells in tumors [67].

Indoleamine 2,3-dioxygenase (IDO) 1, IDO2, and tryptophan 2,3-dioxygenase (TDO) are responsible for degrading tryptophan to its downstream metabolites. Decreased tryptophan levels and increased IDO1 expression correlate with increased levels of immunosuppressive cells such as regulatory T cells and myeloid-derived suppressor cells, decreased levels of tumor infiltrating lymphocytes and NK cells, and up-regulation of PD-1 in cytotoxic T cells [68]. Thus, it seemed natural to combine IDO inhibitor therapy with immune checkpoint blockers. To date, however, the results of clinical trials using indoleamine 2,3-dioxygenase inhibitors and anti-PD-1 have been disappointing [68,69]. Importantly, IDO1 expression levels vary between tumor types and among patients with the same tumor type, which may account for treatment failures [68]. An attractive strategy to improve this association was developed by Zheng et al. They created a self-validating biomedicine based on the photosensitizer chlorin e6, an IDO inhibitor (NLG919), and a PD-1/PD-L1 blocker (BMS-1). PDT with this molecule led to significant cell apoptosis and ICD, while NLG919-induced IDO inhibition and BMS-1-induced PD-1/PD-L1 blockade improved the immunosuppressive tumor microenvironment to enhance the immune activation cascade. The ultimate effect was inhibition of tumor growth and metastasis [70]. In conclusion, improving the immunosuppressive tumor microenvironment and reducing the negative effects of immunosuppressive cells can enhance the efficacy of treatment with immune checkpoint blockers in several ways.

3.2.3. Loss of Tumor Neoantigens and Insufficient Stimulation of the Antitumor Response

It has been shown that loss of neoantigens, which are antigens encoded by the mutant gene of cancer cells, may be the cause of the ineffectiveness of anti-PD-1 therapy [38]. Emerging research supports the critical role of neoantigens in the response to PD-1 blockade therapy [71]. Evidence available to date suggests that chemotherapy promotes tumor antigen release and antigen presentation and stimulates immune factors [38]. Combining checkpoint inhibitors with chemotherapy has proven clinically effective in the treatment of triple-negative breast cancer [72]. The addition of ICD-stimulating PDT to these treatments may therefore be an attractive form of enhancing the immune response. Zhang et al. designed spherical nucleic acids based on the PD-L1 aptamer, consisting of a porphyrin Zr6 MOF core and PD-L1 aptamer shell, with oxaliplatin encapsulated in MOF nanopores. The combination of PDT, chemotherapy, and immunotherapy provided effective induction of immunogenic cell death and activation of the antitumor immune response. Moreover, precise delivery of the immune checkpoint inhibitor enhanced the immune response and minimized systemic toxicity [73]. Also, Feng et al. associated PDT with chemotherapy and immunotherapy. They created mitochondria-targeted nanoparticles capable of improving the efficacy of PDT and delivering the active metabolite of irinotecan SN-38. These particles induced immunogenic cell death and promoted the recruitment and activation of cytotoxic T cells. They were also shown to enhance the antitumor efficacy of anti-PD-1 antibody in a mouse model of a CT26 tumor [74]. It can be speculated that PDT played a key role in the effective immune response, as it was previously shown that the response to irinotecan-based chemotherapy after PD-1 blockade in patients with advanced esophageal squamous cell carcinoma appeared similar to that previously observed in patients who did not receive PD-1 antibodies [75]. A paper by Yao et al. designed a prodrug consisting of acid-activated doxorubicin and an aggregation-induced emission photosensitizer combined with a caspase-3-responsive peptide, and showed that the combination of repetitive PDT with anti-PD-1 therapy further promoted proliferation and intratumor infiltration of cytotoxic

T cells and effectively inhibited tumor growth and lung metastasis [76]. Clinical data available to date indicate that short-term doxorubicin therapy can induce a more favorable tumor microenvironment and increase the likelihood of response to PD-1 blockade in triple-negative breast cancer. However, these data require confirmation [77]. It has been observed that treatment of breast cancer with doxorubicin induced up-regulation of IDO1, so it seems interesting to attach an IDO1 inhibitor to this strategy [78].

Induction of pyroptosis and activation of the cyclic guanosine monophosphate/adenosine synthase/interferon gene stimulator (cGAS/STING) signaling pathway may be a new strategy to break the immunosuppressive tumor environment and improve the efficacy of anti-PD-L1 immunotherapy [79]. Recent studies have demonstrated the feasibility and clinical potential of using pyroptosis as a mechanism of antitumor immunity. Pyroptosis can increase the immune response and enhance the effect of immunotherapy through the release of various tumor antigens, DAMPs, and pro-inflammatory cytokines [80–83]. A limitation of this approach, however, is that pyroptosis can be strongly correlated with the proliferation and migration of various tumor cells [80,81]. The cyclic GMP-AMP synthase (cGAS) and stimulator of interferon genes (STING) pathway has emerged as a critical innate immune pathway [84]. As a DNA receptor, cyclic GMP-AMP synthase recognizes abnormal DNA in the cytoplasm and activates STING. This signaling cascade leads to an immune response produced by type I interferon and other immune mediators, but in some cases chronic STING activation can promote tumorigenesis [85]. STING agonists have been shown to reduce the side effects of drugs and overcome tolerance to cancer treatment [85]. It is also known that immune checkpoint blockade therapy remains limited by an insufficient immune response in "cold" tumors [67]. To address this problem, Yang et al. developed albumin nanospheres with photosensitizer-IR780 encapsulated in the core and cGAS-STING agonists/h2S-ZnS producer placed on the shell. Their results suggest that treatment based on this molecule can remodel the TME by increasing infiltration of CD8 + T cells and decreasing immunosuppressive Treg cells, thus changing triple-negative breast cancer from a "cold" tumor to a "hot" tumor, and thereby improving the efficacy of anti-PD-L1 immunotherapy [79]. Moreover, intracellular zinc ions can produce ROS, which can compensate for the diminished effect of PDT by tumor hypoxia [79,86]. Qu et al. developed a nanoparticle targeting TME of pancreatic ductal adenocarcinoma (PDAC) through tumor-specific midkine nanobodies, which can effectively deliver solid-state polymer nanoparticles to TME PDACs and locally produce abundant reactive oxygen species for precise photoimmunotherapy [87]. PDACs are among the prototypical immunogenically "cold" tumors because they lack the natural infiltration of ICI-responsive effector T cells. It has been found that in order to transform PDACs into ICI-responsive tumors, effective immunotherapy strategies are required to increase antigenicity, enhance T-cell function, and overcome T-cell exclusion and immunosuppressive signals in the tumor microenvironment [88]. PD-1 was shown to be highly expressed in the PDAC TME and was present on 64% and 71% of CD4+ and CD8+ T cells, respectively [89]. Available data indicate that both of these problems can be addressed by PDT. A novel approach by Qu et al. induced immunogenic cell death by remodeling immunosuppressive TME with increased T-cell infiltration. Combined with PD-1 checkpoint blockade, the targeted PDT platform significantly prolonged mouse survival [87]. Tumor immunogenicity was augmented by Su et al. by creating nanoparticles that respond to the tumor microenvironment and near-infrared light to degrade endogenous tumor H2O2 and the ability to deplete glutathione (Figure 4). They show that the use of this molecule with anti-PD-1 can be used to eliminate primary tumors and inhibit the growth of untreated distant tumors and cancer metastases [90].

It has been shown that induction of ferroptosis can overcome resistance to immunotherapy with checkpoint blockers, as it can produce a vaccine-like effect to stimulate antitumor immunity [91,92]. The association of PDT with ICI is therefore particularly attractive because of PDT's ability to induce this cell death [93]. Induction of feroptosis, however, may be prevented by ferroptosis suppressor protein 1 (FSP1) [94]. Zhou et al. developed novel molecules containing FSP1 and the photosensitizer chlorin e6 showing high effi-

ciency in triggering ferroptosis and immunogenic cell death, both in vitro and in vivo. They also observed increased tumor infiltration by CD8+ T cells and efficacy of anti-PD-L1 immunotherapy [93]. On the other hand, increased ROS production in TME can lead to localized neutrophil ferroptosis and release of oxidized lipids that limit T-cell activity [95,96]. In the work of Zhu et al., for the first time, PDT was combined with the ferroptosis inhibitor liproxstatin-1 and ICI. The enhanced photodynamic therapy, mediated by di-iodinated IR780 (Icy7), significantly increased the production of reactive oxygen species with induction of immunogenic cell death. At the same time, liproxstatin-1 effectively inhibited ferroptosis of cancer neutrophils. Ultimately, this strategy enhanced anti-PD-1 treatment [97].

A novel approach to producing tumor-associated antigens and improving PD-L1 efficacy features in the work of Gu et al. They constructed a molecule that contains the photosensitizer IR780, 2,2′-azobis [2-(2-imidazolin-2-yl)propane]-dihydrochloride (AIPH), and an anti-PD-L1 antibody [98]. The use of AIPH enables oxygen-independent generation of nitrogen gas and ROS under thermal excitation, so it can overcome hypoxic TME [99]. This possibility is particularly interesting because hypoxia can induce PD-L1 expression [100,101]. This nanosystem has been shown to effectively inhibit both primary and distant tumors [98].

Through a novel approach, Zhao et al. achieved an effective immune response and elimination of cancer metastasis. They designed a nanoparticle that contains the photosensitizer chlorin e6, an ASCT2 inhibitor (V9302), and a PD-1/PD-L1 blocker [102]. V-9302 is a transmembrane glutamine flow antagonist that selectively and potently targets the amino acid transporter ASCT2. The pharmacological blockade of ASCT2 with V-9302 resulted in attenuated tumor cell growth and proliferation, increased cell death, and enhanced oxidative stress, which together contributed to the antitumor response in vitro and in vivo [103]. It has been shown that the ACST2 blockade can impair T-cell homeostasis in Th1 and Th17 populations, but there was no effect of ASCT2 loss on initial T-cell activation, IL-2 secretion, Treg differentiation, or regulation of T or B cell populations. Since only the effects of chronic loss of ASCT2 were analyzed, it may not be possible to predict the immune effects induced by ASCT2 inhibitors [104].

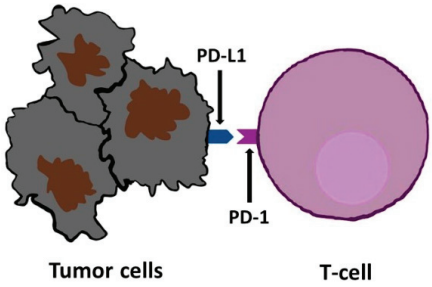


Figure 4. Interaction between PD-1 localized on a T lymphocyte and PD-L1 of a cancer cell.

3.3. Blockade of Immune Checkpoints by PDT Combined with Other Forms of Therapy

Knowledge of the immunosuppressive properties of the PD-1/PD-L1 axis is well established [105]. Not surprisingly, the desirable effects of PDT in anticancer therapy capable of affecting immune checkpoints and stimulating the immune response have been used to associate this method with other forms of therapy. Down-regulation of PD-L1 was achieved by Qiu et al. They designed a photodynamic immunostimulator by encapsulating a signal transducer and activator of transcription 3 (STAT3) inhibitor (Stattic) into a photosensitizer (protoporphyrin IX) and a modified PD-L1 blocking peptide (CVRARTR) by self-assembling the drug [106]. It is known that STAT3 is constitutively activated by abnormal tyrosine kinase activity in a broad spectrum of cancer cell lines and human tumors, and that its activity promotes PD-L1 expression and inhibits the immune response in breast cancer [107,108]. Previous studies have shown that the STAT3 inhibitor,

Stattic, has the ability to induce apoptosis in cancer cells and that it enhances immunogenic cell death induced by chemotherapy [109]. The molecule developed by Qiu et al. can facilitate targeted drug accumulation in breast cancer cells with PD-L1 overexpression to block PD-L1 and inhibit STAT3 phosphorylation to down-regulate PD-L1. Moreover, through photodynamic therapy, it also increases intracellular oxidative stress, exhibiting potent antiproliferative activity, which also induces immunogenic cell death [107].

Jian et al. developed a new nanoparticle by combining the mitochondria-targeted heptamethrin-cyanine PDT dye MHI with tamoxifen via self-assembly with albumin. This strategy showed effective inhibition of PD-L1 protein expression and reversal of tumor hypoxia, which increased tumor T-cell infiltration and immune response, more effectively mitigating the development of lung metastasis [110]. This approach is particularly interesting, as it has previously been shown that tamoxifen administration for breast cancer treatment through direct action on myeloid-derived suppressor cells (MDSCs) enriched the population of leukocytes such as NK cells and NKT cells [111]. PD-1+ NK cells can be inhibited in killing cancer cells which means that PD-1 is an important checkpoint for NK cell activation, and PD-1 blockade can induce an antitumor response in these cells [112]. Moreover, MDSCs can activate the PD-1/PD-L1 axis, inducing a group of PD-1-PD-L1+ Bregs that exert immunosuppressive effects, so blocking MDSCs may provide additional benefits [58]. The association of this molecule with ICI could result in improved treatment efficacy, as tamoxifen has a role in TGF-β inhibition, and numerous preclinical and clinical studies have shown that overactive TGF-β signaling is closely associated with ICI resistance [110,113].

A completely new strategy was developed by Fang et al. by associating PDT with PS indocyanine green with a nanoplex to deliver small interfering RNAs (siRNA) oligo against siPD-L1 encapsulated MPM, in order to give it the ability to reach tumor tissues via Siglec-15, which is expressed on the membrane [114]. The ability of siRNA to silence specific genes has been demonstrated in mammals and anti-cancer therapies based on interfering RNA (RNAi) have been proposed in recent decades as a new technique for cancer treatment [115]. Currently, there are three approved siRNA drugs and seven others are in phase 3 clinical trials [116]. Fang et al. observed that synchronously delivered siPD-L1 attenuated the ROS-induced PDT-generated increase in PD-L1 immunosuppressive expression, thereby effectively inducing a potent antitumor immune response in a mouse model of breast cancer xenografts [114].

As mentioned earlier, there have been reports of PDT's ability to raise PD-1 levels. Anand et al. show that the levels of cells expressing the PD-1 checkpoint receptor were reduced following vitamin D and PDT. Moreover, increased levels of damage-related molecular patterns and infiltration of neutrophils, macrophages, and dendritic cells were observed after combining PDT with vitamin D [37]. However, a recent study showed that vitamin D supplementation may have a bimodal function of increasing serum PD-L1 levels when serum PD-L1 levels are very low and decreasing serum PD-L1 levels when serum PD-L1 levels are very high. Presumably, vitamin D can increase the number of T cells by eliminating the suppressive effect of the PD-1 PD-L1 pathway on T cells [117]. Thus, in the context of this approach, it seems important to establish a limit at which PDT-reduced PD-L1 expression will not be increased by vitamin D.

He et al. developed a molecule containing MnO2, PS IR780, an indoleamine 2,3-dioxygenase inhibitor, and a paclitaxel dimer capable of reducing intra-neoplastic infiltration of M2-type TAMs, alleviating TME hypoxia, reducing PD-L1 expression of tumor cells, consequently eliminating primary tumors, and almost completely preventing tumor recurrence and metastasis of breast cancer [118]. The addition of anti-PD-1 to their strategy seems interesting, as the combination of PD-1 inhibitors and paclitaxel has previously been shown to be effective and may be a potential treatment as second-line therapy in advanced gallbladder cancer [119]. A new approach to the use of IDO inhibitors was proposed by Choi et al. They created multilayer structured upconversion nanoparticles for near-infrared light-induced PDT and immunotherapy combination therapy modified with folic acid, the

photosensitizer chlorin e6, and 1-methyl-tryptophan as an indoleamine 2,3-dioxygenase inhibitor (Table 2). The presence of folic acid ensured targeting of cancer cells and the ROS produced led to their death and activation of immunogenic cell death [120].

Table 2. Summary of recent research papers describing PDT and immunological checkpoints used in this article.

Authors	Results
Gurung et al. [35]	PDT with PS Ce6 inhibit PD-1/PD-L1 axis and induce immune response.
Lobo et al. [36]	PDT with PS redaporphin stimulates CD80 overexpression on cancer cells.
Anand et al. [37]	PDT combined with vitamin D administration shows increased recruitment of immune cells and decreased levels of cells expressing the checkpoint receptor PD-1+.
Delcanale et al. [40]	PDT treatment efficacy does not correlate with PD-L1 expression levels on the surface of tumor cells.
Bhandari et al. [52]	PDT can simultaneously increase α-PD-L1 antibody delivery and induce immunogenic cell death (ICD) in mouse head and neck AT-84 tumors.
Zheng et al. [54]	The new nanoparticle, Combo-NP, exhibits dual functionality by inducing ROS and ICD, leading to increased production of cytotoxic T lymphocytes (CTL) and normalization of tumor blood vessels, inhibiting tumor metastasis.
Sun et al. [62]	The combination of PDT, immune checkpoint blockers, and EV antigens can significantly improve the efficacy of cancer immunotherapy by improving immunosuppressive TME activation of tumor-specific T cells and reversing macrophage depletion.
Wang et al. [67]	Cascade-augmented nanoimmunomodulator with multienzyme-like activities, which includes superoxide dismutase, catalase, peroxidase, and glutathione oxidase, that dissociates under an acidic and abundant GSH TME, leads to stimulation of immune response in "cold" tumors, which may enhance ICI therapy.
Zheng et al. [70]	Novel nanoplatform using cascade of action of multiple enzymes in TME leads to stimulation of immune response in "cold" tumors, which may enhance ICI therapy.
Zhang et al. [73]	A novel therapeutic strategy based on PD-L1 aptamer-based spherical nucleic acids (SNAs) enables simultaneous PDT, chemotherapy, and enhanced immunotherapy, leading to effective inhibition of both primary and untreated distant colorectal tumors in mice, with minimal risk of immune-related side effects.
Feng et al. [74]	A new class of mitochondria-targeted and fluorinated polymers with aggregation-induced emission properties by controlling immune checkpoints in response to therapy may represent a promising strategy for cancer treatment.
Yao et al. [76]	An innovative therapeutic strategy based on a single-molecule prodrug combining acid-activated doxorubicin with aggregation-induced emission photosensitizer (AIEgen) combined with a caspase-3-responsive peptide enables the release of doxorubicin in acidic TME, activation of caspase-3 generation, and aggregation of photosensitizers in situ, leading to effective TNBC ICD and DC maturation, consequently supporting the efficacy of TNBC ICI immunotherapy.
Yang et al. [79]	The construction of albumin nanospheres containing photosensitizer-IR780 in the core and cGAS-STING/producer H2S-ZnS agonists on the shell (IR780-ZnS@HSA), through induction of pyroptosis, activation of the cGAS-STING signaling pathway, and stimulation of the immune response, can lead to inhibition of tumor growth and improved efficacy of anti-PD-L1 antibody immunotherapy, being an innovative therapeutic strategy in TNBC.
Qu et al. [87]	Nanoplatform based on midkine nanobodies and solid-state polymer nanoparticles can lead to inhibition of tumor progression and activation of antitumor immunity through local ROS generation, drug delivery, and activation of immune response and represents a promising strategy for precision photoimmunotherapy of pancreatic ductal adenocarcinoma.
Su et al. [90]	New Cu-MOF@RCD nanoreactors and anti-PD-L1 antibodies through a combination of PDT, PTT, CDT, glutathione depletion, and ICI eliminate primary tumors and inhibit the growth of untreated distant tumors and cancer metastasis.
Zhou et al. [93]	A photoresonant nanocomplex that self-assembles from BODIPY-modified poly(amidoamine) (BMP), enabling stable encapsulation of FSP1 inhibitor (iFSP1) and PS Ce6, can activate ferroptosis via PDT, inhibit ferroptosis suppressor protein 1 (FSP1), stimulate ICD, and thus enhance anti-PD-L1 ICI efficacy.
Zhu et al. [97]	Combination of PDT enhanced with di-iodinated IR780, liposome-containing ferroptase inhibitor liproxstatin-1, and modified photosensitizer Icy7 can induce ICD and inhibit tumor neutrophil ferroptosis, effectively inhibiting gastric cancer growth.
Gu et al. [98]	The functional nanoplatform, containing PS IR780 and 2,2′-azobis[2-(2-imidazolin-2-yl)propane]-dihydrochloride (AIPH), enables PTT and PDT generated by oxygen-independent ROS, which stimulates ICD and blocks immune checkpoints, shows potential in effectively targeting both primary tumors and distant metastases.
Zhao et al. [102]	A photodynamic immunostimulant called BVC, which reprograms tumor metabolism through inhibition of glutamine transport and GSH synthesis, leads to an increased immune response and blockade of the PD-1/PD-L1 immune checkpoint, which in turn leads to effective treatment of metastatic tumors.
Qiu et al. [106]	A photodynamic immunostimulator (PCS) designed to simultaneously down-regulate and block programmed cell death 1 (PD-L1) ligand in tumor cells enables effective inhibition of breast cancer growth by photodynamic therapy (PDT) and activation of the systemic immune response.

Table 2. *Cont.*

Authors	Results
Jiang et al. [110]	Combining PDT with tamoxifen may be an effective approach to simultaneously reduce PD-L1 and TGF-β expression, which may improve the effectiveness of PDT.
Fang et al. [114]	Novel RNA interference (RNAi) therapy strategy by using biomimetic nanoplex with dual redox reactivity, enables efficient delivery of siRNA to cancer cells and improves photodynamic immunotherapy through PDT-induced self-synergistic immunogenic cell death.
He et al. [118]	A novel albumin nanoplatform containing MnO2 efficiently delivers PDT, including PS IR780, as well as NLG919 and paclitaxel dimer, providing a simultaneous solution to the problems of tumor hypoxia, enhancement of ICD induction, and modulation of immunosuppressive TME.
Choi et al. [120]	MSUCN nanocarriers that combine immunotherapy and photodynamic therapy, enabling synergistic anti-cancer therapy by actively targeting tumor cells with folic acid and stimulating the immune response by generating reactive oxygen species and inhibiting the IDO pathway.

3.4. Future Research Directions

Despite the impressive achievements of the research conducted to date, it is necessary to further expand the knowledge of PDT and immune checkpoints (Table 3). As mentioned in earlier sections, the basic issue of the effect of photodynamic therapy on the expression level of immune checkpoints has not been clarified precisely so far. The lack of this knowledge may significantly affect the effectiveness of the association of PDT with ICI. This is due to the fact that in anticancer therapy, on the one hand, it is desirable to reduce the number of PD-1/PD-L1 receptors, while on the other hand, it has been shown that the lack of reactive PD-L1 expression can mean a poor response to PD-1 blockade therapy. The association of PDT with CTLA-4 inhibitors also seems to be an interesting direction of research. So far, reports on this subject are very limited. As mentioned above, the efficacy of ICI delivery can be enhanced by affecting abnormal tumor vasculature. Thus, a solution to increase the efficacy of the PDT–ICI combination would be to develop a new nanoparticle or an optimal PDT regimen to destroy vessels without inducing thrombus formation. It is clear that an apt direction for research to improve the effects of cancer therapy is to increase the efficacy of PDT and to address such problems as the suboptimal properties of photosensitizers and the lack of available oxygen or its depletion, which prevents effective ROS generation. There is already work using associations of different drugs and strategies to improve photosensitizer delivery and break the hypoxic TME. When designing new molecules, attention should also be paid to immunosuppressive cells, which inhibit the immune response in multiple ways. A well-defined therapeutic regimen of PDT capable of most effectively inducing immunogenic cell death has also still not been established. In summary, future research directions should focus on elucidating the precise mechanisms by which PDT affects the PD-1/PD-L1 axis and on designing a molecule with optimal characteristics to improve the multidimensional efficacy of both PDT and ICI, including in combination with other forms of therapy, such as chemotherapy. Particularly promising seems to be the attempt to solve the above-mentioned problems using artificial intelligence, the application of which in medical research has grown rapidly in recent years, yielding promising results in the search for new molecules for therapy.

Table 3. Benefits and limitations of PDT depending on immunological checkpoints.

Advantages
Induction of immunogenic cell death and activation of antitumor immune response
Reduction in the negative impact of the immunosuppressive tumor environment on the effectiveness of the immune response
Reduction in the negative effect of immunosuppressive cells on the effectiveness of the immune response

Table 3. *Cont.*

Promotion of proliferation and intratumor infiltration of cytotoxic T cells by combining repetitive PDT with anti-PD-1 therapy
PDT's ability to enhance delivery of anti-PD-L1 antibodies to the tumor
Disadvantages
Lack of a precisely defined PDT therapeutic regimen capable of most effectively inducing immunogenic cell death
No explanation of the exact mechanisms by which PDT affects the PD-1/PD-L1 axis
Lack of optimal PDT strategy to destroy vessels without simultaneous induction of thrombus formation

4. Conclusions

Based on recently published promising study results, it is suspected that the combination of photodynamic therapy and immune checkpoint blockers may solve the problem of treatment failure in at least some types of cancer. Importantly, this strategy offers the potential to cure metastatic lesions, and the addition of other molecules can provide thoughtful targeting of the tumor's immunosuppressive environment and improve treatment efficacy. Achieving accelerated clinical translation of PDT and ICI combination therapy is possible by focusing on the future research directions listed above. It is important to determine the optimal doses and timing of their administration, and to prove the safety of such therapy, without which it is impossible to introduce PDT with ICI in the clinic. In order to introduce this combination in the clinic on a permanent basis, larger, multicenter clinical trials are needed to confirm the initial optimistic results and to accurately assess the efficacy and safety of combination therapies. It is clear that a deeper understanding of the mechanisms of action of PDT and ICI and their synergies has important implications for the clinical translation of the PDT–ICI combination. In conclusion, photodynamic therapy has the ability to affect the expression levels of immune checkpoints, and its association with their blockers and other molecules may be a promising direction in cancer treatment.

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Abbreviations

Abbreviation	Definition
AIPH	2,2′-azobis [2-(2-imidazolin-2-yl)propane]-dihydrochloride
cGAS/STING	Cyclic guanosine monophosphate/adenosine synthase/interferon gene stimulator
CTL	Cytotoxic T lymphocyte
DAMP	Damage-associated molecular patterns
DC	Dendritic cell
EV	Extracellular vesicles
FGFR	Fibroblast growth factor receptor
FSP1	Ferroptosis suppressor protein 1
ICD	Immunogenic cell death
IDO	Indoleamine 2,3-dioxygenase
ITIMs	Tyrosine inhibitory motifs

KIT	Type III receptor tyrosine kinase
MDSC	Myeloid-derived suppressor cell
PD-1	Programmed cell death protein 1
PDAC	Pancreatic ductal adenocarcinoma
PDGFRB	Platelet-derived growth factor receptor beta
PD-L1	Programmed death-ligand
PDT	Photodynamic therapy
PS	Photosensitizer
RET	Receptor-tyrosine kinase
ROS	Reactive oxygen species
siRNA	Short inhibitor RNA
STAT3	Signal transducer and activator of transcription 3
STING	Stimulator of interferon genes
TAMs	Tumor-associated macrophages
TDO	Tryptophan 2,3-dioxygenase
TGF-β1	Transforming growth factor β1
TME	Tumor microenvironment
VEGFR	Vascular endothelial growth factor receptor

References

1. Cheng, X.; Wei, Y.; Jiang, X.; Wang, C.; Liu, M.; Yan, J.; Zhang, L.; Zhou, Y. Insight into the Prospects for Tumor Therapy Based on Photodynamic Immunotherapy. *Pharmaceuticals* **2022**, *15*, 1359. [CrossRef] [PubMed]
2. Jin, H.; Liao, S.; Yao, F.; Li, J.; Xu, Z.; Zhao, K.; Xu, X.; Sun, S. Insight into the Crosstalk between Photodynamic Therapy and Immunotherapy in Breast Cancer. *Cancers* **2023**, *15*, 1532. [CrossRef] [PubMed]
3. Wang, H.; Ewetse, M.P.; Ma, C.; Pu, W.; Xu, B.; He, P.; Wang, Y.; Zhu, J.; Chen, H. The "Light Knife" for Gastric Cancer: Photodynamic Therapy. *Pharmaceutics* **2022**, *15*, 101. [CrossRef] [PubMed]
4. Zhang, Q.; Li, L. Photodynamic combinational therapy in cancer treatment. *J. BUON* **2018**, *23*, 561–567. [PubMed]
5. Sobhani, N.; Samadani, A.A. Implications of photodynamic cancer therapy: An overview of PDT mechanisms basically and practically. *J. Egypt Natl. Canc. Inst.* **2021**, *33*, 34. [CrossRef]
6. Ma, Y.; Xiao, F.; Lu, C.; Wen, L. Multifunctional Nanosystems Powered Photodynamic Immunotherapy. *Front. Pharmacol.* **2022**, *13*, 905078. [CrossRef] [PubMed]
7. Jung, A.C.; Moinard-Butot, F.; Thibaudeau, C.; Gasser, G.; Gaiddon, C. Antitumor Immune Response Triggered by Metal-Based Photosensitizers for Photodynamic Therapy: Where Are We? *Pharmaceutics* **2021**, *13*, 1788. [CrossRef]
8. Jain, R.; Mohanty, S.; Sarode, I.; Biswas, S.; Singhvi, G.; Dubey, S.K. Multifunctional Photoactive Nanomaterials for Photodynamic Therapy against Tumor: Recent Advancements and Perspectives. *Pharmaceutics* **2022**, *15*, 109. [CrossRef] [PubMed]
9. Overchuk, M.; Weersink, R.A.; Wilson, B.C.; Zheng, G. Photodynamic and Photothermal Therapies: Synergy Opportunities for Nanomedicine. *ACS Nano* **2023**, *17*, 7979–8003. [CrossRef]
10. Itoo, A.M.; Paul, M.; Padaga, S.G.; Ghosh, B.; Biswas, S. Nanotherapeutic Intervention in Photodynamic Therapy for Cancer. *ACS Omega* **2022**, *7*, 45882–45909. [CrossRef]
11. Hamblin, M.R.; Abrahamse, H. Factors Affecting Photodynamic Therapy and Anti-Tumor Immune Response. *Anticancer Agents Med. Chem.* **2021**, *21*, 123–136. [CrossRef] [PubMed]
12. Dinakaran, D.; Wilson, B.C. The use of nanomaterials in advancing photodynamic therapy (PDT) for deep-seated tumors and synergy with radiotherapy. *Front. Bioeng. Biotechnol.* **2023**, *11*, 1250804. [CrossRef] [PubMed]
13. Ulfo, L.; Costantini, P.E.; Di Giosia, M.; Danielli, A.; Calvaresi, M. EGFR-Targeted Photodynamic Therapy. *Pharmaceutics* **2022**, *14*, 241. [CrossRef] [PubMed]
14. Wang, H.; Qiao, C.; Guan, Q.; Wei, M.; Li, Z. Nanoparticle-mediated synergistic anticancer effect of ferroptosis and photodynamic therapy: Novel insights and perspectives. *Asian J. Pharm. Sci.* **2023**, *18*, 100829. [CrossRef] [PubMed]
15. Olszowy, M.; Nowak-Perlak, M.; Woźniak, M. Current Strategies in Photodynamic Therapy (PDT) and Photodynamic Diagnostics (PDD) and the Future Potential of Nanotechnology in Cancer Treatment. *Pharmaceutics* **2023**, *15*, 1712. [CrossRef] [PubMed]
16. Chou, W.; Sun, T.; Peng, N.; Wang, Z.; Chen, D.; Qiu, H.; Zhao, H. Photodynamic Therapy-Induced Anti-Tumor Immunity: Influence Factors and Synergistic Enhancement Strategies. *Pharmaceutics* **2023**, *15*, 617. [CrossRef] [PubMed]
17. Yang, Y.L.; Lin, K.; Yang, L. Progress in Nanocarriers Codelivery System to Enhance the Anticancer Effect of Photodynamic Therapy. *Pharmaceutics* **2021**, *13*, 1951. [CrossRef]
18. Shih, C.Y.; Wang, P.T.; Su, W.C.; Teng, H.; Huang, W.L. Nanomedicine-Based Strategies Assisting Photodynamic Therapy for Hypoxic Tumors: State-of-the-Art Approaches and Emerging Trends. *Biomedicines* **2021**, *9*, 137. [CrossRef]
19. Shrestha, R.; Mallik, S.K.; Lim, J.; Gurung, P.; Magar, T.B.T.; Kim, Y.W. Efficient Synthesis of Chlorin e6 and Its Potential Photodynamic Immunotherapy in Mouse Melanoma by the Abscopal Effect. *Int. J. Mol. Sci.* **2023**, *24*, 3901. [CrossRef]

20. Han, L.; Huang, X.; Zhao, B.; Zhu, H.; Wang, R.; Liu, S.; Lin, H.; Feng, F.; Ma, X.; Liu, F.; et al. TGF-β1 mediates tumor immunosuppression aggravating at the late stage post-high-light-dose photodynamic therapy. *Cancer Immunol. Immunother.* **2023**, *72*, 3079–3095. [CrossRef]
21. Nath, S.; Obaid, G.; Hasan, T. The Course of Immune Stimulation by Photodynamic Therapy: Bridging Fundamentals of Photochemically Induced Immunogenic Cell Death to the Enrichment of T-Cell Repertoire. *Photochem. Photobiol.* **2019**, *95*, 1288–1305. [CrossRef] [PubMed]
22. Kleinovink, J.W.; Ossendorp, F. Measuring the Antitumor T-Cell Response in the Context of Photodynamic Therapy. *Methods Mol. Biol.* **2022**, *2451*, 579–588. [PubMed]
23. Ji, B.; Wei, M.; Yang, B. Recent advances in nanomedicines for photodynamic therapy (PDT)-driven cancer immunotherapy. *Theranostics* **2022**, *12*, 434–458. [CrossRef] [PubMed]
24. Alzeibak, R.; Mishchenko, T.A.; Shilyagina, N.Y.; Balalaeva, I.V.; Vedunova, M.V.; Krysko, D.V. Targeting immunogenic cancer cell death by photodynamic therapy: Past, present and future. *J. Immunother. Cancer* **2021**, *9*, e001926. [CrossRef] [PubMed]
25. Li, Z.; Lai, X.; Fu, S.; Ren, L.; Cai, H.; Zhang, H.; Gu, Z.; Ma, X.; Luo, K. Immunogenic Cell Death Activates the Tumor Immune Microenvironment to Boost the Immunotherapy Efficiency. *Adv. Sci.* **2022**, *9*, e2201734. [CrossRef] [PubMed]
26. Dao, A.; Yadav, A.K.; Wei, L.; Banerjee, S.; Huang, H. Combination of Immunotherapy and Photo-pyroptosis as Novel Anticancer Strategy. *ChemBioChem* **2022**, *23*, e202200201. [CrossRef]
27. Kleinovink, J.W.; Ossendorp, F. Combination of Photodynamic Therapy and Immune Checkpoint Blockade. *Methods Mol. Biol.* **2022**, *2451*, 589–596.
28. Hua, J.; Wu, P.; Gan, L.; Zhang, Z.; He, J.; Zhong, L.; Zhao, Y.; Huang, Y. Current Strategies for Tumor Photodynamic Therapy Combined with Immunotherapy. *Front. Oncol.* **2021**, *11*, 738323. [CrossRef] [PubMed]
29. Yan, S.; Zeng, X.; Tang, Y.; Liu, B.F.; Wang, Y.; Liu, X. Activating Antitumor Immunity and Antimetastatic Effect Through Polydopamine-Encapsulated Core-Shell Upconversion Nanoparticles. *Adv. Mater.* **2019**, *31*, e1905825. [CrossRef]
30. Jin, F.; Liu, D.; Xu, X.; Ji, J.; Du, Y. Nanomaterials-Based Photodynamic Therapy with Combined Treatment Improves Antitumor Efficacy Through Boosting Immunogenic Cell Death. *Int. J. Nanomed.* **2021**, *16*, 4693–4712. [CrossRef]
31. Yin, S.; Chen, Z.; Chen, D.; Yan, D. Strategies targeting PD-L1 expression and associated opportunities for cancer combination therapy. *Theranostics* **2023**, *13*, 1520–1544. [CrossRef] [PubMed]
32. Li, S.; Wang, D.; Cheng, J.; Sun, J.; Kalvakolanu, D.V.; Zhao, X.; Wang, D.; You, Y.; Zhang, L.; Yu, D. A photodynamically sensitized dendritic cell vaccine that promotes the anti-tumor effects of anti-PD-L1 monoclonal antibody in a murine model of head and neck squamous cell carcinoma. *J. Transl. Med.* **2022**, *20*, 505. [CrossRef] [PubMed]
33. Escors, D.; Gato-Cañas, M.; Zuazo, M.; Arasanz, H.; García-Granda, M.J.; Vera, R.; Kochan, G. The intracellular signalosome of PD-L1 in cancer cells. *Signal Transduct. Target Ther.* **2018**, *3*, 26. [CrossRef] [PubMed]
34. Chocarro de Erauso, L.; Zuazo, M.; Arasanz, H.; Bocanegra, A.; Hernandez, C.; Fernandez, G.; Garcia-Granda, M.J.; Blanco, E.; Vera, R.; Kochan, G.; et al. Resistance to PD-L1/PD-1 Blockade Immunotherapy. A Tumor-Intrinsic or Tumor-Extrinsic Phenomenon? *Front. Pharmacol.* **2020**, *11*, 441. [CrossRef] [PubMed]
35. Gurung, P.; Lim, J.; Shrestha, R.; Kim, Y.W. Chlorin e6-associated photodynamic therapy enhances abscopal antitumor effects via inhibition of PD-1/PD-L1 immune checkpoint. *Sci. Rep.* **2023**, *13*, 4647. [CrossRef] [PubMed]
36. Lobo, C.S.; Mendes, M.I.P.; Pereira, D.A.; Gomes-da-Silva, L.C.; Arnaut, L.G. Photodynamic therapy changes tumour immunogenicity and promotes immune-checkpoint blockade response, particularly when combined with micromechanical priming. *Sci. Rep.* **2023**, *13*, 11667. [CrossRef] [PubMed]
37. Anand, S.; Shen, A.; Cheng, C.E.; Chen, J.; Powers, J.; Rayman, P.; Diaz, M.; Hasan, T.; Maytin, E.V. Combination of vitamin D and photodynamic therapy enhances immune responses in murine models of squamous cell skin cancer. *Photodiagnosis Photodyn. Ther.* **2024**, *45*, 103983. [CrossRef] [PubMed]
38. Wang, Q.; Xie, B.; Liu, S.; Shi, Y.; Tao, Y.; Xiao, D.; Wang, W. What Happens to the Immune Microenvironment After PD-1 Inhibitor Therapy? *Front. Immunol.* **2021**, *12*, 773168. [CrossRef] [PubMed]
39. Sugiura, D.; Okazaki, I.M.; Maeda, T.K.; Maruhashi, T.; Shimizu, K.; Arakaki, R.; Takemoto, T.; Ishimaru, N.; Okazaki, T. PD-1 agonism by anti-CD80 inhibits T cell activation and alleviates autoimmunity. *Nat. Immunol.* **2022**, *23*, 399–410. [CrossRef]
40. Delcanale, P.; Alampi, M.M.; Mussini, A.; Fumarola, C.; Galetti, M.; Petronini, P.G.; Viappiani, C.; Bruno, S.; Abbruzzetti, S. A Photoactive Supramolecular Complex Targeting PD-L1 Reveals a Weak Correlation between Photoactivation Efficiency and Receptor Expression Levels in Non-Small-Cell Lung Cancer Tumor Models. *Pharmaceutics* **2023**, *15*, 2776. [CrossRef]
41. Lu, K.; He, C.; Guo, N.; Chan, C.; Ni, K.; Weichselbaum, R.R.; Lin, W. Chlorin-Based Nanoscale Metal-Organic Framework Systemically Rejects Colorectal Cancers via Synergistic Photodynamic Therapy and Checkpoint Blockade Immunotherapy. *J. Am. Chem. Soc.* **2016**, *138*, 12502–12510. [CrossRef] [PubMed]
42. Zhao, Y.; Liu, X.; Liu, X.; Yu, J.; Bai, X.; Wu, X.; Guo, X.; Liu, Z.; Liu, X. Combination of phototherapy with immune checkpoint blockade: Theory and practice in cancer. *Front. Immunol.* **2022**, *13*, 955920. [CrossRef] [PubMed]
43. Li, Q.; Han, J.; Yang, Y.; Chen, Y. PD-1/PD-L1 checkpoint inhibitors in advanced hepatocellular carcinoma immunotherapy. *Front. Immunol.* **2022**, *13*, 070961. [CrossRef] [PubMed]
44. Zaretsky, J.M.; Garcia-Diaz, A.; Shin, D.S.; Escuin-Ordinas, H.; Hugo, W.; Hu-Lieskovan, S.; Torrejon, D.Y.; Abril-Rodriguez, G.; Sandoval, S.; Barthly, L.; et al. Mutations Associated with Acquired Resistance to PD-1 Blockade in Melanoma. *N. Engl. J. Med.* **2016**, *375*, 819–829. [CrossRef] [PubMed]

45. Aebisher, D.; Woźnicki, P.; Bartusik-Aebisher, D. Photodynamic Therapy and Adaptive Immunity Induced by Reactive Oxygen Species: Recent Reports. *Cancers* **2024**, *16*, 967. [CrossRef] [PubMed]
46. Wang, L.; Liu, Y.; Liu, H.; Tian, H.; Wang, Y.; Zhang, G.; Lei, Y.; Xue, L.; Zheng, B.; Fan, T.; et al. The therapeutic significance of the novel photodynamic material TPE-IQ-2O in tumors. *Aging* **2020**, *13*, 1383–1409. [CrossRef] [PubMed]
47. Cramer, G.M.; Moon, E.K.; Cengel, K.A.; Busch, T.M. Photodynamic Therapy and Immune Checkpoint Blockade. *Photochem. Photobiol.* **2020**, *96*, 954–961. [CrossRef] [PubMed]
48. Yue, J.; Mei, Q.; Wang, P.; Miao, P.; Dong, W.F.; Li, L. Light-triggered multifunctional nanoplatform for efficient cancer photo-immunotherapy. *J. Nanobiotechnol.* **2022**, *20*, 181. [CrossRef] [PubMed]
49. Lou, J.; Aragaki, M.; Bernards, N.; Chee, T.; Gregor, A.; Hiraishi, Y.; Ishiwata, T.; Leung, C.; Ding, L.; Kitazawa, S.; et al. Repeated photodynamic therapy mediates the abscopal effect through multiple innate and adaptive immune responses with and without immune checkpoint therapy. *Biomaterials* **2023**, *292*, 121918. [CrossRef]
50. Morad, G.; Helmink, B.A.; Sharma, P.; Wargo, J.A. Hallmarks of response, resistance, and toxicity to immune checkpoint blockade. *Cell* **2021**, *184*, 5309–5337. [CrossRef]
51. Eppihimer, M.J.; Gunn, J.; Freeman, G.J.; Greenfield, E.A.; Chernova, T.; Erickson, J.; Leonard, J.P. Expression and regulation of the PD-L1 immunoinhibitory molecule on microvascular endothelial cells. *Microcirculation* **2002**, *9*, 133–145. [CrossRef] [PubMed]
52. Bhandari, C.; Moffat, A.; Fakhry, J.; Malkoochi, A.; Nguyen, A.; Trinh, B.; Hoyt, K.; Story, M.D.; Hasan, T.; Obaid, G. A single photodynamic priming protocol augments delivery of α-PD-L1 mAbs and induces immunogenic cell death in head and neck tumors. *Photochem. Photobiol.* **2023**, *11*. [CrossRef] [PubMed]
53. Carigga Gutierrez, N.M.; Pujol-Solé, N.; Arifi, Q.; Coll, J.L.; le Clainche, T.; Broekgaarden, M. Increasing cancer permeability by photodynamic priming: From microenvironment to mechanotransduction signaling. *Cancer Metastasis Rev.* **2022**, *41*, 899–934. [CrossRef] [PubMed]
54. Zheng, X.; Shi, Y.; Tang, D.; Xiao, H.; Shang, K.; Zhou, X.; Tan, G. Near-Infrared-II Nanoparticles for Vascular Normalization Combined with Immune Checkpoint Blockade via Photodynamic Immunotherapy Inhibit Uveal Melanoma Growth and Metastasis. *Adv. Sci.* **2023**, *10*, e2206932. [CrossRef] [PubMed]
55. Bertol, B.C.; Bales, E.S.; Calhoun, J.D.; Mayberry, A.; Ledezma, M.L.; Sams, S.B.; Orlicky, D.J.; Donadi, E.A.; Haugen, B.R.; French, J.D. Lenvatinib Plus Anti-PD-1 Combination Therapy for Advanced Cancers: Defining Mechanisms of Resistance in an Inducible Transgenic. Model of Thyroid Cancer. *Thyroid* **2022**, *32*, 153–163. [CrossRef] [PubMed]
56. Makker, V.; Colombo, N.; Casado Herráez, A.; Santin, A.D.; Colomba, E.; Miller, D.S.; Fujiwara, K.; Pignata, S.; Baron-Hay, S.; Ray-Coquard, I.; et al. Study 309–KEYNOTE-775 Investigators. Lenvatinib plus Pembrolizumab for Advanced Endometrial Cancer. *N. Engl. J. Med.* **2022**, *386*, 437–448. [CrossRef] [PubMed]
57. Makker, V.; Taylor, M.H.; Aghajanian, C.; Oaknin, A.; Mier, J.; Cohn, A.L.; Romeo, M.; Bratos, R.; Brose, M.S.; DiSimone, C.; et al. Lenvatinib Plus Pembrolizumab in Patients With. Advanced Endometrial Cancer. *J. Clin. Oncol.* **2020**, *38*, 2981–2992. [CrossRef] [PubMed]
58. Liu, M.; Wei, F.; Wang, J.; Yu, W.; Shen, M.; Liu, T.; Zhang, D.; Wang, Y.; Ren, X.; Sun, Q. Myeloid-derived suppressor cells regulate the immunosuppressive functions of PD-1-PD-L1+ Bregs through PD-L1/PI3K/AKT/NF-κB axis in breast cancer. *Cell Death Dis.* **2021**, *12*, 465. [CrossRef] [PubMed]
59. Lu, C.; Redd, P.S.; Lee, J.R.; Savage, N.; Liu, K. The expression profiles and regulation of PD-L1 in tumor-induced myeloid-derived suppressor cells. *Oncoimmunology* **2016**, *5*, e1247135. [CrossRef]
60. Loeuillard, E.; Yang, J.; Buckarma, E.; Wang, J.; Liu, Y.; Conboy, C.; Pavelko, K.D.; Li, Y.; O'Brien, D.; Wang, C.; et al. Targeting tumor-associated macrophages and granulocytic myeloid-derived suppressor cells augments PD-1 blockade in cholangiocarcinoma. *J. Clin. Investig.* **2020**, *130*, 5380–5396. [CrossRef]
61. Lo Russo, G.; Moro, M.; Sommariva, M.; Cancila, V.; Boeri, M.; Centonze, G.; Ferro, S.; Ganzinelli, M.; Gasparini, P.; Huber, V.; et al. Antibody-Fc/FcR Interaction on Macrophages as a Mechanism for Hyperprogressive Disease in Non-small Cell Lung Cancer Subsequent to PD-1/PD-L1 Blockade. *Clin. Cancer Res.* **2019**, *25*, 989–999. [CrossRef] [PubMed]
62. Sun, Z.; Sun, Z.; Liu, J.; Gao, X.; Jiao, L.; Zhao, Q.; Chu, Y.; Wang, X.; Deng, G.; Cai, L. Engineered Extracellular Vesicles Expressing Siglec-10 Camouflaged AIE Photosensitizer to Reprogram Macrophages to Active M1 Phenotype and Present Tumor-Associated Antigens for Photodynamic Immunotherapy. *Small* **2023**, *20*, e2307147. [CrossRef] [PubMed]
63. Guo, Y.; Ke, S.; Xie, F.; Chen, J.; Liu, X.; Wang, Z.; Xu, D.; Shen, Y.; Zhao, G.; Zhao, W.; et al. SIGLEC10+ macrophages drive gastric cancer progression by suppressing CD8+ T cell function. *Cancer Immunol. Immunother.* **2023**, *72*, 3229–3242. [CrossRef] [PubMed]
64. Wang, H.; Feng, Y.; Zhang, Y.; Wang, T.; Xu, H.; Zhi, Y.; Feng, Y.; Tian, L.; Yuan, K. Siglec10-An immunosuppressor and negative predictor of survival prognosis in gliomas. *Front. Genet.* **2022**, *13*, 873655. [CrossRef]
65. Li, X.; Tian, W.; Jiang, Z.; Song, Y.; Leng, X.; Yu, J. Targeting CD24/Siglec-10 signal pathway for cancer immunotherapy: Recent advances and future directions. *Cancer Immunol Immunother.* **2024**, *73*, 31. [CrossRef] [PubMed]
66. Barkal, A.A.; Brewer, R.E.; Markovic, M.; Kowarsky, M.; Barkal, S.A.; Zaro, B.W.; Krishnan, V.; Hatakeyama, J.; Dorigo, O.; Barkal, L.J.; et al. CD24 signalling through macrophage Siglec-10 is a target for cancer immunotherapy. *Nature* **2019**, *572*, 392–396. [CrossRef] [PubMed]
67. Wang, R.; Qiu, M.; Zhang, L.; Sui, M.; Xiao, L.; Yu, Q.; Ye, C.; Chen, S.; Zhou, X. Augmenting Immunotherapy via Bioinspired MOF-Based ROS Homeostasis Disruptor with Nanozyme-Cascade Reaction. *Adv. Mater.* **2023**, *35*, e2306748. [CrossRef] [PubMed]

68. Fujiwara, Y.; Kato, S.; Nesline, M.K.; Conroy, J.M.; DePietro, P.; Pabla, S.; Kurzrock, R. Indoleamine 2,3-dioxygenase (IDO) inhibitors and cancer immunotherapy. *Cancer Treat. Rev.* **2022**, *110*, 102461. [CrossRef]
69. Zhai, L.; Bell, A.; Ladomersky, E.; Lauing, K.L.; Bollu, L.; Sosman, J.A.; Zhang, B.; Wu, J.D.; Miller, S.D.; Meeks, J.J.; et al. Immunosuppressive IDO in Cancer: Mechanisms of Action, Animal Models, and Targeting Strategies. *Front. Immunol.* **2020**, *11*, 1185. [CrossRef]
70. Zheng, R.R.; Zhao, L.P.; Yang, N.; Chen, Z.X.; Kong, R.J.; Huang, C.Y.; Rao, X.N.; Chen, X.; Cheng, H.; Li, S.Y. Cascade Immune Activation of Self-Delivery Biomedicine for Photodynamic Immunotherapy Against Metastatic Tumor. *Small* **2023**, *19*, e2205694. [CrossRef]
71. Rizvi, N.A.; Hellmann, M.D.; Snyder, A.; Kvistborg, P.; Makarov, V.; Havel, J.J.; Lee, W.; Yuan, J.; Wong, P.; Ho, T.S.; et al. Cancer immunology. Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. *Science* **2015**, *348*, 124–128. [CrossRef] [PubMed]
72. Cortes, J.; Cescon, D.W.; Rugo, H.S.; Nowecki, Z.; Im, S.A.; Yusof, M.M.; Gallardo, C.; Lipatov, O.; Barrios, C.H.; Holgado, E.; et al. Pembrolizumab plus chemotherapy versus placebo plus chemotherapy for previously untreated locally recurrent inoperable or metastatic triple-negative breast cancer (KEYNOTE-355): A randomised, placebo-controlled, double-blind, phase 3 clinical trial. *Lancet* **2020**, *396*, 1817–1828. [CrossRef] [PubMed]
73. Zhang, J.; Li, W.; Qi, Y.; Wang, G.; Li, L.; Jin, Z.; Tian, J.; Du, Y. PD-L1 Aptamer-Functionalized Metal-Organic Framework Nanoparticles for Robust Photo-Immunotherapy against Cancer with Enhanced Safety. *Angew. Chem. Int. Ed. Engl.* **2023**, *62*, e202214750. [CrossRef] [PubMed]
74. Feng, Q.; Xu, J.; Zhuang, C.; Xiong, J.; Wang, H.; Xiao, K. Mitochondria-Targeting and Multiresponsive Nanoplatform Based on AIEgens for Synergistic Chemo-Photodynamic Therapy and Enhanced Immunotherapy. *Biomacromolecules* **2023**, *24*, 977–990. [CrossRef] [PubMed]
75. Zhang, B.; Wang, X.; Li, Q.; Mo, H.; Wang, X.; Song, Y.; Xu, J.; Qu, T.; Huang, J. Efficacy of irinotecan-based chemotherapy after exposure to an anti-PD-1 antibody in patients with advanced esophageal squamous cell carcinoma. *Chin. J. Cancer Res.* **2019**, *31*, 910–917. [CrossRef] [PubMed]
76. Yao, D.; Wang, Y.; Bian, K.; Zhang, B.; Wang, D. A self-cascaded unimolecular prodrug for pH-responsive chemotherapy and tumor-detained photodynamic-immunotherapy of triple-negative breast cancer. *Biomaterials* **2023**, *292*, 121920. [CrossRef]
77. Voorwerk, L.; Slagter, M.; Horlings, H.M.; Sikorska, K.; van de Vijver, K.K.; de Maaker, M.; Nederlof, I.; Kluin, R.J.C.; Warren, S.; Ong, S.; et al. Immune induction strategies in metastatic triple-negative breast cancer to enhance the sensitivity to PD-1 blockade: The TONIC trial. *Nat. Med.* **2019**, *25*, 920–928. [CrossRef] [PubMed]
78. Abd El-Fattah, E.E. IDO/kynurenine pathway in cancer: Possible therapeutic approaches. *J. Transl. Med.* **2022**, *20*, 347. [CrossRef] [PubMed]
79. Yang, J.; Guo, W.; Huang, R.; Bian, J.; Zhang, S.; Wei, T.; He, C.; Hu, Z.; Li, J.; Zhou, C.; et al. Self-assembled albumin nanoparticles induce pyroptosis for photodynamic/photothermal/immuno synergistic therapies in triple-negative breast cancer. *Front. Immunol.* **2023**, *14*, 1173487. [CrossRef]
80. Wei, X.; Xie, F.; Zhou, X.; Wu, Y.; Yan, H.; Liu, T.; Huang, J.; Wang, F.; Zhou, F.; Zhang, L. Role of pyroptosis in inflammation and cancer. *Cell. Mol. Immunol.* **2022**, *19*, 971–992. [CrossRef]
81. Fang, Y.; Tian, S.; Pan, Y.; Li, W.; Wang, Q.; Tang, Y.; Yu, T.; Wu, X.; Shi, Y.; Ma, P.; et al. Pyroptosis: A new frontier in cancer. *Biomed. Pharmacother.* **2020**, *121*, 109595. [CrossRef] [PubMed]
82. Cao, W.; Chen, G.; Wu, L.; Yu, K.N.; Sun, M.; Yang, M.; Jiang, Y.; Jiang, Y.; Xu, Y.; Peng, S.; et al. Ionizing Radiation Triggers the Antitumor Immunity by Inducing Gasdermin E-Mediated Pyroptosis in Tumor Cells. *Int. J. Radiat. Oncol. Biol. Phys.* **2023**, *115*, 440–452. [CrossRef] [PubMed]
83. Wang, W.; Zhang, L.; Sun, Z. Eliciting pyroptosis to fuel cancer immunotherapy: Mechanisms and strategies. *Cancer Biol. Med.* **2022**, *19*, 948–964. [CrossRef] [PubMed]
84. Samson, N.; Ablasser, A. The cGAS-STING pathway and cancer. *Nat. Cancer* **2022**, *3*, 1452–1463. [CrossRef] [PubMed]
85. Gan, Y.; Li, X.; Han, S.; Liang, Q.; Ma, X.; Rong, P.; Wang, W.; Li, W. The cGAS/STING Pathway: A Novel Target for Cancer Therapy. *Front. Immunol.* **2022**, *12*, 795401. [CrossRef] [PubMed]
86. Larue, L.; Myrzakhmetov, B.; Ben-Mihoub, A.; Moussaron, A.; Thomas, N.; Arnoux, P.; Baros, F.; Vanderesse, R.; Acherar, S.; Frochot, C. Fighting Hypoxia to Improve PDT. *Pharmaceuticals* **2019**, *12*, 163. [CrossRef] [PubMed]
87. Qu, C.; Yuan, H.; Tian, M.; Zhang, X.; Xia, P.; Shi, G.; Hou, R.; Li, J.; Jiang, H.; Yang, Z.; et al. Precise Photodynamic Therapy by Midkine Nanobody-Engineered Nanoparticles Remodels the Microenvironment of Pancreatic Ductal Adenocarcinoma and Potentiates the Immunotherapy. *ACS Nano* **2024**, *18*, 4019–4037. [CrossRef]
88. Li, K.; Tandurella, J.A.; Gai, J.; Zhu, Q.; Lim, S.J.; Thomas DL 2nd Xia, T.; Mo, G.; Mitchell, J.T.; Montagne, J.; Lyman, M.; et al. Multi-omic analyses of changes in the tumor microenvironment of pancreatic adenocarcinoma following neoadjuvant treatment with anti-PD-1 therapy. *Cancer Cell.* **2022**, *40*, 1374–1391.e7. [CrossRef]
89. Pearce, H.; Croft, W.; Nicol, S.M.; Margielewska-Davies, S.; Powell, R.; Cornall, R.; Davis, S.J.; Marcon, F.; Pugh, M.R.; Fennell, É.; et al. Tissue-Resident Memory T Cells in Pancreatic Ductal Adenocarcinoma Coexpress PD-1 and TIGIT and Functional Inhibition Is Reversible by Dual Antibody Blockade. *Cancer Immunol. Res.* **2023**, *11*, 435–449. [CrossRef]

90. Su, Z.; Xu, H.; Zhang, Y.; Zhang, H.; Zhang, H.; Bao, Y.; Wu, X.; Yan, R.; Tan, G.; Wang, Z.; et al. A carbon dot-doped Cu-MOF-based smart nanoplatform for enhanced immune checkpoint blockade therapy and synergistic multimodal cancer therapy. *J. Mater. Chem. B* **2023**, *11*, 4211–4226. [CrossRef]
91. Jiang, Z.; Lim, S.O.; Yan, M.; Hsu, J.L.; Yao, J.; Wei, Y.; Chang, S.S.; Yamaguchi, H.; Lee, H.H.; Ke, B.; et al. TYRO3 induces anti-PD-1/PD-L1 therapy resistance by limiting innate immunity and tumoral ferroptosis. *J. Clin. Investig.* **2021**, *131*, e139434. [CrossRef] [PubMed]
92. Zhang, C.; Liu, X.; Jin, S.; Chen, Y.; Guo, R. Ferroptosis in cancer therapy: A novel approach to reversing drug resistance. *Mol. Cancer* **2022**, *21*, 47. [CrossRef] [PubMed]
93. Zhou, Y.; Chen, K.; Lin, W.K.; Liu, J.; Kang, W.; Zhang, Y.; Yang, R.; Jin, L.; Cheng, Y.; Xu, A.; et al. Photo-Enhanced Synergistic Induction of Ferroptosis for Anti-Cancer Immunotherapy. *Adv. Healthc. Mater.* **2023**, *12*, e2300994. [CrossRef] [PubMed]
94. Doll, S.; Freitas, F.P.; Shah, R.; Aldrovandi, M.; da Silva, M.C.; Ingold, I.; Goya Grocin, A.; Xavier da Silva, T.N.; Panzilius, E.; Scheel, C.H.; et al. FSP1 is a glutathione-independent ferroptosis suppressor. *Nature* **2019**, *575*, 693–698. [CrossRef] [PubMed]
95. Kim, R.; Hashimoto, A.; Markosyan, N.; Tyurin, V.A.; Tyurina, Y.Y.; Kar, G.; Fu, S.; Sehgal, M.; Garcia-Gerique, L.; Kossenkov, A.; et al. Ferroptosis of tumour neutrophils causes immune suppression in cancer. *Nature* **2022**, *612*, 338–346. [CrossRef]
96. Xu, S.; Chaudhary, O.; Rodríguez-Morales, P.; Sun, X.; Chen, D.; Zappasodi, R.; Xu, Z.; Pinto, A.F.M.; Williams, A.; Schulze, I.; et al. Uptake of oxidized lipids by the scavenger receptor CD36 promotes lipid peroxidation and dysfunction in CD8+ T cells in tumors. *Immunity* **2021**, *54*, 1561–1577.e7. [CrossRef] [PubMed]
97. Zhu, X.; Zheng, W.; Wang, X.; Li, Z.; Shen, X.; Chen, Q.; Lu, Y.; Chen, K.; Ai, S.; Zhu, Y.; et al. Enhanced Photodynamic Therapy Synergizing with Inhibition of Tumor Neutrophil Ferroptosis Boosts Anti-PD-1 Therapy of Gastric Cancer. *Adv. Sci.* **2024**, *11*, e2307870. [CrossRef]
98. Gu, X.; Wu, B.; Feng, G.; Chen, Z.; Ren, F.; Chen, X.; Hong, W.; Li, W. PD-L1 Blockade Peptide-Modified Polymeric Nanoparticles for Oxygen-Independent-Based Hypoxic Tumor Photo/Thermodynamic Immunotherapy. *Mol. Pharm.* **2023**, *20*, 4007–4020. [CrossRef]
99. Riedinger, A.; Guardia, P.; Curcio, A.; Garcia, M.A.; Cingolani, R.; Manna, L.; Pellegrino, T. Subnanometer local temperature probing and remotely controlled drug release based on azo-functionalized iron oxide nanoparticles. *Nano Lett.* **2013**, *13*, 2399–2406. [CrossRef]
100. Ding, X.C.; Wang, L.L.; Zhang, X.D.; Xu, J.L.; Li, P.F.; Liang, H.; Zhang, X.B.; Xie, L.; Zhou, Z.H.; Yang, J.; et al. The relationship between expression of PD-L1 and HIF-1α in glioma cells under hypoxia. *J. Hematol. Oncol.* **2021**, *14*, 92. [CrossRef]
101. Chen, J.; Jiang, C.C.; Jin, L.; Zhang, X.D. Regulation of PD-L1: A novel role of pro-survival signalling in cancer. *Ann. Oncol.* **2016**, *27*, 409–416. [CrossRef] [PubMed]
102. Zhao, L.; Rao, X.; Zheng, R.; Huang, C.; Kong, R.; Yu, X.; Cheng, H.; Li, S. Targeting glutamine metabolism with photodynamic immunotherapy for metastatic tumor eradication. *J. Control Release* **2023**, *357*, 460–471. [CrossRef] [PubMed]
103. Schulte, M.L.; Fu, A.; Zhao, P.; Li, J.; Geng, L.; Smith, S.T.; Kondo, J.; Coffey, R.J.; Johnson, M.O.; Rathmell, J.C.; et al. Pharmacological blockade of ASCT2-dependent glutamine transport leads to antitumor efficacy in preclinical models. *Nat. Med.* **2018**, *24*, 194–202. [CrossRef] [PubMed]
104. Wahi, K.; Holst, J. ASCT2: A potential cancer drug target. *Expert Opin. Ther. Targets* **2019**, *23*, 555–558. [CrossRef] [PubMed]
105. Tang, Q.; Chen, Y.; Li, X.; Long, S.; Shi, Y.; Yu, Y.; Wu, W.; Han, L.; Wang, S. The role of PD-1/PD-L1 and application of immune-checkpoint inhibitors in human cancers. *Front. Immunol.* **2022**, *13*, 964442. [CrossRef] [PubMed]
106. Qiu, Z.; Lu, Z.; Huang, J.; Zhong, Y.; Yan, N.; Kong, R.; Cheng, H. Self-reinforced photodynamic immunostimulator to downregulate and block PD-L1 for metastatic breast cancer treatment. *Biomaterials* **2023**, *303*, 122392. [CrossRef] [PubMed]
107. Schust, J.; Sperl, B.; Hollis, A.; Mayer, T.U.; Berg, T. Stattic: A small-molecule inhibitor of STAT3 activation and dimerization. *Chem. Biol.* **2006**, *13*, 1235–1242. [CrossRef] [PubMed]
108. Zerdes, I.; Wallerius, M.; Sifakis, E.G.; Wallmann, T.; Betts, S.; Bartish, M.; Tsesmetzis, N.; Tobin, N.P.; Coucoravas, C.; Bergh, J.; et al. STAT3 Activity Promotes Programmed-Death Ligand 1 Expression and Suppresses Immune Responses in Breast Cancer. *Cancers* **2019**, *11*, 1479. [CrossRef] [PubMed]
109. Jafari, S.; Lavasanifar, A.; Hejazi, M.S.; Maleki-Dizaji, N.; Mesgari, M.; Molavi, O. STAT3 inhibitory stattic enhances immunogenic cell death induced by chemotherapy in cancer cells. *Daru* **2020**, *28*, 159–169. [CrossRef]
110. Jiang, X.; Yi, L.; Li, C.; Wang, H.; Xiong, W.; Li, Y.; Zhou, Z.; Shen, J. Mitochondrial Disruption Nanosystem Simultaneously Depressed Programmed Death Ligand-1 and Transforming Growth Factor-β to Overcome Photodynamic Immunotherapy Resistance. *ACS Nano* **2024**, *18*, 3331–3348. [CrossRef]
111. Mohd Idris, R.A.; Mussa, A.; Ahmad, S.; Al-Hatamleh, M.A.I.; Hassan, R.; Tengku Din, T.A.D.A.A.; Wan Abdul Rahman, W.F.; Lazim, N.M.; Boer, J.C.; Plebanski, M.; et al. The Effects of Tamoxifen on Tolerogenic Cells in Cancer. *Biology* **2022**, *11*, 1225. [CrossRef] [PubMed]
112. Hsu, J.; Hodgins, J.J.; Marathe, M.; Nicolai, C.J.; Bourgeois-Daigneault, M.C.; Trevino, T.N.; Azimi, C.S.; Scheer, A.K.; Randolph, H.E.; Thompson, T.W.; et al. Contribution of NK cells to immunotherapy mediated by PD-1/PD-L1 blockade. *J. Clin. Investig.* **2018**, *128*, 4654–4668. [CrossRef] [PubMed]
113. Yi, M.; Li, T.; Niu, M.; Wu, Y.; Zhao, Z.; Wu, K. TGF-β: A novel predictor and target for anti-PD-1/PD-L1 therapy. *Front. Immunol.* **2022**, *13*, 1061394. [CrossRef] [PubMed]

114. Fang, Y.; Chen, S.; Zhang, M.; Lin, X.; Jin, X.; Zhang, M.; Liu, Y.; Wang, Y.; Shi, K. A Trojan Horse Delivery Vehicle Carrying siRNA Nanotherapeutics with Multiple Tumor Microenvironment Responsiveness Elicits Robust Antitumor Immune Responses In Situ via a "Self-Synergistic" Approach. *Adv. Healthc. Mater.* **2023**, *12*, e2301401. [CrossRef]
115. Barati, M.; Mirzavi, F.; Atabaki, M.; Bibak, B.; Mohammadi, M.; Jaafari, M.R. A review of PD-1/PD-L1 siRNA delivery systems in immune T cells and cancer cells. *Int. Immunopharmacol.* **2022**, *111*, 109022. [CrossRef]
116. Zhang, M.M.; Bahal, R.; Rasmussen, T.P.; Manautou, J.E.; Zhong, X.B. The growth of siRNA-based therapeutics: Updated clinical studies. *Biochem. Pharmacol.* **2021**, *189*, 114432. [CrossRef]
117. Morita, M.; Okuyama, M.; Akutsu, T.; Ohdaira, H.; Suzuki, Y.; Urashima, M. Vitamin D Supplementation Regulates Postoperative Serum Levels of PD-L1 in Patients with Digestive Tract Cancer and Improves Survivals in the Highest Quintile of PD-L1: A Post Hoc Analysis of the AMATERASU Randomized Controlled Trial. *Nutrients* **2021**, *13*, 1987. [CrossRef] [PubMed]
118. He, M.; Zhang, M.; Xu, T.; Xue, S.; Li, D.; Zhao, Y.; Zhi, F.; Ding, D. Enhancing photodynamic immunotherapy by reprograming the immunosuppressive tumor microenvironment with hypoxia relief. *J. Control Release.* **2024**, *368*, 233–250. [CrossRef]
119. Tan, S.; Yu, J.; Huang, Q.; Zhou, N.; Gou, H. PD-1 inhibitors plus nab-paclitaxel-containing chemotherapy for advanced gallbladder cancer in a second-line setting: A retrospective analysis of a case series. *Front. Oncol.* **2022**, *12*, 1006075. [CrossRef]
120. Choi, J.; Kim, S.Y. Multi-shell structured upconversion nanocarriers that combine IDO inhibitor-induced immunotherapy with NIR-triggered photodynamic therapy for deep tumors. *Biomater. Sci.* **2023**, *11*, 4684–4699. [CrossRef]

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Review

Targeting β-Cell Plasticity: A Promising Approach for Diabetes Treatment

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Abstract: The β-cells within the pancreas play a pivotal role in insulin production and secretion, responding to fluctuations in blood glucose levels. However, factors like obesity, dietary habits, and prolonged insulin resistance can compromise β-cell function, contributing to the development of Type 2 Diabetes (T2D). A critical aspect of this dysfunction involves β-cell dedifferentiation and transdifferentiation, wherein these cells lose their specialized characteristics and adopt different identities, notably transitioning towards progenitor or other pancreatic cell types like α-cells. This process significantly contributes to β-cell malfunction and the progression of T2D, often surpassing the impact of outright β-cell loss. Alterations in the expressions of specific genes and transcription factors unique to β-cells, along with epigenetic modifications and environmental factors such as inflammation, oxidative stress, and mitochondrial dysfunction, underpin the occurrence of β-cell dedifferentiation and the onset of T2D. Recent research underscores the potential therapeutic value for targeting β-cell dedifferentiation to manage T2D effectively. In this review, we aim to dissect the intricate mechanisms governing β-cell dedifferentiation and explore the therapeutic avenues stemming from these insights.

Keywords: β-cells; dedifferentiation; transdifferentiation; neogenesis; regeneration

1. Introduction

Globally, around 422 million people have Diabetes Mellitus (DM or diabetes), with most living in low- and middle-income countries. Diabetes causes 1.5 million deaths annually worldwide. In the United States, approximately 37.3 million people, or about 11% of the population, have diabetes, with Type 2 Diabetes (T2D) accounting for 90–95% of cases. Projections indicate that the global number of adults with diabetes will rise to 643 million by 2030 and 783 million by 2045.

Findings indicate that changing β-cell plasticity plays an important role in the incidence of all three main forms of diabetes (see Box 1). In T2D, for example, β-cell dedifferentiation and transdifferentiation are more critical than β-cell loss [1,2]. Various factors, including metabolic stress, inflammation [3], and viral infection [4], contribute to these processes. They exert their effects through pathways such as the mTOR pathway, ER stress, and by altering the transcriptional activity of β-cells, targeting key transcription and epigenetic factors (Figure 1).

The concept of β-cell dedifferentiation was introduced by Gershengorn et al. in 2004 after they observed that β-cells in human pancreatic islets vanished and were replaced by fibroblast-like precursor cells. They also noted that these cells could redifferentiate into α- and β-cells under certain growth conditions. This concept was further confirmed by the findings of Dor et al. (2007), Accili et al. (2012), Wang et al. (2014), and Cinti et al. (2016) [5]. β-cell dedifferentiation is characterized by the appearance of progenitor-like biomarkers, such as Ngn3, Oct4, Aldh1a3, Nanog, L-Myc, and Sox9, and the disappearance

or downregulation of β-cell-enriched biomarkers, including *MafA*, *Ins*, *Nkx6.1*, *Glut2*, and *Pdx-1*. This shift results in β-cells losing their functionality, which can ultimately lead to the incidence of diabetes [5,6]. Similar to rodents, β-cell dedifferentiation occurs in human pancreatic islets. However, unlike in rodents, *Ngn3* is not upregulated in human pancreatic islets during dedifferentiation. *Aldh1a3*, on the other hand, is upregulated in both rodents and humans [7]. The transdifferentiation of β-cells into other pancreatic cell types, such as α- and δ-cells, is another form of β-cell plasticity that occurs in both rodents and humans, much like dedifferentiation [6]. The transdifferentiation of β-cells into α-cells is typically marked by the loss of β-cell differentiation markers and the acquisition of α-cell biomarkers, such as glucagon and the Arx transcription factor [8]. The transdifferentiation of pancreatic endocrine cells, including α- and δ-cells, into β-cells is observed alongside reports of pancreatic exocrine cells, like acinar cells, also transdifferentiating into β-cells. This suggests a novel approach for treating diabetes [9].

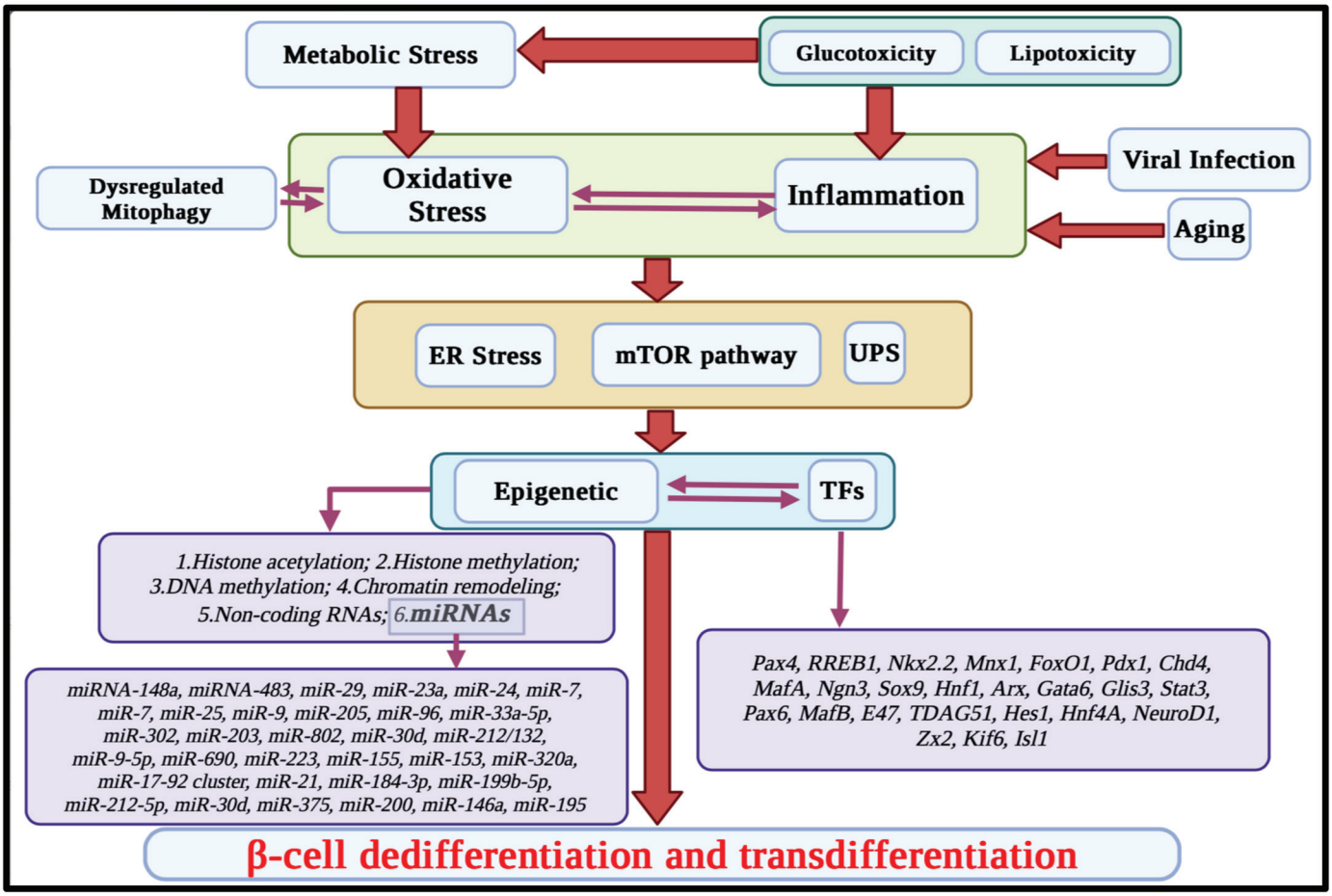


Figure 1. The mechanism of β-cell dedifferentiation and transdifferentiation. The process of β-cell dedifferentiation and transdifferentiation involves metabolic stresses such as glucotoxicity, lipotoxicity, viral infections, and aging. These stresses induce oxidative stress and inflammation, disrupting pathways such as ER stress, the mTOR pathway, and the UPS. This disruption affects the activities of transcription factors and epigenetic factors, including various miRNAs, leading to β-cell dedifferentiation and transdifferentiation. Abbreviations: endoplasmic reticulum: ER; ubiquitin–proteasome system: UPS; transcription factors: TFs. The information included in this figure is based on the research findings cited in this review paper.

The dedifferentiation and transdifferentiation of β-cells are observed across all diabetes types. In Type I Diabetes (T1D), inflammatory reactions are pivotal in triggering these processes, inducing ER stress and modifying gene expression and protein synthesis in β-cells [10]. In T2D, metabolic stresses linked to obesity, insulin resistance, and high

levels of glucose and lipids similarly contribute to β-cell dedifferentiation and transdifferentiation [11].

Mitigating and reversing β-cell dedifferentiation and transdifferentiation offer promising treatments for all three main types of diabetes. Various approaches, including natural products, dietary interventions, and common antidiabetic therapies, can help mitigate β-cell dedifferentiation and transdifferentiation.

In addition, in vitro β-cell neogenesis followed by the transplantation of newly differentiated β-cells is a promising method for treating all types of diabetes, particularly T1D. We have highlighted new findings in this area.

β-cell regeneration and proliferation represent another strategy for treating all types of diabetes. Suppressing Dual-Specificity Tyrosine-Regulated Kinase 1A (DYRK1A) [12], antagonizing glucagon receptors [13–16], and inhibiting polyamine biosynthesis [17] are potential treatments to promote β-cell regeneration and proliferation.

Box 1. Definitions.

β-cell plasticity refers to the ability of β-cells to alter their identity and function in response to specific conditions. This trait is not exclusive to β-cells; other pancreatic cells, such as α-cells, also exhibit this adaptability. The primary aspects of β-cell plasticity are β-cell dedifferentiation and transdifferentiation [18].

β-cell dedifferentiation is triggered by chronic metabolic stresses like hyperglycemia, endoplasmic reticulum (ER) stress, and inflammatory cytokines. This process involves mature β-cells losing their differentiated characteristics and reverting to a progenitor-like or undifferentiated state [2].

β-cell transdifferentiation involves the conversion of mature β-cells into other islet cell types, such as α-cells, under certain conditions.

β-cell regeneration is the process of restoring or increasing the number of functional pancreatic β-cells, which produce insulin. This process can occur through the body's natural mechanisms, involving two main pathways:

1—β-cell proliferation: Existing β-cells divide to increase their numbers.

2—Neogenesis from progenitor cells: Pancreatic stem or progenitor cells differentiate into new β-cells [19].

T1D—Type 1 Diabetes: an autoimmune condition where the immune system attacks insulin-producing β-cells in the pancreas, leading to an inability to produce insulin. It accounts for about 10% of diabetes cases and is usually diagnosed in childhood or adolescence, though it can occur at any age [20].

T2D—Type 2 Diabetes: the most common form caused by insulin resistance and relative insulin deficiency. It is often associated with obesity, a sedentary lifestyle, and genetic factors. While it is typically diagnosed in adulthood, it is increasingly seen in children and teens due to rising obesity rates. Management can involve lifestyle changes, medications, or insulin [21,22].

GDM—Gestational Diabetes Mellitus arises when the body struggles to produce sufficient insulin to counteract the insulin resistance induced by placental hormones such as estrogen, cortisol, and human placental lactogen [23].

In this review, we explore the mechanisms behind β-cell dedifferentiation and transdifferentiation and provide insights into the possibility of mitigating these processes by identifying new therapeutic targets, medications, and treatments. Additionally, we highlight recent discoveries related to β-cell neogenesis and regeneration as potential treatments for all three types of diabetes.

2. The Main Mechanisms Involved in β-Cell Function and Identity

2.1. Inflammation

Inflammation, whether originating from the β-cells themselves or from resident macrophages in the pancreas, plays a critical role in the function and maintenance of β-cells, thereby contributing to the development of diabetes [3]. Pro-inflammatory cytokines like IL-1β, IL-6, and TNFα induce β-cell dedifferentiation in cultured human cells, human islets, and mouse islets, contributing to β-cell dysfunction in T2D. IL-1β is the most potent among them, downregulating *Forkhead Box O1* (*FoxO1*), a key transcription

factor for maintaining β-cell identity. Additionally, treatment with proinflammatory cytokines was associated with a translocation of FoxO1 to the nucleus, the loss of nuclear *Nkx6.1* expression, and the emergence of *Aldh1a3*, indicating β-cell dedifferentiation [24]. Accordingly, in vivo, anti-inflammatory treatments with anti-IL-1β, anti-TNFα, or NF-kB inhibiting sodium salicylate improved insulin secretion from isolated islets. While anti-TNFα therapy partially prevented the loss of β-cell identity gene expression, the combined antagonism of IL-1β and TNFα produced a synergistic effect in restoring insulin secretion. Although inflammation plays a significant role in β-cell dedifferentiation in vitro, its impact on in vivo insulin secretion improvement appears to be more complex, with additional factors potentially involved [25].

The pro-inflammatory factor BMP-2, upregulated in the islets of diabetic individuals, contributes to the inhibition of β-cell function and proliferation. BMP-2 disrupts β-cell maturity through epigenetic changes and decreased NeuroD1 activity, contributing to β-cell dysfunction in diabetes. Exposure to BMP-2 for 10 days impaired glucose-stimulated insulin secretion (GSIS) and β-cell proliferation, correlating with decreased expressions of key β-cell genes like *Ins1*, *Ucn3*, and *Ki67*. BMP-2-induced β-cell dysfunction was also linked to an increased expression of dedifferentiation regulators (*Id1-4*, *Hes-1*, and *Hey-1*), a reduction in H3K27 acetylation, and decreased NeuroD1 DNA binding activity [26].

TGF-β, a versatile cytokine, coordinates various cellular processes such as growth, proliferation, differentiation, apoptosis, and immune responses. It plays a crucial role in regulating β-cell dedifferentiation, preserving β-cell function, and managing β-cell mass [27]. Inhibitors targeting TGF-β receptor I (TGFβ1) have shown potential in restoring β-cell identity by influencing the expression of β-cell-specific transcription factors under severe diabetic conditions [28].

Patients with chronic pancreatitis (CP) are at risk of developing diabetes due to the gradual loss of functional β-cells. In a mouse model for CP-related diabetes (CPRD), researchers found that β-cell loss is driven by TGFβ1-induced epithelial–mesenchymal transition (EMT), not apoptosis. They also observed increased angiogenesis in CP patients' inflamed pancreata and in mice subjected to pancreatic duct ligation, a surgical procedure used in research to induce pancreatitis. However, when angiogenesis was blocked with an anti-vascular endothelial growth factor receptor 2 antibody (DC101), the EMT process reverted to β-cell apoptosis. This indicates that angiogenesis helps β-cells survive in the inflamed pancreas, suggesting a potential therapeutic target for CPRD [29].

Activin is a protein that belongs to the TGF-β superfamily. It plays crucial roles in various biological processes, including embryonic development, cell proliferation, differentiation, and tissue homeostasis. Its signaling influences the balance between α- and β-cell populations, impacting glucose homeostasis. Accordingly, it has been found that Activin can suppress genes in α-cells such as *Arx4* and upregulate β-cell genes such as *Pax4*, which accelerates the process of transdifferentiation from α- to β-cells [30].

In diabetes, stromal cell-derived factor-1 (SDF-1), a chemokine, is implicated in the dedifferentiation of islet β-cells. Elevated SDF-1 levels correlate with the increased dedifferentiation of β-cells in pancreatic tissues. Experimental inhibition of SDF-1 led to a higher proportion of dedifferentiated cells, while overexpression reduced their numbers. Despite the diabetes-induced upregulation of *SDF-1*, its activity may decline, thereby impacting dedifferentiation. SDF-1 exerts its effects through CXCR4, activating AKT and FoxO1 [31].

T2D exhibits hyperglycemia and inflammation, paralleled by the upregulation of prostaglandin E2 (PGE2). A systemic blockade of the PGE2 receptor EP3 promotes β-cell proliferation and mass without affecting α-cells. EP3 blockade reverses islet gene expression changes in diabetic mice, restoring normal islet architecture. Additionally, it increases *Glp1r* expression and activates Nrf2, potentially protecting β-cells from oxidative stress. EP3 blockade shows promise for enhancing β-cell mass in T2D [32].

Insulitis, or inflammation of β-cells, is most commonly associated with T1D. Under this condition, β-cells are usually exposed to a variety of cytokines, including IFNγ, IL-1β, TNFα, and IL-17, all of which are able to trigger ER stress and β-cell dedifferentiation [10].

There is some evidence indicating that the direct interaction of autoimmune T cells with β-cells may be responsible, but not sufficient, for changes in the β-cell identity in T1D patients [33]. However, the islet microenvironment in T1D has been reported to generate CD4 T cells that target proinsulin, suggesting that proinflammatory mediators from islets may cause β-cell dedifferentiation in individuals with T1D [34]. Moreover, it has been found that exposing β-cells to double-stranded RNA, mimicking a viral infection that could trigger T1D, reduces the expressions of β-cell-enriched genes like *MafA* and *Ins* while increasing progenitor markers. This effect is driven by NF-kB within the β-cells and the interferon alpha released by neighboring cells [35,36].

The ablation of β-cells, which is associated with T1D, induces the transdifferentiation of α-cells into β-cells [37]. This indicates that cell plasticity could be used as a potential treatment for T1D. It is worth mentioning that in the context of T1D, the immune system does not attack α-cells [38].

2.2. Metabolic Stress

Metabolic stressors, including glucotoxicity, lipotoxicity, and glucolipotoxicity, have been demonstrated to impair β-cell function and identity by disrupting key metabolic pathways.

Glucotoxicity has been shown to inhibit β-cell regeneration. In a mouse model, where β-cells are ablated, the usual regenerative response includes β-cell proliferation and the normalization of blood glucose levels. However, if the β-cell loss leads to severe hyperglycemia (above 28 mmol/L), regeneration fails, causing permanent diabetes, despite some surviving β-cells entering the cell cycle. Reducing hyperglycemia with insulin, SGLT2 inhibitors, or a ketogenic diet for about three weeks can partially restore β-cell mass and aid in diabetes recovery, indicating that extreme glucotoxicity hinders regeneration. Gene expression analysis suggests that high glucose levels cause metabolic stress and suppress key cell-cycle genes, potentially blocking regeneration [39].

Persistent hyperglycemia maintains a cycle of β-cell dysfunction. Using advanced optical imaging, researchers found that streptozotocin (STZ) predominantly damages larger islets in the pancreatic core, leading to a significant reduction in β-cell mass. However, even in hyperglycemic STZ-treated mice, a considerable number of β-cells remain but with a decreased expression of *Glut2* (*Slc2a2*), indicating impaired β-cell maturity. Islet gene expression studies confirm this downregulation and suggest that hyperglycemia causes β-cell dysfunction more than ablation. Islet transplantation partially restored some markers of islet function but not the overall β-cell mass [40].

The hyperactive isoform of carbohydrate response-element-binding protein (ChREBPβ) plays a crucial role in the response of β-cells to hyperglycemic stress. It is involved in adaptive β-cell expansion when faced with metabolic challenges like prolonged glucose exposure and HFD. However, chronic overexpression of *ChREBPβ* in β-cells can lead to a loss of β-cell identity, increased apoptosis, reduced β-cell mass, and diabetes. Deleting ChREBPβ can prevent β-cell "glucolipotoxicity", while the overexpression of *ChREBPα* or activation of the antioxidant Nrf2 pathway can mitigate ChREBPβ-induced β-cell death [41].

Stearoyl CoA desaturase (SCD), an enzyme pivotal in lipid metabolism, is highly expressed in β-cells and human islets. The silencing of SCD prompts inflammation, ER stress, and elevated *IAPP* mRNA levels. Conversely, treatment with SCD products counteracts these effects. Moreover, when SCD is knocked down and exposed to palmitate, dedifferentiation markers are induced. Notably, SCD knockdown alone disrupts β-cell identity, diminishing mature β-cell markers and compromising insulin secretion [42].

Accordingly, it has been found that the inhibition of stearoyl-CoA desaturase 1 (SCD1) is associated with increased methylation in *Pdx1* and *MafA* promoter regions. This inhibition also skews pancreas development towards α-cell formation, disrupting the α-cell-to-β-cell ratio and islet structure. Consequently, islet dysfunction occurs, characterized by impaired insulin secretion and elevated glucagon levels [43].

Oxidative stress originating from hyperglycemia and hyperlipidemia can cause a significant decline in human pancreatic β-cell function. This decline is characterized by

the activation of ER stress and the nuclear translocation of FoxO1. This stress response results in the downregulation of the maturity genes *MafA* and *Pdx1* along with the upregulation of the progenitor markers *Sox9* and *Hes1*, suggesting a potential process of partial dedifferentiation. These findings highlight the critical importance of targeting antioxidant mechanisms to preserve functional β-cell mass, particularly in the early stages of T1D [44].

During the insulin resistance phase of T2D, β-cells exhibit persistently high calcium levels. Initially, this adaptation may assist β-cells in managing the metabolic stress induced by hyperglycemia. However, prolonged exposure to elevated calcium levels may lead to alterations in β-cell function and identity, as well as diabetic complications. Single-cell RNA sequencing of pancreatic islets from prediabetic and diabetic mice unveiled a diabetes-specific transcriptomic profile. It highlighted *Anxa10*, a gene linked to prediabetes, as being upregulated by persistently high calcium levels in β-cells. Pseudotime analysis (computational ordering of cells in the progression of differentiation) suggested that disturbances in calcium influx during the insulin resistance phase and heightened Anxa10 expression could induce mitochondrial dysfunction, ER stress, and β-cell transdifferentiation into acinar cells, offering new insights into diabetes progression [45].

PARP1 is related to metabolic stress through its involvement in DNA repair mechanisms and cellular responses to stressors such as oxidative stress and inflammation. During metabolic stress, such as high glucose levels or lipid overload, PARP1 can be activated excessively, leading to the increased production of poly(ADP-ribose) polymers (PARylation). Research shows that *PARP1* knockout (KO) mice exhibit resistance to diabetes, and inhibiting PARP1, particularly with PJ34, alleviates complications in type 2 diabetes patients. Complete PARP1 depletion halted β-cell formation, whereas its inhibition enhanced β-cell differentiation and maturation via p38 MAPK phosphorylation and Ngn3 reactivation, implying PARP1's necessity for proper β-cell development [46].

Maintaining lysosomal activity and mitophagy is crucial for the differentiation and maturation of β-cells. The Niemann-Pick C1 protein (NPC1) on the lysosome membrane is essential for intracellular cholesterol transport and lysosome function. Research links NPC1 mutations to pancreatic islet dysfunction in T2D. NPC1 depletion in mice impairs β-cell viability, expansion, and GSIS, disrupting mitochondrial function through compromised lysosomal activity and mitophagy, indicating metabolic stress [47].

Insulin granule maturation in pancreatic β-cells relies heavily on phosphatidylinositol transfer protein α (PITPNA). Reduced PITPNA levels in β-cells from individuals with T2D result in impaired GSIS, diminished β-cell mass, and increased ER stress, reflecting metabolic stress. The conditional deletion of *PITPNA* in mice causes hyperglycemia and compromised insulin processing. Silencing *PITPNA* in human islets yields similar defects that can be reversed by restoring PITPNA levels [48].

2.3. Age-Related Dynamics of β-Cell Dedifferentiation and Transdifferentiation

The impact of age on β-cell dedifferentiation and transdifferentiation is increasingly recognized, with studies revealing a significant rise in dedifferentiated cells among older age groups. This phenomenon is accompanied by diminished FoxO1 activity and reduced *UCN3* expression in aging β-cells along with a decline in adaptive unfolded protein response (UPR) components. Glucose-regulated protein 94 (GRP94), also known as heat shock protein 90B1 (HSP90B1), is an ER chaperone that plays a crucial role in protein folding, quality control, and the maintenance of ER homeostasis. Lowering GRP94 levels has been shown to induce dedifferentiation, while restoring GRP94 levels preserves β-cell identity in aging cells [49].

β-cell regeneration becomes challenging with advancing age, particularly in the context of adult-onset diabetes. In middle-aged rats, β-cell aging is notably influenced by compromised duct cell plasticity and β-cell dedifferentiation. Despite observed increases in hypertrophy and hyperplasia, the regenerative potential of functional β-cell mass is limited. This implies the critical role of β-cell dedifferentiation and potential transdifferentiation in the aging process [50].

Excessive glycolysis may contribute to age-related β-cell dysfunction and the loss of cellular identity. Both aged and diabetic mouse β-cells exhibit common dysfunctional traits, including heightened glucose sensitivity and compromised cellular integrity. They also share an increase in glycolysis driven by elevated *Nmnat2* expression, which is crucial for NAD synthesis. Knocking out *Got1*, a gene associated with glycolysis, replicated these traits, which are observed in aging and diabetes. Restoring the β-cell function and integrity was achieved by modulating glycolysis or the Nmnat2 activity in *Got1* KO β-cells [51].

2.4. Sex-Dependent Variations in Pancreatic Islet Plasticity

Sex influences β-cell plasticity, as evidenced by the differences observed in pancreatic islet structures between male and female mice. Male control mice exhibit larger pancreatic islets and experience a greater reduction in islet and β-cell areas after STZ exposure compared to females. Conversely, female mice treated with STZ show lower rates of β-cell apoptosis. Hydrocortisone (HC) administration results in a higher proportion of α-cells in female islets, which is accompanied by a slight increase in β-cell apoptosis. Healthy female mice demonstrate a higher rate of α-to-β-cell transdifferentiation, which decreases with HC treatment, while STZ treatment increases α-cell transdifferentiation to β-cells in males but not females. These findings underscore the impact of sex hormones on islet morphology and function [52].

2.5. The Impacts of Chemicals on β-Cell Plasticity

Compounds like ethanol, STZ, glucocorticoids, and environmental pollutants impact different aspects of diabetes, including the dedifferentiation and transdifferentiation of β-cells.

Alcohol addiction is a serious global issue, responsible for 5.9% of all deaths worldwide. Regular alcohol consumption heightens the risk of metabolic diseases, including T2DM, compared to nonconsumption. Chronic alcohol consumption results in reduced insulin levels, β-cell apoptosis, and decreased β-cell mass along with upregulated *Fibroblast Growth Factor 21* (*FGF21*) and its receptor mRNA levels, indicating FGF21 resistance. Short-term ethanol exposure does not immediately impair β-cell function, but prolonged exposure progresses to significant dysfunction. Chronic binge drinking is linked to reductions in insulin, the insulin-to-glucagon ratio, the GSIS response, and NKX2.2 and PDX1 levels, highlighting alcohol's impact on β-cell dedifferentiation [53]. Additionally, there is a negative correlation between alcohol consumption and blood adiponectin levels [54], which diminish insulin expression and secretion [55] as well as β-cell survival [56].

STZ is a naturally occurring compound used to induce diabetes in experimental models by selectively destroying insulin-producing β-cells in the pancreas through DNA alkylation and oxidative stress. It targets β-cells specifically due to their high expression of the *Glut2* glucose transporter, which facilitates STZ uptake and leads to cell death. However, some β-cells with lower *Glut2* expression survive the initial damage but experience long-term functional and phenotypic changes, remaining in a low-Glut2 state. It has been found that some interventions, such as islet transplantation and glucose normalization, can partially restore β-cell function and *Glut2* expression [57,58]. STZ treatment potentially promotes β-cell regeneration and transdifferentiation in pancreatic islets. This includes the emergence of vimentin+/MafB+ cells, which can differentiate into insulin-producing β-like cells, and the possibility of α-cells transdifferentiating into β-cells under specific conditions [1,59].

Glucocorticoids such as dexamethasone and hydrocortisone can induce β-cell dedifferentiation through various mechanisms, including triggering ER stress, changes in gene expression [60], impaired β-cell glucose metabolism, and reduced sensitivity to glucose stimulation, leading to altered insulin secretion patterns and decreased GSIS [61,62]. Furthermore, excess glucocorticoid exposure during fetal development can permanently alter β-cell mass and function, resulting in long-term consequences for glucose homeostasis [63].

Bisphenols (BPs) and phthalates are classes of industrial chemicals widely used in the production of plastics and other consumer products. These chemicals have been linked to higher diabetes rates, particularly affecting β-cells. Exposure to these chemicals disrupts electrical activity, mitochondrial function, gene expression, and β-cell function, leading to altered GSIS and impaired β-cell proliferation [64]. Studies show mixed results, with some indicating heightened insulin secretion and content, while others suggest diminished GSIS or insulin levels. These substances can also impact β-cell survival, with higher doses or prolonged exposure often leading to increased cell death [65]. BPs induce ER stress, mitochondrial dysfunction, and oxidative stress in β-cells, contributing to cellular damage, apoptosis, and plasticity [66,67]. The effects on β-cell proliferation vary, with some studies showing increased proliferation at lower doses [68] and others observing reduced growth at higher concentrations [69]. Bisphenol A (BPA) acts as an endocrine disruptor, altering gene expression in β-cells during development and producing long-term metabolic disturbances. Early-life exposure to BPA increases β-cell mass and proliferation through estrogen receptor β (ERβ), resulting in hyperinsulinemia that precedes insulin resistance [70,71].

Phthalates affect insulin secretion in β-cells variably depending on the duration and concentration of exposure. Acute high-concentration exposure increases basal insulin secretion (BIS) without affecting GSIS. In contrast, prolonged exposure typically inhibits both BIS and GSIS, indicating impaired insulin synthesis and β-cell function, often coupled with reduced β-cell survival and increased cellular stress [64,72].

3. Molecular Pathways Involved in β-Cell Plasticity

3.1. ER Stress

ER stress results from the buildup of misfolded proteins, activating the UPR to restore normalcy. Environmental factors like inflammation and oxidative stress can trigger ER stress, which is implicated in T1D and T2D onset [73,74]. ER stress activates the UPR, which is mediated by sensors like IRE1α, PERK, and ATF6, which aim to restore ER homeostasis [75].

Prolonged ER stress can lead to cellular dysfunction and apoptosis, particularly affecting pancreatic β-cells. When β-cells experience ER stress, they need to activate mechanisms to adapt and manage this stress effectively. Chronic stress induces transcriptional and translational changes, impairing β-cell function and identity regulators. Despite having some capacity to adapt to stress, β-cells under prolonged stress can eventually lose their functional identity. This means they no longer produce insulin efficiently, which is crucial for regulating blood sugar levels.

Single-cell RNA sequencing from T1D patients reveals severe disruptions in stress adaptation, indicating a role for β-cell adaptive exhaustion in diabetes progression [76].

A study by Hugo Lee et al. (2020) discovered that the β-cell deletion of IRE1α in Non-Obese Diabetic (NOD) mice, in the T1D model, led to transient β-cell dedifferentiation, temporarily protecting against immune-mediated β-cell destruction. Additionally, IRE1α influences the stability of *TXNIP* mRNA, impacting NLRP3 inflammasome activation and cell death pathways [77].

The X-box binding protein 1 (XBP1) serves as a pivotal regulator of the UPR within the ER. Extensive evidence implies the crucial role of XBP1 in preserving β-cell identity and function, especially during periods of metabolic stress. The depletion of XBP1 results in β-cell dysfunction, dedifferentiation, and heightened vulnerability to diabetes, underscoring its significance in maintaining β-cell homeostasis and regulating insulin secretion [78].

Like T1D, T2D is also characterized by increased ER stress and altered insulin processing, leading to a loss of β-cell identity and function. The transition from impaired glucose tolerance to diabetes, as a hallmark of T2D, is marked by a significant increase in ER stress markers and a decrease in β-cell phenotype markers. β-cell dedifferentiation appears as the disease progresses, with islets showing reduced expressions of key genes such as *Nkx6.1* and *MafA*. Additionally, these changes were accompanied by an increase in

altered proinsulin processing and intracellular localization, indicating a deterioration of the β-cell secretory machinery [73].

ATF4, a key regulator of ER stress responses, contributes to pancreatic β-cell dysfunction in T2D. It induces phosphodiesterase 4D (*PDE4D*) expression, disrupting cAMP signaling and accelerating T2D progression. Pharmacological inhibition of the ATF4 pathway reduces *PDE4D* expression, improving glucose tolerance and insulin secretion. The administration of Sephin1, a selective inhibitor of a holophosphatase with a modulatory impact on ER stress, enhances glucose metabolism in Akita mice by increasing insulin-positive areas and *ATF4* expression. Conversely, β-cell-specific *ATF4* knockout (βAtf4-KO) mice exhibit exacerbated diabetes, with increased dedifferentiation markers and a reduced expression of the Atonal homolog 8 transcription factor (*Atoh8*) [79,80].

ATF4 is also a key player in integrated stress response (ISR). ISR is a complex cellular signaling pathway in eukaryotic cells that manages and mitigates various stress stimuli. Essential for maintaining cellular homeostasis, the ISR can be triggered by intrinsic and extrinsic factors such as hypoxia, amino acid deprivation, glucose deprivation, viral infections, and oxidative stress [81]. The ISR is driven by the phosphorylation of eukaryotic translation initiation factor 2 alpha (eIF2α) by one of four kinases: PERK, GCN2, PKR, or HRI. This phosphorylation reduces global protein synthesis, conserving resources and alleviating the load on the protein-folding machinery. At the same time, it promotes the translation of specific genes, such as the transcription factor ATF4, which, in turn, aids in cellular adaptation to stress by upregulating the genes involved in both protein folding and amino acid metabolism [82]. The activation of the ISR in β-cells is found to regulate oxidative stress, a key player in β-cell dedifferentiation. This supports the observations indicating that the phosphorylation of eIF2α has a protective impact on β-cells against oxidative stress caused by insulin resistance through an ATF4-dependent mechanism [83]. Amino acid deficiency, another inducer of the ISR, stimulates the binding of uncharged tRNA to GCN2, leading to the autophosphorylation of GCN2 and subsequent phosphorylation of eIF2α [84]. This phosphorylation of eIF2α is associated with an increase in ATF4 levels, which upregulates the genes involved in the synthesis and metabolism of amino acids [85]. The upregulation of ATF4 also increases the expression of *sesterin2*, thereby inhibiting mTORC1. It is noteworthy that the chronic activation of mTORC1 during the insulin resistance phase negatively impacts β-cell mass and proliferation [86].

In summary, ATF4 upregulation, whether through ER stress or through the ISR, has a great impact on the β-cell fate.

Both high-glucose- and cytokine-induced ER stress activate intracellular calcium and reactive oxygen species (ROS) signals in pancreatic β-cells. These signals trigger calcineurin (CN)/NFATc2 and PI3K/AKT pathways, regulating gene expression that is critical for β-cell function. While inducing the endocrine differentiation gene *RFX6*, they suppress "disallowed" genes like *MCT1*, which are detrimental to β-cell identity. CN/NFATc2, along with co-activator p300 and co-repressor HDAC1, orchestrates these gene expression changes. Prolonged stress or disrupted CN/NFATc2 signaling leads to β-cell dedifferentiation, marked by reduced *RFX6* and elevated *MCT1* levels [87].

3.2. Angiotensin Receptors

Angiotensin receptors are cell surface receptors that bind and respond to angiotensin peptides produced by the renin–angiotensin system (RAS). There is an interaction between the renin–angiotensin aldosterone system (RAAS) and ER stress in pancreatic β-cells, particularly in the context of T2D [88]. The activation of the Angiotensin II (Ang II) receptor is linked to the initiation of β-cell dedifferentiation and the development of T2D. This occurs through the activation of the RAS and subsequent NFκB activation [89].

The activation of Angiotensin II exerts its negative impact on β-cell plasticity by increasing ER stress markers and reducing insulin secretion in clonal INS-1E β-cells and human islets. Accordingly, it has been found that the inhibition of Ang II production or the blockade of its receptors alleviates ER stress and restores insulin secretion. These findings

elucidate a potential mechanism linking pancreatic RAAS activation to β-cell dysfunction in T2D, highlighting the therapeutic potential of RAAS inhibitors in T2D management [88].

3.3. mTOR Pathway

The protein kinase mTOR (the mechanistic target of rapamycin) regulates cell growth, proliferation, and survival, impacting cellular metabolism and protein synthesis. mTOR activity decreases in mice fed a high-fat diet (HFD), leading to the initial enlargement but subsequent diminishment of pancreatic islets. This decrease in mTOR activity correlates with increased β-cell death, reduced levels of myeloid cell leukemia 1 (Mcl-1), a Bcl-2 family member crucial for pancreatic β-cell survival, and impaired insulin secretion in response to glucose. Additionally, the activation of AMPK by palmitic acid (PA) degrades Mcl-1 via pathways such as ERK and GSK3β, further contributing to β-cell dysfunction and loss of identity [90].

The growth factor receptor-bound protein 10 (GRB10), a negative regulator of insulin and mTORC1 signaling, is significantly elevated in the islets of diabetic mice and insulinoma cells treated with high glucose. β-cell-specific knockout of *GRB10* in mice increased β-cell mass and function along with enhanced mTORC1 signaling and reduced β-cell dedifferentiation, reversible by rapamycin. Conversely, the overexpression of *GRB10* in MIN6 cells induced β-cell dedifferentiation, indicating potential therapeutic targeting of GRB10 to address β-cell dysfunction in diabetes [91].

The deletion of the β-cell-specific gene *Raptor*, a component of mTORC1, resulted in decreased β-cell mass, the adoption of α-cell characteristics, and loss of β-cell identity, even after glucose normalization. A *Raptor* deficiency directly impaired β-cell identity, mitochondrial function, and protein synthesis, leading to β-cell dysfunction. Additionally, *Raptor* loss activated *MafB*, a transcription factor associated with α-cells, and genes specific to α-cells, promoting a transition from β- to α-cell identity. These findings highlight mTORC1's role in maintaining β-cell identity and suppressing α-cell characteristics under normal glucose conditions [92].

Additionally, the mTORC1 pathway regulates β-cell functional maturation through epigenetic mechanisms. Utilizing microarray, MeDIP-seq, and ATAC-seq analyses, it has been uncovered that DNMT3A-dependent DNA methylation and PRC2-dependent H3K27me3 modification are key silencing mechanisms in immature β-cells lacking *Raptor.* Overexpressing *DNMT3A* partially reverses the immature transcriptome pattern and restores impaired GSIS in *Raptor*-deficient β-cells. Furthermore, Raptor directly regulates *PRC2/EED* and *H3K27me3* expressions, suggesting a complex interplay between nutrient sensing, epigenetic modifications, and β-cell function, with implications for T2D [93].

Rictor, a key component of mTORC2, regulates β-cell proliferation and function, with long chain acyl-CoA synthetase 4 (Acsl4) potentially downstream. *Acsl4* is positively regulated by Rictor at both transcriptional and post-translational levels in mouse β-cells. Adenovirus-mediated *Acsl4* expression rescued β-cell dysplasia in βRicKO islets but not dysfunction. However, *Acsl4* could not fully restore glucose oxidation in *Rictor*-deficient β-cells, instead promoting lipid oxidation. Epigenetic analyses revealed signatures of β-cell dedifferentiation and an altered oxidative response in *Rictor*-lacking cells expressing *Acsl4*, suggesting a context-dependent role for *Acsl4* in β-cell dedifferentiation under reduced Rictor/mTORC2 activity [94].

mTORC1 activation under chronic metabolic stress leads to increased levels of the phosphatases PHLPP1 and PHLPP2 in diabetic β-cells. These elevated phosphatases contribute to β-cell failure in diabetes by regulating survival kinases such as AKT and MST1. Inhibiting PHLPPs genetically enhances β-cell survival and function in diabetic models and human T2D islets, suggesting a potential therapeutic target for diabetes [95].

3.4. Role of Ubiquitin–Proteasome System in the Pancreatic β-Cell Plasticity

The ubiquitin–proteasome system (UPS) regulates cellular protein degradation, impacting various biological processes like inflammation and apoptosis. Emerging evidence

suggests its crucial involvement in pancreatic β-cell transdifferentiation in diabetes, presenting a potential treatment avenue [96,97].

The UPS, which includes E3 ligases and deubiquitinases (DUBs), regulates several aspects of β-cell function. The inhibition of deubiquitinase USP1, a key player in β-cell dedifferentiation, either through genetic intervention or using the small-molecule inhibitor ML323, restored the epithelial phenotype of β-cells. Conversely, the overexpression of USP1 induced dedifferentiation, which was mediated by modulating the expression of the inhibitor of differentiation 2 (ID2). In an STZ-induced mouse model of β-cell dedifferentiation, treatment with ML323 improved hyperglycemia [98].

Ubiquitin-Specific Peptidase 7 (USP7) stabilizes Ngn3 by deubiquitination. The conditional knockout of Usp7 in mice reduces islet formation and causes hyperglycemia due to impaired Ngn3-mediated endocrine specification. Pharmacological inhibition of USP7 in human iPSC models also decreases *Ngn3* progenitors and impairs β-cell differentiation, highlighting the importance of the USP7-Ngn3 axis in preventing β-cell dedifferentiation [99].

Ubiquitin-mediated degradation of *Ngn3*, regulated by PRMT1-mediated arginine 65 methylation, is essential for β-cell differentiation. *Protein arginine methyltransferase-1* (*PRMT1*) knockout in human embryonic stem cells (hESCs) leads to Ngn3 accumulation in the cytoplasm, reducing its transcriptional activity and preventing endocrine cell differentiation. This methylation acts as a molecular switch, ensuring Ngn3's proper localization and function, thus preventing β-cell dedifferentiation [100].

4. Transcriptional Regulation in β-Cell Plasticity

Transcription factors are crucial for establishing and maintaining the mature phenotype of β-cells as well as regulating their plasticity through processes such as dedifferentiation, transdifferentiation, and regeneration. Key transcription factors, including Pdx1, Ngn3, Pax4, MafA, FoxO1, and Nkx6.1, are essential for β-cell identity, function, and survival. The dysregulation of these factors can result in dedifferentiation, where β-cells lose their mature state and revert to a progenitor-like condition.

Furthermore, alterations in the transcriptional network can drive the transdifferentiation of β-cells into other islet cell types, such as α- or δ-cells. Modulating transcription factors like Pdx1, Ngn3, and MafA shows potential for reprogramming other cell types into insulin-producing β-like cells, thereby facilitating β-cell regeneration [101]. The information on key transcription factors involved in dedifferentiation (De), transdifferentiation (Tra), neogenesis (Neo), and proliferation (Pro) is summarized in Table 1.

The table is divided into seven columns: "Transcription Factors" lists the names of the transcription factors involved in β-cell dedifferentiation, transdifferentiation, neogenesis, or proliferation (De/Tra/Neo/Pro). "Full Name" provides the full names of these transcription factors. "Target Genes" describes the genes regulated by the transcription factors. "Impact on Genes" explains how each transcription factor affects the target genes (induction (I) or repression (R)). "De/Tra/Neo/Pro" indicates how the transcription factors impact β-cell plasticity and regeneration. "Impact on β-Cell Plasticity/Regeneration" describes whether the transcription factors have a positive (P) or negative (N) effect on β-cell plasticity/regeneration. "References" lists the sources used for each transcription factor's explanation. Abbreviations: P: Positive, N: Negative, NM: Not Mentioned, I: induction, R: Repression.

Table 1. Important transcription factors involved in β-cell dedifferentiation, transdifferentiation, neogenesis, or proliferation (De/Tra/Neo/Pro).

Transcription Factor	Full Name	Targets Genes	Impact on the Genes	De/Tra/Neo/Pro	Impact on β-Cell Plasticity/ Regeneration	References
Pax4	Paired Box 4	*GCG*	R	De	N	[101–104]
		Arx	R			
		Ins	I	Tra	N	
		Bcl-xL	I			
		Gherlin	R	Neo	P	
		SST	R			
		IAPP	R	Pro	P	
		C-myc	I			
Nkx2.2	NK2 homeobox 2	*Ins*	I	De	N	[105–110]
		Pax6	I			
		Nkx6-1	I	Tra	N	
		MafA	I			
		NeuroD1	I	Neo	P	
		Arx	R			
		SST	R	Pro	NM	
		Gherlin	R			
Mnx1 (also known as HLXB9 or HB9)	Motor neuron and pancreas homeobox 1	*Nkx6.1*	I	De	N	[111–115]
				Tra	N	
		Ins	I	Neo	NM	
				Pro	P	
FoxO1	Forkhead box O1	*Ins1*	R/I	De	N and P	[116–123]
		Pdx1	R	Tra	N and P	
		NeuroD	I			
		Superoxide dismutase 1 (Sod1)	I	Neo	N	
		Catalase (Cat)	I			
		MafA	I	Pro	P	
		Glutathione peroxidase 1	I			
Pdx1	Pancreatic and Duodenal Homeobox 1	*Ins*	I	De	N	[124–128]
		Glut2	I			
		Gck	I	Tra	N	
		IAPP	I			
		SST	I	Neo	P	
		Pdx1	I			
		Ngn3	I	Pro	P	
		NFκB	R			
		MafA	I			

Table 1. *Cont.*

Transcription Factor	Full Name	Targets Genes	Impact on the Genes	De/Tra/Neo/Pro	Impact on β-Cell Plasticity/ Regeneration	References
MafA	Musculoaponeurotic Fibrosarcoma Oncogene Family, Protein A	*Ins*	I	De	N	[129–135]
		Glut2	I			
		Gck	I	Tra	N	
		Cacng4	I			
		Nanog	R	Neo	P	
		Pdx1	I			
		NeuroD1	I	Pro	P	
		Nkx6.1	I			
		Glis3	I			
Ngn3	Neurogenin 3	*NeuroD1*	I	De	N	[99,136–139]
		Pax4	I			
		Pax6	I	Tra	N	
		Isl1	I			
		Cdkn1a	I	Neo	P	
		Nkx2-2	I			
		Insm1	I	Pro	N	
		Kcnj11	I			
		Gck	I			
		Slc30a8	I			
		Slc18a2	I			
SOX9	Sex-determining region Y (SRY)-box 9	*Ngn3*	I	De	N	[140–143]
		Nkx6.1	I	Tra	N	
		Pax6	I	Neo	P	
		Ins	I	Pro	P	
HNF1A	Hepatocyte Nuclear Factor 1 A	*HNF4A*	I	De	N	[144–149]
		AS1	I			
		Tmem27	I			
		Kcnj11	I			
		Glut2	I	Tra	N	
		Ins	I	Ne	P	
		NeuroDI	I	Pro	P	
		Gck	I			
		MafB	I			
		Gipr	I			
		Glpr1	I			
		G6pc2	I			
		Slc5a1	I			
		Pdx1	I			
		Bcl2li(Bcl-xL)	I			

Table 1. *Cont.*

Transcription Factor	Full Name	Targets Genes	Impact on the Genes	De/Tra/Neo/Pro	Impact on β-Cell Plasticity/ Regeneration	References
ARX	Aristaless-Related Homeobox	*Gcg*	I	De	P	[150–153]
		Pax4	R			
		SST	R	Tra	P	
				Neo	N	
				Pro		
GATA6	GATA Binding Protein 6	*iNOS*	R	De	N	[154–158]
		Ins	I			
		Glut2	I	Tra	N	
		Abcc8	I			
		Snap-25	I	Neo	P	
		Pdx1	I			
		Nkx6.1	I	Pro	P	
		MafA	I			
GLIS3	GLI Similar 3	*Ins*	I	De	N	[159–163]
		Ngn3	I	Tra	N	
		MafA	I	Neo	P	
		Glut2	I	Pro	P	
PAX6	Paired Box 6	*Ins*	I	De	N/P	[164–167]
		GCG	R			
		Glut2	I			
		Gipr	I			
		Nkx2.2	I			
		PC1/3	I			
		Glp1r	I			
		SST	R	Tra	N/P	
		Gherlin	R			
		Isi	R	Neo	P	
		Foxa2	R/I			
		Ngn3	R/I	Pro	P	
		Pdx1	R/I			
		NeuroD1	R/I			
		MafA	R/I			
		Nkx6.1	R/I			
MafB	Musculoaponeurotic Fibrosarcoma Oncogene Homolog B	*GAST*	R	De	N	[168–171]
		SST	R			
		P2ry1	I	Tra	N	
		Adra2A	I			
		Tph1	I	Neo	P	
		Ins	I			

Table 1. *Cont.*

Transcription Factor	Full Name	Targets Genes	Impact on the Genes	De/Tra/Neo/Pro	Impact on β-Cell Plasticity/ Regeneration	References
MafB	Musculoaponeurotic Fibrosarcoma Oncogene Homolog B	*ChrnA3, ChrnA4, ChrnB4*	I	Pro	P	[168–171]
		Robo1, Robo2, Nrp1, Nrp2	I			
Hes1	Hairy and Enhancer of Split-1	*Ngn3*	R	De	P	[172–175]
		NeuroD	R	Tra	P	
		Ptf1a	R	Neo	N	
		p27Kip1 and p57Kip2	R	Pro	P	
HNF4 A	Hepatocyte Nuclear Factor 4 A	*Aldolase B*	I	De	N	[176,177]
		Glut2	I			
		Gck	I	Tra	N	
		L-pyruvate kinase	I			
		Kir6.2	I	Neo	P	
		Ins	I			
		Pdx1	I	Pro	P	
		Pax4	I			
		Ngn3	I			
		Ki67	I			
NeuroD1	Neurogenic Differentiation 1	*Ins*	I	De	N	[178–180]
		Gck	I			
		Abcc8/Sur1, Kcnj11	I			
		Cacna1a	I	Tra	N	
		Mafa	I			
		Nkx6-1	I			
		Pdx1	I	Neo	P	
		Nkx2-2	I			
		Insm1	I			
		Isl1	I	Pro	P	
		Pax6	I			
		Rfx6	I			
		Cdh4	R			
		Ocln	R			
		Mecom	R			
		ldha	R			

Table 1. *Cont.*

Transcription Factor	Full Name	Targets Genes	Impact on the Genes	De/Tra/Neo/Pro	Impact on β-Cell Plasticity/ Regeneration	References
Nkx6.1	NK6 homeobox 1	*Glut2*	I	De	N	[130,181–184]
		G6pc2	I			
		Arx	R	Tra	N	
		Pdx1	I			
		MafA	I	Neo	P	
		Ero1lb	I	Pro	P	
		Slc30a8	I			
		Nr4a1 and Nr4a3	I			
ISL1	ISL LIM homeobox 1	*MafA*	I	De	N	[185–188]
		Pdx1	I			
		Glut2	I			
		Ins	I			
		NeuroD1	I	Tra	N	
		Pax6	I			
		MafB,	I			
		Nkx6.2	I			
		Insm1	I	Neo	P	
		Sox9	I			
		Arx	I			
		Foxa2	I			
		Nkx6.1	I	Pro	P	
		GCG	I			
		SST	I			
		G6pc2	I			

5. Translational Control

The eukaryotic initiation factor 5A (eIF5A), crucial for β-cell identity and function, undergoes a post-translational modification by deoxyhypusine synthase (DHPS) to activate its translational activity. In mice lacking DHPS, there is a significant reduction in the proteins essential for β-cell identity, leading to rapid-onset diabetes during β-cell maturation. Further investigation revealed that eIF5A regulates the translation of key genes like *Ins1*, *Glut2*, *Ucn3*, and *Chga* during β-cell maturation, underscoring the pathway's significance in β-cell function maintenance. Targeting mRNA translation in β-cells, particularly by modulating the eIF5A pathway, could present a therapeutic approach to correcting diabetes-related defects [189].

6. Epigenetic-Related Targets

6.1. Histon Modifications

Histone modifications act as epigenetic regulators that control the expression of key β-cell genes. The disruption of these histone marks contributes to β-cell dedifferentiation, while resetting them is crucial for transdifferentiation approaches aimed at generating functional β-like cells [190].

6.2. Histone Acetylation

Sirtuin 3 (Sirt3) is a major mitochondrial deacetylase that is synthesized in the cytoplasm and imported into the mitochondrial matrix. It plays a key role in mitochondrial homeostasis by regulating mitochondrial protein acetylation levels and responding to cellular energy status [191]. It inhibits the ATX-LPA pathway to preserve β-cell identity and function. The depletion of Sirt3 enhances autotaxin (ATX) levels, leading to increased lysophosphatidic acid (LPA) production, which induces β-cell dedifferentiation and impairs insulin secretion. Inhibiting ATX in vivo mitigates β-cell dysfunction in HFD-fed mice lacking Sirt3, suggesting a potential therapeutic strategy for managing diabetes-associated β-cell dysfunction [192].

Sirt3 depletion is also attributed to the reduced deacetylation of the *FoxO1* gene and the subsequent decrease in its transcriptional activity. This suggests that Sirt3 downregulation contributes to β-cell dedifferentiation via *FoxO1* [120].

CBP/p300, acting as transcriptional co-activators and histone acetyltransferases, are crucial for β-cell function. Treatment with A-485, a selective inhibitor of CBP/p300 HAT activity, impairs insulin secretion and downregulates β- and α-cell identity genes without inducing dedifferentiation. A-485 also affects genes related to glucose sensing but not glycolysis. In prediabetic db/db mice, decreased CBP/p300 levels impact β-cell function genes, possibly involving histone H3K27 deacetylation and transcription factors like *Hnf1α* and *FoxO1*. This highlights the critical role of CBP/p300 HAT in maintaining β-cell identity [193].

6.3. Histone Methylation

Histone H3 lysine 4 (H3K4) methylation is crucial for sustaining gene expression related to insulin production and glucose responsiveness, with deficient methylation leading to an altered epigenome profile. Moreover, in diabetic models, there is a shift favoring inactive genes, potentially contributing to β-cell dysfunction [194].

Utilizing GSK126 and Tazemetostat (Taz) inhibitors of histone methyltransferase EZH2, researchers induced a phenotypic shift towards a β-like cell identity in exocrine cells from T1D donors. This transition was facilitated by chromatin modifications, particularly H3K27me3 and H3K4me3, suggesting EZH2 to be pivotal regulator of β-cells' regenerative potential. Reprogrammed pancreatic ductal cells demonstrated insulin production and secretion in response to physiological glucose levels ex vivo [195].

6.4. DNA Methylation

Proper DNA methylation patterns are required to maintain the mature β-cell identity. Disruption of these methylation dynamics contributes to β-cell dedifferentiation in diabetes [196].

DNA methylation plays a significant role in regulating tyrosine hydroxylase (TH), an enzyme known to suppress insulin secretion, during β-cell differentiation. The ablation of the de novo DNA methyltransferase *Dnmt3a* in embryonic progenitors increases TH+ β-cells, reducing insulin secretion. However, postnatal β-cell-specific *Dnmt3a* ablation does not have the same effect. This suggests that maintaining a lower proportion of TH+ β-cells is vital for proper insulin secretion and that embryonic DNA methylation patterns regulate β-cell heterogeneity. Chronic overnutrition can lead to a loss of TH promoter methylation, increasing TH+ β-cells and compromising β-cell function, linking embryonic epigenetic regulation to postnatal β-cell identity and diabetes risk [197].

6.5. Chromatin Remodeling

Chromatin remodeling events play a crucial role in facilitating β-cell plasticity by regulating the expression of lineage-specific transcription factors and epigenetic modifiers.

The ARID1A subunit of the SWI/SNF chromatin remodeling complex regulates β-cell regeneration, with its expression naturally decreasing during β-cell proliferation. *ARID1A* knockout mice show increased protection against diabetes, indicating its role

in regeneration. The specific deletion of Arid1a in β-cells enhances regeneration via neuregulin–ERBB–NR4A signaling. Blocking ERBB or NR4A1 disrupts this regeneration, suggesting that mSWI/SNF complex activity limits β-cell regeneration in health and disease [198].

It has also been found that Pdx1 recruits Swi/Snf to regulate pancreas development and β-cell function by influencing gene expression and chromatin structure. The loss of Swi/Snf in β-cells leads to reduced insulin production and impaired β-cell maturation, highlighting its crucial role. The interaction between Pdx1 and Swi/Snf is vital for β-cell proliferation and the expression of insulin-related genes [199].

Chromodomain Helicase DNA-Binding Protein 4 (Chd4), a subunit of the nucleosome remodeling and deacetylase complex, is found to be crucial for maintaining β-cell identity and function. An inducible β-cell-specific *Chd4* knockout mouse model showed that the loss of Chd4 led to glucose intolerance and impaired insulin secretion. Chd4-deficient β-cells exhibited an increased ratio of immature-to-mature insulin granules and elevated proinsulin levels. RNA sequencing revealed that *Chd4* deficiency altered chromatin accessibility and the expressions of key β-cell genes like *MafA*, *Glut2*, *Chga*, and *Chgb*. Similar defects were observed in human β-cells with *Chd44* knockdown, underscoring its importance in β-cell function. Additionally, it has been found that Chd4 is involved in the transcriptional activity of Pdx1 [200,201].

6.6. MicroRNAs in Pancreatic β-Cell Function and Identity

MicroRNAs (miRNAs) are critical for regulating many cellular functions, such as insulin signaling and glucose metabolism. Pancreatic β-cells depend on a network of miRNAs and their target mRNAs, which influence insulin production, secretion, cell growth, differentiation, survival, and apoptosis. Important miRNAs, including miR-7, miR-9, miR-375, miR-130, and miR-124, are abundant in β-cells and significantly impact their function. In diabetes, shifts in miRNA expression can lead to various complications such as β-cell dedifferentiation and transdifferentiation [202,203]. Table 2 provides detailed information on the impacts of various key miRNAs on β-cell dedifferentiation, transdifferentiation, neogenesis, and proliferation.

During the birth-to-weaning period, pancreatic β-cell growth and maturation are influenced by epigenetic programs activated by nutrients in human milk, including milk-derived exosomes (MEXs). These MEXs, containing miRNAs, drive β-cell proliferation, while reducing differentiation and mitochondrial function. Changes in MEX miRNAs during weaning may trigger β-cell differentiation, yet continuous exposure to bovine MEX miRNAs via cow's milk could lead to β-cell dedifferentiation in adults. This suggests a potential link between prolonged bovine MEX exposure and an increased risk of diabetes [204].

Pancreatic β-cells undergo distinct metabolic changes during breastfeeding. Initially, they exhibit an immature state with high proliferation driven by mTORC1 activation and AMPK suppression. Upon weaning, β-cells transition to a mature state with reduced proliferation and altered mTORC1/AMPK activity, facilitating glucose-stimulated insulin secretion.

Table 2. miRNAs involved in β-cell dedifferentiation, transdifferentiation, neogenesis, or proliferation (De/Tra/Pro/Neo).

miRNA	Affected Genes	De/Tra/Pro/Neo	Type of Impact	References
miRNA-148a	*Pten*, *Ampk*	De	P	[205]
microRNA-483	*Aldh1a3*, *Socs3*	De	N	[206–208]
miR-29-a	*Cdc42*, *Irs1*, *AKT*	De	P	[209,210]
		Pro	N	
miR-23a	*SDF-1α*	Tra	N	[211]

Table 2. *Cont.*

miRNA	Affected Genes	De/Tra/Pro/Neo	Type of Impact	References
miR-24	*Ire1α*, *MafA*	De	P	[212]
miR-7	*Pdx1*, *Isl1*	Neo	P	[213,214]
miR-25/miR-92b	*Neurod1*, *Mcl1*	De	P	[215]
miR-9	*Stxbp1*	De/Tra	P	[216]
miR-96	*PAK1*	β-cell dysfunction	N	[217]
miR-33a-5p	*Abca1*	De	Positive and negative	[218]
miR-302s	*NeuroD1 and Kat2b*	De	P	[203]
miR-203	*Zbtb20*	De	P	[219]
miR-802	*NeuroD1*, *Fzd5*	De	P	[220]
miR-30d	*Socs3*, *Glp1r*, *Igf1r*, *MafA*, *Pdx1*, *Nkx6.1*	Tra	P	[221]
miR-212/132	*Fbw7*	Neo	P	[222]
miR-690	*Sox9*, *Ngn3*, *Pdx1*	Neo	N	[223]
miR-223	*FoxO1*, *Sox6*	Pro	P	[121]
miR-155	*Pdx1*	De	P	[224]
miR-153	*SNAREs*, *MafA*, *NeuroD1*, *Bcl2*, *Ero1lb*, *Igf1r*, *Irs2*	De	P	[225]
miR-320a	*MafF*	De	P	[226]
miR-17-92 cluster	*Pten*, *Cdkn1a*, *p57*, *Bcl2L11*	Pro	P	[227]
miR-21	*Tgfb2*, *Fgfr3*, *Smad2*	De	P	[228,229]
miR-184-3p	*Crtc1*, *Slc25A2*	De and loss	P	[230]
miR-199b-5p	*Mlk3*	Pro	P	[231]
miR-212-5p	*Sirt2*	Insulin secretion and function	N	[232]
miR-375	*Motn*, *Mapkap 1*, *Pdx1*, *Pdpk1*, *Notch2*, *Hnf1β*, *Cadm1*, *Pax6*, *Crem*	De	N	[233–239]
		Pro	P	
		Neo	P	
miR-200	*Zeb1*	De	P	[240]
miR-146a	*Numb*	De	N	[241]
miR-195	*Mfn2*	De	P	[242]

The table is divided into five columns: "miRNA" lists the names of the miRNAs involved in β-cell De/Tra/Neo/Pro. "Affected Genes" describes the genes regulated by these miRNAs. "De/Tra/Neo/Pro" indicates the specific processes of β-cell plasticity and regeneration influenced by the miRNAs. "Type of Impact" describes whether the miRNAs have a positive or negative effect on β-cell plasticity and regeneration. "References" lists the sources used for each miRNA's explanation. Abbreviations: P: Positive, N: Negative.

6.7. Long Non-Coding RNAs (LncRNAs)

Long non-coding RNAs (LncRNAs) have become crucial regulators of gene expression and cellular activity in pancreatic β-cells, profoundly influencing their function, adaptability, and growth. Recent studies have revealed several LncRNAs that are vital for preserving β-cell identity, controlling β-cell mass, and facilitating β-cell proliferation and regeneration.

These discoveries highlight the significance of LncRNAs in β-cell biology and their potential as targets for diabetes treatment. Table 3 provides a summary of these key LncRNAs and their roles in β-cell regulation.

Table 3. LncRNAs involved in β-cell dedifferentiation, transdifferentiation, neogenesis, or proliferation (De/Tra/Pro/Neo).

LncRNAs	Direct and Indirect Targets	De/Tra/Pro/Neo	Type of Effect	References
Gm16308		Pro	P	[243]
DANCR	*miR-33a-5p*	Pro	P	[218,244]
H19	*miRNA let-7- miRNA-29a*	Pro	P	[245–247]
TUNAR	*EZH2*	Pro	P	[248]
Meg3	*EZH2*, *Pdx1*, *MafA*	De	N	[249,250]
βFaar	*miR-138-5p*	De	N	[251]
PLUTO	*Pdx1*	De	N	[124]
HILNC25	*Glis3*	De	N	[252]
Gm10451	*miR-338-3p*	De	N	[253]
810019D21Rik (RIOT)	*Nkx6.1*	De	N	[254]
p3134	*Pdx1*	De	N	[124,255]
linc-p21	*miR-766-3p*	Pro	N	[256]
HOTAIR	*Pdx1*	De	N	[124]
LncCplx2	*EZH2*	Pro	N	[257]
MALAT1	*Pdx1*	De	P	[258]
MIR503HG	*CtBP1*	Neo	N	[175]
GAS5	*miR-29a-3p*, *miR-96-3p*, *miR-208a-3p*	De	N	[259,260]
		Pro	P	
LINC01018	*miR-499a-5p*	De	P	[261]
		Pro	N	

The table is organized into five columns: "LncRNAs" lists the names of the lncRNAs involved in β-cell De/Tra/Neo/Pro. "Direct and Indirect Targets" describes the miRNAs and genes regulated by the lncRNAs. "De/Tra/Neo/Pro" specifies the particular processes of β-cell plasticity and regeneration affected by the LncRNAs. "Type of effect" indicates whether the LncRNAs have a positive or negative effect on β-cell plasticity and regeneration. "References" provides the sources used for the information. Abbreviations: P: Positive, N: Negative.

7. Reprogramming and Neogenesis of β-Cells

Evidence suggests that newly emerged cells following β-cell destruction differ from original β-cells, demonstrating bihormonal activity by simultaneously producing both somatostatin and insulin. This dual hormone production may contribute to the management of hyperglycemia. Their rapid appearance following β-cell destruction, regardless of age, indicates a promising avenue for further investigation. Moreover, research indicates that these cells likely originate from Somatostatin 1.1 (sst1.1) δ-cells with a β-cell pre-identity, offering a potential source for β-cell regeneration after β-cell destruction [262]. Using single-cell transcriptomics in zebrafish during diabetes induction and recovery, researchers identified two distinct somatostatin-producing δ-cells, denoted as δ1 and δ2. Additionally, a small subset of glucose-responsive islet cells displayed characteristics of both β- and δ1-cells. These hybrid cells emerged spontaneously and gained glucose responsive-

ness, crucial for insulin expression during recovery. Furthermore, the overexpression of *Dickkopf-related protein 3* (*DKK3*), a member of the Dickkopf (DKK) family involved in Wnt signaling pathway regulation, enhanced the formation of these hybrid cells without harming β-cells [263].

The abundance of these δ-cells, as observed in T1D, could potentially result from α- to β- to δ-cell conversion [264,265]. Supporting this notion, it has been discovered that α-cells could also contribute to β-cell regeneration [264,266,267]. α-cells, especially those secreting glucagon, have the capacity to spontaneously transform into insulin-producing cells, thereby aiding in diabetes recovery, particularly post-puberty. A novel mechanism, partially governed by FoxO1, facilitates this transformation of somatostatin-producing δ-cells, particularly in juvenile individuals [266]. Additionally, a novel β-cell progenitor type displaying mesenchymal characteristics and expressing α-cell markers has been identified, potentially influencing β-cell regeneration in STZ-treated mice. These new β-like cells emerged within 48 h and, initially, were unresponsive to glucose. Insulin treatment normalized the glucose levels, preserving the neogenic β-like cells and gradually maturing the islet function. Intermediate cells expressing vimentin and *MafB* appeared within 16 h, serving as a major source of insulin-producing cells at 24 h [268].

In mice, α-cells and δ-cells can transform into insulin-producing β-cells after β-cell ablation, aiding in diabetes recovery, but whether this occurs in humans is unclear. This study demonstrates that non-β-cells from deceased human donors, including α-cells and γ-cells, can be reprogrammed to produce insulin using the transcription factors Pdx1 and MafA. Transplanted human α-cells reverse diabetes in mice and sustain insulin production for at least six months, retaining their original cell identity markers [269].

In addition to δ- and α-cells, scientists also discovered that ghrelin-producing ε-cells contribute to β-cell regeneration following significant β-cell loss. Augmenting ghrelin levels or expanding ε-cell populations supports this regeneration [270].

Beyond the conventional pancreatic cell types like α-, δ-, and ε-cells, ductal cells also play a role in contributing to the β-cell population, especially under diabetic conditions.

Investigations revealed that there are two distinct niches for β-cell neogenesis: "β duct cells" near ducts and "β vessel cells" near blood vessels. Single-cell transcriptomic analysis revealed five unique populations among newborn β-cells, shedding light on potential regulatory mechanisms, including the role of *MafB*-expressing cells in β-cell specification. Additionally, comparative analysis with human embryonic stem cell-derived β-like cells highlighted similarities in the transcriptional profiles, suggesting shared regulatory pathways in β-cell maturation between mice and humans [271].

It is important to note that the evidence suggests that β-cell transdifferentiation plays a larger role in regenerating β-cells following extreme conditions like partial pancreatectomy, pancreatic duct ligation, and β-cell ablation with STZ, mimicking T1D, compared to neogenesis [264]. Conversely, β-cell neogenesis is more prominent in regenerating β-cells during insulin resistance in T2D, where an increase in β-cell mass is needed to counter hyperglycemia [272].

The process of neogenesis from β duct cells involves the expression of the endocrine master transcription factor Ngn3. Lineage tracing and single-cell sequencing (scRNA-seq) have unveiled that duct-derived somatostatin-expressing cells undergo a transition into β-cells, signifying a physiological mechanism for β-cell maintenance beyond mere proliferation. Moreover, the study has noted the presence of bihormonal cells within ducts, suggesting a ductal origin for certain islet cells and the potential for differentiation into various endocrine cell types. This underscores the significance of ductal cells in pancreatic regeneration and β-cell neogenesis [273].

The inhibition of MAP kinase-interacting serine/threonine kinase 2 (MNK2) with the small molecule CID661578 has been identified as a potent stimulator of β-cell regeneration. This small molecule facilitated β-cell neogenesis from ductal cells across various models, including zebrafish and human pancreatic organoids, by enhancing protein synthesis via modulation of the eIF4E-eIF4G interaction. MNK2 inhibition by CID661578 augmented

eIF4E phosphorylation, promoting hypertranslation and β-cell regeneration, highlighting a potential therapeutic avenue for diabetes management [274].

8. Mitigation of β-Cell Dedifferentiation and Transdifferentiation

β-cell dedifferentiation and transdifferentiation can be reversed through various treatments, including dietary supplements, natural products, dietary interventions, hormones, and antidiabetic medications. These approaches offer promising strategies to restore β-cell function and mitigate diabetes.

8.1. Dietary Supplements

Glutathione is a crucial antioxidant synthesized within cells, playing a vital role in maintaining cellular health and defending against oxidative stress. Prolonged exposure of rats to long-term oscillating glucose (LOsG) triggers β-cell dysfunction through ROS stress. This stress disrupts FoxO1 signaling, leading to an elevation in thioredoxin interacting protein (TXNIP) levels and a reduction in insulin and *SOD-2* expression. Consequently, β-cell dedifferentiation and dysfunction occur. However, the timely administration of glutathione effectively prevents LOsG-induced β-cell failure [275].

Vitamin D3 therapy may offer a promising avenue for attenuating β-cell dedifferentiation. Research indicates that the administration of vitamin D3 and its analogues to the high-glucose-induced mouse insulinoma cell line (MIN6) results in the upregulation of the vitamin D3 receptor. This upregulation enhances β-cell function and maintenance by increasing insulin release and upregulating key transcription factors such as *Pdx1* and *MafA*. Consequently, there is an observed augmentation in the expressions of *Ins-1* and *Ins-2* genes [276].

Omega-3 polyunsaturated fatty acids (PUFAs) are known to have anti-hyperglycemic effects. Transgenic mice (mfat-1) that overexpress an enzyme converting omega-6 to omega-3 polyunsaturated fatty acids (*PUFAs*) were found to have significantly higher levels of eicosapentaenoic acid (EPA) in pancreatic tissues compared to wild-type mice. After STZ treatment, these transgenic mice did not develop diabetes and maintained normal insulin and blood glucose levels. The EPA metabolites from the CYP 450 pathway promoted insulin expression and increased β-cell markers in α-cells. This suggests that omega-3 PUFAs and their metabolites can promote α-to-β-cell transdifferentiation [277].

Folinic acid treatment, or the overexpression of *folate receptor 1* (*folr1*), has been shown to promote β-cell differentiation in a zebrafish model of β-cell loss. This effect extends to cultures of neonatal pig islets, suggesting its potential applicability in mammals. Notably, the source of increased β-cell differentiation in both zebrafish and pig islets was traced back to ductal cells. Additionally, comparative metabolomic analysis suggested that the regenerative effect of folinic acid might be linked to alterations in the pyrimidine, carnitine, and serine pathways [278].

Treatment with nicotinamide (NA), also known as niacinamide, a form of vitamin B3, significantly increased the number of insulin/PCNA, insulin/glucagon, and insulin/somatostatin double-positive cells compared to untreated groups. Notably, NA-treated rats showed enhanced insulin and *Pdx1*-positive cells, located within small clusters or scattered throughout the exocrine tissue and around ducts. Additionally, NA treatment increased *Notch1* expression while decreasing caspase-3 and TUNEL-positive β-cell numbers, suggesting that NA induces β-cell regeneration through ductal progenitor cell differentiation, acinar cell transdifferentiation, and the activation of Notch signaling [279].

Cathelicidin-based peptides like LLKKK18, a class of peptides derived from the cathelicidin family of antimicrobial peptides, have shown promise for T1D therapy. When delivered via poly (lactic-co-glycolic acid) (PLGA) nanoparticles (NPs), these peptides enhance β-cell function and neogenesis. In vitro studies have demonstrated glucose-stimulated insulin release and strong NP association with β-cells. In diabetic rats, these NPs effectively reduced hyperglycemia, improved pancreatic insulin content and glucose tolerance, and notably increased β-cell mass [280].

8.2. Dietary Interventions and β-Cell Function, Dedifferentiation, and Transdifferentiation

Intermittent fasting and calorie restriction have been found to regulate not only blood glucose and insulin levels, triglyceride and cholesterol levels, and insulin resistance but also insulin secretion, GSIS, and the modulation of β-cell transcription factors such as Pdx1, Nkx6.1, and MafA in obesity-induced diabetes [281].

Intermittent protein restriction (IPR) has also been found to significantly reduce hyperglycemia, protect pancreatic islets, and increase β-cell proliferation in diabetic Zucker fatty rats. IPR has been shown to reverse β-cell dedifferentiation, as evidenced by decreased expressions of dedifferentiation markers like *Cd81* and stress-related genes, while increasing markers of β-cell function such as islet amyloid polypeptide and chromogranin. Furthermore, IPR altered the expression of transcription factors, mitigating diabetes-associated changes in D-box binding PAR bZIP transcription factor (Dbp) and immediate-early response genes [282].

The ketogenic diet has also been shown to improve fasting blood glucose and insulin levels, β-cell mass, and identity by restoring the β/α-cell ratio, rearranging β-cells in the pancreas, and modulating β-cell transcription factors in diabetic mice [283].

A Chinese medical nutrition therapy incorporates medicinal herbs, whole grains, and intermittent energy restriction. It has demonstrated effectiveness in alleviating T2D. This therapy reduces fasting blood glucose levels, improves insulin sensitivity, enhances insulin production and secretion, modulates the gut microbiota, reduces pancreatic macrophage infiltration, and restores β-cell identity and morphology [284].

Increasing dietary fiber intake has been demonstrated to alleviate the progression of T2D and β-cell pathophysiological alterations, underscoring the therapeutic potential of dietary interventions. This is supported by research indicating that dietary fiber intake can safeguard and enhance insulin secretion through its influence on the intestinal microbiome. Specifically, it encourages the proliferation of short-chain fatty acid (SCFA)-producing strains and enhances the production of GLP-1, a hormone that stimulates insulin secretion [285].

8.3. Physical Activity and β-Cell Plasticity

Physical activity has been demonstrated to enhance pancreatic β-cell function and may play a crucial role in preventing or reversing β-cell dedifferentiation in the context of T2D. Exercise promotes β-cell function in T2D patients by increasing β-cell mass, improving glucose sensing, enhancing insulin secretion, and augmenting insulin content [285,286]. The protective effects of exercise on β-cells are mediated through multiple mechanisms: 1. Reducing metabolic stress: Physical activity facilitates increased glucose uptake in peripheral tissues, thereby lowering blood glucose levels and reducing the metabolic load on β-cells [287]; 2. Alleviating ER and oxidative stress: Exercise may attenuate ER stress and oxidative stress in β-cells, which are critical contributors to β-cell dysfunction; and 3. Modulating inflammation: Physical activity has the potential to modulate the immune environment of the pancreas, thereby reducing inflammatory processes that contribute to β-cell damage [286]. IL-6, which is produced by muscle cells during contraction, is associated with elevated levels of IL-1RA, IL-10, and GLP-1, which regulate abdominal fat lipolysis and promote β-cell protection, growth, and mass [288,289]. Additionally, certain myokines like angiopoietin and osteoprotegerin, specifically produced by triceps, exhibit anti-inflammatory effects [290]. Exercise can also increase levels of adiponectin, an adipokine synthesized by adipose tissue with anti-inflammatory properties [291]. Adiponectin has been shown to induce a shift of M1 (proinflammatory) macrophages to the M2 (anti-inflammatory) phenotype by reducing proinflammatory cytokines, including TNFα [292]. Notably, inflammatory responses by macrophages resident in the pancreas play a critical role in the fate of β-cells [3].

Besides IL-6, exercise also positively influences brain-derived neurotrophic factor (BDNF) [293] and C-X3-C motif chemokine ligand 1 (CX3CL1) [294]. Similar to GLP-1,

these factors have beneficial effects on insulin secretion, glucose-stimulated insulin secretion (GSIS), and β-cell function [293,294].

Four exerkines elevated during exercise known to enhance β-cell proliferation, function, and maintenance include the following: irisin, an exerkine released by muscle tissue; follistatin, which is induced by exercise and secreted by the liver and muscles; osteocalcin, which is produced by bone marrow; and FGF21, which is produced snd decreted by hepatic tissues [295–300].

β-cell dedifferentiation and exercise: Although direct evidence linking exercise to the prevention or reversal of β-cell dedifferentiation is limited, various studies suggest that exercise may support the maintenance of β-cell identity and function. In animal models, voluntary running has been shown to prevent β-cell failure in susceptible islets [301]. Apelin, an exerkine that is involved in various physiological processes and is produced and secreted by various tissues, including adipose tissue, the heart, and skeletal muscle, has been shown to reverse β-cell dedifferentiation [302]. Exercise is also linked to the activation of the GABAergic system [303]. There is evidence suggesting that GABA and the activation of the GABAergic system might trigger the conversion of α-cells into β-cells, presenting a potential treatment avenue for Type 1 Diabetes (T1D) [304,305].

Combined effects of diet and exercise: The synergistic effects of dietary intervention and physical exercise on β-cell health are notable. Both strategies can alleviate metabolic stress on β-cells, and their combination, particularly through diet-induced weight loss, may enhance the benefits of each intervention. This dual approach has the potential to mitigate the adverse effects of T2D and β-cell identity [286].

Muscle mass reduction can significantly impact glucose metabolism and increase the risk of diabetes. Since approximately 80% of glucose clearance occurs in muscle tissue under normal glycemic conditions, any decrease in muscle mass or quality can adversely affect blood glucose regulation, leading to hyperglycemia, insulin resistance, metabolic stress, and impaired β-cell function and maintenance. Consequently, a correlation has been observed between sarcopenia (age-related loss of muscle mass and function) and the incidence of T2D. To address this issue, dietary supplements containing specific amino acids such as HMB (β-hydroxy β-methylbutyrate), leucine, glutamine, and arginine have been proposed as potential interventions. These supplements may help prevent sarcopenia and, consequently, reduce the risk of developing insulin resistance and T2D. By maintaining muscle mass and function, these amino acids could support better glucose regulation and metabolic health, particularly in aging populations or those at risk for muscle loss [306].

8.4. Antidiabetic Medications

8.4.1. Glucagon-like Peptide-1 Receptor Agonist (GLP-1 RA)

Liraglutide, classified as a GLP-1 RA, stimulates GSIS. Sitagliptin, categorized as a dipeptidyl peptidase-4 inhibitor (DPP-4 inhibitor), elevates endogenous GLP-1 and glucose-dependent insulinotropic peptide (GIP) levels. These actions enhance insulin secretion and inhibit glucagon release. Both liraglutide and sitagliptin treatments have demonstrated the ability to reverse β-cell dedifferentiation in STZ-, high-fat-feeding- or HC-induced mice. Moreover, liraglutide administration has been observed to attenuate β-cell transdifferentiation into α-cells [307].

8.4.2. Sodium–Glucose Co-Transporter 2 (SGLT2) Inhibitors

Dapagliflozin, a medication employed in the treatment of T2D, falls within the category of sodium–glucose co-transporter 2 (SGLT2) inhibitors. Emerging evidence suggests that dapagliflozin administration might mitigate transdifferentiation in diabetic mouse models. Interestingly, this effect appears to occur through a mechanism independent of reducing ambient blood glucose levels [308].

Luseogliflozin, another SGLT2 inhibitor, was tested on the pancreatic β-cell mass in db/db mice of various ages. The 6-week-old mice treated with luseogliflozin showed a significant increase in β-cell mass and upregulation of key genes compared to controls.

While β-cell mass declined with age in the control group, luseogliflozin preserved the β-cell mass, especially in the early stages of diabetes. Immunohistochemistry and gene expression analyses supported these findings [309].

8.4.3. Dipeptidyl Peptidase-4 Inhibitor (DPP-4 Inhibitor)

DPP-4 inhibitor Sitagliptin, known for its β-cell regenerative and immunoregulatory properties, and melatonin, a hormone primarily produced by the pineal gland that regulates sleep–wake cycles and possesses antioxidant, anti-inflammatory, and potential immunomodulatory properties, were studied for their combined effect on β-cell regeneration under glucotoxic stress in diabetic mouse models and humanized islet transplant models. The combination therapy demonstrated additive effects in promoting β-cell regeneration, reducing apoptosis, and improving glucose tolerance. In young diabetic mice, monotherapies induced β-cell transdifferentiation, while in older mice, they promoted β-cell proliferation and reduced apoptosis. Overall, the combination therapy proved superior, suggesting significant potential for future diabetes treatment [310].

8.5. Hormones

Hormones such as insulin, glucagon, and somatostatin play a crucial role in regulating β-cell dedifferentiation and transdifferentiation processes [190,311].

Glucagon has been identified as a key factor triggering the dedifferentiation of β-cells, with FoxO1 playing a partial role in this process. This interaction sheds light on the intriguing interplay between α- and β-cells. Blocking the glucagon receptor with a monoclonal antibody (mAb) has been found to reverse the effects induced by glucagon. Consequently, this intervention leads to improvements in β-cell size and function, while also preventing dedifferentiation in mice with T2D. Conversely, the inhibition of FoxO1 mimics the effects of glucagon, whereas its overexpression counteracts dedifferentiation [123]

Gastrin, a hormone primarily secreted by G cells in the stomach and duodenum, plays a key role in stimulating gastric acid secretion to aid in digestion. Recent studies have revealed that gastrin administration can induce the transformation and reprogramming of regenerative ductal cells in rats with 95% pancreatectomy, leading to the enhanced formation and expansion of β-cells. This process appears to involve the downregulation of *keratin 20* and *β-catenin*, representative of ductal dedifferentiation, and the upregulation of *Ngn 3* and *Nkx6-1*, indicating an endocrine progenitor phenotype [312].

The long-acting xenin analogue xenin-25[Lys13 PAL], a modified form of xenin-25 and peptide hormone that influences gastrointestinal function and glucose metabolism, has antidiabetic effects, promoting insulin secretion and improving pancreatic islet architecture. In high-fat-fed and STZ-induced insulin-deficient mice, xenin-25[Lys13 PAL] treatment reversed STZ-induced body weight loss, increased insulin levels, and reduced blood glucose. It also decreased pancreatic α-cell areas, reduced β-cell apoptosis, and promoted β-cell growth. Additionally, xenin-25[Lys13 PAL] decreased the transition of insulin-positive to glucagon-positive cells, suggesting that it helps maintain β-cell identity and function [313].

Administering T3, a thyroid hormone variant, efficiently counteracts the harmful effects of β-cell dedifferentiation and transdifferentiation. This corrective action not only boosts insulin secretion and restores vital markers but also safeguards pancreatic islet structure by halting β-cell polyploidization. T3 treatment has been shown to diminish the transformation of β-cells into α-cells, thus upholding insulin levels. Conversely, blocking T3 exacerbates transdifferentiation [314].

Angiotensin (1–7) (Ang (1–7)), a peptide hormone derived from the cleavage of Angiotensin II by enzymes such as ACE2, has demonstrated potential in mitigating high-glucose-induced apoptosis and dedifferentiation of pancreatic β-cells through its interaction with MAS receptors. In the presence of high glucose levels, the β-cell morphology deteriorates, insulin secretion diminishes, and markers associated with mature β-cells (*Pdx1* and *MafA*) decrease, while those linked to endocrine progenitor cells (*Oct4* and *Nanog*) increase.

However, treatment with Ang (1–7) significantly reverses these detrimental changes. This reversal is attributed to the specific interaction between Ang (1–7) and its MAS receptors, which subsequently impacts the Wnt/β-catenin/FoxO1 pathway, ultimately safeguarding the β-cell function and identity against the adverse effects of high glucose [315].

Adipsin, also referred to as complement factor D, is a serine protease enzyme encoded by the ADIPOQ gene. Its pivotal role lies within the alternative complement pathway, an integral part of the innate immune system. Predominantly synthesized by adipocytes, adipsin actively participates in adipose tissue metabolism, exerting influence over energy regulation and adipocyte differentiation. Additionally, adipsin contributes to the generation of complement component C3a, which exhibits insulin secretagogue activity. Emerging evidence suggests that elevated levels of blood adipsin/C3a may uphold β-cell identity amidst diabetic conditions [316].

8.6. Natural Products

Sangzhi alkaloids (SZ-A), extracted from *Morus alba* L. (mulberry twig), have shown promise in mitigating hyperglycemia and glucose intolerance in diabetic mice. Moreover, they have been found to restore β-cell identity and function by modulating the levels of β-cell-specific transcription factors under diabetic conditions [317].

Eugenol (4-allyl-2-methoxyphenol) is a naturally derived compound present in clove, basil, cinnamon, and nutmeg. When administered to Ins-1 β-cells exposed to high glucose and lipid levels, eugenol demonstrated the ability to partially restore the expressions of certain β-cell-specific genes, including *Glut2*, *NeuroD1*, and *FoxO1* [318].

Piperine, a compound in black pepper, offers numerous health benefits, including anti-inflammatory, antioxidant, anti-obesity, and antidiabetic effects. In a mouse model of obesity, a 10-week piperine treatment improved glycolipid metabolism, insulin resistance, and metabolic inflammation. Piperine enhanced glucose metabolism, reduced inflammation, and prevented β-cell dedifferentiation while also decreasing M1-like macrophage polarization in islets and visceral adipose tissue [319].

Psilocybin, a natural compound from Psilocybe mushrooms, acts as an agonist for serotonin receptors. It has been shown to reduce β-cell loss induced by high-glucose–high-lipid (HG-HL) conditions by mitigating apoptosis, likely through modulating TXNIP and STAT pathways. Additionally, psilocybin affects key genes related to β-cell dedifferentiation, such as *Pou5f1* and *Nanog*, indicating its potential therapeutic role in protecting against β-cell dysfunction and dedifferentiation in T2D [320].

Phytocannabinoids are a group of secondary metabolites produced by *Cannabis sativa*. We recently studied the impacts of various phytocannabinoids on β-cell survival, function, and dedifferentiation in high-glucose–high-lipid-induced INS-1 β-cells. Our results indicate that certain phytocannabinoids can restore an impaired β-cell identity by reactivating β-cell-enriched transcription factors [321].

Gymnemic acid (GA) is a compound found in the leaves of *Gymnema sylvestre*, a plant native to India and Africa. It is known for its unique property of temporarily suppressing the taste of sweetness, making sweet foods taste less appealing. GA treatment demonstrated promising outcomes by maintaining normoglycemia in diabetic rats through enhanced insulin secretion via the upregulation of β-cell regeneration markers such as *Pdx1*, Neurognin 3, *MafA*, and *NeuroD1*. Additionally, GA facilitated proliferation by activating the PI3K/AKT pathway and promoting cell adhesion through the increased expression of ECAD and β-catenin, thereby preserving islet architecture [322].

Treatment with magainin-AM2, an analog of magainins, which are antimicrobial peptides originally discovered in the skin of the African clawed frog, *Xenopus laevis*, and growth hormone improved glucose tolerance and fasting blood glucose levels post-STZ treatment and increased the total cell counts and insulin+ and glucagon+ cells per islet. The expressions of key factors for α-to-β-like cell conversion and cell proliferation/differentiation were significantly upregulated, suggesting a potential for the pharmaceutical induction of

α-cell production and their transdifferentiation to functional β-like cells, with magainin-AM2 showing the greatest efficacy [323].

The administration of a Fufang-Zhenzhu-Tiaozhi capsule (FTZ), a traditional Chinese medicine prescription commonly used in clinical practice, increased insulin levels, reduced glucose levels, and promoted β-cell regeneration in T1DM mice. Additionally, FTZ inhibited inflammatory cell invasion and islet cell apoptosis, preserving the quantity and quality of β-cells. The promotion of β-cell regeneration by FTZ was associated with the upregulation of key transcription factors (*Pdx1*, *MafA*, and *Ngn3*), suggesting FTZ as a potential therapeutic drug for T1DM [324].

8.7. The Role of the Gut Microbiome in β-Cell Plasticity

The gut microbiome appears to play an important role in shaping β-cell biology through bacterial metabolites, proteins, extracellular vesicles, and immunomodulatory effects [325].

Short-chain fatty acids (SCFAs) produced by gut bacteria help regulate metabolism. SCFAs such as sodium acetate, sodium propionate, and sodium butyrate (SB) did not immediately affect insulin release from pancreatic islets. However, prolonged exposure to SCFAs improved the function of β-cells. An analysis of RNA levels showed that SB-treated islets had lower expression levels of genes related to insulin secretion and β-cell identity, such as *Pdx1* and *MafA*. SB increased oxygen consumption but reduced glucose-stimulated oxygen consumption in islets, with no changes in genes related to glycolysis or the tricarboxylic acid cycle. Additionally, SB lowered the expression of *Kcnj11*, which encodes a channel involved in regulating insulin release, increased calcium levels, and promoted insulin gene expression by affecting the histone modification H3K18bu on its promoter. This suggests that while SB improves islet function, it also alters the expressions of genes related to β-cell identity [326].

Additionally, butyrate demonstrates anti-inflammatory properties, preserving the β-cell function against IL-1β-induced dysfunction. Butyrate exerts these effects through the suppression of IL-1β-induced inflammatory genes and nitric oxide production. Notably, butyrate inhibits NF-κB activity via histone deacetylase inhibition without affecting NF-κB nuclear translocation. These findings highlight butyrate's potential in mitigating β-cell dysfunction through NF-κB pathway modulation, offering insights into T2D prevention and treatment strategies [327].

An altered gut microbiota is linked to T1D, influencing SCFAs and glucose homeostasis. A dietary supplement called HAMSAB, designed to increase serum acetate and butyrate, improved glycemic control in NOD mice and patients with established T1D. Single-cell RNA sequencing on islet cells from NOD mice fed a HAMSAB or control diet revealed that HAMSAB reduced immune-cell infiltration in the pancreas, preserved β-cell function and identity, and decreased cellular stress. This suggests that SCFAs have potential anti-inflammatory effects and that HAMSAB may be a promising approach to managing T1D [328].

8.8. Other Interventions

Following bariatric surgery, rats undergoing Roux-en-Y gastric bypass (RYGB) and sleeve gastrectomy exhibited significant improvements in metabolic parameters and an increase in pancreatic β-cell proportion compared to Sham-operated rats. Both the RYGB and Sleeve groups demonstrated a decrease in the α-to-β-cell ratio. The study identified Gcg+/Ppy+ and Ins+/Gcg+/Ppy+ multiple hormone-expressing cells post-surgery, suggesting potential transdifferentiation events. Notably, the discovery of Ins+/Gcg+/Ppy+ cells, displaying features of both α- and β-cells, implies a role for transdifferentiation in enhancing β-cell function following bariatric surgery [329].

9. Advancements in Stem Cell-Based Therapies for Diabetes Mellitus

Recent studies have detailed the generation of functional β-cells from both embryonic and induced pluripotent stem cells. Adipose-derived mesenchymal stem cells (Ad-MSCs) are potential candidates for generating insulin-producing cells (IPCs), irrespective of donor age, gender, or health status. These cells demonstrated insulin secretion in response to glucose and dysfunction with palmitate exposure. Gene expression analysis showed over 5000 differentially regulated genes, aligning closely with native β-cells. Pathway analysis confirmed relevance to stem cell differentiation and pancreatic development, indicating that adipose-derived β-cells may be a promising clinical therapy for diabetes [330].

Research indicates that manipulating the *FoxO1-1* gene during the final stages of differentiation may enhance IPC generation from Ad-MSCs. While FoxO1 is essential for early β-cell development, its overexpression in mature β-cells can cause glucose intolerance and hyperglycemia. Similarly, in Ad-MSCs, FoxO1 supports early differentiation, but reducing its expression at later stages results in more mature and functional IPCs [331].

In addition to Ad-MSCs, bone marrow-derived mesenchymal stem cells (bm-MSCs) can potentially be transdifferentiated into islet-like cells for treating T1DM. In a research study, this process was induced using high glucose, nicotinamide, β-mercaptoethanol, betacellulin, and IGF-1, and transdifferentiation was confirmed through glucose challenge assays and gene expression analysis. The transdifferentiated cells were then microencapsulated with 1% alginate and cultured in a fluidized-bed bioreactor to ensure stability and proliferation. After transplantation into the omentum of STZ-induced diabetic Wistar rats, blood glucose levels significantly decreased within a week, and the treated rats' weight, insulin, and C-peptide levels returned to normal [332]. Moreover, there is evidence suggesting that exosomes derived from bm-MSCs, known as bm-MSC-derived exosomes (bmMDEs), could alleviate β-cell dedifferentiation and enhance their function in type II diabetic rats as well as primary islets exposed to high glucose levels. This therapeutic effect is facilitated by miR-146a, which acts on the NUMB/β-catenin signaling pathway [241].

A protocol for the transformation of human fibroblasts into endodermal cells using non-integrative episomal reprogramming factors, growth factors, and chemical compounds has been demonstrated. The differentiated cells undergo a progression from definitive endodermal progenitors to posterior foregut-like progenitors, then to pancreatic endodermal progenitors, with significant expansion potential. Through screening, specific compounds were identified that enhance the differentiation of pancreatic endodermal progenitors into functional β-like cells in laboratory settings. When transplanted, these β-like cells exhibit GSIS in animal models, offering protection against chemically induced diabetes [333].

Human umbilical cord mesenchymal stem cells (hUC-MSCs) have shown promising potential in reverting β-cell dedifferentiation when cocultured with purified human T2D mellitus (hT2DM) islets. This reversal is orchestrated through a targeted interaction facilitated by the secretion of interleukin-1 receptor antagonist (IL-1Ra) from hUC-MSCs. This mechanism plays a pivotal role in modulating the overall inflammatory responses within diabetic islets, highlighting the therapeutic significance of hUC-MSC-based interventions in diabetes management [334].

MSC secretomes, which are collections of molecules including proteins, cytokines, and other factors secreted by MSCs, show higher levels of total protein and IL-4 when derived from 3D cultures compared to 2D cultures. In vivo, these secretomes induce regulatory T cells, modulate cytokine release, and increase β-cell regeneration, particularly those from 3D cultures. This demonstrates the dual therapeutic potential of MSC secretomes in T1D, promoting both immunomodulation and β-cell regeneration. Culturing MSCs in 3D scaffolds enhances these effects, suggesting a promising approach for T1D therapy [335].

hTERT-MSC (human telomerase reverse transcriptase MSC) transplantation, both intrapancreatically (IPR) and intravenously (IVR), in pancreatectomized mice, leads to the increased proliferation of pancreatic β-cells. IPR demonstrates moderate advantages over IVR. These cells activate pancreatic progenitor cell characteristics through the AKT/Pdx1/FoxO1 signaling pathway and modulate growth factors and proinflammatory cytokines [119].

10. β-Cell Proliferation and Regeneration

β-cell regeneration and proliferation are essential for diabetes treatment, restoring insulin production, and managing blood glucose levels. Enhancing these processes can potentially reverse diabetes, reducing the need for external insulin therapy. Strategies include stimulating endogenous β-cell proliferation, neogenesis, and transdifferentiation using small molecules and transcription factors, as well as deriving β-cells from pluripotent stem cells [336,337].

10.1. The Suppression of Dual-Specificity Tyrosine-Regulated Kinase 1A (DYRK1A)

The DREAM complex plays a pivotal role in maintaining quiescence in human β-cells. Comprising transcriptionally repressive proteins, this complex forms in response to DYRK1A kinase activity, enforcing cellular quiescence. When DYRK1A activity is inhibited, DREAM subunits transition into the pro-proliferative MMB complex. Small molecule DYRK1A inhibitors stimulate replication in human β-cells by shifting the DREAM complex from its repressive state to the proliferative MMB configuration [338].

Small-scale suppression of DYRK1A holds promise for pharmaceutical intervention in β-cell regeneration for diabetes, addressing a significant therapeutic gap. However, due to DYRK1A's involvement in critical signaling pathways, it is essential to maintain its levels similar to those found in healthy individuals. In silico tests have identified several plant-based compounds as potential ligands for β-cell regeneration. These compounds include 3-[6-(3-methyl-but-2-enyl)-1H-indolyl]-6-(3-methyl-but-2-enyl)-1H-indole, Lanceolatin B, Lysicamine, Pratorinine, Pratorimine, Lanceolatin A, Lanuginosine, Hippacine, (-)-Semiglabrin, Aegyptinone B, 3′-Prenylnaringenin, and 8-C-p-hydroxybenzylluteolin. Computational analyses suggest these compounds have good intestinal absorption and favorable drug-like properties, making them promising candidates for new medications, pending further in vivo validation [12].

Various synthetic small molecules have shown potential for promoting β-cell regeneration by inhibiting DYRK1A activity. AC27, a compound with superior selectivity compared to another DYRK1A inhibitor, harmine, not only inhibits DYRK1A but also reduces its cellular levels, resulting in increased β-cell proliferation and enhanced insulin and C-peptide secretion in response to glucose. These effects were stable over a 12-day period and persisted after the inhibitor was withdrawn. AC27's benefits were validated in insulinoma cell lines, iPSC-derived β-cell organoids, and isolated mouse pancreatic islets, with its effects further enhanced by TGF-β inhibition [339].

Gua et al. conducted a chemical screen in zebrafish to identify small molecules that increase the number of incretin-expressing cells. One of the identified compounds, AZ Dyrk1B 33, was found to be a potent inhibitor of DYRKs and effectively boosted incretin+ cell populations in zebrafish. Moreover, it demonstrated improved glucose regulation in mouse models. The research suggests that AZ Dyrk1B 33 likely influences enteroendocrine cell differentiation through NFAT signaling pathways. Unlike the DYRK1A inhibitors known to enhance β-cell proliferation, AZ Dyrk1B 33 did not affect β-cell mass in this study, highlighting its specific role in enteroendocrine cells regulated by DYRK1B [340].

While DYRK1A inhibitors such as AC27 hold potential for enhancing β-cell proliferation and function by targeting β-cell cycle pathways, significant obstacles persist. These include achieving β-cell specificity, refining evaluation techniques, and overcoming the low proliferation rates of human β-cells. To address these issues, structural modifications are needed to improve targeting and minimize off-target effects. Furthermore, integrating DYRK1A inhibitors with other antidiabetic medications could enhance their therapeutic efficacy [340,341].

Common DYRK1A inhibitors can induce β-cell proliferation, but with low rates and limited specificity. It has been shown that combining any GLP1R agonist with any DYRK1A inhibitor results in a significant synergistic increase in human β-cell replication (5 to 6%), leading to an actual increase in human β-cell count. This combination did not cause β-cell

dedifferentiation and was effective for both normal human β-cells and those derived from individuals with T2D [342].

Molecular docking analysis revealed diosmin, a flavonoid compound found in various plants such as *Zanthoxylum chalybeum* Engl., as a potential inhibitor of DYRK1A. In vitro experiments showed that the extract promoted cell viability and proliferation, particularly under conditions of palmitate exposure. However, in vivo results indicated only mild β-cell regenerative potential, suggesting further investigation is needed to understand the molecular interactions underlying these effects, particularly regarding diosmin's role as a DYRK1A inhibitor [343].

10.2. Glucagon Receptor Antagonism in β-Cell Regeneration

Research indicates that the glucagon receptor antibody (anti-GCGR) has significant potential for promoting β-cell regeneration in diabetes models. Anti-GCGR treatment effectively reverses hyperglycemia, elevates plasma insulin levels, and restores β-cell mass through both β-cell proliferation and α-to-β-cell transdifferentiation [13–16]. Using lineage-tracing techniques, researchers found that GCGR mAb treatment causes α-cells to regress to progenitor states and reactivates Ngn3 progenitors, promoting their transdifferentiation into β-cells. This treatment also upregulates the genes associated with β-cell regeneration and improves insulin secretion in primary mouse islets [14].

Treatment using REMD 2.59, a competitive human GCGR antagonist, lowered blood glucose levels while elevating the plasma levels of glucagon and somatostatin. Notably, the administration of a GCGR monoclonal antibody substantially increased α-cell and δ-cell populations in both normal and T1D mice. This increase in δ-cell mass was linked to enhanced proliferation and neogenesis originating from pancreatic ducts. Remarkably, the observed δ-to-β-cell transdifferentiation in T1D mice post-treatment suggests a promising avenue for restoring β-cell numbers [344].

Additionally, it has been found that REMD 2.59 induces α-cell regression to progenitors, facilitating β-cell neogenesis and upregulating regeneration-related genes in mouse islets. This highlights the GCGR mAb's potential for β-cell regeneration in T2D, supporting α-cell to β-cell conversion and progenitor-driven β-cell formation [345].

GCGR mAb treatment in PANIC-ATTAC mice, which triggers caspase-8–mediated apoptosis specifically within the pancreatic β-cell, stimulates α-cell to β-cell conversion, leading to sustained improvements in glycemic control and effectively curing diabetes in this model. While α-cell hyperplasia may pose challenges in T2D, in T1D, it offers a potential source for generating new insulin-producing cells [346].

FGF21 has been shown to play a crucial role in the regenerative effects of GCGR antagonism on β-cells. The levels of FGF21 increase in both the plasma and liver in response to GCGR antagonism. The inhibition of FGF21 activity diminishes the upregulation of β-cell identity genes induced by the GCGR mAb. Furthermore, the increases in the β-cell number and proliferation driven by the GCGR mAb are less pronounced in mice lacking FGF21 or hepatic FGF21 [16].

Interestingly, anti-GCGR-induced α-cell hyperplasia can occur independently of β-cell regeneration. Furthermore, this therapy has shown efficacy in non-human primates, and when combined with anti-CD3, it significantly enhances β-cell mass and reverses diabetes in T1D models [13].

GCGR antagonism also stimulates the secretion of glucagon and glucagon-like peptide 1 (GLP-1) [347,348], which promotes β-cell regeneration. Blocking GLP-1 receptor (GLP-1R) signaling reduces these regenerative effects, suggesting that GLP-1R activation by glucagon is crucial for the β-cell regenerative effects induced by the GCGR mAb [15].

GLP1 induces insulin–glucagon-positive cells in rat pancreatic islets, potentially indicating α- to β-cell transdifferentiation. Molecular analyses suggest that GLP1 promotes β-cell function through the PI3K/AKT/FOXO1 pathway, enhancing *Pdx1* and *MafA* expressions while reducing *MafB*, suggesting a mechanism for α-cell inhibition and β-cell promotion by GLP1 [349].

The effect of Liraglutide and the GCGR mAb in promoting pancreatic β-cell neogenesis in T1D mice has been examined. Liraglutide treatment expanded β-cell mass through self-replication, differentiation, and transdifferentiation from α-cells, while GCGR mAb treatment significantly increased β-cell mass. However, the combination of both drugs did not further enhance the β-cell area but reduced plasma glucagon levels and increased the proportion of β-cells to α-cells [350].

Dapagliflozin has been found to enhance the expression of pancreatic endocrine progenitor and β-cell markers, including *Pdx1*, in cultured primary rodent islets and αTC1.9 cells under hyperglycemic conditions. This regenerative effect is partially mediated by GLP-1 secreted from α-cells [351].

The stimulatory impact of dapagliflozin on GLP-1 secretion is attributed to the reshaping of the gut microbiota and the modulation of metabolites involved in tryptophan metabolism, particularly l-tryptophan, which augments GLP-1 secretion and insulin levels. These beneficial effects are mediated by gut microbiota and tryptophan metabolism and are attenuated by GLP-1 receptor antagonism or pancreatic *Glp1r* knockout [352].

The GLP-1–oestrogen conjugate yielded significant benefits in managing diabetes. The GLP-1–oestrogen conjugate notably reduced daily insulin needs by 60% and triggered the endoplasmic-reticulum-associated protein-degradation system. This intervention improved β-cell survival and regeneration. Moreover, the GLP-1–oestrogen conjugate shielded human β-cells from dysfunction induced by cytokines [353].

The impacts of γ-aminobutyric acid (GABA) alone or in combination with the GCGR mAb on β-cell and α-cell mass in T1D mice have also been studied. Treatment with GABA or the GCGR mAb individually improved hyperglycemia and increased β-cell mass, with similar effects observed in the combination treatment. Interestingly, the combined treatment attenuated GCGR mAb-induced α-cell hyperplasia [354].

In summary, anti-GCGR treatment and GCGR antagonism promote β-cell regeneration through multiple mechanisms, including α-to-β-cell transdifferentiation, the upregulation of β-cell genes, and involvement of GLP-1 and FGF21 pathways. These findings highlight the potential of GCGR antagonists in enhancing β-cell mass and function in diabetes therapy.

10.3. The Polyamine Biosynthesis Pathway

The polyamine biosynthesis pathway is a conserved metabolic process in all organisms, involving key enzymes like aminopropyltransferases (spermidine and spermine synthases). This pathway is essential for cellular functions, including growth, proliferation, and stress responses. Research has shown that inhibitors of polyamine biosynthesis, such as difluoromethylornithine (DFMO), enhance β-cell regeneration without affecting overall growth or pancreas length, while Imatinib mesylate, a tyrosine kinase inhibitor that inhibits polyamine biosynthesis pathway, has no impact. These findings suggest that targeting polyamine biosynthesis, especially with DFMO, holds promise for stimulating β-cell regeneration in diabetes [17].

In the context of the polyamine pathway, administering spermidine to NOD mice heightened the diabetes occurrence, despite unchanging insulitis levels. This elevation was correlated with increased pancreatic tissue polyamines and markers of autophagy. Remarkably, spermidine induced shifts in the T-cell composition within pancreatic lymph nodes, amplifying pro-inflammatory T-cell populations in diabetic mice and demonstrating differing impacts on regulatory T cells depending on disease onset, ultimately exacerbating T1D incidence [355].

In preclinical models, DFMO has also been shown to delay T1D onset by reducing β-cell stress. A clinical trial with recent-onset T1D patients showed that DFMO is safe and tolerable, reducing urinary putrescine levels and preserving C-peptide at higher doses. Transcriptomic and proteomic analyses suggest DFMO preserves β-cell function by altering mRNA translation, protein transport, and secretion in stressed islets [356].

10.4. Other Pathways Involved in β-Cell Proliferation

In addition to the aforementioned methods to induce β-cell proliferation and regeneration, there are other approaches, such as inhibiting TBK1/IKKε and salt-inducible kinases (SIKs).

Xu et al. found TBK1/IKKε inhibitors (TBK1/IKKε-Is) to be potent enhancers of β-cell regeneration in a zebrafish model of T1D, validated across mammalian systems including human and rat β-cells, and STZ-induced diabetic mice. The mechanism involves TBK1/IKKε inhibition, which promotes β-cell proliferation via the cAMP-PKA-mTOR signaling axis through PDE3, highlighting a novel role for TBK1/IKKε in β-cell mass modulation. Notably, TBK1/IKKε-Is like amlexanox and PIAA selectively enhance β-cell proliferation without inducing proliferation in other cell types, showing promise for targeted therapy [357].

An in vivo study by Charbord et al. identified HG-9-91-01(HG) as a β-cell mitogen that enhances β-cell proliferation in zebrafish, mouse, and human β-cells by inhibiting salt-inducible kinases (SIKs), specifically SIK1–SIK3, members of the AMPK-related protein kinase family. HG stimulates β-cell proliferation through UPR activation via ATF6–IRE1 pathways. Despite showing specificity for SIK1, HG's pharmacokinetic limitations currently preclude its consideration as a drug candidate [358].

11. Conclusions

β-cell dedifferentiation and transdifferentiation are critical factors in the progression of both Type 1 and T2D (T1D and T2D). Metabolic stress and inflammation significantly contribute to β-cell dedifferentiation and transdifferentiation by dysregulating various pathways, including ER stress, the mTOR pathway, the endocannabinoid system, viral infections, and aging. Targeting these processes is considered a potential therapeutic strategy for diabetes. Various natural products, hormones, and dietary treatments have shown promise in mitigating β-cell dedifferentiation and transdifferentiation.

β-cell regeneration and neogenesis present potential treatments for diabetes, particularly T1D. Research has demonstrated that various pancreatic cells, including δ-, ε-, and α-cells, can participate in β-cell regeneration under diabetic conditions. Treatments such as glucagon receptor antagonism, both alone and in combination with GLP-1 agonism and DYRK1A inhibition, have been effective in regenerating ablated β-cells in diabetic models. Additionally, differentiating MSCs into β-cells, followed by the transplantation of these newly differentiated cells, offers a promising approach for diabetes treatment, especially for T1D. It has been shown that specific inducers containing certain components can effectively promote the neogenesis of MSCs into β-cells.

12. Limitations

1. Lack of review on experimental models: In this study, we did not review the experimental models and the methods used to model β-cell dedifferentiation and transdifferentiation. A comprehensive examination of the various approaches to model β-cell dedifferentiation and transdifferentiation in both in vitro and in vivo settings would be highly beneficial.
2. Limited scope on cell plasticity: This review primarily focused on β-cell plasticity and did not cover the plasticities of other cell types. It would be valuable to briefly explore the plasticities of other cells such as hepatocytes, hepatic stellate cells, epidermal stem cells, melanocytes, astrocytes, intestinal stem cells, cardiac fibroblasts, club cells, podocytes, and others. Investigating the plasticities of these diverse cell types could offer insights into the broader applications and implications of cellular plasticity.
3. Context of cell plasticity in other diseases: While this review centered on β-cell plasticity, it would have been beneficial to study cell plasticity in the context of other diseases such as liver diseases, neurodegenerative diseases, and cancer. Understanding how cellular plasticity manifests and contributes to the pathology of these diseases could

inform the development of new therapeutic strategies and enhance our knowledge of disease progression and treatment.

13. Unanswered Questions

Even though the progress in diabetes research and the role of β-cell plasticity in it have been substantial, many questions have remained unanswered:

Combinatorial effects of treatments: What are the combined impacts of various effective natural products and common antidiabetic medications on β-cell dedifferentiation, transdifferentiation, regeneration, and neogenesis?

Endocannabinoid system and β-cell plasticity: How does the endocannabinoid system influence β-cell plasticity? Can the modulation of this system induce β-cell regeneration and neogenesis?

Serotonin signaling pathway and β-cell plasticity: How does the serotonin signaling pathway affect β-cell plasticity? Can targeting this pathway enhance β-cell regeneration and neogenesis?

Long-term effects of β-cell plasticity interventions: What are the long-term effects of interventions aimed at promoting β-cell plasticity on the overall pancreatic function and glucose homeostasis?

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References

1. Bensellam, M.; Jonas, J.-C.; Laybutt, D.R. Mechanisms of β-cell dedifferentiation in diabetes: Recent findings and future research directions. *J. Endocrinol.* **2018**, *236*, R109–R143. [CrossRef]
2. Son, J.; Accili, D. Reversing pancreatic β-cell dedifferentiation in the treatment of type 2 diabetes. *Exp. Mol. Med.* **2023**, *55*, 1652–1658. [CrossRef] [PubMed]
3. Ghasemi-Gojani, E.; Kovalchuk, I.; Kovalchuk, O. Cannabinoids and terpenes for diabetes mellitus and its complications: From mechanisms to new therapies. *Trends Endocrinol. Metab.* **2022**, *33*, 828–849. [CrossRef] [PubMed]
4. Tanday, N.; Tarasov, A.I.; Moffett, R.C.; Flatt, P.R.; Irwin, N. Pancreatic islet cell plasticity: Pathogenic or therapeutically exploitable? *Diabetes Obes. Metab.* **2024**, *26*, 16–31. [CrossRef] [PubMed]
5. Honzawa, N.; Fujimoto, K. The Plasticity of Pancreatic β-Cells. *Metabolites* **2021**, *11*, 218. [CrossRef]
6. Khin, P.-P.; Lee, J.-H.; Jun, H.-S. A Brief Review of the Mechanisms of β-Cell Dedifferentiation in Type 2 Diabetes. *Nutrients* **2021**, *13*, 1593. [CrossRef] [PubMed]
7. Cinti, F.; Bouchi, R.; Kim-Muller, J.Y.; Ohmura, Y.; Sandoval, P.R.; Masini, M.; Marselli, L.; Suleiman, M.; Ratner, L.E.; Marchetti, P.; et al. Evidence of β-Cell Dedifferentiation in Human Type 2 Diabetes. *J. Clin. Endocrinol. Metab.* **2016**, *101*, 1044–1054. [CrossRef]
8. Gao, T.; McKenna, B.; Li, C.; Reichert, M.; Nguyen, J.; Singh, T.; Yang, C.; Pannikar, A.; Doliba, N.; Zhang, T.; et al. Pdx1 Maintains β Cell Identity and Function by Repressing an α Cell Program. *Cell Metab.* **2014**, *19*, 259–271. [CrossRef]
9. Zhou, Q.; Brown, J.; Kanarek, A.; Rajagopal, J.; Melton, D.A. In vivo reprogramming of adult pancreatic exocrine cells to β-cells. *Nature* **2008**, *455*, 627–632. [CrossRef]
10. Eizirik, D.L.; Sammeth, M.; Bouckenooghe, T.; Bottu, G.; Sisino, G.; Igoillo-Esteve, M.; Ortis, F.; Santin, I.; Colli, M.L.; Barthson, J.; et al. The Human Pancreatic Islet Transcriptome: Expression of Candidate Genes for Type 1 Diabetes and the Impact of Pro-Inflammatory Cytokines. *PLoS Genet.* **2012**, *8*, e1002552. [CrossRef]
11. Efrat, S. Beta-cell dedifferentiation in type 2 diabetes: Concise review. *Stem Cells* **2019**, *37*, 1267–1272. [CrossRef] [PubMed]
12. Chikodili, I.M.; Chioma, I.I.; Chinwendu, N.M.; IfedibaluChukwu, E.I. In-silico study for African plants with possible beta-cell regeneration effect through inhibition of DYRK1A. *Sci. Phytochem.* **2022**, *1*, 13–28. [CrossRef]
13. Hædersdal, S.; Andersen, A.; Knop, F.K.; Vilsbøll, T. Revisiting the role of glucagon in health, diabetes mellitus and other metabolic diseases. *Nat. Rev. Endocrinol.* **2023**, *19*, 321–335. [CrossRef] [PubMed]

14. Wei, R.; Gu, L.; Yang, J.; Yang, K.; Liu, J.; Le, Y.; Lang, S.; Wang, H.; Thai, D.; Yan, H.; et al. Antagonistic Glucagon Receptor Antibody Promotes α-Cell Proliferation and Increases β-Cell Mass in Diabetic Mice. *iScience* **2019**, *16*, 326–339. [CrossRef]
15. Wei, T.; Cui, X.; Jiang, Y.; Wang, K.; Wang, D.; Li, F.; Lin, X.; Gu, L.; Yang, K.; Yang, J.; et al. Glucagon Acting at the GLP-1 Receptor Contributes to β-Cell Regeneration Induced by Glucagon Receptor Antagonism in Diabetic Mice. *Diabetes* **2023**, *72*, 599–610. [CrossRef]
16. Xi, Y.; Song, B.; Ngan, I.; Solloway, M.J.; Humphrey, M.; Wang, Y.; Mondal, K.; Wu, H.; Liu, W.; Lindhout, D.A.; et al. Glucagon-receptor-antagonism-mediated β-cell regeneration as an effective anti-diabetic therapy. *Cell Rep.* **2022**, *39*, 110872. [CrossRef]
17. Robertson, M.A.; Padgett, L.R.; Fine, J.A.; Chopra, G.; Mastracci, T.L. Targeting polyamine biosynthesis to stimulate beta cell regeneration in zebrafish. *Islets* **2020**, *12*, 99–107. [CrossRef] [PubMed]
18. Mezza, T.; Cinti, F.; Cefalo, C.M.A.; Pontecorvi, A.; Kulkarni, R.N.; Giaccari, A. β-Cell Fate in Human Insulin Resistance and Type 2 Diabetes: A Perspective on Islet Plasticity. *Diabetes* **2019**, *68*, 1121–1129. [CrossRef]
19. Zhou, Q.; Melton, D.A. Pancreas regeneration. *Nature* **2018**, *557*, 351–358. [CrossRef]
20. DiMeglio, L.A.; Evans-Molina, C.; Oram, R.A. Type 1 diabetes. *Lancet* **2018**, *391*, 2449–2462. [CrossRef]
21. DeFronzo, R.A.; Ferrannini, E.; Groop, L.; Henry, R.R.; Herman, W.H.; Holst, J.J.; Hu, F.B.; Kahn, C.R.; Raz, I.; Shulman, G.I. Type 2 diabetes mellitus. *Nat. Rev. Dis. Prim.* **2015**, *1*, 1–22. [CrossRef]
22. Ahlqvist, E.; Prasad, R.B.; Groop, L. Subtypes of Type 2 Diabetes Determined From Clinical Parameters. *Diabetes* **2020**, *69*, 2086–2093. [CrossRef]
23. McIntyre, H.D.; Catalano, P.; Zhang, C.; Desoye, G.; Mathiesen, E.R.; Damm, P. Gestational diabetes mellitus. *Nat. Rev. Dis. Prim.* **2019**, *5*, 47. [CrossRef]
24. Liang, R.; Liu, N.; Wang, G.; Sun, P.; Liu, Y.; Zou, J.; Wang, L.; Ding, X.; Zhang, B.; Shen, Z.; et al. Cytohistologic analyses of β cell dedifferentiation induced by inflammation in human islets. *Eur. J. Inflamm.* **2021**, *19*. [CrossRef]
25. Nordmann, T.M.; Dror, E.; Schulze, F.; Traub, S.; Berishvili, E.; Barbieux, C.; Böni-Schnetzler, M.; Donath, M.Y. The Role of Inflammation in β-cell Dedifferentiation. *Sci. Rep.* **2017**, *7*, 6285. [CrossRef]
26. Urizar, A.I.; Prause, M.; Ingerslev, L.R.; Wortham, M.; Sui, Y.; Sander, M.; Williams, K.; Barrès, R.; Larsen, M.R.; Christensen, G.L.; et al. Beta cell dysfunction induced by bone morphogenetic protein (BMP)-2 is associated with histone modifications and decreased NeuroD1 chromatin binding. *Cell Death Dis.* **2023**, *14*, 399. [CrossRef]
27. Wang, H.-L.; Wang, L.; Zhao, C.-Y.; Lan, H.-Y. Role of TGF-Beta Signaling in Beta Cell Proliferation and Function in Diabetes. *Biomolecules* **2022**, *12*, 373. [CrossRef]
28. Blum, B.; Roose, A.N.; Barrandon, O.; Maehr, R.; Arvanites, A.C.; Davidow, L.S.; Davis, J.C.; Peterson, Q.P.; Rubin, L.L.; Melton, D.A.; et al. Reversal of β cell de-differentiation by a small molecule inhibitor of the TGFβ pathway. *eLife* **2014**, *3*, e02809. [CrossRef]
29. Qian, J.; Tao, D.; Shan, X.; Xiao, X.; Chen, C. Role of angiogenesis in beta-cell epithelial–mesenchymal transition in chronic pancreatitis-induced diabetes. *Mod. Pathol.* **2022**, *102*, 290–297. [CrossRef]
30. Brown, M.L.; Andrzejewski, D.; Burnside, A.; Schneyer, A.L. Activin enhances α-to β-cell transdifferentiation as a source for β-cells in male FSTL3 knockout mice. *Endocrinology* **2016**, *157*, 1043–1054. [CrossRef]
31. Chen, X.Y.; Shi, Y.X.; Huang, Y.P.; Ding, M.; Shen, Q.L.; Li, C.J.; Lin, J.N. SDF-1 inhibits the dedifferentiation of islet β cells in hyperglycaemia by up-regulating FoxO1 via binding to CXCR4. *J. Cell. Mol. Med.* **2022**, *26*, 750–763. [CrossRef]
32. Bosma, K.J.; Andrei, S.R.; Katz, L.S.; Smith, A.A.; Dunn, J.C.; Ricciardi, V.F.; Ramirez, M.A.; Baumel-Alterzon, S.; Pace, W.A.; Carroll, D.T.; et al. Pharmacological blockade of the EP3 prostaglandin E2 receptor in the setting of type 2 diabetes enhances β-cell proliferation and identity and relieves oxidative damage. *Mol. Metab.* **2021**, *54*, 101347. [CrossRef]
33. Moin, A.S.M.; Dhawan, S.; Shieh, C.; Butler, P.C.; Cory, M.; Butler, A.E. Increased Hormone-Negative Endocrine Cells in the Pancreas in Type 1 Diabetes. *J. Clin. Endocrinol. Metab.* **2016**, *101*, 3487–3496. [CrossRef]
34. Michels, A.W.; Landry, L.G.; McDaniel, K.A.; Yu, L.; Campbell-Thompson, M.; Kwok, W.W.; Jones, K.L.; Gottlieb, P.A.; Kappler, J.W.; Tang, Q.; et al. Islet-Derived CD4 T Cells Targeting Proinsulin in Human Autoimmune Diabetes. *Diabetes* **2016**, *66*, 722–734. [CrossRef]
35. Diedisheim, M.; Oshima, M.; Albagli, O.; Huldt, C.W.; Ahlstedt, I.; Clausen, M.; Menon, S.; Aivazidis, A.; Andreasson, A.-C.; Haynes, W.G.; et al. Modeling human pancreatic beta cell dedifferentiation. *Mol. Metab.* **2018**, *10*, 74–86. [CrossRef]
36. Oshima, M.; Knoch, K.-P.; Diedisheim, M.; Petzold, A.; Cattan, P.; Bugliani, M.; Marchetti, P.; Choudhary, P.; Huang, G.-C.; Bornstein, S.R.; et al. Virus-like infection induces human β cell dedifferentiation. *J. Clin. Investig.* **2018**, *3*, e97732. [CrossRef]
37. Thorel, F.; Népote, V.; Avril, I.; Kohno, K.; Desgraz, R.; Chera, S.; Herrera, P.L. Conversion of adult pancreatic α-cells to β-cells after extreme β-cell loss. *Nature* **2010**, *464*, 1149–1154. [CrossRef]
38. Brissova, M.; Haliyur, R.; Saunders, D.; Shrestha, S.; Dai, C.; Blodgett, D.M.; Bottino, R.; Campbell-Thompson, M.; Aramandla, R.; Poffenberger, G.; et al. α Cell Function and Gene Expression Are Compromised in Type 1 Diabetes. *Cell Rep.* **2018**, *22*, 2667–2676. [CrossRef]
39. Furth-Lavi, J.; Hija, A.; Tornovsky-Babeay, S.; Mazouz, A.; Dahan, T.; Stolovich-Rain, M.; Klochendler, A.; Dor, Y.; Avrahami, D.; Glaser, B. Glycemic control releases regenerative potential of pancreatic beta cells blocked by severe hyperglycemia. *Cell Rep.* **2022**, *41*, 111719. [CrossRef]
40. Hahn, M.; van Krieken, P.P.; Nord, C.; Alanentalo, T.; Morini, F.; Xiong, Y.; Eriksson, M.; Mayer, J.; Kostromina, E.; Ruas, J.L.; et al. Topologically selective islet vulnerability and self-sustained downregulation of markers for β-cell maturity in streptozotocin-induced diabetes. *Commun. Biol.* **2020**, *3*, 541. [CrossRef]

41. Katz, L.S.; Brill, G.; Zhang, P.; Kumar, A.; Baumel-Alterzon, S.; Honig, L.B.; Gómez-Banoy, N.; Karakose, E.; Tanase, M.; Doridot, L.; et al. Maladaptive positive feedback production of ChREBPβ underlies glucotoxic β-cell failure. *Nat. Commun.* **2022**, *13*, 4423. [CrossRef]
42. Oshima, M.; Pechberty, S.; Bellini, L.; Göpel, S.O.; Campana, M.; Rouch, C.; Dairou, J.; Cosentino, C.; Fantuzzi, F.; Toivonen, S.; et al. Stearoyl CoA desaturase is a gatekeeper that protects human beta cells against lipotoxicity and maintains their identity. *Diabetologia* **2019**, *63*, 395–409. [CrossRef]
43. Dobosz, A.M.; Janikiewicz, J.; Krogulec, E.; Dziewulska, A.; Ajduk, A.; Szpila, M.; Nieznańska, H.; Szczepankiewicz, A.A.; Wypych, D.; Dobrzyn, A. Inhibition of stearoyl-CoA desaturase 1 in the mouse impairs pancreatic islet morphogenesis and promotes loss of β-cell identity and α-cell expansion in the mature pancreas. *Mol. Metab.* **2023**, *67*, 101659. [CrossRef]
44. Leenders, F.; Groen, N.; de Graaf, N.; Engelse, M.A.; Rabelink, T.J.; de Koning, E.J.P.; Carlotti, F. Oxidative Stress Leads to β-Cell Dysfunction Through Loss of β-Cell Identity. *Front. Immunol.* **2021**, *12*, 690379. [CrossRef]
45. Motomura, K.; Matsuzaka, T.; Shichino, S.; Ogawa, T.; Pan, H.; Nakajima, T.; Asano, Y.; Okayama, T.; Takeuchi, T.; Ohno, H.; et al. Single-Cell Transcriptome Profiling of Pancreatic Islets From Early Diabetic Mice Identifies Anxa10 for Ca2+ Allostasis Toward β-Cell Failure. *Diabetes* **2023**, *73*, 75–92. [CrossRef]
46. Dadheech, N.; Srivastava, A.; Shah, R.G.; Shah, G.M.; Gupta, S. Role of poly(ADP-ribose) polymerase-1 in regulating human islet cell differentiation. *Sci. Rep.* **2022**, *12*, 21496. [CrossRef]
47. Liu, B.; Hua, D.; Shen, L.; Li, T.; Tao, Z.; Fu, C.; Tang, Z.; Yang, J.; Zhang, L.; Nie, A.; et al. NPC1 is required for postnatal islet β cell differentiation by maintaining mitochondria turnover. *Theranostics* **2024**, *14*, 2058–2074. [CrossRef]
48. Yeh, Y.-T.; Sona, C.; Yan, X.; Li, Y.; Pathak, A.; McDermott, M.I.; Xie, Z.; Liu, L.; Arunagiri, A.; Wang, Y.; et al. Restoration of PITPNA in Type 2 diabetic human islets reverses pancreatic beta-cell dysfunction. *Nat. Commun.* **2023**, *14*, 4250. [CrossRef]
49. Song, J.; Ni, Q.; Sun, J.; Xie, J.; Liu, J.; Ning, G.; Wang, W.; Wang, Q. Aging Impairs Adaptive Unfolded Protein Response and Drives Beta Cell Dedifferentiation in Humans. *J. Clin. Endocrinol. Metab.* **2022**, *107*, 3231–3241. [CrossRef] [PubMed]
50. Téllez, N.; Vilaseca, M.; Martí, Y.; Pla, A.; Montanya, E. β-Cell dedifferentiation, reduced duct cell plasticity, and impaired β-cell mass regeneration in middle-aged rats. *Am. J. Physiol. Endocrinol. Metab.* **2016**, *311*, E554–E563. [CrossRef] [PubMed]
51. Murao, N.; Yokoi, N.; Takahashi, H.; Hayami, T.; Minami, Y.; Seino, S. Increased glycolysis affects β-cell function and identity in aging and diabetes. *Mol. Metab.* **2021**, *55*, 101414. [CrossRef] [PubMed]
52. Tanday, N.; Coulter-Parkhill, A.; Moffett, R.C.; Suruli, K.; Dubey, V.; Flatt, P.R.; Irwin, N. Sex-based impact of pancreatic islet stressors in GluCreERT2/Rosa26-eYFP mice. *J. Endocrinol.* **2023**, *259*, e230174. [CrossRef] [PubMed]
53. Yang, B.C.; Wu, S.Y.; Leung, P.S. Alcohol ingestion induces pancreatic islet dysfunction and apoptosis via mediation of FGF21 resistance. *Ann. Transl. Med.* **2020**, *8*, 310. [CrossRef] [PubMed]
54. Rasineni, K.; Kubik, J.L.; Knight, K.L.; Hall, L.; Casey, C.A.; Kharbanda, K.K. Ghrelin regulates adipose tissue metabolism: Role in hepatic steatosis. *Chem. Interactions* **2020**, *322*, 109059. [CrossRef] [PubMed]
55. Lee, Y.; Ravazzola, M.; Park, B.-H.; Bashmakov, Y.K.; Orci, L.; Unger, R.H. Metabolic mechanisms of failure of intraportally transplanted pancreatic β-cells in rats: Role of lipotoxicity and prevention by leptin. *Diabetes* **2007**, *56*, 2295–2301. [CrossRef] [PubMed]
56. Holland, W.L.; Miller, R.A.; Wang, Z.V.; Sun, K.; Barth, B.M.; Bui, H.H.; Davis, K.E.; Bikman, B.T.; Halberg, N.; Rutkowski, J.M.; et al. Receptor-mediated activation of ceramidase activity initiates the pleiotropic actions of adiponectin. *Nat. Med.* **2011**, *17*, 55–63. [CrossRef]
57. Dufrane, D.; van Steenberghe, M.; Guiot, Y.; Goebbels, R.-M.; Saliez, A.; Gianello, P. Streptozotocin-Induced Diabetes in Large Animals (Pigs/Primates): Role of GLUT2 Transporter and β-cell Plasticity. *Transplantation* **2006**, *81*, 36–45. [CrossRef]
58. Feng, Y.; Qiu, W.-L.; Yu, X.-X.; Zhang, Y.; He, M.-Y.; Li, L.-C.; Yang, L.; Zhang, W.; Franti, M.; Ye, J. Characterizing pancreatic β-cell heterogeneity in the streptozotocin model by single-cell transcriptomic analysis. *Mol. Metab.* **2020**, *37*, 100982. [CrossRef] [PubMed]
59. Cheng, Y.; Kang, H.; Shen, J.; Hao, H.; Liu, J.; Guo, Y.; Mu, Y.; Han, W. Beta-cell regeneration from vimentin+/MafB+ cells after STZ-induced extreme beta-cell ablation. *Sci. Rep.* **2015**, *5*, 11703. [CrossRef]
60. Rafacho, A.; Ortsäter, H.; Nadal, A.; Quesada, I. Glucocorticoid treatment and endocrine pancreas function: Implications for glucose homeostasis, insulin resistance and diabetes. *J. Endocrinol.* **2014**, *223*, R49–R62. [CrossRef]
61. Esguerra, J.L.; Ofori, J.K.; Nagao, M.; Shuto, Y.; Karagiannopoulos, A.; Fadista, J.; Sugihara, H.; Groop, L.; Eliasson, L. Glucocorticoid induces human beta cell dysfunction by involving riborepressor GAS5 LincRNA. *Mol. Metab.* **2020**, *32*, 160–167. [CrossRef] [PubMed]
62. Beaupere, C.; Liboz, A.; Fève, B.; Blondeau, B.; Guillemain, G. Molecular Mechanisms of Glucocorticoid-Induced Insulin Resistance. *Int. J. Mol. Sci.* **2021**, *22*, 623. [CrossRef] [PubMed]
63. Valtat, B.; Dupuis, C.; Zenaty, D.; Singh-Estivalet, A.; Tronche, F.; Bréant, B.; Blondeau, B. Genetic evidence of the programming of beta cell mass and function by glucocorticoids in mice. *Diabetologia* **2010**, *54*, 350–359. [CrossRef] [PubMed]
64. Martínez-Pinna, J.; Sempere-Navarro, R.; Medina-Gali, R.M.; Fuentes, E.; Quesada, I.; Sargis, R.M.; Trasande, L.; Nadal, A. Endocrine disruptors in plastics alter β-cell physiology and increase the risk of diabetes mellitus. *Am. J. Physiol.-Endocrinol. Metab.* **2023**, *324*, E488–E505. [CrossRef] [PubMed]
65. Dos Santos, R.S.; Medina-Gali, R.M.; Babiloni-Chust, I.; Marroqui, L.; Nadal, A. In Vitro Assays to Identify Metabolism-Disrupting Chemicals with Diabetogenic Activity in a Human Pancreatic β-Cell Model. *Int. J. Mol. Sci.* **2022**, *23*, 5040. [CrossRef] [PubMed]

66. Sidarala, V.; Zhu, J.; Levi-D'ancona, E.; Pearson, G.L.; Reck, E.C.; Walker, E.M.; Kaufman, B.A.; Soleimanpour, S.A. Mitofusin 1 and 2 regulation of mitochondrial DNA content is a critical determinant of glucose homeostasis. *Nat. Commun.* **2022**, *13*, 2340. [CrossRef] [PubMed]
67. Barazzuol, L.; Giamogante, F.; Calì, T. Mitochondria Associated Membranes (MAMs): Architecture and physiopathological role. *Cell Calcium* **2021**, *94*, 102343. [CrossRef] [PubMed]
68. Boronat-Belda, T.; Ferrero, H.; Al-Abdulla, R.; Quesada, I.; Gustafsson, J.-A.; Nadal, Á.; Alonso-Magdalena, P. Bisphenol-A exposure during pregnancy alters pancreatic β-cell division and mass in male mice offspring: A role for ERβ. *Food Chem. Toxicol.* **2020**, *145*, 111681. [CrossRef] [PubMed]
69. Azevedo, L.F.; Masiero, M.M.; Cherkaoui, S.; Carneiro, M.H.; Barbosa Jr, F.; Zamboni, N. The alternative analog plasticizer BPS displays similar phenotypic and metabolomic responses to BPA in HepG2 and INS-1E cells. *Food Chem. Toxicol.* **2022**, *167*, 113266. [CrossRef]
70. García-Arévalo, M.; Alonso-Magdalena, P.; Servitja, J.-M.; Boronat-Belda, T.; Merino, B.; Villar-Pazos, S.; Medina-Gómez, G.; Novials, A.; Quesada, I.; Nadal, A. Maternal Exposure to Bisphenol-A During Pregnancy Increases Pancreatic β-Cell Growth During Early Life in Male Mice Offspring. *Endocrinology* **2016**, *157*, 4158–4171. [CrossRef]
71. Bansal, A.; Rashid, C.; Xin, F.; Li, C.; Polyak, E.; Duemler, A.; van der Meer, T.; Stefaniak, M.; Wajid, S.; Doliba, N.; et al. Sex- and Dose-Specific Effects of Maternal Bisphenol A Exposure on Pancreatic Islets of First- and Second-Generation Adult Mice Offspring. *Environ. Heal. Perspect.* **2017**, *125*, 097022. [CrossRef]
72. Yang, R.; Zheng, J.; Qin, J.; Liu, S.; Liu, X.; Gu, Y.; Yang, S.; Du, J.; Li, S.; Chen, B.; et al. Dibutyl phthalate affects insulin synthesis and secretion by regulating the mitochondrial apoptotic pathway and oxidative stress in rat insulinoma cells. *Ecotoxicol. Environ. Saf.* **2023**, *249*, 114396. [CrossRef]
73. Brusco, N.; Sebastiani, G.; Di Giuseppe, G.; Licata, G.; Grieco, G.E.; Fignani, D.; Nigi, L.; Formichi, C.; Aiello, E.; Auddino, S.; et al. Intra-islet insulin synthesis defects are associated with endoplasmic reticulum stress and loss of beta cell identity in human diabetes. *Diabetologia* **2022**, *66*, 354–366. [CrossRef] [PubMed]
74. Rewers, M.; Ludvigsson, J. Environmental risk factors for type 1 diabetes. *Lancet* **2016**, *387*, 2340–2348. [CrossRef]
75. Almanza, A.; Carlesso, A.; Chintha, C.; Creedican, S.; Doultsinos, D.; Leuzzi, B.; Luís, A.; McCarthy, N.; Montibeller, L.; More, S.; et al. Endoplasmic reticulum stress signalling—From basic mechanisms to clinical applications. *FEBS J.* **2018**, *286*, 241–278. [CrossRef]
76. Chen, C.-W.; Guan, B.-J.; Alzahrani, M.R.; Gao, Z.; Gao, L.; Bracey, S.; Wu, J.; Mbow, C.A.; Jobava, R.; Haataja, L.; et al. Adaptation to chronic ER stress enforces pancreatic β-cell plasticity. *Nat. Commun.* **2022**, *13*, 4621. [CrossRef]
77. Lee, H.; Lee, Y.-S.; Harenda, Q.; Pietrzak, S.; Oktay, H.Z.; Schreiber, S.; Liao, Y.; Sonthalia, S.; Ciecko, A.E.; Chen, Y.-G.; et al. Beta Cell Dedifferentiation Induced by IRE1α Deletion Prevents Type 1 Diabetes. *Cell Metab.* **2020**, *31*, 822–836.e5. [CrossRef]
78. Lee, K.; Chan, J.Y.; Liang, C.; Ip, C.K.; Shi, Y.-C.; Herzog, H.; Hughes, W.E.; Bensellam, M.; Delghingaro-Augusto, V.; Koina, M.E.; et al. XBP1 maintains beta cell identity, represses beta-to-alpha cell transdifferentiation and protects against diabetic beta cell failure during metabolic stress in mice. *Diabetologia* **2022**, *65*, 984–996. [CrossRef] [PubMed]
79. Kitakaze, K.; Oyadomari, M.; Zhang, J.; Hamada, Y.; Takenouchi, Y.; Tsuboi, K.; Inagaki, M.; Tachikawa, M.; Fujitani, Y.; Okamoto, Y.; et al. ATF4-mediated transcriptional regulation protects against β-cell loss during endoplasmic reticulum stress in a mouse model. *Mol. Metab.* **2021**, *54*, 101338. [CrossRef] [PubMed]
80. Lee, J.-H.; Ryu, H.; Lee, H.; Yu, H.R.; Gao, Y.; Lee, K.-M.; Kim, Y.-J.; Lee, J. Endoplasmic reticulum stress in pancreatic β cells induces incretin desensitization and β-cell dysfunction via ATF4-mediated PDE4D expression. *Am. J. Physiol. Endocrinol. Metab.* **2023**, *325*, E448–E465. [CrossRef]
81. Kulkarni, A.; Muralidharan, C.; May, S.C.; Tersey, S.A.; Mirmira, R.G. Inside the β Cell: Molecular Stress Response Pathways in Diabetes Pathogenesis. *Endocrinology* **2022**, *164*, bqac184. [CrossRef] [PubMed]
82. Pakos-Zebrucka, K.; Koryga, I.; Mnich, K.; Ljujic, M.; Samali, A.; Gorman, A.M. The integrated stress response. *EMBO Rep.* **2016**, *17*, 1374–1395. [CrossRef] [PubMed]
83. Koromilas, A.E. M(en)TORship lessons on life and death by the integrated stress response. *Biochim. Biophys. Acta (BBA) Gen. Subj.* **2019**, *1863*, 644–649. [CrossRef] [PubMed]
84. Zaborske, J.M.; Narasimhan, J.; Jiang, L.; Wek, S.A.; Dittmar, K.A.; Freimoser, F.; Pan, T.; Wek, R.C. Genome-wide Analysis of tRNA Charging and Activation of the eIF2 Kinase Gcn2p. *J. Biol. Chem.* **2009**, *284*, 25254–25267. [CrossRef]
85. Kilberg, M.S.; Shan, J.; Su, N. ATF4-dependent transcription mediates signaling of amino acid limitation. *Trends Endocrinol. Metab.* **2009**, *20*, 436–443. [CrossRef] [PubMed]
86. Kanno, A.; Asahara, S.-I.; Furubayashi, A.; Masuda, K.; Yoshitomi, R.; Suzuki, E.; Takai, T.; Kimura-Koyanagi, M.; Matsuda, T.; Bartolome, A.; et al. GCN2 regulates pancreatic β cell mass by sensing intracellular amino acid levels. *J. Clin. Investig.* **2020**, *5*, e128820. [CrossRef] [PubMed]
87. Darden, C.M.; Vasu, S.; Mattke, J.; Liu, Y.; Rhodes, C.J.; Naziruddin, B.; Lawrence, M.C. Calcineurin/NFATc2 and PI3K/AKT signaling maintains β-cell identity and function during metabolic and inflammatory stress. *iScience* **2022**, *25*, 104125. [CrossRef]
88. Ramalingam, L.; Sopontammarak, B.; Menikdiwela, K.R.; Moustaid-Moussa, N. Endoplasmic Reticulum (ER) Stress in Part Mediates Effects of Angiotensin II in Pancreatic Beta Cells. *Diabetes Metab. Syndr. Obes. Targets Ther.* **2020**, *13*, 2843–2853. [CrossRef]

89. Chen, H.; Zhou, W.; Ruan, Y.; Yang, L.; Xu, N.; Chen, R.; Yang, R.; Sun, J.; Zhang, Z. Reversal of angiotensin ll-induced β-cell dedifferentiation via inhibition of NF-κb signaling. *Mol. Med.* **2018**, *24*, 43. [CrossRef]
90. Li, M.-Y.; Liu, L.-Z.; Xin, Q.; Zhou, J.; Zhang, X.; Zhang, R.; Wu, Z.; Yi, J.; Dong, M. Downregulation of mTORC1 and Mcl-1 by lipid-oversupply contributes to islet β-cell apoptosis and dysfunction. *Biochim. Biophys. Acta (BBA) Mol. Cell Biol. Lipids* **2023**, *1868*, 159332. [CrossRef]
91. Cai, Z.; Liu, F.; Yang, Y.; Li, D.; Hu, S.; Song, L.; Yu, S.; Li, T.; Liu, B.; Luo, H. GRB10 regulates β-cell mass by inhibiting β-cell proliferation and stimulating β-cell dedifferentiation. *J. Genet. Genom.* **2022**, *49*, 208–216. [CrossRef] [PubMed]
92. Yin, Q.; Ni, Q.; Wang, Y.; Zhang, H.; Li, W.; Nie, A.; Wang, S.; Gu, Y.; Wang, Q.; Ning, G. Raptor determines β-cell identity and plasticity independent of hyperglycemia in mice. *Nat. Commun.* **2020**, *11*, 2538. [CrossRef] [PubMed]
93. Ni, Q.; Sun, J.; Wang, Y.; Wang, Y.; Liu, J.; Ning, G.; Wang, W.; Wang, Q. mTORC1 is required for epigenetic silencing during β-cell functional maturation. *Mol. Metab.* **2022**, *64*, 101559. [CrossRef] [PubMed]
94. Cui, C.; Li, T.; Xie, Y.; Yang, J.; Fu, C.; Qiu, Y.; Shen, L.; Ni, Q.; Wang, Q.; Nie, A. Enhancing Acsl4 in absence of mTORC2/Rictor drove β-cell dedifferentiation via inhibiting FoxO1 and promoting ROS production. *Biochim. Biophys. Acta (BBA) Mol. Basis Dis.* **2021**, *1867*, 166261. [CrossRef] [PubMed]
95. Lupse, B.; Annamalai, K.; Ibrahim, H.; Kaur, S.; Geravandi, S.; Sarma, B.; Pal, A.; Awal, S.; Joshi, A.; Rafizadeh, S. Inhibition of PHLPP1/2 phosphatases rescues pancreatic β-cells in diabetes. *Cell Rep.* **2021**, *36*, 109490. [CrossRef] [PubMed]
96. Liu, Z.; Wang, P.; Zhao, Y.; Lai, K.P.; Li, R. Biomedical importance of the ubiquitin–proteasome system in diabetes and metabolic transdifferentiation of pancreatic duct epithelial cells into β-cells. *Gene* **2023**, *858*, 147191. [CrossRef] [PubMed]
97. Kumar, A.; Francis, M.; Bhaskar, S.; Vishnuvajhala, S.; Prasanna, J. Dynamics of Ubiquitination in Differentiation and Dedifferentiation of Pancreatic β-cells: Putative Target for Diabetes. *Curr. Protein Pept. Sci.* **2022**, *23*, 602–618. [CrossRef]
98. Francis, M.; Bhaskar, S.; Komanduri, S.; Sheshadri, P.; Prasanna, J.; Kumar, A. Deubiquitinase USP1 influences the dedifferentiation of mouse pancreatic β-cells. *iScience* **2023**, *26*, 106771. [CrossRef]
99. Manea, T.; Nelson, J.K.; Garrone, C.M.; Hansson, K.; Evans, I.; Behrens, A.; Sancho, R. USP7 controls NGN3 stability and pancreatic endocrine lineage development. *Nat. Commun.* **2023**, *14*, 2457. [CrossRef]
100. Cho, G.; Hyun, K.; Choi, J.; Shin, E.; Kim, B.; Kim, H.; Kim, J.; Han, Y.-M. Arginine 65 methylation of Neurogenin 3 by PRMT1 is required for pancreatic endocrine development of hESCs. *Exp. Mol. Med.* **2023**, *55*, 1506–1519. [CrossRef]
101. Zhang, T.; Wang, H.; Wang, T.; Wei, C.; Jiang, H.; Jiang, S.; Yang, J.; Shao, J.; Ma, L. Pax4 synergistically acts with Pdx1, Ngn3 and MafA to induce HuMSCs to differentiate into functional pancreatic β-cells. *Exp. Ther. Med.* **2019**, *18*, 2592–2598. [CrossRef]
102. Lorenzo, P.I.; Juárez-Vicente, F.; Cobo-Vuilleumier, N.; García-Domínguez, M.; Gauthier, B.R. The Diabetes-Linked Transcription Factor PAX4: From Gene to Functional Consequences. *Genes* **2017**, *8*, 101. [CrossRef] [PubMed]
103. Brun, T.; Franklin, I.; St-Onge, L.; Biason-Lauber, A.; Schoenle, E.J.; Wollheim, C.B.; Gauthier, B.R. The diabetes-linked transcription factor PAX4 promotes β-cell proliferation and survival in rat and human islets. *J. Cell Biol.* **2004**, *167*, 1123–1135. [CrossRef]
104. Parajuli, K.R.; Zhang, Y.; Cao, A.M.; Wang, H.; Fonseca, V.A.; Wu, H. Pax4 Gene Delivery Improves Islet Transplantation Efficacy by Promoting β Cell Survival and α-to-β Cell Transdifferentiation. *Cell Transplant.* **2020**, *29*. [CrossRef]
105. Auerbach, A.; Cohen, A.; Shlomai, N.O.; Weinberg-Shukron, A.; Gulsuner, S.; King, M.-C.; Hemi, R.; Levy-Lahad, E.; Abulibdeh, A.; Zangen, D. *NKX2-2* Mutation Causes Congenital Diabetes and Infantile Obesity With Paradoxical Glucose-Induced Ghrelin Secretion. *J. Clin. Endocrinol. Metab.* **2020**, *105*, 3486–3495. [CrossRef]
106. Doyle, M.J.; Sussel, L. Nkx2. 2 regulates β-cell function in the mature islet. *Diabetes* **2007**, *56*, 1999–2007. [CrossRef]
107. Papizan, J.B.; Singer, R.A.; Tschen, S.-I.; Dhawan, S.; Friel, J.M.; Hipkens, S.B.; Magnuson, M.A.; Bhushan, A.; Sussel, L. Nkx2. 2 repressor complex regulates islet β-cell specification and prevents β-to-α-cell reprogramming. *Genes Dev.* **2011**, *25*, 2291–2305. [CrossRef] [PubMed]
108. Anderson, K.R.; Torres, C.A.; Solomon, K.; Becker, T.C.; Newgard, C.B.; Wright, C.V.; Hagman, J.; Sussel, L. Cooperative Transcriptional Regulation of the Essential Pancreatic Islet Gene NeuroD1 (Beta2) by Nkx2.2 and Neurogenin 3. *J. Biol. Chem.* **2009**, *284*, 31236–31248. [CrossRef] [PubMed]
109. Gutiérrez, G.D.; Bender, A.S.; Cirulli, V.; Mastracci, T.L.; Kelly, S.M.; Tsirigos, A.; Kaestner, K.H.; Sussel, L. Pancreatic β cell identity requires continual repression of non–β cell programs. *J. Clin. Investig.* **2017**, *127*, 244–259. [CrossRef]
110. Fontcuberta-PiSunyer, M.; García-Alamán, A.; Prades, È.; Téllez, N.; Alves-Figueiredo, H.; Ramos-Rodríguez, M.; Enrich, C.; Fernandez-Ruiz, R.; Cervantes, S.; Clua, L.; et al. Direct reprogramming of human fibroblasts into insulin-producing cells using transcription factors. *Commun. Biol.* **2023**, *6*, 256. [CrossRef]
111. Pan, F.C.; Brissova, M.; Powers, A.C.; Pfaff, S.; Wright, C.V.E. Inactivating the permanent neonatal diabetes gene *Mnx1* switches insulin-producing β-cells to a δ-like fate and reveals a facultative proliferative capacity in aged β-cells. *Development* **2015**, *142*, 3637–3648. [CrossRef] [PubMed]
112. Flanagan, S.E.; De Franco, E.; Allen, H.L.; Zerah, M.; Abdul-Rasoul, M.M.; Edge, J.A.; Stewart, H.; Alamiri, E.; Hussain, K.; Wallis, S.; et al. Analysis of Transcription Factors Key for Mouse Pancreatic Development Establishes NKX2-2 and MNX1 Mutations as Causes of Neonatal Diabetes in Man. *Cell Metab.* **2014**, *19*, 146–154. [CrossRef] [PubMed]
113. Arkhipova, V.; Wendik, B.; Devos, N.; Ek, O.; Peers, B.; Meyer, D. Characterization and regulation of the hb9/mnx1 beta-cell progenitor specific enhancer in zebrafish. *Dev. Biol.* **2012**, *365*, 290–302. [CrossRef] [PubMed]
114. Dalgin, G.; Ward, A.B.; Hao, L.T.; Beattie, C.E.; Nechiporuk, A.; Prince, V.E. Zebrafish *mnx1* controls cell fate choice in the developing endocrine pancreas. *Development* **2011**, *138*, 4597–4608. [CrossRef] [PubMed]

115. Dalgin, G.; Prince, V.E. Mnx1: A gatekeeper of β cell fate. *Islets* **2012**, *4*, 320–322. [CrossRef] [PubMed]
116. Li, X.; Wan, T.; Li, Y. Role of FoxO1 in regulating autophagy in type 2 diabetes mellitus (Review). *Exp. Ther. Med.* **2021**, *22*, 707. [CrossRef] [PubMed]
117. Kitamura, Y.I.; Kitamura, T.; Kruse, J.-P.; Raum, J.C.; Stein, R.; Gu, W.; Accili, D. FoxO1 protects against pancreatic β cell failure through NeuroD and MafA induction. *Cell Metab.* **2005**, *2*, 153–163. [CrossRef]
118. Casteels, T.; Zhang, Y.; Frogne, T.; Sturtzel, C.; Lardeau, C.-H.; Sen, I.; Liu, X.; Hong, S.; Pauler, F.M.; Penz, T.; et al. An inhibitor-mediated beta-cell dedifferentiation model reveals distinct roles for FoxO1 in glucagon repression and insulin maturation. *Mol. Metab.* **2021**, *54*, 101329. [CrossRef]
119. Khatri, R.; Mazurek, S.; Petry, S.F.; Linn, T. Mesenchymal stem cells promote pancreatic β-cell regeneration through downregulation of FoxO1 pathway. *Stem Cell Res. Ther.* **2020**, *11*, 497. [CrossRef]
120. Nie, Y.; Zhang, Y.; Liu, S.; Xu, Z.; Xia, C.; Du, L.; Yin, X.; Wang, J. Downregulation of Sirt3 contributes to β-cell dedifferentiation via FoxO1 in type 2 diabetic mellitus. *Acta Diabetol.* **2023**, *61*, 485–494. [CrossRef]
121. Li, Y.; Deng, S.; Peng, J.; Wang, X.; Essandoh, K.; Mu, X.; Peng, T.; Meng, Z.-X.; Fan, G.-C. MicroRNA-223 is essential for maintaining functional β-cell mass during diabetes through inhibiting both FOXO1 and SOX6 pathways. *J. Biol. Chem.* **2019**, *294*, 10438–10448. [CrossRef] [PubMed]
122. Tang, S.; Xin, Y.; Yang, M.; Zhang, D.; Xu, C. Osteoprotegerin promotes islet β cell proliferation in intrauterine growth retardation rats through the PI3K/AKT/FoxO1 pathway. *Int. J. Clin. Exp. Pathol.* **2019**, *12*, 2324. [PubMed]
123. Wang, K.; Cui, X.; Li, F.; Xia, L.; Wei, T.; Liu, J.; Fu, W.; Yang, J.; Hong, T.; Wei, R. Glucagon receptor blockage inhibits β-cell dedifferentiation through FoxO1. *Am. J. Physiol. Metab.* **2023**, *324*, E97–E113. [CrossRef] [PubMed]
124. Ebrahim, N.; Shakirova, K.; Dashinimaev, E. PDX1 is the cornerstone of pancreatic β-cell functions and identity. *Front. Mol. Biosci.* **2022**, *9*, 1091757. [CrossRef]
125. Chen, F.; Sha, M.; Wang, Y.; Wu, T.; Shan, W.; Liu, J.; Zhou, W.; Zhu, Y.; Sun, Y.; Shi, Y.; et al. Transcription factor Ets-1 links glucotoxicity to pancreatic beta cell dysfunction through inhibiting PDX-1 expression in rodent models. *Diabetologia* **2015**, *59*, 316–324. [CrossRef] [PubMed]
126. Zhang, Y.; Fang, X.; Wei, J.; Miao, R.; Wu, H.; Ma, K.; Tian, J. PDX-1: A Promising Therapeutic Target to Reverse Diabetes. *Biomolecules* **2022**, *12*, 1785. [CrossRef] [PubMed]
127. Weidemann, B.J.; Marcheva, B.; Kobayashi, M.; Omura, C.; Newman, M.V.; Kobayashi, Y.; Waldeck, N.J.; Perelis, M.; Lantier, L.; McGuinness, O.P. Repression of latent NF-κB enhancers by PDX1 regulates β cell functional heterogeneity. *Cell Metab.* **2024**, *36*, 90–102.e107. [CrossRef] [PubMed]
128. Tsuyama, T.; Sato, Y.; Yoshizawa, T.; Matsuoka, T.; Yamagata, K. Hypoxia causes pancreatic β-cell dysfunction and impairs insulin secretion by activating the transcriptional repressor BHLHE40. *EMBO Rep.* **2023**, *24*, e56227. [CrossRef]
129. Vanhoose, A.M.; Samaras, S.; Artner, I.; Henderson, E.; Hang, Y.; Stein, R. MafA and MafB Regulate Pdx1 Transcription through the Area II Control Region in Pancreatic β Cells. *J. Biol. Chem.* **2008**, *283*, 22612–22619. [CrossRef]
130. Aigha, I.I.; Abdelalim, E.M. NKX6. 1 transcription factor: A crucial regulator of pancreatic β cell development, identity, and proliferation. *Stem Cell Res. Ther.* **2020**, *11*, 459. [CrossRef]
131. Luan, C.; Ye, Y.; Singh, T.; Barghouth, M.; Eliasson, L.; Artner, I.; Zhang, E.; Renström, E. The calcium channel subunit gamma-4 is regulated by MafA and necessary for pancreatic beta-cell specification. *Commun. Biol.* **2019**, *2*, 106. [CrossRef] [PubMed]
132. Liang, J.; Chirikjian, M.; Pajvani, U.B.; Bartolomé, A. MafA Regulation in β-Cells: From Transcriptional to Post-Translational Mechanisms. *Biomolecules* **2022**, *12*, 535. [CrossRef] [PubMed]
133. Nishimura, W.; Iwasa, H.; Tumurkhuu, M. Role of the Transcription Factor MAFA in the Maintenance of Pancreatic β-Cells. *Int. J. Mol. Sci.* **2022**, *23*, 4478. [CrossRef] [PubMed]
134. Hang, Y.; Yamamoto, T.; Benninger, R.K.; Brissova, M.; Guo, M.; Bush, W.; Piston, D.W.; Powers, A.C.; Magnuson, M.; Thurmond, D.C.; et al. The MafA Transcription Factor Becomes Essential to Islet β-Cells Soon After Birth. *Diabetes* **2014**, *63*, 1994–2005. [CrossRef] [PubMed]
135. Eto, K.; Nishimura, W.; Oishi, H.; Udagawa, H.; Kawaguchi, M.; Hiramoto, M.; Fujiwara, T.; Takahashi, S.; Yasuda, K. MafA Is Required for Postnatal Proliferation of Pancreatic β-Cells. *PLoS ONE* **2014**, *9*, e104184. [CrossRef] [PubMed]
136. Gradwohl, G.; Dierich, A.; LeMeur, M.; Guillemot, F. *neurogenin3* is required for the development of the four endocrine cell lineages of the pancreas. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 1607–1611. [CrossRef] [PubMed]
137. Zhu, Y.; Liu, Q.; Zhou, Z.; Ikeda, Y. PDX1, Neurogenin-3, and MAFA: Critical transcription regulators for beta cell development and regeneration. *Stem Cell Res. Ther.* **2017**, *8*, 240. [CrossRef] [PubMed]
138. Schreiber, V.; Mercier, R.; Jiménez, S.; Ye, T.; García-Sánchez, E.; Klein, A.; Meunier, A.; Ghimire, S.; Birck, C.; Jost, B.; et al. Extensive NEUROG3 occupancy in the human pancreatic endocrine gene regulatory network. *Mol. Metab.* **2021**, *53*, 101313. [CrossRef]
139. Miyatsuka, T.; Kosaka, Y.; Kim, H.; German, M.S. Neurogenin3 inhibits proliferation in endocrine progenitors by inducing Cdkn1a. *Proc. Natl. Acad. Sci. USA* **2010**, *108*, 185–190. [CrossRef]
140. McDonald, E.; Li, J.; Krishnamurthy, M.; Fellows, G.F.; Goodyer, C.G.; Wang, R. SOX9 regulates endocrine cell differentiation during human fetal pancreas development. *Int. J. Biochem. Cell Biol.* **2012**, *44*, 72–83. [CrossRef]
141. Dettmer, R.; Niwolik, I.; Mehmeti, I.; Jörns, A.; Naujok, O. New hPSC SOX9 and INS Reporter Cell Lines Facilitate the Observation and Optimization of Differentiation into Insulin-Producing Cells. *Stem Cell Rev. Rep.* **2021**, *17*, 2193–2209. [CrossRef] [PubMed]

142. Wang, Q.; Chen, J.; Han, J.; Yang, Z.; Yang, Y.; Li, H.; Wang, S.; Hong, Y. Sox9 is required in regeneration of pancreatic β cells following injury. *Exp. Cell Res.* **2023**, *422*, 113406. [CrossRef] [PubMed]
143. Zhang, M.; Lin, Q.; Qi, T.; Wang, T.; Chen, C.-C.; Riggs, A.D.; Zeng, D. Growth factors and medium hyperglycemia induce Sox9+ ductal cell differentiation into β cells in mice with reversal of diabetes. *Proc. Natl. Acad. Sci. USA* **2016**, *113*, 650–655. [CrossRef] [PubMed]
144. Qian, M.F.; Bevacqua, R.J.; Coykendall, V.M.; Liu, X.; Zhao, W.; Chang, C.A.; Gu, X.; Dai, X.-Q.; MacDonald, P.E.; Kim, S.K. HNF1α maintains pancreatic α and β cell functions in primary human islets. *JCI Insight* **2023**, *8*, e170884. [CrossRef] [PubMed]
145. Sato, Y.; Rahman, M.; Haneda, M.; Tsuyama, T.; Mizumoto, T.; Yoshizawa, T.; Kitamura, T.; Gonzalez, F.J.; Yamamura, K.-I.; Yamagata, K. HNF1α controls glucagon secretion in pancreatic α-cells through modulation of SGLT1. *Biochim. Biophys. Acta (BBA) Mol. Basis Dis.* **2020**, *1866*, 165898. [CrossRef] [PubMed]
146. Low, B.S.J.; Lim, C.S.; Ding, S.S.L.; Tan, Y.S.; Ng, N.H.J.; Krishnan, V.G.; Ang, S.F.; Neo, C.W.Y.; Verma, C.S.; Hoon, S. Decreased GLUT2 and glucose uptake contribute to insulin secretion defects in MODY3/HNF1A hiPSC-derived mutant β cells. *Nat. Commun.* **2021**, *12*, 3133. [CrossRef] [PubMed]
147. Chen, Y.; Jia, J.; Zhao, Q.; Zhang, Y.; Huang, B.; Wang, L.; Tian, J.; Huang, C.; Li, M.; Li, X. Novel Loss-of-Function Variant in HNF1a Induces β-Cell Dysfunction through Endoplasmic Reticulum Stress. *Int. J. Mol. Sci.* **2022**, *23*, 13022. [CrossRef] [PubMed]
148. Cujba, A.-M.; Alvarez-Fallas, M.E.; Pedraza-Arevalo, S.; Laddach, A.; Shepherd, M.H.; Hattersley, A.T.; Watt, F.M.; Sancho, R. An HNF1α truncation associated with maturity-onset diabetes of the young impairs pancreatic progenitor differentiation by antagonizing HNF1β function. *Cell Rep.* **2022**, *38*, 110425. [CrossRef] [PubMed]
149. Sujjitjoon, J.; Charoensuk, C.; Thanyaphon, T.; Kooptiwut, S.; Thamtarana, P.J.; Tangjittipokin, W.; Chongjaroen, N.; Chanprasert, C.; Abubakar, Z.; Lapbenjakul, S.; et al. Defective functions of HNF1A variants on BCL2L1 transactivation and beta-cell growth. *Biochem. Biophys. Res. Commun.* **2020**, *529*, 826–833. [CrossRef]
150. Friedman-Mazursky, O.; Elkon, R.; Efrat, S. Redifferentiation of expanded human islet β cells by inhibition of ARX. *Sci. Rep.* **2016**, *6*, 20698. [CrossRef]
151. Courtney, M.; Rabe, T.; Collombat, P.; Mansouri, A. Pax4 and Arx Represent Crucial Regulators of the Development of the Endocrine Pancreas. *New J. Sci.* **2014**, *2014*, 1–6. [CrossRef]
152. Đorđević, M.; Stepper, P.; Feuerstein-Akgoz, C.; Gerhauser, C.; Paunović, V.; Tolić, A.; Rajić, J.; Dinić, S.; Uskoković, A.; Grdović, N.; et al. EpiCRISPR targeted methylation of Arx gene initiates transient switch of mouse pancreatic alpha to insulin-producing cells. *Front. Endocrinol.* **2023**, *14*, 1134478. [CrossRef] [PubMed]
153. Courtney, M.; Gjernes, E.; Druelle, N.; Ravaud, C.; Vieira, A.; Ben-Othman, N.; Pfeifer, A.; Avolio, F.; Leuckx, G.; Lacas-Gervais, S.; et al. The Inactivation of Arx in Pancreatic α-Cells Triggers Their Neogenesis and Conversion into Functional β-like Cells. *PLoS Genet.* **2013**, *9*, e1003934. [CrossRef] [PubMed]
154. Villamayor, L.; Rodríguez-Seguel, E.; Araujo, R.; Carrasco, M.; Bru-Tarí, E.; Mellado-Gil, J.M.; Gauthier, B.R.; Martinelli, P.; Quesada, I.; Soria, B.; et al. GATA6 Controls Insulin Biosynthesis and Secretion in Adult β-Cells. *Diabetes* **2017**, *67*, 448–460. [CrossRef]
155. Chia, C.Y.; Madrigal, P.; Denil, S.L.; Martinez, I.; Garcia-Bernardo, J.; El-Khairi, R.; Chhatriwala, M.; Shepherd, M.H.; Hattersley, A.T.; Dunn, N.R. GATA6 cooperates with EOMES/SMAD2/3 to deploy the gene regulatory network governing human definitive endoderm and pancreas formation. *Stem Cell Rep.* **2019**, *12*, 57–70. [CrossRef] [PubMed]
156. Tiyaboonchai, A.; Cardenas-Diaz, F.L.; Ying, L.; Maguire, J.A.; Sim, X.; Jobaliya, C.; Gagne, A.L.; Kishore, S.; Stanescu, D.E.; Hughes, N.; et al. GATA6 Plays an Important Role in the Induction of Human Definitive Endoderm, Development of the Pancreas, and Functionality of Pancreatic β Cells. *Stem Cell Rep.* **2017**, *8*, 589–604. [CrossRef] [PubMed]
157. Sanyoura, M.; Jacobsen, L.; Carmody, D.; del Gaudio, D.; Alkorta-Aranburu, G.; Arndt, K.; Hu, Y.Y.; Kobiernicki, F.; Kusmartseva, I.; Atkinson, M.A.; et al. Pancreatic Histopathology of Human Monogenic Diabetes Due to Causal Variants in KCNJ11, HNF1A, GATA6, and LMNA. *J. Clin. Endocrinol. Metab.* **2017**, *103*, 35–45. [CrossRef] [PubMed]
158. Yue, X.; Luo, Y.; Wang, J.; Huang, D. Monogenic Diabetes with GATA6 Mutations: Characterization of a Novel Family and a Comprehensive Analysis of the GATA6 Clinical and Genetics Traits. *Mol. Biotechnol.* **2023**, *66*, 467–474. [CrossRef] [PubMed]
159. Kim, Y.-S.; Kang, H.S.; Takeda, Y.; Hom, L.; Song, H.-Y.; Jensen, J.; Jetten, A.M. Glis3 Regulates Neurogenin 3 Expression in Pancreatic β-Cells and Interacts with Its Activator, Hnf6. *Mol. Cells* **2012**, *34*, 193–200. [CrossRef]
160. Scoville, D.W.; Lichti-Kaiser, K.; Grimm, S.A.; Jetten, A.M. GLIS3 binds pancreatic beta cell regulatory regions alongside other islet transcription factors. *J. Endocrinol.* **2019**, *243*, 1–14. [CrossRef]
161. Amin, S.; Cook, B.; Zhou, T.; Ghazizadeh, Z.; Lis, R.; Zhang, T.; Khalaj, M.; Crespo, M.; Perera, M.; Xiang, J.Z.; et al. Discovery of a drug candidate for GLIS3-associated diabetes. *Nat. Commun.* **2018**, *9*, 2681. [CrossRef] [PubMed]
162. Meulebrouck, S.; Scherrer, V.; Boutry, R.; Toussaint, B.; Vaillant, E.; Dechaume, A.; Loiselle, H.; Balkau, B.; Charpentier, G.; Franc, S.; et al. Pathogenic monoallelic variants in GLIS3 increase type 2 diabetes risk and identify a subgroup of patients sensitive to sulfonylureas. *Diabetologia* **2023**, *67*, 327–332. [CrossRef] [PubMed]
163. Grieve, L.M.; Rani, A.; ZeRuth, G.T. Downregulation of Glis3 in INS1 cells exposed to chronically elevated glucose contributes to glucotoxicity-associated β cell dysfunction. *Islets* **2024**, *16*, 2344622. [CrossRef] [PubMed]
164. Swisa, A.; Avrahami, D.; Eden, N.; Zhang, J.; Feleke, E.; Dahan, T.; Cohen-Tayar, Y.; Stolovich-Rain, M.; Kaestner, K.H.; Glaser, B.; et al. PAX6 maintains β cell identity by repressing genes of alternative islet cell types. *J. Clin. Investig.* **2016**, *127*, 230–243. [CrossRef] [PubMed]

165. Gosmain, Y.; Katz, L.S.; Masson, M.H.; Cheyssac, C.; Poisson, C.; Philippe, J. Pax6 is crucial for β-cell function, insulin biosynthesis, and glucose-induced insulin secretion. *Mol. Endocrinol.* **2012**, *26*, 696–709. [CrossRef] [PubMed]
166. Ashery-Padan, R.; Zhou, X.; Marquardt, T.; Herrera, P.; Toube, L.; Berry, A.; Gruss, P. Conditional inactivation of Pax6 in the pancreas causes early onset of diabetes. *Dev. Biol.* **2004**, *269*, 479–488. [CrossRef] [PubMed]
167. Sheng, J.; Wang, L.; Tang, P.M.-K.; Wang, H.-L.; Li, J.-C.; Xu, B.-H.; Xue, V.W.; Tan, R.-Z.; Jin, N.; Chan, T.-F.; et al. Smad3 deficiency promotes beta cell proliferation and function in *db/db* mice *via* restoring Pax6 expression. *Theranostics* **2021**, *11*, 2845–2859. [CrossRef] [PubMed]
168. Bsharat, S.; Monni, E.; Singh, T.; Johansson, J.K.; Achanta, K.; Bertonnier-Brouty, L.; Schmidt-Christensen, A.; Holmberg, D.; Kokaia, Z.; Prasad, R.B. MafB-dependent neurotransmitter signaling promotes β cell migration in the developing pancreas. *Development* **2023**, *150*, dev201009. [CrossRef] [PubMed]
169. Cyphert, H.A.; Walker, E.M.; Hang, Y.; Dhawan, S.; Haliyur, R.; Bonatakis, L.; Avrahami, D.; Brissova, M.; Kaestner, K.H.; Bhushan, A.; et al. Examining How the MAFB Transcription Factor Affects Islet β-Cell Function Postnatally. *Diabetes* **2018**, *68*, 337–348. [CrossRef] [PubMed]
170. Cha, J.; Tong, X.; Walker, E.M.; Dahan, T.; Cochrane, V.A.; Ashe, S.; Russell, R.; Osipovich, A.B.; Mawla, A.M.; Guo, M.; et al. Species-specific roles for the MAFA and MAFB transcription factors in regulating islet β cell identity. *J. Clin. Investig.* **2023**, *8*, e166386. [CrossRef]
171. Russell, R.; Carnese, P.P.; Hennings, T.G.; Walker, E.M.; Russ, H.A.; Liu, J.S.; Giacometti, S.; Stein, R.; Hebrok, M. Loss of the transcription factor MAFB limits β-cell derivation from human PSCs. *Nat. Commun.* **2020**, *11*, 2742. [CrossRef] [PubMed]
172. Bar, Y.; Russ, H.A.; Sintov, E.; Anker-Kitai, L.; Knoller, S.; Efrat, S. Redifferentiation of Expanded Human Pancreatic β-Cell-derived Cells by Inhibition of the NOTCH Pathway. *J. Biol. Chem.* **2012**, *287*, 17269–17280. [CrossRef]
173. Bar, Y.; Russ, H.A.; Knoller, S.; Ouziel-Yahalom, L.; Efrat, S. HES-1 Is Involved in Adaptation of Adult Human β-Cells to Proliferation In Vitro. *Diabetes* **2008**, *57*, 2413–2420. [CrossRef] [PubMed]
174. Marui, S.; Nishikawa, Y.; Shiokawa, M.; Yokode, M.; Matsumoto, S.; Muramoto, Y.; Ota, S.; Nakamura, T.; Yoshida, H.; Okada, H.; et al. Context-Dependent Roles of Hes1 in the Adult Pancreas and Pancreatic Tumor Formation. *Gastroenterology* **2022**, *163*, 1613–1629.e12. [CrossRef]
175. Xu, Y.; Mao, S.; Fan, H.; Wan, J.; Wang, L.; Zhang, M.; Zhu, S.; Yuan, J.; Lu, Y.; Wang, Z.; et al. LINC MIR503HG Controls SC-β Cell Differentiation and Insulin Production by Targeting CDH1 and HES1. *Adv. Sci.* **2024**, *11*, e2305631. [CrossRef] [PubMed]
176. Barth, R.; Ruoso, C.; Ferreira, S.M.; de Ramos, F.C.; Lima, F.B.; Boschero, A.C.; Dos Santos, G.J. Hepatocyte Nuclear Factor 4-α (HNF4α) controls the insulin resistance-induced pancreatic β-cell mass expansion. *Life Sci.* **2022**, *289*, 120213. [CrossRef]
177. Han, E.H.; Singh, P.; Lee, I.-K.; Urrutia, R.; Chi, Y.-I. ErbB3-binding protein 1 (EBP1) represses HNF4α-mediated transcription and insulin secretion in pancreatic β-cells. *J. Biol. Chem.* **2019**, *294*, 13983–13994. [CrossRef]
178. Romer, A.I.; Singer, R.A.; Sui, L.; Egli, D.; Sussel, L. Murine Perinatal β-Cell Proliferation and the Differentiation of Human Stem Cell–Derived Insulin-Expressing Cells Require NEUROD1. *Diabetes* **2019**, *68*, 2259–2271. [CrossRef]
179. Bohuslavova, R.; Fabriciova, V.; Smolik, O.; Lebrón-Mora, L.; Abaffy, P.; Benesova, S.; Zucha, D.; Valihrach, L.; Berkova, Z.; Saudek, F.; et al. NEUROD1 reinforces endocrine cell fate acquisition in pancreatic development. *Nat. Commun.* **2023**, *14*, 5554. [CrossRef]
180. Gu, C.; Stein, G.H.; Pan, N.; Goebbels, S.; Hörnberg, H.; Nave, K.-A.; Herrera, P.; White, P.; Kaestner, K.H.; Sussel, L.; et al. Pancreatic β Cells Require NeuroD to Achieve and Maintain Functional Maturity. *Cell Metab.* **2010**, *11*, 298–310. [CrossRef]
181. Schaffer, A.E.; Taylor, B.L.; Benthuysen, J.R.; Liu, J.; Thorel, F.; Yuan, W.; Jiao, Y.; Kaestner, K.H.; Herrera, P.L.; Magnuson, M.A.; et al. Nkx6.1 Controls a Gene Regulatory Network Required for Establishing and Maintaining Pancreatic Beta Cell Identity. *PLoS Genet.* **2013**, *9*, e1003274. [CrossRef]
182. Memon, B.; Younis, I.; Abubaker, F.; Abdelalim, E.M. PDX1−/NKX6. 1+ progenitors derived from human pluripotent stem cells as a novel source of insulin-secreting cells. *Diabetes/Metab. Res. Rev.* **2021**, *37*, e3400. [CrossRef] [PubMed]
183. Fujita, Y.; Kozawa, J.; Fukui, K.; Iwahashi, H.; Eguchi, H.; Shimomura, I. Increased NKX6.1 expression and decreased ARX expression in alpha cells accompany reduced beta-cell volume in human subjects. *Sci. Rep.* **2021**, *11*, 17796. [CrossRef]
184. Tessem, J.S.; Moss, L.G.; Chao, L.C.; Arlotto, M.; Lu, D.; Jensen, M.V.; Stephens, S.B.; Tontonoz, P.; Hohmeier, H.E.; Newgard, C.B. Nkx6.1 regulates islet β-cell proliferation via Nr4a1 and Nr4a3 nuclear receptors. *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 5242–5247. [CrossRef] [PubMed]
185. Ratiu, J.; Southard, S.M.; Rust, W.L. The transcription factors ISL-1 and MAFA, but not NKX6-1 Characterize a Stem-cell Derived Population of Endocrine Pancreatic Cells Capable of Controlling blood Glucose in Rodent Models of Diabetes. *Med. Res. Arch.* **2023**, *11*. [CrossRef]
186. Zhang, J.; Zhang, R.; Liu, C.; Ge, X.; Wang, Y.; Jiang, F.; Zhuang, L.; Li, T.; Zhu, Q.; Jiang, Y.; et al. Missense mutation of ISL1 (E283D) is associated with the development of type 2 diabetes. *Diabetologia* **2024**. [CrossRef]
187. Bohuslavova, R.; Fabriciova, V.; Lebrón-Mora, L.; Malfatti, J.; Smolik, O.; Valihrach, L.; Benesova, S.; Zucha, D.; Berkova, Z.; Saudek, F.; et al. ISL1 controls pancreatic alpha cell fate and beta cell maturation. *Cell Biosci.* **2023**, *13*, 53. [CrossRef] [PubMed]
188. Guo, T.; Wang, W.; Zhang, H.; Liu, Y.; Chen, P.; Ma, K.; Zhou, C. ISL1 Promotes Pancreatic Islet Cell Proliferation. *PLoS ONE* **2011**, *6*, e22387. [CrossRef]

189. Connors, C.T.; Villaca, C.B.; Anderson-Baucum, E.K.; Rosario, S.R.; Rutan, C.D.; Childress, P.J.; Padgett, L.R.; Robertson, M.A.; Mastracci, T.L. A Translational Regulatory Mechanism Mediated by Hypusinated Eukaryotic Initiation Factor 5A Facilitates β-Cell Identity and Function. *Diabetes* **2023**, *73*, 461–473. [CrossRef]
190. Wang, W.; Zhang, C. Targeting β-cell dedifferentiation and transdifferentiation: Opportunities and challenges. *Endocr. Connect.* **2021**, *10*, R213–R228. [CrossRef]
191. Wu, Q.-J.; Zhang, T.-N.; Chen, H.-H.; Yu, X.-F.; Lv, J.-L.; Liu, Y.-Y.; Liu, Y.-S.; Zheng, G.; Zhao, J.-Q.; Wei, Y.-F. The sirtuin family in health and disease. *Signal Transduct. Target. Ther.* **2022**, *7*, 402. [PubMed]
192. Cao, H.; Chung, A.C.; Ming, X.; Mao, D.; Lee, H.M.; Cao, X.; Rutter, G.A.; Chan, J.C.; Tian, X.Y.; Kong, A.P. Autotaxin signaling facilitates β cell dedifferentiation and dysfunction induced by Sirtuin 3 deficiency. *Mol. Metab.* **2022**, *60*, 101493. [CrossRef] [PubMed]
193. Zhang, L.; Sheng, C.; Zhou, F.; Zhu, K.; Wang, S.; Liu, Q.; Yuan, M.; Xu, Z.; Liu, Y.; Lu, J.; et al. CBP/p300 HAT maintains the gene network critical for β cell identity and functional maturity. *Cell Death Dis.* **2021**, *12*, 476. [CrossRef] [PubMed]
194. Vanderkruk, B.; Maeshima, N.; Pasula, D.J.; An, M.; McDonald, C.L.; Suresh, P.; Luciani, D.S.; Lynn, F.C.; Hoffman, B.G. Methylation of histone H3 lysine 4 is required for maintenance of beta cell function in adult mice. *Diabetologia* **2023**, *66*, 1097–1115. [CrossRef] [PubMed]
195. Al-Hasani, K.; Marikar, S.N.; Kaipananickal, H.; Maxwell, S.; Okabe, J.; Khurana, I.; Karagiannis, T.; Liang, J.J.; Mariana, L.; Loudovaris, T.; et al. EZH2 inhibitors promote β-like cell regeneration in young and adult type 1 diabetes donors. *Signal Transduct. Target. Ther.* **2024**, *9*, 2. [CrossRef] [PubMed]
196. Parveen, N.; Dhawan, S. DNA Methylation Patterning and the Regulation of Beta Cell Homeostasis. *Front. Endocrinol.* **2021**, *12*, 651258. [CrossRef]
197. Parveen, N.; Wang, J.K.; Bhattacharya, S.; Cuala, J.; Rajkumar, M.S.; Butler, A.E.; Wu, X.; Shih, H.-P.; Georgia, S.K.; Dhawan, S. DNA Methylation-Dependent Restriction of Tyrosine Hydroxylase Contributes to Pancreatic β-Cell Heterogeneity. *Diabetes* **2023**, *72*, 575–589. [CrossRef] [PubMed]
198. Celen, C.; Chuang, J.-C.; Shen, S.; Li, L.; Maggiore, G.; Jia, Y.; Luo, X.; Moore, A.; Wang, Y.; Otto, J.E.; et al. Arid1a loss potentiates pancreatic β-cell regeneration through activation of EGF signaling. *Cell Rep.* **2022**, *41*, 111581. [CrossRef] [PubMed]
199. Spaeth, J.M.; Liu, J.-H.; Peters, D.; Guo, M.; Osipovich, A.B.; Mohammadi, F.; Roy, N.; Bhushan, A.; Magnuson, M.A.; Hebrok, M.; et al. The Pdx1-Bound Swi/Snf Chromatin Remodeling Complex Regulates Pancreatic Progenitor Cell Proliferation and Mature Islet β-Cell Function. *Diabetes* **2019**, *68*, 1806–1818. [CrossRef] [PubMed]
200. Davidson, R.K.; Kanojia, S.; Wu, W.; Kono, T.; Xu, J.; Osmulski, M.; Bone, R.N.; Casey, N.; Evans-Molina, C.; Sims, E.K.; et al. The Chd4 Helicase Regulates Chromatin Accessibility and Gene Expression Critical for β-Cell Function In Vivo. *Diabetes* **2023**, *72*, 746–757. [CrossRef]
201. Davidson, R.K.; Weaver, S.A.; Casey, N.; Kanojia, S.; Hogarth, E.; Aguirre, R.S.; Sims, E.K.; Evans-Molina, C.; Spaeth, J.M. The Chd4 subunit of the NuRD complex regulates Pdx1-controlled genes involved in β-cell function. *J. Mol. Endocrinol.* **2022**, *69*, 329–341. [CrossRef] [PubMed]
202. Soltani, A.; Jafarian, A.; Allameh, A. The Predominant microRNAs in β-cell Clusters for Insulin Regulation and Diabetic Control. *Curr. Drug Targets* **2020**, *21*, 722–734. [CrossRef] [PubMed]
203. Sebastiani, G.; Grieco, G.E.; Brusco, N.; Ventriglia, G.; Formichi, C.; Marselli, L.; Marchetti, P.; Dotta, F. MicroRNA Expression Analysis of In Vitro Dedifferentiated Human Pancreatic Islet Cells Reveals the Activation of the Pluripotency-Related MicroRNA Cluster miR-302s. *Int. J. Mol. Sci.* **2018**, *19*, 1170. [CrossRef]
204. Melnik, B.C.; Schmitz, G. Milk Exosomal microRNAs: Postnatal Promoters of β Cell Proliferation but Potential Inducers of β Cell De-Differentiation in Adult Life. *Int. J. Mol. Sci.* **2022**, *23*, 11503. [CrossRef]
205. Melnik, B.C. Milk exosomal miRNAs: Potential drivers of AMPK-to-mTORC1 switching in β-cell de-differentiation of type 2 diabetes mellitus. *Nutr. Metab.* **2019**, *16*, 85. [CrossRef]
206. Wang, Z.; Mohan, R.; Chen, X.; Matson, K.; Waugh, J.; Mao, Y.; Zhang, S.; Li, W.; Tang, X.; Satin, L.S.; et al. microRNA-483 Protects Pancreatic β-Cells by Targeting ALDH1A3. *Endocrinology* **2021**, *162*, bqab031. [CrossRef]
207. Mohan, R.; Mao, Y.; Zhang, S.; Zhang, Y.-W.; Xu, C.-R.; Gradwohl, G.; Tang, X. Differentially Expressed MicroRNA-483 Confers Distinct Functions in Pancreatic β- and α-Cells. *J. Biol. Chem.* **2015**, *290*, 19955–19966. [CrossRef]
208. Gannon, M. Micro-RNA-mediated Maintenance of Beta-cell Identity Reveals Targets for Reversing Beta-cell Dedifferentiation. *Endocrinology* **2021**, *162*, bqab067. [CrossRef]
209. Sun, Y.; Zhou, Y.; Shi, Y.; Zhang, Y.; Liu, K.; Liang, R.; Sun, P.; Chang, X.; Tang, W.; Zhang, Y.; et al. Expression of miRNA-29 in Pancreatic β Cells Promotes Inflammation and Diabetes via TRAF3. *Cell Rep.* **2021**, *34*, 108576. [CrossRef] [PubMed]
210. Li, J.; Zhang, Y.; Ye, Y.; Li, D.; Liu, Y.; Lee, E.; Zhang, M.; Dai, X.; Zhang, X.; Wang, S. Pancreatic β cells control glucose homeostasis via the secretion of exosomal miR-29 family. *J. Extracell. Vesicles* **2021**, *10*, e12055. [CrossRef]
211. Lang, H.; Lin, N.; Chen, X.; Xiang, J.; Zhang, X.; Kang, C. Repressing miR-23a promotes the transdifferentiation of pancreatic α cells to β cells via negatively regulating the expression of SDF-1α. *PLoS ONE* **2024**, *19*, e0299821. [CrossRef] [PubMed]
212. Zhu, Y.; Sun, Y.; Zhou, Y.; Zhang, Y.; Zhang, T.; Li, Y.; You, W.; Chang, X.; Yuan, L.; Han, X. MicroRNA-24 promotes pancreatic beta cells toward dedifferentiation to avoid endoplasmic reticulum stress-induced apoptosis. *J. Mol. Cell Biol.* **2019**, *11*, 747–760. [CrossRef] [PubMed]

