

Special Issue Reprint

Nutrition in the Liver Damage

Edited by
Aldona Wierzbicka-Rucinska and Zuzanna Goluch

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Guest Editors

Aldona Wierzbicka-Rucinska

Zuzanna Goluch



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Guest Editors

Aldona Wierzbicka-Rucinska
Department of Clinical Biochemistry
The Children's Memorial
Health Institute
Warsaw
Poland

Zuzanna Goluch
Department of Food
Technology and Nutrition
Wroclaw University of
Economics and Business
Wrocław
Poland

Editorial Office

MDPI AG
Grosspeteranlage 5
4052 Basel, Switzerland

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About the Editors

Aldona Wierzbicka-Rucinska

Aldona Wierzbicka-Rucinska is a clinical laboratory diagnostician and researcher affiliated with the Children's Memorial Health Institute in Warsaw, Poland. Her scientific work focuses on pediatric hepatology, laboratory medicine, and translational biomarker research, with particular emphasis on molecular mechanisms of liver injury, microRNA biology, bile acid metabolism, oxidative stress, fibrosis, and inflammation in children. She combines expertise in laboratory diagnostics, molecular biology, and clinical hepatology, integrating biochemical, molecular, histopathological, and clinical data to identify and validate biomarkers of liver diseases. Her research aims to improve understanding of disease mechanisms and support the development of precision medicine approaches in pediatric hepatology. Dr Wierzbicka-Rucińska is the author or co-author of more than 125 peer-reviewed scientific publications and several book chapters, with an H-index of approximately 27. She has participated in numerous national and international research projects, including European collaborative programs focused on pediatric nutrition, metabolism, biomarker discovery, and translational medicine.

Zuzanna Goluch

Zuzanna Goluch specializes in agricultural sciences, the discipline of food and nutrition technology. She works as an associate professor in the Department of Food Technology and Nutrition, Faculty of Production Engineering, Wrocław University of Economics and Business. She conducts research on the impact of dietary supplements and fortified foods on metabolic pathways in humans and model animals; food quality and safety; the diet, nutritional status, and body composition of the population: in various physiological states, using alternative diets, from various professional groups; and the impact of health-promoting nutritional education on changes in population eating behaviors. Additionally, she is the author or co-author of more than 160 peer-reviewed scientific publications, several book chapters and is the editor of six monographs. She is a recognized scientific reviewer.

Preface

Liver diseases represent a growing global health burden closely associated with obesity, metabolic disorders, and unhealthy dietary patterns. As nutrition has emerged as a key factor influencing the development, progression, and management of liver diseases, this Reprint explores current scientific evidence on the relationship between dietary interventions and liver health. The volume covers a broad scope, from molecular mechanisms and genetic susceptibility to clinical nutrition, functional foods, bioactive compounds, and personalized dietary approaches in various liver diseases. Its primary aim is to summarize recent advances in nutritional hepatology and provide an interdisciplinary perspective that integrates experimental and clinical research. The motivation for this work arises from the increasing need for evidence-based nutritional strategies that support the prevention and treatment of liver diseases, particularly metabolic dysfunction-associated steatotic liver disease (MASLD). By bringing together contributions from experts in nutrition, hepatology, and metabolic research, this Reprint serves as a valuable resource for researchers, clinicians, and dietitians, while encouraging further investigation into the role of nutrition as an integral component of liver disease prevention and therapy.

Aldona Wierzbicka-Rucinska and Zuzanna Goluch

Guest Editors



Article

The Role of Acyl-CoA Synthetase 1 in Bioactive Lipid Accumulation and the Development of Hepatic Insulin Resistance

Piotr Zabielski ¹, Monika Imierska ², Kamila Roszczyc-Owsiejczuk ², Mariusz Kuźmicki ³, Paweł Rogalski ⁴, Jarosław Daniluk ⁴ and Agnieszka U. Błachnio-Zabielska ^{2,*}

¹ Medical Biology Department, Medical University of Białystok, 15-222 Białystok, Poland; piotr.zabielski@umb.edu.pl

² Hygiene, Epidemiology and Metabolic Disorders Department, Medical University of Białystok, Mickiewicza 2c, 15-222 Białystok, Poland; monika.imierska@umb.edu.pl (M.I.); kamila.roszczyc-owsiejczuk@umb.edu.pl (K.R.-O.)

³ Gynecology and Gynecological Oncology Department, Medical University of Białystok, 15-276 Białystok, Poland; mariusz.kuzmicki@umb.edu.pl

⁴ Gastroenterology and Internal Medicine Department, Medical University of Białystok, 15-276 Białystok, Poland; pawel.rogalski@umb.edu.pl (P.R.); jaroslaw.daniluk@umb.edu.pl (J.D.)

* Correspondence: agnieszka.blachnio-zabielska@umb.edu.pl; Tel.: +48-(85)-686-52-26

Abstract: The liver plays a crucial role in glucose metabolism. Obesity and a diet rich in fats (HFD) contribute to the accumulation of intracellular lipids. The aim of the study was to explore the involvement of acyl-CoA synthetase 1 (ACSL1) in bioactive lipid accumulation and the induction of liver insulin resistance (InsR) in animals fed an HFD. The experiments were performed on male C57BL/6 mice divided into the following experimental groups: 1. Animals fed a control diet; 2. animals fed HFD; and 3. HFD-fed animals with the hepatic ACSL1 gene silenced through a hydrodynamic gene delivery technique. Long-chain acyl-CoAs, sphingolipids, and diacylglycerols were measured by LC/MS/MS. Glycogen was measured by means of a commercially available kit. The protein expression and phosphorylation state of the insulin pathway was estimated by Western blot. HFD-fed mice developed InsR, manifested as an increase in fasting blood glucose levels (202.5 mg/dL vs. 130.5 mg/dL in the control group) and inhibition of the insulin pathway, which resulted in an increase in the rate of gluconeogenesis (0.420 vs. 0.208 in the control group) and a decrease in the hepatic glycogen content (1.17 µg/mg vs. 2.32 µg/mg in the control group). Hepatic ACSL1 silencing resulted in decreased lipid content and improved insulin sensitivity, accounting for the decreased rate of gluconeogenesis (0.348 vs. 0.420 in HFD_(+/+)) and the increased glycogen content (4.3 µg/mg vs. 1.17 µg/mg in HFD_(+/+)). The elevation of gluconeogenesis and the decrease in glycogenesis in the hepatic tissue of HFD-fed mice resulted from cellular lipid accumulation. Inhibition of lipid synthesis through silencing ACSL1 alleviated HFD-induced hepatic InsR.

Keywords: liver; insulin resistance; obesity; high-fat diet; lipids; gene silencing; mass spectrometry

1. Introduction

The liver, besides skeletal muscle and adipose tissue, plays a crucial role in glucose and lipid metabolism. Obesity and a diet rich in fats (HFD) lead to an excessive elevation of plasma free fatty acid (FFA) and the ectopic accumulation of intracellular lipids, which is associated with occurrence of cardiovascular diseases, insulin resistance (InsR), and type 2 diabetes (T2D) [1,2]. In the physiological state, insulin stimulates hepatic lipogenesis and glycogen synthesis and decreases the plasma blood glucose by inhibiting hepatic gluconeogenesis and glycogenolysis [3]. Upon intracellular translocation, fatty acids (FA) are activated to a long-chain acyl-CoAs (LCACoAs) by a long-chain acyl-CoA synthetase (ACSL). Subsequently, LCACoAs can be directed toward mitochondrial oxidation or be

used as a main substrate for the synthesis of other intracellular lipids [4]. So far, five isoforms of ACSL have been identified. Members of the ACSL family play distinct roles in FA metabolism, accounting for unique substrate preferences and tissue distribution. ACSL1 is a predominant isoenzyme of ACSLs expressed in the hepatic tissue [5]. The content of LCACoAs has been demonstrated to increase in the livers of obese or HFD-fed rats [6–8]. Since LCACoAs are used for the de novo lipid synthesis, those compounds may indirectly inhibit insulin signaling cascade by participating in the synthesis of ceramides (Cer) and diacylglycerols (DAG). The intracellular accumulation of those biologically active lipids may lead to the induction of insulin resistance [9–11].

In the liver, insulin suppresses gluconeogenesis through the phosphorylation-dependent signaling cascade. Ligation of the insulin receptor (IR) results in activation of phosphorylation cascade, namely, IR receptor autophosphorylation; tyrosine phosphorylation of the IRS; and sequential activation of phosphatidylinositol 3-kinase (PI3-K) [12], atypical protein kinase C (aPKC), and protein kinase B (Akt/PKB) [13,14]. Akt/PKB is responsible for the inhibitory phosphorylation of the forkhead box O1 (FOXO1) transcription factor that regulates the expression of glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK). Decreases in the expression of those key gluconeogenic enzymes leads to the suppression of gluconeogenesis [15,16]. Any decrease in Akt/PKB phosphorylation (activity) is often associated with an increase in the ceramide level, which activates phosphatase 2A (PPA2) responsible for the Akt/PKB dephosphorylation [17]. In an insulin-resistant state, insulin is unable to suppress the expression/activity of the aforementioned enzymes. In addition, insulin controls glucose metabolism by promoting glycogen synthesis. This regulation is mediated by Akt/PKB kinase, which phosphorylates and inactivates glycogen synthase kinase 3 (GSK3) [18], the enzyme that controls the activity of glycogen synthase 2 (GYS2). GYS2 is inhibited by phosphorylation and activated by glucose-6-phosphate (G6P) [19, 20]. GYS2 and glycogen phosphorylase are, respectively, responsible for the synthesis and breakdown of hepatic glycogen. Insulin-mediated dephosphorylation regulates the activities of both enzymes [21]. Hepatic lipid accumulation is commonly accepted as a key constituent of hepatic InsR, but despite extensive research, the molecular mechanisms of hepatic InsR still remain incompletely understood. The aim of this study was to explain the role of ACSL1, particularly in terms of bioactive lipid accumulation and the development of hepatic InsR in animals fed an HFD. Since ACSL1 is the predominant hepatic isoform responsible for the synthesis of LCACoAs, and indirectly for the synthesis of signaling lipids, we silenced hepatic ACSL1 to determine its role in the accumulation of the respective bioactive lipids and the induction of hepatic InsR in HFD-fed mice.

2. Materials and Methods

2.1. Animals and Study Design

Consent to conduct the experiment was granted by the appropriate governing body (IACUC equivalent, Lokalna Komisja Etyczna ds. Doświadczeń na Zwierzętach, Ocza-powskiego 13/4 Street, 10-718, Olsztyn, Poland, Approval date 23 September 2020, approval number 48/2020). Experiments were performed on male C57BL/6 mice (approx. 20 g of body weight, Jackson Laboratory, Bar Harbor, ME, USA). Upon arrival from Jackson Laboratory (Bar Harbor, ME, USA), mice were weighed and assigned randomly to cages. The sample size and power analysis, using our previous data regarding HOMA-IR parameters and the hepatic content of TAG and DAG, revealed the optimum group size of 8 ($p < 0.05$ and 90%) whereas the literature data showed n -numbers between 6 and 20 subjects per group. Animals were housed in typical conditions ($21\text{ }^{\circ}\text{C} \pm 2$, 12 h light/12 h dark cycle) with ad libitum access to water and an appropriate diet. After one week of acclimatization, the animals were randomly divided into following groups: the non-silenced control group (control, $n = 10$), fed a control low-fat diet ad libitum (Research Diets INC D12450J; 10% of fat calories, Research Diets, New Brunswick, NJ, USA) which was transfected with scrambled shRNA plasmid; the non-silenced high-fat diet group (HFD_(+/+), $n = 10$), housed on a diet rich in fats (Research Diets INC D12492; 60% of fat

calories, Research Diets, New Brunswick, NJ, USA) and also transfected with non-coding shRNA plasmid; and the silenced high-fat diet group (HFD_(-ACSL1), $n = 13$), transfected with coding, functional shRNA plasmid. In all animal groups, shRNA-mediated plasmid transfection was performed using a hydrodynamic gene delivery (HGD) technique at the 6th week of feeding. Both the non-silenced LFD-fed control and HFD-fed, non-silenced HFD_(+/+) animals underwent the HGD procedure, but with non-coding, scrambled shRNA plasmid. All groups were subsequently housed on the appropriate diet for additional 2 weeks, for a total of 8 weeks of feeding at the end of experiment. Animals underwent glucose tolerance tests (OGTTs) one week before sacrifice. Exclusion criteria at the time of euthanasia included visible ataxia, decreased body weight, any visible wounds or sores, and signs of general distress. As the HGD and other procedures were well tolerated, there were no drop-outs in the study groups. On the day of euthanasia, animals received 2 h of infusion of D-[UL-¹³C₆] glucose (Merck, Lebanon, NJ, USA) through the lateral tail vein at a rate of 5 nmol/min/g of body weight. Fifteen minutes prior to the infusion completion, 0.5 U/kg of insulin was administered through the infusion line. At the end of the experiment, liver samples were harvested, frozen in LN₂, and stored at -80°C for further analysis.

2.2. Plasmids and In Vivo shRNA Plasmids Injection

Bacterial cultures expressing appropriate coding and scrambled shRNA plasmids were obtained from Horizon Discovery (previously Dharmacon, Cambridge, UK). Amplification and isolation of the plasmids was performed according to the manufacturer's guidelines, with the use of GeneJET Plasmid Maxiprep Kit (Thermo Fisher Scientific, Waltham, MA, USA). A silencing mix was prepared using 3 different ACSL1 shRNA sequences (Set Cat. No.: V3SM11244-01EG14081), whereas scrambled shRNA (Cat. No.: VSC11708) was used for the non-silenced group. Gene silencing was performed after 6 weeks of feeding with the appropriate diet. Animals were anesthetized using $\sim 2\%$ isoflurane in oxygen (UNO BV, UNO, Zevenaar, The Netherlands). Hydrodynamic-based transfection was performed by injecting 100 μg of plasmids into Altogen's liver in vivo transfection reagent (Altogen Biosystems, Las Vegas, NV, USA) via the lateral tail vein [22,23]. The volume of the transfectant mixture was adjusted with 150 mM PBS, pH = 7.2 to 10% of equivalent body weight (1 mL/10 g, 2.5 mL for a 25 g mouse, injected within 7 s). All plasmids encoded green fluorescent protein (GFP) as a marker of transfection efficiency (Figure 1C). The feeding with the appropriate diet continued for an additional 2 weeks, until the end of the experiment. The effect of silencing was noticeable just 24 h after the HGD procedure and lasted up to two weeks.

2.3. Lipid Measurements

2.3.1. Sphingolipids

Hepatic sphingolipids levels were measured by UHPLC/MS/MS according to Blachnio-Zabielska et al. [24]. Liver tissue (approx. 15–20 mg) was homogenized in 250 mM sucrose, 25 mM KCl, 50 mM Tris, and 0.5 mM EDTA (pH = 7.4). Afterwards, appropriate ceramide internal standards were added (d18:1/C15:0-d7, d18:1/C16:0-d7, d18:1/C18:1-d7, d18:1/C18:0-d7, d17:1/20:0, d18:1/C24:1-d7, d17:1/20:0C24-d7), (Avanti Polar Lipids, Alabaster, AL, USA) and sphingolipids were extracted through sonication in isopropanol: water:ethyl acetate (30:10:60; $v:v:v$). After vortexing and centrifugation, supernatants were evaporated under N₂ and dissolved in 2 mM ammonium formate/0.1% formic acid in methanol. Samples were resolved on Zorbax SB-C8 reversed-phase column 2.1×150 mm, $1.8 \mu\text{m}$ (Agilent Technologies, Santa Clara, CA, USA) using a binary gradient of 1 mM ammonium formate/0.1% formic acid in water (HPLC solvent A) and 2 mM ammonium formate/0.1% formic acid in methanol (HPLC solvent B). The sphingolipid analysis was performed in MRM mode (with multiple reaction monitoring) on a Sciex QTRAP 6500 + LC/MS/MS system (AB Sciex, Darmstadt, Germany).

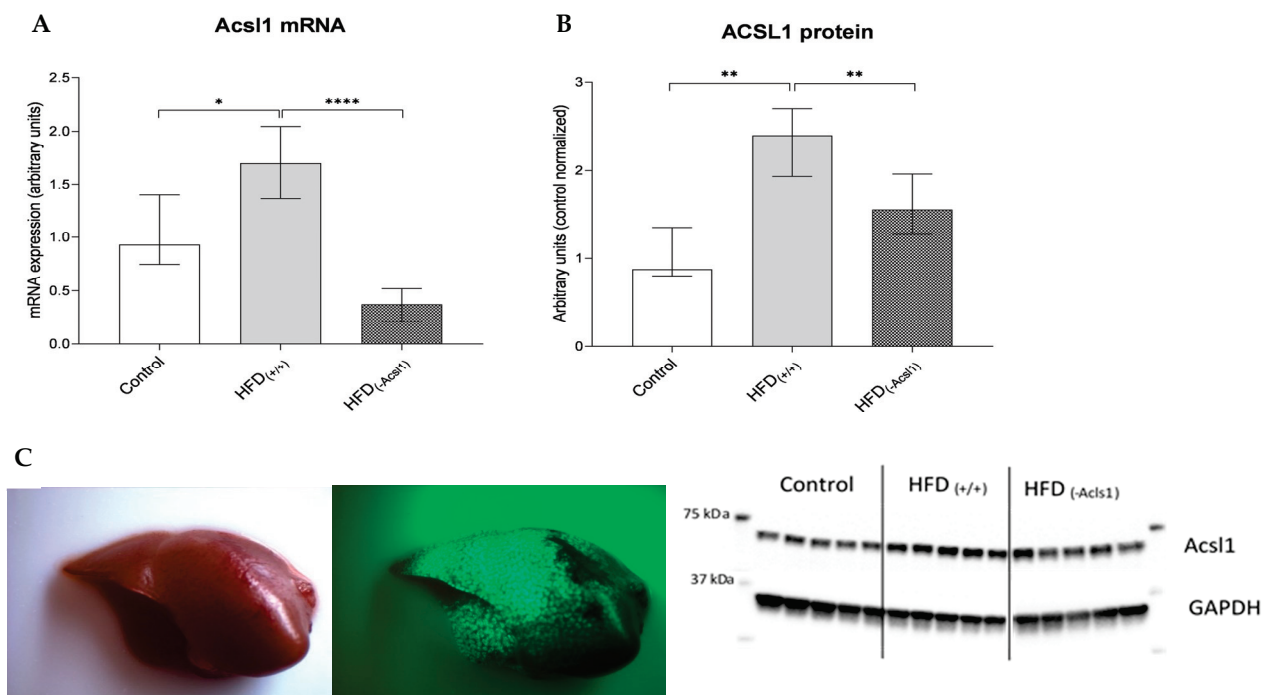


Figure 1. mRNA of Acs11 in liver (A). Protein level of Acs11 (B). Bars represent medians $\pm$ interquartile range; *— $p < 0.05$; **— $p < 0.01$; ****— $p < 0.0001$. Green fluorescent protein in liver after plasmid transfection: visible light of a mouse liver (photo on the left); TurboGFP fluorescence of isolated liver (photo on the right) (C). Fluorescence stereomicroscopy performed with the use of the Nightsea SFA-RB-GO fluorescence adapter (Nightsea, Hatfield, PA, USA)/DeltaPix Invenio 5SIII CMOS camera (DeltaPix, Smorum, Denmark).

2.3.2. Diacylglycerols

Hepatic DAGs were quantified by UHPLC/MS/MS as described in Blachnio-Zabielska et al. [25]. DAG extraction was performed concurrently with sphingolipid extraction, using the same solvent system. Before extraction, a mixture of isotopically labeled internal standards (mixture I and II of deuterated DAG standards—Avanti Polar Lipids) was spiked into each homogenate. Chromatographic separation of DAG was performed on a 2.1×150 mm Zorbax SB-C8 column using the same solvent system as for sphingolipids. Individual DAG species were quantified in MRM mode on a Sciex QTRAP 6500 + LC/MS/MS system.

2.3.3. Long-Chain Acyl-CoA

The level of hepatic LCACoAs was analyzed using UHPLC/MS/MS as described by Blachnio-Zabielska et al. [26]. LCACoA extraction was performed according to Minkler et al. [27] with the use of internal standards (C15:0-, 16:0(d4)-, C17-, C19:0-, C21:0-, C23:0- and 24:0(d4)-CoA) spiked into each sample. Samples were resolved on $2.1 \text{ mm} \times 150 \text{ mm}$ Agilent ZORBAX Extend-C18, using a reverse-phase gradient of ammonium hydroxide (NH_4OH) in water and NH_4OH in ACN. The quantitative analysis of LCACoAs was performed in positive electrospray ionization mode (ESI+) on Sciex QTRAP 6500 + LC/MS/MS system (AB Sciex, Darmstadt, Germany).

2.3.4. Plasma FFA, Hepatic Triacylglycerols and Liver Glycogen Content

The total plasma FFA concentration was estimated with the MAK044 colorimetric assay (Sigma-Aldrich, St. Louis, MO, USA). The total content of hepatic triacylglycerols was measured with the MAK264-1KT fluorometric kit (Sigma Aldrich, St. Louis, MO, USA). Hepatic glycogen was measured with the MAK016 Glycogen Assay Kit (Merck, Lebanon, NJ, USA).

2.4. Liver Glucose-6-Phosphate (G6P) Content and Estimation of Hepatic Gluconeogenesis

The tissue content of G6P was quantified by GC/MS according to Young et al. [28], with modifications. Hepatic gluconeogenesis was measured through plasma glucose isotopomer distribution analysis (MIDA) after infusion of stable-isotope labeled D-[UL-¹³C₆]glucose [29,30]. Glucose isotopomer distribution was measured by means of GC/MS after conversion to di-O-isopropylidene propionate derivatives [31,32]. Isotopomer enrichment was calculated with the use of Python IsoCor v2 software, after correction for tracer purity (99% APE) [33]. The reciprocal pool model of the gluconeogenesis (GNG) by Haymond and Sunehag [34] and Chung et al. [35] was used for estimating the fractional gluconeogenesis (F_{GNG}) and gluconeogenic rate (GNG).

2.5. Protein Expression

Liver proteins prepared in Laemmli buffer were resolved using SDS-PAGE on TGX pre-cast gels (Bio-Rad, Hercules, CA, USA) and transferred to a PVDF membrane via semi-dry transfer (Trans Blot Turbo, Bio-Rad, Hercules, CA, USA). Membranes were blocked with EveryBlot buffer (Bio-Rad Hercules, CA, USA) and incubated with the appropriate primary antibodies: ACSL1, Akt, Phospho-Akt (Ser473), GYS2, PEPCK, pFOXO1(Ser256), FOXO1, and GAPDH (Thermo Fisher Scientific, Waltham, MA, USA). After washing and incubation with an HRP-conjugated secondary antibody, protein bands were detected with an ECL chemiluminescent assay on a Bio-Rad ChemiDoc XRS+ imaging system (Bio-Rad, Hercules, CA, USA). Expression of the target protein was normalized to the GAPDH and expressed as fold changes over the control group value. Original blot pictures and their description are presented in Supplement Figures S1–S8.

2.6. Real-Time PCR

Liver RNA was isolated using a Total RNA Mini kit (A&A Biotechnology, Gdańsk, Poland). First-strand cDNA was synthesized with the TranScriba Kit (A&A Biotechnology, Gdańsk, Poland). Real-time PCR was performed by means of the LightCycler 480 SYBR Green I Master (Roche, Mannheim, Germany) using a LightCycler480 system (Roche, Mannheim, Germany). The nucleotide sequences of the forward and reverse primers for the target genes encoding ACSL1 and GAPDH (which was used as a house-keeping gene) were as follows (5' to 3'): ACSL1 (forward): ACTGTGCAGGAACAAGGATAT, ACSL1 (reverse): TAAGTAAGGCAGTGTTCCTG; Glyceraldehyde-3-phosphate Dehydrogenase (GAPDH) (forward): AGGAGAGTGTTCCTCGTCC, GAPDH (reverse): AGGAGACAACCTGGTCCTCA. GAPDH was used as reference gene and loading control.

2.7. Plasma Insulin and Glucose Concentration

Insulin concentration was measured using an ELISA insulin assay (Crystalchem, Elk Grove Village, IL, USA). Plasma glucose concentration was determined with an AccuChek Aviva glucometer (Roche, Mannheim, Germany).

2.8. OGTT

OGTT was performed on fasting animals. Blood samples were taken at 15, 30, 60, and 120 min after oral gavage of glucose at a dose of 2 g/kg of lean mass. Additionally, at 15 and 60 min, blood was collected for the estimation of insulin concentration.

2.9. Homeostatic Model Assessment for Insulin Resistance (HOMA-IR)

HOMA-IR was calculated with an equation modified for use in rodent-based studies [36]: $\text{HOMA-IR} = [\text{fasting glucose (mg/dL)} \times \text{fasting insulin (}\mu\text{IU/mL)}] / 2430$.

2.10. Protein Concentration

Protein content in homogenates was measured colorimetrically with a Pierce 660 nm protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA) against a standard curve prepared on serum albumin in PBS.

2.11. Statistical Analysis

All values were expressed as medians and interquartile ranges (IQRs). The significance of the observed changes was determined using the non-parametric Mann–Whitney U test. A significance level of $p < 0.05$ was established. The statistical analysis was performed using GraphPad Software's Prism 9.3.1.

3. Results

3.1. Effect of HFD Diet and Silencing on Hepatic Expression of ACSL1 Gene

The high-fat diet feeding significantly increased both the ACSL1 mRNA (by 49%, $p < 0.05$) and protein (over 2.5 times, $p < 0.01$) in the livers of the HFD_(+/+) animals as compared to the controls. Targeted silencing in HFD_(-ACSL1) animals contributed to the reduction in ACSL1 at the mRNA and protein levels by 72% ($p < 0.0001$) and 35% ($p < 0.05$), respectively, vs. HFD_(+/+) values (Figure 1).

3.2. Plasma FFA Concentration and Hepatic Lipid Content

The high-fat diet feeding in HFD_(+/+) animals increased the plasma FFA concentration by 121% vs. the control value ($p < 0.05$) (Table 1). It was accompanied by an elevation in the hepatic LCACoA content (by 20%, $p < 0.01$, Table 2, Figure 2A) and all the measured lipid species. The contents of ceramide and DAG in the livers of the HFD-fed mice were higher by 48% ($p < 0.0001$) and 37% ($p < 0.0001$), respectively, vs. the control (Tables 3 and 4, Figure 2B,C). Hepatic TAG was most affected by HFD consumption, with the levels in the liver of the HFD_(+/+) mice being almost three times higher than in the control (Figure 2D). Regarding individual molecular species, the content of ceramides with oleoyl- (d18:1/C18:1), stearoyl- (d18:1/C18:0), arachidoyl (d18:1/C20:0), and behenoyl-ceramide (d18:1/C22:0) was affected the most under HFD consumption (Table 3). The same was noted for DAG species, which contained 18-carbon chain fatty acids (C16:0/18:0-DAG, C18:0/18:0-DAG, C18:0/18:1-DAG and C18:0/18:2-DAG; Table 4).

Table 1. The impact of the high-fat diet and Acs1 liver silencing on the indices of insulin resistance and plasma FFA.

	Control	HFD _(+/+)	HFD _(-Acs1)
Fasting glucose (mg/dL) ($n = 10$)	130.5 (109.5–149.8)	202.5 (191.8–217.8) *	182.5 (167.8–189.0) #
Fasting insulin (μ U/mL) ($n = 10$)	7.15 (5.60–11.33)	25.25 (13.33–33.30) *	15.00 (11.10–16.73) #
HOMA-IR ($n = 10$)	0.40 (0.200–0.700)	2.25 (1.08–2.58) *	1.00 (0.88–1.33) #
Total FFAs (nmol/mL) ($n = 10$)	220.9 (195.0–232.5)	488.3 (465.3–521.6) *	415.3 (382.4–446.7) #
Baseline body (g) ($n = 10$)	17.45 (16.70–18.73)	18.25 (17.48–9.13)	19.53 (18.7–20.04)
End-point body weights (g) ($n = 10$)	27.18 (26.64–28.36)	30.75 (28.89–34.15)	32.40 (30.64–35.53)

Data expressed as medians and interquartile range; *: $p < 0.05$ vs. control, #: $p < 0.05$ vs. HFD_(+/+).

Table 2. Hepatic content of individual of Acyl-CoA molecular species under Acls1 silencing in mouse livers.

LCACoA	Control	HFD _(+/+)	HFD _(-Acs1)
C14:0	1.55 (1.38–1.75)	1.93 (1.70–2.27) *	1.50 (1.40–1.67) #
C16:0	9.44 (8.71–10.90)	16.32 (13.12–17.74) *	9.03 (8.17–11.00) #
C16:1	10.37 (9.00–11.30)	3.93 (3.51–4.44) *	3.18 (2.68–3.39) #
C18:0	13.72 (11.68–14.36)	18.85 (13.32–19.84) *	10.16 (9.20–11.12) #
C18:1	15.20 (13.81–17.83)	18.01 (13.41–18.79)	10.59 (9.11–10.92) #
C18:2	29.65 (27.34–33.50)	38.39 (35.45–44.53) *	31.37 (28.38–33.41) #
C20:0	1.44 (1.05–1.51)	1.64 (1.44–1.78)	1.23 (1.11–1.36) #

Data expressed as medians (pmol/mg tissue) and interquartile ranges; $n = 10$ per control and HFD_(+/+) group, $n = 13$ per HFD_(-Acs1) group; *: $p < 0.05$ vs. control, #: $p < 0.05$ vs. HFD_(+/+).

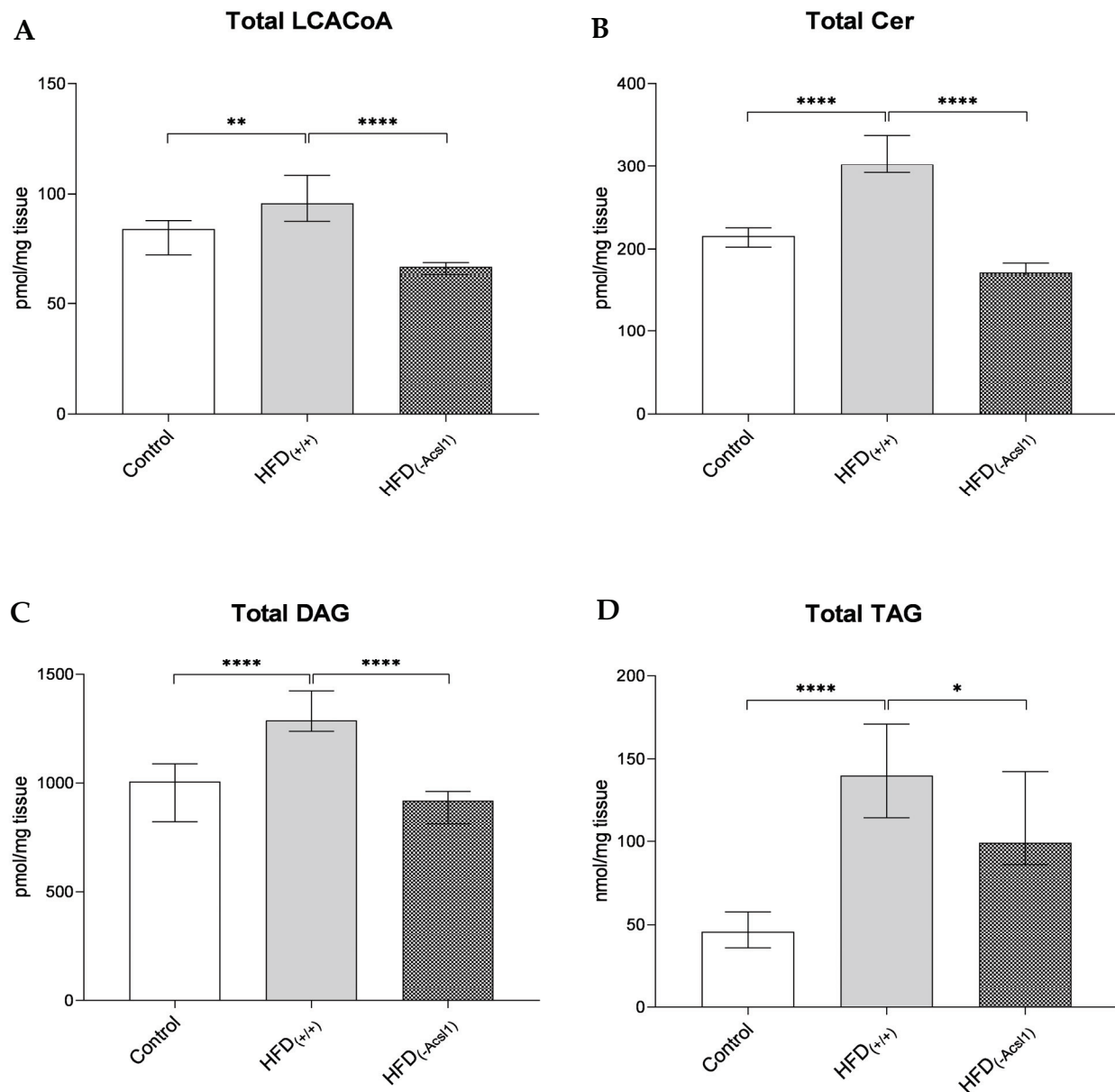


Figure 2. Total content of long-chain acyl-CoA (A), ceramides (B), diacylglycerols (C), and triacylglycerols (D) in the livers of control; HFD-fed (HFD_(+/+)); and HFD-fed, ACSL1-silenced (HFD_(ACSL1−)) mice. Bars represent medians +/− interquartile range; *— $p < 0.05$; **— $p < 0.01$; ****— $p < 0.0001$.

Table 3. The contents of ceramide molecular species in the in the *Acsl1*-silenced mouse livers.

Ceramide	Control	HFD _(+/+)	HFD _(−Acsl1)
d18:1/C14:0	0.043 (0.035–0.049)	0.038 (0.034–0.041)	0.031 (0.029–0.033) #
d18:1/C16:0	6.83 (6.56–7.66)	7.14 (6.89–7.54)	7.25 (6.89–7.81)
d18:1/C18:0	1.50 (1.36–1.62)	3.28 (2.80–3.57) *	2.26 (2.00–2.26) #
d18:1/C18:1	0.148 (0.136–0.173)	0.2817 (0.267–0.316) *	0.212 (0.192–0.233) #
d18:1/C20:0	4.24 (3.91–4.83)	12.48 (11.66–13.61) *	9.82 (8.21–10.62) #
d18:1/C22:0	75.42 (60.19–82.90)	133.8 (129.5–176.0) *	72.40 (64.88–80.42) #
d18:1/C24:0	45.97 (41.58–51.69)	51.24 (47.99–53.49)	32.04 (31.22–34.04) #
d18:1/C24:1	80.60 (74.60–84.78)	87.24 (85.16–91.60) *	47.93 (43.14–55.22) #

Data expressed as medians (pmol/mg tissue) and interquartile ranges; $n = 10$ per control and HFD_(+/+) group, $n = 13$ per HFD_(−Acsl1) group; *: $p < 0.05$ vs. control, #: $p < 0.05$ vs. HFD_(+/+).

Table 4. The contents of DAG molecular species in the ACSL1-silenced mouse livers.

Diacylglycerol	Control	HFD _(+/+)	HFD _(−ACSL1)
C14:0/14:0	0.66 (0.58–0.71)	0.58 (0.51–0.69)	0.61 (0.49–0.70)
C16:0/16:0	16.65 (12.76–17.50)	19.48 (17.67–26.94) *	8.17 (6.99–10.04) #
C16:0/18:0	3.670 (3.50–4.37)	10.08 (8.88–11.65) *	9.23 (7.82–10.57)
C16:0/18:1	395.3 (305.7–446.8)	513.5 (460.2–576.3) *	273.6 (217.7–335.2) #
C16:0/18:2	182.3 (166.1–207.7)	229.7 (210.4–251.4) *	177.1 (160.6–203.4) #
C18:0/18:0	1.76 (1.62–2.06)	3.81 (3.12–4.22) *	2.71 (2.53–3.40) #
C18:0/18:1	17.79 (15.82–19.33)	42.85 (39.36–48.57) *	35.28 (31.85–37.41) #
C18:0/18:2	16.40 (11.92–19.91)	40.90 (36.92–52.96) *	35.01 (32.44–39.59) #
C18:1/18:1	258.0 (194.2–300.9)	292.5 (274.3–315.2)	223.5 (196.6–254.9) #
C18:2/18:2	73.13 (66.38–77.92)	105.0 (84.48–108.5) *	76.21 (63.68–81.41) #
C18:0/20:4	30.23 (28.20–33.92)	49.47 (40.20–54.59) *	39.28 (34.90–44.30) #

Data expressed as medians (pmol/mg tissue) and interquartile ranges; $n = 10$ per control and HFD_(+/+) group, $n = 13$ per HFD_(−ACSL1) group; *, $p < 0.05$ vs. control, #: $p < 0.05$ vs. HFD_(+/+).

The ACSL1 silencing in HFD-fed mice contributed to a slight decrease in the plasma FFA compared to the values in their non-silenced HFD_(+/+) counterparts (by 15%, $p < 0.05$; Table 1). A similar, yet greater, change was observed at the hepatic level in LCACoA, which decreased by 32% ($p < 0.0001$) under ACSL1 silencing (Table 1, Figure 2A). The largest decreases were noticed for C16:0-CoA, C18:0-CoA, and C18:1-CoAs. As far as the ceramide and DAG content was concerned, ACSL1 silencing in the livers of HFD_(−ACSL1) mice significantly decreased the hepatic content of both Cer and DAG by 44% and 33%, respectively, vs. HFD_(+/+) values ($p < 0.0001$ in both cases; Figure 2B,C). Hepatic TAG displayed a similar degree of down-regulation (by 24%, $p < 0.05$, Figure 2D). The most affected ceramide molecular species were d18:1/C18:1, d18:1/C18:0, d18:1/C20:0, d18:1/C22:0, d18:1/C24:1, and d18:1/C24:0 (Table 3), whereas the reduction in hepatic DAG was strongly visible for C16:0/C16:0, C16:0/C18:1, C18:2/C18:2, C18:1/C18:1, and C18:0/C18:0 diacylglycerols (Table 4).

Our results show that the HFD feeding led to an increase in the plasma FFA and subsequent accumulation of both the signaling and neutral lipid classes, whereas the ACSL1 silencing was effective in terms of hindering the accretion of FFA towards hepatic lipids in the HFD-fed C57BL/6J mice.

3.3. Hepatic Response to Insulin

Both the Cer and DAG hepatic accumulation under HFD consumption coincided with down-regulation of insulin-stimulated Akt phosphorylation at the Ser473 moiety (by 27%, $p < 0.05$ vs. control, Figure 3B). A similar trend was observed for the FOXO1 phosphorylation status, yet the change did not reach any significance threshold (Figure 3C). The protein expression of PEPCK, a key regulator of hepatic gluconeogenesis, increased by approx. 3.6-fold ($p < 0.05$) in the hepatic tissue of the HFD_(+/+) animals vs. the values observed in LFD-fed control mice (Figure 3D).

The decrease in hepatic ACSL1 expression in the HFD_(−ACSL1) mice rescued insulin-stimulated Akt phosphorylation (up-regulation by 88%) and significantly decreased liver PEPCK expression (down-regulation by 48% vs. HFD_(+/+), $p < 0.05$ in both cases; Figure 3B,D). Although the ACSL1 silencing resulted in an increase in FOXO1 phosphorylation as compared to the HFD_(+/+) group, it did not reach any statistical significance (Figure 3C).

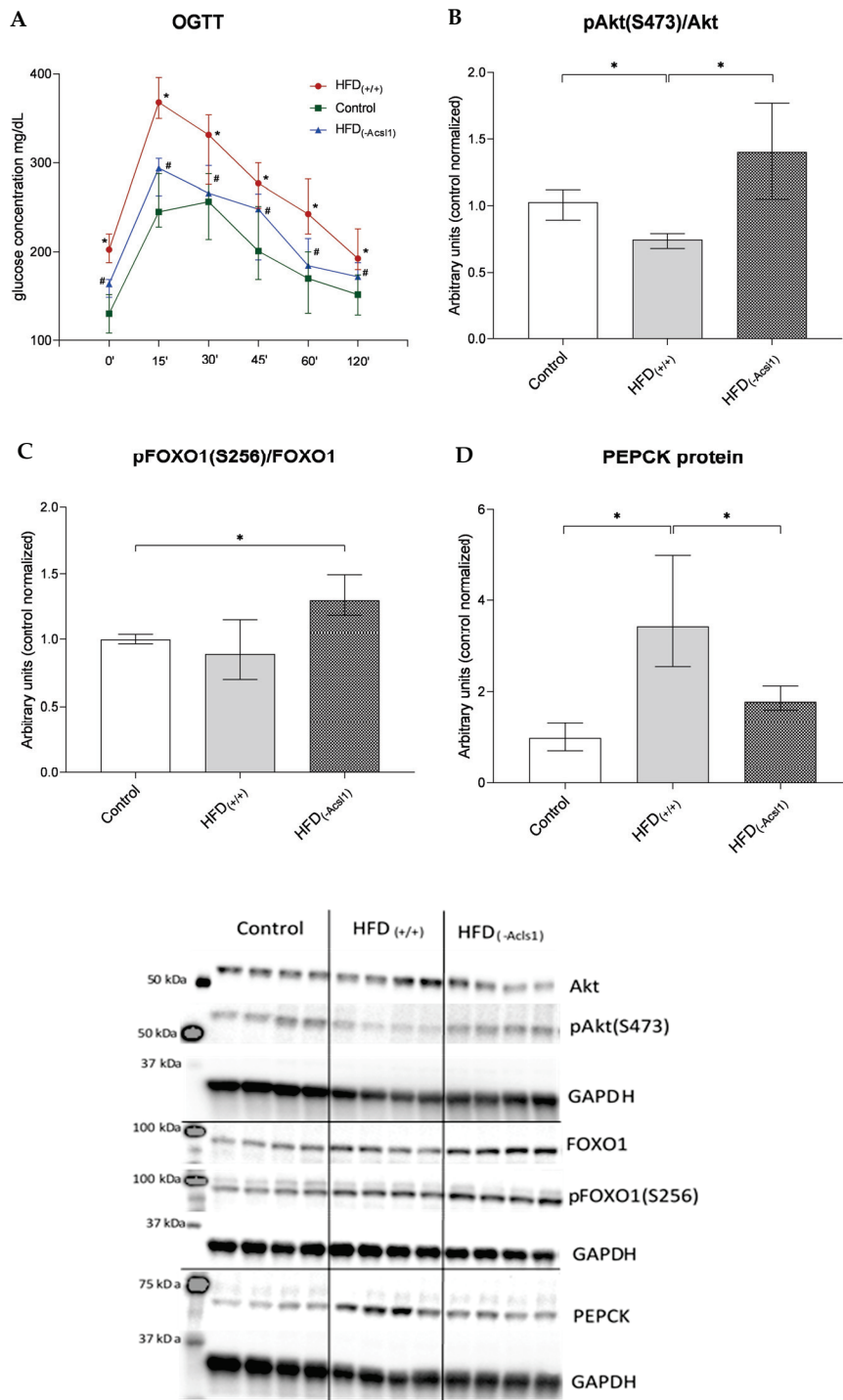


Figure 3. The impact of the HFD consumption and hepatic Acs1 gene silencing on plasma glucose profile during the OGTT (**A**); the phosphorylation of PKB/Akt (pAKT S473) (**B**); the serine phosphorylation of FOXO1 (pFOXO1 S256) (**C**); and the PEPCK level (**D**). Bars represent medians $\pm$ interquartile range; *— $p < 0.05$; Panel (**A**): #— $p < 0.05$ vs. HFD_(+/+).

The presented data indicate that the accumulation of hepatic ceramide and DAG in the HFD-fed animals was accompanied by a decrease in liver insulin sensitivity and an up-regulation of key gluconeogenic enzymes. The hepatic ACSL1 silencing in the HFD_(+/+) group contributed to ameliorating the adverse impact of lipid accumulation on the hepatic insulin signaling and gluconeogenic enzyme expression.

3.4. Hepatic Glucose Metabolism and Indices of Insulin Resistance

The HFD-induced detrimental changes in the hepatic insulin signaling were also visible at the level of the liver G6P content, which decreased by 67% in the HFD_(+/+) group ($p < 0.0001$ vs. control; Figure 4A). Although the expression of glycogen synthase 2 (GYS2) was not affected by HFD feeding (Figure 4C), the insulin-stimulated accumulation of hepatic glycogen decreased by 57% in the HFD_(+/+) mice ($p < 0.01$ vs. control; Figure 2B). Both the fractional gluconeogenesis (fraction of blood glucose derived from hepatic gluconeogenesis) and the gluconeogenic rate increased in the HFD_(+/+) animals (by approx. 100% ($p < 0.01$) and 28% ($p < 0.05$), respectively) vs. the respective values in the control group (Figure 4D,E).

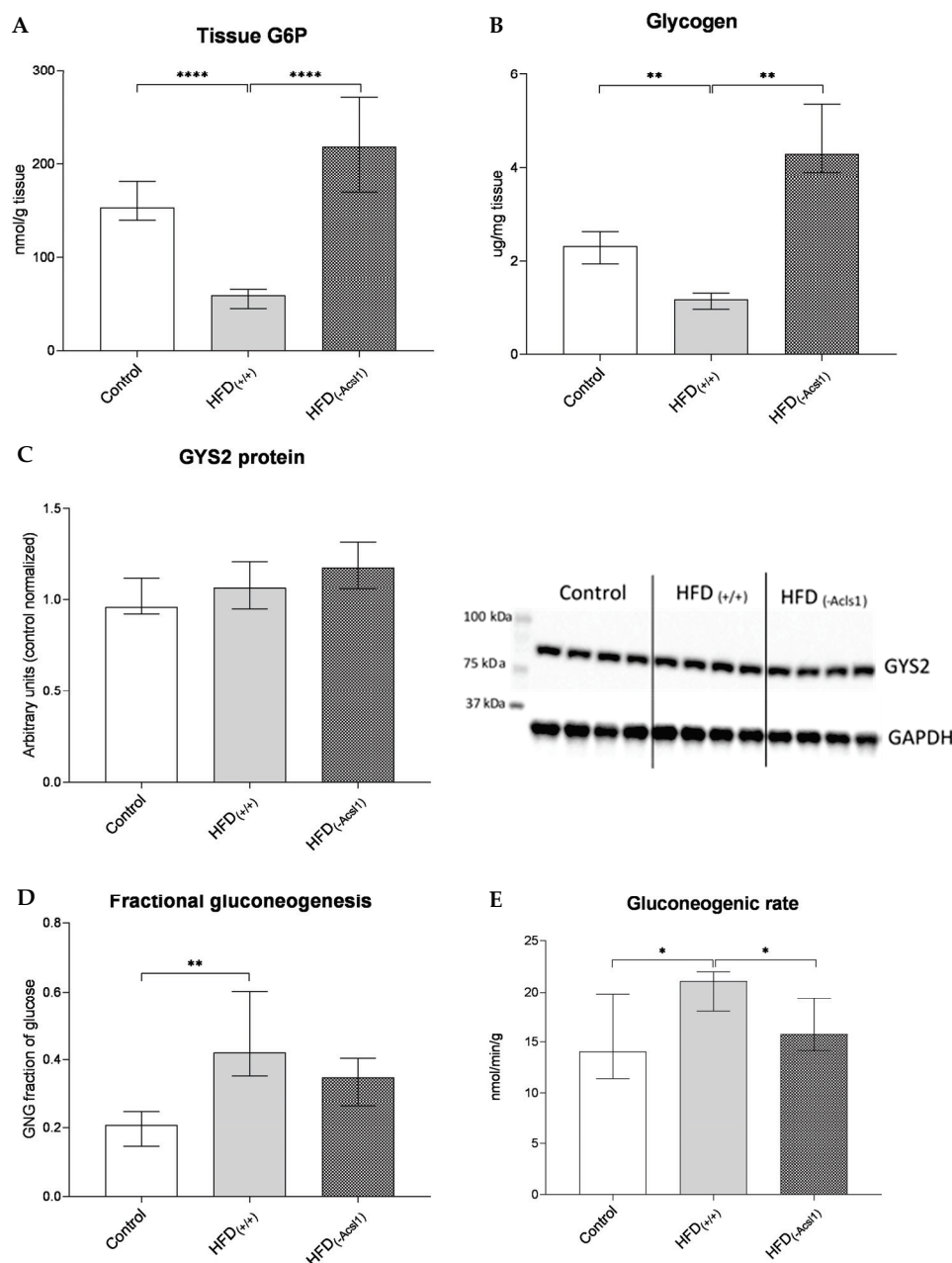


Figure 4. The content of liver glucose-6-phosphate (A); hepatic glycogen (B); liver GYS 2 protein expression (C); fractional gluconeogenesis (D); and gluconeogenic rate (E) under control; HFD-fed (HFD_(+/+)); and HFD-fed, ACSL1-silenced (HFD_(-ACSL1)) conditions. Bars represent medians +/− interquartile ranges; *— $p < 0.05$; **— $p < 0.01$; ****— $p < 0.0001$.

In the livers of mice in the HFD_(-ACSL1) group, the hepatic G6P content increased by more than three times ($p < 0.0001$, Figure 4A) and hepatic glycogen by almost four times ($p < 0.01$, Figure 4B) vs. the non-silenced HFD_(+/+) liver. Interestingly, the GYS2 protein content was not affected by the ACSL1 silencing (Figure 4C). Fractional gluconeogenesis decreased slightly (by 17%) under the ACSL1 silencing, yet the change was insignificant (vs. HFD_(+/+) values, Figure 4D). Contrary to the above parameter, the decrease in the gluconeogenic rate in the HFD_(-ACSL1) mice reached significance, being 15% lower than the values from HFD_(+/+) animals ($p < 0.05$, Figure 4E).

All of the above effects of both the HFD feeding and ACSL1 silencing in the HFD-fed animals significantly altered the systemic indices of IRs. The feeding with the high-fat diet increased plasma glucose and insulin concentration in the HFD_(+/+) mice, resulting in an increase in the HOMA-IR (vs. control, $p < 0.05$, Table 1). In addition, the HFD_(+/+) animals were indicative of the impaired glucose tolerance as compared to the control counterparts, as revealed by OGTT (Figure 3A).

The ACSL1 silencing under HFD consumption resulted in decreases in the glucose, insulin, and HOMA-IR values ($p < 0.05$ for all cases), as well as a significant improvement in OGTT parameters (vs. HFD_(+/+), Figure 3A).

4. Discussion

The liver plays a key in glucose homeostasis by means of both the glucose uptake from the blood in order to store it as glycogen in the postprandial state and the release of glucose from glycogenolysis and gluconeogenesis in the post-absorptive state. The liver is also crucial for the whole-body lipid metabolism. Lipid metabolism disorders in the liver have been repeatedly demonstrated to result in decreased hepatic insulin sensitivity [37,38]. This work is the first to present that the ACSL1 gene silencing in the liver decreases the hepatic content of Cer and DAG and improves the insulin signaling pathway in animals with HFD-induced InsR. Previous works focusing on the silencing or knock-out of the ACSL1 gene in the livers of animals fed a Western diet have not analyzed all the biologically active lipids that we have measured. The work by Singh et al. examined the effect of ACSL1 silencing in the liver on bile acid synthesis in animals fed a Western diet [39]. In the aforementioned study, the silencing of the ACSL1 gene in the liver was found not to affect the plasma fatty acid concentrations, but surprisingly, it triggered a slight, but significant, increase in the liver TAG levels. Although, in our study, we focused on the lipids implicated in the induction of insulin resistance, we also estimated the plasma content of fatty acids at the level of liver TAG. In our work, we noted that the silencing of ACSL1 in the liver under HFD consumption slightly, but significantly, lowered the plasma FFA concentrations and triggered a decrease in the contents of all hepatic lipids, including TAG. However, it is worth noting that the animals in our experiment consumed a lipid-rich diet, in which 60% of the calories came from fats, while in the aforementioned study, the animals consumed a Western-type diet, with approx. 45% of calories in form of fat. Moreover, the other reason for the discrepancy between the aforementioned results and our results may be a different method of silencing the ACSL1 gene. In the work by Singh et al. [39], the silencing of the ACSL1 gene was performed by means of adenovirus infection, which was associated with an intensified immune system response, while in our work, we used the HGD method. Another study used liver-specific ACSL1 knockout animals housed for three months on a Western diet [5]. Despite the observed decrease in the LCACoA levels in the knockout mice, no decrease in the DAG levels was observed, except for TAG, which was the case that proved the increase in the content of those lipids. In our work, we found the levels of all the lipids measured in this study to have increased in the livers of animals fed an HFD. Those data are consistent with those previously obtained by our team [7,8], as well as with the data obtained by other researchers [40–44]. The increase in the content of hepatic lipids overlapped with the elevation in the HOMA-IR value, a weakened response to glucose load, and inhibition of the insulin pathway, manifesting in decreased Akt/PKB phosphorylation and increased protein expression of PEPCK, the rate-limiting enzyme

in gluconeogenesis (Figure 3). The elevated PEPCK content suggested an increased rate of glucose production, which was confirmed by examining the rate of gluconeogenesis through glucose MIDA analysis (mass isotopomer distribution analysis). An increase in hepatic glucose output is a feature of T2D that is commonly attributed to gluconeogenesis rather than glycogenolysis [45,46]. In our work, in the HFD_(+/+) group, we observed not only an increase in the rate of gluconeogenesis, but also an inhibition of glycogen synthesis, which translated into a decrease in the glycogen content, although the content of GYS2, the enzyme responsible for glycogen synthesis, was not affected. It is plausible to state that the most effective GYS2 activator is the G6P. The content of G6P in the liver of HFD_(+/+) animals was significantly lower compared to other groups, which may justify a decrease in GYS2 enzymatic activity and a reduction in hepatic glycogen levels. Similar decreases in the liver glycogen content in insulin-resistant humans and animals have also been observed by other researchers [46,47]. Both increased gluconeogenesis and decreased glycogen synthesis contribute to increases in plasma glucose concentration. The silencing of the ACSL1 gene in the livers of the HFD-fed mice led to decreases in all the measured lipid levels as compared to the HFD_(+/+) group. ACSL1, as the major isoform of ACSL in the liver, is responsible for the availability of the substrate, acyl-CoA, for the de novo synthesis of intracellular lipids. The decrease in the hepatic content of Cer and DAG coincided with an augmentation of the insulin sensitivity at both the systemic and hepatic levels, which manifested as an improved response to glucose load and liver insulin signaling. The level of tissue augmentation of the insulin pathway was visible as an up-regulation of Akt/PKB phosphorylation, as well as by the increase in the inhibitory phosphorylation of the FOXO1 protein as compared to the HFD_(+/+) animals. Phosphorylation of FOXO1 blocks its transcriptional activity and thus inhibits the expression of gluconeogenic genes, as confirmed by the decrease in the PEPCK protein expression in the livers of the HFD_(-ACSL1) mice as compared to the HFD_(+/+) animals. The changes observed in the ACSL1-silenced liver associated with FOXO1 phosphorylation and PEPCK levels translated into a decrease in the rate of gluconeogenesis. Hepatic glycogen synthesis is another important process regulated by insulin. The enzyme directly responsible for the synthesis of glycogen in the liver is GYS2. Interestingly, we did not observe any significant differences in the protein expression of the GYS2 enzyme between the studied groups. However, since G6P is an important allosteric activator of GYS2 [48], an increase in the hepatic level of G6P may also lead to GYS2 activation. This allosteric effect was likely responsible for the accumulation of liver glycogen in the ACSL1-silenced animals, which concurrently displayed significantly higher levels of G6P as compared to the non-silenced HFD-fed counterparts. Our study clearly indicates a significant contribution of ACSL1 to the accumulation of biologically active lipids. Ceramides synthesized de novo from ACSL1-derived acyl-CoAs seem to play an especially key role in the deterioration of the functioning of the insulin pathway in the liver. This results in impaired regulation of gluconeogenesis and glycogenesis, as well as an increase in blood glucose concentration. Consumption of the HFD diet triggered increases in the levels of all the studied lipid groups, and ACSL1 liver-specific gene silencing was able to significantly attenuate this effect. However, the greatest variation was noted in the case of ceramides. Moreover, the ACSL1 silencing resulted in improved functioning of the insulin pathway, primarily at the level of Akt protein phosphorylation. Weakened phosphorylation of that protein often results from ceramide accumulation. The improved functioning of the insulin pathway at this stage is probably an effect of the reduced ceramide content. It can therefore be concluded that the hepatic ceramide pool is more dependent on the ACSL1-supplied acyl-CoAs than the DAG pool.

Although we performed a detailed analysis of LCA-CoA, ceramide, and DAG composition using the LC/MS/MS technique, the study would also benefit from a wider approach to lipid analysis. This is justified by the possible significant impact of ACSL1 silencing on all the cellular lipid classes. Performing non-targeted or semi-targeted lipidomic analysis would reveal the impact of ACSL1 silencing on a wider range of lipid species that are possibly affected in the greater extent. The major rationale behind our study was to observe

the effects of ACSL1 silencing on lipids with proven signaling properties crucial for the induction of lipid resistance. Thus, lipidomic analysis of all identifiable lipid species in the ACSL1 silenced livers of HFD-fed animals warrants its own separate study.

5. Conclusions

In summary, our studies show that the inhibition of LCACoA production can significantly contribute to improving the function of the hepatic insulin pathway and, thus, normalizing glucose metabolism under HFD-induced InsR conditions, which is probably the result of a decrease in both the ceramide and DAG content. It should be noted, however, that ACSL1 activity in the liver determines the level of ceramides to a greater extent than diacylglycerols.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/nu16071003/s1>, Figure S1: Original blot of Acs1, Figure S2: Original blot of Akt, Figure S3: Original blot of pAKT(S473), Figure S4: Original blot of FOXO1, Figure S5: Original blot of pFOXO1(S256), Figure S6: Original blot of PEPCK, Figure S7: Original blot of GYS2, Figure S8: Original blot of GAPDH.

Author Contributions: P.Z.—methodology, conceptualization, investigation, data curation, writing—review and editing; M.I.—investigation, visualization; K.R.-O.—investigation, data curation; M.K.—investigation, data curation; P.R.—investigation; J.D.—investigation; A.U.B.-Z.—conceptualization, methodology, investigation, resources, writing—review and editing, supervision, funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Article

Genetic Risk, BMI Status, BMI Change Patterns, and the Risk of Steatotic Liver Disease and Liver Enzyme Elevation in Chinese Adults

Juan Yang ^{1,†}, Chan Tian ^{1,†}, Maojie Liu ¹, Haiyan Guo ¹, Fei Lin ¹, Yang Ding ¹, Wentao Yao ¹, Jiahao Zhang ¹, Jingyi Fan ², Chengxiao Yu ³, Jing Lu ^{1,*} and Qun Zhang ^{3,*}

¹ Department of Epidemiology, Center for Global Health, School of Public Health, Nanjing Medical University, Nanjing 211166, China; juanyang0223@163.com (J.Y.); tc2419626380@163.com (C.T.); liumaojie99@163.com (M.L.); guohaiyan0227@163.com (H.G.); linfei930@163.com (F.L.); dingy1595@163.com (Y.D.); wentao.yao@foxmail.com (W.Y.); zhangjh0507@126.com (J.Z.)

² Health Management Center, Gusu School, The Affiliated Suzhou Hospital of Nanjing Medical University, Suzhou Municipal Hospital, Suzhou 234099, China; fanjingyi@njmu.edu.cn

³ Health Management Center, The First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, China; yuchengxiao@njmu.edu.cn

* Correspondence: lujing9254@jshp.org.cn (J.L.); lucyqzhang@njmu.edu.cn (Q.Z.); Tel.: +86-25-68303018 (J.L. & Q.Z.)

† These authors contributed equally to this work.

Abstract: Background/Objectives: Whether an increased genetic risk of steatotic liver disease (SLD) can be offset by maintaining a healthy weight remains unknown. We aimed to clarify the associations among the body mass index (BMI) and its change patterns with SLD and assess whether genetic susceptibility can modify these associations in Chinese people. **Methods:** A total of 10,091 and 6124 participants from the Health Omics Preventive Examination (HOPE) Program were enrolled in cross-sectional and follow-up analyses, respectively. BMI change patterns were defined according to the BMI at baseline and the last follow-up visit. Genetic risk was estimated using the polygenic risk score (PRS) derived from variants in *PNPLA3*, *TM6SF2*, *MBOAT7*, and *GCKR*. Data were analyzed using logistic regression models and Cox proportional-hazards models. **Results:** The analyses of the BMI and genetic risk simultaneously showed a dose–response association with the risk of SLD (p -trend < 0.001). Significant interactions between BMI and PRS were found for alanine aminotransferase (ALT) elevation ($p = 0.007$) and aspartate aminotransferase (AST) elevation ($p < 0.001$). Weight loss led to a 71%, 60%, and 67% lower risk of SLD, ALT elevation, and AST elevation, compared with stable overweight/obesity. A significant interaction between the genetic risk and BMI change patterns in ALT elevation was observed ($p = 0.008$). The absolute risk reductions associated with weight loss were greater for participants at a high genetic risk (26.60, 12.29, and 9.31 per 100 person years for SLD, ALT elevation, and AST elevation, respectively). **Conclusions:** Maintaining a healthy weight reduces the liver injury risk among all individuals, and the risk reduction is greater among the subset with a high genetic risk of SLD.

Keywords: steatotic liver disease; liver enzyme elevation; genetic susceptibility; BMI change pattern

1. Introduction

Steatotic liver disease (SLD) is the most prevalent chronic liver disease worldwide, affecting approximately 30% of the population [1]. The prevalence of SLD in the Chinese population is approximately 30% and it has shown an escalating trend over the years [2]. Obesity is an established risk factor for SLD, and more than 50% of obese adults will progress to SLD [3]. Previous studies have shown that trajectories of weight gain during young adulthood are associated with a greater SLD risk in midlife, and weight loss has been shown to not only reduce hepatic steatosis but also to improve biomarkers of hepatic steatosis [4–6]. However, these studies mainly focused on the impact of weight changes

through early and middle adulthood on the SLD risk in American and European populations, and few studies have investigated the association among short-term weight changes and SLD in Asian individuals. A previous Japanese study showed that short-term weight gain was related to an increased risk of SLD [7], but only non-obese people were studied. Therefore, establishing a comprehensive framework to analyze the impact of the BMI and its change patterns on the risk of SLD and liver enzyme elevation will provide a theoretical foundation for the development of targeted prevention and intervention strategies for Chinese people.

While environmental risk factors, such as the BMI, are the primary contributors to the risk of SLD [8], genetic factors have been estimated to account for 20–37% of the variance in the presence of SLD [9]. In the past decade, genome-wide association studies (GWASs) have identified several variants associated with hepatic steatosis [10]. Although an individual single-nucleotide polymorphism only partially explains the genetic variation in SLD, a polygenic risk score (PRS), derived from well-established hereditary variations in *PNPLA3*, *TM6SF2*, *MBOAT7*, and *GCKR*, has been shown to be effective in estimating an individual's genetic predisposition to SLD [11]. Moreover, prior studies found that the PRS for hepatic steatosis was statistically significantly related to higher plasma ALT and AST levels among Caucasian children [12,13]. However, the degree to which a genetic predisposition to hepatic steatosis contributes to SLD and liver enzyme elevation in Chinese adults remains unclear. Although the interactions between genes and adiposity on SLD and ALT levels have been previously explored [14], whether and the extent to which weight loss can offset the increased genetic risk of SLD and liver enzyme elevation is not yet clear among Chinese individuals.

To help to fill these gaps, we conducted a cross-sectional and follow-up study to explore the associations among the BMI, and its short-term change, and the genomic information related to hepatic steatosis and the risk of SLD and aminotransferase elevation among 12,007 participants from the Health Omics Preventive Examination (HOPE) Program. The HOPE study was a multi-center, prospective cohort study conducted in China. The current study enrolled the health examination population who visited the health management center of the First Affiliated Hospital of Nanjing Medical University in Jiangsu. The main objectives of the study were to (1) characterize the independent impacts of the BMI status, BMI change patterns, and genetic factors on liver injuries; (2) test the joint associations of these factors and gene–environment interactions in the risk of SLD and liver enzyme elevation; and (3) assess the degree to which weight loss or a stable normal weight might reduce the risk of SLD and liver enzyme elevation.

2. Materials and Methods

2.1. Study Design and Population

All 12,007 individuals aged 18 and older were invited to participate in the HOPE study from 2019 to 2023, with annual follow-up. The study excluded participants with missing data on genetic factors ($N = 888$), BMI ($N = 328$), liver enzymes ($N = 75$), SLD ($N = 378$), covariates ($N = 92$), and excessive alcohol consumption or other known liver etiologies ($N = 155$). The baseline cross-sectional evaluation therefore included 10,091 eligible participants. After further excluding participants without follow-up BMI or outcome information, as well as those with SLD or ALT or AST elevations at baseline, 6124 participants remained for the SLD analysis, 6550 participants were used for the ALT elevation analysis, and 6989 were used for the AST elevation analysis in the follow-up study. Supplemental Figure S1 displays the inclusion and exclusion process regarding the study population. This study was conducted in compliance with the Declaration of Helsinki and was approved by the ethics committee of Nanjing Medical University. All subjects signed a written informed consent form prior to their involvement in the study.

2.2. Genotyping and PRS Calculation

Genomic DNA was extracted from venous blood from each participant. After extraction, the genomic DNA was immediately stored at $-80\text{ }^{\circ}\text{C}$. The genotyping of *PNPLA3*

rs738409, *TM6SF2* rs58542926, *MBOAT7* rs641738, and *GCKR* rs1260326 was carried out. To examine the overall genetic burden on the SLD risk, the PRS was calculated by summing the number of effect alleles at each locus, each multiplied by its corresponding weight coefficient as reported in published studies [11], and detailed information is provided in Supplemental Table S1. The genetic risk of SLD was categorized as low, intermediate, or high based on the tertiles of the PRS.

2.3. Measurement of Exposure: BMI and BMI Change Pattern

All participants underwent a standardized clinical evaluation at an annual medical visit, including detailed anthropometric measurements, performed by a trained clinical investigator. The body mass index was computed as the weight in kilograms divided by the height in meters squared. Underweight (BMI < 18.5) was rare; thus, it was not considered as a separate category in the main analyses. Therefore, the BMI was classified into 3 categories based on the Chinese guidelines [15]: less than 24 (normal weight), 24 to less than 28 (overweight), and 28 or greater (obese).

BMI change patterns were established based on the BMI at baseline and the last follow-up visit: “stable normal weight” (BMI_{baseline} < 24 kg/m² and BMI_{last visit} < 24 kg/m²), “weight loss” (BMI_{baseline} ≥ 24 kg/m² and BMI_{last visit} < 24 kg/m²), “weight gain” (BMI_{baseline} < 24 kg/m² and BMI_{last visit} ≥ 24 kg/m²), and “stable overweight/obese” (BMI_{baseline} ≥ 24 kg/m² and BMI_{last visit} ≥ 24 kg/m²).

2.4. Measurement of Hepatic Steatosis and Liver Enzymes

In accordance with guidelines and existing studies, SLD was diagnosed when hepatic steatosis was present [16]. In this study, ultrasonography and computed tomography-guided liver examinations were conducted in all participants by trained doctors. A liver-to-spleen ratio (L/S) < 1.0 was used for the diagnosis of hepatic steatosis in the baseline cross-sectional study [17], and US was used as the diagnostic method in the follow-up study. Venous blood was tested by laboratory biochemistry for liver enzyme values. A liver enzyme elevation was defined by adopting sex-specific cut-off values. ALT elevation was defined as having an ALT value of greater than 33 UI/L in males and 25 UI/L in females [18], and AST elevation was defined as having an AST value of greater than 30 UI/L in males and 26 UI/L in females [19].

2.5. Statistical Analyses

In the cross-sectional analysis, logistic regression models were used to estimate the odds ratios (ORs) and 95% confidence intervals (CIs) between the BMI categories, genetic risk, and SLD, as well as the liver enzyme elevation risk. The potential non-linear associations between the SLD risk, genetic risk score, and BMI were evaluated using restricted cubic splines with three knots. In addition, the joint effects of the BMI categories and genetic risk on liver injuries were examined. The interaction was evaluated by including multiplicative interaction terms in the logistic regression models.

In the prospective analysis, a Cox proportional-hazards model was used to estimate the association between BMI change patterns and liver injuries. To explore the combined associations of the BMI change patterns and genetic risk for SLD, eight combined groups were created by crossing four BMI change patterns with two genetic risk categories, using those with a high genetic risk and stable overweight/obesity as the reference group. This study further assessed the interactions between the BMI change patterns and genetic risk of SLD in liver injuries. The absolute risk reduction in SLD and aminotransferase elevation incidence in these given groups were computed to evaluate the benefits of weight loss or a stable normal weight over a 3-year period. To derive the 95% confidence intervals for the absolute risk reduction, 1000 bootstrap samples were generated from the estimation dataset. Sensitivity analyses were conducted to evaluate the stability of the results by (1) reclassifying the genetic risk groups according to the quartiles or quintiles of the PRS

and (2) removing individuals with pre-existing diabetes, hypertension, or dyslipidemia to prevent any potential reverse association.

In the aforementioned analyses, all models were adjusted for age, sex, smoking status, drinking status, education level, regular physical activity, and chronic diseases (hypertension, diabetes, hyperlipidemia). A two-sided *p* value of less than 0.05 was regarded as indicative of a significant difference. R (version 4.3.1) was used to perform all statistical analyses.

3. Results

3.1. Characteristics of Study Participants

The baseline characteristics of the participants are listed in Table 1. Overall, the average age (SD) of all 10,091 participants was 38.29 (10.39) year, and almost half were males (48.79%). During a median follow-up of 2.92 years (interquartile range: 2.25–3.83 years), 1318 out of the 6124 subjects developed SLD, 860 out of the 6550 subjects developed an ALT elevation, and 702 out of the 6989 subjects developed an AST elevation. Supplemental Table S2 shows the baseline characteristics of the subjects participating in the follow-up study according to their BMI change patterns. In general, 4072 (66.49%) participants remained at a normal weight (classified as “stable normal weight” group), 611 (9.98%) became overweight/obese (classified as “weight gain” group), 160 (2.61%) transitioned from being overweight/obese to a normal weight (classified as “weight loss” group), and 1281 (20.92%) remained overweight/obese (classified as “overweight/obesity” group).

Table 1. Baseline characteristics of the study population.

Characteristic	Total (N = 10,091)	Non-Steatotic Liver Disease (N = 8854)	Steatotic Liver Disease (N = 1237)	<i>p</i> Value †
Sex, N(%)				
Male	4923 (48.79)	3952 (44.64)	971 (78.50)	<0.001
Female	5168 (51.21)	4902 (55.36)	266 (21.50)	
Age (years), Mean ± SD	38.29 ± 10.39	38.22 ± 10.40	38.81 ± 10.30	0.058
Education level, N(%)				
High school or below	609 (6.04)	529 (5.97)	80 (6.47)	0.537
College degree or above	9482 (93.96)	8325 (94.03)	1157 (93.53)	
Smoking status, N(%)				
Never	8457 (83.81)	7565 (85.44)	892 (72.11)	<0.001
Ever	1634 (16.19)	1289 (14.56)	345 (27.89)	
Drinking status, N(%)				
Never	8460 (83.84)	7537 (85.13)	923 (74.62)	<0.001
Ever	1631 (16.16)	1317 (14.87)	314 (25.38)	
Regular physical activity, N(%)				
No	6808 (67.47)	5886 (66.48)	922 (74.54)	<0.001
Yes	3283 (32.53)	2968 (33.52)	315 (25.46)	
Body mass index (kg/m ²), Mean ± SD	23.38 ± 3.34	22.85 ± 2.95	27.22 ± 3.45	<0.001
BMI category, N(%)				
<24	6166 (61.10)	5961 (67.33)	205 (16.57)	<0.001
24–28	3021 (29.94)	2449 (27.66)	572 (46.24)	
≥28	904 (8.96)	444 (5.01)	460 (37.19)	
ALT (IU/L), Median (IQR)	16.80 (11.80–25.90)	15.50 (11.30–22.60)	38.80 (26.40–57.50)	<0.001
AST (IU/L), Median (IQR)	19.70 (16.80–23.90)	19.20 (16.50–22.80)	27.00 (21.70–35.00)	<0.001
Hypertension, N(%)				
No	8602 (85.24)	7787 (87.95)	815 (65.89)	<0.001
Yes	1489 (14.76)	1067 (12.05)	422 (34.11)	
Diabetes, N(%)				
No	9833 (97.44)	8706 (98.33)	1127 (91.11)	<0.001
Yes	258 (2.56)	148 (1.67)	110 (8.89)	
Hyperlipidemia, N(%)				
No	7779 (77.09)	7204 (81.36)	575 (46.48)	<0.001
Yes	2312 (22.91)	1650 (18.64)	662 (53.52)	
Genetic risk category, N (%)				
Low	3063 (30.35)	2834 (32.01)	229 (18.51)	<0.001
Intermediate	3406 (33.75)	3026 (34.18)	380 (30.72)	
High	3622 (35.89)	2994 (33.82)	628 (50.77)	

† *p* values were calculated using independent-sample *t* test or Mann–Whitney U test for continuous variables and χ^2 test for categorical variables. BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

3.2. Genetic Associations with SLD and Liver Enzyme Elevation Risk

The associations of *PNPLA3*, *TM6SF2*, *MBOAT7*, and *GCKR* variants and the SLD risk were tested and the four single-nucleotide polymorphisms (SNPs) were all associated with the SLD risk. The strongest evidence of an association was found between rs738409 in the *PNPLA3* variant and SLD, with ORs of 1.93 (1.63–2.28) for CG and 4.65 (3.79–5.70) for the GG genotype (Figure 1a). The result showed that the risk of SLD increased linearly with the increase in the PRS; see Supplemental Figure S2 (p for overall <0.001 , p for non-linearity = 0.142). Participants with SLD exhibited a higher PRS compared to those without SLD ($p < 0.001$) (Figure 1b). After additional adjustment for the BMI, participants in the high-genetic-risk group (top tertile) had a 273% higher risk of SLD (OR: 3.73, 95% CI 3.08–4.51), a 30% higher risk of ALT elevation (OR: 1.30, 95% CI: 1.13–1.49), and a 23% higher risk of AST elevation (OR: 1.23, 95% CI: 1.05–1.43) compared to those in the low-genetic-risk group (bottom tertile) (Figure 1c, Supplemental Table S3).

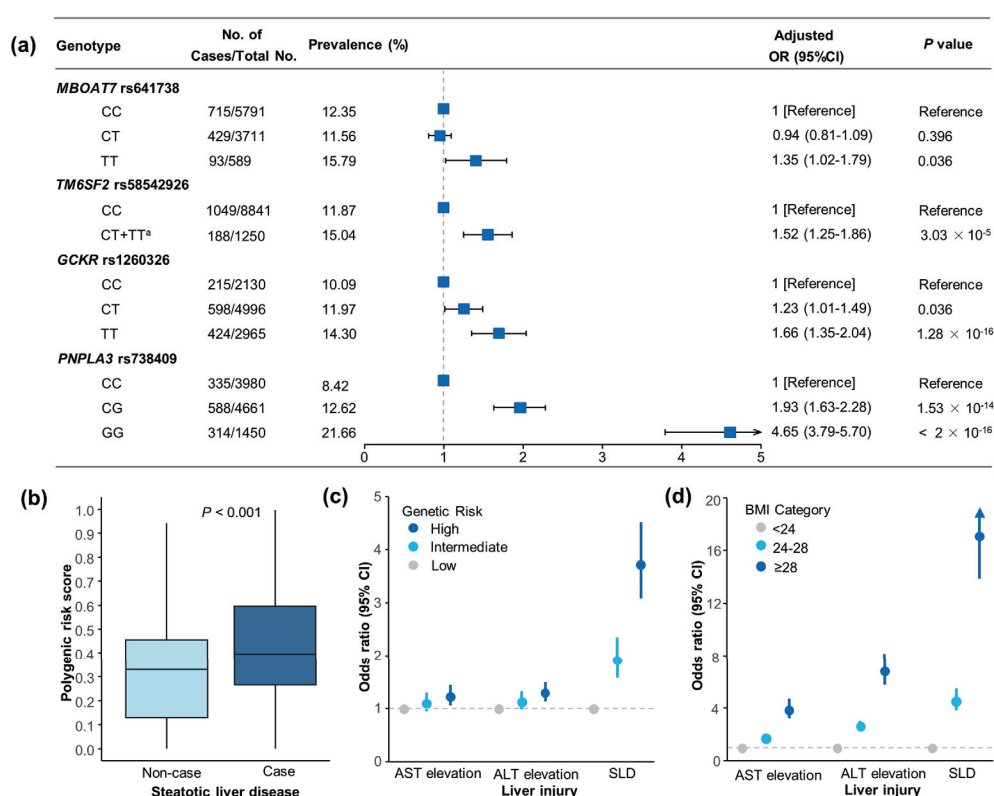


Figure 1. Effects of genetic factor and BMI category on the risk of steatotic liver disease and liver enzyme elevation in the HOPE cohort. (a) Associations between individual SNPs and SLD. (b) Polygenic risk scores among SLD cases versus non-cases. In the boxplot, the median is represented by horizontal lines, the interquartile range is indicated by the upper and lower edges of the box, and the whiskers display the maximum and minimum values for each group. The p value was derived from the Mann–Whitney U test, and $p < 0.001$ indicates that the difference in the polygenic risk score distribution between the case group and the non-case group was statistically significant. (c) Associations of genetic risk categories with liver injuries. (d) Associations of BMI categories with liver injuries. Genetic risk groups were established based on the tertiles of the PRS (low, intermediate, and high). ORs and 95% CIs were determined after controlling for age, sex, smoking status, drinking status, education level, regular physical activity, hypertension, diabetes, hyperlipidemia, BMI (SNP and genetic risk analysis), or genetic risk (BMI category analysis). ^a Because only 39 participants had the *TM6SF2* rs58542926 TT genotype, the CT and TT genotypes were combined into one explanatory variable, “CT+TT”. OR, odds ratio; 95% CI, 95% confidence interval; SLD, steatotic liver disease; BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; SNPs, single-nucleotide polymorphisms.

3.3. Associations of BMI with SLD and Liver Enzyme Elevation Risk

In the analysis of the dose–response relationship, the BMI showed a non-linear association with the SLD risk, following an approximately J-shaped curve (p for non-linear <0.001), with a significantly increasing trend in the OR for each 1 kg/m^2 increase in the BMI when the BMI was greater than 23 kg/m^2 (Supplemental Figure S3). The multivariable-adjusted ORs (95% CI) for the SLD risk were 4.57 (3.81–5.48) for overweight and 17.13 (13.84–21.19) for obese when compared with a normal weight (Figure 1d, Supplemental Table S4).

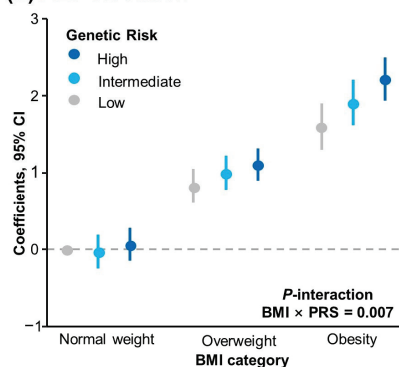
3.4. Joint Association and Interaction of BMI and PRS in SLD and Liver Enzyme Elevation Risk

As the PRS and BMI groups were combined, the SLD and liver enzyme elevation risk grew as both factors rose (Figure 2). Among all of the categories analyzed, individuals with both a high genetic risk and obesity exhibited the highest liver injury risk. Compared with those with a low genetic susceptibility to SLD and normal weight, having a high genetic risk and obesity was linked to an increased risk of SLD (OR: 63.16, 95% CI: 40.86–97.64), ALT elevation (OR: 9.21, 95% CI: 6.96–12.18), and AST elevation (OR: 5.10, 95% CI: 3.86–6.82). Moreover, the risk of SLD and aminotransferase elevation remained consistently lower in individuals with a normal weight than in those with obesity, within each category of genetic risk. Significant interactions between the genetic risk and BMI categories regarding aminotransferase elevation were observed (p for interaction = 0.007 for ALT elevation; p for interaction = 9.39×10^{-7} for AST elevation), indicating that the risk-increasing effect of the genetic risk on aminotransferase elevation was amplified by increasing adiposity. However, no significant interaction was found between the PRS and BMI categories for SLD (p for interaction = 0.933).