213. de Jesus, D.S.; Mak, T.C.; Wang, Y.-F.; von Ohlen, Y.; Bai, Y.; Kane, E.; Chabosseau, P.; Chahrour, C.M.; Distaso, W.; Salem, V. Dysregulation of the Pdx1/Ovol2/Zeb2 axis in dedifferentiated β-cells triggers the induction of genes associated with epithelial–mesenchymal transition in diabetes. *Mol. Metab.* **2021**, *53*, 101248. [CrossRef] [PubMed]
214. Liu, H.; Zhang, D.; Zhou, Y.; Cui, S. MicroRNA-7a inhibits Isl1 expression to regulate insulin secretion by targeting Raf1 and Mapkap1 in NIT-1 cells. *Vitr. Cell. Dev. Biol. Anim.* **2021**, *57*, 817–824. [CrossRef] [PubMed]
215. Shen, Z.; Yu, Y.; Yang, Y.; Xiao, X.; Sun, T.; Chang, X.; Tang, W.; Zhu, Y.; Han, X. miR-25 and miR-92b regulate insulin biosynthesis and pancreatic β-cell apoptosis. *Endocrine* **2022**, *76*, 526–535. [CrossRef] [PubMed]
216. Hu, D.; Wang, Y.; Zhang, H.; Kong, D. Identification of miR-9 as a negative factor of insulin secretion from beta cells. *J. Physiol. Biochem.* **2018**, *74*, 291–299. [CrossRef] [PubMed]
217. Li, L.; Wang, S.; Li, H.; Wan, J.; Zhou, Q.; Zhou, Y.; Zhang, C. microRNA-96 protects pancreatic β-cell function by targeting PAK1 in gestational diabetes mellitus. *Biofactors* **2018**, *44*, 539–547. [CrossRef] [PubMed]
218. Feng, Y.; Qu, X.; Chen, Y.; Feng, Q.; Zhang, Y.; Hu, J.; Li, X. MicroRNA-33a-5p sponges to inhibit pancreatic β-cell function in gestational diabetes mellitus LncRNA DANCR. *Reprod. Biol. Endocrinol.* **2020**, *18*, 1–9. [CrossRef]
219. Li, Y.; Yang, Y.; Sun, Y.; He, L.; Liu, K.; Zhang, Y.; Zhou, Y.; Chang, X.; Liang, R.; Wang, S. The miR-203/ZBTB20/MAFA Axis Orchestrates Pancreatic β-Cell Maturation and Identity During Weaning and Diabetes. *SSRN* **2023**, 4467623. [CrossRef]
220. Zhang, F.; Ma, D.; Zhao, W.; Wang, D.; Liu, T.; Liu, Y.; Yang, Y.; Liu, Y.; Mu, J.; Li, B.; et al. Obesity-induced overexpression of miR-802 impairs insulin transcription and secretion. *Nat. Commun.* **2020**, *11*, 1822. [CrossRef]
221. Mao, Y.; Schoenborn, J.; Wang, Z.; Chen, X.; Matson, K.; Mohan, R.; Zhang, S.; Tang, X.; Arunagiri, A.; Arvan, P.; et al. Transgenic overexpression of microRNA-30d in pancreatic beta-cells progressively regulates beta-cell function and identity. *Sci. Rep.* **2022**, *12*, 11969. [CrossRef] [PubMed]
222. Bai, C.; Ren, Q.; Liu, H.; Li, X.; Guan, W.; Gao, Y. miR-212/132-enriched extracellular vesicles promote differentiation of induced pluripotent stem cells into pancreatic beta cells. *Front. Cell Dev. Biol.* **2021**, *9*, 673231. [CrossRef] [PubMed]
223. Xu, Y.; Huang, Y.; Guo, Y.; Xiong, Y.; Zhu, S.; Xu, L.; Lu, J.; Li, X.; Wan, J.; Lu, Y.; et al. microRNA-690 regulates induced pluripotent stem cells (iPSCs) differentiation into insulin-producing cells by targeting Sox9. *Stem Cell Res. Ther.* **2019**, *10*, 1–13. [CrossRef] [PubMed]
224. Zhang, Y.; Cong, R.; Lv, T.; Liu, K.; Chang, X.; Li, Y.; Han, X.; Zhu, Y. Islet-resident macrophage-derived miR-155 promotes β cell decompensation via targeting PDX1. *iScience* **2024**, *27*, 109540. [CrossRef] [PubMed]
225. Sun, Y.; Zhou, S.; Shi, Y.; Zhou, Y.; Zhang, Y.; Liu, K.; Zhu, Y.; Han, X. Inhibition of miR-153, an IL-1β-responsive miRNA, prevents beta cell failure and inflammation-associated diabetes. *Metabolism* **2020**, *111*, 154335. [CrossRef] [PubMed]
226. Du, H.; Yin, Z.; Zhao, Y.; Li, H.; Dai, B.; Fan, J.; He, M.; Nie, X.; Wang, C.-Y.; Wang, D.W.; et al. miR-320a induces pancreatic β cells dysfunction in diabetes by inhibiting MafF. *Mol. Ther. Nucleic Acids* **2021**, *26*, 444–457. [CrossRef] [PubMed]
227. Wan, S.; Zhang, J.; Chen, X.; Lang, J.; Li, L.; Chen, F.; Tian, L.; Meng, Y.; Yu, X. MicroRNA-17-92 Regulates Beta-Cell Restoration After Streptozotocin Treatment. *Front. Endocrinol.* **2020**, *11*, 9. [CrossRef] [PubMed]
228. Ibrahim, S.M.; Mirmira, R.; Anderson, R.; Sims, E. MiR-21 Contributes to Cytokine-Induced Beta Cell Dysfunction via Inhibition of mRNAs Regulating Beta Cell Identity. *FASEB J.* **2019**, *33*, 694.13. [CrossRef]
229. Ibrahim, S. miR-21 Exacerbates Cytokine Induced Beta Cell Dysfunction via Inhibition of mRNAs Regulating Beta Cell Identity. Ph.D. Thesis, Indiana University-Purdue University Indianapolis, Indianapolis, IN, USA, 2020.
230. Grieco, G.E.; Brusco, N.; Fignani, D.; Nigi, L.; Formichi, C.; Licata, G.; Marselli, L.; Marchetti, P.; Salvini, L.; Tinti, L.; et al. Reduced miR-184-3p expression protects pancreatic β-cells from lipotoxic and proinflammatory apoptosis in type 2 diabetes via CRTC1 upregulation. *Cell Death Discov.* **2022**, *8*, 340. [CrossRef]
231. Sato-Kunisada, R.; Yoshida, N.; Nakamura, S.; Uchiyama, H.; Matsumoto, H. Enhanced Expression of miR-199b-5p Promotes Proliferation of Pancreatic.β-Cells by Down-Regulation of MLK3. *MicroRNA* **2016**, *5*, 57–65. [CrossRef]
232. Qian, B.; Yang, Y.; Tang, N.; Wang, J.; Sun, P.; Yang, N.; Chen, F.; Wu, T.; Sun, T.; Li, Y.; et al. M1 macrophage-derived exosomes impair beta cell insulin secretion via miR-212-5p by targeting SIRT2 and inhibiting Akt/GSK-3β/β-catenin pathway in mice. *Diabetologia* **2021**, *64*, 2037–2051. [CrossRef] [PubMed]
233. Eliasson, L. The small RNA miR-375—A pancreatic islet abundant miRNA with multiple roles in endocrine beta cell function. *Mol. Cell. Endocrinol.* **2017**, *456*, 95–101. [CrossRef]
234. Li, Y.; Xu, X.; Liang, Y.; Liu, S.; Xiao, H.; Li, F.; Cheng, H.; Fu, Z. miR-375 enhances palmitate-induced lipoapoptosis in insulin-secreting NIT-1 cells by repressing myotrophin (V1) protein expression. *Int. J. Clin. Exp. Pathol.* **2010**, *3*, 254–264.
235. Guo, C.; Sun, Y.-Q.; Li, Q.; Zhang, J.-C. MiR-7, miR-9 and miR-375 contribute to effect of Exendin-4 on pancreatic β-cells in high-fat-diet-fed mice. *Clin. Investig. Med.* **2018**, *41*, E16–E24. [CrossRef]
236. Lin, X.; Cheng, L.; Wan, Y.; Yan, Y.; Zhang, Z.; Li, X.; Wu, J.; Wang, X.; Xu, M. Ang II Controls the Expression of Mapkap1 by miR-375 and Affects the Function of Islet β Cells. *Endocrine, Metab. Immune Disord. Drug Targets* **2023**, *23*, 1186–1200. [CrossRef]
237. Lu, Z.; Wang, J.; Wang, X.; Li, Z.; Niu, D.; Wang, M.; Xiang, J.; Yue, Y.; Xia, Y.; Li, X. miR-375 Promotes Pancreatic Differentiation In Vitro by Affecting Different Target Genes at Different Stages. *Stem Cells Int.* **2021**, *2021*, 1–13. [CrossRef] [PubMed]
238. Keller, D.M.; Perez, I.G. Dual regulation of miR-375 and CREM genes in pancreatic beta cells. *Islets* **2022**, *14*, 139–148. [CrossRef] [PubMed]
239. Nathan, G.; Kredo-Russo, S.; Geiger, T.; Lenz, A.; Kaspi, H.; Hornstein, E.; Efrat, S. MiR-375 Promotes Redifferentiation of Adult Human β Cells Expanded In Vitro. *PLoS ONE* **2015**, *10*, e0122108. [CrossRef]

240. Title, A.C.; Silva, P.N.; Godbersen, S.; Hasenöhrl, L.; Stoffel, M. The miR-200–Zeb1 axis regulates key aspects of β-cell function and survival in vivo. *Mol. Metab.* **2021**, *53*, 101267. [CrossRef]
241. He, Q.; Song, J.; Cui, C.; Wang, J.; Hu, H.; Guo, X.; Yang, M.; Wang, L.; Yan, F.; Liang, K. Mesenchymal stem cell-derived exosomal miR-146a reverses diabetic β-cell dedifferentiation. *Stem Cell Res. Ther.* **2021**, *12*, 1–16. [CrossRef]
242. Xu, Y.; Tang, Z.; Dai, H.; Hou, J.; Li, F.; Tang, Z.; Zhang, D. MiR-195 promotes pancreatic β-cell dedifferentiation by targeting Mfn2 and impairing Pi3k/Akt signaling in type 2 diabetes. *Obesity* **2022**, *30*, 447–459. [CrossRef]
243. Sisino, G.; Zhou, A.-X.; Dahr, N.; Sabirsh, A.; Soundarapandian, M.M.; Perera, R.; Larsson-Lekholm, E.; Magnone, M.C.; Althage, M.; Tyrberg, B. Long noncoding RNAs are dynamically regulated during β-cell mass expansion in mouse pregnancy and control β-cell proliferation in vitro. *PLoS ONE* **2017**, *12*, e0182371. [CrossRef]
244. López–Noriega, L.; Rutter, G.A. Long Non-Coding RNAs as Key Modulators of Pancreatic β-Cell Mass and Function. *Front. Endocrinol.* **2021**, *11*, 610213. [CrossRef] [PubMed]
245. Ding, G.-L.; Wang, F.-F.; Shu, J.; Tian, S.; Jiang, Y.; Zhang, D.; Wang, N.; Luo, Q.; Zhang, Y.; Jin, F. Transgenerational glucose intolerance with Igf2/H19 epigenetic alterations in mouse islet induced by intrauterine hyperglycemia. *Diabetes* **2012**, *61*, 1133–1142. [CrossRef]
246. Sanchez-Parra, C.; Jacovetti, C.; Dumortier, O.; Lee, K.; Peyot, M.-L.; Guay, C.; Prentki, M.; Laybutt, D.R.; Van Obberghen, E.; Regazzi, R. Contribution of the Long Noncoding RNA H19 to β-Cell Mass Expansion in Neonatal and Adult Rodents. *Diabetes* **2018**, *67*, 2254–2267. [CrossRef]
247. Alfaifi, M.; Verma, A.K.; Alshahrani, M.Y.; Joshi, P.C.; Alkhathami, A.G.; Ahmad, I.; Hakami, A.R.; Beg, M.M.A. Assessment of Cell-Free Long Non-Coding RNA-H19 and miRNA-29a, miRNA-29b Expression and Severity of Diabetes. *Diabetes Metab. Syndr. Obes. Targets Ther.* **2020**, *13*, 3727–3737. [CrossRef] [PubMed]
248. Zhou, A.-X.; Mondal, T.; Tabish, A.M.; Abadpour, S.; Ericson, E.; Smith, D.M.; Knöll, R.; Scholz, H.; Kanduri, C.; Tyrberg, B.; et al. The long noncoding RNA *TUNAR* modulates Wnt signaling and regulates human β-cell proliferation. *Am. J. Physiol. Metab.* **2021**, *320*, E846–E857. [CrossRef]
249. You, L.; Wang, N.; Yin, D.; Wang, L.; Jin, F.; Zhu, Y.; Yuan, Q.; De, W. Downregulation of Long Noncoding RNA Meg3 Affects Insulin Synthesis and Secretion in Mouse Pancreatic Beta Cells. *J. Cell. Physiol.* **2015**, *231*, 852–862. [CrossRef] [PubMed]
250. Wang, N.; Zhu, Y.; Xie, M.; Wang, L.; Jin, F.; Li, Y.; Yuan, Q.; De, W. Long Noncoding RNA Meg3 Regulates Mafa Expression in Mouse Beta Cells by Inactivating Rad21, Smc3 or Sin3α. *Cell. Physiol. Biochem.* **2018**, *45*, 2031–2043. [CrossRef] [PubMed]
251. Zhang, F.; Yang, Y.; Chen, X.; Liu, Y.; Hu, Q.; Huang, B.; Liu, Y.; Pan, Y.; Zhang, Y.; Liu, D.; et al. The long non-coding RNA βFaar regulates islet β-cell function and survival during obesity in mice. *Nat. Commun.* **2021**, *12*, 3997. [CrossRef]
252. Rashidmayvan, M.; Sahebi, R.; Ghayour-Mobarhan, M. Long non-coding RNAs: A valuable biomarker for metabolic syndrome. *Mol. Genet. Genom.* **2022**, *297*, 1169–1183. [CrossRef] [PubMed]
253. Huang, Y.; Xu, Y.; Lu, Y.; Zhu, S.; Guo, Y.; Sun, C.; Xu, L.; Chen, X.; Zhao, Y.; Yu, B. lncRNA Gm10451 regulates PTIP to facilitate iPSCs-derived β-like cell differentiation by targeting miR-338-3p as a ceRNA. *Biomaterials* **2019**, *216*, 119266. [CrossRef] [PubMed]
254. Zhang, F.F.; Liu, Y.H.; Wang, D.W.; Liu, T.S.; Yang, Y.; Guo, J.M.; Pan, Y.; Zhang, Y.F.; Du, H.; Li, L.; et al. Obesity-induced reduced expression of the lncRNA ROIT impairs insulin transcription by downregulation of Nkx6.1 methylation. *Diabetologia* **2020**, *63*, 811–824. [CrossRef] [PubMed]
255. Ruan, Y.; Lin, N.; Ma, Q.; Chen, R.; Zhang, Z.; Wen, W.; Chen, H.; Sun, J. Circulating LncRNAs Analysis in Patients with Type 2 Diabetes Reveals Novel Genes Influencing Glucose Metabolism and Islet β-Cell Function. *Cell. Physiol. Biochem.* **2018**, *46*, 335–350. [CrossRef] [PubMed]
256. Cao, Z.; Yao, F.; Lang, Y.; Feng, X. Elevated Circulating LINC-P21 Serves as a Diagnostic Biomarker of Type 2 Diabetes Mellitus and Regulates Pancreatic β-cell Function by Sponging miR-766-3p to Upregulate NR3C2. *Exp. Clin. Endocrinol. Diabetes* **2020**, *130*, 156–164. [CrossRef] [PubMed]
257. Wang, L.; Hu, L.; Wang, X.; Geng, Z.; Wan, M.; Hao, J.; Liu, H.; Fan, Y.; Xu, T.; Li, Z. Long non-coding RNA LncCplx2 regulates glucose homeostasis and pancreatic β cell function. *Mol. Metab.* **2024**, *80*, 101878. [CrossRef] [PubMed]
258. Ding, H.; Wang, F.; Shi, X.; Ma, H.; Du, Y.; Hou, L.; Xing, N. LncRNA MALAT1 induces the dysfunction of β cells via reducing the histone acetylation of the PDX-1 promoter in type 1 diabetes. *Exp. Mol. Pathol.* **2020**, *114*, 104432. [CrossRef] [PubMed]
259. Luo, Y.; Guo, J.; Xu, P.; Gui, R. Long non-coding RNA GAS5 maintains insulin secretion by regulating multiple miRNAs in INS-1 832/13 cells. *Front. Mol. Biosci.* **2020**, *7*, 559267. [CrossRef] [PubMed]
260. Jin, F.; Wang, N.; Zhu, Y.; You, L.; Wang, L.; De, W.; Tang, W. Downregulation of Long Noncoding RNA Gas5 Affects Cell Cycle and Insulin Secretion in Mouse Pancreatic β Cells. *Cell. Physiol. Biochem.* **2017**, *43*, 2062–2073. [CrossRef]
261. Liu, L.; Li, Y.; Zhang, X. LncRNA LINC01018 Screens Type 2 Diabetes Mellitus and Regulates β Cell Function Through Modulating miR-499a-5p. *Horm. Metab. Res.* **2023**, *55*, 642–648. [CrossRef]
262. Pardo, C.A.C.; Massoz, L.; Dupont, M.A.; Bergemann, D.; Bourdouxhe, J.; Lavergne, A.; Tarifeño-Saldivia, E.; Helker, C.S.; Stainier, D.Y.; Peers, B.; et al. A δ-cell subpopulation with a pro-β-cell identity contributes to efficient age-independent recovery in a zebrafish model of diabetes. *eLife* **2022**, *11*, e67576. [CrossRef] [PubMed]
263. Singh, S.P.; Chawla, P.; Hnatiuk, A.; Kamel, M.; Silva, L.D.; Spanjaard, B.; Eski, S.E.; Janjuha, S.; Olivares-Chauvet, P.; Kayisoglu, O. A single-cell atlas of de novo β-cell regeneration reveals the contribution of hybrid β/δ-cells to diabetes recovery in zebrafish. *Development* **2022**, *149*, dev199853. [CrossRef] [PubMed]

264. Zhao, H.; Huang, X.; Liu, Z.; Pu, W.; Lv, Z.; He, L.; Li, Y.; Zhou, Q.; Lui, K.O.; Zhou, B. Pre-existing beta cells but not progenitors contribute to new beta cells in the adult pancreas. *Nat. Metab.* **2021**, *3*, 352–365. [CrossRef]
265. Piran, R.; Lee, S.-H.; Li, C.-R.; Charbono, A.; Bradley, L.M.; Levine, F. Pharmacological induction of pancreatic islet cell transdifferentiation: Relevance to type I diabetes. *Cell Death Dis.* **2014**, *5*, e1357. [CrossRef] [PubMed]
266. Chera, S.; Baronnier, D.; Ghila, L.; Cigliola, V.; Jensen, J.N.; Gu, G.; Furuyama, K.; Thorel, F.; Gribble, F.M.; Reimann, F.; et al. Diabetes recovery by age-dependent conversion of pancreatic δ-cells into insulin producers. *Nature* **2014**, *514*, 503–507. [CrossRef]
267. Bru-Tari, E.; Cobo-Vuilleumier, N.; Alonso-Magdalena, P.; Dos Santos, R.S.; Marroqui, L.; Nadal, A.; Gauthier, B.R.; Quesada, I. Pancreatic alpha-cell mass in the early-onset and advanced stage of a mouse model of experimental autoimmune diabetes. *Sci. Rep.* **2019**, *9*, 9515. [CrossRef] [PubMed]
268. Hang, Y.; Stein, R. MafA and MafB activity in pancreatic β cells. *Trends Endocrinol. Metab.* **2011**, *22*, 364–373. [CrossRef] [PubMed]
269. Furuyama, K.; Chera, S.; van Gurp, L.; Oropeza, D.; Ghila, L.; Damond, N.; Vethe, H.; Paulo, J.A.; Joosten, A.M.; Berney, T. Diabetes relief in mice by glucose-sensing insulin-secreting human α-cells. *Nature* **2019**, *567*, 43–48. [CrossRef] [PubMed]
270. Yu, J.; Ma, J.; Li, Y.; Zhou, Y.; Luo, L.; Yang, Y. Pax4-Ghrelin mediates the conversion of pancreatic ε-cells to β-cells after extreme β-cell loss in zebrafish. *Development* **2023**, *150*, dev201306. [CrossRef]
271. Sasaki, S.; Lee, M.Y.Y.; Wakabayashi, Y.; Suzuki, L.; Winata, H.; Himuro, M.; Matsuoka, T.-A.; Shimomura, I.; Watada, H.; Lynn, F.C.; et al. Spatial and transcriptional heterogeneity of pancreatic beta cell neogenesis revealed by a time-resolved reporter system. *Diabetologia* **2022**, *65*, 811–828. [CrossRef]
272. Huang, H.; Yang, K.; Wang, R.; Han, W.H.; Kuny, S.; Zelmanovitz, P.H.; Sauvé, Y.; Chan, C.B. β-Cell compensation concomitant with adaptive endoplasmic reticulum stress and β-cell neogenesis in a diet-induced type 2 diabetes model. *Appl. Physiol. Nutr. Metab.* **2019**, *44*, 1355–1366. [CrossRef]
273. Gribben, C.; Lambert, C.; Messal, H.A.; Hubber, E.-L.; Rackham, C.; Evans, I.; Heimberg, H.; Jones, P.; Sancho, R.; Behrens, A. Ductal Ngn3-expressing progenitors contribute to adult β cell neogenesis in the pancreas. *Cell Stem Cell* **2021**, *28*, 2000–2008.e4. [CrossRef] [PubMed]
274. Karampelias, C.; Watt, K.; Mattsson, C.L.; Ruiz, F.; Rezanejad, H.; Mi, J.; Liu, X.; Chu, L.; Locasale, J.W.; Korbutt, G.S.; et al. MNK2 deficiency potentiates β-cell regeneration via translational regulation. *Nat. Chem. Biol.* **2022**, *18*, 942–953. [CrossRef] [PubMed]
275. Zhang, J.; An, H.; Ni, K.; Chen, B.; Li, H.; Li, Y.; Sheng, G.; Zhou, C.; Xie, M.; Chen, S.; et al. Glutathione prevents chronic oscillating glucose intake-induced β-cell dedifferentiation and failure. *Cell Death Dis.* **2019**, *10*, 321. [CrossRef]
276. John, A.N.; Iqbal, Z.; Colley, S.; Morahan, G.; Makishima, M.; Jiang, F.-X. Vitamin D receptor-targeted treatment to prevent pathological dedifferentiation of pancreatic β cells under hyperglycaemic stress. *Diabetes Metab.* **2018**, *44*, 269–280. [CrossRef]
277. Xing, C.; Tang, M.; Yang, J.; Wang, S.; Xu, Q.; Feng, W.; Mu, Y.; Li, F.; Zhao, A.Z. Eicosapentaenoic acid metabolites promotes the trans-differentiation of pancreatic α cells to β cells. *Biochem. Pharmacol.* **2023**, *216*, 115775. [CrossRef] [PubMed]
278. Karampelias, C.; Rezanejad, H.; Rosko, M.; Duan, L.; Lu, J.; Pazzagli, L.; Bertolino, P.; Cesta, C.E.; Liu, X.; Korbutt, G.S.; et al. Reinforcing one-carbon metabolism via folic acid/Folr1 promotes β-cell differentiation. *Nat. Commun.* **2021**, *12*, 3362. [CrossRef]
279. Kaya-Dagistanli, F.; Ozturk, M. Transdifferentiation of both intra- and extra-islet cells into beta cells in nicotinamide treated neonatal diabetic rats: An in situ hybridization and double immunohistochemical study. *Acta Histochem.* **2020**, *122*, 151612. [CrossRef] [PubMed]
280. Cristelo, C.; Nunes, R.; Pinto, S.; Marques, J.M.; Gama, F.M.; Sarmento, B. Targeting β Cells with Cathelicidin Nanomedicines Improves Insulin Function and Pancreas Regeneration in Type 1 Diabetic Rats. *ACS Pharmacol. Transl. Sci.* **2023**, *6*, 1544–1560. [CrossRef]
281. Patel, S.; Yan, Z.; Remedi, M.S. Intermittent fasting protects β-cell identity and function in a type-2 diabetes model. *Metabolism* **2024**, *153*, 155813. [CrossRef]
282. Li, C.; Zhu, J.; Wei, S.; Ye, X.; Yang, L.; Wang, Z.; Chen, Y. Intermittent protein restriction improves glucose homeostasis in Zucker diabetic fatty rats and single-cell sequencing reveals distinct changes in β cells. *J. Nutr. Biochem.* **2023**, *114*, 109275. [CrossRef]
283. Zhang, M.; Chen, Y.; Sun, S.; Zhang, S.; Yuan, L.; Xu, Y.; Li, X.; Chen, G.; Wei, X.; Liu, C. Ketogenic diet alleviates β-cell dedifferentiation but aggravates hepatic lipid accumulation in db/db mice. *Nutrition* **2024**, *119*, 112284. [CrossRef] [PubMed]
284. Luo, W.; Zhou, J.; Yang, X.; Wu, R.; Liu, H.; Shao, H.; Huang, B.; Kang, X.; Yang, L.; Liu, D. A Chinese medical nutrition therapy diet accompanied by intermittent energy restriction alleviates type 2 diabetes by enhancing pancreatic islet function and regulating gut microbiota composition. *Food Res. Int.* **2022**, *161*, 111744. [CrossRef]
285. Lv, C.; Sun, Y.; Zhang, Z.Y.; Aboelela, Z.; Qiu, X.; Meng, Z.-X. β-cell dynamics in type 2 diabetes and in dietary and exercise interventions. *J. Mol. Cell Biol.* **2022**, *14*, mjac046. [CrossRef]
286. Lyngbaek, M.P.P.; Legaard, G.E.; Bennetsen, S.L.; Feineis, C.S.; Rasmussen, V.; Moegelberg, N.; Brinkløv, C.F.; Nielsen, A.B.; Kofoed, K.S.; Lauridsen, C.A.; et al. The effects of different doses of exercise on pancreatic β-cell function in patients with newly diagnosed type 2 diabetes: Study protocol for and rationale behind the "DOSE-EX" multi-arm parallel-group randomised clinical trial. *Trials* **2021**, *22*, 244. [CrossRef]
287. Chow, L.S.; Gerszten, R.E.; Taylor, J.M.; Pedersen, B.K.; Van Praag, H.; Trappe, S.; Febbraio, M.A.; Galis, Z.S.; Gao, Y.; Haus, J.M. Exerkines in health, resilience and disease. *Nat. Rev. Endocrinol.* **2022**, *18*, 273–289. [CrossRef] [PubMed]
288. Wedell-Neergaard, A.S.; Lehrskov, L.L.; Christensen, R.H.; Legaard, G.E.; Dorph, E.; Larsen, M.K.; Launbo, N.; Fagerlind, S.R.; Seide, S.K.; Nymand, S.; et al. Exercise-induced changes in visceral adipose tissue mass are regulated by IL-6 signaling: A randomized controlled trial. *Yearb. Paediatr. Endocrinol.* **2019**, *29*, 844–855. [CrossRef]

289. Lehrskov, L.L.; Lyngbaek, M.P.; Soederlund, L.; Legaard, G.E.; Ehses, J.A.; Heywood, S.E.; Albrechtsen, N.J.W.; Holst, J.J.; Karstoft, K.; Pedersen, B.K.; et al. Interleukin-6 Delays Gastric Emptying in Humans with Direct Effects on Glycemic Control. *Cell Metab.* **2018**, *27*, 1201–1211.e3. [CrossRef]
290. Rutti, S.; Dusaulcy, R.; Hansen, J.S.; Howald, C.; Dermitzakis, E.T.; Pedersen, B.K.; Pinget, M.; Plomgaard, P.; Bouzakri, K. Angiogenin and Osteoprotegerin are type II muscle specific myokines protecting pancreatic beta-cells against proinflammatory cytokines. *Sci. Rep.* **2018**, *8*, 10072. [CrossRef] [PubMed]
291. Becic, T.; Studenik, C.; Hoffmann, G. Exercise Increases Adiponectin and Reduces Leptin Levels in Prediabetic and Diabetic Individuals: Systematic Review and Meta-Analysis of Randomized Controlled Trials. *Med. Sci.* **2018**, *6*, 97. [CrossRef]
292. Ohashi, K.; Parker, J.L.; Ouchi, N.; Higuchi, A.; Vita, J.A.; Gokce, N.; Pedersen, A.A.; Kalthoff, C.; Tullin, S.; Sams, A.; et al. Adiponectin Promotes Macrophage Polarization toward an Anti-inflammatory Phenotype. *J. Biol. Chem.* **2010**, *285*, 6153–6160. [CrossRef] [PubMed]
293. Fulgenzi, G.; Hong, Z.; Tomassoni-Ardori, F.; Barella, L.F.; Becker, J.; Barrick, C.; Swing, D.; Yanpallewar, S.; Croix, B.S.; Wess, J.; et al. Novel metabolic role for BDNF in pancreatic β-cell insulin secretion. *Nat. Commun.* **2020**, *11*, 1950. [CrossRef] [PubMed]
294. Strömberg, A.; Olsson, K.; Dijksterhuis, J.P.; Rullman, E.; Schulte, G.; Gustafsson, T. CX_3CL1—A macrophage chemoattractant induced by a single bout of exercise in human skeletal muscle. *Am. J. Physiol. Integr. Comp. Physiol.* **2016**, *310*, R297–R304. [CrossRef] [PubMed]
295. Liu, S.; Du, F.; Li, X.; Wang, M.; Duan, R.; Zhang, J.; Wu, Y.; Zhang, Q. Effects and underlying mechanisms of irisin on the proliferation and apoptosis of pancreatic β cells. *PLoS ONE* **2017**, *12*, e0175498. [CrossRef] [PubMed]
296. Natalicchio, A.; Marrano, N.; Biondi, G.; Spagnuolo, R.; Labarbuta, R.; Porreca, I.; Cignarelli, A.; Bugliani, M.; Marchetti, P.; Perrini, S.; et al. The Myokine Irisin Is Released in Response to Saturated Fatty Acids and Promotes Pancreatic β-Cell Survival and Insulin Secretion. *Diabetes* **2017**, *66*, 2849–2856. [CrossRef] [PubMed]
297. Wei, J.; Hanna, T.; Suda, N.; Karsenty, G.; Ducy, P. Osteocalcin Promotes β-Cell Proliferation During Development and Adulthood Through Gprc6a. *Diabetes* **2014**, *63*, 1021–1031. [CrossRef] [PubMed]
298. Kover, K.; Yan, Y.; Tong, P.Y.; Watkins, D.; Li, X.; Tasch, J.; Hager, M.; Clements, M.; Moore, W.V. Osteocalcin protects pancreatic beta cell function and survival under high glucose conditions. *Biochem. Biophys. Res. Commun.* **2015**, *462*, 21–26. [CrossRef] [PubMed]
299. Cui, X.; Feng, J.; Wei, T.; Zhang, L.; Lang, S.; Yang, K.; Yang, J.; Liu, J.; Sterr, M.; Lickert, H.; et al. Pancreatic alpha cell glucagon–liver FGF21 axis regulates beta cell regeneration in a mouse model of type 2 diabetes. *Diabetologia* **2022**, *66*, 535–550. [CrossRef] [PubMed]
300. Davey, J.R.; Estevez, E.; Thomson, R.E.; Whitham, M.; Watt, K.I.; Hagg, A.; Qian, H.; Henstridge, D.C.; Ludlow, H.; Hedger, M.P.; et al. Intravascular Follistatin gene delivery improves glycemic control in a mouse model of type 2 diabetes. *FASEB J.* **2020**, *34*, 5697–5714. [CrossRef]
301. Curran, M.; Drayson, M.T.; Andrews, R.C.; Zoppi, C.; Barlow, J.P.; Solomon, T.P.; Narendran, P. The benefits of physical exercise for the health of the pancreatic β-cell: A review of the evidence. *Exp. Physiol.* **2020**, *105*, 579–589. [CrossRef]
302. Tanday, N.; Irwin, N.; Moffett, R.C.; Flatt, P.R.; O'Harte, F.P.M. Beneficial actions of a long-acting apelin analogue in diabetes are related to positive effects on islet cell turnover and transdifferentiation. *Diabetes Obes. Metab.* **2020**, *22*, 2468–2478. [CrossRef]
303. Li, H.-Q.; Spitzer, N.C. Exercise enhances motor skill learning by neurotransmitter switching in the adult midbrain. *Nat. Commun.* **2020**, *11*, 2195. [CrossRef] [PubMed]
304. Ben-Othman, N.; Vieira, A.; Courtney, M.; Record, F.; Gjernes, E.; Avolio, F.; Hadzic, B.; Druelle, N.; Napolitano, T.; Navarro-Sanz, S.; et al. Long-Term GABA Administration Induces Alpha Cell-Mediated Beta-like Cell Neogenesis. *Cell* **2016**, *168*, 73–85.e11. [CrossRef]
305. Li, J.; Casteels, T.; Frogne, T.; Ingvorsen, C.; Honoré, C.; Courtney, M.; Huber, K.V.; Schmitner, N.; Kimmel, R.A.; Romanov, R.A.; et al. Artemisinins Target GABAA Receptor Signaling and Impair α Cell Identity. *Cell* **2016**, *168*, 86–100.e15. [CrossRef]
306. Maykish, A.; Sikalidis, A.K. Utilization of Hydroxyl-Methyl Butyrate, Leucine, Glutamine and Arginine Supplementation in Nutritional Management of Sarcopenia—Implications and Clinical Considerations for Type 2 Diabetes Mellitus Risk Modulation. *J. Pers. Med.* **2020**, *10*, 19. [CrossRef]
307. Tanday, N.; Flatt, P.R.; Irwin, N.; Moffett, R.C. Liraglutide and sitagliptin counter beta- to alpha-cell transdifferentiation in diabetes. *J. Endocrinol.* **2020**, *245*, 53–64. [CrossRef]
308. Tanday, N.; Irwin, N.; Flatt, P.R.; Moffett, R.C. Dapagliflozin exerts positive effects on beta cells, decreases glucagon and does not alter beta-to alpha-cell transdifferentiation in mouse models of diabetes and insulin resistance. *Biochem. Pharmacol.* **2020**, *177*, 114009. [CrossRef]
309. Takahashi, K.; Nakamura, A.; Miyoshi, H.; Nomoto, H.; Kitao, N.; Omori, K.; Yamamoto, K.; Cho, K.Y.; Terauchi, Y.; Atsumi, T. Effect of the sodium–glucose cotransporter 2 inhibitor luseogliflozin on pancreatic beta cell mass in db/db mice of different ages. *Sci. Rep.* **2018**, *8*, 6864. [CrossRef] [PubMed]
310. Patel, R.; Parmar, N.; Rathwa, N.; Palit, S.P.; Li, Y.; Garcia-Ocaña, A.; Begum, R. A novel therapeutic combination of sitagliptin and melatonin regenerates pancreatic β-cells in mouse and human islets. *Biochim. Biophys. Acta (BBA) Mol. Cell Res.* **2022**, *1869*, 119263. [CrossRef]
311. Moin, A.S.M.; Butler, A.E. Alterations in Beta Cell Identity in Type 1 and Type 2 Diabetes. *Curr. Diabetes Rep.* **2019**, *19*, 83. [CrossRef] [PubMed]

312. Téllez, N.; Montanya, E. Gastrin induces ductal cell dedifferentiation and b-cell neogenesis after 90% pancreatectomy. *J. Endocrinol.* **2014**, *223*, 67–78. [CrossRef] [PubMed]
313. Kucukbas, G.N.; Komuroglu, A.U.; Dirik, D.; Korpe, B.; Kose, C.; Karaaslan, O.; Dirik, Y.; Sahin, H.G. Maternal serum xenin-25 levels in gestational diabetes mellitus. *Eur. Rev. Med. Pharmacol. Sci.* **2023**, *27*, 9902–9907. [CrossRef] [PubMed]
314. Michele, L.A.; Mantzouratou, P.; Cerullo, D.; Sangalli, F.; Corna, D.; Zoja, C.; Remuzzi, G.; Xinaris, C. Thyroid hormone signalling regulates beta-cell pathological growth, dedifferentiation and transdifferentiation in diabetic pancreas. *Endocr. Abstr.* **2023**, *92*, OP13-03. [CrossRef]
315. Guo, D.; He, J.; Guo, H.; Song, G.; Peng, L.; An, M.; Wang, C. Angiotensin (1–7) reverses glucose-induced islet β cell dedifferentiation by Wnt/β-catenin/FoxO1 signalling pathway. *Endokrynol. Pol.* **2023**, *74*, 544–552. [CrossRef] [PubMed]
316. Gómez-Banoy, N.; Guseh, J.S.; Li, G.; Rubio-Navarro, A.; Chen, T.; Poirier, B.; Putzel, G.; Rosselot, C.; Pabón, M.A.; Camporez, J.P.; et al. Adipsin preserves beta cells in diabetic mice and associates with protection from type 2 diabetes in humans. *Nat. Med.* **2019**, *25*, 1739–1747. [CrossRef] [PubMed]
317. Lei, L.; Huan, Y.; Liu, Q.; Li, C.; Cao, H.; Ji, W.; Gao, X.; Fu, Y.; Li, P.; Zhang, R. Morus alba L.(Sangzhi) alkaloids promote insulin secretion, restore diabetic β-cell function by preventing dedifferentiation and apoptosis. *Front. Pharmacol.* **2022**, *13*, 841981. [CrossRef] [PubMed]
318. Gojani, E.G.; Wang, B.; Li, D.-P.; Kovalchuk, O.; Kovalchuk, I. Anti-Inflammatory Properties of Eugenol in Lipopolysaccharide-Induced Macrophages and Its Role in Preventing β-Cell Dedifferentiation and Loss Induced by High Glucose-High Lipid Conditions. *Molecules* **2023**, *28*, 7619. [CrossRef] [PubMed]
319. Yuan, Y.; Zhou, J.; Hu, R.; Zou, L.; Ji, L.; Jiang, G. Piperine protects against pancreatic β-cell dysfunction by alleviating macrophage inflammation in obese mice. *Life Sci.* **2021**, *274*, 119312. [CrossRef] [PubMed]
320. Gojani, E.G.; Wang, B.; Li, D.-P.; Kovalchuk, O.; Kovalchuk, I. The Impact of Psilocybin on High Glucose/Lipid-Induced Changes in INS-1 Cell Viability and Dedifferentiation. *Genes* **2024**, *15*, 183. [CrossRef]
321. Gojani, E.G.; Wang, B.; Li, D.-P.; Kovalchuk, O.; Kovalchuk, I. Effect of THC, CBD, THCV, CBC and CBN Cannabinoids on β-Cells Exposed to High Glucose—High Lipids. *Preprints* **2023**, 2023090973. [CrossRef]
322. Kannan, P.; Raghunathan, M.; Mohan, T.; Palanivelu, S.; Periandavan, K. Gymnemic Acid Ameliorates Pancreatic β-Cell Dysfunction by Modulating Pdx1 Expression: A Possible Strategy for β-Cell Regeneration. *Tissue Eng. Regen. Med.* **2022**, *19*, 603–616. [CrossRef]
323. Moosavi, A.; Yazdanparast, R. Beta cell regeneration upon magainin and growth hormone treatment as a possible alternative to insulin therapy. *FEBS Open Bio.* **2023**, *13*, 447–458. [CrossRef] [PubMed]
324. Zhong, Q.; Yin, J.; Wang, K.; Chen, X.; Wang, H.; Hu, X.; Wang, W.; Wang, L.; Bei, W.; Guo, J. FTZ promotes islet β-cell regeneration in T1DM mice via the regulation of nuclear proliferation factors. *J. Ethnopharmacol.* **2023**, *315*, 116564. [CrossRef]
325. Martínez-Montoro, J.I.; Damas-Fuentes, M.; Fernández-García, J.C.; Tinahones, F.J. Role of the Gut Microbiome in Beta Cell and Adipose Tissue Crosstalk: A Review. *Front. Endocrinol.* **2022**, *13*, 869951. [CrossRef]
326. Wang, S.; Yuan, M.; Zhang, L.; Zhu, K.; Sheng, C.; Zhou, F.; Xu, Z.; Liu, Q.; Liu, Y.; Lu, J.; et al. Sodium butyrate potentiates insulin secretion from rat islets at the expense of compromised expression of β cell identity genes. *Cell Death Dis.* **2022**, *13*, 67. [CrossRef] [PubMed]
327. Pedersen, S.S.; Prause, M.; Williams, K.; Barrès, R.; Billestrup, N. Butyrate inhibits IL-1β-induced inflammatory gene expression by suppression of NF-κB activity in pancreatic beta cells. *J. Biol. Chem.* **2022**, *298*, 102312. [CrossRef]
328. Vandenbempt, V.; Eski, S.E.; Brahma, M.K.; Li, A.; Negueruela, J.; Bruggeman, Y.; Demine, S.; Xiao, P.; Cardozo, A.K.; Baeyens, N.; et al. HAMSAB diet ameliorates dysfunctional signaling in pancreatic islets in autoimmune diabetes. *iScience* **2024**, *27*, 108694. [CrossRef] [PubMed]
329. Liang, Y.; Widjaja, J.; Sun, J.; Li, M.; Qiao, Z.; Cao, T.; Wang, Y.; Zhang, X.; Zhang, Z.; Gu, Y.; et al. Bariatric surgery induces pancreatic cell transdifferentiation as indicated by single-cell transcriptomics in Zucker diabetic rats. *J. Diabetes* **2023**. [CrossRef]
330. Koduru, S.V.; Leberfinger, A.N.; Ozbolat, I.T.; Ravnic, D.J. Navigating the Genomic Landscape of Human Adipose Stem Cell-Derived β-Cells. *Stem Cells Dev.* **2021**, *30*, 1153–1170. [CrossRef]
331. Kamal, M.M.; Ammar, R.A.; Kassem, D.H. Silencing of forkhead box protein O-1 (FOXO-1) enhances insulin-producing cell generation from adipose mesenchymal stem cells for diabetes therapy. *Life Sci.* **2024**, *344*, 122579. [CrossRef]
332. Zaeifi, D.; Azarnia, M. Promoting β-cells function by the recapitulation of in vivo microenvironmental differentiation signals. *Cell Tissue Res.* **2023**, *393*, 37–46. [CrossRef] [PubMed]
333. Zhu, S.; Russ, H.A.; Wang, X.; Zhang, M.; Ma, T.; Xu, T.; Tang, S.; Hebrok, M.; Ding, S. Human pancreatic beta-like cells converted from fibroblasts. *Nat. Commun.* **2016**, *7*, 10080. [CrossRef] [PubMed]
334. Wang, L.; Liu, T.; Liang, R.; Wang, G.; Liu, Y.; Zou, J.; Liu, N.; Zhang, B.; Liu, Y.; Ding, X.; et al. Mesenchymal stem cells ameliorate β cell dysfunction of human type 2 diabetic islets by reversing β cell dedifferentiation. *EBioMedicine* **2020**, *51*, 102615. [CrossRef]
335. Isildar, B.; Ozkan, S.; Ercin, M.; Gezginci-Oktayoglu, S.; Oncul, M.; Koyuturk, M. 2D and 3D cultured human umbilical cord-derived mesenchymal stem cell-conditioned medium has a dual effect in type 1 diabetes model in rats: Immunomodulation and beta-cell regeneration. *Inflamm. Regen.* **2022**, *42*, 55. [CrossRef] [PubMed]
336. Goode, R.A.; Hum, J.M.; Kalwat, M.A. Therapeutic Strategies Targeting Pancreatic Islet β-Cell Proliferation, Regeneration, and Replacement. *Endocrinology* **2023**, *164*, bqac193. [CrossRef] [PubMed]

337. Basile, G.; Qadir, M.; Mauvais-Jarvis, F.; Vetere, A.; Shoba, V.; Modell, A.; Pastori, R.; Russ, H.; Wagner, B.; Dominguez-Bendala, J. Emerging diabetes therapies: Bringing back the β-cells. *Mol. Metab.* **2022**, *60*, 101477. [CrossRef] [PubMed]
338. Wang, P.; Karakose, E.; Argmann, C.; Wang, H.; Balev, M.; Brody, R.I.; Rivas, H.G.; Liu, X.; Wood, O.; Liu, H.; et al. Disrupting the DREAM complex enables proliferation of adult human pancreatic β cells. *J. Clin. Investig.* **2022**, *132*, e157086. [CrossRef] [PubMed]
339. Barzowska, A.; Pucelik, B.; Pustelny, K.; Matsuda, A.; Martyniak, A.; Stępniewski, J.; Maksymiuk, A.; Dawidowski, M.; Rothweiler, U.; Dulak, J.; et al. DYRK1A Kinase Inhibitors Promote β-Cell Survival and Insulin Homeostasis. *Cells* **2021**, *10*, 2263. [CrossRef]
340. Guo, Y.; Li, L.; Yao, Y.; Li, H. Regeneration of Pancreatic β-Cells for Diabetes Therapeutics by Natural DYRK1A Inhibitors. *Metabolites* **2022**, *13*, 51. [CrossRef]
341. Czarna, A.; Wang, J.; Zelencova, D.; Liu, Y.; Deng, X.; Choi, H.G.; Zhang, T.; Zhou, W.; Chang, J.W.; Kildalsen, H.; et al. Novel Scaffolds for Dual Specificity Tyrosine-Phosphorylation-Regulated Kinase (DYRK1A) Inhibitors. *J. Med. Chem.* **2018**, *61*, 7560–7572. [CrossRef]
342. Ackeifi, C.; Wang, P.; Karakose, E.; Fox, J.E.M.; González, B.J.; Liu, H.; Wilson, J.; Swartz, E.; Berrouet, C.; Li, Y.; et al. GLP-1 receptor agonists synergize with DYRK1A inhibitors to potentiate functional human β cell regeneration. *Sci. Transl. Med.* **2020**, *12*. [CrossRef] [PubMed]
343. Kimani, C.N.; Reuter, H.; Kotzé, S.H.; Venter, P.; Ramharack, P.; Muller, C.J.F. Pancreatic beta cell regenerative potential of Zanthoxylum chalybeum Engl. Aqueous stem bark extract. *J. Ethnopharmacol.* **2024**, *320*, 117374. [CrossRef]
344. Gu, L.; Cui, X.; Lang, S.; Wang, H.; Hong, T.; Wei, R. Glucagon receptor antagonism increases mouse pancreatic δ-cell mass through cell proliferation and duct-derived neogenesis. *Biochem. Biophys. Res. Commun.* **2019**, *512*, 864–870. [CrossRef] [PubMed]
345. Cui, X.; Feng, J.; Wei, T.; Gu, L.; Wang, D.; Lang, S.; Yang, K.; Yang, J.; Yan, H.; Wei, R.; et al. Pro-α-cell-derived β-cells contribute to β-cell neogenesis induced by antagonistic glucagon receptor antibody in type 2 diabetic mice. *iScience* **2022**, *25*, 104567. [CrossRef]
346. Wang, M.-Y.; Dean, E.D.; Quittner-Strom, E.; Zhu, Y.; Chowdhury, K.H.; Zhang, Z.; Zhao, S.; Li, N.; Ye, R.; Lee, Y.; et al. Glucagon blockade restores functional β-cell mass in type 1 diabetic mice and enhances function of human islets. *Proc. Natl. Acad. Sci. USA* **2021**, *118*, e2022142118. [CrossRef]
347. Lang, S.; Yang, J.; Yang, K.; Gu, L.; Cui, X.; Wei, T.; Liu, J.; Le, Y.; Wang, H.; Wei, R.; et al. Glucagon receptor antagonist upregulates circulating GLP-1 level by promoting intestinal L-cell proliferation and GLP-1 production in type 2 diabetes. *BMJ Open Diabetes Res. Care* **2020**, *8*, e001025. [CrossRef]
348. Lang, S.; Wei, R.; Wei, T.; Gu, L.; Feng, J.; Yan, H.; Yang, J.; Hong, T. Glucagon receptor antagonism promotes the production of gut proglucagon-derived peptides in diabetic mice. *Peptides* **2020**, *131*, 170349. [CrossRef]
349. Zhang, Z.; Hu, Y.; Xu, N.; Zhou, W.; Yang, L.; Chen, R.; Yang, R.; Sun, J.; Chen, H. A New Way for Beta Cell Neogenesis: Transdifferentiation from Alpha Cells Induced by Glucagon-Like Peptide 1. *J. Diabetes Res.* **2019**, *2019*, 1–11. [CrossRef] [PubMed]
350. Gu, L.; Wang, D.; Cui, X.; Wei, T.; Yang, K.; Yang, J.; Wei, R.; Hong, T. Combination of GLP-1 Receptor Activation and Glucagon Blockage Promotes Pancreatic β-Cell Regeneration In Situ in Type 1 Diabetic Mice. *J. Diabetes Res.* **2021**, *2021*, 1–10. [CrossRef]
351. Wei, R.; Cui, X.; Feng, J.; Gu, L.; Lang, S.; Wei, T.; Yang, J.; Liu, J.; Le, Y.; Wang, H.; et al. Dapagliflozin promotes beta cell regeneration by inducing pancreatic endocrine cell phenotype conversion in type 2 diabetic mice. *Metabolism* **2020**, *111*, 154324. [CrossRef]
352. Jiang, Y.; Yang, J.; Xia, L.; Wei, T.; Cui, X.; Wang, D.; Jin, Z.; Lin, X.; Li, F.; Yang, K. Gut microbiota–tryptophan metabolism–GLP-1 axis participates in β-cell regeneration induced by dapagliflozin. *Diabetes* **2024**, *73*, 926–940. [CrossRef] [PubMed]
353. Sachs, S.; Bastidas-Ponce, A.; Tritschler, S.; Bakhti, M.; Böttcher, A.; Sánchez-Garrido, M.A.; Tarquis-Medina, M.; Kleinert, M.; Fischer, K.; Jall, S.; et al. Targeted pharmacological therapy restores β-cell function for diabetes remission. *Nat. Metab.* **2020**, *2*, 192–209. [CrossRef] [PubMed]
354. Gu, L.; Cui, X.; Lin, X.; Yang, J.; Wei, R.; Hong, T.; Yang, K. γ-aminobutyric acid modulates α-cell hyperplasia but not β-cell regeneration induced by glucagon receptor antagonism in type 1 diabetic mice. *Acta Diabetol.* **2022**, *60*, 19–28. [CrossRef] [PubMed]
355. Karacay, C.; Prietl, B.; Harer, C.; Ehall, B.; Haudum, C.W.; Bounab, K.; Franz, J.; Eisenberg, T.; Madeo, F.; Kolb, D.; et al. The effect of spermidine on autoimmunity and beta cell function in NOD mice. *Sci. Rep.* **2022**, *12*, 4502. [CrossRef] [PubMed]
356. Sims, E.K.; Kulkarni, A.; Hull, A.; Woerner, S.E.; Cabrera, S.; Mastrandrea, L.D.; Hammoud, B.; Sarkar, S.; Nakayasu, E.S.; Mastracci, T.L.; et al. Inhibition of polyamine biosynthesis preserves β cell function in type 1 diabetes. *Cell Rep. Med.* **2023**, *4*, 101261. [CrossRef] [PubMed]
357. Xu, J.; Jia, Y.-F.; Tapadar, S.; Weaver, J.D.; Raji, I.O.; Pithadia, D.J.; Javeed, N.; García, A.J.; Choi, D.-S.; Matveyenko, A.V. Inhibition of TBK1/IKKε promotes regeneration of pancreatic β-cells. *Sci. Rep.* **2018**, *8*, 15587. [CrossRef]
358. Charbord, J.; Ren, L.; Sharma, R.B.; Johansson, A.; Ågren, R.; Chu, L.; Tworus, D.; Schulz, N.; Charbord, P.; Stewart, A.F.; et al. In vivo screen identifies a SIK inhibitor that induces β cell proliferation through a transient UPR. *Nat. Metab.* **2021**, *3*, 682–700. [CrossRef]

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Review

Capsaicin: Emerging Pharmacological and Therapeutic Insights

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Abstract: Capsaicin, the most prominent pungent compound of chilli peppers, has been used in traditional medicine systems for centuries; it already has a number of established clinical and industrial applications. Capsaicin is known to act through the TRPV1 receptor, which exists in various tissues; capsaicin is hepatically metabolised, having a half-life correlated with the method of application. Research on various applications of capsaicin in different formulations is still ongoing. Thus, local capsaicin applications have a pronounced anti-inflammatory effect, while systemic applications have a multitude of different effects because their increased lipophilic character ensures their augmented bioavailability. Furthermore, various teams have documented capsaicin's anti-cancer effects, proven both in vivo and in vitro designs. A notable constraint in the therapeutic effects of capsaicin is its increased toxicity, especially in sensitive tissues. Regarding the traditional applications of capsaicin, apart from all the effects recorded as medicinal effects, the application of capsaicin in acupuncture points has been demonstrated to be effective and the combination of acupuncture and capsaicin warrants further research. Finally, capsaicin has demonstrated antimicrobial effects, which can supplement its anti-inflammatory and anti-carcinogenic actions.

Keywords: capsaicin; bioavailability; physiopathology; anti-cancer effects; local effects; adverse effects

1. Introduction

The most prominent pungent principle in the hot peppers (*Capsicum annuum*) of the genus Capsicum [1] is capsaicin (8-methyl-*N*-vanillyl-6-nonenamide), an organic nitrogenous compound within the lipid group [2]. It must be noted that the name capsaicin was originally used to refer to a multitude of substances originally isolated from *C. oleoresin*; these compounds are now known as capsaicinoids [3], a distinction made after the 1960s [4].

Interestingly, it has been discovered that the cultivation of chilli peppers began around the 5th millennium BC [5], thus rendering them amongst the oldest cultivated plants; their origin is estimated to be somewhere in Bolivia [6]. Chilli peppers came to Europe only after the discovery of the New World and the subsequent Columbian Exchange, which had far-reaching consequences [7]; this is hardly surprising as numerous foodstuffs followed this historical process [8–10]. Subsequently, chilli peppers were swiftly adopted by many cultures and, as such, are ingredients in many local and traditional dishes [11]. It is believed that the synthesis of capsaicin within the plant is part of a defence mechanism developed against consumption by herbivores and micro-organism infestations [12]; however, not all chillies are pungent [13].

Extracted initially as an impure formulation by C.F. Bucholz (1770–1818), it was termed "capsicin" [14,15]. The original compound, isolated almost completely in 1876 by J.C. Thresh (1850–1932) [16–18], was a colourless substance of a crystalline structure—though purified in the 1870s, the first description of its structure is recorded in 1919 [19]—this is not surprising given that the complete isolation of the compound was achieved only in 1898 by K. Micko [20,21]. Based on the original isolation of capsaicin and the identification of its chemical and physical properties, capsaicinoids, of which capsaicin is a member, are defined as chemical compounds which have similar structures and properties as capsaicin [22].

Regarding capsaicin in particular, in its pure form, it is a solid, colourless, hydrophobic, highly volatile and highly pungent substance [23]; if heated to decomposition (80–140 °C), the fumes emitted are toxic nitrogen oxides [24]. Its chemical formula is $C_{18}H_{27}NO_3$ [25]. Capsaicin naturally occurs in its trans form, although a cis-isomer also exists [26].

The biosynthetic path of capsaicin, as described in research [27], involves a chemical reaction between vanillylamine and 7-methyloct-5-ene-1-carboxylic acid chloride; this reaction takes place in the fleshy parts of the fruits. In the seeds of these fruits, no capsaicin is produced; however, the white part of their inner wall contains the highest capsaicin concentration, and it is where the seeds are attached [26]. It is interesting to note that under stress conditions, the capsaicin production of the plant increases [28,29]. Currently, artificial synthesis of capsaicin is possible using a number of different methods [30]. The first artificial synthesis of capsaicin was recorded in 1930 [31]; a number of methods have been developed lately to enhance its production [32–35], given its high demand for research and applications.

The most oft-used scale to measure capsaicin's, or indeed any other compound's, pungency is the Scoville Heat Unit (SHU) scale, proposed in the early 1990s [36]; this is based on the subjective pungency perception of people consuming pungent substances and foods. It is a linear scale, and it can exceed even 10^6 SHU for the hottest peppers containing the highest amounts of capsaicinoids [37].

Due to its properties, capsaicin has a number of already established clinical and industrial uses (Table 1), while a number of novel clinical applications are under discussion. Outside of medical applications, the very potent irritative effect of capsaicin on mucosae [38] means that it constitutes an important component in pepper spray products [39–42].

Table 1. General and medical uses of capsaicin.

Use of Capsaicin and Its Derivatives		References
Animal repellents		[43–46]
Food industry—fragrance ingredient		[47–49]
Pesticides		[50–53]
Veterinary medicine (various uses)		[54–56]
Medical Uses	Chronic pain—cream application (local adm.)	[57–61]
	Gastroprotection in cases of drug administration	[62]
	Post-operative nausea and vomiting	[63–65]
	Post-operative sore throat	[66]
	Pruritus	[67–71]
	Urinary bladder hyperactivity	[72,73]
	Skin conditioning creams	[74,75]

In this review, we will present a comprehensive analysis of the pharmacodynamics and pharmacokinetics of capsaicin and elaborate on its pharmacotoxicity. Given that our study focuses on the pharmacological properties of capsaicin, the most prominent local, systemic and anticarcinogenic applications of capsaicin will be presented in detail. The applications of capsaicin in traditional medicine will also be addressed, and current evidence of the most promising avenues of future research will be reported.

2. Materials and Methods

This review was conducted by systematically searching major electronic databases, including PubMed, Scopus, and Web of Science. The search strategy was developed using a combination of Medical Subject Headings (MeSH) and keywords. MeSH terms included "Capsaicin", "Biochemical Properties", "Therapeutic Applications", and "Pharmacology". Keywords related to these terms, such as "pain management", "analgesic effect", and "TRPV1 receptor", were also used to ensure comprehensive coverage.

Inclusion Criteria: Peer-reviewed articles and studies focused on the biochemical properties, therapeutic applications, or pharmacological insights of capsaicin.

Exclusion Criteria: Studies not specifically addressing capsaicin, non-peer-reviewed literature, such as abstracts, conference proceedings, and grey literature.

The search strategy aimed to capture a wide array of relevant studies to provide an updated and comprehensive overview of capsaicin. Each database was searched using tailored strategies to maximise the retrieval of pertinent studies. The selection process involved screening titles and abstracts, followed by a full-text review to ascertain eligibility based on the predefined inclusion and exclusion criteria.

3. Pharmacokinetics and Pharmacodynamics of Capsaicin

Being liposoluble, capsaicin is well absorbed orally, as well as at the digestive tract level; internal administration means that it will also reach systemic circulation, while systemic administration itself is also possible [76]. The absorption of capsaicin takes place at the level of the stomach and the intestine, varying between 50% and 90%; at any rate, it is invariably a passive process [77]. The intestinal epithelial cells can metabolise a small percentage of the absorbed capsaicin [78]. Despite its lipophilicity, which correlates with good skin absorption [79], capsaicin does not reach sufficiently high levels in the plasma following local or transdermal administration to exert its effects systemically [80].

Following its internal (oral) administration, capsaicin is hepatically metabolised [78], with the small aforementioned exception. Based on in vitro studies, it has been established that, following its rapid metabolisation, the three most important metabolites are 16-hydroxycapsaicin, 17-hydroxycapsaicin, and 16,17-hydroxycapsaicin; vanillin is a minor

metabolite [81,82]. Based on a subsequent study [83], another metabolite of capsaicin was identified, which corresponds to a compound occurring after phase I demethylation and dehydrogenation. A glycine and a glutathione conjugate were also identified. At any rate, a small percentage of capsaicin is fecally excreted, while most of the elimination is renal for the glucuronide metabolites of capsaicin [84].

While it may be assumed that cytochrome P_{450} enzymes are, most probably, involved in capsaicin metabolism [85,86], in human skin cell studies, the biotransformation process has been found to be slow, and most of the administered capsaicin did not undergo any changes [87]—this last fact has important implications for medicinal capsaicin applications. The half-life of capsaicin in the human body was determined to be 25 min [87]; conversely, the local application of a 3% capsaicin solution yielded a value of 24 h [88,89]. More recent research [90] has identified a novel metabolic pathway in the human body, resulting in macrocyclic diene and imide metabolites.

The first physiological action of capsaicin is observed when it binds to the TRPV1 (transient receptor potential cation channel subfamily V member 1) [91]; capsaicin is a potent TRPV1 receptor agonist [92]. Such is the physiological importance of this receptor, and of temperature and mechanically activated channels in general, that research on them resulted in the awarding of the 2021 Nobel Prize in Physiology or Medicine to David Julius and Ardem Patapoutian [93–95] based on their previous research (e.g., [96–101]).

This receptor, which is also called capsaicin or vanilloid receptor 1 [102], can be activated, apart from its agonists, by a temperature higher than 43 °C and a pH lower than 5.2. Some examples of endogenous agonists are bradykinin and prostaglandins [103]. The receptor function is associated either with protein kinase A or protein kinase C [104,105]. The activation of this receptor enables it to exert its modulatory activity; its principal role is body temperature regulation [106,107]. The heat perception properties of TRPV1 have also been proposed by Tominaga et al. [108] who have also noted that TRPV1 is instrumental in peripheral nociception. The nerve signals resulting from its activation reach all up to the spinal cord and eventually the brain.

TRPV1 was identified in the central nervous system as well as in the sensory neurons of the dorsal root ganglion [109]. At the level of the cardiovascular system, it can also be found in vascular smooth muscle cells and endothelial cells [110]; of course, neural TRPV1 activation will also have cardiovascular-related effects [111]. It must be noted that TRPV1 does not seem to be expressed in cardiomyocytes [112], but there is a report indicating that it is possibly expressed in the nerve fibres of the epicardium [113]. At the level of the respiratory system, TRPV1 is found in the airway epithelial cells and in the T cells of the upper and lower airways [114]; interestingly, the expression of TRPV1 in the respiratory system seems to vary in different pathological situations [115,116]. At the level of the gastrointestinal tract (GIT), TRPV1 can be found in the submucosal nerve plexus, myenteric nerve plexus, gastrointestinal mucosal cells, parietal and antral G cells [117].

At the level of the integumentary system, TRPV1 can be found in a number of different cell types, namely unmyelinated type C and thin myelinated A_{δ} sensory nerve fibres, keratinocytes, sebocytes, dermal blood vessels, mast cells, fibroblasts, hair follicles, and vascular smooth muscle cells [118–120]. In the eyes, TRPV1 is present in corneal cells [121] and retinal ganglion cells [122].

Capsaicin induces a variety of TRPV1-mediated sensations with different intensities, from warming and tingling up to burning [123,124]. Another aspect that must be considered is that capsaicin-induced activation of TRPV1 is more persistent compared to the effect of other natural agonists. In fact, capsaicin is a more potent agonist compared to any endogenous TRPV1 agonists—which have been analysed in detail in recent studies [125]—and, although being the most characteristic exogenous TRPV1 agonist [126], there are some more potent such agonists, like resiniferatoxin [127,128] and a number of recently researched compounds [129]. The capsaicin-induced activation of TRPV1 is associated, at least in a number of cases, with a relative desensitisation [130].

Capsaicin exerts a host of different effects at cellular [131–135] and subcellular levels [136,137]. Two pathways are thought to exist via which capsaicin may inhibit nociception: a TRPV1-dependent one and a TRPV1-independent one. The TRPV1-independent effects are associated with changes in the lipid membrane properties, the modulation of voltage-gated ion channels and direct binding to other enzymes and transporters [138–140]. The TRPV-1-dependent pathway implies activation of the receptor and subsequent desensitisation, which can be modulated by various factors, including cAMP/PKA-dependent activation [141,142]. Both the dependent and independent effects are most possibly associated with the reduced nociception caused by capsaicin [138].

The aforementioned blockade of nociceptors, when coupled with the capacity of reducing the inflammation-associated substance P [143], renders capsaicin a good candidate for a non-narcotic analgesic [144,145]; indeed, the new technologies available render the design of pharmacological capsaicin analogues a possible and potent eventuality [126]. In the manifestation of analgesic effects, the indirect blockage of voltage-gated Na^+ channels may also play a role [146,147]. In addition, some other associated capsaicin-induced actions comprise the degeneration of epidermal nerve fibres after prolonged local administration [148]. A number of researchers have presented the most recent developments regarding the novel analgesic capsaicin applications [149–152]. A general outline of the TRPV1-mediated activation by capsaicin is presented in Figure 1.

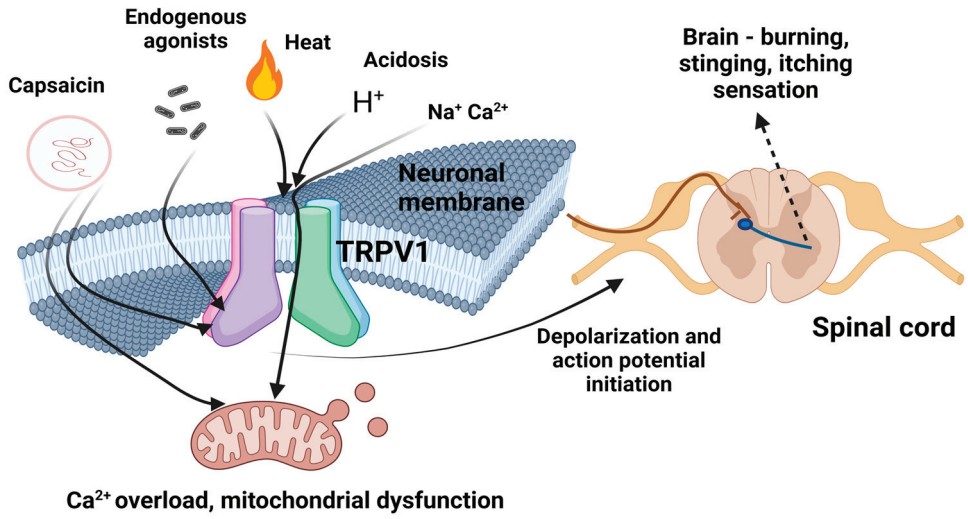


Figure 1. Capsaicin activation of TRPV1. Capsaicin induces sensory neuronal depolarization and local sensitisation to activation by heat, acidosis, and endogenous agonists. Topical application of capsaicin induces sensations of heat, burning, stinging, or itching. When high concentrations of capsaicin are used, or there are repeated applications in cutaneous nociceptors, a de-functionalization process is induced.

4. Indications and Therapeutical Uses of Capsaicin

In general, the uses of capsaicin are numerous and varied, ranging from medicine, either human or veterinary, to uses in agriculture, the food industry, and fragrances. In human medicine, we distinguish between local and systemic applications (Tables 2 and 3).

4.1. Capsaicin as a Local Agent

As discussed above, capsaicin is lipophilic and can hence be absorbed readily, reaching and activating the TRPV1 receptor, which can be found both in nociceptive and non-nociceptive structures. The binding of capsaicin leads to receptor activation, upon which a prolonged desensitisation state prevails; this second state renders the use of capsaicin very promising in chronic pain syndromes, as well as against hyperplasias, inflammation and inflammatory skin diseases, various dermatoses, as well as chemotherapy-induced and radiotherapy-induced mucositis [153].

For local applications, a variety of capsaicin preparations are available, such as creams, gels, liquids and patches [154], while novel formulations comprise nanolipid carriers [155–157], flexible membrane vesicles [158] and alginate microcapsules [159]. These last formulations can be considered better in that they improve the pain threshold in a dose-dependent manner, compared to the older locally-administered drugs; the positive effect is exerted through the reduction of tissue prostaglandin E2 levels, while skin irritation is also reduced [160]. The most prominent local capsaicin applications are presented in Table 2.

Table 2. Local applications of capsaicin.

Indication	Formulation	Effect	Action Mechanism	Type of Study	Year	References
Neuralgia associated with herpes zoster infection	Cream 0.025% 3–4 times/day for 2 days	Antalgic	Substance P depletion/prevention of reuptake	In vivo—human	1988	[161]
Neuralgia-associated periocular and facial pain	15 mg of capsaicin cream 2 daily	Antalgic	Substance P depletion	In vivo—human	1988	[162]
Facial apocrine chromhidrosis	Cream 1–2 times/day	Antalgic and possible vasodilation inhibition	Substance P depletion/prevention of reuptake	In vivo—human	1989	[163]
Reflex sympathetic dystrophy	Cream 0.025% 1–2 times/day for 3 weeks	Antalgic	Substance P depletion	In vivo—human	1990	[164]
Diabetic neuropathy	0.075% capsaicin cream for 8 weeks	Antalgic	Substance P depletion/desensitization of C nociceptal fibers	In vivo—human	1991	[165]
Chronic severe painful diabetic neuropathy unresponsive or intolerant to conventional therapy.	Cream 0.075% 4 times/day for 8 weeks	Antalgic	Substance P depletion/desensitization of warm nociceptors, polymodal nociceptors and nociceptive afferents	In vivo—human	1992	[166]
Osteoarthritis	Cream 0.075%	Antalgic	Unknown	In vivo—human	1992	[167]
Postmastectomy pain syndrome	0.075% capsaicin 4–5 times/day for 4–6 weeks	Antalgic	Unknown	In vivo—human	1992	[57]
Notalgia paresthetica	Cream 0.025 percent for four months	Antalgic, antipruritic	Uncertain	In vivo—human	1992	[168]
Haemodialysis-induced pruritus	Cream 0.025% 4 times/day for 6 weeks	Anti-pruritic	Substance P prevention of reuptake/depletion/desensitization of unmyelinated c fibers of cutaneous nerves	In vivo—human	1992	[67]
Chronic postherpetic neuralgia	0.075% cream	Antalgic	Possible desensitisation of nociceptors	In vivo—human	1993	[169]
Pruritic psoriasis	0.025% cream 4 times/day for 6 weeks	Antipruritic	Substance P depletion	In vivo—human	1993	[170]
Post-mastectomy pain syndrome	0.025% cream 3 times/day for 2 months.	Antalgic	Substance P depletion	In vivo—human	1993	[171]
Cluster headache	Intranasal 3% camphor in 0.025% capsaicin cream for 7 days	Antalgic	Substance P depletion	In vivo—human	1993	[172]

Table 2. *Cont.*

Indication	Formulation	Effect	Action Mechanism	Type of Study	Year	References
Aquagenic pruritus	Cream 0.025%, 0.5% or 1.0% 3 times/day for 4 weeks	Antipruritic	Substance P depletion	In vivo—human	1994	[173]
Erythromelalgia	Cream 0.025% every 12 h for 2 months	Antalgic	Substance P depletion	In vivo—human	1994	[174]
Trigeminal neuralgia manifesting as intraoral pain	Cream 0.025% 4 times/day for 4 weeks	Antalgic	Substance P depletion/desensitization of c nociceptors	In vivo—human	1994	[175]
Chronic neck pain	Cream 0.025% 4 times/day for 5 weeks	Antalgic	Substance P depletion	In vivo—human	1995	[176]
Meralgia paraesthetica	Cream 0.025% 5 times/day for 15 days	Antalgic	Substance P depletion/prevention of reuptake/desensitisation of C-polymodal nociceptors	In vivo—human	1995	[177]
Skin flap survival	Silicongel 0.025%	Increased flap survival	platelet disaggregation	In vivo—animal	1996	[178]
Haemodialysis-induced pruritus	Cream 0.025% 4 times/day	Antipruritic	Substance P depletion	In vivo—human	1996	[68]
Herpes zoster ophthalmicus neuralgia	Cream 0.025% 5 times/day for 4 weeks	Antalgic	Substance P depletion	In vivo—human	1997	[179]
Complex regional pain syndromes and neuropathic pain	Cream 7.5%	Antalgic	Desensitization of C-fiber nociceptors	In vivo—human	1998	[180]
Atopic eczema	Cream 0.05% 3 times/day for 5 days	Antipruritic	Substance P depletion/inhibition	In vivo—human	1998	[181]
Diffuse eosinophilic sinonasal polyposis	3 days 0.5 mL 30 micromol/L capsaicin solution and on days 4 and 5, 100 micromol/L	Improved subjective and endoscopy scores	Possible neurotoxic effect	In vivo—human	2000	[182]
Pain of osteoarthritis	0.025% capsaicin, 1.33% glyceryl trinitrate (one part 0.075% capsaicin, two parts 2% glyceryl trinitrate)	Antalgic	Nociceptive blocking/increase in perfusion of glyceryl trinitrate	In vivo—human	2000	[58]

Table 2. *Cont.*

Indication	Formulation	Effect	Action Mechanism	Type of Study	Year	References
Pain following spinal cord injury	Cream 0.025% 4 times/day	Antalgic	Substance P depletion/desensitization of unmyelinated afferent C fibers	In vivo—human	2000	[183]
Prurigo nodularis	Cream 0.025% to 0.3% 4 to 6 times daily for 2 weeks up to 10 months	Antipruritic	Substance P depletion	In vivo—human	2001	[184]
Complex regional painsyndrome type I	Cream 0.075% 2 times/day for 6 weeks	Antalgic	Desensitization of epidermal C fibers	In vivo—human	2001	[185]
Atopic dermatitis	Lotion 0.025% 2 times/day for 6 weeks	Antipruritic	Possible desensitization or neuroinhibition	In vivo—animal	2002	[186]
Abdominal wall scar pain	Cream 0.075% 3 times/day usually for 2 weeks and after that 2 times/day	Antalgic	Desensitization of vanilloid subtype 1 (VR1) receptors	In vivo—human	2002	[187]
Post-operative nausea and vomiting after abdominal hysterectomy	Capsicum plaster with 345.80 mg of powdered capsicum for at least 30 min before anesthesia and eight hours after surgery on the acupuncture point P6 or the Korean acupuncture point K-D2	Antiemetic	Desensitization of K-D2 hand point zone	In vivo—human	2002	[64]
Haemodialysis related pruritus	Cream 0.05% liniment 3 times/day for 5 days	Antipruritic	Substance P depletion/desensitization of epidermal nerve fibers	In vivo—human	2003	[188]
Meningeal nociception and headache	10 μM topical	Antalgic	Desensitization of afferent fibers	In vivo—animal	2003	[189]
Saphenous neuralgia	Cream 5 times/day for 2 months	Antalgic	Substance P depletion	In vivo—human	2003	[190]
Idiopathic intractable pruritus ani	Capsaicin ointment 0.006–0.012% (depending on dilution) for 4 weeks followed by a week washout and by 4 weeks of placebo (menthol 1%)	Antipruritic	Substance P depletion	In vivo—human	2003	[69]

Table 2. *Cont.*

Indication	Formulation	Effect	Action Mechanism	Type of Study	Year	References
Detrusor hyperreflexia	10 mM topical for 3 months intravesical instillations	Improving continence and bladder function	Possible desensitization of sensory Aδ and unmyelined C fibers	In vivo—human	2004	[191]
Burning mouth syndrome	Oral capsaicin 0.25% 3 times/day for 1 month	Antalgic	Desensitisation of type-C pain receptors	In vivo—human	2004	[192]
Detrusor hyper-reflexia in spinal cord-injured patients	Intravesical instillation of 1 mmol/L CAP diluted in glucidic solvent for 3 months	Improving continence and bladder function	Desensitization or blocking of afferent C-nerve fibres	In vivo—human	2004	[73]
Prevention of post-operative sore throat	Capsicum plaster with powdered capsicum 345.8 mg on the Korean acupuncture point K-A20	Antalgic	Unknown—presumably release of endogenous opioids	In vivo—human	2004	[66]
Post-operative nausea and vomiting after anaesthesia in middle ear surgery	Capsicum plaster withcapsicum oleoresin 1% *w*/*w* on acupuncture point P6	Antiemetic	Release of endogenous opioids/modulation of neurotransmitters of the vestibular system	In vivo—human	2005	[65]
Post-operative nausea and vomiting after laparoscopic cholecystectomy	Capsaicin ointment with oleoresin capsicum equivalent to capsaicin 0.075% *w*/*w* and methyl salicylate I.P. 20% *w*/*w* on the Korean acupuncture point K-D2	Antiemetic	Blocking of synthesis of substance-P from sensory C-fibers/desensitisation of afferent sensory nerves.	In vivo—human	2005	[63]
Acute lobular panniculitis	0.075% capsaicin cream 5 times/day for 3 weeks	Antalgic, antithrombotic	Substance P depletion	In vivo—human	2005	[193]
Acute lipodermatosclerosis	0.075% capsaicin cream for 1–2 weeks followed by a month of continuation	Antalgic, antithrombotic	Substance P depletion	In vivo—human	2005	

Table 2. *Cont.*

Indication	Formulation	Effect	Action Mechanism	Type of Study	Year	References
Post-abdominal hysterctomy pain	Plaster of capsaicin (0.046% *w*/*w*) mixture of powdered capsicum 345.80 mg and capsicum tincture 34.58 mg at ST36 acupuncture point	Antalgic, antiemetic	Release of endogenous opioids (possibly)	In vivo—human	2006	[194]
Skinmorphological changesin patients with growth hormone deficiency and in the elderly	Cream of 0.01% capsaicinoids (dihydrocapsaicin and nordihydrocap-saicin)/0.01% capsinoids (capsiate, dihydrocapsiate and nordihydrocapsiate)	Increased skin elasticity	Increased dermal IGF-I levels	In vivo—human	2007	[195]
Painful HIV-associated distal sensory polyneuropathy (DSP)	Patch 640 microg/cm^2, 8% *w*/*w* 60 min 1 time/day for 12 weeks	Antalgic	Desensitization of cutaneous nociceptors	In vivo—human	2008	[196]
Post-operative pain after orthognathic surgery	Capsicum plaster with 345.80 mg powdered capsicum and 34.58 mg capsicum tincture applied on LI4 acupuncture point	Antalgic, antiemetic	Blocking of transport and synthesis of substance P from sensory C-fibers	In vivo—human	2009	[197]
Migraine pain	Capsaicin jelly with 0.1% capsaicin	Relief and prevention of mild migraines	Substance P depletion	In vivo—human	2010	[198]
Chronic soft tissue pain	0.05% capsaicin cream	Antalgic	Substance P depletion/degeneration of epidermal nerve fibres	In vivo—human	2010	[199]
Haemodialysis-induced uremic pruritus	0.03% capsaicin ointment 4 times/day for 4 weeks	Antipruritic	Substance P depletion	In vivo—human	2010	[70]

Table 2. *Cont.*

Indication	Formulation	Effect	Action Mechanism	Type of Study	Year	References
Cardiac ischemia	5 mL of 0.1% capsaicin cream applied to abdomen; experimental conditions different per animal group	Remote cardioprotective	Release of blood-bornecardioprotective factors	In vivo—animal	2012	[200]
Trigeminal Postherpetic neuralgia	Capsaicin 8% patch; single 60 min application	Antalgic	Substance P depletion/defunctionalization of TRPV1 receptors on sensory nerve endings	In vivo—human	2012	[201]
Visceral obesity	0.075% capsaicin cream for 7 + 7 weeks (pretreatment and post-treatment)	Antiinflammatory, antilipidemic, anti-diabetic	Increased adiponectin, PPARα, PPARγ, visfatin, adipsin and decreased TNF-α and IL-6	In vivo—animal	2013	[202]
Fibromyalgia	0.075% capsaicin cream 3 times/day for 6 weeks	Antalgic	Substance P depletion/desensitization of polymodal nociceptors	In vivo—human	2013	[203]
Peripheral neuropathic pain	8% capsaicin cutaneous patch for 30 min to the feet and 60 min to other parts of the body	Antalgic	Probably substance P related	In vivo—human	2014	[204]
Posttraumatic neuropathic pain	8% capsaicin cutaneous patch for 30 min for the feet and 60 min for other locations every 90 days	Antalgic, anti-inflammatory	Defunctionalisation of nociceptors	In vivo—human	2014	[205]
Arthritis and associated inflammo-musculoskeletal disorders	Topical ethosomal capsaicin	Antalgic, anti-inflammatory	Substance P inhibition	In vivo—animal	2015	[206]
Post-herpetic neuralgia	Liposomal non-ionic capsaicin cream (0.025%) 2–3 times/day for 6 weeks followed by a 2-week cessation	Antalgic	Unclear	In vivo—human	2015	[207]
Intraoral somatosensory sensitivity	30 µL of 5% capsaicin on a paper disc for 15 min	Mechanical desensitization	Desensitization of C-nociceptors	In vivo—human	2015	[208]

Table 2. *Cont.*

Indication	Formulation	Effect	Action Mechanism	Type of Study	Year	References
Lichen amyloidosis	8% capsaicin patch with 179 mg capsaicin for 60 min	Antipruritic	Defunctionalization of transient receptor potential ion channel vanilloid-1	In vivo—human	2016	[209]
Cannabinoid hyperemesis syndrome	Capsaicin cream	Antiemetic	Substance P depletion	In vivo—human	2017	[210]
Burning mouth syndrome	0.01% or 0.025% oral capsaicin gel 3 times/day for 14 days	Antalgic	Substance P depletion/desensitization of transient receptor potential ion channel vanilloid-1	In vivo—human	2017	[211]
Neuropathic pain caused by lumbosacral radiculopathies	8% capsaicin patch for 30 min for the feet and 60 min for other locations	Antalgic	Desensitization of lumbosacral spinal nerves	In vivo—human	2017	[212]
Histamine-induced pruritus on canine skin	3 mL of 0.1% capsaicin solution 2 times/day for 8 days	Antipruritic	Desensitization of the sensory afferents	In vivo—animal	2018	[213]
Neurogenic inflammation	Topical 50 μM of capsaicin for 15 min after the topical application of 200 μM of capsazepine	Neutrophil leukocyte activation	Increased leukocyte rolling and adhesion, increased expression of E-selectin and ICAM-1	In vivo—animal	2018	[214]
Myofascial pain syndrome	10 g capsaicin cream 8%, for 30 min	Antalgic	Substance P depletion/inhibition (probably)	In vivo—human	2019	[215]
Acute musculoskeletal injuries	Capsaicin gel of 0.05% capsaicin 3 times/day for 72 h	Antalgic	Substance P depletion/inhibition (probable)	In vivo—human	2020	[216]
Hepatic staetosis, obesity, dislipidemia and high blood pressure associated with hypoestrogenism	0.75 g/kg capsaicin cream	Anti-obesity, antilipidemic, antihypertensive	Activation of TRPV1 receptors in neurons of the digestive tract/ increased lipid mobilization and oxidation/reduced cholesterol shynthesis	In vivo—animal	2020	[217]
Type 2 diabetic patients with painful peripheral neuropathy	0.075% capsaicin gel	Antalgic	Substance P depletion/defunctionalization of the C fiber nociceptors	In vivo—human	2020	[218]
Trigeminal neuropathic pain	10 μg in 20 μL of vehicle subcutaneously injected	Antalgic	Capsaicin-induced ablation of TRPV1+ afferent terminals	In vivo—animal	2020	[219]

Table 2. *Cont.*

Indication	Formulation	Effect	Action Mechanism	Type of Study	Year	References
Psoriasis	10 μg of Capzasin-HP cream (0.1% capsaicin) for 2 times/day for 8 days	Anti-inflammatory	Desensitization of TRPV1 nerves/denervation-induced inhibition of cutaneous inflammatory responses	In vivo—animal	2021	[220]
Sensory neuropathic cough	Spray of capsaicin 0.02% to 0.04% for 4 times/day for 2 weeks	Antitussive	Substance P depletion/defunctionalization of thermal, mechanical, chemical, and other sensory nerve endings	In vivo—human	2021	[221]
Cannabinoid-induced hyperemesis syndrome	Capsaicin cream 0.025%	Anti-emetic effect	Substance P depletion/defunctionalization of TRPV1	In vivo—human	2021	[222]
UVB-induced cutaneous hyperalgesia	8% transdermal patch or two vehicle patches	Antalgic	Substance P depletion/defunctionalisation of local nociceptors	In vivo—human	2021	[223]
Idiopathic rhinitis	Nasal spray of 0.01 mM capsaicin	Reduction of nasal symptoms	Substance P depletion	In vivo—human	2021	[224]
Hamartoma tumour syndrome	Patch 8%	Pain relief	Substance P depletion/inhibition	In vivo—human	2022	[225]
Acute trauma pain	0.05% capsaicin gel for 3 times/day for 72 h after discharge from the hospital	Antalgic	Substance P depletion/inhibition (probable)	In vivo—human	2022	[226]
Improved dermal blood flow	Cream 8%	Improved skin oxigenation	Local vasodilation induced by TRPV1-mediated release of substance P, CGRP, and other vasoactive peptides	In vivo—human	2022	[227]
Chronic postsurgical pain	8% capsaicin patch every 3 months	Antalgic	Defunctionalization of transient receptor potential vanilloid-1 (probable)	In vivo—human	2022	[228]
Pain during microfocused ultrasound with visualization (MFU-V) treatment	0.025% capsaicin gel	Antalgic	Defunctionalization of transient receptor potential vanilloid-1	In vivo—human	2023	[151]
Peripheral neuropathic pain	One topical high-concentration capsaicin application	Antalgic	Axon reflex vasodilatation associated with pain reduction	In vivo—human	2023	[229]

Most, if not all, of the local applications mentioned in Table 2 can be combined with anti-inflammatory drugs; this enables augmentation of their effects, thus leading to dose reduction, which diminishes their systemic side effects [230]. It may be observed that an abundance of these applications is associated with the inhibition and/or depletion of substance P; substance P, a bioactive peptide of the tachykinin family [231], is secreted by nerve cells and a host of inflammatory cells [232]. Substance P is associated with neurogenic inflammation [233,234] both systemically and at the level of the skin [235–237], the cardiovascular system [238,239], the respiratory [240–242], gastrointestinal [243–245] and genitourinary [246,247] tracts and also in the cerebral arteries [248,249]. The relative ubiquity of substance P in the human body renders it a prime target for pharmacological interventions in inflammatory diseases [250]. Notably, substance P is associated with infection-induced inflammation, a fact proven in both human and animal models [251–254]; taking into account the already proven antimicrobial properties of capsaicin, this could prove an interesting research avenue. In an experimental setting, capsaicin has also been used locally to demonstrate the effects of psychological triggers on vascular responses [255]—this could be a useful future experimental avenue.

4.2. Systemic Applications of Capsaicin

In the recent relevant literature, the tissue-specific and systemic side effects of capsaicin have been rigorously studied. Despite its lipophilicity, local capsaicin administration does not result in any systemic bioavailability, a fact correlating with its poor aqueous solubility properties [256]. Here, it must be remarked again that systemic capsaicin administration correlates with a number of dose-dependent side effects [257]. Since most of these effects are usually GIT-related, they can now be mostly obviated by employing liposomal carriers, which release capsaicin directly into the blood flow [258,259]. In Table 3, based on selected scientific publications, the most notable systemic effects of capsaicin are presented.

Table 3. Systemic effects of capsaicin.

Indication	Formulation	Effect	Action Mechanism	Type of Study	Year	References
Systemic anti-inflammatory effect of somatostatin	Mice: 30, 60 and 90 mg/kg on 3 consecutive days under anaesthesia; guinea pigs: 2% capsaicin solution perineural for 30 min	Anti-inflammatory	Somatostatin release	In vivo—animal	2000	[260]
Burning mouth syndrome	3 capsules of capsaicin (50 mg of powder of red pepper with 0.25% capsaicin) a day for 1 month	Antalgic	Presumed inhibition of substance P	In vivo—human	2003	[261]
Possible protection against cancer, atherosclerosis and age-related diseases	10 μM	Antioxidant	Decrease in malondialdehyde level and protein carbonyl group content	In vitro—erythrocytes	2006	[262]
Helicobacter pylori gastritis	10 μg/mL	Anti-inflammatory	Inhibition of *H. pylori*-induced IL-8 production	In vitro—AGS or MKN45 cells	2007	[263]
Endometriosis	1M solution	Inhibition of proliferation of endometriotic cells	Inhibition of NF-kB	In vitro-immortalized stromal-like and epithelial-like endometriotic cells	2008	[264]
Irritable bowel syndrome	Pills 0.50 mg of capsaicin, 4 pills per day, 6 weeks	Antalgic, antibloating	Desensitisation of nociceptive receptors, depletion of substance P	In vivo—human	2011	[265]
Cardiovascular and metabolic diseases	1% red pepper powder which contains approximately 2.45 mg/g of capsaicin	Antilipidaemic, antiobesity	TRPV1 activation	In vivo—animal	2012	[266]
Chronic unexplained cough triggered by environmental irritants	1 capsule with 0.4 mg pure capsaicin 2 times/day, for 2 weeks, followed by 2 capsules with 0.4 mg pure capsaicin 2 times/day for 2 weeks	Antitussive	Desensitisation of the cough-sensitive TRPV1	In vivo—human	2015	[267]
Atherosclerosis	10, 20, 30, 40, and 50 μM	Antioxidant	Caspase-3 mediated pathways suppression	In vitro—macrophage RAW 264.7 cells	2015	[268]
Anoxia/Reoxygenation injury	10 μM to 40 μM	Cardioprotective	Upregulation of SIRT1 pathway	In vitro—rat cardiomyocytes	2017	[269]

Table 3. *Cont.*

Indication	Formulation	Effect	Action Mechanism	Type of Study	Year	References
Heart failure post myocardial infarction	0.1% cream, 150 μL/25 g	Cardioprotective	Induction of nociceptor-induced conditioning	In vivo—animal	2019	[270]
Hyperlipidaemia, oxidative stress, atherosclerosis	2.5, 5 and 10 mg/kg administered by gavage once daily	Anti-inflammatory, antioxidant, cardioprotective	Decreased total and LDL cholesterol, triglycerides, and apo B-100, and increased HDL cholesterol and SOD	In vivo—animal	2019	[271]
Hepatic steatosis	Cream 0.075%—8 week duration	Antilipidemic, antioxidative	Inhibition of β-oxidation, inhibition of hepatic lipogenesis	In vivo—animal	2020	[272]
Renovascular hypertension	0.006% capsaicin diet for 6 weeks	Antihypertensive	Increased phosphorylation of protein kinase B and endothelial NO synthase	In vivo—animal	2020	[273]
Acute inflammatory demyelinating polyneuropathy (AIDP)/Guillain-Barré syndrome (GBS) and chronic inflammatory demyelinating polyneuropathy (CIDP)	10 μM	Antioxidative, immunomodulatory	Reduction of IFN gamma-induced MHC-II production and decreased TLR4 and ICAM-1 mRNA expression	In vitro—Schwann cells	2020	[274]
Pentylenetetrazole-Induced Seizures	1 or 2 mg/kg	Anticonvulsant, neuroprotective	reduced glutathione (GSH), nitric oxide, and paraoxonase-1 (PON-1)	In vivo—animal	2020	[275]
Hypercholesterolemia	200 μM	Hypolipidemic	Upregulation of LDLR and downregulation of PCSK9 expression	In vitro-HepG2 cells	2022	[276]

In addition to all the aforementioned, in a recent experimental study, it was shown that capsaicin inhibits a series of proteins associated with the Warburg effect in sepsis and also downregulates cyclo-oxygenase 2 (COX-2) in a TRPV-1-independent manner [277]. This is important for a number of reasons; to start with, the Warburg effect, originally proposed in the 1920s [278,279], is essential for the metabolism of cancer cells [280], and its inhibition might provide an avenue for novel therapeutic strategies [268,281,282]. Secondly, the inhibition of COX-2, which is already the target of a number of drugs (e.g., [283–285]), means that capsaicin can be used in conjunction with them to enhance their effect. Finally, in the presence of TRPV-1 agonists other than capsaicin (e.g., [286–288]) or antagonists (e.g., [289–292]), this approach will, theoretically, still be functional.

4.3. Capsaicin as an Anti-Cancer Agent

The anticarcinogenic effect of capsaicin is mainly associated with the activation of TRPV1, which can be considered a probable link between inflammatory, immune and carcinogenic processes, as seen in Table 4.

Table 4. Anti-cancer effects of capsaicin.

Indication	Formulation	Effect	Action Mechanism	Type of Study	Year	References
Hepatocellular carcinoma	200 mM capsaicin or Met-capsaicin	Pro-apoptotic, potential chemopreventive	DNA fragmentation/nuclear condensation/activation of caspase-3	In vitro—SK-Hep-1 hepatocellular carcinoma cells	2001	[131]
Colon cancer	200–300 mM	Proapoptotic, chemopreventive	PPARγ pathway activation (non-TRPV1 related)	In vitro—HT-29 human colon cancer cells	2004	[133]
Gastric adenocarcinoma	1 mmol/L	Proapoptotic	Reduction of Bcl-2 and antiapoptotic protein/DNA fragmentation	In vitro AGS cells	2005	[134]
Prostate cancer	Different doses	Profound antiproliferative effect	Prevention of NF-kappaB activation	In vitro—prostate cancer cell lines/In vivo—prostate cancer cell xenografts on mice	2006	[135]
Gastric cancer	50 μM	Proapoptotic	TRPV6-mediated capsaicin-induced apoptosis/stabilization of p53 through JNK-regulated p53 phosphorylation/increase of Bax and p53 protein without increasing transcription	In vitro—human gastric cancer AGS cells/GES-1 cells	2007	[293]
Melanoma	50–200 μM	Proapoptotic	Down-regulation of Bcl-2 expression, nuclear condensation, internucleosomal DNA fragmentation	In vitro—B16-F10 melanoma cells	2007	[294]
Benzo(a)pyrene induced experimental Lung cancer	10 mg/kg	Chemoprotective	Decreased lung mitochondrial lipid peroxidation	In vivo—animal	2008	[295]
Pancreatic cancer	2.5 mg capsaicin/kg body weight 5 times/week, 5 mg capsaicin/kg 3 times/week by oral gavage (in vivo part)	Proapoptotic	Increased expression of Bax, down-regulation of Bcl-2, significant release of cytochrome c and AIF in the cytosol	In vivo—athymic nude mice; in vitro—AsPC-1 and BxPC-3 cells	2008	[296]

Table 4. *Cont.*

Indication	Formulation	Effect	Action Mechanism	Type of Study	Year	References
Small cell lung cancer	Oral administration of 10 mg capsaicin/kg of mice; 50 Mm capsaicin for cell cultures	Antiproliferative	Decreased expression of E2F-responsive proliferative genes cyclin E, thymidylate synthase, cdc25A and cdc6at	In vivo–animal, in vitro-human SCLC cell lines NCI-H69, NCI-H82, DMS53 and DMS114	2010	[297]
Chronic Pancreatitis and pancreatic intraepithelial neoplasia	Oral administration of 10 ppm. capsaicin or 20 ppm depending on research group	Chemopreventive	Reduction of cell proliferation and suppressed phosphorylation of ERK and c-Jun, blocked Hedgehog/GLI pathway activation	In vivo—animal	2011	[298]
Breast cancer	200 μM	Proapoptotic	Decreased mitochondria membrane potential, induced cleavage of PARP-1, decreased procaspase-7 expression	In vitro—MCF-7 and BT-20 human breast cancer cell lines	2011	[299]
Tongue cancer	50, 100 and 150 μM	Proapoptotic, prooxidant	Activation of caspase 3, DNA fragmentation, induction of G_0/G_1 phase arrest, activation of ROS	In vitro—SCC-4 human tongue cancer cells	2012	[300]
Colorectal cancer	50 and 100 μM	Anti-proliferative, proapoptotic	Suppression of TCF-4 expression and disruption of TCF-4 and β-catenin interaction	In vitro—SW480, LoVo, and HCT-116 colorectal cancer cells	2012	[301]
Bladder cancer	50, 100, 150, 200 μM	Anti-proliferative, proapoptotic	Inhibition of CDK2, CDK4 and CDK6; cell death induction by ROS increase and decreased mitochondrial membrane potential	In vitro—5637 human urinary bladder cancer cell line	2012	[302]
Human KB cancer cells	1, 50, 100, 150, 200 and 250 μM	Anti-proliferative, proapoptotic	Mitochondrial membrane permeabilization and caspase activation	In vitro—human KB cancer cells	2013	[303]
Gastric cancer	10–300 μM	Anti-proliferative (probably), proapoptotic	Decreased expression of phosphorylated ERK 1/2, p38 MAPK or JNK	In vitro—human gastric cancer cells (AGS cells)	2014	[304]
Cholangiocarcinoma	150–200 μM	Impaired cell proliferation, migration, invasion, epithelial to mesenchymal transition growth inhibition in soft agar colonies	Inhibition of Hedgehog signalling pathway	In vitro—human CC cell lines (SZ-1 and TFK-1)	2014	[305]

Table 4. *Cont.*

Indication	Formulation	Effect	Action Mechanism	Type of Study	Year	References
Pancreatic neuroendocrine tumours	10–200 μM	Cytotoxic	Disruption of mitochondrial membrane potential and inhibition of ATP synthesis	In vitro—BON and QGP-1 cells	2014	[306]
Bladder cancer	10–250 μM	Mediation of cancer cell apoptosis	Activation of dendritic cells via CD91	In vitro—T24 and SD48 human urinary bladder cancercell lines	2015	[307]
Prostate cancer	50, 100, 150 and 200 μM	Antiproliferative	Restoration of miR-449a profiling in cancer cells leading to negative modulation of the androgen receptor	In vitro—human C4-2 and LNCaP cells	2015	[308]
Bladder cancer	100 and 200 μM	Antiproliferative, anti-migration, cell cycle prolongation	Inhibition of tNOX and sirtuin 1 (SIRT1)	In vitro—TSGH8301 and T24 urinary bladder cancer cells	2016	[309]
Gastric cancer	0–16 μg/mL	Chemopreventive, antiprofirative, proapoptotic	Reduced hMOF activity	In vitro—colon cancer SW-480, gastric cancer MGC-803 and gastric mucosal GES-1 cells	2016	[310]
Prostate cancer	20, 80 μM	Antiproliferative, induced autophagy	Activation of ROS generation, increased levels of LC3-II, accumulation of p62	In vitro—prostate cancer (LNCaP and PC-3) cells	2016	[311]
Renal cell carcinoma	0–400 μM	Proapoptotic	Up-regulation of pro-apoptotic genes (c-myc, FADD, Bax andcleaved-caspase-3,-8, and-9), down-regulation of Bcl2, activated p38 and JNK MAPK pathways	In vitro—human renal cell carcinoma 786-O, ACHN, Caki-1 cells	2016	[312]
Ovarian cancers	0.1–50 μg/mL	Proapoptotic	Cell cycle arrest	In vitro—SKOV-3 ovarian cancer cells/in vivo-male SD rats	2017	[313]
Nasopharyngeal carcinoma	100, 150, 200 and 300 μM/L	Antiproliferative, proapoptotic, induced autophagy	Increased G_1 phase cell cycle arrest, increased LC3-II and Atg5 levels, decreased p62 and Fap-1 expression, increased caspase-3 activity	In vitro—NPC-TW01 cells	2017	[314]

Table 4. *Cont.*

Indication	Formulation	Effect	Action Mechanism	Type of Study	Year	References
Oesophageal squamous cell carcinoma	120 μM	Antiproliferative, propapoptotic	Inhibition of glycolysis, decreased HK-2 expression	In vitro—Het-1A cell	2018	[315]
Oral squamous cancer	50–350 μM	Antiproliferative, proapoptotic	Disruption of the mitochondrial-membrane potential, activation of caspase-3, -7 and -9, DNA fragmentation	In vitro—ORL-48 cells	2019	[316]
Osteosarcoma	20 μM	Proapoptotic	Mitochondrial dysfunction, overproduction of ROS and JNK, activation of AMPK-p53 pathway	In vitro—MG63 cells	2019	[317]
Breast cancer	0, 10, 50, 100 or 200 μM	Proapoptotic	Induced G_2/M cell cycle arrest, reduced CDK8 expression levels, decreased phosphorylation of PI3K and Akt, downregulation of Wnt and β-catenin	In vitro—MDA MB 231 breast cancer cells	2020	[318]
Prostate cancer	1, 5, 10 μM	Proapoptotic	Decreased expression of Wnt-2, p-GSK3β, β-catenin, c-myc and cyclin D1	In vitro—PC-3 and DU145 prostate cancer stem cells	2020	[319]
Gastric cancer	IC_{50} of 0.6 ± 0.0421 μM	Antiproliferative	Inhibition of histone methylation KDM1A	In vitro—gastric cancer cell line BGC-823	2020	[320]
Glioblastoma	(IC_{50}) values of capsaicin were 325.7 ± 12.4 μM at 24 h and 265.7 ± 10.2 μM at 48 h	Proapoptotic	Upregulation of peroxisome proliferator-activated receptor gamma	In vitro—human glioblastoma LN-18 cell line	2020	[321]
Breast cancer	In vitro: 150 μM/L for 72 h; in vivo: 10 mg/kg 1 time per 3 days for 21 days	Proapoptotic, antiproliferative	Down-regulation of FBI-1-mediated NF-κB pathway	In vivo—female BALB/c athymic nude mice; in vitro—human breast cancer cell lines (MCF-7 and MDA-MB-23)	2021	[322]
Lung cancer cells	0–200 μM	Antiproliferative	Reduced accumulation of HIF-1α protein inhibition of mitochondrial respiration	In vitro—A549, H1299, H2009, and H23 cell lines	2022	[323]
Renal cancer	0, 5, 10, 25, 50, 100 and 200 μM	Inhibition of cell migration, invasion and epithelial-mesenchymal transition Induced autophagy	AMPK/mTOR pathway	In vitro—renal cell carcinoma (RCC) 786-O and CAKI-1 cell lines	2022	[324]

Table 4. *Cont.*

Indication	Formulation	Effect	Action Mechanism	Type of Study	Year	References
Epithelial lung cancer NSCLC	100, 200 and 300 μM/L	Inhibition of proliferation and promotion of ferroptosis	Increase of total iron levels and ferrous ion levels by regulating the SLC7A11/GPX4 axis	In vitro—NSCLC cells (A549 and NCI-H23)	2022	[325]
Hepatocarcinogenesis	100, 200 and 300 μM/L	Significant inhibition of hepatocarcinogenesis	Inhibition of SIRT1/SOX2 signalling; SIRT1 downregulation	In vitro—HepG2 and WB-F344 cells	2022	[326]
Anaplastic thyroid cancer	50, 100 and 200 μM	Stemness-inhibitory effect	Calcium-dependent autophagic degradation of OCT4A, following TRPV1 activation	In vitro—8505C and FRO cells	2022	[327]

There are several events in the anti-cancer trajectory of capsaicin that were documented: antimutagenic activity, anti-oxidative action, anti-inflammatory action, cell cycle regulation and clear involvement in cancer cell death [328]. Out of all the mentioned molecular events associated with capsaicin's anti-cancer action, the induction of cancer cell death is the most important, as capsaicin acts on multiple targets. As outlined in Figure 2, besides TRPV1, another member of the TRPV family involved in the anti-cancer action of capsaicin is TRPV6. Comparable to TRPV1, TRPV6 regulates calcium homeostasis. In in vitro studies, it was shown that capsaicin increases TRPV6 expression and increased levels of intracellular calcium ions that activate the calpain pathway for apoptosis [329]. Moreover, TRPV6 overexpression increased mitochondria permeability through the activation of Bax and p53 through C-jun N-terminal kinase (JNK) activation. Apoptosis can thus be induced by capsaicin in a TRPV1-dependent and independent manner. In the TRPV1 independent pathway, capsaicin activates adenosine 5-monophosphate-activated protein kinase (AMPK), p53 and JNK. When capsaicin binds to the mitochondrial complex I and II in the electron transport chain, the mitochondrial membrane potential is disrupted, and the membrane permeability is increased. Capsaicin increases ROS levels and increases the expression of pro-apoptotic Bcl-2 (Bax), as it was found in the case of neuroendocrine melanoma, a very aggressive and fatal tumour by Jun et al. [294,330,331]. This decreases the anti-apoptotic Bcl-2 and CytC release and induces apoptosis [317].

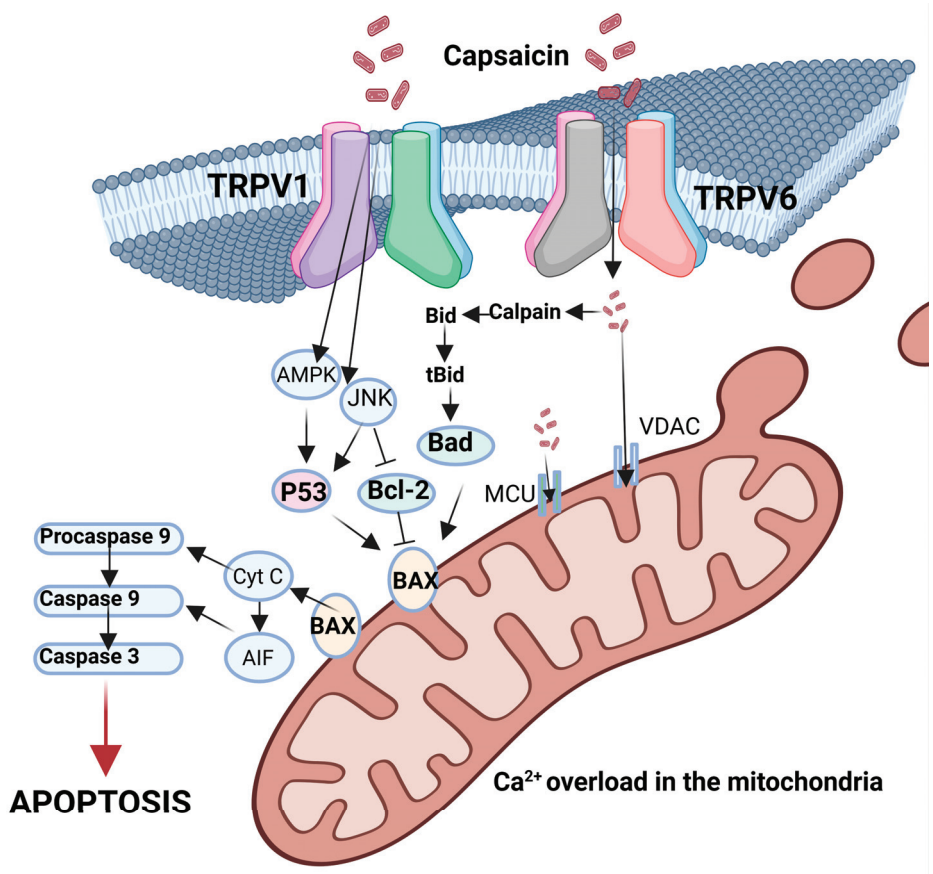


Figure 2. Apoptotic network triggered by capsaicin via TRPV 1 and TRPV6.

Some other anticarcinogenic applications of capsaicin should be mentioned here. It is possible to use capsaicin as a radio-sensitising agent in patients with prostate cancer; this particular use takes advantage of capsaicin-induced inhibition of NFκB signalling [332], resulting in angiogenesis inhibition [333]. More generally, recent studies explore the potential of combining capsaicin with conventional chemotherapeutic agents [334–337].

Other carcinogenesis-related signalling pathways may represent potential targets for future studies [338].

Another aspect we should consider is the increase of serum somatostatin induced by systemic capsaicin administration, which has already been noted by Thán et al. [260] and Szolcsányi et al. [339]. The release of somatostatin is associated with anti-inflammatory [340] and anti-nociceptive effects [341] in rats. The research of [342] has also focused on the somatostatin-induced inhibition of inflammation and nociception.

It is known that somatostatin is linked with such effects in humans [342,343], and somatostatin and its analogues have already been explored as targets for anti-cancer therapies [344–353]. The use of capsaicin in such a manner appears to be a promising avenue in cancer therapy research—a particular application could be in the case of hepatocellular cell carcinoma (HCC) where somatostatin and capsaicin application could be, in theory, effectively combined—the application of capsaicin in the pathogenesis of HCC specifically is explored by Scheau et al. [124].

The anticarcinogenic activity of capsaicin has also been a subject of in vivo studies, where chronic exposure to capsaicin seems to actually promote neoplasia by increasing collagen and elastin deposition [354] and by inhibiting NK cell function [355]. Capsaicin also exhibits a carcinogenic potential when combined with 9, 10-dimethylbenz[a]anthracene/12-O-tetradecanoylphorbol-13-acetate [356]. Finally, long-term capsaicin consumption favours metastasis because it modifies the microbiome of the gut, thus promoting the translocation of bacteria to the liver and altering bile acid metabolism, which ultimately inhibits NK cell function [357]; therefore, it must be examined in detail if and under which circumstances, the use of capsaicin may actually have detrimental effects in human health.

5. Capsaicin in Traditional Medicine Therapies

Originally, the capsaicin-containing plants of the genus Capsicum were native to Central and South America [358,359]. However, after the discovery of the Americas in the 16th century, it was quickly exported, as already mentioned, and gradually became a staple of many different culinary traditions [360].

While this genus comprises about 25 species, only five of them have been domesticated [361–363] and are commonly cultivated [364]; although the species is typically a perennial plant, it can be cultivated as an annual crop in areas with low temperatures [365,366]. Chile peppers, along with a number of other parts, were integral in the Mesoamerican civilisation's agriculture [367] and even later in the formation of traditional Mexican cuisine [368]—the same has happened in a number of other localities, such as Pueblo in Colorado [369].

The traditional medical and even culinary usage of chilli peppers, and therefore capsaicin, is quite diverse [370]. While the domestication of the plant is estimated to have taken place somewhen before the 5th millennium BC [366], it may be assumed that they were also consumed sometime before [371] since the agriculture of many pre-colonial communities was pretty advanced ([372]; and references therein); the domestication process seems to have begun independently in a number of different areas [358]. Its significance is readily apparent from archaeological finds of the pre-Ceramic (ca. 9500–900 BC) and Formative (900 BC–250 AD) periods in South America [373,374] (time frames based on Lanning [375]). The millennia of chilli consumption must have given rise to a number of medicinal applications. In addition, a number of different civilisations that occupied pre-Columbian America, such as the Incas [376], Mayas [377], and Aztecs [378], used chilli peppers as war-related artefacts and for ritualistic purposes [6]. While these last two uses of chilli may be seen as atypical, on the one hand, it must be remarked that the absence of a monetary economy led to natural goods and materials having a more prominent role, a typical example being that of obsidian and other rocks and minerals [379,380]; on the other, a significant number of civilisations have used plants in ritualistic purposes [381–383].

It is known that capsaicin content differs between different Capsicum species [384]. Different foodstuffs also have, as expected, differing capsaicin contents [385], and this

presumably influences their various uses to some extent. In fact, it is even possible to conceive the use of chilli peppers as a food-medicine continuum in the minds of the locals [386,387]. Perhaps the most diverse uses are recorded in Mexico, where chile is native, as is seen in Table 5; interestingly, the increased capsaicin consumption in parts of Mexico seems to correlate positively with adiposity and fat markers [388]. The complete spectrum of the local ethnobotanical use of chilli peppers is provided in a recent study [387]; various uses of different parts of the chilli plants are provided by Meghvansi et al. [389]. Miscellaneous or unverified uses of peppers, and thus capsaicin, also exist, such as those reported by Saleh et al. [390].

Table 5. Some traditional medical applications of capsaicin.

Use	Tribe(s)/Country	Capsicum Species Used	References
Antibacterial/antimicrobial	Pimas/Mexico	*Capsicum* sp.	[391]
Nematicide	Pimas/Mexico	*Capsicum* sp.	[392,393]
Fever alleviation	Zapotecs and Raramuris/Mexico	*Capsicum* sp.	[394,395]
Mental, behavioural and neurological disorders	Raramuris and Mestizos/Mexico	*C. annuum* var. *glabriusculum*	[395]
Ocular pathologies treatment	Mestizos and Zapotecs/Mexico	*C. annuum* var. glabriusculum	[394,396–399]
Auricular pathologies treatment	Nahua/Mexico	*C. annuum* var. glabriusculum	[394,398,399]
Respiratory pathologies treatment	Nahua and Mestizos/Mexico	*Capsicum* sp.	[77,397,400–403]
Pathologies of the gastrointestinal tract	Mestizos, Nahua and Lacandon Maya/Mexico	*Capsicum* sp.	[77,397,400,401,404,405]
Skin pathologies	Mestizos, Zapotecs and Tzotzil/Mexico	*Capsicum* sp.	[77,396,397,403,406,407]
Disease of the musculoskeletal system and associated tissues	Ramamuris/Mexico	*Capsicum* sp.	[395,408]
Pathologies of the genito-urinary system	Local tribes/Mexico	*Capsicum* sp.	[77,400,401]
Labor pain mitigation/ delivery promotion	Local tribes/Mexico	*Capsicum* sp.	[400]
Poisoning treatment (for spider bites)	Mestizos/Mexico	*C. annuum* var. *glabriusculum*	[400,403]
General health benefits	Local people/Eritrea	*Capsicum* sp.	[390]

In addition, chilli is used along with other herbs and plants for a number of diseases or ailments related to the metaphysical concept of soul and evil energy [397,403,406,409–411]. It is possible that a number of medical applications of chilli peppers in traditional medical practice, especially for Native Americans, have been lost to time or have not yet been discovered. It must not be forgotten that Inca medicine, for example, was relatively advanced and possibly superior to contemporary European practises in some fields like surgery [412,413], as evidenced by a variety of findings and mentions in Spanish chronicles [414]. It is, therefore, entirely possible that a number of useful and effective applications of chilli extracts, as well as those of other plants, existed. In order to elucidate the full extent of the intertwining of food, medicine and culture in a local and traditional context, further research and novel practices are required [415,416].

On another note, we would like to point out that, as presented in the tables of the previous sections, based on previous research [63–65], capsaicin cream was applied to acupuncture point P6 or K-D2, which is the Korean equivalent, and also in LI4 [197] and ST36 [194]. Most, if not all, of the effects in these cases, are associated with some form of inhibition of the synthesis, transport and/or action of substance P; indeed, substance P is integral in the modern interpretation of the action of acupuncture in many pain states [417]. Traditional Chinese Medicine (TCM) is one of the most widely used traditional medicine systems in the world, and although it does not incorporate capsaicin-containing plants in its original, ancient phytochemical tradition [418,419], it is interesting to note this, apparently, as of yet, successful combination with capsaicin.

6. Side Effects of Capsaicin

Extensive research has revealed a variety of physiological and pathological effects of capsaicin (Table 6); most but not all of capsaicin's side effects are exerted by the activation of TRPV1. When applied locally, at the level of the skin or other external mucous membranes, it will induce skin erythema, neurogenic inflammation [420], non-blistering associated burning [421], marked lacrimation, blepharospasm and even conjunctivitis [422]. It must be noted here that a specific form of contact dermatitis, the so-called "Hunan hand" was first diagnosed in individuals who handled peppers daily due to their occupation. This is considered a clear and reliable marker of dermal capsaicin toxicity [423,424].

At the level of the CNS, capsaicin toxicity is associated with convulsions, excitement [425,426], disorientation and fear [427]; a host of other generalised symptoms, such as loss of body motor control, including diminished hand-eye coordination, have been reported [427].

In the cardiovascular system, capsaicin causes blood pressure increase and heart rate increase, and, in highly toxic levels, these effects may progress respectively to hypertension and tachycardia, with even ventricular fibrillation having been reported [427]. The blood pressure increase is associated both with the heart rate elevation and with the increased vascular contractility [428], leading to vasoconstriction. A summary of all the hypotheses and determined effects and side effects of capsaicin in the cardiovascular system in different modes of application has been provided in recent research [111]. Particular features of the cardiovascular system might predispose to or aggravate these responses [429–432].

At the level of the respiratory system, it causes bronchoconstriction and coughing [433], while in increased doses, it may even cause oedema of the larynx and the lungs, chemical pneumonitis and even respiratory arrest [434]; these data for capsaicin toxicity are derived from in vitro experiments with capsaicin analogues [434]. Systemic capsaicin toxicity has also been associated with pulmonary oedema and hyperventilation. A particular mechanism of neurogenic toxicity may be beneficial in controlling the neurogenic inflammation associated with nasal polyps, at least in some cases [182].

At the level of the gastrointestinal tract, an increased dose of capsaicin causes a general irritation, ranging from a local warmth sensation to a painful burning sensation [435]. It is also known that capsaicin influences gastric activity [436]. Despite capsaicin having some gastroprotective effects, it also has the potential to induce ulcers [275,437].

Table 6. Pathological effects in cases of capsaicin toxicity per body system.

System	TRPV1-Bearing Cell Types	Toxic Side-Effects	References
CNS	Cerebral neurons, sensory neurons of the dorsal root ganglion	Convulsions, excitement, disorientation, fear, loss of body motor control	[109,425–427]
Cardiovascular	Vascular smooth muscle cells, endothelial cells	Heart rate increase, blood pressure increase, hypertension, tachycardia, ventricular fibrillation, increased vascular contractility, atherosclerosis	[427,428]
Respiratory	Airway epithelial cells, T cells of the upper and lower airways	Bronchoconstriction, coughing, laryngeal oedema, pulmonary oedema, chemical pneumonitis, respiratory arrest	[114,433,434]
Gastrointestinal	Submucosal nerve plexus, myenteric nerve plexus, gastrointestinal mucosal cells, parietal and antral G cells	General irritation and pain, increased ulcer incidence	[117,275,435,437]
Integumentary	Unmyelinated type C and thin myelinated A_δ sensory nerve fibres, keratinocytes, mast cells, dermal blood vessels, fibroblasts, hair follicles, vascular smooth muscle cells, sebocytes and eccrine sweat glands	Skin erythema, non-blistering associated burning, "Hunan hand" (capsaicin-specific contact dermatitis)	[118–120,421,423,424]
Eyes	Corneal cells, retinal ganglion cells	Marked lacrimation, blepharospasm, conjunctivitis	[121,122,422]

From a medical standpoint, in cases of capsaicin overexposure, common adverse effects are painful skin reactions and systemic effects, like nausea, vomiting, abdominal pain and diarrhoea accompanied by a burning sensation [438]; capsaicin is toxic in far lower doses in children compared to adults. In the case of eye exposure, following contact with pepper sprays, marked lacrimation, pain, conjunctivitis, and blepharospasm are common and may be aggravated by the presence of risk factors [439,440]. For local toxic reactions, a thorough decontamination of the skin and mucous membranes is recommended [441], involving water and antiacids [442]; furthermore, the treatment of systemic toxicity is based on the management of symptoms until capsaicin excretion [443].

Finally, a few fringe cases of capsaicin toxicity are reported in the medical literature, namely an acute polyneuropathy, presenting as Guillain-Barre syndrome following pepper spray exposure [444], the death of an infant after a capsaicin-containing traditional medicine was orally administered [445], and an acute MI in a patient with a transdermal capsaicin patch [446].

7. Discussion and Future Research Perspectives

Currently, as a phytomedical compound, capsaicin has been demonstrated to have analgesic, antioxidant, anti-inflammatory, anti-cancer, cardio-protective, and metabolic modulation effects. Capsaicin analogues are also currently evaluated for such properties [447]. A recent study even documented capsaicin-induced inhibition of cell senescence [448], while another proposed that capsaicin may even be a viable management option in cases of schizophrenia [449]; regarding the cardio-protective effects of capsaicin, it might even be possible to use it to alleviate acute myocardial injury [450]. Considering that the majority of such mechanisms are caused by the activation and subsequent inactivation of the TRPV1 receptor, further studies of the role of this receptor may yield useful results regarding both diagnostic and treatment methods. Notably, TRPV1 belongs to a category of receptors recently characterised as extra-oral taste receptors, i.e., oral receptors not found in the oral cavity [451,452]. Outside of the oral cavity, these receptors appear

to have immune system-related and bronchorelaxation-associated properties [453,454]; it is known that taste receptors and their associated effector biomolecules are expressed in tuft-1 cells [455–458]. The characteristic morphology of these cells has been described in detail by Hendel et al. [459]; their localisation is quite diverse [455,460,461]. Moreover, it seems that they are also involved in the regulation of the immune system [462].

A significant challenge of using capsaicin for its potent therapeutic properties is its poor bioavailability due to its quick metabolisation [463]. It is believed that in vivo capsaicin concentrations achieved through conventional routes of administration are inferior to the levels that demonstrated effectiveness in vitro [464]. This is due to variations and limitations in absorption, distribution, and excretion, which limit the permeation of capsaicin to the desired action site. Therefore, in vivo, replication of the effects observed in vitro is an increasing focus of interest, and effective methods are being researched in this regard [465–467]. Furthermore, systemic capsaicin administration is associated with a number of side effects, and so in order to produce the maximum possible therapeutic effect in the target tissue while, at the same time, minimising side effects, it is desirable to control its delivery with precision. This can be performed, as previously alluded to, by employing novel delivery systems, namely liposomes, micelles, micro-emulsions and nano-emulsions [468,469], colloidal capsules and solid nanoparticles [470]; another avenue concerning implant-associated infections [471] would be the integration of capsaicin into 3D printed biomaterials [472]. These improve oral bioavailability for targeted applications, including anti-cancer endeavours [473,474]. Combination of capsaicin with bioenhancing substances such as piperine can prevent its degradation and increase its systemic concentration [475,476]. A number of nanostructured lipid carriers can also be incorporated into transdermal patches to reduce local side effects, such as skin irritation and erythema [477]—the use of capsaicin in the management and treatment of skin pathologies is a promising and rapidly developing field [118]. A future perspective on increasing capsaicin concentration for anti-cancer effects is also its integration into a delivery system that responds to physiologic triggers such as temperature or local pH, therefore optimising its clinical use and expanding its potential as an anti-cancer therapeutic agent [156,478,479]. Capsaicin, as well as other phytochemicals with promising medicinal properties [480], may benefit from such novel delivery methods. A novel way for capsaicin delivery for a particular case of colorectal cancer has recently been explored by Rajput et al. [481].

In cases of inflammation, either local or systemic, capsaicin may offer a good alternative if the common anti-inflammatory drugs are not tolerated due to their side effects. The combination of capsaicin with acupuncture may also be useful in that regard, given that acupuncture is already quite effective in the treatment of pain (e.g., [482–485]) and other inflammatory states (e.g., [486–488]), and the relevant research interest is increasing [489].

Of particular interest is the emerging research on the antibacterial (e.g., [490–492]), antifungal (e.g., [493,494]), antiviral (e.g., [495]) and antiparasitic (e.g., [496]) properties of capsaicin. Already, a number of different phytomedical compounds and their derivatives are being researched for their antimicrobial/antiviral potential, such as kaempferol [497–500], quercetin [501–503], curcumin [504–507], coumarin [508–510], and allicin [511–513]. This is especially important when considering the increasing antimicrobial resistance (e.g., [514–520]) and the occasional severe side-effects like allergies to antimicrobial drugs (e.g., antibiotics [521,522]) and especially some antiparasitic drugs ([523] and references therein).

Furthermore, other research directions could involve capsaicin's role in modulating intestinal microbiota, of whose diversity it increases; this opens new possibilities in combating the complications of various GIT-related illnesses [524,525] through the modulation of the gut-brain axis and immune system interaction [526]. Potential applications of capsaicin in pulmonary and gastrointestinal cancers have also been concisely summarised in recent papers [527,528]. An interesting research direction could also involve the application of capsaicin at acupuncture points, aside from the aforementioned combination of capsaicin and acupuncture; indeed, based on the positive results of three clinical trials [63–65] where capsaicin was applied locally at acupuncture points, larger-scale research for a number

of conditions can be undertaken, following the same principle—similar positive research results were also reported by Kim et al. [194,197]. When considering the proposed special properties of meridians in interstitial fluid—or generally fluid—circulation [529–532], it is compelling to consider the potential for applying specialised cutaneous treatment schemes, using capsaicin or even other bioactive compounds, in this manner.

8. Conclusions

Capsaicin is a potent phytochemical substance that has numerous health benefits. It can be used medicinally both in systemic and local administration. At the same time, the potential toxicity of capsaicin poses an important constraint on its medicinal use, especially in certain sensitive tissues such as the eyes. Already, capsaicin forms part of a number of medical traditions, and such proposed medicinal uses warrant further research. The association between capsaicin and acupuncture must also be explored more thoroughly. Based on the data presented in this paper, we conclude that capsaicin may be used as a monotherapy or adjunct therapy in the treatment or management of a number of pathologies.

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References

1. Cordell, G.A.; Araujo, O.E. Capsaicin: Identification, nomenclature, and pharmacotherapy. *Ann. Pharmacother.* **1993**, *27*, 330–336. [CrossRef] [PubMed]
2. Srinivasan, K. Biological Activities of Red Pepper (*Capsicum annuum*) and Its Pungent Principle Capsaicin: A Review. *Crit. Rev. Food Sci. Nutr.* **2016**, *56*, 1488–1500. [CrossRef] [PubMed]
3. Naves, E.R.; de Ávila Silva, L.; Sulpice, R.; Araújo, W.L.; Nunes-Nesi, A.; Peres, L.E.P.; Zsögön, A. Capsaicinoids: Pungency beyond Capsicum. *Trends Plant Sci.* **2019**, *24*, 109–120. [CrossRef] [PubMed]
4. Chen, K.; Feng, L.; Feng, S.; Yan, Y.; Ge, Z.; Li, Z.; Chen, Z. Multiple quantitative structure-pungency correlations of capsaicinoids. *Food Chem.* **2019**, *283*, 611–620. [CrossRef] [PubMed]
5. Mózsik, G.; Past, T.; Abdel Salam, O.M.; Kuzma, M.; Perjési, P. Interdisciplinary review for correlation between the plant origin capsaicinoids, non-steroidal antiinflammatory drugs, gastrointestinal mucosal damage and prevention in animals and human beings. *Inflammopharmacology* **2009**, *17*, 113–150. [CrossRef] [PubMed]
6. Chiou, K.L.; Hastorf, C.A.; Bonavia, D.; Dillehay, T.D. Documenting Cultural Selection Pressure Changes on Chile Pepper (*Capsicum baccatum* L.) Seed Size Through Time in Coastal Peru (7,600 B.P.–Present). *Econ. Bot.* **2014**, *68*, 190–202. [CrossRef]
7. Nunn, N.; Qian, N. The Columbian exchange: A history of disease, food, and ideas. *J. Econ. Perspect.* **2010**, *24*, 163–188. [CrossRef]
8. Cumo, C. *The Ongoing Columbian Exchange: Stories of Biological and Economic Transfer in World History: Stories of Biological and Economic Transfer in World History*; ABC-CLIO: New York, NY, USA, 2015.
9. Williams, D.E. Agricultural Biodiversity and the Columbian Exchange. In *Routledge Handbook of Agricultural Biodiversity*; Routledge: Oxfordshire, UK, 2017; pp. 192–212.

10. Herniter, I.A.; Muñoz-Amatriaín, M.; Close, T.J. Genetic, textual, and archeological evidence of the historical global spread of cowpea (*Vigna unguiculata* [L.] Walp.). *Legume Sci.* **2020**, *2*, e57. [CrossRef]
11. Basith, S.; Cui, M.; Hong, S.; Choi, S. Harnessing the Therapeutic Potential of Capsaicin and Its Analogues in Pain and Other Diseases. *Molecules* **2016**, *21*, 966. [CrossRef]
12. Tewksbury, J.J.; Manchego, C.; Haak, D.C.; Levey, D.J. Where did the chili get its spice? Biogeography of capsaicinoid production in ancestral wild chili species. *J. Chem. Ecol.* **2006**, *32*, 547–564. [CrossRef]
13. Haak, D.C.; McGinnis, L.A.; Levey, D.J.; Tewksbury, J.J. Why are not all chilies hot? A trade-off limits pungency. *Proc. Biol. Sci.* **2012**, *279*, 2012–2017. [CrossRef] [PubMed]
14. Bucholz, C. Chemische Untersuchung der trockenen reifen spanischen Pfeffers. *Alm. Oder Taschenb. Scheidekünstler Apoth.* **1816**, *37*, 1–30.
15. King, J.; Felter, H.W. *King's American Dispensatory*; Ohio Valley Company: Cincinnati, OH, USA, 1909; Volume 1.
16. Thresh, J.C. Isolation of capsaicin. *Pharm. J. Trans.* **1876**, *6*, 941–947.
17. Thresh, J.C. Capsaicin, the active principle of capsicum fruits. *Pharm. J. Trans.* **1876**, *7*, 259–260.
18. Thresh, J.C. Capsaicin—The active principle of cayenne pepper. *Analyst* **1877**, *2*, 108. [CrossRef]
19. Nelson, E. The constitution of capsaicin, the pungent principle of capsicum. *J. Am. Chem. Soc.* **1919**, *41*, 1115–1121. [CrossRef]
20. Micko, K. Zur Kenntniss des Capsaïcins. *Z. Unters. Nahr.-Und Genußmittel Sowie Gebrauchsgegenstände* **1898**, *1*, 818–829. [CrossRef]
21. Micko, K. Ueber den wirksamen Bestandtheil des Cayennepfeffers. *Z. Unters. Nahr.-Und Genußmittel Sowie Gebrauchsgegenstände* **1899**, *2*, 411–412. [CrossRef]
22. Kosuge, S.; Furuta, M.; Oda, T. Studies on the Pungent Principles of Red Pepper. Part XIII On the pungent principles contents of Japanese red pepper. *Nippon. Shokuhin Kogyo Gakkaishi* **1967**, *14*, 407–410. [CrossRef]
23. Gradinaru, T.C.; Petran, M.; Dragos, D.; Gilca, M. PlantMolecularTasteDB: A Database of Taste Active Phytochemicals. *Front. Pharmacol.* **2021**, *12*, 751712. [CrossRef]
24. Wachtel, R.E. Capsaicin. *Reg. Anesth. Pain. Med.* **1999**, *24*, 361–363. [CrossRef]
25. Alberti, A.; Galasso, V.; Kovac, B.; Modelli, A.; Pichierri, F. Probing the molecular and electronic structure of capsaicin: A spectroscopic and quantum mechanical study. *J. Phys. Chem. A* **2008**, *112*, 5700–5711. [CrossRef] [PubMed]
26. Reyes-Escogido Mde, L.; Gonzalez-Mondragon, E.G.; Vazquez-Tzompantzi, E. Chemical and pharmacological aspects of capsaicin. *Molecules* **2011**, *16*, 1253–1270. [CrossRef]
27. Lu, M.; Chen, C.; Lan, Y.; Xiao, J.; Li, R.; Huang, J.; Huang, Q.; Cao, Y.; Ho, C.T. Capsaicin-the major bioactive ingredient of chili peppers: Bio-efficacy and delivery systems. *Food Funct.* **2020**, *11*, 2848–2860. [CrossRef]
28. Sung, Y.; Chang, Y.-Y.; Ni-Lun, T. Capsaicin biosynthesis in water-stressed hot pepper fruits. *Bot. Bull. Acad. Sin.* **2005**, *46*, 35–42.
29. Arora, R.; Gill, N.; Chauhan, G.; Rana, A. An overview about versatile molecule capsaicin. *Int. J. Pharm. Sci. Drug Res.* **2011**, *3*, 280–286.
30. Katsuragi, H.; Shimoda, K.; Yamamoto, R.; Ohara, T.; Hamada, H. Enzymatic synthesis of capsaicin 4-O-β-xylooligosaccharides by β-xylosidase from *Aspergillus* sp. *Acta Biol. Hung.* **2011**, *62*, 151–155. [CrossRef]
31. Späth, E.; Darling, S.F. Synthese des capsaicins. *Berichte Dtsch. Chem. Ges. (A B Ser.)* **1930**, *63*, 737–743. [CrossRef]
32. Ochoa-Alejo, N.; Ramirez-Malagon, R. In vitro chili pepper biotechnology. *Vitr. Cell. Dev. Biol.-Plant* **2001**, *37*, 701–729. [CrossRef]
33. Castillo, E.; López-González, I.; De Regil-Hernández, R.; Reyes-Duarte, D.; Sánchez-Herrera, D.; López-Munguía, A.; Darszon, A. Enzymatic synthesis of capsaicin analogs and their effect on the T-type Ca^{2+} channels. *Biochem. Biophys. Res. Commun.* **2007**, *356*, 424–430. [CrossRef]
34. Pandhair, V.; Gosal, S. Capsaicin production in cell suspension cultures derived from placenta of *Capsicum annuum* L. fruit. *Indian J. Agric. Biochem.* **2009**, *22*, 78–82.
35. Akhtar, F.; Muhammad Sharif, H.; Arshad Mallick, M.; Zahoor, F.; Abdulmalik, A.; Baig, W.; Shujaat, N.; Gul, S.; Bibi, G.; Ramzan, R.; et al. Capsaicin: Its Biological Activities and In Silico Target Fishing. *Acta Pol. Pharm.* **2017**, *74*, 321–329. [PubMed]
36. Moirangthem, S.S.; Gogoi, S.; Thongbam, P.D.; Ramya, K.T.; Fiyaz, R.A.; Pandey, D.S. Effect of sowing time and crop geometry on the Capsaicinoid content in Bhoot Jolokia (*Capsicum chinense* Jacq.). *J. Food Sci. Technol.* **2014**, *51*, 1974–1981. [CrossRef] [PubMed]
37. Maurya, V.K.; Gothandam, K.M.; Ranjan, V.; Shakya, A.; Pareek, S. Effect of drying methods (microwave vacuum, freeze, hot air and sun drying) on physical, chemical and nutritional attributes of five pepper (*Capsicum annuum* var. annuum) cultivars. *J. Sci. Food Agric.* **2018**, *98*, 3492–3500. [CrossRef]
38. Krishnatreyya, H.; Hazarika, H.; Saha, A.; Chattopadhyay, P. Capsaicin, the primary constituent of pepper sprays and its pharmacological effects on mammalian ocular tissues. *Eur. J. Pharmacol.* **2018**, *819*, 114–121. [CrossRef] [PubMed]
39. Busker, R.W.; van Helden, H.P. Toxicologic evaluation of pepper spray as a possible weapon for the Dutch police force: Risk assessment and efficacy. *Am. J. Forensic Med. Pathol.* **1998**, *19*, 309–316. [CrossRef] [PubMed]
40. Kim, Y.J.; Payal, A.R.; Daly, M.K. Effects of tear gases on the eye. *Surv. Ophthalmol.* **2016**, *61*, 434–442. [CrossRef] [PubMed]
41. Tidwell, R.D.; Wills, B.K. Tear Gas and Pepper Spray Toxicity. In *StatPearls*; StatPearls Publishing LLC.: Treasure Island, FL, USA, 2023.
42. Quiroga-Garza, M.E.; Ruiz-Lozano, R.E.; Azar, N.S.; Mousa, H.M.; Komai, S.; Sevilla-Llorca, J.L.; Perez, V.L. Noxious effects of riot control agents on the ocular surface: Pathogenic mechanisms and management. *Front. Toxicol.* **2023**, *5*, 1118731. [CrossRef]
43. Cowles, R.S.; Keller, J.E.; Miller, J.R. Pungent spices, ground red pepper, and synthetic capsaicin as onion fly ovipositional deterrents. *J. Chem. Ecol.* **1989**, *15*, 719–730. [CrossRef] [PubMed]

44. Sterner, R.T.; Shumake, S.A.; Gaddis, S.E.; Bourassa, J.B. Capsicum oleoresin: Development of an in-soil repellent for pocket gophers. *Pest. Manag. Sci.* **2005**, *61*, 1202–1208. [CrossRef]
45. Kimball, B.A.; Taylor, J.; Perry, K.R.; Capelli, C. Deer responses to repellent stimuli. *J. Chem. Ecol.* **2009**, *35*, 1461–1470. [CrossRef] [PubMed]
46. Stock, B.; Haag-Wackernagel, D. Effectiveness of Gel Repellents on Feral Pigeons. *Animals* **2013**, *4*, 1–15. [CrossRef] [PubMed]
47. Yang, N.; Galves, C.; Racioni Goncalves, A.C.; Chen, J.; Fisk, I. Impact of capsaicin on aroma release: In vitro and in vivo analysis. *Food Res. Int.* **2020**, *133*, 109197. [CrossRef] [PubMed]
48. Hu, X.; Ayed, C.; Chen, J.; Fisk, I.; Yang, N. The role of capsaicin stimulation on the physicochemical properties of saliva and aroma release in model aqueous and oil systems. *Food Chem.* **2022**, *386*, 132824. [CrossRef] [PubMed]
49. Yang, N.; Yang, Q.; Chen, J.; Fisk, I. Impact of capsaicin on aroma release and perception from flavoured solutions. *LWT* **2021**, *138*, 110613. [CrossRef] [PubMed]
50. Maliszewska, J.; Wyszkowska, J.; Kletkiewicz, H.; Rogalska, J. Capsaicin-induced dysregulation of acid-base status in the American cockroach. *J. Environ. Sci. Health B* **2019**, *54*, 676–680. [CrossRef] [PubMed]
51. Li, Y.; Bai, P.; Wei, L.; Kang, R.; Chen, L.; Zhang, M.; Tan, E.K.; Liu, W. Capsaicin Functions as Drosophila Ovipositional Repellent and Causes Intestinal Dysplasia. *Sci. Rep.* **2020**, *10*, 9963. [CrossRef] [PubMed]
52. Xie, D.; Yang, Z.; Hu, X.; Wen, Y. Synthesis, Antibacterial and Insecticidal Activities of Novel Capsaicin Derivatives Containing a Sulfonic Acid Esters Moiety. *Front. Chem.* **2022**, *10*, 929050. [CrossRef] [PubMed]
53. Cui, S.-F.; Wang, J.-W.; Li, H.-F.; Fang, R.; Yu, X.; Lu, Y.-J. Microencapsulation of Capsaicin in Chitosan Microcapsules: Characterization, Release Behavior, and Pesticidal Properties against Tribolium castaneum (Herbst). *Insects* **2023**, *14*, 27. [CrossRef]
54. Hsu, Y.J.; Huang, W.C.; Chiu, C.C.; Liu, Y.L.; Chiu, W.C.; Chiu, C.H.; Chiu, Y.S.; Huang, C.C. Capsaicin Supplementation Reduces Physical Fatigue and Improves Exercise Performance in Mice. *Nutrients* **2016**, *8*, 648. [CrossRef]
55. Adaszek, Ł.; Gadomska, D.; Mazurek, Ł.; Łyp, P.; Madany, J.; Winiarczyk, S. Properties of capsaicin and its utility in veterinary and human medicine. *Res. Vet. Sci.* **2019**, *123*, 14–19. [CrossRef] [PubMed]
56. Mohammad, S.; Mustofa Helmi, E.; Faisal, F.; Muhammad Thohawi Elziyad, P. Role of Capsaicin in the Repair of Cellular Activity in Mice Liver. *Pharmacogn. J.* **2021**, *13*, 1573–1576.
57. Watson, P.N.C.; Evans, R.J. The postmastectomy pain syndrome and topical capsaicin: A randomized trial. *Pain* **1992**, *51*, 375–379. [CrossRef] [PubMed]
58. McCleane, G. The analgesic efficacy of topical capsaicin is enhanced by glyceryl trinitrate in painful osteoarthritis: A randomized, double blind, placebo controlled study. *Eur. J. Pain* **2000**, *4*, 355–360. [CrossRef] [PubMed]
59. Grushka, M.; Epstein, J.B.; Gorsky, M. Burning mouth syndrome. *Am. Fam. Physician* **2002**, *65*, 615–620.
60. Mason, L.; Moore, R.A.; Derry, S.; Edwards, J.E.; McQuay, H.J. Systematic review of topical capsaicin for the treatment of chronic pain. *BMJ* **2004**, *328*, 991. [CrossRef] [PubMed]
61. Saguil, A.; Kane, S.; Mercado, M.; Lauters, R. Herpes Zoster and Postherpetic Neuralgia: Prevention and Management. *Am. Fam. Physician* **2017**, *96*, 656–663.
62. Hayman, M.; Kam, P.C.A. Capsaicin: A review of its pharmacology and clinical applications. *Curr. Anaesth. Crit. Care* **2008**, *19*, 338–343. [CrossRef]
63. Agarwal, A.; Dhiraaj, S.; Tandon, M.; Singh, P.K.; Singh, U.; Pawar, S. Evaluation of capsaicin ointment at the Korean hand acupressure point K-D2 for prevention of postoperative nausea and vomiting. *Anaesthesia* **2005**, *60*, 1185–1188. [CrossRef]
64. Kim, K.S.; Koo, M.S.; Jeon, J.W.; Park, H.S.; Seung, I.S. Capsicum plaster at the korean hand acupuncture point reduces postoperative nausea and vomiting after abdominal hysterectomy. *Anesth. Analg.* **2002**, *95*, 1103–1107. [CrossRef]
65. Misra, M.N.; Pullani, A.J.; Mohamed, Z.U. Prevention of PONV by acustimulation with capsicum plaster is comparable to ondansetron after middle ear surgery. *Can. J. Anaesth.* **2005**, *52*, 485–489. [CrossRef] [PubMed]
66. Park, H.S.; Kim, K.S.; Min, H.K.; Kim, D.W. Prevention of postoperative sore throat using capsicum plaster applied at the Korean hand acupuncture point. *Anaesthesia* **2004**, *59*, 647–651. [CrossRef] [PubMed]
67. Breneman, D.L.; Cardone, J.S.; Blumsack, R.F.; Lather, R.M.; Searle, E.A.; Pollack, V.E. Topical capsaicin for treatment of hemodialysis-related pruritus. *J. Am. Acad. Dermatol.* **1992**, *26*, 91–94. [CrossRef] [PubMed]
68. Tarng, D.C.; Cho, Y.L.; Liu, H.N.; Huang, T.P. Hemodialysis-related pruritus: A double-blind, placebo-controlled, crossover study of capsaicin 0.025% cream. *Nephron* **1996**, *72*, 617–622. [CrossRef] [PubMed]
69. Lysy, J.; Sistiery-Ittah, M.; Israelit, Y.; Shmueli, A.; Strauss-Liviatan, N.; Mindrul, V.; Keret, D.; Goldin, E. Topical capsaicin--a novel and effective treatment for idiopathic intractable pruritus ani: A randomised, placebo controlled, crossover study. *Gut* **2003**, *52*, 1323–1326. [CrossRef] [PubMed]
70. Makhlough, A.; Ala, S.; Haj-Heydari, Z.; Kashi, Z.; Bari, A. Topical capsaicin therapy for uremic pruritus in patients on hemodialysis. *Iran. J. Kidney Dis.* **2010**, *4*, 137–140. [PubMed]
71. Gooding, S.M.; Canter, P.H.; Coelho, H.F.; Boddy, K.; Ernst, E. Systematic review of topical capsaicin in the treatment of pruritus. *Int. J. Dermatol.* **2010**, *49*, 858–865. [CrossRef] [PubMed]
72. Cruz, F. Mechanisms involved in new therapies for overactive bladder. *Urology* **2004**, *63*, 65–73. [CrossRef] [PubMed]
73. de Sèze, M.; Wiart, L.; de Sèze, M.P.; Soyeur, L.; Dosque, J.P.; Blajezewski, S.; Moore, N.; Brochet, B.; Mazaux, J.M.; Barat, M.; et al. Intravesical capsaicin versus resiniferatoxin for the treatment of detrusor hyperreflexia in spinal cord injured patients: A double-blind, randomized, controlled study. *J. Urol.* **2004**, *171*, 251–255. [CrossRef]

74. Botonis, P.G.; Miliotis, P.G.; Kounalakis, S.N.; Koskolou, M.D.; Geladas, N.D. Effects of capsaicin application on the skin during resting exposure to temperate and warm conditions. *Scand. J. Med. Sci. Sports* **2019**, *29*, 171–179. [CrossRef]
75. Rothenberger, J.; Wittwer, M.; Tschumi, C.; Constantinescu, M.A.; Daigeler, A.; Olariu, R. Quantitative impact analysis of remote ischemic conditioning and capsaicin application on human skin microcirculation. *Clin. Hemorheol. Microcirc.* **2019**, *71*, 291–298. [CrossRef] [PubMed]
76. Kawada, T.; Suzuki, T.; Takahashi, M.; Iwai, K. Gastrointestinal absorption and metabolism of capsaicin and dihydrocapsaicin in rats. *Toxicol. Appl. Pharmacol.* **1984**, *72*, 449–456. [CrossRef] [PubMed]
77. Sharma, S.K.; Vij, A.S.; Sharma, M. Mechanisms and clinical uses of capsaicin. *Eur. J. Pharmacol.* **2013**, *720*, 55–62. [CrossRef] [PubMed]
78. Reilly, C.A.; Crouch, D.J.; Yost, G.S.; Fatah, A.A. Determination of capsaicin, nonivamide, and dihydrocapsaicin in blood and tissue by liquid chromatography-tandem mass spectrometry. *J. Anal. Toxicol.* **2002**, *26*, 313–319. [CrossRef] [PubMed]
79. Ilie, M.A.; Caruntu, C.; Tampa, M.; Georgescu, S.R.; Matei, C.; Negrei, C.; Ion, R.M.; Constantin, C.; Neagu, M.; Boda, D. Capsaicin: Physicochemical properties, cutaneous reactions and potential applications in painful and inflammatory conditions. *Exp. Ther. Med.* **2019**, *18*, 916–925. [CrossRef] [PubMed]
80. Zhang, S.; Wang, D.; Huang, J.; Hu, Y.; Xu, Y. Application of capsaicin as a potential new therapeutic drug in human cancers. *J. Clin. Pharm. Ther.* **2020**, *45*, 16–28. [CrossRef] [PubMed]
81. Qin, L.; Wang, Y.; Gong, Y.; Chen, J.; Xu, B.; Tang, L.; Guo, L.; Xie, J. Capsaicin metabolites and GSH-associated detoxification and biotransformation pathways in human liver microsomes revealed by LC-HRMS/MS with data-mining tools. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **2019**, *1133*, 121843. [CrossRef]
82. Chanda, S.; Bashir, M.; Babbar, S.; Koganti, A.; Bley, K. In vitro hepatic and skin metabolism of capsaicin. *Drug Metab. Dispos.* **2008**, *36*, 670–675. [CrossRef] [PubMed]
83. Oyagbemi, A.A.; Saba, A.B.; Azeez, O.I. Capsaicin: A novel chemopreventive molecule and its underlying molecular mechanisms of action. *Indian J. Cancer* **2010**, *47*, 53–58. [CrossRef]
84. Suresh, D.; Srinivasan, K. Tissue distribution & elimination of capsaicin, piperine & curcumin following oral intake in rats. *Indian J. Med. Res.* **2010**, *131*, 682–691.
85. Reilly, C.A.; Yost, G.S. Metabolism of capsaicinoids by P450 enzymes: A review of recent findings on reaction mechanisms, bio-activation, and detoxification processes. *Drug Metab. Rev.* **2006**, *38*, 685–706. [CrossRef]
86. van Eijl, S.; Zhu, Z.; Cupitt, J.; Gierula, M.; Götz, C.; Fritsche, E.; Edwards, R.J. Elucidation of xenobiotic metabolism pathways in human skin and human skin models by proteomic profiling. *PLoS ONE* **2012**, *7*, e41721. [CrossRef]
87. Rollyson, W.D.; Stover, C.A.; Brown, K.C.; Perry, H.E.; Stevenson, C.D.; McNees, C.A.; Ball, J.G.; Valentovic, M.A.; Dasgupta, P. Bioavailability of capsaicin and its implications for drug delivery. *J. Control Release* **2014**, *196*, 96–105. [CrossRef]
88. Babbar, S.; Marier, J.F.; Mouksassi, M.S.; Beliveau, M.; Vanhove, G.F.; Chanda, S.; Bley, K. Pharmacokinetic analysis of capsaicin after topical administration of a high-concentration capsaicin patch to patients with peripheral neuropathic pain. *Ther. Drug Monit.* **2009**, *31*, 502–510. [CrossRef]
89. Zak, A.; Siwinska, N.; Slowikowska, M.; Borowicz, H.; Szpot, P.; Zawadzki, M.; Niedzwiedz, A. The detection of capsaicin and dihydrocapsaicin in horse serum following long-term local administration. *BMC Vet. Res.* **2018**, *14*, 193. [CrossRef]
90. Tian, K.; Zhu, J.; Li, M.; Qiu, X. Capsaicin is efficiently transformed by multiple cytochrome P450s from Capsicum fruit-feeding Helicoverpa armigera. *Pestic. Biochem. Physiol.* **2019**, *156*, 145–151. [CrossRef]
91. Hanson, S.M.; Newstead, S.; Swartz, K.J.; Sansom, M.S.P. Capsaicin interaction with TRPV1 channels in a lipid bilayer: Molecular dynamics simulation. *Biophys. J.* **2015**, *108*, 1425–1434. [CrossRef]
92. Yang, F.; Zheng, J. Understand spiciness: Mechanism of TRPV1 channel activation by capsaicin. *Protein Cell* **2017**, *8*, 169–177. [CrossRef]
93. Cheng, Y. TRPV1 and Piezo: The 2021 Nobel Prize in Physiology or Medicine. *IUCrJ* **2022**, *9*, 4–5. [CrossRef]
94. Earley, S.; Santana, L.F.; Lederer, W.J. The physiological sensor channels TRP and piezo: Nobel Prize in Physiology or Medicine 2021. *Physiol. Rev.* **2022**, *102*, 1153–1158. [CrossRef] [PubMed]
95. Reeh, P.W.; Fischer, M.J. Nobel somatosensations and pain. *Pflügers Arch.-Eur. J. Physiol.* **2022**, *474*, 405–420. [CrossRef] [PubMed]
96. Caterina, M.J.; Schumacher, M.A.; Tominaga, M.; Rosen, T.A.; Levine, J.D.; Julius, D. The capsaicin receptor: A heat-activated ion channel in the pain pathway. *Nature* **1997**, *389*, 816–824. [CrossRef] [PubMed]
97. Tominaga, M.; Caterina, M.J.; Malmberg, A.B.; Rosen, T.A.; Gilbert, H.; Skinner, K.; Raumann, B.E.; Basbaum, A.I.; Julius, D. The cloned capsaicin receptor integrates multiple pain-producing stimuli. *Neuron* **1998**, *21*, 531–543. [CrossRef] [PubMed]
98. Caterina, M.J.; Leffler, A.; Malmberg, A.B.; Martin, W.; Trafton, J.; Petersen-Zeitz, K.; Koltzenburg, M.; Basbaum, A.; Julius, D. Impaired nociception and pain sensation in mice lacking the capsaicin receptor. *Science* **2000**, *288*, 306–313. [CrossRef] [PubMed]
99. McKemy, D.D.; Neuhausser, W.M.; Julius, D. Identification of a cold receptor reveals a general role for TRP channels in thermosensation. *Nature* **2002**, *416*, 52–58. [CrossRef] [PubMed]
100. Peier, A.M.; Moqrich, A.; Hergarden, A.C.; Reeve, A.J.; Andersson, D.A.; Story, G.M.; Earley, T.J.; Dragoni, I.; McIntyre, P.; Bevan, S. A TRP channel that senses cold stimuli and menthol. *Cell* **2002**, *108*, 705–715. [CrossRef] [PubMed]
101. Coste, B.; Mathur, J.; Schmidt, M.; Earley, T.J.; Ranade, S.; Petrus, M.J.; Dubin, A.E.; Patapoutian, A. Piezo1 and Piezo2 are essential components of distinct mechanically activated cation channels. *Science* **2010**, *330*, 55–60. [CrossRef] [PubMed]
102. Frias, B.; Merighi, A. Capsaicin, Nociception and Pain. *Molecules* **2016**, *21*, 797. [CrossRef] [PubMed]

103. Aghazadeh Tabrizi, M.; Baraldi, P.G.; Baraldi, S.; Gessi, S.; Merighi, S.; Borea, P.A. Medicinal Chemistry, Pharmacology, and Clinical Implications of TRPV1 Receptor Antagonists. *Med. Res. Rev.* **2017**, *37*, 936–983. [CrossRef]
104. Frey, E.; Karney-Grobe, S.; Krolak, T.; Milbrandt, J.; DiAntonio, A. TRPV1 Agonist, Capsaicin, Induces Axon Outgrowth after Injury via Ca(2+)/PKA Signaling. *eNeuro* **2018**, *5*, ENEURO.0095-18.2018. [CrossRef]
105. Zhou, Y.; Zhou, Z.S.; Zhao, Z.Q. PKC regulates capsaicin-induced currents of dorsal root ganglion neurons in rats. *Neuropharmacology* **2001**, *41*, 601–608. [CrossRef] [PubMed]
106. Lee, T.H.; Lee, J.W.; Osaka, T.; Kobayashi, A.; Namba, Y.; Inoue, S.; Kimura, S. Lack of integrative control of body temperature after capsaicin administration. *Korean J. Intern. Med.* **2000**, *15*, 103–108. [CrossRef] [PubMed]
107. Rosenberger, D.C.; Binzen, U.; Treede, R.-D.; Greffrath, W. The capsaicin receptor TRPV1 is the first line defense protecting from acute non damaging heat: A translational approach. *J. Transl. Med.* **2020**, *18*, 28. [CrossRef] [PubMed]
108. Tominaga, M.; Tominaga, T. Structure and function of TRPV1. *Pflug. Arch.* **2005**, *451*, 143–150. [CrossRef]
109. Hwang, M.K.; Bode, A.M.; Byun, S.; Song, N.R.; Lee, H.J.; Lee, K.W.; Dong, Z. Cocarcinogenic effect of capsaicin involves activation of EGFR signaling but not TRPV1. *Cancer Res.* **2010**, *70*, 6859–6869. [CrossRef] [PubMed]
110. Zhang, C.; Ye, L.; Zhang, Q.; Wu, F.; Wang, L. The role of TRPV1 channels in atherosclerosis. *Channels* **2020**, *14*, 141–150. [CrossRef] [PubMed]
111. Munjuluri, S.; Wilkerson, D.A.; Sooch, G.; Chen, X.; White, F.A.; Obukhov, A.G. Capsaicin and TRPV1 Channels in the Cardiovascular System: The Role of Inflammation. *Cells* **2021**, *11*, 18. [CrossRef] [PubMed]
112. Hoebart, C.; Rojas-Galvan, N.S.; Ciotu, C.I.; Aykac, I.; Reissig, L.F.; Weninger, W.J.; Kiss, A.; Podesser, B.K.; Fischer, M.J.M.; Heber, S. No functional TRPA1 in cardiomyocytes. *Acta Physiol.* **2021**, *232*, e13659. [CrossRef] [PubMed]
113. Yoshie, K.; Rajendran, P.S.; Massoud, L.; Mistry, J.; Swid, M.A.; Wu, X.; Sallam, T.; Zhang, R.; Goldhaber, J.I.; Salavatian, S.; et al. Cardiac TRPV1 afferent signaling promotes arrhythmogenic ventricular remodeling after myocardial infarction. *JCI Insight* **2020**, *5*, e124477. [CrossRef]
114. Kim, J.H. The Emerging Role of TRPV1 in Airway Inflammation. *Allergy Asthma Immunol. Res.* **2018**, *10*, 187–188. [CrossRef]
115. Baxter, M.; Eltom, S.; Dekkak, B.; Yew-Booth, L.; Dubuis, E.D.; Maher, S.A.; Belvisi, M.G.; Birrell, M.A. Role of transient receptor potential and pannexin channels in cigarette smoke-triggered ATP release in the lung. *Thorax* **2014**, *69*, 1080–1089. [CrossRef] [PubMed]
116. McGarvey, L.P.; Butler, C.A.; Stokesberry, S.; Polley, L.; McQuaid, S.; Abdullah, H.; Ashraf, S.; McGahon, M.K.; Curtis, T.M.; Arron, J.; et al. Increased expression of bronchial epithelial transient receptor potential vanilloid 1 channels in patients with severe asthma. *J. Allergy Clin. Immunol.* **2014**, *133*, 704–712.e4. [CrossRef] [PubMed]
117. Du, Q.; Liao, Q.; Chen, C.; Yang, X.; Xie, R.; Xu, J. The Role of Transient Receptor Potential Vanilloid 1 in Common Diseases of the Digestive Tract and the Cardiovascular and Respiratory System. *Front. Physiol.* **2019**, *10*, 1064. [CrossRef] [PubMed]
118. Caruntu, C.; Negrei, C.; Ilie Ghita, M.; Caruntu, A.; Badarau, B.; ioan Buraga, I.B.; Boda, D.; Albu, A.; Brănişteanu, D. Capsaicin, a hot topic in skin pharmacology and physiology. *Farmacia* **2015**, *63*, 487–491.
119. Inoue, K.; Koizumi, S.; Fuziwara, S.; Denda, S.; Inoue, K.; Denda, M. Functional vanilloid receptors in cultured normal human epidermal keratinocytes. *Biochem. Biophys. Res. Commun.* **2002**, *291*, 124–129. [CrossRef] [PubMed]
120. Ständer, S.; Moormann, C.; Schumacher, M.; Buddenkotte, J.; Artuc, M.; Shpacovitch, V.; Brzoska, T.; Lippert, U.; Henz, B.M.; Luger, T.A.; et al. Expression of vanilloid receptor subtype 1 in cutaneous sensory nerve fibers, mast cells, and epithelial cells of appendage structures. *Exp. Dermatol.* **2004**, *13*, 129–139. [CrossRef] [PubMed]
121. Yang, T.J.; Yu, Y.; Yang, J.Y.; Li, J.J.; Zhu, J.Y.; Vieira, J.A.C.; Jiang, Q. Involvement of transient receptor potential channels in ocular diseases: A narrative review. *Ann. Transl. Med.* **2022**, *10*, 839. [CrossRef] [PubMed]
122. Sappington, R.M.; Sidorova, T.; Long, D.J.; Calkins, D.J. TRPV1: Contribution to retinal ganglion cell apoptosis and increased intracellular Ca2+ with exposure to hydrostatic pressure. *Investig. Ophthalmol. Vis. Sci.* **2009**, *50*, 717–728. [CrossRef] [PubMed]
123. Wang, F.; Xue, Y.; Fu, L.; Wang, Y.; He, M.; Zhao, L.; Liao, X. Extraction, purification, bioactivity and pharmacological effects of capsaicin: A review. *Crit. Rev. Food Sci. Nutr.* **2022**, *62*, 5322–5348. [CrossRef]
124. Scheau, C.; Badarau, I.A.; Caruntu, C.; Mihai, G.L.; Didilescu, A.C.; Constantin, C.; Neagu, M. Capsaicin: Effects on the Pathogenesis of Hepatocellular Carcinoma. *Molecules* **2019**, *24*, 2350. [CrossRef]
125. Benítez-Angeles, M.; Morales-Lázaro, S.L.; Juárez-González, E.; Rosenbaum, T. TRPV1: Structure, Endogenous Agonists, and Mechanisms. *Int. J. Mol. Sci.* **2020**, *21*, 3421. [CrossRef] [PubMed]
126. Caballero, J. A new era for the design of TRPV1 antagonists and agonists with the use of structural information and molecular docking of capsaicin-like compounds. *J. Enzym. Inhib. Med. Chem.* **2022**, *37*, 2169–2178. [CrossRef] [PubMed]
127. Szallasi, A.; Blumberg, P.M. Resiniferatoxin, a phorbol-related diterpene, acts as an ultrapotent analog of capsaicin, the irritant constituent in red pepper. *Neuroscience* **1989**, *30*, 515–520. [CrossRef] [PubMed]
128. Raisinghani, M.; Pabbidi, R.M.; Premkumar, L.S. Activation of transient receptor potential vanilloid 1 (TRPV1) by resiniferatoxin. *J. Physiol.* **2005**, *567*, 771–786. [CrossRef] [PubMed]
129. Duarte, Y.; Cáceres, J.; Sepúlveda, R.V.; Arriagada, D.; Olivares, P.; Díaz-Franulic, I.; Stehberg, J.; González-Nilo, F. Novel TRPV1 Channel Agonists with Faster and More Potent Analgesic Properties Than Capsaicin. *Front. Pharmacol.* **2020**, *11*, 1040. [CrossRef]
130. Căruntu, C.; Boda, D. Evaluation through in vivo reflectance confocal microscopy of the cutaneous neurogenic inflammatory reaction induced by capsaicin in human subjects. *J. Biomed. Opt.* **2012**, *17*, 085003. [CrossRef] [PubMed]

131. Jung, M.Y.; Kang, H.J.; Moon, A. Capsaicin-induced apoptosis in SK-Hep-1 hepatocarcinoma cells involves Bcl-2 downregulation and caspase-3 activation. *Cancer Lett.* **2001**, *165*, 139–145. [CrossRef] [PubMed]
132. Hail, N., Jr.; Lotan, R. Examining the role of mitochondrial respiration in vanilloid-induced apoptosis. *J. Natl. Cancer Inst.* **2002**, *94*, 1281–1292. [CrossRef] [PubMed]
133. Kim, C.S.; Park, W.H.; Park, J.Y.; Kang, J.H.; Kim, M.O.; Kawada, T.; Yoo, H.; Han, I.S.; Yu, R. Capsaicin, a spicy component of hot pepper, induces apoptosis by activation of the peroxisome proliferator-activated receptor gamma in HT-29 human colon cancer cells. *J. Med. Food* **2004**, *7*, 267–273. [CrossRef]
134. Lo, Y.C.; Yang, Y.C.; Wu, I.C.; Kuo, F.C.; Liu, C.M.; Wang, H.W.; Kuo, C.H.; Wu, J.Y.; Wu, D.C. Capsaicin-induced cell death in a human gastric adenocarcinoma cell line. *World J. Gastroenterol.* **2005**, *11*, 6254–6257. [CrossRef]
135. Mori, A.; Lehmann, S.; O'Kelly, J.; Kumagai, T.; Desmond, J.C.; Pervan, M.; McBride, W.H.; Kizaki, M.; Koeffler, H.P. Capsaicin, a component of red peppers, inhibits the growth of androgen-independent, p53 mutant prostate cancer cells. *Cancer Res.* **2006**, *66*, 3222–3229. [CrossRef] [PubMed]
136. Athanasiou, A.; Smith, P.A.; Vakilpour, S.; Kumaran, N.M.; Turner, A.E.; Bagiokou, D.; Layfield, R.; Ray, D.E.; Westwell, A.D.; Alexander, S.P.; et al. Vanilloid receptor agonists and antagonists are mitochondrial inhibitors: How vanilloids cause non-vanilloid receptor mediated cell death. *Biochem. Biophys. Res. Commun.* **2007**, *354*, 50–55. [CrossRef] [PubMed]
137. Pramanik, K.C.; Boreddy, S.R.; Srivastava, S.K. Role of mitochondrial electron transport chain complexes in capsaicin mediated oxidative stress leading to apoptosis in pancreatic cancer cells. *PLoS ONE* **2011**, *6*, e20151. [CrossRef] [PubMed]
138. Yang, R.; Xiong, Z.; Liu, C.; Liu, L. Inhibitory effects of capsaicin on voltage-gated potassium channels by TRPV1-independent pathway. *Cell. Mol. Neurobiol.* **2014**, *34*, 565–576. [CrossRef] [PubMed]
139. Du, X.; Gamper, N. Potassium channels in peripheral pain pathways: Expression, function and therapeutic potential. *Curr. Neuropharmacol.* **2013**, *11*, 621–640. [CrossRef] [PubMed]
140. Oz, M.; Lorke, D.E.; Howarth, F.C. Transient receptor potential vanilloid 1 (TRPV1)-independent actions of capsaicin on cellular excitability and ion transport. *Med. Res. Rev.* **2023**, *43*, 1038–1067. [CrossRef] [PubMed]
141. Mohapatra, D.P.; Nau, C. Regulation of Ca2+-dependent desensitization in the vanilloid receptor TRPV1 by calcineurin and cAMP-dependent protein kinase. *J. Biol. Chem.* **2005**, *280*, 13424–13432. [CrossRef] [PubMed]
142. Shuba, Y.M. Beyond Neuronal Heat Sensing: Diversity of TRPV1 Heat-Capsaicin Receptor-Channel Functions. *Front. Cell. Neurosci.* **2021**, *14*, 612480. [CrossRef] [PubMed]
143. Burks, T.F.; Buck, S.H.; Miller, M.S. Mechanisms of depletion of substance P by capsaicin. *Fed. Proc.* **1985**, *44*, 2531–2534.
144. Dray, A. Mechanism of action of capsaicin-like molecules on sensory neurons. *Life Sci.* **1992**, *51*, 1759–1765. [CrossRef]
145. Ding, H.; Kiguchi, N.; Dobbins, M.; Romero-Sandoval, E.A.; Kishioka, S.; Ko, M.-C. Nociceptin Receptor-Related Agonists as Safe and Non-addictive Analgesics. *Drugs* **2023**, *83*, 771–793. [CrossRef]
146. Liu, L.; Oortgiesen, M.; Li, L.; Simon, S.A. Capsaicin inhibits activation of voltage-gated sodium currents in capsaicin-sensitive trigeminal ganglion neurons. *J. Neurophysiol.* **2001**, *85*, 745–758. [CrossRef] [PubMed]
147. Pasierski, M.; Szulczyk, B. Capsaicin inhibits sodium currents and epileptiform activity in prefrontal cortex pyramidal neurons. *Neurochem. Int.* **2020**, *135*, 104709. [CrossRef]
148. Nolano, M.; Simone, D.A.; Wendelschafer-Crabb, G.; Johnson, T.; Hazen, E.; Kennedy, W.R. Topical capsaicin in humans: Parallel loss of epidermal nerve fibers and pain sensation. *Pain* **1999**, *81*, 135–145. [CrossRef] [PubMed]
149. Guo, R.; Qiu, H.; Li, H.; Ma, D.; Guan, Y.; Wang, Y. The preemptive analgesic effect of capsaicin involves attenuations of epidermal keratinocytes proliferation and expression of pro-inflammatory mediators after plantar incision in rats. *J. Pain. Res.* **2023**, *16*, 141–149. [CrossRef]
150. Goodwin, B.; Chiplunkar, M.; Salerno, R.; Coombs, K.; Sannoh, U.; Shah, V.; Averell, N.; Al-Shebab, U.; Janora, D. Topical capsaicin for the management of painful diabetic neuropathy: A narrative systematic review. *Pain Manag.* **2023**, *13*, 309–316. [CrossRef] [PubMed]
151. Vachiramon, V.; Tanratana, P.; Anuntrangsee, T.; Palakornkitti, P.; Yeesibsean, N.; Kungvalpivat, P.; Fabi, S. The role of topical capsaicin gel in pain management during microfocused ultrasound treatment for neck laxity. *Skin Res. Technol.* **2023**, *29*, e13240. [CrossRef]
152. Peel, J.; John, K.; Page, J.; Jeffries, O.; Heffernan, S.M.; Tallent, J.; Waldron, M. Topical application of isolated menthol and combined menthol-capsaicin creams: Exercise tolerance, thermal perception, pain, attentional focus and thermoregulation in the heat. *Eur. J. Sport. Sci.* **2023**, *23*, 2038–2048. [CrossRef]
153. Berger, A.; Henderson, M.; Nadoolman, W.; Duffy, V.; Cooper, D.; Saberski, L.; Bartoshuk, L. Oral capsaicin provides temporary relief for oral mucositis pain secondary to chemotherapy/radiation therapy. *J. Pain. Symptom Manag.* **1995**, *10*, 243–248. [CrossRef]
154. Derry, S.; Wiffen, P.J.; Kalso, E.A.; Bell, R.F.; Aldington, D.; Phillips, T.; Gaskell, H.; Moore, R.A. Topical analgesics for acute and chronic pain in adults—An overview of Cochrane Reviews. *Cochrane Database Syst. Rev.* **2017**, *5*, Cd008609. [CrossRef]
155. Wang, X.R.; Gao, S.Q.; Niu, X.Q.; Li, L.J.; Ying, X.Y.; Hu, Z.J.; Gao, J.Q. Capsaicin-loaded nanolipoidal carriers for topical application: Design, characterization, and in vitro/in vivo evaluation. *Int. J. Nanomed.* **2017**, *12*, 3881–3898. [CrossRef] [PubMed]
156. Anantaworasakul, P.; Anuchapreeda, S.; Yotsawimonwat, S.; Naksuriya, O.; Lekawanvijit, S.; Tovanabutra, N.; Anantaworasakul, P.; Wattanasri, W.; Buranapreecha, N.; Ampasavate, C. Nanomaterial Lipid-Based Carrier for Non-Invasive Capsaicin Delivery; Manufacturing Scale-Up and Human Irritation Assessment. *Molecules* **2020**, *25*, 5575. [CrossRef] [PubMed]

157. Contri, R.V.; Frank, L.A.; Kaiser, M.; Pohlmann, A.R.; Guterres, S.S. The use of nanoencapsulation to decrease human skin irritation caused by capsaicinoids. *Int. J. Nanomed.* **2014**, *9*, 951–962. [CrossRef]
158. Raza, K.; Singh, B.; Mahajan, A.; Negi, P.; Bhatia, A.; Katare, O.P. Design and evaluation of flexible membrane vesicles (FMVs) for enhanced topical delivery of capsaicin. *J. Drug Target.* **2011**, *19*, 293–302. [CrossRef] [PubMed]
159. Hudita, A.; Galateanu, B.; Costache, M.; Negrei, C.; Ion, R.M.; Iancu, L.; Ginghina, O. In Vitro Cytotoxic Protective Effect of Alginate-Encapsulated Capsaicin Might Improve Skin Side Effects Associated with the Topical Application of Capsaicin. *Molecules* **2021**, *26*, 1455. [CrossRef] [PubMed]
160. Raza, K.; Shareef, M.A.; Singal, P.; Sharma, G.; Negi, P.; Katare, O.P. Lipid-based capsaicin-loaded nano-colloidal biocompatible topical carriers with enhanced analgesic potential and decreased dermal irritation. *J. Liposome Res.* **2014**, *24*, 290–296. [CrossRef] [PubMed]
161. Don, P.C. Topical capsaicin for treatment of neuralgia associated with herpes zoster infection. *J. Am. Acad. Dermatol.* **1988**, *18*, 1135–1136. [CrossRef] [PubMed]
162. Lincoff, N.S.; Rath, P.P.; Hirano, M. The treatment of periocular and facial pain with topical capsaicin. *J. Neuroophthalmol.* **1998**, *18*, 17–20. [CrossRef] [PubMed]
163. Marks, J.G., Jr. Treatment of apocrine chromhidrosis with topical capsaicin. *J. Am. Acad. Dermatol.* **1989**, *21*, 418–420. [CrossRef]
164. Cheshire, W.P.; Snyder, C.R. Treatment of reflex sympathetic dystrophy with topical capsaicin. Case report. *Pain* **1990**, *42*, 307–311. [CrossRef]
165. Treatment of painful diabetic neuropathy with topical capsaicin. A multicenter, double-blind, vehicle-controlled study. The Capsaicin Study Group. *Arch. Intern. Med.* **1991**, *151*, 2225–2229. [CrossRef]
166. Tandan, R.; Lewis, G.A.; Krusinski, P.B.; Badger, G.B.; Fries, T.J. Topical capsaicin in painful diabetic neuropathy. Controlled study with long-term follow-up. *Diabetes Care* **1992**, *15*, 8–14. [CrossRef]
167. McCarthy, G.M.; McCarty, D.J. Effect of topical capsaicin in the therapy of painful osteoarthritis of the hands. *J. Rheumatol.* **1992**, *19*, 604–607. [PubMed]
168. Leibsohn, E. Treatment of notalgia paresthetica with capsaicin. *Cutis* **1992**, *49*, 335–336. [PubMed]
169. Watson, C.P.; Tyler, K.L.; Bickers, D.R.; Millikan, L.E.; Smith, S.; Coleman, E. A randomized vehicle-controlled trial of topical capsaicin in the treatment of postherpetic neuralgia. *Clin. Ther.* **1993**, *15*, 510–526. [CrossRef] [PubMed]
170. Ellis, C.N.; Berberian, B.; Sulica, V.I.; Dodd, W.A.; Jarratt, M.T.; Katz, H.I.; Prawer, S.; Krueger, G.; Rex, I.H., Jr.; Wolf, J.E. A double-blind evaluation of topical capsaicin in pruritic psoriasis. *J. Am. Acad. Dermatol.* **1993**, *29*, 438–442. [CrossRef] [PubMed]
171. Dini, D.; Bertelli, G.; Gozza, A.; Forno, G.G. Treatment of the post-mastectomy pain syndrome with topical capsaicin. *Pain* **1993**, *54*, 223–226. [CrossRef] [PubMed]
172. Marks, D.R.; Rapoport, A.; Padla, D.; Weeks, R.; Rosum, R.; Sheftell, F.; Arrowsmith, F. A double-blind placebo-controlled trial of intranasal capsaicin for cluster headache. *Cephalalgia* **1993**, *13*, 114–116. [CrossRef] [PubMed]
173. Lotti, T.; Teofoli, P.; Tsampau, D. Treatment of aquagenic pruritus with topical capsaicin cream. *J. Am. Acad. Dermatol.* **1994**, *30*, 232–235. [CrossRef]
174. Muhiddin, K.A.; Gallen, I.W.; Harries, S.; Pearce, V.R. The use of capsaicin cream in a case of erythromelalgia. *Postgrad. Med. J.* **1994**, *70*, 841–843. [CrossRef]
175. Epstein, J.B.; Marcoe, J.H. Topical application of capsaicin for treatment of oral neuropathic pain and trigeminal neuralgia. *Oral Surg. Oral Med. Oral Pathol.* **1994**, *77*, 135–140. [CrossRef] [PubMed]
176. Mathias, B.J.; Dillingham, T.R.; Zeigler, D.N.; Chang, A.S.; Belandres, P.V. Topical capsaicin for chronic neck pain. A pilot study. *Am. J. Phys. Med. Rehabil.* **1995**, *74*, 39–44. [CrossRef] [PubMed]
177. Puig, L.; Alegre, M.; de Moragas, J.M. Treatment of meralgia paraesthetica with topical capsaicin. *Dermatology* **1995**, *191*, 73–74. [CrossRef] [PubMed]
178. Iinuma, T.; Sawada, Y. Topical application of capsaicin and flap survival. *Br. J. Plast. Surg.* **1996**, *49*, 319–320. [CrossRef] [PubMed]
179. Frucht-Pery, J.; Feldman, S.T.; Brown, S.I. The use of capsaicin in herpes zoster ophthalmicus neuralgia. *Acta Ophthalmol. Scand.* **1997**, *75*, 311–313. [CrossRef] [PubMed]
180. Robbins, W.R.; Staats, P.S.; Levine, J.; Fields, H.L.; Allen, R.W.; Campbell, J.N.; Pappagallo, M. Treatment of intractable pain with topical large-dose capsaicin: Preliminary report. *Anesth. Analg.* **1998**, *86*, 579–583. [CrossRef] [PubMed]
181. Weisshaar, E.; Heyer, G.; Forster, C.; Handwerker, H.O. Effect of topical capsaicin on the cutaneous reactions and itching to histamine in atopic eczema compared to healthy skin. *Arch. Dermatol. Res.* **1998**, *290*, 306–311. [CrossRef] [PubMed]
182. Baudoin, T.; Kalogjera, L.; Hat, J. Capsaicin significantly reduces sinonasal polyps. *Acta Otolaryngol.* **2000**, *120*, 307–311. [CrossRef]
183. Sandford, P.R.; Benes, P.S. Use of capsaicin in the treatment of radicular pain in spinal cord injury. *J. Spinal Cord. Med.* **2000**, *23*, 238–243. [CrossRef]
184. Ständer, S.; Luger, T.; Metze, D. Treatment of prurigo nodularis with topical capsaicin. *J. Am. Acad. Dermatol.* **2001**, *44*, 471–478. [CrossRef]
185. Ribbers, G.M.; Stam, H.J. Complex regional pain syndrome type I treated with topical capsaicin: A case report. *Arch. Phys. Med. Rehabil.* **2001**, *82*, 851–852. [CrossRef]
186. Marsella, R.; Nicklin, C.F.; Melloy, C. The effects of capsaicin topical therapy in dogs with atopic dermatitis: A randomized, double-blinded, placebo-controlled, cross-over clinical trial. *Vet. Dermatol.* **2002**, *13*, 131–139. [CrossRef] [PubMed]
187. McPartland, J.M. Use of capsaicin cream for abdominal wall scar pain. *Am. Fam. Physician* **2002**, *65*, 2211; author reply 2212.

188. Weisshaar, E.; Dunker, N.; Gollnick, H. Topical capsaicin therapy in humans with hemodialysis-related pruritus. *Neurosci. Lett.* **2003**, *345*, 192–194. [CrossRef] [PubMed]
189. Dux, M.; Sántha, P.; Jancsó, G. Capsaicin-sensitive neurogenic sensory vasodilatation in the dura mater of the rat. *J. Physiol.* **2003**, *552*, 859–867. [CrossRef] [PubMed]
190. Perkins, P.; Morgan, S.; Closs, S.P. Topical capsaicin for saphenous neuralgia. *J. Pain. Symptom Manag.* **2003**, *26*, 785–786. [CrossRef] [PubMed]
191. Lazzeri, M.; Spinelli, M.; Zanollo, A.; Turini, D. Intravesical vanilloids and neurogenic incontinence: Ten years experience. *Urol. Int.* **2004**, *72*, 145–149. [CrossRef] [PubMed]
192. Petruzzi, M.; Lauritano, D.; De Benedittis, M.; Baldoni, M.; Serpico, R. Systemic capsaicin for burning mouth syndrome: Short-term results of a pilot study. *J. Oral. Pathol. Med.* **2004**, *33*, 111–114. [CrossRef] [PubMed]
193. Yosipovitch, G.; Mengesha, Y.; Facliaru, D.; David, M. Topical capsaicin for the treatment of acute lipodermatosclerosis and lobular panniculitis. *J. Dermatol. Treat.* **2005**, *16*, 178–180. [CrossRef] [PubMed]
194. Kim, K.S.; Nam, Y.M. The analgesic effects of capsicum plaster at the Zusanli point after abdominal hysterectomy. *Anesth. Analg.* **2006**, *103*, 709–713. [CrossRef]
195. Harada, N.; Okajima, K. Effect of topical application of capsaicin and its related compounds on dermal insulin-like growth factor-I levels in mice and on facial skin elasticity in humans. *Growth Horm. IGF Res.* **2007**, *17*, 171–176. [CrossRef] [PubMed]
196. Simpson, D.M.; Estanislao, L.; Brown, S.J.; Sampson, J. An open-label pilot study of high-concentration capsaicin patch in painful HIV neuropathy. *J. Pain. Symptom Manag.* **2008**, *35*, 299–306. [CrossRef] [PubMed]
197. Kim, K.S.; Kim, K.N.; Hwang, K.G.; Park, C.J. Capsicum plaster at the Hegu point reduces postoperative analgesic requirement after orthognathic surgery. *Anesth. Analg.* **2009**, *108*, 992–996. [CrossRef] [PubMed]
198. Cianchetti, C. Capsaicin jelly against migraine pain. *Int. J. Clin. Pract.* **2010**, *64*, 457–459. [CrossRef] [PubMed]
199. Chrubasik, S.; Weiser, T.; Beime, B. Effectiveness and safety of topical capsaicin cream in the treatment of chronic soft tissue pain. *Phytother. Res.* **2010**, *24*, 1877–1885. [CrossRef] [PubMed]
200. Redington, K.L.; Disenhouse, T.; Strantzas, S.C.; Gladstone, R.; Wei, C.; Tropak, M.B.; Dai, X.; Manlhiot, C.; Li, J.; Redington, A.N. Remote cardioprotection by direct peripheral nerve stimulation and topical capsaicin is mediated by circulating humoral factors. *Basic. Res. Cardiol.* **2012**, *107*, 241. [CrossRef]
201. Sayanlar, J.; Guleyupoglu, N.; Portenoy, R.; Ashina, S. Trigeminal postherpetic neuralgia responsive to treatment with capsaicin 8% topical patch: A case report. *J. Headache Pain* **2012**, *13*, 587–589. [CrossRef] [PubMed]
202. Lee, G.R.; Shin, M.K.; Yoon, D.J.; Kim, A.R.; Yu, R.; Park, N.H.; Han, I.S. Topical application of capsaicin reduces visceral adipose fat by affecting adipokine levels in high-fat diet-induced obese mice. *Obesity* **2013**, *21*, 115–122. [CrossRef]
203. Casanueva, B.; Rodero, B.; Quintial, C.; Llorca, J.; González-Gay, M.A. Short-term efficacy of topical capsaicin therapy in severely affected fibromyalgia patients. *Rheumatol. Int.* **2013**, *33*, 2665–2670. [CrossRef]
204. Maihöfner, C.G.; Heskamp, M.L. Treatment of peripheral neuropathic pain by topical capsaicin: Impact of pre-existing pain in the QUEPP-study. *Eur. J. Pain* **2014**, *18*, 671–679. [CrossRef]
205. Zis, P.; Apsokardos, A.; Isaia, C.; Sykioti, P.; Vadalouca, A. Posttraumatic and postsurgical neuropathic pain responsive to treatment with capsaicin 8% topical patch. *Pain. Physician* **2014**, *17*, E213–E218.
206. Kumar Sarwa, K.; Rudrapal, M.; Mazumder, B. Topical ethosomal capsaicin attenuates edema and nociception in arthritic rats. *Drug Deliv.* **2015**, *22*, 1043–1052. [CrossRef]
207. Teixeira, M.J.; Menezes, L.M.; Silva, V.; Galhardoni, R.; Sasson, J.; Okada, M.; Duarte, K.P.; Yeng, L.T.; Andrade, D.C. Liposomal topical capsaicin in post-herpetic neuralgia: A safety pilot study. *Arq. Neuropsiquiatr.* **2015**, *73*, 237–240. [CrossRef]
208. Lu, S.; Baad-Hansen, L.; Zhang, Z.; Svensson, P. Spatial and Temporal Effects of Capsaicin and Menthol on Intraoral Somatosensory Sensitivity. *J. Oral. Facial Pain. Headache* **2015**, *29*, 257–264. [CrossRef]
209. Zeidler, C.; Metze, D.; Ständer, S. Successful treatment of lichen amyloidosis using capsaicin 8% patch. *J. Eur. Acad. Dermatol. Venereol.* **2016**, *30*, 1236–1238. [CrossRef] [PubMed]
210. Dezieck, L.; Hafez, Z.; Conicella, A.; Blohm, E.; O'Connor, M.J.; Schwarz, E.S.; Mullins, M.E. Resolution of cannabis hyperemesis syndrome with topical capsaicin in the emergency department: A case series. *Clin. Toxicol.* **2017**, *55*, 908–913. [CrossRef] [PubMed]
211. Jørgensen, M.R.; Pedersen, A.M. Analgesic effect of topical oral capsaicin gel in burning mouth syndrome. *Acta Odontol. Scand.* **2017**, *75*, 130–136. [CrossRef]
212. Baron, R.; Treede, R.D.; Birklein, F.; Cegla, T.; Freynhagen, R.; Heskamp, M.L.; Kern, K.U.; Maier, C.; Rolke, R.; Seddigh, S.; et al. Treatment of painful radiculopathies with capsaicin 8% cutaneous patch. *Curr. Med. Res. Opin.* **2017**, *33*, 1401–1411. [CrossRef]
213. Bae, S.; Yu, J.; Jeong, H.; Oh, T. Anti-pruritic effect of topical capsaicin against histamine-induced pruritus on canine skin. *Pol. J. Vet. Sci.* **2018**, *21*, 789–796. [CrossRef]
214. Járomi, P.; Garab, D.; Hartmann, P.; Bodnár, D.; Nyíri, S.; Sántha, P.; Boros, M.; Jancsó, G.; Szabó, A. Capsaicin-induced rapid neutrophil leukocyte activation in the rat urinary bladder microcirculatory bed. *Neurourol. Urodyn.* **2018**, *37*, 690–698. [CrossRef]
215. Romero, V.; Lara, J.R.; Otero-Espinar, F.; Salgado, M.H.; Modolo, N.S.P.; Barros, G.A.M. [Capsaicin topical cream (8%) for the treatment of myofascial pain syndrome]. *Braz. J. Anesthesiol.* **2019**, *69*, 432–438. [CrossRef]
216. Kocak, A.O.; Dogruyol, S.; Akbas, I.; Menekse, T.S.; Gur, S.T.A.; Kocak, M.B.; Cekmen, B.; Orun, S.; Cakir, Z. Comparison of topical capsaicin and topical piroxicam in the treatment of acute trauma-induced pain: A randomized double-blind trial. *Am. J. Emerg. Med.* **2020**, *38*, 1767–1771. [CrossRef]

217. de Lourdes Medina-Contreras, J.M.; Mailloux-Salinas, P.; Colado-Velazquez, J.I.; Gómez-Viquez, N.; Velázquez-Espejel, R.; Del Carmen Susunaga-Notario, A.; Arias-Chávez, D.J.; Bravo, G. Topical capsaicin cream with moderate exercise protects against hepatic steatosis, dyslipidemia and increased blood pressure in hypoestrogenic obese rats. *J. Sci. Food Agric.* **2020**, *100*, 3212–3219. [CrossRef] [PubMed]
218. Agoons, B.B.; Dehayem Yefou, M.; Katte, J.C.; Etoa Etoga, M.C.; Agoons, D.D.; Yepnjio, F.; Boli, A.; Wasnyo, Y.; Sobngwi, E.; Mbanya, J.C. Effect of Topical Capsaicin on Painful Sensory Peripheral Neuropathy in Patients with Type 2 Diabetes: A Double-Blind Placebo-Controlled Randomised Clinical Trial. *Cureus* **2020**, *12*, e11147. [CrossRef]
219. Wang, S.; Bian, C.; Yang, J.; Arora, V.; Gao, Y.; Wei, F.; Chung, M.K. Ablation of TRPV1+ Afferent Terminals by Capsaicin Mediates Long-Lasting Analgesia for Trigeminal Neuropathic Pain. *eNeuro* **2020**, *7*, ENEURO.0118-20.2020. [CrossRef]
220. Chan, T.C.; Lee, M.S.; Huang, W.C.; Chang, W.Y.; Krueger, J.G.; Tsai, T.F. Capsaicin attenuates imiquimod-induced epidermal hyperplasia and cutaneous inflammation in a murine model of psoriasis. *Biomed. Pharmacother.* **2021**, *141*, 111950. [CrossRef] [PubMed]
221. Hoesli, R.C.; Wingo, M.L.; Wajsberg, B.; Bastian, R.W. Topical Capsaicin for the Treatment of Sensory Neuropathic Cough. *OTO Open* **2021**, *5*, 2473974x211065668. [CrossRef] [PubMed]
222. Kum, V.; Bell, A.; Fang, W.; VanWert, E. Efficacy of topical capsaicin for cannabinoid hyperemesis syndrome in a pediatric and adult emergency department. *Am. J. Emerg. Med.* **2021**, *49*, 343–351. [CrossRef]
223. Lo Vecchio, S.; Andersen, H.H.; Elberling, J.; Arendt-Nielsen, L. Sensory defunctionalization induced by 8% topical capsaicin treatment in a model of ultraviolet-B-induced cutaneous hyperalgesia. *Exp. Brain Res.* **2021**, *239*, 2873–2886. [CrossRef]
224. Van Gerven, L.; Steelant, B.; Cools, L.; Callebaut, I.; Backaert, W.; de Hoon, J.; Ampe, E.; Talavera, K.; Hellings, P.W. Low-dose capsaicin (0.01 mM) nasal spray is equally effective as the current standard treatment for idiopathic rhinitis: A randomized, double-blind, placebo-controlled trial. *J. Allergy Clin. Immunol.* **2021**, *147*, 397–400.e4. [CrossRef]
225. Silva-Clavería, F.; Bernabeu-Wittel, J.; Monserrat, M.T. Topical capsaicin patch for pain management in PTEN hamartoma tumor syndromes. *J. Dtsch. Dermatol. Ges.* **2022**, *20*, 1221–1223. [CrossRef] [PubMed]
226. Cleary, D.; Burton, S.; Cardon, B. Time to consider topical capsaicin for acute trauma pain? *J. Fam. Pract.* **2022**, *71*, 176–177. [CrossRef] [PubMed]
227. Schäfer, R.C.; Sohn, A.; Kersten, A.; Amr, A.; Held, M.; Wenger, A. Quantification of Dermal Microcirculatory Changes after Topical Administration of Capsaicin: A Randomized Placebo-Controlled Study in 46 Subjects. *J. Investig. Surg.* **2022**, *35*, 1673–1678. [CrossRef] [PubMed]
228. Laude-Pagniez, E.; Leclerc, J.; Lok, C.; Chaby, G.; Arnault, J.P. Capsaicin 8% patch as therapy for neuropathic chronic postsurgical pain after melanoma excision surgery: A single center case series. *JAAD Case Rep.* **2022**, *30*, 70–75. [CrossRef] [PubMed]
229. Sendel, M.; Dunst, A.; Forstenpointner, J.; Hüllemann, P.; Baron, R. Capsaicin treatment in neuropathic pain: Axon reflex vasodilatation after 4 weeks correlates with pain reduction. *Pain* **2023**, *164*, 534–542. [CrossRef] [PubMed]
230. Ercan, N.; Uludag, M.O.; Agis, E.R.; Demirel-Yilmaz, E. The anti-inflammatory effect of diclofenac is considerably augmented by topical capsaicinoids-containing patch in carrageenan-induced paw oedema of rat. *Inflammopharmacology* **2013**, *21*, 413–419. [CrossRef] [PubMed]
231. Payan, D.G. Neuropeptides and inflammation: The role of substance P. *Annu. Rev. Med.* **1989**, *40*, 341–352. [CrossRef]
232. O'Connor, T.M.; O'Connell, J.; O'Brien, D.I.; Goode, T.; Bredin, C.P.; Shanahan, F. The role of substance P in inflammatory disease. *J. Cell. Physiol.* **2004**, *201*, 167–180. [CrossRef]
233. Bartold, P.; Kylstra, A.; Lawson, R. Substance P: An immunohistochemical and biochemical study in human gingival tissues. A role for neurogenic inflammation? *J. Periodontol.* **1994**, *65*, 1113–1121. [CrossRef]
234. DeVane, C.L. Substance P: A new era, a new role. *Pharmacother. J. Hum. Pharmacol. Drug Ther.* **2001**, *21*, 1061–1069. [CrossRef]
235. Hägermark, O.; Hökfelt, T.; Pernow, B. Flare and itch induced by substance P in human skin. *Nation* **1978**, *12*, 13. [CrossRef]
236. Simone, D.A.; Nolano, M.; Johnson, T.; Wendelschafer-Crabb, G.; Kennedy, W.R. Intradermal injection of capsaicin in humans produces degeneration and subsequent reinnervation of epidermal nerve fibers: Correlation with sensory function. *J. Neurosci.* **1998**, *18*, 8947–8959. [CrossRef]
237. Caruntu, C.; Boda, D.; Musat, S.; Caruntu, A.; Mandache, E. Stress-induced mast cell activation in glabrous and hairy skin. *Mediat. Inflamm.* **2014**, *2014*, 105950. [CrossRef]
238. Brodin, E.; Nilsson, G. Concentration of substance P-like immunoreactivity (SPLI) in tissues of dog, rat and mouse. *Acta Physiol. Scand.* **1981**, *112*, 305–312. [CrossRef] [PubMed]
239. Mistrova, E.; Kruzliak, P.; Dvorakova, M.C. Role of substance P in the cardiovascular system. *Neuropeptides* **2016**, *58*, 41–51. [CrossRef]
240. Geppetti, P.; Bertrand, C.; Baker, J.; Yamawaki, I.; Piedimonte, G.; Nadel, J.A. Ruthenium red, but not capsazepine reduces plasma extravasation by cigarette smoke in rat airways. *Br. J. Pharmacol.* **1993**, *108*, 646–650. [CrossRef] [PubMed]
241. Figini, M.; Emanueli, C.; Bertrand, C.; Javdan, P.; Geppetti, P. Evidence that tachykinins relax the guinea-pig trachea via nitric oxide release and by stimulation of a septide-insensitive NK1 receptor. *Br. J. Pharmacol.* **1996**, *117*, 1270–1276. [CrossRef] [PubMed]
242. Hauser-Kronberger, C.; Hacker, G.W.; Franz, P.; Albegger, K.; Dietze, O. CGRP and substance P in intraepithelial neuronal structures of the human upper respiratory system. *Regul. Pept.* **1997**, *72*, 79–85. [CrossRef]
243. Barthó, L.; Holzer, P. Search for a physiological role of substance P in gastrointestinal motility. *Neuroscience* **1985**, *16*, 1–32. [CrossRef]

244. Costa, M.; Furness, J.B.; Gibbins, I.L. Chapter 15 Chemical coding of enteric neurons. In *Progress in Brain Research*; Hökfelt, T., Fuxe, K., Pernow, B., Eds.; Elsevier: Amsterdam, The Netherlands, 1986; Volume 68, pp. 217–239.
245. Hökfelt, T.; Pernow, B.; Wahren, J. Substance P: A pioneer amongst neuropeptides. *J. Intern. Med.* **2001**, *249*, 27–40. [CrossRef]
246. Watts, S.W.; Cohen, M.L. Effect of bombesin, bradykinin, substance P and CGRP in prostate, bladder body and neck. *Peptides* **1991**, *12*, 1057–1062. [CrossRef] [PubMed]
247. Maggi, C.A. Tachykinins and calcitonin gene-related peptide (CGRP) as co-transmitters released from peripheral endings of sensory nerves. *Prog. Neurobiol.* **1995**, *45*, 1–98. [CrossRef] [PubMed]
248. Edvinsson, L.; Rosendal-Helgesen, S.; Uddman, R. Substance P: Localization, concentration and release in cerebral arteries, choroid plexus and dura mater. *Cell Tissue Res.* **1983**, *234*, 1–7. [CrossRef] [PubMed]
249. Petersson, J.; Zygmunt, P.M.; Brandt, L.; Högestätt, E.D. Substance P-induced relaxation and hyperpolarization in human cerebral arteries. *Br. J. Pharmacol.* **1995**, *115*, 889. [CrossRef] [PubMed]
250. Harrison, S.; Geppetti, P. Substance P. *Int. J. Biochem. Cell Biol.* **2001**, *33*, 555–576. [CrossRef] [PubMed]
251. Mehboob, R.; Oehme, P.; Pfaff, G. The role of Substance P in the defense line of the respiratory tract and neurological manifestations post COVID-19 infection. *Front. Neurol.* **2023**, *14*, 1052811. [CrossRef] [PubMed]
252. Chen, F.X.; Wan, Q.; Fang, J.; Peng, L.; Li, Q.L.; Hu, J. The Src1-PGC1α-AP1 complex-dependent secretion of substance P induces inflammation and apoptosis in encephalomyocarditis virus-infected mice. *Cytokine* **2023**, *165*, 156186. [CrossRef] [PubMed]
253. Arruda-Vasconcelos, R.; Chiarelli-Neto, V.M.; Louzada, L.M.; Aveiro, E.; Alves-Silva, E.G.; de-Jesus-Soares, A.; Ferraz, C.C.R.; Almeida, J.F.A.; Marciano, M.A.; Pecorari, V.G.A.; et al. Quantitative analysis of culturable bacteria, levels of endotoxins, inflammatory mediators and substance P in teeth with symptomatic irreversible pulpitis and in teeth with vital normal pulp tissues. *Int. Endod. J.* **2023**, *56*, 827–836. [CrossRef] [PubMed]
254. Johnson, M.B.; Suptela, S.R.; Sipprell, S.E.; Marriott, I. Substance P Exacerbates the Inflammatory and Pro-osteoclastogenic Responses of Murine Osteoclasts and Osteoblasts to Staphylococcus aureus. *Inflammation* **2023**, *46*, 256–269. [CrossRef]
255. Scheau, C.; Ilie Ghita, M.; Grigore, O.; Mihailescu, A.; Caruntu, A.; Mihai, L.; Bădărău, I.; Boda, D.; Caruntu, C. Modulation of capsaicin-induced neurogenic vasodilation by acute psychological stress. *Farmacia* **2021**, *69*, 778–784. [CrossRef]
256. Xiang, Y.; Xu, X.; Zhang, T.; Wu, X.; Fan, D.; Hu, Y.; Ding, J.; Yang, X.; Lou, J.; Du, Q.; et al. Beneficial effects of dietary capsaicin in gastrointestinal health and disease. *Exp. Cell Res.* **2022**, *417*, 113227. [CrossRef] [PubMed]
257. Patowary, P.; Pathak, M.P.; Zaman, K.; Raju, P.S.; Chattopadhyay, P. Research progress of capsaicin responses to various pharmacological challenges. *Biomed. Pharmacother.* **2017**, *96*, 1501–1512. [CrossRef] [PubMed]
258. Kunjiappan, S.; Sankaranarayanan, M.; Karan Kumar, B.; Pavadai, P.; Babkiewicz, E.; Maszczyk, P.; Glodkowska-Mrowka, E.; Arunachalam, S.; Ram Kumar Pandian, S.; Ravishankar, V.; et al. Capsaicin-loaded solid lipid nanoparticles: Design, biodistribution, in silico modeling and in vitro cytotoxicity evaluation. *Nanotechnology* **2021**, *32*, 095101. [CrossRef] [PubMed]
259. Ahmady, A.R.; Solouk, A.; Saber-Samandari, S.; Akbari, S.; Ghanbari, H.; Brycki, B.E. Capsaicin-loaded alginate nanoparticles embedded polycaprolactone-chitosan nanofibers as a controlled drug delivery nanoplatform for anticancer activity. *J. Colloid. Interface Sci.* **2023**, *638*, 616–628. [CrossRef] [PubMed]
260. Thán, M.; Németh, J.; Szilvássy, Z.; Pintér, E.; Helyes, Z.; Szolcsányi, J. Systemic anti-inflammatory effect of somatostatin released from capsaicin-sensitive vagal and sciatic sensory fibres of the rat and guinea-pig. *Eur. J. Pharmacol.* **2000**, *399*, 251–258. [CrossRef] [PubMed]
261. Lauritano, D.; Petruzzi, M.; Baldoni, M. [Preliminary protocol for systemic administration of capsaicin for the treatment of the burning mouth syndrome]. *Minerva Stomatol.* **2003**, *52*, 273–278. [PubMed]
262. Luqman, S.; Rizvi, S.I. Protection of lipid peroxidation and carbonyl formation in proteins by capsaicin in human erythrocytes subjected to oxidative stress. *Phytother. Res.* **2006**, *20*, 303–306. [CrossRef]
263. Lee, I.O.; Lee, K.H.; Pyo, J.H.; Kim, J.H.; Choi, Y.J.; Lee, Y.C. Anti-inflammatory Effect of Capsaicin in Helicobacter pylori-Infected Gastric Epithelial Cells. *Helicobacter* **2007**, *12*, 510–517. [CrossRef] [PubMed]
264. Wu, Y.; Starzinski-Powitz, A.; Guo, S.W. Capsaicin inhibits proliferation of endometriotic cells in vitro. *Gynecol. Obstet. Investig.* **2008**, *66*, 59–62. [CrossRef]
265. Bortolotti, M.; Porta, S. Effect of red pepper on symptoms of irritable bowel syndrome: Preliminary study. *Dig. Dis. Sci.* **2011**, *56*, 3288–3295. [CrossRef]
266. Yu, Q.; Wang, Y.; Yu, Y.; Li, Y.; Zhao, S.; Chen, Y.; Waqar, A.B.; Fan, J.; Liu, E. Expression of TRPV1 in rabbits and consuming hot pepper affects its body weight. *Mol. Biol. Rep.* **2012**, *39*, 7583–7589. [CrossRef]
267. Ternesten-Hasséus, E.; Johansson, E.L.; Millqvist, E. Cough reduction using capsaicin. *Respir. Med.* **2015**, *109*, 27–37. [CrossRef] [PubMed]
268. Chen, K.S.; Chen, P.N.; Hsieh, Y.S.; Lin, C.Y.; Lee, Y.H.; Chu, S.C. Capsaicin protects endothelial cells and macrophage against oxidized low-density lipoprotein-induced injury by direct antioxidant action. *Chem. Biol. Interact.* **2015**, *228*, 35–45. [CrossRef]
269. He, H.; Zhou, Y.; Huang, J.; Wu, Z.; Liao, Z.; Liu, D.; Yin, D.; He, M. Capsaicin Protects Cardiomyocytes against Anoxia/Reoxygenation Injury via Preventing Mitochondrial Dysfunction Mediated by SIRT1. *Oxid. Med. Cell. Longev.* **2017**, *2017*, 1035702. [CrossRef]
270. Ren, X.; Roessler, A.E.; Lynch, T.L., 4th; Haar, L.; Mallick, F.; Lui, Y.; Tranter, M.; Ren, M.H.; Xie, W.R.; Fan, G.-C.; et al. Cardioprotection via the skin: Nociceptor-induced conditioning against cardiac MI in the NIC of time. *Am. J. Physiol.-Heart Circ. Physiol.* **2019**, *316*, H543–H553. [CrossRef] [PubMed]

271. Yang, S.; Liu, L.; Meng, L.; Hu, X. Capsaicin is beneficial to hyperlipidemia, oxidative stress, endothelial dysfunction, and atherosclerosis in Guinea pigs fed on a high-fat diet. *Chem. Biol. Interact.* **2019**, *297*, 1–7. [CrossRef] [PubMed]
272. Shin, M.K.; Yang, S.-M.; Han, I.-S. Capsaicin suppresses liver fat accumulation in high-fat diet-induced NAFLD mice. *Anim. Cells Syst.* **2020**, *24*, 214–219. [CrossRef] [PubMed]
273. Segawa, Y.; Hashimoto, H.; Maruyama, S.; Shintani, M.; Ohno, H.; Nakai, Y.; Osera, T.; Kurihara, N. Dietary capsaicin-mediated attenuation of hypertension in a rat model of renovascular hypertension. *Clin. Exp. Hypertens.* **2020**, *42*, 352–359. [CrossRef] [PubMed]
274. Grüter, T.; Blusch, A.; Motte, J.; Sgodzai, M.; Bachir, H.; Klimas, R.; Ambrosius, B.; Gold, R.; Ellrichmann, G.; Pitarokoili, K. Immunomodulatory and anti-oxidative effect of the direct TRPV1 receptor agonist capsaicin on Schwann cells. *J. Neuroinflammation* **2020**, *17*, 145. [CrossRef]
275. Abdel-Salam, O.M.E.; Sleem, A.A.; Sayed, M.; Youness, E.R.; Shaffie, N. Capsaicin Exerts Anti-convulsant and Neuroprotective Effects in Pentylenetetrazole-Induced Seizures. *Neurochem. Res.* **2020**, *45*, 1045–1061. [CrossRef]
276. Nawaka, N.; Wanmasae, S.; Makarasen, A.; Dechtrirat, D.; Techasakul, S.; Jeenduang, N. Allicin and Capsaicin Ameliorated Hypercholesterolemia by Upregulating LDLR and Downregulating PCSK9 Expression in HepG2 Cells. *Int. J. Mol. Sci.* **2022**, *23*, 14299. [CrossRef] [PubMed]
277. Zhang, Q.; Luo, P.; Xia, F.; Tang, H.; Chen, J.; Zhang, J.; Liu, D.; Zhu, Y.; Liu, Y.; Gu, L.; et al. Capsaicin ameliorates inflammation in a TRPV1-independent mechanism by inhibiting PKM2-LDHA-mediated Warburg effect in sepsis. *Cell Chem. Biol.* **2022**, *29*, 1248–1259.e6. [CrossRef] [PubMed]
278. Warburg, O. The metabolism of carcinoma cells. *J. Cancer Res.* **1925**, *9*, 148–163. [CrossRef]
279. Warburg, O.; Wind, F.; Negelein, E. Ueber den stoffwechsel von tumoren im körper. *Klin. Wochenschr.* **1926**, *5*, 829–832. [CrossRef]
280. Liberti, M.V.; Locasale, J.W. The Warburg Effect: How Does it Benefit Cancer Cells? *Trends Biochem. Sci.* **2016**, *41*, 211–218. [CrossRef] [PubMed]
281. Palsson-McDermott, E.M.; O'neill, L.A. The Warburg effect then and now: From cancer to inflammatory diseases. *Bioessays* **2013**, *35*, 965–973. [CrossRef] [PubMed]
282. Kozal, K.; Jóźwiak, P.; Krześlak, A. Contemporary perspectives on the Warburg effect inhibition in cancer therapy. *Cancer Control.* **2021**, *28*, 10732748211041243. [CrossRef] [PubMed]
283. Hawkey, C. COX-2 inhibitors. *Lancet* **1999**, *353*, 307–314. [CrossRef]
284. Stichtenoth, D.O.; Frölich, J.C. The second generation of COX-2 inhibitors: What advantages do the newest offer? *Drugs* **2003**, *63*, 33–45. [CrossRef]
285. Zarghi, A.; Arfaei, S. Selective COX-2 inhibitors: A review of their structure-activity relationships. *Iran. J. Pharm. Res. IJPR* **2011**, *10*, 655.
286. Movahed, P.; Jönsson, B.A.; Birnir, B.; Wingstrand, J.A.; Jørgensen, T.D.; Ermund, A.; Sterner, O.; Zygmunt, P.M.; Högestätt, E.D. Endogenous unsaturated C18 N-acylethanolamines are vanilloid receptor (TRPV1) agonists. *J. Biol. Chem.* **2005**, *280*, 38496–38504. [CrossRef] [PubMed]
287. Wong, G.Y.; Gavva, N.R. Therapeutic potential of vanilloid receptor TRPV1 agonists and antagonists as analgesics: Recent advances and setbacks. *Brain Res. Rev.* **2009**, *60*, 267–277. [CrossRef] [PubMed]
288. Gharat, L.A.; Szallasi, A. Advances in the design and therapeutic use of capsaicin receptor TRPV1 agonists and antagonists. *Expert. Opin. Ther. Pat.* **2008**, *18*, 159–209. [CrossRef]
289. Khairatkar-Joshi, N.; Szallasi, A. TRPV1 antagonists: The challenges for therapeutic targeting. *Trends Mol. Med.* **2009**, *15*, 14–22. [CrossRef] [PubMed]
290. Kym, P.R.; Kort, M.E.; Hutchins, C.W. Analgesic potential of TRPV1 antagonists. *Biochem. Pharmacol.* **2009**, *78*, 211–216. [CrossRef] [PubMed]
291. Kort, M.E.; Kym, P.R. TRPV1 antagonists: Clinical setbacks and prospects for future development. *Prog. Med. Chem.* **2012**, *51*, 57–70.
292. Brandt, M.R.; Beyer, C.E.; Stahl, S.M. TRPV1 antagonists and chronic pain: Beyond thermal perception. *Pharmaceuticals* **2012**, *5*, 114–132. [CrossRef] [PubMed]
293. Chow, J.; Norng, M.; Zhang, J.; Chai, J. TRPV6 mediates capsaicin-induced apoptosis in gastric cancer cells--Mechanisms behind a possible new "hot" cancer treatment. *Biochim. Biophys. Acta* **2007**, *1773*, 565–576. [CrossRef] [PubMed]
294. Jun, H.S.; Park, T.; Lee, C.K.; Kang, M.K.; Park, M.S.; Kang, H.I.; Surh, Y.J.; Kim, O.H. Capsaicin induced apoptosis of B16-F10 melanoma cells through down-regulation of Bcl-2. *Food Chem. Toxicol.* **2007**, *45*, 708–715. [CrossRef]
295. Anandakumar, P.; Kamaraj, S.; Jagan, S.; Ramakrishnan, G.; Vinodhkumar, R.; Devaki, T. Stabilization of pulmonary mitochondrial enzyme system by capsaicin during benzo(a)pyrene induced experimental lung cancer. *Biomed. Pharmacother.* **2008**, *62*, 390–394. [CrossRef]
296. Zhang, R.; Humphreys, I.; Sahu, R.P.; Shi, Y.; Srivastava, S.K. In vitro and in vivo induction of apoptosis by capsaicin in pancreatic cancer cells is mediated through ROS generation and mitochondrial death pathway. *Apoptosis* **2008**, *13*, 1465–1478. [CrossRef] [PubMed]
297. Brown, K.C.; Witte, T.R.; Hardman, W.E.; Luo, H.; Chen, Y.C.; Carpenter, A.B.; Lau, J.K.; Dasgupta, P. Capsaicin displays anti-proliferative activity against human small cell lung cancer in cell culture and nude mice models via the E2F pathway. *PLoS ONE* **2010**, *5*, e10243. [CrossRef] [PubMed]

298. Bai, H.; Li, H.; Zhang, W.; Matkowskyj, K.A.; Liao, J.; Srivastava, S.K.; Yang, G.Y. Inhibition of chronic pancreatitis and pancreatic intraepithelial neoplasia (PanIN) by capsaicin in LSL-KrasG12D/Pdx1-Cre mice. *Carcinogenesis* **2011**, *32*, 1689–1696. [CrossRef] [PubMed]
299. Chang, H.C.; Chen, S.T.; Chien, S.Y.; Kuo, S.J.; Tsai, H.T.; Chen, D.R. Capsaicin may induce breast cancer cell death through apoptosis-inducing factor involving mitochondrial dysfunction. *Hum. Exp. Toxicol.* **2011**, *30*, 1657–1665. [CrossRef] [PubMed]
300. Ip, S.W.; Lan, S.H.; Huang, A.C.; Yang, J.S.; Chen, Y.Y.; Huang, H.Y.; Lin, Z.P.; Hsu, Y.M.; Yang, M.D.; Chiu, C.F. Capsaicin induces apoptosis in SCC-4 human tongue cancer cells through mitochondria-dependent and-independent pathways. *Environ. Toxicol.* **2012**, *27*, 332–341. [CrossRef] [PubMed]
301. Lee, S.H.; Richardson, R.L.; Dashwood, R.H.; Baek, S.J. Capsaicin represses transcriptional activity of β-catenin in human colorectal cancer cells. *J. Nutr. Biochem.* **2012**, *23*, 646–655. [CrossRef] [PubMed]
302. Chen, D.; Yang, Z.; Wang, Y.; Zhu, G.; Wang, X. Capsaicin induces cycle arrest by inhibiting cyclin-dependent-kinase in bladder carcinoma cells. *Int. J. Urol.* **2012**, *19*, 662–668. [CrossRef]
303. Lin, C.H.; Lu, W.C.; Wang, C.W.; Chan, Y.C.; Chen, M.K. Capsaicin induces cell cycle arrest and apoptosis in human KB cancer cells. *BMC Complement. Altern. Med.* **2013**, *13*, 46. [CrossRef] [PubMed]
304. Park, S.Y.; Kim, J.Y.; Lee, S.M.; Jun, C.H.; Cho, S.B.; Park, C.H.; Joo, Y.E.; Kim, H.S.; Choi, S.K.; Rew, J.S. Capsaicin induces apoptosis and modulates MAPK signaling in human gastric cancer cells. *Mol. Med. Rep.* **2014**, *9*, 499–502. [CrossRef]
305. Wutka, A.; Palagani, V.; Barat, S.; Chen, X.; El Khatib, M.; Götze, J.; Belahmer, H.; Zender, S.; Bozko, P.; Malek, N.P.; et al. Capsaicin treatment attenuates cholangiocarcinoma carcinogenesis. *PLoS ONE* **2014**, *9*, e95605. [CrossRef]
306. Skrzypski, M.; Sassek, M.; Abdelmessih, S.; Mergler, S.; Grötzinger, C.; Metzke, D.; Wojciechowicz, T.; Nowak, K.W.; Strowski, M.Z. Capsaicin induces cytotoxicity in pancreatic neuroendocrine tumor cells via mitochondrial action. *Cell Signal.* **2014**, *26*, 41–48. [CrossRef] [PubMed]
307. Gilardini Montani, M.S.; D'Eliseo, D.; Cirone, M.; Di Renzo, L.; Faggioni, A.; Santoni, A.; Velotti, F. Capsaicin-mediated apoptosis of human bladder cancer cells activates dendritic cells via CD91. *Nutrition* **2015**, *31*, 578–581. [CrossRef] [PubMed]
308. Zheng, L.; Chen, J.; Ma, Z.; Liu, W.; Yang, F.; Yang, Z.; Wang, K.; Wang, X.; He, D.; Li, L. Capsaicin causes inactivation and degradation of the androgen receptor by inducing the restoration of miR-449a in prostate cancer. *Oncol. Rep.* **2015**, *34*, 1027–1034. [CrossRef] [PubMed]
309. Lin, M.H.; Lee, Y.H.; Cheng, H.L.; Chen, H.Y.; Jhuang, F.H.; Chueh, P.J. Capsaicin Inhibits Multiple Bladder Cancer Cell Phenotypes by Inhibiting Tumor-Associated NADH Oxidase (tNOX) and Sirtuin1 (SIRT1). *Molecules* **2016**, *21*, 849. [CrossRef] [PubMed]
310. Wang, F.; Zhao, J.; Liu, D.; Zhao, T.; Lu, Z.; Zhu, L.; Cao, L.; Yang, J.; Jin, J.; Cai, Y. Capsaicin reactivates hMOF in gastric cancer cells and induces cell growth inhibition. *Cancer Biol. Ther.* **2016**, *17*, 1117–1125. [CrossRef] [PubMed]
311. Ramos-Torres, Á.; Bort, A.; Morell, C.; Rodríguez-Henche, N.; Díaz-Laviada, I. The pepper's natural ingredient capsaicin induces autophagy blockage in prostate cancer cells. *Oncotarget* **2016**, *7*, 1569–1583. [CrossRef]
312. Liu, T.; Wang, G.; Tao, H.; Yang, Z.; Wang, Y.; Meng, Z.; Cao, R.; Xiao, Y.; Wang, X.; Zhou, J. Capsaicin mediates caspases activation and induces apoptosis through P38 and JNK MAPK pathways in human renal carcinoma. *BMC Cancer* **2016**, *16*, 790. [CrossRef]
313. Lv, L.; Zhuang, Y.X.; Zhang, H.W.; Tian, N.N.; Dang, W.Z.; Wu, S.Y. Capsaicin-loaded folic acid-conjugated lipid nanoparticles for enhanced therapeutic efficacy in ovarian cancers. *Biomed. Pharmacother.* **2017**, *91*, 999–1005. [CrossRef]
314. Lin, Y.T.; Wang, H.C.; Hsu, Y.C.; Cho, C.L.; Yang, M.Y.; Chien, C.Y. Capsaicin Induces Autophagy and Apoptosis in Human Nasopharyngeal Carcinoma Cells by Downregulating the PI3K/AKT/mTOR Pathway. *Int. J. Mol. Sci.* **2017**, *18*, 1343. [CrossRef]
315. Mao, X.; Zhu, H.; Luo, D.; Ye, L.; Yin, H.; Zhang, J.; Zhang, Y.; Zhang, Y. Capsaicin inhibits glycolysis in esophageal squamous cell carcinoma by regulating hexokinase-2 expression. *Mol. Med. Rep.* **2018**, *17*, 6116–6121. [CrossRef]
316. Kamaruddin, M.F.; Hossain, M.Z.; Mohamed Alabsi, A.; Mohd Bakri, M. The Antiproliferative and Apoptotic Effects of Capsaicin on an Oral Squamous Cancer Cell Line of Asian Origin, ORL-48. *Medicina* **2019**, *55*, 322. [CrossRef] [PubMed]
317. Bao, Z.; Dai, X.; Wang, P.; Tao, Y.; Chai, D. Capsaicin induces cytotoxicity in human osteosarcoma MG63 cells through TRPV1-dependent and-independent pathways. *Cell Cycle* **2019**, *18*, 1379–1392. [CrossRef] [PubMed]
318. Wu, D.; Jia, H.; Zhang, Z.; Li, S. Capsaicin suppresses breast cancer cell viability by regulating the CDK8/PI3K/Akt/Wnt/β-catenin signaling pathway. *Mol. Med. Rep.* **2020**, *22*, 4868–4876. [CrossRef] [PubMed]
319. Zhu, M.; Yu, X.; Zheng, Z.; Huang, J.; Yang, X.; Shi, H. Capsaicin suppressed activity of prostate cancer stem cells by inhibition of Wnt/β-catenin pathway. *Phytother. Res.* **2020**, *34*, 817–824. [CrossRef] [PubMed]
320. Jia, G.; Cang, S.; Ma, P.; Song, Z. Capsaicin: A "hot" KDM1A/LSD1 inhibitor from peppers. *Bioorg Chem.* **2020**, *103*, 104161. [CrossRef] [PubMed]
321. Szoka, L.; Palka, J. Capsaicin up-regulates pro-apoptotic activity of thiazolidinediones in glioblastoma cell line. *Biomed. Pharmacother.* **2020**, *132*, 110741. [CrossRef] [PubMed]
322. Chen, M.; Xiao, C.; Jiang, W.; Yang, W.; Qin, Q.; Tan, Q.; Lian, B.; Liang, Z.; Wei, C. Capsaicin Inhibits Proliferation and Induces Apoptosis in Breast Cancer by Down-Regulating FBI-1-Mediated NF-κB Pathway. *Drug Des. Dev. Ther.* **2021**, *15*, 125–140. [CrossRef] [PubMed]
323. Han, T.H.; Park, M.K.; Nakamura, H.; Ban, H.S. Capsaicin inhibits HIF-1α accumulation through suppression of mitochondrial respiration in lung cancer cells. *Biomed. Pharmacother.* **2022**, *146*, 112500. [CrossRef]

324. Que, T.; Ren, B.; Fan, Y.; Liu, T.; Hou, T.; Dan, W.; Liu, B.; Wei, Y.; Lei, Y.; Zeng, J.; et al. Capsaicin inhibits the migration, invasion and EMT of renal cancer cells by inducing AMPK/mTOR-mediated autophagy. *Chem. Biol. Interact.* **2022**, *366*, 110043. [CrossRef]
325. Liu, X.-Y.; Wei, D.-G.; Li, R.-S. Capsaicin induces ferroptosis of NSCLC by regulating SLC7A11/GPX4 signaling in vitro. *Sci. Rep.* **2022**, *12*, 11996. [CrossRef]
326. Xie, Z.Q.; Li, H.X.; Hou, X.J.; Huang, M.Y.; Zhu, Z.M.; Wei, L.X.; Tang, C.X. Capsaicin suppresses hepatocarcinogenesis by inhibiting the stemness of hepatic progenitor cells via SIRT1/SOX2 signaling pathway. *Cancer Med.* **2022**, *11*, 4283–4296. [CrossRef] [PubMed]
327. Wu, L.; Xu, S.; Cheng, X.; Zhang, L.; Wang, Y.; Wu, J.; Bao, J.; Yu, H.; Lu, R. Capsaicin inhibits the stemness of anaplastic thyroid carcinoma cells by triggering autophagy-lysosome mediated OCT4A degradation. *Phytother. Res.* **2022**, *36*, 938–950. [CrossRef] [PubMed]
328. Adetunji, T.L.; Olawale, F.; Olisah, C.; Adetunji, A.E.; Aremu, A.O. Capsaicin: A Two-Decade Systematic Review of Global Research Output and Recent Advances Against Human Cancer. *Front. Oncol.* **2022**, *12*, 908487. [CrossRef] [PubMed]
329. Lau, J.K.; Brown, K.C.; Dom, A.M.; Witte, T.R.; Thornhill, B.A.; Crabtree, C.M.; Perry, H.E.; Brown, J.M.; Ball, J.G.; Creel, R.G.; et al. Capsaicin induces apoptosis in human small cell lung cancer via the TRPV6 receptor and the calpain pathway. *Apoptosis* **2014**, *19*, 1190–1201. [CrossRef] [PubMed]
330. Caruntu, C.; Boda, D.; Constantin, C.; Caruntu, A.; Neagu, M. Catecholamines increase in vitro proliferation of murine B16F10 melanoma cells. *Acta Endocrinol.* **2014**, *10*, 545–558. [CrossRef]
331. Scheau, C.; Draghici, C.; Ilie, M.A.; Lupu, M.; Solomon, I.; Tampa, M.; Georgescu, S.R.; Caruntu, A.; Constantin, C.; Neagu, M.; et al. Neuroendocrine Factors in Melanoma Pathogenesis. *Cancers* **2021**, *13*, 2277. [CrossRef] [PubMed]
332. Venier, N.A.; Colquhoun, A.J.; Sasaki, H.; Kiss, A.; Sugar, L.; Adomat, H.; Fleshner, N.E.; Klotz, L.H.; Venkateswaran, V. Capsaicin: A novel radio-sensitizing agent for prostate cancer. *Prostate* **2015**, *75*, 113–125. [CrossRef] [PubMed]
333. Min, J.K.; Han, K.Y.; Kim, E.C.; Kim, Y.M.; Lee, S.W.; Kim, O.H.; Kim, K.W.; Gho, Y.S.; Kwon, Y.G. Capsaicin inhibits in vitro and in vivo angiogenesis. *Cancer Res.* **2004**, *64*, 644–651. [CrossRef] [PubMed]
334. Vendrely, V.; Peuchant, E.; Buscail, E.; Moranvillier, I.; Rousseau, B.; Bedel, A.; Brillac, A.; de Verneuil, H.; Moreau-Gaudry, F.; Dabernat, S. Resveratrol and capsaicin used together as food complements reduce tumor growth and rescue full efficiency of low dose gemcitabine in a pancreatic cancer model. *Cancer Lett.* **2017**, *390*, 91–102. [CrossRef] [PubMed]
335. Friedman, J.R.; Perry, H.E.; Brown, K.C.; Gao, Y.; Lin, J.; Stevenson, C.D.; Hurley, J.D.; Nolan, N.A.; Akers, A.T.; Chen, Y.C.; et al. Capsaicin synergizes with camptothecin to induce increased apoptosis in human small cell lung cancers via the calpain pathway. *Biochem. Pharmacol.* **2017**, *129*, 54–66. [CrossRef]
336. Dai, N.; Ye, R.; He, Q.; Guo, P.; Chen, H.; Zhang, Q. Capsaicin and sorafenib combination treatment exerts synergistic anti-hepatocellular carcinoma activity by suppressing EGFR and PI3K/Akt/mTOR signaling. *Oncol. Rep.* **2018**, *40*, 3235–3248. [CrossRef] [PubMed]
337. Friedman, J.R.; Richbart, S.D.; Merritt, J.C.; Perry, H.E.; Brown, K.C.; Akers, A.T.; Nolan, N.A.; Stevenson, C.D.; Hurley, J.D.; Miles, S.L.; et al. Capsaicinoids enhance chemosensitivity to chemotherapeutic drugs. *Adv. Cancer Res.* **2019**, *144*, 263–298. [CrossRef] [PubMed]
338. Tampa, M.; Georgescu, S.R.; Mitran, C.I.; Mitran, M.I.; Matei, C.; Scheau, C.; Constantin, C.; Neagu, M. Recent Advances in Signaling Pathways Comprehension as Carcinogenesis Triggers in Basal Cell Carcinoma. *J. Clin. Med.* **2020**, *9*, 3010. [CrossRef] [PubMed]
339. Szolcsányi, J.; Barthó, L. Capsaicin-sensitive afferents and their role in gastroprotection: An update. *J. Physiol. Paris.* **2001**, *95*, 181–188. [CrossRef] [PubMed]
340. Szolcsányi, J.; Helyes, Z.; Oroszi, G.; Németh, J.; Pintér, E. Release of somatostatin and its role in the mediation of the anti-inflammatory effect induced by antidromic stimulation of sensory fibres of rat sciatic nerve. *Br. J. Pharmacol.* **1998**, *123*, 936–942. [CrossRef] [PubMed]
341. Helyes, Z.; Thán, M.; Oroszi, G.; Pintér, E.; Németh, J.; Kéri, G.; Szolcsányi, J. Anti-nociceptive effect induced by somatostatin released from sensory nerve terminals and by synthetic somatostatin analogues in the rat. *Neurosci. Lett.* **2000**, *278*, 185–188. [CrossRef] [PubMed]
342. Pintér, E.; Helyes, Z.; Szolcsányi, J. Inhibitory effect of somatostatin on inflammation and nociception. *Pharmacol. Ther.* **2006**, *112*, 440–456. [CrossRef] [PubMed]
343. Szolcsányi, J. Forty years in capsaicin research for sensory pharmacology and physiology. *Neuropeptides* **2004**, *38*, 377–384. [CrossRef] [PubMed]
344. Evers, B.M.; Parekh, D.; Townsend, C.M., Jr.; Thompson, J.C. Somatostatin and analogues in the treatment of cancer. A review. *Ann. Surg.* **1991**, *213*, 190–198. [CrossRef]
345. Weckbecker, G.; Raulf, F.; Stolz, B.; Bruns, C. Somatostatin analogs for diagnosis and treatment of cancer. *Pharmacol. Ther.* **1993**, *60*, 245–264. [CrossRef]
346. Scarpignato, C.; Pelosini, I. Somatostatin Analogs for Cancer Treatment and Diagnosis: An Overview. *Chemotherapy* **2001**, *47* (Suppl. 2), 1–29. [CrossRef]
347. Lamberts, S.W.J.; de Herder, W.W.; Hofland, L.J. Somatostatin analogs in the diagnosis and treatment of cancer. *Trends Endocrinol. Metab.* **2002**, *13*, 451–457. [CrossRef]
348. Froidevaux, S.; Eberle, A.N. Somatostatin analogs and radiopeptides in cancer therapy. *Pept. Sci.* **2002**, *66*, 161–183. [CrossRef]

349. Klironomos, S.; Notas, G.; Sfakianaki, O.; Kiagiadaki, F.; Xidakis, C.; Kouroumalis, E. Octreotide modulates the effects on fibrosis of TNF-α, TGF-β and PDGF in activated rat hepatic stellate cells. *Regul. Pept.* **2014**, *188*, 5–12. [CrossRef] [PubMed]
350. Ayiomamitis, G.D.; Notas, G.; Zaravinos, A.; Drygiannakis, I.; Georgiadou, M.; Sfakianaki, O.; Mastrodimou, N.; Thermos, K.; Kouroumalis, E. Effects of octreotide and insulin on colon cancer cellular proliferation and correlation with hTERT activity. *Oncoscience* **2014**, *1*, 457. [CrossRef]
351. Kouroumalis, E.; Samonakis, D.; Notas, G. Somatostatin in hepatocellular carcinoma: Experimental and therapeutic implications. *Hepatoma Res.* **2018**, *4*, 34. [CrossRef]
352. Periferakis, A.; Tsigas, G.; Periferakis, A.-T.; Badarau, I.A.; Scheau, A.-E.; Tampa, M.; Georgescu, S.R.; Didilescu, A.C.; Scheau, C.; Caruntu, C. Antitumoral and Anti-inflammatory Roles of Somatostatin and Its Analogs in Hepatocellular Carcinoma. *Anal. Cell. Pathol.* **2021**, *2021*, 1840069. [CrossRef]
353. Kouroumalis, E.; Tsomidis, I.; Voumvouraki, A. Is There a Place for Somatostatin Analogues for the Systemic Treatment of Hepatocellular Carcinoma in the Immunotherapy Era? *Livers* **2022**, *2*, 315–335. [CrossRef]
354. Qin, J.C.; Yu, W.T.; Li, H.X.; Liang, Y.Q.; Nong, F.F.; Wen, B. Cold exposure and capsaicin promote 1,2-dimethylhyrazine-induced colon carcinogenesis in rats correlates with extracellular matrix remodeling. *World J. Gastroenterol.* **2021**, *27*, 6615–6630. [CrossRef]
355. Kim, H.S.; Kwon, H.J.; Kim, G.E.; Cho, M.H.; Yoon, S.Y.; Davies, A.J.; Oh, S.B.; Lee, H.; Cho, Y.K.; Joo, C.H.; et al. Attenuation of natural killer cell functions by capsaicin through a direct and TRPV1-independent mechanism. *Carcinogenesis* **2014**, *35*, 1652–1660. [CrossRef]
356. Liu, Z.; Zhu, P.; Tao, Y.; Shen, C.; Wang, S.; Zhao, L.; Wu, H.; Fan, F.; Lin, C.; Chen, C.; et al. Cancer-promoting effect of capsaicin on DMBA/TPA-induced skin tumorigenesis by modulating inflammation, Erk and p38 in mice. *Food Chem. Toxicol.* **2015**, *81*, 1–8. [CrossRef] [PubMed]
357. Cheng, P.; Wu, J.; Zong, G.; Wang, F.; Deng, R.; Tao, R.; Qian, C.; Shan, Y.; Wang, A.; Zhao, Y.; et al. Capsaicin shapes gut microbiota and pre-metastatic niche to facilitate cancer metastasis to liver. *Pharmacol. Res.* **2023**, *188*, 106643. [CrossRef] [PubMed]
358. Andrews, J. *Peppers: The Domesticated Capsicums*; University of Texas Press: Austin, TX, USA, 1995.
359. Nabhan, G.P. *Why Some Like It Hot: Food, Genes, and Cultural Diversity*; Island Press: Washington, DC, USA, 2004.
360. Kim, S.; Park, M.; Yeom, S.I.; Kim, Y.M.; Lee, J.M.; Lee, H.A.; Seo, E.; Choi, J.; Cheong, K.; Kim, K.T.; et al. Genome sequence of the hot pepper provides insights into the evolution of pungency in Capsicum species. *Nat. Genet.* **2014**, *46*, 270–278. [CrossRef] [PubMed]
361. Basu, S.K.; De, A.K. Capsicum: Historical and botanical perspectives. In *Capsicum*; CRC Press: Boca Raton, FL, USA, 2003; pp. 21–35.
362. Eshbaugh, W.H. Genetic and biochemical systematic studies of chili peppers (Capsicum-Solanaceae). *Bull. Torrey Bot. Club* **1975**, *102*, 396–403. [CrossRef]
363. Eshbaugh, W.H. The taxonomy of the genus Capsicum. In *Peppers: Botany, Production and Uses*; CAB International: Wallingford UK, 2012; pp. 14–28.
364. Heiser, C.B., Jr.; Pickersgill, B. Names for the Cultivated Capsicum Species (Solanaceae). *Taxon* **1969**, *18*, 277–283. [CrossRef]
365. Votava, E.J.; Baral, J.B.; Bosland, P.W. Genetic diversity of chile (*Capsicum annuum* var. annuum L.) landraces from northern New Mexico, Colorado, and Mexico. *Econ. Bot.* **2005**, *59*, 8–17. [CrossRef]
366. Dewitt, D.; Bosland, P.W. *The Complete Chile Pepper Book—A Gardener's Guide to Choosing, Growing, Preserving and Cooking*; Timber Press: London, UK, 2009.
367. Carmack, R.M.; Gasco, J.L.; Gossen, G.H. *The Legacy of Mesoamerica: History and Culture of a Native American Civilization*; Routledge: Oxfordshire, UK, 2016.
368. Pilcher, J.M. Tamales or timbales: Cuisine and the formation of Mexican national identity, 1821–1911. *Americas* **1996**, *53*, 193–216. [CrossRef]
369. Haverluk, T.W. Chile peppers and identity construction in Pueblo, Colorado. *J. Study Food Soc.* **2002**, *6*, 45–59. [CrossRef]
370. Meotti, F.C.; Lemos de Andrade, E.; Calixto, J.B. TRP modulation by natural compounds. *Handb. Exp. Pharmacol.* **2014**, *223*, 1177–1238. [CrossRef]
371. Padilha, H.; Barbieri, R. Plant breeding of chili peppers (Capsicum, Solanaceae)-A review. *Aust. J. Basic. Appl. Sci.* **2016**, *10*, 148–154.
372. Romero Cóndor, C.; Acurio, L.; Paucar-Ayala, S.; Herrera-Robalino, J.; Cabrera, H.; Albán-Villacreces, A.; Sangucho-Montenegro, C.; Veliz-Zambrano, M. Brief Historical review of the geological cartography in Ecuador. *Cienc. Lat. Rev. Científica Multidiscip.* **2023**, *7*, 2584–2621. [CrossRef]
373. Grieder, T.; Bueno Mendoza, A.; Smith, C.E.; Malina, R.M. La Galgada in the World of Its Time. In *La Galgada, Peru: A Preceramic Culture in Transition*; University of Texas Press: Austin, TX, USA, 1988; pp. 192–203.
374. Pearsall, D.M. Plant domestication and the shift to agriculture in the Andes. In *The Handbook of South American Archaeology*; Springer: Berlin/Heidelberg, Germany, 2008; pp. 105–120.
375. Lanning, E.P. *Peru before the Incas*; Prentice-Hall: Saddle River, NJ, USA, 1967.
376. Sarmiento de Gamboa, P. *The History of the Incas*; University of Texas Press: Austin, TX, USA, 2007.
377. Thompson, J.E.S. *Maya History and Religion*; University of Oklahoma Press: Norman, OK, USA, 1990; Volume 99.
378. Smith, M.E. *The Aztecs*; John Wiley & Sons: Hoboken, NJ, USA, 2013.

379. Periferakis, A. Geology and the Aztecs: How the Ore Deposits of Mesoamerica Influenced the Socioeconomic Development of an Empire, from its Emergence to its Downfall. In Proceedings of the 15th International Congress of the Geological Society of Greece, Athens, Greece, 22–24 May 2019; pp. 700–701.
380. Periferakis, A. The Influence of Ore Deposits to the Development and Collapse of the Inca Civilisation between the 15th and the 16th Century. In Proceedings of the 15th International Congress of the Geological Society of Greece, Athens, Greece, 22–24 May 2019; pp. 702–703.
381. De Feo, V. The ritual use of Brugmansia species in traditional Andean medicine in Northern Peru. *Econ. Bot.* **2004**, *58*, S221–S229. [CrossRef]
382. Dafni, A.; Petanidou, T.; Vallianatou, I.; Kozhuharova, E.; Blanché, C.; Pacini, E.; Peyman, M.; Dajić Stevanovic, Z.; Franchi, G.G.; Benítez, G. Myrtle, Basil, Rosemary, and Three-Lobed Sage as Ritual Plants in the Monotheistic Religions: An Historical–Ethnobotanical Comparison. *Econ. Bot.* **2020**, *74*, 330–355. [CrossRef]
383. Kohek, M.; Sánchez Avilés, C.; Romaní, O.; Bouso, J.C. Ancient psychoactive plants in a global village: The ritual use of cannabis in a self-managed community in Catalonia. *Int. J. Drug Policy* **2021**, *98*, 103390. [CrossRef] [PubMed]
384. Yaldiz, G.; Ozguven, M.; Sekeroglu, N. Variation in capsaicin contents of different Capsicum species and lines by varying drying parameters. *Ind. Crops Prod.* **2010**, *32*, 434–438. [CrossRef]
385. Cho, H.; Kwon, Y. Development of a database of capsaicinoid contents in foods commonly consumed in Korea. *Food Sci. Nutr.* **2020**, *8*, 4611–4624. [CrossRef] [PubMed]
386. Ganguly, S.; Praveen, P.K.; Para, P.A.; Sharma, V. Medicinal Properties of chilli pepper in human diet: An editorial. *ARC J. Public. Health Community Med.* **2017**, *2*, 6–7.
387. Aguilar-Meléndez, A.; Vásquez-Dávila, M.A.; Manzanero-Medina, G.I.; Katz, E. Chile (*Capsicum* spp.) as Food-Medicine Continuum in Multiethnic Mexico. *Foods* **2021**, *10*, 2502. [CrossRef]
388. Martínez-Aceviz, Y.; Sobrevilla-Navarro, A.A.; Ramos-Lopez, O. Dietary Intake of Capsaicin and Its Association with Markers of Body Adiposity and Fatty Liver in a Mexican Adult Population of Tijuana. *Healthcare* **2023**, *11*, 3001. [CrossRef] [PubMed]
389. Meghvansi, M.K.; Siddiqui, S.; Khan, M.H.; Gupta, V.K.; Vairale, M.G.; Gogoi, H.K.; Singh, L. Naga chilli: A potential source of capsaicinoids with broad-spectrum ethnopharmacological applications. *J. Ethnopharmacol.* **2010**, *132*, 1–14. [CrossRef]
390. Saleh, B.K.; Omer, A.; Teweldemedhin, B. Medicinal uses and health benefits of chili pepper (Capsicum spp.): A review. *MOJ Food Process Technol.* **2018**, *6*, 325–328. [CrossRef]
391. Montiel-Oseguera, A. *La Persistencia de la Costumbre Pima. Interpretaciones Desde La Antropología Cognitiva*; UAM, INAH y ENAH del Norte de México: Mexico City, Mexico, 2013.
392. Long-Solís, J. *Capsicum y Cultura: La Historia Del Chilli*; Fondo De Cultura Economica: Mexico City, Mexico, 2013.
393. Rakhshandehroo, E.; Asadpour, M.; Jafari, A.; Malekpour, S.H. The effectiveness of Cinnamomum zeylanicum, Punica granatum flower and Capsicum annuum extracts against Parascaris equorum infective larvae. *İstanbul Üniversitesi Vet. Fakültesi Derg.* **2016**, *42*, 132–137. [CrossRef]
394. Frei, B.; Baltisberger, M.; Sticher, O.; Heinrich, M. Medical ethnobotany of the Zapotecs of the Isthmus-Sierra (Oaxaca, Mexico): Documentation and assessment of indigenous uses. *J. Ethnopharmacol.* **1998**, *62*, 149–165. [CrossRef] [PubMed]
395. Irigoyen-Rascón, F.; Paredes, A. *Tarahumara Medicine: Ethnobotany and Healing among the Rarámuri of Mexico*; University of Oklahoma Press: Norman, OK, USA, 2015.
396. Alcorn, J.B. *Huastec Mayan Ethnobotany*; University of Texas Press: Austin, TX, USA, 1984.
397. Martínez, T.H.L. Etnobotánica del Chile Quipín (*Capsicum annuum* var. glabriusculum) en la Sierra Gorda y Semidesierto de Querétaro. Master's Thesis, Colegio de postgraduados, campus Montecillo Botánica, Montecillo, Mexico, 2007.
398. Fiedor, J.; Burda, K. Potential role of carotenoids as antioxidants in human health and disease. *Nutrients* **2014**, *6*, 466–488. [CrossRef] [PubMed]
399. da Silva, L.R.; Silva, B. *Natural Bioactive Compounds from Fruits and Vegetables as Health Promoters Part I*; Bentham Science Publishers: Sharjah, United Arab Emirates, 2016.
400. Roys, R.L. *The Ethno-Botany of the Maya*; Tulane University: New Orleans, LA, USA, 1931.
401. López Austin, A. Textos acerca de las partes del cuerpo humano y de las enfermedades y medicinas en los primeros memoriales de Sahagún. *Estud. Cult. Nahuatl* **1972**, *10*, 129–153. [PubMed]
402. Madero, R.A. *Recetario Totonaco de la Costa de Veracruz*; Dirección General de Culturas Populares de Conaculta: Mexico City, Mexico, 2000.
403. Bañuelos, N.; Salido, P.L.; Gardea, A. Etnobotánica del chiltepín: Pequeño gran señor en la cultura de los sonorenses. *Estud. Soc.* **2008**, *16*, 177–205.
404. Magaña Alejandro, M.A.; Gama Campillo, L.M.; Mariaca Méndez, R. El uso de las plantas medicinales en las comunidades Maya-Chontales de Nacajuca, Tabasco, México. *Polibotánica* **2010**, *29*, 213–262.
405. Cook, S. *The Forest of the Lacandon Maya: An Ethnobotanical Guide*; Springer: Berlin/Heidelberg, Germany, 2016.
406. Aguilar-Meléndez, A.; Vásquez, M.A.; Katz, E.; Colorado, M.R.H. *Los Chiles Que le Dan Sabor al Mundo: Contribuciones MULTIDISCIPLINARIAS*; IRD Éditions: Paris, France, 2018.
407. Breedlove, D.E.; Laughlin, R.M. *Flowering of Man: A Tzotzil Botany of Zinacantán*; Smithsonian Institution Press: Washington, DC, USA, 1993; Volume 2.