(a) SLD

Subgroup	No. of Cases/Total No.	Prevalence (%)	Adjusted OR (95%CI)	P value
Normal weight				
Low genetic risk	30/1880	1.60	1 [Reference]	Reference
Intermediate genetic risk	50/2100	2.38	1.53 (0.97–2.42)	0.069
High genetic risk	125/2186	5.72	3.71 (2.47–5.57)	2.61×10^{-10}
Overweight				
Low genetic risk	101/913	11.06	4.54 (2.96–6.95)	3.66×10^{-12}
Intermediate genetic risk	186/1016	18.31	9.05 (6.05–13.55)	$< 2 \times 10^{-16}$
High genetic risk	285/1092	26.10	14.72 (9.93–21.80)	$< 2 \times 10^{-16}$
Obesity				
Low genetic risk	98/270	36.30	16.40 (10.39–25.89)	$< 2 \times 10^{-16}$
Intermediate genetic risk	144/290	49.66	29.46 (18.89–45.94)	$< 2 \times 10^{-16}$
High genetic risk	218/344	63.37	63.16 (40.86–97.64)	$< 2 \times 10^{-16}$
P-interaction BMI $\times$ PRS = 0.933				

(b) ALT elevation



(c) AST elevation

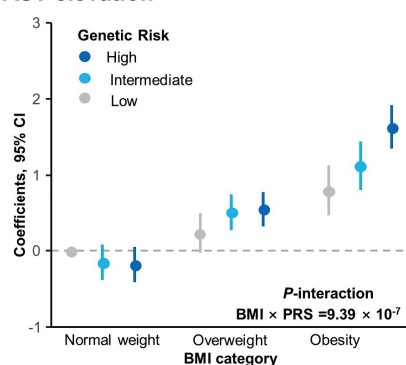


Figure 2. Joint associations of genetic risk, BMI category, and risk of SLD (a), ALT elevation (b), and AST elevation (c). Subjects with low genetic risk and normal weight were used as the reference group. ORs were calculated by logistic regression, adjusted for age, sex, smoking status, drinking status, education level, regular physical activity, hypertension, diabetes, and hyperlipidemia.

3.5. Relations of BMI Change Pattern and SLD and Aminotransferase Elevation

The results showed that individuals who lost weight and transitioned from overweight/obesity to a normal weight had a 71% lower risk of SLD (HR, 0.29, 95% CI, 0.20–0.44), a 60% lower risk of ALT elevation (HR, 0.40, 95% CI, 0.25–0.66), and a 67% lower risk of AST elevation (HR, 0.33, 95% CI, 0.18–0.60) compared with stable overweight/obese individuals. Moreover, participants who gained weight, moving from a normal weight to overweight/obesity, had a 114% higher risk of SLD (HR 2.14, 95% CI, 1.81–2.53) and a 62% higher risk of ALT elevation (HR 1.62, 95% CI, 1.30–2.01) compared with those who maintained a normal weight (Table 2).

Table 2. The association between the BMI change pattern and the risk of liver injury.

Outcome	Risk Reduction: Weight Loss Versus Stable Overweight/Obesity †			Risk Raise: Weight Gain Versus Stable Normal Weight ‡		
	Total No. (Cases)	HR (95%CI) §	p Value	Total No. (Cases)	HR (95%CI) §	p Value
Steatotic liver disease	1441 (635)	0.29 (0.20–0.44)	3.37×10^{-9}	4683 (683)	2.14 (1.81–2.53)	$<2 \times 10^{-16}$
ALT elevation	2072 (404)	0.40 (0.25–0.66)	2.94×10^{-4}	4478 (456)	1.62 (1.30–2.01)	1.77×10^{-5}
AST elevation	2460 (338)	0.33 (0.18–0.60)	2.77×10^{-4}	4529 (364)	1.27 (0.98–1.64)	0.072

† Stable overweight or obese group was designated as the reference group. ‡ Stable normal weight group was designated as the reference group. § HRs and 95% CIs were adjusted for age, sex, smoking status, drinking status, education level, regular physical activity, hypertension, diabetes, hyperlipidemia, and genetic risk category. BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HR, hazard ratio; 95%CI, 95% confidence interval.

3.6. Joint Effect and Interaction of BMI Change Pattern and PRS in Incident SLD and Aminotransferase Elevation Risk

The tendencies of the risk of SLD and aminotransferase elevation among participants with different BMI change patterns and genetic risks of SLD are presented in Figure 3. As compared with those with stable overweight/obesity and a high genetic risk, weight loss was associated with a 78% (HR 0.22, 95% CI, 0.11–0.46) lower risk of SLD among those at a high genetic risk, and a stable normal weight was associated with a 70% (HR 0.30, 95% CI, 0.24–0.37) lower risk of SLD among those at a high genetic risk. Likewise, individuals experienced a gradually decreased liver enzyme elevation risk across the four BMI change patterns in both the low-to-intermediate- and high-genetic-risk categories. An interaction between the BMI change patterns and PRS was observed only for ALT elevation (p for interaction = 0.008).

3.7. Standardized Three-Year Absolute Risk of SLD and Aminotransferase Elevation According to Genetic Risk and BMI Change Patterns

The age- and sex- adjusted 3-year absolute risk of SLD was lower for those with a low-to-intermediate genetic risk (spanning from 7.81 to 23.41 per 100 person years) than for those with a high genetic risk (spanning from 7.91 to 34.51 per 100 person years), but was higher for those with stable overweight/obesity (spanning from 23.41 to 34.51 per 100 person years) than for those with weight loss or a stable normal weight (ranging from 7.81 to 10.07 per 100 person years) at both low-to-intermediate and high genetic risk levels. When those with stable overweight/obesity were used as the reference group, the adjusted 3-year absolute SLD risk reductions associated with weight loss were 13.34 (95% CI, 7.93–19.44) per 100 person years for the low-to-intermediate genetic risk category and 26.60 (95% CI, 18.51–35.87) per 100 person years for the high genetic risk category. Moreover, when people maintained a normal weight, the 3-year absolute SLD risk reductions in the low-to-intermediate and high genetic risk categories were 15.60 (95% CI, 12.02–18.88) and 24.73 (95% CI, 18.17–30.25) per 100 person years, respectively. A comparable pattern of results was observed for the adjusted 3-year absolute risk estimates of ALT and AST elevation. Among individuals who experienced weight loss or maintained a normal weight, the 3-year absolute risk reduction was larger in the high genetic risk category than the low-to-intermediate genetic risk category (Figure 4). The results were successfully repli-

cated in the sensitivity analyses, and similar 3-year absolute risk trends of incident SLD and aminotransferase elevation according to the genetic risk and BMI change patterns were observed (Supplemental Tables S5–S10).

(a) SLD

Subgroup	No. of Cases/Total No.	Incidence / 100 PY	Adjusted HR (95%CI)	P value
Low-to-Intermediate genetic risk				
Weight loss	17/102	5.74	0.32 (0.19–0.52)	5.86×10^{-6}
Stable normal weight	291/2673	3.71	0.26 (0.21–0.31)	$< 2 \times 10^{-16}$
Weight gain	145/403	11.63	0.62 (0.50–0.77)	1.21×10^{-5}
Stable overweight/obesity	404/883	15.66	0.79 (0.67–0.94)	7.58×10^{-3}
High genetic risk				
Weight loss	8/58	4.82	0.22 (0.11–0.46)	3.73×10^{-5}
Stable normal weight	175/1399	4.31	0.30 (0.24–0.37)	$< 2 \times 10^{-16}$
Weight gain	73/208	11.44	0.59 (0.45–0.78)	1.62×10^{-4}
Stable overweight/obesity	206/398	18.04	1.00 [Reference]	Reference
P-interaction BMI change patterns $\times$ PRS = 0.360				

(b) ALT elevation

Subgroup	No. of Cases/Total No.	Incidence / 100 PY	Adjusted HR (95%CI)	P value
Low-to-Intermediate genetic risk				
Weight loss	9/123	2.51	0.30 (0.15–0.59)	4.86×10^{-4}
Stable normal weight	223/2495	3.08	0.39 (0.32–0.49)	$< 2 \times 10^{-16}$
Weight gain	64/403	5.14	0.55 (0.41–0.75)	9.97×10^{-5}
Stable overweight/obesity	234/1246	6.33	0.68 (0.56–0.84)	2.84×10^{-4}
High genetic risk				
Weight loss	8/80	3.46	0.37 (0.18–0.76)	0.010
Stable normal weight	119/1364	2.98	0.39 (0.30–0.50)	1.14×10^{-13}
Weight gain	50/216	7.49	0.80 (0.58–1.10)	0.160
Stable overweight/obesity	153/623	8.42	1.00 [Reference]	Reference
P-interaction BMI change patterns $\times$ PRS = 0.008				

(c) AST elevation

Subgroup	No. of Cases/Total No.	Incidence / 100 PY	Adjusted HR (95%CI)	P value
Low-to-Intermediate genetic risk				
Weight loss	5/141	1.21	0.20 (0.08–0.49)	3.99×10^{-4}
Stable normal weight	178/2490	2.43	0.46 (0.36–0.59)	4.32×10^{-10}
Weight gain	45/429	3.41	0.58 (0.41–0.81)	0.002
Stable overweight/obesity	193/1477	4.43	0.72 (0.58–0.90)	0.004
High genetic risk				
Weight loss	6/86	2.40	0.38 (0.17–0.87)	0.022
Stable normal weight	110/1375	2.74	0.52 (0.40–0.68)	1.27×10^{-6}
Weight gain	31/235	4.26	0.68 (0.46–1.01)	0.054
Stable overweight/obesity	134/756	6.06	1.00 [Reference]	Reference
P-interaction BMI change patterns $\times$ PRS = 0.302				

Figure 3. Joint associations of genetic risk, BMI change pattern, and risk of SLD (a), ALT elevation (b), and AST elevation (c). Individuals having a high genetic risk and stable overweight/obesity served as the reference group. Risk estimates were adjusted for age, sex, smoking status, drinking status, education level, regular physical activity, hypertension, diabetes, and hyperlipidemia. PY, person years.

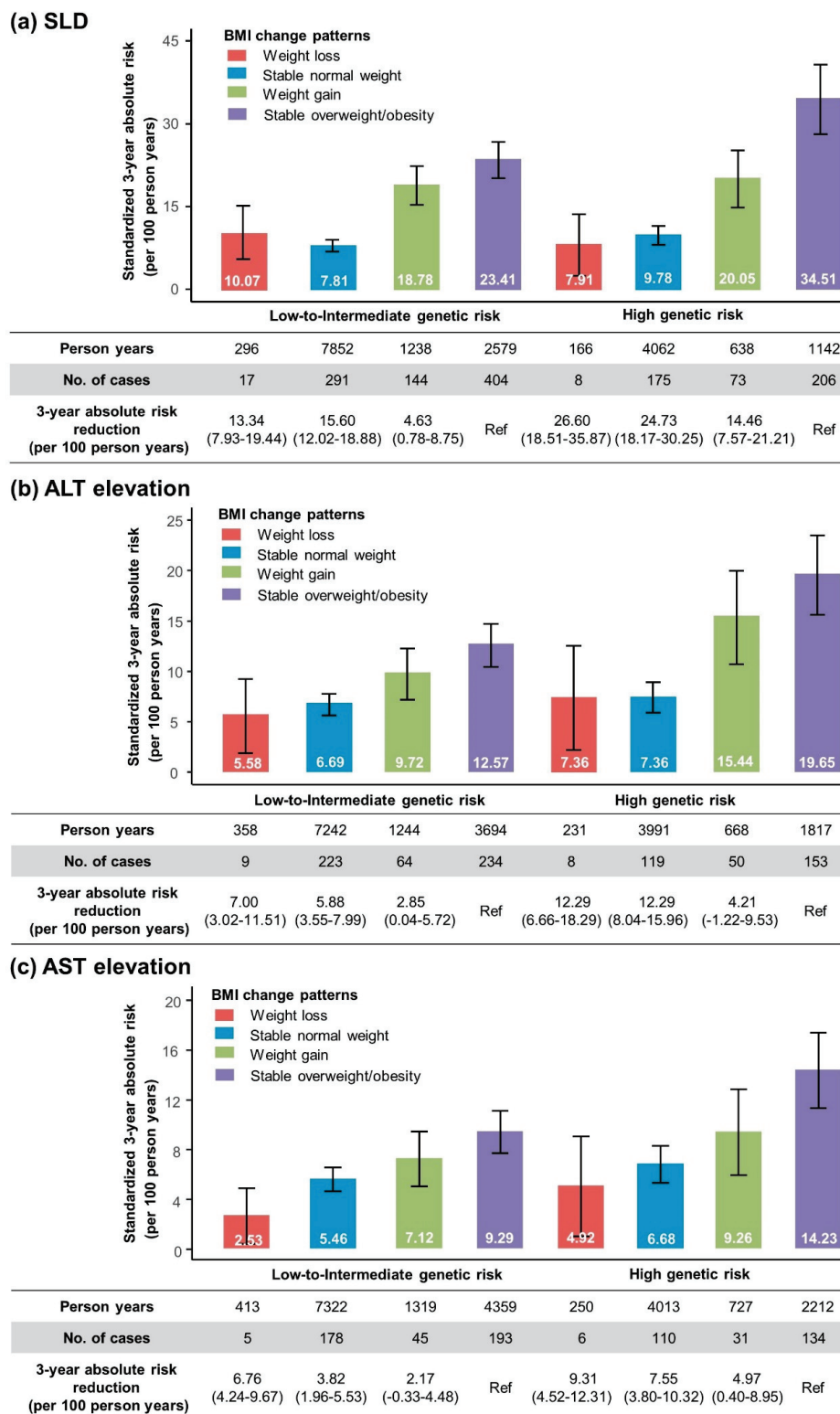


Figure 4. Standardized 3-year absolute risk (per 100 person years) for SLD (a), ALT elevation (b), and AST elevation (c) according to BMI change patterns in different genetic risk groups. The 3-year absolute risks were age- and sex-adjusted. Genetic risk was classified into low-to-intermediate (tertiles 1–2) and high (the top tertile) based on the tertiles of the polygenic risk score. Error bars represent 95%CI of 3-year absolute risk. The HRs were obtained using Cox proportional-hazards regression with adjustment for age, sex, smoking status, drinking status, education level, regular physical activity, hypertension, diabetes, and hyperlipidemia.

4. Discussion

In this study conducted on a population of Chinese adults, the research revealed that (1) obesity and weight gain were positively correlated with a higher risk of SLD and aminotransferase elevation, whereas weight loss was negatively associated; (2) the greater absolute risk reductions for liver injuries associated with maintaining a normal weight or weight loss occurred among those with a high genetic risk of SLD. The current study offers compelling evidence supporting the benefits of maintaining a normal weight for the prevention of SLD and aminotransferase elevation in the whole population, particularly among individuals having a high genetic susceptibility to SLD.

It has been well recognized that the BMI status and absolute weight changes are associated with the risk of SLD. Previous studies showed that absolute weight gain was related to SLD and weight loss was associated with the remission of hepatic steatosis in both obese and non-obese patients [6,20,21]. However, few studies have reported the associations of BMI changes in various patterns defined by the cutoff values of the China BMI classification and liver injury. The current study demonstrated that recent short-term BMI change patterns were closely related to the development of SLD and liver enzyme elevation. Specifically, weight gain from a normal weight to overweight/obesity was a significant risk factor for liver injury, whereas weight loss from overweight/obesity to a normal weight substantially reduced the risk of liver injury. The findings indicate that health examination populations could be categorized based on their BMI change patterns to prevent SLD in healthcare services and in clinical practice.

Previous studies that included subjects of European ancestry found that the PRS-HFC could accurately identify patients with SLD and refined the diagnostic performance for liver damage [11,22,23]. Among East Asian populations, Thomas et al. found that the PRS-HFC derived from Europeans was also useful in assessing the HCC risk [24]. Taken together, the previous findings support that the PRS-HFC, as a robust genetic instrument, is predictive of SLD. In this study with Chinese individuals as the subjects, the PRS-HFC was also independently and robustly associated with a higher risk of SLD, with a high genetic risk score having a 3.73-fold higher risk of SLD compared with a low genetic risk score. Furthermore, our results showed that individuals at a high genetic risk of SLD had a higher risk of ALT and AST elevation than those at a low-to-intermediate genetic risk. This was consistent with a prior study conducted in Denmark that reported that the PRS for hepatic steatosis was robustly associated with higher plasma concentrations of ALT and AST [12].

There is existing evidence that interactions between lifestyle factors (e.g., obesity and alcohol use) and a genetic predisposition to liver damage are present [14,25,26]. In this study, BMI–PRS interactions were present for liver enzyme elevation, and the BMI change pattern–PRS interaction was statistically significant only for ALT elevation. The results suggest that adiposity played a pivotal role in shaping the impact of the genetic risk of SLD on liver enzyme elevation, which was in line with a previous study that found that the association between the PRS and increased ALT was amplified as the BMI increased [27]. In contrast with some observations revealing the interaction between the genetic risk and BMI in SLD [28], no significant interaction for SLD was found. This discrepancy is likely because of the ethnic differences in the allele frequencies and possibly inadequate power to detect it. The biological mechanisms behind the observed interaction results are not clear. Future studies are warranted to further explore the mechanisms.

The results regarding the joint effects of the BMI and PRS demonstrate that having a normal weight could reduce the liver injury risk at all levels of genetic susceptibility to SLD. Among participants with high genetic susceptibility to SLD, this study even found no significant risk effect of the genetic factor on liver enzyme elevation when the subject had a normal weight. Likewise, in the follow-up analysis, individuals who had a high genetic risk of SLD and lost weight or maintained a normal weight had a lower risk of developing SLD and aminotransferase elevation compared with those who had a low-to-intermediate genetic risk but gained weight or maintained overweight/obesity. These findings indicate the potential benefits of a stable normal weight regardless of the genetic risk. Furthermore,

it was observed that, compared with individuals at a low-to-intermediate genetic risk, the beneficial effects of weight loss and a stable normal weight were more evident in subjects at a high genetic risk, who experienced greater 3-year absolute risk reductions regarding liver injuries. The results indicate that maintaining a normal weight could partly offset the deleterious effect of the genetic susceptibility to SLD, which once again underscores the importance of maintaining a normal weight in preventing SLD and aminotransferase elevation, particularly for individuals with a high genetic risk of SLD.

The strength of the current study is that it offers a thorough understanding of the associations of the BMI, short-term BMI change patterns, and genetic risk with liver injuries in Chinese populations, with a cross-sectional and prospective design. This study also has some limitations. First, although the BMI is most widely used and easily obtained in clinical practice, the fat distribution may be more informative [29]. A second limitation is that ultrasound is not the “gold standard” for the identification of hepatic steatosis; however, it is neither feasible nor ethical to undertake liver biopsies in large cohorts of healthy people. Third, the small proportion of the sample that experienced weight loss limited the accuracy of the calculations. Fourth, the beta coefficients associated with the genetic risk used to construct the PRS-HFC were derived from European population data, which may have resulted in racial differences. However, relevant data in Asian populations are lacking. Fifth, the study only included Chinese populations; therefore, our findings cannot be generalized to other populations. Finally, as more genetic variants are identified [10], the variance explained by genetics and PRS estimates will be improved.

5. Conclusions

In Chinese individuals, maintaining a healthy weight could significantly lower the SLD and aminotransferase elevation risk, regardless of the genetic susceptibility to SLD. Weight control could provide a greater benefit for individuals with a high genetic risk of SLD compared to those with a low genetic risk.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/nu16234212/s1>, Figure S1. Flowchart of the study; Figure S2. Linear relationships between polygenic risk score and steatotic liver disease risk.; Figure S3. Linear relationships between BMI and steatotic liver disease risk.; Table S1. Information on four SNPs used to build polygenic risk score; Table S2. Participant characteristics by BMI change pattern among 6124 participants without steatotic liver disease at baseline; Table S3. Association of genetic risk category with SLD, ALT elevation, and AST elevation; Table S4. Association of BMI categories with SLD, ALT elevation, and AST elevation; Table S5. Risk of incident SLD according to BMI change pattern within each genetic risk category based on quartiles or quintiles of polygenic risk score (PRS); Table S6. Risk of incident ALT elevation according to BMI change pattern within each genetic risk category based on quartiles or quintiles of polygenic risk score (PRS); Table S7. Risk of incident AST elevation according to BMI change pattern within each genetic risk category based on quartiles or quintiles of polygenic risk score (PRS); Table S8. Risk of incident SLD according to BMI change pattern within each genetic risk category with exclusion of chronic diseases at baseline; Table S9. Risk of incident ALT elevation according to BMI change pattern within each genetic risk category with exclusion of chronic diseases at baseline; Table S10. Risk of incident AST elevation according to BMI change pattern within each genetic risk category with exclusion of chronic diseases at baseline.

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Institutional Review Board Statement: This study was conducted in accordance with the Declaration of Helsinki and approved by the ethics committee of Nanjing Medical University (code: 2019-SR-209, date: 12 June 2019).

Informed Consent Statement: All participants provided written informed consent.

Data Availability Statement: The datasets of the study are available from the authors upon valid request. The data are not accessible to the public because of laboratory policies.

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Review

Impact of Sexual Dimorphism on Therapy Response in Patients with Metabolic Dysfunction-Associated Steatotic Liver Disease: From Conventional and Nutritional Approaches to Emerging Therapies

Eleonora Dileo [†], Francesca Saba [†], Mirko Parasiliti-Caprino, Chiara Rosso ^{*} and Elisabetta Bugianesi ^{*}

Department of Medical Sciences, University of Turin, 10126 Turin, Italy; eleonora.dileo@unito.it (E.D.); francesca.saba@unito.it (F.S.); mirko.parasiliticaprino@unito.it (M.P.-C.)

^{*} Correspondence: chiara.rosso@unito.it (C.R.); elisabetta.bugianesi@unito.it (E.B.)

[†] These authors contributed equally to this work.

Abstract

Metabolic dysfunction-associated steatotic liver disease (MASLD) represents a spectrum of liver disease ranging from hepatic fat accumulation to steatohepatitis (metabolic dysfunction-associated steatohepatitis, MASH), fibrosis, cirrhosis, and potentially hepatocellular carcinoma in the absence of excessive alcohol consumption. MASLD is characterized by substantial inter-individual variability in terms of severity and rate of progression, with a prevalence that is generally higher in men than in women. Steroids metabolism is characterized by sexual dimorphism and may have an impact on liver disease progression; indeed, several therapeutic strategies targeting hormone receptors are under phase 2/3 development. Despite the fact that the importance of sexual dimorphism in the setting of MASLD is well recognized, the underlying molecular mechanisms that can potentially drive the disease toward progression are not clear. The aim of this review is to delve into the crosstalk between sexual dimorphism and steroid hormone perturbation under nutritional and pharmacological intervention.

Keywords: metabolic dysfunction-associated steatotic liver disease; metabolic dysfunction-associated steatohepatitis; sexual dimorphism; steroids; sex hormones; therapy

1. Introduction

Metabolic dysfunction-associated steatotic liver disease (MASLD) is a liver disease strongly associated with metabolic comorbidities such as obesity, type 2 diabetes mellitus (T2DM), arterial hypertension, and dyslipidemia. Liver damage encompasses a wide and complex spectrum, ranging from simple steatosis to the progressive metabolic dysfunction-associated steatohepatitis (MASH), characterized by ballooning degeneration and lobular inflammation with or without fibrosis [1]. In this context, hepatic fibrosis is the most important negative prognostic factor, driving MASLD toward cirrhosis, which, in turn, increases the risk of hepatocellular carcinoma (HCC) [1]. MASLD prevalence ranges from 25% to 30% in the adult European population [2] and is projected to be the principal etiology for liver transplantation within the decade [2,3]. Despite the high prevalence, only a subset of patients with MASLD progress to advanced liver disease over a period of years, ultimately experiencing major morbidity and mortality [4,5]. In fact, MASLD is characterized by an inter-patient variability in severity and rate of progression, as well as

bi-directional changes in grade of MASH and stage of liver fibrosis, as disease severity ‘waxes and wanes’ over time [6]. The determinants of an individual’s risk of MASH and advanced fibrosis/cirrhosis or HCC are still not clear.

The liver function/physiology is characterized by sexual dimorphism, and the pathogenic mechanisms involved in the onset and progression of liver disease differ, at least in part, according to sex and sex-related factors [7], as seen in Figure 1. This observation has been further discussed in a recent large-scale analysis of hepatic transcriptomic profiles that stressed the sexually dimorphic nature of MASLD and its association with the progression of liver disease, suggesting that sex should be always considered as one of the main non-modifiable risk factors for patients’ stratification [8]. Susceptibility to MASLD is also influenced by a complex interplay between environmental and genetic factors [9]. In the past fifteen years, genome-wide association studies (GWAS) led to the identification of several single nucleotide polymorphisms (SNPs) that are linked to hepatic steatosis and progressive forms of the disease. The majority of the SNPs affect genes encoding for proteins involved in lipid metabolism. Specifically, the risk variants in *PNPLA3*, *TM6SF2*, *MBOAT7*, *GCKR* and the protective variant in *HSD17B13* genes are mostly related to the lipid handling in the hepatocytes and are associated with MASLD onset and progression [10–12]. The genetic variant rs72613567:TA in the gene *HSD17B13*, associated with a reduced risk of MASH and fibrosis, encodes an uncharacterized member of the hydroxysteroid 17-beta dehydrogenase family. The 17beta-hydroxysteroid dehydrogenases are important enzymes in steroid metabolism, particularly in the regulation of the biological effects of steroid hormones, but these enzymes are also involved in the regulation of fatty acid metabolic pathways, affecting several bioactive lipid species (e.g., leukotriene B4), that have been implicated in lipid-mediated inflammation [13]. Although the exact mechanism by which this SNP protects against MASLD is still unclear, anti-*HSD17B13* therapies are ongoing in the setting of clinical trials [14,15].

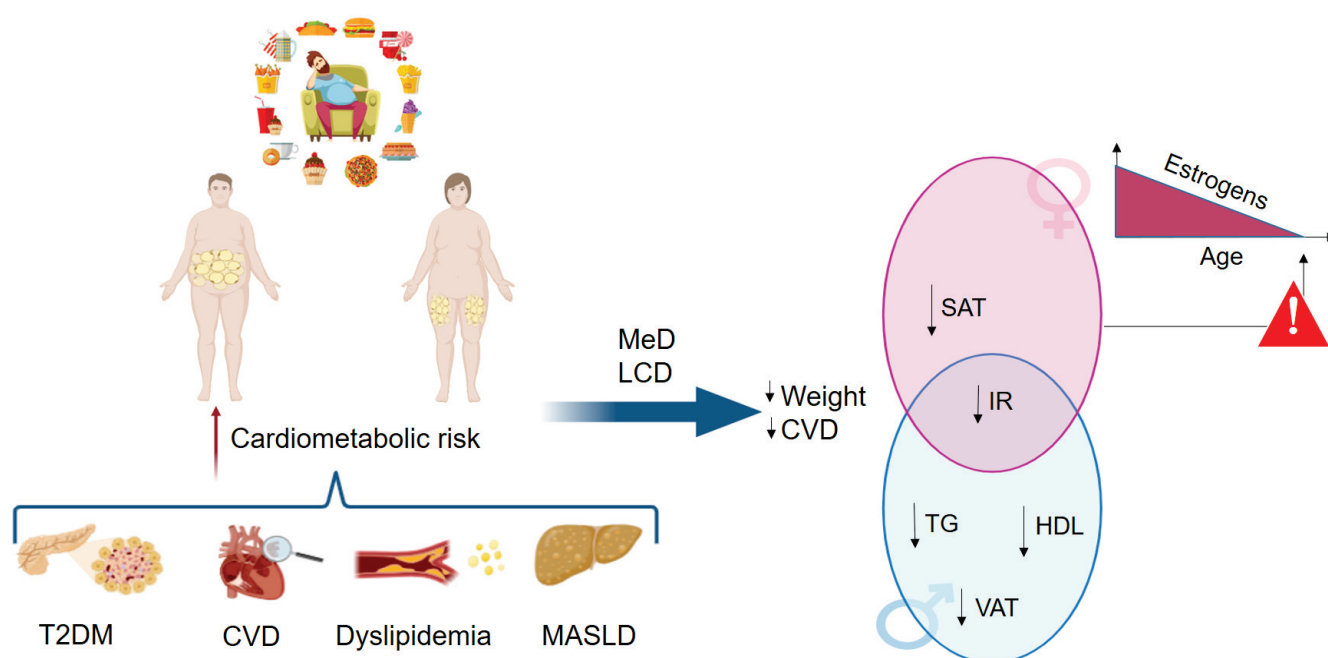


Figure 1. Impact of sexual dimorphism on the beneficial effects of diet.

Unhealthy lifestyle behaviors contribute to weight gain in both sexes, with a predominantly subcutaneous localization in women and visceral localization in men. Overweightness/obesity increase the cardiometabolic risk through the development of type 2 diabetes, arterial hypertension, dyslipidemia, and hepatic steatosis. Mediterranean and the low-

carbohydrate diets are highly recommended to reduce weight and improve metabolic derangement and cardiovascular risk. However, the physiological decrease in estrogens levels later in life predisposes women to a metabolic perturbation that may increase the risk of cardiometabolic events again. Abbreviations: CVD, cardiovascular disease; HDL, high density lipoprotein cholesterol; IR, insulin resistance; LCD, low-carbohydrate diet; MASLD, metabolic-dysfunction associated steatotic liver disease; MeD, Mediterranean diet; SAT, subcutaneous adipose tissue; T2DM, type 2 diabetes mellitus; TG, triglycerides; VAT, visceral adipose tissue.

Among the modifiable risk factors, sedentary lifestyles and changes in dietary patterns have contributed to the dramatic increase in MASLD prevalence, together with the rapidly progressing epidemics of both adult and childhood obesity [16]. In this regard, lifestyle intervention is considered the first-line approach to induce weight loss and to improve liver injury by histology or non-invasive biomarkers, as suggested by the last published joint Clinical Practice Guidelines on the management of MASLD [17]. In this review, we aimed to summarize the available data on the role of sexual dimorphism, in terms of hormonal perturbation, during the onset and progression of MASLD and its impact on conventional and emerging therapies for MASH.

2. The Importance of Sexual Dimorphism in MASLD: The Relevance of Steroids Metabolism

MASLD is a sexually dimorphic disease with a prevalence that is generally higher in men compared to women. Consistently, the prevalence of MASH, advanced hepatic fibrosis, as well as the occurrence of liver-related complications, is 2/4-fold higher in men than women [18]. However, the molecular mechanisms underlying sexual dimorphism in MASLD remains an important unmet question. Glucocorticoids and sex hormones (estrogens and androgens) are primarily responsible for differences among sexes also through the modulation of both glucose and lipid metabolism, but they can exert detrimental effects when their values fall outside the physiological range [19]. Alterations in sex hormones levels in both sexes are associated with lipid metabolism derangement, insulin resistance, T2DM, and hepatic steatosis. Consistently, hormone replacement/deprivation therapies may reverse MASLD [20]. Looking at the metabolic differences between men and women, we observe that premenopausal women display a better metabolic profile compared to men and postmenopausal women, pointing out on the protective effect of estrogens. This effect is mediated by decreasing the rate of lipolysis, the flux of free fatty acids (from the adipose tissue to other organs, including the liver), de novo lipogenesis, and by increasing fatty acids oxidation, thus reducing lipid storage and hepatic steatosis [21]. In women, estrogens prevent metabolic syndrome and the development of cardiovascular diseases (CVD), while low levels of estrogens influence the onset of MASLD [22,23]. In particular, the decrease in estrogens levels after menopause is associated with a high risk of developing MASH and severe hepatic fibrosis regardless of the most representative confounders (i.e., age, weight, visceral adiposity) and several studies reported a similar prevalence of MASLD and MASH in men and postmenopausal women [24–26]. Furthermore, postmenopausal women show a higher prevalence of advanced fibrosis ($F \geq 2$) compared to fertile women, while conflicting data on the impact of estrogens in the progression of liver disease are reported in the latter group [27,28]. Sexually dimorphic role of androgens in human metabolic diseases is an emerging topic, with female androgen excess and male androgen deficiency sharing an overlapping adverse metabolic phenotype, including abdominal overweight/obesity, glucose metabolism impairment, and MASLD [29]. The beneficial effects of androgens are well described in both animal and human studies in which their physiological levels are able to protect against MASLD preventing or attenuating the features of metabolic

syndrome [20]. Conversely, some androgenic species such as dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS) are associated with insulin resistance and with the most important histological features of MASLD [29–31]. High levels of androgens in women exert a detrimental effect on metabolic pathways and can be associated with other diseases. For example, hyperandrogenism is a hallmark in women with polycystic ovary syndrome (PCOS). The prevalence of MASLD in women with PCOS ranges from 15% to 55%, while the prevalence of PCOS in women with MASLD reaches 77%, suggesting that androgen excess in women likely play a role in the onset of MASLD [32,33]. However, the pathogenetic role of androgens in the onset and worsening of MASLD remains to be fully explored. According to the hypogonadal–obesity–adipokine hypothesis, the excess of adipose tissue promotes the conversion of testosterone to estradiol via aromatase activity. Estradiol inhibits the hormone kisspeptin which, in turn, impairs testosterone production. This mechanism is exacerbated by leptin that is released in large amounts from the inflamed and hypertrophic adipose tissue, impacting the gonadal axis, thus impairing testosterone production. Low testosterone levels promote adipocytes differentiation and inflammation, thus enhancing insulin resistance [34,35].

Therapeutic approaches in the setting of MASLD are focused on the improvement of metabolic abnormalities by targeting different metabolic pathways (including lipids, hormones, etc.). In this context, sex is not always considered for patients' risk stratification in clinical trials; therefore, in the following paragraphs, we will summarize the most important studies describing the sexual dimorphism in response to conventional and emerging therapies.

3. Impact of Sexual Dimorphisms on Conventional and Emerging Therapeutic Strategies

3.1. Diet-Based Treatment

Sedentary lifestyle, low fiber diet, high intake of simple sugars, saturated fats, and ultra-processed foods have dramatically contributed to the epidemic of obesity, T2DM, and steatotic liver disease over the years [36]. Several studies demonstrated the benefit of a healthy lifestyle in reducing hepatic steatosis [37–39]. However, the optimal dietary regimen for preventing and managing MASLD in both men and women remains to be defined and, to date, sex-specific nutritional interventions are scanty. From a nutritional perspective, dietary patterns may modulate the risk of metabolic diseases in a sex-specific manner since many organs and tissues are characterized by sexual dimorphism [40,41]. Moreover, distribution of body fat composition has important differences according to gender, with a prevalent subcutaneous localization in pre-menopausal women and with a visceral localization in men [42]. Specifically, visceral adiposity in men is associated with an increased risk of metabolic diseases such as dyslipidemia, T2DM, hepatic steatosis, and CVD. Conversely, CVD risk is lower in pre-menopausal women compared with men [42]. Sex hormones have an impact on fat distribution and cardiometabolic risk as demonstrated in studies on female-to-male transsexual patients, who lose gluteal-femoral fat and gain visceral adipose tissue after the appropriate hormone replacement therapy [43,44]. In addition, as compared to subcutaneous fat, visceral adipocytes are characterized by a high expression of glucocorticoid receptors that, along with a high activation of the hypothalamic–pituitary–adrenal axis, is responsible of the preferential fat storage in visceral depots associated with insulin resistance and with an increased risk of CVD [45,46]. In women, lipolytic activity in the adipose tissue is reduced and post-load fatty acids are stored in the subcutaneous fat as a reservoir. Conversely, visceral fat, which predominates in men, is more metabolically active and more susceptible to lipolysis. This different energy distribution/storage is also due to the different physiological role during life, as men

spend most of their energy on muscular activity, whereas women store energy to maintain reproductive capacity when energy reserves are limited [47,48].

Westerterp-Plantenga et al. explored the effect of diet switch (high protein to carbohydrate) in both sexes, showing that the high-protein diet was associated with greater energy expenditure and fatty acid oxidation in men and with high satiety in women [49]. A study on 782 subjects with overweight/obesity (506 women and 277 men) who underwent a low-calorie diet for six months showed that pre-menopausal women were less responsive to the diet compared with men in terms of cardiometabolic risk parameters such as triglycerides and total cholesterol levels [50]. Similarly, other studies have shown the long-term beneficial effects of the Mediterranean diet on cardiovascular health in men, who maintained a better metabolic profile over time compared with women [51–53]. This sexual dimorphism is supported by the decrease in estrogens levels over time that in post-menopausal women leads to an impairment in lipid metabolism that is typical during the physiological transition toward menopause. Vitale et al. showed that the regulation of glucose homeostasis in 156 adults at high risk of developing T2DM, randomized to receive a high vs. low glycemic index diet, was linked to sex-differences in body composition (fat distribution) and energy metabolism. Specifically, they observed that women were more sensitive to metabolic response due to the protective effects exerted by estrogens [49]. As with other dietary regimens, the ketogenic diet seems to be more effective in men compared with women and, even in this case, sex difference is due to the effect exerted by estrogens [54,55]. A study performed on 42 subjects with obesity who underwent a very low-energy ketogenic nutritional intervention for 45 days displayed no differences among sexes since all the participants significantly reduced weight and improved their inflammatory status. However, after sex and age stratification, men showed a better response in terms of body weight loss compared with fertile women and, among women, those with menopause showed the worst response to the intervention [54]. A summary of the studies described in the paragraph is reported in Table 1.

Table 1. Sexual dimorphism in response to conventional and emerging therapies. Abbreviations. F, females; M, males; na, not available.

Ref.	n, Sex: Age	Intervention and Overall Dosage	Conclusions
Nutritional intervention			
Trouwborst et al. [50]	n = 782 M: 276 (42.5 ± 6.0) F: 506 (41 ± 6.3)	6-month low-calorie diet	Women were less responsive to the diet compared with men in terms of cardiometabolic risk parameters
Bédard et al. [51]	n = 69 M: 37 (42.6 ± 7.3) F: 32 (41.2 ± 7.3)	4-week MeD	Men improved cardiovascular health through the reduction in postprandial insulin concentration
Leblanc et al. [52]	n = 123 M: 64 (41 ± 7.9) F: 59 (41.8 ± 6.7)	12-week MeD	Men showed a significant improvement of metabolic profile
Carruba et al. [53]	n = 115 Post-menopausal women	6-month MeD	Traditional Mediterranean diet significantly reduces endogenous estrogen in healthy postmenopausal women.
Vitale et al. [56]	n = 156 M: 74 (55.7 ± 10.7) F: 82 (55.1 ± 10.7)	12-week high- versus low-GI diet	Women, compared to men, showed a better metabolic improvement
D’Abbondanza et al. [54]	n = 70 M: 28 (20–62) F: 42 (17–67)	25-day very low-carbohydrate ketogenic diet	Men compared to pre-menopausal women, showed a better response in terms of body weight loss and improvement in MASLD
Muscogiuri et al. [55]	n = 42 M: 21 (37.7 ± 10.7) F: 21 (32.7 ± 8.7)	45-day very low-energy ketogenic diet	Men compared to women showed a better response in terms of weight loss and reduction in inflammation

Table 1. Cont.