408. Chemin Bassler, H. *Recetario Pame de San Luis Potosí y Querétaro*; Dirección General de Culturas Populares, Consejo Nacional para la Cultura y las Artes: Coyoacán, México, 2000.
409. Laughlin, R.M. The Huastec. In *Handbook of Middle American Indians*; University of Texas Press: Austin, TX, USA, 1969; Volume 7, pp. 298–311.
410. Fernández, D.L. Medicina indígena y males infantiles entre los nahuas de Texcoco: Pérdida de la guía, caída de mollera, tiricia y mal de ojo. *Proc. An. De Antropol.* **2015**, *49*, 101–148. [CrossRef]
411. Geck, M.S.; Cabras, S.; Casu, L.; Reyes García, A.J.; Leonti, M. The taste of heat: How humoral qualities act as a cultural filter for chemosensory properties guiding herbal medicine. *J. Ethnopharmacol.* **2017**, *198*, 499–515. [CrossRef]
412. Alayza Escardó, F. Historia de la Cirugía en el Perú. In *Historia de la Cirugia en el Peru*; Editorial Monterrico, S.A.: Lima, Peru, 1992.
413. Froeschner, E.H. Two examples of ancient skull surgery. *J. Neurosurg.* **1992**, *76*, 550–552. [CrossRef]
414. Periferakis, A. A review of obsidian source exploitation in pre-columbian south America. *Bull. Geol. Soc. Greece* **2019**, *55*, 65–108. [CrossRef]
415. Schmiedel, U.; Araya, Y.; Bortolotto, M.I.; Boeckenhoff, L.; Hallwachs, W.; Janzen, D.; Kolipaka, S.S.; Novotny, V.; Palm, M.; Parfondry, M.; et al. Contributions of paraecologists and parataxonomists to research, conservation, and social development. *Conserv. Biol.* **2016**, *30*, 506–519. [CrossRef]
416. Nabhan, G.P. *Ethnobiology for the Future: Linking Cultural and Ecological Diversity*; University of Arizona Press: Tucson, AZ, USA, 2016.
417. Fan, Y.; Kim, D.H.; Gwak, Y.S.; Ahn, D.; Ryu, Y.; Chang, S.; Lee, B.H.; Bills, K.B.; Steffensen, S.C.; Yang, C.H.; et al. The role of substance P in acupuncture signal transduction and effects. *Brain Behav. Immun.* **2021**, *91*, 683–694. [CrossRef]
418. Du, G.-h.; Yuan, T.-y.; Du, L.-d.; Zhang, Y.-x. Chapter Twelve—The Potential of Traditional Chinese Medicine in the Treatment and Modulation of Pain. In *Advances in Pharmacology*; Barrett, J.E., Ed.; Academic Press: Cambridge, MA, USA, 2016; Volume 75, pp. 325–361.
419. Li, S.-H.; Li, L.; Yang, R.-N.; Liang, S.-D. Compounds of traditional Chinese medicine and neuropathic pain. *Chin. J. Nat. Med.* **2020**, *18*, 28–35. [CrossRef] [PubMed]
420. Ghiţă, M.A.; Căruntu, C.; Rosca, A.E.; Căruntu, A.; Moraru, L.; Constantin, C.; Neagu, M.; Boda, D. Real-Time Investigation of Skin Blood Flow Changes Induced by Topical Capsaicin. *Acta Dermatovenerol. Croat.* **2017**, *25*, 223–227.
421. Final report on the safety assessment of capsicum annuum extract, capsicum annuum fruit extract, capsicum annuum resin, capsicum annuum fruit powder, capsicum frutescens fruit, capsicum frutescens fruit extract, capsicum frutescens resin, and capsaicin. *Int. J. Toxicol.* **2007**, *26* (Suppl. 1), 3–106. [CrossRef] [PubMed]
422. Surh, Y.-J.; Sup Lee, S. Capsaicin, a double-edged sword: Toxicity, metabolism, and chemopreventive potential. *Life Sci.* **1995**, *56*, 1845–1855. [CrossRef] [PubMed]
423. Weinberg, R.B. Hunan hand. *N. Engl. J. Med.* **1981**, *305*, 1020.
424. Williams, S.R.; Clark, R.F.; Dunford, J.V. Contact Dermatitis Associated with Capsaicin: Hunan Hand Syndrome. *Ann. Emerg. Med.* **1995**, *25*, 713–715. [CrossRef]
425. Jancsó, G.; Király, E.; Such, G.; Joó, F.; Nagy, A. Neurotoxic effect of capsaicin in mammals. *Acta Physiol. Hung.* **1987**, *69*, 295–313.
426. Ritter, S.; Dinh, T.T. Capsaicin-induced neuronal degeneration in the brain and retina of preweanling rats. *J. Comp. Neurol.* **1990**, *296*, 447–461. [CrossRef]
427. Lechner, A.; Alderson, T.; Gautam, S.; Flaker, G. Ventricular fibrillation due to coronary spasm after pepper spray. *Pacing Clin. Electrophysiol.* **2021**, *44*, 548–551. [CrossRef] [PubMed]
428. Xu, Y.; Gu, Q.; Qu, C. Capsaicin pretreatment reversed pulmonary arterial hypertension by alleviating inflammation via p38MAPK pathway. *Exp. Lung Res.* **2017**, *43*, 8–18. [CrossRef] [PubMed]
429. Baz, R.A.; Scheau, C.; Niscoveanu, C.; Bordei, P. Morphometry of the Entire Internal Carotid Artery on CT Angiography. *Medicina* **2021**, *57*, 832. [CrossRef]
430. Augustin, H.G.; Koh, G.Y. Organotypic vasculature: From descriptive heterogeneity to functional pathophysiology. *Science* **2017**, *357*, eaal2379. [CrossRef]
431. MacAlpin, R.N. Contribution of dynamic vascular wall thickening to luminal narrowing during coronary arterial constriction. *Circulation* **1980**, *61*, 296–301. [CrossRef]
432. Liu, B.; Yang, H.; Song, Y.S.; Sorenson, C.M.; Sheibani, N. Thrombospondin-1 in vascular development, vascular function, and vascular disease. *Semin. Cell Dev. Biol.* **2024**, *155*, 32–44. [CrossRef] [PubMed]
433. Bergren, D.R. Capsaicin challenge, reflex bronchoconstriction, and local action of substance P. *Am. J. Physiol.* **1988**, *254*, R845–R852. [CrossRef]
434. Thomas, K.C.; Ethirajan, M.; Shahrokh, K.; Sun, H.; Lee, J.; Cheatham, T.E., 3rd; Yost, G.S.; Reilly, C.A. Structure-activity relationship of capsaicin analogs and transient receptor potential vanilloid 1-mediated human lung epithelial cell toxicity. *J. Pharmacol. Exp. Ther.* **2011**, *337*, 400–410. [CrossRef]
435. Trevisan, G.; Rossato, M.F.; Hoffmeister, C.; Oliveira, S.M.; Silva, C.R.; Matheus, F.C.; Mello, G.C.; Antunes, E.; Prediger, R.D.; Ferreira, J. Mechanisms involved in abdominal nociception induced by either TRPV1 or TRPA1 stimulation of rat peritoneum. *Eur. J. Pharmacol.* **2013**, *714*, 332–344. [CrossRef]

436. Lee, K.J.; Vos, R.; Tack, J. Effects of capsaicin on the sensorimotor function of the proximal stomach in humans. *Aliment. Pharmacol. Ther.* **2004**, *19*, 415–425. [CrossRef] [PubMed]
437. Kwon, Y. Estimation of Dietary Capsaicinoid Exposure in Korea and Assessment of Its Health Effects. *Nutrients* **2021**, *13*, 2461. [CrossRef] [PubMed]
438. Lassen, C.L.; Meyer, K.; Bredthauer, A.; Klier, T.W. Facial and Oral Cross-Contamination of a 3-Year-Old Child with High Concentration Capsaicin: A Case Report. *A A Pract.* **2020**, *14*, e01258. [CrossRef] [PubMed]
439. Forrester, M.B.; Holloway, C. Characteristics of pepper spray-related injuries reported to the National Electronic Injury Surveillance System during 2000–2020. *Clin. Toxicol.* **2022**, *60*, 348–355. [CrossRef]
440. Dragosloveanu, C.D.M.; Celea, C.G.; Dragosloveanu, S. Comparison of 360 degrees circumferential trabeculotomy and conventional trabeculotomy in primary pediatric glaucoma surgery: Complications, reinterventions and preoperative predictive risk factors. *Int. Ophthalmol.* **2020**, *40*, 3547–3554. [CrossRef] [PubMed]
441. Yenigun, O.M.; Thanassi, M. Capsaicin: An Uncommon Exposure and Unusual Treatment. *Clin. Pract. Cases Emerg. Med.* **2019**, *3*, 219–221. [CrossRef] [PubMed]
442. Kim-Katz, S.Y.; Anderson, I.B.; Kearney, T.E.; MacDougall, C.; Hudmon, K.S.; Blanc, P.D. Topical antacid therapy for capsaicin-induced dermal pain: A poison center telephone-directed study. *Am. J. Emerg. Med.* **2010**, *28*, 596–602. [CrossRef] [PubMed]
443. Yeung, M.F.; Tang, W.Y. Clinicopathological effects of pepper (oleoresin capsicum) spray. *Hong Kong Med. J.* **2015**, *21*, 542–552. [CrossRef] [PubMed]
444. Çirakli, S.; Doganay, S.; Kaya Ozcora, G.D.; Canpolat, M.; Kumandas, S. Biber gazı maruziyeti sonucu gelişen Guillain-Barre sendromunu taklit eden polinöropati. *Türk. Pediatri. Arşivi.* **2019**, *54*, 53–56. [CrossRef]
445. Snyman, T.; Stewart, M.J.; Steenkamp, V. A fatal case of pepper poisoning. *Forensic Sci. Int.* **2001**, *124*, 43–46. [CrossRef]
446. Akçay, A.B.; Ozcan, T.; Seyis, S.; Acele, A. Coronary vasospasm and acute myocardial infarction induced by a topical capsaicin patch. *Turk. Kardiyol. Dern. Ars.* **2009**, *37*, 497–500. [PubMed]
447. Xie, M.; Wu, H.; Bian, J.; Huang, S.; Xia, Y.; Qin, Y.; Yan, Z. Synthesis and biological evaluation of capsaicin analogues as antioxidant and neuroprotective agents. *RSC Adv.* **2023**, *13*, 32150–32159. [CrossRef] [PubMed]
448. Dou, F.; Wu, B.; Chen, J.; Liu, T.; Yu, Z.; Chen, C. Capsaicin inhibits A7r5 cell senescence via the mitochondrial carrier protein Slc25a12. *Exp. Cell Res.* **2023**, *433*, 113856. [CrossRef] [PubMed]
449. Xu, S.; Hao, K.; Xiong, Y.; Xu, R.; Huang, H.; Wang, H. Capsaicin alleviates neuronal apoptosis and schizophrenia-like behavioral abnormalities induced by early life stress. *Schizophrenia* **2023**, *9*, 77. [CrossRef] [PubMed]
450. Wang, L.; Liu, Y.; Li, S.; Zha, Z.; Chen, Y.; Wang, Q.; Zhou, S.; Huang, X.; Xu, M. Capsaicin alleviates doxorubicin-induced acute myocardial injury by regulating iron homeostasis and PI3K-Akt signaling pathway. *Aging* **2023**, *15*, 11845–11859. [CrossRef] [PubMed]
451. Dragoș, D.; Petran, M.; Gradinaru, T.C.; Gilca, M. Phytochemicals and Inflammation: Is Bitter Better? *Plants* **2022**, *11*, 2991. [CrossRef] [PubMed]
452. D'Urso, O.; Drago, F. Pharmacological significance of extra-oral taste receptors. *Eur. J. Pharmacol.* **2021**, *910*, 174480. [CrossRef]
453. Töle, J.C.; Behrens, M.; Meyerhof, W. Taste receptor function. *Handb. Clin. Neurol.* **2019**, *164*, 173–185. [CrossRef] [PubMed]
454. Behrens, M.; Lang, T. Extra-Oral Taste Receptors-Function, Disease, and Perspectives. *Front. Nutr.* **2022**, *9*, 881177. [CrossRef]
455. Nevo, S.; Kadouri, N.; Abramson, J. Tuft cells: From the mucosa to the thymus. *Immunol. Lett.* **2019**, *210*, 1–9. [CrossRef]
456. O'Leary, C.E.; Schneider, C.; Locksley, R.M. Tuft Cells-Systemically Dispersed Sensory Epithelia Integrating Immune and Neural Circuitry. *Annu. Rev. Immunol.* **2019**, *37*, 47–72. [CrossRef]
457. Howitt, M.R.; Cao, Y.G.; Gologorsky, M.B.; Li, J.A.; Haber, A.L.; Biton, M.; Lang, J.; Michaud, M.; Regev, A.; Garrett, W.S. The Taste Receptor TAS1R3 Regulates Small Intestinal Tuft Cell Homeostasis. *Immunohorizons* **2020**, *4*, 23–32. [CrossRef]
458. Ki, S.Y.; Jeong, Y.T. Taste Receptors beyond Taste Buds. *Int. J. Mol. Sci.* **2022**, *23*, 9677. [CrossRef]
459. Hendel, S.K.; Kellermann, L.; Hausmann, A.; Bindslev, N.; Jensen, K.B.; Nielsen, O.H. Tuft Cells and Their Role in Intestinal Diseases. *Front. Immunol.* **2022**, *13*, 822867. [CrossRef]
460. Sell, E.A.; Ortiz-Carpena, J.F.; Herbert, D.R.; Cohen, N.A. Tuft cells in the pathogenesis of chronic rhinosinusitis with nasal polyps and asthma. *Ann. Allergy Asthma Immunol.* **2021**, *126*, 143–151. [CrossRef] [PubMed]
461. Xiong, Z.; Zhu, X.; Geng, J.; Xu, Y.; Wu, R.; Li, C.; Fan, D.; Qin, X.; Du, Y.; Tian, Y.; et al. Intestinal Tuft-2 cells exert antimicrobial immunity via sensing bacterial metabolite N-undecanoylglycine. *Immunity* **2022**, *55*, 686–700.e7. [CrossRef] [PubMed]
462. O'Leary, C.E.; Sbierski-Kind, J.; Kotas, M.E.; Wagner, J.C.; Liang, H.E.; Schroeder, A.W.; de Tenorio, J.C.; von Moltke, J.; Ricardo-Gonzalez, R.R.; Eckalbar, W.L.; et al. Bile acid-sensitive tuft cells regulate biliary neutrophil influx. *Sci. Immunol.* **2022**, *7*, eabj1080. [CrossRef]
463. Merritt, J.C.; Richbart, S.D.; Moles, E.G.; Cox, A.J.; Brown, K.C.; Miles, S.L.; Finch, P.T.; Hess, J.A.; Tirona, M.T.; Valentovic, M.A.; et al. Anti-cancer activity of sustained release capsaicin formulations. *Pharmacol. Ther.* **2022**, *238*, 108177. [CrossRef] [PubMed]
464. Chapa-Oliver, A.M.; Mejía-Teniente, L. Capsaicin: From Plants to a Cancer-Suppressing Agent. *Molecules* **2016**, *21*, 931. [CrossRef]
465. Cassano, R.; Serini, S.; Curcio, F.; Trombino, S.; Calviello, G. Preparation and Study of Solid Lipid Nanoparticles Based on Curcumin, Resveratrol and Capsaicin Containing Linolenic Acid. *Pharmaceutics* **2022**, *14*, 1593. [CrossRef]
466. Matei, A.-M.; Caruntu, C.; Tampa, M.; Georgescu, S.R.; Matei, C.; Constantin, M.M.; Constantin, T.V.; Calina, D.; Ciubotaru, D.A.; Badarau, I.A.; et al. Applications of Nanosized-Lipid-Based Drug Delivery Systems in Wound Care. *Appl. Sci.* **2021**, *11*, 4915. [CrossRef]

467. Anantaworasakul, P.; Chaiyana, W.; Michniak-Kohn, B.B.; Rungseevijitprapa, W.; Ampasavate, C. Enhanced Transdermal Delivery of Concentrated Capsaicin from Chili Extract-Loaded Lipid Nanoparticles with Reduced Skin Irritation. *Pharmaceutics* **2020**, *12*, 463. [CrossRef] [PubMed]
468. Zhen, X.; Xie, C.; Jiang, Y.; Ai, X.; Xing, B.; Pu, K. Semiconducting Photothermal Nanoagonist for Remote-Controlled Specific Cancer Therapy. *Nano Lett.* **2018**, *18*, 1498–1505. [CrossRef] [PubMed]
469. Bhagwat, D.A.; Swami, P.A.; Nadaf, S.J.; Choudhari, P.B.; Kumbar, V.M.; More, H.N.; Killedar, S.G.; Kawtikwar, P.S. Capsaicin Loaded Solid SNEDDS for Enhanced Bioavailability and Anticancer Activity: In-Vitro, In-Silico, and In-Vivo Characterization. *J. Pharm. Sci.* **2021**, *110*, 280–291. [CrossRef] [PubMed]
470. Xu, M.; Zhang, J.; Mu, Y.; Foda, M.F.; Han, H. Activation of TRPV1 by capsaicin-loaded CaCO3 nanoparticle for tumor-specific therapy. *Biomaterials* **2022**, *284*, 121520. [CrossRef] [PubMed]
471. Periferakis, A.; Periferakis, A.-T.; Troumpata, L.; Dragosloveanu, S.; Timofticiuc, I.-A.; Georgatos-Garcia, S.; Scheau, A.-E.; Periferakis, K.; Caruntu, A.; Badarau, I.A.; et al. Use of Biomaterials in 3D Printing as a Solution to Microbial Infections in Arthroplasty and Osseous Reconstruction. *Biomimetics* **2024**, *9*, 154. [CrossRef] [PubMed]
472. Timofticiuc, I.-A.; Călinescu, O.; Iftime, A.; Dragosloveanu, S.; Caruntu, A.; Scheau, A.-E.; Badarau, I.A.; Didilescu, A.C.; Caruntu, C.; Scheau, C. Biomaterials Adapted to Vat Photopolymerization in 3D Printing: Characteristics and Medical Applications. *J. Funct. Biomater.* **2024**, *15*, 7. [CrossRef] [PubMed]
473. Yu, T.; Tong, L.; Ao, Y.; Zhang, G.; Liu, Y.; Zhang, H. Novel design of NIR-triggered plasmonic nanodots capped mesoporous silica nanoparticles loaded with natural capsaicin to inhibition of metastasis of human papillary thyroid carcinoma B-CPAP cells in thyroid cancer chemo-photothermal therapy. *J. Photochem. Photobiol. B* **2019**, *197*, 111534. [CrossRef] [PubMed]
474. Lee, J.Y.; Lee, S.Y.; Kim, G.G.; Hur, M.G.; Yang, S.D.; Park, J.H.; Kim, S.W. Development of (68)Ga-SCN-DOTA-Capsaicin as an Imaging Agent Targeting Apoptosis and Cell Cycle Arrest in Breast Cancer. *Cancer Biother. Radiopharm.* **2017**, *32*, 169–175. [CrossRef]
475. Dong, Y.; Yin, Y.; Vu, S.; Yang, F.; Yarov-Yarovoy, V.; Tian, Y.; Zheng, J. A distinct structural mechanism underlies TRPV1 activation by piperine. *Biochem. Biophys. Res. Commun.* **2019**, *516*, 365–372. [CrossRef]
476. McNamara, F.N.; Randall, A.; Gunthorpe, M.J. Effects of piperine, the pungent component of black pepper, at the human vanilloid receptor (TRPV1). *Br. J. Pharmacol.* **2005**, *144*, 781–790. [CrossRef] [PubMed]
477. Arunprasert, K.; Pornpitchanarong, C.; Piemvuthi, C.; Siraprapapornsakul, S.; Sripeangchan, S.; Lertsrimongkol, O.; Opanasopit, P.; Patrojanasophon, P. Nanostructured lipid carrier-embedded polyacrylic acid transdermal patches for improved transdermal delivery of capsaicin. *Eur. J. Pharm. Sci.* **2022**, *173*, 106169. [CrossRef] [PubMed]
478. Kolonko, A.K.; Efing, J.; González-Espinosa, Y.; Bangel-Ruland, N.; van Driessche, W.; Goycoolea, F.M.; Weber, W.M. Capsaicin-Loaded Chitosan Nanocapsules for wtCFTR-mRNA Delivery to a Cystic Fibrosis Cell Line. *Biomedicines* **2020**, *8*, 364. [CrossRef] [PubMed]
479. Manzo, E.; Schiano Moriello, A.; Tinto, F.; Verde, R.; Allarà, M.; De Petrocellis, L.; Pagano, E.; Izzo, A.A.; Di Marzo, V.; Petrosino, S. A Glucuronic Acid-Palmitoylethanolamide Conjugate (GLUPEA) Is an Innovative Drug Delivery System and a Potential Bioregulator. *Cells* **2021**, *10*, 450. [CrossRef]
480. Scheau, C.; Mihai, L.; Badarau, I.A.; Caruntu, C. Emerging applications of some important natural compounds in the field of oncology. *Farmacia* **2020**, *68*, 984–991. [CrossRef]
481. Rajput, H.; Nangare, S.; Khan, Z.; Patil, A.; Bari, S.; Patil, P. Design of lactoferrin functionalized carboxymethyl dextran coated egg albumin nanoconjugate for targeted delivery of capsaicin: Spectroscopic and cytotoxicity studies. *Int. J. Biol. Macromol.* **2023**, *256*, 128392. [CrossRef] [PubMed]
482. Leibing, E.; Leonhardt, U.; Köster, G.; Goerlitz, A.; Rosenfeldt, J.-A.; Hilgers, R.; Ramadori, G. Acupuncture treatment of chronic low-back pain—A randomized, blinded, placebo-controlled trial with 9-month follow-up. *Pain* **2002**, *96*, 189–196. [CrossRef] [PubMed]
483. Mavrommatis, C.I.; Argyra, E.; Vadalouka, A.; Vasilakos, D.G. Acupuncture as an adjunctive therapy to pharmacological treatment in patients with chronic pain due to osteoarthritis of the knee: A 3-armed, randomized, placebo-controlled trial. *Pain* **2012**, *153*, 1720–1726. [CrossRef] [PubMed]
484. Müller, M.; Schmucker, C.; Naumann, J.; Schlueter, N.; Huber, R.; Lederer, A.K. Acupuncture in management of acute dental pain—A systematic review and meta-analysis. *Jpn. Dent. Sci. Rev.* **2023**, *59*, 114–128. [CrossRef]
485. Zhou, M.; Zhang, Q.; Huo, M.; Song, H.; Chang, H.; Cao, J.; Fang, Y.; Zhang, D. The mechanistic basis for the effects of electroacupuncture on neuropathic pain within the central nervous system. *Biomed. Pharmacother.* **2023**, *161*, 114516. [CrossRef]
486. Lu, W.W.; Zhang, J.M.; Lv, Z.T.; Chen, A.M. Update on the Clinical Effect of Acupuncture Therapy in Patients with Gouty Arthritis: Systematic Review and Meta-Analysis. *Evid. Based Complement. Altern. Med.* **2016**, *2016*, 9451670. [CrossRef]
487. Chen, Y.; Chen, X.; Qian, H.; Li, B.; Su, X. Acupuncture alleviates acute peritonitis: A case report. *Heliyon* **2023**, *9*, e15290. [CrossRef]
488. Periferakis, K.; Periferakis, A. Treating gout caused by renal insufficiency with acupuncture and moxibustion: A case report. *Rom. J. Clin. Res.* **2023**, *6*, 21–28.
489. Chen, B.; Liu, D.; Li, T.; Zheng, L.; Lan, L.; Yang, N.; Huang, Y. Research Hotspots and Trends on Acupuncture for Anti-Inflammation: A Bibliometric Analysis from 2011 to 2021. *J. Pain Res.* **2023**, *16*, 1197–1217. [CrossRef]

490. Marini, E.; Magi, G.; Mingoia, M.; Pugnaloni, A.; Facinelli, B. Antimicrobial and Anti-Virulence Activity of Capsaicin Against Erythromycin-Resistant, Cell-Invasive Group A Streptococci. *Front. Microbiol.* **2015**, *6*, 1281. [CrossRef]
491. Das, J.; Deka, M.; Gogoi, K. Antimicrobial activity of chilli extracts (Capsicum chinense) against food borne pathogens Escherichia coli and Staphylococcus aureus. *Int. J. Res. Anal. Rev. (IJRAR)* **2018**, *5*, 717–720.
492. Periferakis, A.-T.; Periferakis, A.; Periferakis, K.; Caruntu, A.; Badarau, I.A.; Savulescu-Fiedler, I.; Scheau, C.; Caruntu, C. Antimicrobial Properties of Capsaicin: Available Data and Future Research Perspectives. *Nutrients* **2023**, *15*, 4097. [CrossRef]
493. Buitimea-Cantúa, G.V.; Buitimea-Cantúa, N.E.; Rocha-Pizaña, M.d.R.; Hernández-Morales, A.; Magaña-Barajas, E.; Molina-Torres, J. Inhibitory effect of Capsicum chinense and Piper nigrum fruits, capsaicin and piperine on aflatoxins production in Aspergillus parasiticus by downregulating the expression of afl D, afl M, afl R, and afl S genes of aflatoxins biosynthetic pathway. *J. Environ. Sci. Health Part B* **2020**, *55*, 835–843. [CrossRef] [PubMed]
494. Behbehani, J.M.; Irshad, M.; Shreaz, S.; Karched, M. Anticandidal Activity of Capsaicin and Its Effect on Ergosterol Biosynthesis and Membrane Integrity of Candida albicans. *Int. J. Mol. Sci.* **2023**, *24*, 1046. [CrossRef] [PubMed]
495. Chang, A.Y.; Mann, T.S.; McFawn, P.K.; Han, L.; Dong, X.; Henry, P.J. Investigating the role of MRGPRC11 and capsaicin-sensitive afferent nerves in the anti-influenza effects exerted by SLIGRL-amide in murine airways. *Respir. Res.* **2016**, *17*, 1–14. [CrossRef]
496. Menezes, R.P.; Bessa, M.A.S.; Siqueira, C.P.; Teixeira, S.C.; Ferro, E.A.V.; Martins, M.M.; Cunha, L.C.S.; Martins, C.H.G. Antimicrobial, Antivirulence, and Antiparasitic Potential of Capsicum chinense Jacq. Extracts and Their Isolated Compound Capsaicin. *Antibiotics* **2022**, *11*, 1154. [CrossRef] [PubMed]
497. Tatsimo, S.J.N.; Tamokou, J.d.D.; Havyarimana, L.; Csupor, D.; Forgo, P.; Hohmann, J.; Kuiate, J.-R.; Tane, P. Antimicrobial and antioxidant activity of kaempferol rhamnoside derivatives from Bryophyllum pinnatum. *BMC Res. Notes* **2012**, *5*, 158. [CrossRef] [PubMed]
498. Periferakis, A.; Periferakis, K.; Badarau, I.A.; Petran, E.M.; Popa, D.C.; Caruntu, A.; Costache, R.S.; Scheau, C.; Caruntu, C.; Costache, D.O. Kaempferol: Antimicrobial Properties, Sources, Clinical, and Traditional Applications. *Int. J. Mol. Sci.* **2022**, *23*, 15054. [CrossRef] [PubMed]
499. Bangar, S.P.; Chaudhary, V.; Sharma, N.; Bansal, V.; Ozogul, F.; Lorenzo, J.M. Kaempferol: A flavonoid with wider biological activities and its applications. *Crit. Rev. Food Sci. Nutr.* **2022**, *63*, 9580–9604. [CrossRef] [PubMed]
500. Periferakis, A.; Periferakis, A.-T.; Troumpata, L.; Periferakis, K.; Scheau, A.-E.; Savulescu-Fiedler, I.; Caruntu, A.; Badarau, I.A.; Caruntu, C.; Scheau, C. Kaempferol: A Review of Current Evidence of Its Antiviral Potential. *Int. J. Mol. Sci.* **2023**, *24*, 16299. [CrossRef] [PubMed]
501. Hirai, I.; Okuno, M.; Katsuma, R.; Arita, N.; Tachibana, M.; Yamamoto, Y. Characterisation of anti-Staphylococcus aureus activity of quercetin. *Int. J. Food Sci. Technol.* **2010**, *45*, 1250–1254. [CrossRef]
502. Shu, Y.; Liu, Y.; Li, L.; Feng, J.; Lou, B.; Zhou, X.; Wu, H. Antibacterial activity of quercetin on oral infectious pathogens. *Afr. J. Microbiol. Res.* **2011**, *5*, 5358–5361.
503. Jaisinghani, R.N. Antibacterial properties of quercetin. *Microbiol. Res.* **2017**, *8*, 6877. [CrossRef]
504. Teow, S.-Y.; Ali, S.A. Synergistic antibacterial activity of Curcumin with antibiotics against Staphylococcus aureus. *Pak. J. Pharm. Sci.* **2015**, *28*, 2109–2114.
505. Yun, D.G.; Lee, D.G. Antibacterial activity of curcumin via apoptosis-like response in Escherichia coli. *Appl. Microbiol. Biotechnol.* **2016**, *100*, 5505–5514. [CrossRef]
506. Gunes, H.; Gulen, D.; Mutlu, R.; Gumus, A.; Tas, T.; Topkaya, A.E. Antibacterial effects of curcumin: An in vitro minimum inhibitory concentration study. *Toxicol. Ind. Health* **2016**, *32*, 246–250. [CrossRef] [PubMed]
507. Liao, Y.; Yao, Y.; Yu, Y.; Zeng, Y. Enhanced antibacterial activity of curcumin by combination with metal ions. *Colloid. Interface Sci. Commun.* **2018**, *25*, 1–6. [CrossRef]
508. Ojala, T.; Remes, S.; Haansuu, P.; Vuorela, H.; Hiltunen, R.; Haahtela, K.; Vuorela, P. Antimicrobial activity of some coumarin containing herbal plants growing in Finland. *J. Ethnopharmacol.* **2000**, *73*, 299–305. [CrossRef] [PubMed]
509. Smyth, T.; Ramachandran, V.N.; Smyth, W.F. A study of the antimicrobial activity of selected naturally occurring and synthetic coumarins. *Int. J. Antimicrob. Agents* **2009**, *33*, 421–426. [CrossRef] [PubMed]
510. Al-Rifai, A.a.A.; Ayoub, M.T.; Shakya, A.K.; Abu Safieh, K.A.; Mubarak, M.S. Synthesis, characterization, and antimicrobial activity of some new coumarin derivatives. *Med. Chem. Res.* **2012**, *21*, 468–476. [CrossRef]
511. Wallock-Richards, D.; Doherty, C.J.; Doherty, L.; Clarke, D.J.; Place, M.; Govan, J.R.W.; Campopiano, D.J. Garlic Revisited: Antimicrobial Activity of Allicin-Containing Garlic Extracts against Burkholderia cepacia Complex. *PLoS ONE* **2014**, *9*, e112726. [CrossRef] [PubMed]
512. Choo, S.; Chin, V.K.; Wong, E.H.; Madhavan, P.; Tay, S.T.; Yong, P.V.C.; Chong, P.P. Review: Antimicrobial properties of allicin used alone or in combination with other medications. *Folia Microbiol.* **2020**, *65*, 451–465. [CrossRef] [PubMed]
513. Nakamoto, M.; Kunimura, K.; Suzuki, J.I.; Kodera, Y. Antimicrobial properties of hydrophobic compounds in garlic: Allicin, vinyldithiin, ajoene and diallyl polysulfides (Review). *Exp. Ther. Med.* **2020**, *19*, 1550–1553. [CrossRef] [PubMed]
514. Neu, H.C. The Crisis in Antibiotic Resistance. *Science* **1992**, *257*, 1064–1073. [CrossRef]
515. Miyakis, S.; Pefanis, A.; Tsakris, A. The Challenges of Antimicrobial Drug Resistance in Greece. *Clin. Infect. Dis.* **2011**, *53*, 177–184. [CrossRef]

516. Rafila, A.; Talapan, D.; Dorobăţ, O.M.; Popescu, G.A.; Piţigoi, D.; Florea, D.; Buicu, F.C. Emergence of Carbapenemase-producing Enterobacteriaceae, a Public Health Threat: A Romanian Infectious Disease Hospital Based Study/Emergenţa Enterobacteriaceaelor producătoare de carbapenemaze, o ameninţare pentru sănătatea publică: Un studiu realizat într-un spital romanesc de boli infectioase. *Rev. Romana De Med. De Lab.* **2015**, *23*, 295–301. [CrossRef]
517. Frieri, M.; Kumar, K.; Boutin, A. Antibiotic resistance. *J. Infect. Public. Health* **2017**, *10*, 369–378. [CrossRef] [PubMed]
518. MacGowan, A.; Macnaughton, E. Antibiotic resistance. *Medicine* **2017**, *45*, 622–628. [CrossRef]
519. Popa, L.I.; Gheorghe, I.; Barbu, I.C.; Surleac, M.; Paraschiv, S.; Măruţescu, L.; Popa, M.; Pîrcălăbioru, G.G.; Talapan, D.; Niţă, M.; et al. Multidrug Resistant Klebsiella pneumoniae ST101 Clone Survival Chain from Inpatients to Hospital Effluent after Chlorine Treatment. *Front. Microbiol.* **2021**, *11*, 610296. [CrossRef] [PubMed]
520. Tălăpan, D.; Rafila, A. Five-Year Survey of Asymptomatic Colonization with Multidrug-Resistant Organisms in a Romanian Tertiary Care Hospital. *Infect. Drug Resist.* **2022**, *15*, 2959–2967. [CrossRef] [PubMed]
521. Jumaah, O.; Abu-Abaa, M.; Huang, K.; Hasan, S. The Rare Adverse Effect of Cefepime-Induced Neutropenia. *Cureus* **2023**, *15*, e38274. [CrossRef] [PubMed]
522. Massoth, C.; Saadat-Gilani, K.; Wenk, M. [allergy to penicillin—No beta-lactam antibiotics?]. *Anasthesiol. Intensivmed. Notfallmed Schmerzther.* **2023**, *58*, 264–266. [CrossRef]
523. Periferakis, A.; Caruntu, A.; Periferakis, A.-T.; Scheau, A.-E.; Badarau, I.A.; Caruntu, C.; Scheau, C. Availability, Toxicology and Medical Significance of Antimony. *Int. J. Environ. Res. Public Health* **2022**, *19*, 4669. [CrossRef]
524. Kang, C.; Wang, B.; Kaliannan, K.; Wang, X.; Lang, H.; Hui, S.; Huang, L.; Zhang, Y.; Zhou, M.; Chen, M.; et al. Gut Microbiota Mediates the Protective Effects of Dietary Capsaicin against Chronic Low-Grade Inflammation and Associated Obesity Induced by High-Fat Diet. *mBio* **2017**, *8*, e00470-17. [CrossRef]
525. Rosca, A.E.; Iesanu, M.I.; Zahiu, C.D.M.; Voiculescu, S.E.; Paslaru, A.C.; Zagrean, A.M. Capsaicin and Gut Microbiota in Health and Disease. *Molecules* **2020**, *25*, 5681. [CrossRef]
526. Weng, G.; Duan, Y.; Zhong, Y.; Song, B.; Zheng, J.; Zhang, S.; Yin, Y.; Deng, J. Plant Extracts in Obesity: A Role of Gut Microbiota. *Front. Nutr.* **2021**, *8*, 727951. [CrossRef] [PubMed]
527. Dumitrache, M.D.; Jieanu, A.S.; Scheau, C.; Badarau, I.A.; Popescu, G.D.A.; Caruntu, A.; Costache, D.O.; Costache, R.S.; Constantin, C.; Neagu, M.; et al. Comparative effects of capsaicin in chronic obstructive pulmonary disease and asthma (Review). *Exp. Ther. Med.* **2021**, *22*, 917. [CrossRef] [PubMed]
528. Popescu, G.D.A.; Scheau, C.; Badarau, I.A.; Dumitrache, M.D.; Caruntu, A.; Scheau, A.E.; Costache, D.O.; Costache, R.S.; Constantin, C.; Neagu, M.; et al. The Effects of Capsaicin on Gastrointestinal Cancers. *Molecules* **2020**, *26*, 94. [CrossRef] [PubMed]
529. Yao, W.; Li, Y.; Ding, G. Interstitial fluid flow: The mechanical environment of cells and foundation of meridians. *Evid. Based Complement. Altern. Med.* **2012**, *2012*, 853516. [CrossRef] [PubMed]
530. Li, H.Y.; Yang, J.F.; Chen, M.; Xu, L.; Wang, W.C.; Wang, F.; Tong, J.B.; Wang, C.Y. Visualized regional hypodermic migration channels of interstitial fluid in human beings: Are these ancient meridians? *J. Altern. Complement. Med.* **2008**, *14*, 621–628. [CrossRef] [PubMed]
531. Ma, W.; Tong, H.; Xu, W.; Hu, J.; Liu, N.; Li, H.; Cao, L. Perivascular space: Possible anatomical substrate for the meridian. *J. Altern. Complement. Med.* **2003**, *9*, 851–859. [CrossRef] [PubMed]
532. Zhang, W.B.; Zhao, Y.; Kjell, F. Understanding propagated sensation along meridians by volume transmission in peripheral tissue. *Chin. J. Integr. Med.* **2013**, *19*, 330–339. [CrossRef]

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Review

Aquaporin Modulation by Cations, a Review

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Abstract: Aquaporins (AQPs) are transmembrane channels initially discovered for their role in water flux facilitation through biological membranes. Over the years, a much more complex and subtle picture of these channels appeared, highlighting many other solutes accommodated by AQPs and a dense regulatory network finely tuning cell membranes' water permeability. At the intersection between several transduction pathways (e.g., cell volume regulation, calcium signaling, potassium cycling, etc.), this wide and ancient protein family is considered an important therapeutic target for cancer treatment and many other pathophysiologies. However, a precise and isoform-specific modulation of these channels function is still challenging. Among the modulators of AQPs functions, cations have been shown to play a significant contribution, starting with mercury being historically associated with the inhibition of AQPs since their discovery. While the comprehension of AQPs modulation by cations has improved, a unifying molecular mechanism integrating all current knowledge is still lacking. In an effort to extract general trends, we reviewed all known modulations of AQPs by cations to capture a first glimpse of this regulatory network. We paid particular attention to the associated molecular mechanisms and pinpointed the residues involved in cation binding and in conformational changes tied up to the modulation of the channel function.

Keywords: AQP; cation; regulations; calcium; mercury

1. Introduction

Aquaporins (AQPs) constitute a wide and ancient family of transmembrane channels dedicated to the facilitation of water passage across cells membranes [1–3]. They also accommodate other small polar solutes such as glycerol (channeled by aquaglyceroporines: AQGP), ammonia (channeled by aqua-ammoniaporines), H_2O_2 or even anions [4–7]. In humans, 13 AQPs are found and are distributed in different tissues and cell types, depending on their substrate selectivity and on their regulations [1,8]. Because AQPs constitute the main pathway for cells to dynamically regulate their membranes water permeability, they are central in the regulation of cell homeostasis (through the regulation of water and ions concentrations), cell volume regulations (regulatory volume decrease [9–12], cell proliferation [13] and cell motility [14]) and trans-cellular water fluxes [8] (e.g., water reabsorption in kidney collecting duct [15], lymph fluxes in the inner ear [16], all types of secretions in dedicated glands [17], etc.). Additionally, other roles involving the other substrates accommodated by AQPs have been described [7,18,19]. Finally, it should be kept in mind that all of the subtleties of the various possible regulations regarding AQPs have not yet been elucidated [20,21]. Obviously, this wide spectrum of functions makes AQP dysregulations a common feature of many different pathophysiologies [22,23], especially in cancer cell development and proliferation [24–26].

AQPs are found in tetrameric assemblies, with each of the four subunits being a functional pore [27]. Each subunit is made out of six transmembrane alpha helices (H1–H6)

that provide the framework for the channel (Figure 1). The angle at which the helices are orientated creates two funnel-shaped vestibules (intra-cellular and extra-cellular) connected together by the conducting pore. This conducting pore is characterized by several water-interacting sites able to complement the hydrophilic nature of water molecules. These sites are mainly constituted by the backbone carbonyl oxygens of loop E of the extra-cellular vestibule and loop B of the intra-cellular vestibule [27]. Both of these two loops contain small alpha helices (HE and HB, respectively) that meet at the center of the subunit. Each of the small helices possesses an NPA motif (for an asparagine–proline–alanine motif) that meets the other's at the center of the pore. At this location, the dipole moments of HB and HE create a positive electrostatic field. This particularity has been shown to be responsible for proton exclusion from the pore and is also a water-interacting site [27,28]. The rest of the conducting pore is hydrophobic, hence forcing water molecules to form a single-file hydrogen network continuum between the ends of the two vestibules. Finally, in the extra-cellular vestibule, three or four residues make the aromatic/arginine (ar/R) constriction, which corresponds to the narrowest part of the channel and from which originates substrate selectivity between classes of aquaporins [27,29]. On top of this structural unit core, flexible loops and N-terminal and C-terminal extremities protrude into the cytoplasm (loop D and loop B, N-ter and C-ter) and in the extra-cellular compartment (loop A, loop E and loop C). The sequence variability is higher in these flexible loops and extremities [1], which are finely involved in the regulation of the core function of AQPs [30].

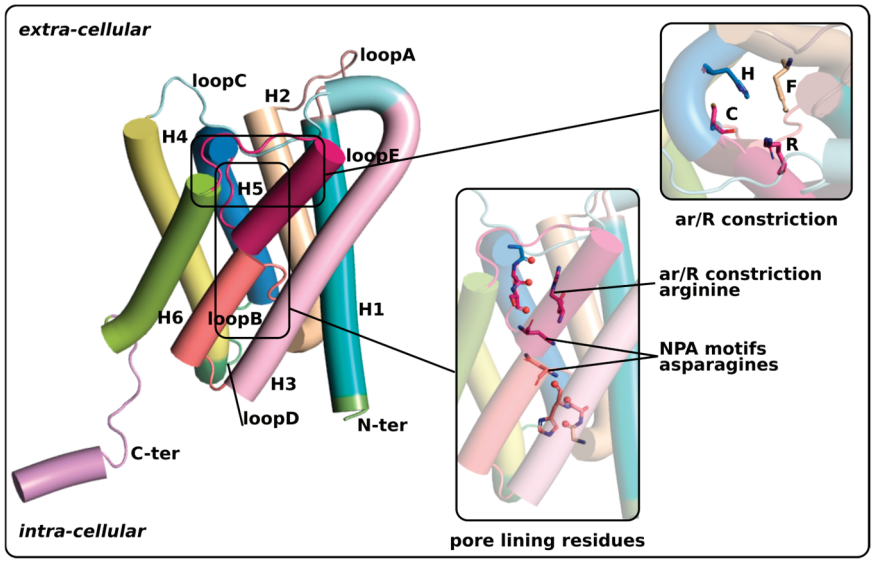


Figure 1. Schematic representation of an AQP. The X-ray structure of human AQP2 was used to make this representation. The two main constrictions within the conducting pore of AQPs are the NPA motifs region and the ar/R constriction. Waters inside the pore (not represented) form single file continuum because of the hydrophobic nature of many pore lining residues. Only a few residues in loop B and loop E have their backbone oxygen (represented by a red sphere) oriented toward the pore lumen, creating punctual polar relays as successive interaction sites for water across the pore. In addition, the polar groups of constitutive sidechains, including NPA motifs asparagines and ar/R constriction polar or charged residues, complete this suite.

AQPs are regulated by many different types of molecules, including protons [31,32], small circulating molecules [33], membrane components [34] or other proteins [35]. In the current review, we intend to summarize the current knowledge about AQP regulation by cations, describe the associated molecular mechanisms and pinpoint the key residues involved.

2. Modulation by Alkaline Earth Metal Cations

2.1. Calcium Ions

Calcium ions are known as one of the most ubiquitous secondary messengers in living cells, transducing many types of signals depending on their concentration, sub-cellular localization [36], route of entry in the cytoplasm [37] and pattern of release (amplitude and frequency) [38]. Many proteins are regulated by calcium, with the most studied family of calcium sensors being the calmodulin family [39]. Calmodulins (CaMs) constitute a highly conserved family of proteins involved in calcium sensing and signaling. Upon calcium binding, changes in conformation induce the opening of the E-F hand motifs, exposing hydrophobic surfaces of the two alpha helices and allowing binding to proteins regulated by calmodulins [39,40].

The first type of regulation involving calcium is indirect and is mediated through the activation of calmodulin by calcium. This regulation was mostly studied for the eye AQP0 [32,41–47]; however, other studies point to other AQPs as being regulated by calmodulin and calcium as well, such as AQP6 [48], AQP4 [49] or AQP2 [50–53]. With the exception of AQP2, whose function is regulated by calmodulin through the modulation of its cellular trafficking [50–53], AQPs (AQP0, AQP4 and AQP6) seem to interact directly with calmodulins [41,44,45,48,49] at a 1-8-14 like motif located on their C-terminal or N-terminal extremities (Figure 2) [41,48,49]. The fixation of calmodulin on the AQP0 tetramer inhibits its water permeability. Upon binding, conformational changes induce an increase in monomer cooperativity associated with a reduction of water pore size at the two constrictions of the channel (R187 and Y149) and near Y23 [45,54,55]. Arginine 156 located on intra-cellular loop D of ovine AQP0 seems to play a key role in this allosteric mechanism [55]. Three phosphorylation sites located at the C-terminal extremities have also been implicated in the regulation of calmodulin binding to human AQP0. Upon the phosphorylation of S229 or S235, calmodulin interaction is prevented (Figure 2). When S231 is phosphorylated, however, calmodulin can bind to AQP0, but the water permeability is not impacted [46].

Additionally, calcium has been directly involved in plant AQP gating [31,56]. More precisely, the experimental X-ray structure of spinach AQP *So*PIP2;1 revealed cadmium cations bound to the intra-cellular N-terminal residues E31 and D28 (Figure 3). In plants, strict AQPs (PIP, for plasma membrane intrinsic protein) possess a long intra-cellular loop D. This flexible loop can switch from an open to a closed conformation in which a few residues create a hydrophobic patch preventing water passage through the intra-cellular vestibule of the AQP. According to the authors, this cation participates in the locking of the closed conformation by joining loop D to the facing N-terminal extremity through a hydrogen bond/salt bridge network [31]. Two residues of loop D (R190 and D191) are also crucial to maintaining the loop in a closed conformation [31,57]. According to the authors that solved the closed conformation of *So*PIP2;1, the closing of the channel by loop D would be triggered and directed by the protonation of H193 [31]. A phosphomimicking mutant study investigated the role of phosphorylation of S115, S188 and S274 [58]. Serine 115 is situated close to the divalent cation binding site, and its mutation into a glutamate disrupted the hydrogen bond and salt bridge lock [31,58]. The serine 274 to glutamate mutant was shown to disorder the C-terminal extremity, but its exact role is still misunderstood [58]. Finally, S188 phosphorylation was associated with the opening of the pore through conformational rearrangements of loop D, disrupting R190 and D191 anchoring to the N-terminal residues [58,59]. As mentioned previously, no direct observation of calcium bound to *So*PIP2;1 was made (i.e., cadmium was observed in interaction with the AQP) however, the authors searched for similar structural motifs and found 13 other experimental structure entries containing calcium ions [31]. Another cadmium ion was found in another structure of *So*PIP2;1, and it interacts directly with T183 and A267 [60]. Following the same idea as for the D28–E31 first cadmium binding site, the authors postulated this second site to correspond to a lower affinity binding site for calcium [60]. Moreover, the phosphorylation of a spinach AQP at S274 was shown to be calcium-dependent in vitro [61], comforting the

reliability of this regulation of spinach AQPs by calcium. In another study, the effects of calcium and other cations were tested on *Arabidopsis thaliana* PIP2;1 (*At*PIP2;1) reconstituted in proteoliposomes [62]. The impact of the cations on the water permeability of *At*PIP2;1 was estimated through the stopped-flow light scattering of the proteoliposomes. Calcium was associated with the strongest inhibition of *At*PIP2;1 after cadmium. The mutation of residues involved in plant AQP gating described for *So*PIP2;1 (D28, E31 and H193) to alanine diminished (for D28A mutant in *At*PIP2;1) or completely canceled (for E31A and H199A mutants in *At*PIP2;1) the sensitivity to calcium of *At*PIP2;1. Interestingly, the mutation of another residue located on loop B (R124 in *At*PIP2;1) also largely reduced *At*PIP2;1 calcium sensitivity [62].

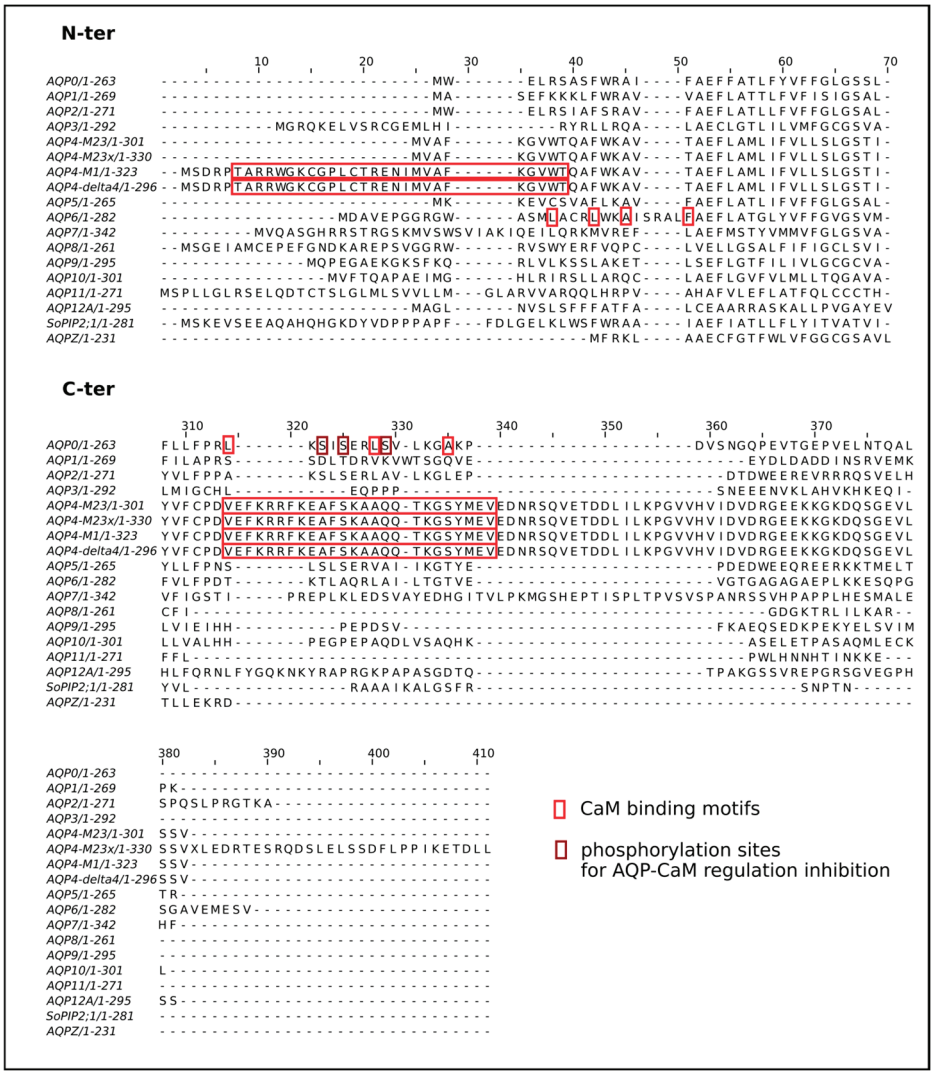


Figure 2. AQP regulation by calmodulin. Multiple sequence alignment of human AQPs, spinach *So*PIP2;1 and *E. coli* AQPZ. CaM binding motifs or fragments used for binding assays extracted from [41,48,49] are reported on the corresponding human sequences. Phosphorylation sites for AQP-CaM regulation inhibition in human AQP0 are extracted from [46].

Similarly to *So*PIP2;1, the human AQP2 experimental structure also contained cadmium ions in interaction with the intra-cellular surface of the tetramer [63]. In the structure, the cation is stabilized by Q57 and E155 [63]. Next to Q57, S148 is located within a casein kinase II consensus site [56]. Hence, in a similar manner as in *So*PIP2;1 regulation, the phosphorylation of S148 could disrupt the salt bridge network stabilizing the cation. Interestingly, a phosphomimicking mutation of S148 induced AQP2 retention in the En-

doplasmic reticulum [64]. Moreover, the mutation of Q57 or A147 (which is close to the cation fixation site) has been identified in patients suffering from nephrogenic diabetes insipidus, a disorder caused by the failure to address AQP2 to the apical membrane [65]. Even though the exact mechanism is still unknown, it is clear that calcium and calmodulin play a significant role in the regulation of AQP2 trafficking [50–53].

2.2. Magnesium Ions

Magnesium is the major intra-cellular divalent cation and plays essential physiological roles (such as in the normal function of the cardiovascular system) [66]. It is involved in proteins and nucleic acid synthesis and is a co-factor of many enzymes. Most of the magnesium in the human body is found in bones (60%), of which about 30% is exchangeable. It is otherwise mainly present within cells (20% in skeletal muscle and 19% in soft tissues), with only 1% in extra-cellular fluids [66].

Several studies have shown that magnesium treatment (magnesium sulfate, magnesium acetate) was associated with a significant increase in human AQP3 transcript levels [67–69]. Moreover, all three studies also observed a significant increase in adenylate cyclase (AC) and protein kinase A (PKA) activity and an increase in phosphorylation of the cAMP response element-binding protein (CREB). Hence, intra-cellular magnesium ion increases seem to activate AC, which in turn triggers CREB phosphorylation through the activation of PKA, eventually promoting AQP3 gene transcription [67–69].

Other studies have highlighted a link between magnesium treatment and AQP4 expression. Studies led on the central nervous system tissues of rats have highlighted a significant decrease of AQP4 proteins through immunohistochemistry after treatment with magnesium sulfate [70,71]. However, another work studying the impact of magnesium sulfate on children with "hand, foot, and mouth" disease found a significant decrease of AQP4 in the serum but not in the cerebrospinal fluid after the magnesium sulfate treatment of patients [72]. Another study led on pregnant rats with acute hypertension indicated a significant reduction of blood–brain barrier permeability by magnesium sulfate treatment in the posterior cerebrum but not in the anterior cerebrum. However, no effect was observed on AQP4 expression [73]. Hence, the exact effect of magnesium sulfate on AQP4 expression and brain water homeostasis is still unclear. Moreover, AQP3 is also expressed in the brain [74]; hence, the physiological effect of magnesium sulfate on water balance observed in brain diseases could also be partly explained by the modulation of AQP3 transcripts level.

3. Transition Metals

3.1. Mercury Ions

Mercury ions are historically associated with the discovery and characterization of AQPs [75]. Initially, the discovery of red blood cell membranes' water permeability inhibition by organic mercurials [76] allowed for later AQP isolation [77], cloning [3,78], membrane transport characterization [79] and mercury sensitivity mutational analysis [80,81]. Mercurial compounds are known to inhibit most AQPs [80,82–84], with the notable exception of human AQP6 [85] and spinach *So*PIP2;1 [60], for which mercury ions induce an increase in their water permeability and the activation of ion conductance in AQP6.

The molecular mechanism associated with AQPs' inhibition by mercury is due to its high affinity with the thiol group found in cysteine residues [82]. Many AQPs display cysteine in their ar/R constriction. Hence, in these cases, mercury binding was hypothesized to directly block the pore sterically [82]. However, depending on where the pore lining cysteine is located, the sensitivity to mercury can be increased. A study of *E. coli* AQPZ wild type and AQPZ mutants' (pore-lining residues T183 and L170 mutated to cysteine) water permeability reconstituted in proteoliposomes coupled to the resolution of their 3D structures through X-ray crystallography highlighted this change in mercury sensitivity [82]. In wild-type AQPZ, the pore lining cysteine (C20) is part of the ar/R constriction and is located on helix 1. This wild-type AQPZ corresponded to the smallest

inhibition by mercury [82]. The aquaglyceroporine AQP3 bears a cysteine at its ar/R constriction on the first alpha helix as well (C40). Another study showed through molecular dynamics that mercury binding to C40 inhibited water and glycerol passage because of small conformational changes in the residues of the ar/R constriction. More precisely, conformational changes of the sidechains of F63 and R218 obstructed the pore [86]. One of the AQPZ mutants corresponded to a cysteine replacing another residue of the same ar/R constriction (T183), which also mimicked the location of cysteine in human AQP1 (C189 in human AQP1) and which is located on loop B. Cysteine in position 183 increased the mercury sensitivity of AQPZ [82]. Moreover, no conformational changes could be observed in the AQPZ-T183C mutant structure when compared to the wild type [82]. Other site-directed mutagenesis studies demonstrated inhibition of AQP1 [80] and AQP2 [87] water permeability by mercury associated with the T183 corresponding cysteine (C189 and C181 in human AQP1 and human AQP2, respectively). In AQP1, however, in another molecular dynamics study, the authors observed small local conformational changes triggered by mercury fixation [88]. In this study, not only steric effects but also the repositioning of loop B backbone explained the inhibition. Indeed, loop B backbone carbonyl oxygens are water interaction sites; hence, their reorientation out of the pore created a hydrophobic patch locally [88]. The AQPZ mutant associated with the highest sensitivity to mercury had the pore-lining leucine 170 replaced by cysteine [82]. This leucine is located at the center of the pore facing the NPA asparagines and corresponds to a position in the pore that was predicted by the authors to maximize steric inhibition upon mercury binding [82]. A molecular dynamics study of this mutant inhibition by mercury revealed that steric inhibition of water passage could be explained by the re-organization of water molecules around mercury cation. According to the study, five to six molecules of water interacted strongly with mercury and were responsible for pore occlusion [89].

However, with more AQPs being studied for their modulation by mercury, it became clear that other molecular mechanisms were involved. AQP4 was initially considered a mercury-insensitive AQP only to later observe mercury inhibition when reconstituted into proteoliposomes [83]. This can be explained by the bi-directional orientation of AQP4 tetramers in proteoliposomes and by the fixation of mercury ions to an intra-cellular cysteine. Indeed, through the site-directed mutagenesis of rat AQP4 cysteines, the authors pinpointed intra-cellular cysteine 178 located on loop D as responsible for the transduction of AQP4 inhibition by mercury [83]. Contrarily to the previous situations, C178 is situated far from the ar/R constriction, and the Van der Waals radius of mercury is not large enough to occlude the pore at this location (i.e., at the entrance of the intra-cellular vestibule). This implies that other molecular mechanisms besides a simple steric blockage of the pore are at work to explain mercury inhibition of AQP4. The authors postulated that the gating of AQP4 through similar conformational changes of loop D as described for plant AQPs could be triggered by mercury fixation (see calcium section) [31,83]. Rat intra-cellular AQP6 water permeability increases and ion channeling activation have been shown to be triggered by mercury ions binding to cysteines 190 and 155 [85,90]. The mutation of C190 to alanine or C155 to alanine resulted in a 50% loss in mercury-inducible water and ions conductance, while the double mutation exhibited negligible mercury activation [85,90]. Similarly to AQP1, C190 is located in the ar/R constriction on the water-interacting sites bearing loop B, while C155 is positioned on the intra-cellular surface at the interface between two sub-units. Electrophysiological measurements coupled to site-directed mutagenesis revealed that changes in water permeability correlated with equivalent changes in ions conductance, indicating both water and ions were very likely to cross AQP6 through the same monomeric conducting pore [85,90]. From molecular dynamics studies, it appeared that the modulation of water flux upon mercury fixation in AQP6 was eventually integrated through the position of the ar/R constriction arginine and through other pore-lining residue methionine 160 (of rat AQP6) sidechains within the pore lumen [91,92]. The exact way in which mercury binding to intra-cellular cysteine 155 resulted in these allosteric modulations of pore-lining residues sidechain position within the pore lumen is still unclear. Finally, in plant AQPs,

the modulation of water permeability is even more complex. Except for two intra-cellular AQPs of Arabidopsis thaliana (located in the tonoplast membrane) [93], no clear link could be found between cysteine residues and the transduction of mercury modulation of the water permeability [60,62,94]. In one study led on spinach AQP *So*PIP2;1, none of the cysteine to serine mutants differed significantly from the wild type in terms of mercury modulation. The water permeability of the wild type and of all of the cysteine mutants were similarly significantly increased by mercury [60]. From this study, it appears that mercury can hence impact AQPs' function through yet another molecular mechanism not related to cysteines. The authors postulated a putative indirect action of the cation through the modulation of phospholipids' bilayer fluidity [60].

Finally, mercurials have also been reported to significantly reduce the transcripts and protein abundance of AQP3, AQP4 and AQP7 in the gastrointestinal tracts of rats [95].

3.2. Zinc Ions

Zinc is one of the most abundant trace elements in the human body [96]. Many biochemical functions have been discovered for zinc cations, including three main roles: a structural role [97], a catalytic role [98] and a role in the maintenance of plasma membranes' functions [99]. Zinc is known to interact with protein motifs called zinc fingers, which are found in many transcription factors. These motifs are characterized by cysteine and histidine residues positioned close to each other and forming the zinc fixation site [100]. Zinc has been shown to significantly increase the water permeability of *Xenopus laevis* oocytes expressing bovine AQP0 [101]. Moreover, the authors concluded that a zinc-mediated increase in water fluxes required positive cooperativity between sub-units associated with a Hill coefficient of 4 [101]. This result was further confirmed by a molecular dynamics study in which zinc fixation at the vicinity of human AQP0 cysteine 144 induced a significant increase in water permeability associated with a cooperative effect between monomers [102]. In this study, the putative zinc-binding site was predicted to be at the interface between sub-units. Upon zinc binding, two adjacent sub-units would hence be more tightly interacting with each other thanks to a salt bridge network between the two sub-units coordinated by zinc ions. This in turn resulted in an increase in the overall AQP0 tetrameric fold stability, which was associated with a re-positioning of R187 of the ar/R constriction sidechain within the pore lumen and higher water permeability [102]. Similar zinc-binding sites (near C145) and associated molecular mechanisms were also described for human AQP5 in the same study [102]. AQP5 is mainly expressed in secretory glands, where it plays a central role in fluid secretion [8,103]. Interestingly, another study has demonstrated that zinc supplementation could induce an increase in these glands' secretory function [104]. However, mutagenesis studies indicate H40 and H122 to be necessary for zinc modulation of bovine AQP0 [101], while zinc-binding sites predicted through molecular dynamics did not incorporate these two histidines [102]. To explain these discrepancies, the authors of the molecular dynamics study formulated the hypothesis that H40 and H122 could be implicated in alternative ways. Histidine 40 could be involved in the accessibility of zinc cations to their binding site through the central pore, and histidine 122 could be involved in the transduction of zinc-binding signals to the ar/R constriction through loop C [102].

In an opposite manner, rat AQP4 reconstituted into proteoliposomes was shown to be transiently inhibited by zinc cations [105]. This inhibition is dependent on intra-cellular zinc (AQP4 was inserted in both directions into proteoliposomes) since no inhibition was observed for AQP4 expressed in *Xenopus laevis* oocytes [101]. Again, this result was comforted by molecular dynamics [102]. AQP4 possesses three intra-cellular cysteines: C87, C178 and C253. All three associated putative zinc-binding sites were tested through molecular dynamics, and only the C253 site was associated with a significant decrease in human AQP4 water permeability [102]. This result contrasted with the suppression of mercury inhibition in the AQP4 C178S mutant. It is, however, worth noting that the other cysteines were not mutated to compare with C178 [105]. According to the authors of the molecular dynamics study, the mutation of C178 to serine could also have induced

small conformational re-arrangements locally. The C178S mutation could hence have hindered zinc binding through its impact on adjacent residue D179, which was involved in zinc stabilization with C253 [102]. Still, in the same molecular dynamics study, zinc fixation to human AQP2 intra-cellular C75 also induced a significant decrease in water permeability [102]. The associated molecular mechanism was similar between AQP4 and AQP2 but differed from the one explaining AQP0 and AQP5 water permeability increase. In this case, zinc binding did not increase the AQP tetrameric fold stability but rather impacted the orientation of the sub-unit dipole moment. Because the arginine of the ar/R sidechain is both positively charged and not stabilized by a salt bridge with a carboxylate residue, the authors postulated zinc fixation to orient this sidechain through long-distance electrostatic interactions [102].

Additionally, zinc was shown to alter transcripts abundance of plant AQPs in soybeans [106], barley [107] and pak choi [108]. In the study led on pak choi, the regulation of AQP genes differential expression was dose-dependent [108].

3.3. Cadmium Ions

As mentioned previously, cadmium was found in several structures of plant AQP *So*PIP2;1 [31,60] and human AQP2 [63]. This cation can be added for methodological purposes to increase the crystal quality [31]. Cadmium ions also induced a 70% water permeability loss in *At*PIP2;1 reconstituted in proteoliposomes [62]. Its toxicity could hence be partly explained by the interference with native calcium regulation of plant AQPs intracellular gating and AQP2 cellular trafficking (see calcium section). The same residues as for calcium-dependent intra-cellular gating E31 and H199 (H193 in *So*PIP2;1) were involved in cadmium sensitivity as well as loop B arginine124 [62].

3.4. Gold Ion Compounds

In the last decade, gold compounds have shown promising results as aquaglyceroporin inhibitors for the development of new therapies and imaging opportunities [109]. Indeed, more and more information has been piling up, indicating AQPs as elusive but interesting therapeutic targets in the fight against several cancers [25,26,109]. A new field of research has therefore emerged in the search for specific AQP inhibitors [110], among which are gold compounds. Several studies have pointed out C40 in AQP3 as the main residue responsible for gold compounds' specific binding to the aquaglyceroporin [109,111–116]. Gold compounds were shown to induce a significant decrease in glycerol permeability of AQP3 through stopped-flow spectroscopy using human red blood cells. Through combined molecular and quantum dynamics, the authors detailed gold compounds' interaction with C40 and highlighted a similar molecular mechanism as for the mercury inhibition of AQP3, i.e., upon gold compound binding, the arginine of the ar/R constriction sidechain undergoes a transconformation and occludes the pore [114]. Another AQGP (AQP7) has also been investigated for gold compound inhibitors. Gold compounds' inhibition of AQP7 was significant and was evaluated by assessing both water and glycerol permeability in murine adipocyte cell line 3T3-L1 [117]. In silico docking highlighted pore-lining methionine 47 as a putative gold compound binding site for AQP7 [117]. Finally, gold particles have been shown to up-regulate AQP1 expression in mouse cerebral endothelium cell line bEnd.3 [118].

3.5. Silver Ions

Similarly to gold compounds, silver compounds were investigated with the aim of finding new AQP-specific inhibitors [119]. The authors tested the effect of silver nitrate on the permeability of the peribacteroid membrane (PBM) of soybean nodules, beetroot plasma membrane and human red blood cell membrane through a stopped-flow fluorimeter coupled with dynamic laser light scattering [119]. Each of the membranes is naturally enriched in one type of AQP: PBM is enriched in Nodulin-like integral protein NOD26 (a class of AQPs dedicated to ammonium uptake and specific to nitrogen-fixing plants [120]); the

beetroot plasma membrane is enriched in plant strict AQPs (PIPs); the human red blood cell membrane is enriched in mammalian strict AQP1 and aquaglyceroporine AQP3. Silver was found to be the most efficient AQP inhibitor, regardless of the type of AQP when compared to other metallic cation compounds such as gold or mercury [119]. However, no mutagenesis or in silico modeling studies were carried out to study the molecular mechanisms involved. Silver nitrate also triggered AQP transcript increases in soybeans [106].

3.6. Copper Ions

Copper is an essential transition metal necessary for the function of several enzymes [121]. Copper sulfate was shown to significantly decrease water and glycerol permeability of human AQP3 but had no effect on human AQP4 or mouse AQP7 function [122]. AQP3 W128A, S152A and H241A mutants lost their sensitivity to copper, highlighting the role of these three residues in copper-mediated AQP3 inhibition. An in vitro study of copper-based AQP inhibitors displayed anti-tumorigenesis effects [123].

3.7. Nickel Ions

In a previous study, the same authors characterized the effect of nickel ions on human AQP3, AQP4 and AQP5 function [124]. Similarly, nickel ions induced significant water permeability inhibition in AQP3 but not in AQP4 or AQP5. Moreover, AQP3 was selectively inhibited by nickel and not by cadmium or zinc. Finally, the same residues as for copper (W128, S152 and H241) were shown to abolish nickel sensitivity when mutated in alanine [124]. Nickel ions also induce a 48% water permeability loss in *At*PIP2;1 reconstituted in proteoliposomes [62].

3.8. Lead Ions

Lead is another transition metal known for its high toxicity [125]. In the central nervous system, acute lead poisoning has been associated with astrocyte swelling [126]. The main AQP of the central nervous system, expressed in astrocytes, is AQP4 [127]. Through the over-expression of mouse AQP4 in an astrocyte cell line initially not expressing AQP4, Gunnarson et al. observed a significant increase in water permeability of these cells when cultivated with lead while no such effect was observed in untransfected cells or cells transfected with human AQP3 [128]. This lead effect seems to be mediated by calmodulin-dependant protein kinase II (CaMKII). Indeed, the inhibition of CaMKII abolished the lead-associated increase of water permeability. Moreover, the mutation to alanine of a serine of AQP4 consensus site for CaMKII phosphorylation (S111) abolished the lead effect on water permeability as well [128].

3.9. Manganese Ions

Manganese is a trace element necessary for the normal physiology of bacteria [129], animals [130,131] or plants [132]. The inhibition of water permeability through direct interaction with AQP has been demonstrated for plant AQP *At*PIP2;1 reconstituted in proteoliposomes [62]. This inhibition was similar to calcium's and corresponded to a 60% loss of water permeability. Alanine mutants highlighted the role of H199 and, to a lesser extent, R124 and E31 in manganese-mediated gating of *At*PIP2;1 [62]. Manganese was also shown to interfere with AQP2 and AQP4 trafficking. Manganese treatment induced AQP2 internalization and intra-cellular accumulation in cultured cells [133]. On the contrary, the treatment of cultured astrocytes with manganese increased AQP4 proteins in the plasma membrane [134].

3.10. Iron Ions

Iron is essential for human physiology [135]. However, its over-accumulation in the brain can lead to brain edema and neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease [136,137]. The upregulation of AQP4 and AQP9 protein levels was observed in cultured astrocytes treated with iron [138]. In another study, cultured astrocytes

were exposed to different concentrations of iron ions [139]. Iron-induced astrocyte swelling and death happened in a dose- and time-dependent manner. AQP4 mRNA and protein levels assessed by RT-qPCR and immunofluorescence staining were also significantly increased by iron treatment [139]. In the same study, it was observed that the inhibition of oxidative stress and Mitogen-activated protein kinases (MAPK) both reduced iron-induced astrocyte death and AQP4 upregulation [139]. Iron effect on AQP4 expression is hence probably mediated by MAPKs. Furthermore, immunohistochemical analysis and real-time quantitative polymerase chain reaction of AQP4 in rat brains showed an association between iron overload, AQP4 expression increase and intracerebral hemorrhage [140,141]. Moreover, hydrocephalus was attenuated by an AQP4 inhibitor (2-(nicotinamide)-1,3,4-thiadiazole: TGN-20) [141].

4. Alkaline Metals

4.1. Lithium Ions

Lithium has been widely used to treat bipolar disorders [142]. Chronic lithium treatment is associated with the development of nephrogenic diabetes insipidus (NDI) [143], which is associated with AQP2 downregulation [144,145]. Several studies have demonstrated a decrease in AQP2 gene expression and protein abundance after lithium exposure [144–150]. The protein abundance of AQP3 was also significantly reduced after the lithium treatment of rats, while AQP1 was not affected [144].

4.2. Sodium Ions

Sodium ions have also been shown to significantly impact transcript and protein levels of AQP2 in rats and humans. Except for one study in which Sprague–Dawley rats subjected to a high NaCl diet displayed a lower abundance of AQP2 transcripts compared to basal NaCl diet [151], all the other studies associated high NaCl diet or treatment with increased AQP2 transcripts or protein abundance in vivo [152–155] or in vitro [156]. Recently, two molecular dynamics studies on the regulation of human AQP2 highlighted a putative direct modulation of AQP2 water permeability by sodium ions [157,158]. The authors observed a preferential interaction of sodium ions with the carboxylates of extra-cellular loop C and loop E (E106, D111, D115, D199 and D200) compared to potassium ions. This interaction interfered with the ability of these carboxylates to establish salt bridges with other charged residues and on the overall stability of loop C and was also associated with ar/R constriction arginine 187 conformational changes and hence water permeability changes as well [157,158]. More research is however needed to assess if AQP2 is directly modulated by sodium ions or not. Supporting a specific regulatory role of sodium in AQP regulation, a study led on the halophytic grass *Puccinellia nuttalliana* highlighted an increase in root cell hydraulic conductivity after sodium and not after potassium treatment, which was abolished by mercury [159].

5. Discussion

Since the discovery of AQPs by Peter Agre in 1992 [3], researchers have been constantly intrigued by the functional complexity underlying this family of channel proteins [20,21]. Initially conceptualized as water channels only, the first challenge was to understand how they could nonetheless exclude protons [28]. It was then discovered over the years that they could also accommodate other small polar molecules [160], reactive oxygen species or even ions for a few of them [161]. On top of the traditional regulation of their gene expression, many other factors have been shown to significantly modulate AQPs' function: sub-cellular trafficking [53,162], membrane composition [34], protein-protein interactions [35], allosteric mechanisms between sub-units of the same tetramer [163] or gating [164]. In this review, we highlighted the significant contribution of cations as well, which interpose in every level of the AQPs regulations previously mentioned. All of the modulation of AQPs by cations are summarized in Table 1, all putative cations-binding-sites are indicated on an archetypal AQP fold in Figure 3 and all of the residues involved in these modulations are indicated on a multiple sequence alignment of human AQPs, *So*PIP2;1 and *Ec*AQPZ in Supplementary Figure S1.

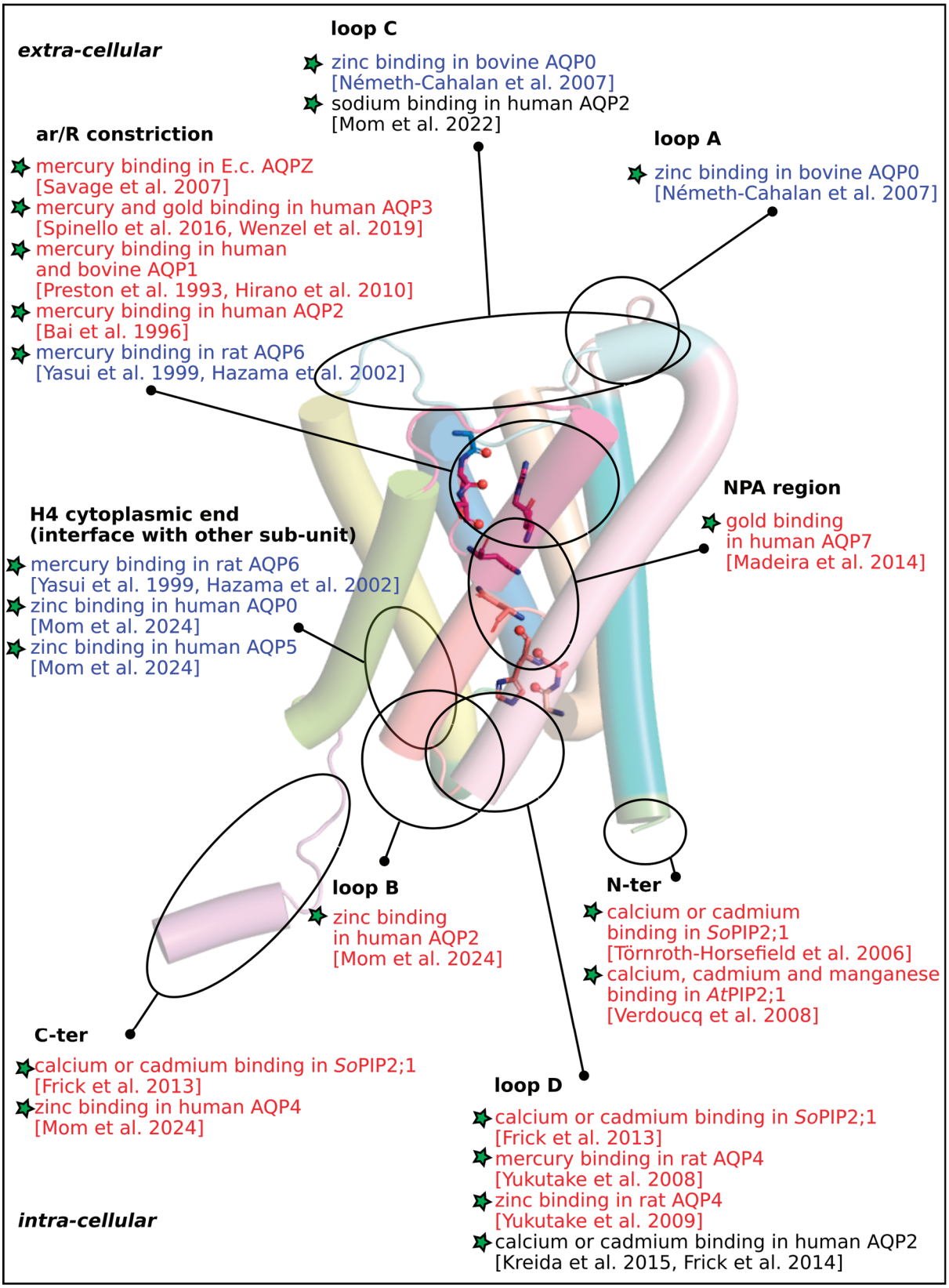


Figure 3. Cation binding sites in AQPs. The cations binding sites mentioned in the current review are indicated on a schematic representation of an archetypal AQP (made from X-ray structure of human AQP2). The reference scientific paper for each binding site is indicated under brackets: Savage et al. 2007 [82]; Spinello et al. 2016 [86]; Wenzel et al. 2019 [114]; Preston et al. 1993 [80]; Hirano et al. 2010 [88];

Bai et al. 1996 [87]; Yasui et al 1999 [85]; Hazama et al. 2002 [90]; Mom et al. 2022 [157]; Mom et al. 2024 [102]; Frick et al. 2013 [60]; Frick et al. 2014 [63]; Yukutake et al. 2008 [83]; Yukutake et al. 2009 [105]; Kreida et al. 2015 [56]; Törnroth-Horsefield et al. 2006 [31]; Verdoucq et al. 2008 [62]; Madeira et al. 2014 [117]; Németh-Cahalan et al. 2007 [101]. Cation binding can be associated with AQP activation (legend written in blue), inhibition (legend written in red) or unknown event (legend written in black).

Table 1. Known modulations of AQPs by cations. All of the modulations of AQPs by cations discussed in the current review are summarized. The corresponding references are indicated under brackets.

Ion Name	Name of the Regulated AQP	Direct Interaction with AQP	Water Channel Function	Residues Involved in Binding	Residues Involved in Molecular Mechanism	Transcripts and/or Protein Abundance
Calcium	*So*PIP2;1	yes	inhibition [31]	D28 and E31 [31] T183 and A267 [60]	D28, E31, R190, R191 and H193 [31,57]	Unknown
Calcium	*So*PIP2;1	No	Activation	None	S115, S188 and S274 [31,58,59]	unknown
Calcium	*At*PIP2;1	Yes	Inhibition	E31, H199 [62]	E31, H199 and R124 [62]	unknown
Calcium	Human AQP2	yes	Unknown	Q57, E155 [56,63]	Unknown	increase [50,165]
Calcium	Ovine AQP0 Human AQP0	No	Inhibition	None	L227, L234, A240 and R156 (CaM binding) [41,55] S229, S231 and S235 (phosphorylation sites) [46] R187, Y149, Y23 (water permeability modulation) [45,54]	unknown
Calcium	Human AQP4	No	Unknown	None	Residues 6-31 in AQP4-M1 residues 256-280 in AQP4-M1 (CaM binding) [49]	unknown
Calcium	Mouse AQP6 rat AQP6 human AQP6	No	Unknown	None	L15, L19, A22 and F28 in human AQP6 [48]	unknown
Magnesium	Human AQP3	Unknown	Unknown	Unknown	Unknown	increase [67–69]
Magnesium	Rat AQP4 human AQP4	Unknown	Unknown	Unknown	Unknown	No effect or decrease [70–73]
Mercury	E. coli AQPZ	yes	Inhibition	C20 [82]	C20 [82] R189 [92]	unknown
Mercury	Human AQP3	yes	Inhibition	C40	F63, R218 [86]	Decrease (rat AQP3) [95]
Mercury	Human AQP1 bovine AQP1	yes	Inhibition	Human: C189 [80] bovine: C191 [88]	For bovine AQP1: R197, H182, F58, I193, G192, C191, G190, E144 [88]	Unknown
Mercury	Human AQP2	yes	Inhibition	C181 [87]	Unknown	unknown
Mercury	rat AQP4	yes	Inhibition	C178 [83]	Unknown	Decrease [95]
Mercury	Rat AQP6	yes	Activation	C155 and C190 [85,90]	R196 [92] and M160 [91]	No effect
Mercury	*At*TIPs	Yes	Inhibition	C116 or C118 [93]	C116 or C118 [93]	unknown
Mercury	Rat AQP7	Unknown	Unknown	Unknown	Unknown	decrease [95]
Zinc	bovine AQP0 human AQP0	yes	Activation	H40 and H122 [101] C144, T54, Q140 [102]	R187 [102]	unknown
Zinc	Human AQP5	yes	Activation	C145, S149, S164, S168, T55, Q58 [102]	Human: R187 [102]	increase [104]

Table 1. *Cont.*

Ion Name	Name of the Regulated AQP	Direct Interaction with AQP	Water Channel Function	Residues Involved in Binding	Residues Involved in Molecular Mechanism	Transcripts and/or Protein Abundance
Zinc	Rat AQP4 Human AQP4	yes	Inhibition	Rat: C178 [105] human: C253, D179, H90 [102]	Human: R216 [102]	unknown
Zinc	Human AQP2	yes	Inhibition	C75 [102]	R216 [102]	unknown
Zinc	Soybean AQPs	Unknown	Unknown	Unknown	Unknown	No effect or increase [106]
Zinc	Barley AQPs (HvPIP1;3, HvPIP2;4 and HvPIP2;5)	Unknown	Unknown	Unknown	Unknown	decrease [107]
Zinc	Pak choi AQPs (PIP1 isoforms)	unknown	Unknown	Unknown	Unknown	increase [108]
Zinc	Human AQP3	No	No effect [124]	None	None	unknown
Cadmium	Human AQP2	Yes	Unknown	Q57, E155 [63]	Unknown	unknown
Cadmium	*So*PIP2;1	Yes	Inhibition	D28 and E31 [31] T183 and A267 [60]	D28, E31, R190, R191 and H193 [31,57]	unknown
Cadmium	*At*PIP2;1	Yes	Inhibition	E31 and H199 [62]	E31, H199 and R124 [62]	unknown
Cadmium	Human AQP3	No	No effect [124]	None	None	Unknown
Gold	Human AQP3	yes	Inhibition	C40 [114]	R218 [114]	unknown
Gold	Human AQP7	yes	Inhibition	Met47 [117]	unknown	Unknown
Gold	mouse AQP1	Unknown	Unknown	Unknown	Unknown	increase [118]
Silver	Soybean NOD26	Unknown	inhibition [119]	Unknown	Unknown	unknown
Silver	Beet root PIPs	Unknown	inhibition [119]	Unknown	Unknown	unknown
Silver	Human AQP1 and human AQP3	Unknown	Inhibition [119]	Unknown	Unknown	unknown
Silver	Soybean AQPs	Unknown	Unknown	Unknown	Unknown	No effect or increase [106]
Copper	Human AQP3	Unknown	inhibition	unknown	W128, S152, H241 [122]	Unknown
Copper	Human AQP4	no	No effect [122]	None	none	unknown
Copper	Mouse AQP7	no	No effect [122]	None	none	Unknown
Nickel	Human AQP3	Unknown	Inhibition	unknown	W128, S152, H241 [124]	Unknown
Nickel	Human AQP4	no	No effect [124]	none	none	unknown
Nickel	Human AQP5	no	No effect [124]	None	None	unknown
Nickel	*At*PIP2;1	Yes	Inhibition [62]	Unknown	unknown	unknown
Lead	Mouse AQP4	unknown	Activation	Unknown	S111 [128]	No effect [128]
Lead	Human AQP3	No	No effect [128]	None	None	unknown
Manganese	*At*PIP2;1	Yes	Inhibition	E31 and H199 [62]	E31, H199 and R124 [62]	unknown
Manganese	Rat AQP2	Unknown	Inhibition [133]	Unknown	Unknown	unknown
Manganese	Rat AQP4	Unknown	Activation [134]	Unknown	Unknown	No effect
Iron	Mouse AQP4 rat AQP4	Unknown	Unknown	Unknown	Unknown	increase [138–141]
Iron	Mouse AQP9	Unknown	Unknown	Unknown	Unknown	increase [138]
Lithium	Human AQP2 [146] rat AQP2 [144,145,148,149] mouse AQP2 [147,150]	Unknown	Unknown	Unknown	Unknown	decrease [144–150]
Lithium	Rat AQP3	Unknown	Unknown	Unknown	Unknown	decrease [144]

Table 1. *Cont.*

Ion Name	Name of the Regulated AQP	Direct Interaction with AQP	Water Channel Function	Residues Involved in Binding	Residues Involved in Molecular Mechanism	Transcripts and/or Protein Abundance
Lithium	Rat AQP1	Unknown	Unknown	Unknown	Unknown	No effect [144]
Sodium	Rat AQP2 [151,154,156] human AQP2 [152,153,155]	yes [157]	Unknown	E106, D111, D115, D199 and D200 in human AQP2 [157]	Unknown	Conflicting results: decrease [151] increase [152–156]

While it appears clearly that cations are relevant physiological regulators of AQPs in all types of organisms, a global understanding of their modes of action is lacking. The initially proposed steric inhibition of water permeability by mercury bound to pore lining cysteine residues is for sure insufficient or too restrictive to explain all types of cation-associated regulations of AQPs. Indeed, there is for instance no clear explanation for allosteric modulations not associated with the targeting of pore-lining residues by cations nor for mercury mode of action in plant AQPs which does not seem to involve cysteines at all. However, from this reviewing work, a few tendencies can be extracted: (i) Most of the described cations-binding-sites are associated with AQP inhibition except for the one located on the helix 4 cytoplasmic end, at the interface between two sub-units (see Figure 3). Indeed, three different studies indicate the fixation of zinc or mercury to this cations-binding site as a trigger for AQP activation. Moreover, this phenomenon is associated with cooperativity between sub-units. (ii) In most cases, cations act upon several layers of regulation, e.g., calcium has been associated with gene expression modulation, sub-cellular trafficking, protein–protein interactions and the gating of AQP2. (iii) Cysteines are far from being the only residues involved in cations binding in AQPs. Other residues such as serines, threonines, glutamines, methionines and carboxylates can also be found in AQPs cations binding sites. (iv) Most of the molecular mechanisms mentioned in this review eventually lead to the fine-tuning of the position of the sidechain of the arginine of the ar/R constriction inside the pore lumen.

The arginine of the ar/R constriction is among the most conserved residues of the AQP fold and is also known to be a determinant of the solute selectivity of the pore. Because of their positively charged sidechains, arginine can act as voltage sensors in voltage-gated channels [166,167]. Considering the perturbations of the protein electrostatic field induced by cation binding, one could hypothesize a similar role for the arginine of the ar/R constriction in AQPs. Several studies have demonstrated the possible modulations of AQPs through the application of external electric fields in silico [168–174]. While other pore-lining residues such as histidines were involved, the ar/R constriction arginine was indeed implicated in external electric field sensing [168,171–174]. Other studies highlighted the modulation of AQPs' water fluxes by non-physiological membrane potentials through all-atom molecular dynamics [175,176]. Once again, the ar/R arginine played a central role. Interestingly, high but realistic concentrations of KCl ions could also induce the same type of modulation [176]. Better understanding how charge repartition at different locations of the AQP fold could impact ar/R constriction arginine conformation inside the pore lumen could hence be a way to propose a unifying molecular mechanism of AQP modulation by cations. To conclude, it appears that cations play a significant role in the regulation of AQPs' function. Moreover, on top of better understanding this regulatory network, more general theoretical knowledge about AQPs and ion channels function could also be gathered from this field of study.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cimb46080470/s1. References [91,93] are cited in the Supplementary Materials.

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References

1. Abascal, F.; Irisarri, I.; Zardoya, R. Diversity and Evolution of Membrane Intrinsic Proteins. *Biochim. Et Biophys. Acta (BBA)—Gen. Subj.* **2014**, *1840*, 1468–1481. [CrossRef] [PubMed]
2. Agre, P.; Sasaki, S.; Chrispeels, M.J. Aquaporins: A Family of Water Channel Proteins. *Am. J. Physiol.* **1993**, *265*, F461. [CrossRef] [PubMed]
3. Preston, G.M.; Carroll, T.P.; Guggino, W.B.; Agre, P. Appearance of Water Channels in Xenopus Oocytes Expressing Red Cell CHIP28 Protein. *Science* **1992**, *256*, 385–387. [CrossRef] [PubMed]
4. Beitz, E. *Aquaporins*; Springer Science & Business Media: Berlin/Heidelberg, Germany, 2008; ISBN 978-3-540-79885-9.
5. Mukherjee, S.; Roy, S.; Corpas, F.J. Aquaporins: A Vital Nexus in H_2O_2-Gasotransmitter Signaling. *Trends Plant Sci.* **2024**, *29*, 681–693. [CrossRef] [PubMed]
6. Bienert, G.P.; Møller, A.L.B.; Kristiansen, K.A.; Schulz, A.; Møller, I.M.; Schjoerring, J.K.; Jahn, T.P. Specific Aquaporins Facilitate the Diffusion of Hydrogen Peroxide across Membranes*. *J. Biol. Chem.* **2007**, *282*, 1183–1192. [CrossRef] [PubMed]
7. Bienert, G.P.; Chaumont, F. Aquaporin-Facilitated Transmembrane Diffusion of Hydrogen Peroxide. *Biochim. Et Biophys. Acta (BBA)—Gen. Subj.* **2014**, *1840*, 1596–1604. [CrossRef] [PubMed]
8. Day, R.E.; Kitchen, P.; Owen, D.S.; Bland, C.; Marshall, L.; Conner, A.C.; Bill, R.M.; Conner, M.T. Human Aquaporins: Regulators of Transcellular Water Flow. *Biochim. Et Biophys. Acta (BBA)—Gen. Subj.* **2014**, *1840*, 1492–1506. [CrossRef] [PubMed]
9. Mola, M.G.; Sparaneo, A.; Gargano, C.D.; Spray, D.C.; Svelto, M.; Frigeri, A.; Scemes, E.; Nicchia, G.P. The Speed of Swelling Kinetics Modulates Cell Volume Regulation and Calcium Signaling in Astrocytes: A Different Point of View on the Role of Aquaporins. *Glia* **2016**, *64*, 139–154. [CrossRef] [PubMed]
10. Akai, M.; Onai, K.; Morishita, M.; Mino, H.; Shijuku, T.; Maruyama, H.; Arai, F.; Itoh, S.; Hazama, A.; Checchetto, V.; et al. Aquaporin AqpZ Is Involved in Cell Volume Regulation and Sensitivity to Osmotic Stress in Synechocystis Sp. Strain PCC 6803. *J. Bacteriol.* **2012**, *194*, 6828–6836. [CrossRef]
11. Kida, H.; Miyoshi, T.; Manabe, K.; Takahashi, N.; Konno, T.; Ueda, S.; Chiba, T.; Shimizu, T.; Okada, Y.; Morishima, S. Roles of Aquaporin-3 Water Channels in Volume-Regulatory Water Flow in a Human Epithelial Cell Line. *J. Membr. Biol.* **2005**, *208*, 55–64. [CrossRef] [PubMed]
12. Chen, Q.; Duan, E. Aquaporins in Sperm Osmoadaptation: An Emerging Role for Volume Regulation. *Acta Pharmacol. Sin.* **2011**, *32*, 721–724. [CrossRef]
13. Di Giusto, G.; Pizzoni, A.; Rivarola, V.; Beltramone, N.; White, A.; Ford, P.; Capurro, C. Aquaporin-2 and Na^+/H^+ Exchanger Isoform 1 Modulate the Efficiency of Renal Cell Migration. *J. Cell. Physiol.* **2020**, *235*, 4443–4454. [CrossRef]
14. Papadopoulos, M.C.; Saadoun, S.; Verkman, A.S. Aquaporins and Cell Migration. *Pflug. Arch—Eur. J. Physiol.* **2008**, *456*, 693–700. [CrossRef] [PubMed]
15. Kwon, T.-H.; Nielsen, J.; Møller, H.B.; Fenton, R.A.; Nielsen, S.; Frøkiaer, J. *Aquaporins in the Kidney. Handbook of Experimental Pharmacology*; Springer: Berlin/Heidelberg, Germany, 2009; pp. 95–132. [CrossRef]
16. Takeda, T.; Taguchi, D. *Aquaporins as Potential Drug Targets for Meniere's Disease and Its Related Diseases. Handbook of Experimental Pharmacology*; Springer: Berlin/Heidelberg, Germany, 2009; pp. 171–184. [CrossRef]
17. Delporte, C. Aquaporins and Gland Secretion. In *Aquaporins*; Yang, B., Ed.; Springer: Dordrecht, The Netherlands, 2017; pp. 63–79. ISBN 978-94-024-1057-0.
18. Kitchen, P.; Day, R.E.; Salman, M.M.; Conner, M.T.; Bill, R.M.; Conner, A.C. Beyond Water Homeostasis: Diverse Functional Roles of Mammalian Aquaporins. *Biochim. Et Biophys. Acta (BBA)—Gen. Subj.* **2015**, *1850*, 2410–2421. [CrossRef] [PubMed]
19. Hibuse, T.; Maeda, N.; Nagasawa, A.; Funahashi, T. Aquaporins and Glycerol Metabolism. *Biochim. Et Biophys. Acta (BBA)—Biomembr.* **2006**, *1758*, 1004–1011. [CrossRef] [PubMed]
20. Verkman, A.S. More than Just Water Channels: Unexpected Cellular Roles of Aquaporins. *J. Cell Sci.* **2005**, *118*, 3225–3232. [CrossRef] [PubMed]
21. Fox, A.R.; Maistriaux, L.C.; Chaumont, F. Toward Understanding of the High Number of Plant Aquaporin Isoforms and Multiple Regulation Mechanisms. *Plant Sci.* **2017**, *264*, 179–187. [CrossRef] [PubMed]
22. Badaut, J.; Fukuda, A.M.; Jullienne, A.; Petry, K.G. Aquaporin and Brain Diseases. *Biochim. Et Biophys. Acta (BBA)—Gen. Subj.* **2014**, *1840*, 1554–1565. [CrossRef] [PubMed]
23. Verkerk, A.O.; Lodder, E.M.; Wilders, R. Aquaporin Channels in the Heart—Physiology and Pathophysiology. *Int. J. Mol. Sci.* **2019**, *20*, 2039. [CrossRef] [PubMed]

24. Ribatti, D.; Ranieri, G.; Annese, T.; Nico, B. Aquaporins in Cancer. *Biochim. Et Biophys. Acta (BBA)—Gen. Subj.* **2014**, *1840*, 1550–1553. [CrossRef] [PubMed]
25. Verkman, A.S.; Hara-Chikuma, M.; Papadopoulos, M.C. Aquaporins—New Players in Cancer Biology. *J. Mol. Med.* **2008**, *86*, 523–529. [CrossRef] [PubMed]
26. Bhattacharjee, A.; Jana, A.; Bhattacharjee, S.; Mitra, S.; De, S.; Alghamdi, B.S.; Alam, M.Z.; Mahmoud, A.B.; Al Shareef, Z.; Abdel-Rahman, W.M.; et al. The Role of Aquaporins in Tumorigenesis: Implications for Therapeutic Development. *Cell Commun. Signal.* **2024**, *22*, 106. [CrossRef] [PubMed]
27. Walz, T.; Fujiyoshi, Y.; Engel, A. *The AQP Structure and Functional Implications. Handbook of Experimental Pharmacology*; Springer: Berlin/Heidelberg, Germany, 2009; pp. 31–56. [CrossRef]
28. de Groot, B.L.; Grubmüller, H. The Dynamics and Energetics of Water Permeation and Proton Exclusion in Aquaporins. *Curr. Opin. Struct. Biol.* **2005**, *15*, 176–183. [CrossRef]
29. Beitz, E.; Wu, B.; Holm, L.M.; Schultz, J.E.; Zeuthen, T. Point Mutations in the Aromatic/Arginine Region in Aquaporin 1 Allow Passage of Urea, Glycerol, Ammonia, and Protons. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 269–274. [CrossRef]
30. Törnroth-Horsefield, S.; Hedfalk, K.; Fischer, G.; Lindkvist-Petersson, K.; Neutze, R. Structural Insights into Eukaryotic Aquaporin Regulation. *FEBS Lett.* **2010**, *584*, 2580–2588. [CrossRef]
31. Törnroth-Horsefield, S.; Wang, Y.; Hedfalk, K.; Johanson, U.; Karlsson, M.; Tajkhorshid, E.; Neutze, R.; Kjellbom, P. Structural Mechanism of Plant Aquaporin Gating. *Nature* **2006**, *439*, 688–694. [CrossRef] [PubMed]
32. Németh-Cahalan, K.L.; Kalman, K.; Hall, J.E. Molecular Basis of pH and Ca^{2+} Regulation of Aquaporin Water Permeability. *J. Gen. Physiol.* **2004**, *123*, 573–580. [CrossRef] [PubMed]
33. Abir-Awan, M.; Kitchen, P.; Salman, M.M.; Conner, M.T.; Conner, A.C.; Bill, R.M. Inhibitors of Mammalian Aquaporin Water Channels. *Int. J. Mol. Sci.* **2019**, *20*, 1589. [CrossRef]
34. Nguyen, A.T.P.; Weigle, A.T.; Shukla, D. Functional Regulation of Aquaporin Dynamics by Lipid Bilayer Composition. *Nat. Commun.* **2024**, *15*, 1848. [CrossRef]
35. Roche, J.V.; Törnroth-Horsefield, S. Aquaporin Protein-Protein Interactions. *Int. J. Mol. Sci.* **2017**, *18*, 2255. [CrossRef]
36. Hardingham, G.E.; Bading, H. Nuclear Calcium: A Key Regulator of Gene Expression. *Biometals* **1998**, *11*, 345–358. [CrossRef] [PubMed]
37. Ghosh, A.; Greenberg, M.E. Calcium Signaling in Neurons: Molecular Mechanisms and Cellular Consequences. *Science* **1995**, *268*, 239–247. [CrossRef] [PubMed]
38. Thomas, A.P.; Bird, G.S.; Hajnóczky, G.; Robb-Gaspers, L.D.; Putney, J.W. Spatial and Temporal Aspects of Cellular Calcium Signaling. *FASEB J.* **1996**, *10*, 1505–1517. [CrossRef] [PubMed]
39. Chin, D.; Means, A.R. Calmodulin: A Prototypical Calcium Sensor. *Trends Cell Biol.* **2000**, *10*, 322–328. [CrossRef] [PubMed]
40. Lewit-Bentley, A.; Réty, S. EF-Hand Calcium-Binding Proteins. *Curr. Opin. Struct. Biol.* **2000**, *10*, 637–643. [CrossRef] [PubMed]
41. Reichow, S.L.; Gonen, T. Noncanonical Binding of Calmodulin to Aquaporin-0: Implications for Channel Regulation. *Structure* **2008**, *16*, 1389–1398. [CrossRef] [PubMed]
42. Németh-Cahalan, K.L.; Hall, J.E. pH and Calcium Regulate the Water Permeability of Aquaporin 0 *. *J. Biol. Chem.* **2000**, *275*, 6777–6782. [CrossRef] [PubMed]
43. Girsch, S.J.; Peracchia, C. Lens Cell-to-Cell Channel Protein: I. Self-Assembly into Liposomes and Permeability Regulation by Calmodulin. *J. Membrain Biol.* **1985**, *83*, 217–225. [CrossRef] [PubMed]
44. Lindsey Rose, K.M.; Wang, Z.; Magrath, G.N.; Hazard, E.S.; Hildebrandt, J.D.; Schey, K.L. Aquaporin 0−Calmodulin Interaction and the Effect of Aquaporin 0 Phosphorylation. *Biochemistry* **2008**, *47*, 339–347. [CrossRef]
45. Reichow, S.L.; Clemens, D.M.; Freites, J.A.; Németh-Cahalan, K.L.; Heyden, M.; Tobias, D.J.; Hall, J.E.; Gonen, T. Allosteric Mechanism of Water-Channel Gating by Ca^{2+}–Calmodulin. *Nat. Struct. Mol. Biol.* **2013**, *20*, 1085–1092. [CrossRef] [PubMed]
46. Kreida, S.; Roche, J.V.; Missel, J.W.; Al-Jubair, T.; Hagströmer, C.J.; Wittenbecher, V.; Linse, S.; Gourdon, P.; Törnroth-Horsefield, S. The Role of Phosphorylation in Calmodulin-Mediated Gating of Human AQP0. *Biochem. J.* **2024**, *481*, 17–32. [CrossRef] [PubMed]
47. Kreida, S.; Virginia Roche, J.; Olsson, C.; Linse, S.; Törnroth-Horsefield, S. Protein–Protein Interactions in AQP Regulation—Biophysical Characterization of AQP0–CaM and AQP2–LIP5 Complex Formation. *Faraday Discuss.* **2018**, *209*, 35–54. [CrossRef] [PubMed]
48. Rabaud, N.E.; Song, L.; Wang, Y.; Agre, P.; Yasui, M.; Carbrey, J.M. Aquaporin 6 Binds Calmodulin in a Calcium-Dependent Manner. *Biochem. Biophys. Res. Commun.* **2009**, *383*, 54–57. [CrossRef] [PubMed]
49. Ishida, H.; Vogel, H.J.; Conner, A.C.; Kitchen, P.; Bill, R.M.; MacDonald, J.A. Simultaneous Binding of the N- and C-Terminal Cytoplasmic Domains of Aquaporin 4 to Calmodulin. *Biochim. Et Biophys. Acta (BBA)—Biomembr.* **2022**, *1864*, 183837. [CrossRef] [PubMed]
50. Bustamante, M.; Hasler, U.; Leroy, V.A.A.; de Seigneux, S.; Dimitrov, M.; Mordasini, D.; Rousselot, M.; Martin, P.-Y.; Fe[Combining Acute Accent]raille, E. Calcium-Sensing Receptor Attenuates AVP-Induced Aquaporin-2 Expression via a Calmodulin-Dependent Mechanism. *J. Am. Soc. Nephrol.* **2008**, *19*, 109. [CrossRef] [PubMed]
51. Chou, C.-L.; Yip, K.-P.; Michea, L.; Kador, K.; Ferraris, J.D.; Wade, J.B.; Knepper, M.A. Regulation of Aquaporin-2 Trafficking by Vasopressin in the Renal Collecting Duct: Roles of Ryanodine-Sensitive Ca^{2+} Stores and Calmodulin *. *J. Biol. Chem.* **2000**, *275*, 36839–36846. [CrossRef] [PubMed]

52. Balasubramanian, L.; Sham, J.S.K.; Yip, K.-P. Calcium Signaling in Vasopressin-Induced Aquaporin-2 Trafficking. *Pflug. Arch—Eur. J. Physiol.* **2008**, *456*, 747–754. [CrossRef] [PubMed]
53. Valenti, G.; Procino, G.; Tamma, G.; Carmosino, M.; Svelto, M. Minireview: Aquaporin 2 Trafficking. *Endocrinology* **2005**, *146*, 5063–5070. [CrossRef] [PubMed]
54. Freites, J.A.; Németh-Cahalan, K.L.; Hall, J.E.; Tobias, D.J. Cooperativity and Allostery in Aquaporin 0 Regulation by Ca^{2+}. *Biochim. Et Biophys. Acta (BBA)—Biomembr.* **2019**, *1861*, 988–996. [CrossRef] [PubMed]
55. Fields, J.B.; Németh-Cahalan, K.L.; Freites, J.A.; Vorontsova, I.; Hall, J.E.; Tobias, D.J. Calmodulin Gates Aquaporin 0 Permeability through a Positively Charged Cytoplasmic Loop *. *J. Biol. Chem.* **2017**, *292*, 185–195. [CrossRef] [PubMed]
56. Kreida, S.; Törnroth-Horsefield, S. Structural Insights into Aquaporin Selectivity and Regulation. *Curr. Opin. Struct. Biol.* **2015**, *33*, 126–134. [CrossRef] [PubMed]
57. Khandelia, H.; Jensen, M.Ø.; Mouritsen, O.G. To Gate or Not to Gate: Using Molecular Dynamics Simulations to Morph Gated Plant Aquaporins into Constitutively Open Conformations. *J. Phys. Chem. B* **2009**, *113*, 5239–5244. [CrossRef] [PubMed]
58. Nyblom, M.; Frick, A.; Wang, Y.; Ekvall, M.; Hallgren, K.; Hedfalk, K.; Neutze, R.; Tajkhorshid, E.; Törnroth-Horsefield, S. Structural and Functional Analysis of SoPIP2;1 Mutants Adds Insight into Plant Aquaporin Gating. *J. Mol. Biol.* **2009**, *387*, 653–668. [CrossRef] [PubMed]
59. Mom, R.; Réty, S.; Mocquet, V.; Auguin, D. Plant Aquaporin Gating Is Reversed by Phosphorylation on Intracellular Loop D—Evidence from Molecular Dynamics Simulations. *Int. J. Mol. Sci.* **2023**, *24*, 13798. [CrossRef] [PubMed]
60. Frick, A.; Järvå, M.; Ekvall, M.; Uzdavinys, P.; Nyblom, M.; Törnroth-Horsefield, S. Mercury Increases Water Permeability of a Plant Aquaporin through a Non-Cysteine-Related Mechanism. *Biochem. J.* **2013**, *454*, 491–499. [CrossRef] [PubMed]
61. Johansson, I.; Larsson, C.; Ek, B.; Kjellbom, P. The Major Integral Proteins of Spinach Leaf Plasma Membranes Are Putative Aquaporins and Are Phosphorylated in Response to Ca^{2+} and Apoplastic Water Potential. *Plant Cell* **1996**, *8*, 1181–1191. [CrossRef] [PubMed]
62. Verdoucq, L.; Grondin, A.; Maurel, C. Structure–Function Analysis of Plant Aquaporin AtPIP2;1 Gating by Divalent Cations and Protons. *Biochem. J.* **2008**, *415*, 409–416. [CrossRef] [PubMed]
63. Frick, A.; Eriksson, U.K.; Mattia, F.d.; Öberg, F.; Hedfalk, K.; Neutze, R.; de Grip, W.J.; Deen, P.M.T.; Törnroth-Horsefield, S. X-Ray Structure of Human Aquaporin 2 and Its Implications for Nephrogenic Diabetes Insipidus and Trafficking. *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 6305–6310. [CrossRef] [PubMed]
64. van Balkom, B.W.M.; Savelkoul, P.J.M.; Markovich, D.; Hofman, E.; Nielsen, S.; van der Sluijs, P.; Deen, P.M.T. The Role of Putative Phosphorylation Sites in the Targeting and Shuttling of the Aquaporin-2 Water Channel*. *J. Biol. Chem.* **2002**, *277*, 41473–41479. [CrossRef] [PubMed]
65. Moeller, H.B.; Rittig, S.; Fenton, R.A. Nephrogenic Diabetes Insipidus: Essential Insights into the Molecular Background and Potential Therapies for Treatment. *Endocr. Rev.* **2013**, *34*, 278–301. [CrossRef] [PubMed]
66. Seo, J.W.; Park, T.J. Magnesium Metabolism. *Electrolytes Blood Press. E BP* **2008**, *6*, 86–95. [CrossRef]
67. Okahira, M.; Kubota, M.; Iguchi, K.; Usui, S.; Hirano, K. Regulation of Aquaporin 3 Expression by Magnesium Ion. *Eur. J. Pharmacol.* **2008**, *588*, 26–32. [CrossRef] [PubMed]
68. Ikarashi, N.; Mochiduki, T.; Takasaki, A.; Ushiki, T.; Baba, K.; Ishii, M.; Kudo, T.; Ito, K.; Toda, T.; Ochiai, W.; et al. A Mechanism by Which the Osmotic Laxative Magnesium Sulphate Increases the Intestinal Aquaporin 3 Expression in HT-29 Cells. *Life Sci.* **2011**, *88*, 194–200. [CrossRef] [PubMed]
69. Ikarashi, N.; Kawai, H.; Shinozaki, Y.; Tabata, K.; Yoshida, R.; Nishinaka, Y.; Kon, R.; Sakai, H.; Hosoe, T. Magnesium Compounds Increase Aquaporin-3 in Human Epidermal Keratinocyte HaCaT Cells. *Dermatol. Ther.* **2023**, *2023*, 8896599. [CrossRef]
70. Ghabriel, M.N.; Thomas, A.; Vink, R. Magnesium Restores Altered Aquaporin-4 Immunoreactivity Following Traumatic Brain Injury to a Pre-Injury State. In Proceedings of the Brain Edema XIII, Ann Arbor, MI, USA, 1–3 June 2005; Hoff, J.T., Keep, R.F., Xi, G., Hua, Y., Eds.; Springer: Vienna, Austria, 2006; pp. 402–406.
71. Li, X.; Liu, H.; Yang, Y. Magnesium Sulfate Attenuates Brain Edema by Lowering AQP4 Expression and Inhibits Glia-Mediated Neuroinflammation in a Rodent Model of Eclampsia. *Behav. Brain Res.* **2019**, *364*, 403–412. [CrossRef] [PubMed]
72. Zhu, L.; Yin, H.; Sun, H.; Qian, T.; Zhu, J.; Qi, G.; Wang, Y.; Qi, B. The Clinical Value of Aquaporin-4 in Children with Hand, Foot, and Mouth Disease and the Effect of Magnesium Sulfate on Its Expression: A Prospective Randomized Clinical Trial. *Eur. J. Clin. Microbiol. Infect. Dis.* **2019**, *38*, 1343–1349. [CrossRef]
73. Euser, A.G.; Bullinger, L.; Cipolla, M.J. Magnesium Sulphate Treatment Decreases Blood–Brain Barrier Permeability during Acute Hypertension in Pregnant Rats. *Exp. Physiol.* **2008**, *93*, 254–261. [CrossRef] [PubMed]
74. Xu, M.; Xiao, M.; Li, S.; Yang, B. Aquaporins in Nervous System. In *Aquaporins*; Yang, B., Ed.; Springer: Dordrecht, The Netherlands, 2017; pp. 81–103. ISBN 978-94-024-1057-0.
75. Carbrey, J.M.; Agre, P. *Discovery of the Aquaporins and Development of the Field. Handbook of Experimental Pharmacology*; Springer: Berlin/Heidelberg, Germany, 2009; pp. 3–28. [CrossRef]
76. Macey, R.I. Transport of Water and Urea in Red Blood Cells. *Am. J. Physiol. -Cell Physiol.* **1984**, *246*, C195–C203. [CrossRef]
77. Zeidel, M.L.; Ambudkar, S.V.; Smith, B.L.; Agre, P. Reconstitution of Functional Water Channels in Liposomes Containing Purified Red Cell CHIP28 Protein. *Biochemistry* **1992**, *31*, 7436–7440. [CrossRef] [PubMed]
78. Fushimi, K.; Uchida, S.; Harat, Y.; Hirata, Y.; Marumo, F.; Sasaki, S. Cloning and Expression of Apical Membrane Water Channel of Rat Kidney Collecting Tubule. *Nature* **1993**, *361*, 549–552. [CrossRef] [PubMed]

79. Javot, H.; Maurel, C. The Role of Aquaporins in Root Water Uptake. *Ann. Bot.* **2002**, *90*, 301–313. [CrossRef] [PubMed]
80. Preston, G.M.; Jung, J.S.; Guggino, W.B.; Agre, P. The Mercury-Sensitive Residue at Cysteine 189 in the CHIP28 Water Channel. *J. Biol. Chem.* **1993**, *268*, 17–20. [CrossRef] [PubMed]
81. Kuang, K.; Haller, J.F.; Shi, G.; Kang, F.; Cheung, M.; Iserovich, P.; Fischbarg, J. Mercurial Sensitivity of Aquaporin 1 Endofacial Loop B Residues. *Protein Sci.* **2001**, *10*, 1627–1634. [CrossRef] [PubMed]
82. Savage, D.F.; Stroud, R.M. Structural Basis of Aquaporin Inhibition by Mercury. *J. Mol. Biol.* **2007**, *368*, 607–617. [CrossRef] [PubMed]
83. Yukutake, Y.; Tsuji, S.; Hirano, Y.; Adachi, T.; Takahashi, T.; Fujihara, K.; Agre, P.; Yasui, M.; Suematsu, M. Mercury Chloride Decreases the Water Permeability of Aquaporin-4-Reconstituted Proteoliposomes. *Biol. Cell* **2008**, *100*, 355–363. [CrossRef] [PubMed]
84. Kuwahara, M.; Gu, Y.; Ishibashi, K.; Marumo, F.; Sasaki, S. Mercury-Sensitive Residues and Pore Site in AQP3 Water Channel. *Biochemistry* **1997**, *36*, 13973–13978. [CrossRef] [PubMed]
85. Yasui, M.; Hazama, A.; Kwon, T.-H.; Nielsen, S.; Guggino, W.B.; Agre, P. Rapid Gating and Anion Permeability of an Intracellular Aquaporin. *Nature* **1999**, *402*, 184–187. [CrossRef] [PubMed]
86. Spinello, A.; de Almeida, A.; Casini, A.; Barone, G. The Inhibition of Glycerol Permeation through Aquaglyceroporin-3 Induced by Mercury(II): A Molecular Dynamics Study. *J. Inorg. Biochem.* **2016**, *160*, 78–84. [CrossRef] [PubMed]
87. Bai, L.; Fushimi, K.; Sasaki, S.; Marumo, F. Structure of Aquaporin-2 Vasopressin Water Channel (∗). *J. Biol. Chem.* **1996**, *271*, 5171–5176. [CrossRef]
88. Hirano, Y.; Okimoto, N.; Kadohira, I.; Suematsu, M.; Yasuoka, K.; Yasui, M. Molecular Mechanisms of How Mercury Inhibits Water Permeation through Aquaporin-1: Understanding by Molecular Dynamics Simulation. *Biophys. J.* **2010**, *98*, 1512–1519. [CrossRef] [PubMed]
89. Zhang, Y.; Cui, Y.; Chen, L.Y. Mercury Inhibits the L170C Mutant of Aquaporin Z by Making Waters Clog the Water Channel. *Biophys. Chem.* **2012**, *160*, 69–74. [CrossRef] [PubMed]
90. Hazama, A.; Kozono, D.; Guggino, W.B.; Agre, P.; Yasui, M. Ion Permeation of AQP6 Water Channel Protein. *J. Biol. Chem.* **2002**, *277*, 29224–29230. [CrossRef] [PubMed]
91. Ma, S.; Xie, H.; Yu, K.; Yang, J. Mechanism of Unusual AQP6 Activation by Mercury Binding to a Pore-External Residue C155. *Biochem. Biophys. Res. Commun.* **2022**, *618*, 1–7. [CrossRef]
92. Xie, H.; Ma, S.; Zhao, Y.; Zhou, H.; Tong, Q.; Chen, Y.; Zhang, Z.; Yu, K.; Lin, Q.; Kai, L.; et al. Molecular Mechanisms of Mercury-Sensitive Aquaporins. *J. Am. Chem. Soc.* **2022**, *144*, 22229–22241. [CrossRef] [PubMed]
93. Daniels, M.J.; Chaumont, F.; Mirkov, T.E.; Chrispeels, M.J. Characterization of a New Vacuolar Membrane Aquaporin Sensitive to Mercury at a Unique Site. *Plant Cell* **1996**, *8*, 587–599. [CrossRef] [PubMed]
94. Secchi, F.; Maciver, B.; Zeidel, M.L.; Zwieniecki, M.A. Functional Analysis of Putative Genes Encoding the PIP2 Water Channel Subfamily in Populus Trichocarpa. *Tree Physiol.* **2009**, *29*, 1467–1477. [CrossRef]
95. Bottino, C.; Vázquez, M.; Devesa, V.; Laforenza, U. Impaired Aquaporins Expression in the Gastrointestinal Tract of Rat after Mercury Exposure. *J. Appl. Toxicol.* **2016**, *36*, 113–120. [CrossRef] [PubMed]
96. Livingstone, C. Zinc. *Nutr. Clin. Pract.* **2015**, *30*, 371–382. [CrossRef] [PubMed]
97. Cassandri, M.; Smirnov, A.; Novelli, F.; Pitolli, C.; Agostini, M.; Malewicz, M.; Melino, G.; Raschellà, G. Zinc-Finger Proteins in Health and Disease. *Cell Death Discov.* **2017**, *3*, 17071. [CrossRef] [PubMed]
98. McCall, K.A.; Huang, C.; Fierke, C.A. Function and Mechanism of Zinc Metalloenzymes. *J. Nutr.* **2000**, *130*, 1437S–1446S. [CrossRef] [PubMed]
99. O'Dell, B.L. Role of Zinc in Plasma Membrane Function. *J. Nutr.* **2000**, *130*, 1432S–1436S. [CrossRef] [PubMed]
100. Klug, A.; Schwabe, J.W.R. Zinc Fingers. *FASEB J.* **1995**, *9*, 597–604. [CrossRef] [PubMed]
101. Németh-Cahalan, K.L.; Kalman, K.; Froger, A.; Hall, J.E. Zinc Modulation of Water Permeability Reveals That Aquaporin 0 Functions as a Cooperative Tetramer. *J. Gen. Physiol.* **2007**, *130*, 457–464. [CrossRef] [PubMed]
102. Mom, R.; Réty, S.; Mocquet, V.; Auguin, D. Deciphering Molecular Mechanisms Involved in the Modulation of Human Aquaporins' Water Permeability by Zinc Cations: A Molecular Dynamics Approach. *Int. J. Mol. Sci.* **2024**, *25*, 2267. [CrossRef] [PubMed]
103. Ma, T.; Song, Y.; Gillespie, A.; Carlson, E.J.; Epstein, C.J.; Verkman, A.S. Defective Secretion of Saliva in Transgenic Mice Lacking Aquaporin-5 Water Channels *. *J. Biol. Chem.* **1999**, *274*, 20071–20074. [CrossRef] [PubMed]
104. Kim, Y.-J.; Jo, Y.; Lee, Y.-H.; Park, K.; Park, H.-K.; Choi, S.-Y. Zn^{2+} Stimulates Salivary Secretions via Metabotropic Zinc Receptor ZnR/GPR39 in Human Salivary Gland Cells. *Sci. Rep.* **2019**, *9*, 17648. [CrossRef]
105. Yukutake, Y.; Hirano, Y.; Suematsu, M.; Yasui, M. Rapid and Reversible Inhibition of Aquaporin-4 by Zinc. *Biochemistry* **2009**, *48*, 12059–12061. [CrossRef] [PubMed]
106. Devi, M.J.; Sinclair, T.R.; Taliercio, E. Silver and Zinc Inhibitors Influence Transpiration Rate and Aquaporin Transcript Abundance in Intact Soybean Plants. *Environ. Exp. Bot.* **2016**, *122*, 168–175. [CrossRef]
107. Gitto, A.; Fricke, W. Zinc Treatment of Hydroponically Grown Barley Plants Causes a Reduction in Root and Cell Hydraulic Conductivity and Isoform-Dependent Decrease in Aquaporin Gene Expression. *Physiol. Plant.* **2018**, *164*, 176–190. [CrossRef] [PubMed]
108. Fatemi, H.; Zaghdoud, C.; Nortes, P.A.; Carvajal, M.; Martínez-Ballesta, M.d.C. Differential Aquaporin Response to Distinct Effects of Two Zn Concentrations after Foliar Application in Pak Choi (*Brassica rapa* L.) Plants. *Agronomy* **2020**, *10*, 450. [CrossRef]

109. Almeida, A.d.; Soveral, G.; Casini, A. Gold Compounds as Aquaporin Inhibitors: New Opportunities for Therapy and Imaging. *Med. Chem. Commun.* **2014**, *5*, 1444–1453. [CrossRef]
110. Verkman, A.S.; Anderson, M.O.; Papadopoulos, M.C. Aquaporins: Important but Elusive Drug Targets. *Nat. Rev. Drug Discov.* **2014**, *13*, 259–277. [CrossRef] [PubMed]
111. Graziani, V.; Marrone, A.; Re, N.; Coletti, C.; Platts, J.A.; Casini, A. A Multi-Level Theoretical Study to Disclose the Binding Mechanisms of Gold(III)–Bipyridyl Compounds as Selective Aquaglyceroporin Inhibitors. *Chem. —A Eur. J.* **2017**, *23*, 13802–13813. [CrossRef] [PubMed]
112. Serna, A.; Galán-Cobo, A.; Rodrigues, C.; Sánchez-Gomar, I.; Toledo-Aral, J.J.; Moura, T.F.; Casini, A.; Soveral, G.; Echevarría, M. Functional Inhibition of Aquaporin-3 With a Gold-Based Compound Induces Blockage of Cell Proliferation. *J. Cell. Physiol.* **2014**, *229*, 1787–1801. [CrossRef] [PubMed]
113. Almeida, A.d.; Mósca, A.F.; Wragg, D.; Wenzel, M.; Kavanagh, P.; Barone, G.; Leoni, S.; Soveral, G.; Casini, A. The Mechanism of Aquaporin Inhibition by Gold Compounds Elucidated by Biophysical and Computational Methods. *Chem. Commun.* **2017**, *53*, 3830–3833. [CrossRef] [PubMed]
114. Wenzel, M.N.; Mósca, A.F.; Graziani, V.; Aikman, B.; Thomas, S.R.; de Almeida, A.; Platts, J.A.; Re, N.; Coletti, C.; Marrone, A.; et al. Insights into the Mechanisms of Aquaporin-3 Inhibition by Gold(III) Complexes: The Importance of Non-Coordinative Adduct Formation. *Inorg. Chem.* **2019**, *58*, 2140–2148. [CrossRef] [PubMed]
115. Martins, A.P.; Marrone, A.; Ciancetta, A.; Cobo, A.G.; Echevarría, M.; Moura, T.F.; Re, N.; Casini, A.; Soveral, G. Targeting Aquaporin Function: Potent Inhibition of Aquaglyceroporin-3 by a Gold-Based Compound. *PLoS ONE* **2012**, *7*, e37435. [CrossRef] [PubMed]
116. Martins, A.P.; Ciancetta, A.; de Almeida, A.; Marrone, A.; Re, N.; Soveral, G.; Casini, A. Aquaporin Inhibition by Gold(III) Compounds: New Insights. *ChemMedChem* **2013**, *8*, 1086–1092. [CrossRef] [PubMed]
117. Madeira, A.; de Almeida, A.; de Graaf, C.; Camps, M.; Zorzano, A.; Moura, T.F.; Casini, A.; Soveral, G. A Gold Coordination Compound as a Chemical Probe to Unravel Aquaporin-7 Function. *ChemBioChem* **2014**, *15*, 1487–1494. [CrossRef] [PubMed]
118. Chen, C.-Y.; Liao, P.-L.; Tsai, C.-H.; Chan, Y.-J.; Cheng, Y.-W.; Hwang, L.-L.; Lin, K.-H.; Yen, T.-L.; Li, C.-H. Inhaled Gold Nanoparticles Cause Cerebral Edema and Upregulate Endothelial Aquaporin 1 Expression, Involving Caveolin 1 Dependent Repression of Extracellular Regulated Protein Kinase Activity. *Part. Fibre Toxicol.* **2019**, *16*, 37. [CrossRef] [PubMed]
119. Niemietz, C.M.; Tyerman, S.D. New Potent Inhibitors of Aquaporins: Silver and Gold Compounds Inhibit Aquaporins of Plant and Human Origin. *FEBS Lett.* **2002**, *531*, 443–447. [CrossRef] [PubMed]
120. Frare, R.; Ayub, N.; Alleva, K.; Soto, G. The Ammonium Channel NOD26 Is the Evolutionary Innovation That Drives the Emergence, Consolidation, and Dissemination of Nitrogen-Fixing Symbiosis in Angiosperms. *J. Mol. Evol.* **2018**, *86*, 554–565. [CrossRef] [PubMed]
121. Shim, H.; Harris, Z.L. Genetic Defects in Copper Metabolism. *J. Nutr.* **2003**, *133*, 1527S–1531S. [CrossRef] [PubMed]
122. Zelenina, M.; Tritto, S.; Bondar, A.A.; Zelenin, S.; Aperia, A. Copper Inhibits the Water and Glycerol Permeability of Aquaporin-3 *. *J. Biol. Chem.* **2004**, *279*, 51939–51943. [CrossRef] [PubMed]
123. Nave, M.; Castro, R.E.; Rodrigues, C.M.; Casini, A.; Soveral, G.; Gaspar, M.M. Nanoformulations of a Potent Copper-Based Aquaporin Inhibitor With Cytotoxic Effect Against Cancer Cells. *Nanomedicine* **2016**, *11*, 1817–1830. [CrossRef] [PubMed]
124. Zelenina, M.; Bondar, A.A.; Zelenin, S.; Aperia, A. Nickel and Extracellular Acidification Inhibit the Water Permeability of Human Aquaporin-3 in Lung Epithelial Cells *. *J. Biol. Chem.* **2003**, *278*, 30037–30043. [CrossRef] [PubMed]
125. Wani, A.L.; Ara, A.; Usmani, J.A. Lead Toxicity: A Review. *Interdiscip. Toxicol.* **2015**, *8*, 55–64. [CrossRef] [PubMed]
126. Selvín-Testa, A.; Capani, F.; Loidl, C.F.; López, E.M.; Pecci-Saavedra, J. Prenatal and Postnatal Lead Exposure Induces 70 kDa Heat Shock Protein in Young Rat Brain Prior to Changes in Astrocyte Cytoskeleton. *Neurotoxicology* **1997**, *18*, 805–817.
127. Papadopoulos, M.C.; Verkman, A.S. Aquaporin Water Channels in the Nervous System. *Nat. Rev. Neurosci.* **2013**, *14*, 265–277. [CrossRef]
128. Gunnarson, E.; Axehult, G.; Baturina, G.; Zelenin, S.; Zelenina, M.; Aperia, A. Lead Induces Increased Water Permeability in Astrocytes Expressing Aquaporin 4. *Neuroscience* **2005**, *136*, 105–114. [CrossRef]
129. Bosma, E.F.; Rau, M.H.; van Gijtenbeek, L.A.; Siedler, S. Regulation and Distinct Physiological Roles of Manganese in Bacteria. *FEMS Microbiol. Rev.* **2021**, *45*, fuab028. [CrossRef]
130. Pankau, C.; Cooper, R.L. Molecular Physiology of Manganese in Insects. *Curr. Opin. Insect Sci.* **2022**, *51*, 100886. [CrossRef] [PubMed]
131. Wachtel, L.W.; Elvehjem, C.A.; Hart, E.B. Studies on the Physiology of Manganese in the Rat. *Am. J. Physiol. -Leg. Content* **1943**, *140*, 72–82. [CrossRef]
132. Campbell, L.C.; Nable, R.O. Physiological Functions of Manganese in Plants. In *Manganese in Soils and Plants: Proceedings of the International Symposium on 'Manganese in Soils and Plants' Held at the Waite Agricultural Research Institute, The University of Adelaide, Glen Osmond, Australia, 22–26 August 1988 as an Australian Bicentennial Event*; Graham, R.D., Hannam, R.J., Uren, N.C., Eds.; Springer: Dordrecht, The Netherlands, 1988; pp. 139–154. ISBN 978-94-009-2817-6.
133. Lei, L.; Huang, M.; Su, L.; Xie, D.; Mamuya, F.A.; Ham, O.; Tsuji, K.; Păunescu, T.G.; Yang, B.; Lu, H.A.J. Manganese Promotes Intracellular Accumulation of AQP2 via Modulating F-Actin Polymerization and Reduces Urinary Concentration in Mice. *Am. J. Physiol. -Ren. Physiol.* **2018**, *314*, F306–F316. [CrossRef] [PubMed]

134. Rama Rao, K.V.; Jayakumar, A.R.; Reddy, P.V.B.; Tong, X.; Curtis, K.M.; Norenberg, M.D. Aquaporin-4 in Manganese-Treated Cultured Astrocytes. *Glia* **2010**, *58*, 1490–1499. [CrossRef] [PubMed]
135. Anderson, G.J.; McLaren, G.D. (Eds.) *Iron Physiology and Pathophysiology in Humans*; Humana Press: Totowa, NJ, USA, 2012; ISBN 978-1-60327-484-5.
136. Belaidi, A.A.; Bush, A.I. Iron Neurochemistry in Alzheimer's Disease and Parkinson's Disease: Targets for Therapeutics. *J. Neurochem.* **2016**, *139*, 179–197. [CrossRef] [PubMed]
137. Ximenes-da-Silva, A. Metal Ion Toxins and Brain Aquaporin-4 Expression: An Overview. *Front. Neurosci.* **2016**, *10*, 233. [CrossRef] [PubMed]
138. Wang, B.; Cui, Z.; Zhong, Z.; Sun, Y.; Sun, Q.; Yang, G.; Bian, L. Curcumin Attenuates Brain Edema in Mice with Intracerebral Hemorrhage through Inhibition of AQP4 and AQP9 Expression. *Acta Pharmacol. Sin.* **2015**, *36*, 939–948. [CrossRef] [PubMed]
139. Zhong, Z.; Sun, Y.; Wang, B.; Sun, Q.; Yang, G.; Bian, L. Involvement of Mitogen-Activated Protein Kinase Pathways in Ferrous Iron-Induced Aquaporin-4 Expression in Cultured Astrocytes. *NeuroToxicology* **2019**, *73*, 142–149. [CrossRef] [PubMed]
140. Qing, W.G.; Dong, Y.Q.; Ping, T.Q.; Lai, L.G.; Fang, L.D.; Min, H.W.; Xia, L.; Heng, P.Y. Brain Edema after Intracerebral Hemorrhage in Rats: The Role of Iron Overload and Aquaporin 4: Laboratory Investigation. *J. Neurosurg.* **2009**, *110*, 462–468. [CrossRef] [PubMed]
141. Li, Y.; Nan, D.; Liu, R.; Li, J.; Zhang, Z.; Deng, J.; Zhang, Y.; Yan, Z.; Hou, C.; Yao, E.; et al. Aquaporin 4 Mediates the Effect of Iron Overload on Hydrocephalus After Intraventricular Hemorrhage. *Neurocrit Care* **2024**, *40*, 225–236. [CrossRef] [PubMed]
142. Freeman, M.P.; Freeman, S.A. Lithium: Clinical Considerations in Internal Medicine. *Am. J. Med.* **2006**, *119*, 478–481. [CrossRef] [PubMed]
143. Timmer, R.T.; Sands, J.M. Lithium Intoxication. *J. Am. Soc. Nephrol.* **1999**, *10*, 666. [CrossRef] [PubMed]
144. Kwon, T.-H.; Laursen, U.H.; Marples, D.; Maunsbach, A.B.; Knepper, M.A.; Frøkiær, J.; Nielsen, S. Altered Expression of Renal AQPs and Na^+transporters in Rats with Lithium-Induced NDI. *Am. J. Physiol. -Ren. Physiol.* **2000**, *279*, F552–F564. [CrossRef] [PubMed]
145. Marples, D.; Christensen, S.; Christensen, E.I.; Ottosen, P.D.; Nielsen, S. Lithium-Induced Downregulation of Aquaporin-2 Water Channel Expression in Rat Kidney Medulla. *J. Clin. Investig.* **1995**, *95*, 1838–1845. [CrossRef]
146. Walker, R.J.; Weggery, S.; Bedford, J.J.; Mcdonald, F.J.; Ellis, G.; Leader, J.P. Lithium-Induced Reduction in Urinary Concentrating Ability and Urinary Aquaporin 2 (AQP2) Excretion in Healthy Volunteers. *Kidney Int.* **2005**, *67*, 291–294. [CrossRef] [PubMed]
147. Kortenoeven, M.L.A.; Schweer, H.; Cox, R.; Wetzels, J.F.M.; Deen, P.M.T. Lithium Reduces Aquaporin-2 Transcription Independent of Prostaglandins. *Am. J. Physiol. -Cell Physiol.* **2012**, *302*, C131–C140. [CrossRef] [PubMed]
148. Nielsen, J.; Hoffert, J.D.; Knepper, M.A.; Agre, P.; Nielsen, S.; Fenton, R.A. Proteomic Analysis of Lithium-Induced Nephrogenic Diabetes Insipidus: Mechanisms for Aquaporin 2 down-Regulation and Cellular Proliferation. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 3634–3639. [CrossRef] [PubMed]
149. Kaiser, M.; Edemir, B. Lithium Chloride and GSK3 Inhibition Reduce Aquaporin-2 Expression in Primary Cultured Inner Medullary Collecting Duct Cells Due to Independent Mechanisms. *Cells* **2020**, *9*, 1060. [CrossRef] [PubMed]
150. Kortenoeven, M.L.A.; Li, Y.; Shaw, S.; Gaeggeler, H.-P.; Rossier, B.C.; Wetzels, J.F.M.; Deen, P.M.T. Amiloride Blocks Lithium Entry through the Sodium Channel Thereby Attenuating the Resultant Nephrogenic Diabetes Insipidus. *Kidney Int.* **2009**, *76*, 44–53. [CrossRef] [PubMed]
151. Roxas, B.; Farjah, M.; Danziger, R.S. Aquaporin-2 Transcript Is Differentially Regulated by Dietary Salt in Sprague–Dawley and Dahl SS/Jr Rats. *Biochem. Biophys. Res. Commun.* **2002**, *296*, 755–758. [CrossRef] [PubMed]
152. Sutters, M.; Duncan, R.; Peart, W.S. Effect of Dietary Salt Restriction on Renal Sensitivity to Vasopressin in Man. *Clin. Sci.* **1995**, *89*, 37–43. [CrossRef] [PubMed]
153. Buemi, M.; Bolignano, D.; Coppolino, G.; Di Pasquale, G.; Cosentini, V.; Campo, S.; Barillà, A.; Aloisi, C. Aquaporin-2 (AQP2) Urinary Excretion and Assumption of Water with Different Mineral Content in Healthy Subjects. *Ren. Fail.* **2007**, *29*, 567–572. [CrossRef] [PubMed]
154. Song, J.; Hu, X.; Shi, M.; Knepper, M.A.; Ecelbarger, C.A. Effects of Dietary Fat, NaCl, and Fructose on Renal Sodium and Water Transporter Abundances and Systemic Blood Pressure. *Am. J. Physiol. Ren. Physiol.* **2004**, *287*, F1204–F1212. [CrossRef] [PubMed]
155. Graffe, C.C.; Bech, J.N.; Pedersen, E.B. Effect of High and Low Sodium Intake on Urinary Aquaporin-2 Excretion in Healthy Humans. *Am. J. Physiol. -Ren. Physiol.* **2012**, *302*, F264–F275. [CrossRef] [PubMed]
156. Storm, R.; Klussmann, E.; Geelhaar, A.; Rosenthal, W.; Maric, K. Osmolality and Solute Composition Are Strong Regulators of AQP2 Expression in Renal Principal Cells. *Am. J. Physiol. -Ren. Physiol.* **2003**, *284*, F189–F198. [CrossRef] [PubMed]
157. Mom, R.; Robert-Paganin, J.; Mom, T.; Chabbert, C.; Réty, S.; Auguin, D. A Perspective for Ménière's Disease: In Silico Investigations of Dexamethasone as a Direct Modulator of AQP2. *Biomolecules* **2022**, *12*, 511. [CrossRef] [PubMed]
158. Mom, R.; Réty, S.; Auguin, D. Cortisol Interaction with Aquaporin-2 Modulates Its Water Permeability: Perspectives for Non-Genomic Effects of Corticosteroids. *Int. J. Mol. Sci.* **2023**, *24*, 1499. [CrossRef] [PubMed]
159. Vaziriyeganeh, M.; Carvajal, M.; Du, N.; Zwiazek, J.J. Salinity Tolerance of Halophytic Grass Puccinellia Nuttalliana Is Associated with Enhancement of Aquaporin-Mediated Water Transport by Sodium. *Int. J. Mol. Sci.* **2022**, *23*, 5732. [CrossRef] [PubMed]
160. Wu, B.; Beitz, E. Aquaporins with Selectivity for Unconventional Permeants. *Cell. Mol. Life Sci.* **2007**, *64*, 2413–2421. [CrossRef] [PubMed]

161. Rambow, J.; Wu, B.; Rönfeldt, D.; Beitz, E. Aquaporins with Anion/Monocarboxylate Permeability: Mechanisms, Relevance for Pathogen–Host Interactions. *Front. Pharmacol.* **2014**, *5*, 199. [CrossRef] [PubMed]
162. Takano, J.; Yoshinari, A.; Luu, D.-T. Plant Aquaporin Trafficking. In *Plant Aquaporins: From Transport to Signaling*; Chaumont, F., Tyerman, S.D., Eds.; Signaling and Communication in Plants; Springer International Publishing: Cham, Switzerland, 2017; pp. 47–81. ISBN 978-3-319-49395-4.
163. Yaneff, A.; Sigaut, L.; Marquez, M.; Alleva, K.; Pietrasanta, L.I.; Amodeo, G. Heteromerization of PIP Aquaporins Affects Their Intrinsic Permeability. *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 231–236. [CrossRef] [PubMed]
164. Hedfalk, K.; Törnroth-Horsefield, S.; Nyblom, M.; Johanson, U.; Kjellbom, P.; Neutze, R. Aquaporin Gating. *Curr. Opin. Struct. Biol.* **2006**, *16*, 447–456. [CrossRef] [PubMed]
165. Li, S.-Z.; McDill, B.W.; Kovach, P.A.; Ding, L.; Go, W.Y.; Ho, S.N.; Chen, F. Calcineurin-NFATc Signaling Pathway Regulates AQP2 Expression in Response to Calcium Signals and Osmotic Stress. *Am. J. Physiol. -Cell Physiol.* **2007**, *292*, C1606–C1616. [CrossRef] [PubMed]
166. Armstrong, C.T.; Mason, P.E.; Anderson, J.L.R.; Dempsey, C.E. Arginine Side Chain Interactions and the Role of Arginine as a Gating Charge Carrier in Voltage Sensitive Ion Channels. *Sci. Rep.* **2016**, *6*, 21759. [CrossRef] [PubMed]
167. Ruta, V.; Chen, J.; MacKinnon, R. Calibrated Measurement of Gating-Charge Arginine Displacement in the KvAP Voltage-Dependent K^+ Channel. *Cell* **2005**, *123*, 463–475. [CrossRef] [PubMed]
168. Bernardi, M.; Marracino, P.; Ghaani, M.R.; Liberti, M.; Del Signore, F.; Burnham, C.J.; Gárate, J.-A.; Apollonio, F.; English, N.J. Human Aquaporin 4 Gating Dynamics under Axially Oriented Electric-Field Impulses: A Non-Equilibrium Molecular-Dynamics Study. *J. Chem. Phys.* **2018**, *149*, 245102. [CrossRef]
169. Burnham, C.J.; English, N.J. Electropumping of Water Through Human Aquaporin 4 by Circularly Polarized Electric Fields: Dramatic Enhancement and Control Revealed by Non-Equilibrium Molecular Dynamics. *J. Phys. Chem. Lett.* **2017**, *8*, 4646–4651. [CrossRef] [PubMed]
170. Marracino, P.; Liberti, M.; Trapani, E.; Burnham, C.J.; Avena, M.; Garate, J.-A.; Apollonio, F.; English, N.J. Human Aquaporin 4 Gating Dynamics under Perpendicularly-Oriented Electric-Field Impulses: A Molecular Dynamics Study. *Int. J. Mol. Sci.* **2016**, *17*, 1133. [CrossRef] [PubMed]
171. Hadidi, H.; Kamali, R. Non-Equilibrium Molecular Dynamics Study of Human Aquaporin-2 in the Static External Electric Fields. *J. Biomol. Struct. Dyn.* **2022**, *40*, 10793–10801. [CrossRef]
172. Reale, R.; English, N.J.; Garate, J.-A.; Marracino, P.; Liberti, M.; Apollonio, F. Human Aquaporin 4 Gating Dynamics under and after Nanosecond-Scale Static and Alternating Electric-Field Impulses: A Molecular Dynamics Study of Field Effects and Relaxation. *J. Chem. Phys.* **2013**, *139*, 205101. [CrossRef] [PubMed]
173. Garate, J.-A.; English, N.J.; MacElroy, J.M.D. Human Aquaporin 4 Gating Dynamics in Dc and Ac Electric Fields: A Molecular Dynamics Study. *J. Chem. Phys.* **2011**, *134*, 055110. [CrossRef] [PubMed]
174. English, N.J.; Garate, J.-A. Near-Microsecond Human Aquaporin 4 Gating Dynamics in Static and Alternating External Electric Fields: Non-Equilibrium Molecular Dynamics. *J. Chem. Phys.* **2016**, *145*, 085102. [CrossRef] [PubMed]
175. Hub, J.S.; Aponte-Santamaría, C.; Grubmüller, H.; de Groot, B.L. Voltage-Regulated Water Flux through Aquaporin Channels In Silico. *Biophys. J.* **2010**, *99*, L97–L99. [CrossRef]
176. Mom, R.; Muries, B.; Benoit, P.; Robert-Paganin, J.; Réty, S.; Venisse, J.-S.; Padua, A.; Label, P.; Auguin, D. Voltage-Gating of Aquaporins, a Putative Conserved Safety Mechanism during Ionic Stresses. *FEBS Lett.* **2021**, *595*, 41–57. [CrossRef] [PubMed]

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Review

Is Copper Still Safe for Us? What Do We Know and What Are the Latest Literature Statements?

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Abstract: Copper (Cu) is a precious metal and one of the three most abundant trace elements in the body (50–120 mg). It is involved in a large number of cellular mechanisms and pathways and is an essential cofactor in the function of cellular enzymes. Both its excess and deficiency may be harmful for many diseases. Even small changes in Cu concentration may be associated with significant toxicity. Consequently, it can be damaging to any organ or tissue in our body, beginning with harmful effects already at the molecular level and then affecting the degradation of individual tissues/organs and the slow development of many diseases, such as those of the immunological system, skeletal system, circulatory system, nervous system, digestive system, respiratory system, reproductive system, and skin. The main purpose of this article is to review the literature with regard to both the healthiness and toxicity of copper to the human body. A secondary objective is to show its widespread use and sources, including in food and common materials in contact with humans. Its biological half-life from diet is estimated to range from 13 to 33 days. The retention or bioavailability of copper from the diet is influenced by several factors, such as age, amount and form of copper in the diet, lifestyle, and genetic background. The upper limit of normal in serum in healthy adults is approximately 1.5 mg Cu/L, while the safe upper limit of average intake is set at 10–12 mg/day, the reference limit at 0.9 mg/day, and the minimum limit at 0.6–0.7 mg/day. Cu is essential, and in the optimal dose, it provides antioxidant defense, while its deficiency reduces the body's ability to cope with oxidative stress. The development of civilization and the constant, widespread use of Cu in all electrical devices will not be stopped, but the health of people directly related to its extraction, production, or distribution can be controlled, and the inhabitants of nearby towns can be protected. It is extremely difficult to assess the effects of copper on the human body because of its ubiquity and the increasing reports in the literature about its effects, including copper nanoparticles.

Keywords: copper; toxicity; use of copper; natural concentration of Cu; healthy/harmful aspects of copper

1. Introduction

Copper (Cu)—*Cuprum* is a chemical element with an atomic number of 29. It is currently estimated that the level of demand in production for this element will virtually double by 2030, in line with current demand. It is one of the three most useful and necessary metals in the world, after iron and aluminum [1–5]. This is likely to be influenced by the tremendous progress in both research work and the milestone development of technological innovations, which is the main focus of many researchers as well as production sources.

For many years, it has been used by practitioners of unconventional medicine (radiesthesists), for example, to make at least jewelry (bracelets, brooches, etc.) or talismans/amulets. Meanwhile, in traditional medicine, it plays a rather important role, including facilitating the incorporation of iron into hemoglobin. Both its excess and deficiency are harmful [6–9]. This article presents a summary of its action in a multifaceted health approach as well as its comprehensive use. It represents an innovative approach to the analysis of this element.

As it is unquestionably a very good and powerful antioxidant, it is necessary to emphasize its other side as well, for it can be toxic, and it is omnipresent in practically every device. Consequently, it can be damaging to any organ or tissue in our body, be-ginning with harmful effects already at the molecular level and then affecting the degradation of individual tissues/organs and the slow development of many diseases, such as those of the immunological system, skeletal system, circulatory system, nervous system, digestive system, respiratory system, reproductive system, and skin (Figure 1).

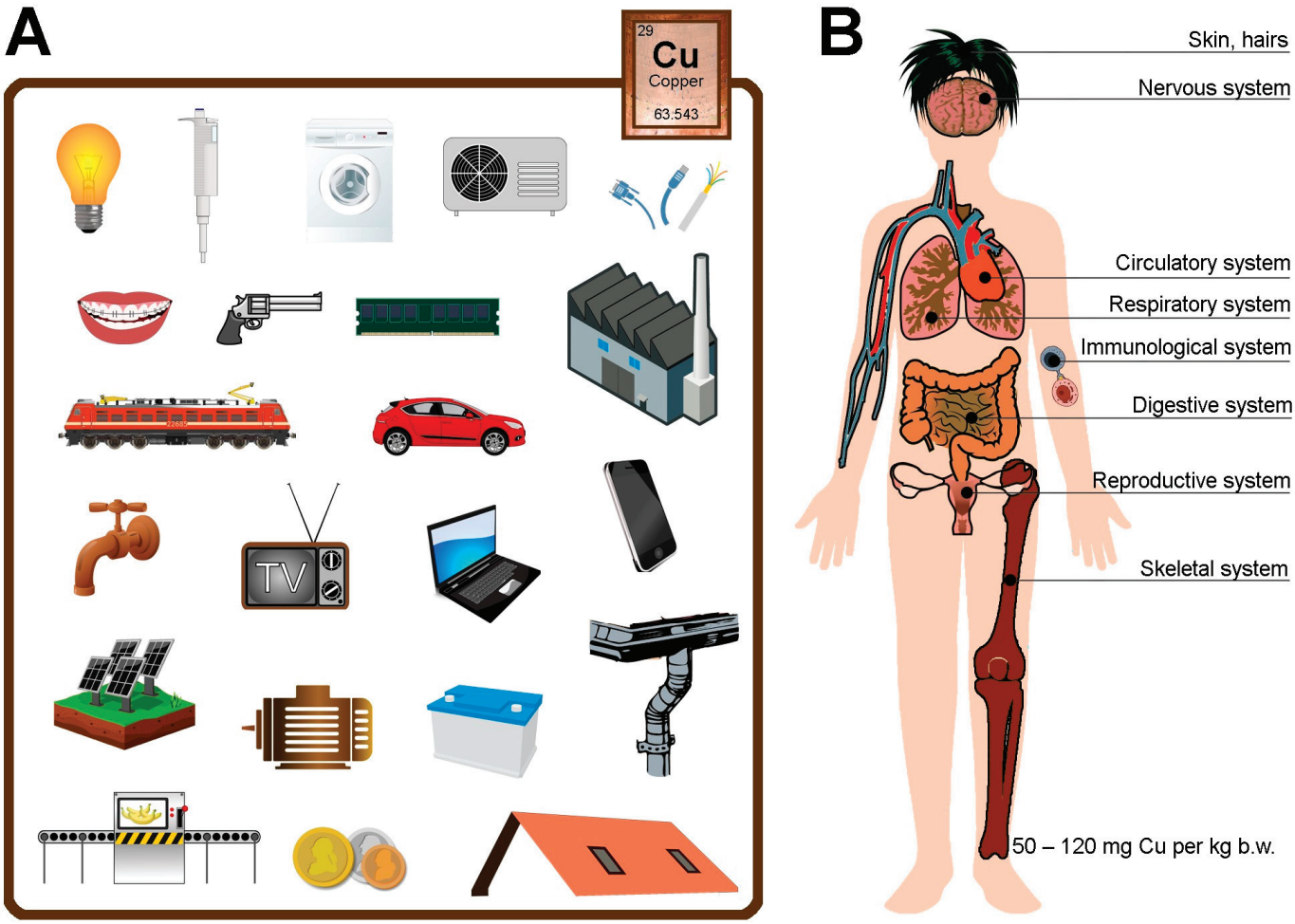


Figure 1. Sources of copper and its most significant effects on different parts of the human body.

Figure 1 illustrates some of the most common sources of Cu (A) and how exposure to Cu leads to the development of many diseases or pathologies in the body (B). It also shows the health effects associated with the long-term accumulation of Cu in organs/tissues and its reaction in the body.

The main purpose of this article is to review the literature with regard to both the healthiness and toxicity of copper to the human body. A secondary objective is to show its widespread use and sources, including in food and common materials in contact with humans.

2. Functions of Copper in the Body

This trace element is found in very small amounts in tissues or cells, while it is most abundant in bone and liver [6,10–13]. It is considered a major player involved in a large number of cellular mechanisms and signaling/cellular pathways, taking part in oxidation as well as reduction processes. It is involved in the neutralization of free radicals as well as altering the sensitivity of cells to their toxic effects [14–17]. It is also an

essential cofactor in the function of cellular enzymes such as cytochrome oxidase, catalase, dopamine-beta-hydroxylase, and peroxidase [5,11,18–20]. It supports the work of many energy-producing enzymes, oxygen metabolism—mitochondrial respiration (cytochrome oxidase (COX)), also building red blood cells, collagen, or connective tissue—collagen cross-linking (lysine oxidase (LOX)), skin pigmentation (tyrosinase), neurotransmitters in the brain (catecholamine biosynthesis (dopamine b-monooxygenase)), antioxidation (zinc–copper superoxide dismutase (SOD)), and, for example, facilitating the absorption or breakdown of iron [6,10,18,19]. It is an important catalyst for heme synthesis and iron absorption and is the third most abundant trace element in the body (after Zn and Fe) [5,13].

Its first deficiencies in humans were documented in the 1950s, where it was shown that a deficiency of this pierivirine causes hypocopperemia, hypoceruloplasminemia, and neutropenia in infants [7,19]. The extent to which it is absorbed depends on its chemical form and the copresentation of other dietary components, lifestyle, or disease entity. It is most readily absorbed with reduced glutathione (GSH) and organic acids (citric, gluconic, lactic, acetic), while its absorption is impaired by zinc, iron, calcium, molybdenum, phosphorus, and vitamin C. Most (30–50%) is absorbed in the small intestine (Cu^{2+}) due to transport with albumin or transcuprein, and least in the stomach and digestive juices [7,8,20,21]. The human body contains 50–120 mg of copper (0.79 to 1.9 mmol) [12,21–23]. The liver stores (18–45 μg Cu/g dry weight) Cu in hepatocytes, which it secretes into the plasma or with bile. Copper is removed via the biliary route (80% from the liver), with feces (80–90% from the diet; 2 mg/day), sweat (50–100 μg/day), urine (10–50 μg/day), and very little with hair. Approximately 60–90% of Cu circulating in the blood is in the form of ceruloplasmin (an antioxidant), transporting it to tissues with histidine, albumin, or transcuprein. Blood contains about 6 mg Cu, and its homeostatic regulation protects the body from both toxicity in the case of increased absorption or Cu deficiency in the case of increased excretion. The upper limit of normal in serum in healthy adults is about 1.5 mg Cu/L, and the safe upper limit of average intake is set at 10 mg/day for women and 12 mg/day for men. Cu poisoning most commonly results in weakness, lethargy, anorexia, erosion of the gastrointestinal epithelium, gastrointestinal disturbances, and hepanism [5,7,21–23]. As recent reports suggest that both copper excess and deficiency can be harmful, it is important to carefully monitor homeostasis, especially in neurological and cardiovascular diseases and liver status [6,15,20]. This element accumulates (Table 1) most often in the liver, kidneys, lungs, bones, muscles, brain, adipose tissue, and skin, inhibiting the activity of many enzymes (Figure 1) [6,9,11,13,14,16–19,22,24–33].

Table 1. Summary of the effect of copper on individual parts of the human body [6,9,11,13,14,16–19,22,24–33].

Human Body	Effects of Exposure to Copper
Immunological system	- occurs in numerous metalloenzymes - has a protective effect on DNA, proteins, or lipids - can catalyze the production of hydroxyl radicals - influences the development and function of the immune system (humoral and cellular and production of immunoreactive substances) - when deficient, can increase host susceptibility to various pathogens - its antibacterial toxicity is used by phagocytic cells to kill pathogens that accumulate at sites of infection
Skeletal system	- is involved in strengthening connective tissue structures, mainly bone - stimulates the differentiation of mesenchymal stem cells toward the osteogenic lineage
Circulatory system	- supports cardiac and vascular function (e.g., through the synthesis of red blood cells) - improves blood morphotic parameters - minimizes the risk of atherosclerotic lesions - severe intoxication with copper sulphate can cause, among other things, intravascular hemolysis - when it accumulates in the heart muscle, it can adversely affect its function

Table 1. *Cont.*

Human Body	Effects of Exposure to Copper
Nervous system	- supports normal brain development - helps eliminate harmful oxygen "free radicals" - has a protective effect on the reduction in beta amyloid levels in the cerebrospinal fluid - controls gene expression in the nucleus accumbens - is involved in myelination of the brain - stimulates brain function and modulates neural transmission - has a positive effect on cognitive functions - participates in the synthesis of ATP - low intake may be associated with mental retardation or impaired central nervous system function
Digestive system	- chronic toxicity affects the liver (cirrhosis with episodes of hemolysis), renal tubular damage, gastritis - partially absorbed in the stomach, duodenum, and ileum - ingestion of concentrations >30 ppm Cu causes gastrointestinal irritation (nausea, vomiting, abdominal pain), and $\geq$25 mg Cu/L is associated with acute gastroenteritis - renal impairment is mild (e.g., anuria or scanty uremia)
Respiratory system	- occurrence of fever caused by metal fumes (chills, muscle aches, headache, malaise, dry throat) - large vapors irritate the airways (mucous membranes of the mouth, eyes, or nose), respiratory tract inflammation, or cause perforation of the nasal septum
Reproductive system	- can potentially affect reduced fertility - in correlation with other elements (e.g., zinc, selenium) is important for maintaining reproductive health in both sexes - essential for the production of male gametes (role in cell division processes–mitotic and meiotic) - deficiencies as well as excesses cause reduced fertility in men
Skin	- component of tyrosinase, a metalloenzyme involved in the synthesis of melanin (skin and hair pigment) - prevents premature greying of hair, alleviates symptoms of vitiligo–protective effect against harmful UV radiation - participation in the synthesis of collagen and elastin - reduction in fine lines and wrinkles on the face - accelerates wound healing - improves the general condition of the skin - deficiencies result in loss of skin, epidermis, or change in hair color - used in face creams (e.g., anti-aging creams) - in excess, causes inflammation, redness, contact eczema, itching
Cancers	- used in cancer therapy by inhibiting, among other things, the activity of the p53 protein - inhibits the growth and proliferation of cancer cells themselves, their metastasis and angiogenesis - there is a correlation between high Cu levels and the metabolic demand of prostate, breast, colon, liver, colorectal, and brain tumors - copper ions are involved in the development, growth, and metastasis of tumors through various pathways - regulates PD-L1 expression by inducing an antitumor immune response

2.1. Immunological System

It is an element required for the proper functioning of the immune system and owes its antioxidant properties to its occurrence in numerous metalloenzymes. It also has a protective effect on DNA, proteins, or lipids [6,18,24]. It can catalyze the production of hydroxyl radicals in a Fenton-like reaction, unfortunately causing oxidative stress and cell damage, subsequently affecting protein aggregation processes or inflammation of the nervous system [6,17]. Cu shows an easy transition between oxidation levels +1 and +2 in biological systems, and the very mechanism of action of these complexes is based on their redox activity and the induction of reactive oxygen species (ROS), which can lead to lethal oxidative stress [34]. It is noteworthy that its deficiency significantly affects the development and function of the immune system, including humoral and cellular immunity and the production and secretion of immunoreactive substances [11,13,19,20,35,36]. Cu homeostasis is essential for healthy brain development and function, which is required

for certain enzymes responsible for various metabolic processes in various tissues, including the brain. It is a cofactor for antioxidant enzymes (e.g., superoxide dismutase and glutathione peroxidase) that provide protection against ROS damage [36]. Among the most common symptoms of this deficiency are increased host susceptibility to various pathogens, reduced numbers and impaired function of neutrophils, reduced proliferation of splenocytes, reduced antimicrobial activity of macrophages, impaired ability of B lymphocytes to produce antibodies, and impaired function of cytotoxic T lymphocytes and helper T lymphocytes [19]. Still, its actual mechanism in many immune processes is not fully defined and understood [35]. Its antimicrobial toxicity is directly exploited by phagocytic cells to kill pathogens (bacteria and fungi by macrophages) that accumulate at sites of infection [11,13]. Its main function is the constitution of enzymes that transfer electrons (oxidases) to reduce molecular oxygen. It is essential for energy metabolism at the cellular as well as molecular level [21]. Copper, including copper nanoparticles, can help in broad-spectrum antiviral therapies, including with bronchitis virus, polio, flu, herpes virus, human immunodeficiency virus (HIV-1), and even SARS-CoV-2 [37,38]. Copper nanoparticles can interact with the thin bacterial cell wall, DNA, nucleic acid, etc. They are also responsible for the production of ROS and oxidative stress, ultimately leading to cell death [39]. A new Cu-ion-induced cell death pathway recently discovered by Tsvetv et al. [40] has been named "cuproptosis". It is a form of metal-regulated cell death induced by this ion, which induces apoptosis-independent cell death through direct binding to TCA (lipoylated tricarboxylic acid cycle) proteins. Importantly, by then inducing the aggregation of lipoylated proteins and destabilization of Fe-S cluster proteins, it leads to proteotic stress [40].

There is no doubt that the effects of copper deficiency on the immune system in humans require further and more detailed studies.

2.2. Skeletal System

Copper is involved in strengthening connective tissue structures, including bones, as it is mainly found there. The level of copper in the blood is correlated with the maintenance of normal bone density and a reduction in the risk of fractures, which in the long term is particularly important in menopausal women [10,22,25]. A value of 5–6 mg/kg sm has been recognized as the physiological level of Cu in bone tissue (its levels vary from 0.16 to 6.30 mg/kg dry weight). Its concentration is highest in the ribs and lowest in the tibia and decreases with age [22]. There are a number of publications and studies confirming the efficacy of copper supplementation (approximately 3 mg per day), resulting in a reduction in the decrease of bone mineral density, characteristic especially during menopause. In elderly patients, regular supplementation may lead to an increase in bone resorption markers such as NTX C (collagen type I cross-linked C-telopeptide) or DPD (deoxypyridinoline) [22,41,42]. Meanwhile, in children, it may result in the reversal of adverse bone changes [43] and have a beneficial effect on joint health [26]. Nevertheless, there is a lack of studies confirming osteoblastic and osteoclastic activity, as well as bone resistance to copper [21,22]. In an analysis by Randanelli et al. [21] on the effect of copper supplementation (2.5–3 mg/day) and its effect on bone metabolism, it was shown that there is a slowing of mineral loss from bone and thus a reduction in resorption rates. Copper stimulates the differentiation of mesenchymal stem cells toward the osteogenic lineage [22,44]. Meanwhile, its severe deficiency due to either low dietary intake or a possible mutation present in the body is associated with weakened connective tissue [20]. Both the effective homeostatic regulation of absorption and retention help protect the body from toxicity as well as copper deficiency and are mainly inhibited by the presence of phytylones, calcium, ascorbic acid, and other trace elements. Interestingly, copper has been shown to have positive effects on cells that regulate bone metabolism and copper ions that can inhibit osteoclastic resorption [21,45]. It is worth noting that low concentrations of Cu improve the viability and growth of osteoblastic cells, while higher concentrations can be cytotoxic [21,46]. In addition, Cu has been shown to stimulate differentiation of

mesenchymal stem cells toward the osteogenic lineage [44]. Also, copper deficiency impairs the strength of the mechanical tissues of the bones, due to reduced elastin and collagen networks [45].

2.3. Circulatory System

Copper has a significant effect on the functioning of the cardiovascular system due to its high antioxidant potential. It supports the function of the heart and blood vessels through the synthesis of red blood cells, improving blood morphotic parameters. It also minimizes the risk of developing atherosclerotic lesions by reducing plasminogen activator inhibitor type 1 (PAI-1) levels by up to 30% with regular supplementation (6 mg/day) [22,27]. Its increased levels can accumulate in the myocardium, adversely affecting its function, both in terms of possible deficiency and toxicity [5,10,30]. In hieprlipidaemic patients also supplementing with copper, a reduction in total and LDL cholesterol and triglycerides has been observed, with a concomitant increase in HDL cholesterol [5,10,47]. Copper also indirectly affects the efficient synthesis of hemoglobin and erythrocytes, aiding in the prevention of iron deficiency anemia, the most common cause of anemia [48]. Its release in large quantities by the liver causes its rapid accumulation in erythrocytes and subsequent oxidant production affecting red blood cell damage [5]. To date, no direct effect on cardiovascular mortality has been confirmed in individuals with higher or lower blood levels, so further research in this direction is important [10]. In the meantime, serum copper levels have been linked to the risk of atherosclerosis in middle-aged men, although a significant potential link to venous thromboembolism (VTE) is still missing [30]. Meanwhile, severe copper sulphate intoxication can cause intravascular hemolysis (anemia, increased plasma hemoglobin concentration, reticulocytosis, hemoglobinuria, hyperbilirubinemia, wrinkled and fragmented erythrocytes, formation of Heinz bodies, among others) [5,12]. There is no evidence to support an association between copper concentrations and CVD risk or the effect of copper supplementation on CVD [12]. Copper deficiency in myocardial cells can lead to impaired myocardial energy consumption, reduced ability of the heart to contract and induce cardiomyopathy, and the accumulation of catalytically active Cu^{2+} in cardiac extracellular muscle induces the toxicity of this element, which is suggested to be an important catalyst for cardiovascular damage. It can also lead to increased mitochondrial density, disruption of the crest, and other unapplied myofibrils with disruption of Z-bands in cardiomyocytes. Interestingly, both copper deficiency and excessive copper accumulation can induce cardiac hypertrophy, leading to redox imbalance through its effects on mitochondrial respiration, protein metabolism, and lipid metabolism. Ultimately, it can induce hypertrophy or loss of these cells [49].

Individual copper nanoparticles are able to move between cells or penetrate cell membranes and eventually enter the circulatory system, spreading from the initial site of exposure to different parts of the body and eventually accumulating in organs [39,50].

Despite the available laboratory tests, more clinical studies need to be conducted, taking into account a range of information about the patient themself.

2.4. Nervous System

Copper supports normal brain development and immune function. It is a component of superoxide dismutase and an antioxidant enzyme that helps eliminate harmful oxygen "free radicals" [10,16]. Copper, through its protective effects, has the effect of reducing the levels of beta amyloid in the cerebrospinal fluid, an abnormal protein whose cerebral deposits are found in Alzheimer's disease. This contradicts previous studies suggesting just the opposite effect of this element on the course of Alzheimer's development. Meanwhile, long-term supplementation (8 mg/day) may have a positive effect on the progression of the disease, as well as, interestingly, improving motorial function but in the course of Parkinson's disease [7,28,51]. Unfortunately, there are still controversial observations regarding dietary copper deficiency versus the etiology and pathophysiology of Alzheimer's disease [12,52]. Some studies show that people with higher copper levels have a lower

risk of Alzheimer's disease [53], while others show that excess dietary copper is involved in the development of the disease [54]. Unfortunately, its accumulation already in damaged brain regions in this disease may not directly show overall copper status or copper intake [55]. Copper plays a role in the onset of Alzheimer's disease, as the hippocampus, cerebral cortex, cerebellum, and brainstem are also pathways for the overall progression of the disease [56–58]. As it is important in controlling gene expression in the nucleus accumbens, it is also involved in brain myelination. And by modulating synaptic activity, as well as excitotoxic cell death and signaling cascades induced by neurotrophic factors, it is important for a wide variety of neuronal functions [6,10]. It should be noted that copper stimulates brain function and modifies neural transmission and has a positive effect on cognitive function. Meanwhile, in school-aged children, its higher levels in the body are associated with, among other things, better memory, concentration, or cognitive techniques [6,20,59]. Copper is also actively involved in mitochondrial synthesis including bioenergetics, dynamics, and mitophagy and how this influences cell fate through metabolic reprogramming [29]. The very mechanism in which Cu is involved in the development of neurodegenerative diseases is not yet fully defined, and oxidative damage in the brain may result from interactions between Cu and homocysteine, or amino acids containing a thiol group. Increased Cu concentrations and/or homocysteine levels in the elderly may promote significant oxidative damage to neurons and may cause Alzheimer's disease or another neurodegenerative disease (Parkinson's or amyotrophic lateral sclerosis) [6,7]. Copper plays an important role in the physiological procs of the brain, although it has not yet been fully elucidated [6]. An inadequate intake of this element, or an existing mutation in the body, may be associated with mental retardation or even impairment of central nervous system (CNS) function [20]. It is worth to mention, first of all, that copper nanoparticles can contribute to the progression of neurodegenerative diseases, including Alzheimer's and Parkinson's diseases [37,60].

2.5. Digestive System

The occurrence of chronic Cu toxicity may mainly affect the liver, manifesting as liver cirrhosis with episodes of hemolysis and damage to the renal tubules, brain, and other organs. As a further consequence, this can lead to coma, hepatic necrosis, vascular collapse, or even death [8,20]. Copper from the diet can be partially absorbed in the stomach. Its strongly acidic environment then releases bound copper ions from partially digested food particles. The largest part of ingested copper passes into the duodenum and ileum, the main site of absorption. Much of it is soluble in the digestive tract due to complexation with amino acids, organic acids, or other chelators. Meanwhile, its ions enter the cells of the mucosa lining the intestine by simple diffusion, and then escape to the basolateral surface by another means of transport via copper-transporting adenosine triphosphatase (ATPase). Copper circulating through the body returns to the liver bound to cerulopasmin and is then secreted into the blood with it [20].

A range of up to approx. 1.5 mg/L serum copper concentrations are present in healthy people, and up to approx. 3 mg Cu/L in people with gastrointestinal symptoms. Meanwhile, ingestion of liquids containing concentrations >30 ppm Cu causing multiple gastrointestinal irritations (nausea, vomiting, abdominal pain), or drinks/foods containing $\geq$25 mg Cu/L are associated with acute gastroenteritis. Renal dysfunction is usually mild and may develop more like anuria or scanty urine [5]. In contrast, consumption of contaminated food or water may result in the development of acute gastrointestinal symptoms [5,7].

An excess of copper occurs very rarely and occurs mainly in the case of its continuous storage and as a result of feeding boiling liquids from corrosive copper or brass vessels, similar to lead or cadmium [10,61,62]. Either accidental or intentional copper overdose can cause acute liver damage. And chronic consumption of excessive amounts may result in copper overload in the body and chronic liver damage [12]. In the case of recommended oral or parenteral supplementation (e.g., in Wilson's disease), its close monitoring in the

body is important [13]. In Wilson's disease, where there is a pathogenic accumulation of hepatic copper that is a source of ROS [12,63,64], copper absorption from the diet is not usually controlled by food absorption but is regulated by copper excretion in the bile. Copper is present in many foods, and its limitation has long been considered an important aspect of treating Wilson's disease. Copper, when it is properly absorbed in the intestine by enterocytes, is transported with proteins via albumin or transcuprein to the sinusoidal plasma membrane in the liver. Copper atoms are then transported into hepatocytes by the copper transporter copper transporter 1 (CTR1) into the cytosol, and in turn, copper chaperones (e.g., superoxide dismutase (CCS), antioxidant protein 1) transport copper to specific intracellular targets. It is noteworthy that if intracellular copper levels are high, then ATP7B moves its cellular compartment to bile-duct-associated structures facilitating the process of the excretion of copper into the bile ducts [64,65].

It is worth emphasizing that copper deficiency in the serum or liver also occurs in many other liver diseases and is very rarely reported by these patients [13]. There is also no doubt that understanding the nature of copper deficiency in liver diseases requires further research and a more holistic understanding of the occurrence of this element in the environment.

2.6. Respiratory System

There are still few data on the absorption of copper into the lungs. One of the most common symptoms is the occurrence of fever caused by metal fumes (chills, muscle aches, headache, malaise, dry throat) when welding copper alone. Then, during the volatilization of metallic copper, the lungs only absorb a certain amount of copper, and it is low due to the high temperatures required for volatilization. It then irritates the respiratory tract, mainly the mucous membranes of the mouth, eyes, or nose. Also, its intensive and regular inhalation may cause perforation of the nasal septum [5,8]. Copper ions quickly enter the circulatory system after inhalation [23]. It should be noted that dysregulation of Cu homeostasis can lead to immune dysregulation, including oxidative stress, followed by chronic inflammation and angiogenic disorders, which may consequently contribute to respiratory diseases. As pointed out by Song et al., the pathological effects of Cu in respiratory diseases mainly include immunity, oxidative stress, chronic inflammation, and angiogenesis [65]. Larger amounts are inhaled in the vicinity of large smelters [8]. In a cohort study of ~0.8 million adults in Toronto, Canada it was shown that long-term exposure to copper in $PM_{2.5}$ (fine particulate matter) and estimated ROS (reactive oxygen species) concentrations in lung fluid were associated with an increased incidence of respiratory disease. This suggests adverse effects of emissions from outside the tailpipe (e.g., brake/rail wear and engine wear) on the respiratory system. An association between Cu and COPD mortality was shown in this long-term study, although this association or increased morbidity was not demonstrated for asthma [66], POChP, PAH and respiratory tract infections, lung cancer, and interstitial lung disease [65]. Meanwhile, Denmark showed no association between the element analyzed and cardiovascular mortality [33], while the Netherlands only showed a worsening of lung function [67]. According to Janssen et al. [23], there is also a potential link between tobacco inhalation and the potential depletion of copper ions in the lungs. The authors speculate that inhalation copper therapy may be used as one treatment option for patients with emphysema. Meanwhile, inhaled copper monotherapy may be associated with fibrosis formation through collagen accumulation [23]. Unfortunately, there are still a small number of studies conducted in humans, which does not mean that it should be ignored. In fact, there is a strong rationale for continuing a long-term study, due to increasing environmental pollution [66].

It is worth adding that the new discovery of cuproptosis will certainly contribute to a deeper understanding of Cu metabolic diseases and their molecular mechanisms, no doubt. Merely understanding the mechanisms of Cu metabolism and this discovery will help usher in the future screening of drugs to treat metabolic diseases in humans. Undoubtedly,

further experimental and clinical studies are needed to guide therapy and understand the effects of Cu on respiratory diseases [65].

2.7. Reproductive System

There is still insufficient work and research on how circulating Cu may be directly related to fertility in humans [16,68,69]. Interestingly, its serum concentration and placental tissue content in women with spontaneous abortion were also assessed. In that case, a significantly higher tissue content was observed in women with spontaneous miscarriage in relation to pregnant women, while a significantly lower serum concentration was observed in women with spontaneous miscarriage in relation to healthy pregnancies [70]. Toxic elements (e.g., cadmium, lead, mercury) can potentially affect reduced fertility [61,62,71–73]. Meanwhile, these trace elements (zinc, copper, selenium) are important for maintaining reproductive health in both sexes [16,31,70]. Copper causes infertility in both sexes through a variety of mechanisms, such as changes in sperm motility, reduced sperm quality, or the effect on the egg [73,74]. In a multicenter, prospective cohort study (Screening for Pregnancy Endpoints—SCOPE), unborn women with singleton pregnancies from Adelaide (Australia), Auckland (New Zealand), Cork (Ireland), Leeds, London, and Manchester (UK) were included (n = 5628). It was then shown that there was no association between serum copper concentration (30.3–30.5 μmol/L) and time to pregnancy [16]. Positive as well as negative associations were also shown between this micronutrient in follicular fluid in both women with and without endometriosis, a common cause of infertility [75]. It is worth noting that it plays a very important role in male fertility, as it is essential for the production of male gametes. It plays a very significant role in cell division processes—mitotic and meiotic. Large amounts of it are found in the sperm-associated fluids in the epididymis and prostate. Both its deficiency and excess causes reduced fertility in men. It is associated with a whole spectrum of abnormalities at the level of spermatozoa, male gonads, hormone production, and the distribution of, for example, zinc and iron [17,18,31,68,69,76]. High concentrations of Cu in seminal plasma are associated with sperm DNA damage, and since it is essential for healthy spermatogenesis, it can cause toxicity and detrimental effects on sperm quality even at higher doses [68]. It is worth noting that some proteins involved in copper homeostasis (e.g., import pump CTR1) are contained in germ cells and mature spermatozoa, which suggests the important role of this element in both the development and function of spermatozoa [77]. Further studies are very important to confirm the impact or potential impediments to pregnancy or even the process of spermatogenesis itself in copper deficiency/excess. Importantly, attention should be paid to its concentration and the possible occurrence of malformations in children.

2.8. Skin

Copper is a component of tyrosinase, a metalloenzyme involved in the synthesis of melanin (skin and hair pigment) [9]. Through its appropriate content, it can prevent premature graying of hair and alleviate the symptoms of vitiligo or show a protective effect against harmful UV radiation. In addition to its beneficial antibacterial action, its beneficial feature is its participation in the synthesis of collagen and elastin. Consequently, it reduces fine lines and wrinkles in the face and, more importantly, accelerates wound healing [9,32,78]. It also improves the overall self-perception of the skin [9,32]. Also, few data are available on the absorption of copper compounds through the skin, as only long-term exposure to copper sulphate or copper azide can be absorbed by the skin [9]. Severe copper deficiency can result in skin loss or change in hair color [21]. Most often, increased copper adsorption is observed in the hair shaft causing a green tinge, especially in damaged parts of the hair. Meanwhile, additional changes also include epidermal loss and, in the case of mechanical damage, can form copper rings. Average copper levels can be around 17.7 ppm in the first 3 cm of the proximal end of scalp hair and 11.9 ppm Cu in pubic hair [5]. Our ancestors already used copper varieties (copper sulphate, malachite etc.) as early as approx. 3000 BC for, among other things, the treatment of difficult-to-heal

wounds, surgical wounds, vaginal disorders, or other skin diseases. Currently, copper is also used in face creams. The risk of adverse effects as a result of skin contact (both damaged and undamaged) with copper is extremely low, and ointments containing up to 20% metallic copper also have no toxic effects [9]. Nanoparticles also accelerate hair follicle and sebaceous gland regeneration and accelerated wound closure in rats but may offer a new therapeutic approach to accelerate diabetic wound healing [37,79].

2.9. Cancers

Emphasizing once again its antioxidant properties, it is important to mention the use of copper in cancer therapy by inhibiting, among other things, the activity of the p53 protein [32,80]. However, this element may also inhibit the growth and proliferation of cancer cells themselves, their metastasis, or the process of angiogenesis [10,16,19,34,81]. Copper plays an important role for several reasons, including promoting the growth of blood vessels supplying the tumor and activating enzymes and signaling proteins used by cancer cells [10,14]. Meanwhile, its levels are higher in aggressive cells, and the targeted blocking of its bioavailability can reduce the energy required for their movement in the body [14]. Currently, some of the new therapeutic strategies available (such as chelators or ionophores) have shown promising results in preclinical studies, and others are already being used to treat [14,34]. Recent reports confirm that abnormal Cu homeostasis can be observed in many cancers and, consequently, its elevated serum and tissue levels, which are correlated with cancer progression [15,34]. Therefore, it is becoming a new treatment strategy to analyze Cu levels in the body and to pay attention to its homeostasis or its possible influence on cancer progression. There are a number of questions regarding the full understanding of its role in cancer. Yet, current analyses and publications show broad prospects for the use of Cu-coordinated compounds as potential therapeutic agents [15,19,34]. It has been shown that the addition of nontoxic concentrations of Cu to healthy and cancer cells can markedly alter changes in cellular metabolism [32,34].

Several reports confirm the correlation between high Cu levels and the metabolic demand of prostate, breast, colon, liver, colorectal, and brain cancers. And importantly, these types of cancers may be sensitive to Cu-modulating therapies [19,34,82,83]. Meanwhile, studies of copper refinery workers have not shown a positive correlation between copper exposure and an increased incidence of lung cancer, possibly due to arsenic contamination of the ores [5]. The first studies analyzing the effects of copper on novae were initiated by de Jorge et al. in 1965 [84]. However, copper ions have been linked to the development of many cancers, including lung cancer, acute lymphatic leukemia, multiple myeloma, and others, in which copper ion levels are significantly elevated. Research over the past 20 years shows that copper ions are involved in the development, growth, and metastasis of cancer through various pathways. Experimental data have shown significantly elevated copper levels in, for example, stage I multiple myeloma, lung cancer, and acute lymphoblastic leukemia [19].

It is noteworthy, however, that Cu has been shown to play a role in inducing an antitumor immune response by regulating the expression of PD-L1 (programmed cell death ligand 1) [19,85]. Understanding the molecular susceptibility of tumors may have important implications for the efficacy of Cu-dependent drug treatments. And, what seems rather important, the depletion as well as overload of this element in the body may be viable therapeutic strategies to be used in cancer diagnosis [19,34]. There is also no doubt that its specific mechanism in tumorigenesis requires further research.

As shown by Borković-Mitićet al., copper in the brain is an essential factor for CuZn-SOD. It mediates the oxidative stress response and neurotransmitter biosynthesis [36].

The use of copper nanoparticles in the treatment of cancer, including breast cancer, can cause a significant reduction in the viability of these cells, morphological deformation of cancer cells, increased production of reactive oxygen species (ROS), and loss of mitochondrial membrane potential. As indicated by studies, the uptake of copper (II) oxide nanoparticles induces apoptosis, but these coppers can also inhibit the viability of cancer

cells by necrosis [37,38,86]. In Ehrlich ascites cancer, in vivo resulted in a reduction in tumor volume [87], and in the treatment of human pancreatic cancer, in vitro and in vivo (mouse model) [88]. The authors of one study showed that the degradation of DNA molecules is dose-dependent for copper nanoparticles, generating singlet oxygen. They also observed that they had cytotoxic effects on human histiocytic lymphoma and cervical cancer cells, triggering apoptosis [39,89,90]

2.10. Copper Transport at the Molecular Level

Each transporter plays an important role, both the very high affinity CTR1, low affinity CTR2, copper chaperones (CCS, Atox1, Cox17, SCO1, and SCO2), and the copper efflux transporters ATP7A and ATP7B. They play a key role in that they are responsible for the uptake, distribution, and export of copper from cells to individual organs or tissue. It is important to understand that cells contain a complex network of soluble regulatory molecules. These enable fine-tuning of the homeostasis of copper itself and the precise temporal and spatial allocation of copper within the cell itself, making it possible to understand the scheme a little better. There is also a strong interaction with dynactin, ADP-ribosylation factor GTPase (Arf)1, phosphatidylinositol-binding protein (COMMD1), the ubiquitin machinery, and other proteins responsible for the metabolism of copper itself. There is a strong need for an in-depth study of the relationship between copper homeostasis and other physiological processes (e.g., lactation, inflammation, lipid metabolism, etc.) to better exploit the therapeutic potential of copper transport regulation in humans in the future [11,38,91]. This molecular mechanism is very complex yet still represents significant interest and probably high therapeutic potential.

3. Effects of a Deficiency as Well as an Excess of Copper in the Body

Both the deficiency and excess of Cu can lead to the appearance of numerous disorders in the body (Table 2). The most common causes of deficiency are an improperly balanced diet (e.g., strict vegetarian diet) and various types of disorders of the body, e.g., malabsorption syndrome, coeliac disease, inflammatory bowel disease (e.g., Crohn's disease), or nephrotic syndrome. It can also result from a specific physiological state with an increased demand (e.g., pregnancy, lactation), or due to excessive loss (e.g., burns, use of copper chelates, penicillamine) [5,6,10,12,21,84]. The most common oxidative damage occurs due to chronic Cu overload or overexposure caused by accidents, occupational hazards, excessive zinc supplementation, or through environmental pollution. Therefore, chronic as well as acute toxicity is very rare [5,7,10,19,20]. However, it should be emphasized that even small changes in Cu concentration are associated with significant toxicity [34], and because of this toxicity, its use as a vomiting agent has been discontinued [5].

Table 2. The most common ailments resulting from copper deficiency and excess in the body [5–7,10,13,22,84,92,93].

Copper Deficiency	Copper Excess
• anemia • Menkes disease • numbness in the limbs • bone weakness • muscle weakness • trouble concentrating • decreased appetite • increased infections • lipid profile disorders • heart arrythmia • increase in blood pressure • optic neuritis • deterioration of the skin condition • hair loss and premature graying • hypothyroidism	• muscle stiffness • involuntary facial grimaces and body movements • lower abdominal pain, ascites, and jaundice developing due to liver cirrhosis • aggressive disorders, psychosis • cardiovascular breakdowns • drooling • lethargy, coma • copper deposits in the cornea of the eye, visible as a brown ring • death

Menkes disease (Menkes syndrome) is an inborn, neurodegenerative defect of copper metabolism occurring predominantly in boys, where a lack of copper injections can be fatal [10,19,23]. Girls are mainly carriers of this mutation. Copper is then not absorbed into the child's body as a result of the genetic abnormality, consequently leading to many serious health problems. One of the characteristic symptoms of this disease is curly, very brittle hair with a steel color, specific facial features, skin lesions, defects of the skeletal, urinary, and vascular systems, and neurological disorders [6,7,22,92]. One of the complications of this disease can be emphysema. This disease is caused by mutations in the P-type ATPase gene [23].

However, the result of over-supplementation and a genetic defect may be Wilson's disease (an autosomal recessive inherited metabolic disease resulting from alterations in the gene labeled ATP7B on chromosome 13), which mainly involves the inability to remove this pietrichrome from the liver through a defect in the enzyme adenosine triphosphatase. As a consequence, copper accumulates in internal organs, causing deleterious effects also on the kidneys, brain, and nervous system [5–7,12,93,94]. Clinical manifestations in chronic Wilson's disease most commonly include mental changes, motor disturbances (dysarthria, dysphagia, ataxia, writing difficulties), hemolytic anemia, renal tubular dysfunction (uricosuria, hypercalciuria), kidney stones, and fulminant liver failure [12,22]. Its accumulation in the liver is then both pathognomonic and pathogenic, providing a source of cellular reactive oxygen species [13]. The disease usually presents in childhood or adolescence with various neurological syndromes. Meanwhile, Kayser–Fleischer rings are present in the cornea of the eye in the Descemet's membrane [5,22]. In this case, early treatment with D-pecylamine prevents death and often restores normal liver status [5,7]. In Wilson's disease, reduced levels (<20 mg/dL) of oxidase-activated ceruloplasmin occur in up to 95% of patients [5].

Environmental exposure to copper and its excessive intake with food are rare in developed countries, but it does occur in developing countries, particularly in India [12]. It is noteworthy that it occurs in rural Indian boys (6 month–5 years), in whom not only a genetic factor is present but there is also an environmental influence (drinking or keeping food in brassware) along with impaired excretion [5,7]. Zn supplementation confirms a beneficial effect, especially in patients with Wilson's disease [7].

It seems interesting to note that in India, copper is a common suicide agent [5,12]. Meanwhile, spirit water (green water) used very frequently and commonly in African rituals induces hemolysis, kidney failure, or even death after ingestion [5].

4. Sources of Copper in Food

The Institute of Medicine, the Food and Nutrition Council, and the Polish Institute of Nutrition recommend a reference intake of Cu for adults of 0.9 mg/day (30 μg/kg body weight per day). The World Health Organization, on the other hand, has set the estimated minimum requirement for Cu at 0.6 mg/day for women and 0.7 mg/day for men. The average daily Cu intake in the USA or Europe (including Poland) is about 1 mg [7,20–22,95,96]. The average absorption of Cu from the diet is about 35–50%, and its absorption increases in the case of Cu deficiency in the body [71]. Regarding the NOAEL, EFSA experts assumed a copper dose for adults of 10 mg/day and a UL value of 5 mg/day [96], with a minimum requirement of 0.4–0.8 mg/day [22]. It should be noted that the lethal dose of copper in an untreated adult is about 10–20 g Cu, and in children, it may occur due to accidental ingestion of coins [5,12]. In the USA, the average daily intake of copper is about 1 mg, and its bioavailability from the diet is estimated to be 65–70%, with a biological half-life of about 13–33 days [5]. US guidelines indicate that drinking water should not contain more than 1.3 mg/L [8]. And the Food and Nutrition Board of the Institute of Medicine recommends an age-dependent dietary allowance (RDA) for copper (1–3 years up to 340 μg/day; 4–8 years up to 440 μg/day; 9–13 years up to 890 μg/day; from 14 years up to 900 μg/day) [8].

The richest dietary sources of copper (Table 3) include oysters, beef and calf liver, seafood, chocolate, nuts, legumes, sunflower seeds, pumpkin seeds, red wine, cocoa, potatoes, spinach, vending machine drinks, and whole grains. Copper is also found in green leafy vegetables, avocados, or mushrooms [5–8,10,12,16,21,22,97].

Table 3. Threshold copper content in selected food products in Poland [97].

Food Product	Threshold Content (mg/kg Fresh Matter)
Milk and milk products	0.00–0.40
Eggs	0.03–0.21
Meat and meat products	0.00–5.50
Fish and fish products	0.02–0.33
Fats and oils	0.00–0.07
Cereal products	0.01–0.95
Vegetables and vegetable products	0.01–1.50
Fruits and fruit products	0.00–0.77
Nuts	0.28–1.29
Seeds and grains	0.40–1.87
Sugar and confectionery	0.01–3.71
Beverages	0.00–0.09
Yeast	0.09
Other products	0.00–0.29
Soups	0.01–0.11
Fish dishes	0.06-.011
Meat dishes	0.01–0.57
Vegetable–meat dishes	0.05–0.10
Vegetable dishes	0.03–0.54
Groat, flour, and potato dishes	0.04–0.25
Eggs dishes	0.04–0.11
Desserts	0.02–0.07
Other dishes	0.02–0.10

However, it is worth noting that the range of copper content in foods may vary depending on the food product, its origin, place of manufacture, etc. Examples of the smallest and largest ranges are given below:

- Milk and milk products (0.00 mg/kg—milk drink with whey and cream; 0.40 mg/kg—cheese "Oscypek");
- Eggs (0.03 mg/kg—chicken eggs white; 0.21 mg/kg—chicken eggs whole dried);
- Meat and meat products (0.00 mg/kg—bone broth; 5.50 mg/kg—calf liver);
- Fish and fish products (0.02 mg/kg—sole raw; 0.33 mg/kg—herring smoked "Pikling");
- Fats and oils (0.00 mg/kg—oil corn; 0.07 mg/kg—oil olive);
- Cereal products (0.01 mg/kg—corn starch; 0.95 mg/kg—wheat bran);
- Vegetables and vegetable products (0.01 mg/kg—aubergine raw; 1.50 mg/kg—soya beans dried);
- Fruits and fruit products (0.00 mg/kg—strawberries sirup; 0.77 mg/kg—apricots dried);
- Nuts (0.28 mg/kg—walnuts; 1.29 mg/kg—hazelnuts);

- Seeds and grains (0.40 mg/kg—linen seeds; 1.87 mg/kg—sunflower seed dried);
- Sugar and confectionery (0.01 mg/kg—fruit gums; 3.71 mg/kg—cocoa powder);
- Beverages (0.00 mg/kg—black tea infusion average without sugar; 0.09 mg/kg—grape white juice);
- Yeast (0.09 mg/kg—yeast baker's compressed);
- Other products (0.00 mg/kg—gelatine; 0.29 mg/kg—potato crisps salted);
- Soups (0.01 mg/kg—soup sauerkraut; 0.11 mg/kg—soup green pea);
- Fish dishes (0.06 mg/kg—cod fillets steamed; 0.11 mg/kg—cod fillets breaded and fried);
- Meat dishes (0.01 mg/kg—chicken fillets steamed; 0.57 mg/kg—pork liver sauté fried);
- Vegetable–meat dishes (0.05 mg/kg—sauerkraut with sausage and meat "Bigos" stewed; 0.10 mg/kg—beans baked with meat in tomato sauce);
- Vegetable dishes (0.03 mg/kg—white cabbage salad; 0.54 mg/kg—mushrooms fried);
- Groat, flour, and potato dishes (0.04 mg/kg—pancakes filled with semi-fat fresh cheese fried; 0.25 mg/kg—milled groats boiled with vegetable fat);
- Eggs dishes (0.04 mg/kg—eggs poached with vegetable fat; 0.11 mg/kg—omelet biscuit baked);
- Desserts (0.02 mg/kg—apple compote with sugar; 0.07 mg/kg—strawberries with whipped cream);
- Other dishes (0.02 mg/kg—cheese Tilsit paste; 0.10 mg/kg—fresh cheese and fish smoked paste) [97].

Natural water contains 4–10 mg Cu/L, while potable water represents 6–13% of the average daily requirement. The human body is able to control excessive amounts of Cu in the body by decreased absorption or increased excretion [12,98]. As water naturally contains copper, the excess is most often the result of its release from old pipes, household taps, standing water, or hot water (it dissolves easily at high temperatures) [6].

Absorption of copper facilitates its binding to several essential amino acids (Cys, His, Asp, Met, Tyr, Gly, and Thr). Its presence in the diet also facilitates binding as ligands to organic acids (gluconic, lactic, citric, and acetic acids) [6]. It is worth noting that Cu is able to catalyze the formation of ROS, and nutrients with antioxidant properties may provide protection against the induction of this element into oxidative damage [7,12,17,18].

Vitamin E may prevent endothelial dysfunction associated with cholesterol metabolism, and vitamin E supplementation alone may be beneficial in preventing ROS-induced functional impairment in atherosclerosis [7]. Meanwhile, vitamin C (an antioxidant) can reduce metals with redox activity, such as Cu and Fe, increasing the pro-oxidant activity of these metals. Also, it can serve as a pro-oxidant (at low concentrations) as well as an antioxidant (at high concentrations) in vitro. Ascorbic acid enhances the cytotoxic effect of Cu ions in human embryonic cells (CLV102 and Lu106) and in exposed human melanoma cells (Mel8). However, it should be emphasized that ascorbic acid from the diet, as well as from supplementation, protects against Cu toxicity and absorption in tissues, mainly through reduced absorption in the small intestine [6,7,12]. Also, a well-balanced diet rich in Se can protect against a toxic version of Cu. This occurs due to the antioxidant properties of Se, which owe their activity to the selenoenzymes in proteins. Meanwhile, Se supplementation does not protect Cu-induced liver damage [7,12]. It is also worth noting that another beneficial effect on oxidative stress levels is the action of Zn, as part of the Cu enzyme Zn-SOD, in which Cu and Zn have a strongly antagonistic effect. High concentrations of Zn prevent absorption in the intestine, liver, and kidneys through the formation of metallothionein. Zinc also helps to reduce the formation of free radicals where different types of Cu bonds are formed, competing with Cu for a place in the molecule. Zn then binds to the substrate in question, probably histidine, stopping the formation of free radicals, and Cu is displaced [7,20]. Interestingly, red wine components (polyphenols) have been shown to prevent LDL oxidation and Cu-dependent and Cu-independent oxidation of LDL and HDL proteins and lipids in a dose-dependent manner [6,7,22]. As shown by Se, beta-carotene,

alpha-lipoic acid, and polyphenols may provide protection against Cu-induced oxidative damage, respectively, although this requires further in vivo studies [7].

5. Copper in the Environment

The natural concentration of this element in soil is about 50 ppm (2–250 ppm) Cu, but it is also released into the air from natural sources (e.g., windblown dust, volcanoes, forest fires, decaying plants, sea spray) or from artificial sources (e.g., iron production, steel production, municipal incinerators), or as a result of human activities (e.g., landfills, wood production, phosphate fertilizers). In the atmosphere alone, it ranges from 5 to 200 ng Cu/m^3, while in the vicinity of smelters, it can reach up to 5000 ng/m^3 [5,7,8]. In turn, its emissions to the environment represent 0.3%, coming mostly from natural sources (wind-borne, dust, volcanoes, forest fires, sea spray), as well as anthropogenic sources (nonferrous metal production, copper smelters, iron and steel production, municipal incinerators) [5,8]. Copper deposits also occur in sedimentary and volcanic rocks in a variety of geological environments, and the resulting deposits can be either layered, massive, or dispersed [3,7,20,99]. It is estimated that municipal wastewater treatment plants remove up to 80% of total copper from the water during pretreatment, where concentrations can be <100 μg Cu/L in the effluent. Water that remains overnight in plumbing fixtures can contain up to 60 mg Cu/L (20–75 ppb). With a taste threshold, a value of 1–5 mg Cu/L is perceptible, and a slight blue-green coloration appears in the water at levels > 5 mg Cu/L [5,8]. Meanwhile, copper concentrations in rivers or lakes range from 0.5 to 1000 ppb, with some groundwater up to 2800 ppb [8].

Almost as much copper is recovered from recycled materials each year as is mined, which contributes to its continually high value in the premium scrap classification. Thanks to its renewability, it is recognized as a sustainable material, i.e., consequently environmentally friendly [4,5].

Copper has always been, is, and will continue to be one of the most important metals traded internationally, and its global trend is confirmed by its largest share in metallurgical products (over 15%) [3,100]. It is well dispersed globally and available, with its supply distributed among the world's top four producers: Chile (28%), Peru (12%), China (9%), and the United States (7%) [4,101]. Meanwhile, in 2020, Poland (35 million tons) was ranked 7th among countries with the largest copper reserves. Chile (200 million tons), Australia and Peru (87 million tons each), Russia (61 million tons), Mexico (53 million tons), and the USA (51 million tons) produce the most, followed by Poland with Indonesia (28 million tons), China (26 million tons), Congo (19 million tons), and the rest together (220 million tons) [102]. Utah (USA) is home to the deepest open-pit copper mine in the world (1200 meters deep, 4 km in diameter) made by human beings [3,100]. All the information collected for some time on copper resources confirms its abundance. It should also be noted that most of this element is mined by large, international companies adhering to strict safety and environmental standards. A mixture of well-known open-pit and underground mining technologies is then used. Primary copper demand in the EU is about 4.2 million tons per year, of which 37% is met through imports, 18% through European copper production (mainly Poland and Sweden), and about 45% through recycling. Thus, the EU has a fairly manageable exposure to its imports and is at the forefront of the circular economy [4,101]. Poland extracted 33.8 billion tons of Cu in 2022 from deep mines (Lubin, Rudna—the largest in Europe, Polkowice). Its largest accumulation in Poland is associated with deposits located on the pre-Sudetic monocline (the New Copper Belt) and in the North Sudetic Basin (the Old Copper Belt), formed about 200 million years ago [3,100]. The most common destinations for Polish electrolytic copper in 2018 included Spain (150 t), Germany (92 t), China (90 t), Italy (33 t), France (31 t), Slovakia (15.6 t), Turkey (14.8 t), Japan (5.3 t), and India (4.5 t) [102].

It is also worth noting that as a biocide (especially for Gram+ and Gram− bacteria, fungi, viruses, and other bacterial spores), copper biocides have become indispensable

worldwide. Many thousands of tons per year are used in agriculture, wood preservation, antifouling paints, and the elimination of unpleasant odors from textiles [9].

6. Source of Copper in Different Materials

Cu comes from Cyprus, where this field precious metal was mined around 2000 BC. Although the first traces of its production and processing were already recorded more than 5,000 years BC in Mesopotamia, the Sinai Peninsula, and the Mediterranean countries. It is worth noting that copper itself forms more than 150 different minerals. Its largest sources are minerals such as chalcopyrite and bornite, and it is obtained by smelting, leaching, and electrolysis. At room temperature, it is resistant to dry air, while it reacts with oxygen. When heated, it can dissolve in oxidizing acids. Meanwhile, in humid conditions, it becomes covered with a patina, or is also used for decorative purposes. Copper also forms complex compounds, and its aqueous solutions of compounds are blue in color. Its soluble compounds are mainly poisonous. Their main use is as an insecticide or fungicide ($CuSO_4$, Cu, O), or for the impregnation of wood (pigments, paint catalysts: CuO, $CuCO_3$, $Cu(OH)_2$.) [2,6]. Noteworthy is the fact that it is the main alloying component of brasses (Cu-Zn), new silver with the addition also of the alloys of some steels, bronzes (Cu-Sn), cast iron, cast steel, aluminum and bearing alloys, zinc, gold, and silver, and binders for joining metals, as well as dental alloys. Copper also guarantees temperature and pressure stability, as well as an aesthetic appearance [1–4].

Because of its distinctive properties, copper is considered a very good material for its widespread use, including its resistance to corrosion (which guarantees its longevity), its biostatic effect (inhibiting the growth of bacteria in the production of doorknobs, kitchen worktops, and tables), and it is nonmagnetic [2,4].

Copper itself is widely used in, among other things,

- roofing, gutters, building fittings, door fittings, locks, architectural decorative elements (also interior design);
- all kinds of installations (electrical, heating, gas, fire-fighting, air-conditioning sprinklers, air conditioners, lighting, heat exchangers, parts of rocket engines, wireless chargers);
- in the hydraulic area, plumbing (cold and hot water pipes, taps);
- in the chemical and food industry apparatus (e.g., coolers, rectification columns, chemical and distillation apparatus, etc.);
- electrical machine components and printed circuits, microelectronic material technology itself as well as optoelectronic materials (solar cells, electro-acoustic relays, batteries);
- in the electrical and energy sector (electrical generators, transformers, wind power stations, types of cabling, wires, sockets);
- in the telecommunications industry (computer connectors, computer chips, graphics cards, hard drives, cables, mobile phones, computers, televisions, household appliances);
- in the automotive industry (trains, aircraft, trucks and cars, including electric vehicles) or mechanical engineering (e.g., sealing of oil injectors);
- in wind and solar energy (supports the proper functioning and efficiency of wind turbines, protects the generator, grounds towers against lightning strikes, conducts electricity);
- in the military sector (weapons, transport);
- in the medical sector (dental products—dental bridges/crowns, intrauterine contraceptive devices—100–150 mg; folk spirit water—13g/L);
- in the mint (Polish one-penny coins are made from manganese brass, containing 59% copper, 40% zinc, 1% manganese);
- in the chemical industry (production of pesticides);
- copper compounds are used in agriculture to treat diseased plants (e.g., molds), to treat water, and as a preservative for wood (impregnation), leather, and textiles [1–9].

7. Prevention

In addition to the abovementioned noted symptoms, through a typical physical examination and history, it is necessary to determine blood levels of total copper and the free fraction, as well as levels of ceruloplasmin (copper transport protein). The determination of copper levels in daily urine collections and blood levels of superoxide dismutase and cytochrome c oxidase are then recommended [5,20,22,92]. A tight regulation of copper metabolism is required to ensure sufficient copper availability for the necessary enzymes, without toxic effects caused by an excess of this element. Knowledge of its regulation allows us to consider a new area of potential therapeutic interventions based on copper supplementation or removal in neurodegenerative diseases, including Wilson's disease, Menkes disease, Alzheimer's disease, Parkinson's disease, and others [5,6,13,20,50,91]. Additional information on copper ions and also on tumor immunity itself may broaden the knowledge on new therapeutic approaches for cancer patients and improve their clinical prognosis [19].

It is recommended to drink only cold water from taps and to use only cold, longer-flowing water from the source for cooking [6,71,72]. It is therefore advisable to drain the water for about 15–30 s to reduce its content [8].

It is worth noting that there are no well-designed studies of workers exposed to copper (e.g., smelters, metallurgy, mechanical industry, etc.) that demonstrate chronic toxicity, acting particularly on the respiratory tract and gastrointestinal tract. There are also currently no data confirming a safe and biological limit in workers exposed to copper. It is only recommended that workers be controlled for copper vapor of 0.2 mg Cu/m^3 and copper dusts and mists of 1 mg Cu/m^3 [5,8].

According to Focarelli et al. [11], bacterial copper detoxification systems may be viable targets for future development of new antibiotic development programs [9]. There is also one novel strategy for treating people with emphysema, through the use of topical supplementation, which would then restore copper levels in the lungs, also improving elastin. Although, in this case, it is important to use high doses, which can be problematic due to entering the circulatory system quickly, thus potentially affecting the brain or liver toxically [23].

Recently discovered and introduced copper nanoparticles even have ideal antibacterial activity against *Staphylococcus aureus* (including methicillin-resistant *S. aureus*), *Bacillus subtilis*, *Proteus vulgaris*, and *Escherichia coli* [50,79]. The characteristics of copper nanomaterials (i.e., antibacterial/antifungal) and the ability to accelerate the healing of infected wounds have enabled applications to other medical fields, such as dentistry. They can be added to dental adhesives, tooth-filling materials, implant coatings, orthodontic abutment coatings, or denture materials [39,50,103].

Thanks to the use of copper oxygen molecules in polymer carriers and their strong biocidal effect, they are now being used in health-promoting as well as consumer products, for example, in the production of hospital linen, nurses' garments, patients' dressing gowns and pajamas, protective breathing masks, wrinkle-reducing pillowcases, socks for the prevention and treatment of foot fungus (athlete's foot) and to reduce the risk of skin pathologies in diabetics, and wound dressings to reduce dressing and wound contamination and promote wound healing [9].

Copper content in biological or environmental samples is mainly determined using atomic absorption spectrometry (flame or graphite) and inductively coupled plasma atomic emission spectroscopy. In the absence of this equipment in the laboratory, rapid colorimetric tests with detection ranges close to 0.1 mg Cu/L are available [80]. As the tests are not available in physicians' offices, samples must be sent to a special laboratory [8].

Only in the case of acute poisoning is the use of chelating agents recommended, abandoning the use of vomiting agents or laxatives. And after, the ingestion of drugs with high levels of CaNa2EDTA or intramuscular BAL is recommended. For Wilson's disease, the treatment of symptomatic neurological, psychiatric, and liver disease with

d-penicillamine chelation is recommended. Foods with a high concentration of copper should also be avoided (shellfish, nuts, liver, mushrooms, chocolate) [5,12].

Examples of preventive interventions:

- governments and major institutions in the country should regularly check Cu levels in food, soil, and air;
- continuously monitor drinkable water systems;
- conduct regular corrosion control, servicing, and regular testing;
- secure drinking sources (especially in schools) with appropriate filtration systems;
- remove old drains, pipes, and sanitation (e.g., taps) from service and replace with new ones;
- use cold water, drained for a minimum of 1 min;
- use appropriate posting if water cannot be consumed in the chosen location (e.g., "Do not use");
- wash hands regularly;
- replace kitchen appliances with glass/porcelain ones;
- use of water treatment technology [39,97,104–106].

8. Conclusions

Undoubtedly, copper is essential for brain cells (as a cofactor and component of many enzymes involved in biochemical pathways); however, excess amounts are harmful. A high concentration of Cu can contribute to many diseases, including neurodegenerative, cardiovascular, immune, bone, respiratory, reproductive, gastrointestinal, skin, and even some cancers. Cu is essential, and in optimal doses provides antioxidant protection, and its deficiency reduces the body's ability to cope with oxidative stress, which is a consequence of many serious diseases. Being involved in a tremendously wide range of many biochemical reactions, providing or accepting electrons, Cu ions also bind to various proteins or enzymes as cofactors or structural components. It is involved in the most important physiological proxies, such as energy metabolism, mitochondrial respiration, inflammatory reactions, and antioxidants, so it is worth focusing attention on it. The development of civilization and the continued widespread use of Cu in all our electrical devices will not be stopped, but the health of those directly involved in its extraction, production, or distribution can be controlled. It is extremely difficult to assess the effects of copper on the human body because of its ubiquity and the increasing reports in the literature about its effects, including copper nanoparticles.

Several factors influence the retention or bioavailability of copper from diet alone, i.e., age, amount and form of copper in the diet, and genetic background. Further research is still needed to better understand how much and to what extent copper affects the health of the body of the current generation, which seems to be already familiar with this element.

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References

1. The Ancient History of Copper. Available online: https://www.thoughtco.com/copper-history-pt-i-2340112 (accessed on 25 January 2020).
2. Copper (Cu) Ore. Available online: https://geologyscience.com/ore-minerals/copper-cu-ore/ (accessed on 23 April 2023).
3. Mining 101: Copper—All You ever Wanted to Know about the Red Metal (and Were too Afraid to Ask). Available online: https://www.proactiveinvestors.co.uk/companies/news/1032168/mining-101-copper-all-you-ever-wanted-to-know-about-the-red-metal-and-were-too-afraid-to-ask-1032168.html (accessed on 8 November 2023).

4. New Copper Technologies. Available online: https://blog.gorozen.com/blog/new-copper-technologies (accessed on 3 May 2024).
5. Barceloux, D.G. Copper. *J. Toxicol. Clin. Toxicol.* **1999**, *37*, 217–230. [CrossRef]
6. Gromadzka, G.; Tarnacka, B.; Flaga, A.; Adamczyk, A. Copper Dyshomeostasis in Neurodegenerative Diseases-Therapeutic Implications. *Int. J. Mol. Sci.* **2020**, *21*, 9259. [CrossRef]
7. Feng, W.; Su, S.; Song, C.; Yu, F.; Zhou, J.; Li, J.; Jia, R.; Xu, P.; Tang, Y. Effects of Copper Exposure on Oxidative Stress, Apoptosis, Endoplasmic Reticulum Stress, Autophagy and Immune Response in Different Tissues of Chinese Mitten Crab (*Eriocheir sinensis*). *Antioxidants* **2022**, *11*, 2029. [CrossRef]
8. Copper. CAS#: 7440-50-8. Available online: https://www.atsdr.cdc.gov/ToxProfiles/tp132-c1-b.pdf (accessed on 26 September 2004).
9. Borkow, G. Using Copper to Improve the Well-Being of the Skin. *Curr. Chem. Biol.* **2014**, *8*, 89–102. [CrossRef] [PubMed]
10. Copper. Available online: https://www.hsph.harvard.edu/nutritionsource/copper/ (accessed on 20 March 2023).
11. Focarelli, F.; Giachino, A.; Waldron, K.J. Copper microenvironments in the human body define patterns of copper adaptation in pathogenic bacteria. *PLoS Pathog.* **2022**, *18*, e1010617. [CrossRef]
12. Copper. Fact Sheet for Health Professionals. Available online: https://ods.od.nih.gov/factsheets/Copper-HealthProfessional/#en3 (accessed on 18 October 2022).
13. Yu, L.; Liou, I.W.; Biggins, S.W.; Yeh, M.; Jalikis, F.; Chan, L.N.; Burkhead, J. Copper Deficiency in Liver Diseases: A Case Series and Pathophysiological Considerations. *Hepatol. Commun.* **2019**, *3*, 1159–1165. [CrossRef]
14. Lelièvre, P.; Sancey, L.; Coll, J.L.; Deniaud, A.; Busser, B. The multifaceted roles of copper in cancer: A trace metal element with dysregulated metabolism, but also a target or a bullet for therapy. *Cancers* **2020**, *12*, 3594. [CrossRef] [PubMed]
15. Li, Y. Copper homeostasis: Emerging target for cancer treatment. *IUBMB Life* **2020**, *72*, 1900–1908. [CrossRef] [PubMed]
16. Grieger, J.A.; Grzeskowiak, L.E.; Wilson, R.L.; Bianco-Miotto, T.; Leemaqz, S.Y.; Jankovic-Karasoulos, T.; Perkins, A.V.; Norman, R.J.; Dekker, G.A.; Roberts, C.T. Maternal Selenium, Copper and Zinc Concentrations in Early Pregnancy, and the Association with Fertility. *Nutrients* **2019**, *11*, 1609. [CrossRef]
17. Herman, S.; Lipiński, P.; Ogórek, M.; Starzyński, R.; Grzmil, P.; Bednarz, A.; Lenartowicz, M. Molecular Regulation of Copper Homeostasis in the Male Gonad during the Process of Spermatogenesis. *Int. J. Mol. Sci.* **2020**, *21*, 9053. [CrossRef]
18. Ogórek, M.; Gąsior, Ł.; Pierzchała, O.; Daszkiewicz, R.; Lenartowicz, M. Role of copper in the process of spermatogenesis. *Postepy Hig. Med. Dosw. (Online)* **2017**, *71*, 663–683. [CrossRef] [PubMed]
19. Cheng, F.; Peng, G.; Lu, Y.; Wang, K.; Ju, Q.; Ju, Y.; Ouyang, M. Relationship between copper and immunity: The potential role of copper in tumor immunity. *Front. Oncol.* **2022**, *12*, 1019153. [CrossRef] [PubMed]
20. National Research Council (US) Committee on Copper in Drinking Water. Physiological Role of Copper. In *Copper in Drinking Water*; National Academies Press (US): Washington, DC, USA, 2000; Chapter 2. Available online: https://www.ncbi.nlm.nih.gov/books/NBK225407/ (accessed on 11 January 2023).
21. Rondanelli, M.; Faliva, M.A.; Infantino, V.; Gasparri, C.; Iannello, G.; Perna, S.; Riva, A.; Petrangolini, G.; Tartara, A.; Peroni, G. Copper as Dietary Supplement for Bone Metabolism: A Review. *Nutrients* **2021**, *13*, 2246. [CrossRef] [PubMed]
22. Ciosek, Ż.; Kot, K.; Rotter, I. Iron, Zinc, Copper, Cadmium, Mercury, and Bone Tissue. *Int. J. Environ. Res. Public Health* **2023**, *20*, 2197. [CrossRef] [PubMed]
23. Janssen, R.; Wouters, E.F.; Janssens, W.; Daamen, W.F.; Hagedoorn, P.; de Wit, H.A.; Serré, J.; Gayan-Ramirez, G.; Franssen, F.M.; Reynaert, N.L.; et al. Copper-Heparin Inhalation Therapy to Repair Emphysema: A Scientific Rationale. *Int. J. Chron. Obstruct. Pulmon Dis.* **2019**, *14*, 2587–2602. [CrossRef] [PubMed]
24. Gaetke, L.; Chow, C. Copper toxicity, oxidative stress, and antioxidant nutrients. *Toxicology* **2003**, *189*, 147–163. [CrossRef] [PubMed]
25. Qu, X. Serum copper levels are associated with bone mineral density and total fracture. *J. Orthop. Transl.* **2018**, *14*, 34–44. [CrossRef] [PubMed]
26. Wang, H.; Zhang, R.; Shen, J.; Jin, Y.; Chang, C.; Hong, M.; Guo, S.; He, D. Circulating Level of Blood Iron and Copper Associated with Inflammation and Disease Activity of Rheumatoid Arthritis. *Biol. Trace Elem. Res.* **2023**, *201*, 90–97. [CrossRef]
27. Copper Supplement (Oral Route, Parenteral Route). Available online: https://www.mayoclinic.org/drugs-supplements/copper-supplement-oral-route-parenteral-route/proper-use/drg-20070120 (accessed on 1 February 2024).
28. Johnson, M.E.; Salvatore, M.F.; Maiolo, S.A.; Bobrovskaya, L. Tyrosine hydroxylase as a sentinel for central and peripheral tissue responses in Parkinson's progression: Evidence from clinical studies and neurotoxin models. *Prog. Neurobiol.* **2018**, *165–167*, 1–25. [CrossRef]
29. Ruiz, L.M.; Libedinsky, A.; Elorza, A.A. Role of Copper on Mitochondrial Function and Metabolism. *Front. Mol. Biosci.* **2021**, *8*, 711227. [CrossRef]
30. Kunutsor, S.K.; Dey, R.S.; Laukkanen, J.A. Circulating serum copper is associated with atherosclerotic cardiovascular disease, but not venous thromboembolism: A prospective cohort study. *Pulse* **2021**, *9*, 109–115. [CrossRef] [PubMed]
31. Tvrda, E.; Peer, R.; Sikka, S.C.; Agarwal, A. Iron and copper in male reproduction: A double-edged sword. *J. Assist. Reprod. Genet.* **2015**, *32*, 3–16. [CrossRef] [PubMed]
32. Pickart, L.; Margolina, A. Skin Regenerative and Anti-Cancer Actions of Copper Peptides. *Cosmetics* **2018**, *5*, 29. [CrossRef]

33. Hvidtfeldt, U.A.; Geels, C.; Sørensen, M.; Ketzel, M.; Khan, J.; Tjønneland, A.; Christensen, J.H.; Brandt, J.; Raaschou-Nielsen, O. Long-term residential exposure to PM2.5 constituents and mortality in a Danish cohort. *Environ. Int.* **2019**, *133 Pt B*, 105268. [CrossRef]
34. Babak, M.V.; Ahn, D. Modulation of Intracellular Copper Levels as the Mechanism of Action of Anticancer Copper Complexes: Clinical Relevance. *Biomedicines* **2021**, *9*, 852. [CrossRef] [PubMed]
35. Oxidation States of Transition Metals. Available online: https://chem.libretexts.org/Bookshelves/Inorganic_Chemistry/Supplemental_Modules_and_Websites_(Inorganic_Chemistry)/Descriptive_Chemistry/Elements_Organized_by_Block/3_d-Block_Elements/1b_Properties_of_Transition_Metals/Oxidation_States_of_Transition_Metals (accessed on 30 July 2023).
36. Borković-Mitić, S.; Stojsavljević, A.; Vujotić, L.; Matić, S.; Mitić, B.; Manojlović, D. Differences between antioxidant defense parameters and specific trace element concentrations in healthy, benign, and malignant brain tissues. *Sci. Rep.* **2021**, *11*, 14766. [CrossRef] [PubMed]
37. Woźniak-Budych, M.J.; Staszak, K.; Staszak, M. Copper and Copper-Based Nanoparticles in Medicine-Perspectives and Challenges. *Molecules* **2023**, *28*, 6687. [CrossRef] [PubMed]
38. Zughaibi, T.A.; Mirza, A.A.; Suhail, M.; Jabir, N.R.; Zaidi, S.K.; Wasi, S.; Zawawi, A.; Tabrez, S. Evaluation of Anticancer Potential of Biogenic Copper Oxide Nanoparticles (CuO NPs) against Breast Cancer. *J. Nanomater.* **2022**, *2022*, 5326355. [CrossRef]
39. Crisan, M.C.; Teodora, M.; Lucian, M. Copper Nanoparticles: Synthesis and Characterization, Physiology, Toxicity and Antimicrobial Applications. *Appl. Sci.* **2022**, *12*, 141. [CrossRef]
40. Tsvetkov, P.; Coy, S.; Petrova, B.; Dreishpoon, M.; Verma, A.; Abdusamad, M.; Joesch-Cohen, L.; Humeidi, R.; Spangler, R.D.; Eaton, J.K.; et al. Copper induces cell death by targeting lipoylated TCA cycle proteins. *Science* **2022**, *375*, 1254–1261. [CrossRef]
41. Su, Y.; Cappock, M.; Dobres, S.; Kucine, A.J.; Waltzer, W.C.; Zhu, D. Supplemental mineral ions for bone regeneration and osteoporosis treatment. *Eng. Regen.* **2023**, *4*, 170–182. [CrossRef]
42. Kawada, E.; Moridaira, K.; Itoh, K.; Hoshino, A.; Tamura, J.; Morita, T. In long-term bedridden elderly patients with dietary copper deficiency, biochemical markers of bone resorption are increased with copper supplementation during 12 weeks. *Ann. Nutr. Metab.* **2006**, *50*, 420–424. [CrossRef] [PubMed]
43. Skrajnowska, D.; Jagielska, A.; Ruszczyńska, A.; Idkowiak, J.; Bobrowska-Korczak, B. Effect of Copper and Selenium Supplementation on the Level of Elements in Rats' Femurs under Neoplastic Conditions. *Nutrients* **2022**, *14*, 1285. [CrossRef] [PubMed]
44. Ding, H.; Gao, Y.-S.; Wang, Y.; Hu, C.; Sun, Y.; Zhang, C. Dimethyloxaloylglycine increases the bone healing capacity of adipose-derived stem cells by promoting osteogenic differentiation and angiogenic potential. *Stem Cells Dev.* **2014**, *23*, 990–1000. [CrossRef] [PubMed]
45. Gaffney-Stomberg, E. The Impact of Trace Minerals on Bone Metabolism. *Biol. Trace Elem. Res.* **2019**, *188*, 26–34. [CrossRef] [PubMed]
46. Milkovic, L.; Hoppe, A.; Detsch, R.; Boccaccini, A.R.; Zarkovic, N. Effects of Cu-doped 45S5 bioactive glass on the lipid peroxidation-associated growth of human osteoblast-like cells in vitro. *J. Biomed. Mater. Res. A* **2014**, *102*, 3556–3561. [CrossRef] [PubMed]
47. Alarcón-Corredor, O.M.; Guerrero, Y.; de Fernández, M.R.; D'Jesús, I.; Burguera, M.; Burguera, J.L.; Di Bernardo, M.L.; García, M.Y.; Alarcón, A.O. Effect of copper supplementation on lipid profile of Venezuelan hyperlipemic patients. *Arch. Latinoam. Nutr.* **2004**, *54*, 413–418. [PubMed]
48. Myint, Z.W.; Oo, T.H.; Thein, K.Z.; Tun, A.M.; Saeed, H. Copper deficiency anemia: Review article. *Ann. Hematol.* **2018**, *97*, 1527–1534. [CrossRef] [PubMed]
49. Cui, X.; Wang, Y.; Liu, H.; Shi, M.; Wang, J.; Wang, Y. The Molecular Mechanisms of Defective Copper Metabolism in Diabetic Cardiomyopathy. *Oxid. Med. Cell Longev.* **2022**, *2022*, 5418376. [CrossRef]
50. Mahmoodi, S.; Elmi, A.; Hallaj-Nezhadi, S. Copper Nanoparticles as Antibacterial Agents. *J. Mol. Pharm. Org. Process Res.* **2018**, *6*, 140. [CrossRef]
51. Kessler, H.; Bayer, T.A.; Bach, D.; Schneider-Axmann, T.; Supprian, T.; Herrmann, W.; Haber, M.; Multhaup, G.; Falkai, P.; Pajonk, F.-G. Intake of copper has no effect on cognition in patients with mild Alzheimer's disease: A pilot phase 2 clinical trial. *J. Neural Transm.* **2008**, *115*, 1181–1187. [CrossRef] [PubMed]
52. Xu, J.; Church, S.J.; Patassini, S.; Begley, P.; Waldvogel, H.J.; Curtis, M.A.; Faull, R.L.M.; Unwin, R.D.; Cooper, G.J.S. Evidence for widespread, severe brain copper deficiency in Alzheimer's dementia. *Metallomics* **2017**, *9*, 1106–1119. [CrossRef] [PubMed]
53. Siotto, M.; Simonelli, I.; Pasqualetti, P.; Mariani, S.; Caprara, D.; Bucossi, S.; Ventriglia, M.; Molinario, R.; Antenucci, M.; Rongioletti, M.; et al. Association between Serum Ceruloplasmin Specific Activity and Risk of Alzheimer's Disease. *J. Alzheimer's Dis.* **2016**, *50*, 1181–1189. [CrossRef] [PubMed]
54. Lanza, V.; Milardi, D.; Di Natale, G.; Pappalardo, G. Repurposing of Copper(II)-chelating Drugs for the Treatment of Neurodegenerative Diseases. *Curr. Med. Chem.* **2018**, *25*, 525–539. [CrossRef] [PubMed]
55. Rembach, A.; Doecke, J.D.; Roberts, B.R.; Watt, A.D.; Faux, N.G.; Volitakis, I.; Pertile, K.K.; Rumble, R.L.; Trounson, B.O.; Fowler, C.J.; et al. Longitudinal analysis of serum copper and ceruloplasmin in Alzheimer's disease. *J. Alzheimer's Dis.* **2013**, *34*, 171–182. [CrossRef] [PubMed]
56. Patel, R.; Aschner, M. Commonalities between Copper Neurotoxicity and Alzheimer's Disease. *Toxics* **2021**, *9*, 4. [CrossRef] [PubMed]

57. Patel, H.; Dobson, R.J.B.; Newhouse, S.J. A Meta-Analysis of Alzheimer's Disease Brain Transcriptomic Data. *J. Alzheimer's Dis.* **2019**, *68*, 1635–1656. [CrossRef] [PubMed]
58. Agarwal, P.; Ayton, S.; Agrawal, S.; Dhana, K.; Bennett, D.A.; Barnes, L.L.; Leurgans, S.E.; Bush, A.I.; Schneider, J.A. Brain copper may protect from cognitive decline and Alzheimer's disease pathology: A community-based study. *Mol. Psychiatry* **2022**, *27*, 4307–4313. [CrossRef] [PubMed]
59. Zhou, G.; Ji, X.; Cui, N.; Cao, S.; Liu, C.; Liu, J. Association between Serum Copper Status and Working Memory in Schoolchildren. *Nutrients* **2015**, *7*, 7185–7196. [CrossRef]
60. Pohanka, M. Copper and copper nanoparticles toxicity and their impact on basic functions in the body. *Bratisl. Lek. Listy* **2019**, *120*, 397–409. [CrossRef]
61. Charkiewicz, A.E.; Omeljaniuk, W.J.; Nowak, K.; Garley, M.; Nikliński, J. Cadmium Toxicity and Health Effects—A Brief Summary. *Molecules* **2023**, *28*, 6620. [CrossRef] [PubMed]
62. Charkiewicz, A.E.; Backstrand, J.R. Lead Toxicity and Pollution in Poland. *Int. J. Environ. Res. Public Health* **2020**, *17*, 4385. [CrossRef] [PubMed]
63. Dzieżyc, K.; Litwin, T.; Sobańska, A.; Członkowska, A. Symptomatic copper deficiency in three Wilson's disease patients treated with zinc sulphate. *Neurol. Neurochir. Pol.* **2014**, *48*, 214–218. [CrossRef] [PubMed]
64. Mohr, I.; Weiss, K.H. Biochemical Markers for the Diagnosis and Monitoring of Wilson Disease. *Clin. Biochem. Rev.* **2019**, *40*, 59–77. [CrossRef] [PubMed]
65. Song, W.; Yue, Y.; Zhang, Q.; Wang, X. Copper homeostasis dysregulation in respiratory diseases: A review of current knowledge. *Front. Physiol.* **2024**, *15*, 1243629. [CrossRef] [PubMed]
66. Zhang, Z.; Weichenthal, S.; Kwong, J.C.; Burnett, R.T.; Hatzopoulou, M.; Jerrett, M.; van Donkelaar, A.; Bai, L.; Martin, R.V.; Copes, R.; et al. A Population-Based Cohort Study of Respiratory Disease and Long-Term Exposure to Iron and Copper in Fine Particulate Air Pollution and Their Combined Impact on Reactive Oxygen Species Generation in Human Lungs. *Environ. Sci. Technol.* **2021**, *55*, 3807–3818. [CrossRef] [PubMed]
67. Boogaard, H.; Fischer, P.H.; Janssen, N.A.; Kos, G.P.; Weijers, E.P.; Cassee, F.R.; van der Zee, S.C.; de Hartog, J.J.; Meliefste, K.; Wang, M.; et al. Respiratory effects of a reduction in outdoor air pollution concentrations. *Epidemiology* **2013**, *24*, 753–761. [CrossRef] [PubMed]
68. Unar, A.; Afridi, H.I.; Ali, A.; Ali, N.; Qureshi, T. Determination of Electrolytes and Trace Elements in Biological Samples from Patients with Altered Semen Parameters: A Correlational Analysis. *Biol. Trace Elem. Res.* **2024**. [CrossRef] [PubMed]
69. Bhardwaj, J.K.; Paliwal, A.; Saraf, P. Effects of heavy metals on reproduction owing to infertility. *J. Biochem. Mol. Toxicol.* **2021**, *35*, e22823. [CrossRef] [PubMed]
70. Omeljaniuk, W.J.; Socha, K.; Borawska, M.H.; Charkiewicz, A.E.; Laudański, T.; Kulikowski, M.; Kobylec, E. Antioxidant status in women who have had a miscarriage. *Adv. Med. Sci.* **2015**, *60*, 329–334. [CrossRef]
71. Charkiewicz, A.E.; Omeljaniuk, W.J.; Orywal, K.; Czygier, M.; Szmitkowski, M.; Mroczko, B.; Maślach, D.; Szpak, A. Concentration of Selected Elements and Antioxidative Potential in a Group of Males Working in the Metal Industry: Elements And Antioxidative Potential In Men. *Am. J. Men's Health* **2019**, *13*, 1557988319851954. [CrossRef] [PubMed]
72. Charkiewicz, A.E.; Jamiołkowski, J.; Pędziński, B.; Krzyżak, M.; Maślach, D.; Szpak, A.; Omeljaniuk, W.J. Changes in Dietary Patterns and the Nutritional Status in Men in the Metallurgical Industry in Poland over A 21-Year Period. *Ann. Nutr. Metab.* **2018**, *72*, 161–171. [CrossRef] [PubMed]
73. Manouchehri, A.; Shokri, S.; Pirhadi, M.; Karimi, M.; Abbaszadeh, S.; Mirzaei, G.; Bahmani, M. The Effects of Toxic Heavy Metals Lead, Cadmium and Copper on the Epidemiology of Male and Female Infertility. *JBRA Assist. Reprod.* **2022**, *26*, 627–630. [CrossRef] [PubMed]
74. Roshankhah, S.; Barani, N.; Salahshoor, M.R.; Rezakhani, L.; Khazaei, M. The Relationship between Body Mass Index, Metal Elements, and Antioxidant Capacity of Semen on the Human Sperm Chromatin. *Int. J. Fertil. Steril.* **2023**, *17*, 287–291. [CrossRef] [PubMed]
75. Turgut, A.; Ozler, A.; Goruk, N.Y.; Tunc, S.Y.; Evliyaoglu, O.; Gul, T. Copper, ceruloplasmin and oxidative stress in patients with advanced-stage endometriosis. *Eur. Rev. Med. Pharmacol. Sci.* **2013**, *17*, 1472–1478.
76. Massányi, P.; Massányi, M.; Madeddu, R.; Stawarz, R.; Lukáč, N. Effects of Cadmium, Lead, and Mercury on the Structure and Function of Reproductive Organs. *Toxics* **2020**, *8*, 94. [CrossRef]
77. Peacey, L.; Peacey, C.; Gutzinger, A.; Jones, C.E. Copper(II) Binding by the Earliest Vertebrate Gonadotropin-Releasing Hormone, the Type II Isoform, Suggests an Ancient Role for the Metal. *Int. J. Mol. Sci.* **2020**, *21*, 7900; Erratum in *Int. J. Mol. Sci.* **2021**, *22*, 3431. [CrossRef]
78. Gupta, P.K. *Fundamentals of Toxicology. Essential Concepts and Applications*, 1st ed.; Academic Press: Cambridge, MA, USA, 2016; ISBN 9780128054260.
79. Geng, X.; Liu, K.; Wang, J.; Su, X.; Shi, Y.; Zhao, L. Preparation of Ultra-Small Copper Nanoparticles-Loaded Self-Healing Hydrogels with Antibacterial, Inflammation-Suppressing and Angiogenesis-Enhancing Properties for Promoting Diabetic Wound Healing. *Int. J. Nanomed.* **2023**, *18*, 3339–3358. [CrossRef] [PubMed]

80. Narayanan, V.S.; Fitch, C.A.; Levenson, C.W. Tumor Suppressor Protein p53 mRNA and Subcellular Localization Are Altered by Changes in Cellular Copper in Human Hep G2 Cells. *J. Nutr.* **2001**, *131*, 1427–1432. [CrossRef]
81. Denoyer, D.; Masaldan, S.; La Fontaine, S.; Cater, M.A. Targeting copper in cancer therapy: 'Copper That Cancer'. *Metallomics* **2015**, *7*, 1459–1476. [CrossRef]
82. Tang, X.; Yan, Z.; Miao, Y.; Ha, W.; Li, Z.; Yang, L.; Mi, D. Copper in cancer: From limiting nutrient to therapeutic target. *Front. Oncol.* **2023**, *13*, 1209156. [CrossRef] [PubMed]
83. Nayak, S.B.; Bhat, V.R.; Upadhyay, D.; Udupa, S.L. Copper and ceruloplasmin status in serum of prostate and colon cancer patients. *Indian J. Physiol. Pharm.* **2003**, *47*, 108–110.
84. Wazir, S.M.; Ghobrial, I. Copper deficiency, a new triad: Anemia, leucopenia, and myeloneuropathy. *J. Community Hosp. Internal Med. Perspect.* **2017**, *7*, 265–268. [CrossRef]
85. Voli, F.; Valli, E.; Lerra, L.; Kimpton, K.; Saletta, F.; Giorgi, F.M.; Mercatelli, D.; Rouaen, J.R.C.; Shen, S.; Murray, J.E.; et al. Intratumoral Copper Modulates PD-L1 Expression and Influences Tumor Immune Evasion. *Cancer Res.* **2020**, *80*, 4129–4144. [CrossRef] [PubMed]
86. Mahmood, R.I.; Kadhim, A.A.; Ibraheem, S.; Albukhaty, S.; Mohammed-Salih, H.S.; Abbas, R.H.; Jabir, M.S.; Mohammed, M.K.A.; Nayef, U.M.; AlMalki, F.A.; et al. Biosynthesis of copper oxide nanoparticles mediated Annona muricata as cytotoxic and apoptosis inducer factor in breast cancer cell lines. *Sci. Rep.* **2022**, *1212*, 16165. [CrossRef] [PubMed]
87. Abdelhakm, L.O.; Kandil, E.I.; Mansour, S.Z.; El-Sonbaty, S.M. Chrysin Encapsulated Copper Nanoparticles with Low Dose of Gamma Radiation Elicit Tumor Cell Death through p38 MAPK/NF-κB Pathways. *Biol. Element Res.* **2023**, *201*, 5278–5297. [CrossRef] [PubMed]
88. Benguigui, M.; Weitz, I.S.; Timaner, M.; Kan, T.; Shechter, D.; Perlman, O.; Sivan, S.; Raviv, Z.; Azhari, H.; Shaked, Y. Copper oxide nanoparticles inhibit pancreatic tumor growth primarily by targeting tumor initiating cells. *Sci. Rep.* **2019**, *99*, 12613. [CrossRef] [PubMed]
89. Mohanraj, V.; Chen, Y. Nanoparticles—A review. *Trop. J. Pharm. Res.* **2006**, *5*, 5561–5573. [CrossRef]
90. Ingle, A.P.; Duran, N.; Rai, M. Bioactivity, mechanism of action, and cytotoxicity of copper-based nanoparticles: A review. *Mikrobiol. Biotechnol* **2014**, *98*, 1001–1009. [CrossRef]
91. Gupta, A.; Lutsenko, S. Human copper transporters: Mechanism, role in human diseases and therapeutic potential. *Future Med. Chem.* **2009**, *1*, 1125–1142. [CrossRef]
92. Miyajima, H. Genetic disorders affecting proteins of iron and copper metabolism: Clinical implications. *Intern. Med.* **2002**, *41*, 762–769. [CrossRef]
93. Liu, J.; Luan, J.; Zhou, X.; Cui, Y.; Han, J. Epidemiology, diagnosis, and treatment of Wilson's disease. *Intractable Rare Dis. Res.* **2017**, *6*, 249–255. [CrossRef]
94. Szeligowska, J.; Ilczuk, T.; Nehring, P.; Górnicka, B.; Litwin, T.; Członkowska, A.; Przybyłkowski, A. Liver injury in Wilson's disease: An immunohistochemical study. *Adv. Med. Sci.* **2022**, *67*, 203–207. [CrossRef] [PubMed]
95. Panel on Micronutrients, and Nutrition Board. *Dietary Reference Intakes for Vitamin A, Vitamin K, Arsenic, Boron, Chromium, Copper, Iodine, Iron, Manganese, Molybdenum, Nickel, Silicon, Vanadium, and Zinc*; Dietary Reference Intakes; National Academies Press: Washington, DC, USA, 2002.
96. Nutrition Standards for the Population of Poland and Their Application. Available online: https://ncez.pzh.gov.pl/wp-content/uploads/2021/03/normy_zywienia_2020web.pdf (accessed on 1 February 2020).
97. Jarosz, M.; Rychlik, E.; Stoś, K.; Charzewska, J. *Food Composition Tables*, 2nd ed.; PZWL Medical Publishers: Warsaw, Poland, 2017.
98. Lead and Copper in School Drinking Water Sampling Results. Summary and Detailed Lead and Copper in Drinking Water Data in School Drinking Water. Available online: https://www.mass.gov/info-details/lead-and-copper-in-school-drinking-water-sampling-results (accessed on 26 April 2016).
99. Ishizuka, O.; Yuasa, M.; Uto, K. Evidence of porphyry copper-type hydrothermal activity from a submerged remnant back-arc volcano of the Izu-Bonin arc implications for the volcanotectonic history of back-arc seamounts. *Earth Planet. Sci. Lett.* **2002**, *198*, 381–399. [CrossRef]
100. Basic Information about Copper (Podstawowe Informacje o Miedzi). Available online: https://www.pgi.gov.pl/psg-1/psg-2/informacja-i-szkolenia/wiadomosci-surowcowe/9795-miedz-i-srebro.html (accessed on 25 January 2019).
101. COPPER—From the 1st Metal Used by Humans to the Essential Element in Technology Development_PART II—How Is Copper Capable to Harness the Technology Development in Digital Age? Available online: https://americanbobcat.blog/2021/05/17/copper-from-the-1st-metal-used-by-humans-to-the-essential-element-in-technology-development_part-ii-how-is-copper-capable-to-harness-the-technology-development-in-digital-age/ (accessed on 17 May 2017).
102. Copper, Silver. Available online: https://www.pgi.gov.pl/images/muzeum/kopalnia_wiedzy/surowce/foldery/miedz_i_srebro.pdf (accessed on 31 July 2020).
103. Ivanova, I.A.; Daskalova, D.S.; Yordanova, L.P.; Pavlova, E.L. Copper and Copper Nanoparticles Applications and Their Role against Infections: A Minireview. *Processes* **2024**, *12*, 352. [CrossRef]
104. Montagnino, E.; Lytle, D.A.; Rose, J.; Cwiertny, D.; Whelton, A.J. School and childcare center drinking water: Copper chemistry, health effects, occurrence, and remediation. *AWWA Water. Sci.* **2022**, *4*, e1270. [CrossRef] [PubMed]

105. Taylor, A.A.; Tsuji, J.S.; Garry, M.R.; McArdle, M.E.; Goodfellow, W.L., Jr.; Adams, W.J.; Menzie, C.A. Critical Review of Exposure and Effects: Implications for Setting Regulatory Health Criteria for Ingested Copper. *Environ. Manag.* **2020**, *65*, 131–159. [CrossRef]
106. Cradock, A.L.; Everett Jones, S.; Merlo, C. Examining differences in the implementation of school water-quality practices and water-access policies by school demographic characteristics. *Prev. Med. Rep.* **2019**, *14*, 100823. [CrossRef]

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Review

Keratins 6, 16, and 17 in Health and Disease: A Summary of Recent Findings

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Abstract: Keratins 6, 16, and 17 occupy unique positions within the keratin family. These proteins are not commonly found in the healthy, intact epidermis, but their expression increases in response to damage, inflammation, and hereditary skin conditions, as well as cancerous cell transformations and tumor growth. As a result, there is an active investigation into the potential use of these proteins as biomarkers for different pathologies. Recent studies have revealed the role of these keratins in regulating keratinocyte migration, proliferation, and growth, and more recently, their nuclear functions, including their role in maintaining nuclear structure and responding to DNA damage, have also been identified. This review aims to summarize the latest research on keratins 6, 16, and 17, their regulation in the epidermis, and their potential use as biomarkers in various skin conditions.

Keywords: keratins; keratin 17; keratin 16; keratin 6; psoriasis; cancer; skin; wound healing

1. Introduction

Keratins are a superfamily of intermediate filamentous proteins that play important roles in the organization of the cytoskeleton. The cytoskeleton of mammalian epithelial cells is formed from pairs of type I and type II keratins. Type I keratins usually have a lower mass (40–56.5 kDa) and a more acidic total charge (pI 4.5–6.0), while type II keratins have a larger mass (50–70 kDa) and a basic neutral charge (pI 6.5–8.5) [1]. The heterodimers of keratin types I and II self-assemble into antiparallel, staggered tetramers, forming intermediate filaments through longitudinal and lateral interactions [2]. These keratin intermediate filaments (KIFs) have a diameter of approximately 10 nanometers and form a network within the cytoskeleton of cells, providing structural support and mechanical resilience.

The patterns of keratin expression in different tissues are not universal and mostly characterize specific types of epithelia. For example, keratins 8 and 18 are expressed in the simple epithelium, along with additional keratins 7, 19, 20, and 23. In contrast, stratified epithelium is characterized by keratins K4/K13 in mucous membranes and keratins K3/K12 in the cornea (reviewed by Kalabusheva et al. [3]). In the epidermis, actively proliferating basal keratinocytes express high levels of K5/K14 and low levels of K15. During epidermal differentiation, the proliferation of keratinocytes decreases, and keratins 5 and 14 are replaced by keratins 1 and 10 as the cells migrate to higher layers of the epidermis [4–6].

Moreover, the expression of certain keratins in the epidermis is highly restricted to specific anatomical sites. Keratin K6 (*KRT6*) and its polymerization partners K16 and K17 (*KRT16* and *KRT17*, respectively) are only constitutively expressed in the palmoplantar epidermis as well as in skin appendages, including the nail plate, hair follicles, and sebaceous and sweat glands [2,7,8]. Keratins K6/16/17 are not detected in intact normal interfollicular epidermis; however, their expression significantly increases in wounds and is maintained at a high level throughout all stages of regeneration until the complete restoration of epithelial barrier function [2,9,10]. At the same time, the expression of K1/K10 rapidly

decreases in keratinocytes at the wound's edge, and K6/K16 induction and dimerization occur, accompanied by an increase in the level of cytoplasmic keratin K17 [10]. K6, K16, and K17 are known as damage-associated keratins, and they are often referred to as "stress keratins" because they play a central role in the process of damage regeneration.

High expression of these keratins has been detected in hyperproliferating keratinocytes in psoriasis and other inflammatory skin conditions [2,11,12]. In addition, in clinical settings, keratin K17 is well known as an oncogenic protein, the high expression of which is often linked to a poor prognosis. Additionally, an increasing number of recent studies have focused on the roles of keratins K16 and K6 in relation to carcinogenesis and tumor progression [13–15].

The study of molecular physiological processes regulated by keratin 6/16/17 has revealed many functions that are atypical for these proteins. These include the regulation of cell migration, growth, proliferation, apoptosis (programmed cell death), nuclear morphology, and responses to DNA damage. This review aimed to summarize the latest research data on the biological functions of these proteins, their encoded genes, and their roles under normal and abnormal conditions. Their potential as clinical markers and targets for therapy are also discussed.

2. Structure and Localization of K6/K16/K17

Keratin 17 is a type I keratin with a molecular weight of 48 kDa. It has a triplet structure consisting of 432 amino acids. Its structure (Figure 1A) includes a nonhelical head region (1–83), an alpha-helical rod region (84–392), and a nonhelical tail region (393–432) [16,17]. Within the core domain, there are segments containing heptadic repeats (coils 1A, 1B, and 2), which are interrupted by linker sequences at two conserved sites. Keratin 16 is also a type I keratin with a molecular weight of 50,924.66 Da. It is structurally similar to keratin 17, but it is slightly larger, consisting of 473 amino acids [1]. A mini family of three genes (*KRT6A*, *KRT6B*, and *KRT6C*) encodes homologous intermediate filaments of type II. The protein encoded by the *KRT6A* gene is dominant in this family. All genes in this mini family encode proteins with 564 amino acids, but the calculated molecular weights of these proteins vary slightly [9].

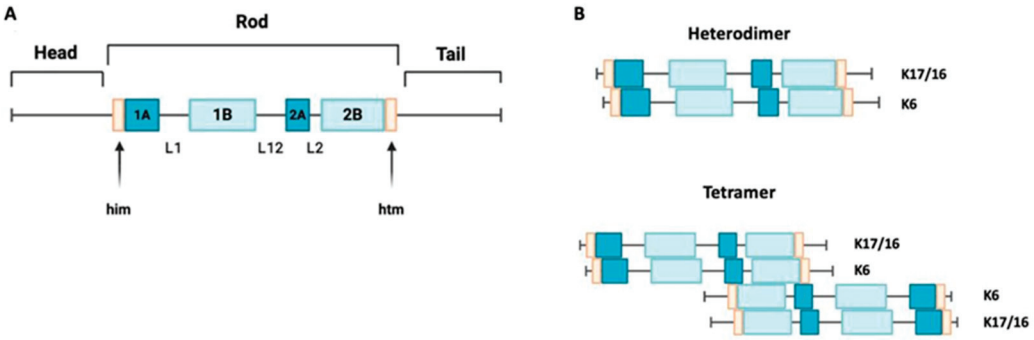


Figure 1. Keratin 17 structure and assembly. (**A**) A schematic structure of keratin 17 showing non-helical domains at the N-terminus and C-terminus ends, with a central helical rod domain. K17's structure consists of four alpha-helical domains (1A, 1B, 2A, and 2B), separated by three non-helical linkers (L1, L12, and L2). K17 also has two motifs, the helix initiation (him) and termination (hit) motifs, located at the ends of domains 1A and 2B, respectively. (**B**) A depiction of the alignment of the acidic type I keratins K17 and K16 along with the basic keratin K6, arranged in parallel to create a heterodimer. These heterodimers align in an antiparallel and staggered fashion to form a tetramer.

Keratins 16 and 17 can form dimers with keratin 6 (Figure 1B), so the expression of these intermediate filaments is often colocalized [18]. To investigate this further, data from the Genotype-Tissue Expression (GTEX) database were analyzed in a recent study. The goal was to reconstruct the tissue-specific expression patterns of keratins in the human

body. This study confirmed the co-expression of several pairs of keratin genes, including *KRT6* and *KRT16*, as well as *KRT6* and *KRT17* [19].

These data are in line with the fact that keratins K6, K16 and K17 are normally co-expressed in the palmar and plantar epidermis, as well as in epithelial appendages such as hair follicles. They are also found in epidermal lesions and in various pathological skin conditions [2,20,21]. In hair follicles, K6/K16/K17 are localized in the cells of the outer root sheath and in the cells of the companion layer and medulla [3,22–24].

In addition to the skin and its derivatives, K17 is expressed in normal basal cells of the respiratory epithelium, urothelial cells, squamous epithelial cells of the mucosal membrane, and basal cells of the oral cavity epithelium [10,25–28]. Keratin K16 has been detected at the protein level in various epithelial tissues and in nonkeratinizing stratified squamous epithelia [25,29,30]. K6 and K16 are also expressed in the duct cells of the mammary glands and sweat glands [31]. It has been reported that keratin 6 is expressed in the multilayered epithelium of the mucous membranes of the oral cavity, esophagus, and glandular epithelium [3,23,25]. However, given its colocalization with keratins K16 and K17, it is reasonable to assume that K6 is expressed in all types of epithelial cells expressing K16 and K17.

3. K6, K16, and K17 Are Essential for Regeneration

Proliferation is the final stage of skin wound healing, during which tissue integrity is restored due to cell division. At the same time, keratinocytes, which are the cells that make up the outermost layer of the skin, fill the damaged area by migrating and proliferating and participate in restoring the epidermal barrier through epidermal differentiation. During the healing process, keratinocytes near the wound temporarily suspend epidermal differentiation and undergo changes in protein translation, cell size, shape, and contact with other cells and the extracellular matrix, preparing for active migration and proliferation. Immediately after injury, keratinocytes at the edge of the wound significantly reduce the expression of keratin K1/K10 and induce the expression of keratins K6, K16, and K17 [2,32–34]. The high expression of keratins K6, K16, and K17 provides keratinocytes with the mechanical resilience required for migration and structural support [35]. However, the role of these keratins in regeneration extends beyond their structural function. The modulation of cell migration and adhesion to substrates is critical for successful regeneration and is an essential physiological function of keratins K6, K16, and K17. To close the defect in the epidermis, keratinocytes at the wound edge must first weaken their adhesion to each other and the basement membrane. This process is facilitated by alterations in the composition of the keratin cytoskeleton and desmosomes, which are considered the main factors determining adhesive strength. Wound healing is accompanied by a temporary reduction in desmosome adhesion, while keratin expression increases. Keratins 5/14 have been shown to contribute to the stabilization of desmosomes, while keratins K6/K17, on the contrary, lead to the destruction of desmosomes through the induction of protein kinase C and subsequent destabilization of epithelial layers [36]. Keratin 6 is able to interact directly with myosin II, which promotes cell adhesion and provides additional resistance to mechanical stress [37,38]. In addition, keratin 6 is expressed in cells at the edge of a wound, where it plays a role in regulating the collective migration of keratinocytes by inhibiting Src [39]. It also regulates cell–matrix interactions and intercellular adhesion [37]. Notably, keratinocytes isolated from *KRT6A*-/*KRT6B*-mice exhibit increased migration [38]. It has been shown in HaCaT keratinocytes that overexpression of keratin 16 leads to a decrease in migration activity. This decrease can be partially reversed by co-transfecting K6A [40]. In contrast, keratin 17 enhances the migratory activity of keratinocytes, as shown by data obtained from HaCaT cells in a scratch wound assay. The knockdown of *KRT17* results in a decrease in the rate of diabetic wound healing in mouse skin [33]. Keratin 17 promotes damage repair by inducing cell proliferation through STAT3 [17] and modulates lipid metabolism in keratinocytes to restore epidermal barrier functions [41]. Generally,

keratins K6/16 provide optimal intercellular adhesion and collective migration for damage repair, while keratin 17 induces cell migration and proliferation.

4. K6, K16, and K17 Are Involved in the Pathogenesis of Various Skin Diseases

4.1. Psoriasis

The involvement of K17 in the development of psoriasis was first identified in 1990 [42]. In 1995, Leigh and his colleagues discovered that K17 can be found in the upper layers of psoriatic skin both in vivo and in vitro [11]. This discovery formed the basis for further research in this area. Since then, the understanding of K17 as a marker for psoriasis has increased, and studies have shown that its level correlates with the severity of the condition [43]. The role of keratin 17 in the pathogenesis of this disease is primarily realized by inducing keratinocyte proliferation through the STAT3 and 14-3-3/mTOR pathways and modulating the immune response [44,45]. Keratin 17 is an autoantigen that stimulates the production of proinflammatory cytokines by T cells [44]. These cytokines, in turn, induce the expression of stress keratins. Interestingly, the a/a sequence (amino acids 102–116 and 188–196) of keratin 17 closely resembles that of the streptococcal M6 protein, a superantigen in psoriasis [44,46]. Keratins 6 and 16 are highly expressed in psoriatic plaques and are characteristic of psoriatic keratinocytes [47]. However, their role in the pathogenesis of psoriasis has been less well studied than that of keratin 17. Keratin 16 has also been shown to contribute to the hyperproliferation of keratinocytes in psoriasis, and silencing keratin 16 leads to a reduction in VEGF secretion by psoriatic keratinocytes [12]. Keratin 16 is also involved in regulating inflammation by modulating damage-associated molecular patterns (DAMPs) and cytokines through the mitogen-activated protein kinase (MAPK) and/or epidermal growth factor receptor (EGFR) pathways [48].

4.2. Atopic Dermatitis

Atopic dermatitis (AD), also known as eczema, is a common chronic inflammatory autoimmune skin disorder. Increased expression of *KRT6A* has been observed in patients with this condition. In keratinocytes from patients with AD, *KRT6A* appears to inhibit inflammation-induced apoptosis and suppress IL-17A expression, making the K6A/IL-17 pathway a potential target for treatment [49]. In addition, a recent study revealed a correlation between variations in the *KRT6A* gene (single nucleotide polymorphisms) and disease severity [50]. Although there is less evidence to support the role of keratin 16 in AD, recent research has shown that increased expression of *KRT16* may be a marker for hyperproliferative keratinocytes in AD, and *KRT16*'s expression is significantly reduced in patients treated with dupilumab for AD [51,52]. Although it is not yet clear whether keratin 17 plays a role in the development of AD, this condition is characterized by an increase in the expression of several cytokines that trigger the production of keratin 17 [53].

4.3. Pachyonychia Congenita

Pachyonychia congenita (PC) is a rare genetic disorder that is inherited in an autosomal dominant pattern. It is characterized by keratoderma in the palms and soles of the feet, nail dystrophy, cyst formation, hyperkeratosis of hair follicles, and leukokeratosis of mucous membranes. Hyperhidrosis is also a common symptom of this condition. Mutations in genes such as *KRT6A*, *KRT16*, and *KRT17* have been found in patients with PC [54–56]. In particular, the Asn92Asp mutation in the *KRT17* gene and the Leu130Pro mutation in *KRT16* (both of which are located in the helix initiation motif) have been linked to this condition. Additionally, mutations in *KRT6A* and *KRT6B* have been identified in some patients with PC [57,58]. Mutations of p.Tyr465His in exon 7 of *KRT6A* and p.Glu413Gln in exon 6 of *KRT16* have also been linked to PC [59].

To date, progress in PC treatment is still unsatisfactory, and many therapies are at different stages of clinical development. Naturally, keratin 6/16/17 have been identified as potential therapeutic targets for PC. Sirolimus (rapamycin), an mTOR inhibitor, has been shown to reduce the expression of keratin 6A in human keratinocytes and reduce

the severity of PC symptoms, although it has some side effects [60,61]. Other approaches include the use of statins for Stat1 repression [62], botulinum toxin injections [63], and siRNA therapy [64,65]. These methods involve complex and painful medical procedures that are not perfect. Therefore, the development of new therapies for treating PC or reducing its symptoms is urgently needed. Since PC serves as a prototype for siRNA-based therapies, genome editing techniques such as CRISPR-based therapeutics could potentially be considered for future research.

4.4. Other Skin Pathologies

In addition to psoriasis and PC, keratin 17 is also involved in the development of other skin conditions, such as lichen planus (LP). The pathogenesis of these conditions shares some similarities, including increased expression of keratin 17. In lichen planus, this expression is triggered by the induction of interferon gamma (IFN-γ) [66]. Similarly, *KRT6A*, *KRT6B*, and *KRT6C* are increased in patients with LP [67]. Elevated levels of *KRT17* have been noted in patients with prurigo nodularis (PN), a chronic inflammatory dermatosis characterized by the presence of multiple hyperkeratotic papules [68]. In addition, in PN, there is increased expression of K6 in all layers of the skin with lesions, particularly in the spinous layer. Conversely, K16 has been detected only in the basal and lower suprabasal layers [69]. In another study, using single-cell RNA profiling, increased expression of the *KRT6A*, *KRT6B*, *KRT6C*, and *KRT16* genes significantly characterized PN keratinocytes [70]. Inflammatory skin diseases are likely to lead to the production of these keratins, as the expression of K6/K16/K17 is associated with skin damage and strongly upregulated by proinflammatory cytokines.

5. Cancer

For many types of cancer, keratin 17 can be used as a diagnostic, prognostic, and predictive marker. Its increased expression often correlates with tumor aggressiveness and has a negative prognosis [71–75]. According to the analysis of data from various types of cancer, based on The Gene Expression Omnibus (GEO), The Cancer Genome Atlas (TCGA), and The Human Protein Atlas (HPA), as well as TIMER2 and other sources, a high level of keratin 17 was noted in most malignant neoplasms compared to normal tissues [76,77]. In addition, high expression of keratin 17 has been correlated with tumor size, depth of invasion, and metastasis in gastric cancer [78]. It has also been found to determine the low level of response to therapy in patients with squamous cell carcinoma of the head and neck (HNSCC), especially in those treated with immune checkpoint blockades [79]. Keratin 17 has been reported to be a negative prognostic biomarker for several types of cancer, including squamous cell carcinoma of the lungs (LSCC) and pulmonary adenocarcinoma (LUAD) [80]. It has also been linked to pancreatic ductal adenocarcinoma (PDAC) [81] and colorectal cancer (CRC) [82]. Keratin 17 has been shown to induce and promote the growth of oral squamous cell carcinoma (OSCC) [83]. Although the expression of keratin 17 is generally associated with an unfavorable prognosis for cancer patients, this trend does not always hold true. For example, in invasive breast carcinoma (BRCA) and chromophobic kidney cancer (KICH), the levels of keratin 17 are lower than those in normal tissue. Additionally, there are no significant differences in the levels of keratin 17 between tumors and normal tissues in urothelial bladder carcinoma (BLCA), papillary carcinoma of the kidney (KIRP), or pancreatic adenocarcinoma (PAAD) [16].

The involvement of K6 and K16 in carcinogenesis and tumor progression has been studied to a lesser extent. However, the number of studies characterizing these oncoproteins has increased in recent years. Keratin 16, in particular, has been identified as a marker for circulating tumor cells. Its high expression in breast cancer samples has been correlated with an intermediate mesenchymal phenotype [14]. Huang and colleagues demonstrated that high levels of keratin 16 are associated with a poorer prognosis, pathological differentiation, and lymph node involvement in patients with oral squamous cell carcinoma (OSCC) [13]. Increased expression of keratin 6A and the involvement of keratin 16 in the

development of this type of tumor have also been reported in LUAD [84]. For keratin 6, a correlation with squamous cell differentiation in As-transformed UROtsa cells has been shown [85]. In addition, increased expression of *KRT6A/B/C* has also been observed in primary melanoma cells, and high levels of these keratins have been associated with a poor prognosis for patients with skin cutaneous melanoma (SKCM) [20]. High levels of keratin 6A are associated with a poorer prognosis and increased invasiveness in colorectal cancer (CRC) [86]. Data on the diagnostic significance of K6, K16, and K17 proteins are summarized in Table 1.

Table 1. Expression of K6/16/17 in various types of cancer.

Cancer Type	Gene			References
	KRT17	*KRT16*	*KRT6*	
Oral squamous cell carcinoma (OSCC)	+	+	+	[13,87]
Lung adenocarcinoma (LUAD)	+	+	+	[15,80,84]
Invasive breast carcinoma (BRCA)	+/−		+	[88,89]
Ovarian cancer	+		+	[90,91]
Urothelial carcinoma (UCC)		+	+	[92]
Bladder cancer			+	[93]
Gastric cancer	+	+		[78,94]
Pancreatic ductal adenocarcinoma (PDAC)	+	+		[81,95,96]
Esophageal cancer (ESCC)	+	+		[97,98]
Tylosis with esophageal cancer (TOC)		+		[98]
Hypopharyngeal squamous carcinoma (HSCC)	−			[99]
Colorectal cancer (CRC)	+		+	[82,86]
Lung squamous cell carcinoma (LSCC)	+			[80]
Cervical cancer (CC)	+			[100]
Endometrial carcinoma	+			[74]
Chromophobe renal cell carcinoma (KICH)	+			[16]
Papillary renal cell carcinoma (KIRP)	n.d.			[16]
Pancreatic adenocarcinoma (PAAD)	n.d.			[16]
Thyroid cancer	+			[101]
Basal cell carcinoma (BCC)	+			[102]
Basaloid follicular hamartoma (BFH)	+			[102]
Head and neck squamous cell carcinoma (HNSCC)	+			[79,103]
Cutaneous squamous cell carcinoma (cSCC)	+	+	+	[104–106]

Symbol explanation: "+"—upregulated, "−"—downregulated, "n.d."—no difference between normal and cancer tissue).

6. The Role of K6, K16, and K17 in Carcinogenesis

Keratin 17 plays a role in the development of cancer through several key processes: (I) promoting cell growth and proliferation; (II) supporting cell survival; and (III) modulating the immune system's response. Keratin 17 has been shown to be involved in regulating the proliferation, migration, and invasion of tumor cells. For example, it has been found to be important in the development of gastric cancer [78] and osteosarcoma [107], among other types of cancer. Thus, keratin 17 promotes cell proliferation and migration by activating Akt/mTOR [83,108], and its inhibition can lead to a reduction in the activity of ERK1/2, Bad, p38 MAPK, and SAPK/JNK [12]. Keratin 17 also contributes to the uncontrolled progression of the cell cycle by promoting the degradation of $p27^{KIP1}$; therefore, K17 may be considered a marker for uncontrolled proliferation [109]. The discovery of the role of K17 in regulating the DDR response and cell survival suggests that one possible mechanism of carcinogenesis is the maintenance of cell viability with DNA damage. This, in turn, could contribute to the accumulation of mutations and genomic instability [110]. Suppression of keratin 17 expression can lead to the induction of tumor cell apoptosis through changes in the levels of Bcl-2 family proteins and enhanced regulation of caspase-3 cleavage. This contributes to stopping the cell cycle at the G1/S phase by reducing the expression of cyclin E1 and cyclin D [78]. Another important aspect of tumor progression is the role of

keratin 17 in regulating glycolysis through its co-expression with HIF1α. When HIF1α is translocated to the nucleus, it binds to the promoters of specific target genes, such as GLUT1 [107]. In addition, keratin 17 covalently binds to α-enolase (ENO1), which supports its phosphorylation by K17-Ser44. This phosphorylation enhances the nuclear translocation of keratin 17 and the transcriptional activation of LDHA, leading to increased glycolysis and cell proliferation [8].

In addition, K17 plays a role in the process of tumor cells evading immune surveillance. CD8+ T cells have been shown to play a role in the recognition and induction of apoptosis in tumor cells, while K17 can block the infiltration of these cells into tumor tissues in some cancers. This has been demonstrated, in particular, in pancreatic cancer and proliferative processes associated with the papilloma virus [111]. At the same time, KRT17 knockdown slows down HNSCC growth and increases the infiltration of CD8+ T cells [103]. In addition, K17 suppresses macrophage-mediated CXCL9/CXCL10 chemokine signaling [112,113]. K17 also plays an oncogenic role by inducing the expression of IFN-γ, tumor necrosis factor alpha (TNF-α), and interleukin 10 (IL-10). These cytokines lead to the activation of different types of macrophages [114,115].

An increasing number of studies have investigated the functions of keratins 6 and 16 in cancer development and progression. One study revealed that silencing *KRT6A* led to a reduction in the expression of TIM-2 and MMP-9, two proteins involved in tumor invasion and metastasis in nasopharyngeal carcinoma [116]. A recent study revealed that KRT6A contributes to the progression of bladder cancer by enhancing cell viability, proliferation, and adhesion while also inhibiting cancer cell apoptosis [93]. In addition, activation of Notch1 in kidney cancer cells may lead to increased expression of *KRT6* [117]. *KRT6A* overexpression in LUAD promotes tumor cell proliferation, epithelial-to-mesenchymal transition (EMT), and metastasis [15]. Keratin 16 has also been reported to promote tumor progression and metastasis by inducing epithelial-to-mesenchymal transition (EMT), as has been demonstrated in LUAD and MCF-7 cells [14,84]. In addition, keratin 16 inhibits the degradation of vimentin, which can also promote metastasis [118]. A recent study showed that iRhom2 acts as a positive regulator of keratin 16. GOF mutations in iRhom2, which are found in tumors with esophageal cancer (TOC), support tumor hyperproliferation by modulating the expression of K16 [97].

7. Cell Functions of K6, K16, and K17

The most studied function of keratin 17 is its positive regulation of cell growth and proliferation. KRT17-deficient keratinocytes are smaller and exhibit reduced translation and suppressed expression of the mTOR/AKT signaling pathway, which is essential for regulating cell growth and synthesis [119]. In addition, keratin 17 promotes the phosphorylation of STAT3 and its nuclear transport, leading to the induction of cyclin D1 and the hyperproliferation of keratinocytes [17]. Phosphorylated keratin 17 regulates cell growth by binding to the adapter protein 14-3-3σ. In contrast, hypophosphorylation of keratin 17 leads to the sequestration of 14-3-3σ in the nucleus, inhibiting protein synthesis and cell growth [119]. Recent studies have revealed the functions of keratin 17 in the nuclear pool. This protein regulates the morphology of the nucleus, the structure of chromatin, RNA processing, and gene expression. It is also involved in regulating the DNA damage response by interacting with various proteins that are involved in DDRs, including Aire [120], hnRNPK [114], H2AX [121], DNA-PKcs [121], 53BP1 [121], and HMGN2 [110]. In addition, recent research has revealed that keratin 17 is essential for the overall regulation of gene expression. However, most of the mechanisms involved are still unknown [122].

The functions of keratin 16 have not been studied in detail, but it is known to play a role in regulating inflammation and innate immunity after damage to the skin barrier. Keratin 16 may influence the MAPK and EGFR signaling pathways, thereby regulating the overall levels of DAMPs [48]. K16 is also thought to be involved in protecting against oxidative stress through its interaction with the Nrf2 pathway. A recent finding was that keratin 16 helps maintain the normal redox balance in cells by activating Nrf2 and

stimulating glutathione synthesis [123]. In addition, keratin 16 and its partner, keratin 6, are involved in regulating mitochondrial morphology, which could explain the signs of oxidative stress observed in PC patients [123].

The currently known functions of keratin 6 mainly involve regulating keratinocyte migration through enhancing cellular adhesion and attachment to the substrate. This is achieved through the interactions between keratin 6a/6b and myosin II and desmoplakin [37]. However, there is still potential for discovering new and unexpected roles for keratin 6. Studies have shown that the addition of recombinant keratin 6A to cell culture media can slow proliferation and stabilize stem cells while also inducing the expression of stem cell-related genes such as *CD133*, *OCT4*, *KLF4*, *SOX2*, *NANOG*, and *ANAX2* [124]. The cell functions of K6, K16, and K17 are illustrated in Figure 2.

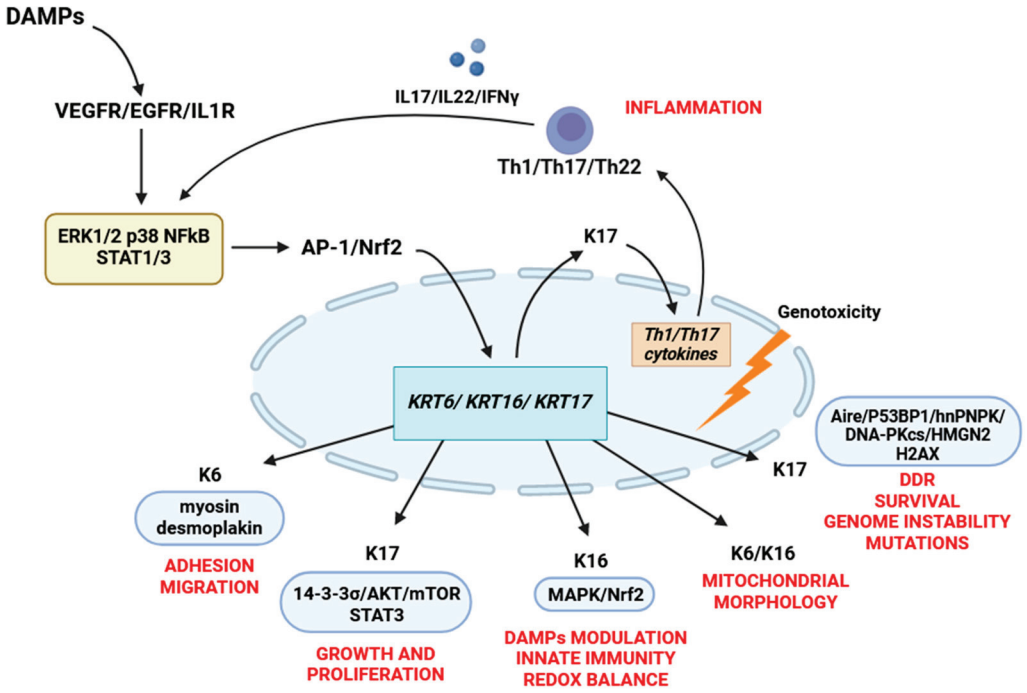


Figure 2. The cell functions of K6, K16, and K17. Damaged keratinocytes release various DAMPs, which induce the expression of K6/16/17 by activating the AP-1, NFkB, Nrf2, MAPK (ERK1/2 and p38) pathways. Altered expression of K17 promotes the secretion of Th1 cytokines (IFNγ, IL17A, and IL22) that maintain K6/16/17 expression through an autoimmune feedback loop. Keratin 17 regulates cell growth and proliferation by binding to the 14-3-3σ/AKT/mTOR pathway and STAT3 phosphorylation. When located in the nucleus, keratin 17 is involved in the DNA damage response by interacting with various DNA damage response proteins, including Aire, hnRNPK, H2AX, DNA-PKcs, 53BP1, and HMGN2. Keratin 16 modulates the secretion of DAMPs through the MAPK and EGFR signaling pathways and plays a role in maintaining the redox balance by interacting with the Nrf2 signaling pathway. Keratins K6 and K16 also play a role in regulating the morphology and functions of mitochondria. Keratin 6 is involved in regulating cell adhesion by directly interacting with myosin IIA and desmosomal proteins, which provide the mechanical properties necessary for wound healing.

8. Regulation of K6, K16, and K17

One of the common properties of all cytokeratins is the ability of their promoters to bind various transcription factors. Among these transcription factors, those involved in the regulation of KRT6/16/17 include proteins from the AP-1 and NFκB signaling pathways. Specifically, in cooperation with Sp1, AP-1 can activate the promoters of *KRT6* and *KRT16* [125,126]. Considering that the AP-1 and NFκB response elements can be physically separated, it is assumed that the interaction between these signaling pathways

regulates the expression of epidermal genes. Activation of these factors leads to the differential expression of cytokeratins during epidermal differentiation, wound healing, and skin diseases.

Another transcription factor that regulates *KRT6/KRT16/KRT17* expression is Nrf2 [47,127]. Nrf2 regulates the expression of keratins 6, 16, and 17 by binding to specific regions of their promoters. These regions, known as ARE (antioxidant response element) domains, are located within the DNA sequences encoding these keratins [47]. Nrf2 is highly expressed in various types of skin cancer and other diseases. Its increased regulation can lead to the hyperproliferation of keratinocytes by inducing the expression of keratins 16 and 17 [47,127]. All three keratins are transcriptionally activated by the Hedgehog-regulated factors GLI1 and GLI2, suggesting a connection between these keratin proteins and the Hedgehog signaling pathway [128–130].

Some members of the p53 family of transcription factors are also involved in the regulation of K6/K16/K17. P53 is essential for the normal differentiation of epithelial cells [131]. The mechanisms by which p53 affects keratin are complex and involve both the activation and suppression of genes. p53 can indirectly suppress the expression of Krt14 in basal keratinocytes, contributing to their differentiation [132]. However, a recent study showed that removing p53 promotes differentiation by partially regulating the expression of *KRT6A* [133]. Therefore, the effect of p53 is complex and multifaceted. It directly binds to the promoter region of the *KRT17* gene and negatively regulates its expression. However, the induction of *KRT17* expression does not depend on the presence of p53 [134]. Additionally, the promoter sequence of *KRT17* contains two p53 response elements [134].

Other important factors involved in the regulation of K6/16/17 include cytokines and growth factors. Moreover, the initial activation of these proteins is carried out autonomously by keratinocytes. Damaged keratinocytes secrete a variety of DAMPs, such as dsRNA, DNA, high mobility group box 1 (HMGB1), uric acid, and heat shock proteins (HSPs) [135–137]. These molecules are recognized by pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs). This leads to the activation of AP-1 and NFkB, which, in turn, activate *KRT6/16/17*. The expression of these keratins triggers the secretion of cytokines (IFN, IL17A, and IL22), which, in turn, support the expression of K6, K16, and K17 [2,37,43,128,134,138]. Epidermal growth factor (EGF) induces the expression of keratin on 6/16/17 through the NFκB and/or MAPK (ERK1/2) pathways [139–141]. TGF-β also activates the expression of keratin 17 in normal keratinocytes [17].

The expression of the K6, K16, and K17 genes can also be regulated in response to various external stimuli such as mechanical stimuli (stretching and scratches). These stimuli lead to the phosphorylation of the EGFR and ERK1/2 proteins, which, in turn, activate the expression of keratin 6 and repress the expression of K10 [142]. The expression of keratin 17 is induced by ionizing radiation (IR), and high levels of keratin 17 are characteristic of IR-induced dermatitis [134]. Keratin 17 is also induced by UV radiation from both UVA and UVB sources [143,144]. Our group reported that the expression of keratin 17 significantly increases in primary human keratinocytes exposed to cadmium in vitro [145]. Notably, a similar response was observed in HaCaT cells with knocked-out mutant p53, but not in wild-type HaCaT cells [146].