Ref.	n, Sex: Age	Intervention and Overall Dosage	Conclusions
Antihyperglycemic treatment			
Gallwitz et al. [57]	n = 3375 M: 1673 (56.8 ± 9.8) F: 1702 (55.9 ± 10.1)	Dulaglutide 1.5 mg and 0.75 mg for 12 months	Significant improvement in glycaemic control irrespective of gender
Onishi et al. [58]	n = 855 M: 649 (56.9 ± 10.1) F: 206 (58.7 ± 11.3)	Dulaglutide 0.75 mg for 26 weeks	Body weight reduction during treatment is more pronounced in women compared to men
Mirabelli et al. [59]	n = 40 (57.5 ± 6.6) M: 18 (na) F: 22 (na)	Liraglutide 1.2 mg or 1.8 mg for a minimum follow-up of 5 years	Prolonging treatment exerted a lasting benefit in women
Buysschaert et al. [60]	n = 184 (59 ± 11) M: 110 (na) F: 74 (na)	Exenatide 5 µg or 10 µg for 9 and 12 months	Body weight reduction during treatment with exenatide is more pronounced in women compared to men
Quan et al. [61]	n = 105 M: 51 (48.4 ± 9.8) F: 54 (49.1 ± 10.4)	Exenatide 5 µg + metformin 0.5 g for 4 weeks Exenatide 10 µg + metformin 0.5 g for 24 weeks	Combination therapy showed better results in women compared with men
Lipid lowering therapy			
Mombelli et al. [62]	n = 337 M: 171 (57.5 ± 9.2) F: 166 (59.4 ± 8.8)	Atorvastatin or rosuvastatin 10 mg/day Pravastatin and simvastatin 20 mg/day Fluvastatin 80 mg/day	Women compared with men showed a greater reduction in LDL-C
Smiderle et al. [63]	n = 495 M: 164 (59.9 ± 11.1) F: 331 (62.3 ± 10.7)	Simvastatin or atorvastatin for approximately 6 months	Adverse drug reactions were more frequent in women than in men
Sever et al. [64]	n = 27,564 M: 20,795 (62 ± 9) F: 6769 (64.1 ± 8.8)	Evolocumab or placebo for 2.2 years	Similar efficacy regardless of age in both men and women
Bittner et al. [65]	n = 18,924 M: 14,162 (mean age 57) F: 4762 (mean age 62)	Alirocumab 75 mg or placebo for a median follow-up of 2.8 years	Improvement of cardiovascular outcomes regardless of sex
Nucleotide-based therapy			
Fabbrini et al. [66]	Study 1: n = 55 (24–65) M: 24 (na) F: 31 (na) Study 2: n = 9 (38–61) M: 8 (na) F: 1 (na)	Study 1: Placebo or JNJ-0795 10/25/75/200/400 mg Study 2: Placebo or 75 mg JNJ-0795	A single dose of GalNac-conjugated <i>PNPLA3</i> siRNA reduced liver fat content, but no sexual dimorphism has been currently described concerning treatment response

From a basic research point of view, the study of the interplay between sexual dimorphism and diet intervention in murine models is biased by the fact that the majority of the studies are limited to the male sex because of the variability of estrogens levels during estrous cycles in females [67–69]. However, studies performed on both sexes revealed conflicting results compared with those observed in humans pointing out the importance of age stratification. For example, a study aimed at exploring sex and age differences in mice fed with a ketogenic diet showed that male mice were more predisposed to develop glucose intolerance and insulin resistance regardless of age, while the reverse happened in female mice who were characterized by an increase in CVD risk but were protected against the risk of developing diabetes [70]. There are several preclinical studies on the effects of a Western diet on both sexes, and the overall results suggest that young male mice are more susceptible to diet-induced obesity when fed with a high fat diet. Conversely, young female mice show a greater capacity to use fat as a source of fuel, increasing energy expenditure, and displaying a better immune response when exposed to a fat diet [71–73]. Data on aged animals are less clear, suggesting a worse response to a high fat diet over time

for both sexes. Specifically, old females gain weight and worsen glucose profile more easily compared with males, and this is in part due to the changes in sex hormones [74–76]. All the evidence suggests the importance of sex and age stratification to understand differences in diet response in preclinical studies. Overall, a regular healthy and varied diet with an adequate nutrient distribution seems to be the best option for improving metabolism in subjects with steatotic liver disease and metabolic comorbidities, probably contributing to the physiological range of hormone levels, thus mitigating their detrimental effects on metabolic pathways.

3.2. Antihyperglycemic Treatment

Sexual dimorphism plays an important role in the pathophysiology of MASLD through insulin resistance and adiposity [77,78]. Generally, men receive a diagnosis of T2DM earlier in life compared to women. Conversely, the prevalence of T2DM in women increases after menopause. The pathophysiologic explanation of these sex specific differences is mainly the loss of estrogens later in life and its impact on glucose metabolism [79]. Knock-out (KO) mice for the estrogen receptor alpha (ER α) (LERKO model) show hyperglycemia during fasting as a consequence of the increased gluconeogenic activity [75]. In the aromatase KO model, sexual dimorphism is more evident, since only male mice show glucose intolerance and insulin resistance, which are restored after estrogens replacement [80–82]. This was also demonstrated in T2DM rat models using estradiol (E2) treatment, which increases insulin levels through the G protein-coupled estrogen receptor (GPER) [83]. Similarly, androgens are able to modulate glucose metabolism. High testosterone levels are associated with an increased risk of T2DM in women compared with men [83]. In addition, androgens improve insulin sensitivity in both sexes, enhancing glucose utilization in the skeletal muscle [84]. A sexual dimorphism has also been described for the androgens receptor (AR) in pancreatic β -cells. Specifically, the testosterone-AR complex in males is able to enhance insulin secretion by potentiating the insulinotropic effect of the glucagon-like peptide 1 (GLP-1), while in females, the excess of androgens led to insulin hypersecretion, thus promoting mitochondrial dysfunction and oxidative injury [85,86].

Among the anti-hyperglycemic drugs, GLP-1 receptor agonists (RAs) are a class of glucose-lowering drugs approved for the treatment of T2DM [87]. Specifically, in patients with T2DM, GLP-1 RAs prevent the occurrence of clinical events over time such as cardiovascular outcomes, diabetes complication, and all-cause mortality by improving glycemic control and by reducing body weight and insulin resistance [87,88]. The main therapeutic goal in the management of T2DM is the reduction in glycated hemoglobin (HbA1c) levels, since HbA1c levels lower than 7% are associated with a lower risk of complications over time [89]. Several studies show that the reduction in HbA1c during exenatide or dulaglutide treatment is similar in men and women and depends on baseline HbA1c levels [57,58,90]. Conversely, when administered in combination with metformin, exenatide exerts a stronger effect in women compared with men, as shown by the reduction in HbA1c levels after treatment (from 8.8% to 6.8% and from 8.9% to 7.5%, respectively), suggesting better glycemic control in women compared with men [57]. However, when stratified by age, women using liraglutide treatments show better glycemic control in the subgroup of those younger than 65 years compared to the counterparts, underlining the importance of age-related estrogens reduction after menopause [59]. The latter consideration is sustained by a study demonstrating that lower baseline levels of HbA1c are associated with a better glycemic control in men but not in women, in which previous treatment of metformin seems to prevent treatment failure with GLP-1 RA [91]. GLP-1 RA induces weight loss and has also been approved for the treatment of obesity [60]. Several studies show that body weight reduction during treatment with exenatide is more pronounced in women

compared to men, and similar results are also reported the same results after dulaglutide and liraglutide treatment [58–61,90,91]. In a recent study, Pelusi et al. showed that in men with low testosterone levels, treatment with semaglutide is safe and may restore androgens levels mainly through the reduction in body weight [92]. The molecular basis of this sexual dimorphism can be explained at least in part by the increased drug exposure observed in women, which is about 30% higher compared to men of comparable weight, suggesting that female sex may be an independent predictor of weight loss [93].

3.3. Lipid Lowering Treatment

Dyslipidemia is a common metabolic dysfunction in subjects with MASLD with a prevalence ranging from 60% to 70% [94]. This atherogenic profile (characterized by high levels of low-density lipoproteins cholesterol (LDL-C), low levels of high-density lipoprotein cholesterol (HDL-C), and high levels of triglycerides) is associated with an increased risk of developing CVD over time regardless of sex [95]. However, pre-menopausal women develop CVD about 10 years later compared to men, highlighting once again the protective role that estrogens exert on lipid metabolism [96]. In addition, women are more responsive to statin treatment compared with men [97]. From a mechanistic point of view, sexual dimorphism may be in part explained by the complex interplay between sex hormones and cardiovascular risk. For example, single nucleotide polymorphisms (SNPs) in the gene *CYP19A1*, encoding for the aromatase enzyme that is involved in the final step of estrogen synthesis in both men and postmenopausal women, are associated with hypertension, apolipoprotein B levels, and insulin resistance [98]. In addition, high estradiol levels in fertile women are associated with an increased expression of LDL receptors and this interaction contributes to the improvement in lipid levels during lipid-lowering treatment [99]. Estrogen signaling pathways may also increase the hepatic reverse transport of cholesterol in women, thus promoting its removal from peripheral tissues and reducing cardiovascular risk [98]. Finally, estrogens may recognize highly conserved genomic sequences, namely estrogen response elements (EREs), in the promoter of several genes by regulating their expression, thus modulating metabolic pathways (as described later in the paragraph on nucleotide-based therapies and *PNPLA3* gene), as show in Figure 2 [100].

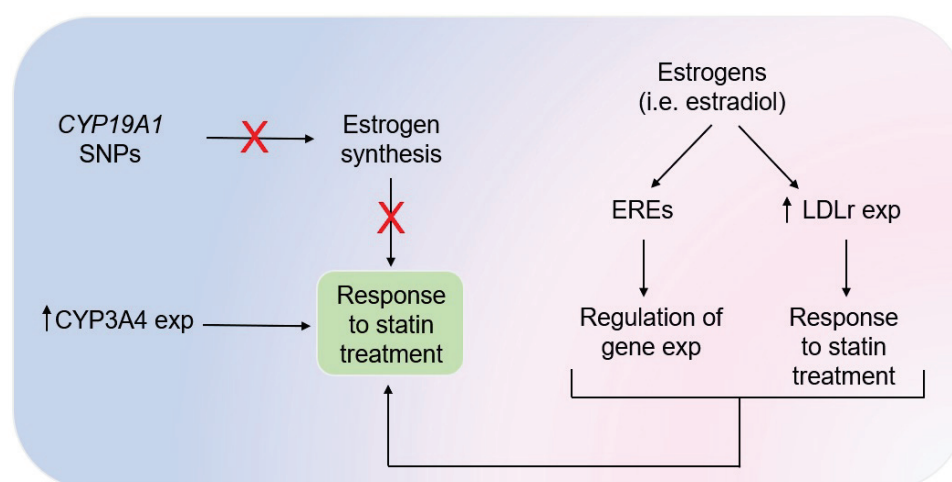


Figure 2. Interplay between sex hormones, lipid lowering drugs, and therapy response. Estrogens exert a protective effect on lipid metabolism in both men and women, but the latter group shows a better response to the statin treatment due to the favorable interaction between sexually dimorphic genes and drugs metabolism as well as the direct interaction between estrogens and sexually dimorphic genes. Abbreviations: *CYP19A1*, CYP19A1-cytochrome P450 family 19 subfamily A member 1 gene; *CYP3A4*, cytochrome P450 3A4 gene; EREs, estrogen response elements; exp, expression; LDLr, low-density lipoprotein receptor; SNPs, single nucleotide polymorphisms.

Statins therapy in MASLD is indicated to prevent CVD, even if their efficacy for treating MASH remains to be proved in large randomized control trials with histological endpoints [101]. The efficacy of statins in improving the plasma lipid profile of women with dyslipidemia is lower compared to men and this can be at least in part explained by the fact that drugs such as atorvastatin and simvastatin are predominantly metabolized by CYP3A4, which is more expressed in women compared to men [62]. In addition, statins improve inflammatory status modulating the expression of several pro-inflammatory cytokines such as interleukin-6 (IL-6), tumor necrosis factor- α (TNF- α), and C reactive protein and, also in this case, women are more responsive than men [97]. A sexual dimorphism has also been described for the occurrence of adverse events. Women experienced myalgia and toxic skeletal muscle injury more frequently compared to men, while the reverse has been observed about liver function impairment [63,102]. This sexual dimorphism can be explained by the different pharmacokinetics that, in turn, depend on the distribution of body fat that affects drug availability [103].

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a key regulator of cholesterol metabolism that increases LDL-C levels through the degradation of the LDL receptors. High PCSK9 concentrations are associated with MASLD and with an increased risk of CVD. PCSK9 inhibitors are able to block the degradation of LDL receptors, thus increasing LDL-C uptake from the circulation [103–105]. Recent studies have observed different PCSK9 levels among sexes, suggesting a related sexual dimorphism. In addition, PCSK9 concentrations differed according to age and to the presence of metabolic abnormalities. However, clinical trials did not show evidence about the existence of sexual dimorphism in response to PCSK9 inhibitors, confirming the same strong effect in reducing LDL-C in both sexes [64,65]. Further studies are needed to better understand the impact of sexual dimorphism on lipid lowering response.

3.4. Nucleotide-Based Therapies

It is well known that genetic background contributes to the onset and progression of MASLD [106]. Genome-wide association studies have identified several genes involved in the regulation of lipid metabolism (i.e., *PNPLA3*, *TM6SF2*, *MBOAT7*, *GCKR*, *HSD17B13*) that contribute to the progression of liver damage [107]. Patatin-like phospholipase domain-containing 3 (*PNPLA3*) is a triacylglycerol lipase located on lipid droplets in both adipose tissue and liver, which is involved in the hydrolysis of triglycerides [108]. *PNPLA3* protein expression is increased in subjects with obesity compared to those without, while the presence of the rs738409 SNP encodes for a loss of function variant that promote triglyceride retention in lipid droplets, favoring the development of hepatic steatosis and enhancing inflammation [109]. The association between the *PNPLA3* SNP and sexual dimorphism emerged over ten years ago in a meta-analysis with the aim to assess the strength of the effect of the *PNPLA3* rs738409 SNP on MASLD and the severity of liver disease across different populations. The analysis of 16 studies revealed the strong transversal association between genetic variants and MASLD [110]. Other studies showed a strong association among the rs738409 variant and increased ALT levels in men regardless of age and in women over 50 years. Considering the differences in estrogen levels in fertile and postmenopausal women, the authors hypothesized the existence of an interaction between the *PNPLA3* SNP and estrogens [110,111]. Recently, in a longitudinal study with a median follow-up of 7 years on a large European cohort of biopsied-proven MASLD patients, the authors found that women without obesity, older than 50 years, and carrying the rs738409 GG risk genotype, displayed a high risk of developing liver-related events over time compared to the other subgroups of patients [112]. This evidence points out the importance of risk stratification in the heterogeneous population of MASLD subjects and

on the importance of *PNPLA3* genotyping as well as sex stratification for the identification of high-risk groups.

Over the last few years, oligonucleotide-based therapies such as small-interfering RNA (siRNA) and antisense oligonucleotide (ASO) have been developed for the treatment of MASLD by reducing the expression of the *PNPLA3* gene [113]. Preclinical studies showed that these new drugs are able to interfere with *PNPLA3* synthesis by silencing the corresponding gene, thus reducing liver disease. For example, the hepatocyte-targeted N-acetylgalactosamine (GalNac)-conjugated ASO improves MASH and hepatic fibrosis in *PNPLA3* rs738409 GG knock-in mice fed with a steatogenic diet; *PNPLA3* silencing through short-hairpin RNA improves liver steatosis in *PNPLA3* rs738409 GG knock-in mice fed a high fructose diet; the administration of lipid nanoparticles containing siRNA against human *PNPLA3* improves histological features of MASH including liver fibrosis, shown in Figure 3 [114–117]. In humans, the results of a phase 1 trial revealed that a single dose of GalNac-conjugated *PNPLA3* siRNA significantly reduced liver fat content [66,118], but no sexual dimorphism has been currently described in the treatment response. It is known that the *PNPLA3* gene is under nutritional control, through the liver X receptor and the steroid regulator element binding protein (SREBP)-1c pathway, which is involved in the regulation of fatty acid synthesis [119]. At the same time, estrogens can modulate lipogenic genes, such as those belonging to the SREBP family, promoting hepatic steatosis [120]. Sex hormones, like estrogens, also modulate lipogenic genes such as the SREBP family, changing lipid metabolism and then contributing to the onset of liver steatosis [116]. Recently, an interaction between estrogens and the *PNPLA3* genetic variant has been described, which seems to negatively affect the progression of MASLD [121]. Specifically, the authors found an increase in *PNPLA3* transcription in immortalized cell line and tissue-derived liver organoids treated with ER α agonists. After cells exposure to an excess of fatty acid, they observed a specific increase in the mutated protein with a contextual accumulation of intracellular lipids and collagen deposition. The removal of the *PNPLA3*-ERE by CRISPR/Cas9 led to a reduced response to ER α treatment, demonstrating the existence of a link between female sex hormones, *PNPLA3* SNP, and impaired lipid metabolism [121].

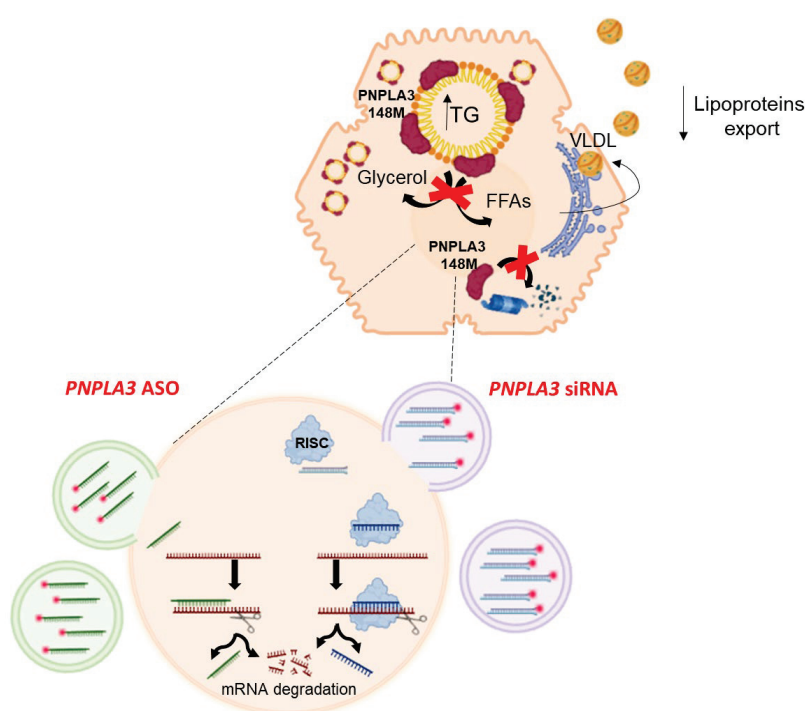


Figure 3. Antisense oligonucleotide and small-interfering RNA mechanisms of action.

The mutant 148 Isoleucine to Methionine (I148M) protein variant in the patatin-like phospholipase domain-containing 3 (*PNPLA3*) gene results in a loss of function of the protein determining triglyceride retention in the hepatocytes, thus resulting in hepatic steatosis. The mutated protein is also resistant to ubiquitylation and accumulates in lipid droplets. Oligonucleotide-based therapies, such as small-interfering RNA and antisense oligonucleotide, have been developed in recent years for the treatment of MASLD by reducing the expression of the *PNPLA3* gene carrying the genetic variant. Abbreviations. ASO, antisense oligonucleotide; FFAs, free fatty acids; *PNPLA3*, patatin-like phospholipase containing domain 3; siRNA, small-interfering RNA; TG, triglycerides; VLDL, very low-density lipoproteins; RISC, RNA-induced silencing complex.

4. Conclusions

MASLD is a sexual dimorphic liver disease tightly associated with metabolic comorbidities, and a multidisciplinary therapeutic approach is required for managing these heterogeneous patients. Lifestyle interventions are highly recommended for all MASLD subjects regardless of sex, along with treatment of metabolic co-morbidities such as dyslipidemia and T2DM. However, pharmacological responses can be different among sexes. Sex hormones play an important role in the modulation of glucose and lipid metabolism, affecting also drug metabolism in the liver. For this reason, an appropriate consideration of sex and hormonal status could be useful to understand sexual dimorphism in MASLD risk and in response to pharmacological treatments. Finally, the study of the interplay between sexual dimorphism, hormones, and sexually dimorphic genes may help to identify novel potential therapeutic targets, leading to a more defined sex-specific personalized therapy.

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Review

The Role of Dietary Ingredients and Herbs in the Prevention of Non-Communicable Chronic Liver Disease

Monika Maćków^{1,2}, Tomasz Dziubyna^{3,*}, Tatiana Jamer⁴, Dmytro Slivinskyi⁵, Tomasz Pytrus⁴, Katarzyna Neubauer⁶, Małgorzata Zwolińska-Wcisło^{3,7}, Andrzej Stawarski⁴, Ewa Piotrowska¹ and Dorian Nowacki¹

- ¹ Department of Human Nutrition, Faculty of Biotechnology and Food Science, Wrocław University of Environmental and Life Sciences, Chelmońskiego 37, 51-630 Wrocław, Poland; monika.mackow@upwr.edu.pl (M.M.); ewa.piotrowska@upwr.edu.pl (E.P.); dorian.nowacki@upwr.edu.pl (D.N.)
- ² Regional Specialist Hospital in Wrocław, Research and Development Center, Kamieńskiego 73A, 51-124 Wrocław, Poland
- ³ Unit of Clinical Dietetics, Department of Gastroenterology and Hepatology, Faculty of Medicine, Jagiellonian University Medical College, M. Jakubowskiego 2, 30-688 Kraków, Poland; m.zwolinska-wcislo@uj.edu.pl
- ⁴ 2nd Department and Clinic of Paediatrics, Gastroenterology and Nutrition, Wrocław Medical University, M. Curie-Skłodowskiej 50/52, 50-367 Wrocław, Poland; tatiana.jamer@umw.edu.pl (T.J.); tomasz.pytrus@umw.edu.pl (T.P.); andrzej.stawarski@umw.edu.pl (A.S.)
- ⁵ Department of Fruit, Vegetable and Plant Nutraceutical Technology, Faculty of Biotechnology and Food Science, Wrocław University of Environmental and Life Sciences, Chelmońskiego 37, 51-630 Wrocław, Poland; dmytro.slivinskyi@upwr.edu.pl
- ⁶ Department and Clinic of Gastroenterology and Hepatology, Wrocław Medical University, Borowska 213, 50-556 Wrocław, Poland; katarzyna.neubauer@umw.edu.pl
- ⁷ Department of Gastroenterology and Hepatology, Faculty of Medicine, Jagiellonian University Medical College, M. Jakubowskiego 2, 30-688 Kraków, Poland
- * Correspondence: tomasz.dziubyna@uj.edu.pl; Tel.: +48-517-666-126

Abstract: Background: Liver diseases are among the most commonly diagnosed conditions, with the main risk factors being inappropriate lifestyles, including poor diet, excessive alcohol consumption, low physical activity and smoking, including electronic cigarettes. Non-communicable chronic liver diseases also often develop as a result of accompanying overweight and obesity, as well as type 2 diabetes. Methods: The literature on risk factors for non-communicable chronic liver diseases, which show a high strong influence on their occurrence, was analysed. Results: Measures to prevent non-communicable chronic liver disease include the selection of suitable food ingredients that have proven protective effects on the liver. Such ingredients include dietary fibre, probiotics, herbs, various types of polyphenols and fatty acids (omega-3). Conclusions: Because of their liver-protective effects, nutritionists recommend consuming vegetables, fruits, herbs and spices that provide valuable ingredients with anti-inflammatory and anti-cancer effects. These components should be provided with food and, in the case of probiotics, supplementation appears to be important. As a preventive measure, a diet rich in these nutrients is therefore recommended, as well as one that prevents overweight and other diseases that can result in liver disease.

Keywords: diet; liver disease; fiber; probiotics; polyphenols; omega-3 fatty acids; herbs

1. Introduction

The World Health Organization (WHO) defines non-communicable diseases (NCDs) as long-term chronic illnesses caused by a combination of genetic, physiological, environmental, and behavioral factors [1]. In this article, non-communicable diseases refer to chronic liver disease. Chronic liver disease (CLD), which includes alcoholic liver disease (ALD), metabolic dysfunction-associated steatotic liver disease (MASLD), autoimmune

liver disease, and liver cancer, is a major liver disease that is increasingly being diagnosed worldwide [2,3]. Liver diseases cause approximately two million deaths per year, ranking it as the 11th most common cause of death worldwide and accounting for approximately 4% of all deaths overall [4]. It is estimated that CLDs caused around 1.32 million deaths in 2017. Among genders, two-thirds were male and one-third were female [5]. The concept of metabolic dysfunction-associated steatotic liver disease recently emerged, instead of the previously used non-alcoholic fatty liver disease (NAFLD). According to experts, the new definition more accurately reflects current knowledge of hepatic steatosis—a disease associated with complex metabolic dysfunction. According to the definition, MASLD is a steatotic liver disease (SLD) in the presence of one or more cardiometabolic risk factor(s) and the absence of harmful alcohol intake. These cardiometabolic factors include hypertension, elevated levels of plasma triglycerides, low levels of high-density lipoprotein (HDL) cholesterol, dyslipidemia or type 2 diabetes (T2D) mellitus, and overweight or obesity. MASLD is currently the most common chronic liver disease in the world [3].

A summary of the most important risk factors for non-communicable chronic liver disease is shown in Figure 1.

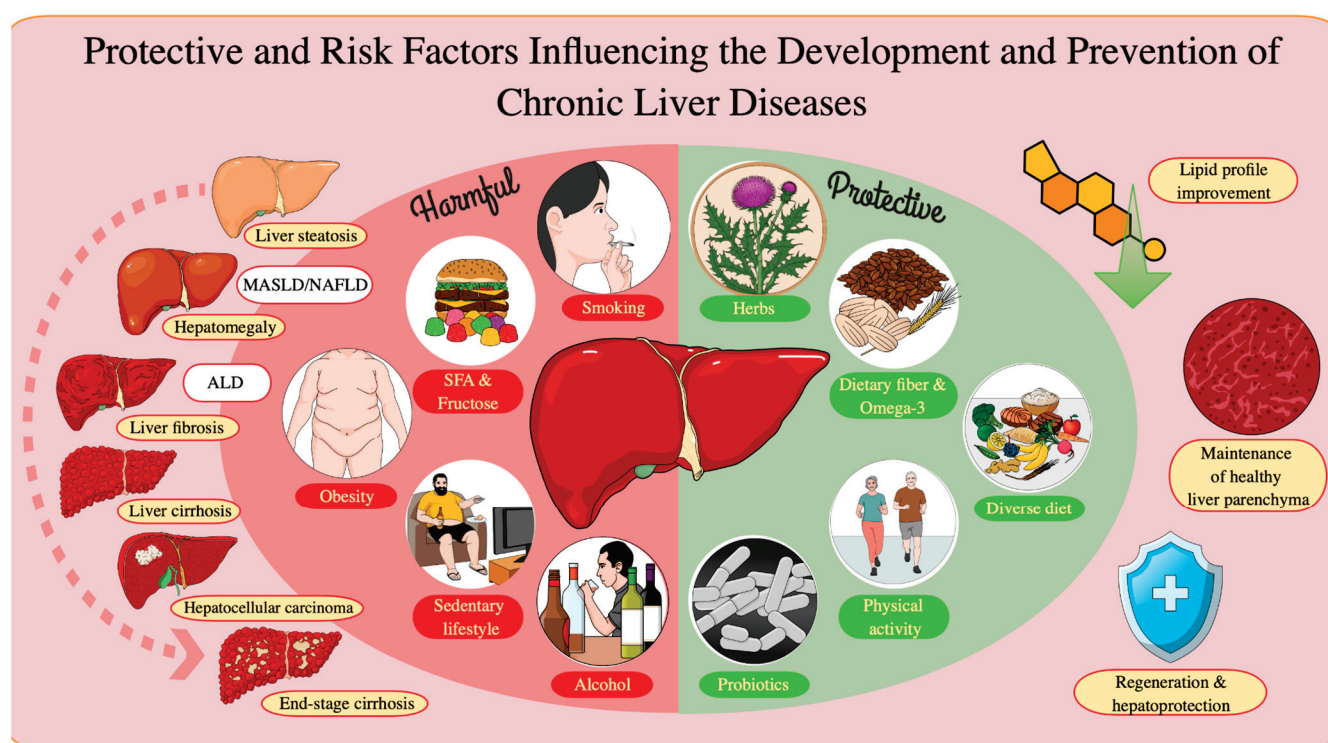


Figure 1. Negative and positive factors influencing the development of non-communicable chronic liver diseases.

2. Risk Factors of Non-Communicable Chronic Liver Diseases

2.1. Cardiometabolic Risk Factors

Risk factors for NCDs are associated with tobacco use, alcohol use, low physical activity, overweight and obesity, food choice, and dental health care [6]. These factors also contribute to the development of liver diseases. Lifestyle factors associated with increased risk of hepatocellular carcinoma (HCC) included past/current smoking, high alcohol consumption, poor diet quality, reduced coffee intake, and prevalent obesity, but not physical activity. The study, conducted on a population living in Hawaii and other regions of the United States, included 753 cases of diagnosed HCC [7]. Factors associated with the occurrence of another liver disease, NAFLD, include high intake of saturated fat and fructose [8], coexistence of central obesity, and insulin resistance, making waist circumference and HOMA index a significant contributor to the occurrence of NAFLD [9].

Changes at the cellular level of adipose tissue increase the production of pro-inflammatory factors (mainly tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), plasminogen activator inhibitor-1 (PAI-1), transforming growth factor beta (TGF- β), C-reactive protein (CRP), enhance the release of significant amounts of free fatty acids, and reduce the secretion of anti-inflammatory factors, such as adiponectin). The consequence of this is the occurrence of increased inflammation that induces the development of MAFLD [10].

The main factors contributing to the development of MASLD include insulin resistance, obesity, or the underlying metabolic syndrome (dyslipidemia, hypertension, and diabetes). Obesity is defined as the excessive accumulation of adipose tissue. Its link with cognitive dysfunction is attributed to the metabolic consequences of visceral adiposity, namely hypertension, dyslipidemia, diabetes type 2, insulin resistance, a constellation referred to as the metabolic syndrome [11,12], and several types of cancer [13]. High BMI increases liver cancer mortality and the incidence of primary liver cancer. Obesity is an independent risk factor for the occurrence and mortality of primary liver cancer [14].

Liver diseases such as MASLD and non-alcoholic steatohepatitis (NASH) are very often causally associated with metabolic disorders such as overweight and obesity, hypertension, dyslipidemia, and diabetes. Obesity is also associated with hemodynamic changes, which may result in changes in the morphology of the heart itself and the functioning of its ventricles. A relationship was identified between the presence and severity of MASLD, in particular NASH and fibrosis, and an increased risk of cardiovascular disease. However, this relationship is dependent on the coexistence of a number of metabolic factors, such as T2D. Importantly, MASLD itself increases the risk of atherosclerosis, cardiomyopathy, arrhythmia, and increases morbidity and mortality from cardiovascular causes [3,15].

2.2. Alcohol

According to the WHO reports for 2016, approximately 5.3% of all deaths worldwide were caused by alcohol consumption, which is equivalent to approximately 3 million deaths worldwide [16]. Globally, two-thirds of alcohol-attributable deaths are NCD deaths, and this index is even higher in the European Union (EU), where alcohol consumption is the highest globally and where 75% of all alcohol-attributable deaths are due to NCDs, with cancer (29%) being the leading cause [17]. The causes of chronic liver disease, including liver cancer, vary from region to region, but the most frequent are typically chronic viral hepatitis and alcohol. Alcohol can initiate the development of liver cancer and is related to tumor progression [18]. Drinking more than 60–100 g of alcohol per day contributes to the development of HCC, and alcohol in amounts of 30–50 g/day contributes to the development of liver cirrhosis [19]. Chronic alcohol consumption is a risk factor in 20% to 50% of liver cirrhosis cases worldwide [20]. This is particularly relevant for non-communicable chronic liver diseases such as ALD. It is a liver disorder caused by excessive alcohol consumption. ALD to be one of the important triggers for other liver diseases, including MAFLD. This is because there is a significant infiltration of T lymphocytes in ALD, but their role is not yet well understood. It was suggested that excessive T-lymphocyte production plays an important role in the development of steatohepatitis associated with MASLD [21].

A study of 175 German patients from primary care centers identified, in addition to alcohol, other risk factors for liver fibrosis and cirrhosis, including concomitant obesity and diabetes [22].

2.3. Smoking

A WHO report published in 2023 shows that 15 years of the MPOWER program helped reduce global smoking prevalence from 22.8% to 17.0% in 2021 [23]. Smoking a cigarette generates over 4000 chemicals that have a deleterious impact on each part of the human body. It produces three main severe effects on the liver: oncogenic, immunological, and indirect or direct toxic effects. It results in the production of cytotoxic substances, which raises fibrosis and necro-inflammation [24]. Tobacco smoking, as shown by studies and meta-analyses, is significantly associated with NAFLD [25–27]. Nicotine, contained

in cigarettes, exacerbates hepatic steatosis through increased oxidative stress, hepatic cell apoptosis, and inactivation of adenosine 5-monophosphate-activated protein kinase, leading to increased hepatic lipogenesis. It is also associated with an increase in low-density lipoprotein (LDL) cholesterol, plasma triglycerides and insulin resistance, as well as a decrease in plasma HDL cholesterol levels, which is associated with the occurrence of diabetes, which is also a risk factor for non-infectious liver disease [28]. As one study [29] shows, people diagnosed with MAFLD who also smoke tobacco are also at risk of liver cancer and cardiovascular disease. Smoking is also associated with the incidence of MAFLD among overweight or obese children whose mothers smoked tobacco during pregnancy and were diagnosed with pre-eclampsia and hypertension [30].

In recent years, there was a significant increase in the use of electronic nicotine-dispensing devices, i.e., the so-called e-cigarettes [31]. E-cigarettes gained exceptional popularity, especially among young people, as e-cigarettes are seen as a healthier alternative to smoking classic cigarettes. Within the TackSHS project, a face-to-face survey was conducted in 2017–2018 in 12 European countries (Bulgaria, England, France, Germany, Greece, Ireland, Italy, Latvia, Poland, Portugal, Romania, and Spain). A total of 11,876 participants, representative of the ~15-year-old population in each country, provided information about the electronic cigarette. A total of 65.1% reported using an electronic cigarette in at least one room where smoking was prohibited [32]. A study to determine the effects of e-cigarette flavoring chemicals on HepG2 liver cell toxicity showed that e-cigarette ingredients such as vanillin, ethylvanillin, and ethyl maltol reduce the viability of HepG2 cells caused by long-term exposure to these ingredients [33].

The studies presented here indicate that it is advisable for pregnant women, those at risk of non-communicable liver disease, or those already diagnosed by a doctor, to stop smoking both traditional cigarettes and e-cigarettes, as smoking is one of the risk factors for liver disease and other associated diseases.

2.4. Low Physical Activity

Factors contributing to many chronic diseases include low levels of physical activity. The risk of NAFLD is associated with obesity, insulin resistance, and metabolic syndrome, all of which are associated with low levels of physical activity. As one Korean study showed, prolonged sitting time and reduced levels of physical activity were positively associated with the incidence of NAFLD among middle-aged individuals [34]. Additionally, among the US population, low levels of physical activity increased the risk of MAFLD [35]. However, it should be noted that research on the effects of exercise on chronic liver disease is relatively new [36]. Scientific reports in recent years mainly focused on NAFLD/NASH, MAFLD, and liver cancer. A study of patients diagnosed with liver disease from UK Biobank best demonstrates the benefits of physical activity for this patient group. The study analyzed data from 96,688 participants who recorded their physical activity using a wrist accelerometer. As a result, participants had a reduced risk of both overall CLD and increasing activity by an additional 2500 steps per person per day was associated with a 38% reduction in CLD and a 47% reduction in the development of NAFLD, regardless of the presence of obesity [37].

3. Dietary Treatment for the Prevention of Non-Communicable Chronic Liver Disease

Considering the risk factors associated with MAFLD, it seems that the overriding role in the treatment of MAFLD should be attributed to lifestyle modifications aimed at inhibiting the progression of the disease. These changes mainly involve reducing body weight, increasing the level of physical activity, and making a number of dietary changes. A reduction in body weight of as little as 5% significantly improves metabolic parameters and the patient's clinical condition [38]. Weight reduction improves serum glucose and prevents T2D, improves blood pressure, and reduces triglycerides (TG) and total cholesterol [39,40]. By implementing physical activity and dietary modifications over a period of 0.5–1 years, it

is possible to significantly reduce liver enzyme levels, increase cellular sensitivity to insulin, and reduce hepatic steatosis [41].

The literature [42–45] mentions the Mediterranean diet (MD) among the dietary interventions used in liver diseases. In particular, the capacity of Mediterranean diet to reduce the risk of development and progression of MASLD or NAFLD is attributed to the nutraceutical effect of bioactive compounds and phytochemicals with antioxidant and anti-inflammatory potential such as fibers, monounsaturated and omega-3 fatty acids, and phytosterols [43]. The TANGO study was conducted on a group of Chinese women diagnosed with NAFLD, divided into three groups: one following a Mediterranean diet, another combining the Mediterranean diet with pentadecanoic acid (C15:0) supplementation, and a control group. C15:0, an odd-chain saturated fatty acid found in dairy fat, was linked in epidemiological studies to a reduced risk of metabolic syndrome, T2D, and NAFLD. Elevated plasma levels of C15:0 are associated with lower liver fat content and favorable effects on the gut microbiome. In this study, the Mediterranean diet groups, particularly those with C15:0 supplementation, achieved significantly greater reductions in body weight, liver proton density fat fraction (PDFF), total cholesterol, gamma-glutamyl transferase, and triglycerides compared to the control group [46]. Similar results were obtained in other studies assessing the impact of MD on liver diseases [47,48]. For this reason, the Mediterranean diet is recommended by guidelines on NAFLD published by various associations [3].

4. Fiber

Various nutritional societies and bodies promulgated different definitions of dietary fiber (DF) worldwide. The EU regulation on the provision of food information to consumers [49] defines the fiber as “carbohydrate polymers with three or more monomeric units, which are neither digested or absorbed in the human small intestine”. DF was categorized as follows: “edible carbohydrate polymers naturally occurring in the food as consumed, edible carbohydrate polymers obtained from food raw material by physical, enzymatic, or chemical means and which have a beneficial physiological effect demonstrated by generally accepted scientific evidence, and edible synthetic carbohydrate polymers which have a beneficial physiological effect demonstrated by generally accepted scientific evidence”. The most popular definition in nutritional science divides DFs into two subgroups based on their solubility in water: soluble and insoluble [50]. Dietary fiber is found primarily in plant foods, such as whole grains, vegetables, fruits, and legumes [51]. In recent years, a number of scientific reports showed that cellulose plays a significant role in carbohydrate metabolism disorders [52,53], hypertension, and circulatory system diseases [54,55] as well as playing an important role in cancer prevention [56–58].

A study of 6613 participants in the United States assessed correlations between fiber intake and the incidence of NAFLD. A reduced risk of NAFLD was found in people who consumed fruits, whole grains, and vegetables which are the source of DF [59]. Other studies also confirm the positive effect of dietary fiber on the occurrence of NAFLD [60,61]. Proper amount of fiber intake, along with a low-energy diet, is linked with weight loss and NAFLD regression [62]. A study conducted in Iran on 158 people with NAFLD showed that fiber, along with other minerals and vitamins, can slow down the NAFLD disease progression [63]. A similar result was obtained by Polish researchers who gave people with NAFLD 28 and 12 g of fiber per day. As a result, a reduction in liver steatosis occurred in both groups, although the effect was similar and more pronounced in patients in the lower fiber group [62]. Other studies also confirmed the effect of fiber on improving liver function in people with NAFLD [64] and reducing the risk of mortality due to NAFLD [65]. Studies show that dietary fiber consumption reduces the risk of MASLD, which is important, especially in people who are at high risk of developing liver disease.

5. Polyphenols

Polyphenols are a group of organic compounds naturally found in plants. To date, at least 10,000 substances classified in this group were discovered. Among foods, rich sources of polyphenols include whole grains, spices, vegetables, fruits, chocolate, and beverages such as tea, coffee, and wine [66]. In terms of chemical structure, they are characterized by one or more aromatic rings and one or more attached hydroxyl functional groups [67]. Polyphenols are formed in plants under the action of pathogens or other stress factors, and thus constitute a defense mechanism for plants. This occurs in a similar way in humans, as it was noted that an increase in endogenous antioxidant levels occurs as a result of polyphenols stimulating the antioxidant system, which removes excess free radicals [68,69]. Classification of polyphenols is difficult due to the heterogeneity of chemical structure, but the number of phenolic rings and some structural elements make it possible to divide them into the following groups: flavonoids, phenolic acids, stilbenes, curcuminoids, and lignans [70]. Polyphenols have a variety of biological activities that support human health and can reduce the risk of diseases, including liver disease.

5.1. Flavonoids

Flavonoids are a diverse group of polyphenols having a C6-C3-C6 carbon skeleton, which consists of a pyran ring and two aromatic rings. Depending on the degree of oxidation and carbon structure, flavonoids are divided into flavones, flavonols, flavanones, isoflavones, flavanols, and anthocyanins [71]. The most common flavonoids in food are flavonols, soy isoflavones, and flavones. These compounds in foods mainly affect taste, color, protect vitamins and enzymes, and counteract fat oxidation [72].