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References

1. Jacob, J.T.; Coulombe, P.A.; Kwan, R.; Omary, M.B. Types I and II Keratin Intermediate Filaments. *Cold Spring Harb. Perspect. Biol.* **2018**, *10*, a018275. [CrossRef] [PubMed]
2. Zhang, X.; Yin, M.; Zhang, L. Keratin 6, 16 and 17—Critical Barrier Alarmin Molecules in Skin Wounds and Psoriasis. *Cells* **2019**, *8*, 807. [CrossRef] [PubMed]

3. Kalabusheva, E.P.; Shtompel, A.S.; Rippa, A.L.; Ulianov, S.V.; Razin, S.V.; Vorotelyak, E.A. A Kaleidoscope of Keratin Gene Expression and the Mosaic of Its Regulatory Mechanisms. *Int. J. Mol. Sci.* **2023**, *24*, 5603. [CrossRef] [PubMed]
4. Rosdy, M.; Clauss, L.C. Terminal epidermal differentiation of human keratinocytes grown in chemically defined medium on inert filter substrates at the air-liquid interface. *J. Investig. Dermatol.* **1990**, *95*, 409–414. [CrossRef] [PubMed]
5. Sanz-Gómez, N.; Freije, A.; Gandarillas, A. Keratinocyte Differentiation by Flow Cytometry. *Methods Mol. Biol.* **2020**, *2109*, 83–92. [CrossRef] [PubMed]
6. Pondeljak, N.; Lugović-Mihić, L.; Tomić, L.; Parać, E.; Pedić, L.; Lazić-Mosler, E. Key Factors in the Complex and Coordinated Network of Skin Keratinization: Their Significance and Involvement in Common Skin Conditions. *Int. J. Mol. Sci.* **2024**, *25*, 236. [CrossRef]
7. Gunnarsson, M.; Larsson, S.; Malak, M.; Ericson, M.B.; Topgaard, D.; Sparr, E. Molecular Mobility in Keratin-Rich Materials Monitored by Nuclear Magnetic Resonance: A Tool for the Evaluation of Structure-Giving Properties. *Biomacromolecules* **2023**, *24*, 2661–2673. [CrossRef]
8. Luo, Y.; Pang, B.; Hao, J.; Li, Q.; Qiao, P.; Zhang, C.; Bai, Y.; Xiao, C.; Chen, J.; Zhi, D.; et al. Keratin 17 covalently binds to alpha-enolase and exacerbates proliferation of keratinocytes in psoriasis. *Int. J. Biol. Sci.* **2023**, *19*, 3395–3411. [CrossRef] [PubMed]
9. Takahashi, K.; Paladini, R.D.; Coulombe, P.A. Cloning and characterization of multiple human genes and cDNAs encoding highly related type II keratin 6 isoforms. *J. Biol. Chem.* **1995**, *270*, 18581–18592. [CrossRef] [PubMed]
10. McGowan, K.M.; Coulombe, P.A. Onset of keratin 17 expression coincides with the definition of major epithelial lineages during skin development. *J. Cell Biol.* **1998**, *143*, 469–486. [CrossRef]
11. Leigh, I.M.; Navsaria, H.; Purkis, P.E.; McKay, I.A.; Bowden, P.E.; Riddle, P.N. Keratins (K16 and K17) as markers of keratinocyte hyperproliferation in psoriasis in vivo and in vitro. *Br. J. Dermatol.* **1995**, *133*, 501–511. [CrossRef] [PubMed]
12. Chen, J.-G.; Fan, H.-Y.; Wang, T.; Lin, L.-Y.; Cai, T.-G. Silencing KRT16 inhibits keratinocyte proliferation and VEGF secretion in psoriasis via inhibition of ERK signaling pathway. *Kaohsiung J. Med. Sci.* **2019**, *35*, 284–296. [CrossRef] [PubMed]
13. Huang, W.-C.; Jang, T.-H.; Tung, S.-L.; Yen, T.-C.; Chan, S.-H.; Wang, L.-H. A novel miR-365-3p/EHF/keratin 16 axis promotes oral squamous cell carcinoma metastasis, cancer stemness and drug resistance via enhancing β5-integrin/c-met signaling pathway. *J. Exp. Clin. Cancer Res.* **2019**, *38*, 89. [CrossRef] [PubMed]
14. Elazezy, M.; Schwentesius, S.; Stegat, L.; Wikman, H.; Werner, S.; Mansour, W.Y.; Failla, A.V.; Peine, S.; Müller, V.; Thiery, J.P.; et al. Emerging Insights into Keratin 16 Expression during Metastatic Progression of Breast Cancer. *Cancers* **2021**, *13*, 3869. [CrossRef] [PubMed]
15. Yang, B.; Zhang, W.; Zhang, M.; Wang, X.; Peng, S.; Zhang, R. KRT6A Promotes EMT and Cancer Stem Cell Transformation in Lung Adenocarcinoma. *Technol. Cancer Res. Treat.* **2020**, *19*, 1533033820921248. [CrossRef] [PubMed]
16. Zhang, H.; Zhang, Y.; Xia, T.; Lu, L.; Luo, M.; Chen, Y.; Liu, Y.; Li, Y. The Role of Keratin17 in Human Tumours. *Front. Cell Dev. Biol.* **2022**, *10*, 818416. [CrossRef] [PubMed]
17. Yang, L.; Jin, L.; Ke, Y.; Fan, X.; Zhang, T.; Zhang, C.; Bian, H.; Wang, G. E3 Ligase Trim21 Ubiquitylates and Stabilizes Keratin 17 to Induce STAT3 Activation in Psoriasis. *J. Investig. Dermatol.* **2018**, *138*, 2568–2577. [CrossRef] [PubMed]
18. Bernot, K.M.; Lee, C.-H.; Coulombe, P.A. A small surface hydrophobic stripe in the coiled-coil domain of type I keratins mediates tetramer stability. *J. Cell Biol.* **2005**, *168*, 965–974. [CrossRef] [PubMed]
19. Ho, M.; Thompson, B.; Fisk, J.N.; Nebert, D.W.; Bruford, E.A.; Vasiliou, V.; Bunick, C.G. Update of the keratin gene family: Evolution, tissue-specific expression patterns, and relevance to clinical disorders. *Hum. Genom.* **2022**, *16*, 1. [CrossRef]
20. Han, W.; Hu, C.; Fan, Z.-J.; Shen, G.-L. Transcript levels of keratin 1/5/6/14/15/16/17 as potential prognostic indicators in melanoma patients. *Sci. Rep.* **2021**, *11*, 1023. [CrossRef]
21. Cohen, E.; Johnson, C.N.; Wasikowski, R.; Billi, A.C.; Tsoi, L.C.; Kahlenberg, J.M.; Gudjonsson, J.E.; Coulombe, P.A. Significance of stress keratin expression in normal and diseased epithelia. *iScience* **2024**, *27*, 108805. [CrossRef] [PubMed]
22. Liu, Y.; Lyle, S.; Yang, Z.; Cotsarelis, G. Keratin 15 promoter targets putative epithelial stem cells in the hair follicle bulge. *J. Investig. Dermatol.* **2003**, *121*, 963–968. [CrossRef] [PubMed]
23. Langbein, L.; Schweizer, J. Keratins of the human hair follicle. *Int. Rev. Cytol.* **2005**, *243*, 1–78. [CrossRef] [PubMed]
24. Hafner, A.; Bulyk, M.L.; Jambhekar, A.; Lahav, G. The multiple mechanisms that regulate p53 activity and cell fate. *Nat. Rev. Mol. Cell Biol.* **2019**, *20*, 199–210. [CrossRef]
25. Moll, R.; Divo, M.; Langbein, L. The human keratins: Biology and pathology. *Histochem. Cell Biol.* **2008**, *129*, 705–733. [CrossRef]
26. Fleckman, P.; Jaeger, K.; Silva, K.A.; Sundberg, J.P. Comparative Anatomy of Mouse and Human Nail Units. *Anat. Rec.* **2013**, *296*, 521–532. [CrossRef] [PubMed]
27. Troyanovsky, S.M.; Leube, R.E.; Franke, W.W. Characterization of the human gene encoding cytokeratin 17 and its expression pattern. *Eur. J. Cell Biol.* **1992**, *59*, 127–137. [PubMed]
28. Troyanovsky, S.M.; Guelstein, V.I.; Tchipysheva, T.A.; Krutovskikh, V.A.; Bannikov, G.A. Patterns of expression of keratin 17 in human epithelia: Dependency on cell position. *J. Cell Sci.* **1989**, *93 Pt 3*, 419–426. [CrossRef] [PubMed]
29. Compton, C.C.; Warland, G.; Nakagawa, H.; Opitz, O.G.; Rustgi, A.K. Cellular characterization and successful transfection of serially subcultured normal human esophageal keratinocytes. *J. Cell. Physiol.* **1998**, *177*, 274–281. [CrossRef]
30. Bernot, K.M.; Coulombe, P.A.; McGowan, K.M. Keratin 16 expression defines a subset of epithelial cells during skin morphogenesis and the hair cycle. *J. Investig. Dermatol.* **2002**, *119*, 1137–1149. [CrossRef]

31. Karantza, V. Keratins in health and cancer: More than mere epithelial cell markers. *Oncogene* **2011**, *30*, 127–138. [CrossRef] [PubMed]
32. Mazzalupo, S.; Wong, P.; Martin, P.; Coulombe, P.A. Role for keratins 6 and 17 during wound closure in embryonic mouse skin. *Dev. Dyn.* **2003**, *226*, 356–365. [CrossRef] [PubMed]
33. Zhou, P.; Feng, H.; Qin, W.; Li, Q. KRT17 from skin cells with high glucose stimulation promotes keratinocytes proliferation and migration. *Front. Endocrinol.* **2023**, *14*, 1237048. [CrossRef] [PubMed]
34. McGowan, K.; Coulombe, P.A. The wound repair-associated keratins 6, 16, and 17. Insights into the role of intermediate filaments in specifying keratinocyte cytoarchitecture. *Subcell. Biochem.* **1998**, *31*, 173–204. [PubMed]
35. Tombultürk, F.K. Effects of keratin6/16 heterodimer on diabetic wound healing treatment with topical metformin. *Front. Life Sci. Relat. Technol.* **2024**, *5*, 65–73. [CrossRef]
36. Loschke, F.; Homberg, M.; Magin, T.M. Keratin Isotypes Control Desmosome Stability and Dynamics through PKCα. *J. Investig. Dermatol.* **2016**, *136*, 202–213. [CrossRef] [PubMed]
37. Wang, F.; Chen, S.; Liu, H.B.; Parent, C.A.; Coulombe, P.A. Keratin 6 regulates collective keratinocyte migration by altering cell–cell and cell–matrix adhesion. *J. Cell Biol.* **2018**, *217*, 4314–4330. [CrossRef] [PubMed]
38. Wong, P.; Coulombe, P.A. Loss of keratin 6 (K6) proteins reveals a function for intermediate filaments during wound repair. *J. Cell Biol.* **2003**, *163*, 327–337. [CrossRef] [PubMed]
39. Rotty, J.D.; Coulombe, P.A. A wound-induced keratin inhibits Src activity during keratinocyte migration and tissue repair. *J. Cell Biol.* **2012**, *197*, 381–389. [CrossRef]
40. Trost, A.; Desch, P.; Wally, V.; Haim, M.; Maier, R.H.; Reitsamer, H.A.; Hintner, H.; Bauer, J.W.; Önder, K. Aberrant heterodimerization of keratin 16 with keratin 6A in HaCaT keratinocytes results in diminished cellular migration. *Mech. Ageing Dev.* **2010**, *131*, 346–353. [CrossRef]
41. Pang, B.; Zhu, Z.; Xiao, C.; Luo, Y.; Fang, H.; Bai, Y.; Sun, Z.; Ma, J.; Dang, E.; Wang, G. Keratin 17 Is Required for Lipid Metabolism in Keratinocytes and Benefits Epidermal Permeability Barrier Homeostasis. *Front. Cell Dev. Biol.* **2022**, *9*, 779257. [CrossRef] [PubMed]
42. Wilson, C.L.; Dean, D.; Lane, E.B.; Dawber, R.P.; Leigh, I.M. Keratinocyte differentiation in psoriatic scalp: Morphology and expression of epithelial keratins. *Br. J. Dermatol.* **1994**, *131*, 191–200. [CrossRef] [PubMed]
43. Shi, X.; Jin, L.; Dang, E.; Chang, T.; Feng, Z.; Liu, Y.; Wang, G. IL-17A upregulates keratin 17 expression in keratinocytes through STAT1- and STAT3-dependent mechanisms. *J. Investig. Dermatol.* **2011**, *131*, 2401–2408. [CrossRef] [PubMed]
44. Jin, L.; Wang, G. Keratin 17: A critical player in the pathogenesis of psoriasis. *Med. Res. Rev.* **2014**, *34*, 438–454. [CrossRef] [PubMed]
45. Lin, Y.; Zhang, W.; Li, B.; Wang, G. Keratin 17 in psoriasis: Current understanding and future perspectives. *Semin. Cell Dev. Biol.* **2022**, *128*, 112–119. [CrossRef] [PubMed]
46. Shen, Z.; Wang, G.; Fan, J.-Y.; Li, W.; Liu, Y.-F. HLA DR B1*04, *07-restricted epitopes on Keratin 17 for autoreactive T cells in psoriasis. *J. Dermatol. Sci.* **2005**, *38*, 25–39. [CrossRef] [PubMed]
47. Yang, L.; Fan, X.; Cui, T.; Dang, E.; Wang, G. Nrf2 Promotes Keratinocyte Proliferation in Psoriasis through Up-Regulation of Keratin 6, Keratin 16, and Keratin 17. *J. Investig. Dermatol.* **2017**, *137*, 2168–2176. [CrossRef] [PubMed]
48. Lessard, J.C.; Piña-Paz, S.; Rotty, J.D.; Hickerson, R.P.; Kaspar, R.L.; Balmain, A.; Coulombe, P.A. Keratin 16 regulates innate immunity in response to epidermal barrier breach. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 19537–19542. [CrossRef] [PubMed]
49. Li, Y.; Wu, Q. KRT6A Inhibits IL-1β-Mediated Pyroptosis of Keratinocytes via Blocking IL-17 Signaling. *Crit. Rev. Eukaryot. Gene Expr.* **2024**, *34*, 1–11. [CrossRef]
50. Zhu, A.Y.; Mitra, N.; Margolis, D.J. Longitudinal association of atopic dermatitis progression and keratin 6A. *Sci. Rep.* **2022**, *12*, 13629. [CrossRef]
51. Leung, D.Y.M.; Calatroni, A.; Zaramela, L.S.; LeBeau, P.K.; Dyjack, N.; Brar, K.; David, G.; Johnson, K.; Leung, S.; Ramirez-Gama, M.; et al. The nonlesional skin surface distinguishes atopic dermatitis with food allergy as a unique endotype. *Sci. Transl. Med.* **2019**, *11*, eaav2685. [CrossRef] [PubMed]
52. Hamilton, J.D.; Suárez-Fariñas, M.; Dhingra, N.; Cardinale, I.; Li, X.; Kostic, A.; Ming, J.E.; Radin, A.R.; Krueger, J.G.; Graham, N.; et al. Dupilumab improves the molecular signature in skin of patients with moderate-to-severe atopic dermatitis. *J. Allergy Clin. Immunol.* **2014**, *134*, 1293–1300. [CrossRef] [PubMed]
53. Pareek, A.; Kumari, L.; Pareek, A.; Chaudhary, S.; Ratan, Y.; Janmeda, P.; Chuturgoon, S.; Chuturgoon, A. Unraveling Atopic Dermatitis: Insights into Pathophysiology, Therapeutic Advances, and Future Perspectives. *Cells* **2024**, *13*, 425. [CrossRef] [PubMed]
54. McLean, W.H.; Rugg, E.L.; Lunny, D.P.; Morley, S.M.; Lane, E.B.; Swensson, O.; Dopping-Hepenstal, P.J.; Griffiths, W.A.; Eady, R.A.; Higgins, C. Keratin 16 and keratin 17 mutations cause pachyonychia congenita. *Nat. Genet.* **1995**, *9*, 273–278. [CrossRef] [PubMed]
55. Leachman, S.A.; Kaspar, R.L.; Fleckman, P.; Florell, S.R.; Smith, F.J.D.; McLean, W.H.I.; Lunny, D.P.; Milstone, L.M.; van Steensel, M.A.M.; Munro, C.S.; et al. Clinical and Pathological Features of Pachyonychia Congenita. *J. Investig. Dermatol. Symp. Proc.* **2005**, *10*, 3–17. [CrossRef] [PubMed]
56. Smith, F.J.D.; Liao, H.; Cassidy, A.J.; Stewart, A.; Hamill, K.J.; Wood, P.; Joval, I.; van Steensel, M.A.M.; Björck, E.; Callif-Daley, F.; et al. The genetic basis of pachyonychia congenita. *J. Investig. Dermatol. Symp. Proc.* **2005**, *10*, 21–30. [CrossRef]

57. McLean, W.H.I.; Smith, F.J.D.; Cassidy, A.J. Insights into genotype-phenotype correlation in pachyonychia congenita from the human intermediate filament mutation database. *J. Investig. Dermatol. Symp. Proc.* **2005**, *10*, 31–36. [CrossRef] [PubMed]
58. Bowden, P.E.; Haley, J.L.; Kansky, A.; Rothnagel, J.A.; Jones, D.O.; Turner, R.J. Mutation of a type II keratin gene (K6a) in pachyonychia congenita. *Nat. Genet.* **1995**, *10*, 363–365. [CrossRef]
59. Gong, L.; Guo, S.; Wang, D.; Wang, T.; Ren, X.; Yuan, Y.; Cui, H. A KRT6A and a Novel KRT16 Gene Mutations in Chinese Patients with Pachyonychia Congenita. *Int. J. Gen. Med.* **2021**, *14*, 903–907. [CrossRef]
60. Teng, J.M.C.; Bartholomew, F.B.; Patel, V.; Sun, G. Novel treatment of painful plantar keratoderma in pachyonychia congenita using topical sirolimus. *Clin. Exp. Dermatol.* **2018**, *43*, 968–971. [CrossRef]
61. Hickerson, R.P.; Leake, D.; Pho, L.N.; Leachman, S.A.; Kaspar, R.L. Rapamycin selectively inhibits expression of an inducible keratin (K6a) in human keratinocytes and improves symptoms in pachyonychia congenita patients. *J. Dermatol. Sci.* **2009**, *56*, 82–88. [CrossRef]
62. Zhao, Y.; Gartner, U.; Smith, F.J.D.; McLean, W.H.I. Statins downregulate K6a promoter activity: A possible therapeutic avenue for pachyonychia congenita. *J. Investig. Dermatol.* **2011**, *131*, 1045–1052. [CrossRef]
63. Koren, A.; Sprecher, E.; Reider, E.; Artzi, O. A treatment protocol for botulinum toxin injections in the treatment of pachyonychia congenita-associated keratoderma. *Br. J. Dermatol.* **2020**, *182*, 671–677. [CrossRef]
64. Leachman, S.A.; Hickerson, R.P.; Hull, P.R.; Smith, F.J.D.; Milstone, L.M.; Lane, E.B.; Bale, S.J.; Roop, D.R.; McLean, W.H.I.; Kaspar, R.L. Therapeutic siRNAs for dominant genetic skin diseases including pachyonychia congenita. *J. Dermatol. Sci.* **2008**, *51*, 151–157. [CrossRef]
65. Leachman, S.A.; Hickerson, R.P.; Schwartz, M.E.; Bullough, E.E.; Hutcherson, S.L.; Boucher, K.M.; Hansen, C.D.; Eliason, M.J.; Srivatsa, G.S.; Kornbrust, D.J.; et al. First-in-human Mutation-targeted siRNA Phase Ib Trial of an Inherited Skin Disorder. *Mol. Ther.* **2010**, *18*, 442–446. [CrossRef] [PubMed]
66. Cui, L.; Zhu, Z.; Feng, Y.; Zhang, Y. Keratin 17 is Not Always a Marker of Proliferation of Keratinocytes in Skin Diseases. *Clin. Cosmet. Investig. Dermatol.* **2021**, *14*, 1247–1251. [CrossRef] [PubMed]
67. Dickman, J.; Howell, M.; Hoopes, R.; Wang, Y.; Dickerson, T.J.; Bottomley, M.; Shamma, H.N.; Rapp, C.M.; Turner, M.J.; Rohan, C.A.; et al. Insights into Lichen Planus Pigmentosus Inversus using Minimally Invasive Dermal Patch and Whole Transcriptome Analysis. *J. Clin. Investig. Dermatol.* **2022**, *10*. [CrossRef]
68. Yang, L.-L.; Huang, H.-Y.; Chen, Z.-Z.; Chen, R.; Ye, R.; Zhang, W.; Yu, B. Keratin 17 is induced in prurigo nodularis lesions. *Open Chem.* **2020**, *18*, 463–471. [CrossRef]
69. Yang, L.L.; Jiang, B.; Chen, S.H.; Liu, H.Y.; Chen, T.T.; Huang, L.H.; Yang, M.; Ding, J.; He, J.J.; Li, J.J.; et al. Abnormal keratin expression pattern in prurigo nodularis epidermis. *Ski. Health Dis.* **2022**, *2*, e75. [CrossRef]
70. Alkon, N.; Assen, F.P.; Arnoldner, T.; Bauer, W.M.; Medjimorec, M.A.; Shaw, L.E.; Rindler, K.; Holzer, G.; Weber, P.; Weninger, W.; et al. Single-cell RNA sequencing defines disease-specific differences between chronic nodular prurigo and atopic dermatitis. *J. Allergy Clin. Immunol.* **2023**, *152*, 420–435. [CrossRef]
71. Baraks, G.; Tseng, R.; Pan, C.-H.; Kasliwal, S.; Leiton, C.V.; Shroyer, K.R.; Escobar-Hoyos, L.F. Dissecting the Oncogenic Roles of Keratin 17 in the Hallmarks of Cancer. *Cancer Res.* **2022**, *82*, 1159–1166. [CrossRef] [PubMed]
72. Torabinia, N.; Razavi, S.M.; Sarrafpour, B.; Ziaei-Rad, E. The comparative evaluation of CK17 expression in histologic and cytological sections of oral squamous-cell carcinoma using immunohistochemistry. *Diagn. Cytopathol.* **2023**, *51*, 251–255. [CrossRef] [PubMed]
73. Sanguansin, S.; Kosanwat, T.; Juengsomjit, R.; Poomsawat, S. Diagnostic Value of Cytokeratin 17 during Oral Carcinogenesis: An Immunohistochemical Study. *Int. J. Dent.* **2021**, *2021*, 4089549. [CrossRef]
74. Bai, J.D.K.; Babu, S.; Roa-Peña, L.; Hou, W.; Akalin, A.; Escobar-Hoyos, L.F.; Shroyer, K.R. Keratin 17 is a negative prognostic biomarker in high-grade endometrial carcinomas. *Hum. Pathol.* **2019**, *94*, 40–50. [CrossRef] [PubMed]
75. Lozar, T.; Wang, W.; Gavrielatou, N.; Christensen, L.; Lambert, P.F.; Harari, P.M.; Rimm, D.L.; Burtness, B.; Grasic Kuhar, C.; Carchman, E.H. Emerging Prognostic and Predictive Significance of Stress Keratin 17 in HPV-Associated and Non HPV-Associated Human Cancers: A Scoping Review. *Viruses* **2023**, *15*, 2320. [CrossRef]
76. Zhang, H.; Zhang, Y.; Feng, Z.; Lu, L.; Li, Y.; Liu, Y.; Chen, Y. Analysis of the Expression and Role of Keratin 17 in Human Tumors. *Front. Genet.* **2022**, *13*, 801698. [CrossRef]
77. Li, C.; Teng, Y.; Wu, J.; Yan, F.; Deng, R.; Zhu, Y.; Li, X. A pan-cancer analysis of the oncogenic role of Keratin 17 (KRT17) in human tumors. *Transl. Cancer Res.* **2021**, *10*, 4489–4501. [CrossRef]
78. Hu, H.; Xu, D.; Huang, X.; Zhu, C.; Xu, J.; Zhang, Z.; Zhao, G. Keratin17 Promotes Tumor Growth and is Associated with Poor Prognosis in Gastric Cancer. *J. Cancer* **2018**, *9*, 346–357. [CrossRef]
79. Lozar, T.; Laklouk, I.; Golfinos, A.E.; Gavrielatou, N.; Xu, J.; Flynn, C.; Keske, A.; Yu, M.; Bruce, J.Y.; Wang, W.; et al. Stress Keratin 17 Is a Predictive Biomarker Inversely Associated with Response to Immune Check-Point Blockade in Head and Neck Squamous Cell Carcinomas and Beyond. *Cancers* **2023**, *15*, 4905. [CrossRef]
80. Babu, S.; Roa-Peña, L.; Akalin, A.; Escobar-Hoyos, L.; Shroyer, K. Keratin 17 is a Negative Prognostic Biomarker in Non-Small Cell Lung Cancer. *FASEB J.* **2022**, *36*. [CrossRef]
81. Roa-Peña, L.; Leiton, C.V.; Babu, S.; Pan, C.-H.; Vanner, E.A.; Akalin, A.; Bandovic, J.; Moffitt, R.A.; Shroyer, K.R.; Escobar-Hoyos, L.F. Keratin 17 identifies the most lethal molecular subtype of pancreatic cancer. *Sci. Rep.* **2019**, *9*, 11239. [CrossRef] [PubMed]

82. Ujiie, D.; Okayama, H.; Saito, K.; Ashizawa, M.; Thar Min, A.K.; Endo, E.; Kase, K.; Yamada, L.; Kikuchi, T.; Hanayama, H.; et al. KRT17 as a prognostic biomarker for stage II colorectal cancer. *Carcinogenesis* **2020**, *41*, 591–599. [CrossRef] [PubMed]
83. Khanom, R.; Nguyen, C.T.K.; Kayamori, K.; Zhao, X.; Morita, K.; Miki, Y.; Katsube, K.; Yamaguchi, A.; Sakamoto, K. Keratin 17 Is Induced in Oral Cancer and Facilitates Tumor Growth. *PLoS ONE* **2016**, *11*, e0161163. [CrossRef] [PubMed]
84. Yuanhua, L.; Pudong, Q.; Wei, Z.; Yuan, W.; Delin, L.; Yan, Z.; Geyu, L.; Bo, S. TFAP2A Induced KRT16 as an Oncogene in Lung Adenocarcinoma via EMT. *Int. J. Biol. Sci.* **2019**, *15*, 1419–1428. [CrossRef] [PubMed]
85. Cao, L.; Zhou, X.D.; Sens, M.A.; Garrett, S.H.; Zheng, Y.; Dunlevy, J.R.; Sens, D.A.; Somji, S. Keratin 6 Expression Correlates to Areas of Squamous Differentiation in Multiple Independent Isolates of As+3-Induced Bladder Cancer. *J. Appl. Toxicol.* **2010**, *30*, 416–430. [CrossRef] [PubMed]
86. Harada-Kagitani, S.; Kouchi, Y.; Shinomiya, Y.; Kodama, M.; Ohira, G.; Matsubara, H.; Ikeda, J.-I.; Kishimoto, T. Keratin 6A is expressed at the invasive front and enhances the progression of colorectal cancer. *Lab. Investig.* **2024**, *104*, 102075. [CrossRef]
87. Kengkarn, S.; Petmitr, S.; Boonyuen, U.; Reamtong, O.; Poomsawat, S.; Sanguansin, S. Identification of Novel Candidate Biomarkers for Oral Squamous Cell Carcinoma Based on Whole Gene Expression Profiling. *Pathol. Oncol. Res.* **2020**, *26*, 2315–2325. [CrossRef] [PubMed]
88. Yadav, S.; Zhou, S.; He, B.; Du, Y.; Garmire, L.X. Deep-learning and transfer learning identify new breast cancer survival subtypes from single-cell imaging data. *medRxiv* **2023**. [CrossRef]
89. Merkin, R.D.; Vanner, E.A.; Romeiser, J.L.; Shroyer, A.L.W.; Escobar-Hoyos, L.F.; Li, J.; Powers, R.S.; Burke, S.; Shroyer, K.R. Keratin 17 is overexpressed and predicts poor survival in estrogen receptor-negative/human epidermal growth factor receptor-2-negative breast cancer. *Hum. Pathol.* **2017**, *62*, 23–32. [CrossRef]
90. Ricciardelli, C.; Lokman, N.A.; Pyragius, C.E.; Ween, M.P.; Macpherson, A.M.; Ruszkiewicz, A.; Hoffmann, P.; Oehler, M.K. Keratin 5 overexpression is associated with serous ovarian cancer recurrence and chemotherapy resistance. *Oncotarget* **2017**, *8*, 17819–17832. [CrossRef]
91. Wang, Y.-F.; Lang, H.-Y.; Yuan, J.; Wang, J.; Wang, R.; Zhang, X.-H.; Zhang, J.; Zhao, T.; Li, Y.-R.; Liu, J.-Y.; et al. Overexpression of keratin 17 is associated with poor prognosis in epithelial ovarian cancer. *Tumour Biol.* **2013**, *34*, 1685–1689. [CrossRef]
92. Hoggarth, Z.E.; Osowski, D.B.; Slusser-Nore, A.; Shrestha, S.; Pathak, P.; Solseng, T.; Garrett, S.H.; Patel, D.H.; Savage, E.; Sens, D.A.; et al. Enrichment of genes associated with squamous differentiation in cancer initiating cells isolated from urothelial cells transformed by the environmental toxicant arsenite. *Toxicol. Appl. Pharmacol.* **2019**, *374*, 41–52. [CrossRef]
93. Chen, Y.; Ji, S.; Ying, J.; Sun, Y.; Liu, J.; Yin, G. *KRT6A* expedites bladder cancer progression, regulated by miR-31-5p. *Cell Cycle* **2022**, *21*, 1479–1490. [CrossRef]
94. Chen, F.; Wang, Y.; Zhang, X.; Fang, J. Five hub genes contributing to the oncogenesis and trastuzumab-resistance in gastric cancer. *Gene* **2023**, *851*, 146942. [CrossRef] [PubMed]
95. Mao, Y.; Shen, J.; Lu, Y.; Lin, K.; Wang, H.; Li, Y.; Chang, P.; Walker, M.G.; Li, D. RNA sequencing analyses reveal novel differentially expressed genes and pathways in pancreatic cancer. *Oncotarget* **2017**, *8*, 42537–42547. [CrossRef]
96. Williams, H.L.; Dias Costa, A.; Zhang, J.; Raghavan, S.; Winter, P.S.; Kapner, K.S.; Ginebaugh, S.P.; Väyrynen, S.A.; Väyrynen, J.P.; Yuan, C.; et al. Spatially-resolved single-cell assessment of pancreatic cancer expression subtypes reveals co-expressor phenotypes and extensive intra-tumoral heterogeneity. *Cancer Res.* **2023**, *83*, 441–455. [CrossRef] [PubMed]
97. Liu, Z.; Yu, S.; Ye, S.; Shen, Z.; Gao, L.; Han, Z.; Zhang, P.; Luo, F.; Chen, S.; Kang, M. Keratin 17 activates AKT signalling and induces epithelial-mesenchymal transition in oesophageal squamous cell carcinoma. *J. Proteom.* **2020**, *211*, 103557. [CrossRef]
98. Maruthappu, T.; Chikh, A.; Fell, B.; Delaney, P.J.; Brooke, M.A.; Levet, C.; Moncada-Pazos, A.; Ishida-Yamamoto, A.; Blaydon, D.; Waseem, A.; et al. Rhomboid family member 2 regulates cytoskeletal stress-associated Keratin 16. *Nat. Commun.* **2017**, *8*, 14174. [CrossRef] [PubMed]
99. Zhou, Z.; Zhang, G.; Li, T.; Ai, J.; Li, W.; Zeng, S.; Ye, M.; Liu, Q.; Xiao, J.; Li, Y.; et al. LncRNA NR120519 Blocks KRT17 to Promote Cell Proliferation and Migration in Hypopharyngeal Squamous Carcinoma. *Cancers* **2023**, *15*, 603. [CrossRef]
100. Escobar-Hoyos, L.F.; Yang, J.; Zhu, J.; Cavallo, J.-A.; Zhai, H.; Burke, S.; Koller, A.; Chen, E.I.; Shroyer, K.R. Keratin 17 in premalignant and malignant squamous lesions of the cervix: Proteomic discovery and immunohistochemical validation as a diagnostic and prognostic biomarker. *Mod. Pathol.* **2014**, *27*, 621–630. [CrossRef] [PubMed]
101. Meng, F.; Dai, L. Transcription factors TP63 facilitates malignant progression of thyroid cancer by upregulating KRT17 expression and inducing epithelial-mesenchymal transition. *Growth Factors* **2023**, *41*, 71–81. [CrossRef] [PubMed]
102. DePianto, D.; Kerns, M.; Dlugosz, A.A.; Coulombe, P.A. Keratin 17 promotes epithelial proliferation and tumor growth by polarizing the immune response in skin. *Nat. Genet.* **2010**, *42*, 910–914. [CrossRef]
103. Wang, W.; Lozar, T.; Golfinos, A.E.; Lee, D.; Gronski, E.; Ward-Shaw, E.; Hayes, M.; Bruce, J.Y.; Kimple, R.J.; Hu, R.; et al. Stress Keratin 17 expression in head and neck cancer contributes to immune evasion and resistance to immune checkpoint blockade. *Clin. Cancer Res.* **2022**, *28*, 2953–2968. [CrossRef] [PubMed]
104. Browning, J.R.; Derr, P.; Derr, K.; Doudican, N.; Michael, S.; Lish, S.R.; Taylor, N.A.; Krueger, J.G.; Ferrer, M.; Carucci, J.A.; et al. A 3D biofabricated cutaneous squamous cell carcinoma tissue model with multi-channel confocal microscopy imaging biomarkers to quantify antitumor effects of chemotherapeutics in tissue. *Oncotarget* **2020**, *11*, 2587–2596. [CrossRef] [PubMed]
105. Hameetman, L.; Commandeur, S.; Bavinck, J.N.B.; Wisgerhof, H.C.; de Gruijl, F.R.; Willemze, R.; Mullenders, L.; Tensen, C.P.; Vrieling, H. Molecular profiling of cutaneous squamous cell carcinomas and actinic keratoses from organ transplant recipients. *BMC Cancer* **2013**, *13*, 58. [CrossRef] [PubMed]

106. Lan, Y.-J.; Chen, H.; Chen, J.-Q.; Lei, Q.-H.; Zheng, M.; Shao, Z.-R. Immunolocalization of vimentin, keratin 17, Ki-67, involucrin, β-catenin and E-cadherin in cutaneous squamous cell carcinoma. *Pathol. Oncol. Res.* **2014**, *20*, 263–266. [CrossRef] [PubMed]
107. Yan, X.; Yang, C.; Hu, W.; Chen, T.; Wang, Q.; Pan, F.; Qiu, B.; Tang, B. Knockdown of KRT17 decreases osteosarcoma cell proliferation and the Warburg effect via the AKT/mTOR/HIF1α pathway. *Oncol. Rep.* **2020**, *44*, 103–114. [CrossRef]
108. Li, D.; Ni, X.-F.; Tang, H.; Zhang, J.; Zheng, C.; Lin, J.; Wang, C.; Sun, L.; Chen, B. KRT17 Functions as a Tumor Promoter and Regulates Proliferation, Migration and Invasion in Pancreatic Cancer via mTOR/S6k1 Pathway. *Cancer Manag. Res.* **2020**, *12*, 2087–2095. [CrossRef] [PubMed]
109. Escobar-Hoyos, L.F.; Shah, R.; Roa-Peña, L.; Vanner, E.A.; Najafian, N.; Banach, A.; Nielsen, E.; Al-Khalil, R.; Akalin, A.; Talmage, D.; et al. Keratin-17 Promotes p27KIP1 Nuclear Export and Degradation and Offers Potential Prognostic Utility. *Cancer Res.* **2015**, *75*, 3650–3662. [CrossRef] [PubMed]
110. Nair, R.R.; Hsu, J.; Jacob, J.T.; Pineda, C.M.; Hobbs, R.P.; Coulombe, P.A. A role for keratin 17 during DNA damage response and tumor initiation. *Proc. Natl. Acad. Sci. USA* **2021**, *118*, e2020150118. [CrossRef]
111. Delgado-Coka, L.; Horowitz, M.; Torrente-Goncalves, M.; Roa-Peña, L.; Leiton, C.V.; Hasan, M.; Babu, S.; Fassler, D.; Oentoro, J.; Bai, J.-D.K.; et al. Keratin 17 modulates the immune topography of pancreatic cancer. *J. Transl. Med.* **2024**, *22*, 443. [CrossRef] [PubMed]
112. Bruni, D.; Angell, H.K.; Galon, J. The immune contexture and Immunoscore in cancer prognosis and therapeutic efficacy. *Nat. Rev. Cancer* **2020**, *20*, 662–680. [CrossRef] [PubMed]
113. Liang, W.; Liu, H.; Zeng, Z.; Liang, Z.; Xie, H.; Li, W.; Xiong, L.; Liu, Z.; Chen, M.; Jie, H.; et al. KRT17 Promotes T-lymphocyte Infiltration Through the YTHDF2-CXCL10 Axis in Colorectal Cancer. *Cancer Immunol. Res.* **2023**, *11*, 875–894. [CrossRef] [PubMed]
114. Chung, B.M.; Arutyunov, A.; Ilagan, E.; Yao, N.; Wills-Karp, M.; Coulombe, P.A. Regulation of C-X-C chemokine gene expression by keratin 17 and hnRNP K in skin tumor keratinocytes. *J. Cell Biol.* **2015**, *208*, 613–627. [CrossRef] [PubMed]
115. Wang, W.; Uberoi, A.; Spurgeon, M.; Gronski, E.; Majerciak, V.; Lobanov, A.; Hayes, M.; Loke, A.; Zheng, Z.-M.; Lambert, P.F. Stress keratin 17 enhances papillomavirus infection-induced disease by downregulating T cell recruitment. *PLoS Pathog.* **2020**, *16*, e1008206. [CrossRef] [PubMed]
116. Chen, C.; Shan, H. Keratin 6A gene silencing suppresses cell invasion and metastasis of nasopharyngeal carcinoma via the β-catenin cascade. *Mol. Med. Rep.* **2019**, *19*, 3477–3484. [CrossRef]
117. Hu, J.; Zhang, L.-C.; Song, X.; Lu, J.-R.; Jin, Z. KRT6 interacting with notch1 contributes to progression of renal cell carcinoma, and aliskiren inhibits renal carcinoma cell lines proliferation in vitro. *Int. J. Clin. Exp. Pathol.* **2015**, *8*, 9182–9188.
118. Wang, W.; Zhu, L.; Zhou, J.; Liu, X.; Xiao, M.; Chen, N.; Huang, X.; Chen, H.; Pei, X.; Zhang, H. Targeting the KRT16-vimentin axis for metastasis in lung cancer. *Pharmacol. Res.* **2023**, *193*, 106818. [CrossRef] [PubMed]
119. Kim, S.; Wong, P.; Coulombe, P.A. A keratin cytoskeletal protein regulates protein synthesis and epithelial cell growth. *Nature* **2006**, *441*, 362–365. [CrossRef]
120. Hobbs, R.P.; DePianto, D.J.; Jacob, J.T.; Han, M.C.; Chung, B.-M.; Batazzi, A.S.; Poll, B.G.; Guo, Y.; Han, J.; Ong, S.; et al. Keratin-dependent regulation of Aire and gene expression in skin tumor keratinocytes. *Nat. Genet.* **2015**, *47*, 933–938. [CrossRef]
121. Jacob, J.T.; Nair, R.R.; Poll, B.G.; Pineda, C.M.; Hobbs, R.P.; Matunis, M.J.; Coulombe, P.A. Keratin 17 regulates nuclear morphology and chromatin organization. *J. Cell Sci.* **2020**, *133*, jcs254094. [CrossRef] [PubMed]
122. Yue, Z.; Lin, J.; Lu, X.; Gao, Q.; Pan, M.; Zhang, Y.; Shen, S.; Zhu, W.-G.; Paus, R. Keratin 17 Impacts Global Gene Expression and Controls G2/M Cell Cycle Transition in Ionizing Radiation–Induced Skin Damage. *J. Investig. Dermatol.* **2023**, *143*, 2436–2446.e13. [CrossRef] [PubMed]
123. Steen, K.; Chen, D.; Wang, F.; Majumdar, R.; Chen, S.; Kumar, S.; Lombard, D.B.; Weigert, R.; Zieman, A.G.; Parent, C.A.; et al. A role for keratins in supporting mitochondrial organization and function in skin keratinocytes. *MBoC* **2020**, *31*, 1103–1111. [CrossRef] [PubMed]
124. Lei, T.; Wang, L.; Zhang, Y.; Pan, Q.; Xie, X.; Du, H. Application of Human Keratin 6A in Stem Cell Culture and Product. CN Patent CN110643571A, 22 October 2019.
125. Wang, Y.-N.; Chang, W.-C. Induction of disease-associated keratin 16 gene expression by epidermal growth factor is regulated through cooperation of transcription factors Sp1 and c-Jun. *J. Biol. Chem.* **2003**, *278*, 45848–45857. [CrossRef] [PubMed]
126. MA, S.; RAO, L.; FREEDBERG, I.M.; BLUMENBERG, M. Transcriptional Control of K5, K6, K14, and K17 Keratin Genes by AP-1 and NF-κB Family Members. *Gene Expr.* **1997**, *6*, 361–370. [PubMed]
127. Zhang, J.; Li, X.; Wei, J.; Chen, H.; Lu, Y.; Li, L.; Han, L.; Lu, C. Gallic acid inhibits the expression of keratin 16 and keratin 17 through Nrf2 in psoriasis-like skin disease. *Int. Immunopharmacol.* **2018**, *65*, 84–95. [CrossRef] [PubMed]
128. Yang, L.; Zhang, S.; Wang, G. Keratin 17 in disease pathogenesis: From cancer to dermatoses. *J. Pathol.* **2019**, *247*, 158–165. [CrossRef] [PubMed]
129. Kurtović, M.; Piteša, N.; Bartoniček, N.; Ozretić, P.; Musani, V.; Čonkaš, J.; Petrić, T.; King, C.; Sabol, M. RNA-seq and ChIP-seq Identification of Unique and Overlapping Targets of GLI Transcription Factors in Melanoma Cell Lines. *Cancers* **2022**, *14*, 4540. [CrossRef]
130. Gu, L.-H.; Coulombe, P.A. Hedgehog Signaling, Keratin 6 Induction, and Sebaceous Gland Morphogenesis. *Am. J. Pathol.* **2008**, *173*, 752–761. [CrossRef] [PubMed]

131. Paramio, J.M.; Segrelles, C.; Laín, S.; Gómez-Casero, E.; Lane, D.P.; Lane, E.B.; Jorcano, J.L. p53 is phosphorylated at the carboxyl terminus and promotes the differentiation of human HaCaT keratinocytes. *Mol. Carcinog.* **2000**, *29*, 251–262. [CrossRef]
132. Cai, B.-H.; Hsu, P.-C.; Hsin, I.-L.; Chao, C.-F.; Lu, M.-H.; Lin, H.-C.; Chiou, S.-H.; Tao, P.-L.; Chen, J.-Y. p53 acts as a co-repressor to regulate keratin 14 expression during epidermal cell differentiation. *PLoS ONE* **2012**, *7*, e41742. [CrossRef]
133. Cottle, D.L.; Kretzschmar, K.; Gollnick, H.P.; Quist, S.R. p53 activity contributes to defective interfollicular epidermal differentiation in hyperproliferative murine skin. *Br. J. Dermatol.* **2016**, *174*, 204–208. [CrossRef]
134. Liao, C.; Xie, G.; Zhu, L.; Chen, X.; Li, X.; Lu, H.; Xu, B.; Ramot, Y.; Paus, R.; Yue, Z. p53 Is a Direct Transcriptional Repressor of Keratin 17: Lessons from a Rat Model of Radiation Dermatitis. *J. Investig. Dermatol.* **2016**, *136*, 680–689. [CrossRef]
135. Piipponen, M.; Li, D.; Landén, N.X. The Immune Functions of Keratinocytes in Skin Wound Healing. *Int. J. Mol. Sci.* **2020**, *21*, 8790. [CrossRef]
136. Cioce, A.; Cavani, A.; Cattani, C.; Scopelliti, F. Role of the Skin Immune System in Wound Healing. *Cells* **2024**, *13*, 624. [CrossRef]
137. Gao, Y.; Gong, B.; Chen, Z.; Song, J.; Xu, N.; Weng, Z. Damage-Associated Molecular Patterns, a Class of Potential Psoriasis Drug Targets. *Int. J. Mol. Sci.* **2024**, *25*, 771. [CrossRef] [PubMed]
138. Zhang, W.; Dang, E.; Shi, X.; Jin, L.; Feng, Z.; Hu, L.; Wu, Y.; Wang, G. The Pro-Inflammatory Cytokine IL-22 Up-Regulates Keratin 17 Expression in Keratinocytes via STAT3 and ERK1/2. *PLoS ONE* **2012**, *7*, e40797. [CrossRef] [PubMed]
139. Bernerd, F.; Magnaldo, T.; Freedberg, I.M.; Blumenberg, M. Expression of the carcinoma-associated keratin K6 and the role of AP-1 proto-oncoproteins. *Gene Expr.* **1993**, *3*, 187–199. [PubMed]
140. Jiang, C.K.; Magnaldo, T.; Ohtsuki, M.; Freedberg, I.M.; Bernerd, F.; Blumenberg, M. Epidermal growth factor and transforming growth factor alpha specifically induce the activation- and hyperproliferation-associated keratins 6 and 16. *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 6786–6790. [CrossRef]
141. Chung, B.-M.; Murray, C.I.; Eyk, J.E.V.; Coulombe, P.A. Identification of Novel Interaction between Annexin A2 and Keratin 17: EVIDENCE FOR RECIPROCAL REGULATION. *J. Biol. Chem.* **2012**, *287*, 7573–7581. [CrossRef]
142. Yano, S.; Komine, M.; Fujimoto, M.; Okochi, H.; Tamaki, K. Mechanical stretching in vitro regulates signal transduction pathways and cellular proliferation in human epidermal keratinocytes. *J. Investig. Dermatol.* **2004**, *122*, 783–790. [CrossRef] [PubMed]
143. Lombardo, G.; Melzi, G.; Indino, S.; Piazza, S.; Sangiovanni, E.; Baruffaldi Preis, F.; Marabini, L.; Donetti, E. Keratin 17 as a Marker of UVB-Induced Stress in Human Epidermis and Modulation by Vitis vinifera Extract. *Cells Tissues Organs* **2022**, *211*, 611–627. [CrossRef] [PubMed]
144. Bernerd, F.; Del Bino, S.; Asselineau, D. Regulation of keratin expression by ultraviolet radiation: Differential and specific effects of ultraviolet B and ultraviolet a exposure. *J. Investig. Dermatol.* **2001**, *117*, 1421–1429. [CrossRef] [PubMed]
145. Romashin, D.; Arzumanian, V.; Poverennaya, E.; Varshaver, A.; Luzgina, N.; Rusanov, A. Evaluation of Cd-induced cytotoxicity in primary human keratinocytes. *Hum. Exp. Toxicol.* **2024**, *43*, 09603271231224458. [CrossRef]
146. Romashin, D.; Rusanov, A.; Tolstova, T.; Varshaver, A.; Netrusov, A.; Kozhin, P.; Luzgina, N. Loss of mutant p53 in HaCaT keratinocytes promotes cadmium-induced keratin 17 expression and cell death. *Biochem. Biophys. Res. Commun.* **2024**, *709*, 149834. [CrossRef]

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Review

Autophagy: Are Amino Acid Signals Dependent on the mTORC1 Pathway or Independent?

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Abstract: Autophagy is a kind of "self-eating" phenomenon that is ubiquitous in eukaryotic cells. It mainly manifests in the damaged proteins or organelles in the cell being wrapped and transported by the autophagosome to the lysosome for degradation. Many factors cause autophagy in cells, and the mechanism of nutrient-deficiency-induced autophagy has been a research focus. It has been reported that amino-acid-deficiency-induced cellular autophagy is mainly mediated through the mammalian rapamycin target protein complex 1 (mTORC1) signaling pathway. In addition, some researchers also found that non-mTORC1 signaling pathways also regulate autophagy, and the mechanism of autophagy occurrence induced by the deficiency of different amino acids is not precisely the same. Therefore, this review aims to summarize the process of various amino acids regulating cell autophagy and provide a narrative review on the molecular mechanism of amino acids regulating autophagy.

Keywords: amino acid; autophagy; molecular mechanism; mTORC1

1. Introduction

Amino acids, as the basic unit of protein synthesis, are necessary for the metabolism of the growth and development of the body [1–3]. Once amino acids are deficient, autophagy and other adverse reactions will be induced [4–6]. However, the relationship between autophagy and the deficiency or supply of different amino acids is multifaceted and it is uncertain if it affects the mammalian target of rapamycin complex 1 (mTORC1) pathway regulation [5,7,8].

Autophagy is characterized by damage in the cytoplasm and mitochondria, endoplasmic reticulum, peroxisome, and other organelles, as well as by the surrounding of intracellular pathogens by autophagosomes and their degradation by lysosomes [9]. It has been long considered as an essential protein degradation pathway. Similarly, the ubiquitin proteasome system (UPS) is another important factor in intracellular protein degradation. Still, the difference between the two is that UPS targets a single short-lived protein, and autophagy as a whole process degrades many long-lived proteins and organelles in cells [10,11]. Autophagy is a crucial mechanism for protein homeostasis and quality control.

Previous studies have reported that autophagy is stimulated by various pathological and physiological stresses, especially undernourishment [12,13]. Starvation-induced autophagy is an evolutionary conservative response of eukaryotes, which degrades proteins, carbohydrates, and lipids, makes cells adapt to their metabolism, and meets energy requirements [5,9]. Furthermore, amino-acid- or other nutrient-deficiency-induced autophagy

effectively protects cells by blocking mitochondrial-induced apoptosis [4,5,7–10,13–16]. As a dynamic circulation system, autophagy provides substrates and energy for producing new proteins and membranes to promote the survival of cells under starvation conditions [17,18].

2. Autophagy

2.1. Classification of Autophagy

Autophagy can be classified into the following three categories (Figure 1) based on the pathways by which degraded substrates are transported into the lysosome: (1) Macroautophagy: the transfer of soluble cytoplasm and damaged organelles to lysosomes by using intermediate organelles called autophagy corpuscles, which are products of membranes [13,14]. Autophagy and lysosomes fuse to form autophagy lysosomes and degrade the substances in them. (2) Micro-autophagy: the lysosome's membrane directly encapsulates long-lived proteins and degrades in the lysosome [9,10]. (3) Chaperone-mediated autophagy (CMA): the lysosome directly engulfs small pieces of cytoplasm through the concave lysosomal membrane [19]. In CMA, the substrate protein containing the KFERQ-like pentapeptide sequence first binds to the cytoplasmic Hsc70 and then translocates to the lysosomal lumen, which is recognized by a lysosomal receptor, the lysosomal-related membrane protein 2A, leading to its unfolding and degradation [20]. The substrate of CMA is a soluble protein molecule, which has selectivity in protein removal, while the former two have no apparent selectivity [21,22]. The three degradation products of autophagy can be used in new protein synthesis, energy production, or gluconeogenesis. Among these, macro-autophagy is the most important form and the most widely studied [4,5,7–10,13–19].

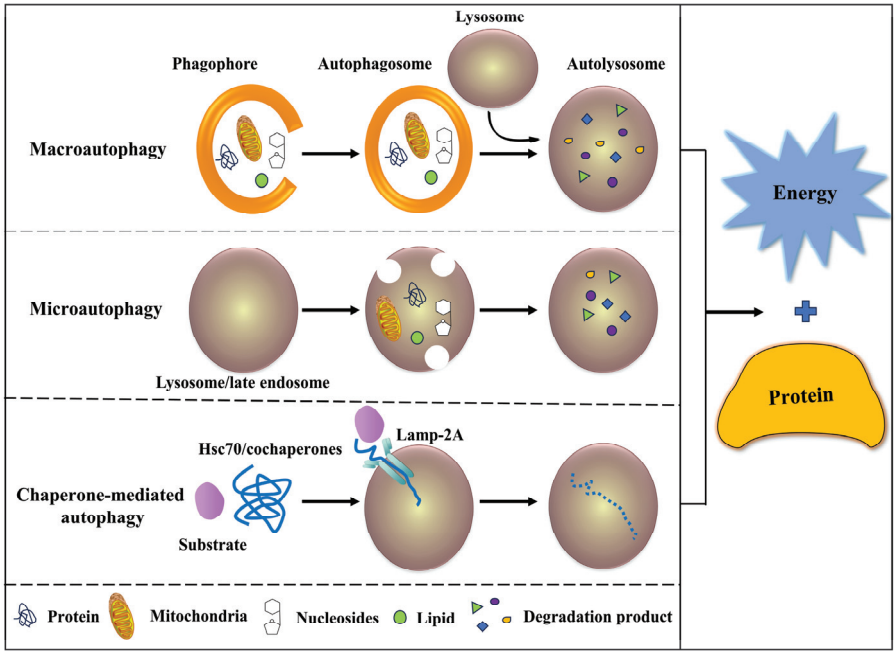


Figure 1. Different categories of autophagy. Macro-autophagy: proteins, lipids, and organelles such as mitochondria, etc., were surrounded by phagophores (also known as isolation membranes) to build an autophagosome, subsequently fused with lysosomes to form an autolysosome, the internal nutrients, and organelles were accordingly degraded. Micro-autophagy: proteins, lipids, and organelles were swallowed by invagination of the lysosome and late endosome. Chaperone-mediated autophagy (CMA): Cytosolic Hsc70 and cochaperones identified the substrate protein (including a KFERQ-like pentapeptide sequence), then translocated into the lysosomal lumen with lysosomal Lamp-2A. All degradation products were used for protein synthesis, energy production, etc.

2.2. Characteristics of Autophagy

(1) Autophagy is a part of the digestion of cells, which seems terrible for cells at first sight. In fact, under normal circumstances, autophagy is performed chiefly at a basal rate unless there are some predisposing factors [23–25]. These factors, such as low nutrient content, hypoxia, and cytokine concentration, may be extracellular. There are also some intracellular ones, such as metabolic stress, aging or broken organelles, misfolding, or accumulation of proteins [4,5,7–9]. Because these factors often exist, cells maintain a low and fundamental autophagy to maintain stability. (2) The process of autophagy is very rapid. The formation of autophagosomes can be observed after 8 min of induction, and autophagy lysosomes are degraded and disappear after 2 h, which is conducive to the rapid adaptation of cells to harsh environments [10,11,13]. (3) Inducible characteristics: these are manifested in two aspects. One is the formation of autophagy-related proteins, which is the preparation stage. The second is the rapid and massive formation of autophagosomes, which is the execution stage [14–18,20]. (4) Batch degradation: batch degradation of autophagosomes is another significant difference from the UPS [10,11]. (5) The specificity of the "capture" cytoplasmic component: because the speed of autophagy is large and the amount is large, specificity is not the first consideration, which is compatible with the emergency characteristics of autophagy [13,14,23,24]. (6) Conservation: autophagy is conducive to the survival of cells, so it is generally preserved regardless of species or between various cell types (including tumor cells) [26].

2.3. Process of Autophagy

Autophagy is a process of phagocytosis of cytoplasmic proteins or organelles and their inclusion into vesicles and fusion with lysosomes to form autolysosomes, which degrade the contents of their inclusion to achieve the metabolic needs of cells themselves and the renewal of some organelles [9–11,13,14]. The specific process could be divided into four steps: (1) after the cell receives the autophagy signal, a membrane structure similar to a "liposome" will be formed somewhere in the cytoplasm, and then it will continue to expand. It is not spherical but flat, like a bowl composed of two layers of phospholipid bimolecular layers, which can be observed under an electron microscope, called a "phagophore". (2) The phagophore extends, wrapping up proteins and organelles in the cytoplasm to form a closed autophagy [19]. (3) After autophagosomes are formed, they can fuse with phagocytic vesicles, pinosomes, and endosomes, but this step is not inevitable. (4) The autophagosome and lysosome are fused to form an autolysosome [17,19,23]. During this period, the inner membrane of the autophagy will be degraded by hydrolase in the lysosome, and the inner membrane of both the autophagy and lysosome will be integrated. The "goods" in autophagy will also be degraded, and the degradation products (amino acids, fatty acids, etc.) will be transported to the cytoplasm for cell reuse [23,24,26]. Some of the residues will be excluded from the cell, while others will be retained in the cell [27–30].

2.4. Regulation of Autophagy

Yeast is a simple eukaryotic cell, which makes it relatively easy to manipulate genes. As a result, many studies on autophagy used yeast as a model. In these studies, it has been found that the autophagy-related gene (Atg) and its corresponding protein play an essential role in different stages of autophagy [4,8,9,19,23].

Moreover, the mammalian target of rapamycin (mTOR), as a sensor of energy and nutritional status, is a typical serine/threonine kinase [31,32]. Under nutrient-rich conditions, mTOR complex 1 (mTORC1) binds to Atg1/Unc-51, like autophagy, activating kinase 1 (ULK1, the Atg1 named in mammalian), which directly inhibits downstream signals of autophagy [4,8]. Meanwhile, mTORC1 allows Atg13 to achieve phosphorylation, inhibiting downstream signals of autophagy. Because the affinity of highly phosphorylated Atg13 and Atg1 will be weakened, thereby reducing Atg1 kinase activity, downstream autophagy signals can be suppressed [33]. However, under starvation, mTORC1 inhibition leads to Atg13 dephosphorylation and close binding with Atg1. The close binding of Atg13

and Atg1 will enhance the activity of Atg1 kinase and activate the down-stream signal of autophagy [34]. In addition, ULK1 induces its phosphorylation by binding to raptor [35], thereby inhibiting the kinase activity of mTORC1.

Adenosine monophosphate-activated protein kinase (AMPK) mediates upstream signals of mTORC1, and mTORC1 controls the cell-energy-sensing pathway [36]. At a high concentration of AMP and its effect on energy consumption, AMPK is activated, which inhibits mTORC1 and promoted autophagy [37]. The activation of autophagy promotes the activation of the ULK1 complex and phosphorylates Beclin1 on Ser14, thereby enhancing the activity of Beclin1-Atg14-Vps34-Vps15 class III PI3K core complexes and promoting the nucleation of autophagosomes [38]. In addition, Bcl-2 binds to the BH3 domain of Beclin1 to inhibit autophagy under the condition of rich nutrition. The phosphorylation of Bcl-2 and Beclin1 destroys this interaction and releases Beclin1 [39]. When nutrient-rich, Atg9 shuttles between Golgi and endosomes, whereas when nutrient insufficient, Atg9 is distributed in the nuclear compartment, providing a membrane source for the formation of autophagosomes [40,41]. The two ubiquitin-like protein systems Atg12-Atg5-Atg16 (localized on the outer membrane of autophagy) and microtubule-associated protein 2 light chain 3 (LC3-II, named Atg8 in yeast and its mammalian homologous protein named LC3)-phosphatidylethanolamine (PE) are involved in the autophagy extension phase [42,43]. Under the catalysis of Atg7 and Atg10 enzymes, Atg12 and Atg5 are tightly bound through isopeptide bonds to form the Atg12-Atg5 complex. Through non-covalent bonding, Atg5 further binds to the spiral-helix region of Atg16 to form the Atg12-Atg5-Atg16 complex [44,45]. Atg16 (containing a coiled–coiled structure) undergoes homo-oligomerization through its unique structure to form a larger complex, which then binds to the pre-autophagosomal structure (PAS) and participates in the extension of the PAS [46]. Through the interaction of Atg7 and Atg3, Atg8/LC3 is covalently linked with PE to form the complex LC3-II-PE, which then binds to the membrane and participates in the extension of PAS [47]. The synthesis and processing of LC3 are often used to monitor the progress of autophagy because LC3-II is in mature autophagy until it merges with lysosome to produce autophagy. The regulation of autophagy is shown in Figure 2.

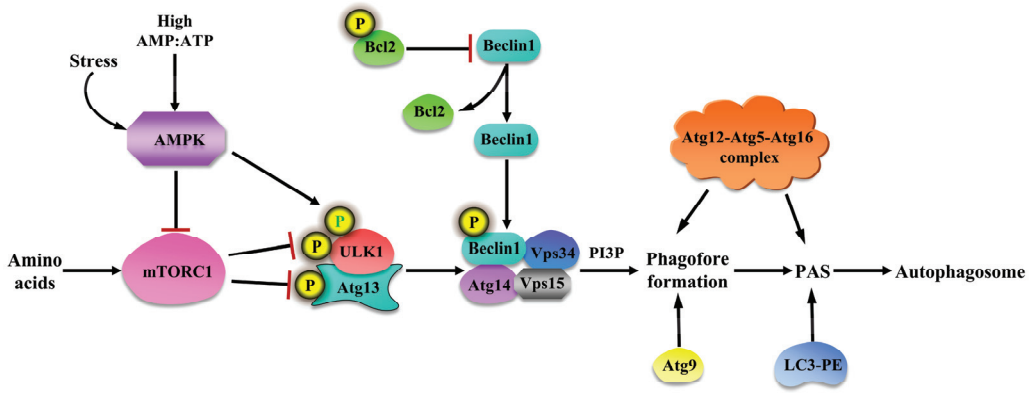


Figure 2. Regulation of autophagy in response to amino acids. High AMP and stress activate AMPK and depresses mTORC1, and AMPK can directly promote autophagy by phosphorylation of ULK1. Whereas rich amino acids could stimulate mTORC1 activation and suppress autophagy, mTORC1 blocks the occurrence of autophagy by phosphorylation of ULK1 and Atg13. Bcl2 is an inhibitor of autophagy. The interaction between Bcl2 and Beclin1 is broken by Bcl2 phosphorylation. Beclin1 is released and moved to the Vps34-Vps15 complex. Phosphorylation Be-clin1 activates Vps34 kinase activity and production of PI3P and contributes to nascent autophagy. Additionally, phagophore formation needs Atg9 and Atg12-Atg5-Atg1 complex, and LC3-PE also participates in pre-autophagosomal structure (PAS) construction and further forms autophagosomes.

3. Autophagy: Amino Acid Signals Dependent on mTORC1 Pathway

Amino acids play an important role in the metabolism of nutrients and the energy supply for the body through the biological activity of themselves or their metabolites, ultimately regulating the growth and development of animals [48]. If the supplementation of amino acids is deficient, autophagy would be the most characteristic phenotype [3–5]. However, the specific mechanisms of autophagy caused by different amino acid deficiencies are not the same. At present, more studies are based on lysine (Lys), leucine (Leu), arginine (Arg), and glutamine (Gln).

mTORC1 is an evolutionarily conserved serine/threonine kinase indispensable in controlling protein metabolism, cell growth, and autophagy [49,50]. Moreover, it negatively regulates autophagy through the phosphorylation of ULK1 and Atg13 [51]. Under nutrient-rich conditions, mTORC1 supports cell growth and inhibits autophagy. In contrast, mTORC1 is inhibited when the cells are under nutrition starvation and induces autophagy to provide energy for its metabolism [52].

As early as 1977, Mortimore et al. found that amino acid deficiency increased autophagy in rat liver [53]. Branched chain amino acids (BCAAs), especially Leu, have an inhibitory effect on the occurrence of autophagy [54]. The mechanism of amino acids to inhibit autophagy is to increase mTORC1 activity in two ways. One way is that amino acids are linked to lysosomes by a complex called the ragulator, which acts as a guanine nucleotide exchange factor and activates the rag triphosphate guanosine (GTP) enzyme in Ras-related small GTPases (Rag) A when it is sufficient [32]. Rag family proteins are members of the Ras family of GTP enzymes. They form heterodimers from four members (RagA, RagB, RagC, and RagD). RagA and RagB bind to GTP, and RagC and RagD bind to guanosine diphosphate (GDP) [32,48]. The other way is that amino acid intake affects mitogen-activated protein kinase kinase kinase kinase 3 (MAP4K3) in Ste proteins. An exogenous increase in amino acid levels improves the activity of Rag GTPase and MAP4K3 [55]. The Rag GTPase complex promotes mTORC1 to connect with the lysosome and collects a large amount of Ras homologous protein (Rheb) rich in the brain to activate mTORC1 [56]. Rheb is negatively regulated by the tuberous sclerosis complex (TSC) 1/2, which is a GTPase-activating protein for Rheb [32,48,52,55,56]. mTORC1 leads to the dissociation of the ULK1 active complex. Unable to form LC3-II, the autophagosome membrane fails to extend, autolysosome formation is blocked, and autophagy is inhibited. In this process, the transcription regulator ribosomal protein S6 kinase (S6K) is also activated to synthesize proteins [4].

3.1. Lys in Regulating Autophagy with mTORC1 Pathway

Lys is an essential component of protein synthesis and promotes the absorption and utilization of other nutrients [2,6,57]. It was found that the growth speed of piglets fed with a low-Lys diet was slower than that of piglets fed with a standard diet [31,57]. The muscle fiber density and muscle quality in the Lys deficiency group were also significantly reduced [58]. Lys deficiency reduces the growth of skeletal muscle, which may be due to insufficient Lys activating the autophagy–lysosomal pathway, resulting in the autophagy of skeletal muscle cells, which makes proteins degraded to resist the lack of intracellular amino acids caused by the external environment and other factors, and then to maintain intracellular amino acid balance in a short time [59]. According to Sato et al., Lys plays a role in preventing the breakdown of skeletal muscle protein [60]. In the absence of Lys, muscle protein decomposition and synthesis will speed up, leading to a decrease in muscle mass [61]. This even results in clinical symptoms such as muscle atrophy and myasthenia.

Studies have shown that the autophagy–lysosomal system is regulated by mTOR activity [62]. Sato et al. analyzed the phosphorylation levels of the mTORC1 downstream target proteins S6K1 and 4EBP1 in Lys-treated C2C12 myotubes [63]. After the C2C12 myotube was treated in a medium containing 10 mmol/L Lys for 30 min, the ratio of LC3-II to LC3-I was significantly reduced by 30% compared to that of the control group (the medium did not contain amino acids). The level of p-4EBP1 significantly increased, suggesting

that Lys regulates the autophagy–lysosomal activity of the C2C12 myotube through the mTORC1-dependent pathway. When the supply of Lys was sufficient, Lys activated the mTORC1 signaling pathway to inhibit the activity of the autophagy–lysosomal system and reduced the degradation of myofibrillar protein in muscle. The synthesis of muscle protein is related to the activation of the mTORC1 pathway. Lang et al. proved that Lys supplementation improved the level of p-4EBP1, which indicates that Lys supplementation inhibits the activity of the autophagy–lysosomal system and promotes the synthesis of proteins by activating the mTORC1 signaling pathway in muscle cells [64]. It has been reported that Lys slowed down the degradation of myofibrillar protein in rats. After feeding 114 mg and 570 mg of Lys for every 100 g of body weight in fasted rats, it was found that the degradation rate of myofibrillar protein was significantly reduced, and the expression of LC3-II was also significantly reduced. However, the mechanisms of Lys inhibiting the degradation of myofibrillar protein are still unclear [59]. Please see Table 1.

3.2. Leu in Regulating Autophagy with mTORC1 Pathway

Leu is a neutral amino acid. As an essential branched chain amino acid, it cannot be synthesized in animals and can only be taken from food to meet the body's need to maintain metabolic balance. Recent studies have shown that Leu not only regulates the metabolic level of proteins in mammals but also plays an essential role in maintaining the body's immune and oxidative functions [65,66]. Many studies have linked protein breakdown caused by Leu deprivation with autophagy. Wu et al. reported that two members of the miR-17 microRNA family, miR-20a and miR-106b, may inhibit the expression of ULK1 in mouse myoblasts (C2C12) involved in the regulation of Leu-deprivation-induced autophagy [67]. Sato et al. found that Leu intake inhibited the activation of the autophagy–lysosomal system and prevented the degradation of myofibrillar protein [60]. Subsequent research also found that the ratio of LC3-II to LC3-I decreased significantly after Leu supplementation in C2C12 cells cultured in Dulbecco's modified Eagle medium (DMEM) for 30 min, suggesting that Leu supplementation has a significant inhibitory effect on the autophagy of C2C12 cells. Amino acid deficiency causes autophagy in cells, and Leu is considered to be the most effective autophagy inhibitor [68].

Leu regulates autophagy mainly because it activates the mTORC1 signaling pathway, and then up-regulates its downstream target protein, thus inhibiting autophagy [69]. Recent studies have shown that the inhibition of Leu on autophagy depends on the mTORC1 signaling pathway, and its upstream target proteins, the Ras homologue enriched in the brain (Rheb) and the regulatory associated protein of mTOR (Raptor), are also necessary. Leu promotes the phosphorylation of mTORC1, and then phosphorylates its downstream target proteins 4EBP1 and S6K1 [70]. It also activates the target protein Rag upstream of mTORC1, so that inactive and diffusely distributed mTORC1 in the cell aggregated around its activating protein. Lang et al. and Yoshizawa et al. found that Leu supplementation enhances the mTORC1 activity of myocytes and then inhibits autophagy [64,71]. When C2C12 myotube cells were treated in Leu containing medium for 30 min, the level of p-4EBP1 could be significantly increased [63]. The microRNA microarray results of Wu et al. showed that 84 microRNAs were differentially expressed in C2C12 myotube cells after 4 h of Leu starvation. The prediction and analysis of these differentially expressed microRNA target genes show that the change in mTOR-autophagy signaling pathways induced by Leu deletion might be indirectly affected by a change in miR-20a and miR-106b [67]. Yan et al. found that the protein breakdown caused by Leu deprivation was related to autophagy. Barkor/Atg14 is a special autophagy binding protein. Under the condition of rich nutrition, its ability of binding to the autophagy membrane is inhibited, and after nutrition deprivation, the inhibition is removed. At the same time, rapamycin effectively reverses the inhibition of nutritional enrichment, suggesting that mTORC1 plays a vital role in the inhibition of autophagy [49]. Please see Table 1.

3.3. Arg in Regulating Autophagy with mTORC1 Pathway

Arg is a functional amino acid, which plays an important role in the body's growth and development, physiological metabolism, and intestinal health [72]. Most melanoma cells require an exogenous supply of Arg for growth and proliferation. Arg deprivation inhibits mTOR signaling but leads to the activation of MEK and ERK. These changes are likely to lead to autophagy, a possible mechanism for survival through the intracellular arginine cycle [73]. Moreover, under conditions of heat stress, Arg induces autophagy with the decreased expression of p62 and p-mTOR/mTOR, and the increased expression of LC3B and Arg can reduce intestinal damage and protect intestinal integrity by promoting autophagy [74]. Additionally, Castejón-Vega et al. investigated the effects of autophagy and lysosomal integrity using skin fibroblasts obtained from Tay-Sachs and Sandhoff patients (GM2 gangliosidosis) with increased lysosome permeability. GM2 fibroblasts showed decreased mTOR signaling and decreased basal mTOR activity. Supplementing with Arg to provide positive nutritional signals could partially restore mTOR activity and alleviate cytopathological abnormalities [75]. These findings suggest that Arg regulates the autophagy process under mTOR modulation. Please see Table 1.

3.4. Gln in Regulating Autophagy with mTORC1 Pathway

Gln is a rich amino acid in the blood. Many tissues, especially skeletal muscle, synthesize Gln as an important carrier of carbon and nitrogen. In addition, it also plays a crucial role in maintaining the intestinal integrity of humans and animals [76,77]. Zhu et al. used pig intestinal epithelial cells to test the hypothesis that Gln deficiency induced autophagy and inhibited autophagy after Gln supplementation [78]. After cultured in normal medium for 2 days, the intestinal epithelial cells were transferred to a Gln-free medium, and the number of cells, the distribution of the autophagy, the expression of LC3, and the protein abundance of mTOR within 8 h were detected. In addition, the complement effect of 5 mmol/L Gln was evaluated. The results showed that Gln deficiency reduced the number of cells and increased the accumulation of autophagy and the expression of LC3-II within 8 h. These results support the view that Gln deficiency induces autophagy, interferes with amino acid metabolism in intestinal epithelial cells, and weakens the mTORC1 signaling pathway to inhibit protein synthesis and cell proliferation. Tan et al. found that during amino acid starvation, Gln needed to reactivate mTORC1 by autophagy, and Gln supplementation alone was sufficient to restore mTORC1 activity and lysosomal localization during long-term amino acid starvation [77]. The inhibition of Gln was eliminated by the treatment of autophagy inhibitors, suggesting that the activation of mTORC1 depended on autophagy. Please see Table 1.

Table 1. Amino-acid-regulated autophagy dependent on mTORC1 signaling pathway.

Amino Acids	Treatment	Samples	Description	References
Lys	10 mM Lys treated for 30 min	C2C12	Suppressed autophagy, activated mTORC1	[60,63]
	Rats fed with 10% casein diet supplemented by Lys	Rat muscle	Suppressed autophagy, activated mTORC1	[79]
Leu	10 mM Leu treated for 30 min	C2C12	Suppressed autophagy, activated mTORC1	[60,63]
	Fasted mice administered an oral gavage of Leu (1.35 g/kg BW)	Mouse muscle	Increased protein synthesis and mTORC1	[64]
	Fasted pigs were infused intra-arterially with Leu (0, 200, or 400 μmol/kg/h)	Pig muscle	Increased protein synthesis and mTORC1	[70]
	Oral administration of Leu in fasted rats	Rat muscle	Increased protein synthesis and mTORC1	[71,80]

Table 1. *Cont.*

Amino Acids	Treatment	Samples	Description	References
Arg	Medium Arg deficiency	Melanoma cells	Induced autophagy, restricted mTORC1	[73]
	Diet Arg supplementation in heat-stress-treated rats	Rat intestine	Induced autophagy, restricted mTORC1	[74]
	Medium Arg supplementation in heat-stress-treated IEC-6	IEC-6	Induced autophagy, restricted mTORC1	[74]
	Arg-treated skin fibroblasts isolated from GM2 gangliosidosis	Skin fibroblasts	Recovered autophagy and mTORC1	[75]
Gln	Gln-treated MEFs and HepG2 cells under amino acid starvation	MEFs and HepG2 cells	Restored autophagy and mTORC1	[77]
	Gln deprivation treated IPEC-1	IPEC-1	Induced autophagy, restricted mTORC1	[78]

4. Autophagy: Amino Acid Signals Independent of mTORC1 Pathway

4.1. Lys in Regulating Autophagy without mTORC1 Pathway

Although the regulation of Lys on cell autophagy is mainly mediated through the mTORC1 signaling pathway, since the role of the mTORC1 pathway is limited, the lack of Lys to induce cell autophagy may also be mediated by signaling molecules other than mTOR [79]. It has been reported that protein kinase B (Akt) regulates protein degradation through the autophagy–lysosomal system [81]. Previous studies have also shown that Leu inhibited the activity of the autophagy–lysosomal system by up-regulating the level of Akt [82], but Sato's research has fully confirmed that Lys is another amino acid that can activate the Akt signaling pathway [79]. Research by Zhao et al. showed that Akt, as a regulator, is indispensable in the process of autophagy in myotubes [83]. Therefore, Akt activation may play a key role in Lys regulating autophagy–lysosomal activity. Sato et al. treated C2C12 cells with Lys and found that the phosphorylation level of the Ser473 and Thr308 sites of Akt significantly increased [60]. When C2C12 cells were treated with Akt-specific inhibitors, Lys inhibited the degradation of myofibrillar protein by activating Akt.

Sato also evaluated the regulatory effect of Lys-induced Akt activation on autophagy–lysosomal activity by the ratio of LC3-II to total LC3. In C2C12 cells treated with Lys, the ratio of LC3-II to total LC-3 decreased, which could be eliminated after being treated with the Akt inhibitor, indicating that Lys inhibition of autophagy–lysosomal system activity depended on Akt activation [79]. Please see Table 2.

4.2. Leu in Regulating Autophagy without mTORC1 Pathway

Although the effect of Leu on autophagy is mainly realized by the mTORC1 signaling pathway, and its effect on protein metabolism and turnover is also dependent on other signaling pathways [80], there are few related studies. In order to determine whether the effect of Leu on autophagy of muscle cells will play a role through a signaling pathway other than mTORC1, Mordier et al. carried out relevant experiments. They treated myotube cells with Leu starvation for 3 h to detect the effect of rapamycin by incubating myotube cells with 50 nmol/L of rapamycin for 3 h. The effect of Leu starvation was examined, and it was found that there was a cumulative effect when two stimuli were applied simultaneously. In addition, the measurement of p-S6 ribosomal protein levels through an 8-h test found that its expression level was not affected by Leu deprivation, thereby further providing that Leu starvation induces muscle cell autophagy through a mTORC1-independent pathway. This indicates that Leu starvation may regulate autophagy through specific signaling pathways unrelated to mTORC1, and also regulate protein synthesis [84]. Please see Table 2.

Table 2. Amino-acid-regulated autophagy independent of mTORC1 signaling pathway.

Amino Acids	Treatment	Samples	Description	References
Lys	Oral administration of Lys (114 mg/100 g BW, sufficient) in fasted rats	Rat muscle	Suppressed autophagy, unchanged mTORC1	[59]
	10 mM Lys treated for 30 min	C2C12	Suppressed autophagy and AMPK	[60]
	SAMP8 fed with 3.0% Lys diet	SAMP8	Suppressed autophagy, unchanged mTORC1	[61]
Leu	10 mM-Leu-treated for 30 min	C2C12	Suppressed autophagy and AMPK	[60]
	MiR-20a- and miR-106b-treated Leu deficiency medium pre-cultured C2C12	C2C12	Suppressed autophagy, unchanged mTORC1	[67]
	Old rats fed a 15% protein diet supplemented with 4.5% leucine	Rat muscle	Suppressed autophagy, unchanged mTORC1	[82]
	Leu-deficiency-treated C2C12	C2C12	Induced autophagy, unchanged mTORC1	[84]
Arg	Medium Arg deficiency	Melanoma cells	Induced autophagy, activated AMPK	[73]
	IFN-γ treated BMECs	BMECs	Decreased Arg content, increased GCN2 pathway and autophagy	[85]
	Arg-deficiency-treated H4-II-E and Hep G2 cells	H4-II-E and Hep G2 cells	Induced autophagy, activated NO pathway	[86,87]
Gln	Atg5 or Atg7 knockdown HeLa cells with Gln supplementation	HeLa cells	Suppressed autophagy, unchanged mTORC1	[88]
	Gln-deficiency-treated porcine-circovirus-type-2-infected mice and PK 15 cells	Mice and PK 15 cells	Induced autophagy, activated JAK2/STAT3	[89]

4.3. Arg in Regulating Autophagy without mTORC1 Pathway

The discovery of Xia et al. proved for the first time that the depletion of Arg and the expression of general control nonderepressible 2 (GCN2) mediate interferonγ (IFN-γ)-induced autophagy, and the continuous activation of autophagy promotes the transformation of bovine mammary epithelial cells [85]. In addition, studies by Sarkar et al. and Angcajas et al. have confirmed that Arg regulates autophagy not only through the mTORC1 signaling pathway, but also through the nitric oxide (NO) signaling pathway [86,87]. NO is a cell signaling molecule commonly found in the cardiovascular, nervous, and immune systems and triggers a variety of physiological responses, such as blood flow regulation and tissue response to hypoxia. Arg is the only source of NO in the human body. In cells, Arg can be degraded into NO by nitric oxide synthase (NOS). Studies by Sarkar et al. have shown that NO reduces the activity of Jun N-terminal kinase 1 (JNK1) and the phosphorylation of B-cell lymphoma-2 (Bcl-2) and inhibits autophagy flux in mammalian cells in a manner dependent on IkB kinase β (IKKβ) and tuberous sclerosis complex 2 (TSC2) [86]. Rat liver cancer cells H4-II-E were starved in an Earl-balanced salt solution, and the ratio of LC3-II/LC3-I decreased by 35% after adding Arg. After treatment with Arg and rapamycin, it was found that the ratio of LC3-II/LC3-I decreased by 33%. There was no significant difference in autophagy inhibition in both cases, indicating that Arg may also inhibit autophagy through other signaling pathways, rather than relying on the mTORC1 signaling pathway [87]. Please see Table 2.

4.4. Gln in Regulating Autophagy without mTORC1 Pathway

Gln is associated with increased enlarged lysosome formation/dissipation by regulating TFEB nuclear/cytoplasmic translocation but has nothing to do with mTORC1 activity [88].

Gln deficiency induces autophagy, which is evident through increased LC3-II conversion and GFP-LC3 dot accumulation, and further triggers porcine circovirus type 2 (PCV2) infection. Subsequent studies have demonstrated that Gln deficiency activates autophagy via the activation of ROS-mediated JAK2/STAT3 signaling [89]. In addition, data in neurons show that in the presence of Gln synthetase1 (GS1), TOR signaling is reduced, even when Gln levels increase, so this may indicate that in terminally differentiated cells, GS1 induces a 'starvation-like' condition that facilitates autophagy without activating TORC1 [90]. Similarly, an earlier study showed that Gln regulated autophagy by growth factor signaling [91]. Therefore, Gln may also inhibit autophagy through other signaling pathways, rather than the mTORC1 pathway. Please see Table 2.

5. Defects in Studies on Amino-Acid-Regulated Autophagy

Many studies on autophagy have fully shown that a lack of nutrition, especially in amino acids, will lead to strong autophagy. However, it is unclear how individual amino acids, such as Lys, Leu, Arg, or Gln, respond to autophagy, which is also the point of investigating the relationship between amino acid nutrition and autophagy. It has been shown that Lys and Leu regulate protein synthesis through the mTORC1 signaling pathway, but Leu stimulates mTORC1 signaling significantly more than Lys [92], and the similarities and differences between Lys and Leu in the regulation of protein metabolism need further study to be clarified.

The effects of nutrients, especially amino acids, on autophagy have been confirmed by studies for many years. However, there are few studies on how amino acids enter the cell to alleviate autophagy and whether amino acids act as a signaling molecule or a nutrient substrate in the process of regulating autophagy. When intracellular nutrients are scarce, cells will initiate autophagy, degrade their own cellular components, and maintain the balance between anabolism and catabolism in a short period of time under special physiological conditions [93]. However, how cells sense the lack of amino acids and then regulate autophagy is still worthy of further study.

6. Summary and Prospects

Autophagy is a kind of dynamic cell process to remove long-lived proteins, damaged organelles, and cytoplasmic components. In recent years, autophagy has been a hot topic in the field of life science and clinical disease research. At present, the relationship between the degree of autophagy and the body's advantages and disadvantages cannot be quantitatively analyzed. The induction of autophagy by amino acid deficiency is mainly achieved by inhibiting the mTORC1 signaling pathway. The study on the molecular mechanism of how mammalian cells perceive amino acid deficiency to promote the formation of autophagy bodies, form autophagy lysosomes, then start the whole process of autophagy, and how autophagy-related-regulators receive and transmit signals downstream, is fiercely in progress. An in-depth study on the effect of amino acids, especially essential amino acids on autophagy, will help us to understand the precise regulation of nutrition more comprehensively and provide a theoretical basis for animal husbandry and medical research.

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References

1. Paulusma, C.C.; Lamers, W.H.; Broer, S.; van de Graaf, S.F.J. Amino acid metabolism, transport and signalling in the liver revisited. *Biochem. Pharmacol.* **2022**, *201*, 115074. [CrossRef] [PubMed]
2. Jin, C.L.; Ye, M.; Song, Z.W.; Zhang, Z.M.; Gao, C.Q.; Yan, H.C.; Wang, X.Q. Lysine interacts with frizzled7 to activate β-catenin in satellite cell-participated skeletal muscle growth. *J. Agric. Food Chem.* **2022**, *70*, 3745–3756. [CrossRef] [PubMed]
3. Chen, J.; Cui, L.; Lu, S.; Xu, S. Amino acid metabolism in tumor biology and therapy. *Cell Death Dis.* **2024**, *15*, 42. [CrossRef] [PubMed]
4. Fukuda, T.; Shiozaki, K. Multiplexed suppression of TOR complex 1 induces autophagy during starvation. *Autophagy* **2021**, *17*, 1794–1795. [CrossRef] [PubMed]
5. Bodineau, C.; Tomé, M.; Murdoch, P.D.S.; Durán, R.V. Glutamine, MTOR and autophagy: A multiconnection relationship. *Autophagy* **2022**, *18*, 2749–2750. [CrossRef] [PubMed]
6. Jin, C.L.; Zhang, Z.M.; Ye, J.L.; Gao, C.Q.; Yan, H.C.; Li, H.C.; Wang, X.Q. Lysine-induced swine satellite cell migration is mediated by the FAK pathway. *Food Funct.* **2019**, *10*, 583–591. [CrossRef] [PubMed]
7. Zhan, Q.; Jeon, J.; Li, Y.; Huang, Y.; Xiong, J.; Wang, Q.; Xu, T.L.; Li, Y.; Ji, F.H.; Du, G.; et al. CAMK2/CaMKII activates MLKL in short-term starvation to facilitate autophagic flux. *Autophagy* **2022**, *18*, 726–744. [CrossRef] [PubMed]
8. Corral-Ramos, C.; Barrios, R.; Ayté, J.; Hidalgo, E. TOR and MAP kinase pathways synergistically regulate autophagy in response to nutrient depletion in fission yeast. *Autophagy* **2022**, *18*, 375–390. [CrossRef] [PubMed]
9. Liu, S.; Yao, S.; Yang, H.; Liu, S.; Wang, Y. Autophagy: Regulator of cell death. *Cell Death Dis.* **2023**, *14*, 648. [CrossRef]
10. Eapen, V.V.; Swarup, S.; Hoyer, M.J.; Paulo, J.A.; Harper, J.W. Quantitative proteomics reveals the selectivity of ubiquitin-binding autophagy receptors in the turnover of damaged lysosomes by lysophagy. *eLife* **2021**, *10*, e72328. [CrossRef]
11. Vargas, J.N.S.; Hamasaki, M.; Kawabata, T.; Youle, R.J.; Yoshimori, T. The mechanisms and roles of selective autophagy in mammals. *Nat. Rev. Mol. Cell Biol.* **2023**, *24*, 167–185. [CrossRef] [PubMed]
12. Inoue, M.; Miyahara, H.; Shiraishi, H.; Shimizu, N.; Tsumori, M.; Kiyota, K.; Maeda, M.; Umeda, R.; Ishitani, T.; Hanada, R.; et al. Leucyl-tRNA synthetase deficiency systemically induces excessive autophagy in zebrafish. *Sci. Rep.* **2021**, *11*, 8392. [CrossRef] [PubMed]
13. Li, W.; He, P.; Huang, Y.; Li, Y.F.; Lu, J.; Li, M.; Kurihara, H.; Luo, Z.; Meng, T.; Onishi, M.; et al. Selective autophagy of intracellular organelles: Recent research advances. *Theranostics* **2021**, *11*, 222–256. [CrossRef] [PubMed]
14. Al-Bari, M.A.A.; Xu, P. Molecular regulation of autophagy machinery by mTOR-dependent and -independent pathways. *Ann. N. Y. Acad. Sci.* **2020**, *1467*, 3–20. [CrossRef] [PubMed]
15. Mohanasundaram, P.; Coelho-Rato, L.S.; Modi, M.K.; Urbanska, M.; Lautenschläger, F.; Cheng, F.; Eriksson, J.E. Cytoskeletal vimentin regulates cell size and autophagy through mTORC1 signaling. *PLoS Biol.* **2022**, *20*, e3001737. [CrossRef] [PubMed]
16. Wang, M.; Lu, Y.; Wang, H.; Wu, Y.; Xu, X.; Li, Y. High ATF4 expression is associated with poor prognosis, amino acid metabolism, and autophagy in gastric cancer. *Front. Oncol.* **2021**, *11*, 740120. [CrossRef] [PubMed]
17. Suzuki, A.; Iwata, J. Amino acid metabolism and autophagy in skeletal development and homeostasis. *Bone* **2021**, *146*, 115881. [CrossRef] [PubMed]
18. Sadria, M.; Layton, A.T. Interactions among mTORC, AMPK and SIRT: A computational model for cell energy balance and metabolism. *Cell Commun. Signal* **2021**, *19*, 57. [CrossRef] [PubMed]
19. Dong, S.; Wang, Q.; Kao, Y.R.; Diaz, A.; Tasset, I.; Kaushik, S.; Thiruthuvanathan, V.; Zintiridou, A.; Nieves, E.; Dzieciatkowska, M.; et al. Chaperone-mediated autophagy sustains haematopoietic stem-cell function. *Nature* **2021**, *591*, 117–123. [CrossRef]
20. Yao, R.; Shen, J. Chaperone-mediated autophagy: Molecular mechanisms, biological functions, and diseases. *MedComm* **2023**, *4*, e347. [CrossRef]
21. Chen, C.; Wang, D.; Yu, Y.; Zhao, T.; Min, N.; Wu, Y.; Kang, L.; Zhao, Y.; Du, L.; Zhang, M.; et al. Legumain promotes tubular ferroptosis by facilitating chaperone-mediated autophagy of GPX4 in AKI. *Cell Death Dis.* **2021**, *12*, 65. [CrossRef] [PubMed]
22. Wang, L.; Cai, J.; Zhao, X.; Ma, L.; Zeng, P.; Zhou, L.; Liu, Y.; Yang, S.; Cai, Z.; Zhang, S.; et al. Palmitoylation prevents sustained inflammation by limiting NLRP3 inflammasome activation through chaperone-mediated autophagy. *Mol. Cell* **2023**, *83*, 281–297. [CrossRef] [PubMed]
23. Cayo, A.; Segovia, R.; Venturini, W.; Moore-Carrasco, R.; Valenzuela, C.; Brown, N. mTOR activity and autophagy in senescent cells, a complex partnership. *Int. J. Mol. Sci.* **2021**, *22*, 8149. [CrossRef] [PubMed]
24. Foerster, E.G.; Mukherjee, T.; Cabral-Fernandes, L.; Rocha, J.D.B.; Girardin, S.E.; Philpott, D.J. How autophagy controls the intestinal epithelial barrier. *Autophagy* **2022**, *18*, 86–103. [CrossRef] [PubMed]

25. Vainshtein, A.; Tryon, L.D.; Pauly, M.; Hood, D.A. Role of PGC-1α during acute exercise-induced autophagy and mitophagy in skeletal muscle. *Am. J. Physiol. Cell Physiol.* **2015**, *308*, C710–C719. [CrossRef] [PubMed]
26. Liu, C.; Ji, L.; Hu, J.; Zhao, Y.; Johnston, L.J.; Zhang, X.; Ma, X. Functional amino acids and autophagy: Diverse signal transduction and application. *Int. J. Mol. Sci.* **2021**, *22*, 11427. [CrossRef] [PubMed]
27. Klionsky, D.J.; Codogno, P. The mechanism and physiological function of macroautophagy. *J. Innate Immun.* **2013**, *5*, 427–433. [CrossRef] [PubMed]
28. Shen, H.M.; Mizushima, N. At the end of the autophagic road: An emerging understanding of lysosomal functions in autophagy. *Trends Biochem. Sci.* **2014**, *39*, 61–71. [CrossRef] [PubMed]
29. Long, B.; Yin, C.; Fan, Q.; Yan, G.; Wang, Z.; Li, X.; Chen, C.; Yang, X.; Liu, L.; Zheng, Z.; et al. Global liver proteome analysis using iTRAQ reveals AMPK-mTOR-autophagy signaling is altered by intrauterine growth restriction in newborn piglets. *J. Proteome Res.* **2016**, *15*, 1262–1273. [CrossRef] [PubMed]
30. Yao, Q.; Ke, Z.Q.; Guo, S.; Yang, X.S.; Zhang, F.X.; Liu, X.F.; Chen, X.; Chen, H.G.; Ke, H.Y.; Liu, C. Curcumin protects against diabetic cardiomyopathy by promoting autophagy and alleviating apoptosis. *J. Mol. Cell. Cardiol.* **2018**, *124*, 26–34. [CrossRef]
31. Jin, C.L.; Ye, J.L.; Yang, J.; Gao, C.Q.; Yan, H.C.; Li, H.C.; Wang, X.Q. mTORC1 mediates lysine-induced satellite cell activation to promote skeletal muscle growth. *Cells* **2019**, *8*, 1549. [CrossRef] [PubMed]
32. Zhu, M.; Wang, X.Q. Regulation of mTORC1 by Small GTPases in response to nutrients. *J. Nutr.* **2020**, *150*, 1004–1011. [CrossRef] [PubMed]
33. Chang, Y.Y.; Neufeld, T.P. An Atg1/Atg13 complex with multiple roles in TOR-mediated autophagy regulation. *Mol. Biol. Cell* **2009**, *20*, 2004–2014. [CrossRef] [PubMed]
34. Pattingre, S.; Espert, L.; Biard-Piechaczyk, M.; Codogno, P. Regulation of macroautophagy by mTOR and Beclin 1 complexes. *Biochimie* **2008**, *90*, 313–323. [CrossRef] [PubMed]
35. Ali, Y.; Gomez-Sanchez, C.E.; Plonczynski, M.; Naray-Fejes-Toth, A.; Fejes-Toth, G.; Gomez-Sanchez, E.P. mTOR Regulates mineralocorticoid receptor transcriptional activity by ULK1-dependent and -independent mechanisms. *Endocrinology* **2024**, *165*, bqae015. [CrossRef] [PubMed]
36. Park, J.M.; Lee, D.H.; Kim, D.H. Redefining the role of AMPK in autophagy and the energy stress response. *Nat. Commun.* **2023**, *14*, 2994. [CrossRef] [PubMed]
37. Rabinowitz, J.D.; White, E. Autophagy and metabolism. *Science* **2010**, *330*, 1344–1348. [CrossRef] [PubMed]
38. Russell, R.C.; Tian, Y.; Yuan, H.; Park, H.W.; Chang, Y.Y.; Kim, J.; Kim, H.; Neufeld, T.P.; Dillin, A.; Guan, K.L. ULK1 induces autophagy by phosphorylating Beclin-1 and activating VPS34 lipid kinase. *Nat. Cell Biol.* **2013**, *15*, 741–750. [CrossRef]
39. Dai, D.F.; Johnson, S.C.; Villarin, J.J.; Chin, M.T.; Nieves-Cintrón, M.; Chen, T.; Marcinek, D.J.; Dorn II, G.W.; Kang, J.; Prolla, T.A.; et al. Mitochondrial oxidative stress mediates angiotensin II-induced cardiac hypertrophy and Galphaq overexpression-induced heart failure. *Circ. Res.* **2011**, *108*, 837–846. [CrossRef]
40. Yen, W.L.; Legakis, J.E.; Nair, U.; Klionsky, D.J. Atg27 is required for autophagy-dependent cycling of Atg9. *Mol. Biol. Cell* **2007**, *18*, 581–593. [CrossRef]
41. Binotti, B.; Ninov, M.; Cepeda, A.P.; Ganzella, M.; Matti, U.; Riedel, D.; Urlaub, H.; Sambandan, S.; Jahn, R. ATG9 resides on a unique population of small vesicles in presynaptic nerve terminals. *Autophagy* **2023**, *26*, 883–901. [CrossRef] [PubMed]
42. Walczak, M.; Martens, S. Dissecting the role of the Atg12-Atg5-Atg16 complex during autophagosome formation. *Autophagy* **2013**, *9*, 424–425. [CrossRef] [PubMed]
43. Alam, J.M.; Maruyama, T.; Noshiro, D.; Kakuta, C.; Kotani, T.; Nakatogawa, H.; Noda, N.N. Complete set of the Atg8-E1-E2-E3 conjugation machinery forms an interaction web that mediates membrane shaping. *Nat. Struct. Mol. Biol.* **2024**, *31*, 170–178. [CrossRef] [PubMed]
44. Martens, S.; Fracchiolla, D. Activation and targeting of ATG8 protein lipidation. *Cell Discov.* **2020**, *6*, 23. [CrossRef] [PubMed]
45. Popelka, H.; Reinhart, E.F.; Metur, S.P.; Leary, K.A.; Ragusa, M.J.; Klionsky, D.J. Membrane binding and homodimerization of atg16 via two distinct protein regions is essential for autophagy in yeast. *J. Mol. Biol.* **2021**, *433*, 166809. [CrossRef] [PubMed]
46. Fujioka, Y.; Noda, N.N.; Nakatogawa, H.; Ohsumi, Y.; Inagaki, F. Dimeric coiled-coil structure of Saccharomyces cerevisiae Atg16 and its functional significance in autophagy. *J. Biol. Chem.* **2010**, *285*, 1508–1515. [CrossRef] [PubMed]
47. Yamaguchi, M.; Noda, N.N.; Nakatogawa, H.; Kumeta, H.; Ohsumi, Y.; Inagaki, F. Autophagy-related protein 8 (Atg8) family interacting motif in Atg3 mediates the Atg3-Atg8 interaction and is crucial for the cytoplasm-to-vacuole targeting pathway. *J. Biol. Chem.* **2010**, *285*, 29599–29607. [CrossRef] [PubMed]
48. Efeyan, A.; Comb, W.C.; Sabatini, D.M. Nutrient-sensing mechanisms and pathways. *Nature* **2015**, *517*, 302–310. [CrossRef] [PubMed]
49. Guo, H.; Ouyang, Y.; Yin, H.; Cui, H.; Deng, H.; Liu, H.; Jian, Z.; Fang, J.; Zuo, Z.; Wang, X.; et al. Induction of autophagy via the ROS-dependent AMPK-mTOR pathway protects copper-induced spermatogenesis disorder. *Redox Biol.* **2022**, *49*, 102227. [CrossRef]
50. Lu, Q.B.; Ding, Y.; Liu, Y.; Wang, Z.C.; Wu, Y.J.; Niu, K.M.; Li, K.X.; Zhang, J.R.; Sun, H.J. Metrnl ameliorates diabetic cardiomyopathy via inactivation of cGAS/STING signaling dependent on LKB1/AMPK/ULK1-mediated autophagy. *J. Adv. Res.* **2023**, *51*, 161–179. [CrossRef]
51. Mizushima, N. The role of the Atg1/ULK1 complex in autophagy regulation. *Curr. Opin. Cell Biol.* **2010**, *22*, 132–139. [CrossRef] [PubMed]

52. Tan, V.P.; Miyamoto, S. Nutrient-sensing mTORC1: Integration of metabolic and autophagic signals. *J. Mol. Cell Cardiol.* **2016**, *95*, 31–41. [CrossRef] [PubMed]
53. Mortimore, G.E.; Schworer, C.M. Induction of autophagy by amino-acid deprivation in perfused rat liver. *Nature* **1977**, *270*, 174–176. [CrossRef] [PubMed]
54. Son, S.M.; Park, S.J.; Stamatakou, E.; Vicinanza, M.; Menzies, F.M.; Rubinsztein, D.C. Leucine regulates autophagy via acetylation of the mTORC1 component raptor. *Nat. Commun.* **2020**, *11*, 3148. [CrossRef] [PubMed]
55. Hsu, C.L.; Lee, E.X.; Gordon, K.L.; Paz, E.A.; Shen, W.C.; Ohnishi, K.; Meisenhelder, J.; Hunter, T.; La Spada, A.R. MAP4K3 mediates amino acid-dependent regulation of autophagy via phosphorylation of TFEB. *Nat. Commun.* **2018**, *9*, 942. [CrossRef] [PubMed]
56. Duan, Y.; Li, F.; Tan, K.; Liu, H.; Li, Y.; Liu, Y.; Kong, X.; Tang, Y.; Wu, G.; Yin, Y. Key mediators of intracellular amino acids signaling to mTORC1 activation. *Amino Acids* **2015**, *47*, 857–867. [CrossRef]
57. Jin, C.L.; Zhang, Z.M.; Song, Z.W.; Gao, C.Q.; Yan, H.C.; Wang, X.Q. mTORC1-Mediated Satellite cell differentiation is required for lysine-induced skeletal muscle growth. *J. Agric. Food Chem.* **2020**, *68*, 4884–4892. [CrossRef] [PubMed]
58. Palma-Granados, P.; Haro, A.; Seiquer, I.; Lara, L.; Aguilera, J.F.; Nieto, R. Similar effects of lysine deficiency in muscle biochemical characteristics of fatty and lean piglets. *J. Anim. Sci.* **2017**, *95*, 3025–3036. [CrossRef] [PubMed]
59. Sato, T.; Ito, Y.; Nagasawa, T. Regulation of skeletal muscle protein degradation and synthesis by oral administration of lysine in rats. *J. Nutr. Sci. Vitaminol.* **2013**, *59*, 412–419. [CrossRef]
60. Sato, T.; Ito, Y.; Nagasawa, T. Lysine suppresses myofibrillar protein degradation by regulating the autophagic-lysosomal system through phosphorylation of Akt in C2C12 cells. *Springerplus* **2014**, *3*, 584. [CrossRef]
61. Sato, T.; Ito, Y.; Nagasawa, T. L-Lysine suppresses myofibrillar protein degradation and autophagy in skeletal muscles of senescence-accelerated mouse prone 8. *Biogerontology* **2017**, *18*, 85–95. [CrossRef] [PubMed]
62. Seibert, M.; Kurrle, N.; Schnütgen, F.; Serve, H. Amino acid sensory complex proteins in mTORC1 and macroautophagy regulation. *Matrix Biol.* **2021**, *100–101*, 65–83. [CrossRef] [PubMed]
63. Sato, T.; Ito, Y.; Nedachi, T.; Nagasawa, T. Lysine suppresses protein degradation through autophagic-lysosomal system in C2C12 myotubes. *Mol. Cell. Biochem.* **2014**, *391*, 37–46. [CrossRef] [PubMed]
64. Lang, S.M.; Kazi, A.A.; Hong-Brown, L.; Lang, C.H. Delayed recovery of skeletal muscle mass following hindlimb immobilization in mTOR heterozygous mice. *PLoS ONE* **2012**, *7*, e38910. [CrossRef] [PubMed]
65. Liu, Y.; Li, J.; Ding, C.; Tong, H.; Yan, Y.; Li, S.; Li, S.; Cao, Y. Leu promotes C2C12 cell differentiation by regulating the GSK3β/β-catenin signaling pathway through facilitating the interaction between SESN2 and RPN2. *J. Sci. Food Agric.* **2024**, *104*, 6696–6705. [CrossRef] [PubMed]
66. Jewell, J.L.; Kim, Y.C.; Russell, R.C.; Yu, F.X.; Park, H.W.; Plouffe, S.W.; Tagliabracci, V.S.; Guan, K.L. Differential regulation of mTORC1 by leucine and glutamine. *Science* **2015**, *347*, 194–198. [CrossRef] [PubMed]
67. Wu, H.; Wang, F.; Hu, S.; Yin, C.; Li, X.; Zhao, S.; Wang, J.; Yan, X. MiR-20a and miR-106b negatively regulate autophagy induced by leucine deprivation via suppression of ULK1 expression in C2C12 myoblasts. *Cell. Signal.* **2012**, *24*, 2179–2186. [CrossRef] [PubMed]
68. Zheng, Z.; Zhong, Q.; Yan, X. YWHAE/14-3-3ε crotonylation regulates leucine deprivation-induced autophagy. *Autophagy* **2023**, *19*, 2401–2402. [CrossRef] [PubMed]
69. Dodd, K.M.; Tee, A.R. Leucine and mTORC1: A complex relationship. *Am. J. Physiol. Endocrinol. Metab.* **2012**, *302*, E1329–E1342. [CrossRef]
70. Escobar, J.; Frank, J.W.; Suryawan, A.; Nguyen, H.V.; Kimball, S.R.; Jefferson, L.S.; Davis, T.A. Physiological rise in plasma leucine stimulates muscle protein synthesis in neonatal pigs by enhancing translation initiation factor activation. *Am. J. Physiol. Endocrinol. Metab.* **2005**, *288*, E914–E921. [CrossRef]
71. Yoshizawa, F. Regulation of protein synthesis by branched-chain amino acids in vivo. *Biochem. Biophys. Res. Commun.* **2004**, *313*, 417–422. [CrossRef] [PubMed]
72. Nüse, B.; Holland, T.; Rauh, M.; Gerlach, R.G.; Mattner, J. L-arginine metabolism as pivotal interface of mutual host-microbe interactions in the gut. *Gut Microbes* **2023**, *15*, 2222961. [CrossRef] [PubMed]
73. Savaraj, N.; You, M.; Wu, C.; Wangpaichitr, M.; Kuo, M.T.; Feun, L.G. Arginine deprivation, autophagy, apoptosis (AAA) for the treatment of melanoma. *Curr. Mol. Med.* **2010**, *10*, 405–412. [CrossRef] [PubMed]
74. Huang, L.; Yin, P.; Liu, F.; Liu, Y.; Liu, Y.; Xia, Z. Protective effects of L-arginine on the intestinal epithelial barrier under heat stress conditions in rats and IEC-6 cell line. *J. Anim. Physiol. Anim. Nutr.* **2020**, *104*, 385–396. [CrossRef] [PubMed]
75. Castejón-Vega, B.; Rubio, A.; Pérez-Pulido, A.J.; Quiles, J.L.; Lane, J.D.; Fernández-Domínguez, B.; Cachón-González, M.B.; Martín-Ruiz, C.; Sanz, A.; Cox, T.M.; et al. L-Arginine ameliorates defective autophagy in GM2 gangliosidoses by mTOR modulation. *Cells* **2021**, *10*, 3122. [CrossRef] [PubMed]
76. Wu, G.; Bazer, F.W.; Johnson, G.A.; Knabe, D.A.; Burghardt, R.C.; Spencer, T.E.; Li, X.L.; Wang, J.J. Triennial growth symposium: Important roles for L-glutamine in swine nutrition and production. *J. Anim. Sci.* **2011**, *89*, 2017–2030. [CrossRef] [PubMed]
77. Tan, H.W.S.; Sim, A.Y.L.; Long, Y.C. Glutamine metabolism regulates autophagy-dependent mTORC1 reactivation during amino acid starvation. *Nat. Commun.* **2017**, *8*, 338. [CrossRef] [PubMed]

78. Zhu, Y.; Lin, G.; Dai, Z.; Zhou, T.; Li, T.; Yuan, T.; Wu, L.; Wu, J.; Wang, J. L-Glutamine deprivation induces autophagy and alters the mTOR and MAPK signaling pathways in porcine intestinal epithelial cells. *Amino Acids* **2015**, *47*, 2185–2197. [CrossRef] [PubMed]
79. Sato, T.; Ito, Y.; Nagasawa, T. Dietary L-lysine suppresses autophagic proteolysis and stimulates Akt/mTOR signaling in the skeletal muscle of rats fed a low-protein diet. *J. Agric. Food Chem.* **2015**, *63*, 8192–8198. [CrossRef]
80. Crozier, S.J.; Kimball, S.R.; Emmert, S.W.; Anthony, J.C.; Jefferson, L.S. Oral leucine administration stimulates protein synthesis in rat skeletal muscle. *J. Nutr.* **2005**, *135*, 376–382. [CrossRef]
81. Mammucari, C.; Milan, G.; Romanello, V.; Masiero, E.; Rudolf, R.; Del Piccolo, P.; Burden, S.T.; Lisi, R.D.; Sandri, C.; Zhao, J.; et al. FoxO3 controls autophagy in skeletal muscle in vivo. *Cell Metab.* **2007**, *6*, 458–471. [CrossRef] [PubMed]
82. Zeanandin, G.; Balage, M.; Schneider, S.M.; Dupont, J.; Hébuterne, X.; Mothe-Satney, I.; Dardevet, D. Differential effect of long-term leucine supplementation on skeletal muscle and adipose tissue in old rats: An insulin signaling pathway approach. *Age* **2012**, *34*, 371–387. [CrossRef]
83. Zhao, J.; Brault, J.J.; Schild, A.; Cao, P.; Sandri, M.; Schiaffino, S.; Lecker, S.H.; Goldberg, A.L. FoxO3 coordinately activates protein degradation by the autophagic/lysosomal and proteasomal pathways in atrophying muscle cells. *Cell Metab.* **2007**, *6*, 472–483. [CrossRef] [PubMed]
84. Mordier, S.; Deval, C.; Béchet, D.; Tassa, A.; Ferrara, M. Leucine limitation induces autophagy and activation of lysosome-dependent proteolysis in C2C12 myotubes through a mammalian target of rapamycin-independent signaling pathway. *J. Biol. Chem.* **2000**, *275*, 29900–29906. [CrossRef] [PubMed]
85. Xia, X.J.; Gao, Y.Y.; Zhang, J.; Wang, L.; Zhao, S.; Che, Y.Y.; Ao, C.J.; Yang, H.J.; Wang, J.Q.; Lei, L.C. Autophagy mediated by arginine depletion activation of the nutrient sensor GCN2 contributes to interferon-γ-induced malignant transformation of primary bovine mammary epithelial cells. *Cell Death Discov.* **2016**, *2*, 15065. [CrossRef] [PubMed]
86. Sarkar, S.; Korolchuk, V.I.; Renna, M.; Imarisio, S.; Fleming, A.; Williams, A.; Garcia-Arencibia, M.; Rose, C.; Luo, S.; Underwood, B.R.; et al. Complex inhibitory effects of nitric oxide on autophagy. *Mol. Cell* **2011**, *43*, 19–32. [CrossRef] [PubMed]
87. Angcajas, A.B.; Hirai, N.; Kaneshiro, K.; Karim, M.R.; Horii, Y.; Kubota, M.; Fujimura, S.; Kadowaki, M. Diversity of amino acid signaling pathways on autophagy regulation: A novel pathway for arginine. *Biochem. Biophys. Res. Commun.* **2014**, *446*, 8–14. [CrossRef] [PubMed]
88. Wilden, A.R.; Molina, J.A.; Feuerborn, M.; Boyle, D.; Lee, S.Y. Glutamine-dependent lysosome homeostatic changes induced by starvation and lysosome inhibition. *Biochim. Biophys. Acta Mol. Cell Res.* **2018**, *1865*, 1356–1367. [CrossRef] [PubMed]
89. Liu, D.; Lin, J.; Su, J.; Chen, X.; Jiang, P.; Huang, K. Glutamine deficiency promotes PCV2 infection through induction of autophagy via activation of ROS-mediated JAK2/STAT3 signaling pathway. *J. Agric. Food Chem.* **2018**, *66*, 11757–11766. [CrossRef]
90. Vernizzi, L.; Paiardi, C.; Licata, G.; Vitali, T.; Santarelli, S.; Raneli, M.; Manelli, V.; Rizzetto, M.; Gioria, M.; Pasini, M.E.; et al. Glutamine synthetase 1 increases autophagy lysosomal degradation of mutant huntingtin aggregates in neurons, ameliorating motility in a drosophila model for Huntington's disease. *Cells* **2020**, *9*, 196. [CrossRef]
91. van der Vos, K.E.; Coffer, P.J. Glutamine metabolism links growth factor signaling to the regulation of autophagy. *Autophagy* **2012**, *8*, 1862–1864. [CrossRef] [PubMed]
92. Martinet, W.; de Meyer, G.R.; Herman, A.G.; Kockx, M.M. Amino acid deprivation induces both apoptosis and autophagy in murine C2C12 muscle cells. *Biotechnol. Lett.* **2005**, *27*, 1157–1163. [CrossRef] [PubMed]
93. Russell, R.C.; Yuan, H.X.; Guan, K.L. Autophagy regulation by nutrient signaling. *Cell Res.* **2014**, *24*, 42–57. [CrossRef] [PubMed]

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Review

The Neuroanatomy of Induced Pluripotent Stem Cells: In Vitro Models of Subcortical Nuclei in Neurodegenerative Disorders

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Abstract: Neuromodulatory subcortical systems (NSSs) are monoaminergic and cholinergic neuronal groups that are markedly and precociously involved in the pathogenesis of many neurodegenerative disorders (NDDs), including Parkinson's and Alzheimer's diseases. In humans, although many tools have been developed to infer information on these nuclei, encompassing neuroimaging and neurophysiological methods, a detailed and specific direct evaluation of their cellular features in vivo has been difficult to obtain until recent years. The development of induced pluripotent stem cell (iPSC) models has allowed research to deeply delve into the cellular and molecular biology of NSS neurons. In fact, iPSCs can be produced easily and non-invasively from patients' fibroblasts or circulating blood monocytes, by de-differentiating those cells using specific protocols, and then be re-differentiated towards neural phenotypes, which may reproduce the specific features of the correspondent brain neurons (including NSS ones) from the same patient. In this review, we summarized findings obtained in the field of NDDs using iPSCs, with the aim to understand how reliably these might represent in vitro models of NSS. We found that most of the current literature in the field of iPSCs and NSSs in NDDs has focused on midbrain dopaminergic neurons in Parkinson's disease, providing interesting results on cellular pathophysiology and even leading to the first human autologous transplantation. Differentiation protocols for noradrenergic, cholinergic, and serotoninergic neurons have also been recently defined and published. Thus, it might be expected that in the near future, this approach could extend to other NSSs and other NDDs.

Keywords: noradrenaline; dopamine; acetylcholine; serotonin; Alzheimer's disease; Parkinson's disease; neurodegeneration; induced pluripotent stem cells

1. Introduction

Neurodegenerative disorders (NDDs) are among the most pressing medical issues of our time. The global burden of Alzheimer's disease (AD) and Parkinson's disease (PD) increases every year, and effective disease-modifying therapies are yet to be developed and introduced into clinical practice [1,2]. At the same time, a precision medicine approach is essential, as the same symptoms and degeneration of similar brain nuclei in patients with the disease may be related to different pathogenetic mechanisms at an individual level. A constant key element of NDD pathogenesis is the involvement of the neuromodulatory subcortical systems (NSSs) from the early stages of the diseases [3]. NSSs are a group of subcortical nuclei distributed along the brainstem and the most phylogenetically ancient part of the telencephalon [4]. They play crucial roles in regulating central nervous system (CNS) homeostasis and modulating its activities both at a microscale and macroscale level. In PD, the degeneration of one of these NSSs, the pars compacta of the mesencephalic nucleus substantia nigra (SNpc), is the main driver of the

disease symptomatology [5]. In AD, the disruption of the cholinergic (Ach) innervation of the limbic cortex, arising from the nucleus basalis of Meynert (NBM) and the medial septal nucleus (MSN), plays a key role in the core symptoms of the disease, i.e., memory impairment [6,7]. Interestingly, in both disorders, the early impairment of the pontine noradrenergic (NA) locus coeruleus (LC) (which represents one of the main NSSs, due to the particularly widespread innervation of the whole brain by its axons, see Section 2.2) has been hypothesized to play a significant pathogenic role by disrupting precociously neuronal homeostasis [8–11]. Furthermore, sleep disturbances and mood alterations, common accompanying symptoms in NDDs, have been linked to the impairment of orexinergic neurons in the lateral hypothalamus (LHA) and serotoninergic (5HT) dorsal raphe nucleus (DRN), respectively [12–14].

In the last decades, our understanding of NDDs' pathogenesis and pathophysiology has remarkably improved, although their etiology remains largely unclear. Arguably, the bottleneck may be the difficulty in directly accessing CNS functioning in vivo in patients. This constraint limits research on NDDs in humans to indirect in vivo tools, such as neuroimaging and neurophysiological measurements, or to static morphological assessments post-mortem of specific brain areas/networks. Concerning NSSs, many neuroimaging tools have been developed to evaluate the integrity of these nuclei in vivo; some have stably entered clinical practice (e.g., Fluoro-DOPA positron emission tomography or DATscan single photon emission computed tomography) [5], while others are limited to research purposes (e.g., neuromelanin-sensitive magnetic resonance imaging for SNpc and LC) [15,16].

In this context, a promising experimental approach that may open a direct window on human NSSs is the development of induced pluripotent stem cell (iPSC) models [17–22]. Fully differentiated mesenchymal cells (such as fibroblasts or circulating blood monocytes) [18,23] can be de-differentiated from iPSCs using specific protocols, and then re-programmed towards neural phenotypes [17], and even further to specific neuronal population phenotypes, such as dopaminergic (DA) or NA cells of the brainstem. This potentially allows researchers to perform experimental studies directly on human cells belonging to one of the NSSs' nuclei, assessing in vivo cellular and molecular pathways that might play a pathogenetic role and/or the effects of specific drugs on those [19–22]. Furthermore, since iPSCs can be developed from the very same cells of the patients, they provide the opportunity to assess any differences in cell physiology and metabolism and to associate them with the characteristics of the donor [19–22].

In this review, we will provide a global perspective on the use of iPSCs as a model for studying NSSs, with the following dual purpose: (a) to assess whether the current state-of-the-art differentiation protocols offer the potential to "replicate" all of the main NSSs (specifically NA LC, DA SNpc, Ach NBM, and 5HT DRN) and (b) to summarize the primary experimental findings obtained thus far, from both pathophysiological and therapeutic viewpoints, in the context of NDD research. To facilitate a comprehensive understanding of our findings, we will begin by briefly introducing the NSSs from anatomical and functional perspectives.

2. The Neuromodulatory Subcortical Systems: Elements of Functional Anatomy

From an anatomical point of view, NSSs belong to the so-called "isodendritic core", which includes several clusters of neurons sparsely distributed along the brainstem and basal telencephalon and classified based on their main neurotransmitter [24]. The key and common denominator feature of NSSs is represented by the isodendritic neuron, characterized by vast dendritic arborization and axonal branching. This allows it to convey a large amount of input stimuli and to reach broad areas of the CNS with its output signal [25]. This specific cytoarchitecture is the morphological counterpart of the main physiological function of the isodendritic core, which is to regulate the activity of the CNS based on the external and internal environment, and, in particular, to modulate the sleep/wake cycle and the state of vigilance [26].

Within this functional framework, even though they show different cytoarchitecture or topography, other neuronal clusters are also classically included in the definition of NSSs, such as the Ach nuclei of the basal forebrain [27] or the orexinergic neurons of the LHA [12]. Additionally, NSSs play specific roles depending on their main neurotransmitter, influencing specific cellular metabolic pathways, neural tissue homeostasis, and neuronal networks.

For the purposes of this review, we summarize the NSSs' main features according to histochemical classification (based on their principal neurotransmitter) [28–30], reporting the nuclei that are more relevant for human neurobiology, in a caudo-rostral order (see also Table 1 for a schematic description).

Table 1. The main neuromodulatory subcortical nuclei.

Anatomical Definition	Histochemical Classification (Neurotransmitter)	CNS Targets	Physiological Effects
Dorsal Raphe Nucleus (DRN)	B8–B9 Serotonin (5HT)	Brainstem, hypothalamus, amygdala, hippocampus, cortex	Wake-promoting nucleus; pain and vegetative function regulation; mood and behavior modulation.
Locus Coeruleus (LC)	A6 Noradrenaline (NA)	Virtually the whole CNS	Wake-promoting nucleus; attention focusing and shifting; learning and memory promotion.
Substantia Nigra pars compacta (SNpc)	A9 Dopamine (DA)	Basal ganglia, thalamus, motor cortex	Movement regulation; planning and execution
Ventral Tegmental Area (VTA)	A10 Dopamine (DA)	Amygdala, hippocampus, nucleus accumbens, frontal cortex	Reward process and reinforced learning promotion; executive functions regulations.
Pedunculopontine Nucleus (PPN) and Lateral Tegmental Nucleus (LTN)	Ch5 and Ch6 Acetylcholine (Ach)	Other brainstem nuclei, hypothalamus	Wake-promoting nuclei
Nucleus Basalis of Meynert (NBM), Medial Septum Nucleus (MSN), horizontal and vertical limbs of the diagonal band of Broca (hDBB and vDBB)	Ch1, Ch2, Ch3 and Ch4 Acetylcholine (Ach)	Isocortex (NBM), hippocampus (MSN and vDBB), and olfactory cortex (hDBB)	Cortical activation and desynchronization; learning and memory promotion.

Legend to table. Ach—acetylcholine; CNS—central nervous system; DA—dopamine; DRN—dorsal raphe nucleus; hDBB—horizontal limb of the diagonal band of Broca; LC—locus coeruleus; LTN—lateral tegmental nucleus; MSN—medial septum nucleus; NA—noradrenaline; NBM—nucleus basalis of Meynert; PPN—pedunculopontine nucleus; SNpc—substantia nigra pars compacta; vDBB—vertical limb of the diagonal band of Broca; VTA—ventral tegmental area; 5HT—serotonin.

2.1. The Raphe Nuclei and the Serotoninergic System

The raphe nuclei are a group of heterogeneous clusters of neurons distributed along the median line of the brainstem, from the medulla oblongata to the midbrain, mainly characterized by the synthesis and release of 5HT. Classified by Dahlstrom and Fuxe into nine different clusters (B1–B9) [28,29], the highest density of 5HT neurons is found in the DRN (B8–B9), which is located at the junction between the pons and the midbrain [28,29]. The DRN projects to several structures of the CNS, including other nuclei of the brainstem, the hypothalamus, the amygdala, the hippocampus, and the cortex [31,32]. Through this wide innervation pattern, 5HT plays pivotal roles in the sleep/wake cycle (promoting wakefulness), pain modulation (projecting onto the periaqueductal gray), vegetative functions, and regulation of mood and affective behavior [31–34].

Functional alterations and structural disruption of the 5HT NSS can be detected in the early stages of many degenerative diseases. Furthermore, both PD and AD patients often

experience prodromal symptoms, including mood and sleep disturbances, that might be associated with 5HT NSS impairment [35].

2.2. The Locus Coeruleus and the Noradrenergic System

Although seven clusters of NA neurons can be detected along the brainstem, the main hub of brain NA system is by far the pontine nucleus LC (A6, according to the Dahlstrom and Fuxe classification), which is placed just below the floor of the fourth ventricle [29,36]. The LC is a tiny tube-shaped nucleus that, through its projections, reaches virtually all areas of the CNS. On a macroscale level, it plays crucial roles in the sleep/wake cycle, acting as a wakefulness and alertness-promoting nucleus, modulating fear and anxiety, and promoting attention [37–39]. At the cellular level, NA exerts a neuroprotective effect by regulating neurovascular coupling, promoting blood–brain barrier integrity, modulating microglial activity, and inducing neural growth factor production [40–43].

The involvement of LC in NDDs has gained increasing attention in recent years. In AD, the LC may be the very first brain structure to show detectable pathological alterations, such as hyperphosphorylated tau accumulation, which has been shown to occur even decades before the clinical onset of the disease [9,44]. In PD and other parkinsonisms, LC degeneration also occurs early and is associated with several non-motor symptoms [45,46]. Furthermore, the loss of its physiological neuroprotective effects is now considered one of the possible pathogenic mechanisms contributing to the progression of these disorders, as the loss of NA innervation has been linked to increased neuroinflammation [47,48], pathological protein accumulation [49], and neurovascular alterations [50].

2.3. The Dopaminergic Nuclei: The Substantia Nigra and the Ventral Tegmental Area

The DA NSS is mainly represented by two nuclei: the SNpc and the ventral tegmental area (VTA), which are both placed in the midbrain close to each other. The DA neurons of the SNpc project to the striatum (nigrostriatal pathway), engage in a complex interplay with the motor cortex, thalamus, and subthalamic nucleus of Lewis to regulate movement planning and execution [4,29]. The VTA projects to both limbic structures, such as the amygdala, hippocampus, and nucleus accumbens (mesolimbic pathway), and prefrontal/frontal cortices (mesocortical pathway). Through its mesolimbic connections, the VTA participates in the reward process and reinforced learning, while the mesocortical pathway allows the modulation of cognitive functions, such as executive function and planning [51–54].

The degeneration of the SNpc is the key pathological feature of PD (together with LC neuronal loss) and, to varying extents, also occurs in other parkinsonisms. The loss of nigrostriatal DA is key in the classical motor symptoms of PD, such as tremors, bradykinesia, and rigidity [5,45]. VTA impairment occurs in PD but can also be detected in AD patients and is involved in psychiatric and behavioral alterations, such as psychosis, hallucinations, and apathy [55,56].

2.4. The Cholinergic Nuclei

The anatomical distribution of the Ach nuclei along the encephalon is more heterogeneous compared with the NSSs described above. According to Mesulam's classification, six main clusters of Ach neurons can be found in the human CNS [27]. Two of them, the pedunculopontine nucleus (Ch5) (PPN) and the lateral tegmental nucleus (Ch6) (LTN), are located at the junction between the pons and the midbrain, laterally to the LC. They mainly participate in the regulation of the sleep/wake cycle, functioning as wake-promoting nuclei [27]. The other four Ach nuclei are placed in the basal forebrain and are the NBM (Ch1), the MSN (Ch2), and the horizontal (Ch3) and vertical (Ch4) limbs of the diagonal band of Broca (hDBB and vDBB, respectively). Despite their position, they are considered as the most rostral extension of the isodendritic core. The hDBB projects to the olfactory cortex, the vDBB and the NSM to the hippocampus, and the NBM to the isocortex [27,30,57]. The Ach nuclei of the basal forebrain play a key role in cortical activation, promoting the desynchronization of neuronal activity

and facilitating memory and learning [58,59]. At the cellular level, Ach also regulates neurovascular coupling and exerts anti-inflammatory effects. The degeneration of these nuclei, particularly the NBM, occurs in AD and is associated with its key symptom, i.e., memory impairments [6,7]. Also, in PD and Lewy body dementia, the involvement of this NSS is strictly linked to cognitive impairment [60,61].

3. Methodology of the Literature Search

We performed a literature search on PubMed.com using "iPSC", "induced pluripotent stem cells", "neurons", "noradrenaline", "dopamine", "acetylcholine", and "serotonin" as key words. We found 1618 records, and 1208 were excluded upon title analysis because they were out of topic. We also excluded 278 papers after abstract analysis and 34 more after text reading. Ninety-eight studies were finally included. While, for the first part of this review, all studies describing NSS iPSCs were considered suitable, for its second part, dedicated to the description of pathophysiological and pharmacological findings, only papers concerning NDDs and related symptoms were considered.

4. NSS iPSCs: Differentiation Protocols

4.1. Reprogramming of Somatic Cells

Human iPSC derivation methods encompass various strategies to reprogram somatic cells into a pluripotent state. In order to obtain neural precursors from somatic cells, mainly fibroblasts [62–84] and peripheral blood mononuclear cells (PBMCs) [85–89], various reprogramming methods have been used over the years to produce iPSCs. One of the first reprogramming approaches was the original method developed by Yamanaka et al. (2006), which involved reprogramming with retroviral vectors by infecting differentiated cells with retroviruses carrying key reprogramming genes, encoding for the transcription factors POU domain class 5 transcription factor 1 (OCT3/4), the sex-determining region Y-box2 (SOX2), Kruppel-like factor 4 (KLF4), and myelocytomatosis oncogene (c-MYC) [90]. While this approach has been successfully employed [72,77–79,81,83,91], further strategies for delivering reprogramming factors into somatic cells have been explored to obtain neural precursors. Lentiviral vectors have been used as a promising alternative [67,69,71,86,92–94], offering significant improvements in safety and efficiency compared with retroviral vectors [95,96]. However, both retroviral and lentiviral vectors share critical limitations due to the integration of reprogramming genes into the cellular genome; for this reason, alternative reprogramming methods that do not depend on integrating viral vectors are used. Among these, Sendai virus has been widely used [64–66,68,75,76,97,98], since its genome does not integrate into the host cell genome, thus avoiding insertional mutagenesis issues, and is not persistent during the post-reprogramming phases. Episomal vectors have also been used [62,63,70,85,87–89,92], although with lower reprogramming efficiency compared with integrating viral vectors [99].

4.2. Induction of Neural Precursors

The initial step of neural induction of iPSC is common for cells that are destined to acquire DA, NA, 5HT, and Ach fate and is based on the SMAD inhibition approach [62–64,66–69,71–74,76,77,81,87–89,91–94,97,98,100–108]. This step allows the generation of neural progenitors with forebrain identity [72,109], using small molecules that suppress transforming growth factor (TGF)-β pathways such as SB431542 [110] and the inhibitors or antagonists of bone morphogenetic proteins (BMPs) LDN193189, dorsomorphin, and noggin [111]. After forebrain induction, in order to generate progenitors with forebrain, midbrain, and hindbrain identity, the modulation of the sonic hedgehog (SHH) and WNT signaling pathways is essential [72,109]. Modulation of the SHH pathway is frequently performed using the same SHH or agonists, including purmorphamine and fibroblast growth factor 8 (FGF8) [62–69,71,72,74,76,77,81–84,87–89,91–94,97,98,101–109,112], and is used in 5HT and Ach neuron phenotype induction, after TGF-β and BMP inhibition, to induce the ventralization of hindbrain precursors [65,72,89,102,105]. For activation of the WNT

pathway, the small compound CHIR99021, a potent inhibitor of GSK3b, is used in many protocols to promote the neural induction of progenitors of a variety of cell types, including DA, 5HT, and NA neurons [62,63,65–69,71–74,87–89,91,92,94,97,98,100,104,106,107,109]. During 5HT neural induction, in order to promote hindbrain precursor generation, rather than CHIR99021, Valiulahi et al. (2021) used retinoic acid, which, through the modulation of *HOX* family genes, controls hindbrain activation in a concentration-dependent manner [113]. In methods aiming at DA neural induction, the activation of the WNT pathway with CHIR99021 is widely used in combination with SHH or the aforementioned agonists [62,63,66,67,69,71,74,87,88,91,92,94,97,98,104,106,107,109], and promotes the expression of transcription factors Foxa2 and Lmx1a, which are involved in the midbrain specification of neural progenitors [114,115]. In this process, WNT signaling plays a crucial role compared with the SHH pathway. Indeed, it has been observed that the administration of SHH or agonists alone, in the absence of WNT activators, leads to a low expression of Foxa2, without any effects on Lmx1a [109]. In order to obtain NA neural progenitors, Tao et al. employed the WNT agonist CHIR99021 during the early phase of neural induction, following SMAD inhibition, to achieve the expression of midbrain differentiation-specific factors. Subsequently, by using different concentrations of the same molecule, they induced neural precursors towards a hindbrain identity [100].

4.3. Dopaminergic Differentiation

Following the neural induction stage, in order to induce DA neuronal phenotypes, precursor cells are cultured in a neural maturation medium supplemented with specific growth factors and other compounds. Among these, commonly used factors are brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), transforming growth factor β3 (TGFβ3), dibutyryl cyclic AMP (dbcAMP), N-[N-(3,5-difluorophenyl acetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT), and ascorbic acid (AA) [62–64,66–69,71,74,76,77,81–84,87,88,91–94,97,98,101,103,104,106–109,112,116]. In particular, the neurotrophic factors GDNF and BDNF are crucial for neuronal maturation by promoting the survival and differentiation of neural progenitors [117]. Similarly, TGFβ3 is thought to be involved in DA neuron survival [118] and functionally interacts with the BDNF and GDNF signaling pathways [119,120]. Omitting TGFβ3 from the neural maturation protocol during phenotype characterization results in the decreased expression of DA markers TH and FOXA2, alongside an increase in the astroglial genesis marker S100B [117]. Interestingly, dbcAMP, a cell-permeable cAMP analog, exhibits a differential effect on DA differentiation compared with the above-described growth factors. Although it has not clearly demonstrated its capability to promote neuronal survival, dbcAMP treatment during DA precursor maturation has been shown to significantly enhance the expression of TH in stem cell-derived neurons [117,121]. Finally, AA is frequently used in DA maturation protocols due to its epigenetic properties on modulating expression of key DA development genes such as FOXA2, LMX1A, and NURR1 through histone and DNA demethylation [122].

4.4. Noradrenergic Differentiation

To the best of our knowledge, the differentiation of iPSCs to NA neurons has been described in only one recent paper until now. Following the acquisition of hindbrain identity through the modulation of the WNT signaling pathway, Tao et al. in 2023 [99] employed Activin A to induce neural progenitors towards an NA phenotype. Activin A, a member of the TGFβ superfamily, promotes the expression of genes crucial for NA fate, including Ascl1, Phox2a, and Phox2b, which exhibit partially overlapping functionalities [123,124]. The authors observed that administering Activin A at varying doses to neurons with rostral hindbrain identity resulted in early activation of Ascl1 at lower doses, and delayed activation of Phox2b at higher doses. In this context, aiming to enhance the expression of genes considered to be essential for acquiring NA fate, cyclopamine, an inhibitor of the SHH signaling pathway, was used. However, this treatment led to a reduction in the activation of

genes necessary for inducing NA fate and a concomitant increase in the activation of genes that promote dorsalization of neural progenitors. Conversely, the administration of DMH1, an antagonist of the BMP signaling pathway, induced an upregulation of genes required for NA development, without causing significant dorsalizing effects. Finally, following the acquisition of NA fate, the growth factors BDNF, GDNF, and TGFβ and the compounds AA and cAMP were employed to facilitate subsequent neuronal maturation.

4.5. Serotoninergic Differentiation

To generate 5HT neurons, it is crucial to establish hindbrain identity in neural precursors during the neural induction phase [65,72,73,89,102]. Protocols involving treatments with SHH and fibroblast growth factor 4 (FGF4) have been developed to induce the ventralization of hindbrain identity precursors [65,72]. Supplementation with FGF4 as an additional ventralization enhances the expression of transcription factor FOXA2 in synergy with SHH activation [65,72], and, in this context, FOXA2 is considered essential for acquiring 5HT fate [72]. Furthermore, FGF4 significantly reduces the expression of transcription factor PHOX2B, which is crucial for NA phenotype acquisition and, in turn, suppresses Fox2a gene expression [65,72]. Supporting the role of FGF4 in 5HT neuron differentiation, an increase in immunoreactivity towards the 5HT marker tryptophan hydroxylase 2 (TPH2) was observed following the differentiation procedure [72]. Subsequent to the acquisition of the 5HT phenotype, further neuronal maturation was achieved by supplementing the culture medium with growth factors, including BDNF, GDNF, TGFβ3, insulin growth factor-1 (IGF-1), and DAPT [65,72,73,89,102].

4.6. Cholinergic Differentiation

Only one paper describing the generation of Ach neurons with basal forebrain identity from induced pluripotent stem cells [105] was included in this report. Following the generation of neural precursors using the SMAD inhibition approach and their expansion using fibroblastic growth factor 2 (FGF2), SHH was used to induce ventralization of neural progenitors. To promote telencephalic identity and Ach differentiation, the culture medium was supplemented with FGF-8 and BMP9, respectively. Lastly, during Ach maturation, BDNF and NGF were supplemented in culture medium until full neuronal development. See Figure 1 for a synthetic schematization of all the protocols described.

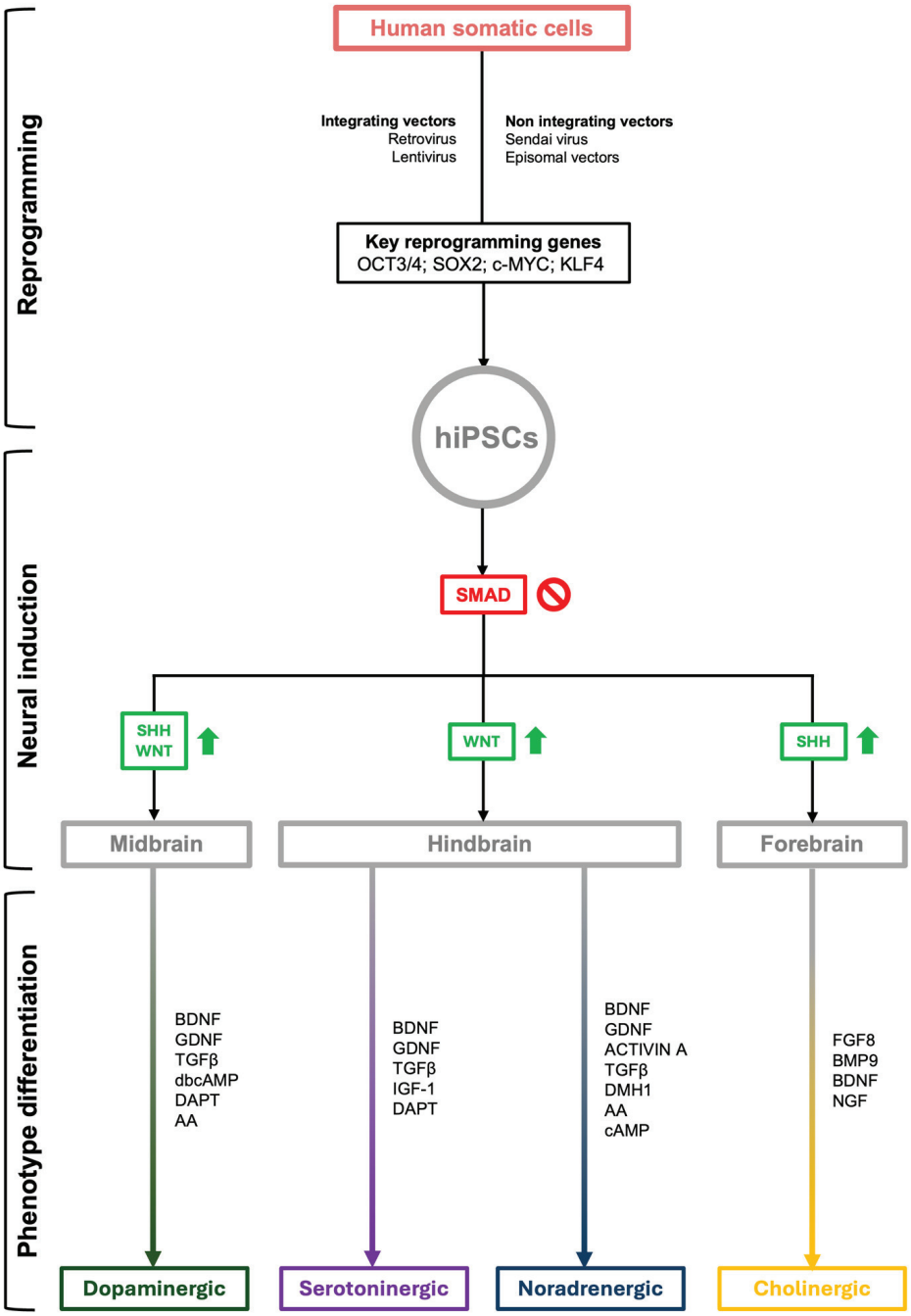


Figure 1. A schematic synthesis of NSS iPCS differentiation protocols. Starting from human somatic cells, either fibroblast or PBMC, a quite standardized reprogramming protocol is applied. Neural induction is obtained by inhibiting SMAD signaling and alternatively enhancing SHH and/or WNT pathways. Specific NSS phenotypes are then developed using standardized approaches, requiring specific growth factors, which are listed sequentially next to each arrow that connects the neural phenotype to the NSS phenotype. Abbreviations. AA—ascorbic acid; BDNF—brain-derived neurotrophic factor; BMP9—bone morphogenetic protein 9; c-MYC—myelocytomatosis oncogene; DAPT—N-[N-(3,5-difluorophenyl acetyl)-L-alanyl]-S-phenylglycine t-butyl ester; dbcAMP—dibutyryl cyclic AMP; FGF8—fibroblastic growth factor 8; GDNF—glial-derived neurotrophic factor; IGF-1—insulin growth factor 1; KLF4—Kruppel-like factor 4; NGF—nerve growth factor; OCT3/4—transcription factor POU domain class 5 transcription factor 1 (OCT3/4); SHH—sonic hedgehog; SOX2—sex-determining region Y-box2; TGFβ—transforming growth factor β.

5. Experimental Findings and Insight into NDDs' Pathophysiology

In this section (and the following one), we describe the findings obtained from iPSC-derived models of NSSs. As a general premise, it should be noted that there is a significant disproportion among the selected studies in terms of neuronal phenotype analyzed, with nearly all of those focusing on the evaluation of the DA system in the context of PD and parkinsonisms, and very few, if any, concerning the other NSSs.

5.1. Dopaminergic IPSC Models

Many studies have been performed on iPSC models of midbrain DA neurons, revealing new elements that may be involved in the cellular pathogenesis of PD, thanks to the extensive manipulability of this model [125]. Interestingly, iPSCs obtained from PD patients' samples in these studies showed morphological alterations consistent with PD pathology, such as alpha-synuclein (alpha-syn) accumulation, neurite arborization disruption, and mitochondrial abnormalities [79,126–128]. In 2012, Sanchez-Danes and colleagues obtained DA iPSCs from both idiopathic and genetic [bearing leucine-rich repeat kinase 2 (*LRRK2*) mutation] PD patients, [79]. They found that, compared with those of controls, DA neurons from patients showed reduced neurite arborization associated with autophagic pathway impairment [79]. These findings were confirmed by Ren and colleagues in a model obtained from *PARK2*-mutated PD patients, where neurite arborization disruption was linked to microtubular instability [126], a relation further confirmed in another iPSC study [127]. When cultured for long enough, DA iPSCs of patients start to accumulate intracellular alfa-syn, which is also released extracellularly [75,79,101,128,129]. According to Diao and colleagues, these might resemble Lewy body formation in case of *SNCA* triplication [101]. Zambon and colleagues found that alfa-syn pathology might be associated with the impairment of mitochondrial disfunction, as observed through the transcriptomic analysis of DA iPSCs obtained from *SNCA* patients [128]. Similar conclusions were drawn in another study on *PARK2*-mutated DA iPSCs, where the authors found impairment of mitochondria, and associated it with the disruption of their biogenesis [130].

DA iPSC models also allow for the dissection of cellular pathogenic mechanisms through which PD-related genetic mutations might cause the onset of the disease. The heterozygous mutation in the glucocerebrosidase gene (*GBA*) is the most significant genetic risk factor for PD and has been extensively studied using iPSC models. In 2016, Fernandes and colleagues developed DA iPSCs from donor patients and found that this mutation led to the misprocessing of beta-glucocerebrosidase within the endoplasmic reticulum, causing its accumulation and the disruption of autophagic pathways, eventually impairing DA synthesis and leading to alpha-syn accumulation [129]. Indeed, in another *GBA* iPSC study, Mazzulli et al. pharmacologically restored the activity of glucocerebrosidase, and showed, in parallel, the reduction of alfa-syn accumulation, the restoration of lysosomal activity, and the enhancement of DA neuron activity [131]. Previously, in 2015, Woodard et al. studied twins carrying a heterozygous mutation of the *GBA*, who were clinically discordant for the disease (one of the brothers was affected, while the other was not) [75]. They found that, while the iPSCs obtained from both twins showed lower levels of GBA activity, DA production, and accumulation of alfa-syn, the ones originating from fibroblasts of the affected brother showed an even lower level of DA synthesis and higher monoamine oxidase B (MAO-B) activity. This highlights how other factors might influence disease development [75], an observation inferred in other studies [132], and cases of different causes of mutation [133].

Mutations in the *LRRK2* gene are the most common cause of genetic PD. In DA iPSCs developed from affected patients, Lopez de Maturana and colleagues found that the abnormal function of this protein causes an alteration in alpha-syn homeostasis, promoting its intracellular accumulation, and it is associated with the impairment of the NF-κB pathway, leading to abnormal activation of neuroinflammation [134]. The role of the latter link was highlighted by Schmidt and colleagues, who found that microglia-derived factors are key in the development of DA iPSCs, suggesting a key neuroprotective effect

on DA neurons [135]. In another study, the authors found that *LRRK2* mutation might be associated with the disruption of calcium homeostasis, impairing cellular metabolisms and exacerbating metabolic stress [136]. This very same pathway might also be recruited in cases of the PD-related mutation of *PINK1*, a gene coding for a serine/threonine kinase involved in mitochondrial activity, which was found to upregulate *LRRK2* activity in DA iPSCs [137]. In PD patients bearing the mutation for *PARK2*, a gene which encodes for E3 ubiquitin ligase parkin, the development of DA iPSCs showed the occurrence of the upregulation of the enzyme catechol-O-methyltransferase (COMT) together with the demethylation of its gene [138], shedding light on an otherwise unclear causative association [139].

Another interesting topic in which iPSCs have been contributing is the association between nicotine assumption and PD development. DA iPSCs express a nicotinic receptor [140], which promotes structural plasticity, increasing the number and the length of DA neuron dendrites and their somata size [141]. Forming a complex with a DA receptor, nicotinic ones exert neuroprotective effects on DA neurons, preventing the accumulation of alfa-syn occurring in cases of cellular distress [69,142] and promoting the transcription of neuronal growth factors [143]. As proof of concept, iPSCs obtained from *LRRK2* patients showed abnormal membrane localization of these receptors, whose neuroprotective function was impaired [144]. This line of research, entirely developed using DA iPSC models, might open a new therapeutic perspective for PD.

Finally, some authors have explored the diagnostic utility of DA iPSC, as transcriptomic analysis performed in this model clearly identified gene and protein networks that can be affected and detected in PD patients [145]. In 2022, Ren and colleagues published a paper in which they reported the results of detailed molecular analysis performed on DA iPSCs obtained from healthy control and PD patients, the latter divided based on the occurrence of resting tremors [146]. They detected significant differences in terms of genetic expression, metabolites, and oxidative stress that allowed them not only to distinguish between patients and controls but also between patients with or without resting tremors, opening a possible scenario for a whole-informative diagnostic test [146].

5.2. Other NSS iPSC Models

For the sake of honesty, we did not find any published papers specifically regarding non-DA NSS iPSC in NDDs. However, the two studies published by Vadodaria and colleagues in 2019, using 5HT iPSC models [147,148], are worth mentioning. In those papers, the authors reported the results of their analysis on major depressive disorder patients, finding an abnormal response to 5HT in forebrain neurons [148] and morphological and developmental alterations in the neurites of 5HT iPSCs [147] when compared with healthy controls. Although included patients were not properly affected by NDDs, depression and mood disturbances are quite common prodromal symptoms in this group of diseases [149]. Thus, these might represent a further potential utility of iPSCs in the study of prodromal stages of NDDs.

As far as we know, there are no published articles yet considering NA and Ach iPSC models of NDDs.

6. Drug Testing and Therapy Development

iPSC models have also been used to open new therapeutic perspectives in NDDs. In this context, two main approaches have been pursued. On the one hand, iPSCs have been applied as in vitro platforms to screen pharmacological compounds and to test gene editing approaches; on the other, the same obtained iPSCs represent pharmacological intervention as in vivo transplantations have been attempted in order to restore the reproduced NSSs disrupted in NDDs.

6.1. In Vitro Evaluation of Drug Safety and Pharmacodynamics

As said, iPSCs can be reprogrammed to achieve the morphological and biochemical characteristics of brain NSS neurons (Sections 4 and 5). Thus, they can be used in vitro

assays to test drug efficacy and safety. DA iPSCs are sensitive to chemical compounds classically used to induce cellular stress and damage, underlining the reliability of this cellular model [98,150,151]. Some molecules have already been tested on these cells, with promising results [106,152–156]. In particular, ketamine and its derivate (2R,6R)-hydroxynorketamine have been shown to promote neurite growth and neural plasticity in DA iPSC models, with minimal or no adverse effects on cultured neurons [106,152, 153], a beneficial effect that was identified also for the combination of L-acetylcarnitine and L-methylfolate [155]. In SNCA-mutated DA iPSCs, felodipine, a calcium antagonist, was found to ameliorate stress signatures, such as autophagy disruption and metabolic impairment [156]. Phorbol esters were tested in DA iPSCs obtained from genetic PD patients and they caused a reduction in alfa-syn pathology by acting as a lysosomal function activator [154]. Similar effects were interestingly observed by stimulating the nicotinic receptors of DA cells, as already reported in the previous section [141,142].

Finally, it is worth mentioning that iPSCs might also be used to evaluate the effectiveness of gene editing in treating genetic diseases. In DA iPSCs, the genetic manipulation through CRISPR-Cas9 of the progenitor can induce the occurrence of PD pathology [157,158], and the same cellular model can be applied to verify the utility of antisense oligonucleotide in silencing causative mutated genes [159].

6.2. In Vivo Transplantation of iPSCs

As expected, all the studies on the opportunity to transplant iPSCs in the human brain have been performed in PD models, as, in this disease, there is a specific degeneration of SNpc DA neurons, and the pharmacological replacement of DA release through L-DOPA administration represents the current standard therapy [160]. Thus, restoring the population of DA neurons would constitute a major achievement in PD treatment. Indeed, many preclinical studies have been performed, in which human-derived iPSCs were transplanted into the SNpc of mice with experimentally induced PD and showed promising results [67,88,92,104,161–167]. In fact, transplanted human DA iPSCs usually survived in the host animal [92,104,161–164,166] and showed neurite growth [88,92,163] and DA innervation of appropriate brain targets [67]. Furthermore, treated animals showed improvement in motor symptoms and behavior, showing that transplanted iPSCs not only can survive and integrate in the host brain but can also restore the original physiological functions of degenerated DA neurons [67,88,92]. Some factors seem to the influence success rate and quality of transplanted neurons, such as the age of the precursor cells or of the same iPSCs [164,165,167] and the vitality of support neurons [161]. Moreover, it should be noted that producing iPSCs from patients might lead to obtaining more fragile DA neurons; in one study, iPSCs were cultured from genetic PD patients and these developed PD pathology once transplanted in the SNpc of the animal model [166]. As we reported above (Section 5.1), DA iPSCs obtained from idiopathic PD patients showed pathological alterations compared with controls, and this might hinder the usefulness of autologous transplantation without any further editing. However, in 2020, this same approach was experimented in a PD patient; DA iPSCs were obtained from skin fibroblasts and implanted bilaterally in the putamen of the subject. The graft survived and the clinical outcome of the patient improved, with the reduction or stabilization of motor symptoms [168].

7. Discussion and Future Perspectives

In this review, we provide a detailed overview of published studies in which the authors either developed specific protocols to obtain iPSC models of NSSs or utilized these models in the context of NDD studies. iPSCs offer a promising opportunity to directly study human neuron cytotypes, whose features and functions would otherwise be extremely difficult to analyze. Our aim was to identify which specific systems these in vitro models are best suited to represent and to describe the state of the art regarding how reliably they can replicate NSS neurons. Indeed, we found that all of the main NSSs can be “replicated”

through iPSC modeling, with refined protocols available for DA SNpc, NA LC, 5HT DRN, and Ach forebrain neurons.

On the other hand, our literature search revealed a clear trend on the use of these models in NDD research, with most published articles focusing on the DA NSS, and only a few papers reporting data about the 5HT, Ach, and NA systems. Studies on DA iPSCs have led to the significant advancement of our understanding of PD pathophysiology, through metabolic, biochemical, and genetic analyses. Moreover, they have led to the first human autologous graft [168], elevating the role of DA iPSCs from that of mere experimental models to the source of potential therapeutic candidates [169].

It might also be supposed that the literature regarding those topics will significantly increase very soon. For instance, in 2023, Tao and colleagues developed a refined protocol to obtain well-differentiated NA iPSCs with characteristics specific to LC-NA neurons [99]. This achievement offers an opportunity to thoroughly study the metabolic and cytological features of a system that is often and early involved in NDDs, such as PD [46] and AD [10]. In particular, in AD, the LC is the first brain structure to show tau-related pathological alterations [44], and many hypotheses have been proposed to explain its sensitivity to degenerative damage [170], including oxidative stress related to catecholamine synthesis and degradation [171], predisposition to tau protein hyperphosphorylation [172–174], and secondary effects of systemic inflammation and xenobiotics [175–178]. All these hypotheses could be tested in NA iPSCs carrying the genetic landscape of patients affected by either sporadic or genetic AD, shedding light on an unclear yet probably crucial step in the pathogenesis of AD.

Nonetheless, we must acknowledge the current lack of information on non-DA iPSC models for NDD research as a limitation of our review. Their pleiotropic roles in NDDs and less refined differentiation protocols may partially explain why only a few studies have focused on other NSSs. However, this limits our ability to assess the potential utility of these in vitro models beyond thae specific case of the DA system in PD. Further extensive studies are required to fully uncover the potential applications of these iPSC models in other NDDs.

One of the most interesting findings is that iPSCs obtained from donors affected by NDDs and reprogrammed show pathological features not only in cases of genetic forms but also in sporadic ones, as reported in [79,145,146]. Some relevant implications are worth noting. From the pathophysiological point of view, it highlights the crucial contribution of polygenetic and epigenetic factors to the occurrence of NDDs, as the iPSCs obtained from sporadic PD patienats showed pathological alterations similar to those developed from the genetic PD ones, even in the absence of any external influence. Methodologically, this characteristic bears a dual meaning. On the one hand, it reinforces the reliability of iPSCs as experimental models for sporadic forms of NDDs, where no clear genetic mutations are present, and a heterogeneous mixture of genetic and environmental factors likely triggers the pathogenic process. On the other hand, it emphasizes the importance of checking for the occurrence of other pathological or para-physiological factors related to the genetic and epigenetic landscape of donor patients. Mutations with low penetrance, even with no or minimal clinical manifestations, might still affect the biochemistry and metabolism of iPSCs, impairing the reliability and reproducibility of the collected results. As these models are further applied and developed, it will become increasingly important to accurately define and consider their genetic and epigenetic backgrounds.

However, extending this topic, it is conceivable that, in the near future, iPSCs might indeed be used as diagnostic tools. They will allow for the easy definition of the genomic and proteomic profiles of affected patients, identifying specific disease subtypes with unprecedented accuracy. Taking this concept to its extreme, one might even anticipate using iPSCs in the future to screen for the propensity to develop NDDs in otherwise asymptomatic individuals. Furthermore, the effects of drugs could be tested directly on target neurons, offering the opportunity to choose therapeutic strategies that best fit the characteristics of each patient, aligning with the personalized medicine approach.

Such future developments will inevitably require reducing or, at the very least, controlling possible variability. This could involve addressing methodological inconsistencies by defining strict and standardized protocols for iPSC differentiation. Indeed, our literature search revealed a notable degree of variability in current approaches to NSS phenotype differentiation across different research groups. Achieving this step will be mandatory to ensure the accuracy and reproducibility necessary for useful diagnostic tools.

In conclusion, NSS iPSCs are a useful and promising in vitro model for the study of NDDs. They have been extensively used in PD research, providing valuable and otherwise elusive findings. Extending their application to other NSSs and NDDs might be an excellent strategy to increase our understanding of the many roles of subcortical structures in this class of neurological disorders.

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Abbreviations

AA—ascorbic acid; Ach—acetylcholine; AD—Alzheimer's disease; Alpha-syn—alpha synuclein; BDNF—brain-derived neurotrophic factor; BMP—bone morphogenetic protein; c-MYC—myelocyt omatosis oncogene; CNS—central nervous system; COMT—catechol-O-methyltransferase; DA—dopamine; dbcAMP—dibutyryl cyclic AMP; DAPT—N-[N-(3,5-difluorophenyl acetyl)-L-alanyl]-S-phenylglycine t-butyl ester; DRN—dorsal raphe nucleus; FGF—fibroblast growth factor; hDBB—horizontal limb of the diagonal band of Broca; GBA—glucocerebrosidase; GDNF—glial-derived neurotrophic factor; IGF—insulin growth factor; iPSCs—induced pluripotent stem cells; KLF4—Kruppel-like factor 4; LC—locus coeruleus; LHA—lateral hypothalamic area; LRRK2—leucine-rich repeat kinase 2; LTN—lateral tegmental nucleus; MAO-B—monoamine oxidase B; MSN—medial septum nucleus; NA—noradrenaline; NBM—nucleus basalis of Meynert; NDD—neurodegenerative disorder; NSS—neuromodulatory subcortical system; OCT3/4—transcription factors POU domain class 5 transcription factor 1; PBMC—peripheral blood mononuclear cell; PD—Parkinson's disease; PPN—pedunculopontine nucleus; SHH—sonic hedgehog; SNpc—substantia nigra pars compacta; SOX2—sex-determining region Y-box2; TGF- β—transforming growth factor β; vDBB—vertical limb of the diagonal band of Broca; VTA—ventral tegmental area; 5HT—serotonin.

References

1. Dorsey, E.R.; Sherer, T.; Okun, M.S.; Bloemd, B.R. The Emerging Evidence of the Parkinson Pandemic. *J. Park. Dis.* **2018**, *8*, S3–S8. [CrossRef]
2. John Wiley & Sons, Ltd. 2023 Alzheimer's disease facts and figures. *Alzheimer's Dement.* **2023**, *19*, 1598–1695. [CrossRef] [PubMed]
3. Ehrenberg, A.J.; Kelberman, M.A.; Liu, K.Y.; Dahl, M.J.; Weinshenker, D.; Falgàs, N.; Dutt, S.; Mather, M.; Ludwig, M.; Betts, M.J.; et al. Priorities for research on neuromodulatory subcortical systems in Alzheimer's disease: Position paper from the NSS PIA of ISTAART. *Alzheimer's Dement.* **2023**, *8*, 21. [CrossRef] [PubMed]
4. Paxinos, G.; Mai, J.K. *The Human Nervous System*, 2nd ed.; Elsevier Inc.: Amsterdam, The Netherlands, 2003. [CrossRef]
5. Lees, A.J.; Hardy, J.; Revesz, T. Parkinson's disease. *Lancet* **2009**, *373*, 2055–2066. [CrossRef]
6. Hampel, H.; Mesulam, M.M.; Cuello, A.C.; Farlow, M.R.; Giacobini, E.; Grossberg, G.T.; Khachaturian, A.S.; Vergallo, A.; Cavedo, E.; Snyder, P.J.; et al. The cholinergic system in the pathophysiology and treatment of Alzheimer's disease. *Brain* **2018**, *141*, 1917–1933. [CrossRef] [PubMed]
7. Mesulam, M.-M. Cholinergic circuitry of the human nucleus basalis and its fate in Alzheimer's disease. *J. Comp. Neurol.* **2013**, *521*, 4124–4144. [CrossRef]
8. Theofilas, P.; Ehrenberg, A.J.; Dunlop, S.; Di Lorenzo Alho, A.T.; Nguy, A.; Leite, R.E.P.; Rodriguez, R.D.; Mejia, M.B.; Suemoto, C.K.; Ferretti-Rebustini, R.E.; et al. Locus coeruleus volume and cell population changes during Alzheimer's disease progression: A stereological study in human postmortem brains with potential implication for early-stage biomarker discovery. *Alzheimers Dement.* **2017**, *13*, 236–246. [CrossRef]

9. Kelly, S.C.; He, B.; Perez, S.E.; Ginsberg, S.D.; Mufson, E.J.; Counts, S.E. Locus coeruleus cellular and molecular pathology during the progression of Alzheimer's disease. *Acta Neuropathol. Commun.* **2017**, *5*, 8. [CrossRef]
10. Galgani, A.; Giorgi, F.S. Exploring the Role of Locus Coeruleus in Alzheimer's Disease: A Comprehensive Update on MRI Studies and Implications. *Curr. Neurol. Neurosci. Rep.* **2023**, *23*, 925–936. [CrossRef]
11. Giorgi, F.S.; Ryskalin, L.; Ruffoli, R.; Biagioni, F.; Limanaqi, F.; Ferrucci, M.; Busceti, C.L.; Bonuccelli, U.; Fornai, F. The Neuroanatomy of the Reticular Nucleus Locus Coeruleus in Alzheimer's Disease. *Front. Neuroanat.* **2017**, *11*, 80. [CrossRef]
12. Giorgi, F.S.; Galgani, A.; Puglisi-Allegra, S.; Busceti, C.L.; Fornai, F. The connections of Locus Coeruleus with hypothalamus: Potential involvement in Alzheimer's disease. *J. Neural Transm.* **2021**, *128*, 589–613. [CrossRef] [PubMed]
13. Eser, R.A.; Ehrenberg, A.J.; Petersen, C.; Dunlop, S.; Mejia, M.B.; Suemoto, C.K.; Walsh, C.M.; Rajana, H.; Oh, J.; Theofilas, P.; et al. Selective Vulnerability of Brainstem Nuclei in Distinct Tauopathies: A Postmortem Study. *J. Neuropathol. Exp. Neurol.* **2018**, *77*, 149–161. [CrossRef] [PubMed]
14. Oh, J.; Eser, R.A.; Ehrenberg, A.J.; Morales, D.; Petersen, C.; Kudlacek, J.; Dunlop, S.R.; Theofilas, P.; Resende, E.D.P.F.; Cosme, C.; et al. Profound degeneration of wake-promoting neurons in Alzheimer's disease. *Alzheimer's Dement.* **2019**, *15*, 1253–1263. [CrossRef]
15. Pavese, N.; Tai, Y.F. Nigrosome Imaging and Neuromelanin Sensitive MRI in Diagnostic Evaluation of Parkinsonism. *Mov. Disord. Clin. Pract.* **2018**, *5*, 131–140. [CrossRef]
16. Galgani, A.; Lombardo, F.; Della Latta, D.; Martini, N.; Bonuccelli, U.; Fornai, F.; Giorgi, F.S. Locus Coeruleus Magnetic Resonance Imaging in Neurological Diseases. *Curr. Neurol. Neurosci. Rep.* **2020**, *21*, 2. [CrossRef]
17. Vadodaria, K.C.; Jones, J.R.; Linker, S.; Gage, F.H. Modeling Brain Disorders Using Induced Pluripotent Stem Cells. *Cold Spring Harb. Perspect. Biol.* **2020**, *12*, a035659. [CrossRef]
18. Malik, N.; Rao, M.S. A Review of the Methods for Human iPSC Derivation. *Methods Mol. Biol.* **2013**, *997*, 23–33.
19. Beghini, D.G.; Kasai-Brunswick, T.H.; Henriques-Pons, A. Induced Pluripotent Stem Cells in Drug Discovery and Neurodegenerative Disease Modelling. *Int. J. Mol. Sci.* **2024**, *25*, 2392. [CrossRef] [PubMed]
20. Yang, J.; Li, S.; He, X.B.; Cheng, C.; Le, W. Induced pluripotent stem cells in Alzheimer's disease: Applications for disease modeling and cell-replacement therapy. *Mol. Neurodegener.* **2016**, *11*, 1–11. [CrossRef]
21. Xu, X.; Huang, J.; Li, J.; Liu, L.; Han, C.; Shen, Y.; Zhang, G.; Jiang, H.; Lin, Z.; Xiong, N.; et al. Induced pluripotent stem cells and Parkinson's disease: Modelling and treatment. *Cell Prolif.* **2016**, *49*, 14–26. [CrossRef]
22. Jungverdorben, J.; Till, A.; Brüstle, O. Induced pluripotent stem cell-based modeling of neurodegenerative diseases: A focus on autophagy. *J. Mol. Med.* **2017**, *95*, 705–718. [CrossRef] [PubMed]
23. Raab, S.; Klingenstein, M.; Liebau, S.; Linta, L. A Comparative View on Human Somatic Cell Sources for iPSC Generation. *Stem Cells Int.* **2014**, *2014*, 768391. [CrossRef] [PubMed]
24. Theofilas, P.; Dunlop, S.; Heinsen, H.; Grinberg, L.T. Turning on the Light Within: Subcortical Nuclei of the Isodentritic Core and their Role in Alzheimer's Disease Pathogenesis. *J. Alzheimers Dis.* **2015**, *46*, 17–34. [CrossRef] [PubMed]
25. Fornai, F.; Lenzi, P. *Sistema Nervoso Vegetativo: Anatomia Funzionale Della Vita Vegetativa*, 1st ed.; Pisa University Press: Pisa, Italy, 2020.
26. Moruzzi, G.; Magoun, H.W. Brain stem reticular formation and activation of the EEG. *Electroencephalogr. Clin. Neurophysiol.* **1949**, *1*, 455–473. [CrossRef] [PubMed]
27. Mesulam, M.-M. The cholinergic innervation of the human cerebral cortex. *Prog. Brain Res.* **2004**, *145*, 67–78.
28. Dahlström, A.; Fuxe, K. Localization of monoamines in the lower brain stem. *Experientia* **1964**, *20*, 398–399. [CrossRef]
29. Dahlstrom, A.; Fuxe, K. Evidence for the existence of monoamine-containing neurons in the central nervous system. I. Demonstration of monoamines in the cell bodies of brain stem neurons. *Acta Physiol. Scand. Suppl.* **1964**, *62* (Suppl. S232), 1–55.
30. Mesulam, M.-M.; Geula, C. Nucleus basalis (Ch4) and cortical cholinergic innervation in the human brain: Observations based on the distribution of acetylcholinesterase and choline acetyltransferase. *J. Comp. Neurol.* **1988**, *275*, 216–240. [CrossRef]
31. Hornung, J.P. The human raphe nuclei and the serotonergic system. *J. Chem. Neuroanat.* **2003**, *26*, 331–343. [CrossRef]
32. Waselus, M.; Valentino, R.J.; Van Bockstaele, E.J. Collateralized dorsal raphe nucleus projections: A mechanism for the integration of diverse functions during stress. *J. Chem. Neuroanat.* **2011**, *41*, 266–280. [CrossRef]
33. Benarroch, E.E. Pain-autonomic interactions. *Neurol. Sci.* **2006**, *27* (Suppl. S2), s130–s133. [CrossRef] [PubMed]
34. Monti, J.M. The structure of the dorsal raphe nucleus and its relevance to the regulation of sleep and wakefulness. *Sleep. Med. Rev.* **2010**, *14*, 307–317. [CrossRef] [PubMed]
35. Šimić, G.; Leko, M.B.; Wray, S.; Harrington, C.R.; Delalle, I.; Jovanov-Milošević, N.; Bažadona, D.; Buée, L.; De Silva, R.; Di Giovanni, G.; et al. Monoaminergic neuropathology in Alzheimer's disease. *Prog. Neurobiol.* **2017**, *151*, 101–138. [CrossRef] [PubMed]
36. Poe, G.R.; Foote, S.; Eschenko, O.; Johansen, J.P.; Bouret, S.; Aston-Jones, G.; Harley, C.W.; Manahan-Vaughan, D.; Weinshenker, D.; Valentino, R.; et al. Locus coeruleus: A new look at the blue spot. *Nat. Rev. Neurosci.* **2020**, *21*, 644–659. [CrossRef]
37. Counts, S.E.; Mufson, E.J. Locus Coeruleus. In *The Human Nervous System*, 3rd ed.; Mai, J.K., Paxinos, G., Eds.; Academic press: Cambridge, MA, USA, 2012; pp. 427–440.
38. Aston-Jones, G.; Waterhouse, B. Locus coeruleus: From global projection system to adaptive regulation of behavior. *Brain Res.* **2016**, *1645*, 75–78. [CrossRef]
39. González, M.M.C.; Aston-Jones, G. Circadian Regulation of Arousal: Role of the Noradrenergic Locus Coeruleus System and Light Exposure. *Sleep* **2006**, *29*, 1327–1336. [CrossRef]

40. Giorgi, F.S.; Saccaro, L.F.; Galgani, A.; Busceti, C.L.; Biagioni, F.; Frati, A.; Fornai, F. The role of Locus Coeruleus in neuroinflammation occurring in Alzheimer's disease. *Brain Res. Bull.* **2019**, *153*, 47–58. [CrossRef]
41. Feinstein, D.L.; Kalinin, S.; Braun, D. Causes, consequences, and cures for neuroinflammation mediated via the locus coeruleus: Noradrenergic signaling system. *J. Neurochem.* **2016**, *139*, 154–178. [CrossRef] [PubMed]
42. Cirelli, C.; Tononi, G. Locus ceruleus control of state-dependent gene expression. *J. Neurosci.* **2004**, *24*, 5410–5419. [CrossRef]
43. Cirelli, C.; Pompeiano, M.; Tononi, G. Neuronal gene expression in the waking state: A role for the locus coeruleus. *Science* **1996**, *274*, 1211–1215. [CrossRef]
44. Braak, H.; Thal, D.R.; Ghebremedhin, E.; Del Tredici, K. Stages of the Pathologic Process in Alzheimer Disease: Age Categories From 1 to 100 Years. *J. Neuropathol. Exp. Neurol.* **2011**, *70*, 960–969. [CrossRef] [PubMed]
45. Braak, H.; Del Tredici, K.; Rüb, U.; De Vos, R.A.I.; Jansen Steur, E.N.H.; Braak, E. Staging of brain pathology related to sporadic Parkinson's disease. *Neurobiol. Aging* **2003**, *24*, 197–211. [CrossRef] [PubMed]
46. Gesi, M.; Soldani, P.; Giorgi, F.S.; Santinami, A.; Bonaccorsi, I.; Fornai, F. The role of the locus coeruleus in the development of Parkinson's disease. *Neurosci. Biobehav. Rev.* **2000**, *24*, 655–668. [CrossRef] [PubMed]
47. Heneka, M.T.; Nadrigny, F.; Regen, T.; Martinez-Hernandez, A.; Dumitrescu-Ozimek, L.; Terwel, D.; Jardanhazi-Kurutz, D.; Walter, J.; Kirchhoff, F.; Hanisch, U.K.; et al. Locus ceruleus controls Alzheimer's disease pathology by modulating microglial functions through norepinephrine. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 6058–6063. [CrossRef] [PubMed]
48. Jardanhazi-Kurutz, D.; Kummer, M.P.; Terwel, D.; Vogel, K.; Dyrks, T.; Thiele, A.; Heneka, M.T. Induced LC degeneration in APP/PS1 transgenic mice accelerates early cerebral amyloidosis and cognitive deficits. *Neurochem. Int.* **2010**, *57*, 375–382. [CrossRef]
49. Heneka, M.T.; Ramanathan, M.; Jacobs, A.H.; Dumitrescu-Ozimek, L.; Bilkei-Gorzo, A.; Debeir, T.; Sastre, M.; Galldiks, N.; Zimmer, A.; Hoehn, M.; et al. Locus Ceruleus degeneration promotes Alzheimer pathogenesis in amyloid precursor protein 23 transgenic mice. *J. Neurosci.* **2006**, *26*, 1343–1354. [CrossRef]
50. Kalinin, S.; Feinstein, D.L.; Xu, H.; Huesa, G.; Pelligrino, D.A.; Galea, E. Degeneration of noradrenergic fibres from the locus coeruleus causes tight-junction disorganisation in the rat brain. *Eur. J. Neurosci.* **2006**, *24*, 3393–3400. [CrossRef]
51. Morales, M.; Margolis, E.B. Ventral tegmental area: Cellular heterogeneity, connectivity and behaviour. *Nat. Rev. Neurosci.* **2017**, *18*, 73–85. [CrossRef]
52. Becker-Krail, D.D.; Walker, W.H.; Nelson, R.J. The Ventral Tegmental Area and Nucleus Accumbens as Circadian Oscillators: Implications for Drug Abuse and Substance Use Disorders. *Front. Physiol.* **2022**, *13*, 886704. [CrossRef]
53. Bariselli, S.; Glangetas, C.; Tzanoulinou, S.; Bellone, C. Ventral tegmental area subcircuits process rewarding and aversive experiences. *J. Neurochem.* **2016**, *139*, 1071–1080. [CrossRef]
54. Oliva, I.; Wanat, M.J. Ventral tegmental area afferents and drug-dependent behaviors. *Front. Psychiatry* **2016**, *7*, 181416. [CrossRef] [PubMed]
55. D'Amelio, M.; Puglisi-Allegra, S.; Mercuri, N. The role of dopaminergic midbrain in Alzheimer's disease: Translating basic science into clinical practice. *Pharmacol. Res.* **2018**, *130*, 414–419. [CrossRef] [PubMed]
56. Latif, S.; Jahangeer, M.; Maknoon Razia, D.; Ashiq, M.; Ghaffar, A.; Akram, M.; El Allam, A.; Bouyahya, A.; Garipova, L.; Ali Shariati, M.; et al. Dopamine in Parkinson's disease. *Clin. Chim. Acta* **2021**, *522*, 114–126. [CrossRef] [PubMed]
57. Smiley, J.F.; Mesulam, M.-M. Cholinergic neurons of the nucleus basalis of Meynert receive cholinergic, catecholaminergic and GABAergic synapses: An electron microscopic investigation in the monkey. *Neuroscience* **1999**, *88*, 241–255. [CrossRef]
58. Bartus, R.T.; Dean, R.L., 3rd; Beer, B.; Lippa, A.S. The cholinergic hypothesis of geriatric memory dysfunction. *Science* **1982**, *217*, 408–414. [CrossRef]
59. Solari, N.; Hangya, B. Cholinergic modulation of spatial learning, memory and navigation. *Eur. J. Neurosci.* **2018**, *48*, 2199–2230. [CrossRef]
60. Abudukeyoumu, N.; Hernandez-Flores, T.; Garcia-Munoz, M.; Arbuthnott, G.W. Cholinergic modulation of striatal microcircuits. *Eur. J. Neurosci.* **2018**, *49*, 604–622. [CrossRef]
61. Grothe, M.J.; Schuster, C.; Bauer, F.; Heinsen, H.; Prudlo, J.; Teipel, S.J. Atrophy of the cholinergic basal forebrain in dementia with Lewy bodies and Alzheimer's disease dementia. *J. Neurol.* **2014**, *261*, 1939–1948. [CrossRef] [PubMed]
62. Carsana, E.V.; Audano, M.; Breviario, S.; Pedretti, S.; Aureli, M.; Lunghi, G.; Mitro, N. Metabolic Profile Variations along the Differentiation of Human-Induced Pluripotent Stem Cells to Dopaminergic Neurons. *Biomedicines* **2022**, *10*, 2069. [CrossRef]
63. Kim, J.; Jeon, J.; Song, B.; Lee, N.; Ko, S.; Cha, Y.; Leblanc, P.; Seo, H.; Kim, K.S. Spotting-based differentiation of functional dopaminergic progenitors from human pluripotent stem cells. *Nat. Protoc.* **2022**, *17*, 890–909. [CrossRef]
64. Novosadova, E.; Dolotov, O.; Inozemtseva, L.; Novosadova, L.; Antonov, S.; Shimchenko, D.; Bezuglov, V.; Vetchinova, A.; Tarantul, V.; Grivennikov, I.; et al. Influence of N-Arachidonoyl Dopamine and N-Docosahex-aenoyl Dopamine on the Expression of Neurotrophic Factors in Neuronal Differentiated Cultures of Human Induced Pluripotent Stem Cells under Conditions of Oxidative Stress. *Antioxidants* **2022**, *11*, 142. [CrossRef] [PubMed]
65. Jansch, C.; Ziegler, G.C.; Forero, A.; Gredy, S.; Wäldchen, S.; Vitale, M.R.; Svirin, E.; Zöller, J.E.M.; Waider, J.; Günther, K.; et al. Serotonin-specific neurons differentiated from human iPSCs form distinct subtypes with synaptic protein assembly. *J. Neural Transm.* **2021**, *128*, 225–241. [CrossRef] [PubMed]
66. Luo, F.; Luo, S.; Qian, W.; Zhang, L.; Chen, C.; Xu, M.; Wang, G.; Wang, Z.; Wang, J.; Wang, W. Developmental deficits and early signs of neurodegeneration revealed by PD patient derived dopamine neurons. *Stem Cell Res.* **2020**, *49*, 102027. [CrossRef]

67. Song, B.; Cha, Y.; Ko, S.; Jeon, J.; Lee, N.; Seo, H.; Park, K.J.; Lee, I.H.; Lopes, C.; Feitosa, M.; et al. Human autologous iPSC-derived dopaminergic progenitors restore motor function in Parkinson's disease models. *J. Clin. Investig.* **2020**, *130*, 904–920. [CrossRef]
68. Mahajani, S.; Raina, A.; Fokken, C.; Kügler, S.; Bähr, M. Homogenous generation of dopaminergic neurons from multiple hiPSC lines by transient expression of transcription factors. *Cell Death Dis.* **2019**, *10*, 1–15. [CrossRef] [PubMed]
69. Bono, F.; Savoia, P.; Guglielmi, A.; Gennarelli, M.; Piovani, G.; Sigala, S.; Leo, D.; Espinoza, S.; Gainetdinov, R.R.; Devoto, P.; et al. Role of Dopamine D2/D3 Receptors in Development, Plasticity, and Neuroprotection in Human iPSC-Derived Midbrain Dopaminergic Neurons. *Mol. Neurobiol.* **2018**, *55*, 1054–1067. [CrossRef]
70. Jung-Klawitter, S.; Blau, N.; Sebe, A.; Ebersold, J.; Göhring, G.; Opladen, T. Generation of an iPSC line from a patient with tyrosine hydroxylase (TH) deficiency: TH-1 iPS.C. *Stem Cell Res.* **2016**, *17*, 580–583. [CrossRef]
71. Sheng, Y.; Filichia, E.; Shick, E.; Preston, K.L.; Phillips, K.A.; Cooperman, L.; Lin, Z.; Tesar, P.; Hoffer, B.; Luo, Y. Using iPSC-derived human DA neurons from opioid-dependent subjects to study dopamine dynamics. *Brain Behav.* **2016**, *6*, e00491. [CrossRef]
72. Lu, J.; Zhong, X.; Liu, H.; Hao, L.; Huang, C.T.L.; Sherafat, M.A.; Jones, J.; Ayala, M.; Li, L.; Zhang, S.C. Generation of serotonin neurons from human pluripotent stem cells. *Nat. Biotechnol.* **2016**, *34*, 89–94. [CrossRef]
73. Vadodaria, K.C.; Mertens, J.; Paquola, A.; Bardy, C.; Li, X.; Jappelli, R.; Fung, L.; Marchetto, M.C.; Hamm, M.; Gorris, M.; et al. Generation of functional human serotonergic neurons from fibroblasts. *Mol. Psychiatry* **2016**, *21*, 49–61. [CrossRef]
74. Konagaya, S.; Iwata, H. Microencapsulation of dopamine neurons derived from human induced pluripotent stem cells. *Biochim. Biophys. Acta Gen. Subj.* **2015**, *1850*, 22–32. [CrossRef] [PubMed]
75. Woodard, C.M.; Campos, B.A.; Kuo, S.H.; Nirenberg, M.J.; Nestor, M.W.; Zimmer, M.; Mosharov, E.V.; Sulzer, D.; Zhou, H.; Paull, D.; et al. IPSC-derived dopamine neurons reveal differences between monozygotic twins discordant for parkinson's disease. *Cell Rep.* **2014**, *9*, 1173–1182. [CrossRef]
76. Sagal, J.; Zhan, X.; Xu, J.; Tilghman, J.; Karuppagounder, S.S.; Chen, L.; Dawson, V.L.; Dawson, T.M.; Laterra, J.; Ying, M. Proneural Transcription Factor Atoh1 Drives Highly Efficient Differentiation of Human Pluripotent Stem Cells Into Dopaminergic Neurons. *Stem Cells Transl. Med.* **2014**, *3*, 888–898. [CrossRef] [PubMed]
77. Hartfield, E.M.; Yamasaki-Mann, M.; Ribeiro Fernandes, H.J.; Vowles, J.; James, W.S.; Cowley, S.A.; Wade-Martins, R. Physiological characterisation of human iPS-derived dopaminergic neurons. *PLoS ONE* **2014**, *9*, e87388. [CrossRef]
78. Theka, I.; Caiazzo, M.; Dvoretskova, E.; Leo, D.; Ungaro, F.; Curreli, S.; Managò, F.; Dell'Anno, M.T.; Pezzoli, G.; Gainetdinov, R.R.; et al. Rapid Generation of Functional Dopaminergic Neurons From Human Induced Pluripotent Stem Cells Through a Single-Step Procedure Using Cell Lineage Transcription Factors. *Stem Cells Transl. Med.* **2013**, *2*, 473–479. [CrossRef] [PubMed]
79. Sánchez-Danés, A.; Richaud-Patin, Y.; Carballo-Carbajal, I.; Jiménez-Delgado, S.; Caig, C.; Mora, S.; Di Guglielmo, C.; Ezquerra, M.; Patel, B.; Giralt, A.; et al. Disease-specific phenotypes in dopamine neurons from human iPS-based models of genetic and sporadic Parkinson's disease. *EMBO Mol. Med.* **2012**, *4*, 380–395. [CrossRef]
80. Caiazzo, M.; Dell'Anno, M.T.; Dvoretskova, E.; Lazarevic, D.; Taverna, S.; Leo, D.; Sotnikova, T.D.; Menegon, A.; Roncaglia, P.; Colciago, G.; et al. Direct generation of functional dopaminergic neurons from mouse and human fibroblasts. *Nature* **2011**, *476*, 224–227. [CrossRef]
81. Nguyen, H.N.; Byers, B.; Cord, B.; Shcheglovitov, A.; Byrne, J.; Gujar, P.; Kee, K.; Schüle, B.; Dolmetsch, R.E.; Langston, W.; et al. LRRK2 mutant iPSC-derived da neurons demonstrate increased susceptibility to oxidative stress. *Cell Stem Cell.* **2011**, *8*, 267–280. [CrossRef]
82. Cooper, O.; Hargus, G.; Deleidi, M.; Blak, A.; Osborn, T.; Marlow, E.; Lee, K.; Levy, A.; Perez-Torres, E.; Yow, A.; et al. Differentiation of human ES and Parkinson's disease iPS cells into ventral midbrain dopaminergic neurons requires a high activity form of SHH, FGF8a and specific regionalization by retinoic acid. *Mol. Cell. Neurosci.* **2010**, *45*, 258–266.
83. Swistowski, A.; Peng, J.; Liu, Q.; Mali, P.; Rao, M.S.; Cheng, L.; Zeng, X. Efficient generation of functional dopaminergic neurons from human induced pluripotent stem cells under defined conditions. *Stem Cells* **2010**, *28*, 1893–1904. [CrossRef]
84. Soldner, F.; Hockemeyer, D.; Beard, C.; Gao, Q.; Bell, G.W.; Cook, E.G.; Hargus, G.; Blak, A.; Cooper, O.; Mitalipova, M.; et al. Parkinson's Disease Patient-Derived Induced Pluripotent Stem Cells Free of Viral Reprogramming Factors. *Cell* **2009**, *136*, 964–977. [CrossRef] [PubMed]
85. Sagar, R.; Zivko, C.; Xydia, A.; Weisman, D.C.; Lyketsos, C.G.; Mahairaki, V. Generation and Characterization of a Human-Derived and Induced Pluripotent Stem Cell (iPSC) Line from an Alzheimer's Disease Patient with Neuropsychiatric Symptoms. *Biomedicines* **2023**, *11*, 3313. [CrossRef] [PubMed]
86. Sheta, R.; Teixeira, M.; Idi, W.; Oueslati, A. Optimized protocol for the generation of functional human induced-pluripotent-stem-cell-derived dopaminergic neurons. *STAR Protoc.* **2023**, *4*, 102486. [CrossRef] [PubMed]
87. Sarkar, A.K.; Nakamura, S.; Nakai, K.; Sato, T.; Shiga, T.; Abe, Y.; Hoashi, Y.; Inoue, T.; Akamatsu, W.; Baba, K. Increased excitability of human iPSC-derived neurons in HTR2A variant-related sleep bruxism. *Stem Cell Res.* **2022**, *59*, 102658. [CrossRef]
88. Doi, D.; Magotani, H.; Kikuchi, T.; Ikeda, M.; Hiramatsu, S.; Yoshida, K.; Amano, N.; Nomura, M.; Umekage, M.; Morizane, A.; et al. Pre-clinical study of induced pluripotent stem cell-derived dopaminergic progenitor cells for Parkinson's disease. *Nat. Commun.* **2020**, *11*, 3369. [CrossRef]
89. Hoashi, Y.; Okamoto, S.; Abe, Y.; Matsumoto, T.; Tanaka, J.; Yoshida, Y.; Imaizumi, K.; Mishima, K.; Akamatsu, W.; Okano, H.; et al. Generation of neural cells using iPSCs from sleep bruxism patients with 5-HT2A polymorphism. *J. Prosthodont. Res.* **2017**, *61*, 242–250. [CrossRef]

90. Takahashi, K.; Yamanaka, S. Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors. *Cell* **2006**, *126*, 663–676. [CrossRef]
91. Komatsu, M.; Konagaya, S.; Egawa, E.Y.; Iwata, H. Maturation of human iPS cell-derived dopamine neuron precursors in alginate-Ca^{2+} hydrogel. *Biochim. Biophys. Acta Gen. Subj.* **2015**, *1850*, 1669–1675. [CrossRef]
92. Li, H.; Jiang, H.; Li, H.; Li, L.; Yan, Z.; Feng, J. Generation of human A9 dopaminergic pacemakers from induced pluripotent stem cells. *Mol. Psychiatry* **2022**, *27*, 4407–4418. [CrossRef]
93. Stanslowsky, N.; Haase, A.; Martin, U.; Naujock, M.; Leffler, A.; Dengler, R.; Wegner, F. Functional differentiation of midbrain neurons from human cord blood-derived induced pluripotent stem cells. *Stem Cell Res. Ther.* **2014**, *5*, 35. [CrossRef]
94. Xi, J.; Liu, Y.; Liu, H.; Chen, H.; Emborg, M.E.; Zhang, S.C. Specification of midbrain dopamine neurons from primate pluripotent stem cells. *Stem Cells* **2012**, *30*, 1655–1663. [CrossRef]
95. Revilla, A.; González, C.; Iriondo, A.; Fernández, B.; Prieto, C.; Marín, C.; Liste, I. Current advances in the generation of human iPS cells: Implications in cell-based regenerative medicine. *J. Tissue Eng. Regen. Med.* **2016**, *10*, 893–907. [CrossRef] [PubMed]
96. Bayart, E.; Cohen-Haguenauer, O. Technological Overview of iPS Induction from Human Adult Somatic Cells. *Curr. Gene Ther.* **2013**, *13*, 73–92. [CrossRef] [PubMed]
97. Katolikova, N.V.; Khudiakov, A.A.; Shafranskaya, D.D.; Prjibelski, A.D.; Masharskiy, A.E.; Mor, M.S.; Golovkin, A.S.; Zaytseva, A.K.; Neganova, I.E.; Efimova, E.V.; et al. Modulation of Notch Signaling at Early Stages of Differentiation of Human Induced Pluripotent Stem Cells to Dopaminergic Neurons. *Int. J. Mol. Sci.* **2023**, *24*, 1429. [CrossRef] [PubMed]
98. Cardo, L.F.; Monzón-Sandoval, J.; Li, Z.; Webber, C.; Li, M. Single-Cell Transcriptomics and In Vitro Lineage Tracing Reveals Differential Susceptibility of Human iPSC-Derived Midbrain Dopaminergic Neurons in a Cellular Model of Parkinson's Disease. *Cells* **2023**, *12*, 2860. [CrossRef] [PubMed]
99. Rao, M.S.; Malik, N. Assessing iPSC reprogramming methods for their suitability in translational medicine. *J. Cell Biochem.* **2012**, *113*, 3061–3068. [CrossRef]
100. Tao, Y.; Li, X.; Dong, Q.; Kong, L.; Petersen, A.J.; Yan, Y.; Xu, K.; Zima, S.; Li, Y.; Schmidt, D.K.; et al. Generation of locus coeruleus norepinephrine neurons from human pluripotent stem cells. *Nat. Biotechnol.* **2023**, 1–13. [CrossRef]
101. Diao, X.; Wang, F.; Becerra-Calixto, A.; Soto, C.; Mukherjee, A. Induced pluripotent stem cell-derived dopaminergic neurons from familial parkinson's disease patients display α-synuclein pathology and abnormal mitochondrial morphology. *Cells* **2021**, *10*, 2402. [CrossRef]
102. Valiulahi, P.; Vidyawan, V.; Puspita, L.; Oh, Y.; Juwono, V.B.; Sittipo, P.; Friedlander, G.; Yahalomi, D.; Sohn, J.W.; Lee, Y.K.; et al. Generation of caudal-type serotonin neurons and hindbrain-fate organoids from hPSCs. *Stem Cell Rep.* **2021**, *16*, 1938–1952. [CrossRef]
103. Amimoto, N.; Nishimura, K.; Shimohama, S.; Takata, K. Generation of striatal neurons from human induced pluripotent stem cells by controlling extrinsic signals with small molecules. *Stem Cell Res.* **2021**, *55*, 102486. [CrossRef]
104. Hiller, B.M.; Marmion, D.J.; Gross, R.M.; Thompson, C.A.; Chavez, C.A.; Brundin, P.; Wakeman, D.R.; McMahon, C.W.; Kordower, J.H. Mitomycin-C treatment during differentiation of induced pluripotent stem cell-derived dopamine neurons reduces proliferation without compromising survival or function in vivo. *Stem Cells Transl. Med.* **2021**, *10*, 278–290. [CrossRef] [PubMed]
105. Sanz Muñoz, S.; Engel, M.; Balez, R.; Do-Ha, D.; Castro Cabral-Da-Silva, M.; Hernández, D.; Berg, T.; Fifita, J.A.; Grima, N.; Yang, S.; et al. A simple differentiation protocol for generation of induced pluripotent stem cell-derived basal forebrain-like cholinergic neurons for alzheimer's disease and frontotemporal dementia disease modeling. *Cells* **2020**, *9*, 2018. [CrossRef] [PubMed]
106. Collo, G.; Cavalleri, L.; Chiamulera, C.; Merlo Pich, E. (2 R,6 R)-Hydroxynorketamine promotes dendrite outgrowth in human inducible pluripotent stem cell-derived neurons through AMPA receptor with timing and exposure compatible with ketamine infusion pharmacokinetics in humans. *Neuroreport* **2018**, *29*, 1425–1430. [CrossRef] [PubMed]
107. Fedele, S.; Collo, G.; Behr, K.; Bischofberger, J.; Müller, S.; Kunath, T.; Christensen, K.; Gündner, A.L.; Graf, M.; Jagasia, R.; et al. Expansion of human midbrain floor plate progenitors from induced pluripotent stem cells increases dopaminergic neuron differentiation potential. *Sci. Rep.* **2017**, *7*, 6036. [CrossRef] [PubMed]
108. Kikuchi, T.; Morizane, A.; Doi, D.; Onoe, H.; Hayashi, T.; Kawasaki, T.; Saiki, H.; Miyamoto, S.; Takahashi, J. Survival of human induced pluripotent stem cell-derived midbrain dopaminergic neurons in the brain of a primate model of Parkinson's disease. *J. Park. Dis.* **2011**, *1*, 395–412. [CrossRef]
109. Kriks, S.; Shim, J.W.; Piao, J.; Ganat, Y.M.; Wakeman, D.R.; Xie, Z.; Carrillo-Reid, L.; Auyeung, G.; Antonacci, C.; Buch, A.; et al. Dopamine neurons derived from human ES cells efficiently engraft in animal models of Parkinson's disease. *Nature* **2011**, *480*, 547–551. [CrossRef]
110. Inman, G.J.; Nicolás, F.J.; Nicolás, N.; Callahan, J.F.; Harling, J.D.; Gaster, L.M.; Reith, A.D.; Laping, N.J.; Hill, C.S. SB-431542 Is a Potent and Specific Inhibitor of Transforming Growth Factor-Superfamily Type I Activin Receptor-Like Kinase (ALK) Receptors ALK4, ALK5, and ALK7. *Mol. Pharmacol.* **2002**, *62*, 65–74. [CrossRef]
111. Boergermann, J.H.; Kopf, J.; Yu, P.B.; Knaus, P. Dorsomorphin and LDN-193189 inhibit BMP-mediated Smad, p38 and Akt signalling in C2C12 cells. *Int. J. Biochem. Cell Biol.* **2010**, *42*, 1802–1807. [CrossRef]
112. Rhee, Y.-H.; Ko, J.-Y.; Chang, M.-Y.; Yi, S.-H.; Kim, D.; Kim, C.-H.; Shim, J.W.; Jo, A.Y.; Kim, B.W.; Lee, H.; et al. Protein-based human iPS cells efficiently generate functional dopamine neurons and can treat a rat model of Parkinson disease. *J. Clin. Investig.* **2011**, *121*, 2326–2335. [CrossRef]

113. Okada, Y.; Shimazaki, T.; Sobue, G.; Okano, H. Retinoic-acid-concentration-dependent acquisition of neural cell identity during in vitro differentiation of mouse embryonic stem cells. *Dev. Biol.* **2004**, *275*, 124–142. [CrossRef]
114. Kittappa, R.; Chang, W.W.; Awatramani, R.B.; McKay, R.D.G. The foxa2 gene controls the birth and spontaneous degeneration of dopamine neurons in old age. *PLoS Biol.* **2007**, *5*, 2875–2884. [CrossRef] [PubMed]
115. Doucet-Beaupré, H.; Ang, S.L.; Lévesque, M. Cell fate determination, neuronal maintenance and disease state: The emerging role of transcription factors Lmx1a and Lmx1b. *FEBS Lett.* **2015**, *589*, 3727–3738. [CrossRef] [PubMed]
116. Moreno, E.L.; Hachi, S.; Hemmer, K.; Trietsch, S.J.; Baumuratov, A.S.; Hankemeier, T.; Vulto, P.; Schwamborn, J.C.; Fleming, R.M. Differentiation of neuroepithelial stem cells into functional dopaminergic neurons in 3D microfluidic cell culture. *Lab. Chip.* **2015**, *15*, 2419–2428. [CrossRef] [PubMed]
117. Sibuea, S.; Ho, J.K.; Pouton, C.W.; Haynes, J.M. TGFβ3, dibutyryl cAMP and a notch inhibitor modulate phenotype late in stem cell-derived dopaminergic neuron maturation. *Front. Cell Dev. Biol.* **2023**, *11*, 1111705. [CrossRef]
118. Flanders, K.C.; Ren, R.F.Â.; Lippa, C.F. Transforming growth factor-βs in neurodegenerative disease. *Prog. Neurobiol.* **1998**, *54*, 71–85. [CrossRef]
119. Schober, A.; Peterziel, H.; von Bartheld, C.S.; Simon, H.; Krieglstein, K.; Unsicker, K. GDNF applied to the MPTP-lesioned nigrostriatal system requires TGF-β for its neuroprotective action. *Neurobiol. Dis.* **2007**, *25*, 378–391. [CrossRef]
120. Luo, S.X.; Timbang, L.; Kim, J.I.; Shang, Y.; Sandoval, K.; Tang, A.A.; Whistler, J.L.; Ding, J.B.; Huang, E.J. TGF-β Signaling in Dopaminergic Neurons Regulates Dendritic Growth, Excitatory-Inhibitory Synaptic Balance, and Reversal Learning. *Cell Rep.* **2016**, *17*, 3233–3245. [CrossRef]
121. Mena, M.A.; Casarejos, M.J.; Bonin, A.; Ramos, J.A.; De Yébenes, G. Effects of Dibutyryl Cyclic AMP and Retinoic Acid on the Differentiation of Dopamine Neurons: Prevention of Cell Death by Dibutyryl Cyclic AMP. *J. Neurochem.* **1995**, *65*, 2612–2620. [CrossRef]
122. Wulansari, N.; Kim, E.H.; Sulistio, Y.A.; Rhee, Y.H.; Song, J.J.; Lee, S.H. Vitamin C-Induced Epigenetic Modifications in Donor NSCs Establish Midbrain Marker Expressions Critical for Cell-Based Therapy in Parkinson's Disease. *Stem Cell Rep.* **2017**, *9*, 1192–1206. [CrossRef]
123. Pattyn, A.; Goridis, C.; Brunet, J.F. Specification of the central noradrenergic phenotype by the homeobox gene Phox2b. *Mol. Cell Neurosci.* **2000**, *15*, 235–243. [CrossRef]
124. Morin, X.; Cremer, H.; Hirsch, M.-R. Defects in Sensory and Autonomic Ganglia and Absence of Locus Coeruleus in Mice Deficient for the Homeobox Gene Phox2a. *Neuron* **1997**, *18*, 411–423. [CrossRef] [PubMed]
125. Vera, E.; Bosco, N.; Studer, L. Generating Late-Onset Human iPSC-Based Disease Models by Inducing Neuronal Age-Related Phenotypes through Telomerase Manipulation. *Cell Rep.* **2016**, *17*, 1184–1192. [CrossRef] [PubMed]
126. Ren, Y.; Jiang, H.; Hu, Z.; Fan, K.; Wang, J.; Janoschka, S.; Wang, X.; Ge, S.; Feng, J. Parkin Mutations Reduce the Complexity of Neuronal Processes in iPSC-Derived Human Neurons. *Stem Cells* **2015**, *33*, 68–78. [CrossRef] [PubMed]
127. Beevers, J.E.; Lai, M.C.; Collins, E.; Booth, H.D.E.; Zambon, F.; Parkkinen, L.; Vowles, J.; Cowley, S.A.; Wade-Martins, R.; Caffrey, T.M. MAPT Genetic Variation and Neuronal Maturity Alter Isoform Expression Affecting Axonal Transport in iPSC-Derived Dopamine Neurons. *Stem Cell Rep.* **2017**, *9*, 587–599. [CrossRef]
128. Zambon, F.; Cherubini, M.; Fernandes, H.J.R.; Lang, C.; Ryan, B.J.; Volpato, V.; Bengoa-Vergniory, N.; Vingill, S.; Attar, M.; Booth, H.D.E.; et al. Cellular α-synuclein pathology is associated with bioenergetic dysfunction in Parkinson's iPSC-derived dopamine neurons. *Hum. Mol. Genet.* **2019**, *28*, 2001–2013. [CrossRef]
129. Fernandes, H.J.R.; Hartfield, E.M.; Christian, H.C.; Emmanoulidou, E.; Zheng, Y.; Booth, H.; Bogetofte, H.; Lang, C.; Ryan, B.J.; Sardi, S.P.; et al. ER Stress and Autophagic Perturbations Lead to Elevated Extracellular α-Synuclein in GBA-N370S Parkinson's iPSC-Derived Dopamine Neurons. *Stem Cell Rep.* **2016**, *6*, 342–356. [CrossRef]
130. Kumar, M.; Acevedo-Cintrón, J.; Jhaldiyal, A.; Wang, H.; Andrabi, S.A.; Eacker, S.; Karuppagounder, S.S.; Brahmachari, S.; Chen, R.; Kim, H.; et al. Defects in Mitochondrial Biogenesis Drive Mitochondrial Alterations in PARKIN-Deficient Human Dopamine Neurons. *Stem Cell Rep.* **2020**, *15*, 629–645. [CrossRef]
131. Mazzulli, J.R.; Zunke, F.; Tsunemi, T.; Toker, N.J.; Jeon, S.; Burbulla, L.F.; Patnaik, S.; Sidransky, E.; Marugan, J.J.; Sue, C.M.; et al. Activation of β-Glucocerebrosidase Reduces Pathological α-Synuclein and Restores Lysosomal Function in Parkinson's Patient Midbrain Neurons. *J. Neurosci.* **2016**, *36*, 7693–7706. [CrossRef]
132. Lang, C.; Campbell, K.R.; Ryan, B.J.; Carling, P.; Attar, M.; Vowles, J.; Perestenko, O.V.; Bowden, R.; Baig, F.; Kasten, M.; et al. Single-Cell Sequencing of iPSC-Dopamine Neurons Reconstructs Disease Progression and Identifies HDAC4 as a Regulator of Parkinson Cell Phenotypes. *Cell Stem Cell* **2019**, *24*, 93–106.e6. [CrossRef] [PubMed]
133. Cukier, H.N.; Kim, H.; Griswold, A.J.; Codreanu, S.G.; Prince, L.M.; Sherrod, S.D.; McLean, J.A.; Dykxhoorn, D.M.; Ess, K.C.; Hedera, P.; et al. Genomic, transcriptomic, and metabolomic profiles of hiPSC-derived dopamine neurons from clinically discordant brothers with identical PRKN deletions. *Npj Park. Dis.* **2022**, *8*, 1–17. [CrossRef]
134. López de Maturana, R.; Lang, V.; Zubiarrain, A.; Sousa, A.; Vázquez, N.; Gorostidi, A.; Águila, J.; López de Munain, A.; Rodríguez, M.; Sánchez-Pernaute, R. Mutations in LRRK2 impair NF-KB pathway in iPSC-derived neurons. *J. Neuroinflam.* **2016**, *13*, 1–15. [CrossRef] [PubMed]
135. Schmidt, S.I.; Bogetofte, H.; Ritter, L.; Agergaard, J.B.; Hammerich, D.; Kabiljagic, A.A.; Wlodarczyk, A.; Lopez, S.G.; Sørensen, M.D.; Jørgensen, M.L.; et al. Microglia-Secreted Factors Enhance Dopaminergic Differentiation of Tissue- and iPSC-Derived Human Neural Stem Cells. *Stem Cell Rep.* **2021**, *16*, 281–294. [CrossRef] [PubMed]

136. Kim, J.W.; Yin, X.; Jhaldiyal, A.; Khan, M.R.; Martin, I.; Xie, Z.; Perez-Rosello, T.; Kumar, M.; Abalde-Atristain, L.; Xu, J.; et al. Defects in mRNA Translation in LRRK2-Mutant hiPSC-Derived Dopaminergic Neurons Lead to Dysregulated Calcium Homeostasis. *Cell Stem Cell* **2020**, *27*, 633–645.e7. [CrossRef] [PubMed]
137. Azkona, G.; López de Maturana, R.; Del Rio, P.; Sousa, A.; Vazquez, N.; Zubiarrain, A.; Jimenez-Blasco, D.; Bolaños, J.P.; Morales, B.; Auburger, G.; et al. LRRK2 expression is deregulated in fibroblasts and neurons from parkinson patients with mutations in PINK1. *Mol. Neurobiol.* **2018**, *55*, 506–516. [CrossRef]
138. Kuzumaki, N.; Suda, Y.; Iwasawa, C.; Narita, M.; Sone, T.; Watanabe, M.; Maekawa, A.; Matsumoto, T.; Akamatsu, W.; Igarashi, K.; et al. Cell-specific overexpression of COMT in dopaminergic neurons of Parkinson's disease. *Brain* **2019**, *142*, 1675–1689. [CrossRef] [PubMed]
139. Lebedeva, O.; Poberezhniy, D.; Novosadova, E.; Gerasimova, T.; Novosadova, L.; Arsenyeva, E.; Stepanenko, E.; Shimchenko, D.; Volovikov, E.; Anufrieva, K.; et al. Overexpression of Parkin in the Neuronal Progenitor Cells from a Patient with Parkinson's Disease Shifts the Transcriptome Towards the Normal State. *Mol. Neurobiol.* **2023**, *60*, 3522–3533. [CrossRef]
140. Oni, E.N.; Halikere, A.; Li, G.; Toro-Ramos, A.J.; Swerdel, M.R.; Verpeut, J.L.; Moore, J.C.; Bello, N.T.; Bierut, L.J.; Goate, A.; et al. Increased nicotine response in iPSC-derived human neurons carrying the CHRNA5 N398 allele. *Sci. Rep.* **2016**, *6*, 1–11. [CrossRef]
141. Collo, G.; Cavalleri, L.; Zoli, M.; Maskos, U.; Ratti, E.; Pich, E.M. Alphα6-containing nicotinic acetylcholine receptors mediate nicotine-induced structural plasticity in mouse and human iPSC-derived dopaminergic neurons. *Front. Pharmacol.* **2018**, *9*, 344362. [CrossRef]
142. Bono, F.; Mutti, V.; Savoia, P.; Barbon, A.; Bellucci, A.; Missale, C.; Fiorentini, C. Nicotine prevents alpha-synuclein accumulation in mouse and human iPSC-derived dopaminergic neurons through activation of the dopamine D3- acetylcholine nicotinic receptor heteromer. *Neurobiol. Dis.* **2019**, *129*, 1–12. [CrossRef]
143. Mutti, V.; Bono, F.; Tomasoni, Z.; Bontempi, L.; Guglielmi, A.; Bolognin, S.; Schwamborn, J.C.; Missale, C.; Fiorentini, C. Structural Plasticity of Dopaminergic Neurons Requires the Activation of the D3R-nAChR Heteromer and the PI3K-ERK1/2/Akt-Induced Expression of c-Fos and p70S6K Signaling Pathway. *Mol. Neurobiol.* **2022**, *59*, 2129–2149. [CrossRef]
144. Bono, F.; Mutti, V.; Devoto, P.; Bolognin, S.; Schwamborn, J.C.; Missale, C.; Fiorentini, C. Impaired dopamine D3 and nicotinic acetylcholine receptor membrane localization in iPSCs-derived dopaminergic neurons from two Parkinson's disease patients carrying the LRRK2 G2019S mutation. *Neurobiol. Aging* **2021**, *99*, 65–78. [CrossRef] [PubMed]
145. Novak, G.; Kyriakis, D.; Grzyb, K.; Bernini, M.; Rodius, S.; Dittmar, G.; Finkbeiner, S.; Skupin, A. Single-cell transcriptomics of human iPSC differentiation dynamics reveal a core molecular network of Parkinson's disease. *Commun. Biol.* **2022**, *5*, 1–19. [CrossRef]
146. Ren, Y.; Jiang, H.; Pu, J.; Li, L.; Wu, J.; Yan, Y.; Zhao, G.; Guttuso, T.J.; Zhang, B.; Feng, J. Molecular Features of Parkinson's Disease in Patient-Derived Midbrain Dopaminergic Neurons. *Mov. Disord.* **2022**, *37*, 70–79. [CrossRef] [PubMed]
147. Vadodaria, K.C.; Ji, Y.; Skime, M.; Paquola, A.C.; Nelson, T.; Hall-Flavin, D.; Heard, K.J.; Fredlender, C.; Deng, Y.; Elkins, J.; et al. Altered serotonergic circuitry in SSRI-resistant major depressive disorder patient-derived neurons. *Mol. Psychiatry* **2019**, *24*, 808–818. [CrossRef]
148. Vadodaria, K.C.; Ji, Y.; Skime, M.; Paquola, A.; Nelson, T.; Hall-Flavin, D.; Fredlender, C.; Heard, K.J.; Deng, Y.; Le, A.T.; et al. Serotonin-induced hyperactivity in SSRI-resistant major depressive disorder patient-derived neurons. *Mol. Psychiatry* **2019**, *24*, 795–807. [CrossRef]
149. Seifan, A.; Ganzer, C.A.; Ryon, K.; Lin, M.; Mahmudur, R.; Adolfo, H.; Shih, C.; Jacobs, A.R.; Greenwald, M.; Isaacson, R.S. Detecting Non-cognitive Features of Prodromal Neurodegenerative Diseases. *Curr. Aging Sci.* **2019**, *11*, 242–249. [CrossRef]
150. Neely, M.D.; Davison, C.A.; Aschner, M.; Bowman, A.B. From the Cover: Manganese and Rotenone-Induced Oxidative Stress Signatures Differ in iPSC-Derived Human Dopamine Neurons. *Toxicol. Sci.* **2017**, *159*, 366–379. [CrossRef] [PubMed]
151. Pamies, D.; Wiersma, D.; Katt, M.E.; Zhao, L.; Burtscher, J.; Harris, G.; Smirnova, L.; Searson, P.C.; Hartung, T.; Hogberg, H.T. Human IPSC 3D brain model as a tool to study chemical-induced dopaminergic neuronal toxicity. *Neurobiol. Dis.* **2022**, *169*, 105719. [CrossRef]
152. Cavalleri, L.; Merlo Pich, E.; Millan, M.J.; Chiamulera, C.; Kunath, T.; Spano, P.F.; Collo, G. Ketamine enhances structural plasticity in mouse mesencephalic and human iPSC-derived dopaminergic neurons via AMPAR-driven BDNF and mTOR signaling. *Mol. Psychiatry* **2017**, *23*, 812–823. [CrossRef]
153. Collo, G.; Cavalleri, L.; Merlo Pich, E. Structural Plasticity Induced by Ketamine in Human Dopaminergic Neurons as Mechanism Relevant for Treatment-Resistant Depression. *Chronic Stress* **2019**, *3*, 2470547019842545. [CrossRef]
154. Laperle, A.H.; Sances, S.; Yucer, N.; Dardov, V.J.; Garcia, V.J.; Ho, R.; Fulton, A.N.; Jones, M.R.; Roxas, K.M.; Avalos, P.; et al. iPSC modeling of young-onset Parkinson's disease reveals a molecular signature of disease and novel therapeutic candidates. *Nat. Med.* **2020**, *26*, 289–299. [CrossRef] [PubMed]
155. Orlando, R.; Ginerete, R.P.; Cavalleri, L.; Aliperti, V.; Imbriglio, T.; Battaglia, G.; Zuena, A.R.; Nicoletti, F.; Merlo Pich, E.; Collo, G. Synergic action of L-acetylcarnitine and L-methylfolate in Mouse Models of Stress-Related Disorders and Human iPSC-Derived Dopaminergic Neurons. *Front. Pharmacol.* **2022**, *13*, 913210. [CrossRef] [PubMed]
156. Fernandes, H.J.R.; Patikas, N.; Foskolou, S.; Field, S.F.; Park, J.E.; Byrne, M.L.; Bassett, A.R.; Metzakopian, E. Single-Cell Transcriptomics of Parkinson's Disease Human In Vitro Models Reveals Dopamine Neuron-Specific Stress Responses. *Cell Rep.* **2020**, *33*, 108263. [CrossRef]

157. Inoue, S.; Nishimura, K.; Gima, S.; Nakano, M.; Takata, K. CRISPR-Cas9-Edited SNCA Knockout Human Induced Pluripotent Stem Cell-Derived Dopaminergic Neurons and Their Vulnerability to Neurotoxicity. *Biol. Pharm. Bull.* **2023**, *46*, 517–522. [CrossRef]
158. Suda, Y.; Kuzumaki, N.; Sone, T.; Narita, M.; Tanaka, K.; Hamada, Y.; Iwasawa, C.; Shibasaki, M.; Maekawa, A.; Matsuo, M.; et al. Down-regulation of ghrelin receptors on dopaminergic neurons in the substantia nigra contributes to Parkinson's disease-like motor dysfunction. *Mol. Brain* **2018**, *11*, 1–9. [CrossRef]
159. Korecka, J.A.; Talbot, S.; Osborn, T.M.; de Leeuw, S.M.; Levy, S.A.; Ferrari, E.J.; Moskites, A.; Atkinson, E.; Jodelka, F.M.; Hinrich, A.J.; et al. Neurite Collapse and Altered ER Ca2+ Control in Human Parkinson Disease Patient iPSC-Derived Neurons with LRRK2 G2019S Mutation. *Stem Cell Rep.* **2019**, *12*, 29–41. [CrossRef]
160. Lewitt, P.A.; Library, W.O. Levodopa therapy for Parkinson's disease: Pharmacokinetics and pharmacodynamics. *Mov. Disord.* **2015**, *30*, 64–72. [CrossRef]
161. Moriarty, N.; Kauhausen, J.A.; Pavan, C.; Hunt, C.P.J.; de Luzy, I.R.; Penna, V.; Ermine, C.M.; Thompson, L.H.; Parish, C.L. Understanding the Influence of Target Acquisition on Survival, Integration, and Phenotypic Maturation of Dopamine Neurons within Stem Cell-Derived Neural Grafts in a Parkinson's Disease Model. *J. Neurosci.* **2022**, *42*, 4995–5006. [CrossRef] [PubMed]
162. Fu, C.L.; Dong, B.C.; Jiang, X.; Li, D.; Yao, J. A cell therapy approach based on iPSC-derived midbrain organoids for the restoration of motor function in a Parkinson's disease mouse model. *Heliyon* **2024**, *10*, e24234. [CrossRef]
163. Zygogianni, O.; Kouroupi, G.; Taoufik, E.; Matsas, R. Engraftable Induced Pluripotent Stem Cell -Derived Neural Precursors for Brain Repair. *Methods Mol. Biol.* **2020**, *2155*, 23–39.
164. Hills, R.; Mossman, J.A.; Bratt-Leal, A.M.; Tran, H.; Williams, R.M.; Stouffer, D.G.; Sokolova, I.V.; Sanna, P.P.; Loring, J.F.; Lelos, M.J. Neurite Outgrowth and Gene Expression Profile Correlate with Efficacy of Human Induced Pluripotent Stem Cell-Derived Dopamine Neuron Grafts. *Stem Cells Dev.* **2023**, *32*, 387–397. [CrossRef] [PubMed]
165. de Luzy, I.R.; Pavan, C.; Moriarty, N.; Hunt, C.P.J.; Vandenhoven, Z.; Khanna, A.; Niclis, J.C.; Gantner, C.W.; Thompson, L.H.; Parish, C.L. Identifying the optimal developmental age of human pluripotent stem cell-derived midbrain dopaminergic progenitors for transplantation in a rodent model of Parkinson's disease. *Exp. Neurol.* **2022**, *358*, 114219. [CrossRef] [PubMed]
166. Shrigley, S.; Nilsson, F.; Mattsson, B.; Fiorenzano, A.; Mudannayake, J.; Bruzelius, A.; Ottosson, D.R.; Björklund, A.; Hoban, D.B.; Parmar, M. Grafts Derived from an α-Synuclein Triplication Patient Mediate Functional Recovery but Develop Disease-Associated Pathology in the 6-OHDA Model of Parkinson's Disease. *J. Park. Dis.* **2021**, *11*, 515–528. [CrossRef]
167. Hiller, B.M.; Marmion, D.J.; Thompson, C.A.; Elliott, N.A.; Federoff, H.; Brundin, P.; Mattis, V.B.; McMahon, C.W.; Kordower, J.H. Optimizing maturity and dose of iPSC-derived dopamine progenitor cell therapy for Parkinson's disease. *Npj Regen. Med.* **2022**, *7*, 1–15. [CrossRef]
168. Schweitzer, J.S.; Song, B.; Herrington, T.M.; Park, T.-Y.; Lee, N.; Ko, S.; Jeon, J.; Cha, Y.; Kim, K.; Li, Q.; et al. Personalized iPSC-Derived Dopamine Progenitor Cells for Parkinson's Disease. *N. Engl. J. Med.* **2020**, *382*, 1926–1932. [CrossRef]
169. Grow, D.A.; McCarrey, J.R.; Navara, C.S. Advantages of nonhuman primates as preclinical models for evaluating stem cell-based therapies for Parkinson's disease. *Stem Cell Res.* **2016**, *17*, 352–366. [CrossRef] [PubMed]
170. Weinshenker, D. Long road to ruin: Noradrenergic dysfunction in neurodegenerative disease. *Trends Neurosci.* **2018**, *41*, 211–223. [CrossRef]
171. Kelly, S.C.; Nelson, P.T.; Counts, S.E. Pontine Arteriolosclerosis and Locus Coeruleus Oxidative Stress Differentiate Resilience from Mild Cognitive Impairment in a Clinical Pathologic Cohort. *J. Neuropathol. Exp. Neurol.* **2021**, *80*, 325–335. [CrossRef]
172. Kang, S.S.; Ahn, E.H.; Liu, X.; Bryson, M.; Miller, G.W.; Weinshenker, D.; Ye, K. ApoE4 inhibition of VMAT2 in the locus coeruleus exacerbates Tau pathology in Alzheimer's disease. *Acta Neuropathol.* **2021**, *142*, 139–158. [CrossRef]
173. Kang, S.S.; Meng, L.; Zhang, X.; Wu, Z.; Mancieri, A.; Xie, B.; Liu, X.; Weinshenker, D.; Peng, J.; Zhang, Z.; et al. Tau modification by the norepinephrine metabolite DOPEGAL stimulates its pathology and propagation. *Nat. Struct. Mol. Biol.* **2022**, *29*, 292–305. [CrossRef]
174. Kang, S.S.; Liu, X.; Ahn, E.H.; Xiang, J.; Manfredsson, F.P.; Yang, X.; Luo, H.R.; Liles, L.C.; Weinshenker, D.; Ye, K. Norepinephrine metabolite DOPEGAL activates AEP and pathological Tau aggregation in locus coeruleus. *J. Clin. Investig.* **2020**, *130*, 422–437. [CrossRef] [PubMed]
175. Capucciati, A.; Zucca, F.A.; Monzani, E.; Zecca, L.; Casella, L.; Hofer, T. Interaction of Neuromelanin with Xenobiotics and Consequences for Neurodegeneration; Promising Experimental Models. *Antioxidants* **2021**, *10*, 824. [CrossRef] [PubMed]
176. Zucca, F.A.; Bellei, C.; Giannelli, S.; Terreni, M.R.; Gallorini, M.; Rizzio, E.; Pezzoli, G.; Albertini, A.; Zecca, L. Neuromelanin and iron in human locus coeruleus and substantia nigra during aging: Consequences for neuronal vulnerability. *J. Neural Transm.* **2006**, *113*, 757–767. [CrossRef] [PubMed]
177. Herrera, A.J.; Castaño, A.; Venero, J.L.; Cano, J.; Machado, A. The single intranigral injection of LPS as a new model for studying the selective effects of inflammatory reactions on dopaminergic system. *Neurobiol. Dis.* **2000**, *7*, 429–447. [CrossRef]
178. Song, S.; Wang, Q.; Jiang, L.; Oyarzabal, E.; Riddick, N.V.; Wilson, B.; Moy, S.S.; Shih, Y.I.; Hong, J.S. Noradrenergic dysfunction accelerates LPS-elicited inflammation-related ascending sequential neurodegeneration and deficits in non-motor/motor functions. *Brain Behav. Immun.* **2019**, *81*, 374–387. [CrossRef]

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Review

Biochemical Pathways Delivering Distinct Glycosphingolipid Patterns in MDA-MB-231 and MCF-7 Breast Cancer Cells

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Abstract: The complex structure of glycosphingolipids (GSLs) supports their important role in cell function as modulators of growth factor receptors and glutamine transporters in plasma membranes. The aberrant composition of clustered GSLs within signaling platforms, so-called lipid rafts, inevitably leads to tumorigenesis due to disturbed growth factor signal transduction and excessive uptake of glutamine and other molecules needed for increased energy and structural molecule cell supply. GSLs are also involved in plasma membrane processes such as cell adhesion, and their transition converts cells from epithelial to mesenchymal with features required for cell migration and metastasis. Glutamine activates the mechanistic target of rapamycin complex 1 (mTORC1), resulting in nucleotide synthesis and proliferation. In addition, glutamine contributes to the cancer stem cell GD2 ganglioside-positive phenotype in the triple-negative breast cancer cell line MDA-MB-231. Thieno[2,3-*b*]pyridine derivative possesses higher cytotoxicity against MDA-MB-231 than against MCF-7 cells and induces a shift to aerobic metabolism and a decrease in S(6)nLc4Cer GSL-positive cancer stem cells in the MDA-MB-231 cell line. In this review, we discuss findings in MDA-MB-231, MCF-7, and other breast cancer cell lines concerning their differences in growth factor receptors and recent knowledge of the main biochemical pathways delivering distinct glycosphingolipid patterns during tumorigenesis and therapy.

Keywords: glycosphingolipids; growth factor receptors; breast cancer; structure; membrane lipid remodeling

1. Introduction

The elucidation of the mechanisms involved in metabolic rewiring is an important milestone for understanding tumorigenesis with the aim of developing effective cancer treatment [1]. Recently, we described a novel thieno[2,3-*b*]pyridine anticancer compound that lowers the triple-negative cancer stem cell (CSC) fraction in the MDA-MB-231 cell line. A mix of both CSC and non-CSC subpopulations shows a metabolic shift [2]. Treatment with thieno[2,3-*b*]pyridine derivative changes the distinct glycosphingolipid (GSL) pattern in MDA-MB-231 and MCF-7 breast cancer cell lines. These lines differ concerning the expression of human epidermal growth factor receptor 3 (HER3), being both present in [3,4] and absent in different cells. The metabolic profiling of two different breast cancer cell lines reveals the inverse effect of thieno[2,3-*b*]pyridine derivative on two metabolites: lactate and glycerylmonostearate decrease in MDA-MB-231 and increase in MCF-7 cells [2]. Therefore, in this review, we aim to explore and discuss the recent knowledge of GSL expression in breast cancer cells and the methods of cancer treatment by targeting its GSLs. We specifically point out the treatment of breast cancer cells with thieno[2,3-*b*]pyridine

compounds. They were discovered via virtual screening targeting phosphoinositide-specific phospholipase C (pi-PLC) [5]. Their antitumor activity was proven in numerous tumor cell lines, where they inhibited the growth of triple-negative breast cancer cell lines at nanomolar concentrations [6,7].

2. The Role of Glycosphingolipids in Membrane Lipid Rafts Is Derived from Their Structure

Glycosphingolipids are essential constituents of the plasma membrane outer leaflet, exposing their sugar residues as their hydrophilic portion in the extracellular space. Their synthesis begins with dihydrosphingosine synthesized from activated palmitic acid (palmitoyl-CoA) [8] and, rarely, stearic acid [9], which binds the amino acid serine, which has a branch chain with an -OH terminal group (Figure 1). Therefore, dihydrosphingosine is an amino alcohol. It binds the -CO group in acyl-CoA (palmitoyl or stearoyl, RCOSCoA) with an amide bond, forming saturated ceramide (dihydroceramide). Dihydroceramide is dehydrogenated within its dihydrosphingosine part, resulting in ceramide containing the long-chain unsaturated amino alcohol sphingosine (Figure 1).

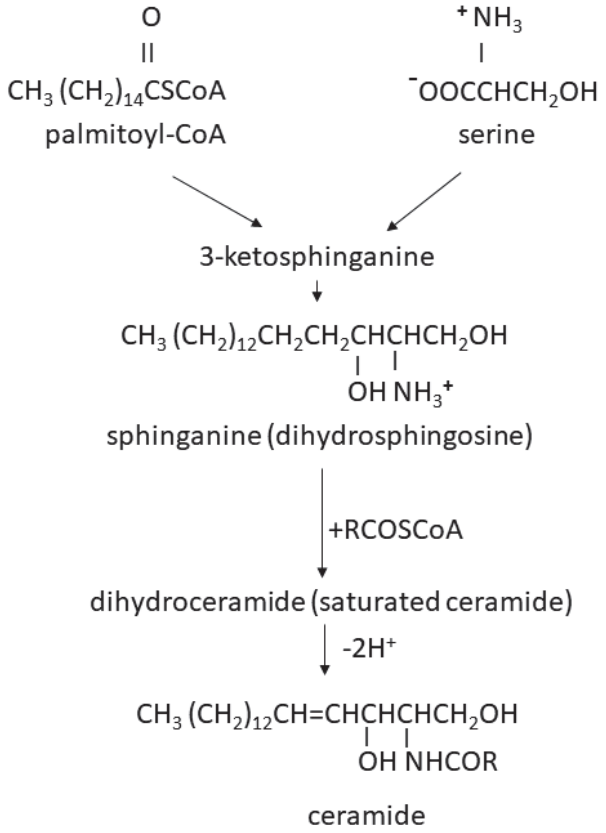


Figure 1. Ceramide synthesis.

The cellular level of ceramide is modulated through the ubiquitination of N-acylsphingosine amidohydrolase 1, which cleaves the acyl residue (represented as COR within the -NHCOR ceramide group in Figure 1) from ceramide. Thereby, ubiquitin domain-containing protein 1 (UBTD1), which performs ubiquitination, indirectly limits the self-phosphorylation of epidermal growth factor receptor (EGFR) by modulating the ceramide balance through the degradation of the N-acylsphingosine amidohydrolase 1 [10]. Specifically, the ceramide level directly affects the GM3 level of GSL, which is a well-known inhibitor of EGFR signal transduction [10].

The binding of sugar residues to ceramide by a glycosidic bond forms glycosphingolipids [11]. Many functions of GSLs are due to their capability to form hydrogen-bound clusters using hydroxyl and acetamide groups from their sugar residues [12,13]. A neutral GSL Gg3Cer, consisting of lactose and N-acetylglucosamine, is shown in Figure 2. It is

called neutral due to its inability to release or accept hydrogen ions in a physiological pH medium.

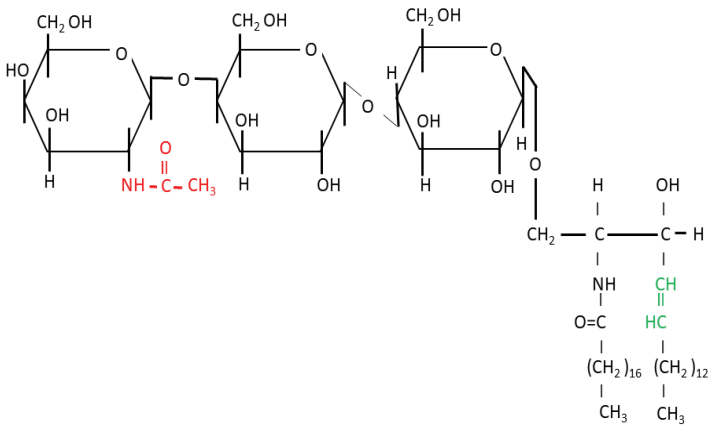


Figure 2. Structure of glycosphingolipid Gg3Cer. Acetamide group of N-acetylglucosamine is marked in red. *Trans* double bond within sphingosine, responsible for lipid raft formation, is marked in green.

On the other hand, acidic GSLs, named gangliosides, contain N-acetylneuraminic acid (Neu5Ac, also called sialic acid) as the third sugar instead of N-acetylglucosamine in Gg3Cer (Figure 2) and release hydrogen ions, leaving negatively charged N-acetyneuraminate residues. The N-acetyneuraminate-lactose part of ganglioside GM3 is essential for the binding of gangliosides to the extracellular domain of EGFR, modulating its activity [14]. The interaction of GSL with other sphingolipids, cholesterol, and transmembrane proteins, including receptors or signal transducers, forms lipid rafts [15]. The hydrophobic ceramide part anchors GSL within the membrane. In sphingolipids, saturated acyl chains such as palmitic and stearic acid are the main components and are present (at least in the nervous system) in sphingolipid-enriched domains and lipid rafts, packed tightly in the hydrophobic core of a bilayer mainly with saturated glycerophospholipid dipalmitoylphosphatidylcholine [16]. In humans with healthy diets, glycerophospholipids with unsaturated fatty acids, which are outside of lipid rafts, contain exclusively *cis*-fatty acids. Hydrogen ions are on the same side of their double bonds, which causes the carbon chain to bend, thereby causing higher membrane fluidity [17]. The sphingosine part of the sphingolipid contains a *trans* double bond (Figure 2). Hydrogens are on the opposite sides of the double bond, causing the carbon chain to remain straight and enabling tight packaging within lipid rafts. The introduction of *cis* conformation into the fatty acyl tail of sphingolipids disrupts the lipid raft ordered domains, whereas *trans* fatty acid conformation promotes raft formation [18].

3. Different Plasma Membrane Lipids Influence Growth Receptor Function

Neoplastic growth of breast cancer cells often relies on the overactivity of members of the ErbB tyrosine kinase receptor family [19]. High expression and/or gene mutations in *ErbB1/EGFR*, *ErbB2*, *ErbB3*, and *ErbB4* constitutively activate receptor kinase signaling [20]. When such a biological event occurs, it generally fosters failure in cell physiology and eventually drives tumor phenotype development. Hence, these receptors have been considered targets of particular interest for therapeutic intervention. In healthy cells, the activity of these receptors is driven by their ligands. When bound to the receptor, each ligand triggers a seemingly overlapping signaling pathway [21]. On the cell surface, the process of signal transduction across the plasma membrane is linked to conformational rearrangement of each receptor domain (extracellular ligand binding, transmembrane, juxtamembrane, and tyrosine kinase) and higher-order assemblies (dimers of EGF-induced dimers) [22].

Over the past 30 years, the complex conformational landscape of these receptors has been described, and the structure–activation interdependency has become more clearly understood [23,24]. Because of these well-characterized ligand–receptor models [25] and

their prominent role in cancer, considerable drug therapy tools have been conceptualized in an attempt to hamper receptor activity [26]. A range of antibodies, allosteric inhibitors, and cyclic peptides have been largely used for therapy, either by preventing a dynamic dimer state, stabilizing an inactive conformation, or targeting sites different from the orthosteric and highly conserved ATP-binding site [27–31].

The role of the plasma membrane lipid environment is of particular interest and is increasingly being explored, considering the clinical significance of heterogeneous and highly dynamic membrane lipid content in cancer cells [32]. Advances pursued in a number of comparative studies, including molecular dynamic simulation, structural modeling, and equally important biophysical and computational studies on lipid-dependent receptor signaling are unfolding and genuinely challenge well-established linear and simple models of receptor activation. It should be noted that the biological role of lipids in this process is not yet well understood and remains incomplete. To date, the emerging picture shows that highly dynamic membrane lipid environment content paradoxically shapes receptor signaling in healthy and cancer cells [33,34]. Because of lipid metabolism, the dynamic membrane environment changes and participates in ligand–receptor signaling. For example, the metabolic enzyme lysophosphatidylcholine acyltransferase, LPCAT1, which is involved in the biosynthetic pathway that controls membrane phospholipid saturation, prevents the internalization and degradation of the receptor and holds the receptors on the cell surface, thereby providing the input for tumor formation and cancer progression [35].

Membrane lipids are involved in the reorganization of signaling molecules in lipid microdomain rafts and structural fluctuations in the conformational landscape of membrane proteins [36–38]. As revealed by several structural studies of full-length receptors, surrounding lipids particularly change the dimerization propensity, stabilization, and orientation of juxtamembrane and kinase domains of receptors [39,40]. For example, phosphatidylinositol 4,5-bisphosphate (PIP_2) regulates transition of the inactive to the active dimer conformers. Under steady-state conditions, PIP_2 membrane acidic lipids, which are highly concentrated in the raft microdomains, interact with the juxtamembrane domain of the receptor and likely stabilize the inactive EGFR dimer. In response to ligand stimulation, positive cooperation between the domains occurs, and the receptor dimer immediately becomes active and displays short-lived kinase activity. Signaling molecules involved in receptor downstream signaling such as protein kinase C (PKC) and membrane-bound phospholipase C gamma (PLCγ) induce phosphorylation in Thr654 and hydrolysis of PIP_2, respectively. These events lead to juxtamembrane helix dimer dissociation, and the whole structure of the receptor returns to the initial, inactive state [41,42].

In addition, ErbB receptors interact with other species of plasma lipids, such as glycosphingolipid GM3. Differences in the interaction energies and dynamics of PIP_2 and GM3 with receptors revealed possible differential modes of receptor structure and function mediation [43]. The retention of ErbB2 within the plasma membrane is due to lipid raft microdomains. Protein MAL2 mediates lipid raft formation, leading to ErbB2 plasma membrane retention and enhanced ErbB2 signaling in breast cancer cells [44]. EGF-induced lipid raft localization of the EGFR-ErbB2 heterodimer is crucial for Akt phosphorylation and breast cancer cell proliferation [45].

Interaction with other membrane lipids, such as cholesterol, which constitutes half of the functional membrane component, has been observed. With advancements in NMR spectroscopy, a tool for lipid membrane investigation, and molecular dynamics studies, a detailed description of the exact mechanistic understanding of cholesterol contribution in the dynamic modulation and rearrangement of the receptor has been reported [46]. The cholesterol-recognition/amino acid consensus (CRAC) sequence within transmembrane and juxtamembrane segments contains two overlapping motifs (A 661-XXX-G 665 and I 659-XXX-V663). It was observed that at high concentrations of cholesterol (16% *w/w*), the interactions between these two motifs are disrupted, helix–helix dimers fall apart, and the whole transmembrane segments separate [47].

Finally, the propensity of different plasma membrane lipids to subtly modify dimer conformity and dynamics presents prospects for new, attractive cancer drug therapy. In general, the gradual profiling of the membrane environment will likely contribute to a better understanding of both physiological and pathological lipid-dependent remodeling signaling pathways. These results may point to the need to prioritize lipid cell-profiling before studying receptor signaling models in general.

Concerning our recent study [2], we can assume that thieno[2,3-*b*]pyridine-derivative-treated MCF-7 cells containing a low level of HER2 and a high level of HER3 are able to transduce the EGF signal using the HER2-HER3 heterodimer according to Balz et al., while MDA-MB-231 cells lack this ability [48,49]. Therefore, after treatment with thieno[2,3-b]pyridine derivative that inhibits PLC, MCF-7 cells maintain anaerobic metabolism and a fraction of cancer stem cells (CSCs), while MDA-MB-231 cells shift to aerobic metabolism and decrease the CSC fraction. In addition to EGF signaling, which results in PLCγ partial activation by binding phosphatidylinositol-3,4,5-trisphosphate (PIP_3) via its N-terminal pH domain [50], these outcomes are expected due to progesterone and estrogen signaling, which are present in MCF-7 and absent in the MDA-MB-231 cell line (Figure 3). Signals from hormones bound to steroid receptors, such as estrogen receptors (ERs), can elicit the phosphorylation of PLCγ and activation of the anticipatory unfolded protein response. Yu et al. showed that Src kinase can trigger the activation of unfolded protein response during stress, enabling the tumor-protective mode in cancer cells [51].

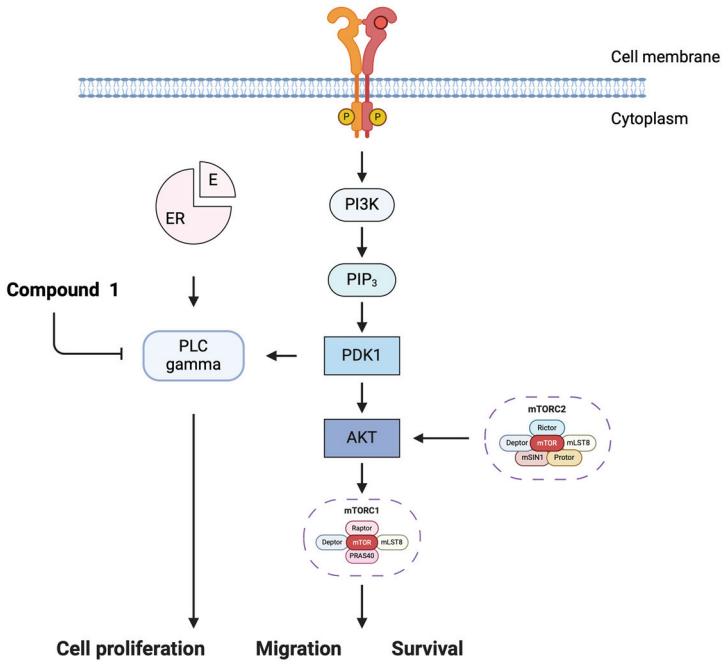


Figure 3. The interplay of growth factor and estrogen signaling in breast cancer cell proliferation, survival, and migration. Higher activation of PLCγ and mTOR is expected in MCF-7 cells containing ER, HER3, and a low level of HER2, which are absent in MDA-MB-231 cells. Compound **1**, as an inhibitor of PLCγ, is effective in lowering the percentage of MDA-MB-231 but not MCF-7 CSCs. Abbreviations: AKT, protein kinase B or Akt; Compound **1** or thieno[2,3-*b*]pyridine derivative, 3-amino-*N*-(3-chloro-2-methylphenyl)-5-oxo-5,6,7,8-tetrahydrothieno[2,3-*b*]quinoline-2-carboxamide; E, estrogen; ER, estrogen receptor; mTORC1 and mTORC2, mechanistic targets of rapamycin complex I and II; PDK1, 3-phosphoinositide-dependent kinase 1; PI3K, phosphoinositide 3-kinase; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; and PLC gamma, phospholipase C gamma. Created with BioRender.com.

Recently, Nguyen et al. proved that casein kinase 1 gamma 2 (CSNK1G2) was the most sensitive target to tamoxifen in MCF-7 cells, while tamoxifen was only slightly toxic in MDA-MB-231 cells [52]. ER silencing almost completely blocked tamoxifen sensitivity

in MCF-7 cells. CSNK1G2 catalyzes the subsequent phosphorylation of phosphatidylinositol 3-kinase (PI3K)/AKT/mechanistic target of rapamycin (mTOR) signaling but not extracellular signal-regulated kinase (ERK) signaling in MCF-7 cells, while in MDA-MB-231 cells, CSNK1G2 activates ERK and slightly activates the PI3K/AKT/mTOR signaling pathway [52].

Different drugs are able to target breast cancer cells via mechanisms that involve lipid raft composition. Lipid-raft-associated PI3K/AKT signaling is a target of phenolic lipid gingerol from ginger in radio-resistant MDA-MB-231/IR cells, implicating a new raft-mediated treatment approach [53]. Membrane raft-localized EGFR expression is altered in MCF-7 and MDA-MB-231 cells by cholesterol metabolite 4-cholesten-3-one, which decreases breast cancer cell viability, reduces lipogenesis, and enhances liver X receptor-dependent cholesterol transporters [54]. Resveratrol treatment increases the fatty acid amount two- and four-fold in MCF-7 and MDA-MB-231 cells, which is accompanied by an increase in the expression of both flotillin-1 and flotillin-2 in breast tumors. Resveratrol induces a change in the pattern of flotillin distribution among lipid raft fractions in both cell lines and induces the nuclear translocation of flotillin-2 [55]. The incorporation of docosahexaenoic acid into whole-cell and lipid rafts differs between MCF-7 and MDA-MB-231 cell lines [56]. Gene expression profiles in MCF-7 and MDA-MB-231 differ concerning cholesterol homeostasis, implicating different lipid raft structures. In MCF-7 cells, miR-128 downregulation is favored because several genes involved in cholesterol synthesis and transport (*HMGCR*, *HMGCS1*, *SREBF1*, *SREBF2*, *CETP*, *LCAT*, and *LDLR*), MDR (*ABCC5* and *UGCG*), and cell signaling (*EGFR*, *IGF1R*, and *ESR1*) are downregulated, while the cholesterol efflux gene is increased (*ABCG1*) [57]. In the MDA-MB-231 cell line, it is demonstrated that adiponectin triggers fatty acid metabolic reprogramming that results in lipid depletion, triggering lipid raft disruption and apoptosis [58].

4. Aberrant Glycosphingolipid Metabolism as a Participant in Tumorigenesis and as a Possible Therapeutic Target

GSLs influence cell proliferation, differentiation, migration, and survival due to their involvement in plasma membrane processes such as cell adhesion, membrane receptor activation, signaling, and endocytosis [59]. The silencing of the rate-limiting enzyme in GSL biosynthesis, glucosylceramide (GlcCer) synthase, encoded by the gene *UGCG* in humans, may thus interfere with cancer cell growth as shown in in vitro cell culture experiments (Figure 4) [60,61]. GlcCer synthase catalyzes the first step of GSL synthesis. Subsequently, GlcCer is elongated by Gal addition via enzyme B4GALT5, forming lactosylceramide (LacCer). The activation of B4GALT5 is a "pivotal" point of convergence for several signaling pathways initiated by growth factors, pro-inflammatory molecules, oxidative stress, diet, and cigarette smoking [62]. Further, formed LacCer can be the substrate of five enzymes: ST3GAL5, catalyzing the synthesis of the simplest negatively charged ganglioside GM3 (containing one sialic acid residue) (Figure 4); B4GALNT1, catalyzing formation of Gg3Cer, which is the first step of neutral ganglioseries GSL synthesis (Gg3, GalNAcβ4Galβ4Glcβ1Cer, shown in Figure 2; Gg4Cer, 4Galβ3GalNAcβ4Galβ4Glcβ1Cer) (Figure 4); A3GALT2 and A4GALT, catalyzing isoglobo-side and globoside synthesis, respectively; and B3GNT5, catalyzing (neo-)lactoseries GSL synthesis (Lc3Cer, GlcNAcβ3Galβ4Glcβ1Cer; nLc4Cer, Galβ4GlcNAcβ3Galβ4Glcβ1Cer; and S(6)nLc4Cer, IV^{6}Neu5Ac Galβ4GlcNAcβ3Galβ4Glcβ1Cer (S, sialic acid residue or Neu5Ac) (Figure 4) [63,64].

Epithelial–mesenchymal transition (EMT) is a process in which epithelial cells lose their cell–cell contacts and apical–basal polarity, being thereby transformed to the mesenchymal phenotype with enhanced cell motility [65]. The EMT is often incomplete, and cells in the intermediate states display mixed epithelial and mesenchymal characteristics, giving them epithelial–mesenchymal plasticity properties [66]. EMT plays an important role in the initiation and development of cancer cell metastasis [67]. Gangliosides derived from the catalytic activity of ST3GAL5 (GM3, GM2, GD3, and GD2 from Figure 4) inhibit transforming growth factor-beta (TGFβ)-induced EMT via degradation of TGFβ type I

receptor in normal mouse mammary gland epithelial NMuMG cells [64]. Consistent with this result, the addition of GM3 inhibits the migration of melanoma B16-F10 cells [68], while the finding of no influence of the treatment with exogenous GD3 on EMT is not consistent with the role of GD3 and GD2 in EMT maintenance [69]. Obviously, the effects of specific gangliosides on EMT vary between different cancer types. The inhibition of GD3 synthase (ST8SIA1, Figure 4) using shRNA or triptolide suppresses the invasion and motility of breast cancer cells and metastasis in mice, while its inhibition does not affect cell proliferation rates in vitro [69]. This finding is in accordance with the finding of inhibited c-Met signaling as a consequence of GD3 synthase inhibition and the known role of c-Met in the sustained proliferation and self-renewal properties of CSCs seeded to different tissues [69,70]. Globosides and ganglioseries inversely correlate and promote the transition between epithelial and mesenchymal cells of ovarian cancer [71]. CRISPR-Cas9-knocking-mediated truncation of endogenous E-cadherin in ovarian cancer cells induces EMT, which results in a decrease in globoside GSLs, while the deletion of ST8SIA1, which synthesizes GD3, induces mesenchymal–epithelial transition [71]. Levels of mRNA for enzymes encoded with ST3GAL2, ST3GAL5, ST8SIA1, and B4GALNT genes are elevated in breast CSCs, resulting in elevated GD2, GD3, GM2, and GD1a gangliosides accompanied by drastically reduced neutral GSLs Fuc-(n)Lc4Cer and the globoside Gb3 [72]. This finding of elevated ganglioside-synthesizing enzyme ST8SIA1 in breast CSCs is consistent with its elevation in ovarian cancer mesenchymal-like samples, predicting poor outcomes [71]. ST8SIA1 has also been suggested to be important for the initiation and maintenance of EMT in MDA-MB-231 breast cancer cells [73]. Namely, GD3 synthase (ST8SIA1) is the rate-limiting enzyme that converts the precursor GM3 to GD3 and further GD3 to GD2 and is known as a specific marker of triple-negative breast CSCs targeted with dinutuximab (Figure 4) [74]. Recently, Liang et al. confirmed in their model of EMT induction that GD3 synthase, together with its common upstream glycosyltransferase (GD2/GM2 synthase or B4GALNT1, Figure 4), maintains the stem cell phenotype in breast CSCs [75]. B4GALNT1 also induces motility and anchorage independence growth in melanoma [76].

Treatment with thieno[2,3-*b*]pyridine derivative, specifically 3-amino-*N*-(3-chloro-2-methylphenyl)-5-oxo-5,6,7,8-tetrahydrothieno[2,3-*b*]quinoline-2-carboxamide (Compound **1**), increases the percentage of ganglioseries GSL GalNAcGM1b (IV^3Neu5Ac-Gg5; Gg5, GalNAcβ4Galβ3GalNAcβ4Galβ4Glcβ1Cer)-positive CSCs in the MCF-7 cell line and decreases the percentage of neolactoseries GSL S(6)nLc4Cer (IV^6Neu5AcGalβ4GlcNAcβ3Galβ4Glcβ1Cer)-positive CSCs in the MDA-MB-231 breast cancer cell line [2].

The same experiment revealed a decreased percentage of $Gg3Cer^+$ in MCF-7 CSCs, indicating that the addition of neuraminic acid (Neu5Ac) and two neutral sugar residues (Gal and GalNAc) to Gg3Cer, forming GalNAcGM1b ganglioside, favors the survival of MCF-7 CSCs. The whole sample of the MDA-MB-231 population (containing both CSCs and non-CSCs) shows a shift from anaerobic to aerobic metabolism. Studies of neolactoseries GSL expression in breast cancer cells are very rare. The deletion of β-1,3-N-acetyl-glucosaminyl-transferase 5 (B3GNT5), which synthesizes Lc3Cer, causes the loss of lacto/neo-lacto series GSLs and reduces α2-6 sialylation on N-glycoproteins in ovarian cancer cells [77]. The profiling of all known α2-6 sialyltransferase-encoding genes shows that the loss of α2-6 sialylation is due to the silencing of *ST6GAL1* expression in Δ*B3GNT5* cells, demonstrating that neolactoseries GSLs affect the complex network of *N*-glycosylation in ovarian cancer cells [77]. Breast CSCs show two-fold higher gene expression of B4GALT1, which catalyzes the synthesis of nLc4Cer, in comparison to non-CSCs [72]. In our earlier report, we showed five times higher expression of S(6)nLc4Cer per one non-treated non-CSC in comparison to CSCs of the MDA-MB-231 cell line [78], with a similar ratio being confirmed recently [2]. In spite of higher *B4GALT1* gene expression in CSCs reported by others [72], the percentage of $S(6)nLc4Cer^+$CSCs decrease in the previous study is shown partially in Figure 4 [2]. This is most probably due to the lack of specific sugar residue substrates (S, sialic acid sugar residue, and Fuc, fucose) caused by thieno[2,3-*b*]pyridine derivative treatment.

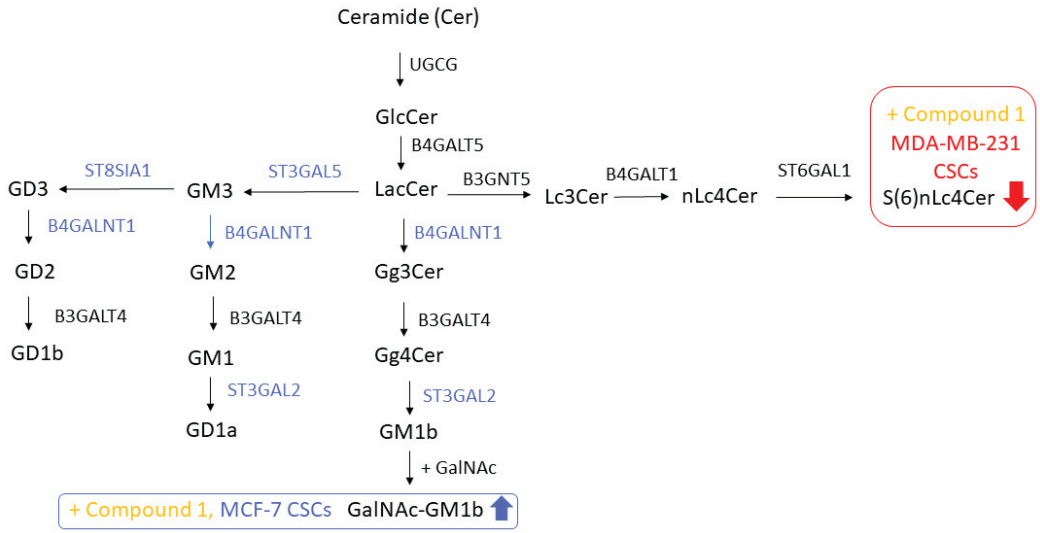


Figure 4. Synthesis of ganglioseries GSLs catalyzed by enzymes, written with blue letters, induced in breast CSCs [72,75] and of neolactoseries GSLs. Percentages of CSCs positive for red and blue framed GSLs were decreased and increased, respectively, after thieno[2,3-*b*]pyridine derivative (Compound **1**, yellow letters) treatment in MDA-MB-231 (red arrow) and MCF-7 (blue arrow) cells, respectively [2]. Abbreviations: In the ganglioside nomenclature, G = ganglioside, with the corresponding number of the sialic acid residues described with letters (M = mono, D = di), and the numbers denote the number of neutral sugar residues that are required to reach the number 5 (1 = GalGalNAcGalGlc, 2 = GalNAcGalGlc, 3 = GalGlc). Glycosidic residues: Gal = galactose, Glc = glucose, GlcNAc = N-acetylglucosamine, GalNAc = N-acetylgalactosamine. Neutral GSLs: Gg3Cer = gangliotriaosylceramide, Gg4Cer = gangliotetraosylceramide, Lc3Cer = lactotriaosylceramide, nLc4Cer = neolactotetraosylceramide. Acidic GSL: S(6)nLc4Cer = sialyl residue bound by α2-3 glycosidic bond to nLc4Cer. UGCG = UDP-glucose ceramide glycosyltransferase. In the nomenclature of other glycosyltransferases, the letter B = β-glycosidic bond, GALNT = N-acetylgalactosaminyltransferase, ST = sialyltransferase. Numbers within the name of β4-N-acetylgalactosaminyltransferase 1, B4GALNT1, represent the glycosidic bond between carbon C1 of the N-acetylgalactosaminyl residue and C4 of the galactosyl residue of LacCer in Gg3Cer, shown precisely in Figure 2.

MDA-MB-231 cells, mostly $CD44^{+}CD24^{-}$, represent triple-negative breast cancer (TNBC) cells, which lack three-hormone receptor expression; hence, there is an urgent need for their targetable markers [79]. CD44 is not a targetable marker owing to its expression in normal tissues. Ly et al. proposed ganglioside GD2 as a tumor-specific marker in TNBC patients due to its significantly higher expression in TNBC tissue compared to normal patient tissues [74]. The overwhelming majority of $CD44^{hi}CD24^{lo}$ cells express GD2 on the cell membrane [73]. CD44 mediates the aggregation of circulating MDA-MB-231 cells after their detachment from cancer tissue and the maintenance of their lipid raft integrity, which further enhances the stemness of aggregated cells [80].

Subsequent studies show the association of GD3 with EGFR and activated EGFR signaling in breast CSCs and breast cancer cell lines. ST8SIA1 (GD3 synthase) knockdown enhances cytotoxicity of gefitinib (an EGFR kinase inhibitor) in resistant MDA-MB468 cells, both in vitro and in vivo, indicating that ST8SIA1 contributes to gefitinib resistance in EGFR-positive breast cancer cells and its potential as a therapeutic target in drug-resistant breast cancers [81].

The enzymes NEU3, neuraminidase, and sialidase, which cleave glycoprotein and ganglioside neuraminic acid residue, enhance EGFR activation without affecting EGFR expression and act on its sialylation levels [82]. The indirect activation of EGFR is achieved via cleavage of neuraminic acid residue from the EGFR inhibitor, ganglioside GM3 [83]. Aberrant NEU3 expression is closely related to tumorigenesis [84]. NEU1 regulates prolif-

eration, apoptosis, and expression in mammary carcinoma cells, but to date, only cadherins have been examined as their substrates and not gangliosides [85].

5. The Interplay of Glycosphingolipid Metabolism with Other Metabolic Pathways during Tumorigenesis and Therapeutical Attempts

To explain the metabolic shift in MDA-MB-231 breast cancer cells, which we noticed after thieno[2,3-*b*]pyridine derivative treatment, it is necessary to review the interplay between GSL metabolism and other lipid and glucose metabolisms. Aerobic metabolism after derivative treatment, proved with lower lactate (Figure 5) and higher pyruvate levels [2], is accompanied by lower glycerylmonostearate (Figure 5), glycerylmonopalmitate, and myo-inositol levels [2]. In MCF-7 cells, derivative treatment increases lactate (Figure 5). Considering the pathway of glycosidic residue synthesis required for GSL synthesis, which shares fructose-6-P with glycolysis in parallel to different glycolysis patterns, a different response in GSL synthesis is expected. MCF-7 cells are able to obtain more glycosidic residues for GalNAcGM1b than MDA-MB-231 cells for S(6)nLc4Cer synthesis in the presence of thieno[2,3-*b*]pyridine derivative during cell cultivation [2]. Citrate transport from the mitochondria to the cytosol is elevated in conditions of elevated NADH in the case of anaerobic glycolysis [86]. Citrate is cleaved in cytosol, yielding acetyl-CoA, the precursor of palmitate and stearate, and consequently glycerylstearate, being increased in MCF-7 cells treated with thieno[2,3-*b*]pyridine derivative (Figure 5). Cancer cells take elevated levels of glucose in spite of decreased oxidative phosphorylation, which can yield maximal amounts of ATP. Their rate of glycolysis is increased due to their ability to produce ATP more quickly, optimizing energy production by maintaining a mix of metabolic capacities to support highly proliferated cancer cells [87]. Increased glucose uptake and lactate production via aerobic glycolysis is known as the Warburg effect [88]. Metabolic enzymes (hexokinase 2, pyruvate kinase type M2, and lactate dehydrogenase) and glucose and lactate (monocarboxylate) transporters are altered by the Warburg effect [89–93], which further increases the quantity of GM3 and sulphated SM4 GSLs required to maintain oncogenic GTPase KRAS in the plasma membrane enabling its signaling [94]. Among breast cancers, KRAS mRNA and GM3 expression have a prognostic role only in the luminal A subtype, which corresponds to the MCF-7 cell line [95–97]. GM3 has been identified and quantified in breast cancer serum with liquid chromatography–Fourier transform mass spectrometry (LC-FTMS) and liquid chromatography–mass spectrometry/mass spectrometry–multiple reaction monitoring (LC-MS/MS-MRM) [97]. More UTP and CTP, energy-rich molecules essential for glycosydic residue activation, and glycosydic residues (Figure 5) are produced in thieno[2,3-*b*]pyridine-derivative-treated MCF-7 cells, which could contribute to the synthesis of GalNAcGM1b^{+}CSCs. On the other hand, aerobic glycolysis in treated MDA-MB-231 resulted in the statistically significant depletion of total CSCs, particularly S(6)nLc4Cer^{+}CSCs [2].

Glucose availability and glycolytic metabolism are recognized as factors dictating glycosphingolipid levels in certain leukemia cells that take up elevated levels of glucose, resulting in increased glycolysis, activation of the pentose phosphate pathway, and biosynthesis of nucleotides, like UTP and CTP, required for GSL synthesis [98]. Increased flux into these pathways yields enough substrates for GSL formation, namely, fatty acyl-CoA for ceramide GSL portion, UDP-glucose, UDP-galactose, UDP-GlcNAc, UDP-GalNAc, and CMP-Neu5Ac (Figure 5). UDP-glucose ceramide glucosyltransferase (UGCG) is the key enzyme in GSL synthesis, and its induction generates GlcCer (Figure 5). It leads to increased proliferation in several cancer types, including proliferation of MCF-7 cells mediated with increased alanine–serine–cysteine transporter 2 (ASCT2) concentrations in GSL-enriched microdomains [99]. Although the acronym suggests otherwise, ASCT2 is a primary glutamine transporter in small-cell lung cancer cells located within lipid rafts rich in GD2 ganglioside [100]. Glutamine is necessary for the formation of aminoglycoside residues, such as GlcNAc and GalNAc, which are incorporated in GSLs (Figure 5), and its uptake is augmented by the induction of UGCG. This plays an important role in cell energy

metabolism, particularly during the adaptation of breast cancer cells to poor nutritional supply. Glutamine serves to reinforce the following: 1, antioxidative capacity via conversion to a reducing agent (glutathione); 2, energy production through conversion to glutamate and further 2-oxo-glutarate, a metabolite of citric cycle; and 3, anti-apoptotic events by inducing proline levels [101]. The role of glutamine in the upregulation of glutathione biosynthesis, which maintains the homeostasis of reactive oxygen species, is also identified by Lampa et al. [102]. Glutamine is recognized as the potent activator of the mammalian target of rapamycin (mTOR), a conserved serine–threonine kinase. It is a part of the mTOR complex 1 (mTORC1), which helps to coordinate cell growth with nutritional status, and it is dysregulated in cancer cells [103]. In addition, glutamine contributes to the CSC GD2 ganglioside-positive phenotype in MDA-MB-231 cells, revealing that targeting of the glutamine transporters could be used as a complement to conventional chemotherapy in TNBC, as shown by the in vivo inhibition of tumor growth in a TNBC patient-derived xenograft mouse model [104]. The pathway of mTOR is highly activated in GD2+ breast CSCs [105]. Anti-GD2 antibody dinutuximab significantly decreases the adhesion and migration of MDA-MB-231 and SUM159 TNBC cells and inhibits mTOR signaling [74]. Dinutuximab significantly inhibits tumor growth in a patient-derived xenograft model in combination with activated natural killer cells [74]. Recently, hexahydrobenzo [4,5]thieno[2,3-*d*]pyrimidine derivatives have been described as potential anticancer agents that downregulate the PI3K/AKT/mTOR signaling pathway [106]. The thieno[2,3-*d*]pyrimidine-4(3H)-one derivative of benzimidazole [107], as well as thieno[2,3-*b*]pyridine derivative in our recent experiment [2], possess higher cytotoxicity against MDA-MB-231 than against MCF-7 cells. In addition to PLCδ1 inhibition, thieno[2,3-*b*]pyridines moderate multiple biological targets: copper-trafficking antioxidant 1 (ATOX1) protein, which induces the proliferation of cancer cells [108]; tyrosyl DNA phosphodiesterase 1 (TDP 1), which is a phospholipase D enzyme involved in repairing DNA damage [109]; the colchicine binding site of tubulin [110], which is a known anticancer target; and adenosine A2A receptor [111]. Due to the expectation of an overlap between the aforementioned targets, it is currently not known which receptor/enzyme is responsible for the noticed anticancer potential of the thieno[2,3-*b*]pyridines or whether a synergistic effect is responsible for the efficacy [112]. The in vivo administration of thieno[2,3-*b*]pyridine 3-amino-*N*-(3-chloro-2-methylphenyl)-5-oxo-5,6,7,8-tetrahydrothieno[2,3-*b*]quinoline-2-carboxamid reviewed in this article [2] had been earlier reported by Reynisson et al. A dose of 10 mg/Kg was injected intraperitoneally and was well tolerated by mice, with no acute reaction observed. The maximal plasma concentration achieved 30 min post-injection was 0.087 μmol/L, and the half-life was over 4 h [6]. The related compound thieno[2,3-d]pyrimidin is toxic against breast cancer MCF-7 cells (IC50 0.074 μM), with lower cytotoxicity towards mouse fibroblast 3T3 cells with an IC50 of 0.20 μM [107]. Doses of 15 different thieno[2,3-*b*]pyridines ranging from 0.75 to 133 mg/kg were proven to be active in the P388 leukemia tumor model (intraperitoneal) in B6D2F1 (BDF1) mice (https://pubchem.ncbi.nlm.nih.gov/bioassay/328#section=Data-Table, accessed on 21 August 2024).

The interaction of transglutaminase 2 and phospholipase C δ1 (PLCδ1) promotes Akt/mTORC1 activation and, consequently, autophagosome degradation in metastatic triple-negative breast cancer [113]. The combined signature of high transglutaminase 2/PLCδ1 levels correlates with lymph node metastasis of ER-negative breast cancer in patients with breast cancer. Blocking the protein–protein interaction of transglutaminase 2 and PLCδ1 inhibits lung metastatic colonies of TNBC cells in vivo [114]. Transglutaminase 2, known to mediate post-translational modifications of intracellular enzymes, is involved in the pathogenesis and progression of cancer. Its inhibition promotes apoptosis in MCF-7 and MDA-MB-231 cell lines, being particularly effective in decreasing MD-MB-231 cell migration and invasiveness [115].

The activation of mTORC1 results in nucleotide synthesis and proliferation, while mTORC2 promotes cancer-forming lipids essential for growth and energy production such as triacylglycerol (from glycerylmonostearate, Figure 5) and particularly sphingolipids (glu-

cosylceramide, GlcCer; Figure 5) and glycerophospholipid (cardiolipin). In the same mTOR-driven mouse model, the prevention of hepatosteatosis from progressing to hepatocellular carcinoma is demonstrated by the inhibition of fatty acid or sphingolipid synthesis [116].

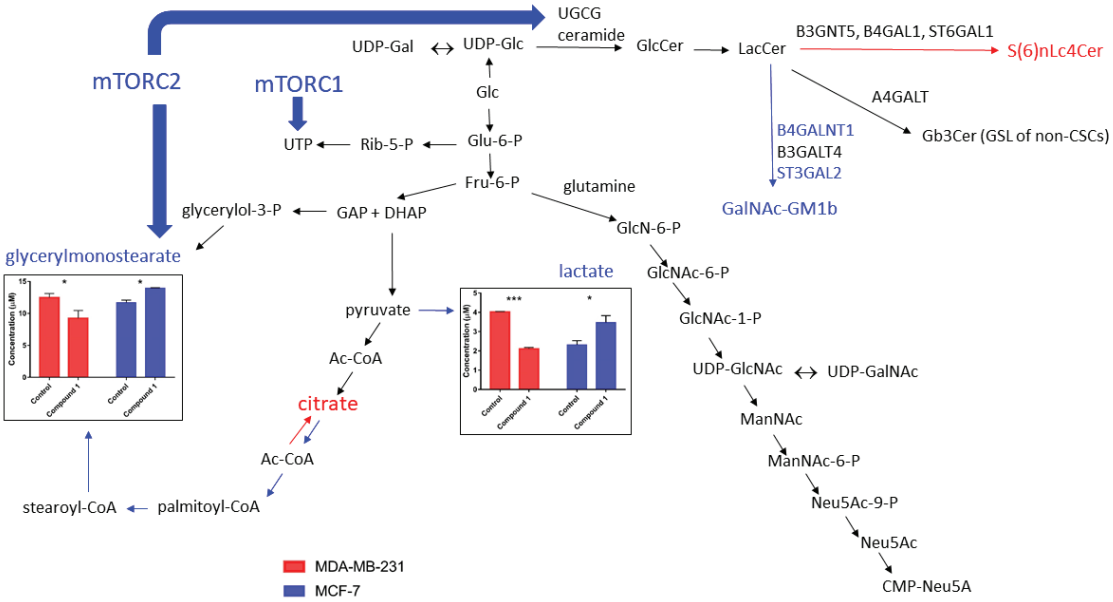


Figure 5. The interplay of glycosphingolipid metabolism with other metabolic pathways, which results in distinct metabolite and GSL expression findings in MDA-MB-231 and MCF-7 breast cancer cells after thieno[2,3-*b*]pyridine derivative treatment. Blue arrows and blue letters indicate the direction of metabolic reactions in MCF-7 cells, while red arrows and red letters indicate reactions in MDA-MB-231 cells. The red arrow from Ac-CoA to the citrate molecule indicates its catabolism in the citric acid cycle for aerobic energy production in the MDA-MB-231 cell line. Black arrows indicate common reactions for both cell lines; * $p < 0.05$; *** $p < 0.001$.

Knockdown of synaptopodin 2, a tumor suppressor inhibiting the transcription cofactors YAP/TAZ, enhances the PI3K/AKT/mTOR signaling pathway in MCF-7 and MDA-MB-231 cells, resulting in their migration and invasion [117]. Knockdown of caveolin-1, a principal structural component of caveolar membrane domains derived from lipid rafts, suppresses cancer development via the inhibition of PI3K/Akt/mTOR signaling, motility, and metastasis of breast carcinoma MDA-MB-231 cells [118]. The inactivation of mTOR and its dissociation from the lysosomal surface happens during starvation, causing transcription factor EB dephosphorylation, translocation to the nucleus, and the subsequent regulation of transcription of the genes involved in autophagy and lysosomal biogenesis [103,119,120]. Recently, Hartwig et al. noticed mTOR inactivation due to treatment with UDP-glucose ceramide glucosyltransferase inhibitors such as 1-phenyl-2-decanoylamino-3-morpholino-1-propanol, which causes the defective export of sphingolipids, lysobisphosphatidic acid, and cholesterol from lysosomes [121].

Signaling via the mTOR pathway in cancer is modulated by the Golgi-localized oncoprotein GOLPH3 [122], which also performs sorting of specific glycosyl transferases of GSL synthesis into vesicles for intra-Golgi retro-transport, enabling the cisternal maturation mechanism and controlling the sub-Golgi enzyme localization and its lysosomal degradation rate [123]. The decrease in GOLPH3 levels is accompanied by a decrease in GD1a and a concomitant increase in GM1 ganglioside expression at the surface of MCF-7 cells [124]. N-terminal domains of ST3GAL2 and B3GALT4 enzymes, which catalyze the last steps of GD1a and GM1 synthesis (Figure 3), respectively, interact to form the glycosyltransferase complex, which improves the enzymatic activity of one of the partners in the case of B3GALT4 [124,125]. GOLPH3 increases the glycosylation of cancer-relevant glycoproteins and regulates EGFR in glioblastoma cells via the modulation of its glycosyla-

tion [126] and has a role in MDA-MB-231 cells, sustaining their bioenergetic function and their mitochondrial fission [127].

Nohara et al. analyzed GSL expression in MDA-MB-231 and MCF-7 cells after their extraction from cells with organic solvents and, using thin-layer chromatography, reported four-fold higher ganglioside content in MDA-MB-231 cells compared to MCF-7 cells [128], which correlate with the values of geometric mean fluorescence intensities found by us using the flow cytometry method [2]. There is a lack of data in the literature concerning different GSL expressions in cell lines for each breast cancer subtype except for GD2 ganglioside. There is growing interest in GD2 investigation due to new discoveries emphasizing its roles in tumorigenesis and therapy [73,74]. Therefore, among all GSLs, only GD2 expression is described among different breast cancer subtypes. A low percent of the basal A subtype of basal-like 1 and basal-like 2 TNBC-subtype (MDA-MB-468, HCC1599 and HCC1806) and luminal (ER+ ZR-75-1, ER+PR+ MCF-7, Her2+ SKBR3, and ER+PR+Her2+ BT-474) cell lines are GD2-positive (up to 6%). The basal B subtype of mesenchymal, mesenchymal stem-like, and basal-like 1 TNBC-subtype TNBC cell lines (BT549, MDA-MB-231, SUM159, HCC38, and Hs 578T) are positive in more than 6% of the population, in 10% of MDA-MB-231, and in up to 99% in the HS 578T cell line [73,74,129]. One-third of several histologic types of breast carcinoma patients (386 in total) were positive for GD2. Positivity was significantly associated with the histologic subtype of the tumor, varying between metaplastic carcinomas (16%), triple-negative, invasive ductal carcinoma of no special type, ER-positive/HER2-negative, invasive lobular carcinoma, and triple-positive tumors (73%) [130]. Therefore, at least a proportion of these tumors may be therapeutically targeted with already available agents such as anti-GD2 antibodies and GD2-GD3 vaccines [131,132].

Serum GM3 is detected as a diagnostic marker of the luminal B subtype in comparison with other subtypes, and it is positively correlated with the Ki-67 status of patients, which indicates the number of mitoses [97]. On the other hand, GM3 expression is important in endothelial cells for the regulation of tumor angiogenesis by suppressing their invasion and oxidative stress tolerance. The GM3 knockout breast cancer mouse model shows an increase in tumor growth and angiogenesis [133]. The metabolomic profiling of blood-derived microvesicles in breast cancer patients reveals that sphingomyelin SM (OH) C16:1 is among eight significant metabolites differentiating breast cancer patients from controls [134]. The combination of the six sphingomyelins with 18 phosphatidylcholines differentiates between luminal A and B breast cancer subtypes [134]. GSLs within blood-derived microvesicles were not analyzed. Nevertheless, due to the distinct sphingomyelin profile, we can also assume that some of the GSLs can be used to differentiate breast cancer subtypes. Sphingolipid and ether lipid expression patterns are altered in breast cancer cells in an estrogen-receptor-dependent manner, being strongly upregulated in SKBr3 (ER-negative and G-protein coupled estrogen receptor 1, GPER1, positive) in comparison to T47D (ER + and GPER1-) and MCF-7 (ER + and GPER1+) cells [135]. It is important to focus on more than one lipid class in breast cancer cells because co-expression of different lipids could influence tumorigenesis and the achievement of improved breast cancer treatment outcomes [135].

6. Conclusions and Perspectives

Glycosphingolipid expression is strongly entwined with cell metabolism. GSLs cause specific shifts of metabolism during tumorigenesis via their effects on growth factor receptors and glutamine transporter (alanine–serine–cysteine transporter 2) concentrations in GSL-enriched plasma membrane microdomains. On the other hand, treatments of cancer stem cells can result in distinct GSL expression, which contributes to the decrease in CSCs.

The finding of higher activity of B3GALT4 and higher GM1 ganglioside level caused by the decrease in GOLPH3 level deserves particular attention due to the expected potential increase in GD1b synthesized by the same enzyme and decrease in its substrate GD2 (Figure 3). Recent clinical evidence of the worse 5-year recurrence-free survival rates among patients with GD2-positive TNBC in comparison to patients with GD2-negative

TNBC brings the novel strategies of GD2 synthesis inhibition into focus within TNBC research. The novel thieno[2,3-*b*]pyridine anticancer compound, being able to target TNBC cells that express glycosphingolipid S(6)nLc4Cer on their surface and diminish the Warburg effect that leads to lower total CSCs, deserves further investigation. Considering that two cell lines (MDA-MB-468 and HCC1599) of the basal A subtype of TNBC basal-like 1 subtype have a low GD2-positive fraction, thieno[2,3-*b*]pyridine needs to be proved in vivo as a therapy for patients who do not respond to anti-GD2 antibodies.

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References

1. Schomel, N.; Geisslinger, G.; Wegner, M.S. Influence of glycosphingolipids on cancer cell energy metabolism. *Prog. Lipid Res.* **2020**, *79*, 101050. [CrossRef] [PubMed]
2. Pervan, M.; Marijan, S.; Markotic, A.; Pilkington, L.I.; Haverkate, N.A.; Barker, D.; Reynisson, J.; Meic, L.; Radan, M.; Cikes Culic, V. Novel Thieno [2,3-b]pyridine Anticancer Compound Lowers Cancer Stem Cell Fraction Inducing Shift of Lipid to Glucose Metabolism. *Int. J. Mol. Sci.* **2022**, *23*, 11457. [CrossRef] [PubMed]
3. Benz, C.C.; Scott, G.K.; Sarup, J.C.; Johnson, R.M.; Tripathy, D.; Coronado, E.; Shepard, H.M.; Osborne, C.K. Estrogen-dependent, tamoxifen-resistant tumorigenic growth of MCF-7 cells transfected with HER2/neu. *Breast Cancer Res. Treat.* **1992**, *24*, 85–95. [CrossRef] [PubMed]
4. Papadimitropoulou, A.; Vellon, L.; Atlas, E.; Steen, T.V.; Cuyas, E.; Verdura, S.; Espinoza, I.; Menendez, J.A.; Lupu, R. Heregulin Drives Endocrine Resistance by Altering IL-8 Expression in ER-Positive Breast Cancer. *Int. J. Mol. Sci.* **2020**, *21*, 7737. [CrossRef] [PubMed]
5. Reynisson, J.; Court, W.; O'Neill, C.; Day, J.; Patterson, L.; McDonald, E.; Workman, P.; Katan, M.; Eccles, S.A. The identification of novel PLC-gamma inhibitors using virtual high throughput screening. *Bioorg. Med. Chem.* **2009**, *17*, 3169–3176. [CrossRef]
6. Reynisson, J.; Jaiswal, J.K.; Barker, D.; D'Mello, S.A.; Denny, W.A.; Baguley, B.C.; Leung, E.Y. Evidence that phospholipase C is involved in the antitumour action of NSC768313, a new thieno[2,3-b]pyridine derivative. *Cancer Cell Int.* **2016**, *16*, 18. [CrossRef] [PubMed]
7. Haverkate, N.A.; van Rensburg, M.; Kumara, S.; Reynisson, J.; Leung, E.; Pilkington, L.I.; Barker, D. Improving the solubility of anti-proliferative thieno[2,3-b]quinoline-2-carboxamides. *Bioorg. Med. Chem.* **2021**, *37*, 116092. [CrossRef]
8. Dasgupta, S.; Ray, S.K. Ceramide and Sphingosine Regulation of Myelinogenesis: Targeting Serine Palmitoyltransferase Using microRNA in Multiple Sclerosis. *Int. J. Mol. Sci.* **2019**, *20*, 5031. [CrossRef]
9. Sonnino, S.; Chigorno, V. Ganglioside molecular species containing C18- and C20-sphingosine in mammalian nervous tissues and neuronal cell cultures. *Biochim. Biophys. Acta* **2000**, *1469*, 63–77. [CrossRef]
10. Torrino, S.; Tiroille, V.; Dolfi, B.; Dufies, M.; Hinault, C.; Bonesso, L.; Dagnino, S.; Uhler, J.; Irondelle, M.; Gay, A.S.; et al. UBTD1 regulates ceramide balance and endolysosomal positioning to coordinate EGFR signaling. *eLife* **2021**, *10*, e68348. [CrossRef]
11. Schnaar, R.L.; Sandhoff, R.; Tiemeyer, M.; Kinoshita, T. Glycosphingolipids. In *Essentials of Glycobiology*, 4th ed.; Varki, A., Cummings, R.D., Esko, J.D., Stanley, P., Hart, G.W., Aebi, M., Mohnen, D., Kinoshita, T., Packer, N.H., Prestegard, J.H., et al., Eds.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, USA, 2022; pp. 129–140.
12. Regina Todeschini, A.; Hakomori, S.I. Functional role of glycosphingolipids and gangliosides in control of cell adhesion, motility, and growth, through glycosynaptic microdomains. *Biochim. Biophys. Acta* **2008**, *1780*, 421–433. [CrossRef] [PubMed]
13. Pascher, I. Molecular arrangements in sphingolipids. Conformation and hydrogen bonding of ceramide and their implication on membrane stability and permeability. *Biochim. Biophys. Acta* **1976**, *455*, 433–451. [CrossRef] [PubMed]
14. Miljan, E.A.; Meuillet, E.J.; Mania-Farnell, B.; George, D.; Yamamoto, H.; Simon, H.G.; Bremer, E.G. Interaction of the extracellular domain of the epidermal growth factor receptor with gangliosides. *J. Biol. Chem.* **2002**, *277*, 10108–10113. [CrossRef] [PubMed]
15. Groux-Degroote, S.; Guerardel, Y.; Julien, S.; Delannoy, P. Gangliosides in breast cancer: New perspectives. *Biochemistry* **2015**, *80*, 808–819. [CrossRef] [PubMed]
16. Prinetti, A.; Loberto, N.; Chigorno, V.; Sonnino, S. Glycosphingolipid behaviour in complex membranes. *Biochim. Biophys. Acta* **2009**, *1788*, 184–193. [CrossRef]
17. Kitagawa, S.; Kotani, K.; Kametani, F. Inhibitory mechanism of cis-polyunsaturated fatty acids on platelet aggregation: The relation with their effects on Ca^{2+} mobilization, cyclic AMP levels and membrane fluidity. *Biochim. Biophys. Acta* **1990**, *1054*, 114–118. [CrossRef]

18. Bryan, A.M.; You, J.K.; Li, G.; Kim, J.; Singh, A.; Morstein, J.; Trauner, D.; Pereira de Sa, N.; Normile, T.G.; Farnoud, A.M.; et al. Cholesterol and sphingomyelin are critical for Fcgamma receptor-mediated phagocytosis of Cryptococcus neoformans by macrophages. *J. Biol. Chem.* **2021**, *297*, 101411. [CrossRef]
19. Drago, J.Z.; Ferraro, E.; Abuhadra, N.; Modi, S. Beyond HER2: Targeting the ErbB receptor family in breast cancer. *Cancer Treat. Rev.* **2022**, *109*, 102436. [CrossRef]
20. Arteaga, C.L. ERBB receptors in cancer: Signaling from the inside. *Breast Cancer Res.* **2011**, *13*, 304. [CrossRef]
21. Okada, T.; Miyagi, H.; Sako, Y.; Hiroshima, M.; Mochizuki, A. Origin of diverse phosphorylation patterns in the ERBB system. *Biophys. J.* **2022**, *121*, 470–480. [CrossRef]
22. Endres, N.F.; Barros, T.; Cantor, A.J.; Kuriyan, J. Emerging concepts in the regulation of the EGF receptor and other receptor tyrosine kinases. *Trends Biochem. Sci.* **2014**, *39*, 437–446. [CrossRef] [PubMed]
23. Kovacs, E.; Zorn, J.A.; Huang, Y.; Barros, T.; Kuriyan, J. A structural perspective on the regulation of the epidermal growth factor receptor. *Annu. Rev. Biochem.* **2015**, *84*, 739–764. [CrossRef]
24. Purba, E.R.; Saita, E.I.; Akhouri, R.R.; Ofverstedt, L.G.; Wilken, G.; Skoglund, U.; Maruyama, I.N. Allosteric activation of preformed EGF receptor dimers by a single ligand binding event. *Front. Endocrinol.* **2022**, *13*, 1042787. [CrossRef]
25. Mudumbi, K.C.; Burns, E.A.; Schodt, D.J.; Petrova, Z.O.; Kiyatkin, A.; Kim, L.W.; Mangiacapre, E.M.; Ortiz-Caraveo, I.; Rivera Ortiz, H.; Hu, C.; et al. Distinct interactions stabilize EGFR dimers and higher-order oligomers in cell membranes. *Cell Rep.* **2024**, *43*, 113603. [CrossRef] [PubMed]
26. Halder, S.; Basu, S.; Lall, S.P.; Ganti, A.K.; Batra, S.K.; Seshacharyulu, P. Targeting the EGFR signaling pathway in cancer therapy: What's new in 2023? *Expert Opin. Ther. Targets* **2023**, *27*, 305–324. [CrossRef] [PubMed]
27. Shrestha, L.; Singh, S.S.; Parajuli, P.; Dahal, A.; Mattheolabakis, G.; Meyer, S.; Bhattacharjee, J.; Jois, S.D. In vivo studies of a peptidomimetic that targets EGFR dimerization in NSCLC. *J. Cancer* **2020**, *11*, 5982–5999. [CrossRef] [PubMed]
28. Arancillo, M.; Taechalertpaisarn, J.; Liang, X.; Burgess, K. Piptides: New, Easily Accessible Chemotypes For Interactions With Biomolecules. *Angew. Chem. Int. Ed. Engl.* **2021**, *60*, 6653–6659. [CrossRef]
29. Singh, S.S.; Mattheolabakis, G.; Gu, X.; Withers, S.; Dahal, A.; Jois, S. A grafted peptidomimetic for EGFR heterodimerization inhibition: Implications in NSCLC models. *Eur. J. Med. Chem.* **2021**, *216*, 113312. [CrossRef]
30. Zheng, M.; Li, C.; Zhou, M.; Jia, R.; Cai, G.; She, F.; Wei, L.; Wang, S.; Yu, J.; Wang, D.; et al. Discovery of Cyclic Peptidomimetic Ligands Targeting the Extracellular Domain of EGFR. *J. Med. Chem.* **2021**, *64*, 11219–11228. [CrossRef]
31. Roskoski, R., Jr. Small molecule inhibitors targeting the EGFR/ErbB family of protein-tyrosine kinases in human cancers. *Pharmacol. Res.* **2019**, *139*, 395–411. [CrossRef]
32. Taborda Ribas, H.; Sogayar, M.C.; Dolga, A.M.; Winnischofer, S.M.B.; Trombetta-Lima, M. Lipid profile in breast cancer: From signaling pathways to treatment strategies. *Biochimie* **2024**, *219*, 118–129. [CrossRef] [PubMed]
33. Pawar, A.B.; Sengupta, D. Effect of Membrane Composition on Receptor Association: Implications of Cancer Lipidomics on ErbB Receptors. *J. Membr. Biol.* **2018**, *251*, 359–368. [CrossRef] [PubMed]
34. Kim, D.H.; Triet, H.M.; Ryu, S.H. Regulation of EGFR activation and signaling by lipids on the plasma membrane. *Prog. Lipid Res.* **2021**, *83*, 101115. [CrossRef] [PubMed]
35. Bi, J.; Ichu, T.A.; Zanca, C.; Yang, H.; Zhang, W.; Gu, Y.; Chowdhry, S.; Reed, A.; Ikegami, S.; Turner, K.M.; et al. Oncogene Amplification in Growth Factor Signaling Pathways Renders Cancers Dependent on Membrane Lipid Remodeling. *Cell Metab.* **2019**, *30*, 525–538.e8. [CrossRef]
36. Lingwood, D.; Simons, K. Lipid rafts as a membrane-organizing principle. *Science* **2010**, *327*, 46–50. [CrossRef]
37. Sezgin, E.; Levental, I.; Mayor, S.; Eggeling, C. The mystery of membrane organization: Composition, regulation and roles of lipid rafts. *Nat. Rev. Mol. Cell Biol.* **2017**, *18*, 361–374. [CrossRef]
38. Rath, A.; Deber, C.M. Protein structure in membrane domains. *Annu. Rev. Biophys.* **2012**, *41*, 135–155. [CrossRef]
39. Gopal, S.M.; Pawar, A.B.; Wassenaar, T.A.; Sengupta, D. Lipid-dependent conformational landscape of the ErbB2 growth factor receptor dimers. *Chem. Phys. Lipids* **2020**, *230*, 104911. [CrossRef]
40. Pawar, A.B.; Sengupta, D. Resolving the conformational dynamics of ErbB growth factor receptor dimers. *J. Struct. Biol.* **2019**, *207*, 225–233. [CrossRef]
41. Maeda, R.; Sato, T.; Okamoto, K.; Yanagawa, M.; Sako, Y. Lipid-Protein Interplay in Dimerization of Juxtamembrane Domains of Epidermal Growth Factor Receptor. *Biophys. J.* **2018**, *114*, 893–903. [CrossRef]
42. Arkhipov, A.; Shan, Y.; Das, R.; Endres, N.F.; Eastwood, M.P.; Wemmer, D.E.; Kuriyan, J.; Shaw, D.E. Architecture and membrane interactions of the EGF receptor. *Cell* **2013**, *152*, 557–569. [CrossRef] [PubMed]
43. Nakano, M.; Hanashima, S.; Hara, T.; Kabayama, K.; Asahina, Y.; Hojo, H.; Komura, N.; Ando, H.; Nyholm, T.K.M.; Slotte, J.P.; et al. FRET detects lateral interaction between transmembrane domain of EGF receptor and ganglioside GM3 in lipid bilayers. *Biochim. Biophys. Acta Biomembr.* **2021**, *1863*, 183623. [CrossRef]
44. Jeong, J.; Shin, J.H.; Li, W.; Hong, J.Y.; Lim, J.; Hwang, J.Y.; Chung, J.J.; Yan, Q.; Liu, Y.; Choi, J.; et al. MAL2 mediates the formation of stable HER2 signaling complexes within lipid raft-rich membrane protrusions in breast cancer cells. *Cell Rep.* **2021**, *37*, 110160. [CrossRef]
45. Maadi, H.; Wang, Z. A Novel Mechanism Underlying the Inhibitory Effects of Trastuzumab on the Growth of HER2-Positive Breast Cancer Cells. *Cells* **2022**, *11*, 4093. [CrossRef] [PubMed]

46. Hasan, M.; Patel, D.; Ellis, N.; Brown, S.P.; Lewandowski, J.R.; Dixon, A.M. Modulation of Transmembrane Domain Interactions in Neu Receptor Tyrosine Kinase by Membrane Fluidity and Cholesterol. *J. Membr. Biol.* **2019**, *252*, 357–369. [CrossRef] [PubMed]
47. Pawar, A.B.; Sengupta, D. Role of Cholesterol in Transmembrane Dimerization of the ErbB2 Growth Factor Receptor. *J. Membr. Biol.* **2021**, *254*, 301–310. [CrossRef] [PubMed]
48. Balz, L.M.; Bartkowiak, K.; Andreas, A.; Pantel, K.; Niggemann, B.; Zanker, K.S.; Brandt, B.H.; Dittmar, T. The interplay of HER2/HER3/PI3K and EGFR/HER2/PLC-gamma1 signalling in breast cancer cell migration and dissemination. *J. Pathol.* **2012**, *227*, 234–244. [CrossRef]
49. Lee, G.S.; Ryu, K.S.; Rha, J.G.; Kim, S.P.; Namkoong, S.E.; Han, K.T. Multiparametric flow cytometric analysis in a breast cancer cell line (MCF-7). *J. Obstet. Gynaecol. Res.* **2002**, *28*, 141–148. [CrossRef]
50. Pasquet, J.M.; Quek, L.; Stevens, C.; Bobe, R.; Huber, M.; Duronio, V.; Krystal, G.; Watson, S.P. Phosphatidylinositol 3,4,5-trisphosphate regulates Ca(2+) entry via btk in platelets and megakaryocytes without increasing phospholipase C activity. *EMBO J.* **2000**, *19*, 2793–2802. [CrossRef]
51. Yu, L.; Wang, L.; Kim, J.E.; Mao, C.; Shapiro, D.J. Src couples estrogen receptor to the anticipatory unfolded protein response and regulates cancer cell fate under stress. *Biochim. Biophys. Acta Mol. Cell Res.* **2020**, *1867*, 118765. [CrossRef]
52. Nguyen Hoang, A.T.; Hoe, K.L.; Lee, S.J. CSNK1G2 differently sensitizes tamoxifen-induced decrease in PI3K/AKT/mTOR/S6K and ERK signaling according to the estrogen receptor existence in breast cancer cells. *PLoS ONE* **2021**, *16*, e0246264. [CrossRef] [PubMed]
53. Ediriweera, M.K.; Moon, J.Y.; Nguyen, Y.T.; Cho, S.K. 10-Gingerol Targets Lipid Rafts Associated PI3K/Akt Signaling in Radio-Resistant Triple Negative Breast Cancer Cells. *Molecules* **2020**, *25*, 3164. [CrossRef] [PubMed]
54. Elia, J.; Carbonnelle, D.; Loge, C.; Ory, L.; Huvelin, J.M.; Tannoury, M.; Diab-Assaf, M.; Petit, K.; Nazih, H. 4-cholesten-3-one decreases breast cancer cell viability and alters membrane raft-localized EGFR expression by reducing lipogenesis and enhancing LXR-dependent cholesterol transporters. *Lipids Health Dis.* **2019**, *18*, 168. [CrossRef] [PubMed]
55. Gomes, L.; Sorgine, M.; Passos, C.L.A.; Ferreira, C.; de Andrade, I.R.; Silva, J.L.; Atella, G.C.; Mermelstein, C.S.; Fialho, E. Increase in fatty acids and flotillins upon resveratrol treatment of human breast cancer cells. *Sci. Rep.* **2019**, *9*, 13960. [CrossRef] [PubMed]
56. Newell, M.; Patel, D.; Goruk, S.; Field, C.J. Docosahexaenoic Acid Incorporation Is Not Affected by Doxorubicin Chemotherapy in either Whole Cell or Lipid Raft Phospholipids of Breast Cancer Cells in vitro and Tumor Phospholipids in vivo. *Lipids* **2020**, *55*, 549–565. [CrossRef]
57. Palma, G.B.H.; Kaur, M. miRNA-128 and miRNA-223 regulate cholesterol-mediated drug resistance in breast cancer. *IUBMB Life* **2023**, *75*, 743–764. [CrossRef]
58. Pham, D.V.; Park, P.H. Adiponectin triggers breast cancer cell death via fatty acid metabolic reprogramming. *J. Exp. Clin. Cancer Res.* **2022**, *41*, 9. [CrossRef]
59. Corsetto, P.A.; Zava, S.; Rizzo, A.M.; Colombo, I. The Critical Impact of Sphingolipid Metabolism in Breast Cancer Progression and Drug Response. *Int. J. Mol. Sci.* **2023**, *24*, 2107. [CrossRef]
60. Wingerter, A.; El Malki, K.; Sandhoff, R.; Seidmann, L.; Wagner, D.C.; Lehmann, N.; Vewinger, N.; Frauenknecht, K.B.M.; Sommer, C.J.; Traub, F.; et al. Exploiting Gangliosides for the Therapy of Ewing's Sarcoma and H3K27M-Mutant Diffuse Midline Glioma. *Cancers* **2021**, *13*, 520. [CrossRef]
61. Jennemann, R.; Volz, M.; Bestvater, F.; Schmidt, C.; Richter, K.; Kaden, S.; Muthing, J.; Grone, H.J.; Sandhoff, R. Blockade of Glycosphingolipid Synthesis Inhibits Cell Cycle and Spheroid Growth of Colon Cancer Cells In Vitro and Experimental Colon Cancer Incidence In Vivo. *Int. J. Mol. Sci.* **2021**, *22*, 10539. [CrossRef]
62. Chatterjee, S.; Yuan, R.; Thapa, S.; Talwar, R. Central Role of beta-1,4-GalT-V in Cancer Signaling, Inflammation, and Other Disease-Centric Pathways. *Int. J. Mol. Sci.* **2023**, *25*, 483. [CrossRef] [PubMed]
63. Schnaar, R.L.; Kinoshita, T. Glycosphingolipids. In *Essentials of Glycobiology*, 3rd ed.; Varki, A., Cummings, R.D., Esko, J.D., Stanley, P., Hart, G.W., Aebi, M., Darvill, A.G., Kinoshita, T., Packer, N.H., Prestegard, J.H., et al., Eds.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, USA, 2015; pp. 125–135.
64. Zhang, J.; van der Zon, G.; Ma, J.; Mei, H.; Cabukusta, B.; Agaser, C.C.; Madunic, K.; Wuhrer, M.; Zhang, T.; Ten Dijke, P. ST3GAL5-catalyzed gangliosides inhibit TGF-beta-induced epithelial-mesenchymal transition via TbetaRI degradation. *EMBO J.* **2023**, *42*, e110553. [CrossRef] [PubMed]
65. Katsuno, Y.; Lamouille, S.; Derynck, R. TGF-beta signaling and epithelial-mesenchymal transition in cancer progression. *Curr. Opin. Oncol.* **2013**, *25*, 76–84. [CrossRef]
66. Yang, J.; Antin, P.; Berx, G.; Blanpain, C.; Brabletz, T.; Bronner, M.; Campbell, K.; Cano, A.; Casanova, J.; Christofori, G.; et al. Guidelines and definitions for research on epithelial-mesenchymal transition. *Nat. Rev. Mol. Cell Biol.* **2020**, *21*, 341–352. [CrossRef]
67. Derynck, R.; Weinberg, R.A. EMT and Cancer: More Than Meets the Eye. *Dev. Cell* **2019**, *49*, 313–316. [CrossRef] [PubMed]
68. Li, T.; Wang, X.; Dong, P.; Yu, P.; Zhang, Y.; Meng, X. Chemoenzymatic synthesis and biological evaluation of ganglioside GM3 and lyso-GM3 as potential agents for cancer therapy. *Carbohydr. Res.* **2021**, *509*, 108431. [CrossRef]
69. Sarkar, T.R.; Battula, V.L.; Werden, S.J.; Vijay, G.V.; Ramirez-Pena, E.Q.; Taube, J.H.; Chang, J.T.; Miura, N.; Porter, W.; Sphyris, N.; et al. GD3 synthase regulates epithelial-mesenchymal transition and metastasis in breast cancer. *Oncogene* **2015**, *34*, 2958–2967. [CrossRef]

70. Li, Y.; Li, A.; Glas, M.; Lal, B.; Ying, M.; Sang, Y.; Xia, S.; Trageser, D.; Guerrero-Cazares, H.; Eberhart, C.G.; et al. c-Met signaling induces a reprogramming network and supports the glioblastoma stem-like phenotype. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 9951–9956. [CrossRef]
71. Cumin, C.; Huang, Y.L.; Rossdam, C.; Ruoff, F.; Cespedes, S.P.; Liang, C.Y.; Lombardo, F.C.; Coelho, R.; Rimmer, N.; Konantz, M.; et al. Glycosphingolipids are mediators of cancer plasticity through independent signaling pathways. *Cell Rep.* **2022**, *40*, 111181. [CrossRef]
72. Liang, Y.J.; Ding, Y.; Levery, S.B.; Lobaton, M.; Handa, K.; Hakomori, S.I. Differential expression profiles of glycosphingolipids in human breast cancer stem cells vs. cancer non-stem cells. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 4968–4973. [CrossRef]
73. Battula, V.L.; Shi, Y.; Evans, K.W.; Wang, R.Y.; Spaeth, E.L.; Jacamo, R.O.; Guerra, R.; Sahin, A.A.; Marini, F.C.; Hortobagyi, G.; et al. Ganglioside GD2 identifies breast cancer stem cells and promotes tumorigenesis. *J. Clin. Investig.* **2012**, *122*, 2066–2078. [CrossRef] [PubMed]
74. Ly, S.; Anand, V.; El-Dana, F.; Nguyen, K.; Cai, Y.; Cai, S.; Piwnica-Worms, H.; Tripathy, D.; Sahin, A.A.; Andreeff, M.; et al. Anti-GD2 antibody dinutuximab inhibits triple-negative breast tumor growth by targeting $GD2^+$ breast cancer stem-like cells. *J. Immunother. Cancer* **2021**, *9*, e001197. [CrossRef] [PubMed]
75. Liang, Y.J. Glycosphingolipids in human embryonic stem cells and breast cancer stem cells, and potential cancer therapy strategies based on their structures and functions. *Glycoconj. J.* **2022**, *39*, 177–195. [CrossRef] [PubMed]
76. Yoshida, H.; Koodie, L.; Jacobsen, K.; Hanzawa, K.; Miyamoto, Y.; Yamamoto, M. B4GALNT1 induces angiogenesis, anchorage independence growth and motility, and promotes tumorigenesis in melanoma by induction of ganglioside GM2/GD2. *Sci. Rep.* **2020**, *10*, 1199. [CrossRef]
77. Alam, S.; Anugraham, M.; Huang, Y.L.; Kohler, R.S.; Hettich, T.; Winkelbach, K.; Grether, Y.; Lopez, M.N.; Khasbiullina, N.; Bovin, N.V.; et al. Altered (neo-) lacto series glycolipid biosynthesis impairs alpha2-6 sialylation on N-glycoproteins in ovarian cancer cells. *Sci. Rep.* **2017**, *7*, 45367. [CrossRef]
78. Marijan, S.; Markotic, A.; Mastelic, A.; Rezic-Muzinic, N.; Pilkington, L.I.; Reynisson, J.; Culic, V.C. Glycosphingolipid expression at breast cancer stem cells after novel thieno[2,3-b]pyridine anticancer compound treatment. *Sci. Rep.* **2020**, *10*, 11876. [CrossRef]
79. Shao, C.; Anand, V.; Andreeff, M.; Battula, V.L. Ganglioside GD2: A novel therapeutic target in triple-negative breast cancer. *Ann. N. Y. Acad. Sci.* **2022**, *1508*, 35–53. [CrossRef]
80. Li, D.; Park, Y.; Hemati, H.; Liu, X. Cell aggregation activates small GTPase Rac1 and induces CD44 cleavage by maintaining lipid raft integrity. *J. Biol. Chem.* **2023**, *299*, 105377. [CrossRef]
81. Liang, Y.J.; Wang, C.Y.; Wang, I.A.; Chen, Y.W.; Li, L.T.; Lin, C.Y.; Ho, M.Y.; Chou, T.L.; Wang, Y.H.; Chiou, S.P.; et al. Interaction of glycosphingolipids GD3 and GD2 with growth factor receptors maintains breast cancer stem cell phenotype. *Oncotarget* **2017**, *8*, 47454–47473. [CrossRef]
82. Mozzi, A.; Forcella, M.; Riva, A.; Difrancesco, C.; Molinari, F.; Martin, V.; Papini, N.; Bernasconi, B.; Nonnis, S.; Tedeschi, G.; et al. NEU3 activity enhances EGFR activation without affecting EGFR expression and acts on its sialylation levels. *Glycobiology* **2015**, *25*, 855–868. [CrossRef]
83. Scaringi, R.; Piccoli, M.; Papini, N.; Cirillo, F.; Conforti, E.; Bergante, S.; Tringali, C.; Garatti, A.; Gelfi, C.; Venerando, B.; et al. NEU3 sialidase is activated under hypoxia and protects skeletal muscle cells from apoptosis through the activation of the epidermal growth factor receptor signaling pathway and the hypoxia-inducible factor (HIF)-1alpha. *J. Biol. Chem.* **2013**, *288*, 3153–3162. [CrossRef] [PubMed]
84. Miyagi, T.; Yamamoto, K. Sialidase NEU3 and its pathological significance. *Glycoconj. J.* **2022**, *39*, 677–683. [CrossRef]
85. Thulasiraman, P.; Kerr, K.; McAlister, K.; Hardisty, S.; Wistner, A.; McCullough, I. Neuraminidase 1 regulates proliferation, apoptosis and the expression of Cadherins in mammary carcinoma cells. *Mol. Cell Biochem.* **2019**, *462*, 207–215. [CrossRef] [PubMed]
86. Menendez, J.A.; Lupu, R. Fatty acid synthase and the lipogenic phenotype in cancer pathogenesis. *Nat. Rev. Cancer* **2007**, *7*, 763–777. [CrossRef] [PubMed]
87. Epstein, T.; Gatenby, R.A.; Brown, J.S. The Warburg effect as an adaptation of cancer cells to rapid fluctuations in energy demand. *PLoS ONE* **2017**, *12*, e0185085. [CrossRef]
88. Liberti, M.V.; Locasale, J.W. The Warburg Effect: How Does it Benefit Cancer Cells? *Trends Biochem. Sci.* **2016**, *41*, 211–218. [CrossRef]
89. Fantin, V.R.; St-Pierre, J.; Leder, P. Attenuation of LDH-A expression uncovers a link between glycolysis, mitochondrial physiology, and tumor maintenance. *Cancer Cell* **2006**, *9*, 425–434. [CrossRef]
90. Mayer, A.; Schmidt, M.; Seeger, A.; Serras, A.F.; Vaupel, P.; Schmidberger, H. GLUT-1 expression is largely unrelated to both hypoxia and the Warburg phenotype in squamous cell carcinomas of the vulva. *BMC Cancer* **2014**, *14*, 760. [CrossRef]
91. Wang, L.; Xiong, H.; Wu, F.; Zhang, Y.; Wang, J.; Zhao, L.; Guo, X.; Chang, L.J.; Zhang, Y.; You, M.J.; et al. Hexokinase 2-mediated Warburg effect is required for PTEN- and p53-deficiency-driven prostate cancer growth. *Cell Rep.* **2014**, *8*, 1461–1474. [CrossRef]
92. Wong, N.; Ojo, D.; Yan, J.; Tang, D. PKM2 contributes to cancer metabolism. *Cancer Lett.* **2015**, *356*, 184–191. [CrossRef]
93. Sun, Z.; Tan, Z.; Peng, C.; Yi, W. HK2 is associated with the Warburg effect and proliferation in liver cancer: Targets for effective therapy with glycyrrhizin. *Mol. Med. Rep.* **2021**, *23*, 343. [CrossRef] [PubMed]

94. Liu, J.; van der Hoeven, R.; Kattan, W.E.; Chang, J.T.; Montufar-Solis, D.; Chen, W.; Wong, M.; Zhou, Y.; Lebrilla, C.B.; Hancock, J.F. Glycolysis regulates KRAS plasma membrane localization and function through defined glycosphingolipids. *Nat. Commun.* **2023**, *14*, 465. [CrossRef] [PubMed]
95. Hwang, K.T.; Kim, B.H.; Oh, S.; Park, S.Y.; Jung, J.; Kim, J.; Choi, I.S.; Jeon, S.Y.; Kim, W.Y. Prognostic Role of KRAS mRNA Expression in Breast Cancer. *J. Breast Cancer* **2019**, *22*, 548–561. [CrossRef] [PubMed]
96. Subik, K.; Lee, J.F.; Baxter, L.; Strzepek, T.; Costello, D.; Crowley, P.; Xing, L.; Hung, M.C.; Bonfiglio, T.; Hicks, D.G.; et al. The Expression Patterns of ER, PR, HER2, CK5/6, EGFR, Ki-67 and AR by Immunohistochemical Analysis in Breast Cancer Cell Lines. *Breast Cancer* **2010**, *4*, 35–41. [CrossRef] [PubMed]
97. Li, Q.; Sun, M.; Yu, M.; Fu, Q.; Jiang, H.; Yu, G.; Li, G. Gangliosides profiling in serum of breast cancer patient: GM3 as a potential diagnostic biomarker. *Glycoconj. J.* **2019**, *36*, 419–428. [CrossRef]
98. Stathem, M.; Marimuthu, S.; O'Neal, J.; Rathmell, J.C.; Chesney, J.A.; Beverly, L.J.; Siskind, L.J. Glucose availability and glycolytic metabolism dictate glycosphingolipid levels. *J. Cell. Biochem.* **2015**, *116*, 67–80. [CrossRef]
99. Schomel, N.; Hancock, S.E.; Gruber, L.; Olzomer, E.M.; Byrne, F.L.; Shah, D.; Hoehn, K.L.; Turner, N.; Grosch, S.; Geisslinger, G.; et al. UGCG influences glutamine metabolism of breast cancer cells. *Sci. Rep.* **2019**, *9*, 15665. [CrossRef] [PubMed]
100. Esaki, N.; Ohkawa, Y.; Hashimoto, N.; Tsuda, Y.; Ohmi, Y.; Bhuiyan, R.H.; Kotani, N.; Honke, K.; Enomoto, A.; Takahashi, M.; et al. ASC amino acid transporter 2, defined by enzyme-mediated activation of radical sources, enhances malignancy of GD2-positive small-cell lung cancer. *Cancer Sci.* **2018**, *109*, 141–153. [CrossRef]
101. Schomel, N.; Gruber, L.; Alexopoulos, S.J.; Trautmann, S.; Olzomer, E.M.; Byrne, F.L.; Hoehn, K.L.; Gurke, R.; Thomas, D.; Ferreiros, N.; et al. UGCG overexpression leads to increased glycolysis and increased oxidative phosphorylation of breast cancer cells. *Sci. Rep.* **2020**, *10*, 8182. [CrossRef]
102. Lampa, M.; Arlt, H.; He, T.; Ospina, B.; Reeves, J.; Zhang, B.; Murtie, J.; Deng, G.; Barberis, C.; Hoffmann, D.; et al. Glutaminase is essential for the growth of triple-negative breast cancer cells with a deregulated glutamine metabolism pathway and its suppression synergizes with mTOR inhibition. *PLoS ONE* **2017**, *12*, e0185092. [CrossRef]
103. Laplante, M.; Sabatini, D.M. mTOR signaling in growth control and disease. *Cell* **2012**, *149*, 274–293. [CrossRef] [PubMed]
104. Jaggupilli, A.; Ly, S.; Nguyen, K.; Anand, V.; Yuan, B.; El-Dana, F.; Yan, Y.; Arvanitis, Z.; Piyarathna, D.W.B.; Putluri, N.; et al. Metabolic stress induces GD2(+) cancer stem cell-like phenotype in triple-negative breast cancer. *Br. J. Cancer* **2022**, *126*, 615–627. [CrossRef] [PubMed]
105. Nguyen, K.; Yan, Y.; Yuan, B.; Dasgupta, A.; Sun, J.; Mu, H.; Do, K.A.; Ueno, N.T.; Andreeff, M.; Battula, V.L. ST8SIA1 Regulates Tumor Growth and Metastasis in TNBC by Activating the FAK-AKT-mTOR Signaling Pathway. *Mol. Cancer Ther.* **2018**, *17*, 2689–2701. [CrossRef] [PubMed]
106. Seif, S.E.; Mahmoud, Z.; Wardakhan, W.W.; Abdou, A.M.; Hassan, R.A. Design and synthesis of novel hexahydrobenzo[4,5]thieno[2,3-d]pyrimidine derivatives as potential anticancer agents with antiangiogenic activity via VEGFR-2 inhibition, and down-regulation of PI3K/AKT/mTOR signaling pathway. *Drug Dev. Res.* **2023**, *84*, 839–860. [CrossRef] [PubMed]
107. Dimov, S.; Mavrova, A.T.; Yancheva, D.; Nikolova, B.; Tsoneva, I. Thieno[2,3-d]pyrimidin-4(3H)-one Derivatives of Benzimidazole as Potential Anti- Breast Cancer (MDA-MB-231, MCF-7) Agents. *Anticancer Agents Med. Chem.* **2021**, *21*, 1441–1450. [CrossRef]
108. Wang, J.; Luo, C.; Shan, C.; You, Q.; Lu, J.; Elf, S.; Zhou, Y.; Wen, Y.; Vinkenborg, J.L.; Fan, J.; et al. Inhibition of human copper trafficking by a small molecule significantly attenuates cancer cell proliferation. *Nat. Chem.* **2015**, *7*, 968–979. [CrossRef]
109. Arabshahi, H.J.; van Rensburg, M.; Pilkington, L.I.; Jeon, C.Y.; Song, M.; Gridel, L.M.; Leung, E.; Barker, D.; Vuica-Ross, M.; Volcho, K.P.; et al. A synthesis, and study of thieno[2,3-]pyridine anticancer analogues. *Medchemcomm* **2015**, *6*, 1987–1997. [CrossRef]
110. Eurtivong, C.; Semenov, V.; Semenova, M.; Konyushkin, L.; Atamanenko, O.; Reynisson, J.; Kiselyov, A. 3-Amino-thieno[2,3-b]pyridines as microtubule-destabilising agents: Molecular modelling and biological evaluation in the sea urchin embryo and human cancer cells. *Bioorg. Med. Chem.* **2017**, *25*, 658–664. [CrossRef]
111. Katritch, V.; Jaakola, V.P.; Lane, J.R.; Lin, J.; Ijzerman, A.P.; Yeager, M.; Kufareva, I.; Stevens, R.C.; Abagyan, R. Structure-based discovery of novel chemotypes for adenosine A(2A) receptor antagonists. *J. Med. Chem.* **2010**, *53*, 1799–1809. [CrossRef]
112. Zafar, A.; Sari, S.; Leung, E.; Pilkington, L.I.; van Rensburg, M.; Barker, D.; Reynisson, J. GPCR Modulation of Thieno[2,3-b]pyridine Anti-Proliferative Agents. *Molecules* **2017**, *22*, 2254. [CrossRef]
113. Lin, H.Y.; Kuei, C.H.; Lee, H.H.; Lin, C.H.; Zheng, J.Q.; Chiu, H.W.; Chen, C.L.; Lin, Y.F. The Galphah/phospholipase C-delta1 interaction promotes autophagosome degradation by activating the Akt/mTORC1 pathway in metastatic triple-negative breast cancer. *Aging (Albany NY)* **2020**, *12*, 13023–13037. [CrossRef] [PubMed]
114. Huang, S.P.; Liu, P.Y.; Kuo, C.J.; Chen, C.L.; Lee, W.J.; Tsai, Y.H.; Lin, Y.F. The Galphah-PLCdelta1 signaling axis drives metastatic progression in triple-negative breast cancer. *J. Hematol. Oncol.* **2017**, *10*, 114. [CrossRef] [PubMed]
115. Gallo, M.; Ferrari, E.; Terrazzan, A.; Brugnoli, F.; Spisni, A.; Taccioli, C.; Aguiari, G.; Trentini, A.; Volinia, S.; Keillor, J.W.; et al. Metabolic characterisation of transglutaminase 2 inhibitor effects in breast cancer cell lines. *FEBS J.* **2023**, *290*, 5411–5433. [CrossRef]
116. Guri, Y.; Colombi, M.; Dazert, E.; Hindupur, S.K.; Roszik, J.; Moes, S.; Jenoe, P.; Heim, M.H.; Riezman, I.; Riezman, H.; et al. mTORC2 Promotes Tumorigenesis via Lipid Synthesis. *Cancer Cell* **2017**, *32*, 807–823.e12. [CrossRef]
117. Xia, E.; Zhou, X.; Bhandari, A.; Zhang, X.; Wang, O. Synaptopodin-2 plays an important role in the metastasis of breast cancer via PI3K/Akt/mTOR pathway. *Cancer Manag. Res.* **2018**, *10*, 1575–1583. [CrossRef] [PubMed]

118. Yang, H.; Guan, L.; Li, S.; Jiang, Y.; Xiong, N.; Li, L.; Wu, C.; Zeng, H.; Liu, Y. Mechanosensitive caveolin-1 activation-induced PI3K/Akt/mTOR signaling pathway promotes breast cancer motility, invadopodia formation and metastasis in vivo. *Oncotarget* **2016**, *7*, 16227–16247. [CrossRef]
119. Settembre, C.; Zoncu, R.; Medina, D.L.; Vetrini, F.; Erdin, S.; Erdin, S.; Huynh, T.; Ferron, M.; Karsenty, G.; Vellard, M.C.; et al. A lysosome-to-nucleus signalling mechanism senses and regulates the lysosome via mTOR and TFEB. *EMBO J.* **2012**, *31*, 1095–1108. [CrossRef] [PubMed]
120. Puertollano, R. mTOR and lysosome regulation. *F1000Prime Rep.* **2014**, *6*, 52. [CrossRef]
121. Hartwig, P.; Hoglinger, D. The Glucosylceramide Synthase Inhibitor PDMP Causes Lysosomal Lipid Accumulation and mTOR Inactivation. *Int. J. Mol. Sci.* **2021**, *22*, 7065. [CrossRef]
122. Scott, K.L.; Kabbarah, O.; Liang, M.C.; Ivanova, E.; Anagnostou, V.; Wu, J.; Dhakal, S.; Wu, M.; Chen, S.; Feinberg, T.; et al. GOLPH3 modulates mTOR signalling and rapamycin sensitivity in cancer. *Nature* **2009**, *459*, 1085–1090. [CrossRef]
123. Rizzo, R.; Russo, D.; Kurokawa, K.; Sahu, P.; Lombardi, B.; Supino, D.; Zhukovsky, M.A.; Vocat, A.; Pothukuchi, P.; Kunnathully, V.; et al. Golgi maturation-dependent glycoenzyme recycling controls glycosphingolipid biosynthesis and cell growth via GOLPH3. *EMBO J.* **2021**, *40*, e107238. [CrossRef] [PubMed]
124. Ruggiero, F.M.; Martinez-Koteski, N.; Cavieres, V.A.; Mardones, G.A.; Fidelio, G.D.; Vilcaes, A.A.; Daniotti, J.L. Correction: Ruggiero et al. Golgi Phosphoprotein 3 Regulates the Physical Association of Glycolipid Glycosyltransferases. Int. J. Mol. Sci. 2022, 23, 10354. *Int. J. Mol. Sci.* **2023**, *24*, 13212. [CrossRef]
125. Spessott, W.; Crespo, P.M.; Daniotti, J.L.; Maccioni, H.J. Glycosyltransferase complexes improve glycolipid synthesis. *FEBS Lett.* **2012**, *586*, 2346–2350. [CrossRef] [PubMed]
126. Arriagada, C.; Cavieres, V.A.; Luchsinger, C.; Gonzalez, A.E.; Munoz, V.C.; Cancino, J.; Burgos, P.V.; Mardones, G.A. GOLPH3 Regulates EGFR in T98G Glioblastoma Cells by Modulating Its Glycosylation and Ubiquitylation. *Int. J. Mol. Sci.* **2020**, *21*, 8880. [CrossRef]
127. Polanco, C.M.; Cavieres, V.A.; Galarza, A.J.; Jara, C.; Torres, A.K.; Cancino, J.; Varas-Godoy, M.; Burgos, P.V.; Tapia-Rojas, C.; Mardones, G.A. GOLPH3 Participates in Mitochondrial Fission and Is Necessary to Sustain Bioenergetic Function in MDA-MB-231 Breast Cancer Cells. *Cells* **2024**, *13*, 316. [CrossRef] [PubMed]
128. Nohara, K.; Wang, F.; Spiegel, S. Glycosphingolipid composition of MDA-MB-231 and MCF-7 human breast cancer cell lines. *Breast Cancer Res. Treat.* **1998**, *48*, 149–157. [CrossRef]
129. Lehmann, B.D.; Bauer, J.A.; Chen, X.; Sanders, M.E.; Chakravarthy, A.B.; Shyr, Y.; Pietenpol, J.A. Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. *J. Clin. Investig.* **2011**, *121*, 2750–2767. [CrossRef] [PubMed]
130. Zhong, E.; Brogi, E.; D'Alfonso, T.M.; Wen, H.; Frosina, D.; Cheung, N.K.; Jungbluth, A.A.; Ross, D.S. Expression Analysis of GD2 by Immunohistochemistry in Invasive Breast Carcinoma: Clinical and Pathologic Correlation. *Appl. Immunohistochem. Mol. Morphol.* **2022**, *30*, 113–118. [CrossRef]
131. Park, J.A.; Cheung, N.V. Targets and Antibody Formats for Immunotherapy of Neuroblastoma. *J. Clin. Oncol.* **2020**, *38*, 1836–1848. [CrossRef]
132. Cheung, I.Y.; Cheung, N.V.; Modak, S.; Mauguen, A.; Feng, Y.; Basu, E.; Roberts, S.S.; Ragupathi, G.; Kushner, B.H. Survival Impact of Anti-GD2 Antibody Response in a Phase II Ganglioside Vaccine Trial Among Patients With High-Risk Neuroblastoma With Prior Disease Progression. *J. Clin. Oncol.* **2021**, *39*, 215–226. [CrossRef]
133. Suzuki, M.; Nagane, M.; Kato, K.; Yamauchi, A.; Shimizu, T.; Yamashita, H.; Aihara, N.; Kamiie, J.; Kawashima, N.; Naito, S.; et al. Endothelial ganglioside GM3 regulates angiogenesis in solid tumors. *Biochem. Biophys. Res. Commun.* **2021**, *569*, 10–16. [CrossRef] [PubMed]
134. Buentzel, J.; Klemp, H.G.; Kraetzner, R.; Schulz, M.; Dihazi, G.H.; Streit, F.; Bleckmann, A.; Menck, K.; Wlochowitz, D.; Binder, C. Metabolomic Profiling of Blood-Derived Microvesicles in Breast Cancer Patients. *Int. J. Mol. Sci.* **2021**, *22*, 13540. [CrossRef] [PubMed]
135. Hahnefeld, L.; Gruber, L.; Schomel, N.; Fischer, C.; Mattjus, P.; Gurke, R.; Beretta, M.; Ferreiros, N.; Geisslinger, G.; Wegner, M.S. Ether lipid and sphingolipid expression patterns are estrogen receptor-dependently altered in breast cancer cells. *Int. J. Biochem. Cell Biol.* **2020**, *127*, 105834. [CrossRef] [PubMed]

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Review

Enhancing Crop Resilience: Insights from Labdane-Related Diterpenoid Phytoalexin Research in Rice (*Oryza sativa* L.)

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Abstract: Rice (*Oryza sativa* L.), as one of the most significant food crops worldwide, holds paramount importance for global food security. Throughout its extensive evolutionary journey, rice has evolved a diverse array of defense mechanisms to fend off pest and disease infestations. Notably, labdane-related diterpenoid phytoalexins play a crucial role in aiding rice in its response to both biotic and abiotic stresses. This article provides a comprehensive review of the research advancements pertaining to the chemical structures, biological activities, and biosynthetic pathways, as well as the molecular regulatory mechanisms, underlying labdane-related diterpenoid phytoalexins discovered in rice. This insight into the molecular regulation of labdane-related diterpenoid phytoalexin biosynthesis offers valuable perspectives for future research aimed at improving crop resilience and productivity.

Keywords: rice; labdane; diterpenoid; phytoalexins; resilience

1. Introduction

As the global population steadily grows, the demand for food escalates accordingly, necessitating greater efforts to ensure sustainable agricultural production and food security worldwide [1,2]. As an important member of the Gramineae family, rice (*Oryza sativa* L.) is one of the most crucial food crops in the world, serving as the staple food for half of the global population, with particular significance in East and Southeast Asian countries [3–5]. Thus, the stable production of rice is directly related to global food security and the livelihoods of hundreds of millions of people.

However, as the severe natural climatic conditions continue to worsen, the situation with pests and diseases has also intensified. These unfavorable factors not only increase the uncertainty in the growth process of rice but also make the prevention and control of pests and diseases more complex and difficult, posing a serious threat to rice yield and quality [6–9]. Therefore, conducting research on rice resistance and cultivating new rice varieties with strong stress tolerance have become urgent requirements for ensuring global food security. This not only helps reduce food production losses caused by natural disasters and pests and diseases but also decreases reliance on chemical pesticides and fertilizers, thereby promoting the sustainable development of agricultural production [10–12].

In recent years, with the rapid development of molecular biology and biotechnology, scientists have gained a deeper understanding of the resistance mechanisms in rice. Among them, the research of phytoalexins in rice is particularly notable. These compounds play an important role in the resistance of rice to pests, diseases, and stress, providing new ideas and methods to improve the resistance of crops [13–15].

Phytoalexins are low-molecular-weight, secondary metabolites produced by plants in response to pathogen attack or other stress stimuli that play an essential role in plant defense mechanisms by inhibiting the growth and proliferation of pathogens, thereby

reducing disease severity and enhancing plant resilience [16–19]. Phytoalexins display a diverse array of chemical structures, encompassing terpenes, phenols, flavonoids, and stilbenes, among others. Their biosynthesis is typically triggered in a localized and swift response following the recognition of a pathogen attack [20–23]. Within this field, the unique biological activities of labdane-related diterpenoid phytoalexins have garnered significant attention in rice resistance research due to their potential to confer targeted and durable resistance against a broad spectrum of pathogens [24,25].

Labdane-related diterpenoid phytoalexins originate from the labdane skeleton, a bicyclic diterpenoid precursor, via a sequence of intricate biosynthetic pathways. These compounds are pivotal in plant innate immunity, functioning as potent toxins or inhibitors that combat invading pathogens, including bacteria, fungi, and viruses [24,25]. The structural complexity of labdane-derived diterpenoids allows for a wide range of bioactivities, making them valuable not only in plant defense but also as potential leads for the development of novel agrochemicals and pharmaceuticals.

This review summarizes the current insights gained from research on labdane-related diterpenoid phytoalexins in rice, highlighting their biosynthesis pathways and key enzymes involved. Furthermore, this review envisions a promising future for leveraging the profound insights gained into labdane-related diterpenoid phytoalexins to devise innovative crop improvement strategies. These strategies, aimed at optimizing the biosynthetic pathways and enhancing the expression of key enzymes, hold the key to developing rice varieties with enhanced innate immunity and resilience. Such advancements, in turn, are poised to contribute substantially to the sustainability of agricultural systems worldwide, ensuring food security for growing populations amidst the challenges posed by climate change and resource constraints. Ultimately, this research endeavor represents a crucial step towards harnessing the full potential of plant natural products for sustainable agriculture and a more resilient global food system.

2. Chemical and Biological Activities of Rice Labdane-Related Diterpene Phytoalexins

2.1. Types and Structural Characteristics

Based on the distinct structures of their hydrocarbon precursors, these phytoalexins in rice can be categorized into three major groups: momilactones, phytocassanes, and oryzalexins (Figure 1). Specifically, the momilactones group encompasses momilactones A–E (**1**–**5**), oryzalactone (**6**) [26–31]; the phytocassanes group comprises phytocassanes A–G (**7**–**13**) [31–34]; and the oryzalexins group includes oryzalexins A–F (**14**–**19**) [35–41] and oryzalexin S (**20**) [34,42,43].

Structurally, momilactones belong to the 9β-H pimarane diterpenoids family, which has continuous chiral centers with a trans-*syn*-cis tricyclic skeleton [44–46]. Among them, momilactone A (**1**) and momilactone B (**2**) are the main components of labdane-type diterpene phytoalexins in rice, bearing a 19,6β-lactone structure [44–46]. These two important phytoalexins were initially isolated by Kato and colleagues from rice husks (*Oryza sativa* cv. Koshihikari) as growth inhibitors of lettuce (*Lactuca sativa*) seedlings [26,27]. In the subsequent 40 years, three more momilactone compounds were isolated and identified from rice blast infected leaves and named momilactones C (**3**), D (**4**), and E (**5**), as well as oryzalactone (**6**) [28–31]. With the development of research, more and more labdane-type diterpenoid phytoalexins have been identified in rice, named phytocassanes and oryzalexins, respectively [31–43].

Phytocassanes are a class of compounds featuring an ent-cassane diterpenoid skeleton with a ketone group at the C11 position [31,34]. Koga et al. isolated and identified four novel diterpenes with a cassane skeleton from the abundant phytoalexins produced in rice leaves infected by the rice blast fungus. These were named phytocassane A (**7**), phytocassane B (**8**), phytocassane C (**9**), and phytocassane D (**10**) [32]. Following this, phytocassane E (1β-hydroxy-12,15-cassadien-3,11-dione) (**11**) and phytocassane F (1α,2α-dihydroxy-ent-12,15-cassadiene-3,11-dione) (**12**) were isolated from rice cells cultured in suspension treated with extracts from the potato pathogen Phytophthora infestans hyphae and from

rice leaves exposed to ultraviolet radiation, respectively [33,34]. As a didehydroisomer of phytocassane A (**7**), phytocassane G (**13**) was isolated and identified by Kariya et al. from the cultivated rice variety Basilanon [31].

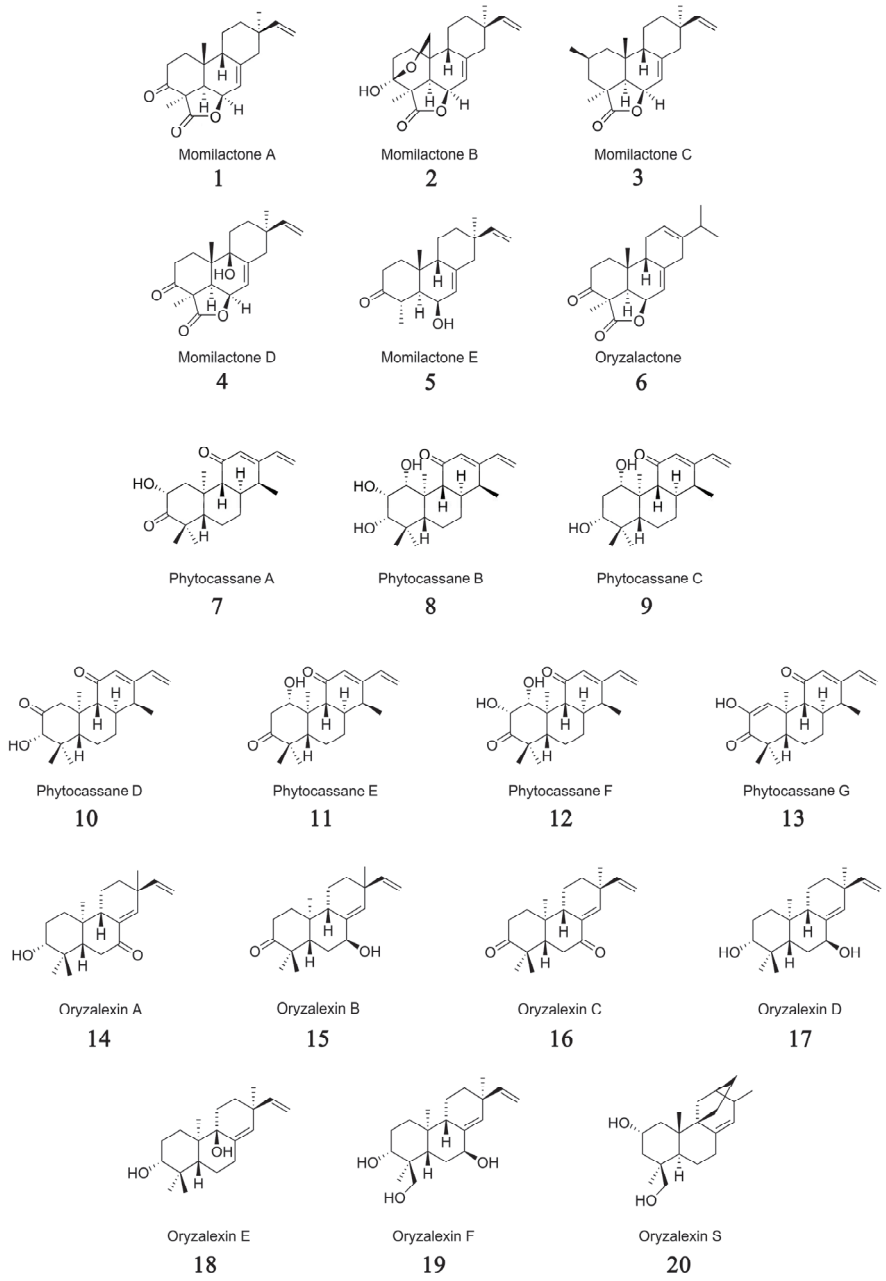


Figure 1. Chemical structures of labdane-type diterpene phytoalexins in rice.

Oryzalexins can be classified into two major groups according to their precursors. Oryzalexins A–F (**14**–**19**) belong to the ent-sandaracopimaradiene diterpene compounds derived from ent-sandaracopimara-8(14),15-diene, and oryzalexin S (**20**) belongs to the astemarane-like diterpene compounds derived from syn-stemar-13-ene via hydroxylation at C2a and C19 [34–44].

Oryzalexin A (3-hydroxy-7-oxo-sandaracopimaradiene) (**14**) was first isolated and identified from rice leaves infected with the rice blast fungus Pyricularia oryzae [35,36]. Subsequently, oryzalexin B (**15**) and oryzalexin C (**16**) were isolated and identified as two new antitoxins from rice leaves infected with the rice blast fungus Pyricularia oryzae by Akatsuka's team in 1985 [37,38]. In 1986, Sekido et al. isolated a new phytoalexin,

named oryzalexin D (**17**), from rice leaves infected with rice blast disease caused by Magnaporthe oryzae. The structure of oryzalexin D (**17**) was confirmed as 3,7-dihydroxy-(+)-sandaracopimaradiene through UV, mass spectrometry, nuclear magnetic resonance, and optical rotation methods. At 100 ppm, oryzalexin D (**17**) significantly inhibited the spore germination of Magnaporthe oryzae [39]. Kato et al. isolated oryzalexin E (**18**) and oryzalexin F (**19**) from rice leaves exposed to ultraviolet light and determined their structures and relative configurations as isopimara-8,(14),15-diene-3β,9α-diol and ent-isopimara-8(14),15-diene-3β,18-diol, respectively, through spectroscopic analysis [40,41]. Then, a novel antifungal phytoalexin, designated as oryzalexin S (**20**), from the rice leaves subjected to ultraviolet light exposure, was isolated and identified [42,43].

2.2. Biological Activities

2.2.1. Antimicrobial

Previous studies have indicated that almost all of the labdane-type diterpene phytoalexins are isolated from leaves infected with blast fungus, suggesting that resistance to pathogens is the primary biological activity of these compounds [28,32,33,37–41,47–50].

Momilactone A (**1**) and momilactone B (**2**) exhibited potent inhibitory effects against the devastating rice blast fungus, *P. oryzae*, achieving 50% inhibition at concentrations as low as 5 μg/mL for momilactone A (**1**) and an even more impressive 1 μg/mL for momilactone B (**2**), thereby highlighting the exceptional efficacy of these compounds in combating this pathogenic threat [47]. What is more, momilactone A (**1**) and momilactone B (**2**) additionally demonstrated strong antifungal activity against *Cladosporium cucumerinum*, further underscoring their broad-spectrum inhibitory potential against fungal pathogens [28].

While momilactone A (**1**) and momilactone B (**2**) have been shown to possess significant inhibitory activity against various pathogens, their efficacy varies depending on the specific type of pathogen [48]. Fukuta et al.'s research underscores the superiority of momilactone B (**2**) over momilactone A (**1**) in terms of antibacterial activity, particularly against fungal pathogens such as *Botrytis cinerea* (IC_{50}: 1.2 μg for momilactone B vs. 78.1 μg for momilactone A), *Fusarium solani* (IC_{50}: 123.9 μg for momilactone B vs. 198.1 μg for momilactone A), and *Colletotrichum gloeosporioides* (IC_{50}: 53.4 μg for momilactone B vs. 95.3 μg for momilactone A). Furthermore, momilactone B (**2**) displays a more pronounced inhibitory effect against bacterial species like *Pseudomonas ovalis*, *Bacillus cereus*, and *Bacillus pumilus* in comparison with momilactone A (**1**). However, when tested against *Escherichia coli*, both compounds exhibit comparable levels of inhibitory activity [48].

The research conducted by Koga et al. reveals that phytocassanes exhibited strong antifungal activity against M. grisea and R. solani, with phytocassanes A, B, C, and D (**7**–**10**) having ED_{50} values of 20, 4, 7, and 25 μg/mL, respectively, in preventing spore germination of *M. grisea* [32]. The research team led by Koga made a discovery in the form of phytocassanes E (**11**), which they identified in rice suspension-cultured cells infected with *Phytophthora infestans*. Their findings indicated that phytocassane E (**11**) possesses remarkable antifungal properties against *Magnaporthe grisea*. Specifically, the ED_{50} values for inhibiting spore germination and germ tube growth of this fungus were astonishingly low, at 6 and 2 μg/mL, respectively. Moreover, they pinpointed the hydroxyl group located at the C1 position as the primary functional group responsible for the exceptionally high antifungal activity exhibited by phytocassane E (**11**), shedding light on the molecular mechanisms underlying its potent effects [33].

Oryzalexins, a class of naturally occurring antimicrobial compounds in rice plants, contribute significantly to the plant's innate defense mechanisms. Previous studies have shown that oryzalexins inhibit the growth of various fungal species, including those responsible for rice diseases like *Pyricularia oryzae* and brown spot (*Bipolaris oryzae*) [37–39,49,50]. When tested against *P. oryzae* spore germination, oryzalexins E (**18**) and F (**19**) exhibited ED_{50} values of 62.5 and 103 ppm, respectively [40,41].

2.2.2. Nematode Resistance

In a study exploring the multifaceted roles of rice diterpenoid phytoalexins (DPs), researchers found that these well-known secondary metabolites, known for their resistance against foliar pathogens, are also produced and released by rice roots. They hypothesized that DPs may play a significant role in plant-nematode interactions, particularly in defending rice plants against phytoparasitic nematodes.

To test this hypothesis, they conducted transcriptome analysis on rice roots, focusing on the effects of infection by the root-knot nematode, Meloidogyne graminicola, and treatment with resistance-inducing chemicals on DP biosynthesis genes. They also evaluated the vulnerability of mutant rice lines with compromised DP biosynthesis to *M. graminicola*. Additionally, the study involved growing these mutants and their wild-type counterparts in field soil and utilizing metabarcoding to assess the influence of impaired DP biosynthesis on rhizosphere and root nematode communities [51].

The results reveal that *M. graminicola* suppresses DP biosynthesis genes early during its invasion, while resistance-inducing stimuli temporarily boost DP biosynthesis. Notably, the absence of DPs increased the rice plants' susceptibility to *M. graminicola*. Metabarcoding analysis of wild-type and DP-deficient plants grown in field soil demonstrated that DPs significantly reshape the composition of rhizosphere and root nematode communities. In conclusion, diterpenoid phytoalexins are crucial players in both constitutive and inducible defense mechanisms against nematode pathogens in rice, and they play a pivotal role in structuring rice-associated nematode communities [51].

2.2.3. Allelopathic

Numerous studies have conclusively demonstrated that momilactone A (**1**) and momilactone B (**2**) are the primary allelochemicals produced by rice. These compounds serve as potent natural weapons, effectively suppressing the growth of weeds [50–52]. These compounds, momilactone A (**1**) and momilactone B (**2**), play a crucial role in rice's competitive ability within its ecological niche. They ensure rice's dominance and healthy growth amidst the surrounding vegetation [52–55]. These intricate compounds, momilactone A (**1**) and momilactone B (**2**), are meticulously synthesized and sequestered within the roots of rice seedlings. They are strategically deployed into the rhizosphere through root exudates, gradually accumulating in the soil. Their presence acts as a formidable barrier, effectively inhibiting the seed germination and overall growth of neighboring weeds, such as barnyard grass and plantain. By disrupting seed germination processes, impeding root elongation, and stunting shoot growth, these allelochemicals ensure that rice thrives in its environment, unencumbered by competing vegetation [56–58]. The exact mechanisms by which momilactone A (**1**) and momilactone B (**2**) inhibit weed growth are complex. They may involve disrupting hormonal balance, inhibiting enzyme activity, or interfering with nutrient uptake.

The research conducted by Kato-Noguchi and his team shows that the growth of seedlings of mustard greens (*Lepidium sativum* L.) and lettuce (*Lactuca sativa* L.) was significantly inhibited when exposed to momilactone B (**2**) solutions. The growth of mustard greens was inhibited at concentrations greater than 3 μM, while lettuce growth was inhibited at concentrations greater than 30 μM [59]. Chung et al. found that momilactones A (**1**) and momilactone B (**2**) also exhibited strong inhibitory activity against duckweed (*Lemna paucicostata*). In further experiments, they showed that when the concentration of momilactone B (**2**) reached 20 ppm, it completely inhibited the germination of three weed species: Chinese sprangletop (*Leptochloa chinensis* L.), redroot pigweed (*Amaranthus retroflexus* L.), and spike rush (*Cyperus difformis* L.) [60]. Upon external application, momilactone A (**1**) and momilactone B (**2**) were found to have notable inhibitory effects on the growth of both shoots and roots of barnyard grass (*E. crus-galli*). The growth was inhibited at concentrations exceeding 30 mmol/L for momilactone A (**1**) and 1 mmol/L for momilactone B (**2**), respectively [52].

2.2.4. Drought Resistance

Apart from their bioactivities, such as resistance against harmful pathogens and allelopathy, momilactones also play a crucial role in enhancing rice's ability to withstand abiotic stresses. Xuan's research team conducted an analysis of the content of momilactone A (**1**) and momilactone B (**2**) in 30 rice varieties from diverse origins, including hybrids of indica and japonica subspecies, as well as exotic, glutinous, local, upland, and drought-tolerant rice. They further compared the correlation between the content of these momilactones and various traits, such as salt tolerance, drought tolerance, weed resistance, total flavonoid content, total phenolic content, and antioxidant capacity. The results reveal a strong correlation between the content of momilactone A and drought tolerance, with a correlation coefficient of 0.65 [61]. Zhang et al. found that *cps2*, *cps4*, and *cps2*×*4* double mutants were more sensitive to drought stress due to reduced stomatal closure under drought conditions compared with the wild type. This suggests that labdane-related diterpenoid phytoalexins may act as a regulatory switch that triggers stomatal closure in rice, potentially reflecting the role of these openings in microbial entry [62].

Figure 2 displays the biological activities of rice labdane-related diterpenoid phytoalexins.

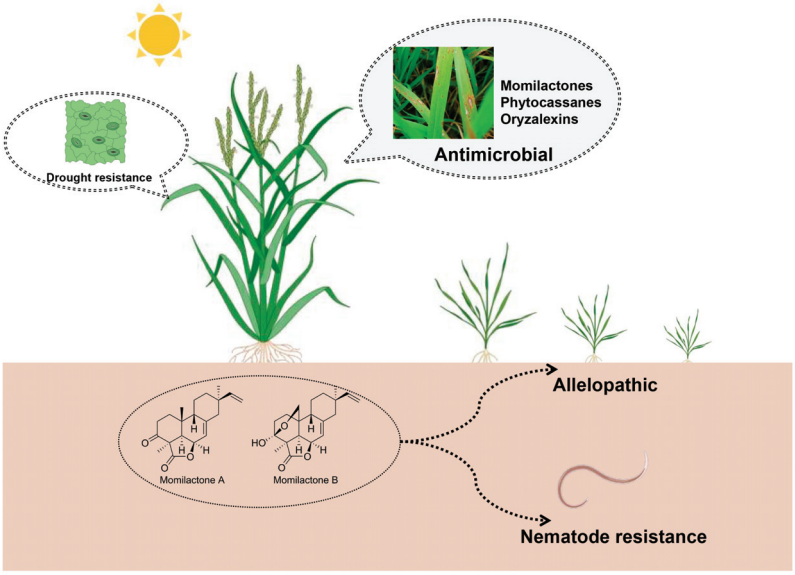


Figure 2. Biological activities of rice labdane-related diterpenoid phytoalexins.

3. Biosynthesis of Rice Labdane-Related Diterpenoid Phytoalexins

Consistent with the biosynthetic pathways of other diterpenoids, the rice labdane-related diterpenoid phytoalexins (Momilactones, Phytocassanes, and Oryzalexins) all initiate from geranylgeranyl diphosphate (GGPP) as the starting material [63,64]. Initially, GGPP undergoes a series of enzymatic transformations catalyzed by a suite of diterpene synthases. These enzymes, each with its unique specificity and catalytic prowess, sculpt the GGPP backbone into intermediate structures that are progressively more complex and specialized. Concurrently, cytochrome P450 enzymes are renowned for their ability to perform oxidative modifications on a wide range of substrates, which intricately decorate the intermediate diterpenes with oxygen-containing functional groups, further diversifying their structures and enhancing their biological activities. This collaborative effort between diterpene synthases and cytochrome P450 enzymes results in the biosynthesis of momilactones, phytocassanes, and oryzalexins—a formidable trio of phytoalexins that arm rice plants against various biotic and abiotic stresses.

3.1. Biosynthesis of Momilactones

A *syn*-CPS enzyme encoded by *OsCPS4* and located on chromosome 4 catalyzes the initial cyclization of the GGPP, converting into syn-copalyl diphosphate (*syn*-CPP)

as a crucial precursor for momilactones [65]. Subsequently, the *syn*-pimara-7,15-diene synthase, encoded by *OsKSL4*, transforms *syn*-CPP into the momilactone tricyclic skeleton, (9β-H)-pimara-7,15-diene directly [66].

CYP99A2 and CYP99A3 occupy a central and crucial role within the intricate momilactone gene cluster, encoding cytochrome enzymes that meticulously orchestrate a vital oxygenation reaction, precisely targeting the C19 position of (9β-H)-pimara-7,15-diene. This enzymatic prowess deftly converts the intermediate into (9β-H)-Pimaradien-19-oic acid, a crucial and direct precursor that paves the way for the biosynthesis of both momilactone C (**3**) and momilactone E (**5**). Subsequently, (9β-H)-Pimaradien-19-oic acid undergoes further enzymatic modifications, elegantly transforming into 3β-hydroxy-(9β-H)-pimara-7,15-dien-19,6β-olide. This intricate and delicate pathway underscores the indispensable and paramount roles that CYP99A2 and CYP99A3 play in the synthesis of these biologically significant and impactful compounds [67].

Additionally, CYP76M8 and CYP701A8, located on the phytocassane gene cluster and associated with gibberellin biosynthesis, have also been shown to participate in momilactone biosynthesis. Research teams led by Kitaoka have elucidated the momilactone biosynthetic pathway and the evolutionary process of key enzymes, confirming the critical roles of CYP76M8 and CYP701A8 in momilactone biosynthesis. These enzymes directly catalyze the conversion of (9β-H)-pimara-7,15-diene into 3β-hydroxy-(9β-H)-pimara-7,15-dien-19,6β-olide [68].

Subsequently, 3β-hydroxy-(9β-H)-pimara-7,15-dien-19,6β-olide could either be directly converted into momilactone D (**4**) or, undergo complete oxidation catalyzed by a short-chain dehydrogenase/reductase (SDR) encoded by *OsMAS*, resulting in the formation of momilactone A (**1**) and momilactone B (**2**) [69,70].

3.2. Biosynthesis of Phytocassanes

Under the catalysis of the diterpene synthase CPS2 which is encoded by *OsCPS2*, GGPP first undergoes a cyclization reaction to form *ent*-CPP. Subsequently, KSL7 catalyzes the conversion of *ent*-CPP into *ent*-cassadiene [71–73]. Following this, the cytochrome P450 enzymes, specifically CYP76M7, CYP76M8 and CYP71Z7, engage in additional oxidative transformations of *ent*-Cassa-12,15-diene. More precisely, CYP76M7 and CYP76M8 are crucial in catalyzing the α-hydroxylation process at the C11 site of 3-hydroxy-cassadiene, whereas CYP71Z7 contributes to the hydroxylation reaction specifically at the C2 position within phytocassanes [74–76]. The sequential cytochrome-mediated hydroxylation modifications ultimately result in the transformation of *ent*-cassadiene into phytocassanes A–E (**7**–**12**).

3.3. Biosynthesis of Oryzalexins

In line with the biosynthetic pathway of the other two labdane-derived diterpenoid phytoalexins found in rice, the synthesis of oryzalexins A–F (**14**–**19**) begins with GGPP as the precursor. This initial substrate undergoes a catalytic transformation catalyzed by OsCPS4, resulting in the formation of *syn*-CPP [65]. Subsequently, OsKSL10 acts on *syn*-CPP, converting it into *ent*-Pimara-7,5-diene, thereby initiating the production of the specified oryzalexins [50].

Under the activity of CYP701A8, the *ent*-Pimara-7,5-diene undergoes a transformation, resulting in the formation of 3α-Hydroxy-ent-sandaracopimaradiene [77]. This intermediate serves as a crucial precursor in the biosynthesis of oryzalexins A-E (**14**–**18**), marking an essential step towards the production of these phytoalexins in rice. The P450 cytochrome enzymes, specifically CYP76M8 and CYP76M6, play pivotal roles in the hydroxylation process of the tricyclic precursor. CYP76M8 selectively hydroxylates the C-7β position, while CYP76M6 targets the C-9β position. These enzymatic modifications lead to the formation of 3α,7β-diol and 3α,9β-diol compounds, respectively, which are definitively identified as oryzalexin D (**17**) and oryzalexin E (**18**). This sequence of enzymatic reactions is crucial for the biosynthesis of these specific phytoalexins in rice [78]. Following the hydroxylation

by CYP76M8 and CYP76M6, the structural modifications in oryzalexin D (**17**) proceed further with the oxidation of the 3α-hydroxy and accompanying 7β-hydroxy groups. This oxidation step is catalyzed by short-chain alcohol dehydrogenase/reductases encoded by *OsSDR110C-MI3* and *OsSDR110C-MS3*, producing oryzalexins A–C (**14**–**16**). This cascade of enzymatic activities in rice underscores the intricate and orchestrated biosynthesis of these phytoalexins [79].

After being synthesized by OsCPS4-catalyzed condensation of GGPP, the resulting *syn*-copalyl pyrophosphate (*syn*-CPP) undergoes a crucial transformation. This conversion is mediated by the *ent*-kaurene synthase enzyme, which is encoded by *OsKSL8* and catalyzes the cyclization of *syn*-CPP into *syn*-stemar-13-*ene*. This step marks a significant advance in the biosynthetic pathway of diterpenoid compounds in rice, leading to the formation of an intermediate that can further undergo diverse modifications to give rise to a variety of biologically active compounds [80,81].

In the subsequent biosynthetic step, the C19 position of *syn*-stemar-13-ene, the product of the *ent*-kaurene synthase reaction catalyzed by OsKLS8, undergoes hydroxylation. This hydroxylation is mediated by the cytochrome P450 enzymes CYP99A3 and its homolog CYP99A2, which specifically target the C19 carbon atom. The outcome of this enzymatic reaction is the formation of *syn*-stemar-13-en-19-ol, a pivotal precursor in the biosynthesis of oryzalexin S (**20**) [82]. This transformation represents a crucial branch point in the diterpenoid biosynthetic pathway, leading toward the production of bioactive compounds that are essential for rice defense mechanisms. In the final step of the biosynthetic pathway leading to oryzalexin S (**20**), the C2α position of *syn*-stemar-13-en-19-ol undergoes hydroxylation catalyzed by the cytochrome P450 enzymes CYP71Z21 and CYP71Z22 [82]. The result of this enzymatic reaction is the formation of oryzalexin S (**20**), a bioactive phytoalexin that plays a crucial role in the defense mechanisms of rice against various biotic and abiotic stresses.

Biosynthetic pathways of rice labdane-related diterpenoid phytoalexins are shown in Figure 3 below.

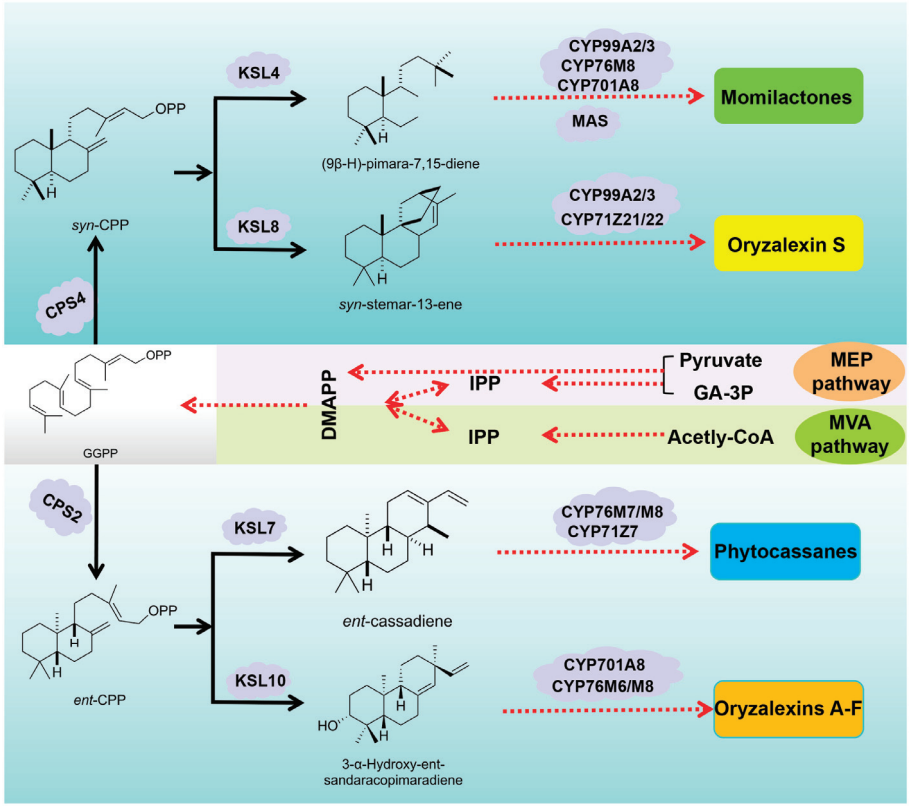


Figure 3. Biosynthetic pathways of rice labdane-related diterpenoid phytoalexins.

4. Molecular Regulation of Labdane-Related Diterpenoid Phytoalexin Biosynthesis in Rice

The biosynthesis of labdane-related diterpenoid phytoalexins in rice embodies a complex molecular machinery, where a diverse array of genes and regulatory factors intertwine intricately to orchestrate sophisticated biochemical reactions. This intricate process not only underscores the profound complexity inherent in plant defense mechanisms but also emphasizes the delicate interplay and intricate interconnection between genetic determinants and environmental cues.

Among the transcription factors implicated in these processes, bZIP, bHLH, and WRKY transcription factors stand out as key regulators. These transcription factors, functioning as molecular switches, bind to specific DNA sequences in the promoter regions of biosynthase genes and modulate their expression levels in response to various stimuli. By doing so, they orchestrate the temporal and spatial expression patterns of biosynthetic enzymes, ensuring the precise and timely production of labdane-related diterpenoid phytoalexins in rice. The molecular regulatory factors of rice labdane-related diterpenoid phytoalexins biosynthesis are shown in Table 1.

Table 1. Molecular regulatory factors of rice labdane-related diterpenoid phytoalexins biosynthesis.

Category	Factors	Regulation Mechanism	Reference
bZIP Transcription Factor	OsTGAP1	Upon induction by chitin oligosaccharide elicitors, OsTGAP1 binds to the *OsKLS4* promoter, positively regulating the expression of genes including *OsCPS4*, *OsKSL4*, *CYP99A2*, *CYP99A3*, and *OsMAS*.	[83]
	OsbZIP79	The expression of *OsCPS4*, *OsCPS2*, *OsKSL4*, *OsKSL7*, *CYP99A2/3*, *CYP76M7*, *CYP76M8*, and *OsMAS* is negatively regulated upon stimulation by chitin oligosaccharide elicitors	[84]
bHLH Transcription Factors	OsbHLH025	OsbHLH025 directly interact with the promoters of the key biosynthetic genes *OsCPS2* and *CYP99A2* to regulate accumulation of momilactones and phytocassanes	[85]
	OsbHLH026	OsbHLH026 protein binds to the promoter regions of OsCPS2 and OsKSL6 and activate the expression of these genes.	[86]
WRKY Transcription Factors	OsWRKY45	Genes encoding enzymes involved in momilactone biosynthesis (*OsCPS4*, *OsKSL4*, *CYP99A2*, *CYP99A3*, and OsMAS), phytocassane biosynthesis (*OsCPS2*, *OsKSL7*, and *CYP71Z7*) and oryzalexin biosynthesis (*OsKSL10*) were upregulated in both *OsWRKY45*-OX rice lines	[87]
	OsWRKY53	Phosphorylation mediated by the OSMKK4-OSMPK3/OsMPK6 signaling cascade mediated *OsWRKY53* positively regulates the expression of key enzyme genes, including *OsCPS4*, *OsMAS*, *CYP99A2* and *CYP99A3*.	[88]
	OsWRKY10	OsWRKY10 protein directly binds to W-boxes and W-box-like cis-elements (WLEs) present in the promoter regions of key biosynthetic enzyme genes, including *OsKSL4*, *OsKSL7*, *OsKSL10*, and *CYP99A3*	[89]
	OsWRKY76	Five genes for momilactones production (*OsCPS4*, *OsKSL4*, *CYP99A2*, *CYP99A3*, and *OsMAS*), along with the three genes central to phytocassanes biosynthesis (*OsCPS2*, *OsKSL7*, and *CYP71Z7*) were down-regulated in *OsWRKY76* overexpressing rice plants.	[90]
Plant Hormone	JA	Mutations disrupting JA signaling lead to reduced accumulation of labdane-related diterpenoid phytoalexins upon *M. oryzae* infection	[91,92]

4.1. bZIP Transcription Factors

Okada et al. reported that a bZIP transcription factor, OsTGAP1, induced by chitin oligosaccharide elicitors, plays a crucial role in the accumulation of monolignols by binding to the *OsKLS4* promoter [83]. Further studies indicated that *OsKSL4*, *OsKSL7*, and *OsDXS3* expression in the *OsTGAP1*-overexpressing lines was upregulated, and the expression levels of *OsCPS4*, *OsKSL4*, *CYP99A2*, *CYP99A3*, and *OsMAS* were nearly absent in *OsTGAP1* knockout mutant plants after induction treatment, resulting in the absence of momilactones accumulation in these mutant plants. The findings indicated that OsTGAP1 emerges as a key regulator in the biosynthesis of diterpenoid phytoalexins, exerting transcriptional control over both the genes directly involved in phytoalexin production and the upstream MEP pathway gene that facilitates the supply of geranylgeranyl diphosphate (GGPP) after elicitor recognition [83].

To deepen their understanding of OsTGAP1's role in regulating labdane-related diterpenoid phytoalexins production, Miyamoto's team screened for OsTGAP1-interacting proteins through yeast two-hybrid. Among candidates, OsbZIP79, a TGA factor, was studied for its physical interaction with OsTGAP1 and potential involvement in phytoalexin regulation. However, in stark contrast to the functional role of OsTGAP1, OsbZIP79 distinctly exhibits an inhibitory influence on the expression of genes that are integral to the biosynthesis of diterpenoid phytoalexins. In plants overexpressing OsbZIP79, the transcriptional levels of key diterpenoid biosynthetic enzymes, such as *OsCPS4*, *OsCPS2*, *OsKSL4*, *OsKSL7*, *CYP99A2*, *CYP99A3*, *CYP76M7*, *CYP76M8*, and *OsMAS*, are significantly downregulated, ultimately resulting in a reduced accumulation of momilactones and phytocassanes. These findings suggested OsbZIP79 acts as a negative regulator of labdane-related diterpenoid phytoalexins production in rice cells stimulated by chitin oligosaccharide elicitors, though the specific conditions for its cooperation with OsTGAP1 remain to be elucidated [84].

It is worth noting that both the expression of *OsTGAP1* and *OsbZIP79* could be stimulated by chitin oligosaccharide elicitor to regulate labdane-related diterpenoid phytoalexins biosynthesis [83,84]. Chitin oligosaccharide elicitors are recognized for their ability to trigger defense responses in plants [93,94]. In rice plants, their stimulation of *OsTGAP1* and *OsbZIP79* underscores an essential component of the plant's innate immune system by regulating the biosynthesis of labdane-related diterpenoid phytoalexins [83,84]. Further research into the precise mechanisms and interactions of these transcription factors will provide valuable insights into the complex regulatory networks underlying plant defense.

4.2. bHLH Transcription Factors

Yamamura and his team have identified the bHLH transcription factor OsbHLH025, which has been named the diterpenoid phytoalexin factor (DPF) [85]. Notably, OsbHLH025 exhibits a remarkable responsiveness to various stimuli, including infection by the rice blast fungus, exposure to copper chloride, and ultraviolet light, all of which induce its expression in rice leaves and panicles [85].

Further research has confirmed that OsbHLH025 can directly bind to N-Box motifs present on the promoters of the key biosynthetic genes *OsCPS2* and *CYP99A2*, exerting direct regulatory control over the biosynthesis of momilactones and phytocassanes. As a result, the accumulation of momilactones and phytocassanes is significantly enhanced in *OsbHLH025*-overexpressed rice plants. Conversely, when *OsbHLH025* expression is knocked down, the accumulation of these compounds decreases correspondingly, further revealing the essential function of OsbHLH025 in regulating the biosynthesis of momilactones and phytocassanes [85].

It is intriguing to observe that rice plants overexpressing *OsbHLH025* (*OsbHLH025*-OX) exhibit increased accumulation of diterpenoids (DPs) even in the absence of stress treatments [85]. The phenomenon of disease-like symptoms displayed by these *OsbHLH025*-OX plants with elevated DP levels poses a challenging question to unravel. Further research is needed to fully understand the molecular mechanisms underlying the cytotoxicity of

high DP levels and to develop strategies to harness the potential of these compounds for crop protection while minimizing adverse effects on plant health.

Liu and her team identified and confirmed that a bHLH transcription factor, OsbHLH026, serves as a key regulator in rice through a comprehensive approach encompassing co-expression analysis, expression quantitative trait locus (eQTL) analysis, and linkage mapping [86]. In order to study the mechanism of OsbHLH026 regulating the biosynthesis of phytoalexins, they demonstrated that OsbHLH026 protein could bind to the promoter region of *OsCPS2* and *OsKSL6* and activate these genes [86]. In the *syn*-CPP pathway, oryzalexin S (**20**), momilactones A (**1**) and B (**2**), and their intermediates *syn*-stemar-13-ene and *syn*-pimara-7,15-diene were significantly upregulated in the *OsbHLH026*-OX line compared with the wild type, whereas in the *osbhlh026-cri* line, only oryzalexin S (**20**) was markedly reduced, with little change observed for the others. Additionally, in the *ent*-CPP pathway, phytocassanes C (**9**), D (**10**), E (**11**), and oryzalexins C (**16**), F (**19**) were detected, with similar upregulation trends in the *OsbHLH026*-OX line as observed in the previous compounds. However, in the osbhlh026-cri line, significant decreases were observed specifically in phytocassanes D (**10**) and E (**11**) [86]. Overall, these experiments revealed that bHLH026 has a significant effect on enhancing disease resistance in rice plants, underscoring its importance in improving the overall health and resilience of the crop.

4.3. WRKY Transcription Factors

Akagi et al. investigated the defense-priming mechanism of benzothiadiazole (BTH) and the function of OsWRKY45 by analyzing the expression of diterpenoid phytoalexin (DP) biosynthetic genes during the interaction between rice and *Magnaporthe oryzae* [87]. They found that while BTH treatment alone slightly upregulated these genes, *M. oryzae* infection rapidly induced their expression in a WRKY45-dependent manner, indicating that BTH primes DP biosynthetic genes through WRKY45. Similarly, rice overexpressing *OsWRKY45* (*OsWRKY45*-OX) showed rapid induction of DP biosynthetic genes upon *M. oryzae* infection, including five genes involved in momilactone biosynthesis (*OsCPS4*, *OsKSL4*, *OsCYP99A2*, *CYP99A3*, and *OsMAS*), as well as those encoding enzymes crucial for phytocassane biosynthesis (*OsCPS2*, *OsKSL7*, and *CYP71Z7*) and oryzalexin biosynthesis (*OsKSL10*). These transcriptional reprogramming resulted in the accumulation of DPs in both *OsWRKY45*-OX and BTH-pretreated rice after infection. In this study, they demonstrated that the SA/CK synergy, mediated by WRKY45, regulates DP biosynthetic genes. Furthermore, they propose that CK acts as a mediator of *M. oryzae* infection signals, triggering the induction of DP biosynthetic genes in BTH-primed plants [87].

The research conducted by Chujo et al. has confirmed that the phosphorylation of the WRKY transcription factor OsWRKY53, facilitated by the OSMKK4-OSMPK3/OsMPK6 signaling cascade, results in a positive regulation of the expression of key enzyme genes, including *OsCPS4*, *OsMAS*, *CYP99A2*, and *CYP99A3* [78]. As a result, the content of momilactones and phytocassanes in rice plants increases, significantly enhancing their defense capabilities [88].

Wang's research team precisely identified OsWRKY10 as an essential transcription factor that orchestrates the crucial upregulation of diterpenoid phytoalexin biosynthesis in rice. Notably, knockout of *OsWRKY10* dramatically enhanced the susceptibility of rice mutants to the *Magnaporthe oryzae*, underscoring its indispensable function in the defense arsenal of rice. Conversely, the amplification of *OsWRKY10* expression in transgenic rice lines robustly strengthened their resistance to rice blast, establishing a clear positive correlation. They found that OsWRKY10 could directly bind to W-boxes and W-box-like *cis*-elements (WLEs) present on the promoter regions of key biosynthetic enzyme genes, such as *OsKSL4*, *OsKSL7*, *OsKSL10*, and *CYP99A3* [89]. This direct interaction underscores the fundamental role of OsWRKY10 in modulating the biosynthesis of labdane-related diterpenoid phytoalexins in rice, thereby reinforcing rice's natural defenses against pathogenic assaults.

Their findings reveal an intriguing phenomenon that knockout of *OsWRKY10* did not completely abolish the induction of biosynthesis genes and accumulation of rice diter-

penoid phytoalexins in response to blast fungus infection or $CuCl_2$ treatment [89]. This redundancy suggests that OsWRKY10, while playing a significant role, is not the sole or exclusive TF responsible for regulating the expression of genes associated with diterpenoid phytoalexin biosynthesis. Rather, there are likely other TFs that possess overlapping or complementary functions, enabling them to partially or fully compensate for the loss of OsWRKY10 activity. This highlights the robustness and flexibility of plant regulatory networks, which often employ multiple layers of redundancy to ensure resilience against perturbations. Future research should aim to unravel the full complement of TFs involved in diterpenoid phytoalexin biosynthesis, as well as their interactions and regulatory hierarchies, to gain a deeper understanding of plant stress responses and potential avenues for crop improvement.

Compared with the aforementioned WRKY transcription factors, OsWRKY76 exhibits a notable transcriptional inhibitory effect on the biosynthetic pathway of labdane-related diterpenoid phytoalexins in rice [90]. Five genes for momilactones production (*OsCPS4*, *OsKSL4*, *CYP99A2*, *CYP99A3*, and *OsMAS*), along with the three genes central to phytocassanes biosynthesis (*OsCPS2*, *OsKSL7*, and *CYP71Z7*), were downregulated significantly in the *OsWRKY76* overexpression rice plants. Consequently, this suppression diminishes the ability of the plants to produce labdane-related diterpenoid phytoalexins, ultimately heightening their vulnerability to rice blast fungus infections [90]. However, overexpression of *OsWRKY76* leads to the upregulation of abiotic stress-related genes, thereby enhancing the cold tolerance of rice [90].

4.4. Other Factors

Previous reports have confirmed that the plant hormone jasmonic acid (JA) is also one of the factors regulating the biosynthesis of labdane-related diterpenoid phytoalexins in rice. Mutations disrupting JA signaling lead to reduced accumulation of labdane-related diterpenoid phytoalexins upon *M. oryzae* infection, enhancing susceptibility to this pathogen [91]. Nevertheless, JA-independent mechanisms regulating DP biosynthesis are also present, as evidenced by JA-deficient mutants producing normal levels of phytocassanes despite reduced momilactones in response to *M. oryzae* [91]. Analogously, an *Osjar1-2* insertion mutant exhibits comparable labdane-related diterpenoid phytoalexins production to wild type under Cu^{2+} exposure or *M. oryzae* infection [92].

In conclusion, studies presented in this passage provide important insights into the roles of these transcription factors in regulating the biosynthesis of labdane-related diterpenoid phytoalexins in rice (Figure 4). However, much work remains to be carried out to fully understand the signaling pathways and transcriptional networks that are regulated by this transcription factor. Future research in this area holds great promise for improving crop resilience and productivity in the face of environmental challenges.

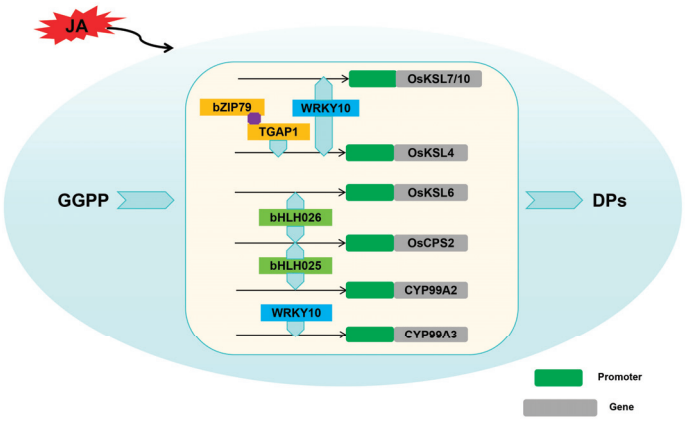


Figure 4. Molecular regulation of rice labdane-related diterpenoid phytoalexins.

5. Genetic Manipulation to Enhance Crop Resistance by Increasing the Production of Diterpenoid Phytoalexins

Currently, there are relatively few reports on attempts to enhance crop resistance by manipulating the expression of genes in the biosynthetic pathway of diterpenoid phytoalexins.

Li et al. confirmed that ectopic expression of the Harpin protein 1 gene (*hrf1*) enhances the resistance of transgenic rice plants to bacterial blight. They discovered that genes related to the biosynthesis of labdane-type diterpenoid antibiotics, specifically *OsCPS2*, *OsCPS4*, *OsKSL4*, *OsKSL7*, *OsKSL8,* and *OsKSL10* in the rice plants transformed with *hrf1*, were significantly activated. This resulted in the rapid and continuous accumulation of labdane-type diterpenoid phytoalexins in these plants, which subsequently significantly inhibited the growth of *Xanthomonas oryzae* strain PXO79 [95].

Lu et al. demonstrate that knocking out or knocking down the *OsCPS4* gene decreases susceptibility to the bacterial leaf blight pathogen *Xanthomonas oryzae*, while manipulating the expression of *OsCPS2*, which encodes the *ent*-CPP synthase, could alter susceptibility to both *M. oryzae* and *X. oryzae* [96].

CYP71Z18 is homologous to rice *CYP71Z6* and *CYP71Z67*, which has been demonstrated to catalyze the formation of phytoalexins, including zealexin A1, sesquiterpenoid phytoalexin, and diterpenoid phytoalexin dolabralexin in maize [97]. To explore the function of CYP71Z18 in rice, Shen and his team created transgenic rice plants that overexpressed *CYP71Z18*. They discovered that the heterologous expression of *CYP71Z18* led to the accumulation of several new diterpenoid compounds in the transgenic rice. Furthermore, compared with wild-type rice plants, the transgenic rice with overexpressed *CYP71Z18* exhibited stronger resistance to *M. oryzae* infection [98].

6. Conclusions, Challenges, and Prospects

6.1. Conclusions

This article reviews the research progress in the past few decades on the biological activities, biosynthetic pathways, and molecular regulation of labdane-type diterpenoid phytoalexins in rice, aiming to provide insights for future crop resistance breeding efforts.

Rice labdane-type diterpenoid phytoalexins, including momilactones, phytocassanes, and oryzalexins, constitute an important component of rice's defense arsenal against a variety of biotic and abiotic stresses. These naturally occurring plant metabolites exhibit diverse biological activities that contribute to the overall resilience of rice plants. Specifically, rice labdane-type diterpenoid phytoalexins display antifungal, allelopathic, and stress-responsive properties, highlighting their crucial role in safeguarding rice crop health and productivity. Their biosynthesis in rice plants primarily initiates with GGPP as the starting compound, constituting a highly orchestrated process that necessitates the intricate interplay and precise regulation of countless enzymes. In this process, some key transcription factors, such as the bZIP transcription factor, bHLH transcription factor, and WRKY transcription factor, play important regulatory roles. They can bind to the promoters of key enzyme genes in the biosynthesis of labdane-type diterpenoid phytoalexins, regulate the expression of these genes, and affect the synthesis and accumulation of these labdane-type diterpenoid phytoalexins.

Further investigation into the biosynthesis and regulation of these compounds holds promise for the development of novel strategies to enhance crop resistance against pathogens and environmental stresses. Therefore, understanding the biosynthesis and regulation of these compounds can facilitate the cultivation of new, eco-friendly crop varieties, thereby reducing the need for pesticides and herbicides, and promoting sustainable agricultural development.

6.2. Challenges

Given their crucial roles in augmenting plants' natural resilience against pests and diseases, phytoalexins have emerged as prime candidates for enhancing agricultural pest and disease defense via plant genetic engineering. By strategically and precisely integrating

genes encoding these phytoalexins into crop genomes, a promising pathway is paved for imparting customized and highly specific resistance mechanisms, thereby effectively protecting plants from specific pests and diseases.

However, despite the successful identification of some key enzymes and genes, the complexity of the entire metabolic network, including the integrity of its components and the dynamic interactions of regulatory mechanisms, remains to be fully elucidated. Moreover, the intricate web of interactions among genes within gene clusters poses a significant challenge. This complexity is compounded by the existence of redundant pathways and sophisticated regulatory mechanisms, which obscure the distinct functions of individual genes. Furthermore, the variations observed in gene expression patterns across different crop varieties introduce an additional layer of complexity to the research endeavor, which necessitates a nuanced and multifaceted approach to fully grasp the underlying biology.

6.3. Prospects

In the future, it is imperative to initially harness systematic biology approaches to meticulously unravel the intricate biosynthetic and regulatory networks governing these defense compounds such as momilactones in rice. This will provide a robust theoretical foundation for precision breeding and biosynthesis endeavors. Following this, synthetic biology techniques can be leveraged to manipulate the rice genome, either by overexpressing or fine-tuning the expression of genes associated with labdane-type diterpenoid phytoalexin biosynthesis, thereby enhancing rice's defenses against diseases and stresses. With the continuous development of CRISPR-Cas9 and other gene editing technologies, the stress resistance potential of labdane-type diterpenoid phytoalexin could be further explored and utilized through precise editing of related genes in rice.

Furthermore, metabolic engineering strategies can be employed to refine the biosynthetic pathways of these defense compounds in rice, optimizing their production and efficacy. This encompasses the introduction of exogenous genes and the modification of endogenous gene expression regulatory elements to precisely control the biosynthetic pathways. In addition, the research of diterpenoids can be extended to other crops, and these favorable genes can be introduced into other crops through gene editing or transgenic technology to improve their overall stress resistance. This not only helps to reduce the use of pesticides and reduce environmental pollution but also improves the yield and quality of crops (Figure 5).

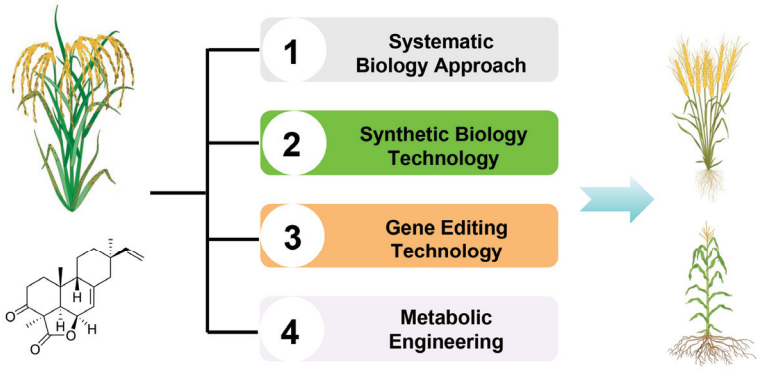


Figure 5. Future research strategies of rice labdane-related diterpenoid phytoalexins.

The implementation of these cutting-edge strategies promises to unlock novel insights and methodologies that will revolutionize rice disease resistance breeding and contribute to agricultural sustainability. By harnessing the full potential of diterpenoid phytoalexins, we can pave the way for more resilient and productive crop systems. In actual agricultural production, promoting the planting of these new varieties of rice with strong stress resistance can reduce the use of pesticides and fertilizers, reduce production costs, and improve

the yield and quality of rice. In addition, these new varieties can also enhance the ability of rice to adapt to extreme climatic conditions and ensure the stability of food production.

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Abbreviations

EC_{50}	Half-maximal effective concentration
IC_{50}	Half-maximal inhibitory concentration
CYPs	Cytochrome P450 enzymes
SDR	Short-chain dehydrogenase reductase
DiTPSs	Diterpene synthases
CPSs	Copalyl diphosphate synthases
KSL	*ent*-kaurene synthase like
GGPP	Geranylgeranyl diphosphate
ent-CPP	*ent*-copalyl diphosphate
syn-CPP	*syn*-copalyl diphosphate
MEP	2-C-methyl-D-erythritol 4-phosphate
MVA	Mevalonate
DMAPP	Dimethylallyl diphosphate
IPP	Isopentenyl diphosphate
GA-3P	Glyceraldehyde 3-phosphate
CoA	Coenzyme A
DPs	Diterpenoid phytoalexins
DPF	Diterpenoid phytoalexin factor
BTH	Benzothiadiazole
JA	Jasmonic acid
SA	Salicylic acid
CK	Cytokinin
UV	Ultraviolet radiation

References

1. Oluwole, O.; Ibidapo, O.; Arowosola, T.; Raji, F.; Zandonadi, R.P.; Alasqah, I.; Lho, L.H.; Han, H.; Raposo, A. Sustainable transformation agenda for enhanced global food and nutrition security: A narrative review. *Front. Nutr.* **2023**, *10*, 1226538. [CrossRef] [PubMed]
2. Roth, J.A.; Galyon, J. Food security: The ultimate one-health challenge. *One Health* **2024**, *19*, 100864. [CrossRef] [PubMed]
3. Muthayya, S.; Sugimoto, J.D.; Montgomery, S.; Maberly, G.F. An overview of global rice production, supply, trade, and consumption. *Ann. N. Y. Acad. Sci.* **2014**, *1324*, 7–14. [CrossRef] [PubMed]
4. Shi, J.; An, G.; Weber, A.P.M.; Zhang, D. Prospects for rice in 2050. *Plant Cell Environ.* **2023**, *46*, 1037–1045. [CrossRef] [PubMed]
5. Alam, M.; Lou, G.; Abbas, W.; Osti, R.; Ahmad, A.; Bista, S.; Ahiakpa, J.K.; He, Y. Improving Rice Grain Quality Through Ecotype Breeding for Enhancing Food and Nutritional Security in Asia-Pacific Region. *Rice* **2024**, *17*, 47. [CrossRef]
6. Cheng, Y.T.; Zhang, L.; He, S.Y. Plant-Microbe Interactions Facing Environmental Challenge. *Cell Host Microbe.* **2019**, *26*, 183–192. [CrossRef]
7. Zhang, H.; Zhu, J.; Gong, Z.; Zhu, J.K. Abiotic stress responses in plants. *Nat. Rev. Genet.* **2022**, *23*, 104–119. [CrossRef]

8. Barrio, I.C.; Rapini, A. Plants under pressure: The impact of environmental change on plant ecology and evolution. *BMC Ecol. Evol.* **2023**, *23*, 13. [CrossRef]
9. Singh, B.K.; Delgado-Baquerizo, M.; Egidi, E.; Guirado, E.; Leach, J.E.; Liu, H.; Trivedi, P. Climate change impacts on plant pathogens, food security and paths forward. *Nat. Rev. Microbiol.* **2023**, *21*, 640–656. [CrossRef]
10. Li, Q.; Wang, B.; Yu, J.; Dou, D. Pathogen-informed breeding for crop disease resistance. *J. Integr. Plant Biol.* **2021**, *63*, 305–311. [CrossRef]
11. Odongo, P.J.; Onaga, G.; Ricardo, O.; Natsuaki, K.T.; Alicai, T.; Geuten, K. Insights Into Natural Genetic Resistance to Rice Yellow Mottle Virus and Implications on Breeding for Durable Resistance. *Front. Plant Sci.* **2021**, *12*, 671355. [CrossRef] [PubMed]
12. Li, C.; Zhong, H.; Ning, W.; Hu, G.; Wu, M.; Liu, Y.; Yan, B.; Ren, H.; Sonne, C. Integrating climate-pest interactions into crop projections for sustainable agriculture. *Nat. Food.* **2024**, *5*, 447–450. [CrossRef] [PubMed]
13. Ahuja, I.; Kissen, R.; Bones, A.M. Phytoalexins in defense against pathogens. *Trends Plant Sci.* **2012**, *17*, 73–90. [CrossRef] [PubMed]
14. Yamane, H. Biosynthesis of phytoalexins and regulatory mechanisms of it in rice. *Biosci. Biotechnol. Biochem.* **2013**, *77*, 1141–1148. [CrossRef]
15. Kariya, K.; Fujita, A.; Ueno, M.; Yoshikawa, T.; Teraishi, M.; Taniguchi, Y.; Ueno, K.; Ishihara, A. Natural variation of diterpenoid phytoalexins in rice: Aromatic diterpenoid phytoalexins in specific cultivars. *Phytochemistry.* **2023**, *211*, 113708. [CrossRef]
16. Hammerschmidt, R. PHYTOALEXINS: What Have We Learned After 60 Years? *Annu. Rev. Phytopathol.* **1999**, *37*, 285–306. [CrossRef]
17. Pedras, M.S.; Yaya, E.E.; Glawischnig, E. The phytoalexins from cultivated and wild crucifers: Chemistry and biology. *Nat. Prod. Rep.* **2011**, *28*, 1381–1405. [CrossRef]
18. Ube, N.; Yabuta, Y.; Tohnooka, T.; Ueno, K.; Taketa, S.; Ishihara, A. Biosynthesis of Phenylamide Phytoalexins in Pathogen-Infected Barley. *Int. J. Mol. Sci.* **2019**, *20*, 5541. [CrossRef]
19. Cárdenas, P.D.; Landtved, J.P.; Larsen, S.H.; Lindegaard, N.; Wøhlk, S.; Jensen, K.R.; Pattison, D.I.; Burow, M.; Bak, S.; Crocoll, C.; et al. Phytoalexins of the crucifer Barbarea vulgaris: Structural profile and correlation with glucosinolate turnover. *Phytochemistry* **2023**, *213*, 113742. [CrossRef]
20. Schmelz, E.A.; Huffaker, A.; Sims, J.W.; Christensen, S.A.; Lu, X.; Okada, K.; Peters, R.J. Biosynthesis, elicitation and roles of monocot terpenoid phytoalexins. *Plant J.* **2014**, *79*, 659–678. [CrossRef]
21. Cho, M.H.; Lee, S.W. Phenolic Phytoalexins in Rice: Biological Functions and Biosynthesis. *Int. J. Mol. Sci.* **2015**, *16*, 29120–29133. [CrossRef] [PubMed]
22. Liang, M.; Ye, H.; Shen, Q.; Jiang, X.; Cui, G.; Gu, W.; Zhang, L.H.; Naqvi, N.I.; Deng, Y.Z. Tangeretin inhibits fungal ferroptosis to suppress rice blast. *J. Integr. Plant. Biol.* **2021**, *63*, 2136–2149. [CrossRef] [PubMed]
23. Maia, M.; Aziz, A.; Jeandet, P.; Carré, V. Profiling and Localization of Stilbene Phytoalexins Revealed by MALDI-MSI during the Grapevine-Botrytis cinerea Interaction. *J. Agric. Food Chem.* **2023**, *71*, 15569–15581. [CrossRef] [PubMed]
24. Peters, R.J. Uncovering the complex metabolic network underlying diterpenoid phytoalexin biosynthesis in rice and other cereal crop plants. *Phytochemistry.* **2006**, *67*, 2307–2317. [CrossRef]
25. Valletta, A.; Iozia, L.M.; Fattorini, L.; Leonelli, F. Rice Phytoalexins: Half a Century of Amazing Discoveries; Part I: Distribution, Biosynthesis, Chemical Synthesis, and Biological Activities. *Plants* **2023**, *12*, 260. [CrossRef]
26. Kato, T.; Kabuto, C.; Sasaki, N.; Tsunagawa, M.; Aizawa, H.; Fujita, K.; Kato, Y.; Kitahara, Y. Momilactones, Growth Inhibitors from Rice, *Oryza sativa* L. *Tetrahedron Lett.* **1973**, *39*, 3861–3864. [CrossRef]
27. Takahashi, N.; Kato, T.; Tsunagawa, M.; Sasaki, N.; Kitahara, Y. Mechanisms of Dormancy in Rice Seeds. II. New Growth Inhibitors, Momilactone-A and -B Isolated from the Hulls of Rice Seeds. *Jpn. J. Breed.* **1976**, *26*, 91–98. [CrossRef]
28. Cartwright, D.W.; Langcake, P.; Pryce, R.J.; Leworthy, D.P.; Ride, J.P. Isolation and characterization of two phytoalexins from rice as momilactones A and B. *Phytochemistry* **1981**, *20*, 535–537. [CrossRef]
29. Tsunakawa, M.; Ohba, A.; Sasaki, N.; Kabuto, C.; Kato, T.; Kitahara, Y.; Takahashi, N. Momilactone-C, a Minor Constituent of Growth Inhibitors in Rice Husk. *Chem. Lett.* **1976**, *5*, 1157–1158. [CrossRef]
30. Cho, J.G.; Cha, B.J.; Lee, S.M.; Shrestha, S.; Jeong, R.H.; Lee, D.S.; Kim, Y.C.; Lee, D.G.; Kang, H.C.; Kim, J.; et al. Diterpenes from the Roots of *Oryza sativa* L. and Their Inhibition Activity on NO Production in LPS-Stimulated RAW264.7 Macrophages. *Chem. Biodivers.* **2015**, *12*, 1356–1364. [CrossRef]
31. Kariya, K.; Ube, N.; Ueno, M.; Teraishi, M.; Okumoto, Y.; Mori, N.; Ueno, K.; Ishihara, A. Natural variation of diterpenoid phytoalexins in cultivated and wild rice species. *Phytochemistry* **2020**, *180*, 112518. [CrossRef] [PubMed]
32. Koga, J.; Shimura, M.; Oshima, K.; Ogawa, N.; Yamauchi, T.; Ogasawara, N. Phytocassanes A, B, C and D, novel diterpene phytoalexins from rice, *Oryza sativa* L. *Tetrahedron* **1995**, *51*, 7907–7918. [CrossRef]
33. Koga, J.; Ogawa, N.; Yamauchi, T.; Kikuchi, M.; Ogasawara, N.; Shimura, M. Functional moiety for the antifungal activity of phytocassane E, a diterpene phytoalexin from rice. *Phytochemistry* **1997**, *44*, 249–253. [CrossRef]
34. Horie, K.; Inoue, Y.; Sakai, M.; Yao, Q.; Tanimoto, Y.; Koga, J.; Toshima, H.; Hasegawa, M. Identification of UV-induced diterpenes including a new diterpene phytoalexin, phytocassane F, from rice leaves by complementary GC/MS and LC/MS approaches. *J. Agric. Food Chem.* **2015**, *63*, 4050–4059. [CrossRef]
35. Akatsuka, T.; Kodama, O.; Kato, H.; Kono, Y.; Takeuchi, S. 3-Hydroxy-7-oxo-sandaracopimaradiene (oryzalexin A), a new phytoalexin isolated from rice blast leaves. *Agric. Biol. Chem.* **1983**, *47*, 445–447. [CrossRef]

36. Kono, Y.; Takeuchi, S.; Kodama, O.; Akatsuka, T. Absolute configuration of oryzalexin A and structures of its related phytoalexins isolated from rice blast leaves infected with *Pyricularia oryzae*. *Agric. Biol. Chem.* **1984**, *48*, 253–255. [CrossRef]
37. Akatsuka, T.; Kodama, O.; Sekido, H.; Kono, Y.; Takeuchi, S. Novel phytoalexins (oryzalexins A, B and C) isolated from rice blast leaves infected with *Pyricularia oryzae*. Part I: Isolation, characterization and biological activities of oryzalexins. *Agric. Biol. Chem.* **1985**, *49*, 1689–1694. [CrossRef]
38. Kono, Y.; Takeuchi, S.; Kodama, O.; Sekido, H.; Akatsuka, T. Novel phytoalexins (oryzalexins A, B and C) isolated from rice blast leaves infected with *Pyricularia oryzae*. Part II: Structural studies of oryzalexins. *Agric. Biol. Chem.* **1985**, *49*, 1695–1701. [CrossRef]
39. Sekido, H.; Endo, T.; Suga, R.; Kodama, O.; Akatsuka, T.; Kono, Y.; Takeuchi, S. Oryzalexin D (3,7-dihydroxy-(+)-sandaracopimaradiene), a new phytoalexin isolated from blast-infected rice leaves. *J. Pestic. Sci.* **1986**, *11*, 369–372. [CrossRef]
40. Kato, H.; Kodama, O.; Akatsuka, T. Oryzalexin E, a diterpene phytoalexin from UV-irradiated rice leaves. *Phytochemistry* **1993**, *33*, 79–81. [CrossRef]
41. Kato, H.; Kodama, O.; Akatsuka, T. Oryzalexin F, a diterpene phytoalexin from UV-irradiated rice leaves. *Phytochemistry* **1994**, *36*, 299–301. [CrossRef]
42. Kodama, O.; Li, W.X.; Tamogami, S.; Akatsuka, T. Oryzalexin S, a novel stemarane-type diterpene rice phytoalexin. *Biosci. Biotechnol. Biochem.* **1992**, *56*, 1002–1003. [CrossRef] [PubMed]
43. Tamogani, S.; Mitani, M.; Kodama, O.; Akatsuka, T. Oryzalexin S Structure: A New Stemarane-Type Rice Plant Phytoalexin and Its Biogenesis. *Tetrahedron* **1993**, *49*, 2025–2032. [CrossRef]
44. Mohan, R.S.; Yee, N.K.; Coates, R.M.; Ren, Y.Y.; Stamenkovic, P.; Mendez, I.; West, C.A. Biosynthesis of Cyclic Diterpene Hydrocarbons in Rice Cell Suspensions: Conversion of 9,10-syn-Labda-8(17),13-dienyl Diphosphate to 9β-Pimara-7,15-diene and Stemar-13-ene. *Arch. Biochem. Biophys.* **1996**, *330*, 33–47. [CrossRef] [PubMed]
45. Zhao, M.; Cheng, J.; Guo, B.; Duan, J.; Che, C.T. Momilactone and Related Diterpenoids as Potential Agricultural Chemicals. *J. Agric. Food Chem.* **2018**, *66*, 7859–7872. [CrossRef]
46. Zhang, Y.; Li, M.; Liu, Q.; Huang, J.; Chen, Y. A Synthetic View on Momilactones and Related 9β-H Pimarane Skeleton Diterpenoids. *Front. Chem.* **2022**, *10*, 882404. [CrossRef]
47. Cartwright, D.; Langcake, P.; Pryce, R.J.; Leworthy, D.P.; Ride, J.P. Chemical activation of host defence mechanisms as a basis for crop protection. *Nature* **1977**, *267*, 511–513. [CrossRef]
48. Fukuta, M.; Xuan, T.D.; Deba, F.; Tawata, S.; Khanh, T.D.; Chung, I.M. Comparative efficacies in vitro of antibacterial, fungicidal, antioxidant, and herbicidal activities of momilatones A and B. *J. Plant Interact.* **2007**, *2*, 245–251. [CrossRef]
49. Reveglia, P.; Cimmino, A.; Masi, M.; Nocera, P.; Berova, N.; Ellestad, G.; Evidente, A. Pimarane diterpenes: Natural source, stereochemical configuration, and biological activity. *Chirality* **2018**, *30*, 1115–1134. [CrossRef]
50. Kariya, K.; Murata, K.; Kokubo, Y.; Ube, N.; Ueno, K.; Yabuta, Y.; Teraishi, M.; Okumoto, Y.; Mori, N.; Ishihara, A. Variation of diterpenoid phytoalexin oryzalexin A production in cultivated and wild rice. *Phytochemistry* **2019**, *166*, 112057. [CrossRef]
51. Desmedt, W.; Kudjordjie, E.N.; Chavan, S.N.; Zhang, J.; Li, R.; Yang, B.; Nicolaisen, M.; Mori, M.; Peters, R.J.; Vanholme, B.; et al. Rice diterpenoid phytoalexins are involved in defence against parasitic nematodes and shape rhizosphere nematode communities. *New Phytol.* **2022**, *235*, 1231–1245. [CrossRef] [PubMed]
52. Kato-Noguchi, H.; Hasegawa, M.; Ino, T.; Ota, K.; Kujime, H. Contribution of momilactone A and B to rice allelopathy. *J. Plant Physiol.* **2010**, *167*, 787–791. [CrossRef] [PubMed]
53. Kato-Noguchi, H. Barnyard grass-induced rice allelopathy and momilactone B. *J. Plant Physiol.* **2011**, *168*, 1016–1020. [CrossRef] [PubMed]
54. Kato-Noguchi, H. The chemical cross talk between rice and barnyardgrass. *Plant Signal. Behav.* **2011**, *6*, 1207–1209. [CrossRef] [PubMed]
55. Kato-Noguchi, H.; Ino, T. The chemical-mediated allelopathic interaction between rice and barnyard grass. *Plant Soil* **2013**, *370*, 267–275. [CrossRef]
56. Serra, N.; Shanmuganathan, R.; Becker, C. Allelopathy in rice: A story of momilactones, kin recognition, and weed management. *J Exp Bot.* **2021**, *72*, 4022–4037. [CrossRef]
57. Rahaman, F.; Shukor Juraimi, A.; Rafii, M.Y.; Uddin, K.; Hassan, L.; Chowdhury, A.K.; Karim, S.M.R.; Yusuf Rini, B.; Yusuff, O.; Bashar, H.M.K.; et al. Allelopathic Potential in Rice: A Biochemical Tool for Plant Defense Against Weeds. *Front. Plant Sci.* **2022**, *13*, 1072723. [CrossRef]
58. Sultana, M.H.; Alamin, M.; Qiu, J.; Fan, L.; Ye, C. Transcriptomic profiling reveals candidate allelopathic genes in rice responsible for interactions with barnyardgrass. *Front Plant Sci.* **2023**, *14*, 1104951. [CrossRef]
59. Kato-Noguchi, H.; Ino, T.; Sata, N.; Yamamura, S. Isolation and identification of a potent allelopathic substance in rice root exudates. *Physiol. Plant* **2002**, *115*, 401–405. [CrossRef]
60. Chung, I.M.; Hahn, S.J.; Ahmad, A. Confirmation of potential herbicidal agents in hulls of rice, *Oryza sativa*. *J. Chem. Ecol.* **2005**, *31*, 1339–1352. [CrossRef]
61. Xuan, T.D.; Minh, T.N.; Anh, L.H.; Khanh, T.D. Allelopathic momilactones A and B are implied in rice drought and salinity tolerance, not weed resistance. *Agron. Sustain. Dev.* **2016**, *36*, 52. [CrossRef]
62. Zhang, J.; Li, R.; Xu, M.; Hoffmann, R.I.; Zhang, Y.; Liu, B.; Zhang, M.; Yang, B.; Li, Z.; Peters, R.J. A (conditional) role for labdane-related diterpenoid natural products in rice stomatal closure. *New Phytol.* **2021**, *230*, 698–709. [CrossRef] [PubMed]

63. Peters, R.J. Two rings in them all: The labdane-related diterpenoids. *Nat. Prod. Rep.* **2010**, *27*, 1521–1530. [CrossRef] [PubMed]
64. De La Peña, R.; Sattely, E.S. Rerouting plant terpene biosynthesis enables momilactone pathway elucidation. *Nat. Chem. Biol.* **2021**, *17*, 205–212. [CrossRef]
65. Toyomasu, T.; Usui, M.; Sugawara, C.; Otomo, K.; Hirose, Y.; Miyao, A.; Hirochika, H.; Okada, K.; Shimizu, T.; Koga, J.; et al. Reverse-genetic approach to verify physiological roles of rice phytoalexins: Characterization of a knockdown mutant of OsCPS4 phytoalexin biosynthetic gene in rice. *Physiol. Plant.* **2014**, *150*, 55–62. [CrossRef]
66. Xu, M.; Galhano, R.; Wiemann, P.; Bueno, E.; Tiernan, M.; Wu, W.; Chung, I.M.; Gershenzon, J.; Tudzynski, B.; Sesma, A.; et al. Genetic evidence for natural product-mediated plant-plant allelopathy in rice (*Oryza sativa*). *New Phytol.* **2012**, *193*, 570–575. [CrossRef]
67. Wang, Q.; Hillwig, M.L.; Peters, R.J. CYP99A3: Functional identification of a diterpene oxidase from the momilactone biosynthetic gene cluster in rice. *Plant J.* **2011**, *65*, 87–95. [CrossRef]
68. Kitaoka, N.; Zhang, J.; Oyagbenro, R.K.; Brown, B.; Wu, Y.; Yang, B.; Li, Z.; Peters, R.J. Interdependent evolution of biosynthetic gene clusters for momilactone production in rice. *Plant Cell* **2021**, *33*, 290–305. [CrossRef]
69. Bajsa-Hirschel, J.; Pan, Z.; Duke, S.O. Rice momilactone gene cluster: Transcriptional response to barnyard grass (Echinochloa crus-galli). *Mol. Biol. Rep.* **2020**, *47*, 1507–1512. [CrossRef]
70. Cho, E.M.; Okada, A.; Kenmoku, H.; Otomo, K.; Toyomasu, T.; Mitsuhashi, W.; Sassa, T.; Yajima, A.; Yabuta, G.; Mori, K.; et al. Molecular cloning and characterization of a cDNA encoding ent-cassa-12,15-diene synthase, a putative diterpenoid phytoalexin biosynthetic enzyme, from suspension-cultured rice cells treated with a chitin elicitor. *Plant J.* **2004**, *37*, 1–8. [CrossRef]
71. Toyomasu, T. Recent advances regarding diterpene cyclase genes in higher plants and fungi. *Biosci. Biotech. Bioch.* **2008**, *72*, 1168–1175. [CrossRef] [PubMed]
72. Toyomasu, T.; Miyamoto, K.; Shenton, M.R.; Sakai, A.; Sugawara, C.; Horie, K.; Kawaide, H.; Hasegawa, M.; Chuba, M.; Mitsuhashi, W.; et al. Characterization and evolutionary analysis of *ent*-kaurene synthase like genes from the wild rice species *Oryza rufipogon*. *Biochem. Biophys. Res. Commun.* **2016**, *480*, 402–408. [CrossRef] [PubMed]
73. Toyomasu, T.; Usui, M.; Sugawara, C.; Kanno, Y.; Sakai, A.; Takahashi, H.; Nakazono, M.; Kuroda, M.; Miyamoto, K.; Morimoto, Y.; et al. Transcripts of two ent-copalyl diphosphate synthase genes differentially localize in rice plants according to their distinct biological roles. *J Exp Bot.* **2015**, *66*, 369–376. [CrossRef] [PubMed]
74. Wang, Q.; Hillwig, M.L.; Okada, K.; Yamazaki, K.; Wu, Y.; Swaminathan, S.; Yamane, H.; Peters, R.J. Characterization of CYP76M5-8 indicates metabolic plasticity within a plant biosynthetic gene cluster. *J. Biol. Chem.* **2012**, *287*, 6159–6168. [CrossRef]
75. Ye, Z.; Yamazaki, K.; Minoda, H.; Miyamoto, K.; Miyazaki, S.; Kawaide, H.; Yajima, A.; Nojiri, H.; Yamane, H.; Okada, K. In planta functions of cytochrome P450 monooxygenase genes in the phytocassane biosynthetic gene cluster on rice chromosome 2. *Biosci. Biotech. Bioch.* **2018**, *82*, 1021–1030. [CrossRef]
76. Li, R.; Zhang, J.; Li, Z.; Peters, R.J.; Yang, B. Dissecting the labdane-related diterpenoid biosynthetic gene clusters in rice reveals directional cross-cluster phytotoxicity. *New Phytol.* **2022**, *233*, 878–889. [CrossRef]
77. Wang, Q.; Hillwig, M.L.; Wu, Y.; Peters, R.J. CYP701A8: A rice ent-kaurene oxidase paralog diverted to more specialized diterpenoid metabolism. *Plant Physiol.* **2012**, *158*, 1418–1425. [CrossRef]
78. Wu, Y.; Wang, Q.; Hillwig, M.L.; Peters, R.J. Picking sides: Distinct roles for CYP76M6 and CYP76M8 in rice oryzalexin biosynthesis. *Biochem. J.* **2013**, *454*, 209–216. [CrossRef]
79. Kitaoka, N.; Wu, Y.; Zi, J.; Peters, R.J. Investigating inducible short-chain alcohol dehydrogenases/reductases clarifies rice oryzalexin biosynthesis. *Plant J.* **2016**, *88*, 271–279. [CrossRef]
80. Nemoto, T.; Cho, E.M.; Okada, A.; Okada, K.; Otomo, K.; Kanno, Y.; Toyomasu, T.; Mitsuhashi, W.; Sassa, T.; Minami, E.; et al. Stemar-13-ene synthase, a diterpene cyclase involved in the biosynthesis of the phytoalexin oryzalexin S in rice. *FEBS Lett.* **2004**, *571*, 182–186. [CrossRef]
81. Toyomasu, T.; Goda, C.; Sakai, A.; Miyamoto, K.; Shenton, M.R.; Tomiyama, S.; Mitsuhashi, W.; Yamane, H.; Kurata, N.; Okada, K. Characterization of diterpene synthase genes in the wild rice species *Oryza brachyatha* provides evolutionary insight into rice phytoalexin biosynthesis. *Biochem. Biophys. Res. Commun.* **2018**, *503*, 1221–1227. [CrossRef] [PubMed]
82. Zhao, L.; Oyagbenro, R.; Feng, Y.; Xu, M.; Peters, R.J. Oryzalexin S biosynthesis: A cross-stitched disappearing pathway. *aBIOTECH* **2023**, *4*, 1–7. [CrossRef] [PubMed]
83. Okada, A.; Okada, K.; Miyamoto, K.; Koga, J.; Shibuya, N.; Nojiri, H.; Yamane, H. OsTGAP1, a bZIP transcription factor, coordinately regulates the inductive production of diterpenoid phytoalexins in rice. *J. Biol. Chem.* **2009**, *284*, 26510–26518. [CrossRef] [PubMed]
84. Miyamoto, K.; Nishizawa, Y.; Minami, E.; Nojiri, H.; Yamane, H.; Okada, K. Overexpression of the bZIP transcription factor OsbZIP79 suppresses the production of diterpenoid phytoalexin in rice cells. *J. Plant Physiol.* **2015**, *173*, 19–27. [CrossRef]
85. Yamamura, C.; Mizutani, E.; Okada, K.; Nakagawa, H.; Fukushima, S.; Tanaka, A.; Maeda, S.; Kamakura, T.; Yamane, H.; Takatsuji, H.; et al. Diterpenoid Phytoalexin Factor, a bHLH Transcription Factor, Plays a Central Role in the Biosynthesis of Diterpenoid Phytoalexins in Rice. *Plant J.* **2015**, *84*, 1100–1113. [CrossRef]
86. Liu, C.; Zhu, X.; Zhang, J.; Shen, M.; Chen, K.; Fu, X.; Ma, L.; Liu, X.; Zhou, C.; Zhou, D.X.; et al. eQTLs Play Critical Roles in Regulating Gene Expression and Identifying Key Regulators in Rice. *Plant Biotechnol. J.* **2022**, *20*, 2357–2371. [CrossRef]

87. Akagi, A.; Fukushima, S.; Okada, K.; Jiang, C.J.; Yoshida, R.; Nakayama, A.; Shimono, M.; Sugano, S.; Yamane, H.; Takatsuji, H. WRKY45-Dependent Priming of Diterpenoid Phytoalexin Biosynthesis in Rice and the Role of Cytokinin in Triggering the Reaction. *Plant Mol. Biol.* **2014**, *86*, 171–183. [CrossRef]
88. Chujo, T.; Miyamoto, K.; Ogawa, S.; Masuda, Y.; Shimizu, T.; Kishi-Kaboshi, M.; Takahashi, A.; Nishizawa, Y.; Minami, E.; Nojiri, H.; et al. Overexpression of Phosphomimic Mutated OsWRKY53 Leads to Enhanced Blast Resistance in Rice. *PLoS ONE* **2014**, *9*, e98737. [CrossRef]
89. Wang, L.; Fu, J.; Shen, Q.; Wang, Q. OsWRKY10 Extensively Activates Multiple Rice Diterpenoid Phytoalexin Biosynthesis Genes to Enhance Rice Blast Resistance. *Plant J.* **2023**, *115*, 758–771. [CrossRef]
90. Yokotani, N.; Sato, Y.; Tanabe, S.; Chujo, T.; Shimizu, T.; Okada, K.; Yamane, H.; Shimono, M.; Sugano, S.; Takatsuji, H.; et al. WRKY76 Is a Rice Transcriptional Repressor Playing Opposite Roles in Blast Disease Resistance and Cold Stress Tolerance. *J. Exp. Bot.* **2013**, *64*, 5085–5097. [CrossRef]
91. Riemann, M.; Haga, K.; Shimizu, T.; Okada, K.; Ando, S.; Mochizuki, S.; Nishizawa, Y.; Yamanouchi, U.; Nick, P.; Yano, M.; et al. Identification of rice Allene Oxide Cyclase mutants and the function of jasmonate for defence against Magnaporthe oryzae. *Plant J.* **2013**, *74*, 226–238. [CrossRef] [PubMed]
92. Shimizu, T.; Miyamoto, K.; Miyamoto, K.; Minami, E.; Nishizawa, Y.; Iino, M.; Nojiri, H.; Yamane, H.; Okada, K. OsJAR1 contributes mainly to biosynthesis of the stress-induced jasmonoyl-isoleucine involved in defense responses in rice. *Biosci. Biotechnol. Biochem.* **2013**, *77*, 1556–1564. [CrossRef]
93. Kaku, H.; Nishizawa, Y.; Ishii-Minami, N.; Akimoto-Tomiyama, C.; Dohmae, N.; Takio, K.; Minami, E.; Shibuya, N. Plant cells recognize chitin fragments for defense signaling through a plasma membrane receptor. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 11086–11091. [CrossRef]
94. Pring, S.; Kato, H.; Imano, S.; Camagna, M.; Tanaka, A.; Kimoto, H.; Chen, P.; Shrotri, A.; Kobayashi, H.; Fukuoka, A.; et al. Induction of plant disease resistance by mixed oligosaccharide elicitors prepared from plant cell wall and crustacean shells. *Physiol. Plant.* **2023**, *175*, e14052. [CrossRef] [PubMed]
95. Li, W.; Shao, M.; Zhong, W.; Yang, J.; Okada, K.; Yamane, H.; Zhang, L.; Wang, G.; Wang, D.; Xiao, S.; et al. Ectopic expression of Hrf1 enhances bacterial resistance via regulation of diterpene phytoalexins, silicon and reactive oxygen species burst in rice. *PLoS ONE.* **2012**, *7*, e43914. [CrossRef] [PubMed]
96. Lu, X.; Zhang, J.; Brown, B.; Li, R.; Rodríguez-Romero, J.; Berasategui, A.; Liu, B.; Xu, M.; Luo, D.; Pan, Z.; et al. Inferring Roles in Defense from Metabolic Allocation of Rice Diterpenoids. *Plant Cell* **2018**, *30*, 1119–1131. [CrossRef] [PubMed]
97. Mao, H.; Liu, J.; Ren, F.; Peters, R.J.; Wang, Q. Characterization of CYP71Z18 indicates a role in maize zealexin biosynthesis. *Phytochemistry* **2016**, *121*, 4–10. [CrossRef]
98. Shen, Q.; Pu, Q.; Liang, J.; Mao, H.; Liu, J.; Wang, Q. CYP71Z18 overexpression confers elevated blast resistance in transgenic rice. *Plant Mol. Biol.* **2019**, *100*, 579–589. [CrossRef]

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Review

L-gulono-γ-lactone Oxidase, the Key Enzyme for L-Ascorbic Acid Biosynthesis

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Abstract: L-ascorbic acid (AsA, vitamin C) plays a vital role in preventing various diseases, particularly scurvy. AsA is known for its antioxidant properties, which help protect against reactive oxygen species generated from metabolic activities; however, at high doses, it may exhibit pro-oxidative effects. The final step in AsA biosynthesis is catalyzed by L-gulono-γ-lactone oxidase (GULO). This enzyme is present in many organisms, but some animals, including humans, guinea pigs, bats, and other primates, are unable to synthesize AsA due to the absence of a functional *GULO* gene. The GULO enzyme belongs to the family of aldonolactone oxidoreductases (AlORs) and contains two conserved domains, an N-terminal FAD-binding region and a C-terminal HWXK motif capable of binding the flavin cofactor. In this review, we explore AsA production, the biosynthetic pathways of AsA, and the localization of GULO-like enzymes in both animal and plant cells. Additionally, we compare the amino acid sequences of AlORs across different species and summarize the findings related to their enzymatic activity. Interestingly, a recombinant C-terminal rat GULO (the cytoplasmic domain of the rat GULO expressed in *Escherichia coli*) demonstrated enzymatic activity. This suggests that the binding of the flavin cofactor to the HWXK motif at the C-terminus is sufficient for the formation of the enzyme's active site. Another enzyme, GULLO7 from *Arabidopsis thaliana*, also lacks the N-terminal FAD-binding domain and is strongly expressed in mature pollen, although its activity has not been specifically measured.

Keywords: ascorbic acid; L-gulono-γ-lactone oxidase; vitamin C; GULO characterization; C-terminal GULO; HWXK motif

1. Introduction

L-ascorbic acid (AsA), also known as vitamin C, is a naturally occurring organic compound belonging to the family of monosaccharides. Its consumption in humans correlates with increased protection against degenerative diseases and cancer [1]. The last stage of AsA production from glucose in mammals requires the enzyme L-gulono-γ-lactone oxidase (GULO), which is produced by the intermediates D-glucuronate and L-gulono-γ-lactone (GUL). Although numerous species are capable of synthesizing vitamin C, this enzymatic activity is lost in several mammals, such as humans, other primates, guinea pigs, some bat species, and insects [2]. The GULO enzyme belongs to the family of aldonolactone oxidoreductases (AlORs), which are a part of the vanillyl alcohol oxidase (VAO) family of flavoenzymes. When hydrogen peroxide is produced as a consequence of the oxidation of an aldonolactone using molecular oxygen as an electron acceptor, this family of enzymes is known as oxidases. If the reaction includes other electron acceptors, such as cytochrome c, phenazine methosulfate, 2,6-dichloroindophenol, or benzoquinone, these enzymes are referred to as dehydrogenases [3]. Plants appear to have multiple pathways for AsA biosynthesis. L-galactono-γ-lactone dehydrogenase (GalDH) catalyzes the conversion of GUL into AsA, while GULO-like enzymes, hereinafter referred to as GULLO, use L-galactono-γ-lactone (GAL) as a substrate [4].

2. The Role of Ascorbic Acid in Biochemical Processes and Its Biosynthetic Pathways

As an enzyme co-factor in hydroxylation reactions, AsA is vital to many processes in animals, including collagen synthesis [5], the demethylation of histones and nucleic acids [6], oxidative protein folding and endoplasmic reticulum (ER) stress [7], cell division and apoptosis [8], activation of the chloride channel regulator epithelial cystic fibrosis transmembrane conductance regulator [9], the production of surfactants in human lungs and immune homeostasis [10], macrophage function [11], and stress resistance. AsA has several uses as a medicinal product for human health, including the management of the common cold, wound healing, cancer, and scurvy prevention [5,12,13]. More recently, it has been found that diabetic complications due to uncontrolled hyperglycemia can be reduced if glucose is converted into AsA through GULO activation, thereby reducing oxidative stress [14]. The study by Kim et al. [15] states that endothelial cells can be stimulated to synthesize AsA intracellularly, by expressing GULO and providing them with the substrate GUL. Additionally, it was discovered that an increase in the amount of accessible tetrahydrobiopterin, which in turn enhanced NO generation in response to an increase in calcium, was caused by an increase in intracellular AsA. Therefore, by increasing NO bioactivity in endothelial cells, the intracellular administration of AsA may have positive effects on vascular physiology.

Grollman and Lehninger initially reported the absence of the GULO enzyme in mammals in 1957 [16], when they discovered that the vitamin C synthesis pathway is present in the kidney of birds and reptiles and the liver of mammals. In particular, they found that the three enzymes and the production of vitamin C were present in rats, mice, rabbits, dogs, cats, pigs, cows, chickens, pigeons, and turtles, but they were unable to detect the GULO enzyme in guinea pigs, rhesus monkeys, cynomolgus monkeys, or five distinct human species [5,16]. The four mammals without the third enzyme, according to Grollman and Lehninger, were required to be vitamin C consumers. However, a few years ago, clinical cases of scurvy in pigs were reported for the first time, thanks to the discovery of a mutant family of Danish pigs that is incapable of producing AsA [2,17].

There are other animal groups in which the deletion of the *GULO* gene has been reported, in addition to mammals. It is true that several species of Teleostei fish and Passeriformes birds do not carry the *GULO* gene. Despite the fact that vitamin C was found in numerous species [4,18,19], it was believed that the majority of protostomes lacked the *GULO* gene. There is little disagreement as to whether vitamin C is crucial for insects, despite the fact that they lack the *GULO* gene. For example, the L-ascorbate levels increase in *Drosophila melanogaster* Meigen, 1830 (Diptera: Drosophilidae), as they age, and vitamin C-enriched diets help flies live longer on average and to their maximum capacity [19,20]. The improved tolerance of the moth *Orgyia leucostigma* (J. E. Smith, 1797) (Lepidoptera: Erebidae) against tannin, a substance that induces reactive oxygen species (ROS), is thought to be caused by the increased amount of L-ascorbate found in its midgut lumen [21]. In response to several ROS-inducing pesticides, L-ascorbate levels decline in a dose-dependent way, potentially through supporting enzymatic antioxidant systems, including catalase, superoxide dismutase, and peroxidase [22]. It was discovered that the genes linked to L-ascorbate metabolism in the ladybird *Cryptolaemus montrouzieri* Mulsant, 1850 (Coleoptera: Coccinellidae) were selectively changed in reaction to cold stress, but not in response to heat stress [23]. *Caenorhabditis elegans* Maupas, 1900 (Rhabditida: Rhabditidae), a nematode, lacks the *GULO* gene; yet, it has been demonstrated that it can synthesize L-ascorbate from *Escherichia coli*, its food source. As a result, it has been suggested that *C. elegans* synthesizes L-ascorbate via a different enzyme or pathway, although the gene causing this has not been found [24].

Because most species lack a functioning *GULO* gene, they are unable to produce AsA. It is interesting to note that animal species lacking the *GULO* gene typically still have the other genes in the pathway, such as the *Regucalcin/SMP30* (Senescence Marker Protein 30) gene, which encodes for a gluconolactonase that generates the substrate of GULO (GUL) [4,25]. This finding implies that the other genes within the pathway have important roles. For instance, regucalcin functions as a transcription factor, a regulator of many

nuclear processes, and a regulator of cellular Ca^{2+} levels in mammals [26,27]. Additionally, it has been noted that flies that grow without L-ascorbate exhibit elevated vitamin C levels in response to a brief 4 °C cold shock (10 min) [19,28].

The animals, including humans, that are unable to synthesize AsA must obtain this vitamin from commercially manufactured synthetic supplements or fresh fruits and green vegetables. The majority of AsA in the human diet comes from plants. In plants, AsA is the most prevalent and well-characterized water-soluble antioxidant [29]. AsA primarily builds up in the photosynthetic organs of plants, as it can be found in the cells of green tissues at concentrations of up to 5 mM, which is 10% of the total soluble carbohydrate pool [30]. In plant physiology, AsA is a critical metabolite that plays a key role in detoxifying ROS, promoting resistance to senescence, and mitigating the effects of a variety of environmental stressors, including high temperatures, dehydration stress, high light levels, ozone pollution, UV-B radiation, and salt stress. AsA also functions as a cofactor, contributing to the control of several essential cellular functions, such as photoprotection, the cell cycle, and cell division, as well as the production of important plant hormones, such as gibberellic acid, ethylene, abscisic acid, and jasmonic acid [31,32].

Prior to the characterization in *Arabidopsis thaliana* (L.) Heynh. (Capparales: Brassicaceae) of mutants (vtc-4-1 and vtc) that impair vitamin C production, the precise mechanism for vitamin C synthesis in plants was not known [30,33]. Currently, the Smirnoff–Wheeler pathway is regarded as the main mechanism used in the synthesis of vitamin C. While some plant species, like *Citrus* sp. and the kiwifruit *Actinidia chinensis* Planch. (Ericales: Actinidiaceae), are well-known for having high vitamin C content [34], the biosynthesis of vitamin C in *A. thaliana* has a well-established mechanism. The enzymes responsible for vitamin C biosynthesis in *A. thaliana* from glucose are hexokinase 1 (At4g29130, HXK1), phosphoglucose isomerase (At4g24620, PGI), phosphomannose isomerase 2 (At1g67070, DIN9, PMI2), phosphomannomutase (At2g45790, PMM), GDP-mannose pyrophosphorylase 1 (At2g39770, VTC1, GMP1), GDP-d-mannose epimerase (At5g28840, GME), GDP-L-galactose phosphorylase (At4g26850, VTC2, GGP), L-galactose 1-phosphate phosphatase (At3g02870, VTC4, GPP), L-galactose dehydrogenase (At4g33670, GalDH), and L-galactono-γ-lactone dehydrogenase (At3g47930, GLDH). The roles of these enzymes and their homologues in plants, and in the yeasts, *Kluyveromyces lactis* and *Saccharomyces cerevisiae,* have been established by research [35–37].

Plants and animals have different AsA biosynthesis routes (Figure 1). The primary and most well-understood mechanism in plants is the "Wheeler–Smirnoff pathway", sometimes referred to as the "D-mannose/L-galactose pathway" or the "plant pathway." AsA biosynthesis from glucose or mannose is the first step [4,38], during which D-mannose is converted into GDP-D-mannose and then into GDP-L-galactose. AsA is a precursor to L-galactose, which is further oxidized to form L-galactono-γ-lactone [38]. Every gene involved in this process has been discovered and, at the end of the pathway, GalDH catalyzes an enzyme reaction that forms AsA from GAL. Plants appear to have alternative pathways for the biosynthesis of AsA that involve galacturonate and glucuronate; however, not all of the enzymes involved in these processes have been identified, and the regulation of these pathways is still unclear [39,40]. Moreover, it appears that a portion of the animal system, often referred to as the myo-inositol pathway, which uses GUL as the last precursor for AsA biosynthesis, is active in plants, albeit the specific enzymes involved have not yet been found [41,42]. GalDH serves as the terminal enzyme in the primary plant AsA biosynthesis pathway, whereas GULO serves this function in animals [4,13]. However, plants possess GULO-like enzymes (e.g., GULLO1-GULLO7 in *A. thaliana*), which may be involved in AsA formation via the alternative pathway involving GUL, as shown in Figure 1. The functionality of this pathway is suggested by the elevation in AsA production, due to the overexpression of rat GULO or *A. thaliana* GULLO5 in plants [43–45].

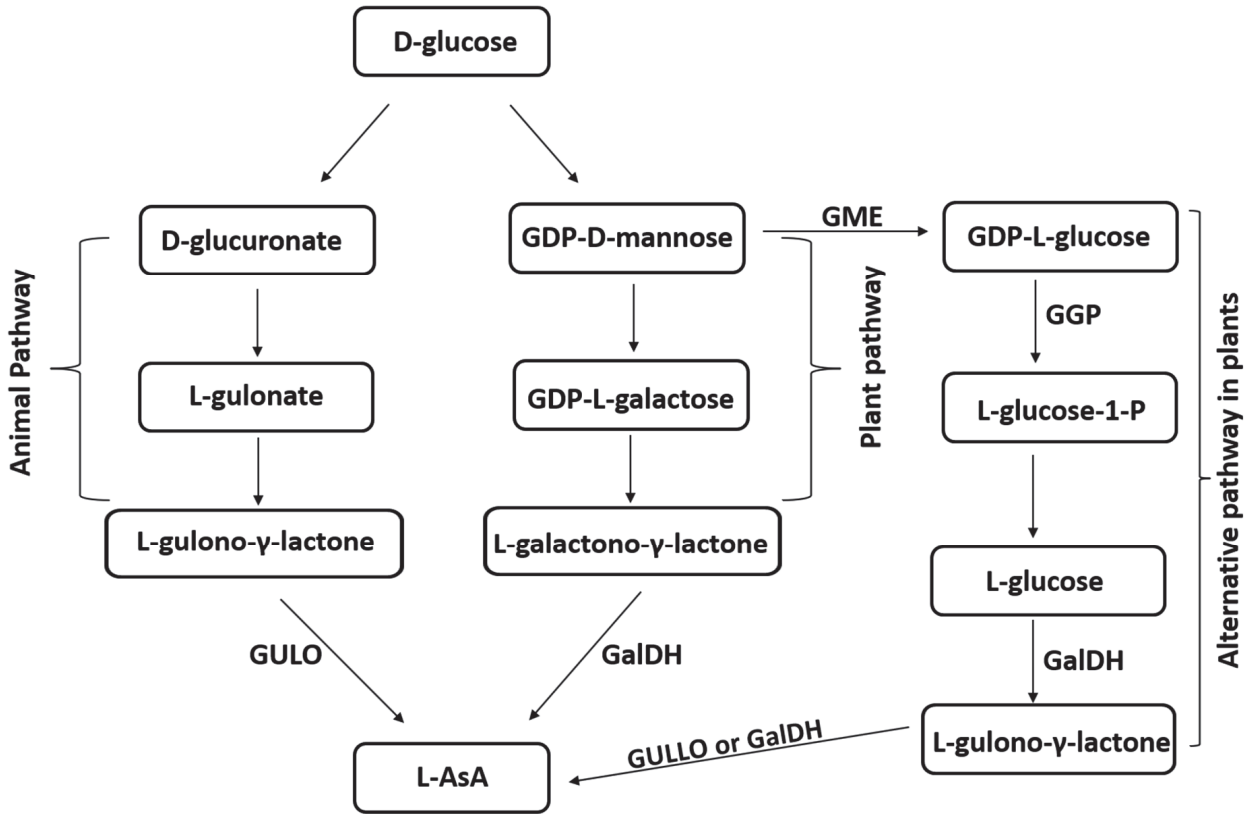


Figure 1. Main pathways for L-ascorbic acid (L-AsA) biosynthesis in animals and plants. GULO, animal L-gulono-γ-lactone oxidase; GULLO, plant L-gulono-γ-lactone oxidase; GalDH, L-galactono-γ-lactone dehydrogenase; GME, GDP-d-mannose epimerase; GGP, GDP-l-galactose phosphorylase [43–45].

3. Methods of Ascorbic Acid Production

3.1. Manufacturing for Commercial Purposes

Among the 13 vitamins, AsA is the one that has the greatest industrial production volume. Every year, the pharmaceutical industry uses about 110 kilotons of vitamin C, which is manufactured industrially. Food and beverage companies use 25% and 15% of these kilotons of vitamin C as antioxidants, respectively. Animal feed applications only consume roughly 10% of the vitamin. This is not the case for any other vitamin, where the primary application for other vitamins is in the feed industry [46]. Chinese manufacturers supply more than 80% of the vitamin C needed by the global market today. The Reichstein process, which combines chemical and microbiological methods, is commonly used to generate commercial vitamin C from D-glucose [47]. Despite being in use for more than 60 years, the process has several inherent drawbacks, including lengthy production time, intricate procedures, high temperature and pressure requirements, and challenges associated with continuous operation. Furthermore, the Reichstein method necessitates the use of numerous hazardous chemicals that are energy intensive and have the potential to significantly contaminate the environment. As a result, scientists have looked for fresh approaches to enhance the effectiveness of the conventional Reichstein technique [48,49]. For instance, a two-step fermentation technique was developed that is easier to use and more ecologically friendly than the Reichstein process [50]. Unfortunately, the two-step fermentation method requires three separate strains of bacteria, which makes it difficult to convert D-glucose directly into vitamin C. A lot of work has gone into creating a one-step fermentation technique for vitamin C [51]. This has, in theory, made it possible to produce vitamin C directly from D-glucose; nevertheless, practically all the existing one-step vitamin C fermentation procedures generate 2-keto-L-gulonic acid (2-KLG) rather than vitamin C [52–54]. Additionally, there

has been report about the direct production of vitamin C from D-glucose by engineering the ten genes involved in the vitamin C biosynthetic pathway in *A. thaliana* [37].

3.2. Recombinant Vanillyl Alcohol Oxidase Family Members Are Usually Produced in Bacterial Cells in Apo Forms Lacking the Flavin Cofactor

GULO was created in both holo and apo forms in riboflavin-rich and free media in insect cells, utilizing the baculovirus system. L-flavin adenine dinucleotide (FAD) only bound non-covalently to the apo form when added externally [55]. However, the enzymatic activity was the same as the holo form when FAD was covalently bound. In contrast, *E. coli* was shown to express protozoan GULO isoenzymes from *Trypanosoma brucei* Dutton, 1902 [56] and *Trypanosoma cruzi* Chagas, 1909 (Trypanosomatida: Trypanosomatidae) [57,58], as well as GalDH from *A. thaliana* [59] and tobacco [60], all of which possess a non-covalent flavin. In more detail, boiling or treating the protein with acid can liberate the redox active flavin cofactor, verifying the non-covalent binding mechanism that was previously inferred from the amino acid sequences. Remarkably, a recombinant *T. cruzi* L-galactono-γ-lactone oxidase (GALO) was efficiently refolded in a reverse micellar system into a native-like structure in the presence of FAD, but not with the flavin mononucleotide [58], which was localized in the inclusion bodies of *E. coli* following overexpression. When expressed in *E. coli*, GALO in *Leishmania donovani* Ross, 1903 (Trypanosomatida: Trypanosomatidae), which was predicted to have non-covalently bound FAD, was determined to be in the apo form, because no activity was seen without the addition of FAD [61]. An enzyme with covalently attached FAD was not produced by AlORS expression in *E. coli*. Given that several members of the vanillyl alcohol oxidase (VAO) family, including VAO itself [62], eugenol oxidase [63], alditol oxidase [64], and chitooligosaccharide oxidase [65], were successfully expressed as holo proteins in *E. coli*, it is possible that the recombinant proteins did not fold correctly. The non-flavoprotein may have been produced by the improper folding of recombinant *Mycobacterium tuberculosis* L-gulono-γ-lactone dehydrogenase (GLDH), expressed in *E. coli*.

3.3. Genetic Engineering of Plants and Animals to Elevate the Ascorbic Acid Level

It has been more than three decades since the idea of employing plants as production machines for different proteins, with biotechnological or medicinal value, was first proposed. Since 1989, biofactory plants have been producing the previously mentioned recombinant human protein. Using mammalian expression models, recombinant protein product yields are poor, and production costs are high [66]. Bacteria have long been utilized as an expression system for the synthesis of recombinant proteins, because of their straightforward molecular biology, the low cost of manufacturing them, and the ease of handling. The absence of post-translational machinery and the creation of inactive proteins as a result of improper disulfide bond formation, which can cause protein misfolding and aggregation into inclusion bodies, represent the biggest obstacles [67]. Instead, plants can produce fully folded functional molecules with nearly identical properties to the native protein and carry out all of the post-translational modifications required for proteins to have the best biological activity, thus plant cells are more attractive for producing eukaryotic proteins [68,69]. Transgenic plants can effectively manufacture recombinant proteins, such as antibodies, cytokines, and vaccines [70,71]. The most often utilized transgenic plants include tobacco, potato, banana, and *A. thaliana* [72].

The expression of even a single gene from the animal pathway can result in a significant increase in the ascorbic acid level in plants. Transgenic plants that express rat cDNA encoding GULO, such tobacco, potatoes, tomatoes, and *A. thaliana*, produce basal levels of ascorbic acid that can be up to seven times higher than those in untransformed plants [73–75]. In an effort to elevate the AsA level, a number of ascorbate biosynthesis pathway genes have recently been inserted into plants using metabolic engineering. These include a human *dehydroascorbate reductase* gene inserted into tobacco [76], GULO cDNA isolated from mice and inserted into tobacco and lettuce [73], and D-galacturonic acid reductase cDNA from strawberry inserted into *A. thaliana* and potato [39,77], and wheat

dehydroascorbate cDNA inserted into tobacco and maize [78]. Recombinant GULLO5 (At2g46740) from *A. thaliana* was also transiently expressed in *Nicotiana benthamiana* Domin (Solanales: Solanaceae) and its activity was verified through ascorbic acid production [38]. In this context, three *Arabidopsis* GULO-like enzymes (GULLO2, 3, 5) overexpressed in tobacco BY-2 cell cultures increased the AsA level after being fed with L-GUL. The GULLO proteins from *A. thaliana* exhibit some similarity to animal GULO [79]. The production of a FAD-containing protein by GULLO-expressing vector-infected cells, matched in size to the expected recombinant GULLO protein, and the conversion of GUL to AsA, indicated the existence of a functional enzyme in cell-free extracts [80]. Also, in plant or animal cells, the expression of the rat or mouse *GULO* gene has been used to restore vitamin C biosynthetic capacity. The examples include the teleost fish, rainbow trout, and guinea pig cells [81], human cells [80], monkey cells [80,82], and the Far Eastern catfish [83,84].

4. Localization and Enzymatic Properties of GULO-like Enzymes from Different Species

It is interesting to note that as a result of evolution, the location of GULO expression appears to have changed from the kidney to the liver; whereas primitive birds, reptiles, and amphibia create GULO in the kidney, mammals and highly evolved birds manufacture it in the liver [2,17]. The GULOs in animals, such as rats [85], goats [3], mice [86], chickens [87], and pigs [2], were localized to microsomes and they are all integral membrane proteins. The rat GULO amino acid sequence has multiple potential membrane-associating sites [88]. The rat GULO was revealed to have an intraluminal active site, and it discharges its products into the ER lumen [89]. Although the exact location of the GULO active site is unknown, it is believed that the enzyme produces H_2O_2 and ascorbate on the lumenal side of the ER membrane [90,91].

GalDH, the final enzyme in the primary AsA biosynthesis pathway in plants, is connected to respiratory Complex 1 and is found in the inner membrane of mitochondria. GalDH may be connected to the respiratory electron transport chain in the mitochondria, based on its location and Cyt c selectivity. Even though a specific ascorbate transporter may be needed for transportation across the inner mitochondrial membrane, which is impermeable to sucrose, but selectively permeable to a limited number of anions depending on the orientation of GalDH, GAL may diffuse through the outer mitochondrial membrane, which is highly permeable to solutes like sucrose, nucleotides, and NAD. GalDH is expected to traverse the inner mitochondrial membrane based on its predicted location, but since its catalytic site faces the intermembrane gap, transporters would not be necessary [45,92–94]. Moreover, in *A. thaliana,* the GULO-like enzyme, GULLO5, localizes to the cell wall [95]. A similar localization to the cell wall occurs in regard to *M. tuberculosis* GalDH [96]. In *A. thaliana,* GalDH, a gatekeeper amino acid residue, was shown to be responsible for determining whether the oxidoreductase was an oxidase or a dehydrogenase. It was discovered that, with a few exceptions, alanine can be found in dehydrogenases, while glycine or proline can be found in oxidases [97]. Despite containing proline and glycine, respectively, *A. thaliana* GULLO5 and *M. tuberculosis* GLDH do not exhibit oxidase activity. It is important to note that the recombinant *M. tuberculosis* GLDH lacks a flavin, which may have contributed to its lack of reactivity with molecular oxygen [84,98].

Trypanosomal D-arabinono-γ-lactone oxidase (ALO) enzymes are found within glycosomes and can be specifically targeted to glycosomes due to a distinctive SHL motif at the C-terminus of their amino acid sequence [56,57]. Along with cytoplasm, *L. donovani* ALO is also found in glycosomes. The SHL motif is absent from the amino acid sequence [43,61].

In contrast, the subcellular localization of a GULLO from the mushroom *Grifola frondosa* (Dicks.) Gray (1821) (Polyporales: Meripilaceae) was not determined, but it may be a membrane protein [99]. This enzyme used GUL as the substrate and oxygen as the electron acceptor under aerobic circumstances to create AsA and H_2O_2. Anaerobic conditions led to the production of AsA, using GUL as the substrate and phenazine methosulfate as the electron acceptor. This means that in addition to being an oxidase, this enzyme also possesses dehydrogenase properties. Even though GULLO from *G. frondosa* has a high substrate specificity comparable to that of GalDHs in plants, its real substrate might be unknown [99,100].

In summary, mammalian GULOs containing a covalently bound 8α-N1-histidyl FAD [101,102] are localized in microsomes, with the active site facing the lumen of the ER [89], protozoan isozymes are localized in glycosomes, while the localization of mushroom isoforms is unknown. In plants, the final step in AsA synthesis via the main pathway (GalDH) is localized in mitochondria [103], while the GULO-like enzymes, presumably responsible for the minor pathway, might be located near the cell wall (Figure 2).

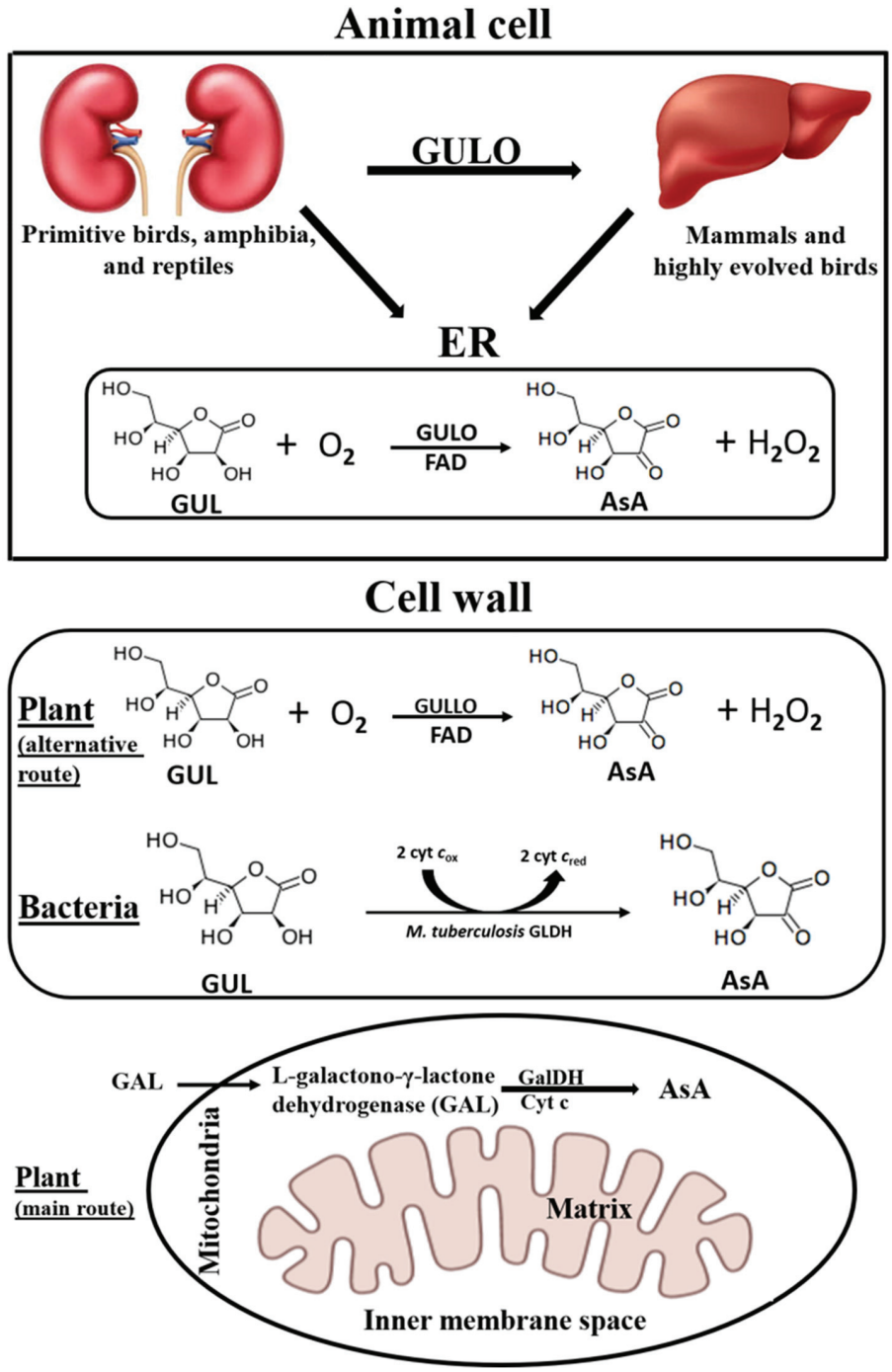


Figure 2. A comparison between the sites for ascorbic acid synthesis in animal, plant, and bacterial cells. AsA, L-ascorbic acid; ER, endoplasmic reticulum; GUL, L-gulono-γ-lactone; GULO, animal L-gulono-γ-lactone oxidase; GULLO, plant L-gulono-γ-lactone oxidase; GLDH, L-gulono-γ-lactone dehydrogenase; GAL, L-galactono-γ-lactone; GalDH, L-galactono-γ-lactone dehydrogenase; Cyt c, cytochrome c.

5. Characterization of L-gulono-γ-lactone Oxidases from Different Sources

The GULO enzyme belongs to the AlORs family and contains two conserved domains: a C-terminal HWXK motif that can bind the flavin cofactor and an N-terminal FAD-binding region. All AlORs found in animals, fungi, and yeast are oxidases, but they also have dehydrogenase-like activity and use phenazine methosulfate as an electron acceptor rather than cytochrome c [104]. Protozoan enzymes consume oxygen and cytochrome c, and they are also oxidases and dehydrogenases [43]. Utilizing phenazine methosulfate and cytochrome c, plant GalDHs are exclusive dehydrogenases. All plant GalDHs contain the same amino acid, which was found to be the cause of oxygen reactivity in *A. thaliana* GalDH [43]. Exclusive dehydrogenases are also seen in bacterial enzymes. An isoenzyme from algae is special in that it can only take electrons in vitro from phenazine methosulfate and not from oxygen or cytochrome c [43]. Animal GULOs are microsomal enzymes that catalyze the interaction by GUL, with oxygen acting as an electron acceptor, to create hydrogen peroxide and L-ascorbate [84,91]. They use FAD as a cofactor in this reaction. It is commonly known that the flavin moiety has a role in the enzymatic catalysis of many AlORs. Reactions containing flavin proceed in two phases. The substrate reduces flavin and the latter is oxidized during the first half of the reductive process. Electron acceptors, like oxygen (oxidase), or other electron acceptors (dehydrogenases), reoxidize flavin in the second half of the process. Electrons from flavin are transferred to oxygen, producing hydrogen peroxide. Because of the distinctive spectrum characteristics displayed throughout the oxidation and reduction cycles involving the flavin group, both half reactions can be investigated independently [43,97]. GUL can be converted into AsA by all known GalDHs from plants [42] and from the protist *Euglena* sp. [104], which use cytochrome c and phenazine methosulfate, respectively, as an electron acceptor. Another example is the *M. tuberculosis* GLDH, which has the ability to receive exogenous electrons from both GUL and cytochrome c [105]. Plant GalDH and animal GULO are both active in terms of the GAL substrate. In the same situation, the *A. thaliana* GalDH uses cytochrome c as an electron acceptor to oxidize GUL, in addition to GAL [59]. In recent studies, Sivadas et al. [14] reported that some phytomolecules, as flavonoids with antioxidant properties, are hypothesized to be promising and selective activators of GULO. In contrast, the majority of the identified AlORs were inhibited by metal ions; Hg^{+2}, Zn^{+2}, and Cu^{+2} were discovered to be prevalent inhibitors of AlORs and were linked to inhibition through sulfhydryl groups [61,106]. By blocking flavin reduction, Hg^{+2} suppresses rat GULO activity, as shown by the absence of a flavin-corresponding peak at 450 nm. On the other hand, sulphite inhibited oxidase activity, but did not inhibit flavin reduction. Although the thiol group is only involved in substrate binding and not in catalysis, it is still essential for the function of these enzymes. This function involves just one sulfhydryl group, which was found to be Cys-340 in the GLDH in *A. thaliana* [43]. The existence of two active sites, one for oxidase activity and the other for dehydrogenase activity was, therefore, postulated by the scientists [84,107]. Reactions involving phenazine methosulfate may also feature a third active site that is independent of flavin, in addition to these two active sites [106]. Similarly, GLDH activity was higher with phenazine methosulfate and lower with cytochrome c, even though *M. tuberculosis* lacked a flavin prosthetic group [84,105].

Burns et al. [108] discovered GULO activity in rat liver microsomes for the first time. The enzyme from rat liver microsomes was partially purified by Eliceiri et al. [85]. Later, using the microsomes from rat and goat liver [3], as well as chicken kidney [87], GULO was isolated to apparent homogeneity and biochemically characterized (Table 1). This helped generate antisera, which in turn helped identify the cDNA sequence [88]. With the use of these instruments, the molecular process behind the deficiency of vitamin C production in scurvy-prone animals, including humans, was studied [109]. Recombinant proteins for both the full-length rat GULO (fGULO) and its C-terminal catalytic domain (cGULO) have recently been purified and biochemically characterized by Gad et al. [110]. A comparison of the biochemical properties of some previously purified and characterized members of the ALO family is shown in Table 1.

Table 1. Comparison of biochemical parameters for ALO family members characterized from different species.

Species	Molecular Mass (kDa)	Optimum pH	Optimum Temperature (°C)	K_m (mM)	V_{max} (μmol/min/mg Protein)	Reference
Rat liver GULO	51	7.8	37	0.066	0.63	[3]
Goat liver GULO	51	Nd	Nd	0.15	2.7	[3]
Chicken kidney GULO	50	7.3	Nd	0.007	139	[87]
Recombinant fGULO expressed in *E. coli*	50	7	40	0.053	780	[110]
Recombinant cGULO expressed in *E. coli*	20	6.5	30	0.042	374	[110]
GfGULLO	69	7	45	24	2.1	[99]
MtGLDH	70	8	40	5.5	0.041	[105]
AtGalDH	60	8.8	25	13.1	Nd	[59]
MsGULLO	70	Nd	Nd	Nd	Nd	[13]
Recombinant (AtGULLO5) expressed in *N. benthamiana*	65	9	40	33.8	4.5×10^{-3}	[43]

Nd, not determined; fGULO, rat full-length L-gulono-γ-lactone oxidase; cGULO, rat C-terminal L-gulono-γ-lactone oxidase; GfGULLO, *Grifola frondosa* L-gulono-γ-lactone oxidase; MtGLDH, *Mycobacterium tuberculosis* L-gulono-γ-lactone dehydrogenase; AtGalDH, *Arabidopsis thaliana* L-galactono-γ-lactone dehydrogenase; MsGULLO, *Mucuna sempervirens* L-gulono-γ-lactone oxidase; AtGULLO5, *Arabidopsis thaliana* homologue of GULLO.

As already mentioned above, GalDH and GULLO are both involved in AsA biosynthesis in plants. The GalDH activity in plants was first identified in the mitochondria of pea and mung bean seeds [111]. The enzyme responsible for this activity was partially purified and characterized from cauliflower floret mitochondria [107]. Since then, isoforms from spinach [112] and white potato tubers [113] have been reported, and cDNA sequences encoding GalDH from cauliflower [114] and sweet potato [115] were identified. The GULLO activity has also been detected in kidney bean hypocotyl homogenates [116], *Grifola frondosa* fruiting bodies [99], *A. thaliana* cell suspension of cytosolic and mitochondrial fractions [41]. The GULLO-like protein from the velvet bean *Mucuna sempervirens* Hemsl. (Fabales: Fabaceae) (homolog of GULO-like enzymes from *A. thaliana*) was purified to near homogeneity from *M. sempervirens* floral nectar and its enzymatic activity was assayed in vitro [13]. Additionally, Maruta et al. [79] expressed four of the seven putative GULLOs from *A. thaliana* in BY2 cell suspension cultures. A recombinant *A. thaliana GULLO5* was expressed in a *N. benthamiana*-based transient expression system and successfully characterized by Aboobucker et al. [43].

6. GULO-like Enzymes from *A. thaliana* and GULO from Rat

Seven GULO-like proteins identified in *A. thaliana* were named as GULLO1 to GULLO7 [79]. A schematic comparison of several representative *A. thaliana* ALO family members, recombinant rat GULO proteins, and GalDH from several plants, is shown in Figure 3. Most of the compared enzymes contain two conserved domains: the FAD-binding domain (Pfam Id: 01565) at the N-terminus and an ALO catalytic domain (Pfam Id: 04030) at the C-terminus [84,108].

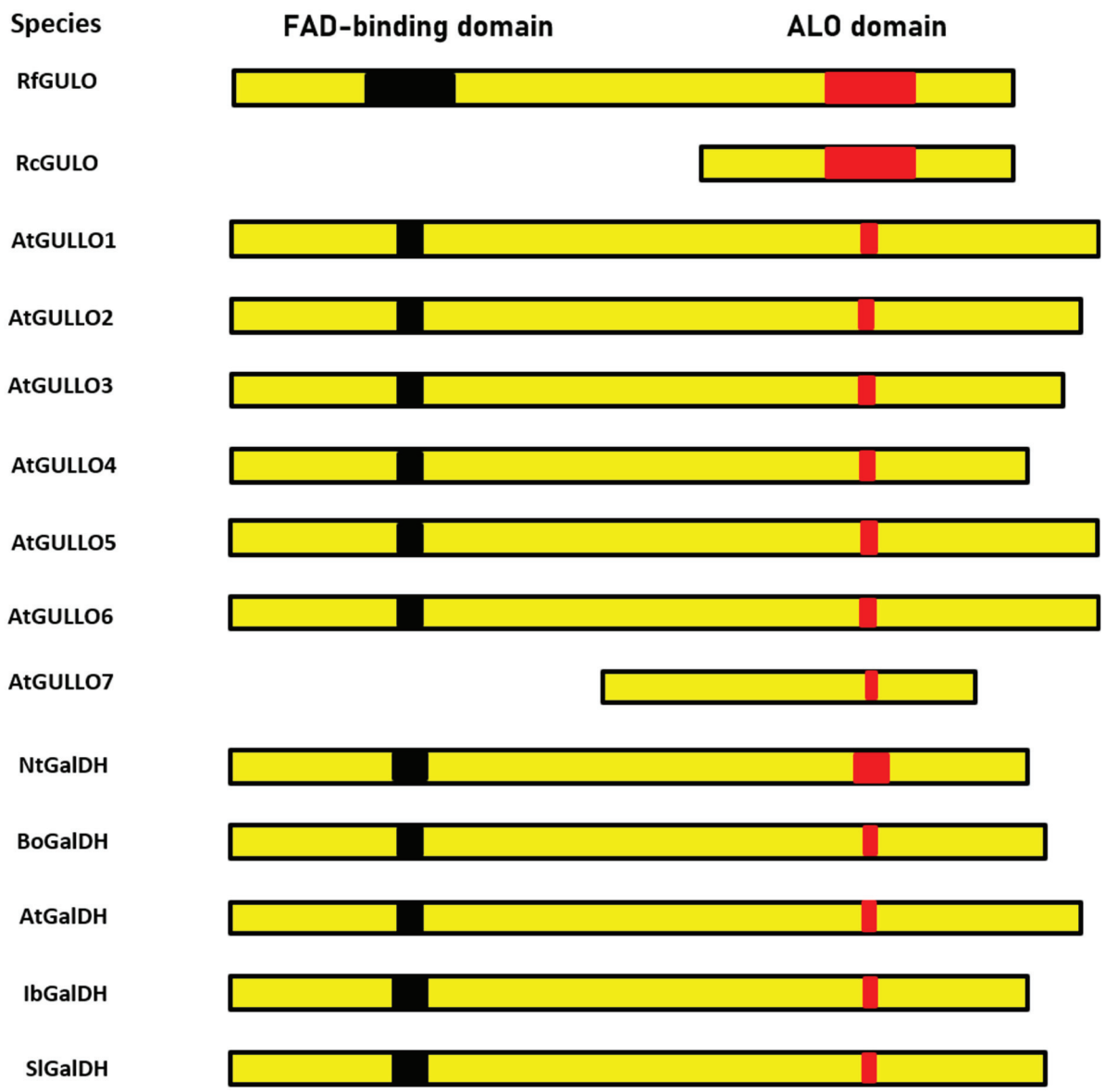


Figure 3. Schematic representation of the FAD-binding and ALO domains found in full-length rat GULO (RfGULO), C-terminal rat L-gulono-γ-lactone oxidase (RcGULO), *Arabidopsis* GULLOs and L-galactono-γ lactone dehydrogenase (GalDH) from different plant sources. Domain sequences of individual proteins were obtained from conserved domains in the NCBI Protein database and compared to that of rat fGULO using the Global Align program. The length of the domains was drawn in relation to RfGULO, which is assumed to be 100%. The accession numbers of the sequences used are as follows: RfGULO (P10867.3), *Arabidopsis* homologs (AtGULLO1 to AtGULLO7), and GalDHs from *Nicotiana tabacum* (NtGalDH; BAB13368.1), *Brassica oleracea* (BoGalDH; O47881.1), *Arabidopsis thaliana* (AtGalDH; NP_190376.1), *Ipomoea batatas* (IbGalDH; BAA34995.1), and *Solanum lycopersicum* (SlGalDH; NP_001234603.1) [43,110].

Additionally, the domain sequences of *A. thaliana* GULLO1-7 were individually compared to those of the rat full-length L-gulono-γ-lactone oxidase (RfGULO), using BLAST2seq to generate a similarity score. The similarity of the ALO domains is 20–33% when compared to rat GULO and that of the FAD-binding domains is 28–36% (with the exception of GULLO7 which is N-terminally truncated). It might be speculated that GULLO7, which lacks the FAD-binding domain, may need a hypothetical effector molecule that is produced under stress conditions or in specific plant parts (mature pollen) where it is presumably present. Different mechanisms of post-translational regulation were discussed in terms of GULLO3

and GULLO5. For example, an effector molecule may increase the catalytic efficiency of GULLO5, while GULLO3 may use another mechanism to maintain its stability in regard to proteolytic degradation [43]. The speculation about the possible catalytic activity of GULLO7 is supported by the fact that the recombinant cytoplasmic domain of rat GULO (cGULO) expressed in *E. coli* is catalytically active, although it lacks the FAD-binding domain [110]. Presumably, only the flavin cofactor, linked to a recently discovered HWXK motif at the C-terminus, is crucial for the formation of the active site of this enzyme [43,110]. However, the enzymatic activity of *A. thaliana* GULLO7 remains to be verified.

7. The Family of Aldonolactone Oxidoreductases with the Conserved HWXK Motif

Specific to the ALO domain, engaged in the last stage of AsA biosynthesis, is the C-terminal HWXK motif. Plants and protozoa contain non-covalently bound FAD, while animals and fungi contain covalently bound FAD. An N-terminal histidine residue in flavoprotein AlORs facilitates the covalent interaction between the FAD cofactor and the C-terminal histidine residue [57,117]. The covalent connection inhibits flavin modification and boosts the enzyme redox power, cofactor saturation, and protein stability [118]. The cofactor shields the enzyme against irreversible unfolding or aggregation, as evidenced by the significant increase in thermal stability observed in the presence of excess FAD [118]. A mutation in the FAD-binding region may be a factor in the activity decrease observed in *Pteropus vampyrus* Linnaeus 1758 (Chiroptera: Pteropodidae) [119]. For this reason, ALO activity could not be detected in *L. donovani* in the absence of FAD [61]. The VAO family includes AlORs, and FAD attaches to a histidine in the C-terminus of VAO. Fraaije et al. [120] found that FAD is covalently linked to the His-422 residue in the C-terminus of VAOs. A histidine is found in all covalently bound FAD-containing isoenzymes, whereas a leucine or lysine is found in non-covalent FAD-containing enzymes.

To highlight the most conserved regions of the ALO family, we performed a multiple sequence alignment of several representative members from different organisms: *A. thaliana* AtGalDH, *Brassica oleracea* BoGalDH, *Nicotiana tabacum* NtGalDH, *Rattus norvegicus* Rn-GULO, *Penicillium griseoroseum* gluconolactone oxidase PgGLO, and *Saccharomyces cerevisiae* ScALO (Figure 4). The FAD-binding domain of the enzymes exhibited the highest level of sequence conservation. The alignment makes it evident that plant GalDHs contain a leucine residue (indicated by the arrow), rather than the histidine residue required for covalent flavinylation in GULO, ALO, and GLO. This suggests that the flavin cofactor is non-covalently attached to the protein. Noticeably, the histidine in the HWXK motif located in the C-termini of all AlORs is evolutionarily conserved [43,57]. The functional significance of the HWXK motif in the *T. cruzi* GALO was investigated through the use of site-directed mutagenesis [57]. Individual mutations of His-447 and Trp-448 to glycine resulted in the loss of flavin binding and enzyme function. While Lys-450 is involved in activity that is not dependent on flavin binding, His-447 and Trp-448 are either directly or indirectly involved in interactions with flavin. After testing the *T. cruzi* GALO with alditol oxidase, Kudryashova et al. [58] postulated that His-447 and Lys-450 are situated near enough to the flavin to allow for a direct interaction. Additionally, they mentioned that His-447 might be bound by the flavin moiety. In conclusion, the flavin cofactor may be connected to the HWXK motif at the C-terminus or the FAD-binding motif at the N-terminus.

Based on the phylogenetic analysis of the AlOR sequences harboring only the HWXK motif [121] it can be concluded that the proteins lacking the HWAK motif are distantly related to GULO. It is easy to identify *GULO* genes in annotated genomes by conducting a basic Blast PHI (pattern-hit initiated) search using the HWAK motif. The HWGK motif is present in GULO encoding sequences outside of the fungi outgroup, with the exception of those from the tick, *Rhipicephalus microplus* (Canestrini, 1888) (Ixodida: Ixodidae), and the cnidarian fish parasite, *Kudoa iwatai* Egusa and Shiomitsu, 1983 (Multivalvulida: Kudoidae). The HWAK motif is present in ten *GULO*-encoding sequences from non-Bilateria and Protostomia species, which are shown as an external group to the fungal sequences in the consensus tree. According to reports, the anticipated structure of the GULO of *Mus*

musculus Linnaeus, 1758 (Rodentia: Muridae) has the GULO FAD-binding domain and the HWAK motif region adjacent to one another. It is possible that this closeness is essential for creating a covalent bond with the FAD cofactor [28]. In light of these findings, it is plausible that, in contrast to the HWGK alternative, where the protein/cofactor interaction can be significantly weaker, the inclusion of an alanine at the HWAK motif confers stronger and permanent covalent binding between GULO and the relevant cofactor. This may, therefore, be connected to the different catalytic characteristics of the proteins containing the two variations of this motif.

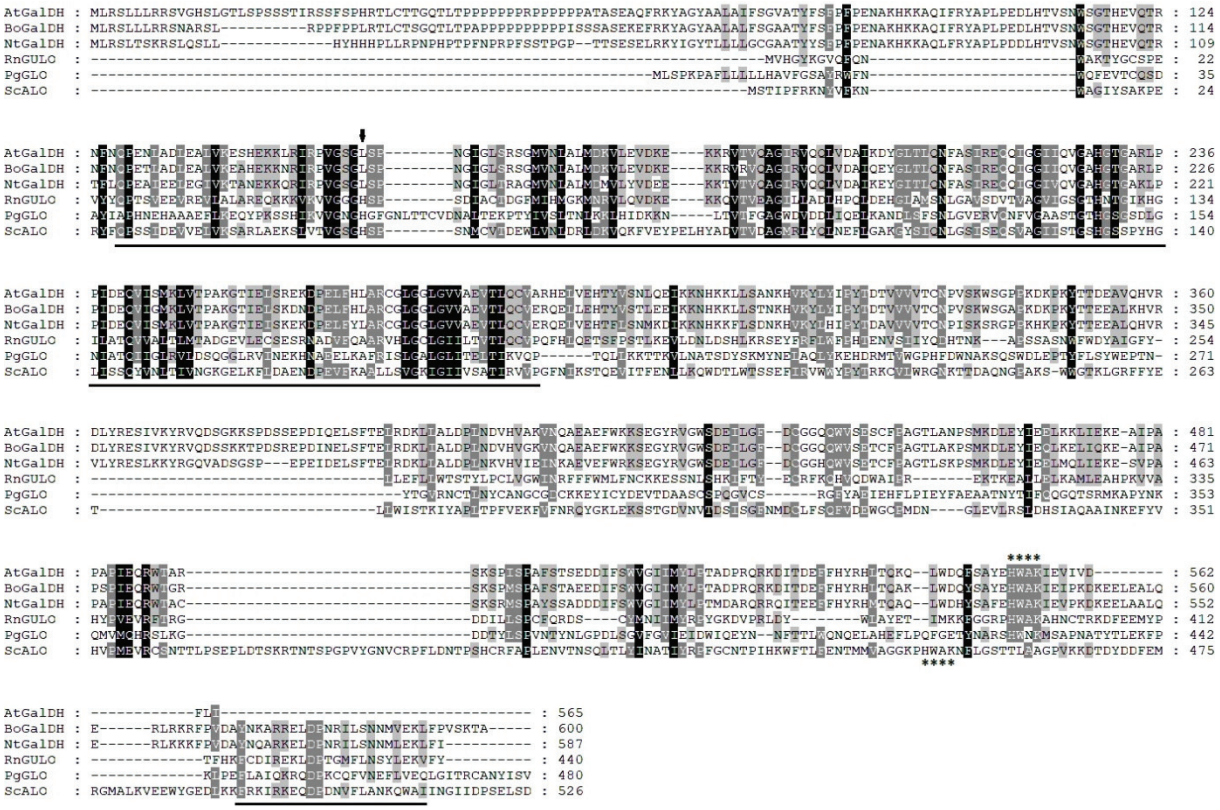


Figure 4. Multiple sequences of the full-length amino acid sequence of *A. thaliana* AtGalDH (At3g47930), with several aldonolactone oxidoreductases. The accession numbers (NCBI Entrez Protein Database) used for the multiple sequence alignment are *Brassica oleracea* BOGalDH (CAB09796), *Nicotiana tabacum* NtGalDH (BAA80870), *Rattus norvegicus* RnGULO (P10867), *Penicillium griseoroseum* gluconolactone oxidase PgGLO (AAT80870), and *Saccharomyces cerevisiae* ScALO (P54783). Alignment was performed using CLUSTAL X2. Amino acid residue numbers are shown on the right. Identical residues are shaded in black; similar residues are shaded in gray. The arrow marks the histidine residue involved in covalent binding of the FAD cofactor in GUO, ALO, and GLO, or the leucine residue in GalDH proteins. The FAD-binding domain in both the N-terminal and C-terminal parts is underlined. The asterisks mark the HWAK motif in RnGULO, ScALO, and all GalDH proteins.

8. Sequence Analysis of Rat L-gulono-γ-lactone Oxidase

The analysis of the nucleotide sequences from the human genome indicates that the *GULO* pseudogene maps to human chromosome 8p21. It accumulated numerous mutations over 40 million years ago, since it stopped being active [28,109]. At the protein level, it shows over 94% similarity to the rat GULO [80]. In terms of its amino acid identity, the vertebrate GULO shares 64–95% of its identity and has fewer conserved exons than the 12 found in the genes of mice and rats (Table 2) [122]. Remarkably, the causative agent of tuberculosis, *M. tuberculosis*, has a protein (Rv1771) encoded in its genome that shares approximately 32% of its identity with the rat GULO protein. Additionally, the *Rv1771*

gene was cloned and expressed in *E. coli*, then affinity chromatography was used to purify and characterize the resulting protein [105].

Table 2. Conservation of genomic structure of *L-gulono-γ-lactone oxidase* gene in selected mammals.

Exon Number	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
Rat	•	•	•	•	•	•	•	•	•	•	•	•
Mouse	•	•	•	•	•	•	•	•	•	•		•
Guinea pig		•	•	•		•	•	•	•	•	•	•
Human								•	•	•		•

The conserved exon structure of mice and rats is shown aligned with the guinea pig and human genes. Missing dots indicate the lack of a corresponding exon.

9. Conclusions and Future Perspectives

This review shortly summarizes the role of ascorbic acid (AsA) in biological processes and discusses various AsA biosynthetic pathways, particularly in plants and animals. The main focus is on L-gulono-γ-lactone oxidase (GULO) from various species, their exact location, chemical characterization, and the kinetic parameters. We also highlight our recent results showing that the recombinant C-terminal cytoplasmic domain in rat GULO, lacking the FAD-binding site and containing only the HWAK motif, is enzymatically active when produced in *E. coli* cells. It demonstrates that the HWAK motif is sufficient for binding the flavin cofactor. This finding might shed more light on the *A. thaliana GULLO7* gene (abundantly expressed in mature pollen) and its protein product. The predicted protein is shorter than other GULLOs and aligns only with the C-terminal part of other GULOs, but might be expected to have enzymatic GULO activity, despite the absence of a FAD-binding domain.

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Conflicts of Interest: The authors declare that there are no conflicts of interest.

Abbreviations

AsA, L-ascorbic acid; NO, nitric oxide; 2-KLG, 2-keto-L-gulonic acid; AlORs, aldonolactone oxidoreductases; ALO, D-arabinono-γ-lactone oxidase; FAD, flavin adenine dinucleotide; ROS, reactive oxygen species; VAO, vanillyl alcohol oxidase; GUL, L-gulono-γ-lactone; GULO, animal L-gulono-γ-lactone oxidase; GULLO, plant L-gulono-γ-lactone oxidase; GLDH, L-gulono-γ-lactone dehydrogenase; GAL, L-galactono-γ-lactone; GalDH, L-galactono-γ-lactone dehydrogenase; GALO, L-galactono-γ-lactone oxidase; Cyt c, cytochrome c.

References

1. Figueroa-Méndez, R.; Rivas-Arancibia, S. Vitamin C in health and disease: Its role in the metabolism of cells and redox state in the brain. *Front. Psychol.* **2015**, *6*, 397–407. [CrossRef] [PubMed]
2. Hasan, L.; Vogeli, P.; Stoll, P.; Špilar Kramer, Š.; Stranzinger, G.; Neuenschwander, S. Intragenic deletion in the gene encoding L-gulonolactone oxidase causes vitamin C deficiency in pigs. *Mamm. Genome* **2004**, *15*, 323–333. [CrossRef] [PubMed]

3. Nishikimi, M. Purification and characterization of L-gulono-γ-lactone oxidase from rat and goat liver. *Arch. Biochem. Biophys.* **1976**, *175*, 427–435. [CrossRef] [PubMed]
4. Wheeler, G.; Ishikawa, T.; Pornsaksit, V.; Smirnoff, N. Evolution of alternative biosynthetic pathways for vitamin C following plastid acquisition in photosynthetic eukaryotes. *eLife* **2015**, *4*, e06369. [CrossRef]
5. Mansueto, A.; Good, D.J. Conservation of a chromosome 8 inversion and exon mutations confirm common gulonolactone oxidase gene evolution among primates, including *H. Neanderthalensis*. *J. Mol. Evol.* **2024**, *92*, 266–277. [CrossRef]
6. Mandl, J.; Szarka, A.; Banhegyi, G. Vitamin C: Update on physiology and pharmacology. *Br. J. Pharmacol.* **2009**, *157*, 1097–1110. [CrossRef]
7. Margittai, E.; Banhegyi, G.; Kiss, A.; Nagy, G.; Mandl, J.; Schaff, Z.; Csala, M. Scurvy leads to endoplasmic reticulum stress and apoptosis in the liver of guinea pigs. *J. Nutr.* **2005**, *135*, 2530–2534. [CrossRef]
8. Dhar-Mascareno, M.; Carcamo, J.M.; Golde, D.W. Hypoxia–reoxygenation-induced mitochondrial damage and apoptosis in human endothelial cells are inhibited by vitamin C. *Free Radic. Biol. Med.* **2005**, *38*, 1311–1322. [CrossRef]
9. Fischer, H.; Schwarzer, C.; Illek, B. Vitamin C controls the cystic fibrosis transmembrane conductance regulator chloride channel. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 3691–3696. [CrossRef]
10. Brown, L.A.S.; Jones, D.P. Antioxidant action of vitamin C in the lung. In *Vitamin C in Health and Disease*; Packer, L., Fuchs, J., Eds.; CRC Press: Boca Raton, FL, USA, 1997; pp. 265–278.
11. May, J.M.; Huang, J.; Qu, Z.C. Macrophage uptake and recycling of ascorbic acid: Response to activation by lipopolysaccharide. Free Radic. *Biol. Med.* **2005**, *39*, 1449–1459. [CrossRef]
12. Chatterjee, I.B. Evolution and the biosynthesis of ascorbic acid. *Science* **1973**, *182*, 1271–1272. [CrossRef] [PubMed]
13. Zhou, H.X.; Milne, R.I.; Ma, X.L.; Song, Y.Q.; Fang, J.Y.; Sun, H.; Zha, H.G. Characterization of a L-gulono-1, 4-lactone oxidase like protein in the floral nectar of *Mucuna sempervirens*, Fabaceae. *Front. Plant Sci.* **2018**, *9*, 1109–1119. [CrossRef] [PubMed]
14. Sivadas, S.; Mohanty, A.K.; Rajesh, S.; Muthuvel, S.K.; Vasanthi, H.R. Molecular modelling and biological evaluation of phytomolecules as potential activators of gluconolactone oxidase (GULO). *J. Biomol. Struct. Dyn.* **2023**, *41*, 15124–15136. [CrossRef] [PubMed]
15. Kim, H.J.; Lee, S.I.; Lee, D.H.; Smith, D.; Jo, H.; Schellhorn, H.E.; Boo, Y.C. Ascorbic acid synthesis due to L-gulono-1, 4-lactone oxidase expression enhances NO production in endothelial cells. *Biochem. Biophys. Res. Commun.* **2006**, *345*, 1657–1662. [CrossRef] [PubMed]
16. Grollman, A.P.; Lehninger, A.L. Enzymic synthesis of L-ascorbic acid in different animal species. *Arch. Biochem. Biophys.* **1957**, *69*, 458–467. [CrossRef]
17. Jensen, P.T.; Basse, A.; Nielsen, D.H.; Larsen, H. Congenital ascorbic acid deficiency in bigs. *Acta Vet. Scand.* **1983**, *24*, 392–402. [CrossRef] [PubMed]
18. Carr, R.S.; Neff, J.M. Determination of ascorbic acid in tissues of marine animals by liquid chromatography with electrochemical detection. *Anal. Chem.* **1980**, *52*, 2428–2430. [CrossRef]
19. Massie, H.R.; Shumway, M.E.; Whitney, S.J.P.; Sternick, S.M.; Aiello, V.R. Ascorbic acid in Drosophila and changes during aging. *Exp. Gerontol.* **1991**, *26*, 487–494. [CrossRef]
20. Bahadorani, S.; Bahadorani, P.; Phillips, J.P.; Hilliker, A.J. The effects of vitamin supplementation on Drosophila life span under normoxia and under oxidative stress. *J. Gerontol. Ser. A Biol. Sci. Med. Sci.* **2008**, *63*, 35–42. [CrossRef]
21. Barbehenn, R.V.; Bumgarner, S.L.; Roosen, E.F.; Martin, M.M. Antioxidant defenses in caterpillars: Role of the ascorbate-recycling system in the midgut lumen. *J. Insect Physiol.* **2001**, *47*, 349–357. [CrossRef]
22. Kolawole, A.O.; Olajuyigbe, F.M.; Ajele, J.O.; Adedire, C.O. Activity of the antioxidant defense system in a typical bioinsecticide- and synthetic insecticide-treated cowpea storage beetle *Callosobrochus maculatus* F. (Coleoptera: *Chrysomelidae*). *Int. J. Insect Sci.* **2014**, *6*, IJIS-S19434. [CrossRef] [PubMed]
23. Zhang, Y.; Wu, H.; Xie, J.; Jiang, R.; Deng, C.; Pang, H. Transcriptome responses to heat and cold-stress in ladybirds (*Cryptolaemus montrouzieri* Mulasnt) analyzed by deep-sequencing. *Biol. Res.* **2015**, *48*, 66. [CrossRef] [PubMed]
24. Patananan, A.N.; Budenholzer, L.M.; Pedraza, M.E.; Torres, E.R.; Adler, L.N.; Clarke, S.G. The invertebrate Caenorhabditis elegans biosynthesizes ascorbate. *Arch. Biochem. Biophys.* **2015**, *569*, 32–44. [CrossRef] [PubMed]
25. Drouin, G.; Godin, J.R.; Page, B. The genetics of vitamin C loss in vertebrates. *Curr. Genom.* **2011**, *12*, 371–378. [CrossRef] [PubMed]
26. Yamaguchi, M.; Yamamoto, T. Purification of calcium binding substance from soluble fraction of Normal rat liver. *Chem. Pharm. Bull.* **1978**, *26*, 1915–1918. [CrossRef]
27. Tsurusaki, Y.; Yamaguchi, M. Role of regucalcin in liver nuclear function: Binding of regucalcin to nuclear protein or DNA and modulation of tumor related gene expression. *Int. J. Mol. Med.* **2004**, *14*, 277–281. [CrossRef]
28. Henriques, S.F.; Duque, P.; López-Fernández, H.; Vázquez, N.; Fdez-Riverola, F.; Reboiro-Jato, M.; Vieira, C.P.; Vieira, J. Multiple independent L-gulonolactone oxidase (GULO) gene losses and vitamin C synthesis reacquisition events in non-Deuterostomian animal species. *BMC Evol. Biol.* **2019**, *19*, 126. [CrossRef]
29. Foyer, C.H.; Shigeoka, S. Understanding oxidative stress and antioxidant functions to enhance photosynthesis. *Plant Physiol.* **2011**, *155*, 93–100. [CrossRef]
30. Smirnof, N.; Wheeler, G.L. Ascorbic acid in plants: Biosynthesis and function. *Crit. Rev. Biochem. Mol. Biol.* **2000**, *35*, 291–314. [CrossRef]

31. Smirnoff, N. Vitamin C: The metabolism and functions of ascorbic acid in plants. In *Biosynthesis of Vitamins in Plants: Vitamins B6, B8, B9, C, E, K*; Rebeille, F., Ed.; Academic Press: Cambridge, MA, USA, 2011; pp. 107–177. [CrossRef]
32. Liang, D.; Zhu, T.; Ni, Z.; Lin, L.; Tang, Y.; Wang, Z.; Wang, X.; Wang, J.; Lv, X.; Xia, H. Ascorbic acid metabolism during sweet cherry (*Prunus avium*) fruit development. *PLoS ONE* **2017**, *12*, e0172818. [CrossRef]
33. Conklin, P.L.; Nprris, S.R.; Wheeler, G.L.; Williams, E.H.; Smirnof, N.; Last, R.L. Genetic evidence for the role of GDP-mannose in plant ascorbic acid (vitamin C) biosynthesis. *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 4198–4203. [CrossRef] [PubMed]
34. Wang, P.; Zeng, W.; Xu, S.; Du, G.; Zhou, J.; Chen, J. Current challenges facing one-step production of l-ascorbic acid. *Biotechnol. Adv.* **2018**, *36*, 1882–1899. [CrossRef] [PubMed]
35. Zhang, L.; Wang, Z.; Xia, Y.; Kai, G.; Chen, W.; Tang, K. Metabolic engineering of plant L-Ascorbic acid biosynthesis: Recent trends and applications. *Crit. Rev. Biotechnol.* **2007**, *27*, 173–182. [CrossRef] [PubMed]
36. Branduardi, P.; Fossati, T.; Sauer, M.; Pagani, R.; Porro, D. Biosynthesis of vitamin C by yeast leads to increased stress resistance. *PLoS ONE* **2007**, *2*, 1092–1100. [CrossRef] [PubMed]
37. Tian, Y.S.; Deng, Y.D.; Zhang, W.H.; Xu, J.; Gao, J.J.; Fu, X.Y.; Han, H.J.; Li, Z.J.; Wang, L.J.; Peng, R.H.; et al. Metabolic engineering of *Escherichia coli* for direct production of vitamin C from D-glucose. *Biotechnol. Biofuels Bioprod.* **2022**, *15*, 86–98. [CrossRef] [PubMed]
38. Wheeler, G.L.; Jones, M.A.; Smirnoff, N. The biosynthetic pathway of vitamin C in higher plants. *Nature* **1998**, *393*, 365–369. [CrossRef]
39. Agius, F.; Lamothe, R.G.; Caballero, J.L.; Blanco, J.M.; Botella, M.A.; Valpuesta, V. Engineering increased vitamin C levels in plants by over-expression of a D-galacturonic acid reductase. *Nat. Biotechnol.* **2003**, *21*, 177–181. [CrossRef]
40. Bulley, S.; Laing, W. The regulation of ascorbate biosynthesis. *Curr. Opin. Plant Biol.* **2016**, *33*, 15–22. [CrossRef]
41. Davey, M.W.; Gilot, C.; Persiau, G.; Østergaard, J.; Han, Y.; Bauw, G.C.; Van Montagu, M.C. Ascorbate biosynthesis in Arabidopsis cell suspension culture. *Plant Physiol.* **1999**, *121*, 535–543. [CrossRef]
42. Wolucka, B.A.; Van Montagu, M. GDP-mannose 3′,5′-epimerase forms GDP-L-gulose, a putative intermediate for the de novo biosynthesis of vitamin C in plants. *J. Biol. Chem.* **2003**, *287*, 47483–47490. [CrossRef]
43. Aboobucker, S.I.; Suza, W.P.; Lorence, A. Characterization of two *Arabidopsis* L-gulono-1, 4-lactone oxidases, AtGulLO3 and AtGulLO5, involved in ascorbate biosynthesis. *React. Oxyg. Species* **2017**, *40*, 389–427. [CrossRef] [PubMed]
44. Lorence, A.; Chevone, B.I.; Mendes, P.; Nessler, C.L. Myoinositol oxygenase offers a possible entry point into plant ascorbate biosynthesis. *Plant Physiol.* **2004**, *134*, 1200–1205. [CrossRef] [PubMed]
45. Smirnoff, N.; Wheeler, G.L. The ascorbate biosynthesis pathway in plants is known, but there is a way to go with understanding control and functions. *J. Exp. Bot.* **2024**, *75*, 2604–2630. [CrossRef] [PubMed]
46. Pappenberger, G.; Hohmann, H.P. Industrial protein of L-ascorbic acid (vitamin C) and D-isoascorbic acid. *Adv. Biochem. Eng. Biotechnol.* **2014**, *143*, 143–188. [CrossRef] [PubMed]
47. Reichstein, T.; Grüssner, A. Eine ergiebige synthese der 1-Ascorbinsäure (C-Vitamin). *Helv. Chim. Acta.* **1934**, *17*, 311–328. [CrossRef]
48. Hancock, R.D.; Viola, R. The use of micro-organisms for l-ascorbic acid production: Current status and future perspectives. *Appl. Microbiol. Biotechnol.* **2001**, *56*, 567–576. [CrossRef] [PubMed]
49. Zhou, J.; Du, G.; Chen, J. Metabolic engineering of microorganisms for vitamin C production. In *Reprogramming Microbial Metabolic Pathways Subcellular Biochemistry*; Chen, J., Quinn, P., Wang, X., Eds.; Springer: Dordrecht, The Netherlands, 2013; pp. 241–259.
50. Yin, G.L. Production of vitamin C precursor-2-keto-l-gulonic acid from d-sorbitol by mixed culture of microorganisms. *Acta Microbiol. Sin.* **2001**, *41*, 709–715.
51. Wang, E.X.; Ding, M.Z.; Ma, Q.; Dong, X.T.; Yuan, Y.J. Reorganization of a synthetic microbial consortium for one-step vitamin C fermentation. *Microb. Cell Fact.* **2016**, *15*, 21. [CrossRef]
52. Lazarus, R.A.; Seymour, J.L.; Staford, R.K.; Dennis, M.S.; Anderson, S. A biocatalytic approach to vitamin C production. In *Biocatalysis Van Nostrand Reinhold Catalysis Series*; Abramowicz, D.A., Ed.; Springer: Dordrecht, The Netherlands, 1990; pp. 135–155.
53. Zou, Y.; Hu, M.; Lv, Y.; Wang, Y.; Song, H.; Yuan, Y.J. Enhancement of 2-ketogulonic acid yield by serial subcultivation of co-cultures of bacillus cereus and ketogulonigenium vulgare. *Bioresour. Technol.* **2013**, *132*, 370–373. [CrossRef]
54. Zeng, W.; Wang, P.; Li, N.; Li, J.; Zhou, J. Production of 2-keto-l-gulonic acid by metabolically engineered *Escherichia coli*. *Bioresour. Technol.* **2020**, *318*, 124069–124078. [CrossRef]
55. Nishikimi, M.; Kobayashi, J.; Yagi, K. Production by a baculovirus expression system of the apoprotein of L-gulono-γ-lactone oxidase, a flavoenzyme possessing a covalently-bound FAD. *Mol. Biol. Int.* **1994**, *33*, 313–320.
56. Wilkinson, S.R.; Prathalingam, S.R.; Taylor, M.C.; Horn, D.; Kelly, J.M. Vitamin C biosynthesis in trypanosomes:A role for the glycosome. *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 11645–11650. [CrossRef] [PubMed]
57. Logan, F.J.; Taylor, M.C.; Wilkinson, S.R.; Kaur, H.; Kelly, J.M. The terminal step in vitamin C biosynthesis in *Trypanosoma cruzi* is mediated by a FMN-dependent galactonolactone oxidase. *Biochem. J.* **2007**, *407*, 419–426. [CrossRef] [PubMed]
58. Kudryashova, E.V.; Leferink, N.G.H.; Slot, I.G.M.; Van Berkel, W.J.H. Galactonolactone oxidoreductase from *Trypanosoma cruzi* employs a FAD cofactor for the synthesis of vitamin C. *Biochim. Biophys. Acta* **2011**, *1814*, 545–552. [CrossRef]
59. Leferink, N.G.H.; Van den Berg, W.A.M.; van Berkel, W.J.H. L-galactono-γ-lactone dehydrogenase from *Arabidopsis thaliana*, a flavoprotein involved in vitamin C biosynthesis. *FEBS J.* **2008**, *275*, 713–726. [CrossRef]

60. Yabuta, Y.; Yoshimura, K.; Takeda, T.; Shigeoka, S. Molecular characterization of tobacco mitochondrial L-galactono-g-dehydrogenase and its expression in *Escherichia coli*. *Plant Cell Physiol.* **2000**, *41*, 666–675. [CrossRef]
61. Biyani, N.; Madhubala, R. *Leishmania donovani* encodes a functional enzyme involved in vitamin C biosynthesis: Arabino-γ-lactone oxidase. *Mol. Biochem. Parasitol.* **2011**, *180*, 76–99. [CrossRef]
62. Benen, J.A.; Sánchez-Torres, P.; Wagemaker, M.J.; Fraaije, M.W.; van Berkel, W.J.; Visser, J. Molecular cloning, sequencing, and heterologous expression of the *vaoA* gene from *Penicillium simplicissimum* CBS 170.90 encoding vanillyl-alcohol oxidase. *J. Biol. Chem.* **1998**, *273*, 7865–7872. [CrossRef] [PubMed]
63. Jin, J.; Mazon, H.; van den Heuvel, R.H.H.; Janssen, D.B.; Fraaije, M.W. Discovery of a eugenol oxidase from Rhodococcus sp.s train RHA1. *FEBS J.* **2007**, *274*, 2311–2321. [CrossRef]
64. Heuts, D.P.H.M.; van Hellemond, E.W.; Janssen, D.B.; Fraaije, M.W. Discovery, characterization, and kinetic analysis of an alditol oxidase from Streptomyces coelicolor. *J. Biol. Chem.* **2007**, *282*, 20283–20291. [CrossRef]
65. Heuts, D.P.H.M.; Janssen, D.B.; Fraaije, M.W. Changing the substrate specificity of a chitooligosaccharide oxidase from *Fusarium graminearum* by modelinspired site-directed mutagenesis. *FEBS Lett.* **2007**, *581*, 4905–4909. [CrossRef] [PubMed]
66. Ganz, P.R.; Dudani, A.K.; Tackaberry, E.S.; Sardana, R.; Sauder, C.; Cheng, X.; Altosaar, I. Expression of human blood proteins in transgenic plants: The cytokine GM-CSF as a model protein. In *Transgenic Plants: A Production System for Industrial and Pharmaceutical Proteins*; Owen, M.R.L., Pen, J., Eds.; Wiley: Hoboken, NJ, USA, 1996; pp. 281–297.
67. Rosano, G.L.; Ceccarelli, E.A. Recombinant protein expression in *Escherichia coli*: Advances and challenges. *Front. Microbiol.* **2014**, *5*, 172–188. [CrossRef] [PubMed]
68. Fischer, R.; Emans, N. Molecular farming of pharmaceutical proteins. *Transgenic Res.* **2000**, *9*, 279–299. [CrossRef] [PubMed]
69. Goldstein, D.A.; Thomas, J.A. Biopharmaceuticals derived from genetically modified plants. *QJM-Int. J. Med.* **2004**, *97*, 705–716. [CrossRef] [PubMed]
70. Sirko, A.; Vaněk, T.; Góra-Sochacka, A.; Redkiewicz, P. Recombinant cytokines from plants. *Int. J. Mol. Sci.* **2011**, *12*, 3536–3552. [CrossRef]
71. Redkiewicz, P.; Sirko, A.; Kamel, K.A.; Góra-Sochacka, A. Plant expression systems for production of hemagglutinin as a vaccine against influenza virus. *Acta Biochim. Pol.* **2014**, *61*, 551–560. [CrossRef]
72. Gregory, J.A.; Topol, A.B.; Doerner, D.Z.; Mayfield, S. Alga-produced cholera toxin-Pfs25 fusion proteins as oral vaccines. *Appl. Environ. Microbiol.* **2013**, *79*, 3917–3925. [CrossRef]
73. Jain, A.K.; Nessler, C.L. Metabolic engineering of an alternative pathway for ascorbic acid biosynthesis in plants. *Mol. Breed.* **2000**, *6*, 73–78. [CrossRef]
74. Lim, M.Y.; Pulla, R.K.; Park, J.M.; Harn, C.H.; Jeong, B.R. Overexpression of l-gulono-γ-lactone oxidase (GLOase) gene leads to ascorbate accumulation with enhanced abiotic stress tolerance in tomato. In Vitro Cell. *Dev. Biol. Plant.* **2012**, *48*, 453–461. [CrossRef]
75. Lisko, K.A.; Torres, R.; Harris, R.S.; Belisle, M.; Vaughan, M.M.; Jullian, B.; Chevone, B.I.; Mendes, P.; Nessler, C.L.; Lorence, A. Elevating vitamin C content via overexpression of myo-inositol oxygenase and L-gulono-γ-lactone oxidase in Arabidopsis leads to enhanced biomass and tolerance to abiotic stresses. *In Vitro Cell. Dev. Biol. Plant.* **2013**, *49*, 643–655. [CrossRef]
76. Kwon, S.Y.; Choi, S.M.; Ahn, Y.O.; Lee, H.S.; Lee, H.B.; Park, Y.M.; Kwak, S.S. Enhanced stress-tolerance of transgenic plants expressing *a human dehydroascorbate reductase* gene. *J. Plant Physiol.* **2003**, *160*, 347–353. [CrossRef] [PubMed]
77. Hemavathi; Upadhyaya, C.P.; Akula, N.; Young, K.E.; Chun, S.C.; Kim, D.H.; Park, S.W. Enhanced ascorbic acid accumulation in transgenic potato confers tolerance to various abiotic stresses. *Biotechnol. Lett.* **2010**, *32*, 321–330. [CrossRef] [PubMed]
78. Chen, Z.; Todd, E.; Ling, Y.J.; Chang, S.C.; Gallie, D.R. Increasing vitamin C content of plants through enhanced ascorbate recycling. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 3525–3530. [CrossRef] [PubMed]
79. Maruta, T.; Ichikawa, Y.; Mieda, T.; Takeda, T.; Tamoi, M.; Yabuta, Y.; Ishikawa, T.; Shigeoka, S. The contribution of Arabidopsis homologs of L-gulono-1, 4-lactone oxidase to the biosynthesis of ascorbic acid, Biosci. *Biotechnol. Biochem.* **2010**, *74*, 1494–1497. [CrossRef] [PubMed]
80. Ha, M.N.; Graham, F.L.; D'ouza, C.K.; Muller, W.J.; Igdoura, S.A.; Schellhorn, H.E. Functional rescue of vitamin C synthesis deficiency in human cells using adenoviral-based expression of murine L-gulono-γ-lactone oxidase. *Genomics* **2004**, *83*, 482–492. [CrossRef] [PubMed]
81. Krasnov, A.; Reinisalo, M.; Pitkänen, T.i.; Nishikimi, M.; Mölsä, H. Expression of rat gene for l-gulono-γ-lactone oxidase, the key enzyme of l-ascorbic acid biosynthesis, in guinea pig cells and in teleost fish rainbow trout (*Oncorhynchus mykiss*). *Biochim. Biophys. Acta BBA Gen. Subj.* **1998**, *1381*, 241–248. [CrossRef]
82. Yagi, K.; Koshizaka, T.; Kito, M.; Ozawa, T.; Nishikimi, M. Expression in monkey cells of the missing enzyme in L-ascorbic acid biosynthesis, L-gulono-gamma-lactone oxidase, Biochem. *Biophys. Res. Commun.* **1991**, *177*, 659–663. [CrossRef]
83. Nam, Y.K.; Cho, Y.S.; Douglas, S.E.; Gallant, J.W.; Reith, M.E.; Kim, D.S. Isolation and transient expression of a cDNA encoding l-gulono-γ-lactone oxidase, a key enzyme for l-ascorbic acid biosynthesis, from the tiger shark *Scyliorhinus torazame*. *Aquaculture* **2002**, *209*, 271–284. [CrossRef]
84. Aboobucker, S.I.; Lorence, A. Recent progress on the characterization of aldonolactone oxidoreductases. *Plant Physiol. Biochem.* **2016**, *98*, 171–185. [CrossRef]
85. Eliceiri, G.L.; Lai, E.K.; Mccay, P.B. Gulonolactone oxidase. Solubilization, properties, and partial purification. *J. Biol. Chem.* **1969**, *244*, 2641–2645. [CrossRef]

86. Braun, L.; Mile, V.; Schaff, Z.; Csala, M.; Kardon, T.; Mandl, J.; Bánhegyi, G. Induction and peroxisomal appearance of gulonolactone oxidase upon clofibrate treatment in mouse liver. *FEBS Lett.* **1999**, *458*, 359–362. [CrossRef] [PubMed]
87. Kiuchi, K.; Nishikimi, M.; Yagi, K. Purification and characterization of L-gulonolactone oxidase from chicken kidney microsomes. *Biochemistry* **1982**, *21*, 5076–5082. [CrossRef] [PubMed]
88. Koshizaka, T.; Nishikimi, M.; Ozawa, T.; Yagi, K. Isolation and sequence analysis of a complementary DNA encoding rat liver L -gulono-gamma-lactone oxidase, a key enzyme for L -ascorbic acid biosynthesis. *J. Biol. Chem.* **1988**, *263*, 1619–1621. [CrossRef] [PubMed]
89. Puskas, F.; Braun, L.; Csala, M.; Kardon, T.; Marcolongo, P.; Benedetti, A.; Mandl, J.; Banhegyi, G. Gulonolactone oxidase activitydependent intravesicular glutathione oxidation in rat liver microsomes. *FEBS Lett.* **1998**, *430*, 293–296. [CrossRef] [PubMed]
90. Linster, C.L.; Van Schaftingen, E. Vitamin C: Biosynthesis, recycling and degradation in mammals. *FEBS J.* **2007**, *274*, 1–22. [CrossRef]
91. Smirnof, N. Ascorbic acid metabolism and functions: A comparison of plants and mammals. *Free Radic Biol Med.* **2018**, *122*, 116–129. [CrossRef]
92. Douce, R. Functions of plant mitochondrial membranes. In *Mitochondria in Higher Plants*; American Society of Plant Physiologists Monograph Series; Academic Press: London, UK, 1985; pp. 77–153.
93. Mackenzie, S.; McIntosh, L. Higher plant mitochondria. *Plant Cell.* **1999**, *11*, 571–585. [CrossRef]
94. Bartoli, C.G.; Pastori, G.M.; Foyer, C.H. Ascorbate biosynthesis in mitochondria is linked to the electron transport chain between complexes III and IV. *Plant Physiol.* **2000**, *123*, 335–344. [CrossRef]
95. Boudart, G.; Jamet, E.; Rossignol, M.; Lafitte, C.; Borderies, G.; Jauneau, A.; Esquerre-Tugaye, M.T.; Pont-Lezica, R. Cell wall proteins in apoplastic fluids of rosettes: Identification by mass spectrometry and bioinformatics. *Proteomics* **2005**, *5*, 212–221. [CrossRef]
96. Mawuenyega, K.G.; Forst, C.V.; Dobos, K.M.; Belisle, J.T.; Chen, J.; Bradbury, E.M.; Bradbury, A.R.; Chen, X. *Mycobacterium tuberculosis* functional network analysis by global subcellular protein profiling. *Mol. Biol. Cell.* **2005**, *16*, 396–404. [CrossRef]
97. Leferink, N.G.; Fraaije, M.W.; Joosten, H.J.; Schaap, P.J.; Mattevi, A.; van Berkel, W.J. Identification of a gatekeeper residue that prevents dehydrogenases from acting as oxidases. *J. Biol. Chem.* **2009**, *284*, 4392–4397. [CrossRef] [PubMed]
98. Sugisawa, T.; Setsuko, O.; Matzinger, P.K.; Hoshino, T. Isolation and characterization of a new vitamin C producing enzyme (L-gulono-g-lactone dehydrogenase) of bacterial origin. *Biosci. Biotech. Biochem.* **1995**, *59*, 190–196. [CrossRef]
99. Okamura, M. Purification and properties of L-gulono-γ-lactone oxidase from *Grifola frondosa*. *J. Nutr. Sci. Vitaminol.* **2001**, *47*, 258–262. [CrossRef] [PubMed]
100. Kazuko, O.; Ishikawa, S.; Nishikawa, M.; Mizuno, H.; Yamamoto, T. Purification and properties of L-galactono-γ-lactone dehydrogenase, a key enzyme for ascorbic acid biosynthesis, from sweet potato roots. *Biochem. J.* **1995**, *117*, 120–124. [CrossRef]
101. Leferink, N.G.H.; Van Berkel, W.J.H. Aldonolactone oxidoreductases. In *Flavins and Flavoproteins: Methods and Protocols*; Methods in Molecular Biology; Weber, S., Schleicher, E., Eds.; Humana Press: New York, NY, USA, 2014; Volume 1146, pp. 95–111. [CrossRef]
102. Kenney, W.C.; Edmondson, D.E.; Singer, T.P. Identification of the covalently bound flavin of L-gulono-γ-lactone oxidase. *Biochem. Biophys. Res. Commun.* **1976**, *71*, 1194–1200. [CrossRef]
103. Bleeg, H.S. L-ascorbic acid in yeast and isolation of L-galactono-gamma-lactone oxidase from the mitochondria. *Enzymologia* **1966**, *31*, 105–112.
104. Shigeoka, S.; Nakano, Y.; Kitaoka, S. Some properties and subcellular localization of l-gulono-γ-lactone dehydrogenase in *Euglena gracilis* z. *Agric. Biol. Chem.* **1979**, *43*, 2187–2188. [CrossRef]
105. Wolucka, B.A.; Communi, D. *Mycobacterium tuberculosis* possesses a functional enzyme for the synhesis of vitamin C, L-gulono-γ-lactone dehydrogenase. *FEBS J.* **2006**, *273*, 4435–4445. [CrossRef]
106. Mapson, L.W.; Breslow, E. Biological synthesis of ascorbic acid: L-Galactono-γ-lactone dehydrogenase. *Biochem. J.* **1958**, *68*, 395–406. [CrossRef]
107. Nakagawa, H.; Asano, A. Ascorbate synthesizing system in rat liver microsomes. I. Gulonolactone-reducible pigment as a prosthetic group of gulonolactone oxidase. *J. Biochem.* **1970**, *68*, 737e–746e. [CrossRef]
108. Burns, J.J.; Peyser, P.; Moltz, A. Missing step in guinea pigs required for the biosynthesis of L-ascorbic acid. *Science* **1956**, *124*, 1148–1149. [CrossRef] [PubMed]
109. Nishikimi, M.; Yagi, K. Molecular basis for the deficiency in humans of gulonolactone oxidase, a key enzyme for ascorbic acid biosynthesis. *Am. J. Clin. Nutr.* **1991**, *4*, l203S–1208S. [CrossRef] [PubMed]
110. Gad, A.A.M.; Gora-Sochacka, A.; Sirko, A. Recombinant C-terminal catalytic domain of rat L-gulono lactone oxidase produced in bacterial cells is enzymatically active. *Curr. Issues Mol. Biol.* **2024**, *46*, 8958–8968. [CrossRef]
111. Mapson, L.W.; Isherwood, F.A.; Chen, Y.T. Biological synthesis of L-ascorbic acid: The conversion of L-galactono-g-lactone into L-ascorbic acid by plant mitochondria. *Biochem. J.* **1954**, *56*, 21–28. [CrossRef]
112. Mutsuda, M.; Ishikawa, T.; Takeda, T.; Shigeoka, S. Subcellular localization and properties of L-galactono-g-lactone dehydrogenase in spinach leaves. *Biosci. Biotechnol. Biochem.* **1995**, *59*, 1983–1984. [CrossRef]

113. Oba, K.; Fukui, M.; Imai, Y.; Iriyama, S.; Nogami, K. L-Galactono-γ-lactone dehydrogenase: Partial characterization, induction of activity and role in the synthesis of ascorbic acid in wounded white potato tuber tissue. *Plant Cell Physiol.* **1994**, *35*, 473–478. [CrossRef]
114. Østergaard, J.; Persiau, G.; Cavey, M.W.; Bauw, G.; Van Montagu, M. Isolation and cDNA cloning for L-galactono-γ-lactone dehydrogenase, an enzyme involved in the biosynthesis of ascorbic acid in plants. *J. Biol. Chem.* **1997**, *272*, 30009–30016. [CrossRef] [PubMed]
115. Imai, T.; Karita, S.; Shratori, G.; Hattori, M.; Nunome, T.; Oba, K.; Hirai, M. L-Galactono-g-lactone dehydrogenase from sweet potato: Purification and cDNA sequence analysis. *Plant Cell Physiol.* **1998**, *39*, 1350–1358. [CrossRef]
116. Siendones, E.; Gonzalez-Reyes, J.A.; Santos-Ocana, C.; Navas, P.; Córdoba, F. Biosynthesis of ascorbic acid in kidney bean. L-galactono-gamma-lactone dehydrogenase is an intrinsic protein located at the mitochondrial inner membrane. *Plant Physiol.* **1999**, *120*, 907–912. [CrossRef]
117. Fraaije, M.W.; Benen, J.A.E.; Visser, J.; Mattevi, A.; van Berkel, W.J.H. A novel oxidoreductase family sharing a conserved FAD-binding domain. *Trends Biochem. Sci.* **1998**, *23*, 206–207. [CrossRef]
118. Leferink, N.G.H.; Heuts, D.P.H.M.; Fraaije, M.W.; Van Berkel, W.J.H. The growing VAO flavoprotein family. *Arch. Biochem. Biophys.* **2008**, *474*, 292–301. [CrossRef] [PubMed]
119. Cui, J.; Yuan, X.; Wang, L.; Jones, G.; Zhang, S. Recent loss of vitamin C biosynthesis ability in bats. *PLoS ONE* **2011**, *6*, e27114. [CrossRef] [PubMed]
120. Fraaije, M.W.; van den Heuvel, R.H.; van Berkel, W.J.; Mattevi, A. Covalent flavinylation is essential for efficient redox catalysis in vanillylalcohol oxidase. *Biol. Chem. J.* **1999**, *274*, 35514–35520. [CrossRef]
121. López-Fernández, H.; Duque, P.; Henriques, S.; Vázquez, N.; Fdez-Riverola, F.; Vieira, C.P.; Reboiro-Jato, M.; Vieira, J. A bioinformatics protocol for quickly creating large-scale phylogenetic trees. In *Practical Applications of Computational Biology and Bioinformatics, 12th International Conference*; Fdez-Riverola, F., Mohamad, M.S., Rocha, M., De Paz, J.F., González, P., Eds.; Springer: Cham, Switzerland, 2019; pp. 88–96. [CrossRef]
122. Yang, H. Conserved or lost: Molecular evolution of the key gene GULO in vertebrate vitamin C biosynthesis. *Biochem. Genet.* **2013**, *51*, 413–425. [CrossRef] [PubMed]

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Review

Desialylation and Apoptosis in Immune Thrombocytopenia: Implications for Pathogenesis and Treatment

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Abstract: Immune thrombocytopenia (ITP) is an autoimmune disease in which platelet autoantibodies play a significant role in its pathogenesis. Regulatory T cell dysfunction and T cell-mediated cytotoxicity also contribute to thrombocytopenia. Current therapies are directed towards immune suppression and modulation as well as stimulation of platelet production with thrombopoietin receptor agonists. Additional mechanisms of the pathogenesis of ITP have been suggested by recent experimental data. One of these processes, known as desialylation, involves antibody-induced removal of terminal sialic acid residues on platelet surface glycoproteins, leading to hepatic platelet uptake and thrombocytopenia. Apoptosis, or programmed platelet death, may also contribute to the pathogenesis of ITP. The extent of the impact of desialylation and apoptosis on ITP, the relative proportion of patients affected, and the role of antibody specificity are still the subject of investigation. This review will discuss both historical and new evidence of the influence of desialylation and apoptosis in the pathogenesis of ITP, with an emphasis on the clinical implications of these developments. Further understanding of both platelet desialylation and apoptosis might change current clinical practice and improve patient outcomes.

Keywords: immune thrombocytopenia; platelets; autoantibodies; desialylation; apoptosis

1. Introduction

Immune thrombocytopenia (ITP) is an acquired autoimmune disease characterised by enhanced platelet destruction and impaired platelet production. Patients can present with no known predisposing condition (primary ITP) or due to an associated disorder such as malignancy, infection, and autoimmune disease (secondary ITP) [1]. Drug-induced ITP is also well recognised [2]. There are three phases of the disease: newly diagnosed (<3 months), persistent (3–12 months), and chronic (>12 months) [3]. ITP affects both children and adults. Adult ITP is typically chronic. Only about 20–30% of cases achieve long-term remission, and many patients require long-term therapy. Primary ITP comprises approximately 80% of all adult ITP cases [4]. On the other hand, paediatric ITP is often triggered by viral infections and is usually acute and self-limiting, with 80% of children experiencing spontaneous remission. In children, treatment is often unnecessary [1,5]. ITP is a heterogeneous disease. Some patients require multiple lines of therapy, while others are managed by observation only. As a result, the quality of life for patients is affected to different degrees.

In addition to its clinical heterogeneity, ITP is a complex disease, and no single proposed mechanism can fully explain its pathogenesis. Early and current data suggest that autoimmunity is responsible for causing thrombocytopenia [6–9]. Over the past decades, further evidence has emerged, showing that the dysregulation of the immune system is multifactorial. In addition to the production of antiplatelet antibodies by B and plasma

cells, regulatory T cell dysfunction and cytotoxic T cell-mediated platelet destruction also contribute to the pathogenesis of ITP. More recent advances further demonstrate that autoimmunity shortens platelet survival through the modification of platelet surface glycans and may hasten platelet programmed cell death.

This review will focus on the key aspects of recent research regarding platelet desialylation and apoptosis, their relevance to ITP pathology, and the diagnostic and treatment implications.

2. Platelet Biogenesis and the Regulation of Lifespan

Platelets are derived from megakaryocytes, which reside in the bone marrow and to a lesser extent in the blood and lungs. It is estimated that 10^{11} platelets are generated each day in healthy adults [10,11]. Thrombopoietin (TPO) is the primary growth factor that drives thrombopoiesis. TPO stimulates the differentiation of haematopoietic stem cells into megakaryocytic precursors and their proliferation and maturation [12,13]. TPO is synthesised primarily in the liver [14], but expression in kidney and bone marrow has been demonstrated [15]. TPO binds to its c-Mpl receptors on haematopoietic stem cells and platelets [14,16], which provides a mechanism for the regulation of circulating TPO levels [17]. During thrombocytosis, more platelets, and therefore more c-Mpl receptors, are available to bind and internalise TPO. This reduces the amount of free TPO, thus decreasing both megakaryopoiesis and platelet formation. In thrombocytopenic states, for example, due to myelosuppressive therapy, less TPO is internalised by platelets and more TPO is available to bind to megakaryocytes, stimulating thrombopoiesis [18]. Curiously, TPO levels are not significantly increased in ITP compared to non-immune thrombocytopenia. This could be due to the markedly increased platelet turnover rate despite the thrombocytopenic state in ITP [19].

2.1. Platelet Desialylation and the Regulation of Lifespan

Another model of TPO regulation is by controlling the TPO mRNA expression. In the last decade, a mechanism of TPO mRNA regulation was described. In a mouse model, Grozovsky and colleagues showed that aged platelets, by losing their terminal sialic acid groups attached to glycoproteins (desialylation), became recognisable by the hepatic Ashwell–Morell receptors (AMR) [20]. Glycoprotein (GP) Ibα, a highly expressed membrane-bound receptor on platelets, has an extracellular domain with an abundance of N-linked glycans (Figure 1) [21,22]. Hence, GPIbα is thought to be a major target of desialylation. The recognition of desialylated GPs by hepatocytes leads to the hepatic internalisation of platelets, which stimulates Janus kinase 2 (JAK2) and activates signal transduction and transcription 3 (STAT3). This signalling drives hepatic TPO mRNA expression (Figure 2). As a result, platelet production is increased, and platelet mass is restored [20]. In fact, a link between desialylation and platelet survival has been known for decades [23] (Table 1).

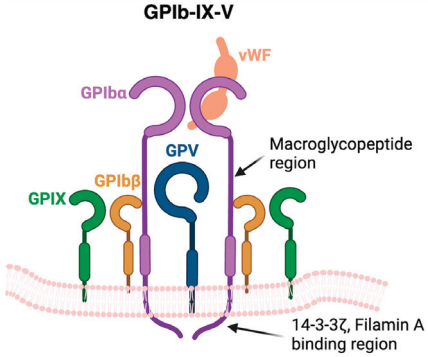


Figure 1. Structure of GPIb-IX-V complex. The GPIbα subunits have a heavily *O* and *N*-linked glycosylated at the N-terminus and at the mucin-like macroglycopeptide domain [24,25]. vWF, von Willebrand factor.

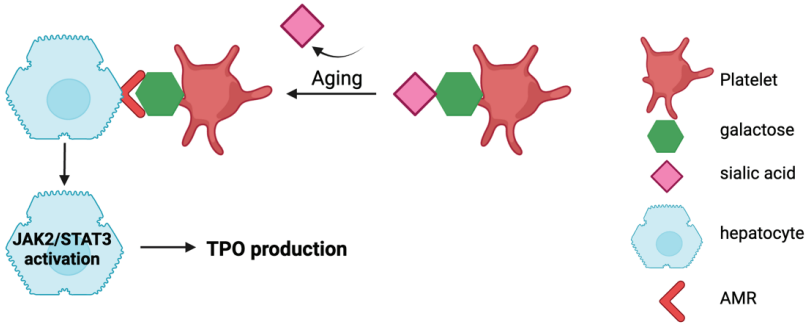


Figure 2. Mechanism of TPO regulation in the liver. Aged platelets lose their terminal sialic acid residues. The desialylated platelets are recognised by AMR and removed by hepatocytes, which activates the JAK2/STAT3 pathway and results in increased TPO mRNA and protein production.

Table 1. Emergence of desialylation in platelet biology.

Comment	Reference; Year
Sialic acid is present on platelets; can be cleaved with neuraminidase	[26]; 1964
Desialylated rabbit platelets are rapidly cleared in vivo; no significant changes to platelet function in vitro	[23]; 1975
Injection of desialylated platelets induced platelet production. Authors suggested that thrombopoiesis may be regulated by uptake of desialylated platelets	[27]; 1980
Sialic acid removal shortens platelet lifespan in primates	[28]; 1993
Platelets lacking sialic acid are recognised by asialoglycoprotein receptors	[29]; 2009
Cold storage leads to platelet desialylation	[30]; 2012
Platelet desialylation by anti GPIb/IX antibody	[31]; 2014
Hepatic Ashwell–Morell receptor binds and removes desialylated platelets	[20]; 2015
Anti GPIbα, not anti GPIIb/IIIa antibodies, induced desialylation and hepatic platelet uptake in mice	[32]; 2015
ITP patient with anti GPIb/IX antibodies successfully treated with oseltamivir	[33]; 2015
TPO-RAs in combination with oseltamivir induced sustained platelet production in patients with anti GPIb antibodies	[34]; 2019
Plasma from patients with ITP affected the sialylation pattern of control platelets	[35]; 2019
Desialylation induced by anti GPIIb/IIIa antibodies and is FcγRIIa-dependent	[36,37]; 2020, 2021
Destruction of human platelets induced by anti-GPIIb/IIIa antibodies was prevented with oseltamivir in a humanised mouse model of ITP	[37,38]; 2021, 2022

2.2. Platelet Apoptosis and Platelet Lifespan

Unlike desialylation (Table 1), the role of apoptosis was only described more recently (Table 2). In the steady state, human platelet lifespan is approximately ten days [39,40]. Intrinsic apoptosis, as triggered by non-receptor-driven events, is probably the most important regulator of platelet life span [41–43]. Two seminal studies showed that proteins of the BCL2 family are the key players in this pathway [42,44]. BCL-X_L is a pro-survival protein that sustains platelet survival by restraining the pro-death activity of the "multidomain killers" BAD, BAK1, and BAX [41]. In the cytosol, 14-3-3ζ exists in complexes with the pro-apoptotic proteins, controlling their activity by maintaining phosphorylation [45]. It has been hypothesised that, as platelets age, they gradually lose BCL-X_L due to degradation. However, BAK1 is degraded more slowly than BCL-X_L, eventually reaching a point when the proapoptotic activity can no longer be restrained [42]. Free BAX transits to the mitochondria and induces mitochondrial membrane permeabilization, cytochrome c release, and activation of caspases, which results in platelet apoptosis [41] (Figure 3). Utilising a potent BCL-X_L antagonist ABT-737 and ^{111}indium-labelled platelets whole body scintigraphy in an animal model, Zhang and colleagues identified the liver as the site of clearance of apoptotic platelets [44].

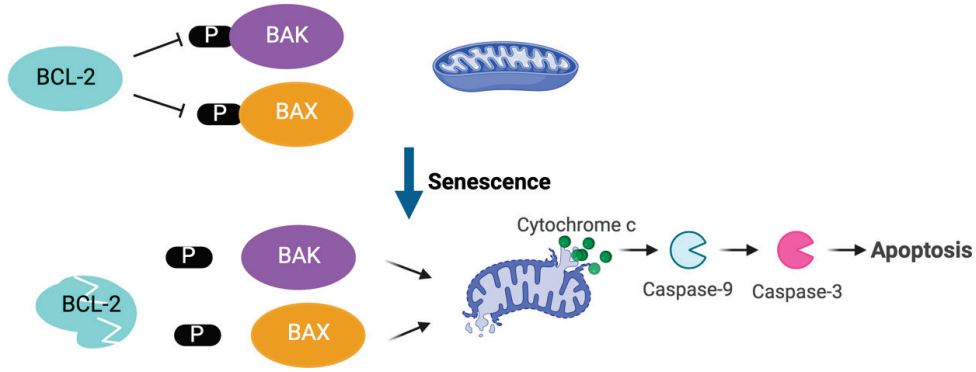


Figure 3. Regulation of platelet lifespan via the intrinsic apoptosis pathway. BCL2 pro-survival protein family restrains BAK and BAX activities by maintaining phosphorylation (p). Platelet senescence resulted in BCL-2 degradation, BAK and BAX dephosphorylation, which causes mitochondrial outer membrane permeabilization and initiates the apoptotic cascade.

It is still unclear if platelet apoptosis and desialylation are mechanistically linked during platelet ageing. Using AMR knockout mice, a preliminary report showed that desialylated platelets are more prone to apoptosis [46]. However, the induction of platelet apoptosis did not induce platelet desialylation [46]. It is possible that the platelet ageing process involves the gradual loss of sialic acid in the circulation, followed by enhanced responsiveness to apoptotic stimuli, which result in the liberation of the pro-death proteins BAK1 and BAX and the eventual platelet apoptosis [46]. Nevertheless, stronger evidence is needed to definitively correlate desialylation with apoptosis in the regulation of platelet lifespan.

Table 2. Evidence for platelet apoptosis.

Comment	Reference; Year
Nucleus is not required for apoptosis	[47]; 1994
Apoptosis-like events associated with platelet activation	[48]; 1999
Anti-platelet antibodies modulate caspase activity and regulate platelet lifespan in mice	[49]; 2002
Apoptosis associated with shortened platelet survival in rabbits	[50]; 2004
Anti GPIIb antibody induces platelet apoptosis in mice	[51]; 2006
Thrombin induces platelet apoptosis	[52,53]; 2007, 2006
Apoptosis program controls platelet lifespan	[42]; 2007
Cold storage leads to platelet apoptosis	[54]; 2010
Apoptotic platelets observed in paediatric patients with ITP	[55]; 2012
Platelet apoptosis in adult ITP	[56,57]; 2018, 2016
The presence of anti-platelet antibodies predicts apoptosis in ITP	[38]; 2022

3. Immune Thrombocytopenia

3.1. Presentation

The main symptoms and signs of ITP are bleeding, ranging from minor petechiae and purpure to severe haemorrhage, such as gastrointestinal and intracranial bleeding. The severity generally correlates with the degree of thrombocytopenia and the patient's age. Older patients with a persistently low platelet count of under 30×10^9/L are at a higher risk of bleeding [58]. Notable exceptions are patients with antiplatelet autoantibodies with platelet function inhibitory effects. Occasionally, these antibodies interfere with fibrinogen binding to platelet GPIIb/IIIa or von Willebrand factor (vWF) binding to GPIb/IX receptors,

resulting in severe bleeding phenotypes from acquired Glanzmann's thrombasthenia or Bernard–Soulier syndrome, respectively [59]. These patients have severe bleeding symptoms regardless of their platelet counts. Unexpectedly, patients with ITP are also more susceptible to both venous and arterial thromboembolism [60]. In addition, patients with ITP frequently suffer from fatigue, impaired quality of life [61], and complications related to treatments [62]. Unfortunately, the mortality rates are consistently higher amongst ITP patients than in the general population [63], ranging from 1.3- to 2.2-fold [62,64,65]. Therefore, ITP is a complex disease, and understanding its pathogenesis is of importance in order to improve the current management.

3.2. Pathogenesis

3.2.1. Fc-Dependent Pathway

It was the seminal Harrington–Hollingsworth experiment of self-infusion of ITP plasma, which led to the discovery of a humoral factor accountable for mediating platelet destruction [6,7]. Subsequently, Shulman identified that such a factor could be adsorbed by platelets and was associated with immunoglobulin G (IgG) [8]. IgM and IgA antiplatelet antibodies in the absence of IgG have been described but are rather uncommon in adult ITP [11]. In contrast, IgM may play a dominant role in acute paediatric ITP [66]. These autoantibodies are commonly directed against the platelet membrane GP complexes, and GPIIb/IIIa and GPIb/IX are most frequently targeted [67,68]. Sole specificity against GPIaIIa and GPVI antibodies is infrequent [69–71]. Regarding antibodies against platelet GPV, which can bind to the GPV subunit independently of the GPIb/IX complex [72], in a recent evaluation, Porcelijn reported that 5% of the 60 patients with ITP tested for anti-platelet antibodies had sole anti-GPV antibodies [73]. In fact, antibodies against more than one GP receptor are much more common in ITP [68,71].

These autoantibodies play a significant role in ITP pathogenesis. One of the most widely accepted mechanisms of platelet destruction is that opsonised platelets are phagocytosed by splenic and/or hepatic macrophages of the reticuloendothelial system via their Fc-gamma receptors (FcγR) [11,74]. Hence, this antibody-mediated platelet clearance is called the Fc-dependent pathway. Like other autoimmune diseases, ITP signifies the loss of self-tolerance. Self-reactive T cells are believed to be one of the central causes of perpetuating autoimmunity, as B cells' ability to produce antibodies is supported by antigen-specific T cells. Studies on $CD4^+FOXP3^+$ T cells or T regulatory cells (Treg) have shown both reduction [75–77] and dysfunction of Treg [77] in patients with untreated ITP. Moreover, in patients with active ITP, the percentage of T-helper (Th) 17 cells is significantly higher than normal controls [78]. Interestingly, T cell clonality has been reported and implicated in treatment failure [79–81]. Large prospective trials are required to determine how clinicians can use this information to tailor ITP treatment strategies.

3.2.2. Fc-Independent Pathway

Platelet Desialylation

Fc-independent platelet clearance has also been strongly suggested for anti-GPIbα antibodies-mediated ITP. Using monoclonal antibodies in a mouse model of ITP, Nieswandt and colleagues first demonstrated that removing the Fc fragment of anti-GPIbα monoclonal antibodies did not prevent thrombocytopenia, whereas the opposite was noted for anti-GPIIb/IIIa antibodies, denoting the Fc independence of anti-GPIbα antibodies in causing thrombocytopenia [82]. Since then, there have been further advances in the understanding of this alternative platelet clearance pathway. In 2003, Hoffmeister and colleagues observed that short-term (2 h) platelet cooling to 4 °C resulted in GPIb receptor clustering and rapid clearance of platelets by Mac-1 receptor-expressing Kupffer cells in the liver [83,84]. From 2009 to 2012, the same group discovered that the sialidase neuraminidase-1 (NEU1) was translocated to the platelet surface following longer-term ($\geq$48 h) refrigeration of platelets. NEU1 mediates removal of terminal sialic acid residues on GPIbα, leading to platelet clearance by hepatocytes via the Ashwell–Morell receptors (AMR) [29,30,85].

More recently, Li and colleagues reported the same FcγR-independent model of platelet clearance by anti-GPIbα antibodies. Using both monoclonal antibodies and plasma from patients with ITP, they reported platelet activation secondary to GPIbα antibodies. This led to NEU1 translocation to platelet surface and platelet desialylation, as well as the resultant platelet removal by AMR on hepatocytes and hence, thrombocytopenia. This finding indicated that detection of platelet desialylation in patients with ITP might have potential diagnostic and therapeutic implications. In vivo evidence suggested that sialidase inhibitors may attenuate thrombocytopenia in ITP secondary to anti-GPIbα antibodies, while splenectomy, which reduces Fc-dependent phagocytosis of opsonised platelets, may be of little benefit for these patients [32]. However, this animal model using monoclonal antibodies may not be directly relevant to human ITP due to the lack of platelet surface FcγRIIa on murine platelets [86] and the polyclonal nature of autoantibodies from primary ITP [87]. In fact, when Cantoni et al. examined the antibody specificities and the location of platelet clearance using indium-111-labelled platelets of 93 patients with ITP, no association between antibody specificities and the site of platelet sequestration was found [88]. These observations are tempered by the fact that only four patients had sole GPIb/IX antibodies. Of those, two patients had splenic uptake while the other two had hepatic and mixed platelet sequestration patterns, respectively [88]. In a later study, Amini and colleagues examined 53 patients and reported an association between the presence of anti-GPIbα and hepatic sequestration only in patients with a platelet count under 50×10^9/L [89]. Again, there were only four patients with anti-GPIbα antibodies without concomitant GPIIb/IIIa antibodies. In fact, when GPV antibodies were excluded, there was only one patient with sole anti-GPIbα antibodies [89].

Whether anti-GPIbα antibodies induce platelet desialylation and hepatic sequestration might be dose-dependent. In a murine model, Morodomi and co-workers reported that high-dose intravenous administration of monoclonal anti-mouse GPIbα antibodies led to platelet clearance by both the spleen and liver, while low-dose subcutaneous administration only led to splenic uptake [90]. Furthermore, the discrepancy between Li's and Cantoni's findings may also be because the true extent to which desialylation was involved in ITP pathology was unknown at the time. Recent research demonstrated that antibodies against GPIIb/IIIa are also capable of inducing platelet and megakaryocyte desialylation [35–38]. Unlike previous reports linking antibody-mediated platelet activation to desialylation [32], these latest findings did not find evidence of platelet activation [36–38]. Instead, when FcγR is inhibited by monoclonal antibody IV.3, desialylation is reduced [36,37]. Hence, anti-GPIIb/IIIa antibodies potentially signal through FcγRIIa to drive neuraminidase surface translocation and platelet desialylation. More importantly, these studies also confirmed the effectiveness of neuraminidase inhibitor oseltamivir in reducing platelet destruction in vivo in the setting of anti-GPIIb/IIIa antibodies-mediated ITP [37,38], providing further evidence that both anti-GPIb/IX and anti-GPIIb/IIIa antibodies may lead to platelet desialylation.