It seems that the majority of ancient medical therapies that achieved success utilized flavonoids, a practice that persisted to the present day. Quercetin represents the flavonoid subgroup of flavones, demonstrating beneficial pharmacological properties in the management of liver conditions such as liver fibrosis, liver steatosis, fatty hepatitis, and liver cancer. The main portion of quercetin-type flavonols in our diets consists of quercetin glycosides, which are compounds where quercetin is bound to one or two glucose residues or to rutinose. Interestingly, the quantity of quercetin in food can be significantly affected by factors such as growing conditions; for instance, tomatoes grown organically tend to have higher levels of quercetin aglycone compared to conventionally cultivated tomatoes [73]. Overall, fruits and vegetables, especially cherries, cranberries, grapes, blueberries, apples, onions, and peppers serve as the primary sources of naturally occurring dietary quercetin in the typical Western diet [74,75]. Moreover, brewed black tea, along with red table wine and various fruit juices, were also identified as rich dietary sources of quercetin [76,77]. This flavonol is recognized to inhibit the advancement of liver fibrosis by modulating nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B)-mediated signaling pathways, decreasing the production of cytokines such as TNF- α , IL-6, IL-1 β , and IL-8 [78]. Moreover, it boosts antioxidant mechanisms mediated by glutathione and IL-10, while also reducing lipid peroxidation in cases of alcoholic liver disease in rats [79].

Procyanidins, epicatechin, and epigallocatechin and its gallate derivative (EGCG) are the most well-known flavanol molecules. Mostly present in dark chocolate and cocoa, epicatechin was shown to control hepatic and serum lipid profiles by upregulating fatty acid synthase, sterol regulatory element binding protein (SREBP), liver X receptor, and sirtuin (SIRT) [80–82]. It was also shown to reduce inflammatory damage and oxidative stress by inhibiting the NF- κ B signaling pathway. One of the most often consumed polyphenols in the public is EGCG, which is mostly found in green tea [83]. Numerous in vitro and animal studies provided evidence in favor of the theory that green tea may protect against liver cancer [84–86]. Regrettably, there are documented cases of negative effects, primarily hepatitis, from using green tea preparations as an herbal supplement to regulate body weight [87,88]. Green tea's potential hepatotoxic effects received a lot of study since then. That being said, the daily equivalent of 10.5–32 cups of green tea is equivalent to the amounts of EGCG that may be harmful to humans [89]. Green tea's protective

effects against fatty liver disease are not surprising, as hyperlipidaemia is thought to be a contributing factor and tea polyphenols may increase the liver's lipid metabolism and lower blood lipid levels [90,91]. It is possible to produce the lipid-lowering effect by preventing lipids from being absorbed through the digestive tract [92]. Moreover, a number of studies demonstrated the protective benefit of tea extracts against cirrhosis and liver fibrosis in rats [93,94]. The study findings show that green tea extract greatly reduced hepatic damage in rats, either through its antioxidant action or by preventing the generation of cytokines caused by lipopolysaccharides (LPS) [95]. However, there is a need for well-designed human clinical trials in this area.

Hesperidin, which belongs to the flavanone group, is found mainly in peppermint and citrus fruits such as grapefruit, lemon, lime, or orange [96,97]. The effectiveness of hesperidin supplementation in the management of NAFLD was assessed in a randomized, double-blind clinical trial. The results indicate that concurrent hesperidin administration and lifestyle modification improves risk factors linked to NAFLD, at least partially, by reducing activation of NF- κ B and enhancing lipid profile and insulin sensitivity [98].

5.2. Phenolic Acids

This class of polyphenols consists of phenolic compounds, which usually exist in bound form as amides, esters, or glycosides and have one carboxylic group. Coffee and numerous plant-based meals, seeds, fruit skins, and vegetable leaves contain them [99]. Meanwhile, there are three different types of phenolic acids: hydroxycinnamic acids, oleuropeunoids, and hydroxibenzoic acids.

Coffee is a rich source of minerals and antioxidants, phenolic compounds, such as cumaric, chlorogenic, caffeic, and ferulic acids and other bioactive substances that may have positive health effects. Specifically, it was suggested that coffee beans' other antioxidant compounds, such as chlorogenic acid, may suppress the development of liver cancer [100]. According to the evidence, coffee drinkers had a 40% lower risk of HCC than non-drinkers, and high drinkers have a risk reduction of more than 50% [101]. Coffee drinking is inversely correlated with liver fibrosis, according to a meta-analysis of studies including coffee drinkers and non-drinkers [102]. Based on NAFLD histologic severity, coffee consumption is also inversely correlated with hepatic fibrosis in patients with NAFLD, as well as those with hepatitis C [103,104].

5.3. Stilbenes

Stilbenes are plant-based molecules found primarily in red wine, berries, peanuts, and grapes. Some of these compounds are referred to as phytoalexins. Resveratrol is the most researched polyphenol in this group. It can be found in grapes, cocoa, peanuts, mulberries, and soy [105]. Preclinical research identified its protective characteristics on several fronts, including the modulation of oxidative stress and hepatocellular damage to mitigate NAFLD by decreasing pro-inflammatory cytokines and free radicals and boosting the activity of antioxidant enzymes such as cytochrome P450 (CYP) 2E1 and glutathione [106,107]. Resveratrol was shown in numerous studies to have a function in tumor chemoprevention by reducing cancer cell growth and triggering apoptosis in these cells. By delaying the cell cycle and increasing nitric oxide synthase, resveratrol was shown to have anti-proliferative and pro-apoptotic effects on HCC cells [108]. Subsequently, Choi, Chong, and Nam (2009) discovered that resveratrol accumulated cells in the G0/G1 phase and caused dose- and time-dependent liver cancer cell death. Additionally, by inhibiting the antioxidant proteins, resveratrol may make cancer cells more susceptible to oxidative stress [109].

5.4. Curcuminoids

One of the main compounds belonging to the curcuminoid subgroup is curcumin. It originates in the rhizomes of the ginger family plant known as turmeric (*Curcuma longa*). Asia is home to naturally occurring turmeric, primarily in India. Because of its strong yellow color, flavor, and aroma, it is frequently used in cooking. Nonetheless, because it

contains curcumin, turmeric has been utilized for thousands of years in medicine [110]. There are numerous positive attributes associated with curcumin. Among its additional qualities include anti-inflammatory, antioxidant, and anti-cancer properties [111]. According to recent research, curcumin and its derivatives greatly lower the metabolism of fat and glucose in the liver and serum, which inhibits inflammatory markers in both organs. Through a number of metabolic pathways, including the peroxidase proliferator-activated receptor (PPAR) signaling system and the adenosine 5'-monophosphate-activated protein kinase (AMPK) signaling network, it also controls the metabolic illness known as NAFLD [112–114]. Furthermore, curcumin activates PPAR- γ , which inhibits hepatic steatosis and hepatic stellate cell activation [115]. A meta-analysis shows that curcumin supplementation alone, in combination with physical activity, dietary guidance, and/or lifestyle modifications, can lower blood levels of total cholesterol, total triglycerides, alanine aminotransferase, aspartate aminotransferase, fasting blood insulin, LDL, and as well as homeostasis model assessment of insulin resistance and waist circumference. Nonetheless, this resulted from the application of doses of curcumin ranging from 80 to 1500 mg and extra advice. So far, research is yet to be able to clearly determine the appropriate dose of curcumin to use alone or in combination with other forms of physical activity, dietary advice, or other lifestyle modifications [116].

5.5. Lignans

A large class of naturally occurring substances known as lignans are produced via the shikimic acid biosynthesis pathway [117]. The structural scaffold of lignans is comprised of two or more phenylpropanoid units [118]. The monomers that constitute lignans are propenyl benzene, allyl benzene, cinnamyl alcohol, and cinnamic acid. The richest sources of lignans are sesame seeds and linseed. In addition, lignans are also found in soy, oats, wheat, fish, meat, wine, coffee, and tea [119]. Studies on rats showed that linseed, which contains a molecule called diglucoside, reduces lipid peroxidation in the liver, the accumulation of lipids in the liver and serum cholesterol [120]. So far, clinical studies are yet to be conducted to evaluate the effect of lignans on the prevention of liver diseases.

6. Herbal Approaches in Liver Disease Prevention

Herbs are usually available as dietary supplements and are often not reliably assessed for safety and effectiveness. Herbal products can protect the liver from liver damage, but their effects must be documented in randomized clinical trials to allow for the rational use of these agents. Improper use can contribute to liver damage, which significantly contributes to serious health consequences.

6.1. Milk Thistle

Milk thistle (*Silybum marianum*) has been used in the treatment of liver diseases in traditional medicine for over 2000 years. The standardized extract of milk thistle (silymarin) contains flavonolignans (silybin A, silybin B, isosilybin A, isosilybin B, silychristin, and silydianin), fatty acids, and polyphenolic compounds. These components have synergistic positive effects on the metabolism of the human body, particularly the liver function [121,122]. The most biologically active compound in milk thistle is silybin, which is mainly found in the seeds and fruits of the plant [122]. Silybin exhibits antioxidant activity, protecting hepatocytes from damage caused by free radicals and lipid peroxidation. Additionally, it has antifibrotic effects by inhibiting the activation of hepatic stellate cells, thereby reducing the fibrosis. Furthermore, silybin acts as a barrier against toxins, preventing them from entering liver cells. Due to these properties, milk thistle has significant potential in the prevention and treatment of liver diseases [122]. Moreover, when silymarin was administered for four weeks to diet-induced obese mice, improvements were observed in intrahepatic lipid accumulation, plasma triglyceride, and levels of HDL and LDL [123]. According to Song et al. in model mice with ALD, silymarin (200 mg/kg) was shown to reduce oxidative stress. This was achieved by giving ethanol at a dosage of 5 g/kg body weight every 12 h

for a total of three doses. Moreover, silymarin therapy stopped reductions in glutathione and lipid peroxidation, as well as elevations in TNF- α and alanine aminotransferase levels [124]. For many years, silymarin has been utilized as a hepatoprotective substance in basic research, and as additional studies demonstrate the substance's significance in a variety of liver illnesses, this use of the substance is only expected to grow. Treatment with silymarin is not commonly utilized, however, as it requires evidence from well-organized trials and standardization of the techniques for assessing therapeutic success, particularly in the promising case of NAFLD [125].

6.2. Greater Burdock

Greater burdock (*Arctium lappa*), similar to milk thistle, belongs to the Asteraceae family. It is a primary component of the KIOM2012H herbal formula due to its wide range of healthy effects, including antioxidant, anticancer, and anti-inflammatory effects. These properties are related to the presence of the lignan arctigenin. Greater burdock exhibits significant hepatoprotective effect, protecting the liver from damage caused by lead and cadmium. This mechanism involves the reduction in oxidative stress and the inhibition of inflammatory processes in the liver. The activation of signaling pathways such as Akt/GSK-3 β (protein kinase B/glycogen synthase kinase 3 beta) and the increased activity of antioxidant enzymes such as SOD (superoxide dismutase) and catalase further enhance the protective effects of this herb on liver tissue [126]. In vitro and in vivo studies show the potential effect of the KIOM2012H herbal formula in the prevention and treatment of NAFLD [127]. It is important to emphasize that this formula also includes *Glycyrrhiza uralensis*, *Magnolia officinalis*, and *Zingiber officinale*, which are yet to be the subjects of clinical trials. However, there is evidence based on in vitro and in vivo studies.

6.3. Ginger

Ginger (*Zingiber officinale*), similar to turmeric, has long been used in traditional medicine and cuisine. Extracts from ginger and its biologically active components, especially gingerol and shogaol, exhibit antioxidant, anticancer, anti-inflammatory, and lipid-lowering effects, which translate into hepatoprotective benefits [128]. Ginger is one of the four components of the KIOM2012H herbal formula with potential hepatoprotective effects by improvement in serum lipid levels, including cholesterol. Furthermore, ginger influences the regulation of LDL oxidation processes [127]. Numerous in vitro and in vivo studies indicate the hepatoprotective potential of ginger by induction of apoptosis, inhibition of the liver cancer cell cycle, reduction in the migration and growth of cancer cells, and the showing of antioxidant effects on hepatocytes [129]. However, there is a lack of clinical studies on the above effects, which seems to be necessary for a complete understanding of ginger's therapeutic potential in the context of liver disease treatment and prevention.

6.4. *Glycyrrhiza Uralensis* and *Magnolia Officinalis*

Glycyrrhiza uralensis and *Magnolia officinalis* are other components of the KIOM2012H herbal formula. They show potential in the prevention and treatment of liver diseases through antioxidant activity and modulation of key signaling pathways associated with lipogenesis and inflammation in the liver [127]. In the case of *Glycyrrhiza uralensis*, its positive effects are primarily attributed to three compounds: formononetin, 3'-methoxyglabridin, and glabridin. Network pharmacology and molecular docking studies identified connections between the active ingredients of *Glycyrrhiza* and their molecular targets in the body, as well as assessed the stability of bonds between these ingredients and target proteins. These studies suggest that *Glycyrrhiza uralensis* affects key signaling pathways, such as mitogen-activated protein kinase 3 (MAPK3), signal transducer and activator of transcription 3 (STAT3), and AKT1. The impact on these pathways suggests a role for *Glycyrrhiza* in the prevention and treatment of alcohol-induced liver damage [130]. However, there are no clinical studies confirming this effect.

Despite the promising results of preclinical studies on *Magnolia officinalis*, there is a clear lack of adequate research on its therapeutic potential. Most available studies focus on mechanisms of action at the cellular and molecular levels. However, comprehensive analyses confirming the effectiveness of this plant in a clinical context are lacking. Therefore, further clinical research is necessary to confirm the therapeutic potential of *Magnolia officinalis* in liver diseases.

6.5. Traditional Chinese Medicine

Traditional Chinese medicine uses a variety of herbs to improve patients' health. In a retrospective cohort study conducted on a population of diabetic patients in Taiwan ($n = 81,105$) aimed at assessing the effect of integrated treatment—Chinese and Western medicine—on the risk of HCC, it was shown that the use of herbal preparations reduces the risk of HCC by 41%. Among the most commonly recommended herbs in traditional Chinese medicine are *Salvia miltiorrhiza*, *Astragalus membranaceus*, *Pueraria lobata*, *Rheum palmatum*, and *Dendrobium nobile*. Their effects are based on antioxidant mechanisms that effectively neutralize free radicals, protecting liver cells from oxidative stress. For example, *Salvia miltiorrhiza* demonstrates the ability to modulate signaling pathways, reducing the levels of pro-inflammatory cytokines such as TNF- α . *Astragalus membranaceus*, in turn, not only has anti-inflammatory effects, but also supports liver cell regeneration. *Pueraria lobata* regulates lipid metabolism and improves the level of cholesterol and TG. *Rheum palmatum* improves liver regeneration. *Dendrobium nobile* shows the ability to inhibit the proliferation of cancer cells, making it a promising agent in the prevention of HCC [131].

The key herbal components that were discussed in this subchapter are summarized in Figure 2. The current knowledge regarding the potential of herbs in liver disease prevention is largely based on preclinical studies, while clinical studies are scarce. Herbs certainly have potential applications for patients at risk of liver diseases, but they should be used with great caution. For example, the prolonged chewing of betel nuts (*Areca catechu* L.), which were used for centuries in traditional medicine, is linked to the development of liver cirrhosis and HCC. The main active compound, arecoline, exhibits genotoxic, hepatotoxic, and carcinogenic effects, raising concerns about the safety of long-term consumption of these nuts. Epidemiological studies indicate a significantly higher risk of liver disease development in individuals who regularly chew betel nuts, regardless of the presence of other risk factors, such as hepatitis B or C virus infection [129]. Therefore, extensive randomized clinical trials are necessary to accurately assess the efficacy of herbs, their active ingredients, and extracts in the treatment and prevention of liver diseases. Clinical studies are essential not only in the context of the therapeutic potential of herbs, but also to determine therapeutic doses, potentially toxic doses, to identify interactions with other medications, and any possible side effects.

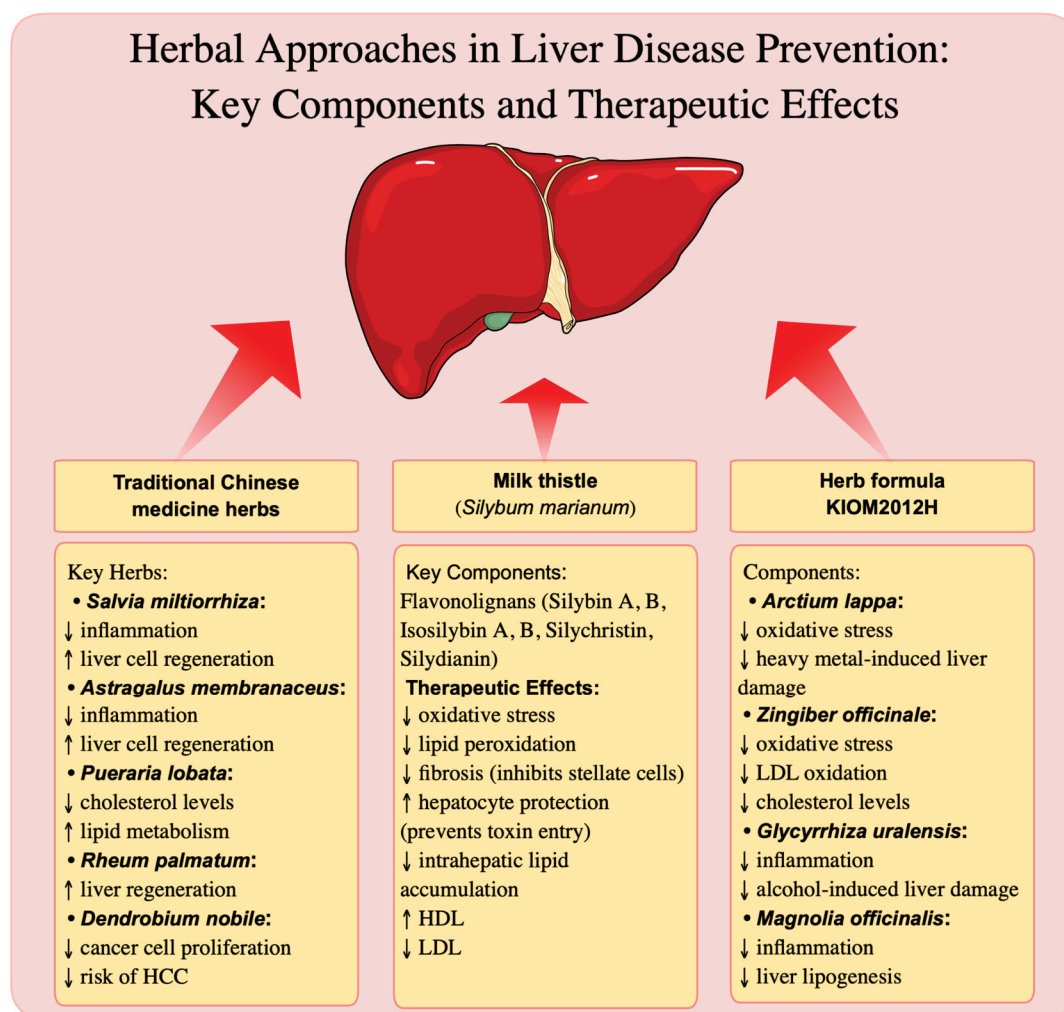


Figure 2. Schematic representation of key herbal components in liver disease prevention. Signs: ↑ increases, ↓ decreases.

7. Unsaturated Fatty Acids and Phospholipids in Liver Disease Prevention

The primary sources of *n*-3 (omega-3) and *n*-6 (omega-6) polyunsaturated fatty acids in the human diet are fatty marine fish, nuts, and vegetable oils. These products also provide fat-soluble vitamins, which have a beneficial impact on metabolic health. Among these vitamins, vitamins D and E are particularly important in the context of health and the main sources are fish as well as nuts that were mentioned earlier [132].

Fatty acids from the *n*-3 group, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), play a crucial role in preventing NAFLD. Their beneficial effect is related to improvement in lipid metabolism and reduction in liver inflammation. EPA and DHA are preferentially oxidized by hepatocytes, which limits their accumulation in the form of TG and reduces the risk of liver steatosis. Additionally, *n*-3 polyunsaturated fatty acids (PUFAs) have the ability to inhibit the expression of genes involved in the de novo lipogenesis (DNL), thereby lowering TG synthesis in the liver. The anti-inflammatory activity of *n*-3 PUFAs are also playing a significant role by reducing the level of pro-inflammatory cytokines and decreasing oxidative stress in liver cells, thereby preventing the progression of NAFLD to more progressed form, such as NASH [133]. An important hepatoprotective mechanism of omega-3 fatty acids is their ability to modulate the ratio of omega-6 to omega-3 fatty acids in the diet. A high omega-6 to omega-3 ratio exhibits pro-inflammatory effects, which seems to be significant in the context of NAFLD, where chronic inflammation is a key risk factor. A randomized, controlled, double-blind clinical trial involving 176 participants

($n = 176$) demonstrated that omega-3 supplementation leads to a reduction in this ratio, contributing to decreased liver inflammation and improved lipid metabolism. A crucial role here is played by omega-3's influence on activity of peroxisome proliferator-activated receptor alpha (PPAR α) and sterol regulatory element-binding proteins (SREBP-1c), which regulate the expression of genes involved in lipogenesis and fatty acid oxidation. PPAR α stimulates fatty acid beta-oxidation, leading to their oxidation instead of accumulation as TG, while omega-3 inhibition of SREBP-1c decreases lipid synthesis in the liver. Additionally, omega-3 improves hepatic insulin sensitivity by improving insulin signaling, which is a key factor of NAFLD prevention and treatment, as insulin resistance is one of the main factors in the development of this disease. Improved insulin sensitivity reduces lipogenesis and supports processes associated with fatty acid oxidation, which translates into a reduction in liver steatosis [134].

Another significant aspect of the hepatoprotective action of omega-3 fatty acids is their impact on metabolism related to fat accumulation and the risk of developing liver diseases. Omega-3 fatty acids, especially EPA and DHA, effectively lower the fatty liver index (FLI), lipid accumulation product (LAP), and visceral adiposity index (VAI). These effects are strongly associated with liver fat content and insulin resistance, which are key elements in the pathophysiology of NAFLD. In a randomized, controlled clinical trial involving 60 patients with T2D and NAFLD, supplementation with 2000 mg of omega-3 for 12 weeks led to significant improvement in FLI, LAP, and VAI compared to the placebo group. The reduction in these factors indicates decreased fat accumulation in the liver and improved overall lipid metabolism [135].

Nuts, such as almonds, walnuts, pistachios, and hazelnuts, exhibit hepatoprotective effects, as proved by numerous epidemiological and clinical studies. A meta-analysis of 12 studies involving over 90,000 participants ($n = 92,621$) found that regular nut consumption is associated with a significant reduction in the risk of developing NAFLD. This effect is primarily attributed to the high content of monounsaturated and polyunsaturated fatty acids (MUFA and PUFA) and antioxidants such as vitamin E and polyphenols. Bioactive substances in nuts, such as ellagic acid and alpha-linolenic acid (ALA), play a key role in modulation of the body's inflammatory response. Nut consumption can lead to a significant reduction in inflammatory markers, such as TNF- α . Another preventive factor in the context of liver diseases is the ability of nuts to support weight control by improving satiety and reducing visceral fat accumulation [136].

In a randomized, controlled study involving 68 participants, it was shown that regular consumption of cashew nuts and cashew nut oil can lead to a significant reduction in cardiovascular risk factors, including reduction in LDL cholesterol, inflammatory markers such as apolipoprotein B (apo B), gamma-glutamyltransferase (GGT), and liver enzymes: aspartate aminotransferase (AST) and alanine aminotransferase (ALT). Additionally, the study found that cashew consumption reduces neck circumference, which is an important indicator of visceral fat reduction. Similar to other nuts, cashews contain high amounts of MUFA, including oleic acid, which is shown to have anti-atherosclerotic activity [137].

Despite the numerous health benefits of nut consumption, there is a risk of contamination with aflatoxins—toxins produced by molds of the *Aspergillus*. In the context of NAFLD, where the risk of disease progression is increased, it is important to choose nuts from controlled sources to minimize exposure to these toxins. Nonetheless, nuts remain an important dietary component that can support the prevention and treatment of liver diseases when the source and quality is appropriate [138].

Fish oil, rich in omega-3 fatty acids, supports lipid metabolism in the liver, reducing TG accumulation by stimulating beta-oxidation. Additionally, fish oil has anti-inflammatory and antioxidant effects, helping to reduce oxidative stress induced by alcohol and supporting the integrity of the intestinal barrier, protecting against endotoxemia [139]. Such hepatoprotective effects in the context of alcohol suggest a significant potential for fish oil in protecting the liver from other toxic factors. However, further research is needed.

Another important component with hepatoprotective potential is phospholipids, especially polyenylphosphatidylcholine (PPC) derived from soybeans. Phospholipids are fundamental components of cell membranes, which not only support the structure and function of hepatocytes, but also participate in the regeneration of these cells. Studies indicate that PPC supplementation improves liver function, which is evidenced by reduced levels of aminotransferases (ALT and AST) and improved lipid metabolism. Phospholipids also show antioxidant and anti-inflammatory activity that reduces the progression of liver disease, including fibrosis. Long-term clinical studies on PPC showed that its use may significantly improve the health of patients with NAFLD and be useful as a supportive therapy [140].

One of the potent sources of omega-3 fatty acids and phospholipids is krill oil. While fish oil contains omega-3 fatty acids in the form of triglycerides, krill oil has them bound to phospholipids, specifically phosphatidylcholine, which improves their absorption and bioavailability. This means that lower doses of krill oil can provide similar or even superior benefits compared to higher doses of fish oil. Additionally, krill oil contains natural antioxidants such as astaxanthin, offering stronger anti-inflammatory and antioxidant effects than fish oil [141]. These factors make krill oil a more efficient option for managing conditions such as NAFLD by reducing triglycerides and improving liver fat metabolism.

Euphausia pacifica, also known as North Pacific krill, similarly to *Euphausia superba* (Antarctic krill), produces oil (*E. pacifica* oil) that is rich in omega-3 fatty acids, but with a higher concentration of phospholipids and a unique compound—8-hydroxyeicosapentaenoic acid (8-HEPE). 8-HEPE demonstrates potent physiological effects, including activating peroxisome proliferator-activated receptors (PPARs), which play a key role in lipid metabolism. This makes *E. pacifica* oil particularly effective in reducing triglyceride accumulation, improving lipid profiles, and mitigating hepatic steatosis [142].

Krill oil is not only beneficial for its high bioavailability and antioxidant properties, but also offers a more sustainable alternative to traditional fish oil sources. The krill fishery, particularly in the Antarctic, is subject to strict environmental regulations that ensure sustainable harvesting practices. With large krill populations and precautionary catch limits, this resource remains abundant and less vulnerable to overfishing. Moreover, advancements in extraction techniques further reduced the environmental footprint of krill oil production, making it a responsible choice for those seeking both health benefits and eco-conscious options [143].

Diet plays a fundamental role in hepatoprotection, as the appropriate selection of nutrients can significantly influence the course and progression of liver diseases. The Mediterranean diet, rich in products that are good sources of unsaturated fatty acids, polyphenols, and vitamins, is an example of a dietary pattern with high potential for modulating metabolic processes in the liver. Additionally, this diet has an anti-inflammatory property, which is an important factor in the pathogenesis of liver diseases [144]. The proper selection of products and dietary patterns can positively impact liver health indicators, making nutrition one of the key elements of hepatoprotection.

8. Probiotics and Microbiota

The gut microbiota can play a crucial role in the development and prevention of various liver diseases. Dysbiosis, or an imbalance in the gut microbiome, can contribute to liver disorders through increased intestinal permeability, chronic inflammation, and metabolic changes [145]. Metabolic dysfunction-associated steatotic liver disease, alcoholic liver disease, and hepatocellular carcinoma are associated with alterations in gut microbiota [146]. Endotoxins (LPS, which are a component of the outer membrane of Gram-negative bacteria) are released into the circulation after bacterial lysis and may reach the liver via the portal vein. LPS can activate complement, the kinin system, leukocytes, platelets, and endothelial cells and increase the synthesis of pro-inflammatory cytokines, inducing oxidative stress contributing to the inflammatory process in MASLD [147,148].

Probiotics can modulate intestinal microbiota, improve intestinal barrier function, and exert immunomodulatory effects, potentially preventing harmful bacteria and their products from entering the circulation and causing liver damage [148,149]. Studies showed that probiotic use can lead to improvements in liver function tests, decreased inflammatory markers, and reduced blood cholesterol levels [148].

It seems important to modify the gastrointestinal microbiota by consuming probiotic bacteria naturally occurring in food or contained in dietary supplements to reduce the progressive inflammation in the liver by modulating the gut microbiome, reducing inflammation, and enhancing intestinal barrier function [148,150].

A randomized study by Alisi et al. to demonstrate the effect of a 4-month supplementation with a composition of 8 probiotic strains (*Bifidobacterium breve*, *Bifidobacterium longum*, *Bifidobacterium infantis*, *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus paracasei*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, and *Streptococcus thermophilus*) in obese children with steatohepatitis showed that the intake of this composition by the study group had a positive effect on the course of the disease compared with placebo. A significant decrease in liver enzymes: ASP and ALT were observed, along with a reduction in the degree of hepatic steatosis in the study group of subjects [151].

A further double-blind study by Duseja et al. [152] also showed a beneficial effect of a probiotic on the course of steatohepatitis. In this study, patients treated for 12 months with lifestyle modification and a multi-strain probiotic (*Lactobacillus paracasei* DSM 24733, *Lactobacillus plantarum* DSM 24730, *Lactobacillus acidophilus* DSM 24735 and *Lactobacillus delbrueckii* ssp. *bulgaricus* DSM 24734, *Bifidobacterium longum* DSM 24736, *Bifidobacterium infantis* DSM 24737, *Bifidobacterium breve* DSM 24732, and *Streptococcus thermophilus* DSM 24731) were observed to reduce inflammation, significantly improve liver histology, as well as reduce alanine transaminase (ALT) activity and improve the profile of pro-inflammatory cytokines. Probiotics administered in MAFLD modify the composition of the intestinal microbiota, improve intestinal barrier function, and the metabolism of lipid compounds and reduce pro-inflammatory cytokines. They may therefore be a beneficial management strategy in patients with fatty liver disease. The literature data do not clearly clarify the long-term effects of probiotics in this group of patients. There is a need for further research in this area, especially in view of the growing global epidemic of MAFLD.

A systematic review and meta-analysis was recently published that also provides convincing evidence that probiotics are beneficial in treating the complication of MAFLD, liver cirrhosis, demonstrating reversal of hepatic encephalopathy, potential improvement in liver function, improvement in quality of life, and regulation of gut dysbiosis. Furthermore, the apparent safety profile suggests that probiotics are a promising intervention in the treatment of liver cirrhosis [153].

While lifestyle modifications remain the primary intervention for NAFLD, probiotics offer a promising adjunct therapy due to their ability to positively influence gut microbiota composition and potentially mitigate liver disease progression [154]. Understanding the complex interactions between the gut microbiota and liver diseases may lead to novel prevention and treatment strategies for these disorders.

9. Conclusions

Non-communicable chronic liver disease is one of the most frequently diagnosed diseases that is a consequence of an inappropriate lifestyle, including poor nutrition, smoking, and alcohol consumption together with inadequate physical activity.

The dietary model associated with the world's most common chronic liver disease, MASLD, is the Western diet. Leading features of this diet are low fruit and vegetable content, a high proportion of animal products, simple sugars, and processed foods. In contrast, the diet recommended for patients diagnosed with MASLD is the Mediterranean diet. The components of this diet, whose sources are primarily fruits and vegetables, are in turn important in the prevention of MASLD. Their preventive effect is usually related to the prevention of cardiometabolic risk factors, which in turn are associated with MASLD.

For instance, the protective effect of dietary fiber on non-communicable chronic liver disease was proven, and its consumption may prevent excessive body weight and MASLD. Dietary fiber can also contribute to beneficial gut microbiota composition, which, together with probiotic supplementation, may in the future also become one of the methods for the prevention of non-communicable liver diseases. Numerous scientific studies observed positive results associated with the use of polyphenols in the prevention of MASLD or liver cancer, such as catechins, silymarin, resveratrol, anthocyanins, and flavonoids. These different types of polyphenols are present in coffee, fruits, and vegetables, among others, and should be part of a balanced diet due to their anti-inflammatory and anti-cancer effects. It is worth noting that coffee already has a place in recommendations for MASLD patients. Fatty acids, especially EPA and DHA, regulate pathways involved in fat metabolism, regulate fatty acid metabolism in tissues, and improve the blood lipid profile. The supply of these fatty acids from fish, plant products, or in the form of supplementation should therefore be recommended to people who are at high risk of non-infectious liver disease. Additionally, the constituents of certain herbs influence liver metabolism and the prevention of various diseases. However, it should be borne in mind that such ingredients should only be used under medical supervision, as inappropriate use, particularly in the form of dietary supplements, can cause liver injury and have other serious health consequences.

In conclusion, a proper diet, rich in products of plant origin, and thus active ingredients that show a protective effect against chronic non-infectious liver diseases, not smoking and not drinking alcohol, along with adequate physical activity, are crucial in the prevention of chronic liver diseases.

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Article

Effects of Egg Consumption on Subjects with SLD or Hypertension: A MICOL Study

Rossella Tatoli ¹, Caterina Bonfiglio ¹, Francesco Cuccaro ², Angelo Campanella ¹, Sergio Coletta ¹, Pasqua Letizia Pesole ¹, Gianluigi Giannelli ^{1,†} and Rossella Donghia ^{1,*,†}

¹ National Institute of Gastroenterology—IRCCS “Saverio de Bellis”, 70013 Castellana Grotte, Italy; rossella.tatoli@irccsdebellis.it (R.T.); catia.bonfiglio@irccsdebellis.it (C.B.); angelo.campanella@irccsdebellis.it (A.C.); sergio.coletta@irccsdebellis.it (S.C.); letizia.pesole@irccsdebellis.it (P.L.P.); gianluigi.giannelli@irccsdebellis.it (G.G.)

² Local Health Unit—Barletta-Andria-Trani, 76121 Barletta, Italy; francescocuccaroepi@gmail.com

* Correspondence: rossella.donghia@irccsdebellis.it

† These authors contributed equally to this work.

Abstract: Background: Steatotic liver disease (SLD) is defined as a fat accumulation in more than 5% of hepatocytes; it can progress to non-alcoholic steatohepatitis (NASH), associated with an increased state of inflammation. The aim of this study was to explore the protective effects of eating eggs and any association with SLD and hypertension (HTN). Methods: The study cohort included 908 participants assessed in the fourth recall of the MICOL study, grouped into four groups, based on NALFD and/or HTN. Results: The prevalence of HTN and SLD among participants was 31.61%. Overall, the results indicated a statistical significance of egg consumption, showing a protective role against the two disease conditions, in both the raw and adjusted models (RRR = 0.34, $p = 0.009$, 0.15 to 0.76 95% C.I.). Conclusions: Many differences were found among the groups, and the protective role of eating eggs was amply demonstrated. We can conclude that it is unwise to demonize the intake of this food and its nutritional properties, in contrast with previous reports in the literature.

Keywords: SLD; HTN; diet; eggs

1. Introduction

Steatotic liver disease (SLD) is defined as a fat accumulation in more than 5% of hepatocytes, in the absence of significant alcohol consumption (30 g per day for men and 20 g per day for women) or other chronic liver diseases [1]. In this condition, hepatocytes present a ballooning appearance; this lesion is characteristic of SLD [1]. The overlap of some risk factors or injuries to the liver may promote the progression to non-alcoholic steatohepatitis (NASH), in which the steatosis is associated with a state of necroinflammation and fast fibrosis progression, unlike in SLD [1].

The increasing prevalence of SLD has made it a significant public health problem worldwide [2]. It is the primary cause of chronic liver disease in Europe [3] and affects 44% of inhabitants of Western countries [4].

SLD is known to be strongly linked to metabolic syndrome and can be considered the hepatic manifestation [5]. The increasing prevalence of metabolic syndrome and its related conditions, such as insulin resistance, hypertension (HTN), and dyslipidemia, predispose many people to the risk of developing SLD [6].

Together with SLD, HTN is another important health problem because it is one of the major causes of cardiovascular disease [7]. Among cardiovascular diseases, HTN is one of the main risk factors predisposing to fatal complications [8], and HTN diagnoses were estimated to increase by about 60% between 2000 and 2025 [9].

Diet and eating habits play a central role in the prevention and management of both SLD and HTN. They are considered modifiable risk factors for the development of HTN [10] and the only recommended treatment for SLD [11].

Dietary components can influence the pathophysiology of diseases [12–15]. Special attention should be paid to dietary cholesterol, which is a modifiable risk factor for HTN [16], while the role of this dietary component concerning the risk and progression of SLD is still controversial [17,18].

Eggs are one of the most important dietary sources of cholesterol, present in the egg yolk at 180–225 mg per egg [19]. Many societies and organizations recommend limiting egg consumption to avoid raising circulating cholesterol levels [20]. The American Heart Association recommends reducing egg intake unless other sources of dietary cholesterol are limited [21].

The high cholesterol content (an average egg provides about 70% of the recommended daily value) has led to eggs often being demonized, despite their being rich in other nutrients that can provide beneficial effects to human health. They are a good source of minerals, vitamins, proteins, and unsaturated fatty acids [22–25].

Very few studies have analyzed the relationship between egg consumption and the risk of diseases, in particular SLD, and the results are inconsistent [26,27].

The present study aims to investigate the effect of egg consumption on the risk of developing steatosis, HTN, or both conditions in an older Southern Italian population.

2. Materials and Methods

2.1. Study Design and Population

This cross-sectional study involved 980 subjects aged over 60 years. The study sample is a sub-sample from the Multicenter Italian study on Cholelithiasis (MICOL). MICOL recruitment started in 2017 among subjects listed on the electoral register of Castellana Grotte, Southern Italy. The present study is based on data from MICOL IV, a recall of MICOL III patients [28]. The methodological details of this population-based study have been described elsewhere [29,30]. All participants provided informed consent before the examination. The study was approved in line with the ethical standards of the institutional research committee of the National Institute of Gastroenterology and Research Hospital “S. de Bellis” in Castellana Grotte, Italy (DDG-CE 782/2013. The date of approval was Prot. n.144/C.E. of 15/04/2019). The IRB of the head institution, the National Institute of Gastroenterology and Research Hospital “S. de Bellis” in Castellana Grotte, Italy, approved the study. The study was conducted in accordance with the Helsinki Declaration of 1975 and adhered to the “Standards for Reporting Diagnostic Accuracy Studies” (STARD) guidelines (<http://www.stard-statement.org/> Accessed on 5 October 2019). The manuscript is organized according to the “Strengthening the Reporting of Observational Studies in Epidemiology-Nutritional Epidemiology” (STROBE-nut) guidelines (<https://www.strobe-nut.org/> Accessed on 5 October 2019).

Our study sample was divided into four categories: No Steatosis and No HTN (a); No Steatosis and Yes HTN (b); Yes Steatosis and No HTN (c); Yes Steatosis and Yes HTN (d). Steatosis was detected with a standardized ultrasound examination [31], while HTN was defined according to specific guidelines [32].

2.2. Lifestyle, Clinical and Dietary Assessment

Lifestyle and anthropometric assessments were evaluated by a physician during an examination at the study center. Smoking status was assessed with the single question, “Are you a current smoker?”. The level of education was expressed as years of schooling.

Body mass index (BMI) was calculated as kg/m². Height and weight measurements were performed using a Seca 220 stadiometer and a Seca 711 scale.

Blood was collected from the subjects the morning after an overnight fast. The serum was separated into aliquots. One aliquot was immediately stored at −80 °C. The second aliquot was used to test serum biochemical markers by standard laboratory techniques in

our laboratory. Other aliquots were stored for use as necessary. Diet and eating habits were investigated with a validated food frequency questionnaire, administered during the visit, and each food (86 validated foods) was converted to mean daily intake in grams [33].