Platelet apoptosis

Platelet apoptosis has also been implicated in the pathogenic processes of ITP. In 2006, Leytin and colleagues reported that anti-GPIIb antibody injection in mice induced thrombocytopenia, platelet caspase-3 activation, enhanced phosphatidylserine (PS) exposure and mitochondrial inner transmembrane potential ($\Delta\Psi m$) depolarisation. Capsase-3, PS exposure and $\Delta\Psi m$ depolarisation are markers of platelet apoptosis [51]. Leytin also demonstrated that treatment with IVIg prior to antibody injection ameliorated the degree of thrombocytopenia, reduced caspase-3 activation and PS exposure but not $\Delta\Psi m$ depolarisation. In 2012, Winkler and colleagues reported similar findings in paediatric patients with ITP [55]. However, the number of patients was small (20) and no correlation with antibody specificities was made.

More recently, Qiao and co-workers showed in vitro data of reduced expression of BCL-X_L and increased BAX in platelets treated with ITP plasma [91]. The researchers employed Western blot and quantitative PCR techniques to evaluate mRNA extracted from washed donor platelets treated from patients with ITP and control plasma. The

results indicated a lower mRNA and protein expression of BCL-X_L but an increased mRNA and protein expression of BAX in platelets treated with plasma from patients with ITP when compared to control plasma [91]. However, as platelets lack nuclei, the authors did not describe the mechanism of the changes in RNA level. Possible explanations include alterations in RNA splicing of immature RNAs [92] and modification of mRNA degradation rates [93]. Nevertheless, the imbalanced expression of BCL-X_L and BAX at the protein level provided additional evidence of the loss of proapoptotic restrain in ITP. Again, antibody specificity was not examined in this study.

In addition to desialylation, platelet cold storage also leads to platelet apoptosis upon rewarming [54]. Like desialylation, cold storage induced platelet apoptosis may be a GPIbα driven event. Upon rewarming, the GPIbα receptor clusters, which leads to phosphorylation of its cytoplasmic domains. These phosphorylated sites have strong affinity for the above-mentioned adaptor protein 14-3-3ζ [94]. Consequently, BAX is released from 14-3-3ζ, dephosphorylated and becomes active, which leads to platelet apoptosis [54,95]. Other events, including binding of ligands such as vWF [96] and thrombin stimulation [52,53] may also result in platelet apoptosis [97].

Furthermore, both desialylated and apoptotic platelets are removed by the liver [32,44,85]. To date, a mechanistic link between platelet desialylation and apoptosis is still not fully explored (Tables 1 and 2). From the ITP perspective, anti-GPIbα antibodies were reported to cause platelet desialylation like cold storage [32]. Yet, Leytin et al. found that monoclonal anti-GPIIb antibodies caused platelet apoptosis in a murine ITP model [51]. Whether there is an association between intrinsic apoptosis and ITP caused by anti-GPIbα and/or anti-GPIIbIIIa antibodies is still unclear. Our group analysed the differential impact of these two antibody subtypes on platelet fate, focusing on platelet desialylation and apoptosis. In contrast to Li and colleagues' report, we found a greater proportion of patients' sera with anti-GPIIb/IIIa antibodies led to positive neuraminidase surface translocation, while a significant number of sera from patients with sole anti-GPIbα antibodies caused platelet apoptosis [38]. Although the number of patients with sole antibody positivity was small (n = 9 for anti-GPIIb/IIIa, n = 5 for anti-GPIbα), similar findings of anti-GPIIb/IIIa antibodies indicate a greater ability to induce desialylation [35] and for anti-GPIbα to induce apoptosis [57] have been described (Table 2). Therefore, not only does the presence of platelet antibodies predict platelet desialylation and apoptosis, but the actual antibody specificity may also have implications on the ultimate platelet clearance pathway. This raises the potential of ITP treatment individualisation if such findings can be confirmed in future collaborative prospective evaluations.

T cell mediated cytotoxicity

Since not all ITP plasma-induced thrombocytopenia in healthy volunteers is from the famous Harrington experiments [6], and only about 50% of ITP patients have detectable anti-platelet antibodies [98], non-antibody-mediated mechanisms of ITP should be highlighted. Specifically, autoreactive $CD8^+$ cytotoxic T cells causing platelet lysis have been reported [99–101]. Interestingly, Zhao et al. found a negative correlation with the detectability of platelet antibodies and the presence of platelet lysis by cytotoxic T cells, which suggested that T cell-mediated cytotoxicity could be a dominant mechanism in those patients without detectable antibodies [101]. Recently, it was shown that cytotoxic $CD8^+$ T cells from patients with ITP induced platelet desialylation and apoptosis [102]. This may be a result of platelet activation through T cell receptor-mediated cytotoxic granule release [103]. Moreover, $CD8^+$ cytotoxic T cells may also affect platelet production in ITP by megakaryocytes by paradoxically increasing megakaryopoiesis and decreasing apoptosis [104]. Changes in the steady state of apoptosis in megakaryocytes seem to correlate with lower platelet production [105].

4. Therapeutic Implications

Treatment can be divided into first-line [steroids, intravenous immunoglobulin (IVIg), and anti-Rh(D) (anti-D)], second-line [TPO receptor agonists (TPO-RA), rituximab, splenec-

tomy] and subsequent therapies [4,106]. The currently available therapies are largely targeting the Fc-dependent pathway (Figure 4). Agents such as corticosteroids [107,108], rituximab [109,110], and cytotoxics (for example, vinca alkaloids and azathioprine [111,112]) are used to reduce antibody production. The Bruton kinase inhibitor rilzabrutinib is a promising novel anti-B cell treatment [113]. IVIg and anti-D are thought to inhibit antibody-mediated platelet clearance by saturating the FcR on macrophages [11,114]. Various combination therapies targeting this pathway have been reported [115,116]. Other novel treatments, such as anti-CD38 monoclonal antibodies to direct against plasma cells and neonatal Fc receptor inhibitors to prevent anti-platelet antibody recycling, are currently in clinical trials [117].

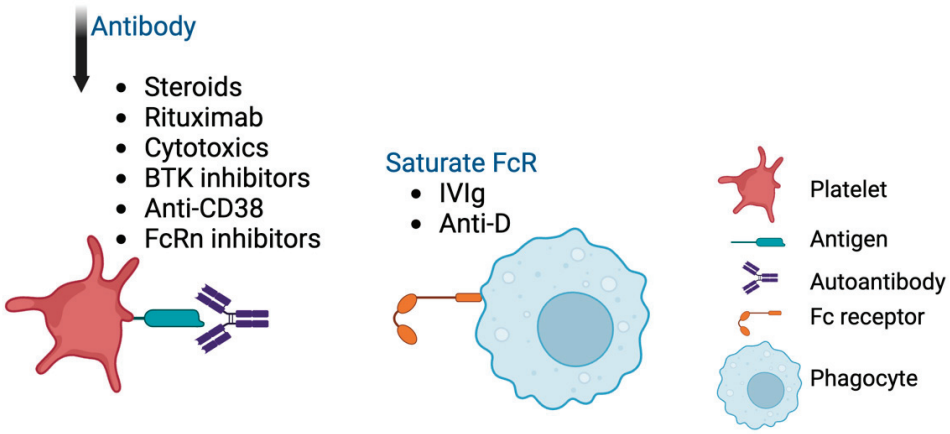


Figure 4. Treatments targeting the Fc-dependent pathway. BTK, Bruton kinase; FcRn, neonatal Fc receptor; IVIg, intravenous immunoglobulin.

The dysregulation of the immune system is multifactorial, and various components of immunity are involved in perpetuating the disease. The underlying pathogenic stimuli of primary ITP often remain unknown [118]. Other mechanisms, such as megakaryocyte injury [119–121], reduced proplatelet formation [122,123], and complement activation [124–126], have implications in ITP treatment. This includes the current use of TPO RA eltrombopag [127], romiplostim [128,129], and avatrombopag [130]. Regarding complement inhibition, the Phase 1 results of a selective inhibitor sutimlimab, have recently been published: five of the 12 patients who received treatment had a durable platelet count response while on sutimlimab, which seemed to be well tolerated [131].

Oseltamivir phosphate is commonly used to treat influenza A and B with a good safety profile. It is a prodrug that, after absorption, is hydrolysed by hepatic esterases to the active form, oseltamivir carboxylate [132]. It inhibits influenza virus neuraminidase, an enzyme known to cleave the budding viral progeny from its cellular envelop attachment point (neuraminic acid) just prior to release [133]. Humans have four sialidases/neuraminidases that are homologues of viral neuraminidase, and the activity may be reduced by oseltamivir [134].

Interestingly, even before the systemic studies of ITP antibody-induced platelet desialylation, there were case reports of oseltamivir improving platelet counts in patients with ITP [33,135–137]. The first report, published in 2010, described a 13-year-old patient with chronic ITP who was hospitalised and treated with oseltamivir for H1N1 infection. The platelet count increased unexpectedly from 32×10^9/L to over 500×10^9/L [135]. In the second case published in 2015, a 69-year-old female with chronic ITP with anti-GPIb-IX antibodies was successfully managed with oseltamivir [33]. The third case described a 46-year-old female with secondary ITP from HIV infection. She was given oseltamivir for a presumed influenza infection. This was followed by normalisation of platelet count to 213×10^9/L from a pre-treatment level of 9×10^9/L [136]. The fourth case also reported a 51-year-old man with HIV infection on antiviral treatment and refractory ITP. His platelet count responded to combination therapy with romiplostim and oseltamivir [137]. Surpris-

ingly, case number four also demonstrated platelet function recovery upon oseltamivir treatment using PAC1 and P-Selectin binding [137]. This is congruent with a later publication by our research group [36]. We described a patient with chronic ITP and acquired Glanzmann thrombasthenia. Not only did this patient's antibodies inhibit platelet function, but they also led to neuraminidase translocation to the platelet surface and desialylation [36]. These findings indicate that certain anti-platelet antibodies potentially cause both platelet desialylation and platelet function inhibition.

Since the availability of more robust scientific data, systematic studies have been conducted in patients with ITP. Revilla and co-workers studied 35 patients with ITP and selected 10 of these patients with evidence of platelet desialylation for oseltamivir treatment [34]. Unlike the animal data, oseltamivir monotherapy was ineffective as none of the patients on oseltamivir-only treatment (n = 4) responded. However, when oseltamivir was given in combination with TPO-RA and/or immunosuppressive therapy, four out of the six patients attained a response. In contrast to the experimental data from Marini et al. [37] and our group [38], all four responders had sole anti-GPIbα antibodies [34]. No solid conclusions can be drawn from this study due to the low number of patients with single antibody specificity.

In 2021, the results of a multicentre, randomised, open-label trial using oseltamivir in combination with dexamethasone in newly diagnosed adult ITP were published [138]. The authors reported significantly improved initial response rate at day 14 in the oseltamivir group, defined as platelet count between 30 and 100 $\times$ 10^9/L without clinically significant bleeding. The 6-month response rate was also higher in this group. However, the 12-month sustained response is not significantly different. In addition, although the duration of response was 9 months in the oseltamivir treatment group and 5 months in the control, this did not reach statistical significance. Such results may be due to the lack of power in the study. Patients with detectable anti-GPIb-IX antibodies had a poorer initial response in the dexamethasone group, but no significant difference was noted in the dexamethasone plus oseltamivir arm. The desialylation status was not examined. A sufficiently powered, well-designed, double-blind randomised clinical trial is needed to examine the potential role of oseltamivir in managing patients with newly diagnosed and refractory ITP. Its potential role in paediatric ITP and its use as a single therapy to minimise immunosuppressive and other steroid side effects are yet to be explored in clinical trials.

5. Conclusions

Since the initial use of splenectomy as the only treatment for ITP in the early 20th century [139], significant progress has been made in understanding the pathogenesis of ITP in recent years. This includes advances into the intricate interplay between B and T cells, as well as in the understanding of platelet antigen and autoantibody interaction. This multifaceted aspect of ITP pathology is connected to one of the most significant challenges in caring for patients with ITP: the lack of treatment individualisation. Clearly defining the platelet clearance pathway, such as desialylation and platelet apoptosis, will have important clinical implications. Patients with different autoantibodies could potentially be directed towards specific therapies. In this regard, platelet antibody testing and measuring platelet desialylation/apoptosis levels could prove clinically helpful in the treatment of ITP. Hence, the characterisation of novel mechanisms will not only contribute to the understanding of ITP's pathogenesis but also potentially guide the development of new diagnostic tests and targeted therapies. Continued dedication to the exploration of personalised treatment should be a priority. Specifically, collaborative, high-powered studies are required to investigate treatments using neuraminidase and/or apoptosis inhibitors in randomised prospective, multicentre ITP clinical trials.

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References

1. Cines, D.B.; Bussel, J.B.; Liebman, H.A.; Luning Prak, E.T. The ITP syndrome: Pathogenic and clinical diversity. *Blood* **2009**, *113*, 6511–6521. [CrossRef] [PubMed]
2. Chong, B.H.; Choi, P.Y.; Khachigian, L.; Perdomo, J. Drug-induced immune thrombocytopenia. *Hematol. Oncol. Clin. N. Am.* **2013**, *27*, 521–540. [CrossRef]
3. Provan, D.; Stasi, R.; Newland, A.C.; Blanchette, V.S.; Bolton-Maggs, P.; Bussel, J.B.; Chong, B.H.; Cines, D.B.; Gernsheimer, T.B.; Godeau, B.; et al. International consensus report on the investigation and management of primary immune thrombocytopenia. *Blood* **2010**, *115*, 168–186. [CrossRef]
4. Lambert, M.P.; Gernsheimer, T.B. Clinical updates in adult immune thrombocytopenia. *Blood* **2017**, *129*, 2829–2835. [CrossRef]
5. Verissimo, V.; Carter, T.; Wright, H.; Robertson, J.; Osborn, M.; Bradbeer, P.; Sabesan, V.; Saxon, B.; Barbaro, P.; Crighton, G.; et al. Australian and New Zealand consensus guideline for paediatric newly diagnosed immune thrombocytopaenia endorsed by Australian New Zealand Children's Haematology and Oncology Group. *J. Paediatr. Child Health* **2023**, *59*, 711–717. [CrossRef] [PubMed]
6. Harrington, W.J.; Minnich, V.; Hollingsworth, J.W.; Moore, C.V. Demonstration of a thrombocytopenic factor in the blood of patients with thrombocytopenic purpura. *J. Lab. Clin. Med.* **1951**, *38*, 1–10.
7. Schwartz, R.S. Immune thrombocytopenic purpura—From agony to agonist. *N. Engl. J. Med.* **2007**, *357*, 2299–2301. [CrossRef] [PubMed]
8. Shulman, N.R.; Marder, V.J.; Weinrach, R.S. Similarities between known antiplatelet antibodies and the factor responsible for thrombocytopenia in idiopathic purpura. Physiologic, serologic and isotopic studies. *Ann. N. Y. Acad. Sci.* **1965**, *124*, 499–542. [CrossRef] [PubMed]
9. Hollenhorst, M.A.; Al-Samkari, H.; Kuter, D.J. Markers of autoimmunity in immune thrombocytopenia: Prevalence and prognostic significance. *Blood Adv.* **2019**, *3*, 3515–3521. [CrossRef]
10. Grozovsky, R.; Giannini, S.; Falet, H.; Hoffmeister, K.M. Regulating billions of blood platelets: Glycans and beyond. *Blood* **2015**, *126*, 1877–1884. [CrossRef]
11. Cines, D.B.; Cuker, A.; Semple, J.W. Pathogenesis of immune thrombocytopenia. *Presse Med.* **2014**, *43*, e49–e59. [CrossRef] [PubMed]
12. Broudy, V.C.; Lin, N.L.; Kaushansky, K. Thrombopoietin (c-mpl ligand) acts synergistically with erythropoietin, stem cell factor, and interleukin-11 to enhance murine megakaryocyte colony growth and increases megakaryocyte ploidy in vitro. *Blood* **1995**, *85*, 1719–1726. [CrossRef] [PubMed]
13. Debili, N.; Wendling, F.; Katz, A.; Guichard, J.; Breton-Gorius, J.; Hunt, P.; Vainchenker, W. The Mpl-ligand or thrombopoietin or megakaryocyte growth and differentiative factor has both direct proliferative and differentiative activities on human megakaryocyte progenitors. *Blood* **1995**, *86*, 2516–2525. [CrossRef] [PubMed]
14. Broudy, V.C.; Lin, N.L.; Sabath, D.F.; Papayannopoulou, T.; Kaushansky, K. Human platelets display high-affinity receptors for thrombopoietin. *Blood* **1997**, *89*, 1896–1904. [CrossRef]
15. Sungaran, R.; Markovic, B.; Chong, B.H. Localization and regulation of thrombopoietin mRNA expression in human kidney, liver, bone marrow, and spleen using in situ hybridization. *Blood* **1997**, *89*, 101–107. [CrossRef] [PubMed]
16. Li, J.; Xia, Y.; Kuter, D.J. Interaction of thrombopoietin with the platelet c-mpl receptor in plasma: Binding, internalization, stability and pharmacokinetics. *Br. J. Haematol.* **1999**, *106*, 345–356. [CrossRef]
17. Deutsch, V.R.; Tomer, A. Megakaryocyte development and platelet production. *Br. J. Haematol.* **2006**, *134*, 453–466. [CrossRef]
18. Kuter, D.J.; Rosenberg, R.D. The reciprocal relationship of thrombopoietin (c-Mpl ligand) to changes in the platelet mass during busulfan-induced thrombocytopenia in the rabbit. *Blood* **1995**, *85*, 2720–2730. [CrossRef] [PubMed]
19. Kuter, D.J.; Gernsheimer, T.B. Thrombopoietin and platelet production in chronic immune thrombocytopenia. *Hematol. Oncol. Clin. N. Am.* **2009**, *23*, 1193–1211. [CrossRef]
20. Grozovsky, R.; Begonja, A.J.; Liu, K.; Visner, G.; Hartwig, J.H.; Falet, H.; Hoffmeister, K.M. The Ashwell-Morell receptor regulates hepatic thrombopoietin production via JAK2-STAT3 signaling. *Nat. Med.* **2015**, *21*, 47–54. [CrossRef]
21. Berndt, M.C.; Gregory, C.; Kabral, A.; Zola, H.; Fournier, D.; Castaldi, P.A. Purification and preliminary characterization of the glycoprotein Ib complex in the human platelet membrane. *Eur. J. Biochem.* **1985**, *151*, 637–649. [CrossRef] [PubMed]
22. Zhang, W.; Deng, W.; Zhou, L.; Xu, Y.; Yang, W.; Liang, X.; Wang, Y.; Kulman, J.D.; Zhang, X.F.; Li, R. Identification of a juxtamembrane mechanosensitive domain in the platelet mechanosensor glycoprotein Ib-IX complex. *Blood* **2015**, *125*, 562–569. [CrossRef] [PubMed]

23. Greenberg, J.; Packham, M.A.; Cazenave, J.P.; Reimers, H.J.; Mustard, J.F. Effects on platelet function of removal of platelet sialic acid by neuraminidase. *Lab. Investig.* **1975**, *32*, 476–484. [PubMed]
24. Eto, K.; Kunishima, S. Linkage between the mechanisms of thrombocytopenia and thrombopoiesis. *Blood* **2016**, *127*, 1234–1241. [CrossRef]
25. Judson, P.A.; Anstee, D.J.; Clamp, J.R. Isolation and characterization of the major oligosaccharide of human platelet membrane glycoprotein GPIb. *Biochem. J.* **1982**, *205*, 81–90. [CrossRef]
26. Madoff, M.A.; Ebbe, S.; Baldini, M. Sialic acid of human blood platelets. *J. Clin. Investig.* **1964**, *43*, 870–877. [CrossRef]
27. Karpatkin, S.; Shulman, S. Asialo platelets enhance thrombopoiesis. *Trans. Assoc. Am. Physicians* **1980**, *93*, 244–250.
28. Kotzé, H.F.; van Wyk, V.; Badenhorst, P.N.; Heyns, A.D.; Roodt, J.P.; Lötter, M.G. Influence of platelet membrane sialic acid and platelet-associated IgG on ageing and sequestration of blood platelets in baboons. *Thromb. Haemost.* **1993**, *70*, 676–680. [CrossRef]
29. Sorensen, A.L.; Rumjantseva, V.; Nayeb-Hashemi, S.; Clausen, H.; Hartwig, J.H.; Wandall, H.H.; Hoffmeister, K.M. Role of sialic acid for platelet life span: Exposure of beta-galactose results in the rapid clearance of platelets from the circulation by asialoglycoprotein receptor-expressing liver macrophages and hepatocytes. *Blood* **2009**, *114*, 1645–1654. [CrossRef]
30. Jansen, A.J.; Josefsson, E.C.; Rumjantseva, V.; Liu, Q.P.; Falet, H.; Bergmeier, W.; Cifuni, S.M.; Sackstein, R.; von Andrian, U.H.; Wagner, D.D.; et al. Desialylation accelerates platelet clearance after refrigeration and initiates GPIbalpha metalloproteinase-mediated cleavage in mice. *Blood* **2012**, *119*, 1263–1273. [CrossRef]
31. Li, J.; Callum, J.L.; Lin, Y.; Zhou, Y.; Zhu, G.; Ni, H. Severe platelet desialylation in a patient with glycoprotein Ib/IX antibody-mediated immune thrombocytopenia and fatal pulmonary hemorrhage. *Haematologica* **2014**, *99*, e61–e63. [CrossRef] [PubMed]
32. Li, J.; van der Wal, D.E.; Zhu, G.; Xu, M.; Yougbare, I.; Ma, L.; Vadasz, B.; Carrim, N.; Grozovsky, R.; Ruan, M.; et al. Desialylation is a mechanism of Fc-independent platelet clearance and a therapeutic target in immune thrombocytopenia. *Nat. Commun.* **2015**, *6*, 7737. [CrossRef] [PubMed]
33. Shao, L.; Wu, Y.; Zhou, H.; Qin, P.; Ni, H.; Peng, J.; Hou, M. Successful treatment with oseltamivir phosphate in a patient with chronic immune thrombocytopenia positive for anti-GPIb/IX autoantibody. *Platelets* **2015**, *26*, 495–497. [CrossRef]
34. Revilla, N.; Corral, J.; Miñano, A.; Mingot-Castellano, M.E.; Campos, R.M.; Velasco, F.; Gonzalez, N.; Galvez, E.; Berrueco, R.; Fuentes, I.; et al. Multirefractory primary immune thrombocytopenia; targeting the decreased sialic acid content. *Platelets* **2019**, *30*, 743–751. [CrossRef] [PubMed]
35. Grodzielski, M.; Goette, N.P.; Glembotsky, A.C.; Constanza Baroni Pietto, M.; Mendez-Huergo, S.P.; Pierdominici, M.S.; Montero, V.S.; Rabinovich, G.A.; Molinas, F.C.; Heller, P.G.; et al. Multiple concomitant mechanisms contribute to low platelet count in patients with immune thrombocytopenia. *Sci. Rep.* **2019**, *9*, 2208. [CrossRef] [PubMed]
36. Zheng, S.S.; Perdomo, J.S.; Leung, H.H.L.; Yan, F.; Chong, B.H. Acquired Glanzmann thrombasthenia associated with platelet desialylation. *J. Thromb. Haemost.* **2020**, *18*, 714–721. [CrossRef] [PubMed]
37. Marini, I.; Zlamal, J.; Faul, C.; Holzer, U.; Hammer, S.; Pelzl, L.; Bethge, W.; Althaus, K.; Bakchoul, T. Autoantibody-mediated desialylation impairs human thrombopoiesis and platelet lifespan. *Haematologica* **2021**, *106*, 196–207. [CrossRef]
38. Zheng, S.S.; Ahmadi, Z.; Leung, H.H.L.; Wong, R.; Yan, F.; Perdomo, J.S.; Chong, B.H. Antiplatelet antibody predicts platelet desialylation and apoptosis in immune thrombocytopenia. *Haematologica* **2022**, *107*, 2195–2205. [CrossRef]
39. Leeksma, C.H.W.; Cohen, J.A. Determination of the Life of Human Blood Platelets using Labelled Diisopropylfluorophosphonate. *Nature* **1955**, *175*, 552–553. [CrossRef]
40. Josefsson, E.C.; Dowling, M.R.; Lebois, M.; Kile, B.T. Chapter 3—The Regulation of Platelet Life Span. In *Platelets*, 3rd ed.; Michelson, A.D., Ed.; Academic Press: Cambridge, MA, USA, 2013; pp. 51–65.
41. Kile, B.T. The role of apoptosis in megakaryocytes and platelets. *Br. J. Haematol.* **2014**, *165*, 217–226. [CrossRef]
42. Mason, K.D.; Carpinelli, M.R.; Fletcher, J.I.; Collinge, J.E.; Hilton, A.A.; Ellis, S.; Kelly, P.N.; Ekert, P.G.; Metcalf, D.; Roberts, A.W.; et al. Programmed anuclear cell death delimits platelet life span. *Cell* **2007**, *128*, 1173–1186. [CrossRef] [PubMed]
43. McArthur, K.; Chappaz, S.; Kile, B.T. Apoptosis in megakaryocytes and platelets: The life and death of a lineage. *Blood* **2018**, *131*, 605–610. [CrossRef] [PubMed]
44. Zhang, H.; Nimmer, P.M.; Tahir, S.K.; Chen, J.; Fryer, R.M.; Hahn, K.R.; Iciek, L.A.; Morgan, S.J.; Nasarre, M.C.; Nelson, R.; et al. Bcl-2 family proteins are essential for platelet survival. *Cell Death Differ.* **2007**, *14*, 943–951. [CrossRef] [PubMed]
45. Gardino, A.K.; Yaffe, M.B. 14-3-3 proteins as signaling integration points for cell cycle control and apoptosis. *Semin. Cell Dev. Biol.* **2011**, *22*, 688–695. [CrossRef]
46. Grozovsky, R.; Fraser, C.; Hoffmeister, K.M.; Sarosiek, K.; Giannini, S. Desialylation and Apoptosis Crosstalk to Modulate Platelet Clearance. *Blood* **2019**, *134*, 1055. [CrossRef]
47. Jacobson, M.D.; Burne, J.F.; Raff, M.C. Programmed cell death and Bcl-2 protection in the absence of a nucleus. *EMBO J.* **1994**, *13*, 1899–1910. [CrossRef]
48. Wolf, B.B.; Goldstein, J.C.; Stennicke, H.R.; Beere, H.; Amarante-Mendes, G.P.; Salvesen, G.S.; Green, D.R. Calpain functions in a caspase-independent manner to promote apoptosis-like events during platelet activation. *Blood* **1999**, *94*, 1683–1692. [CrossRef]
49. Piguet, P.F.; Vesin, C. Modulation of platelet caspases and life-span by anti-platelet antibodies in mice. *Eur. J. Haematol.* **2002**, *68*, 253–261. [CrossRef] [PubMed]
50. Rand, M.L.; Wang, H.; Bang, K.W.A.; Poon, K.S.V.; Packham, M.A.; Freedman, J. Procoagulant surface exposure and apoptosis in rabbit platelets: Association with shortened survival and steady-state senescence. *J. Thromb. Haemost.* **2004**, *2*, 651–659. [CrossRef]

51. Leytin, V.; Mykhaylov, S.; Starkey, A.F.; Allen, D.J.; Lau, H.; Ni, H.; Semple, J.W.; Lazarus, A.H.; Freedman, J. Intravenous immunoglobulin inhibits anti-glycoprotein IIb-induced platelet apoptosis in a murine model of immune thrombocytopenia. *Br. J. Haematol.* **2006**, *133*, 78–82. [CrossRef]
52. Leytin, V.; Allen, D.J.; Mykhaylov, S.; Lyubimov, E.; Freedman, J. Thrombin-triggered platelet apoptosis. *J. Thromb. Haemost.* **2006**, *4*, 2656–2663. [CrossRef] [PubMed]
53. Lopez, J.J.; Salido, G.M.; Gomez-Arteta, E.; Rosado, J.A.; Pariente, J.A. Thrombin induces apoptotic events through the generation of reactive oxygen species in human platelets. *J. Thromb. Haemost.* **2007**, *5*, 1283–1291. [CrossRef]
54. van der Wal, D.E.; Du, V.X.; Lo, K.S.; Rasmussen, J.T.; Verhoef, S.; Akkerman, J.W. Platelet apoptosis by cold-induced glycoprotein Ibalpha clustering. *J. Thromb. Haemost.* **2010**, *8*, 2554–2562. [CrossRef] [PubMed]
55. Winkler, J.; Kroiss, S.; Rand, M.L.; Azzouzi, I.; Annie Bang, K.W.; Speer, O.; Schmugge, M. Platelet apoptosis in paediatric immune thrombocytopenia is ameliorated by intravenous immunoglobulin. *Br. J. Haematol.* **2012**, *156*, 508–515. [CrossRef]
56. Goette, N.P.; Glembotsky, A.C.; Lev, P.R.; Grodzielski, M.; Contrufo, G.; Pierdominici, M.S.; Espasandin, Y.R.; Riveros, D.; García, A.J.; Molinas, F.C.; et al. Platelet Apoptosis in Adult Immune Thrombocytopenia: Insights into the Mechanism of Damage Triggered by Auto-Antibodies. *PLoS ONE* **2016**, *11*, e0160563. [CrossRef] [PubMed]
57. Chen, M.; Yan, R.; Zhou, K.; Li, X.; Zhang, Y.; Liu, C.; Jiang, M.; Ye, H.; Meng, X.; Pang, N.; et al. Akt-mediated platelet apoptosis and its therapeutic implications in immune thrombocytopenia. *Proc. Natl. Acad. Sci. USA* **2018**, *115*, E10682-e10691. [CrossRef]
58. Cohen, Y.C.; Djulbegovic, B.; Shamai-Lubovitz, O.; Mozes, B. The bleeding risk and natural history of idiopathic thrombocytopenic purpura in patients with persistent low platelet counts. *Arch. Intern. Med.* **2000**, *160*, 1630–1638. [CrossRef]
59. Deckmyn, H.; De Reys, S. Functional effects of human antiplatelet antibodies. *Semin. Thromb. Hemost.* **1995**, *21*, 46–59. [CrossRef]
60. Doobaree, I.U.; Nandigam, R.; Bennett, D.; Newland, A.; Provan, D. Thromboembolism in adults with primary immune thrombocytopenia: A systematic literature review and meta-analysis. *Eur. J. Haematol.* **2016**, *97*, 321–330. [CrossRef]
61. Hill, Q.A.; Newland, A.C. Fatigue in immune thrombocytopenia. *Br. J. Haematol.* **2015**, *170*, 141–149. [CrossRef]
62. Portielje, J.E.; Westendorp, R.G.; Kluin-Nelemans, H.C.; Brand, A. Morbidity and mortality in adults with idiopathic thrombocytopenic purpura. *Blood* **2001**, *97*, 2549–2554. [CrossRef] [PubMed]
63. Frederiksen, H.; Maegbaek, M.L.; Norgaard, M. Twenty-year mortality of adult patients with primary immune thrombocytopenia: A Danish population-based cohort study. *Br. J. Haematol.* **2014**, *166*, 260–267. [CrossRef] [PubMed]
64. Norgaard, M.; Jensen, A.O.; Engebjerg, M.C.; Farkas, D.K.; Thomsen, R.W.; Cha, S.; Zhao, S.; Sorensen, H.T. Long-term clinical outcomes of patients with primary chronic immune thrombocytopenia: A Danish population-based cohort study. *Blood* **2011**, *117*, 3514–3520. [CrossRef] [PubMed]
65. Schoonen, W.M.; Kucera, G.; Coalson, J.; Li, L.; Rutstein, M.; Mowat, F.; Fryzek, J.; Kaye, J.A. Epidemiology of immune thrombocytopenic purpura in the General Practice Research Database. *Br. J. Haematol.* **2009**, *145*, 235–244. [CrossRef] [PubMed]
66. Schmidt, D.E.; Heitink-Polle, K.M.J.; Porcelijn, L.; van der Schoot, C.E.; Vidarsson, G.; Bruin, M.C.A.; de Haas, M. Anti-platelet antibodies in childhood immune thrombocytopenia: Prevalence and prognostic implications. *J. Thromb. Haemost.* **2020**, *18*, 1210–1220. [CrossRef]
67. Brighton, T.A.; Evans, S.; Castaldi, P.A.; Chesterman, C.N.; Chong, B.H. Prospective evaluation of the clinical usefulness of an antigen-specific assay (MAIPA) in idiopathic thrombocytopenic purpura and other immune thrombocytopenias. *Blood* **1996**, *88*, 194–201. [CrossRef]
68. Al-Samkari, H.; Rosovsky, R.P.; Karp Leaf, R.S.; Smith, D.B.; Goodarzi, K.; Fogerty, A.E.; Sykes, D.B.; Kuter, D.J. A modern reassessment of glycoprotein-specific direct platelet autoantibody testing in immune thrombocytopenia. *Blood Adv.* **2019**, *4*, 9–18. [CrossRef]
69. He, R.; Reid, D.M.; Jones, C.E.; Shulman, N.R. Spectrum of Ig classes, specificities, and titers of serum antiglycoproteins in chronic idiopathic thrombocytopenic purpura. *Blood* **1994**, *83*, 1024–1032. [CrossRef]
70. Kiefel, V.; Santoso, S.; Kaufmann, E.; Mueller-Eckhardt, C. Autoantibodies against platelet glycoprotein Ib/IX: A frequent finding in autoimmune thrombocytopenic purpura. *Br. J. Haematol.* **1991**, *79*, 256–262. [CrossRef]
71. Vollenberg, R.; Jouni, R.; Norris, P.A.A.; Burg-Roderfeld, M.; Cooper, N.; Rummel, M.J.; Bein, G.; Marini, I.; Bayat, B.; Burack, R.; et al. Glycoprotein V is a relevant immune target in patients with immune thrombocytopenia. *Haematologica* **2019**, *104*, 1237–1243. [CrossRef]
72. Garner, S.F.; Campbell, K.; Metcalfe, P.; Keidan, J.; Huiskes, E.; Dong, J.F.; Lopez, J.A.; Ouwehand, W.H. Glycoprotein V: The predominant target antigen in gold-induced autoimmune thrombocytopenia. *Blood* **2002**, *100*, 344–346. [CrossRef] [PubMed]
73. Porcelijn, L.; Huiskes, E.; Oldert, G.; Schipperus, M.; Zwaginga, J.J.; de Haas, M. Detection of platelet autoantibodies to identify immune thrombocytopenia: State of the art. *Br. J. Haematol.* **2018**, *182*, 423–426. [CrossRef] [PubMed]
74. Johnsen, J. Pathogenesis in immune thrombocytopenia: New insights. *Hematol. Am. Soc. Hematol. Educ. Program* **2012**, *2012*, 306–312. [CrossRef]
75. Stasi, R.; Cooper, N.; Del Poeta, G.; Stipa, E.; Laura Evangelista, M.; Abruzzese, E.; Amadori, S. Analysis of regulatory T-cell changes in patients with idiopathic thrombocytopenic purpura receiving B cell-depleting therapy with rituximab. *Blood* **2008**, *112*, 1147–1150. [CrossRef] [PubMed]
76. Liu, B.; Zhao, H.; Poon, M.C.; Han, Z.; Gu, D.; Xu, M.; Jia, H.; Yang, R.; Han, Z.C. Abnormality of $CD4^+CD25^+$ regulatory T cells in idiopathic thrombocytopenic purpura. *Eur. J. Haematol.* **2007**, *78*, 139–143. [CrossRef]

77. Ling, Y.; Cao, X.; Yu, Z.; Ruan, C. Circulating dendritic cells subsets and CD4+Foxp3+ regulatory T cells in adult patients with chronic ITP before and after treatment with high-dose dexamethasome. *Eur. J. Haematol.* **2007**, *79*, 310–316. [CrossRef]
78. Zhang, J.; Ma, D.; Zhu, X.; Qu, X.; Ji, C.; Hou, M. Elevated profile of Th17, Th1 and Tc1 cells in patients with immune thrombocytopenic purpura. *Haematologica* **2009**, *94*, 1326–1329. [CrossRef]
79. Stasi, R.; Del Poeta, G.; Stipa, E.; Evangelista, M.L.; Trawinska, M.M.; Cooper, N.; Amadori, S. Response to B-cell-depleting therapy with rituximab reverts the abnormalities of T-cell subsets in patients with idiopathic thrombocytopenic purpura. *Blood* **2007**, *110*, 2924–2930. [CrossRef]
80. Zhang, H.; Zhang, B.M.; Guo, X.; Xu, L.; You, X.; West, R.B.; Bussel, J.B.; Zehnder, J.L. Blood transcriptome and clonal T-cell correlates of response and non-response to eltrombopag therapy in a cohort of patients with chronic immune thrombocytopenia. *Haematologica* **2020**, *105*, e129–e132. [CrossRef]
81. Fogarty, P.F.; Rick, M.E.; Zeng, W.; Risitano, A.M.; Dunbar, C.E.; Bussel, J.B. T cell receptor VB repertoire diversity in patients with immune thrombocytopenia following splenectomy. *Clin. Exp. Immunol.* **2003**, *133*, 461–466. [CrossRef]
82. Nieswandt, B.; Bergmeier, W.; Rackebrandt, K.; Gessner, J.E.; Zirngibl, H. Identification of critical antigen-specific mechanisms in the development of immune thrombocytopenic purpura in mice. *Blood* **2000**, *96*, 2520–2527. [CrossRef] [PubMed]
83. Hoffmeister, K.M.; Felbinger, T.W.; Falet, H.; Denis, C.V.; Bergmeier, W.; Mayadas, T.N.; von Andrian, U.H.; Wagner, D.D.; Stossel, T.P.; Hartwig, J.H. The clearance mechanism of chilled blood platelets. *Cell* **2003**, *112*, 87–97. [CrossRef] [PubMed]
84. Hoffmeister, K.M.; Josefsson, E.C.; Isaac, N.A.; Clausen, H.; Hartwig, J.H.; Stossel, T.P. Glycosylation restores survival of chilled blood platelets. *Science* **2003**, *301*, 1531–1534. [CrossRef]
85. Rumjantseva, V.; Grewal, P.K.; Wandall, H.H.; Josefsson, E.C.; Sorensen, A.L.; Larson, G.; Marth, J.D.; Hartwig, J.H.; Hoffmeister, K.M. Dual roles for hepatic lectin receptors in the clearance of chilled platelets. *Nat. Med.* **2009**, *15*, 1273–1280. [CrossRef]
86. Qiao, J.; Al-Tamimi, M.; Baker, R.I.; Andrews, R.K.; Gardiner, E.E. The platelet Fc receptor, FcgammaRIIa. *Immunol. Rev.* **2015**, *268*, 241–252. [CrossRef] [PubMed]
87. Chan, H.; Moore, J.C.; Finch, C.N.; Warkentin, T.E.; Kelton, J.G. The IgG subclasses of platelet-associated autoantibodies directed against platelet glycoproteins IIb/IIIa in patients with idiopathic thrombocytopenic purpura. *Br. J. Haematol.* **2003**, *122*, 818–824. [CrossRef]
88. Cantoni, S.; Carpenedo, M.; Nichelatti, M.; Sica, L.; Rossini, S.; Milella, M.; Popescu, C.; Cairoli, R. Clinical relevance of antiplatelet antibodies and the hepatic clearance of platelets in patients with immune thrombocytopenia. *Blood* **2016**, *128*, 2183–2185. [CrossRef]
89. Amini, S.N.; Nelson, V.S.; Porcelijn, L.; Netelenbos, T.; Zwaginga, J.J.; de Haas, M.; Schipperus, M.R.; Kapur, R. The interplay between GPIb/IX antibodies, platelet hepatic sequestration, and TPO levels in patients with chronic ITP. *Blood Adv.* **2023**, *7*, 1066–1069. [CrossRef]
90. Morodomi, Y.; Kanaji, S.; Won, E.; Ruggeri, Z.M.; Kanaji, T. Mechanisms of anti-GPIbα antibody–induced thrombocytopenia in mice. *Blood* **2020**, *135*, 2292–2301. [CrossRef]
91. Qiao, J.; Liu, Y.; Li, D.; Wu, Y.; Li, X.; Yao, Y.; Niu, M.; Fu, C.; Li, H.; Ma, P.; et al. Imbalanced expression of Bcl-xL and Bax in platelets treated with plasma from immune thrombocytopenia. *Immunol. Res.* **2016**, *64*, 604–609. [CrossRef]
92. Nassa, G.; Giurato, G.; Cimmino, G.; Rizzo, F.; Ravo, M.; Salvati, A.; Nyman, T.A.; Zhu, Y.; Vesterlund, M.; Lehtiö, J.; et al. Splicing of platelet resident pre-mRNAs upon activation by physiological stimuli results in functionally relevant proteome modifications. *Sci. Rep.* **2018**, *8*, 498. [CrossRef] [PubMed]
93. Mills, E.W.; Green, R.; Ingolia, N.T. Slowed decay of mRNAs enhances platelet specific translation. *Blood* **2017**, *129*, e38–e48. [CrossRef] [PubMed]
94. Feng, S.; Christodoulides, N.; Resendiz, J.C.; Berndt, M.C.; Kroll, M.H. Cytoplasmic domains of GpIbalpha and GpIbbeta regulate 14-3-3zeta binding to GpIb/IX/V. *Blood* **2000**, *95*, 551–557. [CrossRef]
95. George, N.M.; Targy, N.; Evans, J.J.; Zhang, L.; Luo, X. Bax contains two functional mitochondrial targeting sequences and translocates to mitochondria in a conformational change- and homo-oligomerization-driven process. *J. Biol. Chem.* **2010**, *285*, 1384–1392. [CrossRef]
96. Li, S.; Wang, Z.; Liao, Y.; Zhang, W.; Shi, Q.; Yan, R.; Ruan, C.; Dai, K. The glycoprotein Ibalpha-von Willebrand factor interaction induces platelet apoptosis. *J. Thromb. Haemost.* **2010**, *8*, 341–350. [CrossRef] [PubMed]
97. Yuan, Y.; Zhang, W.; Yan, R.; Liao, Y.; Zhao, L.; Ruan, C.; Du, X.; Dai, K. Identification of a novel 14-3-3zeta binding site within the cytoplasmic domain of platelet glycoprotein Ibalpha that plays a key role in regulating the von Willebrand factor binding function of glycoprotein Ib-IX. *Circ. Res.* **2009**, *105*, 1177–1185. [CrossRef]
98. Vrbensky, J.R.; Moore, J.E.; Arnold, D.M.; Smith, J.W.; Kelton, J.G.; Nazy, I. The sensitivity and specificity of platelet autoantibody testing in immune thrombocytopenia: A systematic review and meta-analysis of a diagnostic test. *J. Thromb. Haemost.* **2019**, *17*, 787–794. [CrossRef]
99. Olsson, B.; Andersson, P.O.; Jernas, M.; Jacobsson, S.; Carlsson, B.; Carlsson, L.M.; Wadenvik, H. T-cell-mediated cytotoxicity toward platelets in chronic idiopathic thrombocytopenic purpura. *Nat. Med.* **2003**, *9*, 1123–1124. [CrossRef] [PubMed]
100. Zhang, F.; Chu, X.; Wang, L.; Zhu, Y.; Li, L.; Ma, D.; Peng, J.; Hou, M. Cell-mediated lysis of autologous platelets in chronic idiopathic thrombocytopenic purpura. *Eur. J. Haematol.* **2006**, *76*, 427–431. [CrossRef]
101. Zhao, C.; Li, X.; Zhang, F.; Wang, L.; Peng, J.; Hou, M. Increased cytotoxic T-lymphocyte-mediated cytotoxicity predominant in patients with idiopathic thrombocytopenic purpura without platelet autoantibodies. *Haematologica* **2008**, *93*, 1428–1430. [CrossRef]

102. Qiu, J.; Liu, X.; Li, X.; Zhang, X.; Han, P.; Zhou, H.; Shao, L.; Hou, Y.; Min, Y.; Kong, Z.; et al. $CD8^+$ T cells induce platelet clearance in the liver via platelet desialylation in immune thrombocytopenia. *Sci. Rep.* **2016**, *6*, 27445. [CrossRef] [PubMed]
103. Malik, A.; Sayed, A.A.; Han, P.; Tan, M.M.H.; Watt, E.; Constantinescu-Bercu, A.; Cocker, A.T.H.; Khoder, A.; Saputil, R.C.; Thorley, E.V.; et al. The role of $CD8^+$ T cell clones in immune thrombocytopenia. *Blood* **2023**, *141*, 2417–2429. [CrossRef] [PubMed]
104. Li, S.; Wang, L.; Zhao, C.; Li, L.; Peng, J.; Hou, M. CD8+ T cells suppress autologous megakaryocyte apoptosis in idiopathic thrombocytopenic purpura. *Br. J. Haematol.* **2007**, *139*, 605–611. [CrossRef] [PubMed]
105. Vrbensky, J.R.; Nazy, I.; Toltl, L.J.; Ross, C.; Ivetic, N.; Smith, J.W.; Kelton, J.G.; Arnold, D.M. Megakaryocyte apoptosis in immune thrombocytopenia. *Platelets* **2018**, *29*, 729–732. [CrossRef]
106. Choi, P.Y.; Merriman, E.; Bennett, A.; Enjeti, A.K.; Tan, C.W.; Goncalves, I.; Hsu, D.; Bird, R. Consensus guidelines for the management of adult immune thrombocytopenia in Australia and New Zealand. *Med. J. Aust.* **2022**, *216*, 43–52. [CrossRef] [PubMed]
107. Fujisawa, K.; Tani, P.; Piro, L.; McMillan, R. The effect of therapy on platelet-associated autoantibody in chronic immune thrombocytopenic purpura. *Blood* **1993**, *81*, 2872–2877. [CrossRef] [PubMed]
108. Wang, L.; Xu, L.; Hao, H.; Jansen, A.J.G.; Liu, G.; Li, H.; Liu, X.; Zhao, Y.; Peng, J.; Hou, M. First line treatment of adult patients with primary immune thrombocytopenia: A real-world study. *Platelets* **2020**, *31*, 55–61. [CrossRef] [PubMed]
109. Cragg, M.S.; Walshe, C.A.; Ivanov, A.O.; Glennie, M.J. The biology of CD20 and its potential as a target for mAb therapy. *Curr. Dir. Autoimmun.* **2005**, *8*, 140–174. [CrossRef] [PubMed]
110. Arnold, D.M.; Vrbensky, J.R.; Karim, N.; Smith, J.W.; Liu, Y.; Ivetic, N.; Kelton, J.G.; Nazy, I. The effect of rituximab on anti-platelet autoantibody levels in patients with immune thrombocytopenia. *Br. J. Haematol.* **2017**, *178*, 302–307. [CrossRef]
111. Szczepanik, A.B.; Sikorska, A.; Slomkowski, M.; Konopka, L. The use of vinca alkaloids in preparation for splenectomy of corticosteroid refractory chronic immune thrombocytopenic purpura patients. *Int. J. Lab. Hematol.* **2007**, *29*, 347–351. [CrossRef]
112. Quiquandon, I.; Fenaux, P.; Caulier, M.T.; Pagniez, D.; Huart, J.J.; Bauters, F. Re-evaluation of the role of azathioprine in the treatment of adult chronic idiopathic thrombocytopenic purpura: A report on 53 cases. *Br. J. Haematol.* **1990**, *74*, 223–228. [CrossRef] [PubMed]
113. Kuter, D.J.; Efraim, M.; Mayer, J.; Trněný, M.; McDonald, V.; Bird, R.; Regenbogen, T.; Garg, M.; Kaplan, Z.; Tzvetkov, N.; et al. Rilzabrutinib, an Oral BTK Inhibitor, in Immune Thrombocytopenia. *N. Engl. J. Med.* **2022**, *386*, 1421–1431. [CrossRef] [PubMed]
114. Ware, R.E.; Zimmerman, S.A. Anti-D: Mechanisms of action. *Semin. Hematol.* **1998**, *35*, 14–22. [PubMed]
115. Bradbury, C.A.; Pell, J.; Hill, Q.; Bagot, C.; Cooper, N.; Ingram, J.; Breheny, K.; Kandiyali, R.; Rayment, R.; Evans, G.; et al. Mycophenolate Mofetil for First-Line Treatment of Immune Thrombocytopenia. *N. Engl. J. Med.* **2021**, *385*, 885–895. [CrossRef]
116. Choi, P.Y.; Roncolato, F.; Badoux, X.; Ramanathan, S.; Ho, S.J.; Chong, B.H. A novel triple therapy for ITP using high-dose dexamethasone, low-dose rituximab, and cyclosporine (TT4). *Blood* **2015**, *126*, 500–503. [CrossRef]
117. Kuter, D.J. Novel therapies for immune thrombocytopenia. *Br. J. Haematol.* **2022**, *196*, 1311–1328. [CrossRef]
118. Semple, J.W.; Provan, D. The immunopathogenesis of immune thrombocytopenia: T cells still take center-stage. *Curr. Opin. Hematol.* **2012**, *19*, 357–362. [CrossRef]
119. Dameshek, W.; Miller, E.B. The megakaryocytes in idiopathic thrombocytopenic purpura, a form of hypersplenism. *Blood* **1946**, *1*, 27–50. [CrossRef]
120. Stahl, C.P.; Zucker-Franklin, D.; McDonald, T.P. Incomplete antigenic cross-reactivity between platelets and megakaryocytes: Relevance to ITP. *Blood* **1986**, *67*, 421–428. [CrossRef]
121. Houwerzijl, E.J.; Blom, N.R.; van der Want, J.J.; Esselink, M.T.; Koornstra, J.J.; Smit, J.W.; Louwes, H.; Vellenga, E.; de Wolf, J.T. Ultrastructural study shows morphologic features of apoptosis and para-apoptosis in megakaryocytes from patients with idiopathic thrombocytopenic purpura. *Blood* **2004**, *103*, 500–506. [CrossRef]
122. Iraqi, M.; Perdomo, J.; Yan, F.; Choi, P.Y.; Chong, B.H. Immune thrombocytopenia: Antiplatelet autoantibodies inhibit proplatelet formation by megakaryocytes and impair platelet production in vitro. *Haematologica* **2015**, *100*, 623–632. [CrossRef] [PubMed]
123. Lev, P.R.; Grodzielski, M.; Goette, N.P.; Glembotsky, A.C.; Espasandin, Y.R.; Pierdominici, M.S.; Contrufo, G.; Montero, V.S.; Ferrari, L.; Molinas, F.C.; et al. Impaired proplatelet formation in immune thrombocytopenia: A novel mechanism contributing to decreased platelet count. *Br. J. Haematol.* **2014**, *165*, 854–864. [CrossRef]
124. Peerschke, E.I.; Andemariam, B.; Yin, W.; Bussel, J.B. Complement activation on platelets correlates with a decrease in circulating immature platelets in patients with immune thrombocytopenic purpura. *Br. J. Haematol.* **2010**, *148*, 638–645. [CrossRef]
125. Najaoui, A.; Bakchoul, T.; Stoy, J.; Bein, G.; Rummel, M.J.; Santoso, S.; Sachs, U.J. Autoantibody-mediated complement activation on platelets is a common finding in patients with immune thrombocytopenic purpura (ITP). *Eur. J. Haematol.* **2012**, *88*, 167–174. [CrossRef]
126. Peerschke, E.I.; Panicker, S.; Bussel, J. Classical complement pathway activation in immune thrombocytopenia purpura: Inhibition by a novel C1s inhibitor. *Br. J. Haematol.* **2016**, *173*, 942–945. [CrossRef]
127. Stasi, R. Eltrombopag for the treatment of idiopathic thrombocytopenic purpura. *Expert. Rev. Hematol.* **2008**, *1*, 145–152. [CrossRef] [PubMed]
128. Kuter, D.J. New thrombopoietic growth factors. *Blood* **2007**, *109*, 4607–4616. [CrossRef]
129. Cines, D.B.; Gernsheimer, T.; Wasser, J.; Godeau, B.; Provan, D.; Lyons, R.; Altomare, I.; Wang, X.; Lopez, A. Integrated analysis of long-term safety in patients with chronic immune thrombocytopaenia (ITP) treated with the thrombopoietin (TPO) receptor agonist romiplostim. *Int. J. Hematol.* **2015**, *102*, 259–270. [CrossRef] [PubMed]

130. Bussel, J.B.; Kuter, D.J.; Aledort, L.M.; Kessler, C.M.; Cuker, A.; Pendergrass, K.B.; Tang, S.; McIntosh, J. A randomized trial of avatrombopag, an investigational thrombopoietin-receptor agonist, in persistent and chronic immune thrombocytopenia. *Blood* **2014**, *123*, 3887–3894. [CrossRef]
131. Broome, C.M.; Roth, A.; Kuter, D.J.; Scully, M.; Smith, R.; Wang, J.; Reuter, C.; Hobbs, W.E.; Daak, A.A.A. Safety and Efficacy of Classical Complement Pathway Inhibition with Sutimlimab in Chronic Immune Thrombocytopenia. *Blood Adv.* **2023**, *7*, 987–996. [CrossRef]
132. Davies, B.E. Pharmacokinetics of oseltamivir: An oral antiviral for the treatment and prophylaxis of influenza in diverse populations. *J. Antimicrob. Chemother.* **2010**, *65* (Suppl. 2), ii5–ii10. [CrossRef] [PubMed]
133. Moscona, A. Neuraminidase inhibitors for influenza. *N. Engl. J. Med.* **2005**, *353*, 1363–1373. [CrossRef] [PubMed]
134. Li, C.Y.; Yu, Q.; Ye, Z.Q.; Sun, Y.; He, Q.; Li, X.M.; Zhang, W.; Luo, J.; Gu, X.; Zheng, X.; et al. A nonsynonymous SNP in human cytosolic sialidase in a small Asian population results in reduced enzyme activity: Potential link with severe adverse reactions to oseltamivir. *Cell Res.* **2007**, *17*, 357–362. [CrossRef] [PubMed]
135. Alioglu, B.; Tasar, A.; Ozen, C.; Selver, B.; Dallar, Y. An experience of oseltamivir phosphate (tamiflu) in a pediatric patient with chronic idiopathic thrombocytopenic purpura: A case report. *Pathophysiol. Haemost. Thromb.* **2010**, *37*, 55–58. [CrossRef] [PubMed]
136. Bigot, P.; Auffret, M.; Gautier, S.; Weinborn, M.; Ettahar, N.K.; Coupe, P. Unexpected platelets elevation in a patient with idiopathic thrombocytopenia treated with oseltamivir for influenza infection. *Fundam. Clin. Pharmacol.* **2016**, *30*, 483–485. [CrossRef]
137. Álvarez-Román, M.T.; Rivas Pollmar, M.I.; Bernardino, J.I.; Lozano, M.L.; Martín-Salces, M.; Fernández-Bello, I.; Jiménez-Yuste, V.; Butta, N.V. Thrombopoietin receptor agonists in conjunction with oseltamivir for immune thrombocytopenia. *AIDS* **2016**, *30*, 1141–1142. [CrossRef]
138. Sun, L.; Wang, J.; Shao, L.; Yuan, C.; Zhao, H.; Li, D.; Wang, Z.; Han, P.; Yu, Y.; Xu, M.; et al. Dexamethasone plus oseltamivir versus dexamethasone in treatment-naive primary immune thrombocytopenia: A multicentre, randomised, open-label, phase 2 trial. *Lancet Haematol.* **2021**, *8*, e289–e298. [CrossRef]
139. Stasi, R.; Newland, A.C. ITP: A historical perspective. *Br. J. Haematol.* **2011**, *153*, 437–450. [CrossRef]

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Review

Extracellular Vesicles: A Novel Diagnostic Tool and Potential Therapeutic Approach for Equine Osteoarthritis

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Abstract: Osteoarthritis (OA) is a chronic progressive degenerative joint disease that affects a significant portion of the equine population and humans worldwide. Current treatment options for equine OA are limited and incompletely curative. Horses provide an excellent large-animal model for studying human OA. Recent advances in the field of regenerative medicine have led to the exploration of extracellular vesicles (EVs)—cargoes of microRNA, proteins, lipids, and nucleic acids—to evaluate their diagnostic value in terms of disease progression and severity, as well as a potential cell-free therapeutic approach for equine OA. EVs transmit molecular signals that influence various biological processes, including the inflammatory response, apoptosis, proliferation, and cell communication. In the present review, we summarize recent advances in the isolation and identification of EVs, the use of their biologically active components as biomarkers, and the distribution of the gap junction protein connexin 43. Moreover, we highlight the role of mesenchymal stem cell-derived EVs as a potential therapeutic tool for equine musculoskeletal disorders. This review aims to provide a comprehensive overview of the current understanding of the pathogenesis, diagnosis, and treatment strategies for OA. In particular, the roles of EVs as biomarkers in synovial fluid, chondrocytes, and plasma for the early detection of equine OA are discussed.

Keywords: mesenchymal stem cells; extracellular vesicles; osteoarthritis; veterinary medicine

1. Introduction

Osteoarthritis (OA) is a progressive, degenerative, and multifactorial joint disease that constitutes the most common form of musculoskeletal disorder worldwide [1–3]. The disease affects approximately 50 million people in the United States, compared to 100 million reported in Europe, according to [4,5]. As in humans, the disease has a negative impact within veterinary medicine, as reported in horses [6,7], as well as in dogs [8] and cats with hip-joint OA [9]. Achieving satisfactory treatment of damaged articular cartilage has become a major challenge in both human orthopedics and clinical veterinary medicine due to several factors, including the absence of effective pharmacological drugs that provide temporary pain relief and cartilage tissue engineering being insufficient to deliver the optimal therapeutic effect [10]. In this context, the use of mesenchymal stem cells for chondrocyte replenishment in clinical practice has not yet been fully elucidated [11,12]. Moreover, OA is one of the leading causes of lameness in both leisure and sport horses [13,14]. The main symptoms of OA are pain, limited mobility, and stiffness [15]. However, as the disease progresses, the subchondral bone, joint capsule, and adjacent ligaments and muscles may also be damaged [16]. Moreover, in the advanced stage of the disease, OA is accompanied by

alteration of the biochemical markers, along with deterioration of the extracellular matrix and the cellular components of the cartilage [17,18]. Reliable treatment options are urgently needed to repair the deterioration of articular cartilage and minimize the subchondral bone damage, which subsequently leads to progressive locomotor dysfunction and joint pain [16]. None of the currently available pharmacological treatments are able to slow the progression of the disease, being limited to symptom management [16]; additionally, some of them cannot reverse the tissue damage, and their application may be accompanied by adverse effects [4]. In addition, the mechanisms of OA's pathophysiology and development remain poorly understood, involving alterations of several factors and cytokines, such as IL-1β, TNF-α, TGF-β, MMP13, and others [4]. Although the diagnosis is commonly based on clinical examination and radiographic imaging, the insidious nature of the disease causes chronic cartilage damage that becomes less responsive to intrinsic repair due, on the one hand, to the degenerative alterations of the cartilage and loss of type I/II collagen and, on the other hand, to the accumulation of pro-inflammatory cytokines that disrupt the homeostasis of the cartilage matrix [19–21]. It has been reported that several factors have been identified as potential causes of OA, including genetic background, aging, and obesity; a clear understanding of OA's pathogenesis remains to be fully elucidated [4]. A study reported that the progression from joint injury to detectable biological evidence of joint deterioration is difficult to study in humans. The reasons for this include the lack of accurate quantitative assessments of joint injury severity, particularly the biological reaction and tissue alterations, as well as the evaluation of the pathogenesis and the interaction with the other tissues involved in the affected joint. The authors suggested the use of an animal model to detect the biological changes in the course of OA, as well as to evaluate the effects of surgical interference and to optimize the pharmacological treatments [22].

This review provides an overview of the causes, diagnosis, and current therapeutic treatments of OA. We review how to identify and define biomarkers of the disease that can be used to develop rapid and efficient diagnostic tools. We discuss the role of mesenchymal stem cell-derived extracellular vesicles (EVs)/exosomes in modulating the immune response, cellular communication, enhancing autophagy, and inhibiting apoptosis in the joint microenvironment. We also demonstrate the role of MSC secretomes and EVs in OA as potential biomarkers for sensitive and early disease detection, as well as the therapeutic potential of MSC-derived EVs in OA. We selected horses as the large-animal model because the equine stifle joint has a complex anatomical structure similar to that of the human knee, making horses a suitable animal model to evaluate the pathogenesis and clinical findings of OA [23], along with future prospects in equine research regarding the application of EVs for diagnosis and therapy. Thus, EVs offer a novel diagnostic approach, not only because they provide an appropriate biomarker to assess the progression of OA in veterinary medicine through analyzing their contents but because they also aid in understanding the cell-cell and cell-matrix cross-talk to improve OA treatment strategies.

2. Causes and Progression of Equine OA

In general terms, OA is characterized by multifactorial chronic joint degeneration [24]. Clinical surveys have indicated that around 60% of equine lameness is linked to OA [25,26]. OA results from several overlapping diseases with different causes, but they all lead to similar morphological, biological, and clinical outcomes [27]. The progression of OA depends on both the causative agents and the severity of the condition, starting with articular cartilage and extending to the subchondral bone and surrounding tissues (including ligaments and the synovial membrane) [28]. These changes lead to a series of morphological deteriorations from focal articular degeneration, followed by fibrotic lesions and ulcer formation, to complete loss of the articular surface [29]. In horses, factors such as obesity, aging, genetic predisposition, and trauma contribute to chronic inflammation and long-term joint damage [30]. The metacarpophalangeal and femorotibial joints are common sites of OA in horses [6]. The progression of OA is largely dependent on the inflammatory processes [31,32]. In the course of OA, the inflammatory response due to mechanical stress

has been described as altering the balance between anabolic and catabolic processes in the joint [33], thereby causing a predisposition to articular cartilage degeneration, progressive joint damage, and pain [34]. However, an appropriate balance between these processes is essential to maintain cartilage integrity and stop molecular damage during daily activities [33]. Excessive production of pro-inflammatory cytokines such as interleukin 1-β (IL-1β) and tumor necrosis factor-α (TNF-α) drives cartilage matrix degradation, while chemokines (e.g., monocyte chemotactic protein 1, granulocyte-macrophage colony-stimulating factor) and matrix metalloproteinases (MMPs) contribute to the resorption of the subchondral bone [4,35]. It has been reported that a number of inflammatory cytokines play key roles in the development of OA, including IL-1β, TNF-α, IL-6, IL-15, IL-17, and IL-18, as previously reviewed in [36]. One study reported upregulated TNF-α expression in the synovial membrane and the cartilage in equine OA, whereas IL-1β was upregulated only in the cartilage, not in the synovium [37]. Inflammatory cytokines showed different levels of response, which could impact the severity of the disease. While IL-1β plays a key role in both equine and human OA [38], TNF-α is primarily involved in acute OA cases in humans [39]. The inflammatory response in OA disrupts TGF-β's role in preventing chondrocyte hypertrophy, as IL-1β and other cytokines inhibit TGF-β signaling, leading to cartilage damage [40]. Furthermore, IL-1β has been shown to inhibit the SMAD signaling cascade, which prevents TGF-β receptor synthesis in chondrocytes [41]. In addition, IL-1β stimulates the production of reactive oxygen species (ROS), which not only reduces the expression of oxidative enzymes but also damages articular cartilage [42]. Evidence suggests that pro-inflammatory cytokines such as IL-8, TNF-α, and H_2O_2 contribute to chondrocyte hypertrophy, further worsening OA [43–45].

3. Diagnosis of OA

The basic features of OA can be identified either by the clinical symptom of pain, by physical disability and morphological changes of the joint, or by radiological examination to detect a high bone density, reduced joint space, and the presence of osteophytes. In addition, magnetic resonance imaging (MRI) can be used to detect articular cartilage damage [6]. The process of diagnosing OA has evolved in recent years; characterization of the molecular alterations could provide a diagnostic overview regarding the progression of OA. Based on the detection of biomarkers, one study categorized the sequence of events in the course of knee OA, including pre-, early-, progressive-, and end-stage OA. This facilitated the evaluation of therapeutic applications in a homogeneous group of patients [46]. It has been reported that the measurement of biological markers, including 846 epitopes, chondroitin sulfate, and type II procollagen, can provide an indication of osteochondral fragmentation in synovial fluid and serum in horses [47]. In this regard, the presence of numerous M1 macrophages in the synovium is indicative of pro-inflammatory secretion and high cartilage degradation [48,49]. Furthermore, cytokines and adipokines released from the infrapatellar fat pad indicate local inflammation and are involved in the pathogenesis of OA [50]. Such molecular alterations indicate a developing pathological condition in the joint. Characterization of these biomarkers using a less invasive approach would improve the accuracy of diagnosis. It has been proposed that synovial fluid, blood, and urine are valuable resources for the identification of biomarkers [51]. Although urine and blood samples are easily harvested to detect pathological alterations [52,53], synovial fluid is the optimal resource for identifying molecular biomarkers due to its central role in maintaining the cross-talk between all of the joint compartments [54].

4. Current Treatment Strategies for OA

The development of appropriate and effective disease-modifying OA drugs (DMOADs) is currently challenging. The most common treatment options for OA to date are based on non-pharmacological, pharmacological, and surgical approaches to minimize pain and improve joint mobility [55]. However, none of these approaches have shown satisfactory results [56]. Pharmacological therapy is based on the use of nonsteroidal anti-inflammatory

dexamethasone (NSAID) to reduce the inflammatory response and temporarily relieve OA-associated pain in horses [57]. Intra-articular steroid administration has been reported in sport horses, including betamethasone, methylprednisolone acetate, and triamcinolone administration, as reviewed in [6]. Intra-articular injection of sodium hyaluronan or polysulfated glycosaminoglycan has been shown to improve the pathology of OA by reducing cartilage fibrillation, which may be a therapeutic choice for OA in horses [58]. Non-pharmacological treatment strategies include physiotherapy to maintain joint function through physical activity [59]. Moreover, electrophysical therapy approaches, including transcutaneous electrical nerve induction, neuromuscular electrical stimulation, pulsed electromagnetic fields, ultrasound, laser therapy, vibration therapy, and shockwave therapy, have previously been described for horses, as reviewed in [60]. Manual therapy, with a focus on the mobilization of specific target joints, can be used to reduce pain and joint stiffness and increase the range of motion in early OA [61,62]. Physiotherapy can improve locomotor and joint performance, as well reducing the accompanying pain and the inflammatory condition. Several therapeutic options can also be used to treat the affected joints, such as ice, heat, electricity, magnetic fields, compression, and movement, as reviewed in [63]. As in humans, exercise plays an essential role in the physiological performance of the joints in young and old horses. It has been reported that mechanical load has a particular impact on cartilage integrity, joint homeostasis, and vascular flow [64]. Exercise has been shown to improve cartilage regeneration and reduce bone remodeling in experimental models of cartilage lesions in horses, as reviewed in [65]. Moreover, laser therapy has been reported as a rehabilitation therapy in which particular care should be taken regarding the application intensity, frequencies, and target tissue depth. The anti-inflammatory effect of laser therapy arises from the stimulation of prostaglandin, which results in vasodilatation; this approach also reduces pain and joint swelling [66]. Therapeutic ultrasound can sufficiently penetrate horse joints, resulting in improvements in tissue metabolism and oxygenation, as well as enhancements in the infiltration of inflammatory cells to the target lesion [60]. Physical methods using focused extracorporeal shockwave therapy (ESWT) have been reported in equine medicine [67]. The use of ESWT has been shown to activate biomarkers related to bone remodeling [68]. Cryotherapy has previously been recommended to reduce the inflammatory response and improve tissue function. A study investigated the effects of induced cryotherapy on the footprint and biochemical components of rat joints. The authors demonstrated that cryotherapy treatment increased the footprint area while reducing the leukocyte count and inflammatory cytokine concentrations in joints in OA patients compared to controls [69]. Similarly, it was determined that short-term cryotherapy could be used as a conservative treatment to relieve pain and improve functional performance in humans with knee OA [70]. The use of magnetic fields in equine physiotherapy has been proposed, with one study showing that applying a magnetic field for one hour every second day may improve cell regeneration and enhance bone symphysis [71]. Along the same lines, an in vitro study investigated the ability of electromagnetic fields to reverse experimentally induced cartilage degeneration in bovines. The authors revealed that a pulsed electromagnetic field could be applied as an adjuvant treatment to protect cartilage from the negative effects of increased inflammatory cytokine concentrations in OA [72]. However, to the best of our knowledge, there are no studies demonstrating the effectiveness of cryotherapy in horses, especially those with advanced OA, and further studies should be conducted to prove the suitability of this method for clinical application. Gene therapy has been proposed as a treatment option in the same context, with one study demonstrating that administration of the interleukin-1 receptor antagonist was able to eliminate the catabolic effect and reduce the progression of OA in horses [73,74]. The use of the aforementioned conventional therapy options is either inadequate or restricted to severe OA due to severe degradation of the articular cartilage, involvement of the subchondral bone, and a reduction in the joint space. Surgical interventions represent the last choice and have been used in human medicine [75,76], involving the repair of the cartilage surface, arthroplasty, or complete joint replacement [77]. Recently, the use of bone marrow- or adi-

pose tissue-derived mesenchymal stem cells (ASCs, BMSCs) has been proposed as a novel therapeutic intervention. It has been reported that intra-articular administration of BMSCs improves prostaglandin E2 levels in synovial fluid, but further studies are recommended to confirm the utility of BMSVs in therapeutic applications [78].

5. Inflammatory Responses Trigger Stem Cell Activation During OA

Cell-based therapy has become a promising strategy for tissue regeneration. The isolation and characterization of stem cells provide an alternative tool for the treatment of chronic incurable diseases, including OA and tendinopathy. MSCs have become increasingly promising for addressing the requirements for treating degenerative diseases that are unmet through conventional medicine. Self-renewal and multipotency are the key hallmarks of stem cells, allowing them to act as fundamental units to promote growth, homeostasis, and tissue injury repair. These two important characteristics position stem cells as a reasonable tool for regenerative applications [79,80]. Although MSCs have the ability to differentiate into almost all musculoskeletal cell types [81,82], they also have potent immunosuppressive activities [83–85] and the ability to induce vascularization [86]. Therefore, these cells have become an interesting potential choice for tissue engineering applications, especially in veterinary medicine. To date, no conservative therapies have been able to achieve complete tissue repair. Therefore, the application of MSCs in tissue engineering has understandably raised hopes for faster healing and complete repair after musculoskeletal injuries [87]. In addition to their potential differentiation into osteogenic and chondrogenic lineages [88], the use of MSCs has been reported as a potential approach for tendon repair [89]. Despite the use of stem cells as an appropriate resource for cell-based therapeutic applications, several problems have been reported, including loss of guidance cytokines, cellular host rejection, and off-target migration, which collectively could compromise tissue regeneration.

It is well established that the inflammatory response during the development of OA causes chondrocyte and matrix degradation due to the increased release of metalloproteinases, collagenases, PGE2, aggrecans, and free radicals in the joint microenvironment, as reviewed in [6]. A body of evidence has demonstrated the immunomodulatory activity of mesenchymal stem cells [90–92]. The key mechanism behind this activity relies on stem cells' ability to inhibit the proliferation of B and T cells, as well as to decrease the production of pro-inflammatory cytokines and reduce cytotoxicity [93]. Although the ability of MSCs to undergo chondrogenic differentiation has been documented, optimal cartilage repair in OA requires the suppression of cytokines such as IL-1α and TNF-α [94]. It has been observed that MSCs isolated from the synovial membrane have a greater capacity for chondrogenic differentiation compared to BMSCs [95], and these cells were able to improve meniscal cartilage healing in a rabbit model of OA [96]. Similarly, MSCs isolated from the infrapatellar fat pad have proven to be an interesting, easily accessible source for cartilage treatment, as reviewed in [97]. One of the important features of MSCs is their immunomodulatory activity, which helps overcome the negative effects of inflammatory cytokines in the joint microenvironment, thereby facilitating enhanced tissue repair [98]. It has been reported that the action of MSCs is based on the delivery of chemokines and cytokines that not only enable the repair of tissue damage and restoration of normal metabolism but also modulate the inflammatory response. The production of these molecules is relatively dependent on the level of tissue damage and the activity of the host immune system [99]. One study showed that, after MSC treatment, although the cells migrate away from the target site, their chondroprotective and immunomodulatory properties remain active [100]. This effect is probably related to the paracrine effect of MSCs, through which another cell type, such as pericytes, is recruited to enhance tissue repair [101]. Meanwhile, MSCs suppress the proliferation of immune cells at the target site [83]. The chemoattractant also enables the proliferation and differentiation of endogenous progenitors at the site of injury [99]. Identifying the nature of the biological components that regulate networking between the site of injury, immune cells, and MSCs, as well as determining whether these compounds

are soluble molecules or transported in small EVs, remains a challenge. Ideally, OA therapy should result in improved joint mobility, no mechanical restriction, and proper integration with healthy adjacent cartilage [102]. In recent years, the use of MSCs from bone marrow or adipose tissue has become the focus of clinical interest in the treatment of OA. Despite the incomplete integration of the cells into the defects, the in situ differentiation of MSCs [103] has chondroprotective effects that inhibit progressive cartilage degeneration by suppressing inflammatory processes [67,103]. A clinical study concluded that the injection of MSCs isolated from adipose tissue into osteoarthritic joints resulted in significant alleviation of lameness symptoms and improvements in movement [104,105].

6. Extracellular Vesicles (EVs) Are Potential Biomarkers for the Detection and Diagnosis of OA

6.1. Overview of EVs

MSCs are known to play a key role in tissue regeneration. One of the major advantages of stem cells as therapeutic agents is that they can produce biologically active EVs, as EVs can mediate paracrine activity in joint repair in OA [106]. Following the characterization of MSCs as medicinal signaling cells [107], it can be assumed that the therapeutic effects after implantation of MSCs are not based on chondrogenic differentiation in situ but rather on the efficacy of MSC-derived trophic factors released as paracrine-active factors that stimulate regeneration [108]. Although cytokines, growth factors, chemokines, immunomodulatory factors, and signaling lipid mRNAs are involved in this process, regulatory miRNAs are also included within the EVs. EVs play an increasingly important therapeutic role [109]. These vesicles mediate intercellular communication by delivering essential elements such as proteins, lipids, miRNAs, and cytokines to target cells [110–112]. Exosomes are vesicles with a diameter of 50–150 nm that are formed intracellularly in microvesicular bodies. They are surrounded by a lipid double membrane and are constricted by the cells. Microvesicles are thus part of the secretome of the cell [113]. It has been reported that EVs/exosomes deliver bioactive products via three different mechanisms (Figure 1). Briefly, (1) surface proteins of exosomes can attach to the membranes of other cells and modulate their activity, (2) the interaction between exosomes and extracellular matrix proteases can release cell receptor activation ligands, and (3) exosomes can integrate into the cell membrane and release their components into the target cell, as reviewed in [114,115]. EVs play a pivotal role in modulating various biological activities and cellular performance. It has been observed that exosomes contribute to the immune response, antigen detection, cell migration and differentiation, and tumor progression [106]. Assessments of the quality and quantity of EVs could facilitate the early detection of pathological alterations in OA, as well as provide an alarm signal to evaluate the severity of the condition and disease progression. These criteria are useful for selecting the optimal therapeutic approach. It was found that EVs play an important role in the pathogenesis of OA; a study revealed that the exosomes isolated from fibroblast-like synoviocytes (FLSs) and chondrocytes play an essential role in OA development, including in inflammatory response and cartilage degeneration, and the impact of these vesicles' components on the different stages of OA was studied [116]. As part of their function as cargoes of molecular elements in the joint microenvironment, EVs play a pivotal role in mediating intercellular communication, and it has been reported that the assembly of functional Cx43-based channels along the surface of EVs enables the extracellular release of intraluminal contents from these vesicles. This alternative connexin-based mechanism of information transfer to target cells has only been partially elucidated, but according to current knowledge, it has great therapeutic benefits [117]. Exosomal Cx43 has been shown to mediate exosome-based interactions and information transfer between source and target cells [118].

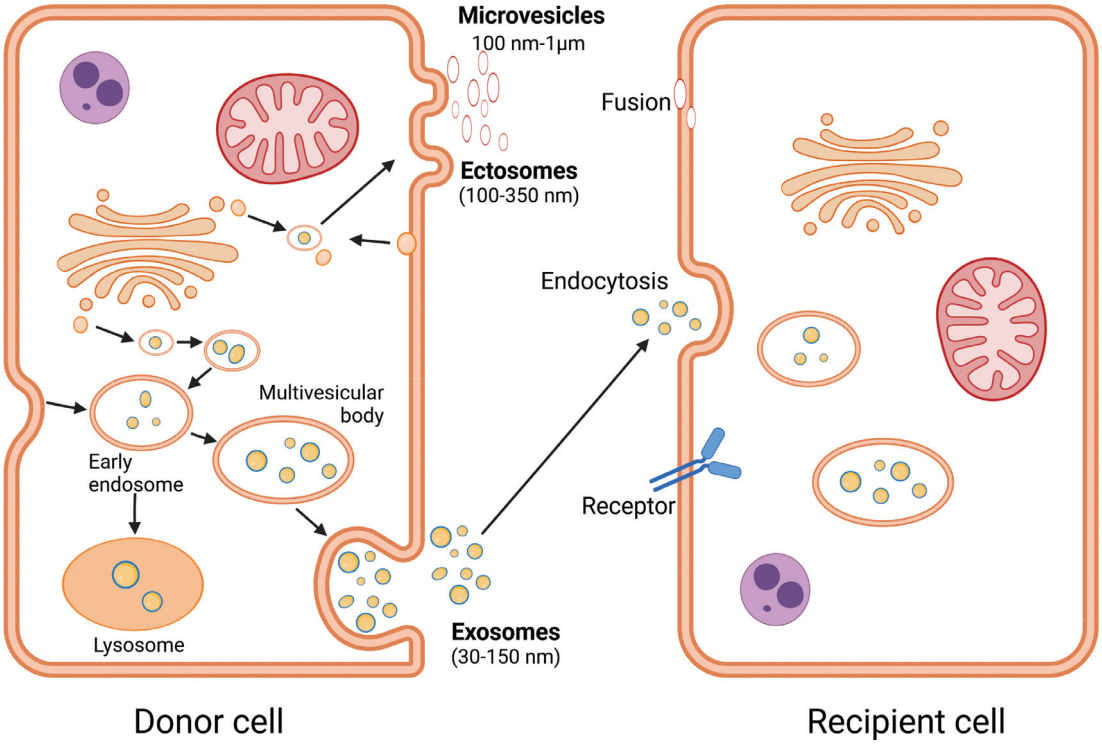


Figure 1. Graphical illustration demonstrating extracellular vesicle biogenesis and secretion and the method by which they target other cells. Exosomes are released from multivesicular bodies (MVBs) via an endosomal process. MVBs bind to the cell membrane to release the exosomes outside the donor cell. Microvesicles are released by budding and detachment from the plasma membrane. The exosomes either internalize the recipient cell via endocytosis, establish ligand-receptor interactions, or fuse into the cell membrane of the recipient cell [119,120].

6.2. The Biological Characteristics of EVs/Exosomes

Within the group of EVs, exosomes are thought to play a special role in intercellular communication. In addition, exosomes contain small, non-coding RNAs and miRNAs, which mediate their activity and maintain cell-cell communication [121]. MSC-derived exosomes regulate the microenvironment and biological activity of the stem cell with their miRNAs by controlling the balance between proliferation and differentiation [122]. The therapeutic efficacy of EVs has been demonstrated in numerous studies and different cell types. A body of evidence has shown improved tissue regeneration after injury in cardiomyocytes, hepatocytes, and keratinocytes after treatment with exosomes. Furthermore, exosomes have been considered nano-communication vehicles for therapeutic signals due to their low immunogenicity, long half-life, and ability to cross biomembranes [123,124]. Exosomes have been isolated from bodily fluids such as urine [125], milk and blood [126], synovial fluid [127], and platelet-rich plasma (PRP) [128]. The components of the latter source of exosomes, such as growth factors, have shown therapeutic efficacy; one study reported that PRP-derived exosomes improved muscle regeneration after injury in a rat model [129].

Once released into the extracellular milieu, exosomes can be internalized by target cells in both micro- and macroenvironments, thereby contributing to cell-cell communication [130]. EVs/exosomes were isolated via ultracentrifugation at 100,000 rpm for two hours and then processed for transmission electron microscopy analysis, as previously reported by our group [131] (Figure 2a,b). Exosomes can be characterized by the expression of surface marker proteins belonging to the tetraspanin family; these include the surface markers CD9, CD63, and CD81 [132]. Using immunogold labeling, EVs positive for the specific markers CD81 and CD9 could be identified compared to a negative control where primary antibodies were omitted (Figure 2b–d). The presence of exosomes in the cell

culture supernatant of MSCs was first described in [133]. In addition, there is evidence for tissue-specific effects of exosomes from different cellular sources [134]. Our recent study showed successful isolation of EVs based on immunogold surface marker detection from equine adipose tissue- or bone marrow-derived stem cells [131,135].

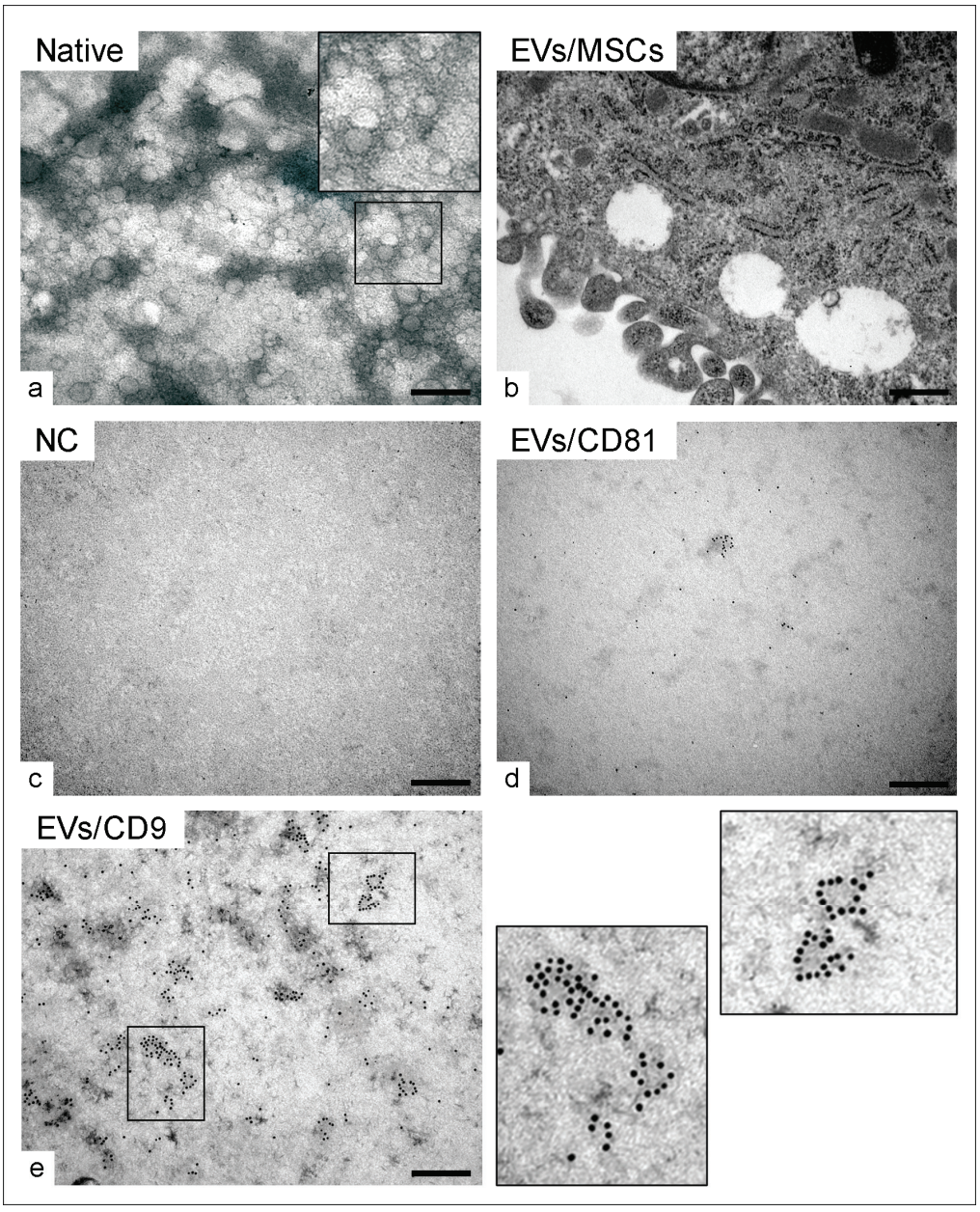


Figure 2. Ultrastructural detection of equine mesenchymal stem cell-derived extracellular vesicles (EVs) using transmission electron microscopy: (**a**) Native EVs/exosomes without immune labeling. (**b**) Mesenchymal stem cell (MSC)-enclosed EVs (white spherical vesicles) in the cytoplasm. EVs were isolated from cell culture supernatant and coupled with 6 nm gold particle antibodies. (**c**–**e**) Immunogold labeling of the specific EV markers, showing (**c**) no primary antibody negative control (NC), (**d**) CD81-positive EVs, and (**e**) CD9-positive EVs. The black squares in (**a**,**e**) indicate a higher magnification of the corresponding area. Scale bars: (**a**,**c**,**d**) 250 nm; (**b**) 500 nm.

6.3. EVs as Potential Biomarkers for OA

Most of the current literature dealing with EVs in the context of OA can be divided into two categories: the first group investigates the potential of EVs isolated from plasma and synovial fluid as biomarkers for the early detection of OA (Table 1), and the second group

investigates the potential of EVs as bioactive agents for the treatment of equine OA (Table 2). A biomarker is defined as a characteristic indicator of normal biological or pathological conditions that can be measured using molecular, morphological, or physiological assays. These indicators can be present in either bodily fluids or blood and include any of the following: molecular indicators such as proteins, lipids, and nucleic acids; changes in gene expression; abnormal metabolic byproducts; and changes in tissue architecture, as reviewed in [136]. In a study investigating the response of MSCs to different apoptotic cell lines, the data revealed an upregulated expression and production of stanniocalcin1, indicating the anti-apoptotic potential of MSCs [137]. In the same context, it has been described that co-culture of autologous ASCs with OA-derived chondrocytes counteracts fibrotic activity, including downregulation of MMP13, alkaline phosphatase, Runx2, collagen I/II/VI, and vimentin, along with increased expression of TGF-β [138]. However, these factors are either soluble in the microenvironment or accumulate in EVs, and they have not been fully described. Therefore, tracking these molecular changes by isolating EVs and identifying their components facilitates the establishment of a biomarker for the detection and diagnosis of OA. Similarly, it was found that the quantification of hepatocyte growth factor (HGF), a potent antifibrotic factor in synovial fluid, is indicative of negative progression of OA, which can be used as a biomarker for OA [139]. It has been documented that the chondrogenic differentiation requires the expression of mir193-3p, while upregulation of mir193-3p was detected with chondrogenesis, and expression was not detected in the damaged cartilage. The author reported a low plasma level of exosome-derived miR193-3p in OA patients, indicating that it may be a suitable biological marker for the diagnosis of OA [140].

Table 1. Contents of EVs and their functions in the context of OA.

Composition	Response to OA/Function	Reference
(1) miRNAs miR-372 miR449a-5p	Increased expression in OA to protect chondrocytes from apoptosis Upregulated in advanced OA patient undergoing total knee replacement	[141,142]
miR-126-3p	Decreases with OA; suppresses apoptosis, inflammation, and osteophyte formation in chondrocytes	[143]
miR-500b, miR-720, miR-4454, miR-199b-5p miR-3154	Upregulated after IL-1β induction	[144]
miR-504-3p	Upregulated with OA	[145]
eca-miR-451, eca-miR-25, eca-miR-215, eca-miR-92a, eca-miR-let-7c, eca-miR-486-5p, and eca-miR-23a; snoRNAs	Involved in cell-cycle inhibition, cell-cycle progression, cell proliferation, and differentiation; potential biomarkers for OA	[146]
miR-372-3p	Upregulated with OA	[141]
miR-449a-5p	Increased expression in chondrocytes following IL-1β treatment	[142]
miR-155-5p	Upregulated with potential induction of an TNF-α-based inflammatory response	[147]
mir193-3p	Upregulated with chondrogenesis; expression was not detected in the damaged cartilage of OA patients	[140]
miR-19a, miR-29c, miR-132, miR-144, miR-183, miR-185, miR-194, miR-195, miR-199a-3p, miR-200a, miR-200b, miR-219-3p, miR-409-3p, miR-411, miR-499-5p, miR-628a, miR-1301, miR-3200, miR-7177b, and miR-9055	Detected in plasma- and synovial fluid-derived EVs of horses with OA	[148]
miR-199a, miR-214, and miR-9094	Differentially expressed, indicating fibrosis, chondrogenesis, and an inflammatory response in post-traumatic OA	[148]
miR-504, miR-146a, miR-26a, miR-200c, and miR-210	Targeting their expression could be diagnostic for OA	[149]
miR-320c	Enhances chondrocyte proliferation, downregulates MMP13 in OA chondrocytes, and indicates pathological improvement	[150]
miR-140	Upregulated in OA; can be used as a biomarker	[151]
miR-146a	Strong expression in early OA cartilage; plays a role in OA cartilage pathogenesis.	[152]

Table 1. *Cont.*

Composition	Response to OA/Function	Reference
miR-145, miR-221	Inhibits inflammation and improves chondrogenesis of the OA joint	[153]
HULC	The amount decreases with OA progression	[141]
lncRNA	Osteoarthritis marker	[154]
PVT1	Regulates OA progression via modulating HMGB1/T1r4/NF-kB signaling	[155]
PCGEM1	Osteoarthritis marker	[154]
Fatty acids (FAs) palmitic acid, stearic acid, and behenic acid	FA alteration level is indicative of OA development, providing a valuable biomarker for OA pathogenesis	[156]
Proteins CD163, myosin regulatory light polypeptide 9, caveolin-1, CD109, ezrin, and moesin	Differentially detected in the EVs of horses with OA	[157]

Table 2. Using EVs for OA management.

Source of EVs	Species	Conclusion	Reference
BMSCs	Horse	Improves the articular cartilage performance including chondrocyte function and migration potential compared to MSC therapy alone	[158]
BMSCs	Horse	Exosomes primed with equine proinflammatory cytokines promote articular cartilage regeneration	[159]
BMSCs	Rat	Reduces the effect of OA through promoting phenotypic transformation of synovial macrophages from M1 to M2	[160]
BMSCs	Rat	Treatment with miR-140-3p-overexpressing exosomes under hypoxia increases the survival rate, migration of inflammatory chondrocytes, and chondrogenesis	[161]
BMSCs	Horse	Delivers an anti-inflammatory effect to artificially inflamed autologous chondrocytes	[135]
BMSCs	Mouse	Demonstrates chondroprotective and anti-inflammatory properties in a murine model of OA	[162]
ASCs	Human	Exosomes overexpressing miR-145 and miR-221 reduce the inflammation response and improve cartilage regeneration	[153]
ASCs	Mouse	Exosomes of TNF-α preconditioned MSCs ameliorate the pathological changes in the joint of OA mice	[163]
ASCs	Human	Inflammatory priming enhanced the healing capacity of ASC-derived EVs in an animal model of OA	[164]
MSCs	Human	Improves cartilage regeneration, restores tissue injuries, and improves the joint performance of OA patient	[165]
MSCs	Human	Improves chondrogenesis and attenuates the negative effect of proinflammatory cytokines	[166]
SMSCs	Mouse	Exosomes overexpressing miR-155-5p enhance the physiological performance of chondrocytes, attenuate apoptosis, and modulate matrix production in an OA mouse model	[167]
PRP	Rabbit	Promotes OA cartilage repair by increasing proliferation and inhibiting apoptosis of chondrocytes	[168]
UCMSCs	Human	Improves cartilage repair in an OA rat model	[169]

A study to evaluate EVs as potential biomarkers was conducted using experimentally induced OA as a model for post-traumatic osteoarthritis. After the isolation of EVs using standard procedures, the EVs were characterized according to the criteria of the MISEV guidelines [170]. Plasma-derived and synovial fluid-derived EVs were compared for the expression of relevant microRNAs, including eca-miR-451, eca-miR-25, eca-miR-215, eca-miR-92a, eca-miR-let-7c, eca-miR-486-5p, and eca-miR-23a, along with four snoRNAs (U3, snord15, snord46, and snord58). Bioinformatics analysis revealed that these differentially expressed miRNAs were involved in cell-cycle inhibition, cell-cycle progression, cell proliferation, increased cell viability, and stem cell differentiation in a time-dependent manner. The authors suggested that these miRNAs can be considered not only as potential biomarkers for OA but also as potential therapeutic targets for degenerative joint diseases [146]. In the same context, EVs were characterized by analyzing the microRNA composition in the plasma and synovial fluid of horses with naturally occurring post-traumatic OA. Transcriptome analysis revealed an increase in the percentage of exosomes and a decrease in the percentage of microvesicles compared to horses without post-traumatic OA, as determined via nanoparticle tracking analysis (NTA). Postmortem evaluation of OA was performed in the radiocarpal, middle carpal, and metacarpophalangeal joints. The results of sRNA sequencing revealed that, out of 658 DE miRNAs, only 67 were known, and 591 novel proteins were identified in the plasma EVs of control and post-traumatic horses. Analysis of synovial fluid EVs revealed 805 DE miRNAs, with approximately 74 known and 731 unknown miRNAs for both groups. Surprisingly, 20 DE miRNAs, including miR-19a, miR-29c, miR-132, miR-144, miR-183, miR-185, miR-194, miR-195, miR-199a-3p, miR-200a, miR-200b, miR-219-3p, miR-409-3p, miR-411, miR-499-5p, miR-628a, miR-1301, miR-3200, miR-7177b, and miR-9055, were detected in both plasma- and synovial fluid-derived EVs [148]. The current report suggests that specific markers that can be used for the diagnosis of OA are carried and transferred by EVs.

A proteomic evaluation was employed to detect the early phase of OA in a study that compared the protein cargo of EVs collected from plasma and SF in a time-dependent manner. The proteomic data analysis demonstrated that EV proteins are a more effective predictive tool for assessing OA progression than small non-coding RNAs. Principal component cluster analysis revealed significant discrepancies in the protein cargo between the two EV sources. Concurrently, clusters derived from SF-derived EVs were linked to immune and inflammatory pathways. Conversely, factor analysis of plasma-derived EVs highlighted an increase in proteins that regulate lipid metabolism. Furthermore, protein analysis of SF-derived EVs revealed an association between intermediate filaments and the supramolecular complexes involved in tissue repair. The authors proposed that there is an interaction between time and the development of OA with regard to CRE, NFkB, SRE, and SRF levels. Additionally, EVs derived from the SF of OA patients have been observed to impact chondrocyte proliferation [171]. Similarly, the extent of OA can be assessed in plasma-derived EVs through a combination of Raman and photothermal infrared spectroscopy (RPIS). Plasma samples were collected from thoroughbred racehorses. The optical photothermal infrared imaging (PPII) method demonstrated a high classification rate of 93.4% in distinguishing between EVs derived from osteoarthritic and healthy samples. In contrast, Raman spectroscopy exhibited a lower classification rate of only 64.3%. The higher classification rate achieved with RPIS is based on the detection of increased levels of proteins, lipids, and nucleic acids in EVs derived from osteoarthritic samples. The authors suggested that the former method is a more suitable diagnostic approach than the PPII method for detecting the early stage of OA by utilizing EVs isolated from patient plasma [151]. An evaluation of alterations to the structural elements of the joint could facilitate the early detection of OA and its progression. A study employed EVs as biomarkers in OA to analyze their hyaluronic acid (HA) content in the synovial fluid (SF). For this purpose, EVs were isolated from SF obtained from OA-affected horses following euthanasia. EVs from the contralateral metacarpophalangeal joint were examined in comparison to those from unaffected control horses. The authors were able to distinguish

between small and large EVs using NTA and laser scanning microscopy (LSM). Small EVs were not affected by OA, whereas the large HA-containing EVs were markedly reduced. The authors proposed that the reduction in the number of large HA-containing EVs could serve as a diagnostic tool for early-stage OA, preceding the emergence of degenerative evidence through either radiological or clinical symptom assessments [172].

The metabolic performance and components of the joint—for example, fatty acids (FA) and their derivatives, as well as pro- and anti-inflammatory properties—have an impact on the condition of joints, especially during the development of OA. In this context, a recent report compared the FA composition in both SF and SF-derived EVs in equine OA metacarpophalangeal joints using gas chromatography. The FA profiles were modified in both SF and EV-enriched pellets as a consequence of spontaneous OA. The presence of linoleic acid, myristic acid, palmitolic acid, and n-3/n-6 polyunsaturated FAs revealed substantial differences in the SF of OA joints compared to the controls. In EV-enriched pellets, the saturated FAs, including palmitic acid, stearic acid, and behenic acid exhibited alterations indicative of the development of OA. The authors suggested that FA alterations in both EVs and SF represent a valuable biomarker that can impact the pathogenesis of OA. Furthermore, the SF may serve as a potential target for the treatment of OA [156]. Similarly, a recent report employed a combined proteomics and phospholipidomics approach to identify potential biomarkers within SF-derived EVs of equine OA with varying severity, utilizing mass spectrometry. While there was no alteration in the number of EVs in the SF of OA joints, approximately 40 differentially expressed proteins were identified, associated with seven essential trend-setting pathways in OA; these included CD163, myosin regulatory light polypeptide 9, caveolin-1, CD109, ezrin, and moesin [157]. The study indicated that despite the absence of any change in the number of these vesicles, mass spectrometry is vital to detect alterations in EV components in OA at the protein level.

Monitoring the expression of biomarkers in bodily fluids, particularly in SF, represents a valuable means of assessing the progression and development of OA in horses. At present, the diagnosis of OA is typically based on a combination of clinical symptoms and radiographic examination tools. A recent report documented that EVs collected from synovial fluid biopsies using a minimally invasive approach, followed by the targeting of common miRNA expression in exosomes, including miR-504, miR-146a, miR-26a, miR-200c, and miR-210, facilitated the diagnosis of OA [149]. A parallel investigation examined the differential expression of microRNA within synovial fluid and plasma-derived EVs. The analysis demonstrated an increase in the exosome concentration and a reduction in the microvesicle concentration in OA horses compared to healthy controls. Additionally, the expression of miR-144, miR-219-3p, and miR-199a-31 was observed in plasma, while the expression of miR-199a, miR-214, and miR-9094 was detected in synovial fluid-derived EVs. The author concluded that these differentially expressed markers provide evidence of fibrosis, chondrogenesis, and an inflammatory response in post-traumatic OA. The authors concluded that EVs offer a valuable diagnostic tool for understanding the pathology and identifying suitable therapeutic applications for OA in horses [148]. The potential of small non-coding RNAs (sncRNAs) in the synovial fluid as diagnostic markers for early OA was subsequently investigated. The authors identified downregulation of miR-223 in early OA, along with upregulation of miR-23b, let-7a-2, snord96A, and snord13, which were associated with various cellular activities, including inflammation, necrosis, apoptosis, and autophagy. Moreover, mRNA transcripts that target biological cell activities such as proliferation and cellular response were observed to be expressed. The authors suggested that sncRNAs may serve not only as biomarkers for the early detection of OA but also for elucidating the underlying molecular mechanisms involved in the progression of OA [19].

The contents and quantity of EVs, their interaction in the joint microenvironment, and the severity of the condition may be critical, not only for understanding the disease progression but also for selecting the most suitable treatment approach for the patient. It has been demonstrated that the synovial fluid of patients with OA contains higher levels of cytokines, indicative of advanced OA. Furthermore, OA-derived exosomes have been

demonstrated to facilitate cartilage degeneration, in addition to an intensified inflammatory response and a reduction in the number of chondrocytes. The author recommended further investigation to detect the composition of EVs as a potential novel diagnostic biological marker for OA [173]. Similarly, the detection of specific molecules could be indicative of OA progression. A study reported that the expression of PCGEM1 was elevated in advanced OA patients compared to those in the early stages, and it also exhibited high expression in the early stages of OA compared to the control group. These findings suggest that PCGEM1 may serve as a valuable biomarker for assessing OA progression and severity [154]. The induction of MSCs using IL-1β leads to the secretion of miR-320c-containing exosomes that enhance chondrocyte proliferation and downregulate MMP13 in OA chondrocytes, indicating pathological improvement [150]. The expression of specific markers in EVs has been demonstrated to impact disease susceptibility, gender differences, and inflammatory progression. It has been demonstrated that the miRNA components of OA exosomes in female patients are estrogen-sensitive and can influence Toll-like receptor signaling. Additionally, the synovial fluid-derived EVs of OA patients modulate the equilibrium between anabolic and catabolic activities in chondrocytes, resulting in the upregulation of catabolic and inflammatory gene expression [145]. Moreover, a study reported that the expression level of miR95-5p was downregulated in exosomes derived from OA cartilage compared to those derived from normal cartilage. Conversely, the treatment of MSCs with articular cartilage-derived miR-95-5p exosomes has been demonstrated to enhance chondrogenic differentiation and matrix expression [174]. These studies indicate that EVs may serve as a valuable source of biomarkers for evaluating the state of inflammation and cartilage deterioration. Moreover, a study was conducted to determine whether cartilage oligomeric matrix protein (COMP) fragments are a potential biomarker for cartilage degeneration in the joints of equines. The authors observed evidence of COMP degradation, fragmentation, and synthesis in the synovial fluid using a monoclonal antibody (14 G4), suggesting that COMP could be a promising marker for OA in equines [175].

A proteomic analysis was conducted to identify the main proteins present in the secretome of articular cartilage, with the aim of mimicking the inflammatory condition observed in OA using 10 ng/mL IL-1β. The authors identified tryptic peptides derived from aggrecan cores, COMP, fibronectin, fibromodulin, thrombospondin-1 (TSP-1), clusterin, cartilage intermediate layer protein-1 (CLIP-1), chondroadherin, and MMP1/3. Furthermore, the induction of inflammation using IL-1β was observed to activate MMP1, MMP3, and TSP-1. Conversely, the level of CLU precursor was found to be reduced, while no change was detected in CLIP-1 or CLU [176]. A similar study monitored the presence of inflammatory cytokines following chondrocyte stimulation with IL-1β for up to 25 days in vitro. The authors identified proteins in the medium that were associated with the inflammatory response, matrix metalloproteinases, acute-phase proteins, IL-6, and complemental components. The authors documented that early-stage OA was characterized by aggrecan and cartilage oligomeric matrix fragmentations after Day 3 and Day 6 post-induction, mimicking the in vivo evidence of early OA. In contrast, the late stage was distinguished by the degradation of the collagenous network after 18 and 22 days [177]. While these studies underscore the alterations in the extracellular matrix secretome, the transfer of these molecules by means of EVs necessitates paying particular attention to the early detection and diagnosis of OA in equines.

A recent study in China investigated a common chronic degenerative disease affecting the joints, known as Kashin–Beck disease (KBD). The objective was to isolate serum- and chondrocyte-derived exosomes for the purpose of evaluating alterations in miRNA. The findings revealed the presence of 53 DE miRNAs in the chondrocyte-derived exosomes. The author employed single-cell RNA sequencing (scRNA-Seq) to identify 115 genes that were targeted by the differentially expressed (DE) miRNA contents of serum- and chondrocyte-derived exosomes. The authors concluded that the analysis of differentially expressed miRNAs using single-cell RNA sequencing is a valuable tool, not only for the diagnosis of the disease but also for the development of therapeutic applications for KBD [178].

A study identified a potential biomarker for the diagnosis of OA based on the presence of autophagy. The study assessed the number and contents of articular cartilage vesicles (ACVs) to determine a correlation between autophagy and OA. The authors reported an increase in autophagy in the ACVs of healthy cartilage, which could be positively induced using rapamycin. In contrast, the ACVs of the OA cartilage exhibited reduced autophagy and a diminished number of ACVs following rapamycin induction. The authors emphasized the importance of autophagy in cartilage regeneration [179]. These findings suggest that the absence of autophagy and the response of ACV numbers to rapamycin may serve as a diagnostic biomarker for OA, distinguishing it from healthy cartilage.

It is now widely accepted that the transmembrane gap junction protein connexin 43 (CX43) plays a pivotal role in mediating intercellular communication in a multitude of cell types within the musculoskeletal system. Our group has demonstrated the indispensable function of Cx43 in bone remodeling [180], myogenic differentiation [181], and osteogenic differentiation of MSCs, as reviewed in [182]. The expression and the pivotal role of Cx43 in EVs, as well as the potential modulation of EVs by Cx43 in the context of OA, remain incompletely characterized. Evidence suggests that Cx43 expression may serve as a biomarker for the detection and diagnosis of OA in equine patients. It has been reported that overexpression of Cx43 was detected in the chondrocytes of OA patients, increasing the exosomal Cx43 level and causing cell senescence of targeted chondrocytes [183]. Moreover, the senescent chondrocytes exhibited augmented degenerative traits, including the formation of a senescence-associated secretory phenotype (SASP), as well as IL-1β, IL-6, and MMPs. The author suggested a double effect of high levels of Cx43 on exosome activity, influencing cell senescence, inflammatory processes, and cellular reprogramming, which collectively facilitate disease progression. Additionally, the potential utility of Cx43 expression as a biomarker in OA has been proposed [184]. Similarly, a recent report demonstrated that exosomes derived from experimentally induced senescence in ASCs could not protect against cartilage damage or subchondral bone degradation, and they exhibited a lower capacity to polarize macrophages towards an anti-inflammatory phenotype [185]. The existing literature on the role of Cx43 in EV production, the transfer of their components into target cells, and their interaction with cells in the microenvironment of the OA joint is limited. Further investigations into this topic are needed, especially in equine OA.

6.4. Immunomodulatory Effect of EVs as a Biological Marker

It is well established that EVs play a role in the inflammatory response during OA, and this response can be used as a biological marker to determine the progression of OA and to modulate the immune microenvironment in the joint. Indeed, exosomes have been demonstrated to contribute to the progression of the pathological profile of OA [186,187]. In the same context, a study reported that adipose-derived MSCs secreted factors that were capable of inhibiting the apoptotic effect of IL-1β on human chondrocytes in vitro [166]. Similarly, a study demonstrated that IL-1β promotes the secretion of exosomes from synovial fluid, resulting in fibroblast cell alteration and subsequent chondrocyte alteration. These alterations are characterized by the upregulation of OA-relative markers, secretion of inflammatory cytokines, extracellular matrix degradation, and antigen presentation, as well as an angiogenic response [188]. The exosomes derived from OA synovial fluid have been observed to promote the secretion of cytokines and chemokines from M1 macrophages [189]. Additionally, incubation of macrophages with OA synovial fluid-derived exosomes has been observed to result in enhanced proliferation and osteoclast formation, independent of the macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor kappa-B ligand (RANKL) [190]. Similarly, a study examined the response of synovial membrane MSCs to the inflammatory cytokine miRNA-140 cultivated in two- and three-dimensional (3D and 2D) culture conditions. The inflammatory condition was induced through the use of IL-1β and TNF-α. The results demonstrated the presence of EVs containing miR-140 in the cells cultivated in a 3D OA condition at 24 and 72 h, respectively. Moreover, the expression of miR-140 was upregulated in the 3D and 2D OA groups at

72 and 120 h, respectively. The authors recommended the utilization of miR-140 as a biomarker for the diagnosis and detection of inflammation in terms of EV tracking for OA in equines [191]. The cross-talk between macrophages and fibroblast-like synoviocytes (FLSs) was examined using a co-culture model, highlighting that lipopolysaccharide activation resulted in the upregulation of IL-1β, IL-6, and ADAMTS4/5 expression in FLSs. The expression of ADAMTS5 was downregulated by DH82 in canine macrophages after incubation with either cells or medium. Moreover, the expression of IL-1β was found to be upregulated following lipopolysaccharide activation in equine FLSs. The authors proposed that macrophages secrete soluble factors that are capable of modulating the inflammatory progression associated with OA in horses [192]. However, the nature of these factors, whether encapsulated in EVs or released directly into the microenvironment, could offer diagnostic value, particularly for the tracking of EVs.

7. Stem Cell-Derived EVs/Exosomes: A Potential Therapeutic Choice for OA

The current standard of care for OA is a highly sophisticated and complex approach that primarily relies on anti-inflammatory medications to manage pain and inflammation. This is because no substantial regeneration of cartilage has been observed. Although numerous studies have investigated the use of MSCs in OA, the success rate remains relatively low due to the observation of inflammatory conditions associated with synovitis as a side effect following stem cell injection [193]. The original concept of local MSC differentiation into recipient tissue cells could not be substantiated by the findings of corresponding investigations. EVs/exosomes represent a promising therapeutic tool for chronic degenerative diseases that are unresponsive to traditional medical treatment. The success of MSC treatment was found to be contingent on the secretome of cells, including growth and anti-inflammatory factors [194]. Furthermore, the secretome of MSCs contains EVs. In recent years, research into the potential diagnostic and therapeutic applications of EVs has received considerable attention. The use of EVs as a treatment option has the advantage of being a cell-free approach that does not entail any adverse side effects [195]. The advantages of using EVs/exosomes can be briefly summarized as follows: firstly, EVs isolated from healthy donors or tissue can be used to deliver regenerative products to target lesions; secondly, EVs offer an alternative cell-free approach that excludes the risk of immunoreactivity; and thirdly, it is possible to load EVs with the required bioactive products by activating MSCs in vitro prior to the isolation of EVs from the medium [196]. The impact of stem cell-derived EVs and exosomes on cellular differentiation has been examined extensively. EVs convey signaling molecules that facilitate intercellular communication between donor and recipient cells. A study demonstrated that exosomes derived from osteogenic differentiated mesenchymal stem cells (MSCs) were capable of stimulating osteogenic differentiation in undifferentiated MSCs [197]. Similarly, the isolation of exosomes from the supernatants of various stem cell sources has been demonstrated to prevent the degradation of cartilage and bone in vitro [162]. The presence of inflammatory mediators has been observed to increase the levels of therapeutic miRNAs in exosomes when compared to similar MSC cultures that have not been induced with an inflammatory condition [164]. In addition to their regenerative properties, exosomes have anti-inflammatory and immunomodulatory properties [198]. There is evidence that MSC-derived exosomes both promote the proliferation of chondrocytes and inhibit their apoptosis, mediated via specific miRNAs such as miR-206/GIT1 [166]. It was observed that EVs pre-labeled with a lipophilic stain such as PKH67 were internalized by treated chondrocytes. In the context of the anti-inflammatory effects, a study examined the impact of MSC-derived EVs from bone marrow on the pro-inflammatory cytokines in chondrocytes. The authors observed a significant downregulation of the inflammatory cytokines in the presence of EVs. A similar effect was observed with respect to ADAMTS4 gene expression, suggesting that EVs exert an anti-inflammatory effect on pre-treated equine chondrocytes [135]. In light of the pivotal role of the MSC secretome, a study demonstrated an improvement in the articular cartilage performance following the administration of an MSC secretome for the treatment of OA.

This included improvements in chondrocyte function and migration potential compared to MSC therapy alone. The authors recommended further investigation to identify the components of the secretome, such as soluble factors and microvesicles [158].

As in humans, OA causes major locomotor problems in horses; thus, PRP could represent an alternative approach to modulate the inflammatory reaction and the progression of OA in horses, as reviewed in [199]. The intra-articular injection of platelet-rich plasma (PRP) is emerging as a promising therapeutic avenue for patients with OA, as reviewed in [200,201]. It is well established that platelets secrete factors and cytokines that modulate the inflammatory response as well as the regeneration capacity in OA [202]. Platelets contain numerous bioactive chemoattractants that are involved in new matrix formation, tissue angiogenesis, and cellular proliferation at the target site [203]. Specifically, platelets are rich in α-granules [204], which produce chemoattractant factors to promote cell migration and enhance matrix formation [205]. α-granules are a valuable source of factor V fibrinogen, angiogenin, vascular endothelial growth factor, angiostatin, platelet factor IV, metalloproteinases, TNF-α, and IL-1β [206]. In addition, platelets produce platelet-derived growth factor (PDGF), which promotes cell proliferation and proteoglycan production [207]. PRP has a positive impact on reducing the inflammatory response in musculoskeletal lesions, as well as enhancing chondrogenesis, promoting autophagy, and decreasing apoptosis in human OA [208]. Interestingly, however, PRP is a source of exosomes and their numerous bioactive contents. A study demonstrated that PRP-derived exosomes can induce chondrogenic gene expression in OA chondrocytes; the authors assessed the effect of PRP-derived exosomes on experimentally induced OA in rabbits, and the results revealed a reduction in chondrocyte apoptosis, inhibition of TNF-α, and improvement in the OA condition via a mechanism including Wnt/catenin signaling, which suggests a novel therapeutic approach for OA [168]. In a parallel investigation, the impact of PRP on OA chondrocytes was assessed by analyzing chondrocyte proliferation, autophagy, apoptosis, and inflammatory cytokines. The authors observed an increase in chondrocyte proliferation and autophagy following treatment with PRP from healthy donors. PRP treatment was observed to reduce cell apoptosis and induce a dose-dependent reduction in the expression of MMP3, MMP13, ADAMTS-5, IL-6, and COX-2. In contrast, the expression of TGF-β, aggrecan, collagen II, and TIMPs was upregulated, as were the intracellular levels of IL-4, IL-10, and IL-13 [208]. Nevertheless, the beneficial impact of PRP on OA could be attributed to the release of soluble factors directly into the plasma, or to the delivery of microvesicles. Therefore, elucidating the nature of these secretory products could enhance the therapeutic potential of PRP, particularly in terms of isolating and characterizing EVs from PRP.

In a more sophisticated approach, MSCs were isolated from adipose tissue and selected for high expression of α10β1 integrin using MACS cell sorting. Following the selection procedure, the cells were injected into an experimentally induced osteochondral lesion in the carpal joints on Day 18 after lesioning. EVs derived from SF were isolated using ultracentrifugation. The authors observed the presence of a multitude of proteins, including serine endopeptidase activity, complement activation, and collagen extracellular matrix, following MSC treatment. The authors concluded that EVs may play a role in mediating the effects of cell therapy due to altered joint homeostasis, highlighting the therapeutic mechanisms of MSC-based OA therapy [209]. This approach provides valuable insights into the analysis of the effects of OA treatments, with or without cells, via the protein equipment of EVs in joint fluid.

In an experiment, equine articular chondrocytes were isolated from the carpal or femoral condyles and cultivated in the presence of MSC-conditioned media in vitro. The expression of markers for cartilage and cell proliferation was examined in equine articular cartilage cells, either in monolayer cultures or in three-dimensional organoid cultures. The authors documented that the treatment of articular cartilage cells with the conditional medium resulted in a positive impact on cartilage markers and cell migration. The authors of this study concluded that cell-free therapy (whereby the conditioned medium is

administered, including EVs) represents a promising approach for the treatment of OA. Nevertheless, additional analysis is necessary to elucidate the mechanisms of action [158].

The method of isolation and purification may influence the therapeutic potential of EVs. It was previously demonstrated that MSC-derived EVs isolated using membrane affinity capture (MAC) exhibited a more pronounced beneficial impact on equine articular cartilage (eAC) cells cultivated as cartilaginous organoids in comparison to EVs isolated via size-exclusion chromatography (SEC). An improvement of eAC was observed, including the synthesis of a hyaline-like matrix, an increase in the expression of type IIB and type I collagen, a modulation of type X collagen, and an alteration in Htra1 and PCNA levels. Subsequently, there was an increase in the production of MMP1 and MMP3. The authors concluded that the reason why MAC-purified exosomes led to a superior improvement in the eAC phenotype compared to SEC-purified EVs may be attributed to the enhanced sample purity achieved through MAC purification [159], as the low vasculature and oxygen concentration in the cartilage microenvironment impedes the regeneration capacity. A study was conducted to investigate the effects of BM-MSC-derived exosomes overexpressing miR-140-3p on chondrocytes that had been previously induced with the inflammatory cytokine IL-1β and maintained under a low oxygen concentration. The authors reported that exosome treatment improved the proliferative and migratory ability of inflammatory chondrocytes and increased chondrogenesis. Furthermore, the healing activity of inflammatory chondrocytes was mediated via the positive effect of hypoxia on miR-140-3p. The authors suggested that hypoxia improves the regenerative ability of chondrocytes, perhaps by regulating the miR-140-3p in BM-MSC-derived exosomes, which improves the healing ability in OA [161].

8. Research and Future Prospects of EVs in Veterinary Medicine

In the field of veterinary medicine, investigations into EVs represent a relatively novel and rapidly expanding area of research. The number of publications on EVs is increasing annually, with studies exploring the diverse roles of EVs in animal physiology and pathology, as well as their potential applications as diagnostic biomarkers and in therapy. The most appealing aspects of EVs are their natural biocompatibility, biodegradability, and low immunogenicity, which make them an attractive option for drug delivery approaches [210]. The tremendous increase in the number of EV studies in veterinary medicine mainly reflects the current global concerns under the concept of "one health", such as the need for high productivity and better welfare in animal production, control of emerging infectious diseases and zoonoses, and environmental monitoring [211–213]. Understanding the performance of EVs in large animals' physiology and pathology could be of great importance for the overall outcomes of EV research targeting OA. Indeed, animals and humans have similar physiology and share diseases such as cancer and musculoskeletal diseases (e.g., OA), which also makes animals good spontaneous and inducible models for research in human medicine [214,215]. It has been reported that OA is common in both horses and humans and has a similar pathogenesis; thus, horses may serve as a large-animal model to confirm theoretical and clinical findings from human orthopedics [216]. This is made even more important by the fact that most studies on the diagnosis and treatment of OA have so far been carried out in small-animal models with limited transferability to human patients [217]. At present, the available literature regarding the identification, diagnostic value, and therapeutic benefit of EVs or exosomes in equine veterinary medicine is scarce. Thus, it is worthwhile to take a closer look at the diagnostic options and therapeutic opportunities presented by the use of EVs/exosomes in equine medicine for the diagnosis and treatment of OA. In addition, the important role of MSC-derived EVs/exosomes in orchestrating the inflammatory response and cartilage regeneration represents a potential cell-free therapeutic tool for OA. However, the lack of essential information impacts the search for an optimal isolation method, the identification of biomarkers relevant to OA progression, and the suitable conditions for EV storage, transfer, and injection for therapeutic use, which require further investigation in equines. EVs' viability and biocompatibility,

either in hydrogels or loaded in microcarriers, as well as their potency following therapeutic application, remain an interesting hot topic, not only for equine OA but also for human OA.

9. Conclusions

The potential of EVs for the diagnosis and treatment of joint diseases in equine orthopedics remains largely unexplored. In veterinary medicine, only a limited number of studies have thus far addressed this significant and promising research area. Initial findings suggest that extracellular vesicles (EVs) obtained from blood plasma and synovial fluid may serve as an early diagnostic tool due to their rich composition of specific microRNAs (miRNAs) and proteins that play a pivotal role in regulating the inflammatory response, cell proliferation, autophagy, and apoptosis associated with OA. The identification of changes in biomarkers within EVs enables the early detection of OA and the selection of the most appropriate treatment strategy. Concerning the therapeutic potential of EVs, the data derived from in vitro studies and preclinical investigations suggest that EVs exert anti-inflammatory and regenerative effects. In light of these findings, EVs represent a promising potential therapeutic agent for the treatment of OA. The efficacy of EVs has been demonstrated in some preliminary, early-stage, and pre-clinical OA studies. In addition, the use of cell-free therapeutic bioproducts avoids the problems and side effects that have been repeatedly reported with the use of MSCs. Therefore, further studies are urgently needed to standardize the isolation methods and to characterize the constituents of EVs more precisely, such that EVs can be offered as a safe therapeutic option as one component of the mesenchymal stem cell secretome.

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Abbreviations

MSCs	Mesenchymal stem cells
ASCs	Adipose tissue-derived mesenchymal stem cells
BMSCs	Bone marrow-derived mesenchymal stem cells
IL-1β	Interleukin 1-β
TNF-α	Tumor necrosis factor-α
MMPs	Matrix metalloproteinases
ROS	Reactive oxygen species
TGF-β	Transforming growth factor-β

NSAID	Nonsteroidal anti-inflammatory dexamethasone
HA	Hyaluronic acid
PGE2	Prostaglandin E2
PRP	Platelet-rich plasma
SncRNA	Small non-coding RNA
COMP	Cartilage oligomeric matrix protein
TSP-1	Thrombospondin-1
CLIP-1	Cartilage intermediate layer protein-1
M-CSF	Macrophage colony-stimulating factor
RANKL	Receptor activator of nuclear factor kappa-B ligand
TIMPs	Tissue inhibitors of matrix metalloproteinase
HGF	Hepatocyte growth factor
HULC	Highly upregulated in liver cancer
SMSCs	Synovial mesenchymal stem cells
UCMSCs	Umbilical cord mesenchymal stem cells

References

1. Buckwalter, J.A.; Martin, J.A.; Brown, T.D. Perspectives on chondrocyte mechanobiology and osteoarthritis. *Biorheology* **2006**, *43*, 603–609. [PubMed]
2. Manoto, S.L.; Maepa, M.J.; Motaung, S.K. Medical ozone therapy as a potential treatment modality for regeneration of damaged articular cartilage in osteoarthritis. *Saudi J. Biol. Sci.* **2018**, *25*, 672–679. [CrossRef]
3. Meng, X.; Ziadlou, R.; Grad, S.; Alini, M.; Wen, C.; Lai, Y.; Qin, L.; Zhao, Y.; Wang, X. Animal Models of Osteochondral Defect for Testing Biomaterials. *Biochem. Res. Int.* **2020**, *2020*, 9659412. [CrossRef] [PubMed]
4. Szponder, T.; Latalski, M.; Danielewicz, A.; Krać, K.; Kozera, A.; Drzewiecka, B.; Nguyen Ngoc, D.; Dobko, D.; Wessely-Szponder, J. Osteoarthritis: Pathogenesis, Animal Models, and New Regenerative Therapies. *J. Clin. Med.* **2022**, *12*, 5. [CrossRef] [PubMed]
5. Chen, D.; Shen, J.; Zhao, W.; Wang, T.; Han, L.; Hamilton, J.L.; Im, H.-J. Osteoarthritis: Toward a comprehensive understanding of pathological mechanism. *Bone Res.* **2017**, *5*, 16044. [CrossRef]
6. McIlwraith, C.W.; Frisbie, D.D.; Kawcak, C.E. The horse as a model of naturally occurring osteoarthritis. *Bone Joint Res.* **2012**, *1*, 297–309. [CrossRef]
7. McIlwraith, C.W.; Kawcak, C.E.; Frisbie, D.D.; Little, C.B.; Clegg, P.D.; Peffers, M.J.; Karsdal, M.A.; Ekman, S.; Laverty, S.; Slayden, R.A.; et al. Biomarkers for equine joint injury and osteoarthritis. *J. Orthop. Res.* **2018**, *36*, 823–831. [CrossRef]
8. Alves, J.C.; Santos, A.; Jorge, P. Platelet-rich plasma therapy in dogs with bilateral hip osteoarthritis. *BMC Vet. Res.* **2021**, *17*, 207. [CrossRef]
9. Kerwin, S.C. Osteoarthritis in cats. *Top. Companion Anim. Med.* **2010**, *25*, 218–223. [CrossRef]
10. Huey, D.J.; Hu, J.C.; Athanasiou, K.A. Unlike bone, cartilage regeneration remains elusive. *Science* **2012**, *338*, 917–921. [CrossRef]
11. Djouad, F.; Bouffi, C.; Ghannam, S.; Noël, D.; Jorgensen, C. Mesenchymal stem cells: Innovative therapeutic tools for rheumatic diseases. *Nat. Rev. Rheumatol.* **2009**, *5*, 392–399. [CrossRef] [PubMed]
12. Johnson, K.; Zhu, S.; Tremblay, M.S.; Payette, J.N.; Wang, J.; Bouchez, L.C.; Meeusen, S.; Althage, A.; Cho, C.Y.; Wu, X.; et al. A stem cell-based approach to cartilage repair. *Science* **2012**, *336*, 717–721. [CrossRef] [PubMed]
13. Marr, C.M.; Love, S.; Boyd, J.S.; McKellar, Q. Factors affecting the clinical outcome of injuries to the superficial digital flexor tendon in National Hunt and point-to-point racehorses. *Vet. Rec.* **1993**, *132*, 476–479. [CrossRef] [PubMed]
14. Silver, I.A.; Brown, P.N.; Goodship, A.E.; Lanyon, L.E.; McCullagh, K.G.; Perry, G.C.; Williams, I.F. A clinical and experimental study of tendon injury, healing and treatment in the horse. *Equine Vet. J. Suppl.* **1983**, *1*, 1–43.
15. Ackerman, I.N.; Cavka, B.; Lippa, J.; Bucknill, A. The feasibility of implementing the ICHOM Standard Set for Hip and Knee Osteoarthritis: A mixed-methods evaluation in public and private hospital settings. *J. Patient Rep. Outcomes* **2017**, *2*, 32. [CrossRef]
16. Olmos Calvo, I.; Kuten-Pella, O.; Kramer, K.; Madár, Á.; Takács, S.; Kardos, D.; Simon, D.; Erdö-Bonyár, S.; Berki, T.; de Luna, A.; et al. Optimization of Lyophilized Hyperacute Serum (HAS) as a Regenerative Therapeutic in Osteoarthritis. *Int. J. Mol. Sci.* **2021**, *22*, 7496. [CrossRef]
17. Feng, X.; Pan, J.; Li, J.; Zeng, C.; Qi, W.; Shao, Y.; Liu, X.; Liu, L.; Xiao, G.; Zhang, H.; et al. Metformin attenuates cartilage degeneration in an experimental osteoarthritis model by regulating AMPK/mTOR. *Aging* **2020**, *12*, 1087–1103. [CrossRef]
18. Clegg, P.D.; Mobasheri, A. Chondrocyte apoptosis, inflammatory mediators and equine osteoarthritis. *Vet. J.* **2003**, *166*, 3–4. [CrossRef]
19. Castanheira, C.; Balaskas, P.; Falls, C.; Ashraf-Kharaz, Y.; Clegg, P.; Burke, K.; Fang, Y.; Dyer, P.; Welting, T.J.M.; Peffers, M.J. Equine synovial fluid small non-coding RNA signatures in early osteoarthritis. *BMC Vet. Res.* **2021**, *17*, 26. [CrossRef]
20. Kuyinu, E.L.; Narayanan, G.; Nair, L.S.; Laurencin, C.T. Animal models of osteoarthritis: Classification, update, and measurement of outcomes. *J. Orthop. Surg. Res.* **2016**, *11*, 19. [CrossRef]
21. Chow, Y.Y.; Chin, K.-Y. The Role of Inflammation in the Pathogenesis of Osteoarthritis. *Mediators Inflamm.* **2020**, *2020*, 8293921. [CrossRef] [PubMed]

22. Anderson, D.D.; Chubinskaya, S.; Guilak, F.; Martin, J.A.; Oegema, T.R.; Olson, S.A.; Buckwalter, J.A. Post-traumatic osteoarthritis: Improved understanding and opportunities for early intervention. *J. Orthop. Res.* **2011**, *29*, 802–809. [CrossRef] [PubMed]
23. McCoy, A.M. Animal Models of Osteoarthritis: Comparisons and Key Considerations. *Vet. Pathol.* **2015**, *52*, 803–818. [CrossRef]
24. Bijlsma, J.W.J.; Berenbaum, F.; Lafeber, F.P.J.G. Osteoarthritis: An update with relevance for clinical practice. *Lancet* **2011**, *377*, 2115–2126. [CrossRef]
25. USDA. *Lameness and Laminitis in U.S. Horses*; USDA:APHIS:VS, CEAH, National Ani mal Health Monitoring System: Fort Collins, CO, USA, 2000.
26. Caron, J.P.; Genovese, R.L. Principles and Practices of Joint Disease Treatment. In *Diagnosis and Managment of Lameness in the Horse*; Ross, M.W., Dyson, S.J., Eds.; Saunders: Philadelphia, PA, USA; London, UK, 2003; pp. 746–764, ISBN 9780721683423.
27. Martel-Pelletier, J.; Barr, A.J.; Cicuttini, F.M.; Conaghan, P.G.; Cooper, C.; Goldring, M.B.; Goldring, S.R.; Jones, G.; Teichtahl, A.J.; Pelletier, J.-P. Osteoarthritis. *Nat. Rev. Dis. Primers* **2016**, *2*, 16072. [CrossRef]
28. Cohen-Solal, M.; Funck-Brentano, T.; Hay, E. Animal models of osteoarthritis for the understanding of the bone contribution. *Bonekey Rep.* **2013**, *2*, 422. [CrossRef] [PubMed]
29. Brandt, K.D.; Dieppe, P.; Radin, E.L. Etiopathogenesis of osteoarthritis. *Rheum. Dis. Clin. N. Am.* **2008**, *34*, 531–559. [CrossRef]
30. Goldring, M.B.; Otero, M. Inflammation in osteoarthritis. *Curr. Opin. Rheumatol.* **2011**, *23*, 471–478. [CrossRef]
31. Bos, S.D.; Kloppenburg, M.; Suchiman, E.; van Beelen, E.; Slagboom, P.E.; Meulenbelt, I. The role of plasma cytokine levels, CRP and Selenoprotein S gene variation in OA. *Osteoarthr. Cartil.* **2009**, *17*, 621–626. [CrossRef]
32. Pelletier, J.-P.; Martel-Pelletier, J.; Abramson, S.B. Osteoarthritis, an inflammatory disease: Potential implication for the selection of new therapeutic targets. *Arthritis Rheum.* **2001**, *44*, 1237–1247. [CrossRef]
33. Mueller, M.B.; Tuan, R.S. Anabolic/Catabolic balance in pathogenesis of osteoarthritis: Identifying molecular targets. *PM R* **2011**, *3*, S3–S11. [CrossRef] [PubMed]
34. Goldring, S.R.; Goldring, M.B. The role of cytokines in cartilage matrix degeneration in osteoarthritis. *Clin. Orthop. Relat. Res.* **2004**, *427*, S27–S36. [CrossRef]
35. Song, J.; Jin, E.-H.; Kim, D.; Kim, K.Y.; Chun, C.-H.; Jin, E.-J. MicroRNA-222 regulates MMP-13 via targeting HDAC-4 during osteoarthritis pathogenesis. *BBA Clin.* **2015**, *3*, 79–89. [CrossRef]
36. Wojdasiewicz, P.; Poniatowski, Ł.A.; Szukiewicz, D. The role of inflammatory and anti-inflammatory cytokines in the pathogenesis of osteoarthritis. *Mediat. Inflamm.* **2014**, *2014*, 561459. [CrossRef] [PubMed]
37. Kamm, J.L.; Nixon, A.J.; Witte, T.H. Cytokine and catabolic enzyme expression in synovium, synovial fluid and articular cartilage of naturally osteoarthritic equine carpi. *Equine Vet. J.* **2010**, *42*, 693–699. [CrossRef]
38. Berenbaum, F. The quest for the Holy Grail: A disease-modifying osteoarthritis drug. *Arthritis Res. Ther.* **2007**, *9*, 111. [CrossRef]
39. Toncheva, A.; Remichkova, M.; Ikonomova, K.; Dimitrova, P.; Ivanovska, N. Inflammatory response in patients with active and inactive osteoarthritis. *Rheumatol. Int.* **2009**, *29*, 1197–1203. [CrossRef]
40. Thielen, N.; Neefjes, M.; Wiegertjes, R.; van den Akker, G.; Vitters, E.; van Beuningen, H.; Blaney Davidson, E.; Koenders, M.; van Lent, P.; van de Loo, F.; et al. Osteoarthritis-Related Inflammation Blocks TGF-β's Protective Effect on Chondrocyte Hypertrophy via (de)Phosphorylation of the SMAD2/3 Linker Region. *Int. J. Mol. Sci.* **2021**, *22*, 8124. [CrossRef] [PubMed]
41. Baugé, C.; Attia, J.; Leclercq, S.; Pujol, J.-P.; Galéra, P.; Boumédiene, K. Interleukin-1beta up-regulation of Smad7 via NF-kappaB activation in human chondrocytes. *Arthritis Rheum.* **2008**, *58*, 221–226. [CrossRef]
42. Afonso, V.; Champy, R.; Mitrovic, D.; Collin, P.; Lomri, A. Reactive oxygen species and superoxide dismutases: Role in joint diseases. *Jt. Bone Spine* **2007**, *74*, 324–329. [CrossRef]
43. Cecil, D.L.; Johnson, K.; Rediske, J.; Lotz, M.; Schmidt, A.M.; Terkeltaub, R. Inflammation-induced chondrocyte hypertrophy is driven by receptor for advanced glycation end products. *J. Immunol.* **2005**, *175*, 8296–8302. [CrossRef] [PubMed]
44. Merz, D.; Liu, R.; Johnson, K.; Terkeltaub, R. IL-8/CXCL8 and growth-related oncogene alpha/CXCL1 induce chondrocyte hypertrophic differentiation. *J. Immunol.* **2003**, *171*, 4406–4415. [CrossRef] [PubMed]
45. Morita, K.; Miyamoto, T.; Fujita, N.; Kubota, Y.; Ito, K.; Takubo, K.; Miyamoto, K.; Ninomiya, K.; Suzuki, T.; Iwasaki, R.; et al. Reactive oxygen species induce chondrocyte hypertrophy in endochondral ossification. *J. Exp. Med.* **2007**, *204*, 1613–1623. [CrossRef] [PubMed]
46. Lv, Z.; Yang, Y.X.; Li, J.; Fei, Y.; Guo, H.; Sun, Z.; Lu, J.; Xu, X.; Jiang, Q.; Ikegawa, S.; et al. Molecular Classification of Knee Osteoarthritis. *Front. Cell Dev. Biol.* **2021**, *9*, 725568. [CrossRef] [PubMed]
47. Frisbie, D.D.; Ray, C.S.; Ionescu, M.; Poole, A.R.; Chapman, P.L.; McIlwraith, C.W. Measurement of synovial fluid and serum concentrations of the 846 epitope of chondroitin sulfate and of carboxy propeptides of type II procollagen for diagnosis of osteochondral fragmentation in horses. *Am. J. Vet. Res.* **1999**, *60*, 306–309. [CrossRef]
48. Robinson, W.H.; Lepus, C.M.; Wang, Q.; Raghu, H.; Mao, R.; Lindstrom, T.M.; Sokolove, J. Low-grade inflammation as a key mediator of the pathogenesis of osteoarthritis. *Nat. Rev. Rheumatol.* **2016**, *12*, 580–592. [CrossRef]
49. Zhang, H.; Cai, D.; Bai, X. Macrophages regulate the progression of osteoarthritis. *Osteoarthr. Cartil.* **2020**, *28*, 555–561. [CrossRef]
50. Zeng, N.; Yan, Z.-P.; Chen, X.-Y.; Ni, G.-X. Infrapatellar Fat Pad and Knee Osteoarthritis. *Aging Dis.* **2020**, *11*, 1317–1328. [CrossRef]
51. Sung, W.-H.; Tsao, Y.-T.; Shen, C.-J.; Tsai, C.-Y.; Cheng, C.-M. Small-volume detection: Platform developments for clinically-relevant applications. *J. Nanobiotechnol.* **2021**, *19*, 114. [CrossRef]

52. Attur, M.; Belitskaya-Lévy, I.; Oh, C.; Krasnokutsky, S.; Greenberg, J.; Samuels, J.; Smiles, S.; Lee, S.; Patel, J.; Al-Mussawir, H.; et al. Increased interleukin-1β gene expression in peripheral blood leukocytes is associated with increased pain and predicts risk for progression of symptomatic knee osteoarthritis. *Arthritis Rheum.* **2011**, *63*, 1908–1917. [CrossRef]
53. Bihlet, A.R.; Byrjalsen, I.; Bay-Jensen, A.-C.; Andersen, J.R.; Christiansen, C.; Riis, B.J.; Karsdal, M.A. Associations between biomarkers of bone and cartilage turnover, gender, pain categories and radiographic severity in knee osteoarthritis. *Arthritis Res. Ther.* **2019**, *21*, 203. [CrossRef] [PubMed]
54. Ingale, D.; Kulkarni, P.; Electricwala, A.; Moghe, A.; Kamyab, S.; Jagtap, S.; Martson, A.; Koks, S.; Harsulkar, A. Synovium-Synovial Fluid Axis in Osteoarthritis Pathology: A Key Regulator of the Cartilage Degradation Process. *Genes* **2021**, *12*, 989. [CrossRef] [PubMed]
55. Sun, D.; Zhuang, X.; Xiang, X.; Liu, Y.; Zhang, S.; Liu, C.; Barnes, S.; Grizzle, W.; Miller, D.; Zhang, H.-G. A novel nanoparticle drug delivery system: The anti-inflammatory activity of curcumin is enhanced when encapsulated in exosomes. *Mol. Ther.* **2010**, *18*, 1606–1614. [CrossRef]
56. Varela-Eirín, M.; Varela-Vázquez, A.; Guitián-Caamaño, A.; Paíno, C.L.; Mato, V.; Largo, R.; Aasen, T.; Tabernero, A.; Fonseca, E.; Kandouz, M.; et al. Targeting of chondrocyte plasticity via connexin43 modulation attenuates cellular senescence and fosters a pro-regenerative environment in osteoarthritis. *Cell Death Dis.* **2018**, *9*, 1166. [CrossRef]
57. Goodrich, L.R.; Nixon, A.J. Medical treatment of osteoarthritis in the horse—A review. *Vet. J.* **2006**, *171*, 51–69. [CrossRef] [PubMed]
58. Frisbie, D.D.; Kawcak, C.E.; McIlwraith, C.W.; Werpy, N.M. Evaluation of polysulfated glycosaminoglycan or sodium hyaluronan administered intra-articularly for treatment of horses with experimentally induced osteoarthritis. *Am. J. Vet. Res.* **2009**, *70*, 203–209. [CrossRef]
59. Zhang, W.; Ouyang, H.; Dass, C.R.; Xu, J. Current research on pharmacologic and regenerative therapies for osteoarthritis. *Bone Res.* **2016**, *4*, 15040. [CrossRef]
60. Schlachter, C.; Lewis, C. Electrophysical Therapies for the Equine Athlete. *Vet. Clin. N. Am. Equine Pract.* **2016**, *32*, 127–147. [CrossRef]
61. Haussler, K.K. Joint Mobilization and Manipulation for the Equine Athlete. *Vet. Clin. N. Am. Equine Pract.* **2016**, *32*, 87–101. [CrossRef]
62. Baccarin, R.Y.A.; Seidel, S.R.T.; Michelacci, Y.M.; Tokawa, P.K.A.; Oliveira, T.M. Osteoarthritis: A common disease that should be avoided in the athletic horse's life. *Anim. Front.* **2022**, *12*, 25–36. [CrossRef]
63. Porter, M. Equine rehabilitation therapy for joint disease. *Vet. Clin. N. Am. Equine Pract.* **2005**, *21*, 599–607. [CrossRef] [PubMed]
64. Te Moller, N.C.R.; van Weeren, P.R. How exercise influences equine joint homeostasis. *Vet. J.* **2017**, *222*, 60–67. [CrossRef] [PubMed]
65. Buchner, H.H.F.; Schildboeck, U. Physiotherapy applied to the horse: A review. *Equine Vet. J.* **2006**, *38*, 574–580. [CrossRef] [PubMed]
66. Atalaia, T.; Prazeres, J.; Abrantes, J.; Clayton, H.M. Equine Rehabilitation: A Scoping Review of the Literature. *Animals* **2021**, *11*, 1508. [CrossRef] [PubMed]
67. Frisbie, D.D.; Kawcak, C.E.; McIlwraith, C.W. Evaluation of the effect of extracorporeal shock wave treatment on experimentally induced osteoarthritis in middle carpal joints of horses. *Am. J. Vet. Res.* **2009**, *70*, 449–454. [CrossRef]
68. Kawcak, C.E.; Frisbie, D.D.; McIlwraith, C.W. Effects of extracorporeal shock wave therapy and polysulfated glycosaminoglycan treatment on subchondral bone, serum biomarkers, and synovial fluid biomarkers in horses with induced osteoarthritis. *Am. J. Vet. Res.* **2011**, *72*, 772–779. [CrossRef]
69. Barbosa, G.M.; Cunha, J.E.; Cunha, T.M.; Martinho, L.B.; Castro, P.A.T.S.; Oliveira, F.F.B.; Cunha, F.Q.; Ramalho, F.S.; Salvini, T.F. Clinical-like cryotherapy improves footprint patterns and reduces synovial inflammation in a rat model of post-traumatic knee osteoarthritis. *Sci. Rep.* **2019**, *9*, 14518. [CrossRef]
70. Dantas, L.O.; Breda, C.C.; Mendes Silva Serrao, P.R.; Serafim Jorge, A.H.; Salvini, T.F. Cryotherapy short-term use relieves pain, improves function and quality of life in individuals with knee osteoarthritis—Randomized controlled trial. *Osteoarthr. Cartil.* **2017**, *25*, S174. [CrossRef]
71. JASTRZĘBSKA, E.W. Use of magnetic field in equine physiotherapy—Preliminary study. *Med. Weter.* **2021**, *77*, 137–141. [CrossRef]
72. Veronesi, F.; Fini, M.; Giavaresi, G.; Ongaro, A.; de Mattei, M.; Pellati, A.; Setti, S.; Tschon, M. Experimentally induced cartilage degeneration treated by pulsed electromagnetic field stimulation; an in vitro study on bovine cartilage. *BMC Musculoskelet. Disord.* **2015**, *16*, 308. [CrossRef]
73. Frisbie, D.D.; Ghivizzani, S.C.; Robbins, P.D.; Evans, C.H.; McIlwraith, C.W. Treatment of experimental equine osteoarthritis by in vivo delivery of the equine interleukin-1 receptor antagonist gene. *Gene Ther.* **2002**, *9*, 12–20. [CrossRef] [PubMed]
74. Frisbie, D.D.; McIlwraith, C.W. Gene therapy: Future therapies in osteoarthritis. *Vet. Clin. N. Am. Equine Pract.* **2001**, *17*, 233–243. [CrossRef] [PubMed]
75. Hunziker, E.B. Articular cartilage repair: Basic science and clinical progress. A review of the current status and prospects. *Osteoarthr. Cartil.* **2002**, *10*, 432–463. [CrossRef] [PubMed]
76. Hunziker, E.B.; Lippuner, K.; Keel, M.J.B.; Shintani, N. Age-Independent Cartilage Generation for Synovium-Based Autologous Chondrocyte Implantation. *Tissue Eng. Part A* **2015**, *21*, 2089–2098. [CrossRef]

77. Kalamegam, G.; Memic, A.; Budd, E.; Abbas, M.; Mobasheri, A. A Comprehensive Review of Stem Cells for Cartilage Regeneration in Osteoarthritis. *Adv. Exp. Med. Biol.* **2018**, *1089*, 23–36. [CrossRef]
78. Frisbie, D.D.; Kisiday, J.D.; Kawcak, C.E.; Werpy, N.M.; McIlwraith, C.W. Evaluation of adipose-derived stromal vascular fraction or bone marrow-derived mesenchymal stem cells for treatment of osteoarthritis. *J. Orthop. Res.* **2009**, *27*, 1675–1680. [CrossRef]
79. Rehman, J. Empowering self-renewal and differentiation: The role of mitochondria in stem cells. *J. Mol. Med.* **2010**, *88*, 981–986. [CrossRef]
80. Wagers, A.J. The stem cell niche in regenerative medicine. *Cell Stem Cell* **2012**, *10*, 362–369. [CrossRef]
81. Caplan, A.I.; Dennis, J.E. Mesenchymal stem cells as trophic mediators. *J. Cell. Biochem.* **2006**, *98*, 1076–1084. [CrossRef]
82. Zhang, M.; Mal, N.; Kiedrowski, M.; Chacko, M.; Askari, A.T.; Popovic, Z.B.; Koc, O.N.; Penn, M.S. SDF-1 expression by mesenchymal stem cells results in trophic support of cardiac myocytes after myocardial infarction. *FASEB J.* **2007**, *21*, 3197–3207. [CrossRef]
83. Aggarwal, S.; Pittenger, M.F. Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood* **2005**, *105*, 1815–1822. [CrossRef] [PubMed]
84. Chen, X.; Armstrong, M.A.; Li, G. Mesenchymal stem cells in immunoregulation. *Immunol. Cell Biol.* **2006**, *84*, 413–421. [CrossRef] [PubMed]
85. Nauta, A.J.; Fibbe, W.E. Immunomodulatory properties of mesenchymal stromal cells. *Blood* **2007**, *110*, 3499–3506. [CrossRef]
86. Martens, T.P.; See, F.; Schuster, M.D.; Sondermeijer, H.P.; Hefti, M.M.; Zannettino, A.; Gronthos, S.; Seki, T.; Itescu, S. Mesenchymal lineage precursor cells induce vascular network formation in ischemic myocardium. *Nat. Clin. Pract. Cardiovasc. Med.* **2006**, *3* (Suppl. S1), S18–S22. [CrossRef]
87. Koch, C.; Brounts, S.H.; Foerner, J.J. What is your diagnosis? Avulsion fracture at the origin of the long digital extensor and fibularis tertius muscles. *J. Am. Vet. Med. Assoc.* **2009**, *234*, 1389–1390. [CrossRef]
88. Nakagami, H.; Maeda, K.; Morishita, R.; Iguchi, S.; Nishikawa, T.; Takami, Y.; Kikuchi, Y.; Saito, Y.; Tamai, K.; Ogihara, T.; et al. Novel autologous cell therapy in ischemic limb disease through growth factor secretion by cultured adipose tissue-derived stromal cells. *Arterioscler. Thromb. Vasc. Biol.* **2005**, *25*, 2542–2547. [CrossRef]
89. Uysal, A.C.; Mizuno, H. Tendon regeneration and repair with adipose derived stem cells. *Curr. Stem Cell Res. Ther.* **2010**, *5*, 161–167. [CrossRef] [PubMed]
90. Wang, M.; Yuan, Q.; Xie, L. Mesenchymal Stem Cell-Based Immunomodulation: Properties and Clinical Application. *Stem Cells Int.* **2018**, *2018*, 3057624. [CrossRef]
91. Gao, F.; Chiu, S.M.; Motan, D.A.L.; Zhang, Z.; Chen, L.; Ji, H.-L.; Tse, H.-F.; Fu, Q.-L.; Lian, Q. Mesenchymal stem cells and immunomodulation: Current status and future prospects. *Cell Death Dis.* **2016**, *7*, e2062. [CrossRef]
92. Trounson, A.; McDonald, C. Stem Cell Therapies in Clinical Trials: Progress and Challenges. *Cell Stem Cell* **2015**, *17*, 11–22. [CrossRef]
93. Contreras, R.A.; Figueroa, F.E.; Djouad, F.; Luz-Crawford, P. Mesenchymal Stem Cells Regulate the Innate and Adaptive Immune Responses Dampening Arthritis Progression. *Stem Cells Int.* **2016**, *2016*, 3162743. [CrossRef] [PubMed]
94. Jorgensen, C.; Noel, D.; Apparailly, F.; Sany, J. Stem cells for repair of cartilage and bone: The next challenge in osteoarthritis and rheumatoid arthritis. *Ann. Rheum. Dis.* **2001**, *60*, 305–309. [CrossRef]
95. Kristjánsson, B.; Honsawek, S. Current perspectives in mesenchymal stem cell therapies for osteoarthritis. *Stem Cells Int.* **2014**, *2014*, 194318. [CrossRef]
96. Horie, M.; Driscoll, M.D.; Sampson, H.W.; Sekiya, I.; Caroom, C.T.; Prockop, D.J.; Thomas, D.B. Implantation of allogenic synovial stem cells promotes meniscal regeneration in a rabbit meniscal defect model. *J. Bone Joint Surg. Am.* **2012**, *94*, 701–712. [CrossRef] [PubMed]
97. Chang, Y.-H.; Liu, H.-W.; Wu, K.-C.; Ding, D.-C. Mesenchymal Stem Cells and Their Clinical Applications in Osteoarthritis. *Cell Transplant.* **2016**, *25*, 937–950. [CrossRef] [PubMed]
98. Roberts, S.; Genever, P.; McCaskie, A.; de Bari, C. Prospects of stem cell therapy in osteoarthritis. *Regen. Med.* **2011**, *6*, 351–366. [CrossRef]
99. Mancuso, P.; Raman, S.; Glynn, A.; Barry, F.; Murphy, J.M. Mesenchymal Stem Cell Therapy for Osteoarthritis: The Critical Role of the Cell Secretome. *Front. Bioeng. Biotechnol.* **2019**, *7*, 9. [CrossRef] [PubMed]
100. ter Huurne, M.; Schelbergen, R.; Blattes, R.; Blom, A.; de Munter, W.; Grevers, L.C.; Jeanson, J.; Noël, D.; Casteilla, L.; Jorgensen, C.; et al. Antiinflammatory and chondroprotective effects of intraarticular injection of adipose-derived stem cells in experimental osteoarthritis. *Arthritis Rheum.* **2012**, *64*, 3604–3613. [CrossRef]
101. Roelofs, A.J.; Zupan, J.; Riemen, A.H.K.; Kania, K.; Ansboro, S.; White, N.; Clark, S.M.; de Bari, C. Joint morphogenetic cells in the adult mammalian synovium. *Nat. Commun.* **2017**, *8*, 15040. [CrossRef]
102. Cokelaere, S.; Malda, J.; van Weeren, R. Cartilage defect repair in horses: Current strategies and recent developments in regenerative medicine of the equine joint with emphasis on the surgical approach. *Vet. J.* **2016**, *214*, 61–71. [CrossRef]
103. Desando, G.; Cavallo, C.; Sartoni, F.; Martini, L.; Parrilli, A.; Veronesi, F.; Fini, M.; Giardino, R.; Facchini, A.; Grigolo, B. Intra-articular delivery of adipose derived stromal cells attenuates osteoarthritis progression in an experimental rabbit model. *Arthritis Res. Ther.* **2013**, *15*, R22. [CrossRef] [PubMed]

104. Guercio, A.; Di Marco, P.; Casella, S.; Cannella, V.; Russotto, L.; Purpari, G.; Di Bella, S.; Piccione, G. Production of canine mesenchymal stem cells from adipose tissue and their application in dogs with chronic osteoarthritis of the humeroradial joints. *Cell Biol. Int.* **2012**, *36*, 189–194. [CrossRef] [PubMed]
105. Bastia, D.; Zaman, S. Mechanism and physiological significance of programmed replication termination. *Semin. Cell Dev. Biol.* **2014**, *30*, 165–173. [CrossRef] [PubMed]
106. Miao, C.; Zhou, W.; Wang, X.; Fang, J. The Research Progress of Exosomes in Osteoarthritis, With Particular Emphasis on the Mediating Roles of miRNAs and lncRNAs. *Front. Pharmacol.* **2021**, *12*, 685623. [CrossRef]
107. Caplan, A.I. Medicinal signalling cells: They work, so use them. *Nature* **2019**, *566*, 39. [CrossRef]
108. Arnhold, S.; Wenisch, S. Adipose tissue derived mesenchymal stem cells for musculoskeletal repair in veterinary medicine. *Am. J. Stem Cells* **2015**, *4*, 1–12.
109. Phinney, D.G.; Pittenger, M.F. Concise Review: MSC-Derived Exosomes for Cell-Free Therapy. *Stem Cells* **2017**, *35*, 851–858. [CrossRef]
110. Fu, Y.; Karbaat, L.; Wu, L.; Leijten, J.; Both, S.K.; Karperien, M. Trophic Effects of Mesenchymal Stem Cells in Tissue Regeneration. *Tissue Eng. Part B Rev.* **2017**, *23*, 515–528. [CrossRef]
111. Vagner, T.; Spinelli, C.; Minciacchi, V.R.; Balaj, L.; Zandian, M.; Conley, A.; Zijlstra, A.; Freeman, M.R.; Demichelis, F.; De, S.; et al. Large extracellular vesicles carry most of the tumour DNA circulating in prostate cancer patient plasma. *J. Extracell. Vesicles* **2018**, *7*, 1505403. [CrossRef]
112. Bellingham, S.A.; Coleman, B.M.; Hill, A.F. Small RNA deep sequencing reveals a distinct miRNA signature released in exosomes from prion-infected neuronal cells. *Nucleic Acids Res.* **2012**, *40*, 10937–10949. [CrossRef]
113. Merino, A.M.; Hoogduijn, M.J.; Borras, F.E.; Franquesa, M. Therapeutic potential of extracellular vesicles. *Front. Immunol.* **2014**, *5*, 658. [CrossRef] [PubMed]
114. Familtseva, A.; Jeremic, N.; Tyagi, S.C. Exosomes: Cell-created drug delivery systems. *Mol. Cell. Biochem.* **2019**, *459*, 1–6. [CrossRef]
115. Jiang, L.; Dong, H.; Cao, H.; Ji, X.; Luan, S.; Liu, J. Exosomes in Pathogenesis, Diagnosis, and Treatment of Alzheimer's Disease. *Med. Sci. Monit.* **2019**, *25*, 3329–3335. [CrossRef]
116. KELLGREN, J.H.; LAWRENCE, J.S. Radiological assessment of osteo-arthrosis. *Ann. Rheum. Dis.* **1957**, *16*, 494–502. [CrossRef]
117. Ribeiro-Rodrigues, T.M.; Martins-Marques, T.; Morel, S.; Kwak, B.R.; Girão, H. Role of connexin 43 in different forms of intercellular communication—Gap junctions, extracellular vesicles and tunnelling nanotubes. *J. Cell Sci.* **2017**, *130*, 3619–3630. [CrossRef] [PubMed]
118. Soares, A.R.; Martins-Marques, T.; Ribeiro-Rodrigues, T.; Ferreira, J.V.; Catarino, S.; Pinho, M.J.; Zuzarte, M.; Isabel Anjo, S.; Manadas, B.; P G Sluijter, J.; et al. Gap junctional protein Cx43 is involved in the communication between extracellular vesicles and mammalian cells. *Sci. Rep.* **2015**, *5*, 13243. [CrossRef]
119. Mathieu, M.; Martin-Jaular, L.; Lavieu, G.; Théry, C. Specificities of secretion and uptake of exosomes and other extracellular vesicles for cell-to-cell communication. *Nat. Cell Biol.* **2019**, *21*, 9–17. [CrossRef] [PubMed]
120. van Niel, G.; Carter, D.R.F.; Clayton, A.; Lambert, D.W.; Raposo, G.; Vader, P. Challenges and directions in studying cell-cell communication by extracellular vesicles. *Nat. Rev. Mol. Cell Biol.* **2022**, *23*, 369–382. [CrossRef]
121. Yu, B.; Kim, H.W.; Gong, M.; Wang, J.; Millard, R.W.; Wang, Y.; Ashraf, M.; Xu, M. Exosomes secreted from GATA-4 overexpressing mesenchymal stem cells serve as a reservoir of anti-apoptotic microRNAs for cardioprotection. *Int. J. Cardiol.* **2015**, *182*, 349–360. [CrossRef]
122. Baglio, S.R.; Rooijers, K.; Koppers-Lalic, D.; Verweij, F.J.; Pérez Lanzón, M.; Zini, N.; Naaijkens, B.; Perut, F.; Niessen, H.W.M.; Baldini, N.; et al. Human bone marrow- and adipose-mesenchymal stem cells secrete exosomes enriched in distinctive miRNA and tRNA species. *Stem Cell Res. Ther.* **2015**, *6*, 127. [CrossRef]
123. Sun, M.M.-G.; Beier, F.; Pest, M.A. Recent developments in emerging therapeutic targets of osteoarthritis. *Curr. Opin. Rheumatol.* **2017**, *29*, 96–102. [CrossRef] [PubMed]
124. Alvarez-Erviti, L.; Seow, Y.; Yin, H.; Betts, C.; Lakhal, S.; Wood, M.J.A. Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. *Nat. Biotechnol.* **2011**, *29*, 341–345. [CrossRef] [PubMed]
125. Royo, F.; Zuñiga-Garcia, P.; Sanchez-Mosquera, P.; Egia, A.; Perez, A.; Loizaga, A.; Arceo, R.; Lacasa, I.; Rabade, A.; Arrieta, E.; et al. Different EV enrichment methods suitable for clinical settings yield different subpopulations of urinary extracellular vesicles from human samples. *J. Extracell. Vesicles* **2016**, *5*, 29497. [CrossRef]
126. Baranyai, T.; Herczeg, K.; Onódi, Z.; Voszka, I.; Módos, K.; Marton, N.; Nagy, G.; Mäger, I.; Wood, M.J.; El Andaloussi, S.; et al. Isolation of Exosomes from Blood Plasma: Qualitative and Quantitative Comparison of Ultracentrifugation and Size Exclusion Chromatography Methods. *PLoS ONE* **2015**, *10*, e0145686. [CrossRef] [PubMed]
127. Li, Z.; Wang, Y.; Xiao, K.; Xiang, S.; Li, Z.; Weng, X. Emerging Role of Exosomes in the Joint Diseases. *Cell. Physiol. Biochem.* **2018**, *47*, 2008–2017. [CrossRef]
128. Aatonen, M.; Valkonen, S.; Böing, A.; Yuana, Y.; Nieuwland, R.; Siljander, P. Isolation of Platelet-Derived Extracellular Vesicles. *Methods Mol. Biol.* **2017**, *1545*, 177–188. [CrossRef]
129. Iyer, S.R.; Scheiber, A.L.; Yarowsky, P.; Henn, R.F.; Otsuru, S.; Lovering, R.M. Exosomes Isolated From Platelet-Rich Plasma and Mesenchymal Stem Cells Promote Recovery of Function After Muscle Injury. *Am. J. Sports Med.* **2020**, *48*, 2277–2286. [CrossRef] [PubMed]

130. Javeed, N.; Mukhopadhyay, D. Exosomes and their role in the micro-/macro-environment: A comprehensive review. *J. Biomed. Res.* **2017**, *31*, 386–394. [CrossRef]
131. Klymiuk, M.C.; Balz, N.; Elashry, M.I.; Heimann, M.; Wenisch, S.; Arnhold, S. Exosomes isolation and identification from equine mesenchymal stem cells. *BMC Vet. Res.* **2019**, *15*, 42. [CrossRef]
132. Hurley, J.H.; Odorizzi, G. Get on the exosome bus with ALIX. *Nat. Cell Biol.* **2012**, *14*, 654–655. [CrossRef]
133. Lai, R.C.; Arslan, F.; Lee, M.M.; Sze, N.S.K.; Choo, A.; Chen, T.S.; Salto-Tellez, M.; Timmers, L.; Lee, C.N.; El Oakley, R.M.; et al. Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury. *Stem Cell Res.* **2010**, *4*, 214–222. [CrossRef] [PubMed]
134. Lopez-Verrilli, M.A.; Caviedes, A.; Cabrera, A.; Sandoval, S.; Wyneken, U.; Khoury, M. Mesenchymal stem cell-derived exosomes from different sources selectively promote neuritic outgrowth. *Neuroscience* **2016**, *320*, 129–139. [CrossRef]
135. Hotham, W.E.; Thompson, C.; Szu-Ting, L.; Henson, F.M.D. The anti-inflammatory effects of equine bone marrow stem cell-derived extracellular vesicles on autologous chondrocytes. *Vet. Rec. Open* **2021**, *8*, e22. [CrossRef] [PubMed]
136. Mobasheri, A.; Thudium, C.S.; Bay-Jensen, A.-C.; Maleitzke, T.; Geissler, S.; Duda, G.N.; Winkler, T. Biomarkers for osteoarthritis: Current status and future prospects. *Best Pract. Res. Clin. Rheumatol.* **2023**, *37*, 101852. [CrossRef]
137. Block, G.J.; Ohkouchi, S.; Fung, F.; Frenkel, J.; Gregory, C.; Pochampally, R.; DiMattia, G.; Sullivan, D.E.; Prockop, D.J. Multipotent stromal cells are activated to reduce apoptosis in part by upregulation and secretion of stanniocalcin-1. *Stem Cells* **2009**, *27*, 670–681. [CrossRef]
138. Maumus, M.; Manferdini, C.; Toupet, K.; Peyrafitte, J.-A.; Ferreira, R.; Facchini, A.; Gabusi, E.; Bourin, P.; Jorgensen, C.; Lisignoli, G.; et al. Adipose mesenchymal stem cells protect chondrocytes from degeneration associated with osteoarthritis. *Stem Cell Res.* **2013**, *11*, 834–844. [CrossRef] [PubMed]
139. Dankbar, B.; Neugebauer, K.; Wunrau, C.; Tibesku, C.O.; Skwara, A.; Pap, T.; Fuchs-Winkelmann, S. Hepatocyte growth factor induction of macrophage chemoattractant protein-1 and osteophyte-inducing factors in osteoarthritis. *J. Orthop. Res.* **2007**, *25*, 569–577. [CrossRef]
140. Meng, F.; Li, Z.; Zhang, Z.; Yang, Z.; Kang, Y.; Zhao, X.; Long, D.; Hu, S.; Gu, M.; He, S.; et al. MicroRNA-193b-3p regulates chondrogenesis and chondrocyte metabolism by targeting HDAC3. *Theranostics* **2018**, *8*, 2862–2883. [CrossRef]
141. Song, J.; Kang, Y.; Chun, C.-H.; Jin, E.-J. Selective loading of exosomal HULC and miR-372 is responsible for chondrocyte death during OA pathogenesis. *Anim. Cells Syst.* **2017**, *21*, 397–403. [CrossRef]
142. Ni, Z.; Kuang, L.; Chen, H.; Xie, Y.; Zhang, B.; Ouyang, J.; Wu, J.; Zhou, S.; Chen, L.; Su, N.; et al. The exosome-like vesicles from osteoarthritic chondrocyte enhanced mature IL-1β production of macrophages and aggravated synovitis in osteoarthritis. *Cell Death Dis.* **2019**, *10*, 522. [CrossRef]
143. Zhou, Y.; Ming, J.; Li, Y.; Li, B.; Deng, M.; Ma, Y.; Chen, Z.; Zhang, Y.; Li, J.; Liu, S. Exosomes derived from miR-126-3p-overexpressing synovial fibroblasts suppress chondrocyte inflammation and cartilage degradation in a rat model of osteoarthritis. *Cell Death Discov.* **2021**, *7*, 37. [CrossRef] [PubMed]
144. Kato, T.; Miyaki, S.; Ishitobi, H.; Nakamura, Y.; Nakasa, T.; Lotz, M.K.; Ochi, M. Exosomes from IL-1β stimulated synovial fibroblasts induce osteoarthritic changes in articular chondrocytes. *Arthritis Res. Ther.* **2014**, *16*, R163. [CrossRef]
145. Kolhe, R.; Hunter, M.; Liu, S.; Jadeja, R.N.; Pundkar, C.; Mondal, A.K.; Mendhe, B.; Drewry, M.; Rojiani, M.V.; Liu, Y.; et al. Gender-specific differential expression of exosomal miRNA in synovial fluid of patients with osteoarthritis. *Sci. Rep.* **2017**, *7*, 2029. [CrossRef] [PubMed]
146. Anderson, J.R.; Jacobsen, S.; Walters, M.; Bundgaard, L.; Diendorfer, A.; Hackl, M.; Clarke, E.J.; James, V.; Peffers, M.J. Small non-coding RNA landscape of extracellular vesicles from a post-traumatic model of equine osteoarthritis. *Front. Vet. Sci.* **2022**, *9*, 901269. [CrossRef] [PubMed]
147. Alexander, M.; Hu, R.; Runtsch, M.C.; Kagele, D.A.; Mosbruger, T.L.; Tolmachova, T.; Seabra, M.C.; Round, J.L.; Ward, D.M.; O'Connell, R.M. Exosome-delivered microRNAs modulate the inflammatory response to endotoxin. *Nat. Commun.* **2015**, *6*, 7321. [CrossRef]
148. Connard, S.S.; Gaesser, A.M.; Clarke, E.J.; Linardi, R.L.; Even, K.M.; Engiles, J.B.; Koch, D.W.; Peffers, M.J.; Ortved, K.F. Plasma and synovial fluid extracellular vesicles display altered microRNA profiles in horses with naturally occurring post-traumatic osteoarthritis: An exploratory study. *J. Am. Vet. Med. Assoc.* **2024**, *262*, S83–S96. [CrossRef]
149. Liu, D.-H.; Jheng, Y.-C.; Chen, P.-Y.; Yarmishyn, A.A.; Huang, S.-E.; Chien, C.-S.; Tsai, P.-H.; Chien, Y.; Yang, Y.-P.; Kao, C.-L. Frontier review of the roles of exosomes in osteoarthritis. *J. Chin. Med. Assoc.* **2021**, *84*, 754–756. [CrossRef]
150. Sun, H.; Hu, S.; Zhang, Z.; Lun, J.; Liao, W.; Zhang, Z. Expression of exosomal microRNAs during chondrogenic differentiation of human bone mesenchymal stem cells. *J. Cell. Biochem.* **2019**, *120*, 171–181. [CrossRef]
151. Clarke, E.J.; Lima, C.; Anderson, J.R.; Castanheira, C.; Beckett, A.; James, V.; Hyett, J.; Goodacre, R.; Peffers, M.J. Optical photothermal infrared spectroscopy can differentiate equine osteoarthritic plasma extracellular vesicles from healthy controls. *Anal. Methods* **2022**, *14*, 3661–3670. [CrossRef]
152. Yamasaki, K.; Nakasa, T.; Miyaki, S.; Ishikawa, M.; Deie, M.; Adachi, N.; Yasunaga, Y.; Asahara, H.; Ochi, M. Expression of MicroRNA-146a in osteoarthritis cartilage. *Arthritis Rheum.* **2009**, *60*, 1035–1041. [CrossRef]
153. Zhao, C.; Chen, J.-Y.; Peng, W.-M.; Yuan, B.; Bi, Q.; Xu, Y.-J. Exosomes from adipose-derived stem cells promote chondrogenesis and suppress inflammation by upregulating miR-145 and miR-221. *Mol. Med. Rep.* **2020**, *21*, 1881–1889. [CrossRef] [PubMed]
154. Zhao, Y.; Xu, J. Synovial fluid-derived exosomal lncRNA PCGEM1 as biomarker for the different stages of osteoarthritis. *Int. Orthop.* **2018**, *42*, 2865–2872. [CrossRef] [PubMed]

155. Meng, Y.; Qiu, S.; Sun, L.; Zuo, J. Knockdown of exosome-mediated lnc-PVT1 alleviates lipopolysaccharide-induced osteoarthritis progression by mediating the HMGB1/TLR4/NF-κB pathway via miR-93-5p. *Mol. Med. Rep.* **2020**, *22*, 5313–5325. [CrossRef]
156. Mustonen, A.-M.; Lehmonen, N.; Paakkonen, T.; Raekallio, M.; Käkelä, R.; Niemelä, T.; Mykkänen, A.; Sihvo, S.P.; Nieminen, P. Equine osteoarthritis modifies fatty acid signatures in synovial fluid and its extracellular vesicles. *Arthritis Res. Ther.* **2023**, *25*, 39. [CrossRef]
157. Clarke, E.; Varela, L.; Jenkins, R.E.; Lozano-Andrés, E.; Cywińska, A.; Przewozny, M.; van Weeren, P.R.; van de Lest, C.H.A.; Peffers, M.; Wauben, M.H.M. Proteome and phospholipidome interrelationship of synovial fluid-derived extracellular vesicles in equine osteoarthritis: An exploratory 'multi-omics' study to identify composite biomarkers. *Biochem. Biophys. Rep.* **2024**, *37*, 101635. [CrossRef]
158. Contentin, R.; Jammes, M.; Bourdon, B.; Cassé, F.; Bianchi, A.; Audigié, F.; Branly, T.; Velot, É.; Galéra, P. Bone Marrow MSC Secretome Increases Equine Articular Chondrocyte Collagen Accumulation and Their Migratory Capacities. *Int. J. Mol. Sci.* **2022**, *23*, 5795. [CrossRef]
159. Jammes, M.; Cassé, F.; Velot, E.; Bianchi, A.; Audigié, F.; Contentin, R.; Galéra, P. Pro-Inflammatory Cytokine Priming and Purification Method Modulate the Impact of Exosomes Derived from Equine Bone Marrow Mesenchymal Stromal Cells on Equine Articular Chondrocytes. *Int. J. Mol. Sci.* **2023**, *24*, 4169. [CrossRef] [PubMed]
160. Zhang, J.; Rong, Y.; Luo, C.; Cui, W. Bone marrow mesenchymal stem cell-derived exosomes prevent osteoarthritis by regulating synovial macrophage polarization. *Aging* **2020**, *12*, 25138–25152. [CrossRef]
161. HU, Y.; LIU, H.X.; XU, D.; XUE, X.; XU, X. The Anti-Inflammatory Effect of miR-140-3p in BMSCs-Exosomes on Osteoarthritis. *ACHOT* **2023**, *90*, 267–276. [CrossRef]
162. Cosenza, S.; Ruiz, M.; Toupet, K.; Jorgensen, C.; Noël, D. Mesenchymal stem cells derived exosomes and microparticles protect cartilage and bone from degradation in osteoarthritis. *Sci. Rep.* **2017**, *7*, 16214. [CrossRef]
163. Wu, J.; Wu, J.; Xiang, W.; Gong, Y.; Feng, D.; Fang, S.; Wu, Y.; Liu, Z.; Li, Y.; Chen, R.; et al. Engineering exosomes derived from TNF-α preconditioned IPFP-MSCs enhance both yield and therapeutic efficacy for osteoarthritis. *J. Nanobiotechnol.* **2024**, *22*, 555. [CrossRef] [PubMed]
164. Ragni, E.; de Luca, P.; Perucca Orfei, C.; Colombini, A.; Viganò, M.; Lugano, G.; Bollati, V.; de Girolamo, L. Insights into Inflammatory Priming of Adipose-Derived Mesenchymal Stem Cells: Validation of Extracellular Vesicles-Embedded miRNA Reference Genes as A Crucial Step for Donor Selection. *Cells* **2019**, *8*, 369. [CrossRef] [PubMed]
165. Lee, Y.-H.; Park, H.-K.; Auh, Q.-S.; Nah, H.; Lee, J.S.; Moon, H.-J.; Heo, D.N.; Kim, I.S.; Kwon, I.K. Emerging Potential of Exosomes in Regenerative Medicine for Temporomandibular Joint Osteoarthritis. *Int. J. Mol. Sci.* **2020**, *21*, 1541. [CrossRef]
166. Liu, Y.; Lin, L.; Zou, R.; Wen, C.; Wang, Z.; Lin, F. MSC-derived exosomes promote proliferation and inhibit apoptosis of chondrocytes via lncRNA-KLF3-AS1/miR-206/GIT1 axis in osteoarthritis. *Cell Cycle* **2018**, *17*, 2411–2422. [CrossRef]
167. Wang, Z.; Yan, K.; Ge, G.; Zhang, D.; Bai, J.; Guo, X.; Zhou, J.; Xu, T.; Xu, M.; Long, X.; et al. Exosomes derived from miR-155-5p-overexpressing synovial mesenchymal stem cells prevent osteoarthritis via enhancing proliferation and migration, attenuating apoptosis, and modulating extracellular matrix secretion in chondrocytes. *Cell Biol. Toxicol.* **2021**, *37*, 85–96. [CrossRef]
168. Liu, X.; Wang, L.; Ma, C.; Wang, G.; Zhang, Y.; Sun, S. Exosomes derived from platelet-rich plasma present a novel potential in alleviating knee osteoarthritis by promoting proliferation and inhibiting apoptosis of chondrocyte via Wnt/β-catenin signaling pathway. *J. Orthop. Surg. Res.* **2019**, *14*, 470. [CrossRef]
169. Cao, H.; Chen, M.; Cui, X.; Liu, Y.; Liu, Y.; Deng, S.; Yuan, T.; Fan, Y.; Wang, Q.; Zhang, X. Cell-Free Osteoarthritis Treatment with Sustained-Release of Chondrocyte-Targeting Exosomes from Umbilical Cord-Derived Mesenchymal Stem Cells to Rejuvenate Aging Chondrocytes. *ACS Nano* **2023**, *17*, 13358–13376. [CrossRef]
170. Théry, C.; Witwer, K.W.; Aikawa, E.; Alcaraz, M.J.; Anderson, J.D.; Andriantsitohaina, R.; Antoniou, A.; Arab, T.; Archer, F.; Atkin-Smith, G.K.; et al. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): A position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *J. Extracell. Vesicles* **2018**, *7*, 1535750. [CrossRef] [PubMed]
171. Anderson, J.R.; Johnson, E.; Jenkins, R.; Jacobsen, S.; Green, D.; Walters, M.; Bundgaard, L.; Hausmans, B.A.C.; van den Akker, G.; Welting, T.J.M.; et al. Multi-Omic Temporal Landscape of Plasma and Synovial Fluid-Derived Extracellular Vesicles Using an Experimental Model of Equine Osteoarthritis. *Int. J. Mol. Sci.* **2023**, *24*, 4888. [CrossRef]
172. Mustonen, A.-M.; Lehmonen, N.; Oikari, S.; Capra, J.; Raekallio, M.; Mykkänen, A.; Paakkonen, T.; Rilla, K.; Niemelä, T.; Nieminen, P. Counts of hyaluronic acid-containing extracellular vesicles decrease in naturally occurring equine osteoarthritis. *Sci. Rep.* **2022**, *12*, 17550. [CrossRef]
173. Gao, K.; Zhu, W.; Li, H.; Ma, D.; Liu, W.; Yu, W.; Wang, L.; Cao, Y.; Jiang, Y. Association between cytokines and exosomes in synovial fluid of individuals with knee osteoarthritis. *Mod. Rheumatol.* **2020**, *30*, 758–764. [CrossRef] [PubMed]
174. Mao, G.; Hu, S.; Zhang, Z.; Wu, P.; Zhao, X.; Lin, R.; Liao, W.; Kang, Y. Exosomal miR-95-5p regulates chondrogenesis and cartilage degradation via histone deacetylase 2/8. *J. Cell. Mol. Med.* **2018**, *22*, 5354–5366. [CrossRef]
175. Arai, K.; Misumi, K.; Carter, S.D.; Shinbara, S.; Fujiki, M.; Sakamoto, H. Analysis of cartilage oligomeric matrix protein (COMP) degradation and synthesis in equine joint disease. *Equine Vet. J.* **2005**, *37*, 31–36. [CrossRef]
176. Clutterbuck, A.L.; Smith, J.R.; Allaway, D.; Harris, P.; Liddell, S.; Mobasheri, A. High throughput proteomic analysis of the secretome in an explant model of articular cartilage inflammation. *J. Proteomics* **2011**, *74*, 704–715. [CrossRef] [PubMed]

177. Svala, E.; Löfgren, M.; Sihlbom, C.; Rüetschi, U.; Lindahl, A.; Ekman, S.; Skiöldebrand, E. An inflammatory equine model demonstrates dynamic changes of immune response and cartilage matrix molecule degradation in vitro. *Connect. Tissue Res.* **2015**, *56*, 315–325. [CrossRef]
178. Wang, X.; Zhang, Y.; Wu, Y.; Wang, C.; Li, S.; Yuan, Y.; Lv, X.; Liu, Y.; Chen, F.; Chen, S.; et al. Integration of miRNA in exosomes and single-cell RNA-seq profiles in endemic osteoarthritis, Kashin-Beck disease. *Biofactors* **2024**, *50*, 725–737. [CrossRef] [PubMed]
179. Rosenthal, A.K.; Gohr, C.M.; Mitton-Fitzgerald, E.; Grewal, R.; Ninomiya, J.; Coyne, C.B.; Jackson, W.T. Autophagy modulates articular cartilage vesicle formation in primary articular chondrocytes. *J. Biol. Chem.* **2015**, *290*, 13028–13038. [CrossRef]
180. Glenske, K.; Eldaey, A.; Schaalo, S.; Arnhold, S.; Heiss, C.; Schnettler, R.; Wenisch, S.; Elashry, M.I. Morphological and Immunohistochemical Characterization of Bone Structure and Cell–Cell Communication in a Rat Osteoporosis Model. *Anatomia* **2024**, *3*, 93–109. [CrossRef]
181. Elashry, M.I.; Heimann, M.; Wenisch, S.; Patel, K.; Arnhold, S. Multipotency of skeletal muscle stem cells on their native substrate and the expression of Connexin 43 during adoption of adipogenic and osteogenic fate. *Acta Histochem.* **2017**, *119*, 786–794. [CrossRef]
182. Arnhold, S.; Elashry, M.I.; Klymiuk, M.C.; Wenisch, S. Biological macromolecules and mesenchymal stem cells: Basic research for regenerative therapies in veterinary medicine. *Int. J. Biol. Macromol.* **2019**, *123*, 889–899. [CrossRef]
183. Varela-Eirín, M.; Varela-Vázquez, A.; Guitián-Caamaño, A.; Bravo-López, S.B.; Paíno, C.; Largo, R.; Fonseca, E.; Kandouz, M.; Aasen, T.; Tabernero, A.; et al. FRI0529 spread of senescence and joint inflammation via connexin43-positive exosomes released by osteoarthritic chondrocytes. In *Cartilage, Synovium and Bone, Proceedings of the Annual European Congress of Rheumatology, EULAR 2019, Madrid, Spain, 12–15 June 2019*; BMJ Publishing Group Ltd. and European League Against Rheumatism: London, UK, 2019; p. 959.
184. Varela-Eirín, M.; Carpintero-Fernández, P.; Guitián-Caamaño, A.; Varela-Vázquez, A.; García-Yuste, A.; Sánchez-Temprano, A.; Bravo-López, S.B.; Yañez-Cabanas, J.; Fonseca, E.; Largo, R.; et al. Extracellular vesicles enriched in connexin 43 promote a senescent phenotype in bone and synovial cells contributing to osteoarthritis progression. *Cell Death Dis.* **2022**, *13*, 681. [CrossRef] [PubMed]
185. Boulestreau, J.; Maumus, M.; Bertolino, G.M.; Toupet, K.; Jorgensen, C.; Noël, D. Correction: Extracellular vesicles from senescent mesenchymal stromal cells are defective and cannot prevent osteoarthritis. *J. Nanobiotechnol.* **2024**, *22*, 418. [CrossRef] [PubMed]
186. Chen, B.-Y.; Sung, C.W.-H.; Chen, C.; Cheng, C.-M.; Lin, D.P.-C.; Huang, C.-T.; Hsu, M.-Y. Advances in exosomes technology. *Clin. Chim. Acta* **2019**, *493*, 14–19. [CrossRef] [PubMed]
187. Zhou, Q.-F.; Cai, Y.-Z.; Lin, X.-J. The dual character of exosomes in osteoarthritis: Antagonists and therapeutic agents. *Acta Biomater.* **2020**, *105*, 15–25. [CrossRef]
188. Withrow, J.; Murphy, C.; Liu, Y.; Hunter, M.; Fulzele, S.; Hamrick, M.W. Extracellular vesicles in the pathogenesis of rheumatoid arthritis and osteoarthritis. *Arthritis Res. Ther.* **2016**, *18*, 286. [CrossRef]
189. Domenis, R.; Zanutel, R.; Caponnetto, F.; Toffoletto, B.; Cifù, A.; Pistis, C.; Di Benedetto, P.; Causero, A.; Pozzi, M.; Bassini, F.; et al. Characterization of the Proinflammatory Profile of Synovial Fluid-Derived Exosomes of Patients with Osteoarthritis. *Mediators Inflamm.* **2017**, *2017*, 4814987. [CrossRef]
190. Song, J.E.; Kim, J.S.; Shin, J.H.; Moon, K.W.; Park, J.K.; Park, K.S.; Lee, E.Y. Role of Synovial Exosomes in Osteoclast Differentiation in Inflammatory Arthritis. *Cells* **2021**, *10*, 120. [CrossRef]
191. Pfeifer, J.P.H.; Stievani, F.d.C.; Da Fernandes, C.J.C.; Rosa, G.D.S.; Apolonio, E.V.P.; Rossi, M.C.; Zambuzzi, W.F.; Alves, A.L.G. Influence of inflammation on the expression of microRNA-140 in extracellular vesicles from 2D and 3D culture models of synovial-membrane-derived stem cells. *Front. Bioeng. Biotechnol.* **2024**, *12*, 1416694. [CrossRef]
192. Morgan, R.E.; Clegg, P.D.; Hunt, J.A.; Innes, J.F.; Tew, S.R. Interaction with macrophages attenuates equine fibroblast-like synoviocyte ADAMTS5 (aggrecanase-2) gene expression following inflammatory stimulation. *J. Orthop. Res.* **2018**, *36*, 2178–2185. [CrossRef]
193. Bertoni, L.; Branly, T.; Jacquet, S.; Desancé, M.; Desquilbet, L.; Rivory, P.; Hartmann, D.-J.; Denoix, J.-M.; Audigié, F.; Galéra, P.; et al. Intra-Articular Injection of 2 Different Dosages of Autologous and Allogeneic Bone Marrow- and Umbilical Cord-Derived Mesenchymal Stem Cells Triggers a Variable Inflammatory Response of the Fetlock Joint on 12 Sound Experimental Horses. *Stem Cells Int.* **2019**, *2019*, 9431894. [CrossRef]
194. da Silva Meirelles, L.; Sand, T.T.; Harman, R.J.; Lennon, D.P.; Caplan, A.I. MSC frequency correlates with blood vessel density in equine adipose tissue. *Tissue Eng. Part A* **2009**, *15*, 221–229. [CrossRef] [PubMed]
195. Jammes, M.; Contentin, R.; Cassé, F.; Galéra, P. Equine osteoarthritis: Strategies to enhance mesenchymal stromal cell-based acellular therapies. *Front. Vet. Sci.* **2023**, *10*, 1115774. [CrossRef] [PubMed]
196. Akyurekli, C.; Le, Y.; Richardson, R.B.; Fergusson, D.; Tay, J.; Allan, D.S. A systematic review of preclinical studies on the therapeutic potential of mesenchymal stromal cell-derived microvesicles. *Stem Cell Rev. Rep.* **2015**, *11*, 150–160. [CrossRef]
197. Narayanan, R.; Huang, C.-C.; Ravindran, S. Hijacking the Cellular Mail: Exosome Mediated Differentiation of Mesenchymal Stem Cells. *Stem Cells Int.* **2016**, *2016*, 3808674. [CrossRef]
198. Tofiño-Vian, M.; Guillén, M.I.; Pérez Del Caz, M.D.; Silvestre, A.; Alcaraz, M.J. Microvesicles from Human Adipose Tissue-Derived Mesenchymal Stem Cells as a New Protective Strategy in Osteoarthritic Chondrocytes. *Cell. Physiol. Biochem.* **2018**, *47*, 11–25. [CrossRef] [PubMed]

199. Garbin, L.C.; Olver, C.S. Platelet-Rich Products and Their Application to Osteoarthritis. *J. Equine Vet. Sci.* **2020**, *86*, 102820. [CrossRef]
200. Rodríguez-Merchán, E.C. Intra-Articular Platelet-Rich Plasma Injections in Knee Osteoarthritis: A Review of Their Current Molecular Mechanisms of Action and Their Degree of Efficacy. *Int. J. Mol. Sci.* **2022**, *23*, 1301. [CrossRef]
201. Salzler, M.J. Editorial Commentary: Platelet-Rich Plasma: Fountain of Youth, Cart Before the Horse, or Both? *Arthroscopy* **2018**, *34*, 1541–1542. [CrossRef]
202. Peng, C.; Yang, L.; Labens, R.; Gao, Y.; Zhu, Y.; Li, J. A systematic review and meta-analysis of the efficacy of platelet-rich plasma products for treatment of equine joint disease. *Equine Vet. J.* **2024**, *56*, 858–869. [CrossRef]
203. Navani, A.; Li, G.; Chrystal, J. Platelet Rich Plasma in Musculoskeletal Pathology: A Necessary Rescue or a Lost Cause? *Pain Physician* **2017**, *20*, E345–E356. [CrossRef]
204. Blair, P.; Flaumenhaft, R. Platelet alpha-granules: Basic biology and clinical correlates. *Blood Rev.* **2009**, *23*, 177–189. [CrossRef] [PubMed]
205. Golebiewska, E.M.; Poole, A.W. Secrets of platelet exocytosis—What do we really know about platelet secretion mechanisms? *Br. J. Haematol.* **2013**, *165*, 204–216. [CrossRef] [PubMed]
206. Coppinger, J.A.; Cagney, G.; Toomey, S.; Kislinger, T.; Belton, O.; McRedmond, J.P.; Cahill, D.J.; Emili, A.; Fitzgerald, D.J.; Maguire, P.B. Characterization of the proteins released from activated platelets leads to localization of novel platelet proteins in human atherosclerotic lesions. *Blood* **2004**, *103*, 2096–2104. [CrossRef] [PubMed]
207. Zhu, Y.; Yuan, M.; Meng, H.Y.; Wang, A.Y.; Guo, Q.Y.; Wang, Y.; Peng, J. Basic science and clinical application of platelet-rich plasma for cartilage defects and osteoarthritis: A review. *Osteoarthr. Cartil.* **2013**, *21*, 1627–1637. [CrossRef] [PubMed]
208. Moussa, M.; Lajeunesse, D.; Hilal, G.; El Atat, O.; Haykal, G.; Serhal, R.; Chalhoub, A.; Khalil, C.; Alaaeddine, N. Platelet rich plasma (PRP) induces chondroprotection via increasing autophagy, anti-inflammatory markers, and decreasing apoptosis in human osteoarthritic cartilage. *Exp. Cell Res.* **2017**, *352*, 146–156. [CrossRef] [PubMed]
209. Clarke, E.J.; Johnson, E.; Caamaño Gutierrez, E.; Andersen, C.; Berg, L.C.; Jenkins, R.E.; Lindegaard, C.; Uvebrant, K.; Lundgren-Åkerlund, E.; Turlo, A.; et al. Temporal extracellular vesicle protein changes following intraarticular treatment with integrin α10β1-selected mesenchymal stem cells in equine osteoarthritis. *Front. Vet. Sci.* **2022**, *9*, 1057667. [CrossRef] [PubMed]
210. Varderidou-Minasian, S.; Lorenowicz, M.J. Mesenchymal stromal/stem cell-derived extracellular vesicles in tissue repair: Challenges and opportunities. *Theranostics* **2020**, *10*, 5979–5997. [CrossRef]
211. Kakkar, M.; Chauhan, A.S.; Bahl, T.; Rahi, M. Opportunities for One Health policies to reduce poverty. *Rev. Sci. Tech.* **2019**, *38*, 135–144. [CrossRef]
212. Amuasi, J.H.; Lucas, T.; Horton, R.; Winkler, A.S. Reconnecting for our future: The Lancet One Health Commission. *Lancet* **2020**, *395*, 1469–1471. [CrossRef]
213. Tomley, F.M.; Shirley, M.W. Livestock infectious diseases and zoonoses. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **2009**, *364*, 2637–2642. [CrossRef] [PubMed]
214. Bongiovanni, L.; Andriessen, A.; Wauben, M.H.M.; Nolte-'t Hoen, E.N.M.; de Bruin, A. Extracellular Vesicles: Novel Opportunities to Understand and Detect Neoplastic Diseases. *Vet. Pathol.* **2021**, *58*, 453–471. [CrossRef] [PubMed]
215. Diomaiuto, E.; Principe, V.; de Luca, A.; Laperuta, F.; Alterisio, C.; Di Loria, A. Exosomes in Dogs and Cats: An Innovative Approach to Neoplastic and Non-Neoplastic Diseases. *Pharmaceuticals* **2021**, *14*, 766. [CrossRef] [PubMed]
216. Branly, T.; Contentin, R.; Desancé, M.; Jacquel, T.; Bertoni, L.; Jacquet, S.; Mallein-Gerin, F.; Denoix, J.-M.; Audigié, F.; Demoor, M.; et al. Improvement of the Chondrocyte-Specific Phenotype upon Equine Bone Marrow Mesenchymal Stem Cell Differentiation: Influence of Culture Time, Transforming Growth Factors and Type I Collagen siRNAs on the Differentiation Index. *Int. J. Mol. Sci.* **2018**, *19*, 435. [CrossRef]
217. O'Brien, T.J.; Hollinshead, F.; Goodrich, L.R. Extracellular vesicles in the treatment and prevention of osteoarthritis: Can horses help us translate this therapy to humans? *Extracell. Vesicles Circ. Nucl. Acids* **2023**, *4*, 151–169. [CrossRef]

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Review

Alcohol Consumption and Breast and Ovarian Cancer Development: Molecular Pathways and Mechanisms

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Abstract: Alcohol consumption has been consistently linked to an increased risk of several cancers, including breast and ovarian cancer. Despite substantial evidence supporting this association, the precise mechanisms underlying alcohol's contribution to cancer pathogenesis remain incompletely understood. This narrative review focuses on the key current literature on the biological pathways through which alcohol may influence the development of breast and ovarian cancer. Key mechanisms discussed include the modulation of estrogen levels, the generation of reactive oxygen species, the production of acetaldehyde, the promotion of chronic inflammation, and the induction of epigenetic changes. Alcohol's impact on estrogenic signaling, particularly in the regulation of estrogen and progesterone, is explored in the context of hormone-dependent cancers. Additionally, the role of alcohol-induced DNA damage, mutagenesis, and immune system modulation in tumor initiation and progression is examined. Overall, this review emphasizes the importance of alcohol as a modifiable risk factor for breast and ovarian cancer and highlights the need for further research to clarify its role in cancer biology.

Keywords: alcohol; cancer; breast; ovaries; DNA; oxidative stress; inflammation

1. Introduction

Alcohol consumption has been linked to several types of cancers in humans [1–4]. Alcohol consumption is prevalent worldwide, with varying patterns of use across different regions and cultures. The data on global alcohol consumption in 2019 shows that an estimated 400 million people aged 15 years and older live with alcohol use disorders, and an estimated 209 million live with alcohol dependence [5]. Alcohol abuse—defined as the harmful or hazardous use of alcohol leading to adverse health effects—is a significant public health concern [6–8].

The global burden of alcohol-related harm is estimated to cause 5.3% of all deaths and 5.1% of the global disease burden, according to the Global Burden of Disease Study [9]. Alcohol abuse is typically characterized by excessive consumption, binge drinking, and alcohol dependence, which have a direct impact on morbidity and mortality [10,11]. Long-term alcohol abuse increases the risk for a range of diseases, including liver cirrhosis, cardiovascular disease, neurological disorders, and several types of cancer, particularly

those of the gastrointestinal tract, liver, and breast [12–14]. Epidemiological studies have demonstrated a clear association between alcohol abuse and the increased incidence of certain malignancies, making it a major modifiable risk factor for cancer [15–17]. There is evidence that alcohol consumption promotes the onset of tumors in many organs, for example, the liver, pancreas, larynx, oral cavity, and esophagus [18,19].

Numerous studies have highlighted an increased risk of colorectal cancer [20–22]. Additionally, alcohol consumption is a well-known risk factor for breast cancer in women [23–26]. Breast cancer is a multifactorial disease whose etiology is associated with multiple risk factors related to diet, lifestyle, and hormones [27–30]. Among these factors, alcohol consumption has been linked to a higher risk of breast cancer [31,32].

The link between alcohol consumption and ovarian cancer has been less thoroughly explored, but emerging evidence suggests a potential correlation [33,34]. The proposed mechanisms may involve hormonal modulation and the effect of alcohol on inflammation and cellular proliferation within ovarian tissue [35]. Alcohol consumption is a well-established risk factor for breast cancer, primarily through its effects on estrogen metabolism, oxidative stress, and inflammation. Alcohol promotes the development of estrogen receptor-positive (ER+) breast cancer by elevating estrogen levels and causing DNA damage [36]. In ovarian cancer, the relationship is less clear but may involve similar mechanisms of hormonal modulation, oxidative stress, and inflammation. Although the role of estrogen in ovarian cancer is less well-defined, alcohol's impact on estrogen levels and its potential to increase oxidative damage and inflammation are key factors in its association with ovarian cancer risk [37].

2. Materials and Methods

Between June and October 2024, for this narrative review, a wide literature exploration was carried out to disclose relevant articles (PubMed, Scopus, and WOS). The papers were chosen with the following keywords: "Alcohol", "Ethanol", "DNA", "Oxidative Stress", "Cancer", "Breast" and "Ovaries". We did not filter the year of publication. The chosen papers were meticulously reviewed and assessed by all authors to disclose studies that potentially encounter the aim of this narrative review.

Key inclusion criteria were (1) original studies that dealt with alcohol, ethanol, DNA, oxidative stress, cancer, breast, and ovaries and (2) articles in English. Editorials, case reports, and letters were excluded from this paper. Any divergences between the authors of this study were resolved through a consensus approach.

3. Molecular Mechanisms of Alcohol-Mediated Carcinogenesis

Alcohol-mediated carcinogenesis involves multiple molecular mechanisms that contribute to cancer development [38]. When ethanol is metabolized, it is converted primarily to acetaldehyde by the enzyme alcohol dehydrogenase (ADH), a potent carcinogen that can form DNA adducts, leading to mutations. The accumulation of reactive oxygen species (ROSs) during alcohol metabolism causes oxidative stress, which can damage cellular components, including DNA, proteins, and lipids, further contributing to genetic mutations and genomic instability [39]. Alcohol also influences hormonal pathways, especially in hormone-sensitive cancers like breast cancer, by increasing levels of estrogen through the upregulation of aromatase activity, promoting cell proliferation [40]. Additionally, alcohol-induced inflammation activates pathways like NF-kB, which can drive tumorigenesis by promoting cell survival, proliferation, and metastasis [41]. These combined molecular effects, including DNA damage, oxidative stress, hormonal modulation, and inflammation, all contribute to the carcinogenic potential of alcohol in various tissues (Figure 1).

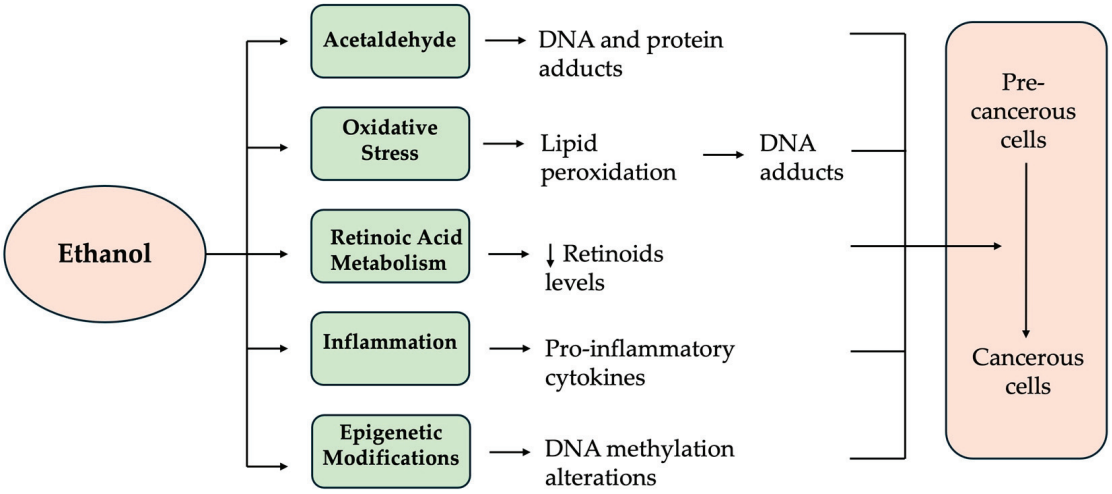


Figure 1. Mechanisms of alcohol-mediated carcinogenesis. Alcohol metabolism produces acetaldehyde, which can bind to DNA and proteins, forming DNA adducts. Another mechanism involves the induction of oxidative stress and the generation of ROS, which can lead to lipid peroxidation and the production of aldehydes capable of binding to DNA and creating etheno-DNA-protein adducts. Furthermore, alcohol can disrupt retinoid metabolism by inhibiting the conversion of vitamin A to retinoic acid. Chronic alcohol consumption can also promote the recruitment of monocytes and macrophages into the tumor microenvironment, producing pro-inflammatory cytokines. Lastly, chronic alcohol consumption can affect the individual's epigenetic profile by depleting intracellular methyl group reserves that support the epigenome. The short down pointing arrow indicates decrease.

3.1. Oxidative Stress

One of the mechanisms by which ethanol contributes to carcinogenesis is through the induction of oxidative stress [42,43].

Oxidative stress can result from the activation of some pathways that produce reactive oxygen species (ROSs), such as hydrogen peroxide and superoxide anion. One of these pathways involves the increase in the activity of CYP2E1, which produces ROS through the oxidation of ethanol to acetaldehyde [44]. It has been shown that excessive alcohol consumption increases CYP2E1 expression in the esophagus. Other sources of ROS resulting from ethanol metabolism are represented by the mitochondrial respiratory chain and some cytosolic enzymes [45].

ROS are highly reactive species that can lead to lipid peroxidation, resulting in the production of aldehydes that can form etheno-DNA adducts through their binding with DNA [46,47]. These DNA adducts are represented by 1,N6-ethenodeoxyadenosine and 3,N4-ethenodeoxycytidine, which are highly mutagenic [48].

Moreover, ROS can lead to the activation of nuclear transcription factor (NF-kB) by acting as messengers in intracellular signaling pathways. ROS interfere with mitogen-activated protein kinase signaling pathways and upregulate vascular endothelial growth factor (VEGF) and monocyte chemoattractant protein-1 (MCP-1), thus increasing cell proliferation and metastasis [49].

3.2. Acetaldehyde Production

Alcohol metabolism leads to the production of acetaldehyde through the action of enzymes such as cytochrome P-450 2E1 (CYP2E1), alcohol dehydrogenase (ADH), and bacterial catalase [50]. Acetaldehyde has a carcinogenic and genotoxic action. Being highly reactive towards DNA, acetaldehyde can bind to DNA and form DNA adducts (such as N2-ethylidene-2-deoxyguanosine, N2-ethyl-2-deoxyguanosine, N2-propano-2-deoxyguanosine (PdG), and N2-etheno-2-deoxyguanosine) [51], which can lead to blocking

of DNA synthesis and repair. These DNA adducts can induce double-strand breaks, DNA point mutations, sister chromatid exchanges, and structural changes in chromosomes [52].

The PdG adduct can lead to the formation of other highly genotoxic structures, including DNA-strand cross-links and DNA-protein cross-links, which may be responsible for the carcinogenesis process [53]. Furthermore, acetaldehyde can bind to proteins directly, causing structural and functional alterations; among these proteins, there is glutathione, a protein involved in the reduction of alcohol-induced oxidative stress, and enzymes involved in DNA repair and methylation. Acetaldehyde can directly inhibit the activity of DNA methyltransferase (DNMT), or it can also reduce DNMT mRNA levels, thereby reducing DNMT production [54]. Furthermore, acetaldehyde and ethanol can inhibit the synthesis of S-adenosyl-L-methionine (SAMe), essential for DNA methylation.

Under normal conditions, acetaldehyde is converted to acetate by acetaldehyde dehydrogenase (ALDH) enzymes. The main enzyme responsible for most of the conversion of acetaldehyde to acetate in the liver is the enzyme ALDH2. The ALDH2*2 allelic variant is a common polymorphism of this enzyme (especially among the East Asian populations [55]) that is responsible for lower ALDH2 activity. Carriers of this polymorphism show a slower acetaldehyde metabolism and, consequently, an accumulation of acetaldehyde that has a greater possibility of exerting the described genotoxic effects.

3.3. Alteration of Retinoic Acid Metabolism

Retinoids play an important function in the regulation of carcinogenesis as they can induce cell growth, cell differentiation, and apoptosis [56]. Retinoic acid primarily acts as a tumor suppressor [57]. Alcohol can interfere with retinoid metabolism by inhibiting the oxidation of vitamin A to retinoic acid [58]. Furthermore, alcohol enhances the activity of CYP2E1, which is involved in the process of metabolizing retinoic acid with the consequent production of toxic metabolites [59].

It has been reported that there is an association between chronic alcohol consumption and reduced retinoid levels in the liver [60]. A link has also been shown between low levels of retinol in the blood and an increased risk of developing head and neck cancers [61].

3.4. Enhancement of Inflammation

Inflammation plays a key role in cancer progression and is increased by alcohol consumption [62]. Chronic alcohol consumption can result in the recruitment of monocytes and macrophages into the tumor microenvironment, resulting in the production of proinflammatory cytokines, such as tumor necrosis factor (TNF-α) and the interleukins IL-1, IL-6, and IL-8 [63]. These proinflammatory cytokines determine the activation of enzymes that generate oxidants leading to the formation of ROS [64].

These cytokines also activate NF-kB, which stimulates ROS-producing enzymes. Furthermore, IL-8 is hypothesized to contribute to the accumulation of neutrophils in the liver, leading to acute inflammation [65]. Elevated levels of IL-8 have been found in patients with alcoholic hepatitis [66]. In addition, the cytokine IL-6 is involved in the production of the anti-apoptotic protein Mcl-1, with the reduction in the apoptotic mechanism and greater exposure of the cell to DNA damage [67].

3.5. DNA Methylation

Chronic alcohol consumption can alter the epigenetic profile of the individual by destroying the intracellular reserves of methyl groups that support the epigenome and 1CM [68]. Methylation of the 5′-cytosine-phosphate-guanine-3′ (CpG) island is an important epigenetic mechanism that controls gene activity [69]. Following alcohol consumption, the DNA methylation status is altered such that some oncogenes are upregulated and tumor suppressor genes are downregulated [6].

DNMTs are de novo methyltransferases; polymorphisms in the DNMT promoter region can increase promoter activity, leading to hypermethylation of genes, including

tumor suppressor genes [70]. Alcohol can "neutralize" or reduce the hypermethylating activity of DNMT genetic variants [71].

Hypomethylation and gene expression can be affected by alcohol at the level of endogenous antioxidants [72]. For example, alcohol can cause a reduction in glutathione levels, acting on the trans-sulfuration pathway, and thus increases ROS-induced stress, which ultimately further shifts from SAM production [73].

4. Mechanisms of Alcohol Action in the Pathogenesis of Breast Cancer

The association between alcohol consumption and increased breast cancer risk is well-established in the literature, but the exact mechanisms underlying this relationship remain complex and multifactorial [74]. Several biological pathways have been proposed to explain how alcohol contributes to the initiation and progression of breast cancer (Figure 2). These mechanisms include alterations in hormonal regulation, the formation of carcinogenic metabolites such as acetaldehyde, the induction of oxidative stress, and the promotion of chronic inflammation [1].

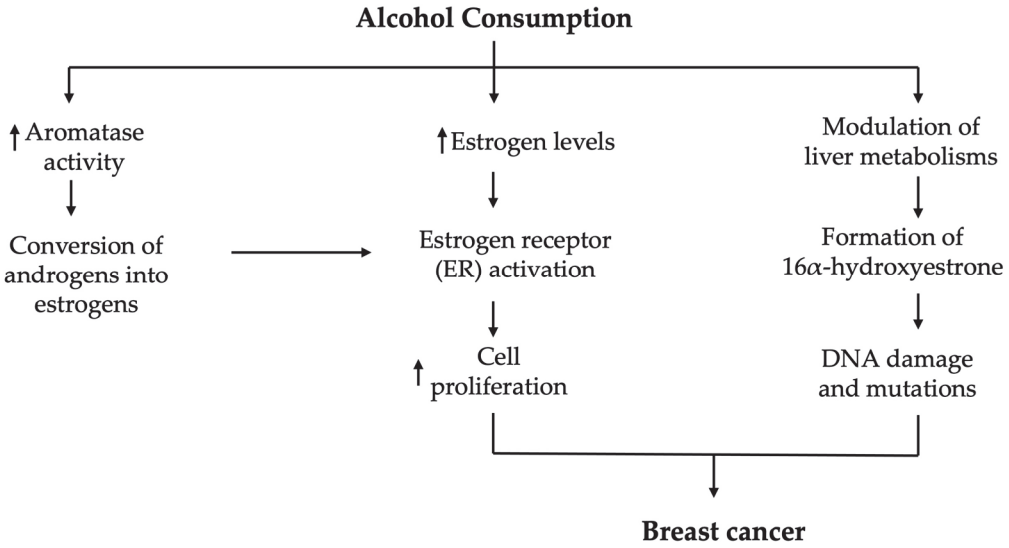


Figure 2. Alcohol consumption can increase the risk of breast cancer by affecting estrogen metabolism. Chronic alcohol intake increases circulating estrogen levels, promoting the growth of estrogen receptor (ER)-positive breast cancer cells. Alcohol also enhances the activity of aromatase, resulting in the conversion of androgens into estrogens, especially in peripheral tissues like adipose tissue. This results in higher local concentrations of estrogen in breast tissue, which further stimulates estrogen receptor signaling and cell proliferation. Additionally, alcohol can alter the metabolism of estrogen in the liver, leading to the production of 16α-hydroxyestrone, which can cause DNA damage and mutations, contributing to the development of breast cancer. The short pointing up/down arrows indicate increase/decrease.

An association has been observed between alcohol abuse and advanced and invasive breast cancer [75], so alcohol might promote the progression of existing tumors and induce more aggressive phenotypes. Many recent experimental studies support this hypothesis and show that alcohol consumption increases the aggressiveness and malignancy of breast cancer [76,77].

4.1. Hormonal Regulation and Estrogenic Effects

One of the primary mechanisms by which alcohol consumption is believed to increase breast cancer risk is through its effects on hormone metabolism, particularly estrogen. One of the key unique mechanisms in alcohol-induced breast cancer is its influence on estrogen metabolism [78]. Estrogens play a key role in the etiology of breast cancer [40]. It has been observed that chronic alcohol consumption is associated with an alteration

in estrogen levels and an increase in the expression and activity of the estrogen receptor (ER-α) [79]. Alcohol has been shown to influence both the synthesis and metabolism of estrogen [80]. Alcohol consumption, especially at higher levels, has been consistently associated with elevated levels of circulating estrogens, which are known to promote the growth of estrogen receptor (ER)-positive breast cancer cells [81]. Estrogen acts as a growth factor for breast tissue by binding to estrogen receptors on the surface of cells, activating signaling pathways that promote cell proliferation.

In women, the majority of breast cancers express estrogen receptors, making them responsive to this hormonal signaling [82]. Moreover, alcohol has been shown to enhance the activity of aromatase, the enzyme responsible for the conversion of androgens (e.g., testosterone) into estrogens in peripheral tissues, including adipose tissue [83]. This increased aromatase activity leads to higher local concentrations of estrogen in breast tissue, further promoting estrogen receptor signaling and cellular proliferation. Alcohol may also affect the metabolism of estrogen, leading to an increased production of estrogen metabolites that are more carcinogenic [84]. In both premenopausal and postmenopausal women, alcohol increases E2 levels by promoting aromatase activity, thereby raising estrogenic stimulation in estrogen receptor-positive (ER+) breast cancer cells. In postmenopausal women, alcohol can also increase the conversion of androstenedione (an androgen) to E1 in peripheral tissues, particularly in adipose tissue, further contributing to elevated estrogen levels [85,86].

The liver, which is the primary organ responsible for estrogen metabolism, converts estradiol into various metabolites, some of which are more likely to form DNA adducts or induce oxidative damage in cells [87]. Alcohol can alter this metabolic pathway by modulating liver enzyme activity, potentially increasing the formation of 16α-hydroxyestrone, a metabolite thought to be more carcinogenic compared to other metabolites like 2-hydroxyestrone. This shift in estrogen metabolism may increase breast cancer risk by promoting DNA damage and mutations [88].

In addition to estrogen receptor-positive breast cancer, alcohol consumption has also been associated with an increased risk of HER2-positive (HER2+) breast cancer [89]. For HER2+ breast cancer, alcohol may contribute to tumor progression by influencing growth factors and signaling pathways. Alcohol-induced inflammation and oxidative stress can activate signaling molecules like NF-kB, which may promote HER2 receptor overexpression, a key feature of HER2+ cancers. In the case of triple-negative breast cancer, which lacks estrogen, progesterone, and HER2 receptors, alcohol's role in promoting carcinogenesis is less understood but still significant [90,91].

4.2. Acetaldehyde and DNA Damage

Another significant mechanism through which alcohol contributes to breast cancer risk involves the formation of acetaldehyde, the primary metabolite of ethanol. Acetaldehyde is a known carcinogen that has been shown to directly damage DNA and interfere with DNA repair mechanisms, promoting mutations and genomic instability, both of which are key events in carcinogenesis [92].

Chronic alcohol consumption can cause DNA damage and genetic mutations [93]. Although alcohol itself is not a direct carcinogen, its metabolism produces acetaldehyde, which can form adducts with proteins and DNA, inducing genetic mutations, DNA cross-linking, and chromosomal aberrations [1,3,94]. The expression of ADH, a key enzyme of alcohol metabolism, has been demonstrated in human breast epithelial cells; therefore, human breast tissue can metabolize alcohol [95]. Due to the reduced ability of breast tissue to detoxify acetaldehyde, it accumulates for prolonged periods, increasing its toxicity [96]. It is therefore hypothesized that alcohol exposure induces breast carcinogenesis through the production of acetaldehyde [77].

4.3. Oxidative Stress and Reactive Oxygen Species

Oxidative stress, caused by an imbalance between ROS production and the body's ability to neutralize them with antioxidants, is another critical mechanism linking alcohol consumption with breast cancer. Alcohol metabolism generates ROS, which can directly damage cellular structures, including DNA, lipids, and proteins [97].

The liver metabolizes ethanol primarily via two enzymes: alcohol dehydrogenase and aldehyde dehydrogenase. The intermediate product, acetaldehyde, is further metabolized to acetate by ALDH [98]. ROS are produced because of the incomplete reduction of oxygen during the enzymatic reactions, including the conversion of ethanol to acetaldehyde by ADH and the subsequent conversion of acetaldehyde to acetate by ALDH. During this process, ROS are generated as by-products, which can accumulate and cause oxidative damage. ROS can induce mutations in the mitochondrial and nuclear DNA, damage cell membranes, and promote inflammatory processes, all of which contribute to carcinogenesis [99]. ROS can directly damage mitochondrial DNA (mtDNA), leading to mutations in mtDNA. Furthermore, ROS can damage cell membranes by oxidizing lipids (lipid peroxidation), leading to membrane fluidity changes, leakage of cellular contents, and ultimately cell death. Thus, alcohol-induced ROS production and mitochondrial dysfunction play a significant role in the initiation and progression of carcinogenesis.

Chronic alcohol consumption has been shown to cause mitochondrial dysfunction, further exacerbating oxidative stress [100]. Mitochondria are a key source of ROS, and their dysfunction can result in an enhanced production of these reactive molecules. Mitochondrial DNA (mtDNA) is particularly vulnerable to oxidative damage, as it lacks the same protective mechanisms as nuclear DNA, such as histone proteins and efficient DNA repair systems. Accumulated mtDNA mutations can disrupt cellular metabolism and contribute to cancer cell proliferation [101].

ROS are capable of inducing DNA strand breaks, base modifications, and cross-linking, leading to mutations in critical genes involved in cell cycle regulation (e.g., TP53), apoptosis (e.g., BAX), and DNA repair (e.g., BRCA1/2) [102]. These mutations can provide a selective advantage for cancerous cells, allowing them to proliferate uncontrollably.

4.4. Epigenetic Modifications

Furthermore, alcohol also contributes to breast carcinogenesis via dysregulation of epigenetic regulation of gene expression, particularly abnormal DNA methylation, as epigenetic dysregulation is a key mechanism for tumor initiation and progression [103].

Recent research has highlighted the role of epigenetic alterations in cancer development, and alcohol-induced epigenetic modifications may contribute to breast cancer risk. Alcohol can influence gene expression through changes in DNA methylation, histone modification, and microRNA expression, all of which can have profound effects on tumor suppression and oncogene activation.

Chronic alcohol consumption has been shown to alter DNA methylation, particularly by inducing genome-wide hypomethylation and tumor suppressor gene hypermethylation [104,105]. Hypermethylation of CpG island promoters of genes involved in central cellular pathways, such as DNA repair (e.g., BRCA1) and cell cycle control (e.g., p16INK4a), can lead to their silencing, thus promoting cell proliferation and carcinogenesis [54,106].

Alcohol may also affect the acetylation and methylation of histone proteins, leading to changes in chromatin structure that alter gene expression. Such modifications can activate oncogenes or silence tumor suppressor genes, contributing to breast cancer development [6,72,107].

5. Mechanisms of Alcohol Action in the Pathogenesis of Ovarian Cancer

While alcohol consumption is a well-established risk factor for several types of cancer, including breast cancer, its role in the pathogenesis of ovarian cancer is less understood and less studied. However, emerging evidence suggests that alcohol may influence ovarian cancer development through several biological mechanisms, including alterations in hor-

monal regulation, the induction of oxidative stress, inflammation, acetaldehyde-induced DNA damage, and epigenetic modifications. Ovarian tumors often exist within a tumor microenvironment rich in inflammatory cytokines, and alcohol's role in promoting inflammation may further support tumor growth and metastasis [108]. Despite this, the exact relationship between alcohol consumption and ovarian cancer risk requires more focused research to determine the strength and nature of this association.

5.1. Hormonal Regulation and Estrogenic Effects

Like breast cancer, ovarian cancer is influenced by hormonal factors, and alcohol's effects on hormone regulation are thought to play a key role in the development of this malignancy. Although the ovary is the primary organ responsible for estrogen production, alcohol can modulate the balance of sex hormones in the body, thereby influencing ovarian cancer risk [34].

Alcohol consumption has been shown to increase circulating levels of estrogens, particularly estradiol, which is the most potent form of estrogen. Elevated estrogen levels can promote the growth of hormone-dependent ovarian cancers, particularly those of the epithelial subtype, which accounts for the majority of ovarian cancer cases [109]. Estrogen acts by binding to estrogen receptors (ER-α and ER-β) on ovarian epithelial cells, activating downstream signaling pathways that regulate cell proliferation, survival, and apoptosis. In cases of excess estrogen, this signaling can become dysregulated, potentially leading to the uncontrolled growth of malignant cells [110].

In addition to estrogens, alcohol consumption may disrupt the balance of other hormones, including progesterone and androgens. Progesterone plays a protective role against ovarian cancer by counteracting the proliferative effects of estrogen. Chronic alcohol consumption can lead to lower levels of progesterone, potentially increasing the mitogenic effects of estrogen and promoting the growth of estrogen-dependent ovarian tumors [111].

5.2. Acetaldehyde and DNA Damage

Acetaldehyde is a potent carcinogen that can contribute to the development of various cancers, including ovarian cancer.

Acetaldehyde can bind to DNA, forming adducts that cause structural changes in the DNA molecule [92]. These DNA adducts can lead to mutations if they are not repaired by the cell's DNA repair mechanisms. These mutations may occur in key tumor suppressor genes (such as TP53) or oncogenes (such as MYC), contributing to the initiation of ovarian cancer [112].

Acetaldehyde also interferes with DNA repair processes by inhibiting the activity of enzymes involved in DNA repair, such as poly(ADP-ribose) polymerase (PARP) [113,114]. Acetaldehyde's inhibitory effect on DNA repair may enhance the likelihood of genetic alterations that promote ovarian cancer [115].

5.3. Oxidative Stress and Reactive Oxygen Species

Alcohol metabolism produces reactive oxygen species, which can induce oxidative damage in tissues throughout the body, including the ovaries. The prolonged exposure to ROS can drive the transformation of normal ovarian cells into malignant ones [116].

Ethanol consumption, particularly chronic or excessive drinking, results in increased ROS generation, leading to oxidative stress in ovarian cells [117]. ROS generated by alcohol metabolism can directly cause DNA damage by inducing base modifications, strand breaks, and cross-linking. The cumulative DNA damage resulting from ROS exposure increases the risk of mutations in oncogenes or tumor suppressor genes, such as KRAS, BRCA1, BRCA2, and TP53, all of which are frequently altered in ovarian cancer [118]. This DNA damage can lead to genetic instability, which allows cancer cells to proliferate uncontrollably.

Alcohol-induced oxidative stress can damage the mitochondria, leading to mitochondrial dysfunction [101]. Dysfunctional mitochondria contribute to further ROS production,

creating a positive feedback loop that exacerbates oxidative stress and accelerates the onset of cancer [119].

6. Discussion and Conclusions

Alcohol consumption has been shown to elevate levels of circulating estrogens, particularly through increased aromatase activity and adipose tissue conversion of androgens to estrogens, leading to enhanced cell proliferation in hormone-sensitive tissues. In the context of ovarian cancer, while the evidence is less definitive, emerging studies suggest that alcohol may also influence risk through similar pathways.

Additionally, the induction of oxidative stress from alcohol metabolism contributes to DNA damage and inflammation, both of which are recognized precursors to cancer development. For instance, the formation of reactive oxygen species and the subsequent oxidative modifications to cellular components can create a microenvironment conducive to tumorigenesis in ovarian tissues. Furthermore, genetic polymorphisms in alcohol-metabolizing enzymes, such as ALDH2, may influence individual susceptibility to alcohol-related cancer risks. These genetic factors, combined with environmental influences, underscore the complexity of the relationship between alcohol and cancer.

These findings highlight the urgent need for further research to fully elucidate the mechanisms by which alcohol influences cancer development in breast and ovarian tissues. The epidemiological evidence firmly supports the association between alcohol abuse and an increased risk of breast cancer, with a dose–response relationship that emphasizes the risks of both moderate and heavy drinking. The mechanisms underlying this association involve hormonal changes, oxidative stress, acetaldehyde formation, and inflammation, all of which contribute to carcinogenesis in breast tissue. Although the evidence linking alcohol consumption to ovarian cancer is less conclusive, emerging studies suggest a possible modest association, warranting further investigation into the underlying biological mechanisms.

Given the public health implications of alcohol abuse, particularly concerning cancer risk, public health strategies aimed at reducing alcohol consumption could play a significant role in the prevention of both breast and ovarian cancers.

Controlling alcohol-related breast and ovarian cancers requires a multifaceted approach, incorporating pharmacological agents, lifestyle interventions, and early detection strategies. Furthermore, alcohol metabolism generates reactive oxygen species (ROSs) that induce DNA damage, contributing to genetic mutations and tumor progression. By incorporating antioxidant therapies to counteract oxidative stress, the damaging effects of ROS could be mitigated, potentially decreasing cancer risk and progression.

Reducing alcohol intake remains one of the most effective preventive measures, particularly for individuals with a family history of these cancers or known genetic predispositions. Combining these strategies with a greater understanding of the molecular mechanisms of alcohol-mediated carcinogenesis provides a promising avenue for reducing the burden of alcohol-associated breast and ovarian cancers.

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References

1. Rumgay, H.; Murphy, N.; Ferrari, P.; Soerjomataram, I. Alcohol and Cancer: Epidemiology and Biological Mechanisms. *Nutrients* **2021**, *13*, 3173. [CrossRef] [PubMed]
2. Jun, S.; Park, H.; Kim, U.-J.; Choi, E.J.; Lee, H.A.; Park, B.; Lee, S.Y.; Jee, S.H.; Park, H. Cancer Risk Based on Alcohol Consumption Levels: A Comprehensive Systematic Review and Meta-Analysis. *Epidemiol. Health* **2023**, *45*, e2023092. [CrossRef]
3. Ferraguti, G.; Terracina, S.; Petrella, C.; Greco, A.; Minni, A.; Lucarelli, M.; Agostinelli, E.; Ralli, M.; de Vincentiis, M.; Raponi, G.; et al. Alcohol and Head and Neck Cancer: Updates on the Role of Oxidative Stress, Genetic, Epigenetics, Oral Microbiota, Antioxidants, and Alkylating Agents. *Antioxidants* **2022**, *11*, 145. [CrossRef] [PubMed]
4. Ciafrè, S.; Ferraguti, G.; Greco, A.; Polimeni, A.; Ralli, M.; Ceci, F.M.; Ceccanti, M.; Fiore, M. Alcohol as an Early Life Stressor: Epigenetics, Metabolic, Neuroendocrine and Neurobehavioral Implications. *Neurosci. Biobehav. Rev.* **2020**, *118*, 654–668. [CrossRef] [PubMed]
5. World Health Organization. *Global Status Report on Alcohol and Health and Treatment of Substance Use Disorders*; World Health Organization: Geneva, Switzerland, 2024.
6. Fanfarillo, F.; Ferraguti, G.; Lucarelli, M.; Fuso, A.; Ceccanti, M.; Terracina, S.; Micangeli, G.; Tarani, L.; Fiore, M. The Impact of Alcohol-Induced Epigenetic Modifications in the Treatment of Alcohol Use Disorders. *Curr. Med. Chem.* **2023**, *31*, 5837–5855. [CrossRef] [PubMed]
7. Terracina, S.; Ferraguti, G.; Fanfarillo, F.; Coriale, G.; Ceccanti, M.; Fiorentino, D.; Tarani, L.; Piccioni, M.G.; Fiore, M. Italian Guidelines for the Diagnosis and Treatment of Fetal Alcohol Spectrum Disorders: Structural Abnormalities. *Riv. Psichiatr.* **2024**, *59*, 221–229. [CrossRef] [PubMed]
8. Ceccanti, M.; Coriale, G.; Hamilton, D.A.; Carito, V.; Coccurello, R.; Scalese, B.; Ciafrè, S.; Codazzo, C.; Messina, M.P.; Chaldakov, G.N.; et al. Virtual Morris Task Responses in Individuals in an Abstinence Phase from Alcohol. *Can. J. Physiol. Pharmacol.* **2018**, *96*, 128–136. [CrossRef] [PubMed]
9. Abbafati, C.; Abbas, K.M.; Abbasi, M.; Abbasifard, M.; Abbasi-Kangevari, M.; Abbastabar, H.; Abd-Allah, F.; Abdelalim, A.; Abdollahi, M.; Abdollahpour, I.; et al. Global Burden of 369 Diseases and Injuries in 204 Countries and Territories, 1990–2019: A Systematic Analysis for the Global Burden of Disease Study 2019. *Lancet* **2020**, *396*, 1204–1222. [CrossRef]
10. Ferraguti, G.; Pascale, E.; Lucarelli, M. Alcohol Addiction: A Molecular Biology Perspective. *Curr. Med. Chem.* **2015**, *22*, 670–684. [CrossRef] [PubMed]
11. Koob, G.F.; Volkow, N.D. Neurocircuitry of Addiction. *Neuropsychopharmacology* **2010**, *35*, 217–238. [CrossRef]
12. Fiore, M.; Minni, A.; Cavalcanti, L.; Raponi, G.; Puggioni, G.; Mattia, A.; Gariglio, S.; Colizza, A.; Meliante, P.G.; Zoccali, F.; et al. The Impact of Alcohol Consumption and Oral Microbiota on Upper Aerodigestive Tract Carcinomas: A Pilot Study. *Antioxidants* **2023**, *12*, 1233. [CrossRef] [PubMed]
13. Carito, V.; Ceccanti, M.; Ferraguti, G.; Coccurello, R.; Ciafrè, S.; Tirassa, P.; Fiore, M. NGF and BDNF Alterations by Prenatal Alcohol Exposure. *Curr. Neuropharmacol.* **2019**, *17*, 308–317. [CrossRef] [PubMed]
14. Varghese, J.; Dakhode, S. Effects of Alcohol Consumption on Various Systems of the Human Body: A Systematic Review. *Cureus* **2022**, *14*, e30057. [CrossRef]
15. Gapstur, S.M.; Bandera, E.V.; Jernigan, D.H.; LoConte, N.K.; Southwell, B.G.; Vasiliou, V.; Brewster, A.M.; Naimi, T.S.; Scherr, C.L.; Shield, K.D. Alcohol and Cancer: Existing Knowledge and Evidence Gaps across the Cancer Continuum. *Cancer Epidemiol. Biomark. Prev.* **2022**, *31*, 5–10. [CrossRef] [PubMed]
16. Anderson, B.O.; Berdzuli, N.; Ilbawi, A.; Kestel, D.; Kluge, H.P.; Krech, R.; Mikkelsen, B.; Neufeld, M.; Poznyak, V.; Rekve, D.; et al. Health and Cancer Risks Associated with Low Levels of Alcohol Consumption. *Lancet Public Health* **2023**, *8*, e6–e7. [CrossRef]
17. Brunet, A.; Bonni, A.; Zigmond, M.J.; Lin, M.Z.; Juo, P.; Hu, L.S.; Anderson, M.J.; Arden, K.C.; Blenis, J.; Greenberg, M.E. Akt Promotes Cell Survival by Phosphorylating and Inhibiting a Forkhead Transcription Factor. *Cell* **1999**, *96*, 857–868. [CrossRef] [PubMed]
18. Pakzad, R.; Nedjat, S.; Salehiniya, H.; Mansournia, N.; Etminan, M.; Nazemipour, M.; Pakzad, I.; Mansournia, M.A. Effect of Alcohol Consumption on Breast Cancer: Probabilistic Bias Analysis for Adjustment of Exposure Misclassification Bias and Confounders. *BMC Med. Res. Methodol.* **2023**, *23*, 157. [CrossRef] [PubMed]
19. Scherübl, H. Alcohol Use and Gastrointestinal Cancer Risk. *Visc. Med.* **2020**, *36*, 175–181. [CrossRef]
20. Fedirko, V.; Tramacere, I.; Bagnardi, V.; Rota, M.; Scotti, L.; Islami, F.; Negri, E.; Straif, K.; Romieu, I.; La Vecchia, C.; et al. Alcohol Drinking and Colorectal Cancer Risk: An Overall and Dose-Response Meta-Analysis of Published Studies. *Ann. Oncol.* **2011**, *22*, 1958–1972. [CrossRef] [PubMed]
21. Ben, Q.; Wang, L.; Liu, J.; Qian, A.; Wang, Q.; Yuan, Y. Alcohol Drinking and the Risk of Colorectal Adenoma: A Dose-Response Meta-Analysis. *Eur. J. Cancer Prev.* **2015**, *24*, 286–295. [CrossRef] [PubMed]

22. Chen, X.; Li, H.; Guo, F.; Hoffmeister, M.; Brenner, H. Alcohol Consumption, Polygenic Risk Score, and Early- and Late-Onset Colorectal Cancer Risk. *eClinicalMedicine* **2022**, *49*, 101460. [CrossRef] [PubMed]
23. Freudenheim, J.L. Alcohol's Effects on Breast Cancer in Women. *Alcohol Res. Curr. Rev.* **2020**, *40*, 11. [CrossRef] [PubMed]
24. Scoccianti, C.; Lauby-Secretan, B.; Bello, P.Y.; Chajes, V.; Romieu, I. Female Breast Cancer and Alcohol Consumption: A Review of the Literature. *Am. J. Prev. Med.* **2014**, *46* (Suppl. S1), S16–S25. [CrossRef] [PubMed]
25. Shield, K.D.; Soerjomataram, I.; Rehm, J. Alcohol Use and Breast Cancer: A Critical Review. *Alcohol Clin. Exp. Res.* **2016**, *40*, 1166–1181. [CrossRef]
26. McDonald, J.A.; Goyal, A.; Terry, M.B. Alcohol Intake and Breast Cancer Risk: Weighing the Overall Evidence. *Curr. Breast Cancer Rep.* **2013**, *5*, 208–221. [CrossRef] [PubMed]
27. Obeagu, E.I.; Obeagu, G.U. Breast Cancer: A Review of Risk Factors and Diagnosis. *Medicine* **2024**, *103*, E36905. [CrossRef]
28. Satpathi, S.; Gaurkar, S.S.; Potdukhe, A.; Wanjari, M.B. Unveiling the Role of Hormonal Imbalance in Breast Cancer Development: A Comprehensive Review. *Cureus* **2023**, *15*, e41737. [CrossRef] [PubMed]
29. González-Palacios Torres, C.; Barrios-Rodríguez, R.; Muñoz-Bravo, C.; Toledo, E.; Dierssen, T.; Jiménez-Moleón, J.J. Mediterranean Diet and Risk of Breast Cancer: An Umbrella Review. *Clin. Nutr.* **2023**, *42*, 600–608. [CrossRef]
30. Urzì, A.G.; Tropea, E.; Gattuso, G.; Spoto, G.; Marsala, G.; Calina, D.; Libra, M.; Falzone, L. Ketogenic Diet and Breast Cancer: Recent Findings and Therapeutic Approaches. *Nutrients* **2023**, *15*, 4357. [CrossRef] [PubMed]
31. Cohen, S.Y.; Stoll, C.R.; Anandarajah, A.; Doering, M.; Colditz, G.A. Modifiable Risk Factors in Women at High Risk of Breast Cancer: A Systematic Review. *Breast Cancer Res.* **2023**, *25*, 45. [CrossRef]
32. Brown, R.B.; Bigelow, P.; Dubin, J.A.; Neiterman, E. Breast Cancer, Alcohol, and Phosphate Toxicity. *J. Appl. Toxicol.* **2024**, *44*, 17–27. [CrossRef]
33. L'Espérance, K.; Grundy, A.; Abrahamowicz, M.; Arseneau, J.; Gilbert, L.; Gotlieb, W.H.; Provencher, D.; Koushik, A. Alcohol Intake and the Risk of Epithelial Ovarian Cancer. *Cancer Causes Control.* **2023**, *34*, 533–541. [CrossRef] [PubMed]
34. Webb, P.M.; Purdie, D.M.; Bain, C.J.; Green, A.C. Alcohol, Wine, and Risk of Epithelial Ovarian Cancer. *Cancer Epidemiol. Biomark. Prev.* **2004**, *13*, 592–599. [CrossRef]
35. Wu, D.; Yang, H.; Winham, S.J.; Natanzon, Y.; Koestler, D.C.; Luo, T.; Fridley, B.L.; Goode, E.L.; Zhang, Y.; Cui, Y. Mediation Analysis of Alcohol Consumption, DNA Methylation, and Epithelial Ovarian Cancer. *J. Hum. Genet.* **2018**, *63*, 339–348. [CrossRef] [PubMed]
36. Vieira, R.; Tobar, J.S.S.; Dardes, R.; Claudio, L.; Thuler, S. Alcohol Consumption as a Risk Factor for Breast Cancer Development: A Case-Control Study in Brazil. *Asian Pac. J. Cancer Prev.* **2018**, *19*, 703–707. [CrossRef] [PubMed]
37. Chang, E.T.; Canchola, A.J.; Lee, V.S.; Clarke, C.A.; Purdie, D.M.; Reynolds, P.; Bernstein, L.; Stram, D.O.; Anton-Culver, H.; Deapen, D.; et al. Wine and Other Alcohol Consumption and Risk of Ovarian Cancer in the California Teachers Study Cohort. *Cancer Causes Control* **2007**, *18*, 91–103. [CrossRef] [PubMed]
38. Johnson, C.H.; Golla, J.P.; Dioletis, E.; Singh, S.; Ishii, M.; Charkoftaki, G.; Thompson, D.C.; Vasiliou, V. Molecular Mechanisms of Alcohol-Induced Colorectal Carcinogenesis. *Cancers* **2021**, *13*, 4404. [CrossRef]
39. Orywal, K.; Szmitkowski, M. Alcohol Dehydrogenase and Aldehyde Dehydrogenase in Malignant Neoplasms. *Clin. Exp. Med.* **2017**, *17*, 131–139. [CrossRef]
40. Al-Shami, K.; Awadi, S.; Khamees, A.; Alsheikh, A.M.; Al-Sharif, S.; Ala' Bereshy, R.; Al-Eitan, S.F.; Banikhaled, S.H.; Al-Qudimat, A.R.; Al-Zoubi, R.M.; et al. Estrogens and the Risk of Breast Cancer: A Narrative Review of Literature. *Heliyon* **2023**, *9*, e20224. [CrossRef] [PubMed]
41. Hibino, S.; Kawazoe, T.; Kasahara, H.; Itoh, S.; Ishimoto, T.; Sakata-Yanagimoto, M.; Taniguchi, K. Inflammation-Induced Tumorigenesis and Metastasis. *Int. J. Mol. Sci.* **2021**, *22*, 5421. [CrossRef]
42. Fiore, M. Oxidative Stress in Alcohol Abuse: An Unfortunately Still Open Question. *Antioxidants* **2024**, *13*, 934. [CrossRef]
43. Barve, S.; Chen, S.Y.; Kirpich, I.; Watson, W.H.; McClain, C. Development, Prevention, and Treatment of Alcohol-Induced Organ Injury: The Role of Nutrition. *Alcohol Res. Curr. Rev.* **2017**, *38*, 289–302.
44. Millonig, G.; Wang, Y.; Homann, N.; Bernhardt, F.; Qin, H.; Mueller, S.; Bartsch, H.; Seitz, H.K. Ethanol-Mediated Carcinogenesis in the Human Esophagus Implicates CYP2E1 Induction and the Generation of Carcinogenic DNA-Lesions. *Int. J. Cancer* **2011**, *128*, 533–540. [CrossRef] [PubMed]
45. Albano, E. Alcohol, Oxidative Stress and Free Radical Damage. *Proc. Nutr. Soc.* **2006**, *65*, 278–290. [CrossRef]
46. Linhart, K.; Bartsch, H.; Seitz, H.K. The Role of Reactive Oxygen Species (ROS) and Cytochrome P-450 2E1 in the Generation of Carcinogenic Etheno-DNA Adducts. *Redox Biol.* **2014**, *3*, 56–62. [CrossRef] [PubMed]
47. Haorah, J.; Ramirez, S.H.; Floreani, N.; Gorantla, S.; Morsey, B.; Persidsky, Y. Mechanism of Alcohol-Induced Oxidative Stress and Neuronal Injury. *Free Radic. Biol. Med.* **2008**, *45*, 1542–1550. [CrossRef]
48. Seitz, H.K.; Stickel, F. Molecular Mechanisms of Alcohol-Mediated Carcinogenesis. *Nat. Rev. Cancer* **2007**, *7*, 599–612. [CrossRef]
49. Ratna, A.; Mandrekar, P. Alcohol and Cancer: Mechanisms and Therapies. *Biomolecules* **2017**, *7*, 61. [CrossRef]
50. Rossi, M.; Anwar, M.J.; Usman, A.; Keshavarzian, A.; Bishehsari, F. Colorectal Cancer and Alcohol Consumption—Populations to Molecules. *Cancers* **2018**, *10*, 38. [CrossRef] [PubMed]
51. Wang, M.; McIntee, E.J.; Cheng, G.; Shi, Y.; Villalta, P.W.; Hecht, S.S. Identification of DNA Adducts of Acetaldehyde. *Chem. Res. Toxicol.* **2000**, *13*, 1149–1157. [CrossRef] [PubMed]

52. Garaycoechea, J.I.; Crossan, G.P.; Langevin, F.; Mulderrig, L.; Louzada, S.; Yang, F.; Guilbaud, G.; Park, N.; Roerink, S.; Nik-Zainal, S.; et al. Alcohol and Endogenous Aldehydes Damage Chromosomes and Mutate Stem Cells. *Nature* **2018**, *553*, 171–177. [CrossRef] [PubMed]
53. Brooks, P.J.; Theruvathu, J.A. DNA Adducts from Acetaldehyde: Implications for Alcohol-Related Carcinogenesis. *Alcohol* **2005**, *35*, 187–193. [CrossRef]
54. Varela-Rey, M.; Woodhoo, A.; Martinez-Chantar, M.L.; Mato, J.M.; Lu, S.C. Alcohol, DNA Methylation, and Cancer. *Alcohol Res. Curr. Rev.* **2012**, *35*, 25–35.
55. Chang, J.S.; Hsiao, J.R.; Chen, C.H. ALDH2 Polymorphism and Alcohol-Related Cancers in Asians: A Public Health Perspective Tse-Hua Tan. *J. Biomed. Sci.* **2017**, *24*, 19. [CrossRef] [PubMed]
56. Chen, Q.; Ross, A.C. Retinoic Acid Regulates Cell Cycle Progression and Cell Differentiation in Human Monocytic THP-1 Cells. *Exp. Cell Res.* **2004**, *297*, 68–81. [CrossRef]
57. Connolly, R.M.; Nguyen, N.K.; Sukumar, S. Molecular Pathways: Current Role and Future Directions of the Retinoic Acid Pathway in Cancer Prevention and Treatment. *Clin. Cancer Res.* **2013**, *19*, 1651–1959. [CrossRef]
58. Clugston, R.D.; Blaner, W.S. The Adverse Effects of Alcohol on Vitamin A Metabolism. *Nutrients* **2012**, *4*, 356–371. [CrossRef] [PubMed]
59. Jiang, Y.; Zhang, T.; Kusumanchi, P.; Han, S.; Yang, Z.; Liangpunsakul, S. Alcohol Metabolizing Enzymes, Microsomal Ethanol Oxidizing System, Cytochrome P450 2E1, Catalase, and Aldehyde Dehydrogenase in Alcohol-Associated Liver Disease. *Biomedicines* **2020**, *8*, 50. [CrossRef] [PubMed]
60. Ferdouse, A.; Agrawal, R.R.; Gao, M.A.; Jiang, H.; Blaner, W.S.; Clugston, R.D. Alcohol Induced Hepatic Retinoid Depletion Is Associated with the Induction of Multiple Retinoid Catabolizing Cytochrome P450 Enzymes. *PLoS ONE* **2022**, *17*, e0261675. [CrossRef]
61. Hada, M.; Mondul, A.M.; Weinstein, S.J.; Albanes, D. Serum Retinol and Risk of Overall and Site-Specific Cancer in the ATBC Study. *Am. J. Epidemiol.* **2020**, *189*, 532–542. [CrossRef] [PubMed]
62. Singh, N.; Baby, D.; Rajguru, J.; Patil, P.; Thakkannavar, S.; Pujari, V. Inflammation and Cancer. *Ann. Afr. Med.* **2019**, *18*, 121–126. [CrossRef]
63. Zhao, H.; Wu, L.; Yan, G.; Chen, Y.; Zhou, M.; Wu, Y.; Li, Y. Inflammation and Tumor Progression: Signaling Pathways and Targeted Intervention. *Signal Transduct. Target. Ther.* **2021**, *6*, 263. [CrossRef] [PubMed]
64. Checa, J.; Aran, J.M. Reactive Oxygen Species: Drivers of Physiological and Pathological Processes. *J. Inflamm. Res.* **2020**, *13*, 1057–1073. [CrossRef] [PubMed]
65. Guo, Q.; Jin, Y.; Chen, X.; Ye, X.; Shen, X.; Lin, M.; Zeng, C.; Zhou, T.; Zhang, J. NF-KB in Biology and Targeted Therapy: New Insights and Translational Implications. *Signal Transduct. Target. Ther.* **2024**, *9*, 53. [CrossRef]
66. Feng, D.; Hwang, S.; Guillot, A.; Wang, Y.; Guan, Y.; Chen, C.; Maccioni, L.; Gao, B. Inflammation in Alcohol-Associated Hepatitis: Pathogenesis and Therapeutic Targets. *Cell. Mol. Gastroenterol. Hepatol.* **2024**, *18*, 101352. [CrossRef]
67. Sancho, M.; Leiva, D.; Lucendo, E.; Orzáez, M. Understanding MCL1: From Cellular Function and Regulation to Pharmacological Inhibition. *FEBS J.* **2022**, *289*, 6209–6234. [CrossRef] [PubMed]
68. Kruman, I.I.; Fowler, A.K. Impaired One Carbon Metabolism and DNA Methylation in Alcohol Toxicity. *J. Neurochem.* **2014**, *129*, 770–780. [CrossRef] [PubMed]
69. Moore, L.D.; Le, T.; Fan, G. DNA Methylation and Its Basic Function. *Neuropsychopharmacology* **2013**, *38*, 23–38. [CrossRef] [PubMed]
70. Shen, H.; Wang, L.; Spitz, M.R.; Hong, W.K.; Mao, L.; Wei, Q. A Novel Polymorphism in Human Cytosine DNA-Methyltransferase-3B Promoter Is Associated with an Increased Risk of Lung Cancer. *Cancer Res.* **2002**, *62*, 4992–4995. [PubMed]
71. Cheng, T.Y.D.; Makar, K.W.; Neuhouser, M.L.; Miller, J.W.; Song, X.; Brown, E.C.; Beresford, S.A.A.; Zheng, Y.; Poole, E.M.; Galbraith, R.L.; et al. Folate-Mediated One-Carbon Metabolism Genes and Interactions with Nutritional Factors on Colorectal Cancer Risk: Women's Health Initiative Observational Study. *Cancer* **2015**, *121*, 3684–3691. [CrossRef]
72. Ciafrè, S.; Carito, V.; Ferraguti, G.; Greco, A.; Chaldakov, G.N.; Fiore, M.; Ceccanti, M. How Alcohol Drinking Affects Our Genes: An Epigenetic Point of View. *Biochem. Cell Biol.* **2019**, *97*, 345–356. [CrossRef] [PubMed]
73. Zakhari, S. Alcohol Metabolism and Epigenetics Changes. *Alcohol Res. Curr. Rev.* **2012**, *35*, 6–16.
74. Zakhari, S.; Hoek, J.B. Epidemiology of Moderate Alcohol Consumption and Breast Cancer: Association or Causation? *Cancers* **2018**, *10*, 349. [CrossRef] [PubMed]
75. Jordahl, K.M.; Malone, K.E.; Baglia, M.L.; Flanagan, M.R.; Tang, M.T.C.; Porter, P.L.; Li, C.I. Alcohol Consumption, Smoking, and Invasive Breast Cancer Risk after Ductal Carcinoma in Situ. *Breast Cancer Res. Treat.* **2022**, *193*, 477–484. [CrossRef] [PubMed]
76. Williams, L.A.; Olshan, A.F.; Tse, C.K.; Bell, M.E.; Troester, M.A. Alcohol Intake and Invasive Breast Cancer Risk by Molecular Subtype and Race in the Carolina Breast Cancer Study. *Cancer Causes Control* **2016**, *27*, 259–269. [CrossRef]
77. Wang, Y.; Xu, M.; Ke, Z.J.; Luo, J. Cellular and Molecular Mechanisms Underlying Alcohol-Induced Aggressiveness of Breast Cancer. *Pharmacol. Res.* **2017**, *115*, 299–308. [CrossRef] [PubMed]
78. Corti, C.; Binboğa Kurt, B.; Koca, B.; Rahman, T.; Conforti, F.; Pala, L.; Bianchini, G.; Criscitiello, C.; Curigliano, G.; Garrido-Castro, A.C.; et al. Estrogen Signaling in Early-Stage Breast Cancer: Impact on Neoadjuvant Chemotherapy and Immunotherapy. *Cancer Treat. Rev.* **2025**, *132*, 102852. [CrossRef]

79. Baweja, S.; Mittal, A.; Thangariyal, S.; Subudhi, P.D.; Gautam, S.; Kaul, R. Unveiling the Effect of Estrogen Receptors in Alcoholic Liver Disease: A Novel Outlook. *Liver Res.* **2023**, *7*, 333–341. [CrossRef]
80. Łukasiewicz, S.; Czeczelewski, M.; Forma, A.; Baj, J.; Sitarz, R.; Stanisławek, A. Breast Cancer—Epidemiology, Risk Factors, Classification, Prognostic Markers, and Current Treatment Strategies—An Updated Review. *Cancers* **2021**, *13*, 4287. [CrossRef]
81. Huang, C.; Zhang, Y.; Zhong, S.; Goncaves, R.V. Alcohol Intake and Abnormal Expression of Brf1 in Breast Cancer. *Oxid. Med. Cell. Longev.* **2019**, *2019*, 4818106. [CrossRef] [PubMed]
82. Clusan, L.; Ferrière, F.; Flouriot, G.; Pakdel, F. A Basic Review on Estrogen Receptor Signaling Pathways in Breast Cancer. *Int. J. Mol. Sci.* **2023**, *24*, 6834. [CrossRef] [PubMed]
83. Jenzer, H.; Sadeghi-Reeves, L. Nutrigenomics-Associated Impacts of Nutrients on Genes and Enzymes with Special Consideration of Aromatase. *Front. Nutr.* **2020**, *7*, 37. [CrossRef]
84. Finn, D.A. The Endocrine System and Alcohol Drinking in Females. *Alcohol Res. Curr. Rev.* **2020**, *40*, 2. [CrossRef] [PubMed]
85. Folkerd, E.; Dowsett, M. Sex Hormones and Breast Cancer Risk and Prognosis. *Breast* **2013**, *22*, S38–S43. [CrossRef]
86. Playdon, M.C.; Coburn, S.B.; Moore, S.C.; Brinton, L.A.; Wentzensen, N.; Anderson, G.; Wallace, R.; Falk, R.T.; Pfeiffer, R.; Xu, X.; et al. Alcohol and Oestrogen Metabolites in Postmenopausal Women in the Women's Health Initiative Observational Study. *Br. J. Cancer* **2018**, *118*, 448–457. [CrossRef]
87. Germain, D. Estrogen Carcinogenesis in Breast Cancer. *Endocrinol. Metab. Clin. N. Am.* **2011**, *40*, 473–484. [CrossRef]
88. Ginsburg, E.S. Estrogen, Alcohol and Breast Cancer Risk. *J. Steroid Biochem. Mol. Biol.* **1999**, *69*, 299–306. [CrossRef] [PubMed]
89. Kabat, G.C.; Kim, M.; Phipps, A.I.; Li, C.I.; Messina, C.R.; Wactawski-Wende, J.; Kuller, L.; Simon, M.S.; Yasmeen, S.; Wassertheil-Smoller, S.; et al. Smoking and Alcohol Consumption in Relation to Risk of Triple-Negative Breast Cancer in a Cohort of Postmenopausal Women. *Cancer Causes Control* **2011**, *22*, 775–783. [CrossRef]
90. Hirko, K.A.; Chen, W.Y.; Willett, W.C.; Rosner, B.A.; Hankinson, S.E.; Beck, A.H.; Tamimi, R.M.; Eliassen, A.H. Alcohol Consumption and Risk of Breast Cancer by Molecular Subtype: Prospective Analysis of the Nurses' Health Study after 26 Years of Follow-Up. *Int. J. Cancer* **2016**, *138*, 1094–1101. [CrossRef]
91. Furrer, D.; Jacob, S.; Michaud, A.; Provencher, L.; Lemieux, J.; Diorio, C. Association of Tobacco Use, Alcohol Consumption and HER2 Polymorphisms With Response to Trastuzumab in HER2-Positive Breast Cancer Patients. *Clin. Breast Cancer* **2018**, *18*, e687–e694. [CrossRef] [PubMed]
92. Mizumoto, A.; Ohashi, S.; Hirohashi, K.; Amanuma, Y.; Matsuda, T.; Muto, M. Molecular Mechanisms of Acetaldehyde-Mediated Carcinogenesis in Squamous Epithelium. *Int. J. Mol. Sci.* **2017**, *18*, 1943. [CrossRef]
93. Sun, J.K.L.; Wu, D.; Wong, G.C.N.; Lau, T.M.; Yang, M.; Hart, R.P.; Kwan, K.M.; Chan, H.Y.E.; Chow, H.M. Chronic Alcohol Metabolism Results in DNA Repair Infidelity and Cell Cycle-Induced Senescence in Neurons. *Aging Cell* **2023**, *22*, e13772. [CrossRef] [PubMed]
94. Zheng, Q.; Wang, H.; Yan, A.; Yin, F.; Qiao, X. DNA Methylation in Alcohol Use Disorder. *Int. J. Mol. Sci.* **2023**, *24*, 10130. [CrossRef] [PubMed]
95. Xia, J.; Li, S.; Liu, S.; Zhang, L. Aldehyde Dehydrogenase in Solid Tumors and Other Diseases: Potential Biomarkers and Therapeutic Targets. *MedComm* **2023**, *4*, e195. [CrossRef] [PubMed]
96. Starek-Świechowicz, B.; Budziszewska, B.; Starek, A. Alcohol and Breast Cancer. *Pharmacol. Rep.* **2023**, *75*, 69–84. [CrossRef]
97. Prokopieva, V.D.; Vetlugina, T.P. Features of Oxidative Stress in Alcoholism. *Biomeditsinskaya Khimiya* **2023**, *69*, 83–96. [CrossRef]
98. Contreras-Zentella, M.L.; Villalobos-García, D.; Hernández-Muñoz, R. Ethanol Metabolism in the Liver, the Induction of Oxidant Stress, and the Antioxidant Defense System Ethanol Metabolism in the Liver, the Induction of Oxidant Stress, and the Antioxidant Defense System. *Antioxidants* **2022**, *11*, 1258. [CrossRef]
99. Nakamura, H.; Takada, K. Reactive Oxygen Species in Cancer: Current Findings and Future Directions. *Cancer Sci.* **2021**, *112*, 3945–3952. [CrossRef]
100. Hoek, J.B.; Cahill, A.; Pastorino, J.G. Alcohol and Mitochondria: A Dysfunctional Relationship. *Gastroenterology* **2002**, *122*, 2049–2063. [CrossRef]
101. Siggins, R.W.; McTernan, P.M.; Simon, L.; Souza-Smith, F.M.; Molina, P.E. Mitochondrial Dysfunction: At the Nexus between Alcohol-Associated Immunometabolic Dysregulation and Tissue Injury. *Int. J. Mol. Sci.* **2023**, *24*, 8650. [CrossRef]
102. Nagini, S.; Thiyagarajan, P.; Rao, K.S. Interplay Between Reactive Oxygen Species and Key Players in the DNA Damage Response Signaling Network. In *Handbook of Oxidative Stress in Cancer: Mechanistic Aspects*; Chakraborti, S., Ray, B.K., Roychoudhury, S., Eds.; Springer Nature: Singapore, 2022; Volume 2, pp. 1005–1022. [CrossRef]
103. Zhang, S.; Xiao, X.; Yi, Y.; Wang, X.; Zhu, L.; Shen, Y.; Lin, D.; Wu, C. Tumor Initiation and Early Tumorigenesis: Molecular Mechanisms and Interventional Targets. *Signal Transduct. Target. Ther.* **2024**, *9*, 149. [CrossRef]
104. Wilson, L.E.; Xu, Z.; Harlid, S.; White, A.J.; Troester, M.A.; Sandler, D.P.; Taylor, J.A. Alcohol and DNA Methylation: An Epigenome-Wide Association Study in Blood and Normal Breast Tissue. *Am. J. Epidemiol.* **2019**, *188*, 1055–1065. [CrossRef] [PubMed]
105. Liu, J.; Huang, B.; Ding, F.; Li, Y. Environment Factors, DNA Methylation, and Cancer. *Environ. Geochem. Health* **2023**, *45*, 7543–7568. [CrossRef] [PubMed]
106. Aref-Eshghi, E.; McGee, J.D.; Pedro, V.P.; Kerkhof, J.; Stuart, A.; Ainsworth, P.J.; Lin, H.; Volodarsky, M.; McLachlin, C.M.; Sadikovic, B. Genetic and Epigenetic Profiling of BRCA1/2 in Ovarian Tumors Reveals Additive Diagnostic Yield and Evidence of a Genomic BRCA1/2 DNA Methylation Signature. *J. Hum. Genet.* **2020**, *65*, 865–873. [CrossRef]

107. Terracina, S.; Tarani, L.; Ceccanti, M.; Vitali, M.; Francati, S.; Lucarelli, M.; Venditti, S.; Verdone, L.; Ferraguti, G.; Fiore, M. The Impact of Oxidative Stress on the Epigenetics of Fetal Alcohol Spectrum Disorders. *Antioxidants* **2024**, *13*, 410. [CrossRef]
108. Yang, Y.; Yang, Y.; Yang, J.; Zhao, X.; Wei, X.; Suarez, C.A. Tumor Microenvironment in Ovarian Cancer: Function and Therapeutic Strategy. *Front. Cell Dev. Biol.* **2020**, *8*, 758. [CrossRef] [PubMed]
109. Mungenast, F.; Thalhammer, T. Estrogen Biosynthesis and Action in Ovarian Cancer. *Front. Endocrinol.* **2014**, *5*, 192. [CrossRef] [PubMed]
110. Kozieł, M.J.; Piastowska-Ciesielska, A.W. Estrogens, Estrogen Receptors and Tumor Microenvironment in Ovarian Cancer. *Int. J. Mol. Sci.* **2023**, *24*, 14673. [CrossRef]
111. Rachdaoui, N.; Sarkar, D.K. Pathophysiology of the Effects of Alcohol Abuse on the Endocrine System. *Alcohol Res. Curr. Rev.* **2017**, *38*, 255.
112. Zhang, Y.; Cao, L.; Nguyen, D.; Lu, H. TP53 Mutations in Epithelial Ovarian Cancer. *Transl. Cancer Res.* **2016**, *5*, 650–663. [CrossRef] [PubMed]
113. Tomasova, K.; Cumova, A.; Seborova, K.; Horak, J.; Koucka, K.; Vodickova, L.; Vaclavikova, R.; Vodicka, P. DNA Repair and Ovarian Carcinogenesis: Impact on Risk, Prognosis and Therapy Outcome. *Cancers* **2020**, *12*, 1713. [CrossRef]
114. Hou, D.; Liu, Z.; Xu, X.; Liu, Q.; Zhang, X.; Kong, B.; Wei, J.J.; Gong, Y.; Shao, C. Increased Oxidative Stress Mediates the Antitumor Effect of PARP Inhibition in Ovarian Cancer. *Redox Biol.* **2018**, *17*, 99–111. [CrossRef] [PubMed]
115. Grimley, E.; Cole, A.J.; Luong, T.T.; McGonigal, S.C.; Sinno, S.; Yang, D.; Bernstein, K.A.; Buckanovich, R.J. Aldehyde Dehydrogenase Inhibitors Promote DNA Damage in Ovarian Cancer and Synergize with ATM/ATR Inhibitors. *Theranostics* **2021**, *11*, 3540–3551. [CrossRef]
116. Liang, J.; Gao, Y.; Feng, Z.; Zhang, B.; Na, Z.; Li, D. Reactive Oxygen Species and Ovarian Diseases: Antioxidant Strategies. *Redox Biol.* **2023**, *62*, 102659. [CrossRef] [PubMed]
117. Jelic, M.D.; Mandic, A.D.; Maricic, S.M.; Srdjenovic, B.U. Oxidative Stress and Its Role in Cancer. *J. Cancer Res. Ther.* **2021**, *17*, 22–28. [CrossRef]
118. Zhao, Y.; Ye, X.; Xiong, Z.; Ihsan, A.; Ares, I.; Martínez, M.; Lopez-Torres, B.; Martínez-Larrañaga, M.R.; Anadón, A.; Wang, X.; et al. Cancer Metabolism: The Role of ROS in DNA Damage and Induction of Apoptosis in Cancer Cells. *Metabolites* **2023**, *13*, 796. [CrossRef]
119. Xu, L.; Wu, T.; Lu, S.; Hao, X.; Qin, J.; Wang, J.; Zhang, X.; Liu, Q.; Kong, B.; Gong, Y.; et al. Mitochondrial Superoxide Contributes to Oxidative Stress Exacerbated by DNA Damage Response in RAD51-Depleted Ovarian Cancer Cells. *Redox Biol.* **2020**, *36*, 101604. [CrossRef] [PubMed]

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Review

Photodynamic Therapy in Cancer: Insights into Cellular and Molecular Pathways

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Abstract: Photodynamic therapy is a non-ionizing radiation treatment that utilizes a photosensitizer in combination with light to produce singlet oxygen. This singlet oxygen induces anti-cancer effects by causing apoptotic, necrotic, or autophagic cell death in tumor cells. Currently, photodynamic therapy is employed in oncology to treat various cancers. In the presence of oxygen, this non-invasive approach leads to direct tumor cell death, damage to microvasculature, and the induction of a local inflammatory response. These effects allow photodynamic therapy to be effective in treating early-stage tumors, extending survival in cases where surgery is not feasible, and significantly improving quality of life. In this paper, we provide a state of the art on cytomolecular mechanisms and associated pathways involved in photodynamic therapy. By integrating these mechanistic insights with the most recent advancements in nanotechnology, this phototherapeutic approach has the potential to become a prevalent treatment option within conventional cancer therapies, enhancing its application in precision medicine.

Keywords: photodynamic therapy; cancer; cytomolecular mechanisms; inflammation; oxidative stress; stress response molecules; cytokines; nanomedicine; immunotherapy

1. Introduction

Photodynamic therapy (PDT) is a promising non-invasive phototherapeutic approach widely studied in oncology, with applications extending to other clinical settings. Its origins date back to 1900 [1]. The procedure involves administering a photosensitizing pro-drug, which, when photoactivated by light of a specific wavelength (ideally between 650 and 850 nm) in the presence of oxygen, triggers the formation of reactive oxygen species (ROS). These ROS are highly cytotoxic to cells where the photosensitizer (PS) accumulates [2].

Biochemically, PDT operates through two types of reactions (Type 1 and Type 2) initiated when the PS absorbs a photon and is electronically excited to a singlet state. The excited PS transitions to a triplet state via intersystem crossing. In a Type 1 reaction, an electron or hydrogen is transferred from the triplet state of the PS to nearby biomolecules or directly to oxygen, resulting in the generation of radical species such as hydrogen peroxide, hydroperoxyl, nitric oxide, superoxide ions, and hydroxyl radicals. Alternatively, in a Type 2 reaction, there is direct energy transfer from the excited PS to molecular oxygen, leading to the production of singlet oxygen. Such direct transfer occurs when PS and molecular

oxygen have aligned electronic spins. The latter is a property of molecules that determines their quantum state [2,3]. Singlet oxygen is highly reactive towards biomolecules, but its intracellular lifetime does not exceed 15–30 μs, and its propagation distance is up to 0.5–1 μm. On the other hand, among the various ROS produced, hydrogen peroxide, a precursor of the hydroxyl radical, has a longer intracellular lifetime (up to 1 ms) and, in addition to inducing the oxidation of membrane lipids and proteins, plays a regulatory role by interacting with proteins involved in various cellular signaling pathways, thus triggering either cytoprotective mechanisms or cell death processes [4].

In this context of redox reactions, most of the reactive species formed interconvert with each other; furthermore, both radicals and singlet oxygen can attack PS, leading to its degradation [5]. An important consideration is the influence of the intracellular localization of PS in determining therapeutic efficacy and the type of cytomolecular events elicited by PDT [6]. More specifically, photodamage to the endoplasmic reticulum induces a massive intracellular calcium release, which plays a major role in triggering necroptosis. Additionally, photodamage to other organelles such as mitochondria and lysosomes is equally crucial in inducing various cell death mechanisms. Beyond the well-known induction of the apoptotic process (via cytoplasmic release of cytochrome c), the mitochondrial localization of PS also induces necrosis and autophagy depending on the PDT dose (which is defined by PS concentration and light intensity). Specifically, high PDT doses induce necrosis, mild PDT doses induce apoptosis, and low PDT doses induce autophagy as a cytoprotective mechanism for mitochondrial repair ("mitophagy"), which, if unsuccessful, leads to autophagic cell death. Similarly, lysosomal photodamage depends on the PDT dose; high PDT doses cause complete organelle destruction with massive release of lysosomal enzymes into the cytoplasm and induction of necrosis. On the other hand, partial lysosomal damage results in the release of hydrolases, which induce apoptosis and autophagic cell death [5].

PDT offers several advantages, including its curative potential, especially in early-stage tumors, and its ability to extend survival in cases of inoperable cancers, thereby improving quality of life.

Additional factors that support the use of this therapy include its low tissue toxicity, minimal systemic reactions, significant reduction in long-term morbidity, and excellent cosmetic outcomes, as well as the preservation of organ function [7].

The biological behavior of cancer is characterized by mechanisms that ensure its survival, primarily through two crucial processes: tumor neoangiogenesis and evasion of immune surveillance. Tumors achieve this evasion by presenting or secreting molecules that inhibit the immune response [8]. PDT offers an advantage over conventional therapies by targeting these two distinct biological processes. Specifically, PDT's ability to induce cell death through direct damage is complemented by its targeting of neoangiogenesis. The accumulation of PSs within the endothelial cells leads to indirect cytotoxic effects that destroy the tumor vasculature, causing hypoxia, nutrient deprivation, and accumulation of toxic metabolites, which collectively trigger cell death. Additionally, PDT-induced direct cytotoxicity can stimulate the release of inflammatory cytokines and alarmins into the bloodstream, thereby eliciting an innate immune response that is further enhanced by the activation of a sustained adaptive immune response [8–10]. Among the major PSs being commercially approved for clinical use are m-tetrahydroxyphenylchlorin (mTHPC), porfimer sodium (Photofrin), hemoporfin, 5-aminolevulinic acid (5-ALA), methylamine levulinate hydrochloride (MAL), verteporfin, talaporfin, hexylamine levulinate hydrochloride (HAL), chlorin e6 (Ce6), foscan (Temoporfin), 3-Devinyl-3-(1′-hexyloxyethyl) pyrylporphyrin-a (HPPH), and pheophorbide a [3,11].

Understanding the molecular and therapeutic mechanisms of PDT suggests that combining PDT with other therapeutic strategies could enhance the overall therapeutic index of treatments in modern oncology.

Based on this premise, we conducted a narrative review of the current state of the art regarding the cytomolecular mechanisms modulated by PDT in various neoplastic pathologies. Moving into basic research, this paper highlights how understanding these mechanisms, particularly when supported by the new frontiers of combination therapy in nanomedicine, is steering medicine towards a more precision-focused approach.

Search Strategy: we performed a comprehensive search in PubMed electronic database for relevant cytomolecular studies, without restrictions on study type.

Inclusion Criteria: only English-language papers from the past decade that elucidate pathways directly influenced by PDT were included.

Exclusion Criteria: papers addressing comparative efficacy studies, as well as cytomolecular mechanisms resulting from the combination of PDT with other anti-cancer therapeutic strategies, were excluded.

2. Breast Cancer

Breast cancer is the most prevalent form of cancer and the leading cause of cancer-related mortality worldwide. It predominantly affects women, with its incidence increasing with age. Modifiable risk factors include obesity, alcohol consumption, physical inactivity, and hormone therapy, while non-modifiable factors encompass age, genetic predisposition, and endogenous hormonal exposure. Protective factors include breastfeeding, physical activity, and the use of aspirin and nonsteroidal anti-inflammatory drugs. Tumorigenesis is rooted in the dysregulation of pathways controlling apoptosis and cellular proliferation [12].

In recent decades, PDT has emerged as a promising novel approach for the treatment of this malignancy, proving to be effective, minimally invasive, and associated with negligible adverse reactions. In clinical settings, its application has been proposed for managing breast cancers refractory to standard treatments, with the added potential of restoring cancer cell sensitivity to conventional therapies [13]. The mechanisms triggered by PDT include tumor cell killing via direct phototoxic damage, leading to cell death through apoptosis, necrosis, or autophagy. Additionally, PDT induces vascular damage within the tumor and stimulates host immunity, resulting in an antitumor inflammatory response [13]. More specifically, the type of cell death induced by PDT depends on the intracellular localization of the PS. Mitochondrial or cytoplasmic localization of PS triggers apoptosis, activating the intrinsic mitochondrial pathway through cytochrome c (cyt-c) release or damaging the nuclear factor kappa B (NFκB) pathway when localized in the cytoplasm. Endoplasmic reticulum (ER) localization induces autophagy via the activation of Beclin-1 and the mechanistic target of rapamycin (mTOR) protein. Necrosis, on the other hand, occurs following the disruption of the cell membrane when PS localizes in this region. Indirect cytotoxicity results from PS uptake and activation in endothelial cells of the tumor vasculature, leading to vascular destruction. This deprives tumor cells of essential oxygen and nutrients while causing metabolic waste accumulation, culminating in cell death [9]. Following PDT-induced cytotoxicity (both direct and indirect), the release of cytokines and tumor necrosis factors (TNFs) triggers an antitumor inflammatory response and immunogenic cell death (ICD) of cancer cells. Interestingly, the activation of the innate immune response, followed by the adaptive immune response, leads to the development of memory T cells and so to a long-lasting antitumor immunity which can effectively control metastasis and prevent tumor recurrence after PDT [9]. Dos Santos et al. evaluated the efficacy of methylene blue-mediated PDT (MB-PDT) in three breast epithelial cell lines, both tumorigenic and non-tumorigenic, demonstrating its ability to selectively induce

massive tumor cell destruction. Moreover, they observed that apoptosis does not appear to be the predominant cell death pathway triggered by MB-PDT, not even through a caspase-independent mechanism. Interestingly, autophagy emerges as an initial pro-survival response to oxidative damage induced by MB-PDT in triple-negative breast cancer (TNBC) cells, but not in luminal A cells [14].

Cutting-edge is the emerging research on the PDT potential to modulate breast cancer epigenetics in an antitumor direction, specifically by suppressing the expression of oncogenic microRNAs (miRNAs) and upregulating tumor-suppressing ones. Notably, the PDT-induced suppression of oncogenic miR-21, miR-221, and miR-27a in female Wistar rats—miRNAs typically overexpressed in this malignancy—paves the way for further in vivo studies on the subject [15].

Moreover, an in-depth investigation into the effects of PDT on breast cancer epigenetics could elucidate novel mechanisms of tumor cell survival directly linked to PDT-induced oxidative damage and the consequent impaired biogenesis and maturation of tumor-suppressor miRNAs. This has been preliminarily observed for Hsa-miR-16 in a TNBC cell model treated with δ-aminolevulinic acid (ALA)-PDT [16]. On the other hand, the latest nanotechnological optimizations of ALA-PDT in breast cancer cells highlight the efficacy of this approach in inhibiting the phosphatidylinositol 3-kinase (Pi3K)/protein kinase B (AKT)/mTOR signaling pathway, a key cellular survival mechanism, thereby inducing mitochondrial apoptotic events [17].

Highlights:

- Apoptosis (primarily caspase-dependent), necrosis, autophagy and immunogenic cell death are the main PDT-induced cell death pathways in breast cancer.
- The presumed role of autophagy as an initial survival mechanism has been revealed.
- Type of cell death is strictly dependent on the intracellular PS localization.
- Potential epigenetic regulatory role of PDT through stimulation of oncosuppressive miRNAs and downregulation of tumorigenic ones would be emerging.
- PDT impacts Pi3K/AKT/mTOR signaling, a crucial survival pathway.

3. Skin Cancer

Skin cancer is the most frequently diagnosed form of cancer worldwide. The most prevalent types include keratinocyte carcinomas, such as squamous cell carcinoma (SCC) and basal cell carcinoma (BCC), as well as cutaneous melanoma. Since 1990, these cancers have shown a global increase in prevalence and disability-adjusted life years, despite ongoing prevention efforts, particularly those aimed at precise risk stratification [18,19].

Within a context of an even greater need for novel therapeutic strategies, the clinical application of PDT offers significant advantages, including precise targeting, minimal trauma, synergy with conventional treatments, and repeatability. Currently, PDT is an established alternative treatment for non-melanoma skin cancers (BCC and SCC), potentially applicable in neoadjuvant settings. Additionally, a growing body of literature supports its effectiveness in the treatment of superficial and localized forms of cutaneous melanoma [20,21].

The promising efficacy of PDT relies on the elicitation of three distinct phenomena: cytotoxicity, microvascular killing, and immune response activation. The cytotoxic effect stems from ROS production, leading to the induction of apoptosis, necrosis, and ferroptosis as cell death pathways. Among these, mitochondria-dependent apoptosis, characterized by cyt c release and subsequent downstream events, is considered the main mechanism of cell death. Necrosis, on the other hand, arises from the activation of receptor-interacting protein kinase 1 (RIPK1), lysosomal damage, and intracellular calcium overload [22].

Specifically, massive ROS production is thought to elicit necrosis, whereas lower oxidative stress (OS) primarily induces apoptosis as the main mechanism of cell death [22–24]. Molecularly, in SCC, the antiproliferative effect of topical ALA-PDT is primarily attributed to the inhibition of Signal transducer and activator of transcription 3 (STAT3) expression [25].

In parallel, PDT-induced ICD occurs through the release of damage-associated molecular patterns (DAMPs) or alarmins such as heat shock protein (HSP)-70, HSP-90, High-Mobility Group Box 1 (HMGB1), and Interleukin (IL)-1β. The microvascular killing effect is mediated by direct damage to endothelial cells, resulting in endothelial necrosis. Noteworthy is the PDT-induced antitumor immune response, manifested as an acute local inflammatory reaction that is sustained or amplified through the concomitant inhibition of anti-inflammatory cytokines, such as IL-10 and transforming growth factor (TGF)-β. Interestingly, in BCC patients treated with PDT, an increased immunoreactivity of circulating leukocytes against the BCC-related tumor antigen Hedgehog-interacting protein 1 (Hip-1) has been observed. Similarly, preliminary evidence suggests the immunogenic potential of ALA-PDT in SCC through the increased release of DAMPs [22,25]. Concerning cutaneous melanoma, its high resistance to conventional therapies makes PDT a promising treatment strategy. Valli et al. investigated the effectiveness of cationic zinc (II) phthalocyanine Pc13 as a PS in various melanoma cell lines. Pc13 treatment activates several pathways, including Mitogen-activated protein kinase (MAPK) p38, Extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and the PI3K/AKT. Pc13 irradiation also triggers an autophagic response, evidenced by increased levels of Beclin-1, LC3-II, and generating green fluorescent protein (GFP)-LC3 punctate staining. Autophagy is promoted by JNK and negatively regulated by the PI3K/AKT pathway. Blocking autophagy enhances Pc13 phototoxicity, suggesting that this event protects against apoptotic cell death. Moreover, repeated cycles of Pc13 PDT in A375 cells led to reduced susceptibility to treatment and increased autophagy activation. Thus, modulating autophagy may be a potential strategy to enhance PDT efficacy in melanoma [26].

Highlights:

- Cytotoxicity, microvascular killing, and immune response activation are the basic therapeutic PDT induced mechanisms in skin cancer.
- Apoptosis, necrosis, ferroptosis, and ICD are the main PDT-induced cell death pathways in skin cancer.
- Apoptosis and necrosis would appear to be dependent on the grade of OS.
- In melanoma, PDT-induced autophagy would be recognized as having a presumed cytoprotective role.

4. Pancreatic Cancer

Pancreatic ductal adenocarcinoma (PDAC) is predicted to become the second leading cause of cancer-related deaths in the United States by 2030. This grim prognosis is primarily due to late-stage diagnosis, occurring in approximately 90% of cases, and as a result metastatic spread, which is observed in over 50% of patients. In this critical epidemiological context, there is an urgent need for early diagnostic strategies and effective therapies to target and eliminate metastatic PDAC cells [27].

In clinical settings, PDT shows promise for pancreatic cancer, particularly for palliative care in advanced stages or as an adjuvant therapy. It can be used preoperatively to downstage the tumor or to address micrometastatic lesions, aiding in the achievement of an R0 resection post-operatively [11]. Mechanistically, the efficacy of PDT in pancreatic cancer relies on its ability to induce necrosis and apoptosis of cancer cells, damage tumor vasculature, and stimulate antitumor immunity. Specifically, integrin-targeted PDT approaches

have been shown to cause S-phase cell cycle retardance and inhibit cell proliferation, ultimately leading to apoptosis induction. Moreover, maltotriose-chlorin has been identified as the first PS capable of inhibiting ascites formation by inducing irreversible apoptosis in peritoneal disseminated tumor cells. Additionally, verteporfin-mediated PDT is employed in this malignancy due to its ability to trigger apoptosis through the oxidation of key molecules involved in cell proliferation [11]. Beyond its pro-oxidant potential, verteporfin has recently been discovered to induce ferroptosis independently of light activation in pancreatic cancer cells [28]. The rationale for using verteporfin as a PS in this malignancy is also justified by its role as a small molecular inhibitor of the Hippo-Yes-associated protein-1 (YAP) pathway, which is critically involved in the proliferation of human PDAC cells. Mechanistically, this inhibition occurs by disrupting the interaction between YAP and TEAD, leading to suppressed expression of their target genes. Additionally, verteporfin exerts inhibitory effects on angiogenesis and vasculogenic mimicry by suppressing the expression of Angiopoietin-2 (Ang2), matrix metalloproteinase (MMP)-2, vascular endothelial (VE)-cadherin, and α-Smooth Muscle Actin (SMA), both in vitro and in vivo [29].

In addition to apoptosis, MB-PDT-induced necroptosis has recently emerged as a novel cell death mechanism in human PDAC cells. This process is associated with enhanced cytotoxicity, potentially opening new clinical applications for this approach in the treatment of microscopic residual disease and in minimizing the likelihood of local and metastatic recurrence [30].

Even more intriguingly, an emerging area of research focuses on PDT's ability to impact the fibrotic stroma of PDAC, which is notoriously a barrier to drug delivery and a critical hub for pro-tumorigenic signaling. Preliminary evidence suggests that PDT may play a role in depleting both cellular (fibroblasts, pancreatic stellate cells) and non-cellular (ECM breakdown) stromal components of PDAC. This disruption could interfere with the stromal signaling crosstalk, essential for tumor survival [31–33].

Groundbreaking and highly promising are also the initial in vivo studies on murine models of PDAC, which explore the ability of fibroblast activation protein (FAP)-targeted PDT to induce localized depletion of FAP-expressing stromal cells, thereby exerting an even more significant impact on the tumor-supporting stroma [34,35].

Concerning the stimulation of anti-cancer immunity recent studies have highlighted PDT's ability to induce pyroptosis, a highly inflammatory form of ICD, in pancreatic cancer. This process promotes M1 macrophage polarization, dendritic cell (DC) maturation, and cytotoxic T lymphocyte (CTL) activation as hallmarks of a systemic immune response. These effects, alongside regulatory T cell (Treg) suppression, facilitate the conversion of the tumor microenvironment from "cold" (immunosuppressive) to "hot" (immunogenic), thereby inhibiting both primary tumor growth and distant metastasis [36,37]. On the other hand, the pro-metastatic potential associated with low (sublethal) doses of PDT in peripheral pancreatic cancer cells cannot be overlooked. This effect is thought to occur through the upregulation of MMP-2/9 expression, as well as the enhanced motility and invasion of residual pancreatic cancer cells [38].

Highlights:

- Direct cytotoxicity, damage of tumor vasculature, and stimulation of antitumor immunity are the three mechanisms underlying the efficacy of PDT in pancreatic cancer.
- Apoptosis, necrosis, pyroptosis, and ferroptosis (independent of light activation) are the main PDT-induced cell death pathways in pancreatic cancer.
- Promising role of Verteporfin-PDT through its anti-angiogenic effects and inhibition of Hippo-YAP pathway involved in PDAC cell proliferation.
- The ability of FAP-targeted PDT to impact the dense tumor stroma represents an innovative and promising line of research.

5. Cholangiocarcinoma (CCA)

CCA is a rare neoplasm with an increasing incidence, characterized by insidious clinical progression and challenging diagnosis. Palliative treatments, including biliary drainage, chemotherapy, radiotherapy, targeted therapy, and immunotherapy, are the primary approaches for managing CCA. However, these treatments often offer limited improvements in quality of life and survival [39,40]. Preliminary clinical studies suggest that PDT may be beneficial for palliative care in CCA. Additional clinical advantages include a reduction in biliary ductal stenosis in unresectable cases, as well as improved quality of life and extended survival without significant additional adverse events. These findings were supported by a recent meta-analysis focused on hilar CCA [41].

The clinical application potential of PDT is highly promising, particularly in gemcitabine-resistant CCA. At the molecular level, recent studies have highlighted the effect of PDT on the Krueppel-like factor 10 (KLF10)/epidermal growth factor receptor (EGFR) axis. Specifically, PDT promotes KLF10 expression, which binds to the EGFR promoter region, thereby inhibiting EGFR transcription. This results in suppressed cell viability, G1 cell cycle arrest, and the induction of cellular apoptosis [42].

The induction of apoptosis also involves epigenetic mechanisms, with a critical role played by miR-34a-5p. In CCA cells, PDT-induced p53 upregulation has been shown to mediate the transactivation of miR-34a-5p, which subsequently downregulates Wnt Family Member 7B (WNT7B) expression. This, in turn, inhibits Wnt signaling, a pathway associated with cell proliferation [43].

Even more interestingly, Yan et al., in a recent in vivo and in vitro study, demonstrated the ability of HiPorfin-mediated PDT to trigger multiple mechanisms of cell death in CCA cell lines. In addition to the well-known apoptosis, ferroptosis has emerged as a complementary cell death pathway, which appears to be associated with enhanced therapeutic efficacy. This was in vivo evidenced by a reduction in tumor tissue and malignancy grade. The underlying molecular mechanisms involve the activation of the P53/Solute Carrier Family 7 Member 11 (SLC7A11)/Glutathione (GSH) peroxidase 4 (GPX4) ferroptosis signaling pathway through the inhibition of GSH synthesis-related pathways [44]. Intriguingly, in CCA cells, Chlorin A-PDT has also been shown to induce autophagy as an additional cell death mechanism essential for triggering apoptosis. At the molecular level, the activation of the ER stress-related Protein Kinase RNA-Like ER Kinase (PERK)/Phosphorylation of the eukaryotic initiation factor 2 (p-EIF)2α/CCAAT-enhancer-binding protein homologous protein (CHOP) pathway plays a crucial role in initiating these processes [45]. In addition to its previously discussed antitumor effects, the PDT-induced extensive activation of survival pathways in CCA cells should not be overlooked, especially in sublethally afflicted tumor cells. These include activator protein 1 (AP-1), NF-κB, hypoxia-inducible factor 1-alpha (HIF-1α), nuclear factor erythroid 2-related factor 2 (NFE2L2), and pathways mediated by the unfolded protein response (UPR), responsible for a suboptimal therapeutic response. Indeed, all these pathways are essentially involved in the transcriptional regulation of pro-tumoral cellular events, including proliferation, energy metabolism, detoxification, inflammation/angiogenesis, and metastasis [46,47].

Highlights:

- Apoptosis, ferroptosis, and autophagy are the main PDT-induced cell death pathways in CCA.
- In gemcitabine-resistant CCA, apoptosis is mainly related to the PDT-induced KLF10/EGFR axis.
- In CCA cells, apoptosis is also epigenetically PDT-induced via Wnt signaling inhibition.

6. Hepatocellular Carcinoma (HCC)

HCC is a significant global health issue, especially in the context of the obesity epidemic, which has made non-alcoholic fatty liver disease a major risk factor. According to the latest epidemiological data, HCC represents the sixth most common malignancy and the fourth leading cause of cancer-associated mortality in the world. However, prognosis has been improving over the years, thanks to the development of more effective systemic and locoregional therapies [48]. In this scenario, there is justified optimism regarding the therapeutic prospects offered by PDT, especially considering its well-known immunoregulatory role, which suggests its potential use in the adjuvant setting. Specifically, the intra-operative administration of PS and the fluorescence generated from their excitation enhance visual detection, allowing the surgeon to identify subclinical nodules and guide optimal liver resection. Following this, PDT can target any residual lesions and activate an antitumor immune response [49]. Given this context, the growing interest in understanding the underlying mechanisms behind this promising new therapeutic frontier is well-justified. Assuming that the escape of cancer cells from apoptosis is a major driver of their uncontrolled proliferation, the antiproliferative effects of PDT, which can trigger pro-apoptotic pathways, has been widely investigated. Radachlorin-based PDT has been shown to primarily induce apoptosis in human liver cancer HepG2 cells [50]. In the same cell line, ALA-based PDT has also been demonstrated to induce deoxyribonucleic acid (DNA) damage, leading to cell death via apoptosis and/or necrosis [51]. Further evidence supports that necrosis is a significant PDT-induced cell death mechanism alongside apoptosis [52].

Molecularly, the upstream target of this PDT tumor-killing activity appears to be the suppression of the proliferative MAPK-signaling pathway [53].

Moreover, a recent notion is that the diverse molecular and infectious characteristics of HCC tumor cells influence their PDT-induced pro-apoptotic response. In studies using 5-ALA-based PDT on HCC cell lines Huh-7 and SNU-449, an antiproliferative effect and subsequent induction of apoptosis were observed, with SNU-449 cells showing significantly higher sensitivity compared to Huh-7 cells. This differential response appears to depend on the hepatitis B virus status and specific molecular characteristics of the cells, such as point mutation in the p53 gene in Huh-7 cells and a deletion of the cyclin dependent kinase (CDK) inhibitor 2A gene in SNU-449 cells [54].

Concerning the recently emerged anti-inflammatory potential, it is important to note that the inflammatory tumor microenvironment (TME) plays a significant role in various stages of HCC development, with NF-κB acting as a crucial proinflammatory modulator through its transcriptional activation. In this inflammatory setting, neutrophils are activated, migrate, and accumulate in the tumor tissue. Building on these pathophysiological insights, the use of neutrophil membrane-Hypocrellin B nanoparticle-mediated PDT represents an interesting development in HCC treatment. This approach has shown a significant reduction in expression levels of TNF-α and IL-6, indicating a systemic anti-inflammatory effect. Additionally, it induces mitochondrial ROS generation and depolarization in HepG2 cells, via inhibited junB expression and subsequent apoptosis [55].

Last but not least, it is well established that most anti-cancer therapies cause a reorganization of the cellular immune landscape within the TME, either promoting or inhibiting tumor growth. An ideal anti-cancer therapy should not only have destructive effects on the tumor but also enhance the immune system against the neoplasm itself. In this view, the immunostimulatory ability of PDT in HCC is significant and warrants further investigation. Recent in vivo studies have reviewed the effects of deuteporfin-mediated PDT on mice hepatomas, demonstrating its ability to induce maturation and activation of DCs, which in turn can significantly reduce tumor growth. This immunogenic potential is further supported

by evidence showing that pheophorbide (Pheo)-mediated PDT induces ICD by activating macrophage phagocytosis of cancer cells. Consequently, this leads to the processing and presentation of tumor-associated antigens, stimulating an antigen-specific T cell response. PDT also promotes the clonal expansion of human peripheral blood mononuclear cells, further supporting its role in enhancing immune responses against cancer [49,56].

Highlights:

- Apoptosis and necrosis may both be major mechanisms of PDT-induced cell death in HCC.
- PDT-induced ICD is part of a more extensive antitumor immunostimulatory role of PDT in HCC.
- PDT-modulated cell death-related pathways: apoptosis—MAPK-signaling pathway;

ICD—macrophage phagocytosis/processing and exposure of tumor-associated antigens/antigen-specific T cell response.

7. Gastric Cancer (GC)

GC is the fifth most common cancer and the third leading cause of cancer-related death worldwide. Due to its phenotypic and molecular variability, it is considered a highly heterogeneous disease [57]. Recent epidemiological revisions indicate that this heterogeneity extends to its geographical distribution. East Asia has the highest incidence rates, followed by Eastern and Central Europe [58]. In the therapeutic setting, PDT is emerging as a promising "light knife" approach for treating this neoplasm at various clinical stages, thanks to its non-invasive nature and minimal side effects. Encouraging clinical results have been observed with the use of PDT in treating early gastric cancer, as well as in its application as an adjunct treatment during endoscopy. In cases of inoperable advanced gastric cancer, particularly when associated with peritoneal metastases, as well as cardiac and pyloric obstructions, PDT may prove valuable for palliative care, thereby improving the patient's quality of life [59]. Current research trends focus on elucidating the cell death pathways engaged and the role of PDT in stimulating antitumor immunity.

Specifically, in various GC cell lines, several PSs-mediated PDT approaches have been observed to induce a dose-and energy-dependent antiproliferative effect. This is achieved through cell cycle arrest in the S-phase and subsequent induction of apoptotic cell death via the mitochondrial pathway, as indicated by the upregulation of caspase-9 and caspase-3 protein levels [60–62].

Additionally, apoptosis appears to be mediated not only by mitochondrial mechanisms but also by ER stress. It is known that cellular stress related to the accumulation of unfolded or misfolded proteins at the ER level is a powerful driver for the onset of various chronic diseases, including cancer. In this context, the UPR is the signaling pathway deputed to ensure surveillance of ER proteostasis. However, chronic cell damage can lead this pathway to induce apoptotic cell death [63]. This pathophysiological rationale is exploited by PDT in cancer. In the human GC cell line MKN45, 2-(1 hexyloxyethyl)-2-devinyl porphin e6 trisodium salt (HCE6)-mediated PDT induces mitochondria and ER-mediated apoptosis. Specifically, the mitochondrial pathway is activated via upregulation of proapoptotic ankyrin repeat domain 1 and downstream release of cyt-c. Concurrently, ER-mediated apoptosis is triggered via upregulation of the glucagon receptor and junctional sarcoplasmic reticulum protein 1, along with increased levels of HSP70, HSPA6, HSPA7, and antisense ribonucleic acid (RNA) IDI2-AS1 [64].

In addition to apoptosis, necrosis and autophagy have recently emerged as additional PDT-induced cell death pathways in RGK1 gastric cancer-like cells [65].

As previously mentioned, a promising PDT role in stimulating antitumor immunity is being actively investigated for GC. This is particularly relevant in the era of immunotherapy, as advancements in this area could enhance the clinical management of this malignancy by synergizing with existing immune checkpoint inhibitors. Such progress could improve therapeutic efficacy by converting "cold tumors" into "hot tumors", characterized by increased cellular immune infiltration. PDT's role in promoting intratumoral immune cell infiltration is noteworthy, as it fosters specific clonal expansion of CTLs while reducing the presence of Tregs. Moreover, PDT enhances interactions between tumor cells and effector cells, while disfavoring interactions between Tregs and other immune cells. In this context, PDT not only promotes co-stimulatory signaling but also disrupts co-inhibitory signaling [66].

Highlights:

- Apoptosis, both mitochondria-and ER-mediated, appears to be the main mechanism of PDT-induced cell death in GC. However, there is recent evidence of alternative pathways which have been identified in autophagy and necrosis.
- PDT-induced cell death-related pathways: intrinsic mitochondrial apoptosis—ankyrin repeat domain 1/Cyt-c/caspase-9/caspase-3; ER-mediated apoptosis—glucagon receptor/junctional sarcoplasmic reticulum protein 1/HSPs.
- The emerging antitumor immunity-stimulating role of PDT in GC relies on CTLs clonal expansion and simultaneous Treg restriction.

8. Esophageal Cancer

Esophageal cancer is the sixth most common cause of cancer-related death worldwide, with esophageal squamous cell carcinoma (ESCC) being the predominant histotype. Over the last decade, new treatment strategies have emerged and are replacing traditional approaches in all stages of this neoplasm [67]. In this era of announced therapeutic revolution, PDT is already an approved endoscopic ablative practice for this neoplasm in some countries. It is also approved for dysplastic Barrett's esophagus and is used for palliative purposes in symptomatic obstructive esophageal cancer. A further impetus for its use comes from diminished phototoxicity thanks to the use of second-generation PSs [68]. In this context, there is a growing body of preliminary evidence aimed at elucidating the cytomolecular mechanisms underlying its therapeutic efficacy. Research is focusing on three main areas: the effects of PDT on the induction of cell death mechanisms and its anti-migratory properties, the stimulation of antitumor immunity, and the suppression of cancerous aerobic glycolysis (also known as the Warburg effect) [68].

Regarding its immunoregulatory role, evidence suggests that PDT has a stimulatory potential on both innate and acquired host immunity. In a clinical setting, these cellular events lead to tumor regression and inhibited tumor recurrence, contributing to the improved long-term survival rates reported after PDT. A significant aspect of this process involves the modulation of immune cells which are crucial in maintaining immune homeostasis. In patients with invasive ESCC, PDT has been shown to inhibit the immunosuppressive functions of peripheral cluster of differentiation (CD)4 + CD25 + CD127-forkhead box P3 (foxp3) + Tregs. Consequently, there is a substantial increase in circulating neutrophilic granulocytes and monocytes [69].

Beyond this immunomodulatory role, PDT's applicative potential in esophageal cancer also includes its direct antitumor action on cancer cells, which is further enhanced by the use of novel PSs designed to minimize side effects on surrounding healthy tissues. Hu et al. revealed the in vitro antiproliferative efficacy of Sinoporphyrin sodium (DVDMS)-mediated PDT on human esophageal cancer Eca-109 cells. Their study points out a significant

induction of ROS production, leading to reduced cell viability, DNA damage, and loss of mitochondrial membrane potential [70].

Further molecular findings in Eca-109 cells concern the ability of DVDMS-mediated PDT in inducing a ROS-dependent activation of p38 MAPK, JNK, and eme-oxygenase 1 (HO-1) upregulation. This activation is responsible for inducing apoptosis, which is also functionally supported by the induction of autophagy [71].

However, the induction of the apoptotic process is not limited to the above-mentioned molecular involvement but also involves NF-κB. This factor is known to be a key modulator of various cellular processes such as proliferation, apoptosis, immunity, angiogenesis, and embryonic development. Upon activation by various stimuli, including cytokines, OS, DNA damage, infection, or others, NF-κB is transported from the cytoplasm to the nucleus, where it triggers the transcription of target genes. In human esophageal cancer cell lines Eca109 and Ec9706, dihydroartemisinin (DHA)-mediated PDT has been shown to inactivate ROS-induced NF-κB activity. This results in significant downstream effects, including the downregulation of the anti-apoptotic Bcl-2 protein, upregulation of Bax, and increased activation of caspase-3 and caspase-9 [72]. Specifically, the DHA-PDT antitumor role was also revealed in the suppressing ability of NF-κB/HIF-1α/vascular endothelial growth factor (VEGF) pathway [73].

Furthermore, there is substantial evidence of EGFR overexpression in esophageal cancer, leading to the upregulation of its two downstream PI3K/AKT and Ras/Raf/MAPK signaling pathways. Both pathways play significant roles in the malignant biological behavior of cancer cells, including proliferation, apoptosis, angiogenesis, invasion, and metastasis. In ALA-PDT treated esophageal carcinoma cells Eca-109, the governing antitumoral mechanism was identified as the downregulation of certain molecules within the EGFR/PI3K/AKT signaling pathway [74].

The involvement of the PI3K/AKT pathway in other cancer cell functions includes a key role in cell migration, which is associated with epithelial–mesenchymal transition (EMT). A crucial marker of this event is the so-called "cadherin switch", characterized by downregulation of E-cadherin and concomitant N-cadherin up-regulation. This results in reduced epithelial intercellular adhesion and subsequent promoted cell migration. In ESCC cells, hematoporphyrin derivative-mediated PDT showed significant ROS-generating potential, associated with a reduction in cell viability and migration. This effect is achieved by inhibiting the EMT process via the upregulation of E-cadherin and downregulation of N-cadherin. The anti-migratory effect was further supported by the induction of cell apoptosis and autophagy. The molecular mechanism governing these cellular phenomena is identified in the PDT-mediated downregulation of the PI3K/AKT/mTOR signaling pathway [75].

A further field of research worthy of attention is the influence of PDT on aerobic glycolysis in cancer cells. This metabolic process, characterized by a dramatic increase in glycolysis even in the presence of oxygen, is a hallmark of cancer metabolism and a promising therapeutic target. Known as the Warburg effect, this phenomenon reflects an adaptive mechanism that cancer cells use to meet the demands of uncontrolled proliferation. Despite having functional mitochondria and the presence of oxygen, cancer cells exhibit increased glucose uptake and lactate production. Gan et al. evaluated the influence of 5-ALA-PDT on the Warburg effect in esophageal cancer cells. Their study highlighted that 5-ALA-PDT downregulates pyruvate kinase M2 (PKM2), a key speed-limiting enzyme of cancer cell glycolysis, together with inhibition of glucose uptake and lactate production. This initial (after 4 h) antiglycolytic effect was followed, at a late post-PDT period (after 24 h), by significant PKM2 upregulation, thus highlighting the time-dependent regulatory role of ALA-PDT on cancer cell glycolysis [76].

Furthermore, closely related to PKM2 downregulation is the induction of pyroptosis as a novel mechanism of cell death. In Eca109 and Ec9706 cancer cell lines, PDT has been shown to downregulate PKM2, resulting in the activation of caspase-8 and caspase-3, and downstream release of N-gasdermin E (GSDME) activating pyroptosis. This is revealed by an increase in proinflammatory factors TNF-α, IL-1α, IL-1β, IL-6, and interferon (IFN)-γ) [77].

In addition to pyroptosis, necrosis and ferroptosis also represent key cell death pathways induced by PDT in esophageal cancer cells. Recent studies have highlighted the role of ALA-PDT in inducing various cell death pathways, including apoptosis, necrosis, and ferroptosis, in esophageal Kyse 450 carcinoma cells. Notably, ALA-PDT was observed to alter local extracellular levels of DAMPs. Specifically, there was an increase in adenosine triphosphate (ATP) amounts and a decrease in HMGB1, alongside an increase in extracellular lipid peroxidation products. These changes may influence macrophage phenotype and potentially activate antitumor immunity, highlighting an area of exploration for future research [78].

Highlights:

- Apoptosis (also functionally supported by autophagy) is configured as the main mechanism of PDT-induced cell death in esophageal cancer cells. On the other hand, PDT-induced PKM2 downregulation is responsible for GSDME-mediated pyroptosis.
- PDT-influenced cell death-related pathways:
  Apoptosis: NF-κB/HIF-1α/VEGF and PI3K/AKT/mTOR signaling.
  Pyroptosis: PKM2/caspase-8/caspase-3/GSDME.
- There is emerging evidence on the antitumor immunoregulatory role of PDT via inhibition of Tregs' suppressive functions as well as by supposed alterations in extracellular levels of DAMPs.
- An antiglycolytic (even time-dependent) role of various PDT approaches is emerging. This anti-Warburg effect is molecularly founded on PKM2 downregulation.

9. Colorectal Cancer (CRC)

CRC is the third most common cancer and the second leading cause of cancer-related death in the world [79]. Beyond its classical definition applicable to late-onset CRC, clinical interest has been progressively increasing in recent decades due to the increased incidence rates of early-onset CRC (<50 years old). The use of PDT for CRC shows promise, particularly as a new palliative treatment for advanced cases. However, significant challenges remain, including issues related to PDT resistance [80–82]. In this clinical-epidemiological setting, the importance resulting from an increased cytomolecular understanding of PDT's antitumor potential is crucial. The three main research strands focus on (1) PDT-induced cell death phenomena, (2) the role of PDT in cytoskeletal reorganization for antimigratory purposes, and (3) PDT's stimulatory action on antitumor immunity.

The involvement of MAPKs on elicited cell death pathways is essential. MAPKs are activated in response to various cellular stimuli and are essentially involved in the regulation of cellular processes, including apoptosis and autophagy. Among MAPKs, p38 MAPK is specifically activated by stress stimuli like OS, ultraviolet irradiation, or hyperthermia, leading to apoptosis [83]. This underscores the importance of p38 MAPK as a key molecular target for PDT in cancer therapy.

Xue et al. studied the in vitro effects of chlorin e6 (Ce6)-mediated PDT in human colorectal cancer SW620 cells. Its ability for ROS generation and downstream p38 MAPK activation was identified, leading to the induction of apoptosis and autophagy. Notably, autophagy may represent a cytoprotective mechanism, as p38 MAPK activation appears to mitigate Ce6-PDT-induced cell apoptosis [84].

The regulatory role of p38 MAPK was also investigated in tetra-α-(4-carboxyphenoxy) phthalocyanine zinc (TαPcZn)-PDT-in LoVo human colon carcinoma cells. The study revealed a key involvement of p38 MAPK in the crosstalk with caspase-9 during TαPcZn-PDT-induced mitochondria-mediated apoptosis [83].

Interestingly, PDT's molecular targeting can extend to the epigenetic level, thanks to advances in the discovery of an increasing number of CRC-related non-coding RNAs and miRNAs. Among these, miR-124 is well known for its tumor suppressive role on CRC, as well as for its interaction with various oncogenic transcripts, including Nuclear Enriched Abundant Transcript 1 (NEAT1). Recent studies have shown that 5-ALA-PDT induces apoptosis in colorectal cancer p53 wild-type HCT116 and RKO cells. This effect includes the downregulation of NEAT1 (via the downregulation of c-myc) and the upregulation of miR-124 expression, which is associated with a decrease in proliferating cell nuclear antigen levels [81].

In addition to apoptosis, a newly emerged PDT-induced cell death pathway has been identified in pyroptosis. In CT26 and HCT116 cells, mitochondria-targeted PDT induces pyroptosis via ROS formation and the promotion of downstream p38 phosphorylation. This process leads to the active caspase 3 (CASP3)-mediated cleavage of GSDME within the ROS/p38/CASP3/GSDME axis, thereby inhibiting CRC progression [85]

The cytoskeleton is a crucial target in anti-cancer therapy, as are all signaling molecules and processes that contribute to cancer cell migration and metastasis. The cytoskeleton is specifically supported by the combined action of pseudopodia, cytoskeletal aggregation, and depolymerization. Key regulatory events involved in cytoskeletal reorganization and cell migration are controlled by Ras homology (Rho) family proteins, of which Ras-related C3 botulinum toxin substrate 1 (Rac1) is a highly expressed member in many CRC cells and plays a key role in cell migration [82].

Wufuer et al. evaluated the in vitro anti-migratory effects of Ce6-PDT on SW620 human CRCs. They identified the downregulation of the Rac1/p21 (Rac1) activated kinase 1/LIM domain kinase 1/cofilin signaling pathway as a key factor in the depolymerizing ability of the microfilament structure. This was combined with a significant reduction in F-actin expression, in turn responsible for the decrease or disappearance of cell pseudopodia [82]. These cytoskeletal events were further corroborated by observations of a marked decrease in F-actin, α-tubulin, β-tubulin, and vimentin, along with an increase in E-cadherin. All these mechanisms were found to be responsible for the inhibition of proliferation and migration of SW480 cells following Ce6-PDT [86]. Building on preliminary evidence, the effects of hypericin (Hyp)-mediated PDT on metastatic CRC cells have recently been evaluated in vitro. It has been revealed Hyp-PDT's anti-invasive and anti-migratory ability, evidenced by decreased F-actin formation, destruction of pseudopodia formation, upregulation of E-cadherin, and downregulation of N-cadherin and vimentin. Additionally, Hyp-PDT suppressed EMT. These events are linked to the downregulation of RhoA/Rho-associated, coiled-coil-containing protein kinase 1 signaling pathway in metastatic colorectal HCT116 and SW620 cells [87].

Furthermore, research into the antitumor immunostimulatory potential of PDT is gaining traction, especially in the context of immunotherapy. This is particularly relevant given the potential synergy between PDT and immune checkpoint inhibitors, which have already shown success in treating various malignancies, including CRC. In a mouse model of CRC, Ce6-PDT was found to increase CD8 + activated T cells, CD8 + CTLs, and CD8 + naïve T cells while decreasing exhausted CD8 + T cells and CD4 + effector memory T cells. PDT also promoted CD8 + T cell infiltration and their functional activation in TME, evidenced by increased release of key effector molecules, including granzyme B (Gzmb)

and perforin 1. Notably, this antitumor immune activation appears to be systemic, as indicated by elevated serum levels of Gzmb, IFN-γ, and IL-2 [88].

Highlights:

- Apoptosis, besides the newly emerged pyroptosis, is the main mechanism of PDT-induced cell death in CRC, while PDT-induced autophagy performs a tumor cytoprotective role.
- PDT-influenced cell death-related pathways:
  Mitochondrial apoptosis: p38 MAPK/caspase-9/caspase-3.
  Pyroptosis: ROS/p38/CASP3/GSDME.
- There is growing evidence for a PDT-reorganizing role of cytoskeletal architecture for anti-migratory purposes in CRC cells.
- There is encouraging evidence of an antitumor immunostimulatory role for PDT, primarily involving CD8 + T cells.

10. Osteosarcoma (OS)

OS is the most common primary bone malignancy. Its incidence is now characterized by a unimodal distribution, with a significant peak occurring in the second decade of life [89]. Over the past decade, there has been a growing interest in systematically re-evaluating data to better understand the effectiveness and safety of PDT for this neoplasm. To date, there are no clinical reports demonstrating the curative potential of PDT for human OS. However, it could potentially serve primarily as a palliative treatment [90]. A thorough evaluation of the signaling pathways being PDT modulated has been lacking, with preliminary evidence only addressing molecular pathways that either promote or inhibit various mechanisms of cell death.

Among second-generation PSs, hiporfin-mediated PDT has been widely investigated. In OS, the induction of various cell death pathways differs depending on the cell line under investigation. Apoptosis has been observed in DLM-8, 143B and HOS cells; necroptosis has been observed in DLM-8 cells; and autophagy has been observed in 143B and HOS cells. Molecularly, apoptosis is indicated by increased levels of cleaved-caspase-3, cleaved PARP-1 and Bax, along with reduced expression of Bcl-2. Increased levels of receptor-interacting protein1 indicate necroptosis, while LC3I to LC3II conversion is a marker for autophagy, which is believed to have a protective role [91].

Both apoptosis and autophagy are pathways induced by ROS-JNK pathway activation. Janus signaling exhibits a "Yin and Yang" effect, which is particularly evident in PDT treated OS. JNK plays a mediating role, functioning both as a pro-apoptotic agent and as a mediator of cancer cell survival via an autophagic process. Moreover, this pro-autophagic effect has been associated with the development of chemotherapeutic resistance [92].

In the human OS cell line MG-63 treated with AE-PDT, there was a notable overexpression of both the apoptotic protein cleaved caspase-3 and the autophagic proteins LC-3II and Beclin-1. This molecular change is attributed to a time-dependent p-JNK overexpression, indicating essential ROS-JNK pathway involvement. However, a presumed autophagic protective mechanism occurs only in the early stage of therapy [93]. A similar study on the effects of Pheo-α methyl ester (MPPa)-mediated PDT on human OS MG-63 cells evidenced comparable ROS-JNK pathway activation ability, leading to both autophagic and mitochondrial apoptotic responses [94].

Additionally, the ROS-JNK pathway is not the only survival pathway being PDT-modulated. In HOS cells treated with MPPα-PDT, a further resistance mechanism recognizes the crucial involvement of p21, an effector of the PERK-activating transcription Factor 4 (Atf4) pathway [95].

Beyond the dichotomous role of the ROS-JNK pathway, a clear pro-survival role involving the PI3K/AKT/mTOR pathway highlights this pathway as a potential PDT target. In the human OS cell line MG-63, MPPa-PDT inhibits proteins in the PI3K/AKT/mTOR pathway, further contributing to the suppression of tumor cell proliferation, angiogenesis, and metastasis. Specifically, its antiproliferative action involves arresting the cell cycle in the G2M phase, combined with ER stress-induced apoptosis. Additionally, MPPa-PDT-induced antitumor effects are enhanced by inhibiting cell invasion and migration via the downregulation of MMP-2 and MMP-9 [96].

However, the functional dualism of PDT involves various other molecular actors, with a close association observed between the promotion of antioxidant signaling and cancer cell survival mechanisms.

Zhan et al. provided additional evidence supporting a pro-apoptotic role of MPPa-PDT in HOS cells. Nevertheless, the MPPa-PDT-mediated induction of YAP represents an emerging and significant mechanism of therapeutic resistance. YAP's role in suppressing apoptosis contributes to enhanced cancer cell proliferation [97]. Additionally, in human HOS cells, MPPα-PDT also activates X-box binding protein 1 (XBP1), a downstream product of the inositol-requiring enzyme 1 α (IRE1α)-XBP1 pathway, which is a key mediator of resistance mechanism to PDT. This activation occurs via MPPα-PDT-induced upregulation of antioxidant-related proteins, such as catalase (CAT) and superoxide dismutase (SOD)-1 [98].

Besides the conflicting PDT-induced molecular functions, a functional synergism between nanoparticle-guided PDT induced apoptosis and ferroptosis has also been observed in HOS cells. This antitumor synergism is elicited by PDT-induced inactivation of glutathione peroxidase 4, alongside the activation of nuclear receptor coactivator 4-mediated ferritinophagy [99].

Highlights:

- In OS, hiporfin-mediated PDT induces various cell death pathways depending on the cell lines used.
- Autophagy is a presumed tumor protective mechanism against PDT.
- PDT-influenced cell death-related pathways.
  ROS-JNK pathway activation by various PDT approaches is crucial for the downstream activation of apoptotic and autophagic pathways.
- In OS, the activation of PI3K/AKT/mTOR and ROS-JNK pathways is crucially induced by MPPa-PDT for antitumor purposes. Conversely, MPPα-PDT-induced activation of IRE1α-XBP1 and PERK-Atf4 pathways is a mechanism of tumor resistance.

11. Blood Cancer

In the oncohematological field, particularly for adult T-cell leukemia/lymphoma (ATL), PDT represents a promising new approach due to its restricted side effects compared to radiotherapy and chemotherapy. The current era is moving towards clinical applications for PDT in this field. Previous reviews have mainly highlighted the efficacy of ALA-PDT in eliminating ATL leukemic cells by inducing leukemic cell death via apoptosis and/or necrosis. Clinically, this would lead to the inhibition of ATL progression from an indolent to an aggressive form [100].

Given these promising prospects, the last decade has witnessed a growing international scientific interest in elucidating both the underlying antitumor and pro-tumor mechanisms induced by PDT in lymphoma- and leukemia-derived cell lines.

Among the various PSs investigated, significant scientific attention has been directed toward the excellent photochemical properties of hypericin. This plant-derived compound has demonstrated selective accumulation in tumor tissue through mechanism such as

diffusion, endocytosis, or pinocytosis. Its efficacy as a PS is attributed to its ability to generate high singlet oxygen. In Jurkat cells, Hyp-PDT pro-apoptotic effects have been linked to the activation of the TNF-related apoptosis-inducing ligand (TRAIL)/TRAIL-receptor system and caspase-8, while in ATL cells, it induces mitochondrial apoptotic activation. In U937 cells, Hyp-PDT led to reduced cell viability and apoptosis, as evidenced by altered levels of Bcl-2, Bax, and PARP. Furthermore, in a P388 murine lymphoma model, Hyp-PDT induced tumor vasculature damage [101].

Among the several pathophysiological pathways regulating apoptosis, the JNK pathway plays an essential role. JNK, activated by various cellular stressors can act in a bimodally fashion, either promoting or inhibiting apoptosis depending on the type of stressor event and the specific cell type involved. In K562 leukemia cells, Hyp-PDT triggers mitochondria-caspase-dependent apoptosis via the upstream activation of P-JNK and subsequent upregulation of cleaved caspase-9 and cleaved caspase-3 [102]. Further support for this apoptotic mechanism was provided by studies on phenalenone-induced PDT in the human tumor cell line HL60 (acute promyelocytic leukemia). Notably, ROS generation via both type I and type II mechanisms induced both extrinsic (direct activation of caspase-3) and intrinsic (mitochondrial depolarization) apoptotic pathways, initiated by upstream activation of caspase-8/tBid and p38-MAPK. This process showed a clear predominance of apoptosis over resistance pathways mediated by PI3K/AKT and JNK, simultaneously activated by PDT [103].

Based on the reviewed evidence, mitochondrial apoptotic activation remains the primary mechanism of PDT-induced cell death in the oncohematologic setting. This was further supported by findings in the rat acute myeloid leukemia cell line LT12, where PsD007-mediated PDT led to the upregulation of cleaved caspase-7, caspase-9, PARP, and cleaved caspase-3 [104].

Nevertheless, evidence for PDT-induced caspase-independent apoptosis is limited, despite a broader activation of non-apoptotic cell death pathways. Specifically, PDT-induced lysosomal ROS generation can lead to the cytoplasmic release of cathepsins, which are crucial for the initiation of various cell death pathways. For instance, in mouse leukemia cells L1210 treated with the novel Acridin-3,6-dialkyldithiourea hydrochlorides-mediated PDT, ROS formation and cell cycle arrest in the subG0 phase were observed, as along with induction of multiple cell death types, such as autophagy, caspase-independent apoptosis, and/or necrosis [105].

Highlights:

- Lymphoma and leukemia are the hematological malignancies mainly investigated concerning molecular and cellular pro/antitumor mechanisms elicited by PDT.
- Various PDT approaches appear to elicit apoptosis, both caspase-dependent and caspase-independent, as the predominant mechanism of cell death. Minor evidence of autophagy and necrosis as alternative/complementary pathways of cell death is also evidenced.
- PDT-influenced cell death-related pathways:
  Apoptosis: TRAIL/TRAIL-receptor system/caspase-8, intrinsic (P-JNK/caspase-9/caspase-3) and extrinsic (caspase-8/tBid/caspase-3).
- There is preliminary evidence for the prevalence of the apoptotic pathway over the resistance pathway, equally and simultaneously activated by PDT.

12. Prostate Cancer

Prostate cancer is the leading cause of cancer mortality in Western countries, albeit with epidemiological differences observed between countries, caused by the interplay of multiple influencing factors, such as genetic, environmental and social factors. Moreover,

each canonical treatment available currently is associated with severe side effects, along with the development of resistance to the initial treatment. This explains the need to move towards new therapeutic strategies that are both effective and inexpensive and well-tolerated. Nanomedicine, gene therapy and phytotherapy offer promising perspectives in this regard [106]. The PDT shows promise as a focal therapy for men with localized low-and intermediate-risk prostate cancer, and clinical trials are ongoing. However, its success depends on a detailed understanding of the cellular and molecular mechanisms driving or hindering its antitumor efficacy [107].

On this subject, a noteworthy functional dichotomy essentially involves nitric oxide (NO). This mediator, produced not only by macrophages and endothelial cells in the tumor vasculature but also by the tumor cells, exhibits a complex role. There is a large body of evidence on the pro-cancerous role of NO at low concentrations, also through the promotion of aerobic glycolysis. Conversely, high NO concentrations promote its antitumor role. This dual role is influenced by various factors, such as the source of NO in TME, the characteristics of the TME-including local oxygen level- and NO generation rate [108]. In prostate cancer, the effect of ALA induced protoporphyrin IX-mediated PDT has been extensively studied. This treatment dramatically increases inducible NO synthase (iNOS/NOS2) activity, thus NO, resulting in a synergistic effect of resistance to cell death pathways (primarily apoptosis) while simultaneously promoting tumor growth, migration, and invasiveness [108,109].

Concerning PDT-induced NO-mediated cytoprotective molecular mechanisms, a further in vitro study on prostate cancer PC3 and LNCaP cells revealed key pathways in the activation of pro-survival/anti-apoptotic NF-κB and Yin Yang 1 (YY1). Concurrently, there was concomitant inhibition of anti-survival/pro-apoptotic factors and the metastasis suppressor RAF-kinase inhibitor protein (RKIP). These effects were observed at low PDT-induced NO levels, whereas at high PDT-induced NO levels, the diametrically opposite molecular events (inhibition of NF-κB and YY1 with RKIP activation) explain the antitumoral role of NO [110].

Far beyond these dichotomic mechanisms, PDT plays an essential antitumor role in prostate cancer through its classically induced cytotoxic events. These effects lead, in turn, to the activation of various cell death mechanisms. Specifically, in primary prostate epithelial cells cultured from prostate cancer patient samples, the in vitro cytotoxic effects of porphyrin-based PDT are characterized by a ROS-dependent reduction in cell viability and colony-forming ability, along with DNA damage and subsequent induction of both autophagy and necrosis, with apoptosis being less prominent as cell death mechanism [111]. Furthermore, in human prostate cancer PC-3 cells treated with Pheo-mediated PDT, an anti-invasive and anti-metastatic potential is observed via the inhibition of EMT and downregulation of MMPs [112].

However, aside from the well-known imbalance caused by massive ROS generation, PDT-induced tumor-killing effects are also significantly associated with the suppression of antioxidant signaling. This redox homeostasis disruption represents a promising new molecular target in anticancer therapy [113].

In a comparative study on the therapeutic efficacy of Pheo-mediated PDT in prostate cancer cells, a 75% rate of apoptotic cells was observed. This apoptosis resulted from PDT-induced increased levels of ROS and malondialdehyde, as well as the downregulation of SOD and decreased levels of GSH and CAT [114]. These antitumoral molecular mechanisms have recently been reproduced in PC3 cells using an innovative PDT model involving quaternized derivatives (Q-Zn1c, Q-Zn2c) [115]. Beyond apoptosis, autophagy-induced necroptosis has emerged as another mechanism of cell death in PC3-cells treated with MB-PDT. This was molecularly revealed by increased levels of LC3 (a marker of

autophagy), and elevated phospho-mixed lineage kinase domain-like pseudokinase (a marker of necroptosis). The involvement of ROS in this process was further supported by higher lipidic peroxidation as well as by the downregulation of CAT and total antioxidant potential [116].

A new therapeutic frontier for prostate cancer may involve the use of PDT in functional synergy with the established antitumor efficacy of androgen deprivation therapy in castration-sensitive prostate cancer [117].

In androgen-dependent prostate cancer, Pheo-PDT has demonstrated the ability to induce G0/G1 cell cycle arrest and exhibit anti-migratory and anti-invasive properties, contributing to its anti-metastatic effects. This antitumor potential is ERS-dependent and attributable to the rapid Pheo-PDT-induced ROS generation [118]. Furthermore, similar cytotoxic and antitumor effects of Pheo-PDT have been confirmed in vitro on androgen-independent metastatic prostate cancer cell lines, such as DU-145 and C4-2 [119].

In recent years, the anti-cancer efficacy of phthalocyanine derivative-based PDT has been evaluated in prostate cancer DU145 and BPH-1 cells through non-canonical mechanisms. It has been identified that these derivatives can alter the activity of several signaling pathways, including PI3K, JNK, ERK1/2 and notch homolog protein 1(Notch1) as an alternative anticancer mechanism to the traditional ROS generation. This non-canonical notion is supported by the significant roles these pathways play in regulating apoptosis, proliferation, cell cycle, carcinogenesis, angiogenesis, metastasis, and immunomodulation [120].

Highlights:

- PDT-induced activation of iNOS was identified as a key cytoprotective and thus protumoral mechanism in prostate cancer cells. Interestingly, these events only occur at low PDT-induced NO levels, whereas at high PDT-induced NO levels, the antitumor regulatory role of NO prevails.
- Necroptosis and autophagy are PDT-induced cell death mechanism alternatives to apoptosis, which is well represented in prostate cancer. Nevertheless, when induced, apoptosis remains a phenomenon that is closely related to PDT-induced ROS generation and mitochondrial involvement.
  PDT-influenced cell death-related pathways:
  Apoptosis: iNOS/NOS2/NO/RKIP.
- In a non-canonical manner, some PDT approaches act in an antitumor mode by regulating signaling pathways as an alternative to classical ROS generation.

13. Bladder Cancer (BC)

BC is the leading malignancy of the urinary tract and, according to the most recent epidemiological data, ranks as the tenth most common cancer and thirteenth most common cause of cancer-related mortality worldwide [121,122]. In a clinical setting, PDT represents a pioneering approach for urothelial carcinoma. PDT holds promise for use in both adjuvant and neoadjuvant settings to reduce local recurrence and has potential for integration with other well-established therapeutic regimens [122].

However, despite its clinical promise, there is, to date, a lack of comprehensive understanding regarding the molecular and cellular mechanisms underlying the efficacy of PDT in this malignancy.

Much of the scientific attention is on elucidating the various PDT-elicited cell death pathways. Similar to findings in other neoplasms, preliminary evidence in BC suggests that apoptosis and autophagy may play functionally opposite roles.

Chlorophyllin f-based PDT in human BC 5637 and T24 cells has demonstrated significant pro-apoptotic and autophagic effects. The autophagic response, evidenced by the upregulation of the autophagy-related protein Beclin1 and LC3-I to LC3-II conversion, is

thought to contribute to tumor resistance [123]. Additional preliminary evidence supports this functional dualism of apoptosis and autophagy in BC, involving other molecular actors such as autophagy-related enzyme 3. In contrast, apoptosis induction appears to be driven by massive ROS production alongside inhibited SOD activity [124,125].

An even more interesting aspect of cellular response involves the dysfunction of the autophagic process, which is believed to trigger necrosis as the main ROS-dependent cell death mechanism (80%), while significantly suppressing apoptosis (0.68%) [126].

Highlights:

- Various PDT strategies in BC induce apoptosis and necrosis as major pathways of cell death.
- Autophagy is ascribed a putative role as a cancer survival mechanism.
- According to current evidence, it is still unclear whether PDT-induced ROS generation induces apoptosis or necrosis as the major cell death mechanism in BC.

14. Cervical Cancer

Cervical cancer is the fourth most common cancer among women worldwide. However, despite persistent geographical disparities, epidemiological data from the last two decades offer some reassurance due to the implementation of cytological screening programs [127]. In the current era of promising new therapeutic approaches for this neoplasm, PDT plays a prominent clinical role, especially in the treatment of early-stage cervical cancer. PDT is also correlated with higher rates of human papillomavirus (HPV) eradication [128,129].

A substantial body of in vitro evidence identifies apoptosis as the primary mechanism of cell death induced by various PDT approaches in cervical cancer cells and epithelial cells transfected with high-risk HPV [130–133]. This apoptotic activation is primarily driven by ROS generation through simultaneous type I and type II photodynamic reactions, with type II reactions likely playing a predominant role [132]. Apoptosis predominantly involves the mitochondrial pathway, characterized by a transient upregulation of caspase-9 and a more sustained caspase-3 activation [130]. Moreover, PDT is associated with the upregulation of several oncosuppressors, such as p53, the CDK inhibitor p21, and retinoblastoma-associated protein 48 [131]. Alongside apoptosis, PDT also induces the downregulation of Notch-1, leading to the suppression of its downstream pathways, such as NF-κB and VEGF-A [134].

In addition to apoptosis, which is also ERS-dependent, preliminary evidence suggests that ALA-PDT-induced autophagy-likewise ERS-dependent-may contribute to PDT resistance in HeLa cells. The molecular basis for this dual activation involves the upregulation of the Ras/Raf/MEK/ERK pathway and the concomitant downregulation of the PI3K/AKT/mTOR pathway [135,136]. Additionally, necrosis also plays a role in the PDT-induced cell death pathways, affecting both HPV-immortalized and non-HPV-immortalized cervical cancer-derived cell lines [137].

Another field worth investigating is the influence of PDT on epigenetics and the subsequent modulation of alarmins, which play a key role in inducing antitumor immunity. Preliminary evidence suggests that, in HPV-positive cervical cancer cells, ALA-PDT downregulates miR-34a. Such downregulation leads to the upregulation of HMGB1, which in turn enhances antitumor immunity by increasing the percentage of mature DCs in peripheral blood and by elevating levels of proinflammatory cytokines such as IL-6, IL-12, IL-18, IFN-α, and TNF-α. In this immune setting, HPV viral load seems to play a key role in ALA-PDT-mediated modulation of the miR-34a/HMGB1 axis [138].

Additionally, in cervical intraepithelial neoplasia grade 2, ALA-PDT also promotes local immune infiltration of cytotoxic CD8 + T cells expressing GrzB, which can influence prognosis [139].

Highlights:

- In cervical cancer cells, PDT-induced apoptosis is the main mechanism of cell death and is consequent to both massive ROS generation and the upregulation of oncosuppressors p53 and RbAp48 as well as cell cycle inhibitors. In contrast, some investigations suggest that necrosis is the main mechanism of cell death, even in non-HPV-immortalized cervical cancer cells.
- PDT-influenced cell death-related pathways:
  Intrinsic apoptosis: ROS/Cyt-c/APAF1/caspase-9/caspase-3.
  Autophagy: Ras/Raf/MEK/ERK and PI3K/AKT/mTOR.
- There is preliminary evidence of the autophagic process as a PDT-resistance mechanism in cervical cancer cells.
- Very promising and deserving of further investigation is the role of ALA-PDT in promoting antitumor immunity both by upregulating HMGB1 alarmin levels and by directly targeting CD8 + T-cells.

15. Ovarian Cancer

Ovarian cancer has the highest mortality rate among the gynecological cancers in the world and is the second most common gynecological cancer in the United States. Its insidious clinical course results in more than half of all advanced or metastatic diagnoses [140]. In recent years, there has been increasing interest in the use of PDT for ovarian cancer, particularly in the context of a detailed cytomolecular characterization of the TME and emerging therapeutic strategies [141].

Recent research has focused on elucidating the resistance mechanisms directly or indirectly induced by PDT, especially in cancer cells that are already resistant to other canonical treatments. Preliminary evidence suggests the importance of intracellular PS accumulation for the cytotoxic effects of PDT. Furthermore, the PS accumulation level is directly proportional to the sensitivity of ovarian cancer cells to PDT [142].

In doxorubicin-resistant ovarian cancer cells, the activation of the c-methylnitronitrosoguanidine HOS transforming gene (MET)/PI3K/AKT signaling pathway is a putative mechanism of resistance to Pheo-PDT. Specifically, this signaling was responsible for overexpression of breast cancer resistance protein/ATP-binding cassette subfamily G member (ABCG2), which leads to the extracellular export of Pheo, thus reducing its cytotoxic effects [143]. Another emerging mechanism of tumor cell survival to PDT involves the PDT-induced HSP27 overexpression, which inhibits active apoptosome formation [144]. Apoptosis is the main PDT-induced cell death mechanism in ovarian cancer cells, closely dependent on down-regulation of nuclear factor erythroid 2-related factor 2 (Nrf2)-ABCG2 or Nrf2-HO-1 antioxidant signaling, leading to massive ROS generation [145–147]. Consequently, lysosomes become the primary targets, with disruption of their membrane integrity. This disruption results in the cytoprotective downregulation of the proton pump ATPase H + transporting V1/T cell immune regulator 1, and the consequent degradation of most intralysosomal acid hydrolases, aiming to prevent their destructive activity on other cellular structures [148] (Table 1) (Figure 1).

Highlights:

- In ovarian cancer, non-negligible tumor cell survival mechanisms are ascribed to the activation of specific pathways, such as c-MET/PI3K/AKT signaling, as well as to the PDT-induced action of specific alarmins such as HSP27.
- Apoptosis is reported to be the main PDT-induced cell death mechanism via massive ROS production and simultaneous PDT-induced downregulation of antioxidant signaling pathways.

- PDT-influenced cell death-related pathways: apoptosis: Nrf2-ABCG2 or Nrf2-HO-1.

Table 1. Schematization of cytochemical events and related signaling pathways being involved in each PDT-targeted tumor. Abbreviations: Ribonucleic acid (RNA), endoplasmic reticulum (ER), Rat sarcoma (Ras), B-cell lymphoma 2 Homology 3 interacting-domain death agonist (Bid), B-cell lymphoma 2-associated X protein (Bax), cytochrome c (Cyt-c), apoptotic protease activating factor-1 (APAF-1), mechanistic target of rapamycin (mTOR), Hippo-Yes-associated protein-1 (HIPPO-YAP), Krueppel-like factor 10 (KLF10), epidermal growth factor receptor (EGFR), protein kinase RNA-Like ER kinase (PERK), phosphorylation of the eukaryotic initiation factor 2 (p-EIF), CCAAT-enhancer-binding protein homologous protein (CHOP), mitogen-activated protein kinase (MAPK), immunogenic cell death (ICD), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), hypoxia-inducible factor 1-alpha (HIF-1α), vascular endothelial growth factor (VEGF), phosphatidylinositol 3-kinase (Pi3K), protein kinase B (AKT), pyruvate kinase M2 (PKM2), p38 protein (p38), reactive oxygen species (ROS), caspase 3 (CASP3), N-gasdermin E (GSDME), cellular myelocytomatosis oncogene (c-myc), nuclear enriched abundant transcript 1 (NEAT1), microRNA (miR), Ras-related C3 botulinum toxin substrate 1 (Rac1), p21 protein (p21), Lin-11, Isl1, and MEC-3 (LIM), c-Jun N-terminal kinase (JNK), inositol-requiring enzyme 1 α (IRE1α), X-box binding protein 1 (XBP1), truncated Bid (tBid), nuclear factor erythroid 2-related factor 2 (Nrf2), adenosine triphosphate-binding cassette subfamily G member (ABCG2), eme-oxygenase 1 (HO-1).

Tumors	PDT-Influenced Cytological and Biochemical Events	Molecular Pathways Involved
Breast cancer [9,14]	Apoptosis, necrosis, autophagy, ICD	Intrinsic apoptosis: Bid/Bax/Cyt-c/APAF-1/caspase-9. Autophagy: mTOR/ Beclin-1.
Skin cancer [22,23,25,26]	Apoptosis, necrosis, autophagy, ICD	Apoptosis: mitochondria-dependent Autophagy: RIPK1, JNK and PI3K/AKT/mTOR
Pancreatic cancer [11,28–31,36,37]	Apoptosis, necrosis, ferroptosis, necroptosis, pyroptosis, fibroblast depletion, neoangiogenesis and vasculogenic mimicry inhibition	Apoptosis - Intrinsic pathway. - Hippo-YAP pathway.
Cholangiocarcinoma [42–45]	Apoptosis, autophagy, ferroptosis	Extrinsic apoptosis: procaspase-8/caspase-8/Caspase-3. Transcriptional regulation: KLF10/EGFR axis. Autophagy: PERK)/(p-EIF)2α/ CHOP pathway.
Hepatocellular carcinoma [48–50,52,56]	Apoptosis, necrosis, ICD, clonal expansion of peripheral blood mononuclear cells	Apoptosis: MAPK-signaling pathway. ICD: macrophage phagocytosis/processing and exposure of tumor-associated antigens/antigen-specific T cell response.
Gastric cancer [60–66]	Apoptosis, autophagy, necrosis, CTLs clonal expansion and Tregs restriction	Apoptosis - Mitochondria-mediated: ankyrin repeat domain 1/Cyt-c/caspase-9/caspase-3. - ER-mediated: glucagon receptor/junctional sarcoplasmic reticulum protein 1/HSPs.

Table 1. *Cont.*

Tumors	PDT-Influenced Cytological and Biochemical Events	Molecular Pathways Involved
Esophageal cancer [68,69,71–78]	Apoptosis, autophagy, pyroptosis, Tregs inhibition, anti-Warburg effect, EMT suppression	Apoptosis: NF-κB/HIF-1α/VEGF and PI3K/AKT/mTOR signaling. Pyroptosis: PKM2/caspase-8/caspase-3/GSDME. EMT: PI3K/AKT/mTOR. Anti-Warburg effect: PKM2 downregulation.
Colorectal cancer [81–85,87,88]	Apoptosis, pyroptosis, autophagy, CTLs activation, cytoskeletal anti-migratory reorganization, anti-oncogenic epigenetic regulation, EMT suppression	Intrinsic apoptosis: p38 MAPK/caspase-9/caspase-3. Pyroptosis: ROS/p38/CASP3/GSDME. Epigenetic regulation: C-myc/NEAT-1/miR-124. Cytoskeletal reorganization: Rac1/p21 (Rac1)-activated kinase 1/LIM domain kinase 1/cofilin.
Osteosarcoma [91–99]	Apoptosis, necroptosis, autophagy, antioxidant signaling upregulation	Extrinsic apoptosis: ROS-JNK. ER-mediated apoptosis: PI3K/AKT/mTOR. Autophagy: ROS-JNK. Antioxidant signaling: inositol-requiring enzyme 1 α (IRE1α)-XBP1.
Blood cancer [101–105]	Apoptosis, autophagy, necrosis	Apoptosis: TRAIL/TRAIL-receptor system/caspase-8. - Intrinsic: P-JNK/caspase-9/caspase-3. - Extrinsic: caspase-8/tBid/caspase-3.
Prostate cancer [108–110,113,116,118,120]	Apoptosis, necroptosis, autophagy, EMT suppression, antioxidant signaling downregulation	Apoptosis: iNOS/NOS2/NO/RKIP
Bladder cancer [123–126]	Apoptosis, necrosis, autophagy, antioxidant signaling downregulation	Autophagy: beclin-1/autophagy-related enzyme 3
Cervical cancer [130,132,134–136,138,139]	Apoptosis, necrosis, autophagy, antitumor immunity (CTLs an DCs stimulation)	Intrinsic apoptosis: ROS/Cyt-c/APAF1/caspase-9/caspase-3. Autophagy: Ras/Raf/MEK/ERK and PI3K/AKT/mTOR. Epigenetic regulation: miR-34a/HMGB1.
Ovarian cancer [143–145,147,148]	Apoptosis, antioxidant signaling downregulation	Apoptosis: Nrf2-ABCG2 or Nrf2-HO-1

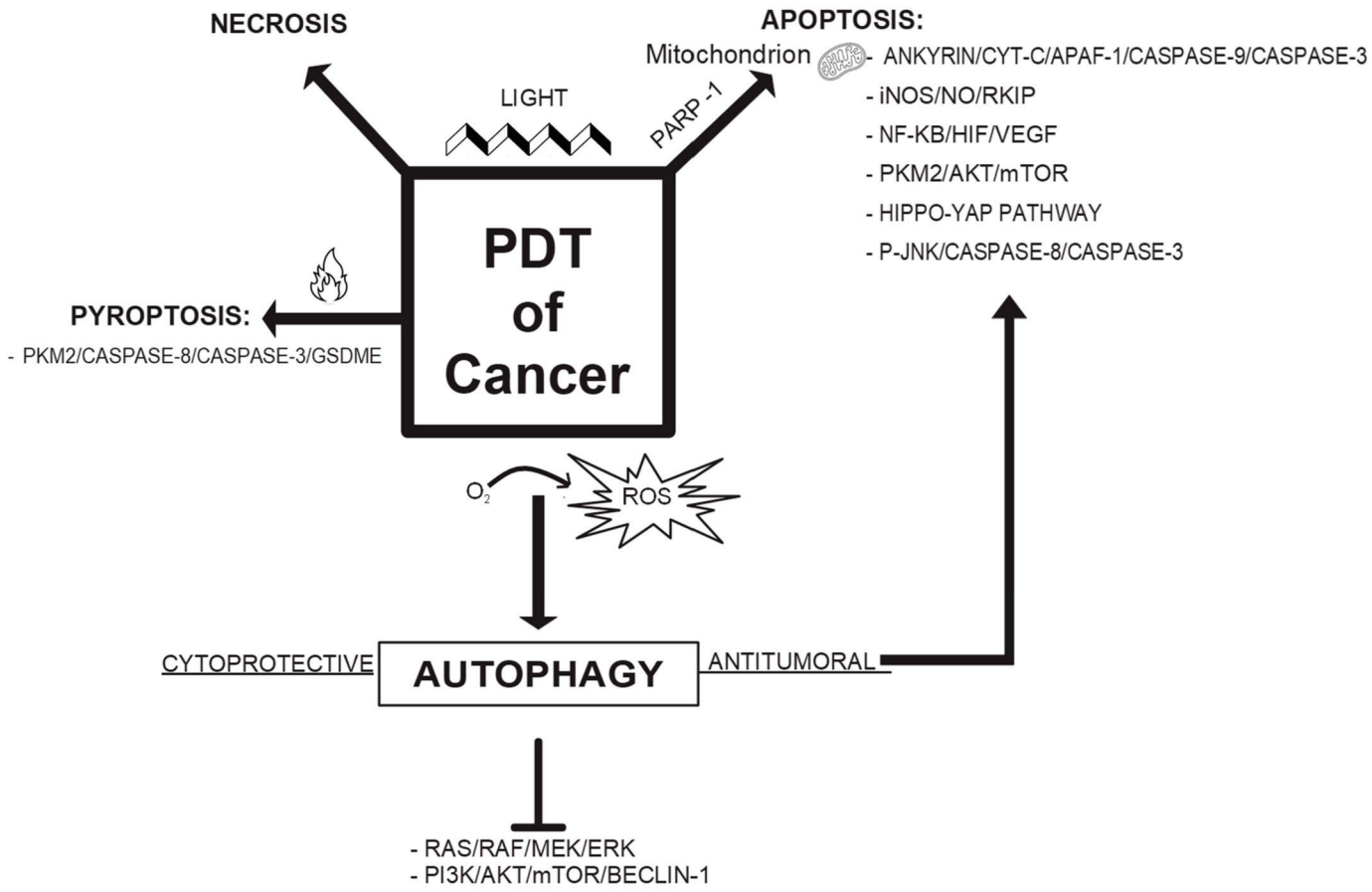


Figure 1. A comprehensive overview of the signaling pathways governing various cell death processes that are PDT-modulated. Abbreviations: cytochrome c (CYT-C), apoptotic protease activating factor-1 (APAF-1), nitric oxide (NO), inducible NO synthase (iNOS), rapidly accelerated fibrosarcoma (RAF)-kinase inhibitor protein (RKIP), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), hypoxia-inducible factor (HIF), vascular endothelial growth factor (VEGF), pyruvate kinase M2 (PKM2), protein kinase B (AKT), mechanistic target of rapamycin (mTOR), Hippo-Yes-associated protein-1 (HIPPO-YAP), phosphorylated c-Jun N-terminal kinase (p-JNK), N-gasdermin E (GSDME), rat sarcoma (RAS), rapidly accelerated fibrosarcoma (RAF), mitogen-activated protein kinase kinase (MEK), extracellular signal-regulated kinase (ERK), phosphatidylinositol 3-kinase (Pi3K).

16. Discussion

16.1. General Highlights

16.1.1. PDT-Induced Cytological Phenomena

Apoptosis: This innate process is the primary mechanism of cell death observed across various tumors. It is triggered by high concentrations of ROS. In certain cancers, such as prostate cancer and ovarian cancer, PDT promotes apoptosis by downregulating antioxidant signaling [113,114,116,145,147]. Apoptosis involves both intrinsic (mitochondrial and ER-mediated) and extrinsic pathways. However, in some tumors, such as esophageal cancer (Eca-109 cells), apoptosis is functionally supported by autophagy [71]. In acute promyelocytic leukemia, apoptosis is the predominant cell death mechanism compared to PDT resistance pathways [103].

Autophagy: Triggered by increased ROS and influenced by PS intracellular localization, autophagy can either support apoptosis as an additional cell death mechanism or act as a survival mechanism. In several cancers, including breast cancer, melanoma, CRC, osteosarcoma (OS), BC, and cervical cancer, autophagy plays a protective role by inhibiting apoptosis, which can lead to PDT resistance [14,26,84,92,123,135].

Necrosis: Characterized by cell membrane disintegration and the release of cytosolic contents into the extracellular space [111]. In many tumors, necrosis occurs alongside apoptosis as a significant PDT-induced and ROS-dependent cell death mechanism. In bladder cancer, for instance, impaired autophagy and suppressed apoptosis may shift necrosis to the primary PDT-induced cell death mechanism [126].

Necroptosis: Observed in OS and prostate cancer cells, where it serves as an alternative cell death mechanism to apoptosis and is functionally supported by autophagy [30,91,116].

Ferroptosis: Preliminary evidence suggests that ferroptosis is induced by PDT in skin cancer, pancreatic cancer, CCA, esophageal carcinoma, and OS cells [22,28,44,78,99].

Pyroptosis: A newly identified PDT-induced cell death mechanism recently observed in pancreatic cancer, esophageal cancer, and CRC [36,77,85].

Immunogenic Cell Death (ICD): PDT-induced ICD has been reported in breast cancer, skin cancer, and HCC [9,22,48]. ICD is part of PDT's broader antitumor immunostimulatory role, involving macrophage activation, which leads to the phagocytosis and presentation of tumor antigens to CTLs [55].

Antitumor Immune Response: Seen in various cancers (pancreatic cancer, HCC, gastric cancer, esophageal cancer, CRC, cervical cancer), this response is primarily driven by the clonal expansion of CTLs and concurrent restriction of Tregs [37,48,65,69,88,139].

16.1.2. PDT-Influenced Biochemical Phenomena

Anti-Warburg Effect: A reduction in aerobic glycolysis has been observed in esophageal cancer cells [76].

Antioxidant Signaling: This can be either upregulated, as seen in OS cells (indicating a resistance mechanism to PDT), or downregulated in OS cells, prostate cancer cells, and ovarian cancer cells [98,114,116,145–147,149].

16.1.3. PDT and Nanomedicine

PDT is crucial in killing tumor cells and stimulating various immune responses related to apoptotic and necrotic tumor cells, as well as inflammatory cells [150]. Nanoparticle-based PDT plays a key role in targeting undetectable and metastatic tumors, overcoming drug resistance, and regulating the TME [151]. Nanomaterials can be applied to enhance the antitumor immunity of PDT [152]. Nanoparticles, including liposomes, polymers, metallic nanomaterials, and porous silicon nanoparticles, offer several advantages that enhance pharmacological efficacy. These features include improved tumor penetration, prevention of drug degradation, enhanced drug solubility, and the ability to be tailored for tissue-specific targeting [106,153]. However, a significant limitation is the challenge posed by immune system surveillance and blood vessel barriers, which can impede the delivery of nanoplatform-based PSs to tumor-specific tissues.

A promising approach to overcome this challenge is the use of biomimicry in nanomedicine. For example, targeting the inflammatory TME is beneficial, as it plays a role in various stages of tumor development, particularly in cancers such as HCC. Biomimicking nanoparticles, functioning as a "Trojan horse", can effectively deliver PSs into the inflammatory microenvironment [55].

16.1.4. PDT Limitations

Although PDT is considered a groundbreaking therapeutic approach in oncology, it does have several biochemical, biophysical, and clinical limitations. Biochemically, the effectiveness of oxygen-dependent PDT can be compromised by the hypoxic tumor environment, which is exacerbated by therapeutic oxygen consumption, thereby creating a detrimental cycle that promotes tumor growth.

From a biophysical perspective, the activation of many PSs requires short-wavelength light (400–700 nm), which limits the use of PDT to peripheral anatomical regions (such as the skin) or areas accessible via endoscopy. This limitation arises from the poor tissue penetration of light, making it challenging to target deep-seated tumors (such as osteosarcoma). Additionally, the optical properties of PSs can be diminished by aggregation-caused quenching when PSs are administered at high concentrations.

Clinically, there is a risk of phototoxicity due to the off-target distribution and accumulation of systemically administered PSs [106,153,154]. Another critical issue is the potential development of resistance to PDT. This resistance has been observed in various previously treated malignancies (such as osteosarcoma, acute promyelocytic leukemia, prostate cancer, breast cancer, cervical cancer, and ovarian cancer) and is often linked to PDT-induced activation of autophagic processes, which can contribute to cancer cell survival.

16.1.5. PDT-Based Combination Therapies

PDT is just one of the many anticancer phototherapy modalities currently available and increasingly studied, including, among others, photothermal, photoimmune, photogas, and radiotherapeutic approaches [155]. The combination of two or more of these approaches is revolutionizing cancer treatment strategies, resulting in continuously improving therapeutic efficacy. For example, both PDT and photothermal therapy (PTT) are capable of eliciting an antitumor immune response, thus effectively cooperating with immunotherapy. Moreover, PDT and PTT enhance each other's therapeutic efficacy. In fact, while PDT increases the sensitivity of tumor cells to PTT, the latter, through heat generation and increased blood flow, improves oxygen supply, which is crucial for the photodynamic therapeutic effect [156].

An important issue concerns the inability of PDT alone to stimulate an immune response strong enough to counterbalance the release of immunosuppressive cytokines by cancer cells. This highlights the need to combine PDT with other therapeutic strategies that boost antitumor host immunity.

A promising new therapeutic approach is the combination of PDT with cancer immunotherapy, including checkpoint blockade immunotherapy, adoptive cell therapy, and cancer vaccines. The goal of immune-based therapies is to "awaken" the host immune system to mount an effective antitumor response. However, a significant limitation is the poor immunogenicity of many tumors, which can result in non-responsiveness and therapeutic failure when these therapies are used alone.

In this context, PDT's ability to stimulate both innate and adaptive antitumor immunity and induce ICD is particularly valuable. ICD is considered a crucial link between PDT and cancer immunotherapy. Specifically, enhancing ICD via the combination of local PDT treatment with systemic inhibition of the Programmed Death (PD)-1/Programmed Death-Ligand 1 (PD-L1) immune checkpoint (which are negative regulators of immune response) would clinically be associated with both regression of primary tumors and regression of distant metastases [157]. Further impetus for the development of such combination therapeutic strategies comes from the use of new nanotechnologies that ensure effective delivery of both PSs and immunotherapeutic agents to the TME [158,159].

Additionally, conventional therapies such as chemotherapy, radiotherapy, and PTT have also been recognized for their capacity to activate ICD [154]. An innovative and highly promising anticancer therapy that activates the antitumor host immunity is near-infrared (NIR) photoimmunotherapy (PIT). NIR-PIT is a photochemistry-based cancer therapy consisting of the injection of a conjugate of a near-infrared, water-soluble silicon-phthalocyanine derivative, IRdye700DX (IR700), and a monoclonal antibody targeting a specific tumor-associated antigen expressed on the surface of cancer cells. Subsequent

local exposure to NIR light triggers a rapid and highly selective ICD of the targeted cancer cell, which in turn results in the rapid maturation of immature dendritic cells adjacent to dying cancer cells, thereby activating the antitumor host immunity through the priming of polyclonal tumor-infiltrating CD8 + T cells against the released tumor antigens [160]. Interestingly, beyond its cytotoxic effects limited to cancer cells, NIR-PIT can also be repurposed to target and deplete a wide repertoire of non-cancerous immune-suppressive cells, such as Tregs, myeloid-derived suppressor cells, and cancer-associated fibroblasts, further enhancing the antitumor host immunity. In this regard, the therapeutic synergy between NIR-PIT and other immune-activating treatments is highly promising [161].

Emerging nanomedicines offer a revolutionary perspective by addressing the limitations of PDT. These advanced nanomedicines can effectively co-deliver PSs, immunomodulators, and chemotherapeutics to targeted sites, paving the way for a new era of combination therapy in oncology [154]. Even more so in this context, the emerging concept of "ROS science" not only aims to investigate the chemical mechanisms and biological effects related to ROS but also seeks to advance the understanding of ROS-based nanotherapies in order to improve therapeutic outcomes [162].

16.2. Remarks and Future Goals

The growing understanding of the cytomolecular mechanisms being directly PDT-modulated aligns with its clinical application across various cancer types.

16.3. State of the Art

- PDT's Dual Mechanism: PDT exerts its effects through both the destruction of tumor vasculature (indirect cytotoxicity) and the stimulation of the immune system (direct cytotoxicity and release of alarmins). This approach effectively counteracts key carcinogenic processes, including tumor neoangiogenesis and the suppression of antitumor immune responses.
- Immunogenic Potential: The emerging immunogenic properties of PDT, particularly through the activation of ICD, offer a promising avenue to address the limitations of cancer immunotherapy. This potential supports the development of novel combination therapies in nanomedicine.
- Autophagy and Resistance: PDT-induced autophagy has been identified as a significant mechanism of cancer cell survival across various tumor types, contributing to PDT resistance. Understanding the signaling pathways involved—whether cytoprotective or pro-apoptotic—is a critical area for further research. This knowledge could lead to more effective strategies for targeting PDT resistance in precision medicine (Figure 2).

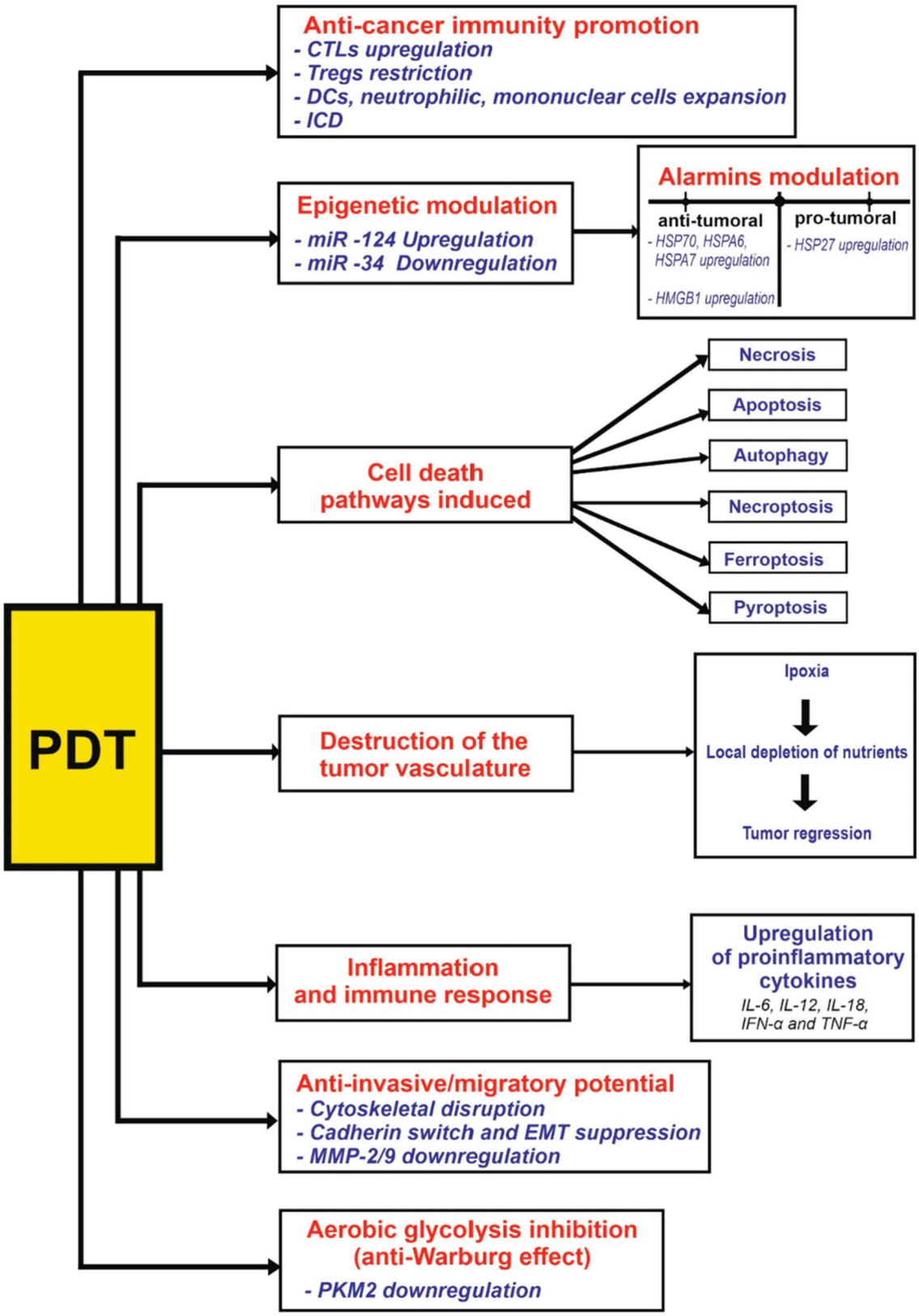


Figure 2. A schematic illustration of PDT's impact on cytomolecular pathways. Abbreviations: photodynamic therapy (PDT), cytotoxic T lymphocytes (CTLs), dendritic cells (DCs), immunogenic cell death (ICD), microRNA (miR), heat shock protein (HSP) 70/A6/A7/27, High Mobility Group Box 1 (HMGB1), matrix metalloproteinase (MMP)-2/9, pyruvate kinase M2 (PKM2), Interleukin (IL)-6/12/18, interferon (IFN)-α, tumor necrosis factor (TNF)-α.

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References

1. Kolarikova, M.; Hosikova, B.; Dilenko, H.; Barton-Tomankova, K.; Valkova, L.; Bajgar, R.; Malina, L.; Kolarova, H. Photodynamic Therapy: Innovative Approaches for Antibacterial and Anticancer Treatments. *Med. Res. Rev.* **2023**, *43*, 717–774. [CrossRef] [PubMed]
2. Donohoe, C.; Senge, M.O.; Arnaut, L.G.; Gomes-da-Silva, L.C. Cell Death in Photodynamic Therapy: From Oxidative Stress to Anti-Tumor Immunity. *Biochim. Biophys. Acta (BBA)-Rev. Cancer* **2019**, *1872*, 188308. [CrossRef] [PubMed]
3. Aebisher, D.; Szpara, J.; Bartusik-Aebisher, D. Advances in Medicine: Photodynamic Therapy. *Int. J. Mol. Sci.* **2024**, *25*, 8258. [CrossRef] [PubMed]
4. Peskova, N.N.; Brilkina, A.A.; Gorokhova, A.A.; Shilyagina, N.Y.; Kutova, O.M.; Nerush, A.S.; Orlova, A.G.; Klapshina, L.G.; Vodeneev, V.V.; Balalaeva, I.V. The Localization of the Photosensitizer Determines the Dynamics of the Secondary Production of Hydrogen Peroxide in Cell Cytoplasm and Mitochondria. *J. Photochem. Photobiol. B* **2021**, *219*, 112208. [CrossRef] [PubMed]
5. Bacellar, I.; Tsubone, T.; Pavani, C.; Baptista, M. Photodynamic Efficiency: From Molecular Photochemistry to Cell Death. *Int. J. Mol. Sci.* **2015**, *16*, 20523–20559. [CrossRef]
6. Mokoena, D.R.; George, B.P.; Abrahamse, H. Photodynamic Therapy Induced Cell Death Mechanisms in Breast Cancer. *Int. J. Mol. Sci.* **2021**, *22*, 10506. [CrossRef]
7. Agostinis, P.; Berg, K.; Cengel, K.A.; Foster, T.H.; Girotti, A.W.; Gollnick, S.O.; Hahn, S.M.; Hamblin, M.R.; Juzeniene, A.; Kessel, D.; et al. Photodynamic Therapy of Cancer: An Update. *CA Cancer J. Clin.* **2011**, *61*, 250–281. [CrossRef]
8. Nath, S.; Obaid, G.; Hasan, T. The Course of Immune Stimulation by Photodynamic Therapy: Bridging Fundamentals of Photochemically Induced Immunogenic Cell Death to the Enrichment of T-Cell Repertoire. *Photochem. Photobiol.* **2019**, *95*, 1288–1305. [CrossRef]
9. Chou, W.; Sun, T.; Peng, N.; Wang, Z.; Chen, D.; Qiu, H.; Zhao, H. Photodynamic Therapy-Induced Anti-Tumor Immunity: Influence Factors and Synergistic Enhancement Strategies. *Pharmaceutics* **2023**, *15*, 2617. [CrossRef]
10. Mroz, P.; Yaroslavsky, A.; Kharkwal, G.B.; Hamblin, M.R. Cell Death Pathways in Photodynamic Therapy of Cancer. *Cancers* **2011**, *3*, 2516–2539. [CrossRef]
11. Wang, Y.; Wang, H.; Zhou, L.; Lu, J.; Jiang, B.; Liu, C.; Guo, J. Photodynamic Therapy of Pancreatic Cancer: Where Have We Come from and Where Are We Going? *Photodiagn. Photodyn. Ther.* **2020**, *31*, 101876. [CrossRef] [PubMed]
12. Katsura, C.; Ogunmwonyi, I.; Kankam, H.K.; Saha, S. Breast Cancer: Presentation, Investigation and Management. *Br. J. Hosp. Med.* **2022**, *83*, 1–7. [CrossRef] [PubMed]
13. Gustalik, J.; Aebisher, D.; Bartusik-Aebisher, D. Photodynamic Therapy in Breast Cancer Treatment. *J. Appl. Biomed.* **2022**, *20*, 98–105. [CrossRef] [PubMed]
14. dos Santos, A.F.; Terra, L.F.; Wailemann, R.A.M.; Oliveira, T.C.; Gomes, V.D.M.; Mineiro, M.F.; Meotti, F.C.; Bruni-Cardoso, A.; Baptista, M.S.; Labriola, L. Methylene Blue Photodynamic Therapy Induces Selective and Massive Cell Death in Human Breast Cancer Cells. *BMC Cancer* **2017**, *17*, 194. [CrossRef]
15. Kazakov, O.V.; Kabakov, A.V.; Poveshchenko, A.F.; Kononchuk, V.V.; Strunkin, D.N.; Gulyaeva, L.F.; Konenkov, V.I. Effect of Photodynamic Therapy on the MicroRNA Level in Breast Cancer Tissues of Female Wistar Rats. *Bull. Exp. Biol. Med.* **2022**, *173*, 444–447. [CrossRef]
16. Cupido-Sánchez, M.G.; Herrera-González, N.E.; Mendoza, C.C.B.; Hernández, M.L.M.; Ramón-Gallegos, E. In Silico Analysis of the Association of Hsa-MiR-16 Expression and Cell Survival in MDA-MB-231 Breast Cancer Cells Subjected to Photodynamic Therapy. *Photodiagn. Photodyn. Ther.* **2021**, *33*, 102106. [CrossRef]
17. Vinita, N.M.; Devan, U.; Durgadevi, S.; Anitha, S.; Prabhu, D.; Rajamanikandan, S.; Govarthanan, M.; Yuvaraj, A.; Biruntha, M.; Antony Joseph Velanganni, A.; et al. Triphenylphosphonium Conjugated Gold Nanotriangles Impact Pi3K/AKT Pathway in Breast Cancer Cells: A Photodynamic Therapy Approach. *Sci. Rep.* **2023**, *13*, 2230. [CrossRef]
18. Perez, M.; Abisaad, J.A.; Rojas, K.D.; Marchetti, M.A.; Jaimes, N. Skin Cancer: Primary, Secondary, and Tertiary Prevention. Part I. *J. Am. Acad. Dermatol.* **2022**, *87*, 255–268. [CrossRef]

19. Rojas, K.D.; Perez, M.E.; Marchetti, M.A.; Nichols, A.J.; Penedo, F.J.; Jaimes, N. Skin Cancer: Primary, Secondary, and Tertiary Prevention. Part II. *J. Am. Acad. Dermatol.* **2022**, *87*, 271–288. [CrossRef]
20. Zhang, P.; Han, T.; Xia, H.; Dong, L.; Chen, L.; Lei, L. Advances in Photodynamic Therapy Based on Nanotechnology and Its Application in Skin Cancer. *Front. Oncol.* **2022**, *12*, 836397. [CrossRef]
21. Hua, Y.; Tian, X.; Zhang, X.; Song, G.; Liu, Y.; Zhao, Y.; Gao, Y.; Yin, F. Applications and Challenges of Photodynamic Therapy in the Treatment of Skin Malignancies. *Front. Pharmacol.* **2024**, *15*, 1476228. [CrossRef] [PubMed]
22. Sun, J.; Zhao, H.; Fu, L.; Cui, J.; Yang, Y. Global Trends and Research Progress of Photodynamic Therapy in Skin Cancer: A Bibliometric Analysis and Literature Review. *Clin. Cosmet. Investig. Dermatol.* **2023**, *16*, 479–498. [CrossRef] [PubMed]
23. Allegra, A.; Pioggia, G.; Tonacci, A.; Musolino, C.; Gangemi, S. Oxidative Stress and Photodynamic Therapy of Skin Cancers: Mechanisms, Challenges and Promising Developments. *Antioxidants* **2020**, *9*, 448. [CrossRef] [PubMed]
24. Gugu Nkosi, P.W.; Chandran, R.; Abrahamse, H. Hypocrellin: A Natural Photosensitizer and Nano-Formulation for Enhanced Molecular Targeting of PDT of Melanoma. *WIREs Nanomed. Nanobiotechnol.* **2024**, *16*, e1997. [CrossRef]
25. Yu, X.; Zheng, H.; Chan, M.T.V.; Wu, W.K.K. Immune Consequences Induced by Photodynamic Therapy in Non-Melanoma Skin Cancers: A Review. *Environ. Sci. Pollut. Res.* **2018**, *25*, 20569–20574. [CrossRef]
26. Valli, F.; García Vior, M.C.; Roguin, L.P.; Marino, J. Crosstalk between Oxidative Stress-Induced Apoptotic and Autophagic Signaling Pathways in Zn(II) Phthalocyanine Photodynamic Therapy of Melanoma. *Free Radic. Biol. Med.* **2020**, *152*, 743–754. [CrossRef]
27. Wood, L.D.; Canto, M.I.; Jaffee, E.M.; Simeone, D.M. Pancreatic Cancer: Pathogenesis, Screening, Diagnosis, and Treatment. *Gastroenterology* **2022**, *163*, 386–402.e1. [CrossRef]
28. Zhou, W.; Lim, A.; Elmadbouh, O.H.M.; Edderkaoui, M.; Osipov, A.; Mathison, A.J.; Urrutia, R.; Liu, T.; Wang, Q.; Pandol, S.J. Verteporfin Induces Lipid Peroxidation and Ferroptosis in Pancreatic Cancer Cells. *Free Radic. Biol. Med.* **2024**, *212*, 493–504. [CrossRef]
29. Wei, H.; Wang, F.; Wang, Y.; Li, T.; Xiu, P.; Zhong, J.; Sun, X.; Li, J. Verteporfin Suppresses Cell Survival, Angiogenesis and Vasculogenic Mimicry of Pancreatic Ductal Adenocarcinoma via Disrupting the YAP–TEAD Complex. *Cancer Sci.* **2017**, *108*, 478–487. [CrossRef]
30. de Almeida, D.R.Q.; dos Santos, A.F.; Wailemann, R.A.M.; Terra, L.F.; Gomes, V.M.; Arini, G.S.; Bertoldi, E.R.M.; Reis, E.M.; Baptista, M.S.; Labriola, L. Necroptosis Activation Is Associated with Greater Methylene Blue-Photodynamic Therapy-Induced Cytotoxicity in Human Pancreatic Ductal Adenocarcinoma Cells. *Photochem. Photobiol. Sci.* **2022**, *22*, 729–744. [CrossRef]
31. Karimnia, V.; Slack, F.J.; Celli, J.P. Photodynamic Therapy for Pancreatic Ductal Adenocarcinoma. *Cancers* **2021**, *13*, 4354. [CrossRef] [PubMed]
32. Lintern, N.; Smith, A.M.; Jayne, D.G.; Khaled, Y.S. Photodynamic Stromal Depletion in Pancreatic Ductal Adenocarcinoma. *Cancers* **2023**, *15*, 4135. [CrossRef] [PubMed]
33. Liu, Y.; Wu, X.; Chen, F.; Li, H.; Wang, T.; Liu, N.; Sun, K.; Zhou, G.; Tao, K. Modulating Cancer-Stroma Crosstalk by a Nanoparticle-Based Photodynamic Method to Pave the Way for Subsequent Therapies. *Biomaterials* **2022**, *289*, 121813. [CrossRef] [PubMed]
34. Dorst, D.N.; Smeets, E.M.M.; Klein, C.; Frielink, C.; Geijs, D.; Trajkovic-Arsic, M.; Cheung, P.F.Y.; Stommel, M.W.J.; Gotthardt, M.; Siveke, J.T.; et al. Fibroblast Activation Protein-Targeted Photodynamic Therapy of Cancer-Associated Fibroblasts in Murine Models for Pancreatic Ductal Adenocarcinoma. *Mol. Pharm.* **2023**, *20*, 4319–4330. [CrossRef] [PubMed]
35. Smeets, E.M.M.; Dorst, D.N.; Franssen, G.M.; van Essen, M.S.; Frielink, C.; Stommel, M.W.J.; Trajkovic-Arsic, M.; Cheung, P.F.; Siveke, J.T.; Wilson, I.; et al. Fibroblast Activation Protein-Targeting Minibody-IRDye700DX for Ablation of the Cancer-Associated Fibroblast with Photodynamic Therapy. *Cells* **2023**, *12*, 1420. [CrossRef]
36. Wang, M.; Wu, M.; Liu, X.; Shao, S.; Huang, J.; Liu, B.; Liang, T. Pyroptosis Remodeling Tumor Microenvironment to Enhance Pancreatic Cancer Immunotherapy Driven by Membrane Anchoring Photosensitizer. *Adv. Sci.* **2022**, *9*, 202202914. [CrossRef]
37. Zhang, D.; Xie, Q.; Liu, Y.; Li, Z.; Li, H.; Li, S.; Li, Z.; Cui, J.; Su, M.; Jiang, X.; et al. Photosensitizer IR700DX-6T- and IR700DX-Mbc94-Mediated Photodynamic Therapy Markedly Elicits Anticancer Immune Responses during Treatment of Pancreatic Cancer. *Pharmacol. Res.* **2021**, *172*, 105811. [CrossRef]
38. Shen, Y.; Li, M.; Sun, F.; Zhang, Y.; Qu, C.; Zhou, M.; Shen, F.; Xu, L. Low-Dose Photodynamic Therapy-Induced Increase in the Metastatic Potential of Pancreatic Tumor Cells and Its Blockade by Simvastatin. *J. Photochem. Photobiol. B* **2020**, *207*, 111889. [CrossRef]
39. Elvevi, A.; Laffusa, A.; Scaravaglio, M.; Rossi, R.E.; Longarini, R.; Stagno, A.M.; Cristoferi, L.; Ciaccio, A.; Cortinovis, D.L.; Invernizzi, P.; et al. Clinical Treatment of Cholangiocarcinoma: An Updated Comprehensive Review. *Ann. Hepatol.* **2022**, *27*, 100737. [CrossRef]
40. Li, Y.; Li, Y.; Song, Y.; Liu, S. Advances in Research and Application of Photodynamic Therapy in Cholangiocarcinoma (Review). *Oncol. Rep.* **2024**, *51*, 53. [CrossRef]

41. Chen, P.; Yang, T.; Shi, P.; Shen, J.; Feng, Q.; Su, J. Benefits and Safety of Photodynamic Therapy in Patients with Hilar Cholangiocarcinoma: A Meta-Analysis. *Photodiagn. Photodyn. Ther.* **2022**, *37*, 102712. [CrossRef] [PubMed]
42. Yang, Y.; Li, J.; Yao, L.; Wu, L. Effect of Photodynamic Therapy on Gemcitabine-Resistant Cholangiocarcinoma in Vitro and in Vivo Through KLF10 and EGFR. *Front. Cell Dev. Biol.* **2021**, *9*, 710721. [CrossRef] [PubMed]
43. Han, Y.; Yang, Y.; Huang, S.; Yao, L.; Wu, L. The MiR-34a/WNT7B Modulates the Sensitivity of Cholangiocarcinoma Cells to P53-Mediated Photodynamic Therapy Toxicity. *Biochem. Biophys. Res. Commun.* **2022**, *591*, 54–61. [CrossRef] [PubMed]
44. Yan, X.; Li, Z.; Chen, H.; Yang, F.; Tian, Q.; Zhang, Y. Photodynamic Therapy Inhibits Cancer Progression and Induces Ferroptosis and Apoptosis by Targeting P53/GPX4/SLC7A11 Signaling Pathways in Cholangiocarcinoma. *Photodiagn. Photodyn. Ther.* **2024**, *47*, 104104. [CrossRef]
45. He, C.; Xia, J.; Gao, Y.; Chen, Z.; Wan, X. Chlorin A-Mediated Photodynamic Therapy Induced Apoptosis in Human Cholangiocarcinoma Cells via Impaired Autophagy Flux. *Am. J. Transl. Res.* **2020**, *12*, 5080–5094.
46. Weijer, R.; Broekgaarden, M.; van Golen, R.F.; Bulle, E.; Nieuwenhuis, E.; Jongejan, A.; Moerland, P.D.; van Kampen, A.H.C.; van Gulik, T.M.; Heger, M. Low-Power Photodynamic Therapy Induces Survival Signaling in Perihilar Cholangiocarcinoma Cells. *BMC Cancer* **2015**, *15*, 1014. [CrossRef]
47. Weijer, R.; Clavier, S.; Zaal, E.A.; Pijls, M.M.E.; van Kooten, R.T.; Vermaas, K.; Leen, R.; Jongejan, A.; Moerland, P.D.; van Kampen, A.H.C.; et al. Multi-OMIC Profiling of Survival and Metabolic Signaling Networks in Cells Subjected to Photodynamic Therapy. *Cell. Mol. Life Sci.* **2017**, *74*, 1133–1151. [CrossRef]
48. Brown, Z.J.; Tsilimigras, D.I.; Ruff, S.M.; Mohseni, A.; Kamel, I.R.; Cloyd, J.M.; Pawlik, T.M. Management of Hepatocellular Carcinoma. *JAMA Surg.* **2023**, *158*, 410. [CrossRef]
49. Kumar, A.; Moralès, O.; Mordon, S.; Delhem, N.; Boleslawski, E. Could Photodynamic Therapy Be a Promising Therapeutic Modality in Hepatocellular Carcinoma Patients? A Critical Review of Experimental and Clinical Studies. *Cancers* **2021**, *13*, 5176. [CrossRef]
50. Mirzaei, H.; Djavid, G.E.; Hadizadeh, M.; Jahanshiri-Moghadam, M.; Hajian, P. The Efficacy of Radachlorin-Mediated Photodynamic Therapy in Human Hepatocellular Carcinoma Cells. *J. Photochem. Photobiol. B* **2015**, *142*, 86–91. [CrossRef]
51. Abo-Zeid, M.A.M.; Abo-Elfadl, M.T.; Mostafa, S.M. Photodynamic Therapy Using 5-Aminolevulinic Acid Triggered DNA Damage of Adenocarcinoma Breast Cancer and Hepatocellular Carcinoma Cell Lines. *Photodiagn. Photodyn. Ther.* **2018**, *21*, 351–356. [CrossRef] [PubMed]
52. Liu, C.; Wu, T.; Wang, S.; Zhou, W.; Li, Y.; Chen, X.; Li, W.; Huang, Z.; Li, T.; Yang, L.; et al. Anticancer Effect of LS-HB-Mediated Photodynamic Therapy on Hepatocellular Carcinoma in Vitro and in Vivo. *Photodiagn. Photodyn. Ther.* **2020**, *30*, 101718. [CrossRef] [PubMed]
53. Ogbodu, R.O.; Nitzsche, B.; Ma, A.; Atilla, D.; Gürek, A.G.; Höpfner, M. Photodynamic Therapy of Hepatocellular Carcinoma Using Tetra-Triethyleneoxysulfonyl Zinc Phthalocyanine as Photosensitizer. *J. Photochem. Photobiol. B* **2020**, *208*, 111915. [CrossRef] [PubMed]
54. Ozten, O.; Guney Eskiler, G.; Sonmez, F.; Yıldız, M.Z. Investigation of the Therapeutic Effect of 5-Aminolevulinic Acid Based Photodynamic Therapy on Hepatocellular Carcinoma. *Lasers Med. Sci.* **2022**, *37*, 1325–1332. [CrossRef]
55. Zhang, Z.; Li, D.; Cao, Y.; Wang, Y.; Wang, F.; Zhang, F.; Zheng, S. Biodegradable Hypocrellin B Nanoparticles Coated with Neutrophil Membranes for Hepatocellular Carcinoma Photodynamics Therapy Effectively via JUNB/ROS Signaling. *Int. Immunopharmacol.* **2021**, *99*, 107624. [CrossRef]
56. Kumar, A.; Pecquenard, F.; Baydoun, M.; Quilbé, A.; Moralès, O.; Leroux, B.; Aoudjehane, L.; Conti, F.; Boleslawski, E.; Delhem, N. An Efficient 5-Aminolevulinic Acid Photodynamic Therapy Treatment for Human Hepatocellular Carcinoma. *Int. J. Mol. Sci.* **2023**, *24*, 10426. [CrossRef]
57. Smyth, E.C.; Nilsson, M.; Grabsch, H.I.; van Grieken, N.C.; Lordick, F. Gastric Cancer. *Lancet* **2020**, *396*, 635–648. [CrossRef]
58. López, M.J.; Carbajal, J.; Alfaro, A.L.; Saravia, L.G.; Zanabria, D.; Araujo, J.M.; Quispe, L.; Zevallos, A.; Buleje, J.L.; Cho, C.E.; et al. Characteristics of Gastric Cancer around the World. *Crit. Rev. Oncol. Hematol.* **2023**, *181*, 103841. [CrossRef]
59. Wang, H.; Ewetse, M.P.; Ma, C.; Pu, W.; Xu, B.; He, P.; Wang, Y.; Zhu, J.; Chen, H. The "Light Knife" for Gastric Cancer: Photodynamic Therapy. *Pharmaceutics* **2022**, *15*, 101. [CrossRef]
60. Nakayama, T.; Kobayashi, T.; Shimpei, O.; Fukuhara, H.; Namikawa, T.; Inoue, K.; Hanazaki, K.; Takahashi, K.; Nakajima, M.; Tanaka, T.; et al. Photoirradiation after Aminolevulinic Acid Treatment Suppresses Cancer Cell Proliferation through the HO-1/P21 Pathway. *Photodiagn. Photodyn. Ther.* **2019**, *28*, 10–17. [CrossRef]
61. Tan, H.; Gao, S.; Zhuang, Y.; Dong, Y.; Guan, W.; Zhang, K.; Xu, J.; Cui, J. R-Phycoerythrin Induces SGC-7901 Apoptosis by Arresting Cell Cycle at S Phase. *Mar. Drugs* **2016**, *14*, 166. [CrossRef] [PubMed]
62. Lin, H.-D.; Li, K.-T.; Duan, Q.-Q.; Chen, Q.; Tian, S.; Chu, E.S.M.; Bai, D.-Q. The Effect of Aloe-Emodin-Induced Photodynamic Activity on the Apoptosis of Human Gastric Cancer Cells: A Pilot Study. *Oncol. Lett.* **2017**, *13*, 3431–3436. [CrossRef]
63. Hetz, C.; Zhang, K.; Kaufman, R.J. Mechanisms, Regulation and Functions of the Unfolded Protein Response. *Nat. Rev. Mol. Cell Biol.* **2020**, *21*, 421–438. [CrossRef] [PubMed]

64. Tan, Y.-K.; Liu, K.-J.; Zou, H.; Yao, H.-L.; Jin, J.-Y.; Zhang, C.-Y.; Li, Q.-Z.; Xiong, L.; Lei, S.-L.; Chen, W. Inhibitory Effect and Molecular Mechanism of the New Phorphyrin-Based HCE6 Photosensitizer on the Activity of MKN45 Human Gastric Cancer Cells. *J. Biomed. Nanotechnol.* **2019**, *15*, 1345–1353. [CrossRef] [PubMed]
65. Taninaka, A.; Kurokawa, H.; Kamiyanagi, M.; Ochiai, T.; Arashida, Y.; Takeuchi, O.; Matsui, H.; Shigekawa, H. Polphylipoprotein-Induced Autophagy Mechanism with High Performance in Photodynamic Therapy. *Commun. Biol.* **2023**, *6*, 1212. [CrossRef] [PubMed]
66. Yu, Y.; Xu, B.; Xiang, L.; Ding, T.; Wang, N.; Yu, R.; Gu, B.; Gao, L.; Maswikiti, E.P.; Wang, Y.; et al. Photodynamic Therapy Improves the Outcome of Immune Checkpoint Inhibitors via Remodelling Anti-Tumour Immunity in Patients with Gastric Cancer. *Gastric. Cancer* **2023**, *26*, 798–813. [CrossRef]
67. Waters, J.K.; Reznik, S.I. Update on Management of Squamous Cell Esophageal Cancer. *Curr. Oncol. Rep.* **2022**, *24*, 375–385. [CrossRef]
68. Wu, H.; Minamide, T.; Yano, T. Role of Photodynamic Therapy in the Treatment of Esophageal Cancer. *Dig. Endosc.* **2019**, *31*, 508–516. [CrossRef]
69. Reginato, E.; Lindenmann, J.; Langner, C.; Schweintzger, N.; Bambach, I.; Smolle-Jüttner, F.; Wolf, P. Photodynamic Therapy Downregulates the Function of Regulatory T Cells in Patients with Esophageal Squamous Cell Carcinoma. *Photochem. Photobiol. Sci.* **2014**, *13*, 1281–1289. [CrossRef]
70. Hu, J.; Wang, X.; Liu, Q.; Zhang, K.; Xiong, W.; Xu, C.; Wang, P.; Leung, A.W. Antitumor Effect of Sinoporphyrin Sodium-Mediated Photodynamic Therapy on Human Esophageal Cancer Eca-109 Cells. *Photochem. Photobiol.* **2014**, *90*, 1404–1412. [CrossRef]
71. Shi, Y.; Zhang, B.; Feng, X.; Qu, F.; Wang, S.; Wu, L.; Wang, X.; Liu, Q.; Wang, P.; Zhang, K. Apoptosis and Autophagy Induced by DVDMs-PDT on Human Esophageal Cancer Eca-109 Cells. *Photodiagn. Photodyn. Ther.* **2018**, *24*, 198–205. [CrossRef] [PubMed]
72. Li, Y.J.; Zhou, J.H.; Du, X.X.; Jia, D.X.; Wu, C.L.; Huang, P.; Han, Y.; Sui, H.; Wei, X.L.; Liu, L.; et al. Dihydroartemisinin Accentuates the Anti-Tumor Effects of Photodynamic Therapy via Inactivation of NF-κB in Eca109 and Ec9706 Esophageal Cancer Cells. *Cell. Physiol. Biochem.* **2014**, *33*, 1527–1536. [CrossRef] [PubMed]
73. Li, Y.; Sui, H.; Jiang, C.; Li, S.; Han, Y.; Huang, P.; Du, X.; Du, J.; Bai, Y. Dihydroartemisinin Increases the Sensitivity of Photodynamic Therapy Via NF-KB/HIF-1α/VEGF Pathway in Esophageal Cancer Cell in Vitro and in Vivo. *Cell. Physiol. Biochem.* **2018**, *48*, 2035–2045. [CrossRef] [PubMed]
74. Zhang, X.; Cai, L.; He, J.; Li, X.; Li, L.; Chen, X.; Lan, P. Influence and Mechanism of 5-Aminolevulinic Acid-Photodynamic Therapy on the Metastasis of Esophageal Carcinoma. *Photodiagn. Photodyn. Ther.* **2017**, *20*, 78–85. [CrossRef]
75. Wei, X.; Ni, J.; Yuan, L.; Li, X. Hematoporphyrin Derivative Photodynamic Therapy Induces Apoptosis and Suppresses the Migration of Human Esophageal Squamous Cell Carcinoma Cells by Regulating the PI3K/AKT/MTOR Signaling Pathway. *Oncol. Lett.* **2023**, *27*, 17. [CrossRef]
76. Gan, J.; Li, S.; Meng, Y.; Liao, Y.; Jiang, M.; Qi, L.; Li, Y.; Bai, Y. The Influence of Photodynamic Therapy on the Warburg Effect in Esophageal Cancer Cells. *Lasers Med. Sci.* **2020**, *35*, 1741–1750. [CrossRef]
77. Li, L.; Song, D.; Qi, L.; Jiang, M.; Wu, Y.; Gan, J.; Cao, K.; Li, Y.; Bai, Y.; Zheng, T. Photodynamic Therapy Induces Human Esophageal Carcinoma Cell Pyroptosis by Targeting the PKM2/Caspase-8/Caspase-3/GSDME Axis. *Cancer Lett.* **2021**, *520*, 143–159. [CrossRef]
78. Čunderlíková, B.; Klučková, K.; Babál, P.; Mlkvý, P.; Teplický, T. Modifications of DAMPs Levels in Extracellular Environment Induced by Aminolevulinic Acid-Based Photodynamic Therapy of Esophageal Cancer Cells. *Int. J. Radiat. Biol.* **2024**, *100*, 802–816. [CrossRef]
79. Baidoun, F.; Elshiwy, K.; Elkeraie, Y.; Merjaneh, Z.; Khoudari, G.; Sarmini, M.T.; Gad, M.; Al-Husseini, M.; Saad, A. Colorectal Cancer Epidemiology: Recent Trends and Impact on Outcomes. *Curr. Drug Targets* **2021**, *22*, 998–1009. [CrossRef]
80. Nfonsam, V.; Wusterbarth, E.; Gong, A.; Vij, P. Early-Onset Colorectal Cancer. *Surg. Oncol. Clin. N. Am.* **2022**, *31*, 143–155. [CrossRef]
81. Liu, K.; Lei, S.; Kuang, Y.; Jin, Q.; Long, D.; Liu, C.; Jiang, Y.; Zhao, H.; Yao, H. A Novel Mechanism of the C-Myc/NEAT1 Axis Mediating Colorectal Cancer Cell Response to Photodynamic Therapy Treatment. *Front. Oncol.* **2021**, *11*, 652831. [CrossRef] [PubMed]
82. Wufuer, R.; Ma, H.-X.; Luo, M.-Y.; Xu, K.-Y.; Kang, L. Downregulation of Rac1/PAK1/LIMK1/Cofilin Signaling Pathway in Colon Cancer SW620 Cells Treated with Chlorin E6 Photodynamic Therapy. *Photodiagn. Photodyn. Ther.* **2021**, *33*, 102143. [CrossRef] [PubMed]
83. Wang, Y.; Xia, C.; Lun, Z.; Lv, Y.; Chen, W.; Li, T. Crosstalk between P38 MAPK and Caspase-9 Regulates Mitochondria-Mediated Apoptosis Induced by Tetra-α-(4-Carboxyphenoxy) Phthalocyanine Zinc Photodynamic Therapy in LoVo Cells. *Oncol. Rep.* **2017**, *39*, 61–70. [CrossRef] [PubMed]
84. Xue, Q.; Wang, X.; Wang, P.; Zhang, K.; Liu, Q. Role of P38MAPK in Apoptosis and Autophagy Responses to Photodynamic Therapy with Chlorin E6. *Photodiagn. Photodyn. Ther.* **2015**, *12*, 84–91. [CrossRef] [PubMed]

85. Zhou, Y.; Zhang, W.; Wang, B.; Wang, P.; Li, D.; Cao, T.; Zhang, D.; Han, H.; Bai, M.; Wang, X.; et al. Mitochondria-Targeted Photodynamic Therapy Triggers GSDME-Mediated Pyroptosis and Sensitizes Anti-PD-1 Therapy in Colorectal Cancer. *J. Immunother. Cancer* **2024**, *12*, e008054. [CrossRef]
86. Ma, H.; Yang, K.; Li, H.; Luo, M.; Wufuer, R.; Kang, L. Photodynamic Effect of Chlorin E6 on Cytoskeleton Protein of Human Colon Cancer SW480 Cells. *Photodiagn. Photodyn. Ther.* **2021**, *33*, 102201. [CrossRef]
87. Hu, J.; Wen, X.; Song, J. Hypericin-Mediated Photodynamic Therapy Inhibits Metastasis and EMT of Colorectal Cancer Cells by Regulating RhoA–ROCK1 Signaling Pathway. *Photochem. Photobiol. Sci.* **2024**, *23*, 1361–1372. [CrossRef]
88. Lee, E.-J.; Choi, J.-G.; Han, J.H.; Kim, Y.-W.; Lim, J.; Chung, H.-S. Single-Cell RNA Sequencing Reveals Immuno-Oncology Characteristics of Tumor-Infiltrating T Lymphocytes in Photodynamic Therapy-Treated Colorectal Cancer Mouse Model. *Int. J. Mol. Sci.* **2023**, *24*, 13913. [CrossRef]
89. Kar, E.; Ammanamanchi, A.; Yousif, M.; Geetha, S.D.; Schwartz, K.; Mishra, A.S.; Ling, J.; Nonyelu, K.N.; Kannadath, B.S. From Bimodal to Unimodal: The Transformed Incidence of Osteosarcoma in the United States. *J. Bone Oncol.* **2024**, *47*, 100613. [CrossRef]
90. Tan, G.; Xu, J.; Yu, Q.; Yang, Z.; Zhang, H. The Safety and Efficiency of Photodynamic Therapy for the Treatment of Osteosarcoma: A Systematic Review of in Vitro Experiment and Animal Model Reports. *Photodiagn. Photodyn. Ther.* **2022**, *40*, 103093. [CrossRef]
91. Sun, M.; Zhou, C.; Zeng, H.; Puebla-Osorio, N.; Damiani, E.; Chen, J.; Wang, H.; Li, G.; Yin, F.; Shan, L.; et al. Hiporfin-Mediated Photodynamic Therapy in Preclinical Treatment of Osteosarcoma. *Photochem. Photobiol.* **2015**, *91*, 533–544. [CrossRef] [PubMed]
92. Wu, Q.; Wu, W.; Fu, B.; Shi, L.; Wang, X.; Kuca, K. JNK Signaling in Cancer Cell Survival. *Med. Res. Rev.* **2019**, *39*, 2082–2104. [CrossRef] [PubMed]
93. TU, P.; HUANG, Q.; OU, Y.; DU, X.; LI, K.; TAO, Y.; YIN, H. Aloe-Emodin-Mediated Photodynamic Therapy Induces Autophagy and Apoptosis in Human Osteosarcoma Cell Line MG-63 through the ROS/JNK Signaling Pathway. *Oncol. Rep.* **2016**, *35*, 3209–3215. [CrossRef]
94. Huang, Q.; Ou, Y.-S.; Tao, Y.; Yin, H.; Tu, P.-H. Apoptosis and Autophagy Induced by Pyropheophorbide-α Methyl Ester-Mediated Photodynamic Therapy in Human Osteosarcoma MG-63 Cells. *Apoptosis* **2016**, *21*, 749–760. [CrossRef]
95. Zhong, S.; Zhang, Y.; Mou, H.; Jian, C.; Huang, Q.; Ou, Y. Targeting PERK-ATF4-P21 Axis Enhances the Sensitivity of Osteosarcoma HOS Cells to Mppα-PDT. *Aging* **2024**, *16*, 2789. [CrossRef]
96. Chen, Y.; Yin, H.; Tao, Y.; Zhong, S.; Yu, H.; Li, J.; Bai, Z.; Ou, Y. Antitumor Effects and Mechanisms of Pyropheophorbide-α Methyl Ester-mediated Photodynamic Therapy on the Human Osteosarcoma Cell Line MG-63. *Int. J. Mol. Med.* **2020**, *45*, 971–982. [CrossRef] [PubMed]
97. Zhan, F.; Zhang, Y.; Zuo, Q.; Xie, C.; Li, H.; Tian, L.; Wu, C.; Chen, Z.; Yang, C.; Wang, Y.; et al. YAP Knockdown in Combination with Ferroptosis Induction Increases the Sensitivity of HOS Human Osteosarcoma Cells to Pyropheophorbide-α Methyl Ester-Mediated Photodynamic Therapy. *Photodiagn. Photodyn. Ther.* **2022**, *39*, 102964. [CrossRef]
98. Yu, H.; Zhang, Y.; Zuo, Q.; Zhong, S.; Chen, Y.; Zhang, M.; Zhan, F.; Ou, Y. Targeting X Box-Binding Protein-1 (XBP1) Enhances the Sensitivity of HOS Osteosarcoma Cells to Pyropheophorbide- α Methyl Ester-Mediated Photodynamic Therapy. *Photodiagn. Photodyn. Ther.* **2022**, *37*, 102646. [CrossRef]
99. Wang, Y.; Zhang, L.; Zhao, G.; Zhang, Y.; Zhan, F.; Chen, Z.; He, T.; Cao, Y.; Hao, L.; Wang, Z.; et al. Homologous Targeting Nanoparticles for Enhanced PDT against Osteosarcoma HOS Cells and the Related Molecular Mechanisms. *J. Nanobiotechnol.* **2022**, *20*, 83. [CrossRef]
100. Oka, T.; Matsuoka, K.; Utsunomiya, A. Sensitive Photodynamic Detection of Adult T-Cell Leukemia/Lymphoma and Specific Leukemic Cell Death Induced by Photodynamic Therapy: Current Status in Hematopoietic Malignancies. *Cancers* **2020**, *12*, 335. [CrossRef]
101. Dong, X.; Zeng, Y.; Zhang, Z.; Fu, J.; You, L.; He, Y.; Hao, Y.; Gu, Z.; Yu, Z.; Qu, C.; et al. Hypericin-Mediated Photodynamic Therapy for the Treatment of Cancer: A Review. *J. Pharm. Pharmacol.* **2021**, *73*, 425–436. [CrossRef] [PubMed]
102. Xu, Y.; Wang, D.; Zhuang, Z.; Jin, K.; Zheng, L.; Yang, Q.; Guo, K. Hypericin-Mediated Photodynamic Therapy Induces Apoptosis in K562 Human Leukemia Cells through JNK Pathway Modulation. *Mol. Med. Rep.* **2015**, *12*, 6475–6482. [CrossRef] [PubMed]
103. Salmerón, M.L.; Quintana-Aguiar, J.; De La Rosa, J.V.; López-Blanco, F.; Castrillo, A.; Gallardo, G.; Tabraue, C. Phenalenone-photodynamic Therapy Induces Apoptosis on Human Tumor Cells Mediated by Caspase-8 and P38-MAPK Activation. *Mol. Carcinog.* **2018**, *57*, 1525–1539. [CrossRef]
104. Yin, H.; Ye, X.; Niu, Q.; Wang, C.; Li, Y. Time Course of Apoptosis Induced by Photodynamic Therapy with PsD007 in LT12 Acute Myeloid Leukemia Cells. *Lasers Med. Sci.* **2016**, *31*, 817–824. [CrossRef] [PubMed]
105. Cisáriková, A.; Barbieriková, Z.; Janovec, L.; Imrich, J.; Hunáková, L.; Bačová, Z.; Paulíková, H. Acridin-3,6-Dialkyldithiourea Hydrochlorides as New Photosensitizers for Photodynamic Therapy of Mouse Leukemia Cells. *Bioorg. Med. Chem.* **2016**, *24*, 2011–2022. [CrossRef]
106. Sekhoacha, M.; Riet, K.; Motloung, P.; Gumenku, L.; Adegoke, A.; Mashele, S. Prostate Cancer Review: Genetics, Diagnosis, Treatment Options, and Alternative Approaches. *Molecules* **2022**, *27*, 5730. [CrossRef]

107. Xue, Q.; Zhang, J.; Jiao, J.; Qin, W.; Yang, X. Photodynamic Therapy for Prostate Cancer: Recent Advances, Challenges and Opportunities. *Front. Oncol.* **2022**, *12*, 980239. [CrossRef]
108. Girotti, A.W. Tumor-Generated Nitric Oxide as an Antagonist of Photodynamic Therapy. *Photochem. Photobiol. Sci.* **2015**, *14*, 1425–1432. [CrossRef]
109. Girotti, A.W. Role of Endogenous Nitric Oxide in Hyperaggressiveness of Tumor Cells That Survive a Photodynamic Therapy Challenge. *Crit. Rev. Oncog.* **2016**, *21*, 353–363. [CrossRef]
110. Rapozzi, V.; Varchi, G.; Della Pietra, E.; Ferroni, C.; Xodo, L.E. A Photodynamic Bifunctional Conjugate for Prostate Cancer: An in Vitro Mechanistic Study. *Investig. New Drugs* **2017**, *35*, 115–123. [CrossRef]
111. Frame, F.M.; Savoie, H.; Bryden, F.; Giuntini, F.; Mann, V.M.; Simms, M.S.; Boyle, R.W.; Maitland, N.J. Mechanisms of Growth Inhibition of Primary Prostate Epithelial Cells Following Gamma Irradiation or Photodynamic Therapy Include Senescence, Necrosis, and Autophagy, but Not Apoptosis. *Cancer Med.* **2016**, *5*, 61–73. [CrossRef] [PubMed]
112. Liu, L.-Y.; Man, X.-X.; Yao, H.-X.; Tan, Y.-Y. Effects of Pheophorbide A-Mediated Photodynamic Therapy on Proliferation and Metastasis of Human Prostate Cancer Cells. *Eur. Rev. Med. Pharmacol. Sci.* **2017**, *21*, 5571–5579. [CrossRef] [PubMed]
113. Zhang, Z.; Tan, Y.; Huang, C.; Wei, X. Redox Signaling in Drug-Tolerant Persister Cells as an Emerging Therapeutic Target. *EBioMedicine* **2023**, *89*, 104483. [CrossRef] [PubMed]
114. Aksel, M.; Bozkurt-Girit, O.; Bilgin, M.D. Pheophorbide A-Mediated Sonodynamic, Photodynamic and Sonophotodynamic Therapies against Prostate Cancer. *Photodiagn. Photodyn. Ther.* **2020**, *31*, 101909. [CrossRef] [PubMed]
115. Kocaağa, N.; Türkkol, A.; Bilgin, M.D.; Erdoğmuş, A. The Synthesis of Novel Water-Soluble Zinc (II) Phthalocyanine Based Photosensitizers and Exploring of Photodynamic Therapy Activities on the PC3 Cancer Cell Line. *Photochem. Photobiol. Sci.* **2023**, *22*, 2037–2053. [CrossRef]
116. de Melo Gomes, L.C.; de Oliveira Cunha, A.B.; Peixoto, L.F.F.; Zanon, R.G.; Botelho, F.V.; Silva, M.J.B.; Pinto-Fochi, M.E.; Góes, R.M.; de Paoli, F.; Ribeiro, D.L. Photodynamic Therapy Reduces Cell Viability, Migration and Triggers Necroptosis in Prostate Tumor Cells. *Photochem. Photobiol. Sci.* **2023**, *22*, 1341–1356. [CrossRef]
117. Desai, K.; McManus, J.M.; Sharifi, N. Hormonal Therapy for Prostate Cancer. *Endocr. Rev.* **2021**, *42*, 354–373. [CrossRef]
118. Gheewala, T.; Skwor, T.; Munirathinam, G. Photodynamic Therapy Using Pheophorbide and 670 Nm LEDs Exhibits Anti-Cancer Effects in-Vitro in Androgen Dependent Prostate Cancer. *Photodiagn. Photodyn. Ther.* **2018**, *21*, 130–137. [CrossRef]
119. Gheewala, T.; Ali Hussein, M.; Munirathinam, G. Photoactivation of Pheophorbide-a Utilizing 670 Nm LEDs Elicits Cancer Suppressive Effects in Androgen-Independent Prostate Cancer Cellular Models. *J. Pharm. Pharmacol.* **2023**, *75*, 1544–1559. [CrossRef]
120. Ayaz, F.; Yetkin, D.; Yüzer, A.; Demircioğlu, K.; Ince, M. Non-Canonical Anti-Cancer, Anti-Metastatic, Anti-Angiogenic and Immunomodulatory PDT Potentials of Water Soluble Phthalocyanine Derivatives with Imidazole Groups and Their Intracellular Mechanism of Action. *Photodiagn. Photodyn. Ther.* **2022**, *39*, 103035. [CrossRef]
121. Dobruch, J.; Oszczudłowski, M. Bladder Cancer: Current Challenges and Future Directions. *Medicina* **2021**, *57*, 749. [CrossRef] [PubMed]
122. Kochergin, M.; Fahmy, O.; Asimakopoulos, A.; Theil, G.; Zietz, K.; Bialek, J.; Tiberi, E.; Gakis, G. Photodynamic Therapy: Current Trends and Potential Future Role in the Treatment of Bladder Cancer. *Int. J. Mol. Sci.* **2024**, *25*, 960. [CrossRef] [PubMed]
123. Lihuan, D.; Jingcun, Z.; Ning, J.; Guozeng, W.; Yiwei, C.; Wei, L.; Jing, Q.; Yuanfang, Z.; Gang, C. Photodynamic Therapy with the Novel Photosensitizer Chlorophyllin f Induces Apoptosis and Autophagy in Human Bladder Cancer Cells. *Lasers Surg. Med.* **2014**, *46*, 319–334. [CrossRef] [PubMed]
124. Berndt-Paetz, M.; Weimann, A.; Sieger, N.; Schastak, S.; Riyad, Y.M.; Griebel, J.; Arthanareeswaran, V.K.A.; Stolzenburg, J.-U.; Neuhaus, J. Tetrahydroporphyrin-Tetratosylat (THPTS): A near-Infrared Photosensitizer for Targeted and Efficient Photodynamic Therapy (PDT) of Human Bladder Carcinoma. An in Vitro Study. *Photodiagn. Photodyn. Ther.* **2017**, *18*, 244–251. [CrossRef] [PubMed]
125. Zhuo, Z.; Song, Z.; Ma, Z.; Zhang, Y.; Xu, G.; Chen, G. Chlorophyllin e6-mediated Photodynamic Therapy Inhibits Proliferation and Induces Apoptosis in Human Bladder Cancer Cells. *Oncol. Rep.* **2019**, *41*, 2181–2193. [CrossRef]
126. Robeldo, T.; Ribeiro, L.S.; Manrique, L.; Kubo, A.M.; Longo, E.; Camargo, E.R.; Borra, R.C. Modified Titanium Dioxide as a Potential Visible-Light-Activated Photosensitizer for Bladder Cancer Treatment. *ACS Omega* **2022**, *7*, 17563–17574. [CrossRef]
127. Buskwofie, A.; David-West, G.; Clare, C.A. A Review of Cervical Cancer: Incidence and Disparities. *J. Natl. Med. Assoc.* **2020**, *112*, 229–232. [CrossRef]
128. Sharma, S.; Deep, A.; Sharma, A.K. Current Treatment for Cervical Cancer: An Update. *Anticancer Agents Med. Chem.* **2020**, *20*, 1768–1779. [CrossRef]
129. Afanasiev, M.S.; Dushkin, A.D.; Grishacheva, T.G.; Afanasiev, S.S.; Karaulov Academician, A.V. Photodynamic Therapy for Early-Stage Cervical Cancer Treatment. *Photodiagn. Photodyn. Ther.* **2022**, *37*, 102620. [CrossRef]
130. Miao, F.; Lv, T.; Zhang, Y.; Huang, Z.; Wang, X.; Wang, H. Induction of Apoptosis in HPV16 E7 Transfected Human Keratinocyte by ALA-Mediated Photodynamic Therapy. *Photodiagn. Photodyn. Ther.* **2016**, *13*, 205–210. [CrossRef]

131. Wu, S.X.; Ren, X.Y.; Pan, Y.L.; Guan, D.D.; Liu, S.; Zou, X.Y.; Shi, C.G.; Li, Y.Z.; Zhang, Y.Z. Effects of ALA-PDT on HPV16-Immortalized Cervical Epithelial Cell. *Neoplasma* **2017**, *64*, 175–181. [CrossRef] [PubMed]
132. Cheng, J.; Li, W.; Tan, G.; Wang, Z.; Li, S.; Jin, Y. Synthesis and in Vitro Photodynamic Therapy of Chlorin Derivative 131-Ortho-Trifluoromethyl-Phenylhydrazone Modified Pyropheophorbide-a. *Biomed. Pharmacother.* **2017**, *87*, 263–273. [CrossRef] [PubMed]
133. Li, Z.; Xiao, Z.; Feng, Y.; Wang, Q.; Teng, M. Mechanism of a New Photosensitizer (TBZPy) in the Treatment of High-Risk Human Papillomavirus-Related Diseases. *Photodiagn. Photodyn. Ther.* **2022**, *37*, 102591. [CrossRef] [PubMed]
134. He, G.; Mu, T.; Yuan, Y.; Yang, W.; Zhang, Y.; Chen, Q.; Bian, M.; Pan, Y.; Xiang, Q.; Chen, Z.; et al. Effects of Notch Signaling Pathway in Cervical Cancer by Curcumin Mediated Photodynamic Therapy and Its Possible Mechanisms In Vitro and In Vivo. *J. Cancer* **2019**, *10*, 4114–4122. [CrossRef]
135. Xie, J.; Wang, S.; Li, Z.; Ao, C.; Wang, J.; Wang, L.; Peng, X.; Zeng, K. 5-Aminolevulinic Acid Photodynamic Therapy Reduces HPV Viral Load via Autophagy and Apoptosis by Modulating Ras/Raf/MEK/ERK and PI3K/AKT Pathways in HeLa Cells. *J. Photochem. Photobiol. B* **2019**, *194*, 46–55. [CrossRef]
136. Li, Z.; Teng, M.; Wang, Y.; Wang, Q.; Feng, Y.; Xiao, Z.; Li, C.; Zeng, K. The Mechanism of 5-aminolevulinic Acid Photodynamic Therapy in Promoting Endoplasmic Reticulum Stress in the Treatment of HR-HPV-infected HeLa Cells. *Photodermatol. Photoimmunol. Photomed.* **2021**, *37*, 348–359. [CrossRef]
137. Damke, G.M.Z.F.; Damke, E.; de Souza Bonfim-Mendonça, P.; Ratti, B.A.; de Freitas Meirelles, L.E.; da Silva, V.R.S.; Gonçalves, R.S.; César, G.B.; de Oliveira Silva, S.; Caetano, W.; et al. Selective Photodynamic Effects on Cervical Cancer Cells Provided by P123 Pluronic®-Based Nanoparticles Modulating Hypericin Delivery. *Life Sci.* **2020**, *255*, 117858. [CrossRef]
138. Jin, Y.; Guan, Z.; Wang, X.; Wang, Z.; Zeng, R.; Xu, L.; Cao, P. ALA-PDT Promotes HPV-Positive Cervical Cancer Cells Apoptosis and DCs Maturation via MiR-34a Regulated HMGB1 Exosomes Secretion. *Photodiagn. Photodyn. Ther.* **2018**, *24*, 27–35. [CrossRef]
139. Wu, A.; Niu, J.; Hong, Z.; Gu, L.; Huang, Y.; Qiu, L. The Effects of 5-Aminolevulinic Acid Photodynamic Therapy on the Local Immune Response of Women with Cervical Intraepithelial Neoplasia Grade 2. *Front. Immunol.* **2023**, *14*, 1211114. [CrossRef]
140. Thull, T.; Kempton, D. Ovarian Cancer. *JAAPA* **2024**, *37*, 32–36. [CrossRef]
141. Schoutrop, E.; Moyano-Galceran, L.; Lheureux, S.; Mattsson, J.; Lehti, K.; Dahlstrand, H.; Magalhaes, I. Molecular, Cellular and Systemic Aspects of Epithelial Ovarian Cancer and Its Tumor Microenvironment. *Semin. Cancer Biol.* **2022**, *86*, 207–223. [CrossRef] [PubMed]
142. Yokoyama, Y.; Shigeto, T.; Miura, R.; Kobayashi, A.; Mizunuma, M.; Yamauchi, A.; Futagami, M.; Mizunuma, H. Differences in the Sensitivity of Ovarian Cancer to Photodynamic Therapy and the Mechanisms for Those Differences. *Oncol. Lett.* **2017**, *13*, 4933–4938. [CrossRef] [PubMed]
143. Jung, K.-A.; Choi, B.; Kwak, M.-K. The C-MET/PI3K Signaling Is Associated with Cancer Resistance to Doxorubicin and Photodynamic Therapy by Elevating BCRP/ABCG2 Expression. *Mol. Pharmacol.* **2015**, *87*, 465–476. [CrossRef] [PubMed]
144. Saczko, J.; Choromańska, A.; Rembiałkowska, N.; Dubińska-Magiera, M.; Bednarz-Misa, I.; Bar, J.; Marcinkowska, A.; Kulbacka, J. Oxidative Modification Induced by Photodynamic Therapy with Photofrin®II and 2-Methoxyestradiol in Human Ovarian Clear Carcinoma (OvBH-1) and Human Breast Adenocarcinoma (MCF-7) Cells. *Biomed. Pharmacother.* **2015**, *71*, 30–36. [CrossRef]
145. Tian, S.; Yong, M.; Zhu, J.; Zhang, L.; Pan, L.; Chen, Q.; Li, K.-T.; Kong, Y.-H.; Jiang, Y.; Yu, T.-H.; et al. Enhancement of the Effect of Methyl Pyropheophorbide-a-Mediated Photodynamic Therapy Was Achieved by Increasing ROS through Inhibition of Nrf2-HO-1 or Nrf2-ABCG2 Signaling. *Anticancer Agents Med. Chem.* **2018**, *17*, 1824–1836. [CrossRef]
146. Wang, Q.; Suo, Y.; Wang, X.; Wang, Y.; Tian, X.; Gao, Y.; Liu, N.; Liu, R. Study on the Mechanism of Photodynamic Therapy Mediated by 5-Aminoketovalerate in Human Ovarian Cancer Cell Line. *Lasers Med. Sci.* **2021**, *36*, 1873–1881. [CrossRef]
147. Sun, Q.; Suo, Y.; Lv, H.; Wang, Q.; Yin, H. Porphin E6 Complex Loaded with Gold Nanorod Mesoporous Silica Enhances Photodynamic Therapy in Ovarian Cancer Cells in Vitro. *Lasers Med. Sci.* **2023**, *38*, 115. [CrossRef]
148. Qi, D.; Wang, Q.; Li, H.; Zhang, T.; Lan, R.; Kwong, D.W.J.; Wong, W.-K.; Wong, K.-L.; Li, S.; Lu, F. SILAC-Based Quantitative Proteomics Identified Lysosome as a Fast Response Target to PDT Agent Gd-N Induced Oxidative Stress in Human Ovarian Cancer IGROV1 Cells. *Mol. Biosyst.* **2015**, *11*, 3059–3067. [CrossRef]
149. Morabito, F.; Cristani, M.; Saija, A.; Stelitano, C.; Callea, V.; Tomaino, A.; Minciullo, P.L.; Gangemi, S. Lipid Peroxidation and Protein Oxidation in Patients Affected by Hodgkin's Lymphoma. *Mediat. Inflamm.* **2004**, *13*, 381–383. [CrossRef]
150. Liu, H.; Hu, Y.; Sun, Y.; Wan, C.; Zhang, Z.; Dai, X.; Lin, Z.; He, Q.; Yang, Z.; Huang, P.; et al. Co-Delivery of Bee Venom Melittin and a Photosensitizer with an Organic–Inorganic Hybrid Nanocarrier for Photodynamic Therapy and Immunotherapy. *ACS Nano* **2019**, *13*, 12638–12652. [CrossRef]
151. Hou, Y.; Yang, X.; Liu, R.; Zhao, D.; Guo, C.; Zhu, A.; Wen, M.; Liu, Z.; Qu, G.; Meng, H. Pathological Mechanism of Photodynamic Therapy and Photothermal Therapy Based on Nanoparticles. *Int. J. Nanomed.* **2020**, *15*, 6827–6838. [CrossRef] [PubMed]
152. Doix, B.; Trempolec, N.; Riant, O.; Feron, O. Low Photosensitizer Dose and Early Radiotherapy Enhance Antitumor Immune Response of Photodynamic Therapy-Based Dendritic Cell Vaccination. *Front. Oncol.* **2019**, *9*, 811. [CrossRef] [PubMed]

153. Gunaydin, G.; Gedik, M.E.; Ayan, S. Photodynamic Therapy—Current Limitations and Novel Approaches. *Front. Chem.* **2021**, *9*, 691697. [CrossRef] [PubMed]
154. Ji, B.; Wei, M.; Yang, B. Recent Advances in Nanomedicines for Photodynamic Therapy (PDT)-Driven Cancer Immunotherapy. *Theranostics* **2022**, *12*, 434–458. [CrossRef]
155. Wang, Y.; Ma, K.; Kang, M.; Yan, D.; Niu, N.; Yan, S.; Sun, P.; Zhang, L.; Sun, L.; Wang, D.; et al. A New Era of Cancer Phototherapy: Mechanisms and Applications. *Chem. Soc. Rev.* **2024**, *53*, 12014–12042. [CrossRef]
156. Kong, C.; Chen, X. Combined Photodynamic and Photothermal Therapy and Immunotherapy for Cancer Treatment: A Review. *Int. J. Nanomed.* **2022**, *17*, 6427–6446. [CrossRef]
157. Warszyńska, M.; Repetowski, P.; Dąbrowski, J.M. Photodynamic Therapy Combined with Immunotherapy: Recent Advances and Future Research Directions. *Coord. Chem. Rev.* **2023**, *495*, 215350. [CrossRef]
158. Jia, J.; Wu, X.; Long, G.; Yu, J.; He, W.; Zhang, H.; Wang, D.; Ye, Z.; Tian, J. Revolutionizing Cancer Treatment: Nanotechnology-Enabled Photodynamic Therapy and Immunotherapy with Advanced Photosensitizers. *Front. Immunol.* **2023**, *14*, 1219785. [CrossRef]
159. Thiruppathi, J.; Vijayan, V.; Park, I.-K.; Lee, S.E.; Rhee, J.H. Enhancing Cancer Immunotherapy with Photodynamic Therapy and Nanoparticle: Making Tumor Microenvironment Hotter to Make Immunotherapeutic Work Better. *Front. Immunol.* **2024**, *15*, 1375767. [CrossRef]
160. Kobayashi, H.; Choyke, P.L. Near-Infrared Photoimmunotherapy of Cancer. *Acc. Chem. Res.* **2019**, *52*, 2332–2339. [CrossRef]
161. Fukushima, H.; Furusawa, A.; Okada, R.; Fujii, Y.; Choyke, P.L.; Kobayashi, H. Antitumor Host Immunity Enhanced by Near-infrared Photoimmunotherapy. *Cancer Sci.* **2024**. *ahead of print*. [CrossRef] [PubMed]
162. Yang, B.; Chen, Y.; Shi, J. Reactive Oxygen Species (ROS)-Based Nanomedicine. *Chem. Rev.* **2019**, *119*, 4881–4985. [CrossRef] [PubMed]

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Review

The Impact of Ozone on Periodontal Cell Line Viability and Function

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Abstract: Periodontal diseases, including gingivitis and periodontitis, are chronic inflammatory conditions of the teeth' supporting structures that can lead to progressive tissue destruction and loss if left untreated. Basic treatments like scaling and root planing, alone or combined with antimicrobial agents, are the standard of care. However, with the increasing prevalence of antibiotic resistance and the need for new ideas in therapy, adjunctive treatments like ozone therapy have gained attention. Ozone (O_3), a triatomic oxygen molecule, is used because of its strong antimicrobial, anti-inflammatory, and regenerative activity and, hence, as a potential tool in periodontal therapy. This review of the use of ozone therapy in periodontal disease breaks down the multifaceted mechanism of ozone therapy, which includes the selective antimicrobial action against biofilm-associated pathogens, immunomodulatory effects on host cells, and stimulation of tissue repair. O_3 therapy disrupts microbial biofilms, enhances immune cell function, and promotes healing by activating Nuclear Factor Erythroid 2-Related Factor 2 (Nrf2) and Mitogen-Activated Protein Kinase (MAPK) signaling pathways that regulate oxidative stress, inflammation, and apoptosis. Additional findings include its ability to upregulate growth factors and extracellular matrix proteins, which is significant for periodontal tissue regeneration. This review also discusses the application of O_3 therapy in periodontal cell lines, emphasizing its impact on cell viability, proliferation, and differentiation. Advances in periodontal regenerative techniques, combined with the antimicrobial and healing properties of O_3, have demonstrated significant clinical benefits. Challenges, including the need for standardized dosages, effective delivery systems, and long-term studies, are also addressed to ensure safe and effective clinical integration. O_3 therapy, with its dual antimicrobial and regenerative capabilities, offers an innovative adjunctive approach to periodontal treatment. Future research focusing on optimized protocols and evidence-based guidelines is essential to fully realize its potential in enhancing periodontal health and improving patient outcomes.

Keywords: O_3 therapy; periodontal disease; antimicrobial; inflammation modulation; tissue regeneration; periodontal cell lines

1. Introduction

Periodontal diseases, including gingivitis and periodontitis, are a class of oral health issues that impact the supporting structures of the teeth, including the periodontal ligament, cementum, and alveolar bone [1]. Periodontitis is a chronic inflammatory condition, usually of bacterial etiology, resulting in the gradual loss of the soft tissues and bone that support the teeth. Periodontitis may result in permanent tissue deterioration if ignored or insufficiently treated, potentially causing tooth mobility and, eventually, tooth loss. Periodontitis may negatively impact facial aesthetics, functionality, and nutrition, affecting an individual's quality of life and psychological well-being [2]. The management of periodontitis is essential for oral and overall health since there is growing evidence connecting periodontal disease to many systemic conditions, including cardiovascular disease, diabetes, respiratory disease, and adverse pregnancy outcomes [3]. These results underscore the need for efficacious strategies for treatment to mitigate periodontal inflammation and tissue deterioration [4]. The conventional treatment for periodontitis is scaling and root planing (mechanical debridement) to eliminate plaque and calculus in conjunction with antibiotic medication [5]. Nonetheless, the growing issue of antibiotic resistance, along with the need for novel, effective, and sustainable treatment modalities, has compelled researchers to investigate other therapeutic strategies, such as ozone therapy [6,7]. (O_3) is a triatomic oxygen molecule with strong oxidative characteristics that have a substantial antibacterial impact. O_3 not only eradicates bacterial cell walls but also diminishes inflammation and facilitates tissue healing, making it a viable option for periodontal therapy [8]. Ozone may cure infections and facilitate healing in periodontal disease by eliminating harmful bacteria and reducing oxidative stress [9]. Recent investigations have validated the use of O_3 as a therapeutic adjunct in dentistry [10,11]. Evidence indicates that it may impede biofilm development and improve fibroblast activity in vitro; hence, ozone is regarded as an essential instrument in regenerative periodontal treatment [12,13]. Furthermore, periodontal cell lines serve as valuable models in periodontal research for examining cellular responses and molecular processes [14]. These cell lines provide a controlled environment to examine the impacts of pharmaceutical agents and facilitate the comprehension of biological mechanisms, including proliferation, differentiation, and apoptosis [15]. Recent advancements in cell culture techniques, particularly studies on the interaction between O_3 and periodontal ligament stem cells (PDLSCs), have shown their ability to enhance cellular differentiation and matrix formation, both crucial for tissue regeneration [16]. Consequently, periodontal cell lines have emerged as valuable resources in the research of novel pharmaceuticals, the advancement of pharmacological investigations, and the examination of tissue engineering approaches intended to regenerate lost or damaged periodontal tissues [17]. These cellular models are under investigation and may result in enhancements to diagnostic methodologies and therapeutic strategies. Research demonstrated that O_3 treatment promotes the proliferation and migration of periodontal ligament fibroblasts under an oxidative stress environment, elucidating its molecular mode of action [18,19]. These results contribute to the accumulating data endorsing the use of O_3 in addressing inflammatory and bacterial infections, as well as facilitating regenerative healing in periodontitis [20]. This review looks into the impact of O_3 on periodontal cell lines concerning cell viability, proliferation, and differentiation, as well as its use as an adjunctive therapy in the management of periodontal disease.

1.1. Mechanisms of Ozone Action

O_3 therapy is presented as a new way to treat periodontal diseases because of its strong antimicrobial activity. It can be an alternative or supplementary method to conventional antimicrobial agents [21]. O_3, a strong oxidizing agent, is effective against all types of microbial pathogens, including bacteria, viruses, fungi, and protozoa, and can be used to decrease the microbial load in periodontal pockets and may lead to better clinical results. Its interaction with the host eukaryotic cells is also involved in immunomodulation and tissue regeneration, which makes it a good candidate for therapy [22]. Recent advancements in research have expanded our understanding O_3's mechanisms, particularly in distinguishing its effects on prokaryotic versus eukaryotic cells and exploring microbial resistance mechanisms [23–27]. (Table 1).

Table 1. Comparison of ozone's effects on prokaryotic and eukaryotic cells, highlighting targeted structures, mechanisms, and clinical implications.

Aspect	Prokaryotic Cells	Eukaryotic Cells
Targeted Structures	Cell membrane lipids, proteins, and DNA	Host cell signaling pathways, antioxidant systems, and growth factors
Mechanism of Action	Lipid peroxidation, protein oxidation, nucleic acid damage	Activation of Nrf2 and MAPK pathways; modulation of cytokine and growth factor expression
Antioxidant Defense	Limited antioxidant defenses; higher susceptibility to ROS	Robust antioxidant systems, including enzymes like superoxide dismutase and catalase
Effects on Cell Survival	Disruption of cellular integrity, leading to cell lysis and death	Promotes cell survival via ERK pathway; induces controlled apoptosis via JNK and p38 pathways
Effects on DNA	DNA damage through strand breaks and mutations; inhibits replication	Minimal DNA damage due to enhanced antioxidant response; protects genomic stability
Resistance Mechanisms	Upregulation of oxidative stress defense genes in biofilm-associated pathogens	No significant resistance noted; mechanisms focus on repair and modulation
Clinical Implications	Effective for biofilm disruption and microbial eradication; may require combination therapies to address resistance	Promotes tissue repair and regeneration; enhances host immune modulation

1.2. The Multifaceted Mechanisms and Clinical Applications of Ozone Therapy in Periodontal Treatment

Oxidative stress, in which reactive oxygen species (ROSs) such as peroxides, superoxides, and hydroxyl radicals are produced upon contact with microbial cells, is the main antibacterial mechanism of ozone [28]. ROSs provoke lipid peroxidation in microbial membranes, undermining their structural integrity and enhancing permeability. This results

in osmotic imbalances and cellular lysis. Furthermore, ROSs induce protein oxidation, inhibiting enzyme functionality essential for microbial metabolism, and inflict damage on nucleic acids via strand breakage and mutations, thereby compromising microbial reproduction and survival [29,30] (Figure 1).

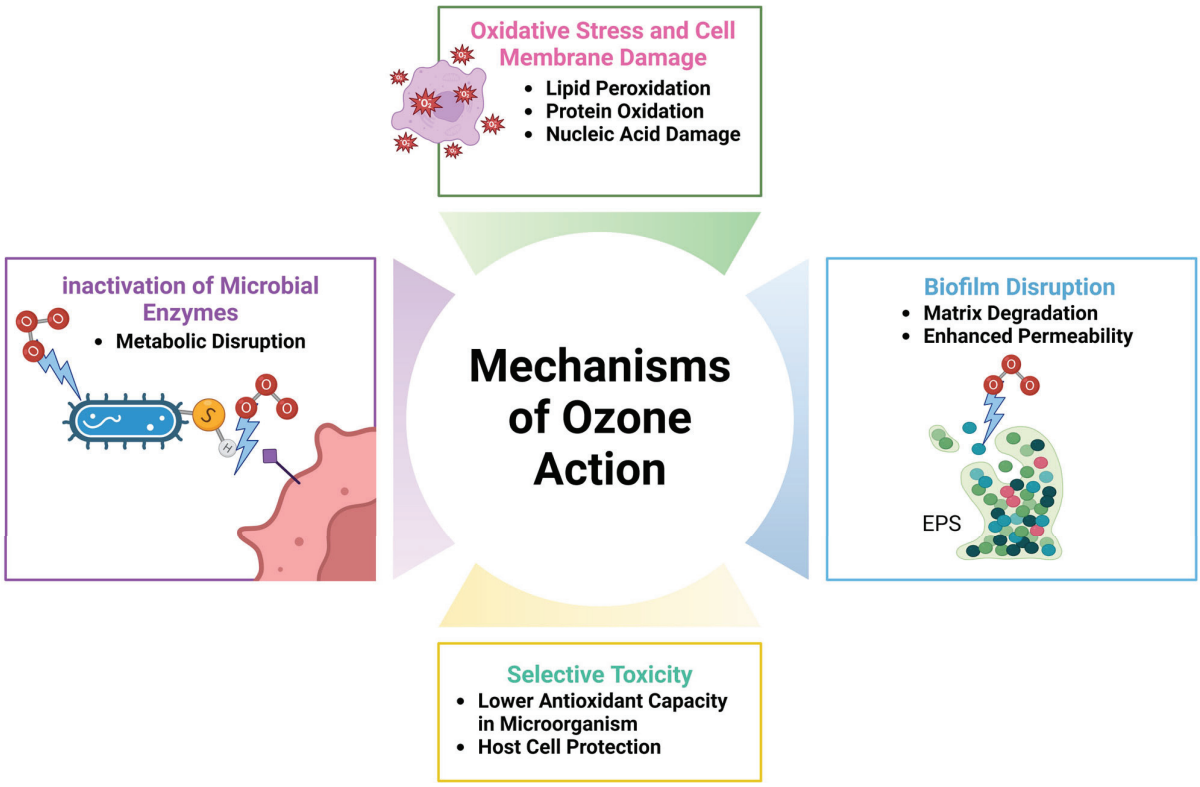


Figure 1. Highlights the mechanisms of O_3 action in microbial control and tissue repair. Ozone induces oxidative stress, leading to lipid peroxidation, protein oxidation, and nucleic acid damage, compromising cell membranes. It disrupts microbial biofilms by degrading the matrix and enhancing permeability. O_3 inactivates microbial enzymes, causing metabolic disruption. Its selective toxicity targets microorganisms with lower antioxidant capacity, sparing host cells and enhancing their protection. These combined actions underscore ozone's therapeutic potential in reducing microbial burden and promoting healing.

The impacts of O_3 impact biofilms, which are dense microbial populations that exhibit resistance to standard treatments [31]. O_3 disrupts the biofilm matrix by degrading extracellular polymeric compounds, increasing biofilm permeability, and facilitating the penetration of antimicrobial drugs to eradicate embedded bacteria [32]. Moreover, O_3's selective toxicity mostly affects bacteria with diminished antioxidant defenses while preserving host cells, which are equipped with strong antioxidant systems like superoxide dismutase and catalase [33]. (Figure 1).

In eukaryotic cells, ozone has immunomodulatory effects by affecting critical signaling pathways and facilitating tissue healing. O_3-induced reactive oxygen species activate the Nrf2 pathway, resulting in the transcription of antioxidant and cytoprotective genes, including glutathione S-transferase and heme oxygenase-1, which safeguard host cells from oxidative damage [34]. The MAPK and (Extracellular Signal-Regulated Kinase (ERK), c-Jun N-terminal Kinase (JNK), and p38 Mitogen-Activated Protein Kinase (p38) pathways simultaneously modulate cellular responses to stress, inflammation, and apoptosis, maintaining equilibrium between cell survival and regulated cell death. For instance, JNK and

p38 facilitate the removal of injured cells by apoptosis, while ERK promotes cell survival and aids in tissue regeneration [35]. (Figure 2).

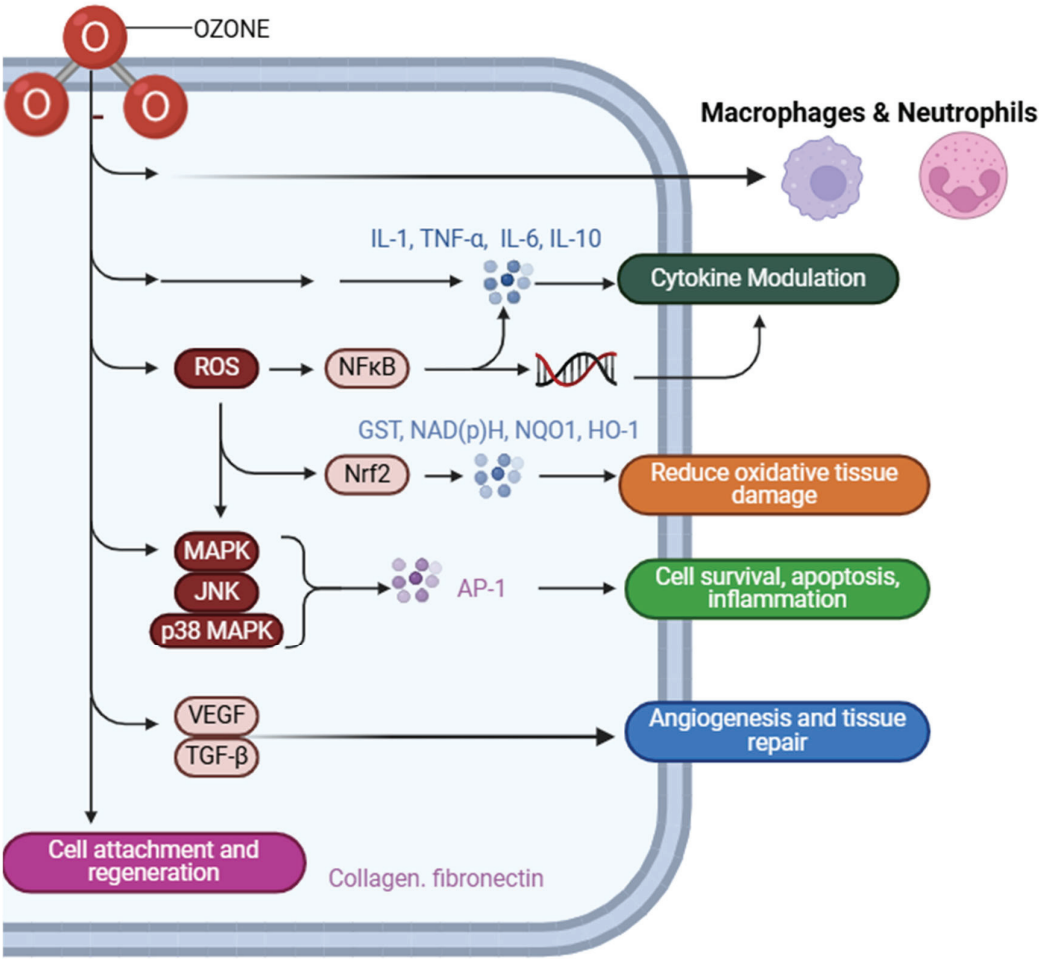


Figure 2. Illustrates the cellular and molecular mechanisms activated by O_3 therapy. ROS stimulate NF-κB and MAPK pathways, leading to cytokine modulation (IL-1, TNF-α, IL-6, IL-10) and promoting cell survival, apoptosis, and inflammation. Concurrently, the Nrf2 pathway enhances antioxidant responses, reducing oxidative tissue damage through upregulation of GST, NAD(P)H, NQO1, and HO-1. VEGF and TGF-β mediate angiogenesis and tissue repair, while collagen and fibronectin contribute to cell attachment and regeneration. The interplay of macrophages and neutrophils further supports immune modulation and tissue healing.

O_3 amplifies the function of macrophages and neutrophils in immune cells, which are essential elements of the innate immune system. It enhances these cells' phagocytic activity and the synthesis of antimicrobial agents, including reactive nitrogen species and cytokines, therefore augmenting the host's capacity to battle periodontal infections. O_3 influences dendritic cells, enhancing antigen presentation and promoting adaptive immune responses [36,37]. Moreover, O_3 regulates cytokine synthesis, equilibrating pro-inflammatory and anti-inflammatory responses. It downregulates cytokines such as Interleukin-1 Beta (IL-1β), Interleukin-6 (IL-6), and Tumor Necrosis Factor Alpha (TNF-α) to mitigate excessive inflammation while enhancing Interleukin-10 (IL-10), an anti-inflammatory cytokine, to foster an environment favorable to healing and tissue repair [26,38,39]. (Figure 2).

O_3 furthermore influences gene expression associated with inflammation, apoptosis, and repair. It first enhances the production of pro-inflammatory cytokines to facilitate pathogen elimination [40]. Nonetheless, its stimulation of the Nrf2 pathway mitigates this reaction, resulting in the transcription of anti-inflammatory genes and diminishing

collateral tissue damage. Moreover, O_3 activates growth factors such as Vascular Endothelial Growth Factor (VEGF) and Transforming Growth Factor Beta (TGF-β), facilitating angiogenesis, collagen synthesis, and extracellular matrix creation, which are critical for wound healing and the regeneration of periodontal tissues [41]. Through the activation of essential pathways of signaling, such as the Nrf2, ozone regulates the transcription of genes involved in oxidative stress response, inflammation, and tissue repair. The Nrf2 pathway promotes the expression of antioxidant enzymes like glutathione S-transferase (GST) and heme oxygenase-1 (HO-1), which mitigate oxidative damage and enhance cellular resilience [42,43].

The MAPK pathways further mediate cellular stress responses, with the ERK pathway promoting cell survival and tissue regeneration, while the JNK and p38 pathways facilitate controlled apoptosis to eliminate damaged or infected cells [44]. Controlled cell death, or apoptosis, is a crucial aspect of ozone's effect on gene expression. O_3 regulates apoptosis by activating MAPK pathways, including JNK and p38 MAPK, which upregulate pro-apoptotic genes such as *Bax* and *caspases.* This process ensures the removal of damaged or infected cells, preventing their persistence and promoting tissue homeostasis. At the same time, the ERK pathway is activated, enhancing the expression of anti-apoptotic genes like *Bcl-2* [45]. This action prevents premature apoptosis of healthy cells, preserving their functionality and contributing to tissue stability. By carefully modulating apoptotic processes, O_3 ensures that only compromised cells are eliminated while supporting the survival of essential ones, facilitating repair and regeneration [46].

O_3's ability to promote tissue repair is mediated through the upregulation of growth factors and extracellular matrix proteins. For instance, O_3 stimulates the expression of VEGF and TGF-β, which are vital for angiogenesis, fibroblast proliferation, and collagen. synthesis. These processes enhance wound healing and facilitate the regeneration of periodontal tissues [47]. Additionally, O_3 upregulates genes responsible for the production of matrix proteins such as collagen and fibronectin, which provide the structural framework necessary for tissue remodeling and regeneration [47]. These processes facilitate the restoration of periodontal architecture and functionality, emphasizing O_3 's role in periodontal tissue recovery [48]. The literature has offered profound insights into O_3 's impact on gene expression. Studies indicate that ozone-induced Nrf2 activation boosts antioxidant defenses and promotes the expression of genes related to extracellular matrix synthesis, enhancing cellular resilience and facilitating tissue healing [49,50].

O_3's antibacterial, immunomodulatory, and regenerative capabilities make it a notable adjunct in periodontal therapy. O_3 therapy has dual advantages by improving immune cell functionality, regulating the inflammatory response, and facilitating tissue regeneration, thus aiding in infection control and healing enhancement [9,51].

Nevertheless, individualized application tactics are essential to combat the increasing rates of microbial resistance and to attain improved clinical results, hence enhancing the treatment of periodontal diseases [52]. Recent studies have further elucidated the immunomodulatory effects of O_3. These investigations corroborate its capacity to activate the immune system without inflicting considerable harm to surrounding tissues, providing novel insights into its use in clinical environments [38,53]. According to Ma et al., periodontal tissues offer a protective–regenerative function via ozone-induced Nrf2 activation, which decreases oxidative stress and stimulates cellular proliferation [54].

1.3. Periodontal Cell Line in Periodontal Disease

Periodontal cell lines are laboratory-cultured cells derived from the tissues that comprise the periodontium, including the gingiva, periodontal ligament, cementum, and alveo-

lar bone [55]. These cell lines offer an essential framework for examining the pathogenesis of periodontal disease and developing new therapeutic strategies [55].

Each cell type within the periodontium plays a distinct role in maintaining the integrity of periodontal tissues [55]. Gingival fibroblasts are essential for producing extracellular matrix components and collagen, which contribute to the resilience and repair of gingival tissues while defending against bacterial invasion and inflammation. Periodontal ligament fibroblasts anchor teeth to the surrounding alveolar bone, mediate responses to mechanical forces, and facilitate tissue repair and regeneration [56]. Cementoblasts are responsible for forming cementum, the mineralized tissue that anchors the periodontal ligament to the tooth root, while osteoblasts and osteoclasts regulate alveolar bone remodeling, ensuring structural stability and adaptation to physiological or pathological changes (Figure 3) [57,58]. Recent studies have utilized periodontal cell lines, including gingival fibroblasts, periodontal ligament (PDL) cells, and stem cells derived from periodontal tissues, to explore the molecular mechanisms driving these pathological processes and to create targeted therapies that reflect our evolving understanding of the disease [17,59].

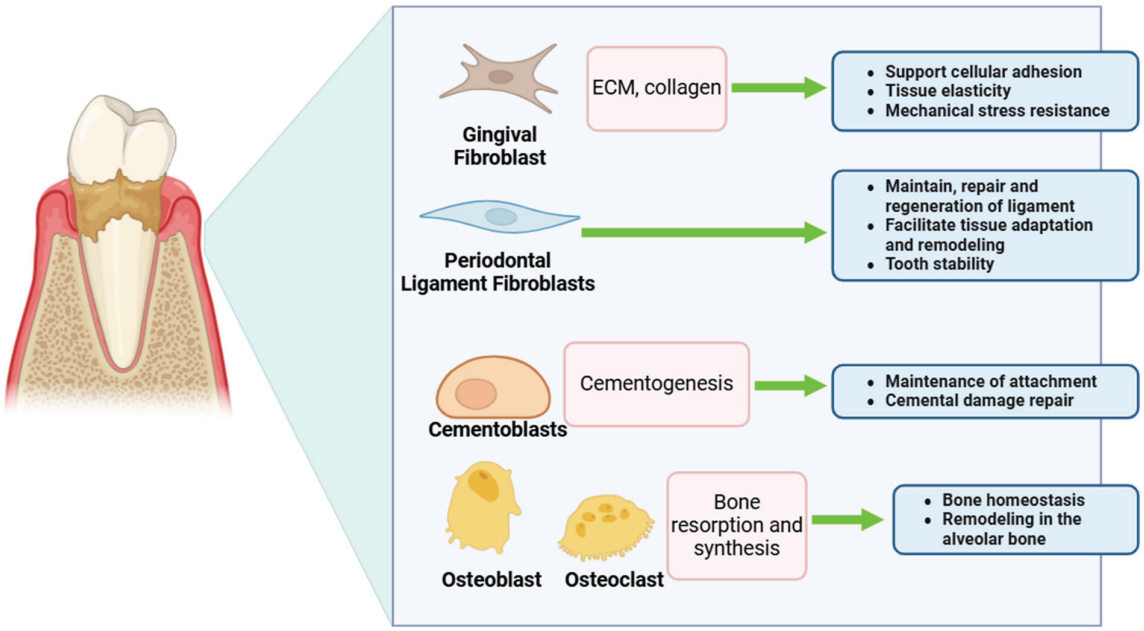


Figure 3. Illustrates the roles of different cells in periodontal tissue health and repair. Gingival fibroblasts produce (ECM) and collagen, essential for cellular adhesion, tissue elasticity, and mechanical stress resistance. Periodontal ligament fibroblasts maintain, repair, and regenerate ligament tissue, promoting adaptation and tooth stability. Cementoblasts support cementogenesis, ensuring attachment maintenance and cemental damage repair. Osteoblasts and osteoclasts coordinate bone resorption and synthesis, maintaining bone homeostasis and remodeling in the alveolar bone. Together, these cellular interactions preserve periodontal structure and function.

Gingival fibroblasts have a dual role in the inflammatory phase of periodontal disease. They not only participate in the inflammatory process but also produce pro-inflammatory cytokines like IL-6 and TNF-α in response to microbial pathogens. Additionally, these fibroblasts are vital for the integrity and repair of tissue through the production of extracellular matrix (ECM) components, including collagen and fibronectin, which are essential for maintaining tissue structure [60]. Understanding how these cells alternate between destructive and reparative functions has revealed new therapeutic targets for regulating their behavior throughout disease progression [60].

PDL cells, which anchor teeth to the alveolar bone, are a key focus of periodontal research. These cells are directly affected during disease progression, as inflammatory mediators diminish their ability to maintain periodontal attachment and support homeostasis [61]. Adaptation and repair under favorable settings have been shown in recent research employing PDL cell lines, which have focused on their involvement in reacting to mechanical stress and inflammatory stimuli [61,62]. New possibilities for periodontal regeneration have emerged as a result of cellular and molecular biology discoveries that show PDL cells may develop into osteoblasts and cementoblasts. Molecular mechanisms such as Wnt, MAPK, and TGF-β signaling control this differentiation and are now being investigated as potential therapeutic targets for repairing periodontal tissues that have been injured [61,63].

A fundamental aspect of regenerative periodontal therapy is stem cells derived from periodontal tissues, known as PDLSCs. Osteoblasts, cementoblasts, and fibroblasts are three critical cell types involved in periodontal tissue regeneration, and these multipotent cells have shown the ability to differentiate into all three [64]. The potential of PDLSCs to upregulate mineralization-related genes, such as alkaline phosphatase and osteocalcin, has been linked to enhanced bone and ligament repair in preclinical models [65]. Alveolar bone loss and periodontal attachment issues may be effectively addressed through tissue engineering techniques made possible by the regenerative capabilities of these cells. These techniques encompass scaffold-based treatments and the administration of bioactive molecules [66]. By incorporating these discoveries into our understanding of the molecular and cellular processes that contribute to periodontal disease, periodontal cell lines are a priceless tool for bridging the gap between the laboratory and the clinical application [65,66]. These cell models have not only advanced our understanding of disease pathogenesis but have also expedited the development of innovative treatments aimed at regulating inflammation, restoring tissue integrity, and preventing disease recurrence [67]. With ongoing advancements in cellular and molecular research, periodontal cell lines continue to lead efforts to combat periodontal disease and enhance patient outcomes [67] (Table 2).

Table 2. Highlights the diverse research applications of periodontal cell lines, encompassing pathophysiological mechanisms, therapeutic development, tissue engineering, and genetic studies. Each application provides insights and potential outcomes for advancing periodontal disease management and treatment strategies.

Research Area	Applications	Potential Outcomes
Pathophysiological Mechanisms	Study cellular responses to bacterial infections, inflammation, and oxidative stress.	Identify therapeutic targets to halt or reverse periodontal disease progression.
Therapeutic Development	Test new drugs and therapeutic strategies in vitro before animal models or clinical trials.	Improve drug safety and effectiveness for potential periodontal treatments.
Tissue Engineering and Regeneration	Develop biocompatible scaffolds and biomaterials to support periodontal tissue regeneration.	Enhance tissue recovery and repair periodontal defects with innovative regenerative strategies.
Genetic Studies	Investigate the roles of specific genes in periodontal health and disease.	Highlight genetic risk factors and develop gene therapies for periodontal disease management.

1.4. Effect of Ozone on Periodontal Cell Lines

O_3 therapy, recognized for its antimicrobial, anti-inflammatory, and oxidative stress-modulating properties, has demonstrated promising results in enhancing the functional capabilities of PDLSCs [68]. For example, O_3 exposure has been linked to increased proliferation and differentiation of PDLSCs into osteogenic and fibroblastic lineages, facilitating periodontal regeneration [69]. Moreover, ozone has been shown to upregulate the production of growth factors and extracellular matrix components, improving the regenerative microenvironment necessary for tissue repair [70]. These findings indicate that O_3 therapy could serve as a valuable adjunct in periodontal treatments aimed at harnessing the regenerative capacity of PDLSCs [69,70]. To maximize the therapeutic outcomes of O_3 while minimizing potential adverse effects, careful attention must be given to the dosage and application duration. Based on the literature, an O_3 concentration of 10–40 μg/mL applied for short intervals (30–60 s per treatment cycle) has been shown to stimulate cellular proliferation and differentiation without inducing cytotoxicity [71]. Prolonged or excessive ozone exposure, however, can lead to oxidative damage, highlighting the need for precise control in clinical applications [72].

Implementing these advancements to restore damaged tissues and regenerate periodontal structures offers hope for improved outcomes in patients with periodontitis.

1.5. Therapeutic Applications of Ozone in Periodontal Therapy

The ability of O_3 to selectively target pathogenic microorganisms, modulate host immune responses and promote tissue repair makes it a promising tool for managing periodontal disease, characterized by chronic inflammation and microbial biofilms [73]. Numerous studies have highlighted O_3's effectiveness in periodontal therapy, particularly for its role in reducing bacterial loads in periodontal pockets. Controlled O_3 application has demonstrated significant reductions in periodontal pathogens such as *Porphyromonas gingivalis* and *Tannerella forsythia*, which are key contributors to disease progression [74–78]. This targeted antimicrobial action not only disrupts biofilms but also prevents recolonization, making it an effective adjunct to scaling and root planing (SRP), the gold standard in periodontal therapy [77].

In addition to its antimicrobial effects, ozone has been shown to modulate inflammatory responses in periodontal tissues. It reduces pro-inflammatory cytokines IL-1β, IL-6, and TNF-α while enhancing anti-inflammatory mediators such as IL-10 [19,38]. This dual action contributes to a balanced immune response, which is critical for mitigating tissue destruction and promoting healing in periodontal therapy [79].

While O_3 therapy offers several advantages, it is not without limitations. The lack of long-term clinical trials evaluating its efficacy in comparison to standard treatments like chlorhexidine and SRP limits its widespread adoption [80]. Additionally, adverse effects such as transient tissue irritation have been reported, emphasizing the importance of adhering to established protocols. Nevertheless, O_3's unique ability to combine antimicrobial, anti-inflammatory, and regenerative properties positions it as a valuable tool in the comprehensive management of periodontal disease [81].

Integrating O_3 therapy into periodontal practice provides a promising opportunity to enhance therapeutic outcomes, particularly in cases of refractory periodontitis or patients with contraindications to conventional antimicrobials [80,81]. Future research should focus on standardized protocols, comparative studies with established treatments, and patient-reported outcomes to solidify O_3's role in evidence-based periodontal care.

1.6. Therapeutic Applications of Ozone in Dentistry

1.6.1. Endodontic Treatment

While this review is primarily about periodontal therapy, it is important to note ozone's use in endodontics as well. The problem of eliminating biofilms in a complex root canal structure has made ozone a potential additional tool in root canal disinfection [82]. Ozone can penetrate biofilm matrices and has very strong antimicrobial action that is very efficient in reducing bacterial numbers, including resistant species like Enterococcus faecalis [83–85]. It has been shown that including ozone in endodontic treatments lowers the chance of reinfection, enhances the success rate of treatments, and benefits patients. For instance, Sinha, et al. found that microbial numbers were greatly reduced after ozone treatment than with conventional chemical irrigants [86].

1.6.2. Oral Surgery

Ozone therapy in oral surgery is useful in preventing postoperative infections, enhancing wound healing, and increasing patient comfort. The antimicrobial and regenerative properties of ozone are particularly valuable in surgical procedures that involve tissue resection or implant placement [87,88].

Clinical studies have shown that ozone decreases the occurrence of postoperative problems by decreasing bacterial numbers and enhancing the rate of tissue regeneration. For example, the application of ozonated oils has been found to stimulate angiogenesis and improve fibroblast activity, thus enhancing the healing rate and reducing the healing time. Furthermore, the use of gaseous ozone in implant surgeries has been linked with better osseointegration, providing additional advantages in surgical outcomes [89–92].

1.6.3. Balancing Ozone Exposure in Therapeutic Applications

Ozone therapy presents a powerful tool in periodontal treatment, offering significant antimicrobial, anti-inflammatory, and regenerative benefits. However, its therapeutic efficacy depends heavily on precise dosage control, localized application, and a clear understanding of its cellular mechanisms. This balance ensures maximum therapeutic outcomes while minimizing potential cytotoxic effects [10].

1.6.4. Controlled Dosage

Ozone's therapeutic efficacy and safety hinge on carefully controlling dosage and exposure time [78].

According to research, maintaining ozone concentrations within the therapeutic window is essential for optimizing its benefits without overtaxing the tissue's antioxidant mechanisms. For example, studies have shown that gaseous ozone concentrations of 10–40 μg/mL and exposure durations of 30–60 s effectively generate antibacterial activity while preserving cellular viability. Ozone may overcome cellular defenses and have cytotoxic effects at higher concentrations due to its ability to induce excessive oxidative stress. On the other hand, its hormetic benefits, which boost cellular resilience and repair processes, are enhanced at regulated, lower dosages [71,93].

In periodontal treatment, for example, clinicians must follow precise, well-established protocols to maximize results and minimize risks. Implementing real-time concentration monitoring and other innovations in ozone delivery systems allows practitioners to apply ozone consistently and safely [94].

1.6.5. Localized Application

Localized application of ozone is a critical strategy to enhance its specificity and reduce systemic exposure. In periodontal therapy, targeted ozone delivery into periodontal

pockets ensures concentrated antimicrobial action at the site of infection without affecting surrounding healthy tissues [95]. Recent clinical studies demonstrate that localized ozone application reduces bacterial counts within periodontal pockets while improving clinical attachment levels and pocket-depth reduction [20,96].

Beyond periodontal therapy, localized ozone application has shown efficacy in various clinical conditions, including peri-implantitis and endodontic infections [97,98]. For example, ozonated water irrigation has been used successfully in endodontic therapy to reduce biofilm-associated pathogens, while topical ozone application has demonstrated wound-healing benefits in oral surgical sites [99]. Such studies emphasize the versatility of localized ozone therapy in diverse clinical scenarios, highlighting its ability to target specific areas with precision and minimal adverse effects [96–98].

1.6.6. Induction of Protective Cellular Responses

Inducing defensive cellular responses via hormesis is one of the most fascinating elements of ozone treatment. Low-dose ozone exposure triggers adaptive cellular pathways that produce moderate oxidative stress to improve tissue resilience and recovery [93,100]. Crucial to this process is the Nrf2 (Nuclear Factor Erythroid 2-Related Factor 2) pathway, which increases the expression of genes that code for antioxidant enzymes including SOD, catalase, and glutathione peroxidase. Protecting cellular components and aiding in tissue repair, these enzymes fight oxidative stress [101].

New studies have shown that different kinds of tissues react differently to oxidative stress, and this has led researchers to focus on the tissue-specific processes of ozone-induced hormesis [102]. For instance, periodontal ligament fibroblasts may increase matrix production and proliferation when exposed to low levels of ozone, yet epithelial cells can demonstrate higher antioxidant activity and less inflammation. These anticipated outcomes may suggest that the hormetic effects of ozone are strongly dependent on the specific tissue type and local microenvironment [103]. By looking into these processes, we may approach the use of ozone in regenerative periodontal treatments from a novel point of view. Through precise dosing and administration, clinicians may tap into ozone's capacity to promote defensive responses, hasten wound healing, and heighten periodontal tissue regeneration.

2. Challenges and Considerations

Despite its potential, ozone therapy faces several challenges that must be addressed for safe and effective integration into clinical practice:

- Standardization of Dosage: One of the primary challenges with ozone therapy is determining the precise concentration and exposure time required for optimal effects on periodontal tissues. While lower ozone concentrations are associated with beneficial effects on cellular proliferation and antimicrobial activity, higher concentrations can be cytotoxic, causing damage to host tissues. Rigorous research is required to establish dosage parameters that balance therapeutic efficacy with cellular safety, ensuring reproducible and predictable results in clinical settings.
- Development of Effective Delivery Systems: To harness the benefits of ozone therapy, it is essential to design and develop advanced delivery systems that allow for precise, controlled, and targeted application. Effective delivery methods would ensure that ozone reaches specific areas within periodontal pockets or damaged tissues without impacting surrounding healthy structures. Various forms, such as gaseous ozone, ozonated water, and ozone oils, have been explored, yet more research is needed to identify the most efficient and practical application methods that maximize therapeutic impact while minimizing systemic exposure.

- Assessment of Long-term Effects: While short-term benefits of ozone therapy in periodontal treatment have been documented, there is a lack of extensive longitudinal studies that evaluate its long-term impact on periodontal tissues and overall oral health. Understanding the effects of repeated ozone applications on tissue integrity, cellular health, and periodontal stability is crucial to determining its safety and efficacy over extended periods. Long-term studies will provide insight into potential risks, cumulative effects, and the possibility of chronic tissue alterations or adaptive responses to ozone exposure.

Further investigation into these areas will be instrumental in overcoming the current challenges associated with ozone therapy, refining its application, and unlocking its full potential in the management and treatment of periodontal disease. Addressing these considerations could establish ozone therapy as a valuable component of comprehensive periodontal care, expanding therapeutic options and enhancing patient outcomes in the field of dental medicine.

3. Conclusions

Ozone therapy has emerged as a promising adjunctive treatment in periodontal care, primarily due to its potent antimicrobial properties, as well as its ability to stimulate cell proliferation, differentiation, and modulate inflammatory responses. By leveraging ozone's capacity to reduce bacterial load, promote healing, and support tissue regeneration, clinicians may enhance the effectiveness of traditional periodontal treatments, potentially reducing treatment duration and improving clinical outcomes. Current evidence points to significant benefits of ozone therapy in periodontal applications; however, the need for a more comprehensive understanding of its biological mechanisms and optimization of its therapeutic protocols remains essential. Establishing evidence-based guidelines for ozone application could pave the way for its widespread adoption in periodontal therapy, offering an innovative approach to managing periodontal diseases and improving patient outcomes.

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References

1. Könönen, E.; Gursoy, M.; Gursoy, U.K. Periodontitis: A Multifaceted Disease of Tooth-Supporting Tissues. *J. Clin. Med.* **2019**, *8*, 1135. [CrossRef] [PubMed]
2. Łasica, A.; Golec, P.; Laskus, A.; Zalewska, M.; Gędaj, M.; Popowska, M. Periodontitis: Etiology, Conventional Treatments, and Emerging Bacteriophage and Predatory Bacteria Therapies. *Front. Microbiol.* **2024**, *15*, 1469414. [CrossRef] [PubMed]
3. Isola, G.; Santonocito, S.; Lupi, S.M.; Polizzi, A.; Sclafani, R.; Patini, R.; Marchetti, E. Periodontal Health and Disease in the Context of Systemic Diseases. *Mediat. Inflamm.* **2023**, *2023*, 9720947. [CrossRef] [PubMed]
4. Hashim, N.T.; Babiker, R.; Rahman, M.M.; Mohamed, R.; Priya, S.P.; Chaitanya, N.C.; Islam, M.S.; Gobara, B. Natural Bioactive Compounds in the Management of Periodontal Diseases: A Comprehensive Review. *Molecules* **2024**, *29*, 3044. [CrossRef]
5. Munasur, S.L.; Turawa, E.B.; Chikte, U.M.E.; Musekiwa, A. Mechanical Debridement with Antibiotics in the Treatment of Chronic Periodontitis: Effect on Systemic Biomarkers—A Systematic Review. *Int. J. Environ. Res. Public Health* **2020**, *17*, 5601. [CrossRef]
6. Nwobodo, D.C.; Ugwu, M.C.; Anie, C.O.; Al-Ouqaili, M.T.S.; Ikem, J.C.; Victor, U.C.; Saki, M. Antibiotic Resistance: The Challenges and Some Emerging Strategies for Tackling a Global Menace. *J. Clin. Lab. Anal.* **2022**, *36*, e24655. [CrossRef]
7. Serra, M.E.G.; Baeza-Noci, J.; Mendes Abdala, C.V.; Luvisotto, M.M.; Bertol, C.D.; Anzolin, A.P. The Role of Ozone Treatment as Integrative Medicine: An Evidence and Gap Map. *Front. Public Health* **2023**, *10*, 1112296. [CrossRef]
8. Meenakshi, P.S.; Rajasekar, A. A Review on Ozone Therapy in Periodontitis. *Bioinformation* **2022**, *18*, 634. [CrossRef]
9. Dengizek, S.E.; Serkan, D.; Abubekir, E.; Aysun Bay, K.; Onder, O.; Arife, C. Evaluating Clinical and Laboratory Effects of Ozone in Non-Surgical Periodontal Treatment: A Randomized Controlled Trial. *J. Appl. Oral Sci.* **2019**, *27*, e20180108. [CrossRef]
10. El Meligy, O.A.; Elemam, N.M.; Talaat, I.M. Ozone Therapy in Medicine and Dentistry: A Review of the Literature. *Dent. J.* **2023**, *11*, 187. [CrossRef]

11. Goswami, P.; Sharma, K.; Maiti, N.; Yadav, S.; Verma, V.; Puthenkandathil, R. Ozone: An Adjunct in Dental Treatment. *J. Pharm. Bioallied Sci.* **2024**, *16*, S2–S4. [CrossRef] [PubMed]
12. Russo, C.; Curcio, G.; Graziani, A.; Mencacci, A.; Pietrella, D. Antibiofilm, Anti-Inflammatory, and Regenerative Properties of a New Stable Ozone-Gel Formulation. *Pharmaceutics* **2024**, *16*, 1580. [CrossRef] [PubMed]
13. Veneri, F.; Filippini, T.; Consolo, U.; Vinceti, M.; Generali, L. Ozone Therapy in Dentistry: An Overview of the Biological Mechanisms Involved. *Biomed. Rep.* **2024**, *21*, 115. [CrossRef]
14. Marchesan, J.T.; Scanlon, C.S.; Soehren, S.; Matsuo, M.; Kapila, Y.L. Implications of Cultured Periodontal Ligament Cells for the Clinical and Experimental Setting: A Review. *Arch. Oral Biol.* **2011**, *56*, 933–943. [CrossRef] [PubMed]
15. Gordon, J.L.; Brown, M.A.; Reynolds, M.M. Cell-Based Methods for Determination of Efficacy for Candidate Therapeutics in the Clinical Management of Cancer. *Diseases* **2018**, *6*, 85. [CrossRef]
16. Inchingolo, A.M.; Inchingolo, A.D.; Nardelli, P.; Latini, G.; Trilli, I.; Ferrante, L.; Malcangi, G.; Palermo, A.; Inchingolo, F.; Dipalma, G. Stem Cells: Present Understanding and Prospects for Regenerative Dentistry. *J. Funct. Biomater.* **2024**, *15*, 308. [CrossRef]
17. Swanson, W.B.; Yao, Y.; Mishina, Y. Novel Approaches for Periodontal Tissue Engineering. *Genesis* **2022**, *60*, e23499. [CrossRef]
18. Medeiros Cardoso, J.E.; Ervolino, E.; Miyasawa, E.M.; Theodoro, L.H.; Padovan, L.E.M.; Pereira, E.L.; de Molon, R.S.; Garcia, V.G. Unveiling the Therapeutic Potential of Systemic Ozone on Skin Wound Repair: Clinical, Histological, and Immunohistochemical Study in Rats. *Biomed. Res. Int.* **2024**, *2024*, 6623114. [CrossRef]
19. Travagli, V.; Iorio, E. The Biological and Molecular Action of Ozone and Its Derivatives: State-of-the-Art, Enhanced Scenarios, and Quality Insights. *Int. J. Mol. Sci.* **2023**, *24*, 8465. [CrossRef]
20. Alsakr, A.; Gufran, K.; Alqahtani, A.S.; Alasqah, M.; Alnufaiy, B.; Alzahrani, H.G.; Alahmari, A.A.; Alhumaidani, F.K.; Alhumaidani, R.K.; Althobiti, M.J. Ozone Therapy as an Adjuvant in the Treatment of Periodontitis. *J. Clin. Med.* **2023**, *12*, 7078. [CrossRef]
21. Uraz, A.; Karaduman, B.; Isler, S.Ç.; Gönen, S.; Çetiner, D. Ozone Application as Adjunctive Therapy in Chronic Periodontitis: Clinical, Microbiological, and Biochemical Aspects. *J. Dent. Sci.* **2019**, *14*, 27–37. [CrossRef] [PubMed]
22. Xue, W.; Macleod, J.; Blaxland, J. The Use of Ozone Technology to Control Microorganism Growth, Enhance Food Safety, and Extend Shelf Life: A Promising Food Decontamination Technology. *Foods* **2023**, *12*, 814. [CrossRef] [PubMed]
23. Rangel, K.; Cabral, F.O.; Lechuga, G.C.; Carvalho, J.P.R.S.; Villas-Bôas, M.H.S.; Midlej, V.; De-Simone, S.G. Detrimental Effect of Ozone on Pathogenic Bacteria. *Microorganisms* **2021**, *10*, 40. [CrossRef] [PubMed]
24. Breidablik, H.J.; Lysebo, D.E.; Johannessen, L.; Skare, Å.; Andersen, J.R.; Kleiven, O. Effects of Hand Disinfection with Alcohol Hand Rub, Ozonized Water, or Soap and Water: Time for Reconsideration? *J. Hosp. Infect.* **2020**, *105*, 213–215. [CrossRef]
25. Mohammad, Z.; Kalbasi-Ashtari, A.; Riskowski, G.; Juneja, V.; Castillo, A. Inactivation of Salmonella and Shiga Toxin-Producing Escherichia coli (S.T.E.C.) from the Surface of Alfalfa Seeds and Sprouts by Combined Antimicrobial Treatments Using Ozone and Electrolyzed Water. *Food Res. Int.* **2020**, *136*, 109488. [CrossRef]
26. De Sire, A.; Marotta, N.; Ferrillo, M.; Agostini, F.; Sconza, C.; Lippi, L.; Respizzi, S.; Giudice, A.; Invernizzi, M.; Ammendolia, A. Oxygen-Ozone Therapy for Reducing Pro-Inflammatory Cytokine Serum Levels in Musculoskeletal and Temporomandibular Disorders: A Comprehensive Review. *Int. J. Mol. Sci.* **2022**, *23*, 2528. [CrossRef]
27. Milillo, C.; Falcone, L.; Di Carlo, P.; Aruffo, E.; Del Boccio, P.; Cufaro, M.C.; Patruno, A.; Pesce, M.; Ballerini, P. Ozone Effect on the Inflammatory and Proteomic Profile of Human Macrophages and Airway Epithelial Cells. *Respir. Physiol. Neurobiol.* **2023**, *307*, 103979. [CrossRef]
28. Sagai, M.; Bocci, V. Mechanisms of Action Involved in Ozone Therapy: Is Healing Induced via a Mild Oxidative Stress? *Med. Gas Res.* **2011**, *1*, 29. [CrossRef]
29. Juan, C.A.; Pérez de la Lastra, J.M.; Plou, F.J.; Pérez-Lebeña, E. The Chemistry of Reactive Oxygen Species (ROS) Revisited: Outlining Their Role in Biological Macromolecules (DNA, Lipids, and Proteins) and Induced Pathologies. *Int. J. Mol. Sci.* **2021**, *22*, 4642. [CrossRef]
30. Su, L.J.; Zhang, J.H.; Gomez, H.; Murugan, R.; Hong, X.; Xu, D.; Jiang, F.; Peng, Z.Y. Reactive Oxygen Species-Induced Lipid Peroxidation in Apoptosis, Autophagy, and Ferroptosis. *Oxid. Med. Cell. Longev.* **2019**, *2019*, 5080843. [CrossRef]
31. Uruén, C.; Chopo-Escuin, G.; Tommassen, J.; Mainar-Jaime, R.C.; Arenas, J. Biofilms as Promoters of Bacterial Antibiotic Resistance and Tolerance. *Antibiotics* **2020**, *10*, 3. [CrossRef] [PubMed]
32. Panebianco, F.; Rubiola, S.; Di Ciccio, P.A. The Use of Ozone as an Eco-Friendly Strategy against Microbial Biofilm in Dairy Manufacturing Plants: A Review. *Microorganisms* **2022**, *10*, 162. [CrossRef] [PubMed]
33. Galiè, M.; Covi, V.; Tabaracci, G.; Malatesta, M. The Role of Nrf2 in the Antioxidant Cellular Response to Medical Ozone Exposure. *Int. J. Mol. Sci.* **2019**, *20*, 4009. [CrossRef] [PubMed]
34. Franzini, M.; Valdenassi, L.; Pandolfi, S.; Tirelli, U.; Ricevuti, G.; Chirumbolo, S. The Role of Ozone as an Nrf2-Keap1-ARE Activator in the Anti-Microbial Activity and Immunity Modulation of Infected Wounds. *Antioxidants* **2023**, *12*, 1985. [CrossRef]
35. Iqbal, M.J.; Kabeer, A.; Abbas, Z.; Siddiqui, H.A.; Calina, D.; Sharifi-Rad, J.; Cho, W.C. Interplay of Oxidative Stress, Cellular Communication, and Signaling Pathways in Cancer. *Cell Commun. Signal.* **2024**, *22*, 7. [CrossRef]

36. Al-Hegelan, M.; Tighe, R.M.; Castillo, C.; Hollingsworth, J.W. Ambient Ozone and Pulmonary Innate Immunity. *Immunol. Res.* **2011**, *49*, 173–191. [CrossRef]
37. Shafqat, A.; Khan, J.A.; Alkachem, A.Y.; Sabur, H.; Alkattan, K.; Yaqinuddin, A.; Sing, G.K. How Neutrophils Shape the Immune Response: Reassessing Their Multifaceted Role in Health and Disease. *Int. J. Mol. Sci.* **2023**, *24*, 17583. [CrossRef]
38. Cenci, A.; Macchia, I.; La Sorsa, V.; Sbarigia, C.; Di Donna, V.; Pietraforte, D. Mechanisms of Action of Ozone Therapy in Emerging Viral Diseases: Immunomodulatory Effects and Therapeutic Advantages with Reference to SARS-CoV-2. *Front. Microbiol.* **2022**, *13*, 871645. [CrossRef]
39. Rivas-Arancibia, S.; Hernández-Orozco, E.; Rodríguez-Martínez, E.; Valdés-Fuentes, M.; Cornejo-Trejo, V.; Pérez-Pacheco, N.; Dorado-Martínez, C.; Zequeida-Carmona, D.; Espinosa-Caleti, I. Ozone Pollution, Oxidative Stress, Regulatory T Cells, and Antioxidants. *Antioxidants* **2022**, *11*, 1553. [CrossRef]
40. Li, Z.; Tighe, R.M.; Feng, F.; Ledford, J.G.; Hollingsworth, J.W. Genes of Innate Immunity and the Biological Response to Inhaled Ozone. *J. Biochem. Mol. Toxicol.* **2013**, *27*, 3–16. [CrossRef]
41. Valacchi, G.; Sticozzi, C.; Zanardi, I.; Belmonte, G.; Cervellati, F.; Bocci, V.; Travagli, V. Ozone Mediators Effect on "In Vitro" Scratch Wound Closure. *Free Radic. Res.* **2016**, *50*, 1022–1031. [CrossRef] [PubMed]
42. Pecorelli, A.; Bocci, V.; Acquaviva, A.; Belmonte, G.; Gardi, C.; Virgili, F.; Ciccoli, L.; Valacchi, G. NRF2 Activation Is Involved in Ozonated Human Serum Up-Regulation of HO-1 in Endothelial Cells. *Toxicol. Appl. Pharmacol.* **2013**, *267*, 30–40. [CrossRef] [PubMed]
43. Delgado-Roche, L.; Riera-Romo, M.; Mesta, F.; Hernández-Matos, Y.; Barrios, J.M.; Martínez-Sánchez, G.; Al-Dalaien, S.M. Medical Ozone Promotes Nrf2 Phosphorylation Reducing Oxidative Stress and Pro-Inflammatory Cytokines in Multiple Sclerosis Patients. *Eur. J. Pharmacol.* **2017**, *811*, 148–154. [CrossRef]
44. Yue, J.; López, J.M. Understanding MAPK Signaling Pathways in Apoptosis. *Int. J. Mol. Sci.* **2020**, *21*, 2346. [CrossRef]
45. Tang, S.; Xu, B.; Li, J.; Zhong, M.; Hong, Z.; Zhao, W.; Zeng, T.; He, X. Ozone Induces BEL7402 Cell Apoptosis by Increasing Reactive Oxygen Species Production and Activating JNK. *Ann. Transl. Med.* **2021**, *9*, 1257. [CrossRef]
46. Mustafa, M.; Ahmad, R.; Tantry, I.Q.; Ahmad, W.; Siddiqui, S.; Alam, M.; Abbas, K.; Hassan, M.I.; Habib, S.; Islam, S. Apoptosis: A Comprehensive Overview of Signaling Pathways, Morphological Changes, and Physiological Significance and Therapeutic Implications. *Cells* **2024**, *13*, 1838. [CrossRef]
47. Xiao, W.; Tang, H.; Wu, M.; Liao, Y.; Li, K.; Li, L.; Xu, X. Ozone Oil Promotes Wound Healing by Increasing the Migration of Fibroblasts via PI3K/Akt/mTOR Signaling Pathway. *Biosci. Rep.* **2017**, *37*, BSR20170658. [CrossRef]
48. Srikanth, A.; Sathish, M.; Sri Harsha, A.V. Application of Ozone in the Treatment of Periodontal Disease. *J. Pharm. Bioallied Sci.* **2013**, *5*, S89–S94. [CrossRef]
49. Cisterna, B.; Costanzo, M.; Nodari, A.; Galiè, M.; Zanzoni, S.; Bernardi, P.; Covi, V.; Tabaracci, G.; Malatesta, M. Ozone Activates the Nrf2 Pathway and Improves Preservation of Explanted Adipose Tissue In Vitro. *Antioxidants* **2020**, *9*, 989. [CrossRef]
50. Wang, Z.; Zhang, A.; Meng, W.; Wang, T.; Li, D.; Liu, Z.; Liu, H. Ozone Protects the Rat Lung from Ischemia-Reperfusion Injury by Attenuating NLRP3-Mediated Inflammation, Enhancing Nrf2 Antioxidant Activity, and Inhibiting Apoptosis. *Eur. J. Pharmacol.* **2018**, *835*, 82–93. [CrossRef]
51. Ramirez-Peña, A.M.; Sánchez-Pérez, A.; Campos-Aranda, M.; Hidalgo-Tallón, F.J. Ozone in Patients with Periodontitis: A Clinical and Microbiological Study. *J. Clin. Med.* **2022**, *11*, 2946. [CrossRef] [PubMed]
52. Yao, J.; Zou, P.; Cui, Y.; Quan, L.; Gao, C.; Li, Z.; Gong, W.; Yang, M. Recent Advances in Strategies to Combat Bacterial Drug Resistance: Antimicrobial Materials and Drug Delivery Systems. *Pharmaceutics* **2023**, *15*, 1188. [CrossRef] [PubMed]
53. Chirumbolo, S.; Valdenassi, L.; Simonetti, V.; Bertossi, D.; Ricevuti, G.; Franzini, M.; Pandolfi, S. Insights on the Mechanisms of Action of Ozone in the Medical Therapy Against COVID-19. *Int. Immunopharmacol.* **2021**, *96*, 107777. [CrossRef] [PubMed]
54. Ma, F.; Luo, S.; Lu, C.; Jiang, X.; Chen, K.; Deng, J.; Ma, S.; Li, Z. The Role of Nrf2 in Periodontal Disease by Regulating Lipid Peroxidation, Inflammation, and Apoptosis. *Front. Endocrinol.* **2022**, *13*, 963451. [CrossRef]
55. Zakrzewski, W.; Dobrzyński, M.; Szymonowicz, M.; Rybak, Z. Stem Cells: Past, Present, and Future. *Stem Cell Res. Ther.* **2019**, *10*, 68. [CrossRef]
56. Wielento, A.; Lagosz-Cwik, K.B.; Potempa, J.; Grabiec, A.M. The Role of Gingival Fibroblasts in the Pathogenesis of Periodontitis. *J. Dent. Res.* **2023**, *102*, 489–496. [CrossRef]
57. Bosshardt, D.D. Are Cementoblasts a Subpopulation of Osteoblasts or a Unique Phenotype? *J. Dent. Res.* **2005**, *84*, 390–406. [CrossRef]
58. Omi, M.; Mishina, Y. Roles of Osteoclasts in Alveolar Bone Remodeling. *Genesis* **2022**, *60*, e23490. [CrossRef]
59. Bharuka, T.; Reche, A. Advancements in Periodontal Regeneration: A Comprehensive Review of Stem Cell Therapy. *Cureus* **2024**, *16*, e54115. [CrossRef]
60. Naruishi, K. Biological Roles of Fibroblasts in Periodontal Diseases. *Cells* **2022**, *11*, 3345. [CrossRef]
61. Wen, S.; Zheng, X.; Yin, W.; Liu, Y.; Wang, R.; Zhao, Y.; Liu, Z.; Li, C.; Zeng, J.; Rong, M. Dental Stem Cell Dynamics in Periodontal Ligament Regeneration: From Mechanism to Application. *Stem Cell Res. Ther.* **2024**, *15*, 389. [CrossRef] [PubMed]

62. Wang, T.; Liu, X.; Li, J.; Yue, Y.; Li, J.; Wang, M.; Wei, N.; Hao, L. Mechanisms of Mechanical Force in Periodontal Homeostasis: A Review. *Front. Immunol.* **2024**, *15*, 1438726. [CrossRef] [PubMed]
63. Na Nan, D.; Klincumhom, N.; Trachoo, V.; Everts, V.; Ferreira, J.N.; Osathanon, T.; Pavasant, P. Periostin-Integrin Interaction Regulates Force-Induced TGF-β1 and α-SMA Expression by hPDLSCs. *Oral Dis.* **2023**, *30*, 2570–2579. [CrossRef]
64. Iwayama, T.; Sakashita, H.; Takedachi, M.; Murakami, S. Periodontal Tissue Stem Cells and Mesenchymal Stem Cells in the Periodontal Ligament. *Jpn. Dent. Sci. Rev.* **2022**, *58*, 172–178. [CrossRef]
65. Alves, L.; Machado, V.; Botelho, J.; Mendes, J.J.; Cabral, J.M.S.; da Silva, C.L.; Carvalho, M.S. Enhanced Proliferative and Osteogenic Potential of Periodontal Ligament Stromal Cells. *Biomedicines* **2023**, *11*, 1352. [CrossRef] [PubMed]
66. Ge, S.; Zhao, N.; Wang, L.; Yu, M.; Liu, H.; Song, A.; Huang, J.; Wang, G.; Yang, P. Bone Repair by Periodontal Ligament Stem Cell-Seeded Nanohydroxyapatite-Chitosan Scaffold. *Int. J. Nanomed.* **2012**, *7*, 5405–5414. [CrossRef] [PubMed]
67. Albougha, M.S.; Sugii, H.; Adachi, O.; Mardini, B.; Soeno, S.; Hamano, S.; Hasegawa, D.; Yoshida, S.; Itoyama, T.; Obata, J.; et al. Exosomes from Human Periodontal Ligament Stem Cells Promote Differentiation of Osteoblast-like Cells and Bone Healing in Rat Calvarial Bone. *Biomolecules* **2024**, *14*, 1455. [CrossRef]
68. Jeyaraman, M.; Jeyaraman, N.; Ramasubramanian, S.; Balaji, S.; Nallakumarasamy, A.; Patro, B.P.; Migliorini, F. Ozone Therapy in Musculoskeletal Medicine: A Comprehensive Review. *Eur. J. Med. Res.* **2024**, *29*, 398. [CrossRef]
69. Choi, B.; Choi, J.-H.; Kang, T.-H.; Lee, S.-J.; Kim, G.-C. Enhancement of Osteoblast Differentiation Using No-Ozone Cold Plasma on Human Periodontal Ligament Cells. *Biomedicines* **2021**, *9*, 890. [CrossRef]
70. Pasalkar, L.; Chavan, M.; Kharat, D.-A.; Sanap, A.; Kheur, S.; Bhonde, R. Gaseous Ozone Treatment Augments Chondrogenic and Osteogenic Differentiation but Impairs Adipogenic Differentiation in Human Dental Pulp Stem Cells In Vitro. *J. Orofac. Sci.* **2022**, *14*, 36–44. [CrossRef]
71. Viebahn-Haensler, R.; León Fernández, O.S. Ozone in Medicine: The Low-Dose Ozone Concept and Its Basic Biochemical Mechanisms of Action in Chronic Inflammatory Diseases. *Int. J. Mol. Sci.* **2021**, *22*, 7890. [CrossRef] [PubMed]
72. Clavo, B.; Rodríguez-Esparragón, F.; Rodríguez-Abreu, D.; Martínez-Sánchez, G.; Llontop, P.; Aguiar-Bujanda, D.; Fernández-Pérez, L.; Santana-Rodríguez, N. Modulation of Oxidative Stress by Ozone Therapy in the Prevention and Treatment of Chemotherapy-Induced Toxicity: Review and Prospects. *Antioxidants* **2019**, *8*, 588. [CrossRef] [PubMed]
73. Takizawa, F.; Domon, H.; Hiyoshi, T.; Tamura, H.; Shimizu, K.; Maekawa, T.; Tabeta, K.; Ushida, A.; Terao, Y. Ozone Ultrafine Bubble Water Exhibits Bactericidal Activity Against Pathogenic Bacteria in the Oral Cavity and Upper Airway and Disinfects Contaminated Healthcare Equipment. *PLoS ONE* **2023**, *18*, e0284115. [CrossRef]
74. Failor, K.C.; Silver, B.; Yu, W.; Heindl, J.E. Biofilm Disruption and Bactericidal Activity of Aqueous Ozone Coupled with Ultrasonic Dental Scaling. *JADA Found. Sci.* **2022**, *1*, 100003. [CrossRef]
75. Palma, P.V.; Cunha, R.O.; Leite, I.S.G. Effectiveness of Ozone Therapy in the Treatment of Periodontal Diseases: A Systematic Review. *RGO Rev. Gaúcha Odontol.* **2023**, *71*, e20230004. [CrossRef]
76. Saeed, S.S.M.; Afify, O.; El-Moula, E.M.; El-ZamaranY, E.A. Clinical and Microbiological Evaluation of Oleozone Gel in the Treatment of Chronic Periodontitis. *EC Dent. Sci.* **2017**, *12*, 227–236.
77. Huth, K.C.; Quirling, M.; Lenzke, S.; Paschos, E.; Kamereck, K.; Brand, K.; Hickel, R.; Ilie, N. Effectiveness of Ozone Against Periodontal Pathogenic Microorganisms. *Eur. J. Oral Sci.* **2011**, *119*, 204–210. [CrossRef]
78. Bocci, V.A. Scientific and Medical Aspects of Ozone Therapy: State of the Art. *Arch. Med. Res.* **2006**, *37*, 425–435. [CrossRef]
79. Giuliani, G.; Ricevuti, G.; Galoforo, A.; Franzini, M. Microbiological Aspects of Ozone: Bactericidal Activity and Antibiotic/Antimicrobial Resistance in Bacterial Strains Treated with Ozone. *Ozone Ther.* **2018**, *3*, 7971. [CrossRef]
80. Puletic, M.; Velikic, G.; Maric, D.M.; Supic, G.; Maric, D.L.; Radovic, N.; Avramov, S.; Vojvodic, D. Clinical Efficacy of Extracellular Vesicle Therapy in Periodontitis: Reduced Inflammation and Enhanced Regeneration. *Int. J. Mol. Sci.* **2024**, *25*, 5753. [CrossRef]
81. Nambiar, S.; Malothu, S.; Karmakar, S.; Varkey, A.; Chandra, D.; Chava, V.K. Comparison of Ozonated Olive Oil and Chlorhexidine Gel as an Adjunct to Nonsurgical Periodontal Therapy for the Treatment of Chronic Periodontitis: A Randomized Controlled Clinical Trial. *J. Pharm. Bioallied Sci.* **2022**, *14*, S94–S98. [CrossRef] [PubMed]
82. Tetè, G.; D'Amicantonio, T.; Polizzi, E. Efficacy of Ozone Therapy in Reducing Periodontal Disease. *Materials* **2023**, *16*, 2375. [CrossRef] [PubMed]
83. Swimberghe, R.C.; Coenye, T.; De Moor, R.J.G.; Meire, M.A. Biofilm Model Systems for Root Canal Disinfection: A Literature Review. *Int. Endod. J.* **2019**, *52*, 604–628. [CrossRef] [PubMed]
84. Case, P.D.; Bird, P.S.; Kahler, W.A.; George, R.; Walsh, L.J. Treatment of Root Canal Biofilms of Enterococcus faecalis with Ozone Gas and Passive Ultrasound Activation. *J. Endod.* **2012**, *38*, 523–526. [CrossRef]
85. Huth, K.C.; Quirling, M.; Maier, S.; Kamereck, K.; Alkhayer, M.; Paschos, E.; Welsch, U.; Miethke, T.; Brand, K.; Hickel, R. Effectiveness of ozone against endodontopathogenic microorganisms in a root canal biofilm model. *Int. Endod. J.* **2009**, *42*, 3–13. [CrossRef]

86. Bitter, K.; Vlassakids, A.; Niepel, M.; Hoedke, D.; Schulze, J.; Neumann, K.; Moter, A.; Noetzel, J. Effects of diode laser gaseous ozone and medical dressings on Enterococcus faecalis biofilms in the root canal ex vivo. *Biomed. Res. Int.* **2017**, *2017*, 6321850. [CrossRef]
87. Sinha, N.; Asthana, G.; Parmar, G.; Langaliya, A.; Shah, J.; Kumbhar, A.; Singh, B. Evaluation of Ozone Therapy in Endodontic Treatment of Teeth with Necrotic Pulp and Apical Periodontitis: A Randomized. *Clin. Trial. J. Endod.* **2021**, *47*, 1820–1828. [CrossRef]
88. Tonon, C.C.; Panariello, B.H.D.; Spolidorio, D.M.P.; Gossweiler, A.G.; Duarte, S.J. Antibiofilm Effect of Ozonized Physiological Saline Solution on Peri-Implant-Related Biofilm. *Periodontology* **2021**, *92*, 1151–1162. [CrossRef]
89. Isler, S.C.; Unsal, B.; Soysal, F.; Ozcan, G.; Peker, E.; Karaca, I.R. The Effects of Ozone Therapy as an Adjunct to the Surgical Treatment of Peri-Implantitis. *J. Periodontal Implant Sci.* **2018**, *48*, 136–151. [CrossRef]
90. Campanati, A.; De Blasio, S.; Giuliano, A.; Ganzetti, G.; Giuliodori, K.; Pecora, T.; Consales, V.; Minnetti, I.; Offidani, A. Topical Ozonated Oil versus Hyaluronic Gel for the Treatment of Partial- to Full-Thickness Second-Degree Burns: A Prospective, Comparative, Single-Blind, Non-Randomised, Controlled Clinical Trial. *Burns* **2013**, *39*, 1178–1183. [CrossRef]
91. Solovăstru, L.G.; Stîncanu, A.; De Ascentii, A.; Capparé, G.; Mattana, P.; Vâţă, D. Randomized, Controlled Study of Innovative Spray Formulation Containing Ozonated Oil and α-Bisabolol in the Topical Treatment of Chronic Venous Leg Ulcers. *Adv. Ski. Wound Care* **2015**, *28*, 406–409. [CrossRef] [PubMed]
92. Ripamonti, C.I.; Cislaghi, E.; Mariani, L.; Maniezzo, M. Efficacy and Safety of Medical Ozone (O_3) Delivered in Oil Suspension Applications for the Treatment of Osteonecrosis of the Jaw in Patients with Bone Metastases Treated with Bisphosphonates: Preliminary Results of a Phase I-II Study. *Oral Oncol.* **2011**, *47*, 185–190. [CrossRef] [PubMed]
93. Bocci, V.A.; Zanardi, I.; Travagli, V. Ozone Acting on Human Blood Yields a Hormetic Dose-Response Relationship. *J. Transl. Med.* **2011**, *9*, 66. [CrossRef] [PubMed]
94. Bocci, V. Is It True That Ozone Is Always Toxic? The End of a Dogma. *Toxicol. Appl. Pharmacol.* **2006**, *216*, 493–504. [CrossRef]
95. Deepthi, R.; Bilichodmath, S. Ozone Therapy in Periodontics: A Meta-Analysis. *Contemp. Clin. Dent.* **2020**, *11*, 108–115. [CrossRef]
96. Barahim, A.A.; Shemais, N.; Mousa, A.; Darhous, M. Clinical and Radiographic Evaluation of Non-Surgical Therapy with and without Ozone Gel Application in Controlled Type 2 Diabetic Patients with Periodontitis: A Randomized Controlled Clinical Trial. *BMC Oral Health* **2024**, *24*, 1435. [CrossRef]
97. Salve, A.; Ansari, S.; Gattani, D.; Kar, N.; Heda, P. Ozone—A Versatile Therapy in Implant Dentistry. *J. Glob. Oral Health* **2022**, *5*, 124–127. [CrossRef]
98. Makeeva, M.K.; Daurova, F.Y.; Byakova, S.F.; Turkina, A.Y. Treatment of an Endo-Perio Lesion with Ozone Gas in a Patient with Aggressive Periodontitis: A Clinical Case Report and Literature Review. *Clin. Cosmet. Investig. Dent.* **2020**, *12*, 447–464. [CrossRef]
99. Barczyk, I.; Masłyk, D.; Walczuk, N.; Kijak, K.; Skomro, P.; Gronwald, H.; Pawlak, M.; Rusińska, A.; Sadowska, N.; Gronwald, B.; et al. Potential Clinical Applications of Ozone Therapy in Dental Specialties—A Literature Review, Supported by Own Observations. *Int. J. Environ. Res. Public Health* **2023**, *20*, 2048. [CrossRef]
100. Bondy, S.C. The Hormesis Concept: Strengths and Shortcomings. *Biomolecules* **2023**, *13*, 1512. [CrossRef]
101. Ngo, V.; Duennwald, M.L. Nrf2 and Oxidative Stress: A General Overview of Mechanisms and Implications in Human Disease. *Antioxidants* **2022**, *11*, 2345. [CrossRef] [PubMed]
102. Franzini, M.; Valdenassi, L.; Pandolfi, S.; Tirelli, U.; Ricevuti, G.; Simonetti, V.; Berretta, M.; Vaiano, F.; Chirumbolo, S. The Biological Activity of Medical Ozone in the Hormetic Range and the Role of Full Expertise Professionals. *Front. Public Health* **2022**, *10*, 979076. [CrossRef] [PubMed]
103. Floare, A.D.; Scurtu, A.D.; Balean, O.I.; Chioran, D.; Buzatu, R.; Sava Rosianu, R.; Alexa, V.T.; Jumanca, D.; Rusu, L.-C.; Racea, R.C.; et al. The Biological Effects of Ozone Gas on Soft and Hard Dental Tissues and the Impact on Human Gingival Fibroblasts and Gingival Keratinocytes. *Processes* **2021**, *9*, 1978. [CrossRef]

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Review

The Cross-Talk Between the Peripheral and Brain Cholesterol Metabolisms

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Abstract: Cholesterol is an essential element for the development and normal function of the central nervous system. While peripheral cholesterol is influenced by liver metabolism and diet, brain cholesterol metabolism takes place in an isolated system due to the impermeability of the blood–brain barrier (BBB). However, cross-talk occurs between the brain and periphery, specifically through metabolites such as oxysterols that play key roles in regulating cholesterol balance. Several neurodegenerative conditions such as Alzheimer's disease or Parkinson's disease are considered to be affected by the loss of this balance. Also, the treatment of hypercholesterolemia needs to consider these discrete interferences between brain and peripheral cholesterol and the possible implications of each therapeutic approach. This is particularly important because of 27-hydroxycholesterol and 24-hydroxycholesterol, which can cross the BBB and are involved in cholesterol metabolism. This paper examines the metabolic pathways of cholesterol metabolism in the brain and periphery and focuses on the complex cross-talk between these metabolisms. Also, we emphasize the regulatory role of the BBB and the need for an integrated approach to cholesterol management.

Keywords: cholesterol; 24-hydroxycholesterol; 27-hydroxycholesterol; statins; PCSK9i; metabolism; physiology; physiopathology; brain; peripheral metabolism; preoperative management

1. Introduction

All plasma membranes of eukaryotic cells contain sterols. Cholesterol synthesis is considered an evolutionary step from prokaryotes to eukaryotes, as long as cholesterol synthesis does not occur in the absence of oxygen [1]. Each cell of the human body is capable of synthesizing cholesterol [2], with endogenous cholesterol representing approximately 90% of the body's total cholesterol; this grounds the assertion that cholesterol is a fundamental compound of the human body and essentially involved in cell functions and physiological processes [3]. Cholesterol is found mostly but not exclusively in intracellular organelles, as well as in plasma membranes, contributing to cell membrane fluidity, permeability, and integrity. Cholesterol is also found in the blood in plasma lipoproteins, which represent the major blood cholesterol carriers.

Cholesterol is presented in unesterified and esterified versions, with different distributions throughout the body. Most of the cellular cholesterol is unesterified and found mainly in plasma membrane leaflets, representing 20–25% of lipid molecules within these structures [4–6]. However, cholesterol is also found, in small amounts, in the endoplasmic reticulum (ER), Golgi apparatus, and nucleus. Some plasma membrane cells contain specialized microdomains rich in cholesterol, known as lipid rafts or lipid domains [7,8]. The circulating forms of cholesterol are represented by esterified cholesterol, found mainly in low-density lipoproteins (LDLs) (about 60%) but also in very low-density lipoproteins (VLDLs) and high-density lipoproteins (HDLs). LDLs transport cholesterol from the liver to the cells, while HDLs transport cholesterol in the opposite direction, from the tissues to the liver [9].

Oxidized cholesterol derivatives (i.e., oxysterols) are excretion cholesterol products also involved in cholesterol homeostasis and represent metabolic intermediates in steroid hormones, vitamin D3, and bile acids biosynthesis [10].

The widely used paradigm is that higher cholesterol levels are strongly associated with higher cardiovascular morbidity and mortality. However, the optimal range for total cholesterol levels associated with the lowest incidence of cardiovascular and overall events remains a topic of debate and continuously evolves based on clinical evidence.

The first point to emphasize regarding "optimal" cholesterol levels is that these levels fluctuate with age. Early life, especially while suckling, represents a critical phase in brain growth and myelination [11,12]. Plasma cholesterol levels are physiologically elevated at this time, rising quickly across all species, highlighting its crucial role in these processes [13]. Deficits in cholesterol synthesis during this period may lead to significant abnormalities in brain development, with major functional consequences [14]. On the contrary, in adults, hypercholesterolemia is associated with increased cardiovascular risk and general mortality [15].

In 2019, the European Society of Cardiology and the European Society for Atherosclerosis Research reported that the target for LDLs varies according to the cardiovascular risk, as follows: very high risk < 55 mg/dL, high risk < 70 mg/dL, moderate risk < 100 mg/dL, and low risk < 116 mg/dL [16]. To reach these targets, aggressive and sustained lipid-lowering treatments are mandatory [17].

In adulthood, both hypercholesterolemia and low cholesterol levels are linked to increased general and cardiovascular mortality, as well as to detrimental behaviors, including aggression and suicidal tendencies [18–22].

A long-term follow-up study showed that sustained low serum cholesterol for more than two decades is related to the most increased level of mortality [23], including high rates of cancer-associated deaths. Treatment with statins was also shown to increase cancer incidence in several studies [24–26].

A large United States cohort study involving 30,687 patients followed for 15 years (between 1999 and 2014) [27] found that the relationship between plasma cholesterol levels and all-cause mortality is not linear; instead, it follows a U-shaped pattern. This indicates that both high and low cholesterol levels are associated with increased general and cardiovascular mortality. The study concluded that low cholesterol levels correlate with higher mortality rates, with total cholesterol levels below 200 mg/dL strongly linked to general mortality, and levels below 120 mg/dL significantly associated with higher mortality and cardiovascular morbidity. Conversely, cholesterol levels of 280 mg/dL or higher were primarily related to deaths from cardiovascular diseases. The same study [23] established cutoff values for total cholesterol of 172 mg/dL, 267 mg/dL, and 205 mg/dL for all-cause mortality, respectively cardiovascular disease mortality and cancer mortality. Another interesting conclusion was that people with higher education levels were more likely to have higher total cholesterol values. While it is often perceived that lower education correlates with poorer health outcomes, this finding might indicate that those with higher education may be more prone to certain dietary habits or lifestyle choices that increase cholesterol levels. Also, individuals with a higher level of education often have better access to healthcare information and resources, but this does not necessarily mean healthier choices are made [23].

2. Cholesterol Level Regulation

Sterols are essential for membrane structure, fluidity, and permeability; therefore, their concentration within plasma membranes needs to be tightly regulated [28]. This constant level is tissue-specific and maintained through a dynamic equilibrium between the accumulation and excretion of cholesterol [29].

2.1. Cholesterol Biosynthesis and Uptake

Cholesterol originates from the following two sources: endogenous and exogenous. The endogenous source is dominant and is represented by cell biosynthesis from acetyl coenzyme A under the action of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoAR). The essential element in cholesterol synthesis is represented by mevalonate [30]. Mevalonate is converted in a few steps to lanosterol, after which cholesterologenesis occurs differently for neurons and astrocytes; in neurons, cholesterol derives from 7-dehydrocholesterol (on the Kandutsch–Russell pathway) while in astrocytes from desmosterol (on the Bloch pathway) [31] (Figure 1) [30]. The other source that ensures up to 22% of total plasma cholesterol is represented by food [32]. Cholesterol biosynthesis occurs via the isoprenoid pathway which also produces other active molecules, such as coenzyme Q10 (CoQ10), heme-A, and dolichol, with important cellular functions. Briefly, CoQ10 and heme-A are involved in aerobic cellular respiration and in adenosine triphosphate generation, CoQ10 being particularly important as an antioxidant, and dolichol is required for glycoproteins synthesis [33].

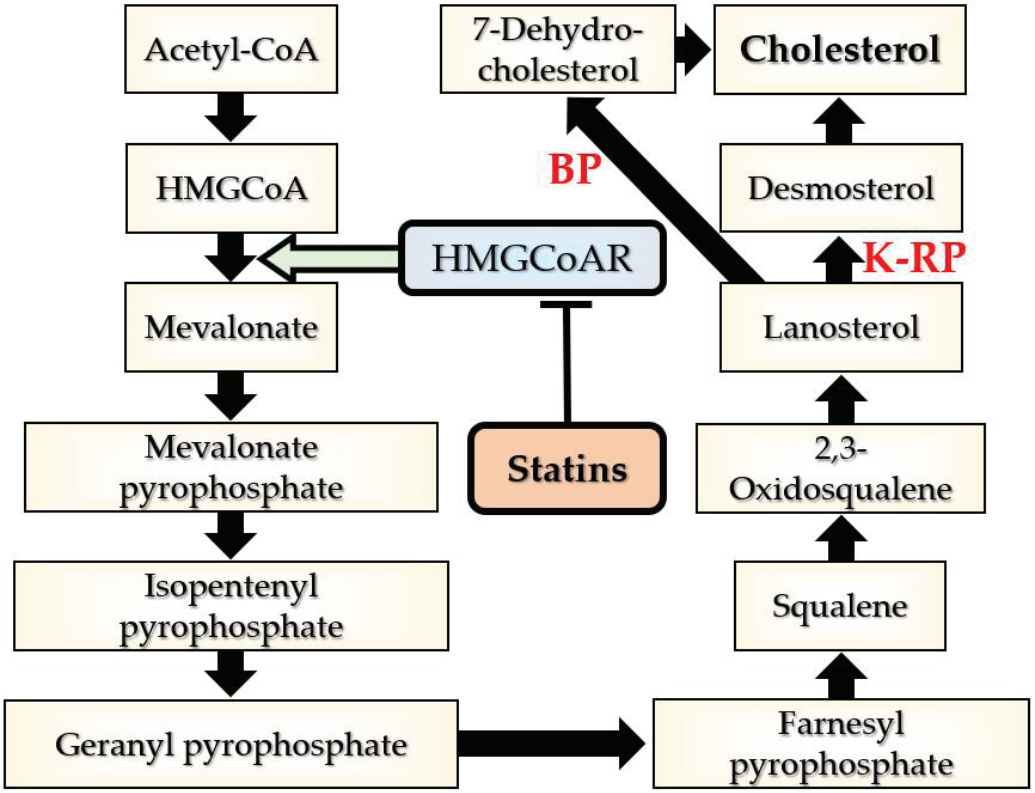


Figure 1. The mevalonate pathway leading to cholesterol synthesis. Green arrow = catalytic role; Acetyl-CoA = acetyl coenzyme A; BP = Bloch pathway; HMGCoA = β-hydroxy β-methylglutaryl-CoA; HMGCoAR = HMG-CoA reductase; K-RP = Kandutsch–Russell pathway.

Cholesterol synthesis is regulated by HMGCoAR, a rate-limiting enzyme in the mevalonate–cholesterol biosynthetic pathway which presents a sterol-sensing domain sensitive to sterol levels within the ER membrane. The cross-talk between HMGCoAR and ER is essential for cholesterol synthesis. With increased sterol levels, HMGCoAR interacts with the ER-resident protein insulin-induced gene (INSIG), undergoing proteasomal degradation [34–36].

Sterol regulatory element-binding proteins (SREBPs) transcription factors are located on the ER membrane and modulate the synthesis and uptake of cholesterol depending on the levels of intracellular sterol content [37,38].

Circulating cholesteryl esters enter the cell via LDL receptors (LDLrs) and scavenger receptor class B type 1 (SR-B1). LDLrs are responsible for binding VLDLs and LDLs, which contain apolipoproteins such as apoE and apoB100. SR-B1 is found in hepatocytes and endocrine cells and binds HDL particles that contain ApoA1 apolipoprotein [39–42]. ApoA1 is largely distributed in the cerebrospinal fluid (CSF), despite not being produced or located in the brain [43]. ApoA1 is delivered to the CSF at the level of the choroid plexus and is involved in cholesterol transfer from the peripheral nervous system [44,45]. Low levels of ApoA1 and HDL were correlated with lower cognitive performances [46].

Hydrolysis of cholesteryl esters contained in LDLs generates free cholesterol delivered to cellular compartments via membrane proteins involved in cholesterol trafficking in mammals, called Niemann–Pick disease types C1 and C2 (NPC1 and NPC2) [47,48].

2.2. Cholesterol Excess Regulation: Cholesterol Efflux and Cholesterol Stores

Because of the cellular toxicity of cholesterol accumulation, the excessive cholesterol needs to be excreted (unesterified or as oxysterols) or stored as cholesteryl-ester droplets.

There are several mechanisms involved in cellular cholesterol efflux. The central nervous system tissue expresses various types of ATP-binding cassette (ABC) transporters, such as ABCA1, ABCG1, and ABCG4, all expressed by neurons [49]. ABCA1 are expressed in higher levels in neurons, as well as in glial cells [50–52]. ABC transporters excrete unesterified cholesterol from the membrane or cellular cytosol to the external environment [29].

Acting as cholesterol receptors, the extracellular apolipoproteins [32] uptake the cholesterol under a chemical gradient [53,54]. ApoA1-containing lipoproteins are taken up by various receptors belonging to the LDLr family, highly expressed in glial cells (astrocytes), endothelial brain capillary cells, and neurons [29,55–58].

Oxysterols are products of cholesterol oxidation [59], more polar than cholesterol and, subsequently, more prone to being absorbed by cells compared to cholesterol [60]. They also activate LXRs, leading to a reduction in the intracellular cholesterol content [61]. Oxysterols seem to be stronger promoters of atherosclerosis than cholesterol itself [62–64]. Atherosclerotic lesions develop more rapidly in animals fed with a diet high in oxidized fatty acids and oxidized cholesterol [63,65]. Oxysterols are generated through enzymatic cholesterol oxidation (e.g., 24S-hydroxycholesterol (24-OHC) in the brain, 27-hydroxycholesterol (27-OHC) in several tissues, and 7-hydroxycholesterol (7-OHC) in the liver) or are generated from non-enzymatic cholesterol oxidation upon oxidative stress (e.g., 25-hydroxycholesterol (25-OHC), cholesterol 5β6β-epoxide, 7-ketocholesterol, and 7β-hydroxycholesterol) [66–68]. Oxysterols are transported mainly by HDLs, unlike cholesterol which is transported mainly by the LDL/VLDL system [69]. Oxysterols play a crucial role in cholesterol transport out of cells and are essential for maintaining cholesterol homeostasis. Acting as ligands for SREBP2, oxysterols downregulate endogenous cholesterol synthesis, through a mechanism similar to statins, decreasing HMGCoAR expression [70]. They also have various other roles, including the contribution to bile acid synthesis following uptake by the liver.

The most studied metabolites of cholesterol are 24-OHC, 25-OHC, and 27-OHC. 27-OHC is the most abundant plasmatic cholesterol metabolite and is generated in peripheral circulation from cholesterol enzymatic oxidation under the action of the CYP27A1 enzyme. 24-OHC is found peripherally, reflects only the brain cholesterol turnover, and is generated exclusively in the central nervous system (CNS) [71,72]. Plasmatic levels of 24-OHC are increased in high brain cholesterol turnover and low in neurodegenerative processes [73]. 25-OHC is produced in all cells, in response to cholesterol-enriched foods.

24-OHC, 25-OHC, and very small amounts of 27-OHC that cross the blood–brain barrier (BBB) are present in the brain. Brain 27-OHC levels are very low if the BBB is intact [73].

The interaction between 24-OHC and 27-OHC in the brain plays a particularly important role in maintaining brain cholesterol homeostasis [74–78]. At the moment, this relation is a topic of debate; some authors support the hypothesis that 27-OHC downregulates brain cholesterol synthesis and 24-OHC production [79], while other studies enforced the opposite concept, that 27-OHC, via stimulating CYP46A1 activity, increases the 24-OHC levels [80].

The ratio between 24-OHC and 27-OHC (24-OHC/27-OHC) in serum or plasma is considered a marker for cholesterol metabolism [81]. The ratio of 27-OHC to 24-OHC is functionally significant and is tightly regulated. 24-OHC/27-OHC have differing metrics in different brain areas [82].

3. Oxysterols Within the Brain

3.1. 27-Hydroxycholesterol

27-OHC is generated in both hepatic and extrahepatic tissues. Within the brain, cholesterol is converted to 27-OHC to a lesser extent than to 24-OHC, because CYP27A1 is

expressed at lower levels in neurons, astrocytes, and oligodendrocytes [83]. In the brain, CYP7B1 converts 27-OHC into 7alpha-hydroxy-3-oxo-4-cholestenoic acid (7-HOCA) [84].

If the BBB is intact, the level of 27-OHC in the CSF reflects the plasmatic 27-OHC levels [85]. Low plasmatic cholesterol levels are followed by decreased 27-OHC production and reduced 27-OHC influx into the brain [86]. On the contrary, hypercholesterolemia is associated with increased levels of 27-OHC, including within the CNS [87]. Higher levels of 27-OHC within the brain are related not only to the increased BBB permeability [88] but also to higher CYP27A1 expression in glial cells [89].

Peripheral injection of 27-OHC in rats upregulates ABCA1 in the brain and downregulates HMGCoAR and LDLr [75].

27-OHC has numerous deleterious effects on CNS through various mechanisms. Firstly, increasing levels of 27-OHC downregulate HMGCoAR, decreasing brain cholesterol levels. At the CNS level, 27-OHC reduces brain cholesterol synthesis and also 24-OHC production [90]. Secondly, 27-OHC facilitates the transport of cholesterol between astrocytes and neurons leading to its accumulation in neurons [91]. Moreover, 27-OHC promotes neuroinflammation [92], which holds special relevance regarding Alzheimer's disease (AD). The detrimental effect of neuroinflammation is sustained by the observation of a direct relationship between neuronal and astrocytic inflammatory markers, such as IL-1, IL-6, and tumor necrosis factors alpha and amyloid beta (Ab) levels [93]. Another notable effect of 27-OHC is that it induces overactivation of the brain renin–angiotensin-aldosterone system (RAAS) [94] and increases the oxidative stress [95], with negative consequences on cognition. Brains of deceased individuals with AD displayed elevated levels of 27-OHC compared to the brains of those who died without the diagnosis of AD [82].

Most 27-OHC results from processed and ultra-processed animal food (mainly microwave and grill preparation), excessive baking, cooking, or heating of cooked refrigerated products [96,97]. 27-OHC is inversely correlated with HDL levels [98].

3.2. 24S-Hydroxycholesterol

As 24-OHC results exclusively from brain cholesterol oxidation under 24-hydroxylase (CYP46A1) action, its plasmatic and CSF levels are directly correlated to brain cholesterol metabolism [84]. However, the level of 24-OHC (cerebrosterol) is influenced not only by the brain cholesterol level but also by the number and integrity of neurons. The CSF levels of 24-OHC more accurately reflect its expression because of the independence of hepatic clearance [85]. In healthy individuals, 24-OHC plasma levels are maintained throughout most of their lifetime. After the seventh decade of life, as a consequence of a natural decline in neuronal mass, serum levels of 24-OHC decrease [84]. In the early stages of neurodegenerative diseases, as a consequence of myelin loss, 24-OHC levels are elevated but decrease in advanced stages [99]. 24-OHC traverses the BBB almost integrally with only a very small percent (1%) entering the CSF [72,100].

24-OHC has important contributions in cholesterol elimination and synthesis, and in cholesterol transport between astrocytes and neurons [101]. Another important function of 24-OHC is modulating NMDA receptors, contributing to synaptic plasticity and learning [102–104], of particular importance in hippocampal neurons [105].

Neuronal cholesterol levels are primarily influenced by the efflux of cholesterol from astrocytes because of significant limitations in cholesterol production in neural cells [106]. 24-OHC migrates from neurons to astrocytes, activating LXR and its target genes ABCA1 and ABCG1, which cause the efflux of ApoE-bound cholesterol from astrocytes. ApoE is released by astrocytes under stimulation from 24-OHC and will bind cholesterol, which is subsequently taken up by neurons [107]. 24-OHC binds to α and β LXR receptors [108,109], inhibiting brain cholesterol synthesis mainly in astrocytes [110]. LXR receptor activation

determines astrocyte synthesis of ApoE and ABCA1/ABCG1, stimulating the transport of cholesterol from the neurons to the astrocytes [107,111] (Figure 2). LXR activation increases ApoE expression in neurons and also induces ABCA1/ABCG1 expression in the endothelial cells of brain capillaries, as shown by experimental studies [112].

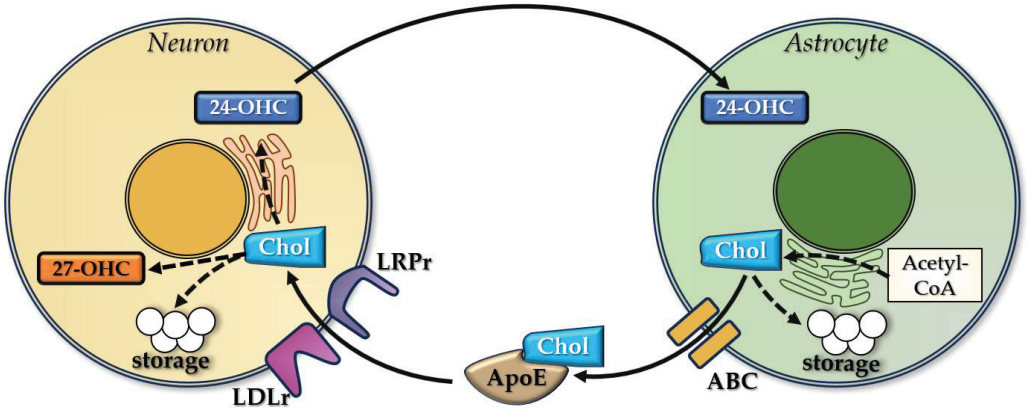


Figure 2. The role of 24-OHC in cholesterol transport between astrocytes and neurons.

In normal human brain tissue, 24-hydroxylase is primarily expressed in neurons, which are the main site for cholesterol turnover [75]. It is also present in lower concentrations in oligodendrocytes and microglia [113]. Moreover, the highest CYP46A1 levels are found in cortical, hippocampal, and cerebellar neurons [80,114,115], suggesting either special metabolic rates or a particular sensitivity to high cholesterol levels of the neurons located in these regions. Decreased 24-hydroxylase activity (experimentally through administration in mice of voriconazole, an inhibitor of CYP46A1) is followed by cognition improvement, likely due to preservation of hippocampal cholesterol [116,117]. But also, 24-hydroxylase hyper-expression is linked to neuronal cholesterol loss and impaired cognition, as seen in aging or stress [118]. In mice deficient in 24-hydroxylase, cholesterol excretion significantly slows, and its synthesis is suppressed. Furthermore, noticeable deficiencies in motor learning related to cognitive function may be identified [119]. Oxidative stress induces CYP46A1 hyperexpression [120].

There is only an apparent contradiction between the 24-hydroxylase levels, 24-OHC levels, and cognition, as 24-OHC functions as a double-edged sword; low levels are harmful to cognition, while excessive accumulation can become neurotoxic [121]. These observations support the concept that brain cholesterol homeostasis is finely tuned, especially in cerebral regions engaged in higher cognitive functions. Therefore, brain cholesterol metabolism is in a continuous adjustment toward equilibrium.

24-OHC plays a dual role for neurons, acting as a pro-survival or pro-death factor, depending on its concentration. At physiological concentrations, 24-OHC is neuroprotective, through LXRs activation [68,122]. This effect is lost at higher concentrations, due to the inhibition of LXR transcriptional activity [122], becoming a promoter of neuronal loss [123,124]. In older people, cholesterol synthesis and 24-OHC levels are concordant and decreased at the hippocampal level [125], explaining the low performances in learning and memory [119,126].

24-OHC has beneficial effects, such as anti-inflammatory and anti-atherogenic properties [127], but also deleterious effects, inducing cell death at high concentrations [73]. In cultured cells, 24-OHC promotes the non-amyloidogenic processing of soluble amyloid precursor protein (APP) [128,129] and impairs neuronal accumulation of hyperphosphorylated tau protein [130]. Toxic accumulation of oxysterols within the brain is fostered in

conditions that increase BBB permeability, the most common conditions being aging and hypercholesterolemia, both linked to oxidative stress [131].

3.3. 25-Hydroxycholesterol

25-OHC is produced mainly in microglia [132] and regulated by the activity of cholesterol 25-hydroxylase (CH-25H) [133], an enzyme localized in the ER. Higher cholesterol levels in the ER lead to higher 25-OHC levels, in return inhibiting several intracellular cholesterol mechanisms, such as degradation of HMGCoAR, prevention of SREBP2, and activation of LXR receptors [29,34,134]. These actions explain why 25-OHC exhibits neuroprotective effects, limiting neuroinflammation and neuronal loss, improving synaptic transmission and lipid rafts formation [135].

25-OHC has the capacity to cross membranes and vascular barriers [85] (Figure 3).

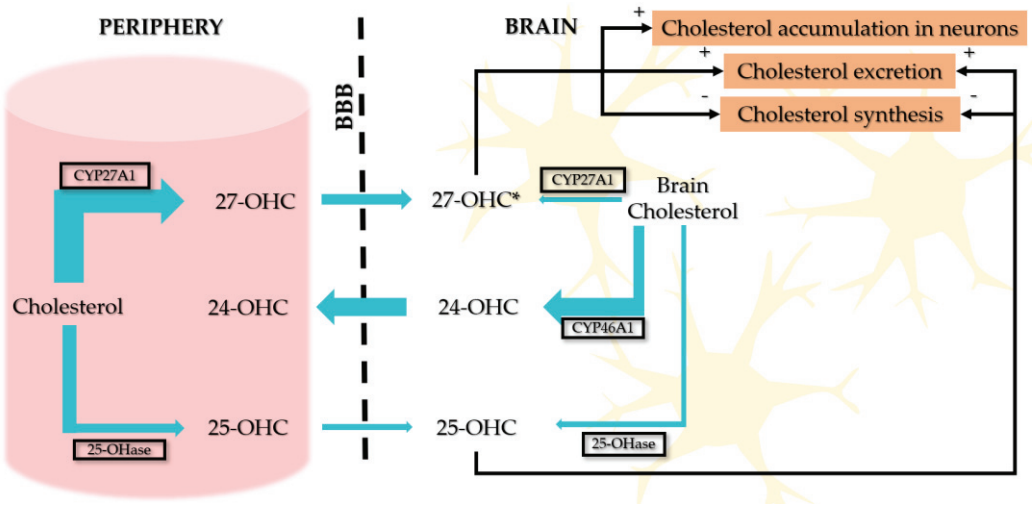


Figure 3. The exchange of cholesterol between plasma and brain via the BBB. Cholesterol homeostasis in neurons. * 27-OHC levels depend on the BBB's integrity; hypercholesterolemia increases the BBB's permeability and CYP27A1 in glial cells.

Through the action of sulfotransferases, cholesterol and oxysterols become cholesterol derivatives with increased water solubility, like cholesterol sulphate and oxysterol 3-sulfates, respectively [136]. These sulphated derivatives exhibit neuroprotective effects, such as stimulation of neuroprotective neurosteroids synthesis [137], and limit brain oxysterol accumulation [138]. Levels of oxysterol sulphate are significantly reduced in the prefrontal cortex of patients with AD [138].

4. Brain Cholesterol Metabolism

4.1. Role of Cholesterol in the Central Nervous System

In humans, but also in other species (e.g., primates and mice), total body cholesterol is ~2200 mg/kg body weight (2.2 mg/g fresh tissue) [28]. In all species, the brain's cholesterol concentration is approximately 15–20 mg/g of brain matter [14,139–144], but the amount of brain cholesterol differs among species and is significantly greater in humans. The organ with the highest cholesterol content is the adult human brain, where it accounts for approximately 25% of the body's total cholesterol [84,111].

Cholesterol is the major lipid compound in the brain [139,145], and it is not equally distributed, differing among brain regions [146].

In hypercholesterolemia, the permeability of the BBB is increased, resulting in higher plasma levels of 24-OHC compared to individuals with normal plasma cholesterol levels. However, in hypercholesterolemic patients, higher 27-OHC levels and the cross-talk

between 24-OHC and 27-OHC contribute, to some extent, to increased 24-OHC plasma levels [147]. Therefore, a reasonable conclusion is that the separation between the CNS and the body is largely restricted to cholesterol molecules, not cholesterol metabolism.

There are significant differences in the metabolic and turnover rates of cholesterol between the periphery and the brain. In the periphery, the basal metabolic rate of cholesterol and the cholesterol turnover rate are directly related to each other. In contrast, while the metabolic rate of cholesterol in the CNS is high, its turnover rate is slow, at around 0.03% per day compared, with 0.7% per day in the periphery [145]. Another notable difference is between the short half-time of plasma cholesterol (of few days) [14,145], and the half-time of brain cholesterol, which ranges between 6 months and 5 years [111,148,149]. This is yet another indication that for proper functioning, the brain requires a stable cholesterol level.

The observed differences between the adult and the very young animal brains refer not only to turnover rates, low in the adult brain and high during the development period but also to the type of cholesterol. In the adult brain, 99.5% of cholesterol is unesterified [150], unlike the very young animal brain, where the amount of cholesteryl esters is higher [140–144].

While a large part of the literature suggests that cholesterol metabolism in the adult brain is very slow, some studies indicate that it might not be as static as previously thought [151,152]. Nonetheless, it is still slower than in younger individuals, where both cholesterol synthesis and degradation are more active [153].

4.2. Regulatory Mechanisms of Cholesterol Metabolism Homeostasis

Most of the adult brain's cholesterol (around 70–80%) is found in myelin sheaths, while the remaining quantity is identified in astrocytes and plasma membranes of neural cells [14]. The concentration of cholesterol is low in the cytosol and CSF [150].

The majority of brain cholesterol originates from local synthesis in the ER within the nerve cells [154,155], with highly increased levels during the early period of development, when myelination and neuronal growth are essential [144]. Following these events, the production of cholesterol carries on at significantly lower rates [156]. Unlike early life, when neurons are the primary source of brain cholesterol, after myelination, glial cells become the main source of cholesterol in the brain [144]. Cholesterol homeostasis relies on the cross-talk between astrocytes (main producer) and neurons (main consumer) in the adult brain [31]. The astrocyte-synthetized cholesterol is carried out by membrane ABC transporters. After, cholesterol is taken up by ApoE and ApoI lipoproteins (also synthetized by astrocytes), which further interact with their receptors belonging to LDLr and LRP, localized on neuronal plasma membranes. The final step of this cross-talk is neuronal internalization of cholesterol [75,157]. ApoE is the main apolipoprotein transporter in the CNS, being a key player in brain cholesterol metabolism. The main source of ApoE is represented by astrocytes [158].

Both astrocytes and neurons use glucose and lactate as a metabolic source for cellular processes, but neurons prefer the oxidative metabolism [159] over lactate production which is primarily used by astrocytes as an energy source [160]. These two pathways are complementary and essential for glutamate–glutamine metabolism [161].

Alongside regulating the BBB, astrocytes play complex roles in numerous brain functions, as well as neural growth and support [162,163]. The neurovascular unit is composed of astrocytes alongside endothelial cells, pericytes, and vascular smooth muscle cells; this unit is essential for the regulation of cerebral blood flow and underlines the critical function played by the astrocytes [164].

4.3. Role of the Blood–Brain Barrier in Brain Cholesterol Metabolism

In the periphery, cholesterol needs are met through the following two main sources: de novo synthesis and circulating lipoproteins. Conversely, the pool of cholesterol in the CNS is mainly supplied by local synthesis and recirculation.

CNS cholesterol metabolism is tightly regulated [165] to maintain a stable absolute brain cholesterol level throughout life [33]. BBB integrity plays a key role in the preservation of brain cholesterol and the metabolic autonomy of the brain. BBB prevents cholesterol uptake from the systemic circulation; therefore, brain cholesterol metabolism relies on recycling [166].

Cholesterol is essential for neuronal functions, and astrocytes play a major function in brain cholesterol homeostasis. Neuronal cholesterol is sufficient for survival and growth but insufficient for building efficient synapses, essential for all physiological brain functions, including learning, memory, and behavior [150,167]. For this last purpose, neurons need extra-neuronal cholesterol provided by astrocytes [168–170].

Cholesterol is a key component of myelin [165] and a capital element for dendrite formation and synapse development [171]. It is also important for mitochondrial function and neurotransmitter receptor expression [172], exhibiting neuroprotective effects [173] and antioxidant properties [171].

Maintaining normal levels of brain cholesterol is required for proper brain function, making the regulation of excess cholesterol crucial to this need. Cholesterol excess is prevented through active secretion (excretion) and storage mechanisms.

Cholesterol is removed daily from the brain across the BBB either as hydroxycholesterol derivatives (daily elimination of 6–7 mg of 24-OHC) [72] or as ApoA1- and ApoE-bound cholesterol in the CSF (1–2 mg/day) [174,175]. Cholesterol efflux from neurons to ApoA1 and ApoE [49] is mediated by ABC transporters, resulting in lipoproteins that are released into the CSF [176–178] and selectively taken up by LDL receptor-related protein 1 (LRP1), mainly expressed in neurons [29] and SR-B1 receptors expressed in endothelial cells within brain capillaries [55,56]. ApoA1 is found in HDL, and HDL particles can cross the BBB [179,180] while ApoE cannot.

ApoE represents the main mechanism of cholesterol transport between astrocytes and neurons [181], as well as a pathway for cholesterol excretion into the CSF [182]. The highest ApoE expression level is recorded in the liver, followed by the brain [183]. Under normal conditions, neurons only produce small amounts of ApoE, while the main source of ApoE (around 80%) is represented by astrocyte cells [184]. During stress or injuries, neuronal ApoE production may increase [185].