2.3. Statistical Analysis

Patient characteristics are reported as mean and standard deviation ($M \pm SD$) for continuous variables, and as frequency and percentages (%) for categorical variables. To test the association between the independent groups (SLD (No)/HTN (No), SLD (No)/HTN (Yes), SLD (Yes)/HTN (No), and SLD (Yes)/HTN (Yes)), a Chi-square test was used for categorical variables, while the Kruskal–Wallis equality rank test was used to compare more than two independent groups. Dunn’s test and the proportion test were performed for multiple pairwise comparisons.

We estimated a multiple multinomial logistic regression model using the four-category outcome variable described above as the dependent variable and egg consumption (both continuous and categorical) determinants as predictors. The models were also adjusted for some covariates (Model 1—for age and gender; Model 2—for age, gender, and daily kcal intake), and estimated coefficients were transformed to relative-risk ratios (RRR).

To test the null hypothesis of non-association, the two-tailed probability level was set at 0.05. The analyses were conducted with StataCorp (2023. Stata Statistical Software: Release 18. College Station, TX, USA: StataCorp LLC.), while RStudio (“Prairie Trillium” Release) was used for the plots.

3. Results

Cohort characteristics stratified by the disease group combinations are shown in Table 1.

Males are more prevalent in the SLD and no-HTN group compared to both the healthy group (60.29% vs. 49.15%, $p = 0.019$) and the HTN-only group (60.29% vs. 50.00%, $p = 0.04$). The mean age is higher in the diseased group than in both the healthy group (69.24 ± 8.44 vs. 61.34 ± 12.16 , $p < 0.0001$) and the SLD-only group (69.24 ± 8.44 vs. 61.72 ± 10.35 , $p < 0.0001$). The same applies for the HTN-only group compared to the healthy group (70.54 ± 9.33 vs. 61.34 ± 12.16 , $p < 0.0001$), while there was a statistically significant number of younger subjects in the NALFD-only group compared to the HTN-only group (61.72 ± 10.35 vs. 70.54 ± 9.33 , $p < 0.0001$).

The level of education was statistically significantly associated with the disease conditions in all multiple comparisons with the exception of the group with both disease conditions versus HTN-only subjects. BMI was lower in healthy subjects and was statistically higher values in the groups with the disease conditions, and the highest value was found in the group with both (31.56 ± 5.39). The same trend was also observed for diabetes and metabolic syndrome. Diabetes was more prevalent in the group of patients with both conditions, showing a prevalence of 22.65%, except in comparison with subjects with SLD and with only HTN (8.13% vs. 13.64%, $p = 0.08$). Subjects with metabolic syndrome were less prevalent in the group with neither disease condition, to a statistically significant degree ($p < 0.005$), with the exception of the NALFD vs. HTN groups (40.19% vs. 43.75%, $p = 0.48$). As shown in Supplementary Table S1, blood test results showed higher glucose concentrations in the group with both conditions than in the other groups, while HDL tended toward a statistically significant decrease ($p < 0.0001$).

Hematocrit (HCT), mean corpuscular volume (MCV), white blood cells (WBCs), neutrophils, hemoglobin A1c (HbA1c), gamma-glutamyl transferase (GGT), and C-reactive protein (CRP) tended toward a statistically significant increase in the last group, both in general and in multiple comparisons.

The other parameters showed no defined trend, even if the variations were significant, and even in multiple comparisons.

Figure 1 and Table 2 illustrate egg consumption both as grams/day and as the number of eggs per week.

Table 1. Epidemiological and clinical characteristics of patients with SLD or HTN (*n* = 908).

Parameters *	Disease Condition				<i>p</i> †	Multiple Comparisons ‡			
	SLD (No)/ HTN (No) (<i>n</i> = 236) (a)	SLD (No)/ HTN (Yes) (<i>n</i> = 176) (b)	SLD (Yes)/ HTN (No) (<i>n</i> = 209) (c)	SLD (Yes)/ HTN (Yes) (<i>n</i> = 287) (d)		(b) vs. (a)	(c) vs. (a)	(d) vs. (a)	(d) vs. (b)
Gender (M) (%)	116 (49.15)	88 (50.00)	126 (60.29)	164 (57.14)	0.05 ‡	0.86 †	0.01 †	0.48 †	0.13 †
Age (yrs)	61.34 ± 12.16	70.54 ± 9.33	61.72 ± 10.35	69.24 ± 8.44	0.001	<0.0001	0.44	<0.0001	0.07
Degree of Education (%)					<0.001 ‡				
No	59 (25.11)	77 (43.75)	47 (22.71)	114 (40.00)		0.0001 †	<0.0001 †	0.0001 †	0.04 †
Elementary School	68 (28.94)	43 (24.43)	81 (39.13)	89 (31.23)		0.31 †	0.002 †	0.07 †	0.12 †
Secondary School	75 (31.91)	29 (16.48)	52 (25.12)	45 (15.79)		0.0004 †	0.04 †	0.01 †	0.04 †
High School	27 (11.49)	6 (3.41)	17 (8.21)	10 (3.51)		0.003 †	0.05 †	0.02 †	0.05 †
Degree	6 (2.55)	21 (11.93)	10 (4.83)	27 (9.47)		0.0001 †	0.01 †	0.05 †	0.01 †
Smoker (Yes) (%)	36 (15.32)	18 (10.29)	33 (15.79)	31 (10.84)	0.18 ‡	0.14 †	0.89 †	0.13 †	0.11 †
BMI (kg/m ²)	25.27 ± 3.32	27.32 ± 4.20	28.80 ± 4.33	31.56 ± 5.39	0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Diabetes (Yes) (%)	10 (4.26)	24 (13.64)	17 (8.13)	65 (22.65)	<0.001 ‡	0.0006 †	0.09 †	<0.0001 †	0.08 †
MetS (Yes) (%)	37 (15.68)	77 (43.75)	84 (40.19)	198 (68.99)	<0.001 ‡	<0.0001 †	<0.0001 †	<0.0001 †	<0.0001 †

* As mean and standard deviation for continuous variables, and as frequency and percentage (%) for categorical variables. † Kruskal–Wallis equality of populations rank test; ‡ Chi-square test; ‡ Dunn’s test of multiple comparisons; † Proportion test. Abbreviations: SLD, Steatotic Liver Disease; HTN, Hypertension; BMI, Body Mass Index; MetS, Metabolic Syndrome.

Table 2. Eggs consumption stratified by SLD or HTN.

Parameters *	Disease Condition				<i>p</i> †	Multiple Comparisons ‡			
	SLD (No)/ HTN (No) (<i>n</i> = 236) (a)	SLD (No)/ HTN (Yes) (<i>n</i> = 176) (b)	SLD (Yes)/ HTN (No) (<i>n</i> = 209) (c)	SLD (Yes)/ HTN (Yes) (<i>n</i> = 287) (d)		(b) vs. (a)	(c) vs. (a)	(d) vs. (a)	(d) vs. (b)
Eggs (g/die)	10.43 ± 9.85	7.23 ± 6.98	9.45 ± 8.77	7.82 ± 7.63	0.001	0.0005	0.12	0.0007	0.02
Eggs (n#/week) (%)					0.004 ‡				
<2	168 (71.19)	146 (82.95)	147 (70.33)	227 (79.09)		0.005 †	0.84 †	0.04 †	0.004 †
2–3	46 (19.49)	26 (14.77)	48 (22.97)	50 (17.42)		0.21 †	0.37 †	0.55 †	0.04 †
>3	22 (9.32)	4 (2.27)	14 (6.70)	10 (3.48)		0.004 †	0.31 †	0.005 †	0.07 †
									0.64 †
									0.10 †

* As Mean and Standard Deviation for continuous variables, and as frequency and percentage (%) for categorical variables. † Kruskal–Wallis equality of populations rank test; ‡ Fisher’s exact test; ‡ Dunn’s test of multiple comparisons; † Proportions test.

Table 3. Multinomial logistic regression analysis of disease condition categories using egg daily intake or eggs per week.

Parameters	SLD (No) and HTN (Yes) ‡				SLD (Yes) and HTN (No) ‡				SLD (Yes) and HTN (Yes) ‡			
	RRR	se (RRR)	p	95% C.I.	RRR	se (RRR)	p	95% C.I.	RRR	se (RRR)	p	95% C.I.
Model 1												
Eggs (g/die)	0.95	0.01	<0.001	0.93 to 0.98	0.99	0.01	0.29	0.97 to 1.01	0.96	0.01	0.001	0.94 to 0.98
Eggs (n#/week)	--	--	--	--	--	--	--	--	--	--	--	--
<2 [Ref.]	0.65	0.17	0.11	0.38 to 1.10	1.19	0.28	0.45	0.75 to 1.89	0.80	0.18	0.34	0.51 to 1.26
2–3	0.21	0.12	0.005	0.07 to 0.62	0.73	0.26	0.38	0.36 to 1.47	0.34	0.13	0.006	0.15 to 0.73
>3												
Model 2												
Eggs (g/die)	0.96	0.01	0.006	0.94 to 0.99	0.99	0.01	0.33	0.97 to 1.01	0.97	0.01	0.01	0.95 to 0.99
Eggs (n#/week)	--	--	--	--	--	--	--	--	--	--	--	--
<2 [Ref.]	0.93	0.26	0.79	0.53 to 1.62	1.22	0.29	0.40	0.76 to 1.96	1.10	0.26	0.70	0.68 to 1.76
2–3	0.27	0.15	0.02	0.09 to 0.81	0.71	0.26	0.36	0.35 to 1.46	0.40	0.16	0.03	0.18 to 0.90
>3												
Model 3												
Eggs (g/die)	0.96	0.01	0.009	0.94 to 0.99	0.99	0.01	0.22	0.97 to 1.01	0.97	0.01	0.008	0.95 to 0.99
Eggs (n#/week)	--	--	--	--	--	--	--	--	--	--	--	--
<2 [Ref.]	0.99	0.29	0.96	0.56 to 1.75	1.17	0.28	0.51	0.73 to 1.89	1.03	0.25	0.91	0.63 to 1.67
2–3	0.27	0.16	0.02	0.09 to 0.84	0.62	0.23	0.19	0.30 to 1.28	0.34	0.14	0.009	0.15 to 0.76
>3												

Abbreviations: SLD, Steatotic Liver Disease (SLD); HTN, Hypertension; RRR, Relative-Risk Ratios; se (RRR), standard error of RRR; 95% C.I., Confidential Interval at 95%. ‡ Reference category: SLD (No) and HTN (No). Model 1, Row Model; Model 2, Adjusted for age, and gender; Model 3, adjusted for age, gender, and kcal daily intake.

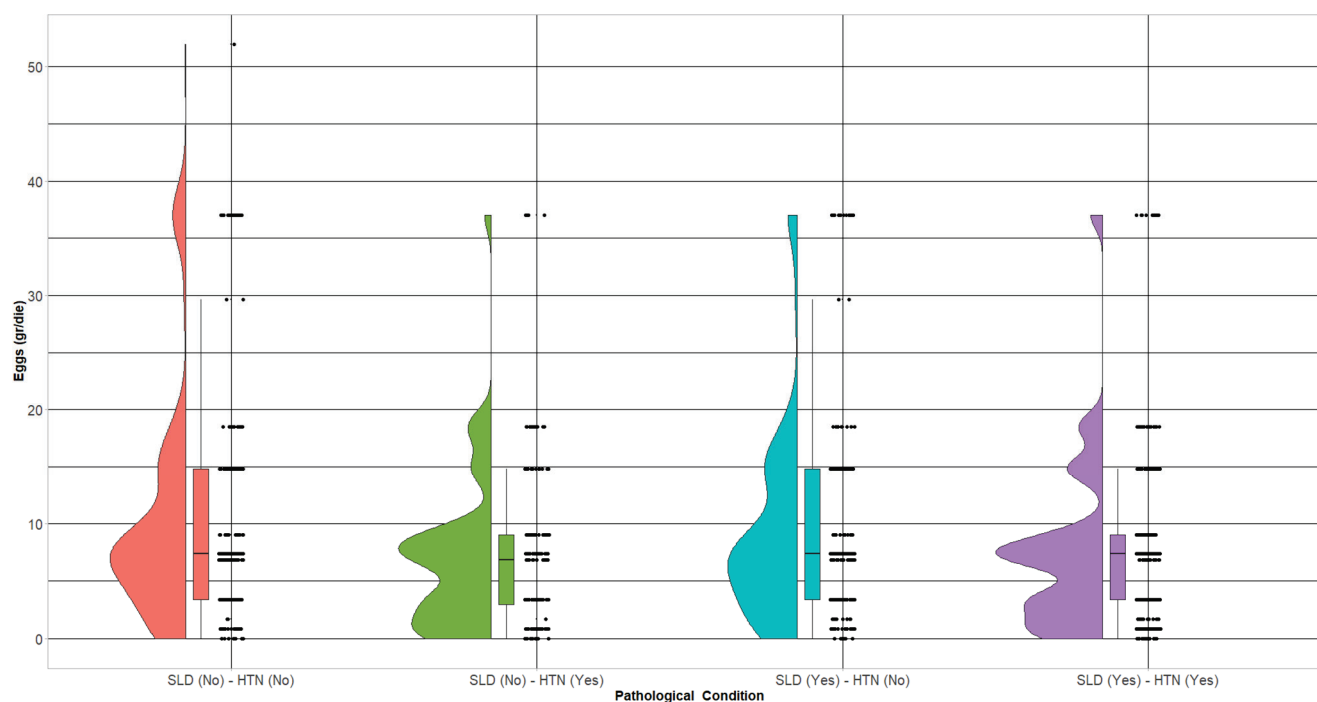


Figure 1. Half-violin plot of daily egg consumption by group.

There was a significant difference between the groups in terms of daily consumption ($p = 0.0001$) which was also reflected in multiple comparisons, where the greatest consumption was in healthy subjects without either disease, followed by those affected by SLD; weekly intake was also statistically significantly different ($p = 0.004$).

The relative nutrients, fatty acids, and amino acid intake is reported in Supplementary Table S2.

The multinomial logistic regression models are reported in Table 3.

Egg intake (g/die) showed a protective role in subjects with only HTN (RRR = 0.95, $p < 0.001$, 0.93 to 0.98 C.I. 95%) and with SLD (RRR = 0.96, $p = 0.001$, 0.94 to 0.98 C.I. 95%), and a consumption > 3 (week) was more protective for these groups (RRR = 0.21, $p = 0.005$, 0.07 to 0.62 C.I. 95%, and RRR = 0.34, $p = 0.006$, 0.15 to 0.73 C.I. 95%, respectively) in the unadjusted model. The positive association was confirmed in the models adjusted for covariates.

4. Discussion

The present study shows a positive effect of egg consumption in an older population living in the Southern Mediterranean area. Our analyses revealed a protective power of eggs, which increases with dietary consumption. This relationship was also significant after adjustment for age, gender, and daily kcal intake.

In the scientific literature, data on the link between eggs consumption and SLD are few, inconsistent, and controversial.

Some previous studies associated egg consumption with an increased risk of NALFD [30]. In these, the positive link between egg consumption and SLD was explained by the high cholesterol content of eggs [34,35].

Some studies have shown that a higher cholesterol consumption is associated with SLD and its exacerbation to NASH [35,36]. Baumgartner et al. [26] has shown that daily egg consumption increases serum cholesterol and LDL-C concentrations in women [37]. On the contrary, some randomized controlled trials observed that egg intake did not substantially change plasma TC, low-density lipoprotein cholesterol (LDL-C), HDL-C levels [38,39] or the TC/HDL-C ratio [39,40].

Therefore, reducing egg consumption as a single food may not be so important for reducing the risk of disease. In our opinion, it is more important to frame egg consumption

within the individual's eating habits. Egg preparation methods and concurrent consumption of other foods (e.g., bacon vs. vegetables) may partly account for the differences. Dietary fiber is known to reduce the intestinal absorption of cholesterol [41,42]. For this reason, consuming a dish with mixed vegetables and eggs has a different effect on serum cholesterol concentrations than a low-fiber dish such as eggs and bacon. Mokhtari et al. [26] used this argument to explain how consumption of more than four eggs per week was not significantly associated with the risk of SLD [43]. Our study population is linked to food traditions within the Mediterranean dietary pattern. They have healthy eating habits, and vegetables are a constant in their diet. The cooking methods used are simple, healthy, and without added fat [40]. For example, the preparation of vegetable timbale, a dish made of eggs and vegetables (zucchini, mushrooms, spinach, etc.), baked in the oven or in a non-stick pan without adding fat, is extremely frequent in our study population's diet. Genetic factors also appear to affect cholesterol absorption [44]. Homeostatic control of cholesterol absorption adapts to dietary cholesterol intake, decreasing the amount of cholesterol absorbed at higher dietary intake levels and downregulating endogenous cholesterol synthesis [45,46]. Cholesterol aside, eggs are nutritionally an excellent source of beneficial nutrients. Eggs provide high biological value protein, monounsaturated and polyunsaturated fatty acids, arginine—a precursor to nitric oxide, carotenoids, minerals, and vitamins [47,48].

A current topic of interest is the effect of methyl donor food deficiency, including VIT B12 and folic acid, on the development of SLD [49]. A recent randomized, double-blind controlled trial evaluated the effects of vitamin B12 supplementation on serum liver enzymes, the degree of hepatic steatosis, and metabolic profiles in patients with SLD [50]. A reduction in the steatosis score and liver enzymes, especially ALT, was observed in the VIT B12-treated group. In another study, patients with liver steatosis had lower VIT B12 levels than healthy subjects [51]. A study conducted in rats suggested that the supplementation of folates and VIT B12 could improve obesogenic diet-induced hepatic triglyceride accumulation [52].

Chicken eggs have a non-negligible content of folates and VIT B12 [www.bda-ieo.it. (accessed on 9 November 2023)]. This could explain the protective effect of regular, moderate consumption.

A protective role of eggs has also been found in HTN [53]. Previous studies considered peptides with antihypertensive properties that can stimulate the release of nitric oxide and prostacyclins and inhibit angiotensin-converting enzyme (ACE) activity and endothelium-dependent vasodilatory activity [53]. However, despite the number of studies already published, more well-designed prospective cohort studies are needed to further examine and support this finding [53].

5. Conclusions

In conclusion, the results from our study show a protective role of eggs both against HTN only and associated with SLD. In the study population, the risk of developing these pathological conditions decreased linearly with increased egg consumption (consumption of >3 eggs per week).

In our view, it is unwise to demonize a nutritionally noble food like eggs. It is necessary to inform people about the wise consumption of eggs in order to take full advantage of their nutritional properties. However, further studies are needed to support our results and also to define egg consumption in populations other than those living in Mediterranean areas.

Strengths and Limitations

Among the strengths of the present study are the ample cohort and the generalizability of the results to populations of the Southern Mediterranean. To the best of our knowledge, the scientific literature lacks data and consistent results on the link between egg consumption and SLD. There are currently few studies evaluating this topic, and none were conducted on an older Mediterranean population.

Another important strength is the FFQ, which is the dietary assessment method most commonly used to study diet and eating habits. The FFQ is very complete and detailed, allowing us to obtain all the necessary information about the eating habits of our study population.

However, there are also some limitations. The main one is the nature of the study, which, being cross-sectional, does not clarify the directionality of an association. In addition, the present study only considered the effect of whole-egg consumption; it would be interesting to study the effect of the separate consumption of egg whites and yolks.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/nu16030430/s1>, Table S1: Blood characteristics of patients with SLD or HTN; Table S2: Nutrients (mg/die) eggs intake by disease condition.

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Review

***Vaccinium* spp. Berries in the Prevention and Treatment of Non-Alcoholic Fatty Liver Disease: A Comprehensive Update of Preclinical and Clinical Research**

Ewelina Książek ^{1,*}, Zuzanna Goluch ² and Marta Bochniak ¹

¹ Department of Agroengineering and Quality Analysis, Faculty of Production Engineering, Wrocław University of Economics and Business, Komandorska 118-120, 53-345 Wrocław, Poland; marta.bochniak@ue.wroc.pl

² Department of Food Technology and Nutrition, Faculty of Production Engineering, Wrocław University of Economics and Business, Komandorska 118-120, 53-345 Wrocław, Poland; zuzanna.goluch@ue.wroc.pl

* Correspondence: ewelina.ksiazek@ue.wroc.pl

Abstract: Non-alcoholic fatty liver disease (NAFLD) is a common chronic liver disorder marked by the buildup of triacylglycerols (TGs) in the liver. It includes a range of conditions, from simple steatosis to more severe forms like non-alcoholic steatohepatitis (NASH), which can advance to fibrosis, cirrhosis, and hepatocellular carcinoma. NAFLD's prevalence is rising globally, estimated between 10% and 50%. The disease is linked to comorbidities such as obesity, type 2 diabetes, insulin resistance, and cardiovascular diseases and currently lacks effective treatment options. Therefore, researchers are focusing on evaluating the impact of adjunctive herbal therapies in individuals with NAFLD. One herbal therapy showing positive results in animal models and clinical studies is fruits from the *Vaccinium* spp. genus. This review presents an overview of the association between consuming fruits, juices, and extracts from *Vaccinium* spp. and NAFLD. The search used the following keywords: ((*Vaccinium* OR blueberry OR bilberry OR cranberry) AND ("non-alcoholic fatty liver disease" OR "non-alcoholic steatohepatitis")). Exclusion criteria included reviews, research notes, book chapters, case studies, and grants. The review included 20 studies: 2 clinical trials and 18 studies on animals and cell lines. The findings indicate that juices and extracts from *Vaccinium* fruits and leaves have significant potential in addressing NAFLD by improving lipid and glucose metabolism and boosting antioxidant and anti-inflammatory responses. In conclusion, blueberries appear to have the potential to alleviate NAFLD, but more clinical trials are needed to confirm these benefits.

Keywords: non-alcoholic fatty liver disease; NAFLD; NASH; liver steatosis; *Vaccinium* spp.; blueberry; bilberry; PRISMA; fruits; juices; extract; supplement; animal model; cell line

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is a prevalent and escalating health concern with significant implications for liver-related morbidity and mortality [1]. This condition includes a range of metabolic disorders associated with insulin resistance (IR), progressing from simple fatty liver (NAFL) to more severe forms such as non-alcoholic steatohepatitis (NASH), cirrhosis, and hepatocellular carcinoma (HCC) [2–4]. The prevalence of NAFLD in the general population worldwide varies from 9% to 30%, with the highest rates observed in industrialized communities [5,6]. In Europe, the prevalence of NAFLD reaches 25% [1]. The prevalence is higher among men (40%) than women (26%). It is projected that the prevalence of NAFLD will significantly increase in many world regions by 2030 [7].

NAFLD is induced by increased accumulation of triacylglycerols (TGs), leading to hepatic steatosis, excluding alcohol abuse as a pathogenic factor, although with a histopathological picture resembling alcohol-induced liver damage [8]. The pathogenesis of NAFLD is multifactorial and involves factors such as insulin resistance, oxidative stress, inflammation, and mitochondrial dysfunction [9,10]. Furthermore, the gut–liver axis and gut

microbiota have been linked to the progression of NAFLD by influencing gut homeostasis [9,11]. In NAFLD, disrupted insulin signaling and heightened lipid accumulation are key contributors to hepatocyte injury, leading to increased levels of liver enzymes such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT), which serve as serum indicators of liver damage [12].

The disease often progresses asymptotically in its early stages, which poses challenges for early detection and intervention. NAFLD has emerged as the most prevalent form of chronic liver disease globally, concurrent with the rise in obesity and type 2 diabetes. It is associated with an elevated risk of cardiovascular complications, underscoring its impact on overall health [13]. Additionally, NAFLD is linked to diminished quality of life and heightened mortality risk, with cardiovascular disease being the primary cause of mortality, followed by extrahepatic cancer, liver-related mortality, and diabetes [14–16]. NAFLD represents a complex and multifaceted disease with profound implications extending beyond liver health. Understanding the diverse etiological factors contributing to NAFLD, including metabolic, inflammatory, and genetic factors, is pivotal for developing effective prevention and treatment strategies for this increasingly prevalent disorder.

Multiple pharmacological approaches have been explored in the treatment of NAFLD. Current drug therapies targeting comorbidities associated with metabolic syndrome primarily involve combinations of various agents. These treatment approaches include antioxidants like vitamins E and C and betaine; insulin sensitizers such as thiazolidinediones and metformin; lipid-lowering medications like statins, orlistat, and probucol; cytoprotective agents like ursodeoxycholic acid; and anti-inflammatory or antifibrotic drugs, including pentoxifylline and angiotensin receptor blockers [8,17]. The threat of NAFLD to human health is gradually increasing, underscoring the need for continued research into potential therapeutic targets. Numerous studies indicate that patients with NAFLD suffer from various metabolic disorders, such as dyslipidemia and glucose regulation disturbances, which further exacerbate the condition. The close relationship between metabolism and NAFLD highlights the necessity of metabolic therapy. It is worth considering the use of drugs targeting related diseases in combination with those aimed at NAFLD. Although many compounds have shown promising potential in preclinical studies, their outcomes in clinical trials remain unsatisfactory [18–20]. Despite the registration of over 1400 clinical trials as of August 2024 (<https://clinicaltrials.gov/>), progress in the treatment of NAFLD and NASH remains limited. The first drug approved by the Food and Drug Administration (FDA) in March 2024 is resmetirom, which was authorized for use in the United States under an accelerated approval process in combination with diet and exercise for the treatment of adults with NASH and moderate to advanced liver fibrosis (stages F2 to F3). In the European Union, resmetirom is currently under regulatory review for treating NASH. Resmetirom also reduced NAFLD activity by ≥ 2 points and improved fibrosis by at least one stage without worsening the NAFLD activity score. Resmetirom is an oral THR- β agonist. Hepatotoxicity and adverse effects, such as diarrhea, pruritus, and nausea, have been observed during resmetirom treatment [21,22].

Dietary interventions and physical activity (PA) are broadly recognized as cornerstones in the management of NAFLD/NASH [23]. In routine clinical practice, maintaining a healthy weight, adhering to a well-balanced diet with adequate energy intake, and engaging in physical activity or resistance training are key strategies for preventing and treating NAFLD [24]. Current AASLD, EASL, and ESPEN guidelines recommend weight loss through hypocaloric diets combined with increased physical activity as a primary approach to managing NAFLD/NASH [25]. Although current treatment methods combined with lifestyle changes have proven effective for NAFLD, patient adherence to recommendations is often inadequate. As a result, there is an urgent need to develop innovative therapies that provide high efficacy with minimal side effects for the treatment of NAFLD.

In recent years, natural products have played a significant role in drug development and design, as an increasing number of them demonstrate considerable therapeutic potential in treating various diseases, including NAFLD [25]. Progress in the development of

natural products for NAFLD is evident in clinical research, with 63 out of 1407 studies focusing on natural products (August 2024, according to <https://clinicaltrials.gov/>). To date, studies have shown that berberine and silymarin improve lipid accumulation in the liver of patients and are currently in phase IV clinical trials, potentially leading to new treatments for NAFLD [26–28].

Compounds such as flavonoids, polyphenols, and phytochemicals present in fruits, vegetables, and medicinal plants have exhibited promising therapeutic effects in NAFLD, including attenuation of liver enzymes, antioxidant properties, and anti-inflammatory actions [29]. Plant-derived foods rich in bioactive compounds represent potential natural interventions for preventing and mitigating fatty liver disease [30]. Consequently, researchers are evaluating the impact of adjunctive herbal therapies on individuals with NAFLD [8,30]. One such herbal therapy demonstrating favorable outcomes in preclinical and clinical studies is *Vaccinium* spp. blueberry. Blueberry supplementation has been shown to mitigate hepatic lipid accumulation, oxidative stress, and inflammatory responses by modulating Notch1 signaling, a key regulator of liver lipid metabolism [31,32]. Similarly, cranberry supplementation has been associated with ameliorating high-fat-diet-induced NAFLD in murine models, attributed to its anti-inflammatory and antioxidant properties [33]. Notably, a study by Hormoznejad et al. observed a significant reduction in fibrosis severity among NAFLD patients receiving cranberry supplementation compared to placebo [34]. Current knowledge regarding the full utilization of *Vaccinium* fruits and leaves remains limited, and conclusive clinical results have not yet been achieved. Therefore, significant efforts are still needed to thoroughly elucidate the mechanisms and targets of these compounds, including their hepatoprotective effects, and to develop research strategies that will provide a foundation for creating new drugs.

Studies indicate that blueberry consumption does not lead to significant side effects, making it safe for daily intake and beneficial to health [35,36]. The extract from *Vaccinium arctostaphylos* berries has been shown to be safe, with no adverse effects on the hematopoietic system, liver, or kidneys and no undesirable interactions in patients [37]. Similarly, extracts from *Vaccinium myrtillus* have been well-tolerated in toxicity studies on dogs and rodents, where no adverse effects were reported [38].

Due to the prevalence of NAFLD and the lack of effective treatment modalities, we hypothesized that blueberry supplementation could effectively mitigate the severity of hepatic steatosis in NAFLD patients. To elucidate the mechanisms of action of *Vaccinium* spp. fruits in NAFLD and their therapeutic potential, a comprehensive literature review encompassing in vitro, in vivo, and clinical studies was conducted. This review aims to present an overview of the relationship between the consumption of *Vaccinium* spp. fruits—including juices and extracts—and NAFLD in the context of current achievements.

2. Materials and Methods

2.1. Search Strategy

The current systematic review was conducted following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines [39]. Electronic searches were performed in the academic libraries of Cochrane, Ebsco, PubMed, Embase, Scopus, and Web of Science. The searches combined MeSH terms and keywords, utilizing quotation marks and field tags with Boolean operators. The following keywords were employed for the search: ((*Vaccinium* OR blueberry OR bilberry OR cranberry) AND (“non-alcoholic fatty liver disease” OR “non-alcoholic steatohepatitis”).

Furthermore, to ensure the comprehensiveness of the searches, references of included studies were examined for additional potential sources.

2.2. Selection Criteria

For all databases, the fundamental stages of exclusion were determined as follows: 1. keyword search; 2. publication years (2011–2024); 3. language (English); 4. publication type (article). Selection occurred across the following domains: title, abstract, keywords (Cochrane, Ebsco, Embase, PubMed, Scopus), and topic (Web of Science). The search and selection

process engaged three independent researchers operating concurrently. Results from each database were exported to files in CSV or Excel format, and summaries containing publication information, including abstracts, were generated. Exclusion criteria comprised reviews, research notes, book chapters, case studies, and grants. Three independent researchers scrutinized titles and abstracts. Articles failing to meet inclusion criteria underwent exclusion through deliberation. Two independently operating researchers analyzed the acquired results to mitigate errors. Any discrepancies were resolved through discussion.

Selected studies adhered to the following criteria: (a) experimental investigations involving cellular models, animals, and randomized controlled trials (RCTs); (b) evaluation of the effects of extracts, beverages, powders, or juices on hepatic enzyme levels; (c) provision of adequate data about hepatic enzyme levels.

2.3. Methodological Quality Assessment

Three researchers independently reviewed the methodology of the included animal studies using the Stroke Therapy Academic Industry Roundtable guidelines [40] and assessed clinical trials with the Jadad scale [41]. The researchers evaluated the quality of each animal study based on criteria such as sample size calculation, inclusion/exclusion criteria, randomization, allocation concealment, exclusion reporting, blinded outcome assessment, and disclosure of conflicts of interest and funding. The Jadad scale rated clinical trials on randomization (2 points), double blinding (2 points), and withdrawals/dropouts (1 point), with scores above 4 indicating high quality.

2.4. Study Selection

Given the rapidly evolving number and diversity of databases, information retrieval specialists trained in systematic literature searching were engaged to ensure the high quality of the search process. Each team member independently conducted systematic searches to identify as many eligible studies as possible. To ensure the reliability of the findings, the abstracts were reviewed independently and in duplicate. Following discussions, the researchers decided to include preclinical studies in addition to randomized controlled trials (RCTs) due to the minimal number of clinical studies available on the use of Vaccinium berries in the alleviation of NAFLD.

The team opted not to perform a meta-analysis for three reasons: (1) the existing clinical literature was insufficient, with only two studies available; (2) the included studies differed significantly in terms of dosage, duration, sample size, and method of administration of the active substance; and (3) the review focused on processes, theory development, and a qualitative description of the studies. Data analysis and quality assessment of the articles were conducted independently by three researchers, with the results of each study recorded in an evidence table.

The article selection process was carried out in three stages. In the first stage, three researchers initially screened titles and abstracts. In the second stage, a detailed assessment of the articles was performed based on predefined selection criteria, developed according to the research question, with the results organized in a table. In the third stage, one researcher integrated all results into a single document, and the articles were subjected to detailed analysis to determine their inclusion in the study. The selected studies were then organized by year of publication and alphabetically by the first author's surname. Any disagreements were discussed and resolved through consultation among the researchers. The preliminary searches produced 150 results, and after removing duplicates, 46 peer-reviewed articles were selected for further consideration based on relevance (Figure 1).

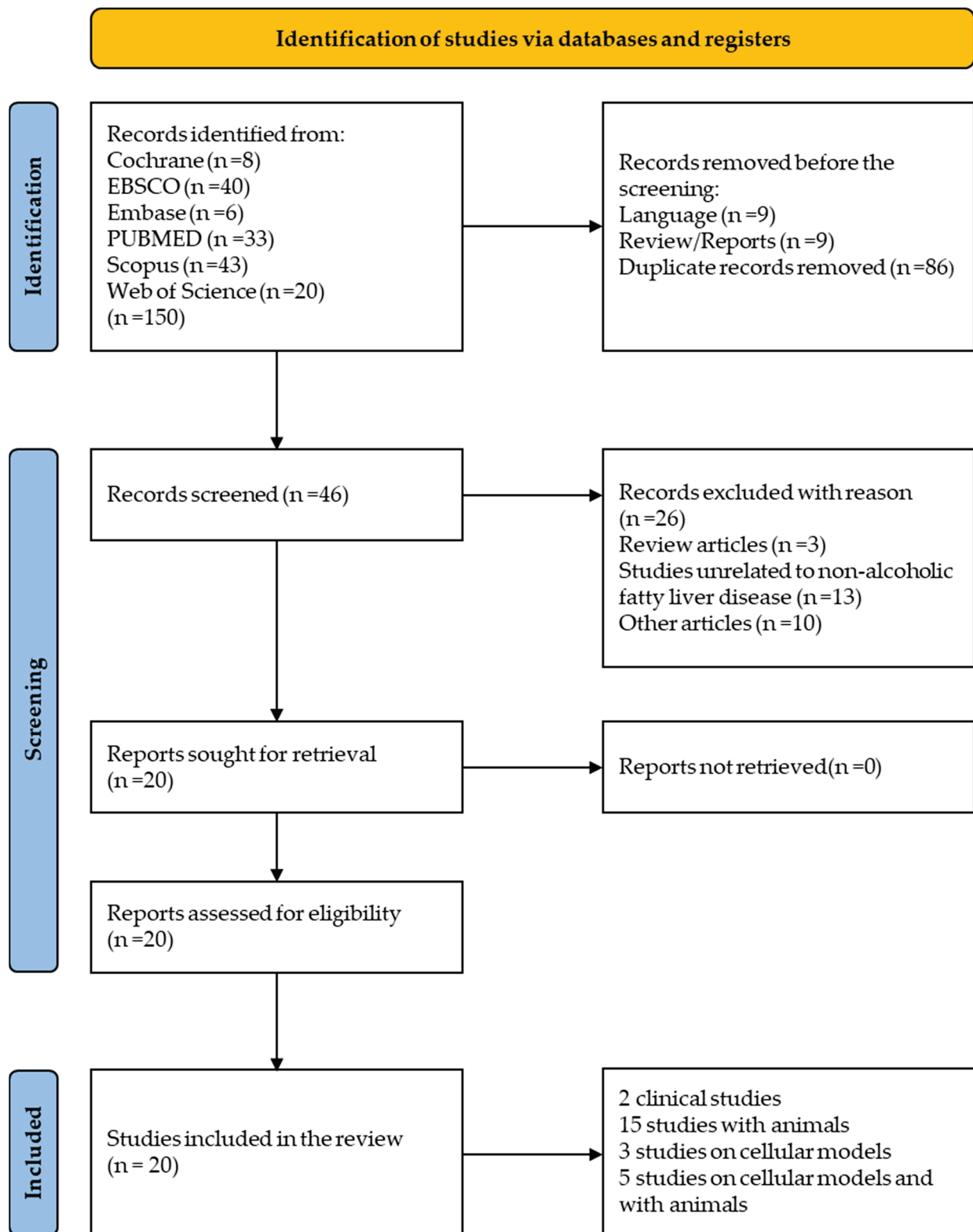


Figure 1. Flow diagram of the study selection process.

3. Results

3.1. Overview of *Vaccinium* spp.

The *Vaccinium* species, belonging to the *Ericaceae* family and the *Rhododendron* genus, complement the morphologically dominant taxon, which encompasses 4250 species. In Europe, the species of this genus include *Vaccinium myrtillus* L., *Vaccinium vitis-idaea* L., *Vaccinium oxycoccus* L.,

and *Vaccinium uliginosum* L. The flowers, leaves, and fruits of these plants are widely used in traditional medicine [42]. Due to their numerous health benefits, these plants are utilized in the prevention and treatment of various conditions, including cardiovascular diseases, neurodegenerative disorders, infections, rheumatoid arthritis, and cancer [43–48]. Their primary health effects are attributed to their antioxidant, antimicrobial, and detoxifying properties. It is also noteworthy that these plants, mainly those rich in polyphenols such as anthocyanins, exhibit a particularly beneficial impact on health [49,50]. Anthocyanins are the primary compounds isolated and identified in berries, constituting 0.1 to 0.25% of the fresh fruit weight, as well as in leaves, alongside other active components such as resveratrol, flavonols (e.g., quercetin, catechins), phenolic acids, ellagitannins, and iridoids [42]. The presence of various types of anthocyanins characterizes the fruits of the *Vaccinium* genus. Specifically, compounds such as cyanidin 3-O-galactoside, cyanidin 3-O-glucoside, cyanidin 3-O-arabinoside, delphinidin 3-O-galactoside, delphinidin 3-O-arabinoside, delphinidin 3-O-glucoside, malvidin 3-O-galactoside, malvidin 3-O-arabinoside, malvidin 3-O-glucoside, petunidin 3-O-galactoside, petunidin 3-O-arabinoside, petunidin 3-O-acetylglucoside, peonidin 3-O-galactoside, and peonidin 3-O-arabinoside have been identified [51,52].

Studies suggest that anthocyanins may have a beneficial impact on liver health, particularly in the context of NAFLD, by balancing lipid storage and metabolism. Incubation of HepG2 cells, treated with oleic acid, with petunidin-3-O-galactoside (100 μ M), petunidin-3-O-glucoside (50 and 100 μ M), and malvidin-3-O-galactoside (100 μ M) derived from *Vaccinium virgatum* fruits for 12 h significantly reduced intracellular TC and TG levels compared to the model group. Malvidin and petunidin exhibited more significant hypolipidemic activity than delphinidins in HepG2 cells [53]. Among the anthocyanins present in blueberries, malvidin-3-O-glucoside and malvidin-3-O-galactoside significantly alleviated FFA-induced lipid accumulation. Additionally, malvidin-3-O-glucoside (M3G) and malvidin-3-O-galactoside inhibited oxidative stress by suppressing ROS, increasing glutathione levels, and enhancing antioxidant enzyme activity [54]. The study on the effects of malvidin, malvidin-3-O-glucoside, and malvidin-3-O-galactoside from *Vaccinium ashei* in the human HepG2 cell line and in streptozotocin-induced diabetic mice demonstrated that anthocyanins reduced ROS levels by 80%, 76%, and 91% and increased HepG2 cell viability by 79%, 73%, and 98%, respectively. They also inhibited hyperglycemia and hyperlipidemia by decreasing the expression levels of enzymes involved in gluconeogenesis and lipogenesis and increasing those engaged in glycogenolysis and lipolysis via the AMPK signaling pathway in HepG2 cells [55].

Anthocyanins may exhibit potential in alleviating NAFLD due to their antioxidant properties and effectiveness in controlling lipid metabolism, glucose homeostasis, and transcription factors [56]. Unfortunately, these compounds are highly susceptible to degradation when exposed to high pH, light, heat, and oxygen during processing and storage and to interactions with other food components and additives, leading to poor bioavailability and reduced bioactivity [49]. Anthocyanins typically exist in the form of a flavylium cation, which predominates in highly acidic aqueous solutions (pH < 2). Under the pH conditions found in plants, food, and the gastrointestinal tract (from pH 2 to pH 8), anthocyanins transform into a mixture of colored and colorless forms in equilibrium through acid–base reactions, water addition–elimination processes, and isomerization. Each chemical species is characterized by specific features (such as charge, electron distribution, planarity, and shape), which affect its reactivity and interactions with plant or food components, including other phenolic compounds [57]. It is believed that the bioavailability of anthocyanins is among the lowest of all flavonoids, amounting to only 1–2% [58]. However, studies by Czank K. et al. [59] report that anthocyanins have a minimal relative bioavailability of $12.3 \pm 1.3\%$, with their metabolites reaching peak serum concentrations 42 times higher. Anthocyanins are typically consumed as a mixture of various compounds and structural forms, which can undergo further transformation, metabolism, and degradation in different sections of the gastrointestinal tract and other body compartments. Human intervention studies on absorption, distribution, metabolism, and elimination suggest that peak con-

centrations of anthocyanins and phase II anthocyanidin conjugates in serum are reached after approximately 1.5 h, with levels around 100 nmol/l following doses of ≤ 500 mg of anthocyanins. This indicates anthocyanins are significantly less bioavailable than other flavonoids [58–60]. Anthocyanins are likely absorbed across the intestinal membrane via transporters such as OATP 2B1 (for all forms), GLUT2 (for both aglycones and glycosylated forms), and SGLT1 (for glycosylated forms), though these absorption pathways require further in-depth validation through research. Given the diversity of anthocyanins in nature, depending on the aglycone structures and types of sugar molecules, it is essential to conduct additional studies on structure–activity relationships to understand better their bioavailability in the human body [61,62].

Low bioavailability, primarily due to limited stability, is a significant drawback associated with anthocyanins. Therefore, preserving these compounds present in blueberries and enhancing their bioavailability are crucial [61]. Consequently, efforts are being made to enhance the limited bioavailability of phytochemicals present in blueberries. These strategies include the use of various delivery systems such as encapsulation in lipid nanocarriers or liposomes, emulsions, micelles, incorporation into polymeric nanoparticles, solid dispersions, and nanocrystals [63]. In recent years, research has focused on nanoencapsulation, microencapsulation, and protein complexes. Macromolecular components are currently considered effective encapsulating carriers that protect anthocyanins from degradation during digestion [61]. Studies by Lang X. et al. [64] have demonstrated that the two main casein monomers, α -casein and β -casein, can preserve the stability and antioxidant capacity of blueberry anthocyanins under processing conditions. Further research by Lang X. et al. [65] showed that both α -casein and β -casein can enhance the stability of blueberry anthocyanins during intestinal digestion and protect their antioxidant properties. Additionally, the inclusion of α -casein or β -casein could improve the bioavailability of blueberry anthocyanins. To enhance the bioavailability and applications of anthocyanins, nanocomplexes have also been employed, which may exert a protective effect on the gastrointestinal tract and other processes. Ge et al. observed that anthocyanin nanocomplexes, prepared from chitosan hydrochloride and carboxymethyl chitosan, exhibited improved stability when stored at various temperatures and pH levels. The bioavailability of anthocyanins in these nanocomplexes reached 40.1%, compared to 17.2% for free anthocyanins, indicating a significant improvement in bioavailability due to encapsulation in nanocomplexes [66,67]. In contrast, nanoliposomes containing lecithin and cholesterol, prepared using enhanced supercritical carbon dioxide, provided additional stability to anthocyanins during storage and simulated gastrointestinal digestion. Protection of anthocyanins was observed during simulated intestinal digestion, where encapsulated anthocyanins decreased to 72.76% compared to 52.01% for free anthocyanins. Unfortunately, the use of nanoliposomes as protectors for anthocyanins appears to be limited by their instability when in contact with pancreatin [68]. Microencapsulation effectively stabilizes anthocyanins against degradation caused by light and oxygen, enhancing their utility in functional products [69]. Encapsulation using a combination of carboxymethyl starch and xanthan gum has demonstrated enhanced thermal stability of blueberry anthocyanins. Additionally, storage stability results showed that the stability of anthocyanins increased to 76.11% after 30 days of storage at 37 °C. The anthocyanins were also primarily retained within the microcapsules in the stomach and released in the intestine [70]. Nanoparticle systems, microcapsules, and protein complexes contribute to enhancing the stability and bioavailability of anthocyanins. However, the results of research and technological innovations require further application to develop safe products that provide health benefits to consumers.

3.2. Study Selection and Characteristics

A comprehensive search across multiple databases, including Cochrane, Ebsco, Embase, PubMed, Scopus, and Web of Science, yielded 150 articles. After removing duplicates, 64 articles remained. Of these, 18 were excluded due to being in a language other than English or not being original research. An additional 18 articles were excluded because they did not pertain to NAFLD or failed to meet the inclusion criteria. Finally, 20 articles

met the inclusion criteria and were included in the analysis. The characteristics of the included studies are presented in Tables 1–3. All studies were published between 2011 and 2024. The selected articles included clinical studies (2), animal studies (15), cell line studies (3), and studies involving both animal and cell line research (5).

In the clinical studies, the Kolmogorov–Smirnov test was used to assess data distribution. For comparing variables within groups, either the paired *t*-test or the Wilcoxon test was employed, while for comparing variables between two groups, either the independent *t*-test or the Mann–Whitney U test was used. A significance level of $p < 0.05$ was applied in the studies. In the studies using cellular and animal models, statistical differences in data were assessed using the independent samples *t*-test and one-way analysis of variance (ANOVA) [31–33,35,71–80], with post hoc comparisons performed using Duncan’s multiple-range tests [81]. Multiple comparisons were also conducted using Tukey’s test for parametric variables and the Kruskal–Wallis test for non-parametric variables [72,82,83]. In one study, the statistical significance of differences was determined using Tukey’s test [84]. In the study by Sotelo-Gonzales et al. [82], principal component analysis (PCA), sparse partial least squares discriminant analysis (sPLS-DA), and K-means clustering were also performed based on the urinary metabolite profiles of each experimental group. Statistical differences at $p < 0.05$ were considered significant in all the cited studies.

3.3. Clinical Study

Clinical studies evaluating the effects of plants from the genus *Vaccinium* are limited. There are only two studies assessing the impact of cranberry tablets on patients with NAFLD, which are summarized in Table 1 [34,85]. Both human studies are randomized controlled trials conducted directly on patients with NAFLD, diagnosed based on liver ultrasonography. Pregnant and breastfeeding individuals, as well as those with diabetes, other liver diseases, heart failure, renal insufficiency, pulmonary insufficiency, alcohol abuse, and those using antioxidant or vitamin supplements other than vitamin E were excluded from both studies. The duration of the trials ranged from 12 weeks and 6 months.

In the study conducted by Hormoznejad R. et al. [34], patients with NAFLD (18 females and 23 males) were randomly allocated to receive either a cranberry supplement or a placebo for 12 weeks, administered twice daily. Participants in the cranberry group received tablets containing 144 mg of *Vaccinium macrocarpon* extract, providing at least 36 mg of proanthocyanidins, equivalent to 13 g of dried cranberry fruit. The remaining composition of the tablets was unspecified. Placebo tablets contained 288 mg of starch. All patients followed a hypocaloric diet, consuming 500–1000 kcal less than their estimated energy needs. Fasting insulin levels significantly decreased in both groups; however, the reduction was more significant in the cranberry group compared to the placebo group (from 10.55 to 8.20 μ /mL and from 10.66 to 9.80 μ /mL, respectively, $p = 0.005$). The homeostatic model assessment of insulin resistance (HOMA-IR) index showed significant improvement in the cranberry group, decreasing to 1.88. No significant differences between the cranberry and placebo groups were observed in the TG, TC, and LDL-C levels ($p > 0.05$). Significant improvements in ALT levels were noted in both groups posttreatment compared to baseline, with a markedly more significant reduction in the cranberry group compared to the placebo group (from 58.35 to 36.90 IU/L and from 55.33 to 45.42 IU/L, respectively; $p = 0.040$). Ultrasound assessments revealed a significant decrease in liver steatosis in both groups after the intervention ($p < 0.001$), with no significant differences between the postintervention data of the two groups.

In the study by Shirazi M.K. et al. [85], 110 patients diagnosed with NAFLD were initially enrolled, with data ultimately analyzed from 94 participants (49 females, 45 males) due to dropouts from nine in the intervention group and seven in the control group. The diagnosis was confirmed via liver ultrasound by gastroenterology specialists. The study included adults over 18 who followed a hypocaloric diet, reduced their daily intake by 500 kcal below their estimated energy needs, and received vitamin E supplementation. The intervention group, comprising 46 patients, was given one cranberry capsule (144 mg) daily, while the placebo

group, consisting of 48 patients, received a placebo with the same base formula minus the active ingredient for a duration of six months. The cranberry capsules contained 144 mg of *Vaccinium macrocarpon*, equivalent to 13 g of dried cranberry fruit. Out of one hundred and ten patients with NAFLD, nine from the intervention group and seven from the control group did not continue with the observation. In lipid profile measurements, average levels of TC (189.02 mmol/L; $p < 0.001$) and TG (190.54 mmol/L; $p = 0.01$) were significantly lower in the cranberry group compared to the placebo group (200.29 mmol/L and 188.54 mmol/L, respectively). At the end of the intervention, average levels of insulin and HOMA-IR were significantly lower in the cranberry group (5.62 $\mu\text{L}/\text{mL}$ and 1.39, respectively) compared to the placebo group (10.06 $\mu\text{L}/\text{mL}$ and 2.51, respectively).

3.4. Preclinical Study

Preclinical studies evaluated the effects of standardized aqueous or alcoholic extracts [31,32,74,82], juices [75,76], powders [33,35,80,84], nutraceuticals [71,72,81], and preparations containing monomers [77,78] from berry fruits in various animal models, primarily in male Sprague Dawley rats and male C57BL/6N mice. The animal models in these studies were categorized into (1) dietary supplementation, (2) pharmacological intervention, (3) genetic mutation, or (4) a combination of these approaches. Various diets were employed to simulate NAFLD or NASH, including Western, high-fat, and high-cholesterol diets, all containing elevated sucrose, fructose, soybean oil, or lard levels. These dietary regimes resulted in liver alterations, weight gain, and insulin resistance, critical preclinical research issues. The summary of preclinical studies is presented in Table 2.

3.4.1. *Vaccinium* and the Alleviation of Liver Steatosis and Hepatocellular Damage

In the analyzed studies, the use of extracts or juices from *Vaccinium* berries in combination with diet-induced NAFLD in animal models was investigated. In the studies by Ren et al. [75], the administration of berry juice to Sprague Dawley rats (200 to 250 g) at a dose of 15 g/kg body weight once daily contributed to mitigating the severity of NAFLD and lipid degeneration of hepatocytes. In the control group of the NAFLD model, hepatocyte lobular structures were disrupted, and hepatic plates were scattered with numerous lipid droplets. The disrupted liver structures were effectively restored and organized around the central vein in the berry juice experimental model. Several small vacuoles and numerous small red lipid droplets were also observed. In the study by Morrison et al. [72], administering Mirtoselect at a 0.1% (w/w) concentration helped slow down the progression of liver steatosis caused by a Western-type diet, which includes 15% cocoa butter, 1% corn oil, 40.5% sucrose, 20% acid casein, 10% corn starch, and 6.2% cellulose. The treatment significantly reduced macrovesicular steatosis ($p < 0.001$) and also decreased microvesicular steatosis ($p = 0.027$). Histological examination of the mice indicated fewer enlarged hepatocytes lacking cytokeratin 18, indicating a protective effect against liver cell damage. In research conducted by Glisan et al. [33], dietary supplementation with blueberry extract led to a 31.2% reduction in plasma ALT levels in obese mice on a high-fat diet compared to those on a high-fat diet alone. Although the total number of hepatic lipid droplets did not significantly differ between the groups, histological analysis showed a notable decrease in both the total lipid droplet area and the overall hepatic lipid area in the livers of mice that received the blueberry extract. In the studies by Ren et al. [76], hepatocytes in healthy Sprague Dawley rats regularly surrounded the tubular section, with all lipid droplets being small. In the control group of the NAFLD model, liver cell structures were destroyed and widely dispersed with numerous lipid droplets. The administration of berry juice at a dose of 10 mL/kg body weight of rats reduced the size of lipid droplets. Liver cords exhibited a radial arrangement around the central vein, accompanied by a decrease in the number of lipid droplets. Flow cytometry analysis demonstrated that treatment with berry juice decreased apoptosis rates in NASH models. In the study by Shimizu et al. [84], serum ALT levels in mice fed a high-fat diet supplemented with cranberry and those fed a high-fat diet did not show statistically significant differences. However, ALT levels were lower in mice supplemented with 1% cranberry powder. The same study observed a reduction in lipid

droplets and hepatocyte ballooning in mice fed a cranberry-supplemented diet compared to those on a high-fat diet. Haga et al. [74] explored the impact of 5% and 10% bilberry (*Vaccinium myrtillus* L.) fruit extracts on liver steatosis and damage in mice. Their study found that bilberry extracts significantly reduced liver fat accumulation and triglyceride content, as shown by histological analysis and hepatic TG measurements. While a high-fat, high-cholesterol diet caused mild fibrosis in the liver around the portal area, mice treated with bilberry extracts exhibited significantly less fibrosis. Histological analysis of mice on a high-fat diet revealed substantial lipid droplets, ballooning degeneration, and inflammatory cell infiltration, indicating hepatic steatosis and inflammation. In contrast, liver sections from the group receiving lyophilized bilberry leaf extracts at a dose of 400 mg/kg per day showed very mild steatosis and inflammatory foci, suggesting that high doses effectively alleviated diet-induced hepatic steatosis and inflammatory infiltration [35]. The administration of a 2% anthocyanin extract from bilberries to laboratory animals on a Western diet significantly reduced lipid content in the liver compared to both the normal diet and Western diet groups [81]. Faheem et al. [71] observed that liver sections of rats treated exclusively with cranberry showed normal liver architecture. Meanwhile, animals on a high-fat and high-cholesterol diet concurrently treated with cranberry (100 mg/kg) exhibited only mild congestion in the portal vein associated with mild ballooning degeneration in hepatocytes and no signs of fibrosis. Nanoparticles resembling exosomes, isolated from blueberries and administered to mice concurrently with a high-fat diet, demonstrated the ability to reduce lipid droplet accumulation and liver mass. Additionally, qRT-PCR analyses revealed that these exosomes decreased mRNA levels of FAS and ACC1 enzymes in the livers of high-fat-diet-fed mice [78]. In the study conducted by Hewage et al. [31], supplementation with lingonberry significantly reduced hepatic TG accumulation and total cholesterol levels. Additionally, a decreased number and size of vacuoles were observed in liver sections of mice fed a high-fat diet supplemented with lingonberry (5% *w/w*). In the study by Zhu et al. [77], supplementation with blueberry monomers (TEC) via gavage at doses of 7.5, 15.0, or 30.0 mg/kg once daily for 6 weeks significantly alleviated hepatic steatosis in mice on a high-fat diet.

3.4.2. *Vaccinium* and the Alleviation of Hepatic Fibrosis

NAFLD encompasses a range of liver damage, from clinically mild intrahepatic fat accumulation (steatosis) to the more advanced non-alcoholic steatohepatitis (NASH), which can progress further to fibrosis, cirrhosis, and hepatocellular carcinoma.

Histological observations by Morrison et al. [72] demonstrated that liver collagen content was significantly lower ($p = 0.034$) in the diet supplemented with Mirtoselect. A significant reduction in Colla1 expression was also observed compared to the high-cholesterol diet. The expression of the hepatic stellate cell activation marker gene Acta2 (D), as well as the induction of profibrotic cytokines Tgfb1 (E) and Tnf (F), was less pronounced with Mirtoselect supplementation. Mirtoselect strongly inhibited TGF- β signaling activation and suppressed hepatic stellate cell activation. It was concluded that Mirtoselect significantly mitigated disease progression. Faheem et al. [71] observed that cranberry supplementation in the diet reduced TGF- β levels by 28% and 49%, α -SMA levels by 32% and 45%, and hydroxyproline levels by 17% and 37% at doses of 50 mg/kg and 100 mg/kg, respectively. Rats fed a high-fat, high-cholesterol diet and simultaneously treated with cranberry at doses of 50 mg/kg and 100 mg/kg exhibited a significant reduction in collagen deposition. The activation of hepatic stellate cells and liver fibrosis may be driven by the activation of the NLRP3 inflammasome. In the study by Zhu et al. [77], blueberry TEC monomers significantly inhibited pyroptosis and reduced NLRP3 inflammasome activation, suggesting that TEC may improve NASH. Ryyti et al. [80] found at the functional level that lingonberry supplementation prevents the high-fat-diet-induced upregulation of inflammatory response genes, specifically Cxcl14, which is one of the genes involved in liver inflammation and fibrosis.

Table 1. Characteristics of the included randomized clinical trials regarding the impact of *Vaccinium macrocarpon* on liver enzyme levels.

Study	Study Design	Study Population	Type of Intervention	Dose (mg/day)	Trial Duration	ALT Levels (U/L)	AST Levels (U/L)	Insulin (μ mL)	HOMA IR	Jadad Scale
Hormoznejad et al. 2020 [34]	Randomized double-blind, placebo-controlled clinical trial	Age $\geq$ 18 years; BMI 25 ± 5 kg/m ² , N = 41 (groups: cranberry n = 20, placebo n = 21)	The placebo and cranberry groups received either placebo or cranberry tablets (two tablets; one tablet after lunch and another one after dinner)	288	12 weeks	Before: in the cranberry group: 58.35 ± 18.03 ; in the placebo group: 55.33 ± 26.10 ; after: in the cranberry group: 36.90 ± 9.00 ; in the placebo group: 45.42 ± 15.59	Before: in the cranberry group: 26.85 ± 10.30 ; in the placebo group: 29.95 ± 15.02 ; after: in the cranberry group: 22.60 ± 7.68 ; in the placebo group: 24.90 ± 15.79	Before: in the cranberry group: 10.55 ± 1.43 ; in the placebo group: 10.66 ± 1.55 ; after: in the cranberry group: 8.20 ± 0.61 ; in the placebo group: 9.80 ± 1.36	Before: in the cranberry group: 2.59 ± 0.86 ; in the placebo group: 2.38 ± 0.71 ; after: in the cranberry group: 1.88 ± 0.20 ; in the placebo group: 2.20 ± 0.45	5
Shirazi et al. 2021 [85]	Randomized double-blind, placebo-controlled clinical trial (parallel)	Age $\geq$ 18 years, N = 110 (groups: cranberry n = 46, placebo n = 48)	The cranberry capsule includes 144 mg <i>Vaccinium macrocarpon</i> (equal to 13 g dried cranberry fruit)	144	6 months	Before: in the cranberry group: 42.74 ± 15.04 ; in the placebo group: 47.48 ± 18.35 ; after: in the cranberry group: 39.54 ± 16.95 ; in the placebo group: 38.69 ± 14.20	Before: in the cranberry group: 37.22 ± 13.51 ; in the placebo group: 41.17 ± 16.69 ; after: in the cranberry group: 32.98 ± 14.33 ; in the placebo group: 31.98 ± 12.48	Before: in the cranberry group: 10.38 ± 3.09 ; in the placebo group: 10.65 ± 3.02 ; after: in the cranberry group: 5.62 ± 2.04 ; in the placebo group: 10.06 ± 2.94	Before: in the cranberry group: 2.78 ± 0.99 ; in the placebo group: 2.84 ± 0.98 ; after: in the cranberry group: 1.39 ± 0.62 ; in the placebo group: 2.51 ± 0.85	5

ALT—alanine aminotransferase; AST—aspartate transaminase; HOMA-IR—homeostatic model assessment for insulin resistance.

3.4.3. Anti-Inflammatory Effects of *Vaccinium*

In individuals with NAFLD, lipotoxicity, insulin resistance, and endotoxins trigger the activation of proinflammatory cytokines such as tumor necrosis factor- α (TNF- α), interleukin-1 α (IL-1 α), interleukin-1 β (IL-1 β), interleukin-6 (IL-6), and resistin [3]. This inflammation plays a crucial role in the progression from steatosis to NASH. Furthermore, TNF- α and its receptor impede insulin receptors and activate the NF- κ B transcription factor [86,87].

A significant reduction in liver inflammation was observed in studies investigating the effects of a high-cholesterol diet combined with the nutraceutical Mirtoselect ($p < 0.001$). Mirtoselect did not influence the expression of *Emr1* or *Ccl2*, indicating its anti-inflammatory effect might limit the influx of other immune cells. Additionally, there was no increase in the hepatic gene expression of the neutrophil marker *Mpo*, suggesting a decrease in neutrophil infiltration. Supporting this, the expression of the neutrophil chemoattractants *Cxcl1* and *Cxcl2* was also reduced by the high-cholesterol diet and Mirtoselect combination. Moreover, Mirtoselect mitigated the disease-related increase in hepatic free cholesterol. The high-cholesterol diet significantly enhanced TNF- α and IL-1 β signaling, which activated the proinflammatory transcription factor NF- κ B. There was a positive correlation between liver-free cholesterol levels and p65-NF- κ B transcriptional activation, linking cholesterol to inflammation. Conversely, Mirtoselect helped reduce p65-NF- κ B activity [72]. Glisan et al. [33] examined the impact of cranberry extract supplementation on liver inflammation in obese C57BL/6J mice on a high-fat diet. They discovered that the dietary inclusion of cranberry extract lowered the hepatic mRNA expression of TNF- α , *Ccl3*, and *Cox2*, as well as the hepatic protein levels of CCL2 and TNF- α when compared to mice on a high-fat diet without the extract. The study also revealed that cranberry extract significantly reduced hepatic mRNA levels of Tlr4 and NF- κ B. These findings, alongside the decrease in TNF- α protein levels and the reduction in the liver's *Cox2* and TNF- α mRNA levels, indicate that the extract partially mitigates liver inflammation by influencing the TLR4/NF- κ B signaling pathway. Ren et al. [76] investigated the impact of blueberry juice on the development of NASH. In this study, qRT-PCR analysis indicated that mRNA levels of IL-6 and TNF- α were lower with dietary supplementation of blueberry juice in laboratory animals. Treatment with blueberry juice reduced the levels of IL-6 and TNF- α . Similar findings were obtained by Haga et al. [74], who investigated extracts from *Vaccinium myrtillus* L. in a murine model of liver steatosis. The research results demonstrated reduced levels of proinflammatory cytokines TNF- α , IL-9, IL-1 β , and IFN- γ . Treatment with cranberry showed a tendency to suppress IL-6 expression, as observed in the study by Shimizu et al. [84]. Cranberry at 50 and 100 mg/kg doses significantly reduced TNF- α levels by 48% and 72%, respectively. Serum levels of IL-6 also significantly decreased by 36% and 73% at doses of 50 and 100 mg/kg, respectively. Cranberry at both doses reduced the activation of NF- κ B, which regulates the production of several proinflammatory cytokines, including TNF- α and IL-6 [71]. In the study by Hewage et al. [32], a high-fat diet induced the formation of inflammatory foci in the livers of mice and increased hepatic mRNA levels of IL-6, MCP-1, and TNF- α . Inflammatory foci were not observed in mice supplemented with cranberry. Furthermore, supplementation with cranberry reduced the expression of IL-6, MCP-1, and TNF- α mRNA [80].

Lingonberry supplementation decreased the expression of inflammation-related genes (*Saa1*, *Saa2*) that were upregulated by a high-fat diet. In mice, the serum amyloid A (*Saa*) gene family plays a role in the development of chronic inflammation, fibrosis, and secondary amyloidosis [80].

Recent research involving mice has underscored the importance of hepatocyte inflammasomes in linking early metabolic stress to subsequent hepatocyte death and fibrogenesis in NASH. These inflammasomes, which are multiprotein complexes within the cytoplasm, respond to danger-associated molecular patterns (DAMPs)—such as saturated fatty acids from de novo lipogenesis (DNL)—and pathogen-associated molecular patterns (PAMPs) from gut microbiota that enter the liver via the portal vein. Data indicate that activating

inflammasomes in the liver produces proinflammatory cytokines like interleukin (IL)-1 β and IL-18 and initiates apoptosis through caspase-1 (Casp1) activation [3,88]. In experiments conducted by Glisan et al. [33], mice given cranberry extract showed a substantial decrease in hepatic expression of NLRP3 (43%) and thioredoxin-interacting protein (Txnip, 30%) compared to control mice fed a high-fat diet. Furthermore, cranberry supplementation resulted in a significant reduction in the expression of PPAR- α , a transcription factor involved in regulating Txnip, and a 35% decrease in hepatic Casp1 expression.

Studies conducted on various animal models have shown that extracts from cranberry and other plants of the *Vaccinium* genus can significantly reduce levels of proinflammatory cytokines such as TNF- α and IL-6 and modulate signaling pathways, including NF- κ B and TLR4 (Figure 2), which are key in the pathogenesis of NAFLD and NASH.

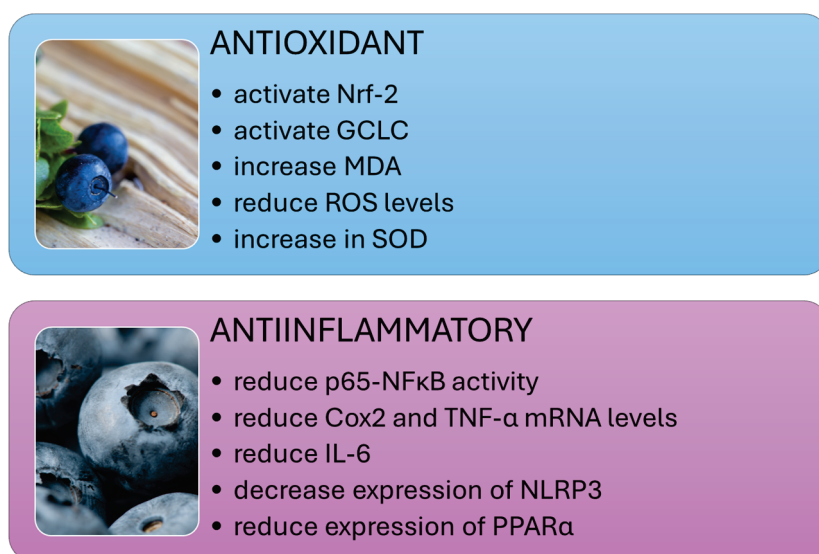


Figure 2. Systemic anti-inflammatory and antioxidant effects of dietary polyphenols.

3.4.4. Antioxidant Effects of *Vaccinium*

Elevated free radicals, lipid peroxidation products, and reduced antioxidant levels characterize NAFLD. The accumulation of fat in the liver exacerbates lipotoxicity through high concentrations of free fatty acids (FFAs), free cholesterol, and other lipid metabolites. This leads to oxidative stress, increased reactive oxygen species, and the activation of endoplasmic reticulum stress and mitochondrial dysfunction. The transcription factor nuclear factor-erythroid 2-related factor 2 (Nrf-2) plays a vital role in maintaining cellular redox balance. Research has shown that extracts from *Vaccinium* plants can activate Nrf-2, as well as heme oxygenase-1 (HO-1), NADPH quinone dehydrogenase (NQO1), and glutamate-cysteine ligase catalytic (GCLC) (Figure 2). This activation results in increased production of antioxidant defense enzymes and reduced oxidative stress [56,75,89,90].

For instance, in the livers of high-fat-diet-fed subjects supplemented with *Vaccinium corymbosum* L. leaf extract, a notable increase in the levels of ERR α , Nrf-1, and Nrf-2 genes was observed [35]. Nakano et al. assessed certain antioxidant factors and enzyme levels in the liver. The level of Nrf-2, a key transcription factor regulating the expression of antioxidant proteins, decreased 0.7-fold with a Western diet and returned 1.3-fold with the same diet supplemented with blueberry extract. Additionally, the ubiquitinated Nrf-2 (Ub-Nrf-2) level increased 3.6-fold in animals whose diet included blueberry extracts. Conversely, the level of TBARSs in the liver, a sensitive marker of lipid peroxidation in animal tissues, significantly decreased following supplementation with black currant extracts [81]. In the study by Zhao et al., the distribution of Nrf-2 in hepatocytes of mice fed a high-fat diet supplemented with exosome-like nanoparticles from blueberry was determined. The supplementation accelerated the translocation of Nrf-2 from the cytoplasm to the nuclei in the livers of mice fed a high-fat diet. The expression of Bcl-2

and HO-1 increased in the livers of C57BL/6 mice fed a high-fat diet supplemented with blueberry, while the level of Bax protein decreased [78]. Conversely, the studies by Hewage et al. [32] demonstrated that supplementing with 2% or 5% (*w/w*) *Vaccinium vitis-idaea* L. (lingonberry) restored nuclear Nrf-2 protein levels in the liver. Additionally, the study measured the levels of genes and proteins involved in glutathione synthesis, specifically glutamate-cysteine ligase (Gclc—the catalytic subunit and Gclm—the modifier subunit) and glutathione synthetase. Lingonberry supplementation increased the expression of Gclc and the mRNA levels of Gclm in the liver.

Oxidative stress biomarkers encompass malondialdehyde (MDA) and reduced glutathione (GSH). During oxidative stress, intracellular superoxide species are produced, contributing to liver damage. The liver's antioxidant enzyme, superoxide dismutase (SOD), is essential for removing these harmful species [91,92]. Antioxidant enzymes, including SOD, catalase (CAT), and glutathione peroxidase (GPx), increased in response to supplementation with extracts from plants of the *Vaccinium* genus [32,35,75,76,78,81]. Rats administered cranberry exhibited a marked increase in SOD and catalase activities, as well as depleted GSH levels. A higher dose of cranberry (100 mg/kg) significantly restored depleted GSH by 257% compared to the high-fat diet group, in which SOD and GSH levels had decreased by nearly 80% [71]. Similarly, in the studies by Li et al., blueberry leaf extract inhibited the increase in MDA and reduced ROS levels in the liver [35]. Feeding mice a high-fat diet supplemented with lingonberry reduced MDA levels and restored GSH levels. Lingonberry supplementation also decreased hepatic GSSG levels and restored the GSH/GSSG ratio [32].

Additionally, Shimizu et al. measured the mRNA levels of IL-6, a proinflammatory cytokine, as an indicator of oxidative stress. Their results indicated that cranberry treatment tended to suppress IL-6 expression and reduce oxidative stress in the liver [84].

3.4.5. *Vaccinium*, Lipid Metabolism, and NAFLD

Recent research on NAFLD and lipid metabolism disorders has indicated that disruptions in hepatic lipid metabolism lead to lipid accumulation, resulting in hepatotoxicity and NAFLD. Dyslipidemia, marked by elevated levels of FFA, ox-LDL, and TG in the plasma, contributes to inflammation, oxidative stress, lipotoxicity, and liver damage. This condition can manifest at any stage of NAFLD and can worsen its progression [3]. Peroxisome proliferator-activated receptors (PPARs) and sterol regulatory element-binding proteins (SREBPs) are crucial for regulating lipid metabolism and are significant in the pathogenesis of NAFLD. Studies indicate that certain *Vaccinium* species, such as blueberries and cranberries, may affect these transcription factors and potentially offer therapeutic benefits in NAFLD by modulating AMPK signaling (Figure 3) [9,31,32,35,71,75,93].

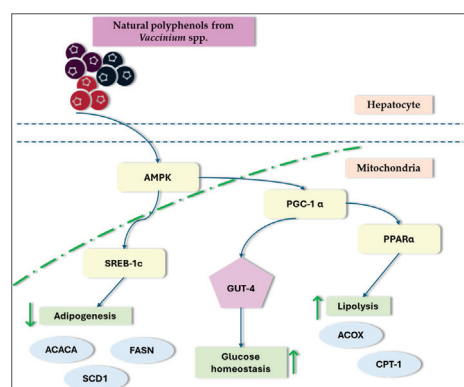


Figure 3. General overview of the impact of *Vaccinium* spp. on transcription factors. ACC—acetyl-coenzyme A carboxylase; ACOX1—acyl-CoA oxidase1; ADP—adenosine-5'-diphosphate; AMPK—AMP-activated protein kinase; CPT1α—carnitine palmitoyltransferase-I-alpha; PPAR—peroxisome proliferator-activated receptor; SREBP—sterol regulatory element-binding protein; ↑—increase; ↓—decrease;.

Impact of *Vaccinium* on Peroxisome Proliferator-Activated Receptors (PPARs)

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors in the nuclear receptor family. They regulate lipid metabolism, glucose homeostasis, energy balance, inflammation, and atherosclerosis. PPARs are divided into three isoforms: alpha (α), beta (β)/delta (δ), and gamma (γ), each with distinct expression patterns across different tissues [94]. The three PPAR isotypes have unique lipid and glucose metabolism roles, crucial in developing NAFLD. PPAR- α is a crucial regulator of fatty acid breakdown in the liver, overseeing the expression of genes involved in the body's overall balance of fatty acids [95]. Evidence suggests that PPAR- α can regulate the expression of key transcription factors SREBP-1c and liver X receptor α (LXR α), thereby enhancing the transcription levels of *Scd-1* and other lipogenic genes, ultimately affecting hepatic lipogenesis [96].

In the liver, PPAR- β/δ regulates lipid and glucose metabolism, with its expression significantly reduced during fasting but rapidly restored upon refeeding. The activation of PPAR- β/δ enhances insulin sensitivity in diabetic mice, mainly through the modulation of genes involved in hepatic fatty acid production and the pentose phosphate pathway [95]. PPAR- β/δ may play a crucial role in regulating lipid metabolism [88,94,97].

PPAR- γ promotes insulin sensitivity by regulating lipid storage in adipose tissue and the differentiation of adipocytes. PPAR- γ is predominantly active during the postprandial state and regulates fat storage in adipose tissue. As a transcription factor, PPAR- γ controls the expression of genes involved in adipogenesis, adipose tissue differentiation, and lipid metabolism, including fatty acid uptake and triacylglycerol lipolysis in adipose tissue. Consequently, tissue-specific deficiency of PPAR- γ leads to significant loss of adipose tissue and severe insulin resistance, resulting in hepatic fat accumulation [88,98].

In an immunohistochemical analysis conducted by Ren et al. [75] to assess the impact of blueberry juice on the expression of PPAR- α in the liver of NAFLD rats, PPAR- α in the NAFLD group exhibited weak expression in the cytoplasm with a very light brown color. In contrast, in the blueberry-juice-supplemented group, PPAR- α showed strong expression in the cytoplasm with a brown color. Regarding the expression of SIRT1, SREBP-1c, and PPAR- α in the control group, the SIRT1 and PPAR- α proteins were high, while the level of SREBP-1c was low. In contrast, in the liver of NAFLD rats, SIRT1 and PPAR- α protein levels were low, while SREBP-1c levels were high. In the blueberry-juice-treated group, the SIRT1 and PPAR- α protein levels were high, whereas the level of SREBP-1c was low. In a separate study, Ren et al. [76] validated the impact of blueberry juice on PPAR- α and subsequently on the SREBP-1c/PNPLA-3 pathway. Their findings indicate that blueberry juice elevated PPAR- α protein levels, whereas blocking PPAR- α activity increased SREBP-1c and PNPLA-3 levels. Thus, blueberry juice may help inhibit the progression of NASH by modulating the SREBP-1c-PNPLA-3 pathway via PPAR- α . The hepatic expression of peroxisome proliferator-activated receptor α was significantly reduced by 24% due to supplementation with *Vaccinium macrocarpon* extract [33]. In the study by Shimizu et al., hepatic mRNA levels of PPAR- γ and MCP-1, which are detected in patients with NAFLD, were significantly lower in mice fed a high-fat diet supplemented with 5% cranberry powder compared to mice on a high-fat diet alone. This implies that supplementing with cranberry powder can mitigate NAFLD caused by a high-fat diet [84]. In studies investigating the impact of *Vaccinium vitis-idaea* L. supplementation on hepatic gene expression in mice fed a high-fat diet, it was confirmed that this diet significantly increased the expression of PPAR- γ and its target genes, such as monoacylglycerol O-acyltransferase 1 (*Mogat1*), the cluster of differentiation 36 (*CD36*), and death effector domain-containing similar to DFFA inducing apoptosis c (*Cidec*). Supplementation with cranberry inhibited the increase in expression of the monoacylglycerol O-acyltransferase 1 (*Mogat1*) gene, which plays a role in lipid metabolism and is activated by PPAR- γ [80].

Impact of *Vaccinium* on Sterol Regulatory Element-Binding Proteins (SREBPs)

Sterol regulatory element-binding proteins are transcription factors involved in cholesterol, TG, and FFA biosynthesis. SREBPs are categorized into three subtypes: SREBP-2,

SREBP-1c, and SREBP-1a [99]. These factors are integral to the development of conditions such as non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, and liver cancer. In their phosphorylated state, SREBPs remain inactive in the cytoplasm, but once dephosphorylated, they move to the nucleus, promoting lipogenic enzyme production. Among them, SREBP-1c is a vital regulator of liver lipogenesis [100,101]. PPAR- γ and CCAAT/enhancer-binding proteins alpha (C/EBP α) are other transcription factors involved in the differentiation of adipocytes and lipid accumulation [102,103].

Ren et al. [75] suggest that blueberry juice may improve NAFLD by activating the SIRT1-mediated signaling pathway. Their research demonstrated that blueberry juice can enhance the expression of SIRT1, increase the levels of PPAR- α protein, and reduce the levels of SREBP-1c. The same authors' studies showed that blueberry juice increased PPAR- α protein levels and inhibited PPAR- α activity, which increased SREBP-1c and PNPLA-3 levels. This indicates that blueberry juice could potentially slow the advancement of NASH by modulating the SREBP-1c/PNPLA-3 pathway via PPAR- α [76]. Peroxisome proliferator-activated receptor, gamma coactivator 1a (PGC-1a), and sirtuin-3 (SIRT3) are regulators of AMP-activated protein kinase (AMPK), increasing their expression through AMPK-mediated phosphorylation. PGC-1a further enhances the activity of various nuclear receptors, such as estrogen-related receptor alpha (ERR α), which is crucial for mitochondrial biogenesis and Nrf-1 and Nrf-2. Additionally, SIRT3 is involved in the activation of antioxidant enzymes. In a particular study, administering 100 or 400 mg/kg body weight per day of a 70% ethanol extract from *Vaccinium corymbosum* L. leaves by oral gavage activated the AMPK/PGC-1a/SIRT3 signaling pathway in rats that were on a high-fat diet for 9 weeks [35]. A high-fat diet induces lipid accumulation in the liver, leading to increased expression of SREBP-1c and ACC-1. Lingonberry supplementation attenuated the high-fat-diet-induced expression of SREBP-1c and ACC-1, reduced plasma lipid levels, and improved hepatic steatosis. The study findings imply that the decrease in hepatic lipid accumulation observed with lingonberry supplementation could be attributed to the suppression of SREBP-1c [32]. In the study by Hewage et al. [31], lingonberry supplementation was found to inhibit the expression of SREBP-1c and ACC1 in the livers of mice on a high-fat diet, leading to a reduction in lipogenesis. The study also noted a decrease in the expression of the enzymes DGAT1 and DGAT2, which may contribute to lower triglyceride levels in the livers of these mice. Furthermore, lingonberry supplementation reduced the expression of CD36, a receptor crucial for fatty acid uptake, which could further explain the decrease in hepatic fatty acid absorption.

Influence of *Vaccinium* on AMP-Activated Protein Kinase (AMPK)

AMP-activated protein kinase is a key sensor and regulator of cellular energy balance. It plays an essential role in managing lipid and glucose metabolism, as well as controlling oxidative stress and inflammatory responses within the body [104]. Previous research has shown that the inhibition of AMPK signaling is associated with the progression of NAFLD [105]. AMPK plays a crucial role in regulating lipid metabolism by influencing various processes such as the oxidative breakdown of FFA and TG and their synthesis. This regulation supports cellular metabolism and proliferation, making AMPK a key player in the development of fatty liver disease [106]. AMPK reduces hepatic fat accumulation through two mechanisms. The first mechanism involves the phosphorylation and inactivation of acetyl-CoA carboxylase (ACC) by activated AMPK, preventing ACC dimerization and reducing fatty acid synthesis. The second mechanism is related to malonyl-CoA, which serves as a precursor for fatty acid synthesis and a potent carnitine palmitoyltransferase 1 (CPT1) inhibitor. AMPK phosphorylates ACC, decreasing malonyl-CoA levels, which promotes CPT1 expression and thus enhances fatty acid oxidation [106,107].