Within the CNS, astrocytes express LDLrs, which are an important functional receptor for ApoE and Ab [186–188]. ApoE is involved in Ab degradation and clearance [189] and plays a role in mitochondrial dysfunction [181,189]. Of the various isoforms, ApoE4 is linked to a higher risk for the development of AD and shows detrimental effects on BBB permeability [190,191].

A minor (less than 1%) part of the cholesterol surplus is stored in lipid droplets as esterified cholesterol [192]. Cholesterol esterification occurs as a result of cholesterol acyltransferase activity; while these enzymes are expressed in glial cells, they are mainly present in neurons and are sensitive to higher free cholesterol levels [193].

The free exchange of cholesterol between plasma and the brain is prevented by an intact BBB. It was shown that cholesterol derivatives can cross the BBB [194], while lipoproteins and free cholesterol do not exhibit this ability [72,111]. However, only an intact BBB restricts the passage of free cholesterol.

The BBB impermeability to lipoproteins was demonstrated more than 50 years ago. Connor [195] characterized the peripheral and brain cholesterol metabolism by prelabeling

the sterol in the yolk sac and administrating C^{14} cholesterol to hens. In newborn chickens, the labeled sterol was found largely in peripheral tissues (95–98% from the reference yolk sac level) but also in the brain tissue in small amounts (around 11% of the reference level). We note that there are significant differences between the two percentages but also that the brain-specific activity was not null, underlining the importance of cholesterol biosynthesis in the brain. Another study, on terminally ill patients, showed that administration of C^{14}-labeled cholesterol is followed by the detection of approximately 3% of the labeled cholesterol in CNS structures [196]. One alternative explanation for the flow across the BBB might be represented by cholesterol transport at the choroid plexus [197].

The permeability of circumventricular organs (CVOs) is dependent on the molecule size, as shown by murine immunohistochemistry studies. The permeability differs according to the low molecular weight of the tracer, and molecules heavier than 10 KDa cannot cross the barrier [198–200]. While the molecular weight of cholesterol is only 386.7 g/mol or 0.3867 kDa, there remains the question of whether free cholesterol can pass through CVOs and reach the brain tissue [93,201,202]. There are limited data regarding this hypothesis as long as the permeability of statins across CVOs is also incompletely understood.

Brain endothelial cells express large amounts of LDLr, SR-B1, and ABCA1 [55,203], which raises the possibility that small amounts of CNS cholesterol originate in the plasma. The primary source of cholesterol in the brain is its synthesis within the CNS, although a small amount of brain cholesterol does originate from plasma [204]. Suckling animals have high levels of circulating lipoproteins during the accumulation of sterol in the brain [14]. This may hint at the possibility that lipoproteins can cross the BBB; however, there are limited data in this regard.

The paradigm is that brain and peripheral cholesterol homeostases are independent. Some authors modulate this paradigm, concluding that brain and peripheral cholesterol metabolism are largely independent but not completely isolated, sustaining the existence of low cholesterol transport from the circulation into the brain [205,206].

Experimental murine studies have demonstrated that cholesterol can cross the BBB in small amounts, a process that is amplified in hypercholesterolemia [207]. Rabbits receiving a high-fat diet for 10 weeks presented higher BBB permeability compared to rabbits fed a normal diet [208]. Therefore, the BBB's permeability enables peripheral cholesterol to pass through [209].

The increase in BBB permeability in hypercholesterolemia translates to higher plasma 24-OHC levels compared to persons with normal plasma cholesterol levels. However, in hypercholesterolemic patients, higher 27-OHC levels and the cross-talk between 24-OHC and 27-OHC may partly contribute to increased 24-OHC plasma levels [210].

The most reasonable conclusion is that the separation between the CNS and the periphery is largely restricted to the cholesterol molecule, not to its metabolism. Brain cholesterol metabolism is tightly regulated but integrated into general body metabolic processing.

5. The Effects of Lipid-Lowering Therapy on Brain Cholesterol Metabolism

5.1. Statins and Central Cholesterol Regulation

Statins decrease cholesterol synthesis by inhibiting HMGCoAR, upregulating LDLr, and increasing LDL-cholesterol (LDL-C) clearance via apolipoprotein B-rich hepatic absorption facilitation [211]. Statins also reduce triglyceride-rich lipoprotein biosynthesis in the liver [212]. Within the brain, the highest levels of HMGCoAR are recorded in cholinergic cortical and hippocampal neurons [213].

Downregulating the mevalonate pathway is accompanied by a decrease in molecules produced, including CoQ10, and non-steroid isprenoid compounds (e.g., farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP)) [154].

CoQ10 plays a major role in cellular integrity and ATP formation and has antioxidant properties [214–216]. The highest level of CoQ10 is found in tissues with high metabolic activity, such as the heart, brain, muscles, and kidneys [217,218]. The circulating form of CoQ10 mainly binds to LDLs and VLDLs, while small amounts are bound to HDLs [219]. Although it is unclear whether CoQ10 can cross the BBB, most studies conclude that CoQ10 can flow to the brain side only if the tight junctions of the barrier are disrupted. Meanwhile, in an integer BBB, there is no transport of CoQ10 across the barrier [220].

While dolichol inhibition is related to various myopathies, neuropathies, and diabetes [214,221], it is not obvious to what extent the adverse effects of statins are related to brain cholesterol reduction or to the reduction in other mevalonate metabolites [222]. Statins exhibit numerous intricate effects such as anti-inflammatory and antioxidant properties [118,223–226]. However, statins also present deleterious effects on the CNS, such as impairing synaptic vesicle release and reducing synapse density after exposure to low concentrations of lovastatin for a short period of 7–14 days [227]; blocking dendritic growth and dendritic retraction [228]; promoting apoptosis [229]; reducing astrocyte viability after only 3 days of administration [230]; reducing ApoE expression [185]; and increasing serotonin uptake, through direct effects on serotonin transporters [231].

The effects of statins in the CNS primarily depend on their structure and molecular weight, as well as the presence of specific transporters [232–234]. Also relevant for their ability to cross the BBB is their concentration, with higher levels associated with increased permeability [235].

Statins may be either lipophilic (atorvastatin, simvastatin, cerivastatin, pitavastatin, fluvastatin, and lovastatin) or hydrophilic (rosuvastatin and pravastatin), and their ability to affect the synthesis of brain cholesterol depends on their capacity to cross the BBB. While lipophilic statins easily pass through the BBB, hydrophilic statins only manage to traverse it in small quantities. Not all lipophilic statins have the same BBB permeability, with simvastatin and fluvastatin having higher BBB penetration capacities (around 25%) followed by atorvastatin [233,236].

Atorvastatin and simvastatin are defined as potent lipophilic statins and cross the BBB, suppressing brain cholesterol synthesis with subsequent decreases in plasma 24-OHC levels [237]. Decreases in circulating levels of 24-OHC were also observed for less lipophilic statins (lovastatin) and some hydrophilic statins (pravastatin), thus implicating another mechanism that is probably related to circulatory LDL clearance [127].

The central effects of statins depend on the treatment's duration. Most studies concluded that short-term statin treatment has no effect on the levels of cholesterol in the brain [238], but long-term treatment does. Statin administration for more than 6 months was shown to decrease cholesterol levels in the CSF [239]. One study evaluating the effects of simvastatin on cholesterol and 24-OHC levels in patients with hypercholesterolemia evaluated the effects at 6 and 24 weeks [237]. The results show that hepatic cholesterol synthesis (evoked by the plasma lathosterol/cholesterol ratio) was reduced similarly at the two timepoints, and so were the plasma 24-OHC levels and 24-OHC/total cholesterol ratio [73].

5.2. Statins and Potential Effects on Cognition

It is considered that statins promote cardiovascular and cerebrovascular health through their exhibited antioxidant and anti-inflammatory actions [240]. However, it is unclear whether statins yield beneficial or adverse effects on cognition. While most authors consider

that initiating statin therapy in late life does not negatively impact cognition, it is uncertain whether long-term administration has favorable effects.

Statins increase the levels of LRP1 at the BBB [241] while also increasing the binding capacity for LDLs, ApoE, and Ab [242]. Conversely, statins decrease FPP and GGPP levels [243], with opposite effects on cognition. Lower FPP levels are associated with beneficial effects on cognition, acting as a protective mechanism against glutamate toxicity [244,245]. However, GGPP reduction leads to serotonin uptake [231], demonstrating how statin administration may lead to depression and anxiety, and, on a structural level, to neurite loss [119,126,246].

Another effect linked to lower isoprenoid levels are the promotion of endothelial protection [247,248], alongside the anti-inflammatory [244], antithrombotic, and antioxidant effects [249]. Statins also exhibit antiapoptotic effects. Simvastatin upregulates the antiapoptotic gene Bcl2, which is essential for neuronal survival [250].

There is a lack of consensus regarding the relationship between cognitive function and plasma lipids [251–254]. Observational studies and randomized controlled trials in AD have shown contradictory results regarding statin administration [255–257]. This might be explained by the heterogeneity of studies but also by the high dependence on individual factors. It might be possible that dementia and, possibly, AD are not associated with statin intake as reported in a study on 18,846 subjects over 65 years old [208].

Cholesterol is involved in the metabolism of APP, with the processing occurring at the level of lipid rafts [258,259]. High-fat diet administration in animal models of AD was shown to promote the synthesis of Ab [207,260–266] and impede the production of APP [267]. Conversely, low cholesterol levels were followed by the inhibition of β-secretase activity (involved in Ab production) and an increase in α-secretase activity, a proteolytic enzyme involved in APP metabolism [268].

A connection between cognitive impairment and increased levels of LDL and triglycerides was identified [269,270]. The risk of AD occurrence in patients with dyslipidemia varies according to age; higher cholesterol levels during midlife are associated with late-life AD [271]. A high-cholesterol diet contributes to increased amyloid concentration in the hippocampus, associated with oxidative stress and memory deficits [272]. This demonstrates how lowering cholesterol levels through statins in midlife might decrease the risk of AD later in life [273]. However, studies on older adults showed a positive association between LDL levels and cognitive performance [274]. A recent longitudinal study involving patients with AD underlined that LDL-C in higher concentrations translates to improved cognitive function [275]. Conversely, lower cholesterol levels in late life correlate with cognitive deterioration [268,276,277]. Higher cholesterol levels in older individuals are associated with lower risks for AD [278,279].

Importantly, multiple studies have indicated that lower cholesterol levels are associated with violent behavior, including aggression, suicide, and homicide [18,20,280,281]. In the Cardiovascular Prevention Program study, which included 1365 participants, dyslipidemia seems to not be associated with cognitive impairment [282].

The effects of statins on brain cholesterol levels are under debate. Murine studies assessed variable concentrations of simvastatin or pravastatin, and high concentrations were followed by a reduction in plasma cholesterol and lathosterol (an indicator of endogenous cholesterol synthesis), as well as a reduction in fraction synthesis rate (FSR) of cholesterol synthesis in the brain [145]. However, the brain can only maintain cholesterol homeostasis either through suppression of cholesterol outflow, by increasing HMGCoAR activity, or through concordant decreases in 24-OHC and lathosterol [283].

Numerous studies, including the MIRAGE study, have found that statins are linked to a lower risk of dementia and a slower progression of AD [284–287]. High doses of rosu-

vastatin or atorvastatin over long periods of time reduce the incidence of dementia [288], as shown by a longitudinal study with a follow-up period of 12 years. Statins may have positive effects on cognitive functions, including verbal memory, logical reasoning, and working memory [273,289]. The positive effects on cognition, particularly in women, may primarily be attributed to the beneficial effects of statins on the vascular system [290].

Other clinical trials [24,291,292] found no decrease in the risk of dementia in patients treated with statins. The Heart Protection Study concluded that dementia occurs with comparable incidence in patients receiving either simvastatin or placebo. The PROSPER (Pravastatin in Elderly Individuals at Risk of Vascular Disease) trial also revealed that cognitive function is no different in patients using statins compared with ones not undergoing lipid-lowering treatment; there was also no proof that statins could prevent vascular dementia or alleviate AD progression [293]. Additionally, statin administration, regardless of the lipophilic quality, had no influence on dementia, as shown by the Three-City with a 7-year follow-up [294].

The greatest benefit of statin in delaying AD progression is obtained by patients under 65 years old and who started statin therapy in midlife [295], while poorer benefits are recorded in patients older than 65 years [296]. However, a contradictory finding is that high doses of statins in diabetic patients or the use of statins in ApoE4 carriers are associated with an increased risk of dementia [297,298].

Simvastatin administration for 7 days triggered a significant decrease in dopamine levels at the level of the nigrostriatum in murine studies [299]. This leads to the question of whether the chronic use of statins is associated with a decrease in dopamine in specific brain synapses [228].

In 2012, the Food and Drug Administration (FDA) announced that statins cause reversible cognitive impairment [300]. Anyway, the FDA updated the beneficial statin effects on cognition. The JUPITER (Justification for the Use of Statins in Primary Prevention) trial warned of a higher risk for reversible decline in cognitive function [273].

Problems in cognition typically arise within a year after the debut of statin treatment [86]. Fortunately, cognitive impairment is short-term and quickly reversible (3 weeks) after statin treatment discontinuation [301] or statin replacement (exchange of a lipophilic statin with a hydrophilic one [302]). There are reports of a recurrence of cognitive impairment upon reintroducing statins after discontinuation [303]. Cognitive changes are more pronounced in potent and lipophilic statins, such as simvastatin and atorvastatin [273,304], and affect patients with a history of alcohol abuse or psychiatric disease to a greater extent [273]. Cognitive impairment appears to be dosage-dependent [301], with better diffusion into the CNS and more impactful effects on brain cholesterol synthesis at higher doses. Alongside their effect on cholesterol synthesis, statins might be responsible for cognitive impairment through their detrimental effects on brain mitochondrial function [301], via lowering CoQ10 levels. Overall, the reductions in CoQ10 and CNS cholesterol may contribute to the cognitive impairment associated with statin use observed in some patients. However, a clear conclusion on the beneficial or deleterious effects of statins on cognitive performance cannot be drawn at this point. Studies are reporting no evidence of statins affecting cognition [305–307].

5.3. Novel Agents and Their Effects on Central Cholesterol Metabolism and Cognition

Because lowering LDL-C directly correlates with cardiovascular risk reduction, actual recommendations are to decrease LDL-C to new target levels. This is possible because of newer agents, such as ezetimibe or proprotein convertase subtilin/kexin type 9 (PCSK9) inhibitors like evolocumab, alirocumab, or inclisiran [308].

PCSK9 catalyzes LDLr breakdown and determines a decrease in the number of LDLrs [309]; this effect occurs mainly in hepatocytes, where the highest levels of PCSK9 are expressed [310,311]. Within the brain, PCSK9 is only expressed in neurons [312], with the highest levels observed during the developmental period [297], and contributes to cholesterol levels' stabilization within the CSF by reducing the expression of LDLr, LRP1, VLDLr, and ApoE [313–315].

PCSK9 and PCSK9 monoclonal antibodies do not traverse the intact BBB [316,317]. In increases in BBB permeability, PCSK9 traverses to the CNS and proceeds to LDLr degradation [318,319], decreasing the capacity for internalization of LDL-containing particles. PCSK9 interacts with multiple proteins involved in neuronal metabolism and functions, such as neuronal differentiation, migration, and survival.

PCSK9 inhibitors (PCSK9i) increase circulating LDLrs and lead to a robust LDL-C reduction of approximately 60% in monotherapy [320,321]. PCSK9i are indicated mainly for the reduction in LDL-C levels in patients who do not tolerate statins or when statins cannot control LDL levels. PCSK9i enhances LDLr capacity and lowers serum concentrations of 24-OHC, 27-OHC, and cholesterol itself, mainly affecting total cholesterol and 27-OHC levels, and, to a lesser degree, 24-OHC. Therefore, PCSKi does not alter the ratio between 27-OHC and cholesterol, but 24-OHC/total cholesterol and 24-OHC/27-OHC are significantly increased [210].

PCSK9 might be involved in the pathogenesis of AD [318]. PCSK9 is higher in the CSF of AD patients [322,323], with the highest levels being detected in ApoE4 carriers [323]. The expression of PCSK9 in the frontal cortex was higher in patients with AD than in controls [324,325], and several mechanisms could be involved. Firstly, PCSK9 interferes with neuronal cholesterol uptake [326]. Other possible mechanisms are represented by the pro-apoptotic effect of PCSK9 [327,328], neuronal cholesterol depletion (by decreasing ApoE and LDLr), reduction of Ab clearance [324], and the pro-inflammatory effect on cerebral cells exerted by PCSK9 [329–332]. Conversely, the absence of PCSK9 is associated with better hippocampal spatial memory [325]. PCSK9 levels are also higher in patients with depression [323,333], correlating with depression score [334].

Most analyses of PCSK9i on cognitive functions show no effect on cognition using this medication class. The FOURIER trial (Further Cardiovascular Outcomes Research with PCSK9 Inhibitors in Subjects with Elevated Risk) compared evolocumab with placebo for the secondary prevention in patients on optimized statin therapy, receiving or not ezetimibe; the study revealed no adverse cognition events in patients taking evolocumab [320]. A derived sub-study, named EBBINGHAUS (Evaluating PCSK9 Binding Antibody Influence on Cognitive Health in High Cardiovascular Risk Subjects), was the first to assess cognitive changes associated with PCSK9i as an endpoint and showed no adverse cognitive events in young patients over a 19-month follow-up period [154].

OSLER-1 and OSLER-2 studies compared evolocumab added to standard therapy vs. standard therapy in patients with hypercholesterolemia. They reported that neurocognitive adverse events were more frequent in patients taking evolocumab compared to standard therapy alone [335].

The efficacy and safety of alirocumab compared to statins at the maximal tolerated dose were analyzed in the ODYSSEY trial [336]. The ODDYSEY LONG TERM trial included patients with heterozygous familial hypercholesterolemia with or without cardiovascular disease on an optimized statin dose [337]. The study concluded that neurocognitive adverse events were more frequent in patients receiving alirocumab for 70 weeks than those that received a placebo, including symptoms such as confusion, amnesia, and memory impairment [325]. In the ODYSSEY COMBO I study, alirocumab was compared to a placebo,

over 52 weeks, in patients receiving an optimized statin dose, and no difference between the study groups was recorded in regard to neurocognitive adverse events [338].

MEMOGAL was a prospective study that included adult patients with a first prescription of evolocumab or alirocumab, followed for 24 months for cognitive impairment; it showed no changes in neurocognitive performances and no differences between the two PCSK9i in this respect [339].

The effects of evolocumab and alirocumab on cerebral oxysterol production were analyzed after 1 month and 3 months of treatment. In the first assessment, 27-OHC was significantly lower while 24-OHC/27-OHC increased, and the ratio reached even higher levels after 3 months of treatment [81,210]. This finding might reflect an increased brain cholesterol synthesis.

The Individual Case Safety Reports (ICSRs) of the pharmaco-vigilance database on the secondary neuropsychiatric effects of evolocumab and alirocumab were analyzed. Close to 23% of cases showed nervous or psychiatric symptoms, the majority of which were severe. Approximately 60% of patients were not taking concomitant medication, while the rest were taking other drugs including statins or ezetimibe [340].

Oxysterols detected in the peripheral blood correlate with cholesterol absorption, as well as cholesterol synthesis [90,341]. One study found that 24-OHC, 27-OHC, and 25-OHC, along with other sterols, have a direct relationship with seric levels of campesterol and lathosterol; they also concluded that 27-OHC is positively regulated by cholesterol synthesis, while 24-OHC and 25-OHC correlate with cholesterol absorption and synthesis [342].

Ezetimibe limits the intestinal absorption of cholesterol by blocking the Niemann–Pick C1-like 1 transporter [343]. This transporter is involved in oxysterol level regulation through intestinal dietary oxysterol absorption [344].

5.4. Lipid-Lowering Therapies and Neurodegenerative Diseases

The most prevalent neurodegenerative diseases are AD and vascular dementia. Diets rich in saturated fats were associated with a higher risk of developing AD [345], while a Mediterranean diet decreased this risk [346]. However, the potential connection between total cholesterol or LDL levels and AD is still under debate.

Recent studies demonstrated a direct correlation between cholesterol levels and cognitive performance [347]. Conversely, there are data showing that persistent increased cholesterol levels do not associate with a higher risk of cognitive impairment compared with decreased cholesterol levels [348]. Another hypothesis states that cholesterol variation, regardless of the direction, leads to cognitive decline; therefore, this should be accounted for in the management of patients with major risks for vascular disease [348].

As previously mentioned, oxidized cholesterol metabolites can cross the BBB and abnormal levels of oxysterols in the brain are associated with increased Ab production [349] and increased neuroinflammation [350]. Gut microbiota plays a role in regulating neuroinflammation and lipid metabolism, and recent data emphasize the cholesterol-lowering effects of probiotics [351,352]. Modulation of gut microbiota or the administration of probiotic supplements may be a promising approach in the prevention of cognitive decline and determent or delay of AD [353]. Murine administration of the SLAB51 probiotic is associated with a decrease in HMGCoAR activity, with subsequent upregulation of LXRs expression within the brain and in the liver and significant decreases in plasmatic and central 27-OHC concentrations [354].

It is clear from the above findings that the nuanced interplay between lipid-lowering therapies and brain cholesterol metabolism imposes the need for further research to clearly establish the long-term neurocognitive implications and to refine management strategies for cholesterol-related disorders. While statins have caused controversy regarding their

effects on cognition and brain function, to date PCSK9i did not exhibit a significant risk for the same adverse cognitive effects in recent studies. An overview of the lipid-lowering therapies is provided in Table 1 [16,355].

Table 1. Overview of lipid-lowering therapy and implications for cholesterol management [16,355].

Lipid-Lowering Therapy	Average LDL-C Reduction	Indication	Mechanism of Action	Adverse Effects
Moderate-intensity statins	30% (monotherapy)	First-line treatment for lipid-lowering therapy and ASCVD risk reduction	↓ Hepatic cholesterol production by blocking HMGCoAR → ↑ LDLr expression → higher uptake of LDLs from the bloodstream	Myopathy Rhabdomyolysis Hepatotoxicity Diabetes mellitus Hemorrhagic stroke Proteinuria
High-intensity statins	50% (monotherapy)	Recommended up to the highest tolerated dose to reach the goals set for the specific level of risk		
Ezetimibe	65% (in combination with high-intensity statins) 85% (in combination with high-intensity statins and PCSK9 inhibitors)	Second-line therapy in association with statins when the therapeutic goal is not achieved at the maximal tolerated statin dose or when statins cannot be prescribed	Blocks intestinal absorption of dietary and biliary cholesterol → ↑ LDLr expression → ↑ LDL clearance from the bloodstream	Gastrointestinal adverse effects
Bile acid sequestrants	18–25% (monotherapy) 10–25% further decrease in LDL-C in combination with statins 18% further decrease in LDL-C when added to statins and ezetimibe	Second-line therapy in association with statins when the therapeutic goal is not achieved at the maximal tolerated statin dose or in cases of statin intolerance in combination with ezetimibe. Third-line therapy as an addition to statin plus ezetimibe therapy when the therapeutic goal is not achieved	Prevent the reabsorbtion of both the drug and cholesterol in the blood by binding the bile acids in the intestinum → liver synthesizes more bile acids from hepatic cholesterol → ↑ demand for cholesterol and a ↑ LDL-R expression	Gastrointestinal adverse effects Increased circulating TG levels (contraindicated in baseline TG > 400 mg/dL) Affects the absorption of many drugs and fat-soluble vitamins
PCSK9 inhibitors	60% (monotherapy) 75% (in combination with high-intensity statin) 85% (in combination with high-intensity statin plus ezetimibe)	Third-line therapy for the following: • Primary prevention in patients at very high risk but without FH if the LDL-C goal is not achieved on a maximum tolerated dose of a statin and ezetimibe • Secondary prevention for patients at very high risk not achieving their goal on a maximum tolerated dose of a statin and ezetimibe • Very-high-risk FH patients who do not achieve their goal on a maximum tolerated dose of a statin and ezetimibe If a statin-based regimen is not tolerated at any dosage, added to ezetimibe	PCSK9 binds to the LDL-R and promotes its degradation → ↑ LDL concentration in the plasma. PCSK9 inhibitors increase LDL-R expression by reduction in the plasma levels of PCSK9 → ↑ clearance of LDLs → decrease in LDL-C levels PCSK9 inhibitors also decrease Lp(a) levels	Itching, erythema, swelling, pain at the site of injection Allergic reactions—flu-like symptoms Increased risk of new, onset diabetes mellitus or neurocognitive dysfunction have been suspected but not demonstrated Occurrence of antidrug antibodies—very rare
Inclisiran	No data	Investigational	Stimulates the catalytic breakdown of PCSK9 mRNA in hepatocytes → reduction in hepatic synthesis of PCSK9 → increase in LDL-R expression → increased clearance of LDLs → decrease in LDL-C levels	Injection-site adverse reactions
Bempedroic acid	21.4% (monotherapy) 18% (in combination with statins) 38% (in combination with ezetimibe)	Reducing the risk of myocardial infarction and coronary revascularization in adults at risk and unable to take statins therapy. Adjunct to diet in combination with other LDL-C lowering therapies or alone when concomitant LDL-C lowering therapy is not possible	Inhibits ACLY which catalyzes the formation of acetyl-CoA → decreased cholesterol synthesis in liver → increased LDL-R expression → decrease in LDL-C levels	Hyperuricemia Tendon rupture Renal toxicity Cholelithiasis Benign prostatic hyperplasia Combination with simvastatin or pravastatin causes an increase in the concentrations of these drugs and, therefore, may increase the risk of myopathy

Table 1. *Cont.*

Lipid-Lowering Therapy	Average LDL-C Reduction	Indication	Mechanism of Action	Adverse Effects
Mipomersen	No data	Adjunct to lipid-lowering medications and diet for the treatment of statin-intolerant patients with severe HoFH	Inhibits of ApoB 100 production in the liver by binding with ApoB mRNA and preventing its translation → lowering LDL and VLDL levels	Adverse reactions at the injection site Liver toxicity
Lomitapide	30–50% (dose-dependent)	Indicated as once daily oral treatment for lowering LDL-C levels in adults with HoFH	Inhibits MTP → hinders the production of VLDLs in the liver and chylomicrons in the intestine	Gastrointestinal adverse effects Reduced absorption of fat-soluble vitamins and essential fatty acids Hepatotoxicity
Fibrates	50% reduction in the TG levels <20% reduction in the LDL-C levels	Indicated for patients with elevated TG levels and low HDL-C levels	Agonists of PPARs, acting via transcription factors regulating various steps in lipid and lipoprotein metabolisms	Renal dysfunction Liver disease Gallbladder disease Increased risk of pancreatitis

↑—increase/stimulate, ↓—decrease/inhibit, →—leads to; ApoB—apolipoprotein B; ACLY—ATP-citrate lyase; ASCVD—atherosclerotic cardiovascular disease; FH—familial hypercholesterolemia; HDL-C—HDL cholesterol; HoFH—homozygous familial hypercholesterolemia; HMG-CoAR—enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase; LDL-C—low-density lipoprotein cholesterol; LDL-Rs—receptors; Lp(a)—lipoprotein A; MTP—microsomal triglyceride transfer protein; mRNA—messenger RNA; PCSK9—proprotein convertase subtilisin/kexin type 9; PPARs—peroxisome proliferator-activated receptors; TG—triglyceride; VLDL—very-low-density lipoprotein.

6. Future Research Directions

LXRs agonists could be instrumental in the modulation of cholesterol metabolism with effects on AD based on their ability to attenuate amyloid pathology and microglial inflammation, as demonstrated in animal models [356]. However, the beneficial effects are overweighed by fatty acid and triglyceride upregulation [357,358].

Some statins, such as lovastatin, simvastatin, and pravastatin, are derivatives of microbial cultures, and this fact supports the increased interest for the study of microbiota in respect to dyslipidemia and cognitive functions [359]. Experimental data show that fecal microbiota transplantation might be effective against cognitive decline in animals exposed to a high-fat/high-cholesterol diet [360]. Fermented dairy products present numerous benefits alongside the reductions in cholesterol levels, such as antioxidant, anti-tumoral, and anti-inflammatory effects [361]. Short-chain fatty acids may play a role in the pathogenesis of AD and are involved in the maintenance of BBB integrity as well as neuroinflammation inhibition [362].

Future directions in lipid-lowering therapy include plant foods, marine algae, probiotics, and prebiotics to respond to both needs to lower cholesterol level and to improve cognition.

Healthy diets are increasingly promoted and plant foods have shown effectiveness in cardiovascular disease prevention due to their low amounts of fats and bioactive compounds, which act as cholesterol-lowering agents via different mechanisms [363]. Among them, soy proteins impair cholesterol absorption, lowering liver cholesterol levels and reducing the expression of several genes regulating lipid transport proteins [364]. Dietary fibers decrease cholesterol levels because of their capacity to bind and excrete bile acids while decreasing cholesterol absorption; short-chain fatty acids formation contributes to this effect, increasing cholesterol excretion and inhibiting cholesterol production [365]. Polysaccharide-based ingredients such as b-glucans exhibit beneficial properties in terms of cholesterol metabolism and are accepted by the European Food Safety Agency and the FDA [366]. These compounds conduct to bile salts sequestration and to active moieties resulted from polysaccharide fermentation by gut microbiota [367].

Marine algae exhibit neuroprotective effects through their bioactive compounds [368]. An increasingly studied molecule with hypolipidemic properties is naringenin (Nar) [369]. Citrus juices, especially orange, grapefruit, and bergamot juices, are rich in Nar, which inhibits acyl-coenzyme A cholesterol acyltransferase, HMGCoAR, and PCSK9 [370–374]. Beneficial effects on the lipid profile were confirmed for bergamot juice, which lowers total cholesterol, LDL-C, and triglycerides and increases HDL levels, as shown in animal models [375], as well as in patients with dyslipidemia [376,377]. Overall, the use of Nar in lowering cholesterol levels may represent an effective and novel approach that is not burdened by the adverse effects related to statins or PCSK9i.

7. Conclusions

Cholesterol plays a central role in CNS development and regulation, with a tightly regulated metabolism. The impermeability of the BBB keeps brain cholesterol operating within an isolated system. However, some metabolites such as oxysterols, enable essential cross-talk between brain and peripheral cholesterol systems, serving as key regulators of cholesterol balance.

Neurodegenerative conditions, including Alzheimer's and Parkinson's diseases, imply disruptions of this balance and bring attention to the importance of maintaining proper cholesterol homeostasis. The regulatory role of the BBB is essential in these processes, given its influence in determining both the passage of oxysterols and the broader interplay between brain and peripheral cholesterol. BBB permeability increases in various conditions, most commonly represented by aging and hypercholesterolemia, both associated with toxic accumulation of oxysterols within the brain.

The effects of lipid-lowering therapies raise critical considerations. While statins and PCSK9 inhibitors are effective in managing hypercholesterolemia, their impacts on brain cholesterol and cognitive health require further investigation.

An integrated approach taking into account the interdependence of brain and peripheral cholesterol systems is essential. It is expected that future research will improve our understanding of these complex pathways, advance cholesterol management, and address associated neurological outcomes.

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References

1. Bloch, K.E. Sterol, Structure and Membrane Function. *Crit. Rev. Biochem.* **1983**, *14*, 47–92. [CrossRef] [PubMed]
2. Dietschy, J.M.; Turley, S.D.; Spady, D.K. Role of Liver in the Maintenance of Cholesterol and Low Density Lipoprotein Homeostasis in Different Animal Species, Including Humans. *J. Lipid Res.* **1993**, *34*, 1637–1659. [CrossRef] [PubMed]
3. Ebner, E. Critical Considerations on Statin Therapy. *J. Clin. Biomed. Res.* **2020**, 1–2. [CrossRef]
4. Pike, L.J. Lipid Rafts: Bringing Order to Chaos. *J. Lipid Res.* **2003**, *44*, 655–667. [CrossRef] [PubMed]
5. Simons, K.; Van Meer, G. Lipid Sorting in Epithelial Cells. *Biochemistry* **1988**, *27*, 6197–6202. [CrossRef] [PubMed]
6. van Meer, G. The Different Hues of Lipid Rafts. *Science (1979)* **2002**, *296*, 855–857. [CrossRef] [PubMed]
7. Sezgin, E.; Levental, I.; Mayor, S.; Eggeling, C. The Mystery of Membrane Organization: Composition, Regulation and Roles of Lipid Rafts. *Nat. Rev. Mol. Cell Biol.* **2017**, *18*, 361–374. [CrossRef] [PubMed]

8. Burger, K.; Gimpl, G.; Fahrenholz, F. Regulation of Receptor Function by Cholesterol. *Cell Mol. Life Sci.* **2000**, *57*, 1577–1592. [CrossRef]
9. Brzeska, M.; Szymczyk, K.; Szterk, A. Current Knowledge about Oxysterols: A Review. *J. Food Sci.* **2016**, *81*, R2299–R2308. [CrossRef] [PubMed]
10. Miller, D.W. Fallacies in Modern Medicine: Statins and the Cholesterol-Heart Hypothesis. *J. Am. Physicians Surg.* **2015**, *20*, 54–56.
11. Lifshitz, F.; Moses, N. Growth Failure. A Complication of Dietary Treatment of Hypercholesterolemia. *Am. J. Dis. Child.* **1989**, *143*, 537–542. [CrossRef] [PubMed]
12. Barness, L.A. Nutritional Requirements of Infants and Children with Respect to Cholesterol and Related Compounds. *Am. J. Med. Genet.* **1994**, *50*, 353–354. [CrossRef] [PubMed]
13. Wong, W.W.; Hachey, D.L.; Insull, W.; Opekun, A.R.; Klein, P.D. Effect of Dietary Cholesterol on Cholesterol Synthesis in Breast-Fed and Formula-Fed Infants. *J. Lipid Res.* **1993**, *34*, 1403–1411. [CrossRef] [PubMed]
14. Dietschy, J.M.; Turley, S.D. Thematic Review Series: Brain Lipids. Cholesterol Metabolism in the Central Nervous System during Early Development and in the Mature Animal. *J. Lipid Res.* **2004**, *45*, 1375–1397. [CrossRef]
15. Makover, M.E.; Shapiro, M.D.; Toth, P.P. There Is Urgent Need to Treat Atherosclerotic Cardiovascular Disease Risk Earlier, More Intensively, and with Greater Precision: A Review of Current Practice and Recommendations for Improved Effectiveness. *Am. J. Prev. Cardiol.* **2022**, *12*, 100371. [CrossRef] [PubMed]
16. Mach, F.; Baigent, C.; Catapano, A.L.; Koskinas, K.C.; Casula, M.; Badimon, L.; Chapman, M.J.; De Backer, G.G.; Delgado, V.; Ference, B.A.; et al. 2019 ESC/EAS Guidelines for the Management of Dyslipidaemias: Lipid Modification to Reduce Cardiovascular Risk. *Eur. Heart J.* **2020**, *41*, 111–188. [CrossRef] [PubMed]
17. Sakuma, M.; Iimuro, S.; Shinozaki, T.; Kimura, T.; Nakagawa, Y.; Ozaki, Y.; Iwata, H.; Miyauchi, K.; Daida, H.; Suwa, S.; et al. Optimal Target of LDL Cholesterol Level for Statin Treatment: Challenges to Monotonic Relationship with Cardiovascular Events. *BMC Med.* **2022**, *20*, 441. [CrossRef] [PubMed]
18. Golomb, B.A.; Stattin, H.; Mednick, S. Low Cholesterol and Violent Crime. *J. Psychiatr. Res.* **2000**, *34*, 301–309. [CrossRef] [PubMed]
19. Virkkunen, M.; Penttinen, H. Serum Cholesterol in Aggressive Conduct Disorder: A Preliminary Study. *Biol. Psychiatry* **1984**, *19*, 435–439. [PubMed]
20. Golomb, B.A. Cholesterol and Violence: Is There a Connection? *Ann. Intern. Med.* **1998**, *128*, 478. [CrossRef]
21. Jenkins, D.C.; Hames, C.G.; Zyzanski, S.J.; Rosenman, R.H.; Friedman, M. Psychological Traits and Serum Lipids. *Psychosom. Med.* **1969**, *31*, 115–128. [CrossRef]
22. Engelberg, H. Low Serum Cholesterol and Suicide. *Lancet* **1992**, *339*, 727–729. [CrossRef]
23. Schatz, I.J.; Masaki, K.; Yano, K.; Chen, R.; Rodriguez, B.L.; Curb, J.D. Cholesterol and All-Cause Mortality in Elderly People from the Honolulu Heart Program: A Cohort Study. *Lancet* **2001**, *358*, 351–355. [CrossRef]
24. Shepherd, J.; Blauw, G.J.; Murphy, M.B.; Bollen, E.L.; Buckley, B.M.; Cobbe, S.M.; Ford, I.; Gaw, A.; Hyland, M.; Jukema, J.W.; et al. Pravastatin in Elderly Individuals at Risk of Vascular Disease (PROSPER): A Randomised Controlled Trial. *Lancet* **2002**, *360*, 1623–1630. [CrossRef]
25. Sacks, F.M.; Pfeffer, M.A.; Moye, L.A.; Rouleau, J.L.; Rutherford, J.D.; Cole, T.G.; Brown, L.; Warnica, J.W.; Arnold, J.M.O.; Wun, C.-C.; et al. The Effect of Pravastatin on Coronary Events after Myocardial Infarction in Patients with Average Cholesterol Levels. *N. Engl. J. Med.* **1996**, *335*, 1001–1009. [CrossRef] [PubMed]
26. Alsheikh-Ali, A.A.; Maddukuri, P.V.; Han, H.; Karas, R.H. Effect of the Magnitude of Lipid Lowering on Risk of Elevated Liver Enzymes, Rhabdomyolysis, and Cancer. *J. Am. Coll. Cardiol.* **2007**, *50*, 409–418. [CrossRef]
27. He, G.; Liu, X.; Liu, L.; Yu, Y.; Chen, C.; Huang, J.; Lo, K.; Huang, Y.; Feng, Y. A Nonlinear Association of Total Cholesterol with All-Cause and Cause-Specific Mortality. *Nutr. Metab.* **2021**, *18*, 25. [CrossRef] [PubMed]
28. Dietschy, J.M.; Wilson, J.D. Cholesterol Synthesis in the Squirrel Monkey: Relative Rates of Synthesis in Various Tissues and Mechanisms of Control. *J. Clin. Investig.* **1968**, *47*, 166–174. [CrossRef] [PubMed]
29. Luo, J.; Yang, H.; Song, B.-L. Mechanisms and Regulation of Cholesterol Homeostasis. *Nat. Rev. Mol. Cell Biol.* **2020**, *21*, 225–245. [CrossRef] [PubMed]
30. Andronie-Cioară, F.L.; Jurcău, A.; Jurcău, M.C.; Nistor-Cseppentö, D.C.; Simion, A. Cholesterol Management in Neurology: Time for Revised Strategies? *J. Pers. Med.* **2022**, *12*, 1981. [CrossRef] [PubMed]
31. Nieweg, K.; Schaller, H.; Pfrieger, F.W. Marked Differences in Cholesterol Synthesis between Neurons and Glial Cells from Postnatal Rats. *J. Neurochem.* **2009**, *109*, 125–134. [CrossRef]
32. Martini, C.; Pallottini, V. Cholesterol: From Feeding to Gene Regulation. *Genes. Nutr.* **2007**, *2*, 181–193. [CrossRef]
33. Reháková, R.; Cebová, M.; Matúšková, Z.; Košútová, M.; Kovácsová, M.; Pecháňová, O. Brain Cholesterol and the Role of Statins in Neuroprotection. *Act. Nerv. Super. Rediviva* **2016**, *58*, 11–17.
34. Sever, N.; Yang, T.; Brown, M.S.; Goldstein, J.L.; DeBose-Boyd, R.A. Accelerated Degradation of HMG CoA Reductase Mediated by Binding of Insig-1 to Its Sterol-Sensing Domain. *Mol. Cell* **2003**, *11*, 25–33. [CrossRef]

35. Theesfeld, C.L.; Pourmand, D.; Davis, T.; Garza, R.M.; Hampton, R.Y. The Sterol-Sensing Domain (SSD) Directly Mediates Signal-Regulated Endoplasmic Reticulum-Associated Degradation (ERAD) of 3-Hydroxy-3-Methylglutaryl (HMG)-CoA Reductase Isozyme Hmg2. *J. Biol. Chem.* **2011**, *286*, 26298–26307. [CrossRef] [PubMed]
36. Jo, Y.; DeBose-Boyd, R.A. Control of Cholesterol Synthesis through Regulated ER-Associated Degradation of HMG CoA Reductase. *Crit. Rev. Biochem. Mol. Biol.* **2010**, *45*, 185–198. [CrossRef]
37. DeBose-Boyd, R.A.; Brown, M.S.; Li, W.-P.; Nohturfft, A.; Goldstein, J.L.; Espenshade, P.J. Transport-Dependent Proteolysis of SREBP. *Cell* **1999**, *99*, 703–712. [CrossRef] [PubMed]
38. Nohturfft, A.; Yabe, D.; Goldstein, J.L.; Brown, M.S.; Espenshade, P.J. Regulated Step in Cholesterol Feedback Localized to Budding of SCAP from ER Membranes. *Cell* **2000**, *102*, 315–323. [CrossRef]
39. Goldstein, J.L.; Dana, S.E.; Faust, J.R.; Beaudet, A.L.; Brown, M.S. Role of Lysosomal Acid Lipase in the Metabolism of Plasma Low Density Lipoprotein. Observations in Cultured Fibroblasts from a Patient with Cholesteryl Ester Storage Disease. *J. Biol. Chem.* **1975**, *250*, 8487–8495. [CrossRef] [PubMed]
40. Ishibashi, S.; Herz, J.; Maeda, N.; Goldstein, J.L.; Brown, M.S. The Two-Receptor Model of Lipoprotein Clearance: Tests of the Hypothesis in "Knockout" Mice Lacking the Low Density Lipoprotein Receptor, Apolipoprotein E, or Both Proteins. *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 4431–4435. [CrossRef] [PubMed]
41. Acton, S.; Rigotti, A.; Landschulz, K.T.; Xu, S.; Hobbs, H.H.; Krieger, M. Identification of Scavenger Receptor SR-BI as a High Density Lipoprotein Receptor. *Science (1979)* **1996**, *271*, 518–520. [CrossRef] [PubMed]
42. Landschulz, K.T.; Pathak, R.K.; Rigotti, A.; Krieger, M.; Hobbs, H.H. Regulation of Scavenger Receptor, Class B, Type I, a High Density Lipoprotein Receptor, in Liver and Steroidogenic Tissues of the Rat. *J. Clin. Investig.* **1996**, *98*, 984–995. [CrossRef] [PubMed]
43. Gardner, L.A.; Levin, M.C. Importance of Apolipoprotein A-I in Multiple Sclerosis. *Front. Pharmacol.* **2015**, *6*, 278. [CrossRef]
44. Choroszyński, M.; Barcikowska, M.; Barczak, A. Metabolism and the Effect of Animal-Derived Oxysterols in the Diet on the Development of Alzheimer's Disease. *Ann. Nutr. Metab.* **2022**, *78*, 125–132. [CrossRef] [PubMed]
45. Cuperlovic-Culf, M.; Badhwar, A. Recent Advances from Metabolomics and Lipidomics Application in Alzheimer's Disease Inspiring Drug Discovery. *Expert. Opin. Drug Discov.* **2020**, *15*, 319–331. [CrossRef] [PubMed]
46. Liu, S.; Suzuki, H.; Ito, H.; Korenaga, T.; Akatsu, H.; Meno, K.; Uchida, K. Serum Levels of Proteins Involved in Amyloid-β Clearance Are Related to Cognitive Decline and Neuroimaging Changes in Mild Cognitive Impairment. *Alzheimer's Dement. Diagn. Assess. Dis. Monit.* **2019**, *11*, 85–97. [CrossRef] [PubMed]
47. Li, J.; Pfeffer, S.R. Lysosomal Membrane Glycoproteins Bind Cholesterol and Contribute to Lysosomal Cholesterol Export. *Elife* **2016**, *5*, e21635. [CrossRef] [PubMed]
48. Kwon, H.J.; Abi-Mosleh, L.; Wang, M.L.; Deisenhofer, J.; Goldstein, J.L.; Brown, M.S.; Infante, R.E. Structure of N-Terminal Domain of NPC1 Reveals Distinct Subdomains for Binding and Transfer of Cholesterol. *Cell* **2009**, *137*, 1213–1224. [CrossRef]
49. Kim, W.S.; Weickert, C.S.; Garner, B. Role of ATP-binding Cassette Transporters in Brain Lipid Transport and Neurological Disease. *J. Neurochem.* **2008**, *104*, 1145–1166. [CrossRef]
50. Wellington, C.L.; Walker, E.K.Y.; Suarez, A.; Kwok, A.; Bissada, N.; Singaraja, R.; Yang, Y.-Z.; Zhang, L.-H.; James, E.; Wilson, J.E.; et al. ABCA1 MRNA and Protein Distribution Patterns Predict Multiple Different Roles and Levels of Regulation. *Lab. Investig.* **2002**, *82*, 273–283. [CrossRef] [PubMed]
51. Koldamova, R.P.; Lefterov, I.M.; Ikonomovic, M.D.; Skoko, J.; Lefterov, P.I.; Isanski, B.A.; DeKosky, S.T.; Lazo, J.S. 22R-Hydroxycholesterol and 9-Cis-Retinoic Acid Induce ATP-Binding Cassette Transporter A1 Expression and Cholesterol Efflux in Brain Cells and Decrease Amyloid β Secretion. *J. Biol. Chem.* **2003**, *278*, 13244–13256. [CrossRef]
52. Fukumoto, H.; Deng, A.; Irizarry, M.C.; Fitzgerald, M.L.; Rebeck, G.W. Induction of the Cholesterol Transporter ABCA1 in Central Nervous System Cells by Liver X Receptor Agonists Increases Secreted Aβ Levels. *J. Biol. Chem.* **2002**, *277*, 48508–48513. [CrossRef] [PubMed]
53. Yokoyama, S. Release of Cellular Cholesterol: Molecular Mechanism for Cholesterol Homeostasis in Cells and in the Body. *Biochim. Et Biophys. Acta (BBA)-Mol. Cell Biol. Lipids* **2000**, *1529*, 231–244. [CrossRef]
54. Fielding, C.J.; Fielding, P.E. Cellular Cholesterol Efflux. *Biochim. Et Biophys. Acta (BBA)-Mol. Cell Biol. Lipids* **2001**, *1533*, 175–189. [CrossRef]
55. Panzenboeck, U.; Balazs, Z.; Sovic, A.; Hrzenjak, A.; Levak-Frank, S.; Wintersperger, A.; Malle, E.; Sattler, W. ABCA1 and Scavenger Receptor Class B, Type I, Are Modulators of Reverse Sterol Transport at an In Vitro Blood-Brain Barrier Constituted of Porcine Brain Capillary Endothelial Cells. *J. Biol. Chem.* **2002**, *277*, 42781–42789. [CrossRef] [PubMed]
56. Gosselet, F.; Candela, P.; Sevin, E.; Berezowski, V.; Cecchelli, R.; Fenart, L. Transcriptional Profiles of Receptors and Transporters Involved in Brain Cholesterol Homeostasis at the Blood–Brain Barrier: Use of an In Vitro Model. *Brain Res.* **2009**, *1249*, 34–42. [CrossRef] [PubMed]
57. Li, Y.; Lu, W.; Marzolo, M.P.; Bu, G. Differential Functions of Members of the Low Density Lipoprotein Receptor Family Suggested by Their Distinct Endocytosis Rates. *J. Biol. Chem.* **2001**, *276*, 18000–18006. [CrossRef]

58. William Rebeck, G.; Reiter, J.S.; Strickland, D.K.; Hyman, B.T. Apolipoprotein E in Sporadic Alzheimer's Disease: Allelic Variation and Receptor Interactions. *Neuron* **1993**, *11*, 575–580. [CrossRef]
59. Karuna, R.; von Eckardstein, A.; Rentsch, K.M. Dopant Assisted-Atmospheric Pressure Photoionization (DA-APPI) Liquid Chromatography–Mass Spectrometry for the Quantification of 27-Hydroxycholesterol in Plasma. *J. Chromatogr. B* **2009**, *877*, 261–268. [CrossRef]
60. Poli, G.; Biasi, F.; Leonarduzzi, G. Oxysterols in the Pathogenesis of Major Chronic Diseases. *Redox Biol.* **2013**, *1*, 125–130. [CrossRef] [PubMed]
61. Zhu, R.; Ou, Z.; Ruan, X.; Gong, J. Role of Liver X Receptors in Cholesterol Efflux and Inflammatory Signaling (Review). *Mol. Med. Rep.* **2012**, *5*, 895–900. [CrossRef]
62. Jacobson, M.S.; Price, M.G.; Shamoo, A.E.; Heald, F.P. Atherogenesis in White Carneau Pigeons Effects of Low-Level Cholestane-Triol Feeding. *Atherosclerosis* **1985**, *57*, 209–217. [CrossRef] [PubMed]
63. Staprans, I.; Pan, X.-M.; Rapp, J.H.; Grunfeld, C.; Feingold, K.R. Oxidized Cholesterol in the Diet Accelerates the Development of Atherosclerosis in LDL Receptor– and Apolipoprotein E–Deficient Mice. *Arterioscler. Thromb. Vasc. Biol.* **2000**, *20*, 708–714. [CrossRef] [PubMed]
64. Meynier, A.; Lherminier, J.; Demaison-Meloche, J.; Ginies, C.; Grandgirard, A.; Demaison, L. Effects of Dietary Oxysterols on Coronary Arteries in Hyperlipidaemic Hamsters. *Br. J. Nutr.* **2002**, *87*, 447–458. [CrossRef] [PubMed]
65. Staprans, I.; Pan, X.-M.; Rapp, J.H.; Feingold, K.R. Oxidized Cholesterol in the Diet Accelerates the Development of Aortic Atherosclerosis in Cholesterol-Fed Rabbits. *Arterioscler. Thromb. Vasc. Biol.* **1998**, *18*, 977–983. [CrossRef] [PubMed]
66. Dias, I.H.K.; Mistry, J.; Fell, S.; Reis, A.; Spickett, C.M.; Polidori, M.C.; Lip, G.Y.H.; Griffiths, H.R. Oxidized LDL Lipids Increase β-Amyloid Production by SH-SY5Y Cells through Glutathione Depletion and Lipid Raft Formation. *Free Radic. Biol. Med.* **2014**, *75*, 48–59. [CrossRef]
67. Olkkonen, V.M.; Béaslas, O.; Nissilä, E. Oxysterols and Their Cellular Effectors. *Biomolecules* **2012**, *2*, 76–103. [CrossRef]
68. Okabe, A.; Urano, Y.; Itoh, S.; Suda, N.; Kotani, R.; Nishimura, Y.; Saito, Y.; Noguchi, N. Adaptive Responses Induced by 24S-Hydroxycholesterol through Liver X Receptor Pathway Reduce 7-Ketocholesterol-Caused Neuronal Cell Death. *Redox Biol.* **2014**, *2*, 28–35. [CrossRef]
69. Babiker, A.; Diczfalusy, U. Transport of Side-Chain Oxidized Oxysterols in the Human Circulation. *Biochim. Et Biophys. Acta (BBA)-Lipids Lipid Metab.* **1998**, *1392*, 333–339. [CrossRef]
70. Dias, I.H.K.; Milic, I.; Lip, G.Y.H.; Devitt, A.; Polidori, M.C.; Griffiths, H.R. Simvastatin Reduces Circulating Oxysterol Levels in Men with Hypercholesterolaemia. *Redox Biol.* **2018**, *16*, 139–145. [CrossRef]
71. Leoni, V.; Masterman, T.; Mousavi, F.S.; Wretlind, B.; Wahlund, L.-O.; Diczfalusy, U.; Hillert, J.; Björkhem, I. Diagnostic Use of Cerebral and Extracerebral Oxysterols. *Clin. Chem. Lab. Med. (CCLM)* **2004**, *42*, 186–191. [CrossRef] [PubMed]
72. Lütjohann, D.; Breuer, O.; Ahlborg, G.; Nennesmo, I.; Sidén, A.; Diczfalusy, U.; Björkhem, I. Cholesterol Homeostasis in Human Brain: Evidence for an Age-Dependent Flux of 24S-Hydroxycholesterol from the Brain into the Circulation. *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 9799–9804. [CrossRef] [PubMed]
73. Sodero, A.O. 24S-hydroxycholesterol: Cellular Effects and Variations in Brain Diseases. *J. Neurochem.* **2021**, *157*, 899–918. [CrossRef] [PubMed]
74. Pfrieger, F.W. Cholesterol Homeostasis and Function in Neurons of the Central Nervous System. *Cell Mol. Life Sci.* **2003**, *60*, 1158–1171. [CrossRef] [PubMed]
75. Zhang, J.; Liu, Q. Cholesterol Metabolism and Homeostasis in the Brain. *Protein Cell* **2015**, *6*, 254–264. [CrossRef]
76. Guizzetti, M.; Costa, L. Cholesterol Homeostasis in the Developing Brain: A Possible New Target for Ethanol. *Hum. Exp. Toxicol.* **2007**, *26*, 355–360. [CrossRef] [PubMed]
77. Meir, K.; Kitsberg, D.; Alkalay, I.; Szafer, F.; Rosen, H.; Shpitzen, S.; Avi, L.B.; Staels, B.; Fievet, C.; Meiner, V.; et al. Human Sterol 27-Hydroxylase (CYP27) Overexpressor Transgenic Mouse Model. Evidence against 27-Hydroxycholesterol as a Critical Regulator of Cholesterol Homeostasis. *J. Biol. Chem.* **2002**, *277*, 34036–34041. [CrossRef]
78. Lund, E.G.; Guileyardo, J.M.; Russell, D.W. CDNA Cloning of Cholesterol 24-Hydroxylase, a Mediator of Cholesterol Homeostasis in the Brain. *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 7238–7243. [CrossRef]
79. Reitz, C.; Mayeux, R. Alzheimer Disease: Epidemiology, Diagnostic Criteria, Risk Factors and Biomarkers. *Biochem. Pharmacol.* **2014**, *88*, 640–651. [CrossRef]
80. Sodero, A.O.; Vriens, J.; Ghosh, D.; Stegner, D.; Brachet, A.; Pallotto, M.; Sassoè-Pognetto, M.; Brouwers, J.F.; Helms, J.B.; Nieswandt, B.; et al. Cholesterol Loss during Glutamate-Mediated Excitotoxicity. *EMBO J.* **2012**, *31*, 1764–1773. [CrossRef]
81. Wang, H.-L.; Wang, Y.-Y.; Liu, X.-G.; Kuo, S.-H.; Liu, N.; Song, Q.-Y.; Wang, M.-W. Cholesterol, 24-Hydroxycholesterol, and 27-Hydroxycholesterol as Surrogate Biomarkers in Cerebrospinal Fluid in Mild Cognitive Impairment and Alzheimer's Disease: A Meta-Analysis. *J. Alzheimers Dis.* **2016**, *51*, 45–55. [CrossRef] [PubMed]

82. Heverin, M.; Bogdanovic, N.; Lütjohann, D.; Bayer, T.; Pikuleva, I.; Bretillon, L.; Diczfalusy, U.; Winblad, B.; Björkhem, I. Changes in the Levels of Cerebral and Extracerebral Sterols in the Brain of Patients with Alzheimer's Disease. *J. Lipid Res.* **2004**, *45*, 186–193. [CrossRef] [PubMed]
83. Björkhem, I.; Cedazo-Minguez, A.; Leoni, V.; Meaney, S. Oxysterols and Neurodegenerative Diseases. *Mol. Aspects Med.* **2009**, *30*, 171–179. [CrossRef] [PubMed]
84. Björkhem, I. Crossing the Barrier: Oxysterols as Cholesterol Transporters and Metabolic Modulators in the Brain. *J. Intern. Med.* **2006**, *260*, 493–508. [CrossRef]
85. Leoni, V.; Masterman, T.; Patel, P.; Meaney, S.; Diczfalusy, U.; Björkhem, I. Side Chain Oxidized Oxysterols in Cerebrospinal Fluid and the Integrity of Blood-Brain and Blood-Cerebrospinal Fluid Barriers. *J. Lipid Res.* **2003**, *44*, 793–799. [CrossRef]
86. Petek, B.; Villa-Lopez, M.; Loera-Valencia, R.; Gerenu, G.; Winblad, B.; Kramberger, M.G.; Ismail, M.-A.-M.; Eriksdotter, M.; Garcia-Ptacek, S. Connecting the Brain Cholesterol and Renin-Angiotensin Systems: Potential Role of Statins and RAS-Modifying Medications in Dementia. *J. Intern. Med.* **2018**, *284*, 620–642. [CrossRef]
87. Michikawa, M. Role of Cholesterol in Amyloid Cascade: Cholesterol-Dependent Modulation of Tau Phosphorylation and Mitochondrial Function. *Acta Neurol. Scand. Suppl.* **2006**, *185*, 21–26. [CrossRef]
88. Dias, I.H.K.; Polidori, M.C.; Griffiths, H.R. Hypercholesterolaemia-Induced Oxidative Stress at the Blood-Brain Barrier. *Biochem. Soc. Trans.* **2014**, *42*, 1001–1005. [CrossRef] [PubMed]
89. Gamba, P.; Staurenghi, E.; Testa, G.; Giannelli, S.; Sottero, B.; Leonarduzzi, G. A Crosstalk Between Brain Cholesterol Oxidation and Glucose Metabolism in Alzheimer's Disease. *Front. Neurosci.* **2019**, *13*, 556. [CrossRef]
90. Björkhem, I.; Leoni, V.; Svenningsson, P. On the Fluxes of Side-Chain Oxidized Oxysterols across Blood-Brain and Blood-CSF Barriers and Origin of These Steroids in CSF (Review). *J. Steroid Biochem. Mol. Biol.* **2019**, *188*, 86–89. [CrossRef] [PubMed]
91. Wang, Y.; Zhang, X.; Wang, T.; Liu, W.; Wang, L.; Hao, L.; Ju, M.; Xiao, R. 27-Hydroxycholesterol Promotes the Transfer of Astrocyte-Derived Cholesterol to Neurons in Co-Cultured SH-SY5Y Cells and C6 Cells. *Front. Cell Dev. Biol.* **2020**, *8*, 580599. [CrossRef] [PubMed]
92. Ma, W.-W.; Li, C.-Q.; Zhao, L.; Wang, Y.-S.; Xiao, R. NF-KB-Mediated Inflammatory Damage Is Differentially Affected in SH-SY5Y and C6 Cells Treated with 27-Hydroxycholesterol. *Food Sci. Nutr.* **2019**, *7*, 1685–1694. [CrossRef]
93. Chen, Z.; Li, Y.; Xu, H.; Ma, F.; Li, J.; Zhao, L.; Xu, Y. Elevated Ischaemia-Associated Lysyl Oxidase Activity in Delayed Graft Failure 6-12 Months after Renal Transplantation. *Exp. Physiol.* **2017**, *102*, 282–287. [CrossRef]
94. Ismail, M.-A.-M.; Mateos, L.; Maioli, S.; Merino-Serrais, P.; Ali, Z.; Lodeiro, M.; Westman, E.; Leitersdorf, E.; Gulyás, B.; Olof-Wahlund, L.; et al. 27-Hydroxycholesterol Impairs Neuronal Glucose Uptake through an IRAP/GLUT4 System Dysregulation. *J. Exp. Med.* **2017**, *214*, 699–717. [CrossRef]
95. Infante, J.; Rodríguez-Rodríguez, E.; Mateo, I.; Llorca, J.; Vázquez-Higuera, J.L.; Berciano, J.; Combarros, O. Gene–Gene Interaction between Heme Oxygenase-1 and Liver X Receptor-β and Alzheimer's Disease Risk. *Neurobiol. Aging* **2010**, *31*, 710–714. [CrossRef]
96. Khan, M.I.; Min, J.-S.; Lee, S.-O.; Yim, D.G.; Seol, K.-H.; Lee, M.; Jo, C. Cooking, Storage, and Reheating Effect on the Formation of Cholesterol Oxidation Products in Processed Meat Products. *Lipids Health Dis.* **2015**, *14*, 89. [CrossRef]
97. Min, J.-S.; Lee, S.-O.; Khan, M.I.; Yim, D.G.; Seol, K.-H.; Lee, M.; Jo, C. Monitoring the Formation of Cholesterol Oxidation Products in Model Systems Using Response Surface Methodology. *Lipids Health Dis.* **2015**, *14*, 77. [CrossRef] [PubMed]
98. Nunes, V.S.; Panzoldo, N.B.; Leança, C.C.; Parra, E.S.; Zago, V.S.; da Silva, E.J.; Cazita, P.M.; Nakandakare, E.R.; de Faria, E.C.; Quintão, E.C.R. Increased 27-Hydroxycholesterol Plasma Level in Men with Low High Density Lipoprotein-Cholesterol May Circumvent Their Reduced Cell Cholesterol Efflux Rate. *Clin. Chim. Acta* **2014**, *433*, 169–173. [CrossRef] [PubMed]
99. Hughes, T.M.; Rosano, C.; Evans, R.W.; Kuller, L.H. Brain Cholesterol Metabolism, Oxysterols, and Dementia. *J. Alzheimer's Dis.* **2013**, *33*, 891–911. [CrossRef]
100. Leoni, V. Oxysterols as Markers of Neurological Disease—A Review. *Scand. J. Clin. Lab. Investig.* **2009**, *69*, 22–25. [CrossRef] [PubMed]
101. Leoni, V.; Solomon, A.; Lövgren-Sandblom, A.; Minthon, L.; Blennow, K.; Hansson, O.; Wahlund, L.-O.; Kivipelto, M.; Björkhem, I. Diagnostic Power of 24S-Hydroxycholesterol in Cerebrospinal Fluid: Candidate Marker of Brain Health. *J. Alzheimer's Dis.* **2013**, *36*, 739–747. [CrossRef] [PubMed]
102. Collingridge, G.L.; Volianskis, A.; Bannister, N.; France, G.; Hanna, L.; Mercier, M.; Tidball, P.; Fang, G.; Irvine, M.W.; Costa, B.M.; et al. The NMDA Receptor as a Target for Cognitive Enhancement. *Neuropharmacology* **2013**, *64*, 13–26. [CrossRef] [PubMed]
103. Paul, S.M.; Doherty, J.J.; Robichaud, A.J.; Belfort, G.M.; Chow, B.Y.; Hammond, R.S.; Crawford, D.C.; Linsenbardt, A.J.; Shu, H.-J.; Izumi, Y.; et al. The Major Brain Cholesterol Metabolite 24(S)-Hydroxycholesterol Is a Potent Allosteric Modulator of *N*-Methyl-d-Aspartate Receptors. *J. Neurosci.* **2013**, *33*, 17290–17300. [CrossRef]
104. Sun, M.-Y.; Izumi, Y.; Benz, A.; Zorumski, C.F.; Mennerick, S. Endogenous 24 *S*-Hydroxycholesterol Modulates NMDAR-Mediated Function in Hippocampal Slices. *J. Neurophysiol.* **2016**, *115*, 1263–1272. [CrossRef] [PubMed]

105. Wei, X.; Nishi, T.; Kondou, S.; Kimura, H.; Mody, I. Preferential Enhancement of GluN2B-Containing Native NMDA Receptors by the Endogenous Modulator 24S-Hydroxycholesterol in Hippocampal Neurons. *Neuropharmacology* **2019**, *148*, 11–20. [CrossRef] [PubMed]
106. Pfrieger, F.W. Outsourcing in the Brain: Do Neurons Depend on Cholesterol Delivery by Astrocytes? *BioEssays* **2003**, *25*, 72–78. [CrossRef]
107. Abildayeva, K.; Jansen, P.J.; Hirsch-Reinshagen, V.; Bloks, V.W.; Bakker, A.H.F.; Ramaekers, F.C.S.; de Vente, J.; Groen, A.K.; Wellington, C.L.; Kuipers, F.; et al. 24(S)-Hydroxycholesterol Participates in a Liver X Receptor-Controlled Pathway in Astrocytes That Regulates Apolipoprotein E-Mediated Cholesterol Efflux. *J. Biol. Chem.* **2006**, *281*, 12799–12808. [CrossRef] [PubMed]
108. Janowski, B.A.; Willy, P.J.; Devi, T.R.; Falck, J.R.; Mangelsdorf, D.J. An Oxysterol Signalling Pathway Mediated by the Nuclear Receptor LXRα. *Nature* **1996**, *383*, 728–731. [CrossRef]
109. Lehmann, J.M.; Kliewer, S.A.; Moore, L.B.; Smith-Oliver, T.A.; Oliver, B.B.; Su, J.-L.; Sundseth, S.S.; Winegar, D.A.; Blanchard, D.E.; Spencer, T.A.; et al. Activation of the Nuclear Receptor LXR by Oxysterols Defines a New Hormone Response Pathway. *J. Biol. Chem.* **1997**, *272*, 3137–3140. [CrossRef] [PubMed]
110. Han, M.; Wang, S.; Yang, N.; Wang, X.; Zhao, W.; Saed, H.S.; Daubon, T.; Huang, B.; Chen, A.; Li, G.; et al. Therapeutic Implications of Altered Cholesterol Homeostasis Mediated by Loss of CYP46A1 in Human Glioblastoma. *EMBO Mol. Med.* **2020**, *12*, e10924. [CrossRef] [PubMed]
111. Czuba, E.; Steliga, A.; Lietzau, G.; Kowiański, P. Cholesterol as a Modifying Agent of the Neurovascular Unit Structure and Function under Physiological and Pathological Conditions. *Metab. Brain Dis.* **2017**, *32*, 935–948. [CrossRef] [PubMed]
112. Saint-Pol, J.; Candela, P.; Boucau, M.-C.; Fenart, L.; Gosselet, F. Oxysterols Decrease Apical-to-Basolateral Transport of Aß Peptides via an ABCB1-Mediated Process in an In Vitro Blood-Brain Barrier Model Constituted of Bovine Brain Capillary Endothelial Cells. *Brain Res.* **2013**, *1517*, 1–15. [CrossRef]
113. Bogdanovic, N.; Bretillon, L.; Lund, E.G.; Diczfalusy, U.; Lannfelt, L.; Winblad, B.; Russell, D.W.; Björkhem, I. On the Turnover of Brain Cholesterol in Patients with Alzheimer's Disease. Abnormal Induction of the Cholesterol-Catabolic Enzyme CYP46 in Glial Cells. *Neurosci. Lett.* **2001**, *314*, 45–48. [CrossRef] [PubMed]
114. Russell, D.W.; Halford, R.W.; Ramirez, D.M.O.; Shah, R.; Kotti, T. Cholesterol 24-Hydroxylase: An Enzyme of Cholesterol Turnover in the Brain. *Annu. Rev. Biochem.* **2009**, *78*, 1017–1040. [CrossRef]
115. Sodero, A.O.; Weissmann, C.; Ledesma, M.D.; Dotti, C.G. Cellular Stress from Excitatory Neurotransmission Contributes to Cholesterol Loss in Hippocampal Neurons Aging In Vitro. *Neurobiol. Aging* **2011**, *32*, 1043–1053. [CrossRef] [PubMed]
116. Palomer, E.; Martín-Segura, A.; Baliyan, S.; Ahmed, T.; Balschun, D.; Venero, C.; Martin, M.G.; Dotti, C.G. Aging Triggers a Repressive Chromatin State at Bdnf Promoters in Hippocampal Neurons. *Cell Rep.* **2016**, *16*, 2889–2900. [CrossRef]
117. Pérez-Cañamás, A.; Sarroca, S.; Melero-Jerez, C.; Porquet, D.; Sansa, J.; Knafo, S.; Esteban, J.A.; Sanfeliu, C.; Ledesma, M.D. A Diet Enriched with Plant Sterols Prevents the Memory Impairment Induced by Cholesterol Loss in Senescence-Accelerated Mice. *Neurobiol. Aging* **2016**, *48*, 1–12. [CrossRef]
118. Loera-Valencia, R.; Goikolea, J.; Parrado-Fernandez, C.; Merino-Serrais, P.; Maioli, S. Alterations in Cholesterol Metabolism as a Risk Factor for Developing Alzheimer's Disease: Potential Novel Targets for Treatment. *J. Steroid Biochem. Mol. Biol.* **2019**, *190*, 104–114. [CrossRef] [PubMed]
119. Kotti, T.J.; Ramirez, D.M.O.; Pfeiffer, B.E.; Huber, K.M.; Russell, D.W. Brain Cholesterol Turnover Required for Geranylgeraniol Production and Learning in Mice. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 3869–3874. [CrossRef] [PubMed]
120. Ohyama, Y.; Meaney, S.; Heverin, M.; Ekström, L.; Brafman, A.; Shafir, M.; Andersson, U.; Olin, M.; Eggertsen, G.; Diczfalusy, U.; et al. Studies on the Transcriptional Regulation of Cholesterol 24-Hydroxylase (CYP46A1). *J. Biol. Chem.* **2006**, *281*, 3810–3820. [CrossRef] [PubMed]
121. Kölsch, H.; Lütjohann, D.; Tulke, A.; Björkhem, I.; Rao, M.L. The Neurotoxic Effect of 24-Hydroxycholesterol on SH-SY5Y Human Neuroblastoma Cells. *Brain Res.* **1999**, *818*, 171–175. [CrossRef] [PubMed]
122. Moutinho, M.; Nunes, M.J.; Gomes, A.Q.; Gama, M.J.; Cedazo-Minguez, A.; Rodrigues, C.M.P.; Björkhem, I.; Rodrigues, E. Cholesterol 24S-Hydroxylase Overexpression Inhibits the Liver X Receptor (LXR) Pathway by Activating Small Guanosine Triphosphate-Binding Proteins (SGTPases) in Neuronal Cells. *Mol. Neurobiol.* **2015**, *51*, 1489–1503. [CrossRef] [PubMed]
123. Noguchi, N.; Saito, Y.; Urano, Y. Diverse Functions of 24(S)-Hydroxycholesterol in the Brain. *Biochem. Biophys. Res. Commun.* **2014**, *446*, 692–696. [CrossRef] [PubMed]
124. Noguchi, N.; Urano, Y.; Takabe, W.; Saito, Y. New Aspects of 24(S)-Hydroxycholesterol in Modulating Neuronal Cell Death. *Free Radic. Biol. Med.* **2015**, *87*, 366–372. [CrossRef]
125. Thelen, K.M.; Falkai, P.; Bayer, T.A.; Lütjohann, D. Cholesterol Synthesis Rate in Human Hippocampus Declines with Aging. *Neurosci. Lett.* **2006**, *403*, 15–19. [CrossRef]
126. Kotti, T.; Head, D.D.; McKenna, C.E.; Russell, D.W. Biphasic Requirement for Geranylgeraniol in Hippocampal Long-Term Potentiation. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 11394–11399. [CrossRef]

127. Vega, G.L.; Weiner, M.F.; Lipton, A.M.; von Bergmann, K.; Lütjohann, D.; Moore, C.; Svetlik, D. Reduction in Levels of 24S-Hydroxycholesterol by Statin Treatment in Patients With Alzheimer Disease. *Arch. Neurol.* **2003**, *60*, 510. [CrossRef] [PubMed]
128. Famer, D.; Meaney, S.; Mousavi, M.; Nordberg, A.; Björkhem, I.; Crisby, M. Regulation of α- and β-Secretase Activity by Oxysterols: Cerebrosterol Stimulates Processing of APP via the α-Secretase Pathway. *Biochem. Biophys. Res. Commun.* **2007**, *359*, 46–50. [CrossRef] [PubMed]
129. Prasanthi, J.R.; Huls, A.; Thomasson, S.; Thompson, A.; Schommer, E.; Ghribi, O. Differential Effects of 24-Hydroxycholesterol and 27-Hydroxycholesterol on β-Amyloid Precursor Protein Levels and Processing in Human Neuroblastoma SH-SY5Y Cells. *Mol. Neurodegener.* **2009**, *4*, 1. [CrossRef] [PubMed]
130. Testa, G.; Staurenghi, E.; Giannelli, S.; Gargiulo, S.; Guglielmotto, M.; Tabaton, M.; Tamagno, E.; Gamba, P.; Leonarduzzi, G. A Silver Lining for 24-Hydroxycholesterol in Alzheimer's Disease: The Involvement of the Neuroprotective Enzyme Sirtuin 1. *Redox Biol.* **2018**, *17*, 423–431. [CrossRef]
131. Corley, J.; Shivappa, N.; Hébert, J.R.; Starr, J.M.; Deary, I.J. Associations between Dietary Inflammatory Index Scores and Inflammatory Biomarkers among Older Adults in the Lothian Birth Cohort 1936 Study. *J. Nutr. Health Aging* **2019**, *23*, 628–636. [CrossRef] [PubMed]
132. Wong, M.Y.; Lewis, M.; Doherty, J.J.; Shi, Y.; Cashikar, A.G.; Amelianchik, A.; Tymchuk, S.; Sullivan, P.M.; Qian, M.; Covey, D.F.; et al. 25-Hydroxycholesterol Amplifies Microglial IL-1β Production in an ApoE Isoform-Dependent Manner. *J. Neuroinflamm.* **2020**, *17*, 192. [CrossRef] [PubMed]
133. Lund, E.G.; Kerr, T.A.; Sakai, J.; Li, W.-P.; Russell, D.W. CDNA Cloning of Mouse and Human Cholesterol 25-Hydroxylases, Polytopic Membrane Proteins That Synthesize a Potent Oxysterol Regulator of Lipid Metabolism. *J. Biol. Chem.* **1998**, *273*, 34316–34327. [CrossRef] [PubMed]
134. Brown, M.S.; Goldstein, J.L. Cholesterol Feedback: From Schoenheimer's Bottle to Scap's MELADL. *J. Lipid Res.* **2009**, *50*, S15–S27. [CrossRef]
135. Kobierski, J.; Wnętrzak, A.; Chachaj-Brekiesz, A.; Filiczkowska, A.; Petelska, A.D.; Dynarowicz-Latka, P. How the Replacement of Cholesterol by 25-Hydroxycholesterol Affects the Interactions with Sphingolipids: The Langmuir Monolayer Study Complemented with Theoretical Calculations. *J. R. Soc. Interface* **2021**, *18*, rsif.2021.0050. [CrossRef]
136. Javitt, N.B.; Lee, Y.C.; Shimizu, C.; Fuda, H.; Strott, C.A. Cholesterol and Hydroxycholesterol Sulfotransferases: Identification, Distinction from Dehydroepiandrosterone Sulfotransferase, and Differential Tissue Expression. *Endocrinology* **2001**, *142*, 2978–2984. [CrossRef] [PubMed]
137. Borowicz, K.K.; Piskorska, B.; Banach, M.; Czuczwar, S.J. Neuroprotective Actions of Neurosteroids. *Front. Endocrinol.* **2011**, *2*, 50. [CrossRef]
138. Vaňková, M.; Hill, M.; Velíková, M.; Včelák, J.; Vacínová, G.; Lukášová, P.; Vejražková, D.; Dvořáková, K.; Rusina, R.; Holmerová, I.; et al. Reduced Sulfotransferase SULT2A1 Activity in Patients With Alzheimer's Disease. *Physiol. Res.* **2015**, *64*, S265–S273. [CrossRef] [PubMed]
139. Björkhem, I.; Meaney, S. Brain Cholesterol: Long Secret Life Behind a Barrier. *Arterioscler. Thromb. Vasc. Biol.* **2004**, *24*, 806–815. [CrossRef]
140. Davison, A.N. Brain Sterol Metabolism. *Adv. Lipid Res.* **1965**, *3*, 171–196.
141. Kabara, J.J. A Critical Review of Brain Cholesterol Metabolism. *Prog. Brain Res.* **1973**, *40*, 363–382.
142. Jurevics, H.; Morell, P. Cholesterol for Synthesis of Myelin Is Made Locally, Not Imported into Brain. *J. Neurochem.* **1995**, *64*, 895–901. [CrossRef]
143. Turley, S.D.; Burns, D.K.; Dietschy, J.M. Preferential Utilization of Newly Synthesized Cholesterol for Brain Growth in Neonatal Lambs. *Am. J. Physiol.-Endocrinol. Metab.* **1998**, *274*, E1099–E1105. [CrossRef]
144. Quan, G.; Xie, C.; Dietschy, J.M.; Turley, S.D. Ontogenesis and Regulation of Cholesterol Metabolism in the Central Nervous System of the Mouse. *Dev. Brain Res.* **2003**, *146*, 87–98. [CrossRef] [PubMed]
145. Dietschy, J.M.; Turley, S.D. Cholesterol Metabolism in the Brain. *Curr. Opin. Lipidol.* **2001**, *12*, 105–112. [CrossRef] [PubMed]
146. Zhang, Y.; Appelkvist, E.-L.; Kristensson, K.; Dallner, G. The Lipid Compositions of Different Regions of Rat Brain during Development and Aging. *Neurobiol. Aging* **1996**, *17*, 869–875. [CrossRef] [PubMed]
147. Cicolari, S.; Pavanello, C.; Olmastroni, E.; Puppo, M.D.; Bertolotti, M.; Mombelli, G.; Catapano, A.L.; Calabresi, L.; Magni, P. Interactions of Oxysterols with Atherosclerosis Biomarkers in Subjects with Moderate Hypercholesterolemia and Effects of a Nutraceutical Combination (Bifidobacterium Longum BB536, Red Yeast Rice Extract) (Randomized, Double-Blind, Placebo-Controlled Study). *Nutrients* **2021**, *13*, 427. [CrossRef] [PubMed]
148. Andersson, M.; Elmberger, P.O.; Edlund, C.; Kristensson, K.; Dallner, G. Rates of Cholesterol, Ubiquinone, Dolichol and Dolichyl-P Biosynthesis in Rat Brain Slices. *FEBS Lett.* **1990**, *269*, 15–18. [CrossRef] [PubMed]
149. Bjorkhem, I.; Heverin, M.; Leoni, V.; Meaney, S.; Diczfalusy, U. Oxysterols and Alzheimer's Disease. *Acta Neurol. Scand.* **2006**, *114*, 43–49. [CrossRef] [PubMed]

150. Orth, M.; Bellosta, S. Cholesterol: Its Regulation and Role in Central Nervous System Disorders. *Cholesterol* **2012**, *2012*, 1–19. [CrossRef]
151. Ulusoy, E.K. Correlations between the Monocyte to High-Density Lipoprotein Cholesterol Ratio and White Matter Hyperintensities in Migraine. *Neurol. Res.* **2020**, *42*, 126–132. [CrossRef] [PubMed]
152. Lopez, A.M.; Ramirez, C.M.; Taylor, A.M.; Jones, R.D.; Repa, J.J.; Turley, S.D. Ontogenesis and Modulation of Intestinal Unesterified Cholesterol Sequestration in a Mouse Model of Niemann–Pick C1 Disease. *Dig. Dis. Sci.* **2020**, *65*, 158–167. [CrossRef]
153. Martin, M.; Dotti, C.G.; Ledesma, M.D. Brain Cholesterol in Normal and Pathological Aging. *Biochim. Et Biophys. Acta (BBA)-Mol. Cell Biol. Lipids* **2010**, *1801*, 934–944. [CrossRef]
154. Goldstein, J.L.; Brown, M.S. Regulation of the Mevalonate Pathway. *Nature* **1990**, *343*, 425–430. [CrossRef]
155. Di Paolo, G.; Kim, T.-W. Linking Lipids to Alzheimer's Disease: Cholesterol and Beyond. *Nat. Rev. Neurosci.* **2011**, *12*, 284–296, Erratumed in *Nat. Rev. Neurosci.* **2011**, *12*, 484. [CrossRef] [PubMed]
156. Lopez, A.M.; Chuang, J.-C.; Turley, S.D. Measurement of Rates of Cholesterol and Fatty Acid Synthesis in Vivo Using Tritiated Water. *Cholest. Homeost. Methods Protoc.* **2017**, *1583*, 241–256.
157. Vitali, C.; Wellington, C.L.; Calabresi, L. HDL and Cholesterol Handling in the Brain. *Cardiovasc. Res.* **2014**, *103*, 405–413. [CrossRef] [PubMed]
158. Ho, W.Y.; Hartmann, H.; Ling, S. Central Nervous System Cholesterol Metabolism in Health and Disease. *IUBMB Life* **2022**, *74*, 826–841. [CrossRef]
159. Bélanger, M.; Allaman, I.; Magistretti, P.J. Brain Energy Metabolism: Focus on Astrocyte-Neuron Metabolic Cooperation. *Cell Metab.* **2011**, *14*, 724–738. [CrossRef]
160. Lovatt, D.; Sonnewald, U.; Waagepetersen, H.S.; Schousboe, A.; He, W.; Lin, J.H.-C.; Han, X.; Takano, T.; Wang, S.; Sim, F.J.; et al. The Transcriptome and Metabolic Gene Signature of Protoplasmic Astrocytes in the Adult Murine Cortex. *J. Neurosci.* **2007**, *27*, 12255–12266. [CrossRef] [PubMed]
161. Henn, R.E.; Noureldein, M.H.; Elzinga, S.E.; Kim, B.; Savelieff, M.G.; Feldman, E.L. Glial-Neuron Crosstalk in Health and Disease: A Focus on Metabolism, Obesity, and Cognitive Impairment. *Neurobiol. Dis.* **2022**, *170*, 105766. [CrossRef] [PubMed]
162. Joe, E.-H.; Choi, D.-J.; An, J.; Eun, J.-H.; Jou, I.; Park, S. Astrocytes, Microglia, and Parkinson's Disease. *Exp. Neurobiol.* **2018**, *27*, 77–87. [CrossRef] [PubMed]
163. Varcianna, A.; Myszczynska, M.A.; Castelli, L.M.; O'Neill, B.; Kim, Y.; Talbot, J.; Nyberg, S.; Nyamali, I.; Heath, P.R.; Stopford, M.J.; et al. Micro-RNAs Secreted through Astrocyte-Derived Extracellular Vesicles Cause Neuronal Network Degeneration in C9orf72 ALS. *EBioMedicine* **2019**, *40*, 626–635. [CrossRef]
164. Kisler, K.; Nelson, A.R.; Montagne, A.; Zlokovic, B.V. Cerebral Blood Flow Regulation and Neurovascular Dysfunction in Alzheimer Disease. *Nat. Rev. Neurosci.* **2017**, *18*, 419–434. [CrossRef] [PubMed]
165. Brown, M.S.; Goldstein, J.L. A Receptor-Mediated Pathway for Cholesterol Homeostasis. *Science (1979)* **1986**, *232*, 34–47. [CrossRef] [PubMed]
166. Björkhem, I.; Lövgren-Sandblom, A.; Leoni, V.; Meaney, S.; Brodin, L.; Salveson, L.; Winge, K.; Pålhagen, S.; Svenningsson, P. Oxysterols and Parkinson's Disease: Evidence That Levels of 24S-Hydroxycholesterol in Cerebrospinal Fluid Correlates with the Duration of the Disease. *Neurosci. Lett.* **2013**, *555*, 102–105. [CrossRef]
167. Martín, M.G.; Pfrieger, F.; Dotti, C.G. Cholesterol in Brain Disease: Sometimes Determinant and Frequently Implicated. *EMBO Rep.* **2014**, *15*, 1036–1052. [CrossRef]
168. Pfrieger, F.W.; Barres, B.A. Synaptic Efficacy Enhanced by Glial Cells In Vitro. *Science (1979)* **1997**, *277*, 1684–1687. [CrossRef] [PubMed]
169. Mauch, D.H.; Nägler, K.; Schumacher, S.; Göritz, C.; Müller, E.-C.; Otto, A.; Pfrieger, F.W. CNS Synaptogenesis Promoted by Glia-Derived Cholesterol. *Science (1979)* **2001**, *294*, 1354–1357. [CrossRef]
170. Ullian, E.M.; Sapperstein, S.K.; Christopherson, K.S.; Barres, B.A. Control of Synapse Number by Glia. *Science (1979)* **2001**, *291*, 657–661. [CrossRef]
171. Li, R.; Wang, T.-J.; Lyu, P.-Y.; Liu, Y.; Chen, W.-H.; Fan, M.-Y.; Xu, J. Effects of Plasma Lipids and Statins on Cognitive Function. *Chin. Med. J.* **2018**, *131*, 471–476. [CrossRef] [PubMed]
172. Evans, M.A.; Golomb, B.A. Statin-Associated Adverse Cognitive Effects: Survey Results from 171 Patients. *Pharmacother. J. Hum. Pharmacol. Drug Ther.* **2009**, *29*, 800–811. [CrossRef] [PubMed]
173. Dziedzic, T.; Slowik, A.; Gryz, E.A.; Szczudlik, A. Lower Serum Triglyceride Level Is Associated With Increased Stroke Severity. *Stroke* **2004**, *35*, e151–e152. [CrossRef] [PubMed]
174. Lange, Y.; Ye, J.; Strebel, F. Movement of 25-Hydroxycholesterol from the Plasma Membrane to the Rough Endoplasmic Reticulum in Cultured Hepatoma Cells. *J. Lipid Res.* **1995**, *36*, 1092–1097. [CrossRef] [PubMed]
175. Meaney, S.; Bodin, K.; Diczfalusy, U.; Björkhem, I. On the Rate of Translocation In Vitro and Kinetics in Vivo of the Major Oxysterols in Human Circulation. *J. Lipid Res.* **2002**, *43*, 2130–2135. [CrossRef]

176. Roheim, P.S.; Carey, M.; Forte, T.; Vega, G.L. Apolipoproteins in Human Cerebrospinal Fluid. *Proc. Natl. Acad. Sci. USA* **1979**, *76*, 4646–4649. [CrossRef]
177. Pitas, R.E.; Boyles, J.K.; Lee, S.H.; Hui, D.; Weisgraber, K.H. Lipoproteins and Their Receptors in the Central Nervous System. Characterization of the Lipoproteins in Cerebrospinal Fluid and Identification of Apolipoprotein B,E(LDL) Receptors in the Brain. *J. Biol. Chem.* **1987**, *262*, 14352–14360. [CrossRef] [PubMed]
178. Koch, S.; Donarski, N.; Goetze, K.; Kreckel, M.; Stuerenburg, H.J.; Buhmann, C.; Beisiegel, U. Characterization of Four Lipoprotein Classes in Human Cerebrospinal Fluid. *J. Lipid Res.* **2001**, *42*, 1143–1151. [CrossRef]
179. Fung, K.Y.; Wang, C.; Nyegaard, S.; Heit, B.; Fairn, G.D.; Lee, W.L. SR-BI Mediated Transcytosis of HDL in Brain Microvascular Endothelial Cells Is Independent of Caveolin, Clathrin, and PDZK1. *Front. Physiol.* **2017**, *8*, 841. [CrossRef] [PubMed]
180. Elliott, D.A.; Weickert, C.S.; Garner, B. Apolipoproteins in the Brain: Implications for Neurological and Psychiatric Disorders. *Clin. Lipidol.* **2010**, *51*, 555–573. [CrossRef] [PubMed]
181. Liu, C.-C.; Kanekiyo, T.; Xu, H.; Bu, G. Apolipoprotein E and Alzheimer Disease: Risk, Mechanisms and Therapy. *Nat. Rev. Neurol.* **2013**, *9*, 106–118; Corrected in *Nat. Rev. Neurol.* **2013**, *9*, 184. [CrossRef] [PubMed]
182. Pitas, R.E.; Boyles, J.K.; Lee, S.H.; Foss, D.; Mahley, R.W. Astrocytes Synthesize Apolipoprotein E and Metabolize Apolipoprotein E-Containing Lipoproteins. *Biochim. Et Biophys. Acta (BBA)-Lipids Lipid Metab.* **1987**, *917*, 148–161. [CrossRef]
183. Linton, M.F.; Gish, R.; Hubl, S.T.; Bütler, E.; Esquivel, C.; Bry, W.I.; Boyles, J.K.; Wardell, M.R.; Young, S.G. Phenotypes of Apolipoprotein B and Apolipoprotein E after Liver Transplantation. *J. Clin. Investig.* **1991**, *88*, 270–281. [CrossRef]
184. Budny, V.; Knöpfli, Y.; Meier, D.; Zürcher, K.; Bodenmann, C.; Peter, S.L.; Müller, T.; Tardy, M.; Cortijo, C.; Tackenberg, C. APOE4 Increases Energy Metabolism in APOE-Isogenic IPSC-Derived Neurons. *Cells* **2024**, *13*, 1207. [CrossRef]
185. Dong, W.; Vuletic, S.; Albers, J.J. Differential Effects of Simvastatin and Pravastatin on Expression of Alzheimer's Disease-Related Genes in Human Astrocytes and Neuronal Cells. *J. Lipid Res.* **2009**, *50*, 2095–2102. [CrossRef]
186. Benilova, I.; Karran, E.; De Strooper, B. The Toxic Aβ Oligomer and Alzheimer's Disease: An Emperor in Need of Clothes. *Nat. Neurosci.* **2012**, *15*, 349–357. [CrossRef]
187. Hardy, J.; Selkoe, D.J. The Amyloid Hypothesis of Alzheimer's Disease: Progress and Problems on the Road to Therapeutics. *Science (1979)* **2002**, *297*, 353–356. [CrossRef]
188. Basak, J.M.; Verghese, P.B.; Yoon, H.; Kim, J.; Holtzman, D.M. Low-Density Lipoprotein Receptor Represents an Apolipoprotein E-Independent Pathway of Aβ Uptake and Degradation by Astrocytes. *J. Biol. Chem.* **2012**, *287*, 13959–13971. [CrossRef] [PubMed]
189. Kim, J.; Basak, J.M.; Holtzman, D.M. The Role of Apolipoprotein E in Alzheimer's Disease. *Neuron* **2009**, *63*, 287–303. [CrossRef]
190. Banks, W.A.; Reed, M.J.; Logsdon, A.F.; Rhea, E.M.; Erickson, M.A. Healthy Aging and the Blood–Brain Barrier. *Nat. Aging* **2021**, *1*, 243–254. [CrossRef] [PubMed]
191. Narayan, P.; Sienski, G.; Bonner, J.M.; Lin, Y.-T.; Seo, J.; Baru, V.; Haque, A.; Milo, B.; Akay, L.A.; Graziosi, A.; et al. PICALM Rescues Endocytic Defects Caused by the Alzheimer's Disease Risk Factor APOE4. *Cell Rep.* **2020**, *33*, 108224. [CrossRef]
192. Bryleva, E.Y.; Rogers, M.A.; Chang, C.C.Y.; Buen, F.; Harris, B.T.; Rousselet, E.; Seidah, N.G.; Oddo, S.; LaFerla, F.M.; Spencer, T.A.; et al. ACAT1 Gene Ablation Increases 24(S)-Hydroxycholesterol Content in the Brain and Ameliorates Amyloid Pathology in Mice with AD. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 3081–3086. [CrossRef]
193. Sakashita, N.; Miyazaki, A.; Takeya, M.; Horiuchi, S.; Chang, C.C.Y.; Chang, T.-Y.; Takahashi, K. Localization of Human Acyl-Coenzyme A:Cholesterol Acyltransferase-1 (ACAT-1) in Macrophages and in Various Tissues. *Am. J. Pathol.* **2000**, *156*, 227–236. [CrossRef]
194. Brankatschk, M.; Eaton, S. Lipoprotein Particles Cross the Blood-Brain Barrier in Drosophila. *J. Neurosci.* **2010**, *30*, 10441–10447. [CrossRef] [PubMed]
195. Connor, W.E.; Johnston, R.; Lin, D.S. Metabolism of Cholesterol in the Tissues and Blood of the Chick Embryo. *J. Lipid Res.* **1969**, *10*, 388–394. [CrossRef]
196. Chobanian, A.V.; Hollander, W. Body Cholesterol Metabolism in Man. I. The Equilibration of Serum and Tissue Cholesterol. *J. Clin. Investig.* **1962**, *41*, 1732–1737. [CrossRef] [PubMed]
197. Pifferi, F.; Laurent, B.; Plourde, M. Lipid Transport and Metabolism at the Blood-Brain Interface: Implications in Health and Disease. *Front. Physiol.* **2021**, *12*, 645646. [CrossRef] [PubMed]
198. Morita, S.; Furube, E.; Mannari, T.; Okuda, H.; Tatsumi, K.; Wanaka, A.; Miyata, S. Heterogeneous Vascular Permeability and Alternative Diffusion Barrier in Sensory Circumventricular Organs of Adult Mouse Brain. *Cell Tissue Res.* **2016**, *363*, 497–511. [CrossRef] [PubMed]
199. Miyata, S. New Aspects in Fenestrated Capillary and Tissue Dynamics in the Sensory Circumventricular Organs of Adult Brains. *Front. Neurosci.* **2015**, *9*, 390. [CrossRef]
200. Willis, C.L.; Garwood, C.J.; Ray, D.E. A Size Selective Vascular Barrier in the Rat Area Postrema Formed by Perivascular Macrophages and the Extracellular Matrix. *Neuroscience* **2007**, *150*, 498–509. [CrossRef] [PubMed]
201. Gross, P.M.; Weindl, A.; Knigge, K.M. Peering through the Windows of the Brain. *J. Cereb. Blood Flow Metab.* **1987**, *7*, 663–672. [CrossRef]

202. Cameron, O.G. Visceral Brain–Body Information Transfer. *Neuroimage* **2009**, *47*, 787–794. [CrossRef] [PubMed]
203. Dehouck, B.; Fenart, L.; Dehouck, M.P.; Pierce, A.; Torpier, G.; Cecchelli, R. A New Function for the LDL Receptor: Transcytosis of LDL across the Blood-Brain Barrier. *J. Cell Biol.* **1997**, *138*, 877–889. [CrossRef]
204. Rhea, E.M.; Banks, W.A. Interactions of Lipids, Lipoproteins, and Apolipoproteins with the Blood-Brain Barrier. *Pharm. Res.* **2021**, *38*, 1469–1475. [CrossRef]
205. Lütjohann, D.; Stroick, M.; Bertsch, T.; Kühl, S.; Lindenthal, B.; Thelen, K.; Andersson, U.; Björkhem, I.; von Bergmann, K.; Fassbender, K. High Doses of Simvastatin, Pravastatin, and Cholesterol Reduce Brain Cholesterol Synthesis in Guinea Pigs. *Steroids* **2004**, *69*, 431–438. [CrossRef] [PubMed]
206. Serougne, C.; Lefevre, C.; Chevallier, F. Cholesterol Transfer between Brain and Plasma in the Rat: A Model for the Turnover of Cerebral Cholesterol. *Exp. Neurol.* **1976**, *51*, 229–240. [CrossRef] [PubMed]
207. Refolo, L.M.; Pappolla, M.A.; Malester, B.; LaFrancois, J.; Bryant-Thomas, T.; Wang, R.; Tint, G.S.; Sambamurti, K.; Duff, K. Hypercholesterolemia Accelerates the Alzheimer's Amyloid Pathology in a Transgenic Mouse Model. *Neurobiol. Dis.* **2000**, *7*, 321–331; Corrected in **2000**, *9*, 690–691. [CrossRef]
208. Jiang, X.; Guo, M.; Su, J.; Lu, B.; Ma, D.; Zhang, R.; Yang, L.; Wang, Q.; Ma, Y.; Fan, Y. Simvastatin Blocks Blood-Brain Barrier Disruptions Induced by Elevated Cholesterol Both In Vivo and In Vitro. *Int. J. Alzheimers Dis.* **2012**, *2012*, 1–7. [CrossRef]
209. Zhao, X.; Peng, J.; Wu, Q.; Ren, Z.; Pan, L.; Tang, Z.; Jiang, Z.; Wang, G.; Liu, L. Imbalanced Cholesterol Metabolism in Alzheimer's Disease. *Clin. Chim. Acta* **2016**, *456*, 107–114. [CrossRef]
210. Lütjohann, D.; Stellaard, F.; Bölükbasi, B.; Kerksiek, A.; Parhofer, K.G.; Laufs, U. Anti-PCSK 9 Antibodies Increase the Ratios of the Brain-specific Oxysterol 24S-hydroxycholesterol to Cholesterol and to 27-hydroxycholesterol in the Serum. *Br. J. Clin. Pharmacol.* **2021**, *87*, 4252–4261. [CrossRef]
211. Shitara, Y.; Sugiyama, Y. Pharmacokinetic and Pharmacodynamic Alterations of 3-Hydroxy-3-Methylglutaryl Coenzyme A (HMG-CoA) Reductase Inhibitors: Drug–Drug Interactions and Interindividual Differences in Transporter and Metabolic Enzyme Functions. *Pharmacol. Ther.* **2006**, *112*, 71–105. [CrossRef] [PubMed]
212. Ginsberg, H.N. Effects of Statins on Triglyceride Metabolism. *Am. J. Cardiol.* **1998**, *81*, 32B–35B. [CrossRef] [PubMed]
213. Korade, Z.; Mi, Z.; Portugal, C.; Schor, N.F. Expression and P75 Neurotrophin Receptor Dependence of Cholesterol Synthetic Enzymes in Adult Mouse Brain. *Neurobiol. Aging* **2007**, *28*, 1522–1531. [CrossRef] [PubMed]
214. Gravelin, D. Cholesterol-Lowering Injectables: More Harm than Good? *J. Am. Physicians Surg.* **2015**, *20*, 119–121.
215. Beltowski, J.; Wojcicka, G.; Jamroz-Wisniewska, A. Adverse Effects of Statins—Mechanisms and Consequences. *Curr. Drug Saf.* **2009**, *4*, 209–228. [CrossRef] [PubMed]
216. Ladea, L.; Zemba, M.; Calancea, M.I.; Călțaru, M.V.; Dragosloveanu, C.D.M.; Coroleucă, R.; Catrina, E.L.; Brezean, I.; Dinu, V. Corneal Epithelial Changes in Diabetic Patients: A Review. *Int. J. Mol. Sci.* **2024**, *25*, 3471. [CrossRef] [PubMed]
217. Åberg, F.; Appelkvist, E.-L.; Dallner, G.; Ernster, L. Distribution and Redox State of Ubiquinones in Rat and Human Tissues. *Arch. Biochem. Biophys.* **1992**, *295*, 230–234. [CrossRef]
218. Miles, M.V.; Horn, P.S.; Morrison, J.A.; Tang, P.H.; DeGrauw, T.; Pesce, A.J. Plasma Coenzyme Q10 Reference Intervals, but Not Redox Status, Are Affected by Gender and Race in Self-Reported Healthy Adults. *Clin. Chim. Acta* **2003**, *332*, 123–132. [CrossRef] [PubMed]
219. Fong, C.W. Coenzyme Q 10 and Vitamin E Synergy, Electron Transfer, Antioxidation in Cell Membranes, and Interaction with Cholesterol. Eigenenergy Adelaide South Australia Australia. 2023. Available online: https://hal.science/hal-03976270/ (accessed on 16 December 2024).
220. Wainwright, L.; Hargreaves, I.P.; Georgian, A.R.; Turner, C.; Dalton, R.N.; Abbott, N.J.; Heales, S.J.R.; Preston, J.E. CoQ10 Deficient Endothelial Cell Culture Model for the Investigation of CoQ10 Blood–Brain Barrier Transport. *J. Clin. Med.* **2020**, *9*, 3236. [CrossRef]
221. Neagu, M.; Constantin, C.; Surcel, M.; Munteanu, A.; Scheau, C.; Savulescu-Fiedler, I.; Caruntu, C. Diabetic Neuropathy: A NRF2 Disease? *J. Diabetes* **2024**, *16*, e13524. [CrossRef] [PubMed]
222. Meljon, A.; Wang, Y.; Griffiths, W.J. Oxysterols in the Brain of the Cholesterol 24-Hydroxylase Knockout Mouse. *Biochem. Biophys. Res. Commun.* **2014**, *446*, 768–774. [CrossRef] [PubMed]
223. Wanamaker, B.L.; Swiger, K.J.; Blumenthal, R.S.; Martin, S.S. Cholesterol, Statins, and Dementia: What the Cardiologist Should Know. *Clin. Cardiol.* **2015**, *38*, 243–250. [CrossRef]
224. Bayorh, M.; Ganafa, A.; Eatman, D.; Walton, M.; Feuerstein, G. Simvastatin and Losartan Enhance Nitric Oxide and Reduce Oxidative Stress in Salt-Induced Hypertension. *Am. J. Hypertens.* **2005**, *18*, 1496–1502. [CrossRef] [PubMed]
225. Wu, H.; Lu, D.; Jiang, H.; Xiong, Y.; Qu, C.; Li, B.; Mahmood, A.; Zhou, D.; Chopp, M. Simvastatin-Mediated Upregulation of VEGF and BDNF, Activation of the PI3K/Akt Pathway, and Increase of Neurogenesis Are Associated with Therapeutic Improvement after Traumatic Brain Injury. *J. Neurotrauma* **2008**, *25*, 130–139. [CrossRef]
226. Savulescu-Fiedler, I.; Mihalcea, R.; Dragosloveanu, S.; Scheau, C.; Baz, R.O.; Caruntu, A.; Scheau, A.-E.; Caruntu, C.; Benea, S.N. The Interplay between Obesity and Inflammation. *Life* **2024**, *14*, 856. [CrossRef] [PubMed]

227. Mailman, T.; Hariharan, M.; Karten, B. Inhibition of Neuronal Cholesterol Biosynthesis with Lovastatin Leads to Impaired Synaptic Vesicle Release Even in the Presence of Lipoproteins or Geranylgeraniol. *J. Neurochem.* **2011**, *119*, 1002–1015. [CrossRef] [PubMed]
228. Sodero, A.O.; Barrantes, F.J. Pleiotropic Effects of Statins on Brain Cells. *Biochim. Et Biophys. Acta (BBA)-Biomembr.* **2020**, *1862*, 183340. [CrossRef]
229. García-Román, N.; Álvarez, A.M.; Toro, M.J.; Montes, A.; Lorenzo, M.J. Lovastatin Induces Apoptosis of Spontaneously Immortalized Rat Brain Neuroblasts: Involvement of Nonsterol Isoprenoid Biosynthesis Inhibition. *Mol. Cell. Neurosci.* **2001**, *17*, 329–341. [CrossRef]
230. van der Kant, R.; Langness, V.F.; Herrera, C.M.; Williams, D.A.; Fong, L.K.; Leestemaker, Y.; Steenvoorden, E.; Rynearson, K.D.; Brouwers, J.F.; Helms, J.B.; et al. Cholesterol Metabolism Is a Druggable Axis That Independently Regulates Tau and Amyloid-β in IPSC-Derived Alzheimer's Disease Neurons. *Cell Stem Cell* **2019**, *24*, 363–375.e9. [CrossRef] [PubMed]
231. Deveau, C.M.; Rodriguez, E.; Schroering, A.; Yamamoto, B.K. Serotonin Transporter Regulation by Cholesterol-Independent Lipid Signaling. *Biochem. Pharmacol.* **2021**, *183*, 114349. [CrossRef] [PubMed]
232. Shepardson, N.E. Cholesterol Level and Statin Use in Alzheimer Disease. *Arch. Neurol.* **2011**, *68*, 1239. [CrossRef] [PubMed]
233. Sierra, S.; Ramos, M.C.; Molina, P.; Esteo, C.; Vázquez, J.A.; Burgos, J.S. Statins as Neuroprotectants: A Comparative In Vitro Study of Lipophilicity, Blood-Brain-Barrier Penetration, Lowering of Brain Cholesterol, and Decrease of Neuron Cell Death. *J. Alzheimer's Dis.* **2011**, *23*, 307–318. [CrossRef]
234. Niemi, M. Transporter Pharmacogenetics and Statin Toxicity. *Clin. Pharmacol. Ther.* **2010**, *87*, 130–133. [CrossRef]
235. King, D.S.; Wilburn, A.J.; Wofford, M.R.; Harrell, T.K.; Lindley, B.J.; Jones, D.W. Cognitive Impairment Associated with Atorvastatin and Simvastatin. *Pharmacother. J. Hum. Pharmacol. Drug Ther.* **2003**, *23*, 1663–1667. [CrossRef]
236. Ott, B.R.; Daiello, L.A.; Dahabreh, I.J.; Springate, B.A.; Bixby, K.; Murali, M.; Trikalinos, T.A. Do Statins Impair Cognition? A Systematic Review and Meta-Analysis of Randomized Controlled Trials. *J. Gen. Intern. Med.* **2015**, *30*, 348–358. [CrossRef]
237. Locatelli, S.; Lütjohann, D.; Schmidt, H.H.-J.; Otto, C.; Beisiegel, U.; von Bergmann, K. Reduction of Plasma 24S-Hydroxycholesterol (Cerebrosterol) Levels Using High-Dosage Simvastatin in Patients With Hypercholesterolemia. *Arch. Neurol.* **2002**, *59*, 213. [CrossRef] [PubMed]
238. Botti, R.E.; Triscari, J.; Pan, H.Y.; Zayat, J. Concentrations of Pravastatin and Lovastatin in Cerebrospinal Fluid in Healthy Subjects. *Clin. Neuropharmacol.* **1991**, *14*, 256–261. [CrossRef]
239. Fassbender, K.; Stroick, M.; Bertsch, T.; Ragoschke, A.; Kuehl, S.; Walter, S.; Walter, J.; Brechtel, K.; Muehlhauser, F.; von Bergmann, K.; et al. Effects of Statins on Human Cerebral Cholesterol Metabolism and Secretion of Alzheimer Amyloid Peptide. *Neurology* **2002**, *59*, 1257–1258. [CrossRef] [PubMed]
240. Xu, X.; Wang, B.; Ren, C.; Hu, J.; Greenberg, D.A.; Chen, T.; Xie, L.; Jin, K. Recent Progress in Vascular Aging: Mechanisms and Its Role in Age-Related Diseases. *Aging Dis.* **2017**, *8*, 486. [CrossRef] [PubMed]
241. Shinohara, M.; Sato, N.; Kurinami, H.; Takeuchi, D.; Takeda, S.; Shimamura, M.; Yamashita, T.; Uchiyama, Y.; Rakugi, H.; Morishita, R. Reduction of Brain β-Amyloid (Aβ) by Fluvastatin, a Hydroxymethylglutaryl-CoA Reductase Inhibitor, through Increase in Degradation of Amyloid Precursor Protein C-Terminal Fragments (APP-CTFs) and Aβ Clearance. *J. Biol. Chem.* **2010**, *285*, 22091–22102. [CrossRef]
242. Storck, S.E.; Pietrzik, C.U. Endothelial LRP1—A Potential Target for the Treatment of Alzheimer's Disease. *Pharm. Res.* **2017**, *34*, 2637–2651. [CrossRef]
243. Schachter, M. Chemical, Pharmacokinetic and Pharmacodynamic Properties of Statins: An Update. *Fundam. Clin. Pharmacol.* **2005**, *19*, 117–125. [CrossRef]
244. Zhao, L.; Chen, T.; Wang, C.; Li, G.; Zhi, W.; Yin, J.; Wan, Q.; Chen, L. Atorvastatin in Improvement of Cognitive Impairments Caused by Amyloid β in Mice: Involvement of Inflammatory Reaction. *BMC Neurol.* **2016**, *16*, 18. [CrossRef]
245. Jin, H.; Chen, T.; Li, G.; Wang, C.; Zhang, B.; Cao, X.; Sha, S.; Wan, Q.; Chen, L. Dose-Dependent Neuroprotection and Neurotoxicity of Simvastatin through Reduction of Farnesyl Pyrophosphate in Mice Treated with Intracerebroventricular Injection of Aβ 1-42. *J. Alzheimer's Dis.* **2016**, *50*, 501–516. [CrossRef] [PubMed]
246. Baytan, S.H.; Alkanat, M.; Okuyan, M.; Ekinci, M.; Gedikli, E.; Ozeren, M.; Akgun, A. Simvastatin Impairs Spatial Memory in Rats at a Specific Dose Level. *Tohoku J. Exp. Med.* **2008**, *214*, 341–349. [CrossRef] [PubMed]
247. Giannopoulos, S.; Katsanos, A.H.; Tsivgoulis, G.; Marshall, R.S. Statins and Cerebral Hemodynamics. *J. Cereb. Blood Flow Metab.* **2012**, *32*, 1973–1976. [CrossRef] [PubMed]
248. Giannopoulos, S.; Katsanos, A.H.; Kosmidou, M.; Tsivgoulis, G. Statins and Vascular Dementia: A Review. *J. Alzheimer's Dis.* **2014**, *42*, S315–S320. [CrossRef]
249. Ahmadi, Y.; Ghorbanihaghjo, A.; Argani, H. The Balance between Induction and Inhibition of Mevalonate Pathway Regulates Cancer Suppression by Statins: A Review of Molecular Mechanisms. *Chem. Biol. Interact.* **2017**, *273*, 273–285. [CrossRef]
250. Akhtar, R.S.; Ness, J.M.; Roth, K.A. Bcl-2 Family Regulation of Neuronal Development and Neurodegeneration. *Biochim. Et Biophys. Acta (BBA)-Mol. Cell Res.* **2004**, *1644*, 189–203. [CrossRef]

251. Yasuno, F.; Tanimukai, S.; Sasaki, M.; Hidaka, S.; Ikejima, C.; Yamashita, F.; Kodama, C.; Mizukami, K.; Michikawa, M.; Asada, T. Association Between Cognitive Function and Plasma Lipids of the Elderly After Controlling for Apolipoprotein E Genotype. *Am. J. Geriatr. Psychiatry* **2012**, *20*, 574–583. [CrossRef] [PubMed]
252. Wellington, C.L.; Frikke-Schmidt, R. Relation between Plasma and Brain Lipids. *Curr. Opin. Lipidol.* **2016**, *27*, 225–232. [CrossRef] [PubMed]
253. McNamara, D.J. Dietary Cholesterol, Heart Disease Risk and Cognitive Dissonance. *Proc. Nutr. Soc.* **2014**, *73*, 161–166. [CrossRef]
254. Banach, M.; Rizzo, M.; Nikolic, D.; Howard, G.; Howard, V.J.; Mikhailidis, D.P. Intensive LDL-Cholesterol Lowering Therapy and Neurocognitive Function. *Pharmacol. Ther.* **2017**, *170*, 181–191. [CrossRef]
255. Vu, M.; Kettunen, R.; Tolppanen, A.-M.; Hartikainen, S.; Taipale, H. Statin Discontinuation in Persons with and without Alzheimer's Disease. *Eur. J. Clin. Pharmacol.* **2022**, *78*, 1145–1153. [CrossRef]
256. Olmastroni, E.; Molari, G.; De Beni, N.; Colpani, O.; Galimberti, F.; Gazzotti, M.; Zambon, A.; Catapano, A.L.; Casula, M. Statin Use and Risk of Dementia or Alzheimer's Disease: A Systematic Review and Meta-Analysis of Observational Studies. *Eur. J. Prev. Cardiol.* **2022**, *29*, 804–814. [CrossRef]
257. Rajan, K.B.; Mcaninch, E.A.; Wilson, R.S.; Dhana, A.; Evans-Lacko, S.; Evans, D.A. Statin Initiation and Risk of Incident Alzheimer Disease and Cognitive Decline in Genetically Susceptible Older Adults. *Neurology* **2024**, *102*, e209168. [CrossRef] [PubMed]
258. Cho, Y.Y.; Kwon, O.-H.; Chung, S. Preferred Endocytosis of Amyloid Precursor Protein from Cholesterol-Enriched Lipid Raft Microdomains. *Molecules* **2020**, *25*, 5490. [CrossRef] [PubMed]
259. Cho, Y.Y.; Kwon, O.-H.; Park, M.K.; Kim, T.-W.; Chung, S. Elevated Cellular Cholesterol in Familial Alzheimer's Presenilin 1 Mutation Is Associated with Lipid Raft Localization of β-Amyloid Precursor Protein. *PLoS ONE* **2019**, *14*, e0210535. [CrossRef]
260. Shie, F.-S.; Jin, L.-W.; Cook, D.G.; Leverenz, J.B.; LeBoeuf, R.C. Diet-Induced Hypercholesterolemia Enhances Brain Aβ Accumulation in Transgenic Mice. *Neuroreport* **2002**, *13*, 455–459. [CrossRef]
261. Mateos, L.; Akterin, S.; Gil-Bea, F.; Spulber, S.; Rahman, A.; Björkhem, I.; Schultzberg, M.; Flores-Morales, A.; Cedazo-Mínguez, A. Activity-Regulated Cytoskeleton-Associated Protein in Rodent Brain Is Down-Regulated by High Fat Diet in Vivo and by 27-Hydroxycholesterol In Vitro. *Brain Pathol.* **2009**, *19*, 69–80. [CrossRef] [PubMed]
262. Park, S.H.; Kim, J.H.; Choi, K.H.; Jang, Y.J.; Bae, S.S.; Choi, B.T.; Shin, H.K. Hypercholesterolemia Accelerates Amyloid β-Induced Cognitive Deficits. *Int. J. Mol. Med.* **2013**, *31*, 577–582. [CrossRef]
263. Svobodová, H.; Kosnáč, D.; Balázsiová, Z.; Tanila, H.; Miettinen, P.O.; Sierra, A.; Vitovič, P.; Wagner, A.; Polák, Š.; Kopáni, M. Elevated age-related cortical iron, ferritin and amyloid plaques in APP(swe)/PS1(deltaE9) transgenic mouse model of Alzheimer's disease. *Physiol. Res.* **2019**, *68* (Suppl. 4), S445–S451. [CrossRef]
264. Sparks, D.L.; Scheff, S.W.; Hunsaker, J.C.; Liu, H.; Landers, T.; Gross, D.R. Induction of Alzheimer-like β-Amyloid Immunoreactivity in the Brains of Rabbits with Dietary Cholesterol. *Exp. Neurol.* **1994**, *126*, 88–94. [CrossRef]
265. Lodeiro, M.; Puerta, E.; Ismail, M.-A.-M.; Rodriguez-Rodriguez, P.; Rönnbäck, A.; Codita, A.; Parrado-Fernandez, C.; Maioli, S.; Gil-Bea, F.; Merino-Serrais, P.; et al. Aggregation of the Inflammatory S100A8 Precedes Aβ Plaque Formation in Transgenic APP Mice: Positive Feedback for S100A8 and Aβ Productions. *J. Gerontol. A Biol. Sci. Med. Sci.* **2016**, *72*, glw073. [CrossRef]
266. Rahman, A.; Akterin, S.; Flores-Morales, A.; Crisby, M.; Kivipelto, M.; Schultzberg, M.; Cedazo-Mínguez, A. High Cholesterol Diet Induces Tau Hyperphosphorylation in Apolipoprotein E Deficient Mice. *FEBS Lett.* **2005**, *579*, 6411–6416. [CrossRef] [PubMed]
267. Bergmann, C.H. Diversification of Gamma-Secretase Activity versus Beta-Secretase Inhibition by Cholesterol Depletion. *Neurobiol. Aging* **2000**, *21*, 278. [CrossRef]
268. He, Q.; Li, Q.; Zhao, J.; Wu, T.; Ji, L.; Huang, G.; Ma, F. Relationship between Plasma Lipids and Mild Cognitive Impairment in the Elderly Chinese: A Case-Control Study. *Lipids Health Dis.* **2016**, *15*, 146. [CrossRef] [PubMed]
269. Ma, C.; Yin, Z.; Zhu, P.; Luo, J.; Shi, X.; Gao, X. Blood Cholesterol in Late-Life and Cognitive Decline: A Longitudinal Study of the Chinese Elderly. *Mol. Neurodegener.* **2017**, *12*, 24. [CrossRef]
270. Panza, F.; Frisardi, V.; Seripa, D.; Imbimbo, B.P.; Sancarlo, D.; D'Onofrio, G.; Addante, F.; Paris, F.; Pilotto, A.; Solfrizzi, V. Metabolic Syndrome, Mild Cognitive Impairment and Dementia. *Curr. Alzheimer Res.* **2011**, *8*, 492–509. [CrossRef]
271. Power, M.C.; Rawlings, A.; Sharrett, A.R.; Bandeen-Roche, K.; Coresh, J.; Ballantyne, C.M.; Pokharel, Y.; Michos, E.D.; Penman, A.; Alonso, A.; et al. Association of Midlife Lipids with 20-Year Cognitive Change: A Cohort Study. *Alzheimers Dement.* **2018**, *14*, 167–177. [CrossRef] [PubMed]
272. Gamba, P.; Testa, G.; Gargiulo, S.; Staurenghi, E.; Poli, G.; Leonarduzzi, G. Oxidized Cholesterol as the Driving Force behind the Development of Alzheimer's Disease. *Front. Aging Neurosci.* **2015**, *7*, 119. [CrossRef]
273. Schultz, B.G.; Patten, D.K.; Berlau, D.J. The Role of Statins in Both Cognitive Impairment and Protection against Dementia: A Tale of Two Mechanisms. *Transl. Neurodegener.* **2018**, *7*, 5. [CrossRef] [PubMed]
274. Lv, Y.-B.; Yin, Z.X.; Chei, C.-L.; Brasher, M.S.; Zhang, J.; Kraus, V.B.; Qian, F.; Shi, X.; Matchar, D.B.; Zeng, Y. Serum Cholesterol Levels within the High Normal Range Are Associated with Better Cognitive Performance among Chinese Elderly. *J. Nutr. Health Aging* **2016**, *20*, 280–287. [CrossRef]

275. de Oliveira, F.F.; Chen, E.S.; Smith, M.C.; Bertolucci, P.H.F. Longitudinal Lipid Profile Variations and Clinical Change in Alzheimer's Disease Dementia. *Neurosci. Lett.* **2017**, *646*, 36–42. [CrossRef]
276. Reitz, C.; Tang, M.-X.; Manly, J.; Schupf, N.; Mayeux, R.; Luchsinger, J.A. Plasma Lipid Levels in the Elderly Are Not Associated with the Risk of Mild Cognitive Impairment. *Dement. Geriatr. Cogn. Disord.* **2008**, *25*, 232–237. [CrossRef]
277. Kivipelto, M.; Solomon, A. Cholesterol as a Risk Factor for Alzheimer's Disease—Epidemiological Evidence. *Acta Neurol. Scand.* **2006**, *114*, 50–57. [CrossRef] [PubMed]
278. Mielke, M.M.; Zandi, P.P.; Sjögren, M.; Gustafson, D.; Östling, S.; Steen, B.; Skoog, I. High Total Cholesterol Levels in Late Life Associated with a Reduced Risk of Dementia. *Neurology* **2005**, *64*, 1689–1695. [CrossRef]
279. Solfrizzi, V.; Panza, F.; D'Introno, A.; Colacicco, A.M.; Capurso, C.; Basile, A.M.; Capurso, A. Lipoprotein(a), Apolipoprotein E Genotype, and Risk of Alzheimer's Disease. *J. Neurol. Neurosurg. Psychiatry* **2002**, *72*, 732–736. [CrossRef]
280. Partonen, T.; Haukka, J.; Virtamo, J.; Taylor, P.R.; Lönnqvist, J. Association of Low Serum Total Cholesterol with Major Depression and Suicide. *Br. J. Psychiatry* **1999**, *175*, 259–262. [CrossRef] [PubMed]
281. Repo-Tiihonen, E.; Halonen, P.; Tiihonen, J.; Virkkunen, M. Total Serum Cholesterol Level, Violent Criminal Offences, Suicidal Behavior, Mortality and the Appearance of Conduct Disorder in Finnish Male Criminal Offenders with Antisocial Personality Disorder. *Eur. Arch. Psychiatry Clin. Neurosci.* **2002**, *252*, 8–11. [CrossRef] [PubMed]
282. Vicario, A.; Del Sueldo, M.; Fernández, R.A.; Enders, J.; Zilberman, J.; Cerezo, G.H. Cognition and Vascular Risk Factors: An Epidemiological Study. *Int. J. Hypertens.* **2012**, *2012*, 1–6. [CrossRef]
283. Cibickova, L.; Radomir, H.; Stanislav, M.; Norbert, C.; Helena, Z.; Daniel, J.; Alena, T.; Eva, B.; Vladimir, P. The Influence of Simvastatin, Atorvastatin and High-Cholesterol Diet on Acetylcholinesterase Activity, Amyloid Beta and Cholesterol Synthesis in Rat Brain. *Steroids* **2009**, *74*, 13–19; Corrected in *Steroids* **2009**, *74*, 721. [CrossRef]
284. Wolozin, B. Decreased Prevalence of Alzheimer Disease Associated With 3-Hydroxy-3-Methyglutaryl Coenzyme A Reductase Inhibitors. *Arch. Neurol.* **2000**, *57*, 1439. [CrossRef] [PubMed]
285. Jick, H.; Zornberg, G.; Jick, S.; Seshadri, S.; Drachman, D. Statins and the Risk of Dementia. *Lancet* **2000**, *356*, 1627–1631. [CrossRef] [PubMed]
286. Wong, W.B.; Lin, V.W.; Boudreau, D.; Devine, E.B. Statins in the Prevention of Dementia and Alzheimer's Disease: A Meta-analysis of Observational Studies and an Assessment of Confounding. *Pharmacoepidemiol. Drug Saf.* **2013**, *22*, 345–358. [CrossRef] [PubMed]
287. Green, R.C.; McNagny, S.E.; Jayakumar, P.; Cupples, L.A.; Benke, K.; Farrer, L.A. Statin Use and the Risk of Alzheimer's Disease: The MIRAGE Study. *Alzheimer's Dement.* **2006**, *2*, 96–103. [CrossRef] [PubMed]
288. Wu, C.-K.; Yang, Y.-H.; Lin, T.-T.; Tsai, C.-T.; Hwang, J.-J.; Lin, J.-L.; Chen, P.-C.; Chiang, F.-T.; Lin, L.-Y. Statin Use Reduces the Risk of Dementia in Elderly Patients: A Nationwide Data Survey and Propensity Analysis. *J. Intern. Med.* **2015**, *277*, 343–352. [CrossRef]
289. Zhu, X.-C.; Dai, W.-Z.; Ma, T. Overview the Effect of Statin Therapy on Dementia Risk, Cognitive Changes and Its Pathologic Change: A Systematic Review and Meta-Analysis. *Ann. Transl. Med.* **2018**, *6*, 435. [CrossRef] [PubMed]
290. Lee, J.-W.; Choi, E.-A.; Kim, Y.-S.; Kim, Y.; You, H.-S.; Han, Y.-E.; Kim, H.-S.; Bae, Y.-J.; Kim, J.; Kang, H.-T. Statin Exposure and the Risk of Dementia in Individuals with Hypercholesterolaemia. *J. Intern. Med.* **2020**, *288*, 689–698. [CrossRef]
291. Mora, S.; Ridker, P.M. Justification for the Use of Statins in Primary Prevention: An Intervention Trial Evaluating Rosuvastatin (JUPITER)—Can C-Reactive Protein Be Used to Target Statin Therapy in Primary Prevention? *Am. J. Cardiol.* **2006**, *97*, 33–41. [CrossRef] [PubMed]
292. Coats, A. MRC/BHF Heart Protection Study of Cholesterol Lowering with Simvastatin in 20 536 High-Risk Individuals: A Randomised Placebocontrolled Trial. *Lancet* **2002**, *360*, 7–22. [CrossRef]
293. Appleton, J.P.; Scutt, P.; Sprigg, N.; Bath, P.M. Hypercholesterolaemia and Vascular Dementia. *Clin. Sci.* **2017**, *131*, 1561–1578. [CrossRef]
294. Ancelin, M.-L.; Carrière, I.; Barberger-Gateau, P.; Auriacombe, S.; Rouaud, O.; Fourlanos, S.; Berr, C.; Dupuy, A.-M.; Ritchie, K. Lipid Lowering Agents, Cognitive Decline, and Dementia: The Three-City Study. *J. Alzheimers Dis.* **2012**, *30*, 629–637. [CrossRef]
295. Alsehli, A.M.; Olivo, G.; Clemensson, L.E.; Williams, M.J.; Schiöth, H.B. The Cognitive Effects of Statins Are Modified by Age. *Sci. Rep.* **2020**, *10*, 6187. [CrossRef] [PubMed]
296. Davis, K.A.S.; Bishara, D.; Perera, G.; Molokhia, M.; Rajendran, L.; Stewart, R.J. Benefits and Harms of Statins in People with Dementia: A Systematic Review and Meta-Analysis. *J. Am. Geriatr. Soc.* **2020**, *68*, 650–658. [CrossRef]
297. Dagliati, A.; Peek, N.; Brinton, R.D.; Geifman, N. Sex and APOE Genotype Differences Related to Statin Use in the Aging Population. *Alzheimer's Dement. Transl. Res. Clin. Interv.* **2021**, *7*, e12156. [CrossRef] [PubMed]
298. Roy, S.; Hyman, D.; Ayyala, S.; Bakhshi, A.; Kim, S.H.; Anoruo, N.; Weinstock, J.; Balogun, A.; D'Souza, M.; Filatova, N.; et al. Cognitive Function Assessment in Patients on Moderate- or High-Intensity Statin Therapy. *J. Clin. Med. Res.* **2020**, *12*, 255–265. [CrossRef]

299. Ghosh, A.; Roy, A.; Matras, J.; Brahmachari, S.; Gendelman, H.E.; Pahan, K. Simvastatin Inhibits the Activation of $P21^{ras}$ and Prevents the Loss of Dopaminergic Neurons in a Mouse Model of Parkinson's Disease. *J. Neurosci.* **2009**, *29*, 13543–13556. [CrossRef] [PubMed]
300. FDA—US Food and Drug Administration. *FDA Drug Safety Communication: Important Safety Label Changes to Cholesterol-Lowering Statin Drugs*; US Food and Drug Administration: Rockville, MD, USA, 2012.
301. Posvar, E.L.; Radulovic, L.L.; Cilla, D.D.; Whitfield, L.R.; Sedman, A.J. Tolerance and Pharmacokinetics of Single-Dose Atorvastatin, a Potent Inhibitor of HMG-CoA Reductase, in Healthy Subjects. *J. Clin. Pharmacol.* **1996**, *36*, 728–731. [CrossRef]
302. Rojas-Fernandez, C.H.; Cameron, J.-C.F. Is Statin-Associated Cognitive Impairment Clinically Relevant? A Narrative Review and Clinical Recommendations. *Ann. Pharmacother.* **2012**, *46*, 549–557. [CrossRef]
303. Wagstaff, L.R.; Mitton, M.W.; Arvik, B.M.; Doraiswamy, P.M. Statin-Associated Memory Loss: Analysis of 60 Case Reports and Review of the Literature. *Pharmacother. J. Hum. Pharmacol. Drug Ther.* **2003**, *23*, 871–880. [CrossRef] [PubMed]
304. Andreone, B.J.; Chow, B.W.; Tata, A.; Lacoste, B.; Ben-Zvi, A.; Bullock, K.; Deik, A.A.; Ginty, D.D.; Clish, C.B.; Gu, C. Blood-Brain Barrier Permeability Is Regulated by Lipid Transport-Dependent Suppression of Caveolae-Mediated Transcytosis. *Neuron* **2017**, *94*, 581–594.e5. [CrossRef]
305. Swiger, K.J.; Manalac, R.J.; Blumenthal, R.S.; Blaha, M.J.; Martin, S.S. Statins and Cognition: A Systematic Review and Meta-Analysis of Short- and Long-Term Cognitive Effects. *Mayo Clin. Proc.* **2013**, *88*, 1213–1221. [CrossRef] [PubMed]
306. Richardson, K.; Schoen, M.; French, B.; Umscheid, C.A.; Mitchell, M.D.; Arnold, S.E.; Heidenreich, P.A.; Rader, D.J.; deGoma, E.M. Statins and Cognitive Function. *Ann. Intern. Med.* **2013**, *159*, 688. [CrossRef] [PubMed]
307. Liang, T.; Li, R.; Cheng, O. Statins for Treating Alzheimer's Disease: Truly Ineffective? *Eur. Neurol.* **2015**, *73*, 360–366. [CrossRef]
308. Seo, W.-K.; Hosseini, M.B.; Bang, O.Y.; Liebeskind, D.S. Recent Updates in Dyslipidemia Management: Perspectives in Stroke-Specific Situation. *Precis. Future Med.* **2020**, *4*, 9–20. [CrossRef]
309. Schulz, R.; Schlüter, K.-D.; Laufs, U. Molecular and Cellular Function of the Proprotein Convertase Subtilisin/Kexin Type 9 (PCSK9). *Basic. Res. Cardiol.* **2015**, *110*, 4. [CrossRef] [PubMed]
310. Seidah, N.G.; Benjannet, S.; Wickham, L.; Marcinkiewicz, J.; Jasmin, S.B.; Stifani, S.; Basak, A.; Prat, A.; Chrétien, M. The Secretory Proprotein Convertase Neural Apoptosis-Regulated Convertase 1 (NARC-1): Liver Regeneration and Neuronal Differentiation. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 928–933. [CrossRef] [PubMed]
311. Lagace, T.A.; Curtis, D.E.; Garuti, R.; McNutt, M.C.; Park, S.W.; Prather, H.B.; Anderson, N.N.; Ho, Y.K.; Hammer, R.E.; Horton, J.D. Secreted PCSK9 Decreases the Number of LDL Receptors in Hepatocytes and Inlivers of Parabiotic Mice. *J. Clin. Investig.* **2006**, *116*, 2995–3005. [CrossRef]
312. Papotti, B.; Adorni, M.P.; Marchi, C.; Zimetti, F.; Ronda, N.; Panighel, G.; Lupo, M.G.; Vilella, A.; Giuliani, D.; Ferri, N.; et al. PCSK9 Affects Astrocyte Cholesterol Metabolism and Reduces Neuron Cholesterol Supplying In Vitro: Potential Implications in Alzheimer's Disease. *Int. J. Mol. Sci.* **2022**, *23*, 12192. [CrossRef] [PubMed]
313. Witte, M.E.; Geurts, J.J.G.; de Vries, H.E.; van der Valk, P.; van Horssen, J. Mitochondrial Dysfunction: A Potential Link between Neuroinflammation and Neurodegeneration? *Mitochondrion* **2010**, *10*, 411–418. [CrossRef]
314. Yadav, R.; Weng, H.-R. EZH2 Regulates Spinal Neuroinflammation in Rats with Neuropathic Pain. *Neuroscience* **2017**, *349*, 106–117. [CrossRef] [PubMed]
315. Crisby, M.; Rahman, S.M.A.; Sylvén, C.; Winblad, B.; Schultzberg, M. Effects of High Cholesterol Diet on Gliosis in Apolipoprotein E Knockout Mice. *Neurosci. Lett.* **2004**, *369*, 87–92. [CrossRef] [PubMed]
316. O'Connell, E.M.; Lohoff, F.W. Proprotein Convertase Subtilisin/Kexin Type 9 (PCSK9) in the Brain and Relevance for Neuropsychiatric Disorders. *Front. Neurosci.* **2020**, *14*, 609. [CrossRef]
317. Bandyopadhyay, D.; Ashish, K.; Hajra, A.; Qureshi, A.; Ghosh, R.K. Cardiovascular Outcomes of PCSK9 Inhibitors: With Special Emphasis on Its Effect beyond LDL-Cholesterol Lowering. *J. Lipids* **2018**, *2018*, 1–13. [CrossRef]
318. Adorni, M.P.; Ruscica, M.; Ferri, N.; Bernini, F.; Zimetti, F. Proprotein Convertase Subtilisin/Kexin Type 9, Brain Cholesterol Homeostasis and Potential Implication for Alzheimer's Disease. *Front. Aging Neurosci.* **2019**, *11*, 120. [CrossRef]
319. de Oliveira, J.; Engel, D.F.; de Paula, G.C.; dos Santos, D.B.; Lopes, J.B.; Farina, M.; Moreira, E.L.G.; de Bem, A.F. High Cholesterol Diet Exacerbates Blood-Brain Barrier Disruption in LDLr−/− Mice: Impact on Cognitive Function. *J. Alzheimer's Dis.* **2020**, *78*, 97–115. [CrossRef] [PubMed]
320. Sabatine, M.S.; Giugliano, R.P.; Keech, A.C.; Honarpour, N.; Wiviott, S.D.; Murphy, S.A.; Kuder, J.F.; Wang, H.; Liu, T.; Wasserman, S.M.; et al. Evolocumab and Clinical Outcomes in Patients with Cardiovascular Disease. *N. Engl. J. Med.* **2017**, *376*, 1713–1722. [CrossRef]
321. Robinson, J.G.; Farnier, M.; Krempf, M.; Bergeron, J.; Luc, G.; Averna, M.; Stroes, E.S.; Langslet, G.; Raal, F.J.; El Shahawy, M.; et al. Efficacy and Safety of Alirocumab in Reducing Lipids and Cardiovascular Events. *N. Engl. J. Med.* **2015**, *372*, 1489–1499. [CrossRef] [PubMed]

322. Courtemanche, H.; Bigot, E.; Pichelin, M.; Guyomarch, B.; Boutoleau-Bretonnière, C.; Le May, C.; Derkinderen, P.; Cariou, B. PCSK9 Concentrations in Cerebrospinal Fluid Are Not Specifically Increased in Alzheimer's Disease. *J. Alzheimer's Dis.* **2018**, *62*, 1519–1525. [CrossRef]
323. Zimetti, F.; Caffarra, P.; Ronda, N.; Favari, E.; Adorni, M.P.; Zanotti, I.; Bernini, F.; Barocco, F.; Spallazzi, M.; Galimberti, D.; et al. Increased PCSK9 Cerebrospinal Fluid Concentrations in Alzheimer's Disease. *J. Alzheimer's Dis.* **2016**, *55*, 315–320. [CrossRef]
324. Mazura, A.D.; Ohler, A.; Storck, S.E.; Kurtyka, M.; Scharfenberg, F.; Weggen, S.; Becker-Pauly, C.; Pietrzik, C.U. PCSK9 Acts as a Key Regulator of Aβ Clearance across the Blood–Brain Barrier. *Cell. Mol. Life Sci.* **2022**, *79*, 212. [CrossRef]
325. Vilella, A.; Bodria, M.; Papotti, B.; Zanotti, I.; Zimetti, F.; Remaggi, G.; Elviri, L.; Potì, F.; Ferri, N.; Lupo, M.G.; et al. PCSK9 Ablation Attenuates Aβ Pathology, Neuroinflammation and Cognitive Dysfunctions in 5XFAD Mice. *Brain Behav. Immun.* **2024**, *115*, 517–534. [CrossRef]
326. Mahley, R.W. Central Nervous System Lipoproteins. *Arterioscler. Thromb. Vasc. Biol.* **2016**, *36*, 1305–1315. [CrossRef] [PubMed]
327. Liu, L.-S.; Bai, X.-Q.; Gao, Y.; Wu, Q.; Ren, Z.; Li, Q.; Pan, L.-H.; He, N.-Y.; Peng, J.; Tang, Z.-H. PCSK9 Promotes OxLDL-Induced PC12 Cell Apoptosis Through the Bcl-2/Bax-Caspase 9/3 Signaling Pathway. *J. Alzheimer's Dis.* **2017**, *57*, 723–734. [CrossRef] [PubMed]
328. Poirier, S.; Mayer, G.; Benjannet, S.; Bergeron, E.; Marcinkiewicz, J.; Nassoury, N.; Mayer, H.; Nimpf, J.; Prat, A.; Seidah, N.G. The Proprotein Convertase PCSK9 Induces the Degradation of Low Density Lipoprotein Receptor (LDLR) and Its Closest Family Members VLDLR and ApoER2. *J. Biol. Chem.* **2008**, *283*, 2363–2372. [CrossRef]
329. Badimon, L.; Luquero, A.; Crespo, J.; Peña, E.; Borrell-Pages, M. PCSK9 and LRP5 in Macrophage Lipid Internalization and Inflammation. *Cardiovasc. Res.* **2021**, *117*, 2054–2068. [CrossRef] [PubMed]
330. Jaén, R.I.; Povo-Retana, A.; Rosales-Mendoza, C.; Capillas-Herrero, P.; Sánchez-García, S.; Martín-Sanz, P.; Mojena, M.; Prieto, P.; Boscá, L. Functional Crosstalk between PCSK9 Internalization and Pro-Inflammatory Activation in Human Macrophages: Role of Reactive Oxygen Species Release. *Int. J. Mol. Sci.* **2022**, *23*, 9114. [CrossRef]
331. Ricci, C.; Ruscica, M.; Camera, M.; Rossetti, L.; Macchi, C.; Colciago, A.; Zanotti, I.; Lupo, M.G.; Adorni, M.P.; Cicero, A.F.G.; et al. PCSK9 Induces a Pro-Inflammatory Response in Macrophages. *Sci. Rep.* **2018**, *8*, 2267. [CrossRef] [PubMed]
332. Tang, Z.-H.; Peng, J.; Ren, Z.; Yang, J.; Li, T.-T.; Li, T.-H.; Wang, Z.; Wei, D.-H.; Liu, L.-S.; Zheng, X.-L.; et al. New Role of PCSK9 in Atherosclerotic Inflammation Promotion Involving the TLR4/NF-KB Pathway. *Atherosclerosis* **2017**, *262*, 113–122. [CrossRef] [PubMed]
333. Picard, C.; Poirier, A.; Bélanger, S.; Labonté, A.; Auld, D.; Poirier, J. Proprotein Convertase Subtilisin/Kexin Type 9 (PCSK9) in Alzheimer's Disease: A Genetic and Proteomic Multi-Cohort Study. *PLoS ONE* **2019**, *14*, e0220254. [CrossRef] [PubMed]
334. Macchi, C.; Favero, C.; Ceresa, A.; Vigna, L.; Conti, D.M.; Pesatori, A.C.; Racagni, G.; Corsini, A.; Ferri, N.; Sirtori, C.R.; et al. Depression and Cardiovascular Risk—Association among Beck Depression Inventory, PCSK9 Levels and Insulin Resistance. *Cardiovasc. Diabetol.* **2020**, *19*, 187. [CrossRef]
335. Sabatine, M.S.; Giugliano, R.P.; Wiviott, S.D.; Raal, F.J.; Blom, D.J.; Robinson, J.; Ballantyne, C.M.; Somaratne, R.; Legg, J.; Wasserman, S.M.; et al. Efficacy and Safety of Evolocumab in Reducing Lipids and Cardiovascular Events. *N. Engl. J. Med.* **2015**, *372*, 1500–1509. [CrossRef]
336. Ray, K.K.; Colhoun, H.M.; Szarek, M.; Baccara-Dinet, M.; Bhatt, D.L.; Bittner, V.A.; Budaj, A.J.; Diaz, R.; Goodman, S.G.; Hanotin, C.; et al. Effects of Alirocumab on Cardiovascular and Metabolic Outcomes after Acute Coronary Syndrome in Patients with or without Diabetes: A Prespecified Analysis of the ODYSSEY OUTCOMES Randomised Controlled Trial. *Lancet Diabetes Endocrinol.* **2019**, *7*, 618–628; Corrected in *Lancet Diabetes Endocrinol.* **2019**, *7*, E21. [CrossRef]
337. Goodman, S.G.; Steg, P.G.; Poulouin, Y.; Bhatt, D.L.; Bittner, V.A.; Diaz, R.; Garon, G.; Harrington, R.A.; Jukema, J.W.; Manvelian, G.; et al. Long-Term Efficacy, Safety, and Tolerability of Alirocumab in 8242 Patients Eligible for 3 to 5 Years of Placebo-Controlled Observation in the ODYSSEY OUTCOMES Trial. *J. Am. Hear. Assoc.* **2023**, *12*, e029216; Corrected in *J. Am. Hear. Assoc.* **2023**, *12*, e027745. [CrossRef] [PubMed]
338. Kereiakes, D.J.; Robinson, J.G.; Cannon, C.P.; Lorenzato, C.; Pordy, R.; Chaudhari, U.; Colhoun, H.M. Efficacy and Safety of the Proprotein Convertase Subtilisin/Kexin Type 9 Inhibitor Alirocumab among High Cardiovascular Risk Patients on Maximally Tolerated Statin Therapy: The ODYSSEY COMBO I Study. *Am. Heart J.* **2015**, *169*, 906–915.e13. [CrossRef] [PubMed]
339. de Carvalho, L.S.F.; Campos, A.M.; Sposito, A.C. Proprotein Convertase Subtilisin/Kexin Type 9 (PCSK9) Inhibitors and Incident Type 2 Diabetes: A Systematic Review and Meta-Analysis With Over 96,000 Patient-Years. *Diabetes Care* **2018**, *41*, 364–367. [CrossRef] [PubMed]
340. di Mauro, G.; Zinzi, A.; Scavone, C.; Mascolo, A.; Gaio, M.; Sportiello, L.; Ferrajolo, C.; Rafaniello, C.; Rossi, F.; Capuano, A. PCSK9 Inhibitors and Neurocognitive Adverse Drug Reactions: Analysis of Individual Case Safety Reports from the Eudravigilance Database. *Drug Saf.* **2021**, *44*, 337–349. [CrossRef]
341. Mutemberezi, V.; Guillemot-Legris, O.; Muccioli, G.G. Oxysterols: From Cholesterol Metabolites to Key Mediators. *Prog. Lipid Res.* **2016**, *64*, 152–169. [CrossRef] [PubMed]

342. Akiyama, Y.; Katsuki, S.; Matoba, T.; Nakano, Y.; Takase, S.; Nakashiro, S.; Yamamoto, M.; Mukai, Y.; Inoue, S.; Oi, K.; et al. Association of Serum Oxysterols with Cholesterol Metabolism Markers and Clinical Factors in Patients with Coronary Artery Disease: A Covariance Structure Analysis. *Nutrients* **2023**, *15*, 2997. [CrossRef] [PubMed]
343. Altmann, S.W.; Davis, H.R.; Zhu, L.; Yao, X.; Hoos, L.M.; Tetzloff, G.; Iyer, S.P.N.; Maguire, M.; Golovko, A.; Zeng, M.; et al. Niemann-Pick C1 Like 1 Protein Is Critical for Intestinal Cholesterol Absorption. *Science (1979)* **2004**, *303*, 1201–1204. [CrossRef] [PubMed]
344. Yamanashi, Y.; Takada, T.; Tanaka, Y.; Ogata, Y.; Toyoda, Y.; Ito, S.M.; Kitani, M.; Oshida, N.; Okada, K.; Shoda, J.; et al. Hepatic Niemann-Pick C1-Like 1 Exacerbates Non-Alcoholic Fatty Liver Disease by Re-Absorbing Specific Biliary Oxysterols. *Biomed. Pharmacother.* **2022**, *156*, 113877. [CrossRef]
345. Fieldhouse, J.L.P.; Doorduijn, A.S.; de Leeuw, F.A.; Verhaar, B.J.H.; Koene, T.; Wesselman, L.M.P.; de van der Schueren, M.A.E.; Visser, M.; van de Rest, O.; Scheltens, P.; et al. A Suboptimal Diet Is Associated with Poorer Cognition: The NUDAD Project. *Nutrients* **2020**, *12*, 703. [CrossRef] [PubMed]
346. Scarmeas, N.; Stern, Y.; Tang, M.; Mayeux, R.; Luchsinger, J.A. Mediterranean Diet and Risk for Alzheimer's Disease. *Ann. Neurol.* **2006**, *59*, 912–921. [CrossRef]
347. Zhou, F.; Deng, W.; Ding, D.; Zhao, Q.; Liang, X.; Wang, F.; Luo, J.; Zheng, L.; Guo, Q.; Hong, Z. High Low-Density Lipoprotein Cholesterol Inversely Relates to Dementia in Community-Dwelling Older Adults: The Shanghai Aging Study. *Front. Neurol.* **2018**, *9*, 952. [CrossRef]
348. Liu, H.; Zou, L.; Zhou, R.; Zhang, M.; Gu, S.; Zheng, J.; Hukportie, D.N.; Wu, K.; Huang, Z.; Yuan, Z.; et al. Long-Term Increase in Cholesterol Is Associated With Better Cognitive Function: Evidence From a Longitudinal Study. *Front. Aging Neurosci.* **2021**, *13*, 691423. [CrossRef] [PubMed]
349. Liu, Q.; An, Y.; Ma, W.; Feng, L.; Wang, C.; Lu, Y.; Xiao, R. High-cholesterol Diet Results in Elevated Amyloid-β and Oxysterols in Rats. *Mol. Med. Rep.* **2017**, *17*, 1235–1240. [CrossRef] [PubMed]
350. Testa, G.; Staurenghi, E.; Zerbinati, C.; Gargiulo, S.; Iuliano, L.; Giaccone, G.; Fantò, F.; Poli, G.; Leonarduzzi, G.; Gamba, P. Changes in Brain Oxysterols at Different Stages of Alzheimer's Disease: Their Involvement in Neuroinflammation. *Redox Biol.* **2016**, *10*, 24–33. [CrossRef] [PubMed]
351. Bonfili, L.; Cecarini, V.; Gogoi, O.; Berardi, S.; Scarpona, S.; Angeletti, M.; Rossi, G.; Eleuteri, A.M. Gut Microbiota Manipulation through Probiotics Oral Administration Restores Glucose Homeostasis in a Mouse Model of Alzheimer's Disease. *Neurobiol. Aging* **2020**, *87*, 35–43. [CrossRef] [PubMed]
352. Jones, M.L.; Martoni, C.J.; Parent, M.; Prakash, S. Cholesterol-Lowering Efficacy of a Microencapsulated Bile Salt Hydrolase-Active *Lactobacillus Reuteri* NCIMB 30242 Yoghurt Formulation in Hypercholesterolaemic Adults. *Br. J. Nutr.* **2012**, *107*, 1505–1513. [CrossRef]
353. Bonfili, L.; Cecarini, V.; Gogoi, O.; Gong, C.; Cuccioloni, M.; Angeletti, M.; Rossi, G.; Eleuteri, A.M. Microbiota Modulation as Preventative and Therapeutic Approach in Alzheimer's Disease. *FEBS J.* **2021**, *288*, 2836–2855. [CrossRef]
354. Bonfili, L.; Cuccioloni, M.; Gong, C.; Cecarini, V.; Spina, M.; Zheng, Y.; Angeletti, M.; Eleuteri, A.M. Gut Microbiota Modulation in Alzheimer's Disease: Focus on Lipid Metabolism. *Clin. Nutr.* **2022**, *41*, 698–708. [CrossRef]
355. Feingold, K.R. Cholesterol Lowering Drugs. In *Endotext [Internet]*; MDText.com, Inc.: South Dartmouth, MA, USA, 2000.
356. Zelcer, N.; Khanlou, N.; Clare, R.; Jiang, Q.; Reed-Geaghan, E.G.; Landreth, G.E.; Vinters, H.V.; Tontonoz, P. Attenuation of Neuroinflammation and Alzheimer's Disease Pathology by Liver x Receptors. *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 10601–10606. [CrossRef] [PubMed]
357. Kirchgessner, T.G.; Sleph, P.; Ostrowski, J.; Lupisella, J.; Ryan, C.S.; Liu, X.; Fernando, G.; Grimm, D.; Shipkova, P.; Zhang, R.; et al. Beneficial and Adverse Effects of an LXR Agonist on Human Lipid and Lipoprotein Metabolism and Circulating Neutrophils. *Cell Metab.* **2016**, *24*, 223–233. [CrossRef]
358. Muse, E.D.; Yu, S.; Edillor, C.R.; Tao, J.; Spann, N.J.; Troutman, T.D.; Seidman, J.S.; Henke, A.; Roland, J.T.; Ozeki, K.A.; et al. Cell-Specific Discrimination of Desmosterol and Desmosterol Mimetics Confers Selective Regulation of LXR and SREBP in Macrophages. *Proc. Natl. Acad. Sci. USA* **2018**, *115*, E4680–E4689. [CrossRef] [PubMed]
359. Baria, D.; Shah, U.; Egbuna, C.; Mtewa, A. Secondary Metabolites and Toxins of Microbial Origin for the Treatment of Diseases. Research Gate. In *Poisonous Plants and Phytochemicals in Drug Discovery*; John Wiley & Sons, Inc.: Hoboken, NJ, USA, 2024; pp. 225–248.
360. Li, R.; Liu, R.; Chen, L.; Wang, G.; Qin, L.; Yu, Z.; Wan, Z. Microbiota from Exercise Mice Counteracts High-Fat High-Cholesterol Diet-Induced Cognitive Impairment in C57BL/6 Mice. *Oxid. Med. Cell. Longev.* **2023**, *2023*, 1–17. [CrossRef] [PubMed]
361. García-Burgos, M.; Moreno-Fernández, J.; Alférez, M.J.M.; Díaz-Castro, J.; López-Aliaga, I. New Perspectives in Fermented Dairy Products and Their Health Relevance. *J. Funct. Foods* **2020**, *72*, 104059. [CrossRef]
362. Chen, H.; Meng, L.; Shen, L. Multiple Roles of Short-Chain Fatty Acids in Alzheimer Disease. *Nutrition* **2022**, *93*, 111499. [CrossRef] [PubMed]

363. Diab, A.; Dastmalchi, L.N.; Gulati, M.; Michos, E.D. A Heart-Healthy Diet for Cardiovascular Disease Prevention: Where Are We Now? *Vasc. Health Risk Manage* **2023**, *19*, 237–253. [CrossRef] [PubMed]
364. Udenigwe, C.C.; Rouvinen-Watt, K. The Role of Food Peptides in Lipid Metabolism during Dyslipidemia and Associated Health Conditions. *Int. J. Mol. Sci.* **2015**, *16*, 9303–9313. [CrossRef] [PubMed]
365. Vorobyeva, V.M.; Vorobyeva, I.S.; Kochetkova, A.A.; Mazo, V.K.; Zorin, S.N.; Khaider, K.; Sha-Rafetdinov, K.K. Specialize Hypocholesterolemic Foods: Ingredients, Technology, Effects. *Foods Raw Mater.* **2020**, *8*, 20–29. [CrossRef]
366. Knodel, L.C.; Talbert, R.L. Adverse Effects of Hypolipidaemic Drugs. *Med. Toxicol.* **1987**, *2*, 10–32. [CrossRef]
367. Silva, I.M.V.; Machado, F.; Moreno, M.J.; Nunes, C.; Coimbra, M.A.; Coreta-Gomes, F. Polysaccharide Structures and Their Hypocholesterolemic Potential. *Molecules* **2021**, *26*, 4559. [CrossRef]
368. Menaa, F.; Wijesinghe, U.; Thiripuranathar, G.; Althobaiti, N.A.; Albalawi, A.E.; Khan, B.A.; Menaa, B. Marine Algae-Derived Bioactive Compounds: A New Wave of Nanodrugs? *Mar. Drugs* **2021**, *19*, 484. [CrossRef] [PubMed]
369. Kurowska, E.M.; Spence, J.D.; Jordan, J.; Wetmore, S.; Freeman, D.J.; Piché, L.A.; Serratore, P. HDL-Cholesterol-Raising Effect of Orange Juice in Subjects with Hypercholesterolemia. *Am. J. Clin. Nutr.* **2000**, *72*, 1095–1100. [CrossRef] [PubMed]
370. Wilcox, L.J.; Borradaile, N.M.; de Dreu, L.E.; Huff, M.W. Secretion of Hepatocyte ApoB Is Inhibited by the Flavonoids, Naringenin and Hesperetin, via Reduced Activity and Expression of ACAT2 and MTP. *J. Lipid Res.* **2001**, *42*, 725–734. [CrossRef] [PubMed]
371. Grande, F.; Occhiuzzi, M.A.; Perri, M.R.; Ioele, G.; Rizzuti, B.; Statti, G.; Garofalo, A. Polyphenols from Citrus Tacle® Extract Endowed with HMGCR Inhibitory Activity: An Antihypercholesterolemia Natural Remedy. *Molecules* **2021**, *26*, 5718. [CrossRef]
372. Bok, S.-H.; Lee, S.-H.; Park, Y.-B.; Bae, K.-H.; Son, K.-H.; Jeong, T.-S.; Choi, M.-S. Plasma and Hepatic Cholesterol and Hepatic Activities of 3-Hydroxy-3-Methyl-Glutaryl-CoA Reductase and Acyl CoA: Cholesterol Transferase Are Lower in Rats Fed Citrus Peel Extract or a Mixture of Citrus Bioflavonoids. *J. Nutr.* **1999**, *129*, 1182–1185. [CrossRef] [PubMed]
373. Poirier, S.; Mayer, G. The Biology of PCSK9 from the Endoplasmic Reticulum to Lysosomes: New and Emerging Therapeutics to Control Low-Density Lipoprotein Cholesterol. *Drug Des. Dev. Ther.* **2013**, *7*, 1135–1148. [CrossRef]
374. Mollace, R.; Macrì, R.; Tavernese, A.; Gliozzi, M.; Musolino, V.; Carresi, C.; Maiuolo, J.; Fini, M.; Volterrani, M.; Mollace, V. Comparative Effect of Bergamot Polyphenolic Fraction and Red Yeast Rice Extract in Rats Fed a Hyperlipidemic Diet: Role of Antioxidant Properties and PCSK9 Expression. *Nutrients* **2022**, *14*, 477. [CrossRef] [PubMed]
375. Miceli, N.; Mondello, M.R.; Monforte, M.T.; Sdrafkakis, V.; Dugo, P.; Crupi, M.L.; Trovato, A. Hypolipidemic Effects of Citrus Bergamia Risso et Poiteau Juice in Rats Fed a Hypercholesterolemic Diet. *J. Agric. Food Chem.* **2007**, *55*, 10671–10677. [CrossRef] [PubMed]
376. Lamiquiz-Moneo, I.; Giné-González, J.; Alisente, S.; Bea, A.M.; Pérez-Calahorra, S.; Marco-Benedí, V.; Baila-Rueda, L.; Jarauta, E.; Cenarro, A.; Civeira, F.; et al. Effect of Bergamot on Lipid Profile in Humans: A Systematic Review. *Crit. Rev. Food Sci. Nutr.* **2020**, *60*, 3133–3143. [CrossRef]
377. Mollace, V.; Scicchitano, M.; Paone, S.; Casale, F.; Calandruccio, C.; Gliozzi, M.; Musolino, V.; Carresi, C.; Maiuolo, J.; Nucera, S.; et al. Hypoglycemic and Hypolipemic Effects of a New Lecithin Formulation of Bergamot Polyphenolic Fraction: A Double Blind, Randomized, Placebo- Controlled Study. *Endocr. Metab. Immune Disord. Drug Targets* **2019**, *19*, 136–143. [CrossRef]

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Review

Mitochondrial Dysfunction and Its Potential Molecular Interplay in Hypermobile Ehlers–Danlos Syndrome: A Scoping Review Bridging Cellular Energetics and Genetic Pathways

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Abstract: Hypermobile Ehlers–Danlos Syndrome (hEDS) is a hereditary connective tissue disorder characterized by joint hypermobility, skin hyperextensibility, and systemic manifestations such as chronic fatigue, gastrointestinal dysfunction, and neurological symptoms. Unlike other EDS subtypes with known genetic mutations, hEDS lacks definitive markers, suggesting a multifactorial etiology involving both mitochondrial dysfunction and non-mitochondrial pathways. This scoping review, conducted in accordance with the PRISMA-ScR guidelines, highlights mitochondrial dysfunction as a potential unifying mechanism in hEDS pathophysiology. Impaired oxidative phosphorylation (OXPHOS), elevated reactive oxygen species (ROS) levels, and calcium dysregulation disrupt cellular energetics and extracellular matrix (ECM) homeostasis, contributing to the hallmark features of hEDS. We reviewed candidate genes associated with ECM remodeling, signaling pathways, and immune regulation. Protein–protein interaction (PPI) network analyses revealed interconnected pathways linking mitochondrial dysfunction with these candidate genes. Comparative insights from Fabry disease and fragile X premutation carriers underscore shared mechanisms such as RNA toxicity, matrix metalloproteinases (MMP) activation, and ECM degradation. These findings may suggest that mitochondrial dysfunction amplifies systemic manifestations through its interplay with non-mitochondrial molecular pathways. By integrating these perspectives, this review provides a potential framework for understanding hEDS pathogenesis while highlighting latent avenues for future research into its molecular basis. Understanding the potential role of mitochondrial dysfunction in hEDS not only sheds light on its complex molecular etiology but also opens new paths for targeted interventions.

Keywords: hypermobile Ehlers–Danlos Syndrome; mitochondrial dysfunction; oxidative phosphorylation; reactive oxygen species; genetic mutations; molecular pathways; extracellular matrix; mitochondrial DNA

1. Introduction

Hypermobile Ehlers–Danlos Syndrome (hEDS) stands out as the most common form of EDS, yet it remains frequently underdiagnosed due to its diverse clinical manifestations and the absence of specific genetic markers [1,2]. This hereditary connective tissue disorder is characterized by joint hypermobility, tissue fragility, and wide-ranging systemic effects [1,2]. Recent epidemiological data suggest hEDS may be more prevalent than previously thought [3,4]. While global estimates indicate that EDS affects approximately 1

in 5000 individuals [3], localized studies in Wales have reported a much higher incidence of 1 in 500 for hEDS and related Hypermobility Spectrum Disorders (HSDs) [4]. Notably, females account for about 70% of diagnosed cases, potentially due to estrogen's influence on collagen metabolism and pain perception [5–8]. hEDS exhibits an autosomal dominant inheritance pattern with significant phenotypic variability within families [9,10]. This heterogeneity, coupled with the lack of definitive biomarkers, often leads to prolonged diagnostic delays [11]. Unlike other EDS variants with known collagen gene mutations, hEDS's genetic basis remains elusive [1,2]. Current research explores potential links to genes involved in extracellular matrix (ECM) regulation, mitochondrial function, and neuromuscular signaling [2]. This genetic complexity underscores the need for a multifaceted approach to understanding hEDS pathophysiology, integrating connective tissue biology with broader systemic processes. Recent advances in molecular biology and cellular energetics have highlighted mitochondrial dysfunction as a potential key player in hEDS pathophysiology [2,5]. Mitochondria play a crucial role in maintaining connective tissue homeostasis through several key mechanisms. Firstly, mitochondrial oxidative phosphorylation (OXPHOS) provides the ATP necessary for collagen biosynthesis, post-translational modifications, proteoglycan synthesis, ECM assembly, and fibroblast migration during tissue repair [2,5,12]. Secondly, mitochondria regulate redox balance, with balanced ROS production maintaining ECM integrity by modulating matrix metalloproteinases (MMPs) and their inhibitors (TIMPs), while excessive mitochondrial reactive oxygen species (ROS) can lead to collagen fragmentation and elastin degradation [13–15]. Thirdly, mitochondrial calcium buffering is essential for integrin-mediated cell–ECM interactions, Transforming Growth Factor Beta (TGF-β) signaling in fibroblast activation, and smooth muscle contractility [16,17]. Recent research has shown the potential role of mitochondrial DNA (mtDNA) mutations and mitochondrial dysfunctions in the development of connective tissue disorders [18–20]. This discovery may suggest a novel mechanism linking mitochondrial dysfunction to the widespread connective tissue abnormalities observed in disorders such as hEDS, opening new avenues for understanding the complex interplay between mitochondrial function and connective tissue homeostasis. Additionally, sex-specific modulation occurs through estrogen–mitochondria interactions via ERα receptors, influencing collagen cross-linking enzymes and hyaluronic acid synthesis [21], possibly explaining the 3:1 female predominance in hEDS [6–8]. MtDNA inheritance patterns and sex-specific hormonal influences on mitochondrial function could contribute to this sexual dimorphism [22]. Mitochondrial dysfunction can potentially impair connective tissue in hEDS through bioenergetic failure, oxidative stress, and calcium mishandling, leading to reduced ECM component synthesis, the degradation of collagen and elastin networks, and disrupted cellular signaling [12–17]. Proteomic analyses have revealed significant alterations in mitochondrial proteins involved in OXPHOS, including ATP synthase components and enzymes critical for energy metabolism [23].

Mitochondrial dysfunction may also underlie several poorly understood aspects of hEDS, including chronic fatigue, gastrointestinal dysfunction, and neurological manifestations [5,24]. Recent studies have demonstrated that impaired mitochondrial energy production correlates with exercise intolerance and post-exertional malaise in hEDS patients [25,26]. The high energy demands of the gastrointestinal smooth muscle and enteric nervous system make them particularly vulnerable to mitochondrial dysfunction [27].

This review examines the emerging evidence linking mitochondrial dysfunction to hEDS pathophysiology, analyzing recent advances in understanding how mitochondrial abnormalities can potentially influence connective tissue homeostasis, energy metabolism, and cellular signaling pathways.

2. Materials and Methods

This review utilized a systematic and comprehensive approach to identify, evaluate, and synthesize existing evidence on the role of mitochondrial dysfunction in the pathogenesis of hEDS. The methodology was designed to ensure a rigorous selection of high-quality studies and provide a robust foundation for exploring the interplay between mitochondrial pathways and hEDS pathophysiology. This scoping review was conducted in accordance with the PRISMA-ScR guidelines (supplementary material) to ensure comprehensive reporting and methodological rigor [28].

2.1. Literature Search Strategy

A systematic search was conducted across four major databases: PubMed, Web of Science, Google Scholar, and Scopus. The search terms included combinations of keywords such as "*Mitochondria*", "*Mitochondrial dysfunction*", "*Mitochondrial pathway*", "*Mitochondrial mutation*", "*hypermobile Ehlers-Danlos Syndrome*", "*Ehlers-Danlos Syndrome type 3*", and "*hEDS*". Boolean operators (AND/OR) were used to refine the search strategy. The search was not restricted by publication year but was limited to peer-reviewed articles published in English.

2.1.1. Inclusion Criteria

The eligibility criteria for study inclusion were as follows:

- Studies explicitly addressing mitochondrial dysfunction, mitochondrial mutations, or mitochondrial pathways in the context of hEDS.
- Articles exploring genetic mutations linked to mitochondrial or ECM pathways relevant to hEDS.
- Primary research articles, case studies, or experimental studies with original data.

2.1.2. Exclusion Criteria

- Review articles, books, or conference abstracts without original data.
- Studies not published in English.
- Articles focused on unrelated EDS subtypes or conditions without relevance to mitochondrial dysfunction.

2.1.3. Study Selection Process

The initial search yielded 73 articles. These were imported into Zotero version 7.0.11 (64-bit) for reference management. Duplicate entries were removed, reducing the dataset to 68 unique articles. A subsequent screening process excluded 12 review articles and 32 studies deemed non-relevant based on their titles and abstracts. Two articles not published in English were also excluded. This process resulted in a preliminary pool of four eligible studies. To ensure comprehensive coverage, citation tracking was performed on the selected studies, identifying an additional three relevant articles. This culminated in a final pool of eight original studies for detailed review and analysis. The study selection process is detailed in Figure 1, which follows the PRISMA flow diagram format to illustrate the identification, screening, eligibility, and inclusion of studies [28]. Articles were screened using Zotero for duplicates; titles and abstracts were reviewed for relevance, followed by full-text reviews to finalize eligible studies. The process for selecting sources of evidence involved several steps: A systematic search was conducted across four major databases: PubMed, Web of Science, Google Scholar, and Scopus. Duplicate entries were removed using Zotero reference management software. Titles and abstracts were screened for relevance based on predefined inclusion criteria, which focused on studies addressing mitochondrial dysfunction in hEDS or related pathways. Full-text articles were reviewed to confirm eligibility. Exclusion criteria included non-English publications, studies unrelated

to hEDS or mitochondrial dysfunction, and reviews without original data. Citation tracking was performed on eligible studies to identify additional relevant articles.

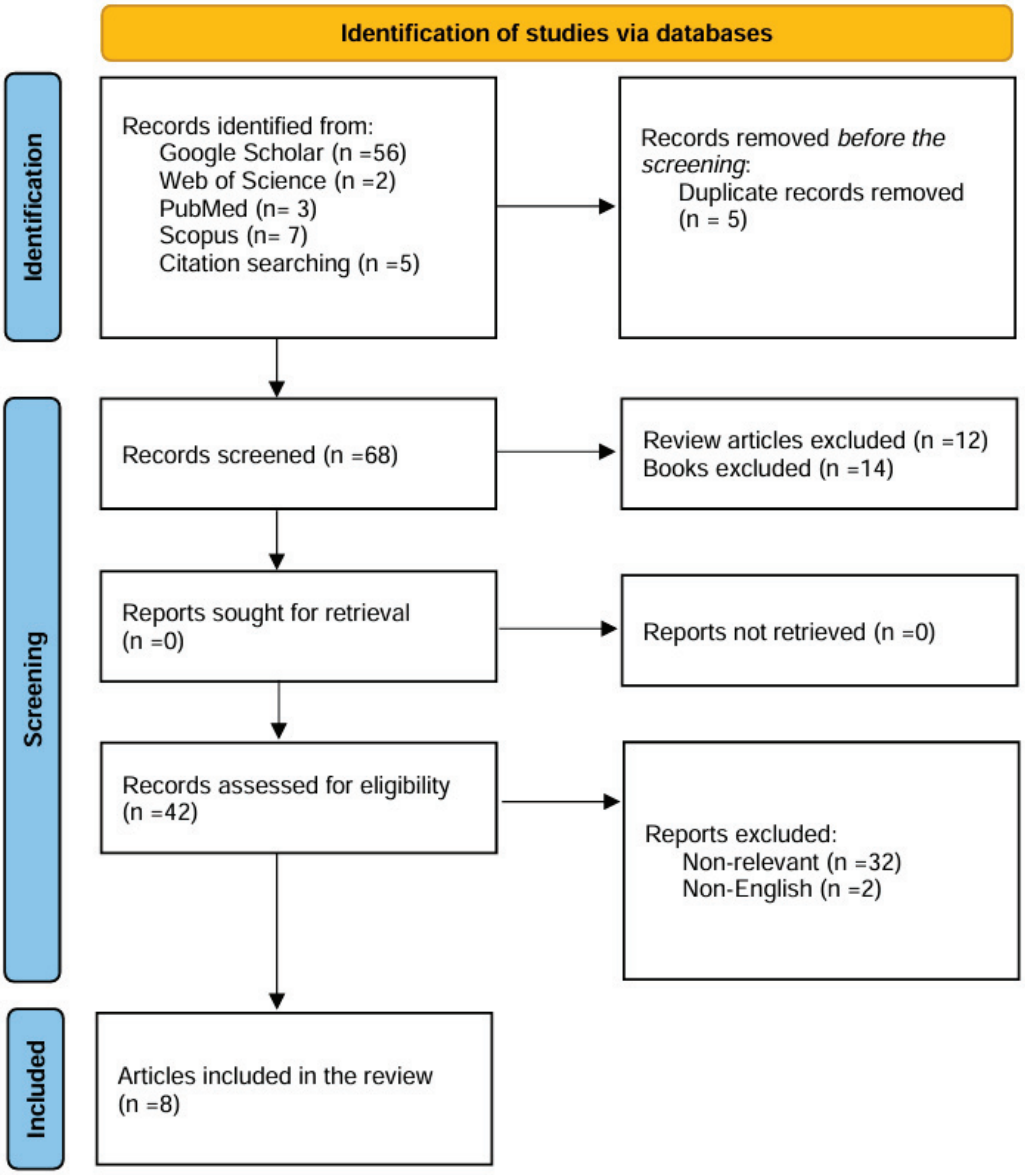


Figure 1. PRISMA flow diagram illustrating the systematic workflow for identifying studies on mitochondrial dysfunction in hypermobile Ehlers–Danlos Syndrome (hEDS) [28]. This figure outlines the systematic approach used to identify and evaluate relevant studies on the role of mitochondrial dysfunction in hEDS pathogenesis. The workflow begins with a comprehensive search across four major databases (PubMed, Web of Science, Google Scholar, and Scopus), yielding an initial pool of 68 articles. Following the removal of duplicates, reviews, and non-English publications, 42 primary research articles were screened for relevance and quality. After excluding non-relevant studies, a final selection of 8 original articles was included for in-depth analysis. Citation tracking was employed to ensure comprehensive coverage. This rigorous methodology highlights the limited but focused evidence base available for exploring mitochondrial dysfunction in hEDS.

2.2. Extraction and Analysis

Two independent reviewers extracted data from the selected studies using a standardized data extraction form. The extracted information included the following:

- Study design and methodology.

- Key findings related to mitochondrial dysfunction (e.g., OXPHOS defects, reactive oxygen species production).
- Genetic variations associated with mitochondrial or ECM pathways.
- Systemic manifestations linked to mitochondrial dysfunction in hEDS patients.
- Discrepancies between reviewers were resolved through discussion or consultation with a third reviewer.

2.3. Protein–Protein Interaction (PPI) Network Analysis

To explore the molecular interplay between mitochondrial dysfunction and non-mitochondrial pathways in hEDS, PPI network analyses using the STRING database (version 12) were performed. Identified genes from reviewed studies were mapped onto the network. We performed functional enrichment analysis to identify overrepresented biological processes and pathways, focusing on Gene Ontology (GO) terms related to mitochondrial function and cellular energetics. The STRING database integrates various sources of protein–protein interaction data, including experimental data, computational prediction methods, and public text collections, to create a comprehensive network of functional protein associations [29,30]. K-means clustering was applied to group genes into distinct functional clusters, providing insights into the interconnected nature of mitochondrial dysfunction in hEDS. The K-means algorithm partitions the genes into K clusters, where each gene belongs to the cluster with the nearest mean [29,30]. We iteratively adjusted the number of clusters (K) to optimize the separation of functional groups while maintaining biological relevance. This clustering approach allowed us to identify coherent groups of genes with similar functional properties or involvement in related biological processes. This network analysis approach allowed us to visualize and interpret the complex relationships between mitochondrial and non-mitochondrial pathways, offering a systems-level perspective on hEDS pathophysiology. The resulting clusters and enriched pathways provide a framework for understanding the multifaceted nature of mitochondrial involvement in hEDS and highlight potential areas for further investigation.

2.4. Comparative Analysis with Related Disorders

Insights from disorders with overlapping features, such as Fabry disease and fragile X premutation carriers, were integrated into the review. These conditions provided comparative data on shared mechanisms like RNA toxicity, ECM remodeling, and oxidative stress, offering additional context for understanding mitochondrial dysfunction in hEDS.

3. Results

This review synthesizes findings from recent studies to explore the intricate relationship between mitochondrial dysfunction and the pathogenesis of hEDS. Table 1 summarizes the key findings from the studies reviewed in this article.

Table 1. Summary of reviewed studies on potential mitochondrial dysfunction in hypermobile Ehlers–Danlos Syndrome (hEDS).

Reference	Sample Size (hEDS)	Potential Mitochondrial Dysfunction [1]	Proteins/ Genes [1]	Measurements	Suggested Pathways
Shirvani et al. (2024) [2]	18 hEDS and 7 first-degree relatives as controls	Mitochondrial complex I assembly model OXPHOS system	*MT-CYB*, *MT-ND1*, *EMC1*, and *ACAD9*.	Whole-genome sequencing	Electron transport chain dysfunction

Table 1. *Cont.*

Reference	Sample Size (hEDS)	Potential Mitochondrial Dysfunction [1]	Proteins/ Genes [1]	Measurements	Suggested Pathways
Chiarelli et al. (2021) [23]	Six hEDS dermal myofibroblasts were compared to 12 control fibroblasts	Altered glycolysis, citric acid cycle, respiratory electron transport and oxidative phosphorylation, increased oxidative stress, and impaired protein folding	GAPDH, PGAM1, PKM, LDHB, ATP5MG, ATP5O, ATP5F1C, FH, MDH2, ACO2, and ETFA	Proteome profiling/dermal myofibroblast pathways	Fibroblast-to-myofibroblast transition (FMT), integrin-linked signaling, and redox balance
Barbey et al. (2019) [31]	24 Fabry patients with the diagnostic criteria of hEDS	Lyso-Gb3 accumulation linked to impaired collagen synthesis, suggesting mitochondrial toxicity	*GLA* mutation and plasma lyso-Gb3	Genetic mutations and plasma evaluation	Lysosomal dysfunction pathways
Wilson and Tonk, (2024) [32,33]	834 hEDS of 1261 EDS patients	Mitochondrial depletion	*POLG/MT-DNA, MT-CO2, MT-TK*, and *MT-ND5, MT-RNA1/2, MT-tRNA, MT-ATP6/8, MT-CYB*	Clinical genomic analysis	Mitochondrial respiratory chain and mitochondrial ribosomal–transfer RNA
Wilson and Tonk, (2020) [34]	A case report of Mitochondrial Dysfunction and dysautonomia symptoms with history of EDS	Possibly complex V	*MT-ATP6, PLOD1, FLNA*, and *ATP2A*	Mt DNA and whole exome sequencing in blood and Mt DNA qPCR analysis in muscle biopsy	Mitochondrial respiratory chain and ATP synthesis
Tassanakijpanich et al. (2022) [35]	Five FMR1 fXPCs cases with characteristics of hEDS	Mitochondrial dysfunction and inflammation	*FMR1* gene	CGG repeat range (55–200 repeats) of the FMR1 gene	RNA toxicity also leads to Ca^{2+} dysregulation, mitochondrial dysfunction, enhanced production of reactive oxygen species, and inflammation
Jahanbani et al. (2024) [36]	A case report of a ME/CFS patient with hypermobility spectrum disorder and with a family history of hEDS	Mitochondrial respiration	TP53	Longitudinal cytokine profiling	Mitochondrial respiration, autophagy, and stress response

This table summarizes findings from studies exploring potential mitochondrial dysfunction in hypermobile Ehlers–Danlos Syndrome (hEDS). These studies investigate various mechanisms, including altered energy metabolism (e.g., glycolysis and oxidative phosphorylation), lysosomal dysfunction, RNA toxicity, and mtDNA mutations. The findings highlight pathways such as fibroblast-to-myofibroblast transition (FMT), redox balance regulation, lysosomal dysfunction, electron transport chain (ETC) dysfunction, autophagy, and calcium dysregulation as potential contributors to hEDS pathogenesis. [1] Abbreviations and key terms: GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; a key enzyme in glycolysis. PGAM1: Phosphoglycerate mutase 1; involved in glycolysis. PKM: Pyruvate kinase M1/2; catalyzes the final step of glycolysis. LDHB: Lactate dehydrogenase B; converts pyruvate to lactate during anaerobic metabolism. ATP5MG, ATP5O, and ATP5F1C: Subunits of ATP synthase, essential for oxidative phosphorylation in mitochondria. FH: Fumarate hydratase; an enzyme in the tricarboxylic acid (TCA) cycle. MDH2: Malate dehydrogenase 2; catalyzes the conversion of malate to oxaloacetate in the TCA cycle. ACO2: Aconitase 2; involved in the TCA cycle. ETFA: Electron transfer flavoprotein subunit alpha; participates in mitochondrial fatty acid oxidation. lyso-Gb3: Globotriaosylsphingosine. *POLG*: Mitochondrial DNA polymerase gamma. *GLA*: Gene encoding α-galactosidase A; mutations of which cause Fabry disease and potentially are linked to mitochondrial dysfunction and impaired collagen synthesis. *FMR1*: Fragile X mental retardation 1 gene; associated with mitochondrial dysfunction and inflammation in fragile X premutation carriers (fXPCs). *MT-CYB, MT-ND1, MT-ND5, MT-RNA1/2, MT-tRNA*, and *MT-ATP6/8*: Mitochondrial genes involved in ETC function and mitochondrial ribosomal RNA synthesis. ME/CFS: Myalgic encephalomyelitis/chronic fatigue syndrome.

3.1. Mitochondrial Dysfunction

Studies reveal that mitochondrial abnormalities disrupt OXPHOS and metabolic pathways, leading to bioenergetic deficits that may underlie key clinical manifestations such as chronic fatigue, muscle hypotonia, and systemic dysregulation [2,23]. Table 1 summarizes findings from studies exploring potential mitochondrial dysfunction in hEDS. These studies investigate various mechanisms, including altered energy metabolism (e.g., glycolysis and OXPHOS), lysosomal dysfunction, RNA toxicity, and mtDNA mutations. The findings highlight pathways such as fibroblast-to-myofibroblast transition (FMT), redox balance regulation, lysosomal dysfunction, ETC dysfunction, autophagy, and calcium dysregulation as potential contributors to hEDS pathogenesis. In this section, we detail the relationship between hEDS and potential mitochondrial dysfunction across these studied pathways.

3.1.1. Mitochondrial Energy Metabolism

- Impaired OXPHOS: Proteomic analyses of hEDS fibroblasts have identified significant alterations in mitochondrial proteins critical for OXPHOS. The subunits of ATP synthase, including ATP5O, ATP5C1, and ATP5L, were found to be dysregulated, indicating compromised ATP production capacity [23]. Additionally, enzymes involved in the TCA cycle, such as aconitase 2 (ACO2), fumarate hydratase (FH), and malate dehydrogenase 2 (MDH2), exhibited altered expression levels in hEDS cells [23]. Studies have demonstrated that genetic variations in genes such as *MT-CYB*, *MT-ND1*, and *ACAD9*, which are involved in the mitochondrial OXPHOS system, are associated with hEDS [2,32,33]. These genes play critical roles in mitochondrial energy metabolism, and their dysregulation may contribute to the bioenergetic deficits observed in hEDS [2,32,33]. Specifically, mutations in *MT-CYB* and *MT-ND1* can impair ETC function, while variations in *ACAD9* disrupt mitochondrial dynamics and fatty acid beta-oxidation [37]. These disruptions may suggest a reduced ability of mitochondria to generate sufficient energy to meet cellular demands, particularly in tissues with high energy requirements like muscles and connective tissues.
- Metabolic Reprogramming Toward Aerobic Glycolysis: Evidence suggests that hEDS fibroblasts undergo metabolic reprogramming characterized by a shift from OXPHOS to aerobic glycolysis [23]. The upregulation of glycolytic enzymes—including glyceraldehyde-3-phosphate dehydrogenase (GAPDH), pyruvate kinase M1/2 (PKM), and lactate dehydrogenase B (LDHB)—has been observed [23]. This shift may represent an adaptive response to mitochondrial inefficiency but results in increased lactate production and reduced glucose oxidation. Such metabolic changes are consistent with the "Warburg effect", commonly seen in cells under stress or with altered energy metabolism [23,38,39].
- Fatty Acid Oxidation and Beta-Oxidation Deficits: Enzymes involved in fatty acid beta-oxidation, such as acyl-CoA dehydrogenase very long chain (ACADVL) and enoyl-CoA hydratase 1 (ECHS1), were also dysregulated in hEDS fibroblasts [23]. Impaired beta-oxidation limits the ability of cells to utilize fatty acids as an alternative energy source, further exacerbating energy deficits [26]. This limitation may contribute to the exercise intolerance and post-exertional malaise frequently reported by hEDS patients [25,40].
- Mitochondria–ER Crosstalk and Calcium Homeostasis: Mitochondria actively communicate with the endoplasmic reticulum (ER) to regulate calcium signaling and energy production [41]. In hEDS fibroblasts, the upregulation of voltage-dependent anion channels (VDAC1 and VDAC2), which mediate the calcium flux between the ER and mitochondria, suggests altered calcium homeostasis [23]. Dysregulated calcium

signaling can impair mitochondrial function, leading to further reductions in ATP synthesis and disruptions in redox balance [23,42].

3.1.2. Elevated ROS and S100A4

Studies reported that increased ROS production in hEDS fibroblasts may be due to mitochondrial dysfunction [23]. This was accompanied by the upregulation of antioxidant enzymes, including superoxide dismutase 1 (SOD1), catalase (CAT), and glutathione peroxidase 1 (GPX1), indicating a compensatory response to oxidative damage [23,43–45]. The ROS-mediated activation of MMPs, such as MMP9 [46], was linked to ECM disorganization [23]. hEDS myofibroblasts showed high levels of S100A4, which is implicated in the disease states of different proinflammatory conditions [23]. S100A4 can cause mitochondrial disruption and oxidative stress in mast cells [47]. Silencing S100A4 can inhibit mitochondrial complex I activity and reduce cellular ATP levels [48].

3.1.3. Comparative Insights from Mitochondrial-Related Diseases

The findings of potential mitochondrial dysfunction in hEDS can be further contextualized by examining parallels with Fabry disease (FD), a lysosomal storage disorder characterized by the accumulation of glycosphingolipids such as globotriaosyl ceramide (Gb3) and its deacylated form, lyso-Gb3 [31,49]. In FD, mitochondrial dysfunction is a significant downstream consequence of lyso-Gb3 accumulation, which disrupts cellular homeostasis through multiple pathways [49–51]. Studies have shown that lyso-Gb3 accumulation impairs mitochondrial energy metabolism by interfering with the autophagy-lysosomal pathway, including the dysregulation of the mechanistic target of rapamycin (mTOR) signaling [50,51]. The inhibition of mTOR signaling in FD leads to reduced mitochondrial efficiency and diminished ATP production [51,52]. Additionally, lyso-Gb3 toxicity has been linked to lysosomal and endothelial impairments, further exacerbating mitochondrial dysfunction [51]. These abnormalities contribute to systemic manifestations in FD, including chronic fatigue, musculoskeletal pain, and connective tissue fragility [23,31]. The toxic effects of lyso-Gb3 also extend to fibroblasts, where it disrupts collagen synthesis and ECM integrity [23,51]. This disruption mirrors the ECM abnormalities observed in hEDS, suggesting a shared potential role for mitochondrial dysfunction in connective tissue pathologies. The insights from FD underscore the broader implications of mitochondrial dysfunction as a unifying mechanism in multisystemic disorders [23]. In both hEDS and FD, a potentially impaired mitochondrial function may contribute to bioenergetic deficits, oxidative stress, and tissue fragility [23,31]. These parallels highlight the need for further research into potential mitochondrial-targeted therapies that could benefit patients with hEDS and other mitochondrial-related diseases.

A recent study on fragile X premutation carriers (fXPCs) revealed molecular mechanisms that overlap with those implicated in hEDS, particularly concerning mitochondrial dysfunction and its downstream effects [35]. An expansion of CGG characterizes fragile X premutation repeats in the FMR1 gene, leading to elevated levels of FMR1 mRNA and subsequent RNA toxicity [35,53]. This RNA toxicity has been shown to disrupt cellular homeostasis through several pathways, including calcium dysregulation, mitochondrial dysfunction, the enhanced production of ROS, and inflammation [35,53].

3.1.4. Systemic Manifestations Linked to Mitochondrial Dysfunction

Mitochondrial dysfunction in hEDS contributes to a wide range of systemic manifestations due to the potential role of mitochondria in energy production, calcium homeostasis, and redox balance. These effects are particularly pronounced in tissues with high energy demands or those reliant on precise cellular signaling. Below, we expand on key systemic

manifestations linked to mitochondrial dysfunction, incorporating recent findings and adding bone fractures as another significant manifestation.

- Chronic Fatigue: Reduced ATP production due to impaired OXPHOS might be a hallmark of mitochondrial dysfunction in hEDS [23,32]. Proteomic analyses of hEDS fibroblasts have revealed the dysregulation of ATP synthase subunits and enzymes involved in the TCA cycle, such as ACO2 and FH, leading to bioenergetic deficits [23]. This energy insufficiency manifests as chronic fatigue and post-exertional malaise, symptoms frequently reported by hEDS patients [25,26,40]. Similar findings are observed in fXPCs, where RNA toxicity disrupts mitochondrial function and reduces energy availability [35]. These shared mechanisms highlight the central role of mitochondrial dysfunction in fatigue-related symptoms.
- Neurological Symptoms: Mitochondrial dysfunction may also contribute to neurological manifestations such as migraine headaches, ataxia, and neuropathic pain in hEDS [54,55]. Dysregulated calcium signaling between ER and mitochondria impairs neuronal signaling and ATP synthesis [56]. Elevated ROS levels further exacerbate neuroinflammation and oxidative damage, contributing to cognitive impairments and pain sensitivity [57]. In fXPCs, RNA toxicity-induced calcium dysregulation and ROS overproduction have been linked to similar neurological symptoms, including brain fog and chronic pain [35]. These findings suggest that potential mitochondrial dysfunction may underlie the overlapping neurological features observed in both conditions.
- Gastrointestinal Dysfunction: The gastrointestinal system is particularly vulnerable to mitochondrial dysfunction due to the high energy demands of smooth muscle cells and enteric neurons [58]. Impaired OXPHOS has been associated with gastrointestinal motility [59]. These symptoms are frequently observed in hEDS patients and contribute significantly to their reduced quality of life.
- Bone Fractures: Bone fragility is an emerging systemic manifestation that may be linked to mitochondrial dysfunction in hEDS [2]. Mitochondria play a critical role in maintaining bone homeostasis by regulating osteoblast activity, osteoclast resorption, and calcium metabolism [60–62]. Disrupted mitochondrial dynamics and reduced ATP production impair osteoblast differentiation and mineralization while promoting oxidative stress that accelerates bone resorption [63]. Studies have shown that hereditary connective tissue disorders like hEDS are associated with an increased risk of fractures due to compromised ECM integrity and reduced bone density [5,64,65]. Additionally, mitochondrial dysfunction contributes to osteoporosis by increasing ROS levels which disrupt the balance between bone formation and resorption [60,61,66,67]. This mechanism may explain the heightened vulnerability to fractures observed in hEDS patients.
- Connective Tissue Fragility: Mitochondrial dysfunction may play a potential role in disrupting ECM homeostasis, contributing to the hallmark features of hEDS such as joint hypermobility, skin hyperextensibility, and tissue fragility. Mitochondria are essential for energy production required during collagen biosynthesis [16,20,68]. Impaired OXPHOS in hEDS fibroblasts leads to reduced ATP availability [23], limiting the energy necessary for collagen production. Studies have shown that mitochondrial dysfunction also affects the post-translational modification of collagen, such as hydroxylation, which is critical for stabilizing the triple-helical structure of collagen molecules [24]. This results in weakened connective tissue that is more prone to mechanical stress and injury [68].

3.1.5. Sex-Specific Differences

The predominance of hEDS in females may be partially explained by sex-specific bioenergetic differences mediated by mitochondrial genetics and hormonal influences [6–8,22]. Estrogen has been shown to modulate mitochondrial biogenesis and function through ERα signaling pathways [21]. This hormonal regulation may influence ECM turnover and connective tissue resilience, which may contribute to the higher prevalence of hEDS in females.

3.2. Genetic Variations and Non-Mitochondrial Molecular Pathways

Unlike other subtypes of EDS with well-defined genetic causes, hEDS currently lacks a single causative gene [10]. Instead, recent studies suggest that hEDS arises from the interplay of multiple genetic variants and molecular pathways, which collectively contribute to its pathogenesis. The suggested genes involved in hEDS are summarized in Table 2, which provides a comprehensive overview of the genes and molecular pathways implicated in the pathogenesis of hEDS. It categorizes the genes into functional groups, such as primary candidate genes, ECM-related genes, signaling pathway genes, cell–matrix interaction genes, immune system-related genes, DNA damage response/stress genes, and emerging candidates. Each gene is accompanied by a brief description of its role or function in relation to hEDS (Table 2).

Table 2. Genes and molecular pathways associated with hypermobile Ehlers–Danlos Syndrome (hEDS).

Category	Gene	Function/Role
Primary Candidate	*KLK15*	Missense variant (p.Gly226Asp) found in ~2% of hEDS patients; affects connective tissue structure [69].
	MIA3	Implicated in collagen transport; identified as a promising candidate [70].
	LZTS1	Identified through genome-wide linkage analysis; linked to hEDS in some families [71].
Collagen and ECM	*TNXB*	Heterozygous mutations affecting collagen organization and ECM integrity [72].
	COL3A1	Includes variants like p.Gly637Ser, affecting collagen synthesis and organization [5,73].
	COL5A1, COL12A1	Variants affecting type V collagen and ECM structure [38,74,75].
	HSPG2, POSTN	Involved in ECM maintenance and structural stability [74].
	MMP1, MMP8, MMP13	Matrix metalloproteinases regulating ECM turnover and remodeling [74].
Signaling Pathway	*LAMC1, ROBO2*	Play roles in ECM organization and vascular integrity [74].
	ADAM7, ADAM27	Implicated in ECM remodeling processes [74].
	TGFB3, SMAD2, SMAD6, TGFB2	Key regulators of TGF-β signaling involved in tissue repair and remodeling [74].
	PIEZO1	Associated with mechanosensing and joint mobility [74].
	ULK4, KCNH1	Linked to novel mechanisms in tissue maintenance and cellular signaling.
	SORBS3	Regulates ECM stiffness and cellular mechanotransduction [74].
Cell–Matrix Interaction	*ITGA3, ITGB4, ITGA8, ITGAV*	Mediate cell adhesion to the ECM and integrin signaling pathways [76].
	ROBO4, PCNT	Associated with vascular stability and cell–matrix interactions [74].
Immune System	*HLA-B, HLA-DRB1*	Involved in antigen processing pathways; linked to immune dysregulation in hEDS.
	TPSAB1	Encodes alpha-tryptase; associated with mast cell activation disorders seen in hEDS patients [2,76].
DNA Damage/Stress	*BCL6, TP53*	Involved in DNA damage responses and cellular stress regulation mechanisms [36].
Other ECM Proteins	*COL9A3*	Affects collagen integrity; potential contributor to hEDS features.
	ALDH18A1, EFEMP1	Associated with structural stability of connective tissues [74].
Emerging Candidates	*ZNF717*	Emerging evidence suggests a role in transcriptional regulation linked to connective tissue health [77].
	RHBG	Potential involvement in cellular ion transport mechanisms relevant to tissue homeostasis [77].

3.2.1. Primary Candidate Genes

Several genes have been identified as potential contributors to hEDS. For example, the *KLK15* gene has been associated with a missense variant (p.Gly226Asp) found in approximately 2% of hEDS patients, potentially affecting connective tissue structure [69]. Additionally, *MIA3*, a gene involved in collagen transport, has emerged as a promising candidate in recent studies [70]. Genome-wide linkage analyses have also implicated *LZTS1* in a small percentage of hEDS patients [71], while heterozygous mutations in *TNXB* are known to disrupt collagen organization and ECM integrity [72]. Furthermore, variants in *COL3A1*, such as the p.Gly637Ser mutation, have been linked to collagen synthesis abnormalities in a single family with the autosomal dominant hEDS phenotype that was identified which led to reduced collagen secretion and the over-modification of collagen [5,73].

3.2.2. Collagen and ECM-Related Genes

ECM plays a central role in maintaining connective tissue integrity, and several collagen-related genes have been implicated in hEDS. Variants in *COL5A1* and *COL12A1* disrupt type V and XII collagen, respectively, leading to ECM instability [2,74,75]. Other genes like *HSPG2* and *POSTN* are involved in ECM maintenance, while *MMPs* such as *MMP1*, *MMP2*, *MMP4*, *MMP8*, *MMP13*, and *MMP25* regulate ECM turnover and remodeling [74].

3.2.3. Signaling Pathway Genes

Disruptions in key signaling pathways have also been associated with hEDS. Genes such as *TGFB3*, *SMAD2*, and *TGFB2* regulate TGF-β signaling, which is critical for tissue repair and remodeling [74]. The mechanosensing gene *PIEZO1* has been linked to joint mobility abnormalities [74], while novel mechanisms involving genes like *ULK4* and *KCNH1* suggest additional roles in tissue maintenance [74]. The gene *SORBS3* has also been implicated in regulating ECM stiffness [74].

3.2.4. Cell–Matrix Interaction Genes

Genes involved in cell adhesion to ECM are crucial for maintaining tissue integrity. For instance, integrin-related genes such as *ITGA3*, *ITGB4*, *ITGA8*, and *ITGAV* mediate cell–ECM adhesion and signaling pathways [74]. Additionally, genes like *ROBO4* and *PCNT* contribute to vascular stability and cell–matrix interactions [74].

3.2.5. Immune System Genes

Immune dysregulation is often observed in hEDS patients, with several immune-related genes being implicated. For example, variants in the antigen-processing genes *HLA-B* and *HLA-DRB1* have been linked to immune system involvement in hEDS [2]. Additionally, the tryptase-encoding gene *TPSAB1* is associated with mast cell activation disorders commonly seen in hEDS patients [2,74].

3.2.6. Emerging Candidate Genes

Recent studies have identified additional genes that may contribute to the molecular basis of hEDS. These include transcriptional regulators like *ZNF717*, which may influence connective tissue health, and ion transport-related genes like *RHBG*, which could impact tissue homeostasis [2,78]. Other emerging candidates include structural protein-related genes such as ALDH18A1 and signaling regulators like DSE, which require further investigation [74,79].

3.3. Protein–Protein Interaction Network Highlights Interplay Between Mitochondrial and Non-Mitochondrial Pathways in hEDS

A PPI network analysis revealed significant interactions between mitochondrial genes (Table 1) and non-mitochondrial genes (Table 2), highlighting a complex interplay that may underlie the pathophysiology of hEDS (Figure 2) [29,30].

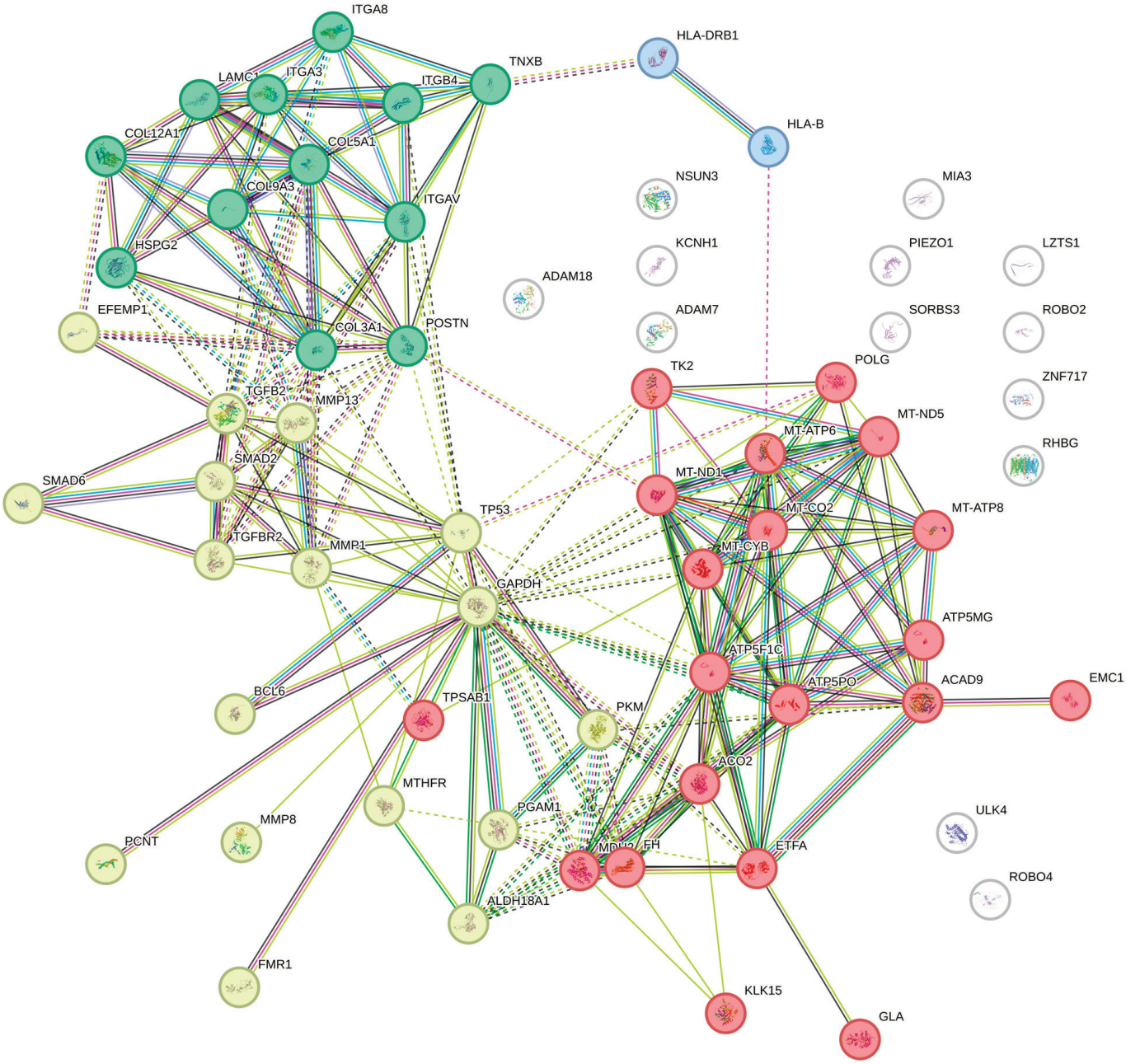


Figure 2. Protein–Protein interaction (PPI) network with functional clustering of mitochondrial and non-mitochondrial genes in hEDS. The PPI network depicted in this figure highlights the functional relationships among mitochondrial and non-mitochondrial genes implicated in hEDS. The network was analyzed using STRING [29,30], and the PPI enrichment *p*-value was calculated as $<1.0 \times 10^{-16}$, indicating that the observed interactions are significantly more frequent than expected by chance. This enrichment suggests that the proteins are biologically connected as a group, sharing common pathways or functions [29,30]. To further analyze the network, k-means clustering was applied, a machine learning algorithm that partitions data into distinct groups based on similarity. In this context,

k-means clustering grouped the PPI network into four distinct clusters, each representing a unique set of biological processes: Cluster 1 (red nodes) includes mitochondrial genes such as MT-CYB, MT-ND5, and ATP5F1C, which are central to energy production through oxidative phosphorylation. These genes play a critical role in ATP synthesis and maintaining cellular bioenergetics. Cluster 2 (blue nodes) encompasses genes like COL3A1, COL5A1, and ITGB4, which are involved in extracellular matrix (ECM) remodeling, collagen biosynthesis, and integrin-mediated cell adhesion. These processes are essential for maintaining connective tissue integrity. Cluster 3 (yellow nodes) represents TGF-β signaling and elastic fiber formation. Genes such as TGFB2, SMAD2, and MMP13 dominate this cluster, highlighting their roles in TGF-β signaling, matrix metalloproteinase activity, and elastic fiber assembly. The dysregulation of these pathways contributes to tissue fragility and abnormal ECM turnover in hEDS. Cluster 4 (green nodes) includes immune-related genes like HLA-B and HLA-DRB1, which are involved in antigen presentation and immune system regulation. These interactions suggest a link between mitochondrial dysfunction and immune dysregulation commonly observed in hEDS patients. This clustering analysis provides a systems-level view of how mitochondrial dysfunction interacts with non-mitochondrial pathways to drive the pathophysiology of hEDS. It underscores the interconnected nature of energy metabolism, ECM remodeling, signaling pathways, and immune regulation in the disorder.

Functional Pathway Enrichment of PPI Network Clusters

To further elucidate the biological significance of the identified clusters in the PPI network [29,30], functional enrichment analysis was performed (Figure 2). This analysis aimed to identify overrepresented biological processes, molecular functions, and pathways associated with mitochondrial and non-mitochondrial genes implicated in hEDS. The results provide deeper insights into how mitochondrial dysfunction interacts with non-mitochondrial pathways to drive the systemic manifestations of hEDS. These interactions may suggest that mitochondrial dysfunction not only disrupts cellular energetics but also influences ECM remodeling, signaling pathways, and immune regulation through its crosstalk with non-mitochondrial pathways.

- Cluster 1 represents mitochondrial energy metabolism and oxidative stress. Functional enrichment revealed that genes in this cluster are predominantly involved in OXPHOS, ATP synthesis, and redox balance regulation. Key pathways include the following: The ETC, critical for ATP production. ROS detoxification processes, highlighting the role of antioxidant enzymes such as SOD2 and GPX1 in mitigating oxidative stress. These findings underscore the role of impaired mitochondrial bioenergetics in symptoms such as chronic fatigue and muscle hypotonia observed in hEDS (Figure 2; red nodes).
- Cluster 2 represents ECM remodeling and cell–matrix interactions. Genes in this cluster showed significant enrichment for ECM-related pathways, including collagen biosynthesis and post-translational modifications. ECM-receptor interaction pathways involving integrins like ITGB4 that mediate cell adhesion. The dysregulation of these processes may explain hallmark features of hEDS, such as joint hypermobility and tissue fragility (Figure 2; yellow nodes).
- Cluster 3 represents TGF-β signaling and ECM turnover. This cluster was enriched for pathways related to the following: TGF-β signaling, which regulates tissue repair and fibrosis. Matrix metalloproteinase (MMP) activity, particularly MMP13, which degrades ECM components like collagen. Elastic fiber formation is essential for maintaining connective tissue elasticity (Figure 2; green nodes). These findings suggest that mitochondrial dysfunction may amplify TGF-β signaling dysregulation, contributing to abnormal ECM turnover in hEDS.
- Cluster 4 represents immune regulation and antigen presentation. Enrichment analysis identified immune-related pathways, including antigen processing and presenta-

tion via major histocompatibility complex (MHC) proteins (HLA-B, HLA-DRB1) [2]. Peptide antigen binding, which may link mitochondrial dysfunction to immune dysregulation commonly observed in hEDS patients. This highlights a potential role for mitochondrial ROS in modulating immune responses and contributing to conditions like mast cell activation syndrome (MCAS) seen in hEDS (Figure 2; blue nodes).

- Emerging pathways of interest: Novel interactions identified in the PPI network suggest additional pathways requiring further investigation including ion transport regulation by genes like *RHBG*, which may influence tissue homeostasis. Transcriptional regulation by ZNF717 potentially affects gene expression patterns relevant to connective tissue health [2,77].

3.4. Therapeutic Implications: Targeting Mitochondrial Dysfunction in hEDS

Mitochondrial dysfunction, as highlighted in this review, may play a potential role in the pathophysiology of hEDS. This dysfunction could provide a basis for exploring therapeutic strategies aimed at alleviating the systemic manifestations of the disorder. While these approaches remain speculative and require further validation, they offer promising avenues for future research and clinical application.

3.4.1. Enhancing Mitochondrial Bioenergetics

Impaired OXPHOS and reduced ATP production are hallmark features of mitochondrial dysfunction in mitochondrial dysfunction [80]. Strategies to support mitochondrial energy production might include the following:

- Coenzyme Q10 (CoQ10): As an electron carrier in the ETC, CoQ10 supplementation could improve mitochondrial efficiency and reduce fatigue, as observed in other mitochondrial disorders [81].
- Nicotinamide riboside (NR) or nicotinamide mononucleotide (NMN): These NAD+ precursors may enhance mitochondrial function by supporting cellular energy metabolism [82,83].
- Exercise-based interventions: Although exercise has been shown to promote mitochondrial biogenesis, its application in hEDS patients requires careful consideration due to the risk of post-exertional malaise [84,85].

3.4.2. Reducing Oxidative Stress

Elevated ROS levels associated with mitochondrial dysfunction may contribute to ECM degradation and systemic inflammation in hEDS [86,87]. Antioxidant therapies could mitigate these effects:

- Mitochondria-targeted antioxidants: Compounds such as MitoQ or SkQ1 might specifically target mitochondrial ROS, reducing oxidative damage [88].
- Dietary antioxidants: Nutrients like vitamin C, vitamin E, and glutathione precursors could provide additional support for redox balance [89].

3.4.3. Modulating ECM Remodeling

The interplay between mitochondrial dysfunction and ECM instability might be a key feature of hEDS. Therapies targeting ECM remodeling may help restore tissue integrity:

- Matrix metalloproteinase (MMP) inhibitors: These compounds are a family of zinc-dependent proteinases involved in the degradation of the ECM that could prevent excessive collagen degradation driven by ROS-mediated MMP activation [90–92].
- Tissue inhibitors of metalloproteinases (TIMPs): Enhancing TIMP activity might restore the balance between ECM synthesis and degradation [93].

- Collagen supplements or enhancers: These could support ECM repair by providing substrates for collagen biosynthesis [94].

4. Discussion

Unlike other EDS subtypes, hEDS lacks specific genetic markers, making it challenging to establish causative molecular pathways [2,5]. The absence of a single unifying genetic mutation suggests that hEDS may involve a multifactorial etiology, including both mitochondrial and non-mitochondrial mechanisms [2,78]. A recent survey revealed that individuals with clinically confirmed hEDS received an average of 10 years of alternative diagnoses during their diagnostic journey, with conditions such as anxiety, depression, and migraines being the most common [11]. This delay not only exacerbates patient distress but also delays appropriate management, potentially worsening outcomes. This underscores the challenge of distinguishing hEDS from other disorders with overlapping symptoms, such as fibromyalgia, functional neurological disorders, and multiple sclerosis [11]. Notably, many patients rejected these initial diagnoses upon receiving their hEDS diagnosis, reflecting a significant burden of misdiagnosis and prolonged diagnostic uncertainty [11]. The complexity of hEDS, involving multiple bodily systems and diverse manifestations such as chronic pain, dysautonomia, and gastrointestinal dysfunction, further complicates timely diagnosis [95]. Misdiagnosis can have profound consequences for vulnerable populations, particularly children [64,65,96,97]. Case studies have documented instances where symptoms of hEDS such as easy bruising and fractures due to connective tissue fragility were mistaken for signs of child abuse [64,65,96,97]. The uncertainty surrounding hEDS diagnosis can lead to significant psychosocial distress for patients [95]. A narrative review identified illness uncertainty (IU) as a key factor contributing to emotional distress in hEDS patients, driven by ambiguities in symptom attribution, diagnosis, and treatment options [95]. Studies suggest that healthcare providers may lack awareness of specific hEDS phenotypes or dismiss symptoms as psychosomatic [65,95–97]. This complexity underscores the need for more comprehensive genetic studies to identify candidate genes and their roles in hEDS pathogenesis.

This review underscores the hypothetical interplay between mitochondrial dysfunction and the systemic manifestations of hEDS. Mitochondrial dysfunction may act as a potential contributor to hEDS pathophysiology through several interconnected mechanisms [2]. OXPHOS and elevated reactive oxygen species levels could disrupt cellular energetics, leading to bioenergetic deficits that manifest as chronic fatigue and muscle hypotonia [98,99]. A meta-analysis of 17 patient cohorts with mitochondrial diseases suggests a potential link between OXPHOS defects and signs of hypermetabolism [98]. Clinically, these defects are associated with a broad range of multisystem disorders, including symptoms such as fatigue and exercise intolerance [98,100,101]. Consequently, many individuals with mitochondrial diseases tend to avoid physical activity and exercise, likely due to the exacerbation of these symptoms [98,102,103].

Furthermore, mitochondrial dysfunction may exacerbate ECM instability by influencing collagen biosynthesis and post-translational modifications [16,104,105], thereby possibly contributing to hallmark features of hEDS such as joint hypermobility, skin hyperextensibility, and tissue fragility. Mitochondria dysfunction influences ECM composition and vice versa [106]. Our review highlights potential crosstalk between mitochondrial abnormalities and non-mitochondrial pathways, including ECM remodeling, immune regulation, and signaling cascades (Figure 2). PPI network analyses suggest that mitochondrial dysfunction could amplify systemic manifestations by interacting with key molecular pathways, such as TGF-β signaling and matrix metalloproteinase activation (Figure 2). A recent study showed that the TGF-β receptor mediates communication between the

ECM and mitochondria [16]. These interactions may further disrupt ECM homeostasis and tissue integrity [16]. Additionally, the systemic implications of mitochondrial dysfunction extend to neurological [107] and gastrointestinal symptoms [58] commonly observed in hEDS. Altered calcium homeostasis and increased ROS production may impair neuronal signaling and smooth muscle function [108], contributing to migraines [109], cognitive impairments [110,111], gastrointestinal dysmotility, and other multisystemic symptoms. The proposed relationship between mitochondrial dysfunction and these diverse clinical features provides a framework for future research aimed at elucidating the molecular underpinnings of hEDS.

While this review provides valuable insights into the potential role of mitochondrial dysfunction in the pathogenesis of hEDS, several limitations must be acknowledged. First, the evidence base is notably sparse, with only eight original studies directly addressing the relationship between mitochondria and hEDS. This limited dataset restricts the ability to draw robust conclusions and highlights the need for further research to substantiate these preliminary findings. A significant limitation in existing studies is the small sample sizes in genetic investigations, which reduce power and may fail to capture the full spectrum of genetic variability associated with hEDS. Many identified genetic variants lack functional validation, leaving their biological significance unclear. Without experimental confirmation, it remains speculative whether these variants directly contribute to mitochondrial dysfunction or broader hEDS pathophysiology. Additionally, while this review proposes mechanisms linking potential mitochondrial dysfunction to specific hEDS symptoms such as fatigue, connective tissue fragility, and gastrointestinal dysmotility, the exact molecular pathways remain poorly defined. The interplay between mitochondrial abnormalities and non-mitochondrial pathways, such as ECM remodeling and immune dysregulation, is suggested but not yet fully elucidated. This gap underscores the need for mechanistic studies using advanced tools like proteomics, bioinformatics, and functional assays. Another limitation is the lack of longitudinal data on disease progression in hEDS patients. Mitochondrial dysfunction may evolve over time or vary across different stages of hEDS, but current cross-sectional studies do not capture these dynamics. Longitudinal research could provide critical insights into how mitochondrial abnormalities contribute to disease onset, progression, and symptom variability. Finally, the heterogeneity of hEDS as a clinical entity presents challenges in interpreting findings. Future research should aim to standardize diagnostic frameworks and stratify patients based on molecular or clinical subtypes to improve study reproducibility and relevance. While these findings are based on emerging evidence and remain hypothetical, they emphasize the need for further investigation into the role of mitochondrial pathways in hEDS. To advance our understanding, future research should focus on the direct measurements of mitochondrial function and larger-scale genetic studies. Specifically, researchers should consider implementing techniques such as seahorse extracellular flux analysis to quantify the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in patient-derived cells [112]. Additionally, assessing mitochondrial membrane potential using fluorescent dyes like JC-1 [113] or TMRE [114] in live cells from hEDS patients could provide valuable insights. Quantifying ATP production [115] in hEDS patient cells compared to controls using luminescence-based assays would further elucidate the role of mitochondrial pathways in hEDS pathogenesis. Analyzing mtDNA copy number and integrity through qPCR and long-range PCR techniques [116] in hEDS patient samples could reveal potential mitochondrial DNA alterations associated with the condition. Furthermore, investigating mitochondrial morphology and dynamics using high-resolution microscopy techniques in patient-derived cells would provide crucial information about the structural and functional changes in mitochondria [117] in hEDS.

Despite the limitations discussed, the novelty of this review lies in its pioneering exploration of mitochondrial dysfunction as a potential unifying mechanism in the pathophysiology of hEDS. This novel perspective opens a new era in hEDS research by integrating mitochondrial pathways into the broader understanding of this complex disorder. By proposing mitochondria as a potential player, this review provides a fresh framework for investigating hEDS, shifting the focus from solely ECM abnormalities to include cellular energetics and mitochondrial dynamics. The current review not only highlights the potential role of mitochondrial dysfunction in hEDS but also suggests that it may address several unresolved research gaps.

First, it offers an explanation for the wide spectrum of hEDS phenotypes, ranging from mild to severe. Variability in mitochondrial function across tissues and individuals could contribute to differences in symptom severity and progression [118]. Second, it provides a rationale for the lack of a single causative gene for hEDS. The interplay between multiple genes and their interaction with mitochondrial pathways or just mitochondrial genes may underlie the multifactorial nature of hEDS. Recent research suggests the potential role of mitochondrial DNA (mtDNA) mutations and mitochondrial dysfunctions in the development of connective tissue disorders [18–20]. A groundbreaking study by Schaefer et al. revealed that common mtDNA mutations, when present in an incompatible mtDNA background, can have far-reaching effects beyond the mitochondria themselves [20]. These mutations were found to alter the nuclear epigenome and influence the expression of numerous extracellular matrix genes [20]. This discovery provides a novel mechanism linking mitochondrial dysfunction to the widespread connective tissue abnormalities observed in disorders such as hEDS, opening new avenues for understanding the complex interplay between mitochondrial function and connective tissue homeostasis. This complexity suggests that future studies should prioritize examining mtDNA and its heteroplasmy through tissue-specific biopsies, rather than relying solely on genomic DNA from blood samples [119]. Such approaches may uncover tissue-specific mitochondrial dysfunctions that are masked in broader genomic analyses [120]. Additionally, our review proposes that the predominance of hEDS in females [6–8] could be partially explained by hormonal influences on mitochondrial function [21,22]. Estrogen has been shown to regulate mitochondrial biogenesis and activity, potentially amplifying sex-specific differences in bioenergetics and ECM turnover [21,22]. This hypothesis warrants further investigation into the role of sex hormones in modulating mitochondrial pathways and their contribution to hEDS pathophysiology. Importantly, the current review lays the groundwork for future research directions. It encourages studies aimed at validating the proposed links between mitochondria and hEDS through functional assays, advanced proteomics, and longitudinal analyses. Furthermore, it highlights the potential for clinical trials targeting mitochondrial function as a therapeutic strategy. Interventions such as mitochondrial antioxidants, coenzyme Q10 supplementation, or NAD+ precursors could be explored as potential treatments to alleviate systemic manifestations like fatigue, connective tissue fragility, and dysautonomia.

5. Conclusions

This review highlights mitochondrial dysfunction as a potentially unifying mechanism in the complex pathophysiology of hEDS. By integrating evidence from mitochondrial biology, ECM remodeling, and systemic manifestations, this work provides a novel framework for understanding the multifactorial etiology of hEDS. Impaired OXPHOS, elevated ROS, and calcium dysregulation emerge as central contributors to cellular energetics deficits and ECM instability, which may underlie hallmark features of hEDS such as joint hypermobility, skin hyperextensibility, and systemic symptoms like chronic fatigue and gastrointestinal

dysfunction. Despite the limitations of the current evidence base such as small sample sizes, a lack of functional validation for identified genetic variants, and limited longitudinal data, this review opens new avenues for research. It addresses key gaps in hEDS studies by proposing that potential mitochondrial dysfunction could explain the wide phenotypic spectrum of hEDS, the absence of a single causative gene, and the predominance of female patients due to hormonal influences on mitochondrial function. These insights suggest that future investigations should prioritize tissue-specific mitochondrial analyses, including assessments of mtDNA heteroplasmy and functional studies in relevant tissues. The proposed relationship between mitochondria and hEDS not only advances our understanding of its molecular basis but also sets the stage for clinical trials that could transform patient care.

In conclusion, this work represents a significant step forward in unraveling the molecular complexity of hEDS. By bridging mitochondrial dysfunction, it lays the groundwork for a more comprehensive understanding of hEDS pathogenesis while offering a promising direction for future research and therapeutic development.

Supplementary Materials: Supplementary-PRISMA-ScR-Checklist. The supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cimb47020134/s1.

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References

1. Shirvani, P.; Shirvani, A.; Holick, M.F. Decoding the Genetic Basis of Mast Cell Hypersensitivity and Infection Risk in Hypermobile Ehlers-Danlos Syndrome. *Curr. Issues Mol. Biol.* **2024**, *46*, 11613–11629. [CrossRef]
2. Malfait, F.; Francomano, C.; Byers, P.; Belmont, J.; Berglund, B.; Black, J.; Bloom, L.; Bowen, J.M.; Brady, A.F.; Burrows, N.P.; et al. The 2017 international classification of the Ehlers-Danlos syndromes. *Am. J. Med. Genet. C Semin. Med. Genet.* **2017**, *175*, 8–26. [CrossRef] [PubMed]
3. Steinmann, B.; Royce, P.M.; Superti-Furga, A. *The Ehlers-Danlos syndrome In Connective Tissue and Its Heritable Disorders: Molecular, Genetic, and Medical Aspects*; Royce, P.M., Steinmann, B., Eds.; Wiley: Hoboken, NJ, USA, 2002; pp. 431–523.
4. Demmler, J.C.; Atkinson, M.D.; Reinhold, E.J.; Choy, E.; Lyons, R.A.; Brophy, S.T. Diagnosed prevalence of Ehlers-Danlos syndrome and hypermobility spectrum disorder in Wales, UK: A national electronic cohort study and case-control comparison. *BMJ Open* **2019**, *9*, e031365. [CrossRef]
5. Gensemer, C.; Burks, R.; Kautz, S.; Judge, D.P.; Lavallee, M.; Norris, R.A. Hypermobile Ehlers-Danlos syndromes: Complex phenotypes, challenging diagnoses, and poorly understood causes. *Dev. Dyn.* **2021**, *250*, 318–344. [CrossRef] [PubMed] [PubMed Central]
6. Castori, M.; Camerota, F.; Celletti, C.; Grammatico, P.; Padua, L. Ehlers-Danlos syndrome hypermobility type and the excess of affected females: Possible mechanisms and perspectives. *Am. J. Med. Genet.* **2010**, *152A*, 2406–2408. [CrossRef]
7. Castori, M. Ehlers-danlos syndrome, hypermobility type: An underdiagnosed hereditary connective tissue disorder with mucocutaneous, articular, and systemic manifestations. *ISRN Dermatol.* **2012**, *2012*, 751768. [CrossRef]
8. Rodgers, K.R.; Gui, J.; Dinulos, M.B.; Chou, R.C. Ehlers-Danlos syndrome hypermobility type is associated with rheumatic diseases. *Sci Rep.* **2017**, *7*, 39636. [CrossRef] [PubMed] [PubMed Central]

9. Levy, H.P. Hypermobile Ehlers-Danlos Syndrome: Clinical Description and Natural History. *Genet. Med.* **2017**, *19*, 552–557.
10. Ritelli, M.; Colombi, M. Molecular Genetics and Pathogenesis of Ehlers-Danlos Syndrome and Related Connective Tissue Disorders. *Genes* **2020**, *11*, 547. [CrossRef]
11. Halverson, C.M.E.; Cao, S.; Perkins, S.M.; Francomano, C.A. Comorbidity, misdiagnoses, and the diagnostic odyssey in patients with hypermobile Ehlers-Danlos syndrome. *Genet. Med. Open* **2023**, *1*, 100812. [CrossRef] [PubMed] [PubMed Central]
12. Albaugh, V.L.; Mukherjee, K.; Barbul, A. Proline Precursors and Collagen Synthesis: Biochemical Challenges of Nutrient Supplementation and Wound Healing. *J Nutr.* **2017**, *147*, 2011–2017. [CrossRef] [PubMed] [PubMed Central]
13. Eble, J.A.; de Rezende, F.F. Redox-relevant aspects of the extracellular matrix and its cellular contacts via integrins. *Antioxid. Redox Signal.* **2014**, *20*, 1977–1993. [CrossRef] [PubMed] [PubMed Central]
14. Cabral-Pacheco, G.A.; Garza-Veloz, I.; Castruita-De la Rosa, C.; Ramirez-Acuña, J.M.; Perez-Romero, B.A.; Guerrero-Rodriguez, J.F.; Martinez-Avila, N.; Martinez-Fierro, M.L. The Roles of Matrix Metalloproteinases and Their Inhibitors in Human Diseases. *Int. J. Mol. Sci.* **2020**, *21*, 9739. [CrossRef] [PubMed] [PubMed Central]
15. Chen, X.; Yang, C.; Jiang, G. Research progress on skin photoaging and oxidative stress. *Postepy Dermatol. Alergol.* **2021**, *38*, 931–936. [CrossRef] [PubMed] [PubMed Central]
16. Zhang, H.; Tsui, C.K.; Garcia, G.; Joe, L.K.; Wu, H.; Maruichi, A.; Fan, W.; Pandovski, S.; Yoon, P.H.; Webster, B.M.; et al. The extracellular matrix integrates mitochondrial homeostasis. *Cell* **2024**, *187*, 4289–4304.e26. [CrossRef] [PubMed]
17. Mogensen, T.H.; Melchjorsen, J.; Höllsberg, P.; Paludan, S.R. Activation of NF-kappa B in virus-infected macrophages is dependent on mitochondrial oxidative stress and intracellular calcium: Downstream involvement of the kinases TGF-beta-activated kinase 1, mitogen-activated kinase/extracellular signal-regulated kinase kinase 1, and I kappa B kinase. *J. Immunol.* **2003**, *170*, 6224–6233. [CrossRef] [PubMed]
18. Pu, W.; Gu, Z. Incompatibilities between common mtDNA variants in human disease. *Proc. Natl. Acad. Sci. USA* **2023**, *120*, e2217452119. [CrossRef] [PubMed] [PubMed Central]
19. Jaeger, V.K.; Lebrecht, D.; Nicholson, A.G.; Wells, A.; Bhayani, H.; Gazdhar, A.; Tamm, M.; Venhoff, N.; Geiser, T.; Walker, U.A. Mitochondrial DNA mutations and respiratory chain dysfunction in idiopathic and connective tissue disease-related lung fibrosis. *Sci. Rep.* **2019**, *9*, 5500. [CrossRef] [PubMed] [PubMed Central]
20. Schaefer, P.M.; Scherer Alves, L.; Lvova, M.; Huang, J.; Rathi, K.; Janssen, K.; Butic, A.; Yardeni, T.; Morrow, R.; Lott, M.; et al. Combination of common mtDNA variants results in mitochondrial dysfunction and a connective tissue dysregulation. *Proc. Natl. Acad. Sci. USA* **2022**, *119*, e2212417119. [CrossRef] [PubMed] [PubMed Central]
21. Klinge, C.M. Estrogenic control of mitochondrial function. *Redox Biol.* **2020**, *31*, 101435. [CrossRef] [PubMed] [PubMed Central]
22. Sultanova, R.F.; Schibalski, R.; Yankelevich, I.A.; Stadler, K.; Ilatovskaya, D.V. Sex differences in renal mitochondrial function: A hormone-gous opportunity for research. *Am. J. Physiol. Renal. Physiol.* **2020**, *319*, F1117–F1124. [CrossRef] [PubMed] [PubMed Central]
23. Chiarelli, N.; Zoppi, N.; Ritelli, M.; Venturini, M.; Capitanio, D.; Gelfi, C.; Colombi, M. Biological insights in the pathogenesis of hypermobile Ehlers-Danlos syndrome from proteome profiling of patients' dermal myofibroblasts. *Biochim. Biophys. Acta Mol. Basis Dis.* **2021**, *1867*, 166051. [CrossRef] [PubMed]
24. Zong, Y.; Li, H.; Liao, P.; Chen, L.; Pan, Y.; Zheng, Y.; Zhang, C.; Liu, D.; Zheng, M.; Gao, J. Mitochondrial dysfunction: Mechanisms and advances in therapy. *Signal Transduct. Target Ther.* **2024**, *9*, 124. [CrossRef] [PubMed] [PubMed Central]
25. Ganesh, R.; Munipalli, B. Long COVID and hypermobility spectrum disorders have shared pathophysiology. *Front. Neurol.* **2024**, *15*, 1455498. [CrossRef] [PubMed] [PubMed Central]
26. Hakim, A. Hypermobile Ehlers-Danlos Syndrome. In *GeneReviews® [Internet]*; Adam, M.P., Feldman, J., Mirzaa, G.M., Pagon, R.A., Wallace, S.E., Amemiya, A., Eds.; University of Washington: Seattle, WA, USA, 2024. [PubMed]
27. Nezami, B.G.; Srinivasan, S. Enteric nervous system in the small intestine: Pathophysiology and clinical implications. *Curr. Gastroenterol. Rep.* **2010**, *12*, 358–365. [CrossRef] [PubMed] [PubMed Central]
28. Tricco, A.C.; Lillie, E.; Zarin, W.; O'Brien, K.K.; Colquhoun, H.; Levac, D.; Moher, D.; Peters, M.D.; Horsley, T.; Weeks, L.; et al. PRISMA extension for scoping reviews (PRISMA-ScR): Checklist and explanation. *Ann. Intern Med.* **2018**, *169*, 467–473. [CrossRef] [PubMed]
29. Data Were Retrieved from STRING v12.0. Available online: https://string-db.org/cgi/network?taskId=b9273dA6mdQd&sessionId=b0a5Hv0IYW6a (accessed on 5 January 2025).
30. Szklarczyk, D.; Kirsch, R.; Koutrouli, M.; Nastou, K.; Mehryary, F.; Hachilif, R.; Annika, G.L.; Fang, T.; Doncheva, N.T.; Pyysalo, S.; et al. The STRING database in 2023: Protein–protein association networks and functional enrichment analyses for any sequenced genome of interest. *Nucleic Acids Res.* **2023**, *51*, D638–D646. [CrossRef]
31. Barbey, F.; Lidove, O.; Dormond, O. Hypermobile Ehlers-Danlos-like syndrome in Fabry disease. *Clin. Genet.* **2019**, *95*, 627–628. [CrossRef] [PubMed]
32. Wilson, G.N.; Tonk, V.S. Clinical-Genomic Analysis of 1261 Patients with Ehlers-Danlos Syndrome Outlines an Articulo-Autonomic Gene Network (Entome). *Curr. Issues Mol. Biol.* **2024**, *46*, 2620–2643. [CrossRef] [PubMed] [PubMed Central]

33. Wilson, G.; Tonk, V. Clinical-DNA Correlates of Anxiety in Patients with Ehlers-Danlos Syndrome. *Open J. Psychiatry* **2024**, *14*, 319–333. [CrossRef]
34. Wilson, G.; Tonk, V. Mitochondrial Dysfunction Contributes to Ehlers-Danlos Syndrome—A Patient Presentation. *J. Biol. Life Sci.* **2020**, *11*, 190–202. [CrossRef]
35. Tassanakijpanich, N.; McKenzie, F.J.; McLennan, Y.A.; Makhoul, E.; Tassone, F.; Jasoliya, M.J.; Romney, C.; Petrasic, I.C.; Napalinga, K.; Buchanan, C.B.; et al. Hypermobile Ehlers-Danlos syndrome (hEDS) phenotype in fragile X premutation carriers: Case series. *J. Med. Genet.* **2022**, *59*, 687–690. [CrossRef] [PubMed] [PubMed Central]
36. Jahanbani, F.; Sing, J.C.; Maynard, R.D.; Jahanbani, S.; Dafoe, J.; Dafoe, W.; Jones, N.; Wallace, K.J.; Rastan, A.; Maecker, H.T.; et al. Longitudinal cytokine and multi-modal health data of an extremely severe ME/CFS patient with HSD reveals insights into immunopathology, and disease severity. *Front. Immunol.* **2024**, *15*, 1369295. [CrossRef] [PubMed] [PubMed Central]
37. He, M.; Rutledge, S.L.; Kelly, D.R.; Palmer, C.A.; Murdoch, G.; Majumder, N.; Nicholls, R.D.; Pei, Z.; Watkins, P.A.; Vockley, J. A new genetic disorder in mitochondrial fatty acid beta-oxidation: ACAD9 deficiency. *Am. J. Hum. Genet.* **2007**, *81*, 87–103. [CrossRef] [PubMed] [PubMed Central]
38. Eisner, V.; Picard, M.; Hajnóczky, G. Mitochondrial dynamics in adaptive and maladaptive cellular stress responses. *Nat. Cell Biol.* **2018**, *20*, 755–765. [CrossRef] [PubMed] [PubMed Central]
39. Gibb, A.A.; Lazaropoulos, M.P.; Elrod, J.W. Myofibroblasts and fibrosis: Mitochondrial and metabolic control of cellular differentiation. *Circ. Res.* **2020**, *127*, 427–447. [CrossRef]
40. Guerra, I.M.S.; Ferreira, H.B.; Melo, T.; Rocha, H.; Moreira, S.; Diogo, L.; Domingues, M.R.; Moreira, A.S.P. Mitochondrial Fatty Acid β-Oxidation Disorders: From Disease to Lipidomic Studies—A Critical Review. *Int. J. Mol. Sci.* **2022**, *23*, 13933. [CrossRef] [PubMed] [PubMed Central]
41. Tsuda, M.; Fukushima, A.; Matsumoto, J.; Takada, S.; Kakutani, N.; Nambu, H.; Yamanashi, K.; Furihata, T.; Yokota, T.; Okita, K.; et al. Protein acetylation in skeletal muscle mitochondria is involved in impaired fatty acid oxidation and exercise intolerance in heart failure. *J. Cachexia Sarcopenia Muscle* **2018**, *9*, 844–859. [CrossRef] [PubMed] [PubMed Central]
42. Leung, A.; Ohadi, D.; Pekkurnaz, G.; Rangamani, P. Systems modeling predicts that mitochondria ER contact sites regulate the postsynaptic energy landscape. *NPJ Syst. Biol. Appl.* **2021**, *7*, 26. [CrossRef] [PubMed] [PubMed Central]
43. Alevriadou, B.R.; Shanmughapriya, S.; Patel, A.; Stathopulos, P.B.; Madesh, M. Mitochondrial Ca^{2+} transport in the endothelium: Regulation by ions, redox signalling and mechanical forces. *J. R. Soc. Interface* **2017**, *14*, 20170672. [CrossRef] [PubMed] [PubMed Central]
44. Jomova, K.; Alomar, S.Y.; Alwasel, S.H.; Nepovimova, E.; Kuca, K.; Valko, M. Several lines of antioxidant defense against oxidative stress: Antioxidant enzymes, nanomaterials with multiple enzyme-mimicking activities, and low-molecular-weight antioxidants. *Arch. Toxicol.* **2024**, *98*, 1323–1367. [CrossRef] [PubMed] [PubMed Central]
45. Veal, E.; Jackson, T.; Latimer, H. Role/s of 'antioxidant' enzymes in ageing. In *Subcellular Biochemistry*; Springer: New York, NY, USA, 2018; pp. 425–450. [CrossRef]
46. Mori, K.; Uchida, T.; Yoshie, T.; Mizote, Y.; Ishikawa, F.; Katsuyama, M.; Shibanuma, M. A mitochondrial ROS pathway controls matrix metalloproteinase 9 levels and invasive properties in RAS-activated cancer cells. *FEBS J.* **2019**, *286*, 459–478. [CrossRef] [PubMed] [PubMed Central]
47. Mo, S.; Yan, S.; Li, J.; He, J.; Wu, T.; Tao, T.; Wang, C.; Yu, F. S100A4 reprofiles lipid metabolism in mast cells via RAGE and PPAR-γ signaling pathway. *Int. Immunopharmacol.* **2024**, *128*, 111555, Erratum in: *Int. Immunopharmacol.* **2025**, *145*, 113741. https://doi.org/10.1016/j.intimp.2024.113741. [CrossRef] [PubMed]
48. Liu, L.; Qi, L.; Knifley, T.; Piecoro, D.W.; Rychahou, P.; Liu, J.; Mitov, M.I.; Martin, J.; Wang, C.; Wu, J.; et al. S100A4 alters metabolism and promotes invasion of lung cancer cells by up-regulating mitochondrial complex I protein NDUFS2. *J. Biol. Chem.* **2019**, *294*, 7516–7527. [CrossRef] [PubMed] [PubMed Central]
49. Kok, K.; Zwiers, K.C.; Boot, R.G.; Overkleeft, H.S.; Aerts, J.M.F.G.; Artola, M. Fabry Disease: Molecular Basis, Pathophysiology, Diagnostics and Potential Therapeutic Directions. *Biomolecules* **2021**, *11*, 271. [CrossRef] [PubMed] [PubMed Central]
50. Emecen Sanli, M.; Kilic, A.; Inci, A.; Okur, I.; Ezgu, F.; Tumer, L. Endocrinological, immunological and metabolic features of patients with Fabry disease under therapy. *J. Pediatr. Endocrinol. Metab.* **2023**, *36*, 650–658. [CrossRef] [PubMed]
51. Tuttolomondo, A.; Simonetta, I.; Riolo, R.; Todaro, F.; Di Chiara, T.; Miceli, S.; Pinto, A. Pathogenesis and Molecular Mechanisms of Anderson-Fabry Disease and Possible New Molecular Addressed Therapeutic Strategies. *Int. J. Mol. Sci.* **2021**, *22*, 10088. [CrossRef] [PubMed] [PubMed Central]
52. Laplante, M.; Sabatini, D.M. mTOR signaling at a glance. *J. Cell Sci.* **2009**, *122*, 3589–3594. [CrossRef] [PubMed]
53. Hagerman, R.; Hagerman, P. Advances in clinical and molecular understanding of the FMR1 premutation and fragile X-associated tremor/ataxia syndrome. *Lancet Neurol.* **2013**, *12*, 786–798. [CrossRef]
54. Pieczenik, S.R.; Neustadt, J. Mitochondrial dysfunction and molecular pathways of disease. *Exp. Mol. Pathol.* **2007**, *83*, 84–92. [CrossRef] [PubMed]

55. Bénistan, K.; Martinez, V. Pain in hypermobile Ehlers-Danlos syndrome: New insights using new criteria. *Am. J. Med. Genet. A* **2019**, *179*, 1226–1234. [CrossRef] [PubMed]
56. Matuz-Mares, D.; González-Andrade, M.; Araiza-Villanueva, M.G.; Vilchis-Landeros, M.M.; Vázquez-Meza, H. Mitochondrial Calcium: Effects of Its Imbalance in Disease. *Antioxidants* **2022**, *11*, 801. [CrossRef] [PubMed] [PubMed Central]
57. Picca, A.; Calvani, R.; Coelho-Junior, H.J.; Landi, F.; Bernabei, R.; Marzetti, E. Mitochondrial Dysfunction, Oxidative Stress, and Neuroinflammation: Intertwined Roads to Neurodegeneration. *Antioxidants* **2020**, *9*, 647. [CrossRef] [PubMed] [PubMed Central]
58. Haque, P.S.; Kapur, N.; Barrett, T.A.; Theiss, A.L. Mitochondrial function and gastrointestinal diseases. *Nat. Rev. Gastroenterol. Hepatol.* **2024**, *21*, 537–555. [CrossRef] [PubMed]
59. Chitkara, D.K.; Nurko, S.; Shoffner, J.M.; Buie, T.; Flores, A. Abnormalities in gastrointestinal motility are associated with diseases of oxidative phosphorylation in children. *Am. J. Gastroenterol.* **2003**, *98*, 871–877. [CrossRef] [PubMed]
60. Yan, C.; Shi, Y.; Yuan, L.; Lv, D.; Sun, B.; Wang, J.; Liu, X.; An, F. Mitochondrial quality control and its role in osteoporosis. *Front. Endocrinol.* **2023**, *14*, 1077058. [CrossRef] [PubMed] [PubMed Central]
61. Liu, J.; Zhang, X.; Hou, H.; Ouyang, J.; Dai, J. Advances in Osteoblast and Mitochondrial Dynamics and Their Transfer in Osteoporosis. *J. Cell Mol. Med.* **2024**, *28*, e70299. [CrossRef] [PubMed] [PubMed Central]
62. Ding, P.; Gao, C.; Zhou, J.; Mei, J.; Li, G.; Liu, D.; Li, H.; Liao, P.; Yao, M.; Wang, B.; et al. Mitochondria from osteolineage cells regulate myeloid cell-mediated bone resorption. *Nat. Commun.* **2024**, *15*, 5094. [CrossRef] [PubMed] [PubMed Central]
63. Wang, F.S.; Wu, R.W.; Chen, Y.S.; Ko, J.Y.; Jahr, H.; Lian, W.S. Biophysical Modulation of the Mitochondrial Metabolism and Redox in Bone Homeostasis and Osteoporosis: How Biophysics Converts into Bioenergetics. *Antioxidants* **2021**, *10*, 1394. [CrossRef] [PubMed] [PubMed Central]
64. Holick, M.F.; Shirvani, A.; Charoenngam, N. Fetal Fractures in an Infant with Maternal Ehlers-Danlos Syndrome, CCDC134 Pathogenic Mutation and a Negative Genetic Test for Osteogenesis Imperfecta. *Children* **2021**, *8*, 512. [CrossRef] [PubMed] [PubMed Central]
65. Holick, M.F.; Hossein-Nezhad, A.; Tabatabaei, F. Multiple fractures in infants who have Ehlers-Danlos/hypermobility syndrome and or vitamin D deficiency: A case series of 72 infants whose parents were accused of child abuse and neglect. *Dermato-Endocrinology* **2017**, *9*, e1279768. [CrossRef] [PubMed] [PubMed Central]
66. Zhang, L.; Gan, X.; He, Y.; Zhu, Z.; Zhu, J.; Yu, H. Drp1-dependent mitochondrial fission mediates osteogenic dysfunction in inflammation through elevated production of reactive oxygen species. *PLoS ONE* **2017**, *12*, e0175262. [CrossRef] [PubMed]
67. Liu, J.; Gao, Z.; Liu, X. Mitochondrial dysfunction and therapeutic perspectives in osteoporosis. *Front. Endocrinol.* **2024**, *15*, 1325317. [CrossRef] [PubMed] [PubMed Central]
68. Pathria, M.N.; Chung, C.B.; Resnick, D.L. Acute and Stress-related Injuries of Bone and Cartilage: Pertinent Anatomy, Basic Biomechanics, and Imaging Perspective. *Radiology* **2016**, *280*, 21–38. [CrossRef] [PubMed] [PubMed Central]
69. Gensemer, C.; Beck, T.; Guo, L.; Petrucci, T.; Morningstar, J.; Kornblau, I.; Byerly, K.; Biggs, R.; Weintraub, A.; Moore, K.; et al. Variants in the Kallikrein Gene Family and Hypermobile Ehlers-Danlos Syndrome. *Res. Sq.* **2024**. [CrossRef]
70. Junkiert-Czarnecka, A.; Pilarska-Deltow, M.; Bąk, A.; Heise, M.; Haus, O. A novel mutation in collagen transport protein, MIA3 gene, detected in a patient with clinical symptoms of Ehlers-Danlos hypermobile syndrome. *Adv. Clin. Exp. Med.* **2023**, *32*, 777–781. [CrossRef]
71. Syx, D.; Symoens, S.; Steyaert, W.; De Paepe, A.; Coucke, P.J.; Malfait, F. Ehlers-Danlos Syndrome, Hypermobility Type, Is Linked to Chromosome 8p22-8p21.1 in an Extended Belgian Family. *Dis. Markers.* **2015**, *2015*, 828970. [CrossRef] [PubMed]
72. Zweers, M.C.; Bristow, J.; Steijlen, P.M.; Dean, W.B.; Hamel, B.C.; Otero, M.; Kucharekova, M.; Boezeman, J.B.; Schalkwijk, J. Haploinsufficiency of TNXB is associated with hypermobility type of Ehlers-Danlos syndrome. *Am J Hum Genet.* **2003**, *73* 1, 214–217. [CrossRef] [PubMed] [PubMed Central]
73. Narcisi, P.; Richards, A.; Ferguson, S.; Pope, M.F. A family with Ehlers-Danlos syndrome type III/articular hypermobility syndrome has a glycine 637 to serine substitution in type III collagen. *Hum. Mol. Genet.* **1994**, *3*, 1617–1620. [CrossRef]
74. Vandersteen, A.M.; Weerakkody, R.A.; Parry, D.A.; Kanonidou, C.; Toddie-Moore, D.J.; Vandrovcova, J.; Darlay, R.; Santoyo-Lopez, J.; Meynert, A.; NIHR BioResource; et al. Genetic complexity of diagnostically unresolved Ehlers-Danlos syndrome. *J. Med. Genet.* **2024**, *61*, 232–238. [CrossRef]
75. Vera, A.M.; Peterson, L.E.; Dong, D.; Haghshenas, V.; Yetter, T.R.; Delgado, D.A.; McCulloch, P.C.; Varner, K.E.; Harris, J.D. High Prevalence of Connective Tissue Gene Variants in Professional Ballet. *Am. J. Sports Med.* **2020**, *48* 1, 222–228. [CrossRef] [PubMed]
76. Courseault, J.; Umar, M.; Bordnick, P.; Simons, J.; Volic, M.; Stock, A.; Bix, G. Prevalence of MTHFR Polymorphisms in Patients With Hypermobile Ehlers-Danlos Syndrome and Hypermobile Spectrum Disorders in a US Hypermobility Clinic. *ACR Open Rheumatol.* **2024**, *6*, 399–402. [CrossRef]
77. Vazquez, M.; Chovanec, J.; Kim, J.; DiMaggio, T.; Milner, J.D.; Francomano, C.A.; Gurnett, C.A.; Ritelli, M.; Colombi, M.; Lyons, J.J. Hereditary alpha-tryptasemia modifies clinical phenotypes among individuals with congenital hypermobility disorders. *HGG Adv.* **2022**, *3*, 100094. [CrossRef] [PubMed]

78. Alanis-Funes, G.J.; Lira-Albarrán, S.; Hernández-Pérez, J.; Garza-Elizondo, M.A.; Ortíz-López, R.; Elizondo, C.V.; Rojas-Martinez, A.; Chávez-Santoscoy, R.A.; Rangel-Escareño, C. Genomic Characterization by Whole-Exome Sequencing of Hypermobility Spectrum Disorder. *Genes* **2022**, *13*, 1269. [CrossRef]
79. Alazami, A.M.; Al-Qattan, S.M.; Faqeih, E.; Alhashem, A.; Alshammari, M.; Alzahrani, F.; Al-Dosari, M.S.; Patel, N.; Alsagheir, A.; Binabbas, B.; et al. Expanding the clinical and genetic heterogeneity of hereditary disorders of connective tissue. *Hum. Genet.* **2016**, *135*, 525–540. [CrossRef] [PubMed]
80. Vardhana, S.A.; Hwee, M.A.; Berisa, M.; Wells, D.K.; Yost, K.E.; King, B.; Smith, M.; Herrera, P.S.; Chang, H.Y.; Satpathy, A.T.; et al. Impaired mitochondrial oxidative phosphorylation limits the self-renewal of T cells exposed to persistent antigen. *Nat. Immunol.* **2020**, *21*, 1022–1033. [CrossRef] [PubMed] [PubMed Central]
81. Suárez-Rivero, J.M.; Pastor-Maldonado, C.J.; Povea-Cabello, S.; Álvarez-Córdoba, M.; Villalón-García, I.; Munuera-Cabeza, M.; Suárez-Carrillo, A.; Talaverón-Rey, M.; Sánchez-Alcázar, J.A. Coenzyme Q10 Analogues: Benefits and Challenges for Therapeutics. *Antioxidants* **2021**, *10*, 236. [CrossRef] [PubMed] [PubMed Central]
82. Cantó, C.; Menzies, K.J.; Auwerx, J. NAD(+) Metabolism and the Control of Energy Homeostasis: A Balancing Act between Mitochondria and the Nucleus. *Cell Metab.* **2015**, *22*, 31–53. [CrossRef] [PubMed] [PubMed Central]
83. Alegre, G.F.S.; Pastore, G.M. NAD+ Precursors Nicotinamide Mononucleotide (NMN) and Nicotinamide Riboside (NR): Potential Dietary Contribution to Health. *Curr. Nutr. Rep.* **2023**, *12*, 445–464. [CrossRef] [PubMed] [PubMed Central]
84. Perry, C.G.R.; Hawley, J.A. Molecular Basis of Exercise-Induced Skeletal Muscle Mitochondrial Biogenesis: Historical Advances, Current Knowledge, and Future Challenges. *Cold Spring Harb. Perspect. Med.* **2018**, *8*, a029686. [CrossRef] [PubMed] [PubMed Central]
85. Palmer, S.; Bailey, S.; Barker, L.; Barney, L.; Elliott, A. The effectiveness of therapeutic exercise for joint hypermobility syndrome: A systematic review. *Physiotherapy* **2014**, *100*, 220–227. [CrossRef] [PubMed]
86. Stanworth, M.; Zhang, S.D. Elucidating the roles of SOD3 correlated genes and reactive oxygen species in rare human diseases using a bioinformatic-ontology approach. *PLoS ONE* **2024**, *19*, e0313139. [CrossRef] [PubMed] [PubMed Central]
87. Kuz'mina, N.S.; Shipaeva, E.V.; Semyachkina, A.N.; Vasil'eva, I.M.; Kovalenko, L.P.; Durnev, A.D.; Zasukhina, G.D. Polymorphism of detoxification genes and cell resistance to mutagens in patients with Ehlers-Danlos syndrome. *Bull. Exp. Biol. Med.* **2007**, *144*, 717–721. [CrossRef] [PubMed]
88. Jiang, Q.; Yin, J.; Chen, J.; Ma, X.; Wu, M.; Liu, G.; Yao, K.; Tan, B.; Yin, Y. Mitochondria-Targeted Antioxidants: A Step towards Disease Treatment. *Oxid. Med. Cell Longev.* **2020**, *2020*, 8837893. [CrossRef] [PubMed] [PubMed Central]
89. Janciauskiene, S. The Beneficial Effects of Antioxidants in Health And Diseases. *Chronic Obstr. Pulm. Dis.* **2020**, *7*, 182–202. [CrossRef] [PubMed] [PubMed Central]
90. Manuel Hidalgo, S. Gail Eckhardt, Development of Matrix Metalloproteinase Inhibitors in Cancer Therapy. *J. Natl. Cancer Inst.* **2001**, *93*, 178–193. [CrossRef]
91. Levin, M.; Udi, Y.; Solomonov, I.; Sagi, I. Next generation matrix metalloproteinase inhibitors—Novel strategies bring new prospects. *Biochim. Biophys. Acta Mol. Cell Res.* **2017**, *1864 Pt A*, 1927–1939. [CrossRef] [PubMed]
92. Kim, I.S.; Yang, W.S.; Kim, C.H. Physiological Properties, Functions, and Trends in the Matrix Metalloproteinase Inhibitors in Inflammation-Mediated Human Diseases. *Curr. Med. Chem.* **2023**, *30*, 2075–2112. [CrossRef] [PubMed]
93. Arpino, V.; Brock, M.; Gill, S.E. The role of TIMPs in regulation of extracellular matrix proteolysis. *Matrix Biol.* **2015**, *44–46*, 247–254. [CrossRef] [PubMed]
94. Rahman, A.; Rehmani, R.; Pirvu, D.G.; Huang, S.M.; Puri, S.; Arcos, M. Unlocking the Therapeutic Potential of Marine Collagen: A Scientific Exploration for Delaying Skin Aging. *Mar. Drugs* **2024**, *22*, 159. [CrossRef] [PubMed] [PubMed Central]
95. Feldman, E.C.H.; Homan, K.J.; Williams, S.E.; Ting, T.V.; Goldschneider, K.R.; Kashikar-Zuck, S. A narrative review of the literature on illness uncertainty in hypermobile ehlers-danlos syndrome: Implications for research and clinical practice. *Pediatr. Rheumatol. Online J.* **2023**, *21*, 121. [CrossRef] [PubMed] [PubMed Central]
96. van Gemert, M.J.C.; Zwinderman, A.H.; Koppen, P.J.V.; Neumann, H.A.M.; Vlaming, M. Child Abuse, Misdiagnosed by an Expertise Center-Part II-Misuse of Bayes' Theorem. *Children* **2023**, *10*, 843. [CrossRef] [PubMed] [PubMed Central]
97. Vlaming, M.; Sauer, P.J.J.; Janssen, E.P.F.; van Koppen, P.J.; Bruijninckx, C.M.A.; Akkerman-Zaalberg van Zelst, M.W.M.; Neumann, H.A.M.; van Gemert, M.J.C. Child Abuse, Misdiagnosed by an Expertise Center: Part I—Medico-Social Aspects. *Children* **2023**, *10*, 963. [CrossRef]
98. Sturm, G.; Karan, K.R.; Monzel, A.S.; Santhanam, B.; Taivassalo, T.; Bris, C.; Ware, S.A.; Cross, M.; Towheed, A.; Higgins-Chen, A.; et al. OxPhos defects cause hypermetabolism and reduce lifespan in cells and in patients with mitochondrial diseases. *Commun Biol.* **2023**, *6*, 22. [CrossRef] [PubMed] [PubMed Central]
99. Nolfi-Donegan, D.; Braganza, A.; Shiva, S. Mitochondrial electron transport chain: Oxidative phosphorylation, oxidant production, and methods of measurement. *Redox Biol.* **2020**, *37*, 101674. [CrossRef] [PubMed] [PubMed Central]
100. Gorman, G.S.; Elson, J.L.; Newman, J.; Payne, B.; McFarland, R.; Newton, J.L.; Turnbull, D.M. Perceived fatigue is highly prevalent and debilitating in patients with mitochondrial disease. *Neuromuscul. Disord.* **2015**, *25*, 563–566. [CrossRef] [PubMed]

101. Kaufmann, P.; Engelstad, K.; Wei, Y.; Kulikova, R.; Oskoui, M.; Battista, V.; Koenigsberger, D.Y.; Pascual, J.M.; Sano, M.; Hirano, M.; et al. Protean phenotypic features of the A3243G mitochondrial DNA mutation. *Arch. Neurol.* **2009**, *66*, 85–91. [CrossRef] [PubMed]
102. Porcelli, S.; Marzorati, M.; Morandi, L.; Grassi, B. Home-based aerobic exercise training improves skeletal muscle oxidative metabolism in patients with metabolic myopathies. *J. Appl. Physiol.* **2016**, *121*, 699–708. [CrossRef]
103. Apabhai, S.; Gorman, G.S.; Sutton, L.; Elson, J.L.; Plötz, T.; Turnbull, D.M.; Trenell, M.I. Habitual physical activity in mitochondrial disease. *PLoS ONE* **2011**, *6*, e22294. [CrossRef] [PubMed]
104. Bernardi, P.; Bonaldo, P. Mitochondrial dysfunction and defective autophagy in the pathogenesis of collagen VI muscular dystrophies. *Cold Spring Harb. Perspect. Biol.* **2013**, *5*, a011387. [CrossRef] [PubMed] [PubMed Central]
105. Luo, S.; Wang, D.; Zhang, Z. Post-translational modification and mitochondrial function in Parkinson's disease. *Front. Mol. Neurosci.* **2024**, *16*, 1329554. [CrossRef] [PubMed] [PubMed Central]
106. Siekacz, K.; Piotrowski, W.J.; Iwański, M.A.; Górski, P.; Białas, A.J. The Role of Interaction between Mitochondria and the Extracellular Matrix in the Development of Idiopathic Pulmonary Fibrosis. *Oxid. Med. Cell Longev.* **2021**, *2021*, 9932442. [CrossRef] [PubMed] [PubMed Central]
107. Norat, P.; Soldozy, S.; Sokolowski, J.D.; Gorick, C.M.; Kumar, J.S.; Chae, Y.; Yağmurlu, K.; Prada, F.; Walker, M.; Levitt, M.R.; et al. Mitochondrial dysfunction in neurological disorders: Exploring mitochondrial transplantation. *NPJ Regen. Med.* **2020**, *5*, 22, Erratum in: *NPJ Regen. Med.* **2021**, *6*, 13. https://doi.org/10.1038/s41536-021-00125-3. [CrossRef] [PubMed] [PubMed Central]
108. Gleichmann, M.; Mattson, M.P. Neuronal calcium homeostasis and dysregulation. *Antioxid. Redox Signal.* **2011**, *14*, 1261–1273. [CrossRef] [PubMed] [PubMed Central]
109. Wang, Y.; Wang, Y.; Yue, G.; Zhao, Y. Energy metabolism disturbance in migraine: From a mitochondrial point of view. *Front. Physiol.* **2023**, *14*, 1133528. [CrossRef] [PubMed] [PubMed Central]
110. Griffioen, G. Calcium Dyshomeostasis Drives Pathophysiology and Neuronal Demise in Age-Related Neurodegenerative Diseases. *Int. J. Mol. Sci.* **2023**, *24*, 13243. [CrossRef] [PubMed] [PubMed Central]
111. Doser, R.L.; Hoerndli, F.J. Regulation of neuronal excitability by reactive oxygen species and calcium signaling: Insights into brain aging. *Curr. Res. Neurobiol.* **2021**, *2*, 100012. [CrossRef] [PubMed] [PubMed Central]
112. Yoo, I.; Ahn, I.; Lee, J.; Lee, N. Extracellular flux assay (Seahorse assay): Diverse applications in metabolic research across biological disciplines. *Mol. Cells* **2024**, *47*, 100095. [CrossRef] [PubMed] [PubMed Central]
113. Sivandzade, F.; Bhalerao, A.; Cucullo, L. Analysis of the Mitochondrial Membrane Potential Using the Cationic JC-1 Dye as a Sensitive Fluorescent Probe. *Bio Protoc.* **2019**, *9*, e3128. [CrossRef] [PubMed] [PubMed Central]
114. Teodoro, J.S.; Machado, I.F.; Castela, A.C.; Rolo, A.P.; Palmeira, C.M. The Evaluation of Mitochondrial Membrane Potential Using Fluorescent Dyes or a Membrane-Permeable Cation (TPP^+) Electrode in Isolated Mitochondria and Intact Cells. *Methods Mol. Biol.* **2020**, *2184*, 197–213. [CrossRef] [PubMed]
115. Desousa, B.R.; Kim, K.K.; Jones, A.E.; Ball, A.B.; Hsieh, W.Y.; Swain, P.; Morrow, D.H.; Brownstein, A.J.; Ferrick, D.A.; Shirihai, O.S.; et al. Calculation of ATP production rates using the Seahorse XF Analyzer. *EMBO Rep.* **2023**, *24*, e56380. [CrossRef] [PubMed] [PubMed Central]
116. Gonzalez-Hunt, C.P.; Rooney, J.P.; Ryde, I.T.; Anbalagan, C.; Joglekar, R.; Meyer, J.N. PCR-Based Analysis of Mitochondrial DNA Copy Number, Mitochondrial DNA Damage, and Nuclear DNA Damage. *Curr. Protoc. Toxicol.* **2016**, *67*, 20.11.1–20.11.25. [CrossRef] [PubMed] [PubMed Central]
117. Chin, M.Y.; Joy, D.A.; Samaddar, M.; Rana, A.; Chow, J.; Miyamoto, T.; Calvert, M. Novel high-content and open-source image analysis tools for profiling mitochondrial morphology in neurological cell models. *SLAS Discov.* **2025**, *31*, 100208. [CrossRef] [PubMed]
118. Moreira, J.D.; Gopal, D.M.; Kotton, D.N.; Fetterman, J.L. Gaining Insight into Mitochondrial Genetic Variation and Downstream Pathophysiology: What Can i(PSCs) Do? *Genes* **2021**, *12*, 1668. [CrossRef] [PubMed] [PubMed Central]
119. Ferreira, T.; Rodriguez, S. Mitochondrial DNA: Inherent Complexities Relevant to Genetic Analyses. *Genes* **2024**, *15*, 617. [CrossRef] [PubMed] [PubMed Central]
120. Gómez-Serrano, M.; Camafeita, E.; Loureiro, M.; Peral, B. Mitoproteomics: Tackling Mitochondrial Dysfunction in Human Disease. *Oxid. Med. Cell Longev.* **2018**, *2018*, 1435934. [CrossRef] [PubMed] [PubMed Central]

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