Extracts from *Vaccinium* species may influence the activity of fatty acid synthase (FAS) and ACC. In vivo, studies demonstrated that a high-fat diet significantly reduced pAMPK α , PGC-1 α , and SIRT3 proteins in rat livers, an effect that was effectively countered by the administration of blueberry leaf extracts [35]. In a study on de novo hepatic lipogenesis, the phosphorylation of AMPK was evaluated by measuring the ratio of phosphorylated

AMPK (pAMPK) to total AMPK protein levels in the liver. A high-fat diet significantly decreased both pAMPK levels and the pAMPK/AMPK ratio compared to animals on a control diet. However, supplementation with lingonberry restored both pAMPK levels and the pAMPK/AMPK ratio [32].

3.4.6. The Effect of *Vaccinium* on Glucose Metabolism

Abnormal glucose metabolism and insulin resistance (IR) are also recognized as key risk factors in the development and progression of NAFLD [108]. Insulin resistance is marked by a diminished capacity of tissues outside the liver, such as adipose tissue and muscles, to use glucose effectively. In adipose tissue, this resistance results in abnormal fatty acid release due to disrupted lipolysis, exacerbating insulin signaling impairment across the entire body [3].

In research conducted by Glisan et al. [33], cranberry supplementation did not significantly affect fasting plasma insulin levels, fasting blood glucose levels, or HOMA-IR. Similarly, in research by Shimizu et al. [84], fasting blood glucose levels were higher in mice fed a high-fat diet, and glucose levels did not differ significantly between the groups receiving 1% and 5% doses of cranberry. In contrast, in studies by Haga et al. [74], plasma glucose and TC levels in the group receiving a 10% extract of *Vaccinium myrtillus* L. were significantly lower than those in the high-fat and high-cholesterol diet group. Nakano et al. [81] observed a significant reduction in serum insulin levels and insulin resistance with supplementation of *Vaccinium myrtillus* L. extracts; however, glucose levels did not differ significantly across all studied groups. HOMA-IR and TyG index, FGIR index values, and pancreatic β -cell function did not show statistically significant differences for the high-fat and high-fructose diets. Conversely, studies by Faheem et al. [36] demonstrated a significant reduction in the HOMA-IR index in rats treated with cranberry at doses of 50 and 100 mg/kg, showing decreases of 54% and 77%, respectively. Additionally, the TyG index was notably lowered by 6% and 11% in the same cranberry-treated groups. Blueberry-derived exosome-like nanoparticles (BELNs) improved insulin resistance, reduced fasting glucose levels, and decreased serum insulin content in mice fed a high-fat diet. The results demonstrated that C57BL/6 mice could take up BELNs and they were rapidly distributed into several organs, including the small intestine, liver, and spleen. These findings suggest that the administration of BELNs may be beneficial in ameliorating the pathological features of NAFLD [78].

Table 2. Studies evaluating the influence of *Vaccinium* spp. on non-alcoholic fatty liver disease in vivo.

Study	Study Types		Effects		STAIR
	Animal Model	Dosage and Duration	Type of Intervention	Metabolism/Molecular	
Ren, Huang, and Cheng 2014 [75]	Male Sprague Dawley rats (200 to 250 g)	Blueberry juice (15 g/kg, once a day) / 8 weeks	Rats were divided into 2 groups: (1) HFD—50 rats; (2) control group—8 rats	↓ The degrees of NAFLD and degenerated hepatocytes; ↓ Serum activities of AST and ALT; ↓ The ratio of TG/HDL-C; ↑ The mRNA levels of SREBP-1c and PPAR-α; ↓ The levels of SREBP-1c.	5
Morrison et al. 2015 [72]	Female ApoE 31.eiden mice	0.1% (<i>w/w</i>) Mirtoselect—standardized <i>Vaccinium myrtillus</i> L. extract (66% anthocyanins) / 20 weeks	Mice were divided into 3 treatment groups: (1) HCD; (2) HCD and 0.1% (<i>w/w</i>) Mirtoselect; and (3) Western-type diet without cholesterol supplementation	↓ The development of hepatic steatosis; ↓ Microvesicular steatosis; ↓ An accumulation of lipids esterified to cholesterol (cholesteryl esters); ↓ The hepatic free cholesterol; ↓ p65-NF-κB activity; - Expression of <i>Emr1</i> or <i>Cd2</i>—not significant; ↓ Neutrophil infiltration and the expression of two neutrophil chemoattractants—<i>Cxcl1</i> and <i>Cxcl2</i>; ↓ The pronounced increase in collagen and significantly reduced <i>Col1a1</i> expression.	4
Glisan et al. 2016 [33]	Male C57BL/6J mice (4 weeks old)	0.8% CBE—4 g per day (CBE, D13051702) (CBE—macerated sulfite-free dried cranberries) / 21 weeks	Mice were divided into 2 treatment groups: (1) HFD (n = 24) and (2) CBE diet (n = 24) for 10 weeks	- Blood glucose levels, plasma insulin levels, and HOMA-IR—not significant; ↓ The plasma levels of free fatty acids; ↓ The serum levels of IL-1β; ↓ The serum levels of ALT levels; ↓ The total lipid droplet area in the liver and the total hepatic lipid area; ↓ The hepatic expression of the NF-κB-dependent proinflammatory genes TNF-α (47%) and Cox2 (46%); ↓ The hepatic mRNA expression of Il1b (55%) and Ucp2 (57%); ↓ The hepatic mRNA levels of C-C chemokine receptor 2 (Ccr2), the CCL2 receptor expressed on recruited monocytes, by 56% and Cclb by 55%; ↓ The hepatic expression of Nlrp3 (43%) and Txnlp (30%); ↑ The gene expression of hepatic PPAR-α; ↓ The transcription factor responsible for regulating Txnlp expression (24%).	5
Ren et al. 2017 [76]	Male Sprague Dawley rats (6 to 8 weeks old)	10 mL/kg blueberry juice (1 kg blueberries were thawed, milled, and pressed) / 12 weeks	Rats were divided into 2 groups: (1) blueberry juice group (injected with 50 μ L/kg saline solution and orally received 10 mL/kg blueberry juice and 10 mL/kg liquid placebo daily); (2) blueberry juice and PPAR- α inhibitor group (injected with 50 μ L/kg PPAR- α in saline solution and orally received 10 mL/kg blueberry juice and 10 mL/kg liquid placebo daily)	- Blueberry juice: ↓ The serum levels of ALT and AST; ↑ The levels of SOD and GSH; ↓ The serum levels of MDA, TG, TC, and HDL-C; ↓ The mRNA levels of PPAR-α and reduced the level of SREBP-1c and PNPLA3; - Blueberry juice and PPAR-α inhibitor: ↓ the mRNA levels of SREBP-1c and PNPLA3-α.	5

Table 2. Cont.

Study	Study Types		Effects		STAIR
	Animal Model	Dosage and Duration	Type of Intervention	Metabolism/Molecular	
Shimizu et al. 2019 [84]	Male mice C57BL/6 (6 weeks old)	1% cranberry powder or 5% cranberry powder (anthocyanin 120 mg/100 g and proanthocyanidin 2600 mg/100 g)/8 weeks	Mice were divided into 4 treatment groups: (1) ND, (2) HFD, (3) HFD + 1% cranberry powder, and (4) HFD + 5% cranberry powder	↓ Body weight and concomitantly triggered hyperphagia; ↓ Oxidative stress and proinflammatory cytokine expression (IL-6); The serum levels of glucose—not significant; ↓ The serum levels of TG; ↓ The serum level of ALT; ↓ The serum level of hepatic mRNA of PPAR-γ and MCP-1.	4
				↓ Fat accumulation and TG contents in mouse liver; ↓ Less fibrosis; ↓ The serum levels of ALT and AST; ↓ The plasma levels of GLU and TG; ↓ Proinflammatory cytokine levels (TNF-α, IL-5, IL-1β, and IFN-γ).	
Haga et al. 2019 [74]	Male homozygous leptin receptor-deficient (BKS.Cg- ⁺ Lepr ^{db} /J; db/db) mice (10 weeks old)	5% and 10% bilberry fruits extracts (≥36% anthocyanin glycosides)/8 weeks	Mice were divided into 4 treatment groups: (1) ND, (2) HFD + HCD, (3) HFD + HCD + 5% bilberry fruit extracts, and (4) HFD + HCD + 10% bilberry fruit extracts	↓ The hepatic TC, TC, L-LDL, ALT, and AST levels; ↓ The hepatic steatosis and inflammatory infiltration; ↓ The generation of hepatic malondialdehyde (MDA); ↓ The hepatic ROS levels; Protection against hepatic oxidative stress; ↓ pAMPK α, PGC-1 α, and SIRT3 proteins in liver; ↑ ERR α, Nrf-1, and Nrf-2 genes in liver.	4
Li et al. 2020 [85]	Male Sprague Dawley rats (8 weeks old)	Freeze-dried leaf extract of <i>Vaccinium corymbosum</i> L. (PBL)/8 weeks	Mice were divided into 4 groups: (1) ND, (2) HFD (3) HFD + high dose PBL (H-PBL); (4) HFD + low dose PBL (L-PBL). Rats received: PBL at a dose of 400 mg/kg/day (H-PBL group) or 100 mg/kg/day (L-PBL group) or an equal volume of vehicle (0.9% NaCl, ND, and HFD groups) by gavage for 9 weeks	↓ Body weight, liver weight, epididymal fat mass, liver-to-body-weight ratio, and hepatic fat mass; ↓ The serum level of AST and ALT; ↓ The serum level of MCP-1; ↓ The serum level of insulin; ↓ The serum levels of HDL- α , TG, and glucose—not significantly different; ↑ The level of lactic acid in the gut; ↑ The levels of Nrf-2 and SOD2; ↓ The level of Keap1 and TBARS in the liver.	5
				↓ Body weight; ↓ The serum levels of ALT and AST; ↓ The serum levels of TG; ↓ HOMA IR; ↑ SOD and GSH; ↑ ADP levels; ↓ The serum level of TNF- α , IL-6, NF- κ B, TGF- β and α -SMA tissue levels; ↓ Reduction of collagen deposition; ↑ IRS-2 expression.	
Nakano et al. 2020 [81]	Male mice C57BL/6N (5 weeks old)	2% bilberry anthocyanin extract powder (Mirtoselect)/18 weeks	Mice were randomly divided into 4 groups: (1) ND group, (2) ND + 2% bilberry anthocyanins, (3) WD group, and (4) WD + 2% bilberry anthocyanins	↓ Body weight; ↓ The serum levels of ALT and AST; ↓ The serum levels of TG; ↓ SOD and GSH; ↑ ADP levels; ↓ The serum level of TNF- α , IL-6, NF- κ B, TGF- β and α -SMA tissue levels; ↓ Reduction of collagen deposition; ↑ IRS-2 expression.	5
Fahem et al. 2020 [71]	Male albino Wistar rats (12 weeks old)	Cranberry nutraceutical (186c1025) diluted in water (40 mg/mL)/8 weeks	Mice were divided into 5 groups: control group: (1) ND for 8 weeks and received 1 mL/kg distilled water orally three weekly; HfCD group: (2) HFCD for 8 weeks and received 1 mL/kg distilled water orally three times weekly; 30/ HFCD group: (3) HFCD and cranberry (30 mg/kg/day) orally three times weekly; 100/ HFCD group: (4) HFCD and cranberry (100 mg/kg/day) orally three times weekly; treated group: (5) ND and cranberry (100 mg/kg/day) orally three times weekly	↓ Body weight; ↓ The serum levels of ALT and AST; ↓ The serum levels of TG; ↓ HOMA IR; ↑ SOD and GSH; ↑ ADP levels; ↓ The serum level of TNF- α , IL-6, NF- κ B, TGF- β and α -SMA tissue levels; ↓ Reduction of collagen deposition; ↑ IRS-2 expression.	5

Table 2. Cont.

Study	Study Types		Effects		STAIR
	Animal Model	Dosage and Duration	Type of Intervention	Metabolism/Molecular	
Zhao et al. 2021 [78]	Male mice C57BL/6 (6–8 weeks old)	Blueberry-derived exosome-like nanoparticles (BELNs) at 25, 50, or 100 mg/kg/4 weeks	Mice in the 3 HFD groups received intragastric administration of blueberry-derived exosome-like nanoparticles at doses of 25, 50, or 100 mg/kg, administered once every other day	↓ The serum level of insulin, fasting glucose; ↑ Insulin resistance; ↓ The accumulation of lipid droplets in the liver and the liver weight; ↓ The contents of TG and TC; the levels of ALT and AST; ↓ The content of HDL-C; ↑ The activities of SOD and GSH; ↓ The content of MDA in the liver; Accelerated the translocation of Nrf-2 from the cytoplasm to nuclei in the liver; ↓ The mRNA levels of FAS and ACC1 in the liver; ↓ The expression of Bel-2, Bax, and HO-1 in the liver.	5
Hewage et al. 2021 [32]	Male C57BL/6J mice (6 weeks old)	(5% w/w) Manitoba lingonberry <i>Vaccinium vitis-idaea</i> L./freeze-dried berry powder/12 weeks	Mice were divided into 3 groups: (1) control (D12450) diet, (2) HFD (D12492), or (3) HFD supplemented with (5% w/w) Manitoba lingonberry	↓ The serum levels of ALT and AST; ↓ The hepatic accumulation of TG and TC; ↓ MDA levels and restored GSH levels; ↓ The hepatic GSSG level and restored GSH/GSSG ratio; ↓ The hepatic ACC1, SREBP-1c mRNA expression, and SREBP-1c protein level of expression; and ↓ The expression of Gclc in the liver; ↓ The serum levels of IL-6, MCP-1, and TNF-α mRNA expression; ↑ pAMPK level and pAMPK/AMPK ratio; ↑ Nuclear Nrf-2 protein level in the liver.	4
Ryti et al. 2021 [80]	Male C57BL/6N mice (8 weeks old)	20% w/w air-dried lingonberry <i>Vaccinium vitis-idaea</i> L. powder (900 g of fresh lingonberries were used to produce 100 g of berry powder)/6 weeks	Mice were divided into 3 groups: (1) LFD (10 kcal% fat); (2) HFD (46 kcal% fat); (3) HFD with air-dried lingonberry powder (20% w/w)	↓ The serum levels of ALT; ↓ The expression of the acute phase inflammatory factors Saa1 and Saa2; ↓ The expression of Cyp16a1; ↑ The expression of hydroxysteroid (17-βeta) dehydrogenase 6 (Hsd17b6) and insulin-like growth factor binding protein 2 (Igfbp2); ↓ The expression of genes associated with lipid metabolic process (Mogat1, Plin4), inflammatory/immune response or cell migration (Lgals, Saa1, Saa2, Cxcl14, Ccp1, S100a10), and cell cycle regulation (Cdkn1a, Tubb2a, Tubb6).	4
Hewage et al. 2022 [31]	Male C57BL/6J mice (6 weeks old)	5% w/w Manitoba wild lingonberry/12 weeks	Mice were divided into 3 groups: (1) a control diet (D12450) containing 11% kcal fat, 18% kcal protein, and 71% kcal carbohydrate, or (2) an HFD (D12492) containing 62% kcal fat, 18% kcal protein, and 20% kcal carbohydrate, or (3) an HFD supplemented with (5% w/w) Manitoba wild lingonberry	↓ The hepatic accumulation of TG and TC; ↓ Notox1 expression in the liver; ↓ Liver NICD1 protein and HES1 mRNA levels; ↓ The expression of SREBP-1c and ACC1; ↑ The hepatic mRNA levels of ACOX1 and CPT1α; ↓ Gene expressions of CD36, DGAT1 and DGAT2.	3

Table 2. Cont.

Study	Study Types		Effects		STAIR
	Animal Model	Dosage and Duration	Type of Intervention	Metabolism/Molecular	
Zhu et al. 2022 [77]	Male C57BL/6 mice (8–10 weeks old)	TEC—blueberry monomers were prepared as 1000 ppb, 800, 600, 400, 200, and 100 ppb with 0.1% formic acid methanol solution/16 weeks	Mice were treated with TEC via gavage at doses of 7.5, 15.0, or 30.0 mg/kg daily for 6 weeks following 10 weeks on a high-fat diet (HFD). To achieve IR-47 knockdown in vivo, a tRF-47 antagonist (5 µg/mouse in 1.5 mL saline) was injected into the tail vein of NASH mice three times a week for 2 weeks, starting after 9 weeks on the HFD	↓ The serum levels of ALT, AST and MDA; ↑ The level of autophagy marker LC3B in the liver ↓ The activation of inflammasomes and TLR4; TEC relies on tRF-47 (dRF-47-582ZJ)Q/5WRY/MMV5B0) to promote autophagy and weaken pyroptosis	5
	Male Wistar rats	10% (w/v) blueberry aqueous extracts/18 weeks	Mice were divided into 3 groups: (1) standard-diet-fed group; (2) HFFD (standard diet added with 20% lard and 18% fructose); (3) HFFD with blueberry beverage	↓ The serum level of TC; ↓ The accumulation of lipid vacuoles; ↓ The accumulation of saturated, monounsaturated, and polyunsaturated fatty acids; ↓ FAS and ACC expression.	

ACC—acetyl-coenzyme A carboxylase; ACOX1—acyl-CoA oxidase1; ADP—adenosine-5'-diphosphate; ALT—alanine aminotransferase; AMPK—AMP-activated protein kinase; AST—aspartate transaminase; CD36—fatty acid translocase; CPT1α—carnitine palmitoyltransferase-1-alpha; DGAT (1 and 2)—diacylglycerol O-acyltransferase; FAS—fatty acid synthase; GSH—glutathione; GPx—glutathione peroxidase; HCD—high-cholesterol diet; HDL—high-density lipoprotein; HES1—hairly and enhancer of split-1; HFD—high-fat diet; HFCD—high-fat and -cholesterol diet; HFFD—high-fat and -fructose diet; HOMA-IR—homeostatic model assessment for insulin resistance; IL—interleukin; IFN-γ—interferon gamma; IRS-2—insulin receptor substrate; Keap1—Kelch-like ECH-associated protein 1; LDL—low-density lipoprotein; MCP-1/CCL2—monocyte chemoattractant protein-1; MDA—malondialdehyde; ND—normal diet; NOS—nitric oxide synthase; NF-κB—nuclear factor kappa B; NLRP3—NLR family pyrin domain containing 3; Notch 1—neurogenic locus notch homolog protein 1; Nrf-1—nuclear respiratory factor 1; Nrf-2—nuclear factor erythroid 2-related factor 2; PNPLA—patatin like phospholipase domain containing; PPAR—peroxisome proliferator-activated receptor; SAA—circulating serum amyloid A; SOD—superoxide dismutase; SREBP—sterol regulatory element-binding protein; STAT3—signal transducer and activator of transcription; TC—total cholesterol; TG—triacylglycerol; TGF-β—transforming growth factor β; Txnip—thioredoxin-interacting protein; TLR4—Toll-like receptor 4; TNF-α—tumor necrosis factor alpha; WD—Western diet; ↑—increase; ↓—decrease.

3.5. Cell Culture Experiments

Studies evaluating the effects of *Vaccinium* species in vitro on lipid metabolism and oxidative stress in hepatocytes, typical of NAFLD and NASH, are presented in Table 3. Most studies were conducted on human hepatoma HepG2 cells [31,35,74,77,79,109,110]. In all studies, extracts rich in polyphenols from *Vaccinium* berries were utilized. Unfortunately, the interpretation of results is complicated by variations in the cell models, experimental protocols, and molecular pathways assessed. Despite these differences, most studies agree that extracts from blueberries or cranberries help reduce hepatocyte lipid accumulation by inhibiting lipogenesis and likely enhancing lipolysis. However, not all studies have explored all facets of lipid metabolism. Despite the variability in cell models and experimental methods, most research consistently supports the beneficial effects of these extracts on lipid reduction and the enhancement of mitochondrial function in hepatocytes.

Liu et al. [79] investigated the inhibitory effect of blueberry polyphenol-rich extract and its fractions on TG accumulation in HepG2 cells. They found that at a concentration of 80 µg/mL, the extract achieved a maximum inhibition of TG synthesis of approximately 60%. To identify the most effective component in reducing lipid accumulation, the extract was divided into three fractions: an anthocyanin-rich fraction, a phenolic-acid-rich fraction, and an ethyl acetate extract. The phenolic-acid-rich fraction demonstrated the greatest efficacy in TG clearance, reducing levels by $58.6 \pm 4.7\%$ at a 100 µg/mL concentration. The anthocyanin-rich fraction reduced TG levels by 30% at the same concentration. In contrast, the ethyl acetate extract showed the least bioactivity in inhibiting TG accumulation in HepG2 cells. Wang et al. [109] also assessed the inhibitory effects of wild blueberry extracts on TG accumulation and ROS levels in HepG2 cells. In their study, HepG2 cells were treated with blueberry extract at concentrations of 10, 20, and 40 µg/mL alongside oleic acid for 24 h to assess the impact on lipid accumulation. The blueberry extracts led to a 24% reduction in TG levels and decreased ROS levels in liver cells. Additionally, bilberry extract enhanced the growth of steatotic hepatocytes induced by oleic and linoleic acids or the nuclear receptor LXRα agonist, with optimal effects at a concentration of 5 µg/mL. The extracts reduced lipid deposition in steatotic hepatocytes, promoted cell growth, protected against oxidative stress, and significantly inhibited lipid accumulation. Moreover, when AML12 cells were exposed to fatty acids, Rubicon expression was markedly increased. The addition of bilberry extracts notably reduced Rubicon expression and significantly increased p62/SQSTM expression, indicating enhanced autophagy and a subsequent reduction in lipid accumulation [74]. Blueberry leaf extracts effectively alleviated the reduction of mitochondrial membrane potential in the mitochondria of fatty-acid-induced cells. Additionally, the extracts improved mitochondrial OCR, ATP production, and basal and maximal respiration in HepG2 cells. Blueberry extracts support the mitochondrial energy supply in the liver by increasing the pools of mitochondrial NTP and NMP in HepG2 cells. The balance of mitochondrial deoxyribonucleotide triphosphate (dNTP) pools is critical for the fidelity of DNA synthesis during mtDNA replication and repair. The extracts significantly mitigated the reduction of mtDNA in both cells and the livers of rats. In the same study, blueberry leaf extracts significantly increased the phosphorylation of AMPKα, the expression of SIRT3 protein, the expression of PGC-1α protein, and the expression of ERRα, Nrf-1, and Nrf-2 genes in fatty-acid-induced HepG2 cells [35]. In the study by Wang et al. [110], HepG2 cells treated with blueberry leaf extract at concentrations ranging from 0.78 to 25 µg/mL showed increased viability compared to NAFLD-modeled cells. Additionally, the extracts reduced lipid accumulation in the cells. The results indicated that PBL treatment might suppress NAFLD-induced apoptosis, as it decreased the number of apoptotic cells. Specifically, the leaf extract at a concentration of 25 µg/mL significantly reduced the expression of the caspase-3 protein and slightly increased the expression of the antiapoptotic protein Bcl-2. Blueberry-derived exosome-like nanoparticles (BELNs) notably increased mitochondrial content and reduced ROS production in a dose-dependent manner in HepG2 cells treated with rotenone. Under rotenone treatment, these cells displayed a decreased mitochondrial membrane potential (MMP), but preincubation with BELNs

effectively mitigated this effect. Mitochondria, key organelles for energy production via oxidative phosphorylation, depend on MMP as a crucial driving force. Dose-dependent preincubation with BELNs also counteracted the effects of rotenone on the protein levels of Bcl-2, Bax, and HO-1 in HepG2 cells, significantly decreasing rotenone-induced apoptosis. This suggests that Nrf-2 is an essential transcription factor that activates the expression of various antioxidant enzymes and proteins by relocating from the cytoplasm to the nucleus in mammalian cells exposed to oxidative stress [78]. Hewage et al. [31] investigated the impact of lingonberry on Notch1 signaling and lipid metabolism by exposing hepatocytes to palmitic acid. They observed that palmitic acid induced the expression of the Notch1 gene. Subsequently, they examined the effects of lingonberry extract and C3Glu (cyanidin-3-O-glucoside) on Notch signaling and the induction of genes associated with lipid synthesis. Treatment with lingonberry extract or C3Glu significantly reduced palmitic-acid-induced Notch1 mRNA expression. Furthermore, lingonberry extract or C3Glu lowered the mRNA expression levels of HES1, SREBP-1c, and ACC1 and reduced palmitic-acid-induced intracellular triacylglycerol accumulation. Moderate concentrations of anthocyanin monomers (TEC) enhanced cell viability in the NASH model, decreased the secretion of IL-6, TNF- α , IL-10, IL-17, and IL-4, and normalized the elevated expression of TLR4 associated with the NASH model. Additionally, TEC mitigated pyroptosis by stimulating autophagy, which reduced NLRP3 and GSDMD expression and inhibited the fatty-acid-induced release of LDH [77].

Table 3. Studies evaluating the influence of *Vaccinium* spp. on non-alcoholic fatty liver disease in vitro.

Study	Study Types		Effects
	Model	Dosage and Duration	
Liu et al. 2011 [79]	Human hepatocellular cancer cell line (HepG2)	Anthocyanin-rich and phenolic-acid-rich fractions from fresh blueberries (<i>Vaccinium</i> spp.)	– ↓ TG deposition in HepG2 cells; – achieved a maximum TG synthesis inhibition rate of approximately 60% (80 μg/mL); the phenolic-acid-rich fraction was the most effective for TG clearance, with a maximum of $58.6 \pm 4.7\%$ at 100 μg/mL; – the anthocyanin-rich fraction achieved a maximum TG clearance of 30% at 100 μg/mL.
Wang et al. 2016 [109]	Human hepatocellular cancer cell line (HepG2)	purified ACNs from wild blueberries (<i>Vaccinium</i> spp.); –10, 20, and 40 μ g/mL	– ↓ increase in TG levels; – ↓ the rise in ROS in hepatic cells; – ↓ the number of lipid droplets in cells treated with Dp-3-Glu; – ↓ oleic acid-induced lipid accumulation in hepatic cells.
Haga et al. 2019 [74]	Alpha mouse liver 12 cells	A 90% ethanolic extract of bilberry fruits ($\geq 36\%$ anthocyanin glycosides)	– ↓ cellular lipid accumulation in both types of hepatic steatosis (FA and T090); – ↑ cell survival/proliferation with a peak effect at 1 μg/mL; – ↓ Rubicon (protein inhibiting late-stage autophagy) – ↑ p62/SQSTM1 (autophagy marker); – ↓ the cellular FA synthesis pathway stimulated by the LXR agonist (nuclear receptor transcription factor involved in lipid synthesis);

Table 3. Cont.

Study	Study Types		Effects
	Model	Dosage and Duration	
Li et al. 2020 [35]	Human hepatocellular cancer cell line (HepG2)	Blueberry (<i>Vaccinium corymbosum</i> L.) leaves (PBL); 3 doses of PBL at 10 (high), 5 (medium), and 2.5 (low) µg/mL	– ↓ FFA induced lipid accumulation and cellular injury in HepG2 cells; – ↓ FFA induced increases in TNF-α, IL-1β, and IL-6 mRNA levels in HepG2 cells; – ↓ mitochondrial membrane potential (MMP) observed in FFA-treated cells; – ↑ in mitochondrial NTP and NMP pools following FFA treatment, suggesting an improvement in hepatic mitochondrial energy supply; – ↑ AMPKα phosphorylation in HepG2 cells in a dose-dependent manner, leading to increased PGC-1α expression; – ↑ PGC-1α protein expression in a dose-dependent manner; – ↑ the expression of ERRα, Nrf-1, and Nrf-2 genes in FFA-induced cells.
Zhao et al. 2021 [78]	Human hepatocellular cancer cell line (HepG2)	Blueberry-derived exosome-like nanoparticles (BELNs)—100 µg/mL	– ↑ mitochondrial content and inhibited ROS production dose-dependently in HepG2 cells; – ↓ MMP in HepG2 cells; – ↓ rotenone-induced apoptosis in HepG2 cells; – ↑ the translocation of Nrf-2 from the cytoplasm to the nucleus in HepG2 cells.
Wang et al. 2021 [110]	Human hepatocellular cancer cell line (HepG2)	Blueberry leaves (PBL)—6.25, 12.5, 25 µg/mL for 48 h	– ↓ apoptosis population in a dose-dependent manner compared to NAFLD-modeled cells; – ↓ caspase-3 protein expression and slightly upregulated antiapoptotic protein Bcl-2.
Zhu et al. 2022 [77]	Human hepatoma cell line HepG2	25, 50, and 75 µM C3Glu, myricetin, myricetin 3-o-galactoside, delphinidin, and blueberry monomers (TEC)	– ↓ lipid droplet formation (TEC being more effective than C3Glu); – ↓ the release of IL-6, TNF-α, IL-10, IL-17, and IL-4; – ↑ TLR4 expression in the NASH model compared to the control group, but TEC effectively reversed this increase; – ↓ inflammatory mediators, TLR4, and LDH release levels.
Hewage et al. 2022 [31]	Human hepatoma cells (HepG2, cell line: HB-8065)	5% w/w Manitoba wild lingonberry	– ↓ palmitic-acid-induced HES1, SREBP-1c, and ACC1 mRNA expression; – ↓ palmitic-acid-induced intracellular TG accumulation; – ↓ cellular lipid droplets compared to cells treated with palmitic acid alone.

ACC—acetyl-coenzyme A carboxylase; AMPK—AMP-activated protein kinase; BCL2—B-cell CLL/lymphoma 2; ERRα—estrogen-related receptor alpha; FFA—fatty acid synthase; C3Glu—cyanidin 3-glucoside; Dp-3-glu—delphinidin-3-glucoside; HDL—high-density lipoprotein; HES1—hairly and enhancer of split-1; IL—interleukin; LDL—low-density lipoprotein; NF-κB—nuclear factor kappa B; NLRP3—NLR family pyrin domain containing 3; NMP—nucleoside monophosphate; Nrf-1—nuclear respiratory factor 1; Nrf-2—nuclear factor erythroid 2-related factor 2; NTP—nucleotide triphosphate; PGC-1α—proliferator-activated receptor γ coactivator 1α; SREBP—sterol regulatory element-binding protein; TC—total cholesterol; TG—triacylglycerol; TGF-β—transforming growth factor β; TLR4—Toll-like receptor 4; TNF-α—tumor necrosis factor alpha; ↑—increase; ↓—decrease.

4. Discussion

NAFLD is the most prevalent chronic liver disease globally [111]. The onset and advancement of NAFLD are driven by various interconnected factors that work together in individuals with a genetic predisposition. This condition is linked not only to poor lifestyle and dietary choices but also to metabolic disorders, genetic factors, oxidative stress, and disturbances in the gut–liver axis, all of which can impact the disease’s development

and progression. Recent studies describe these mechanisms as part of the “multiple-hit hypothesis” in understanding NAFLD pathogenesis and progression [112]. Insulin resistance is one of the critical factors in the development of NAFLD/NASH, leading to increased hepatic de novo lipogenesis and impaired inhibition of adipose tissue lipolysis, consequently resulting in an increased influx of fatty acids to the liver [113]. Fat accumulates in the liver as TG, which occurs simultaneously with increased lipotoxicity due to high levels of free fatty acids, free cholesterol, and other lipid metabolites [114,115]. Free fatty acids are central to the development of fatty liver disease. They mainly derive from the breakdown of TG in adipose tissue and are transported to the liver through the bloodstream. Another critical source of FFA is de novo lipogenesis, where hepatocytes convert excess carbohydrates, especially fructose, into fatty acids. These fatty acids are metabolized in hepatocytes through mitochondrial beta-oxidation or re-esterified into TG. The TG stored in lipid droplets can undergo lipolysis, releasing fatty acids back into the hepatocyte’s free fatty acid pool. However, when the capacity for beta-oxidation or TG synthesis is exceeded, FFAs can generate lipotoxic compounds, leading to endoplasmic reticulum stress, oxidative stress, and inflammasome activation. These processes contribute to hepatocyte injury, inflammation, and the progressive buildup of extracellular matrix [3,5].

In the case of NAFLD, there is no established pharmacological therapy, and the primary treatment approach involves lifestyle-related therapies, including dietary modifications and physical activity, which are gaining increasing clinical significance. Efforts are underway to discover new treatment options for this disease. This article reviews preclinical and clinical studies evaluating the efficacy of extracts isolated from fruits or leaves of plants from the *Vaccinium* genus in treating NAFLD. Both clinical trials received a score of 5 points, indicating that they meet high methodological standards in terms of randomization, blinding, and reporting of withdrawals. In this context, there is no clear distinction in quality between these studies based on the Jadad scale. Preclinical studies have received ratings ranging from 4 to 5 according to the STAIR guidelines. Results from studies rated 4 suggest that these studies are well-conducted but may have minor limitations that do not significantly affect the overall reliability of the results. Such studies are considered solid and may be a good indicator of the potential efficacy of the therapy, but additional validation is recommended before advancing to clinical trials. Studies rated 5 are regarded as high quality, and further research should focus on confirming the results across different models or conditions and potentially initiating the planning of clinical trials.

The analysis of the collected results suggests that berry leaf and fruit extracts may prevent liver steatosis and its progression to NASH. Additionally, compounds in these extracts, such as anthocyanins, may act through various mechanisms to improve NAFLD. Hepatocytes, which contain many mitochondria, are susceptible to dysfunction from excessive production of ROS. This ROS overproduction can cause lipid peroxidation and damage mitochondrial DNA and membranes [89]. Analysis of the results showed that extracts from blueberry, bilberry, and lingonberry, exosome-like nanoparticles derived from blueberry, and blueberry monomers reduced ROS production, increased mitochondrial content and membrane potential, and prevented cell apoptosis by regulating the expression of Bcl-2, Bax, and HO-1 in HepG2 cells [31,35,74,78,109]. Furthermore, extracts from *Vaccinium* plants effectively reduced MDA content, increased SOD and GSH activity, enhanced Bcl-2 and HO-1 protein expression, and decreased Bax protein levels in the livers of mice fed a high-fat diet [9,32,35,71,78,81]. The analysis of the collected studies suggests that *Vaccinium* fruits may be beneficial in treating NAFLD due to their antioxidant and antiapoptotic effects.

Nrf-2 is a crucial transcription factor that orchestrates the adaptive antioxidant response and the cellular reaction to xenobiotic stress. It is pivotal in managing ROS generated during various stress conditions. By regulating the expression of several antioxidant enzymes, including HO-1, SOD, GPx, and CAT, Nrf-2 is thought to contribute to the mitigation of NAFLD [116]. This review demonstrated that blueberry leaf extracts increase Nrf-2 expression, while BELNs enhance Nrf-2 translocation from the cytoplasm to the nucleus in HepG2 cells [35,78]. Studies indicate the beneficial effects of supplementation

with various berry extracts on the levels of crucial antioxidant factors and liver enzymes in animals fed a high-fat diet. Supplementation with *Vaccinium corymbosum* L. leaf extract significantly increased the levels of $\text{ERR}\alpha$, Nrf-1, and Nrf-2 genes in the liver. The nuclear level of Nrf-2 increased after supplementation with berry extracts and berry-derived exosome nanoparticles [71,78,81]. These findings suggest that the antioxidant activity of *Vaccinium* may modulate Nrf-2 distribution to induce the expression of antioxidant proteins and enzymes. Li et al. [35] suggest that treating NAFLD with blueberry leaf extract may involve directly neutralizing ROS and activating the AMPK/PGC-1 α /SIRT3 signaling pathway, which enhances mitochondrial function and antioxidant defenses. Their research found that blueberry leaf extract significantly increased hepatic pAMPK, PGC-1 α , and SIRT3 protein levels and upregulated $\text{ERR}\alpha$, Nrf-1, and Nrf-2 gene expression. This gene activation promotes mitochondrial DNA replication, β -oxidation, antioxidant defense, and oxidative phosphorylation. These combined effects help reduce lipotoxicity, mitigate oxidative damage, and lower inflammation, thereby alleviating NAFLD.

In the pathophysiology of NAFLD, elevated release of proinflammatory cytokines is a frequent occurrence and plays a crucial role in advancing from liver steatosis to more severe stages of the disease [3]. Preclinical studies have shown that extracts from *Vaccinium* fruits and leaves, in various doses, reduce the levels of proinflammatory cytokines in both serum and liver, especially IL-6, IL-1 β , and TNF- α [32,35,71,74,77]. IL-6 is expressed in many inflammatory cells, regulating various biological processes, including inflammation and insulin resistance. The analysis of the applied doses of *Vaccinium* extracts or nutraceuticals indicated a decrease in serum IL-6 levels [32,35,71,74,77,84].

The regulator of the metabolic inflammatory signaling pathway in the liver is NF- κ B, which controls the production of various proinflammatory cytokines, including TNF- α and IL-6. Moreover, NF- κ B influences liver fibrogenesis by controlling hepatocyte damage, which is a significant factor driving fibrogenic responses in these cells [3,71,72]. Morrison et al. [72] observed that a high-cholesterol diet activated inflammatory signaling pathways (IL-1 β , TNF- α , TGF- β) and identified a positive correlation between free cholesterol levels and NF- κ B activity, indicating NF- κ B as an effector of cholesterol-induced inflammation. Similarly, Faheem et al. [71] found that cranberry supplementation resulted in a significant reduction in hepatic NF- κ B expression. Glisan et al. [33] found that cranberry may alleviate liver inflammation by influencing the TLR4/NF- κ B signaling pathway and its subsequent effects on TNF- α and the mRNA levels of Cox2 and TNF- α in the liver. The TLR4/NF- κ B pathway is essential for regulating the expression of proinflammatory genes, including IL-1 β , COX-2, and TNF- α .

According to data analysis, *Vaccinium* extracts administered in various forms improved liver dysfunction by reducing AST and ALT activity, as well as diminishing lipid droplet accumulation and decreasing the expression of FAS and ACC1 in the livers of mice fed a high-fat diet [31,32,78,82]. This indicates their influence on de novo lipogenesis and the potential benefits for NAFLD. Fatty acids can also be synthesized from non-lipid precursors through de novo lipogenesis, a process regulated at the transcriptional level by ACC1 and FAS. SREBP-1c and carbohydrate response element-binding protein primarily mediate this regulation [78].

Studies have shown that high-fat-diet-induced lipid accumulation in the liver is associated with increased expression of SREBP-1c [100,101]. Supplementation with lingonberry, as demonstrated in the studies by Hewage et al. [31,32], reduced the expression of SREBP-1c, decreased plasma lipid levels, and improved hepatic steatosis. Notably, lingonberry supplementation attenuated Notch1 expression in the liver of mice fed a high-fat diet and inhibited Notch1 signaling, lipogenesis, and palmitic-acid-induced lipid accumulation in HepG2 cells. Notch1 signaling stimulates lipid accumulation in the liver by inducing de novo hepatic lipogenesis in NAFLD through the upregulation of transcriptional activation of fatty acid synthesis genes. Inhibiting Notch significantly reduced cellular lipid accumulation by alleviating SREBP-1c-dependent lipogenesis and increasing fatty acid oxidation [31]. Studies by Ren et al. [75] showed that blueberry juice increased PPAR- α levels, reduced SREBP-1c

levels, and decreased PNPLA-3 levels. Other studies by Ren et al. [76] indicated that treatment with blueberry juice resulted in high levels of SIRT1 and PPAR- α proteins, while SREBP-1c levels remained low. Cranberry treatment also reduced hepatic SREBP-1c expression in mice receiving a 5% cranberry dose, though this inhibitory effect disappeared after two weeks [84]. Activation of PPAR- α prevents TG synthesis in liver cells by inhibiting SREBP-1c activity, which is known to be associated with hepatic steatosis. PNPLA-3 is linked to lipid accumulation in liver tissues, particularly in the progression of hepatic steatosis [75]. SIRT1 regulates lipid homeostasis through the upregulation of PPAR- α . It is crucial to understand the pathways by which PPAR- α inhibits SREBP-1c and PNPLA-3 activity and how SIRT1 controls the SREBP-1c/PNPLA-3 pathway [76]. Hewage et al. [32] indicated that supplementation with 1% (*w/w*) *Vaccinium vitis-idaea* L. could reduce liver lipid accumulation by downregulating SREBP-1c and restoring AMPK. One of the mechanisms of AMPK action involves the phosphorylation of SREBP-1c, which inhibits its proteolytic cleavage and nuclear translocation, thereby suppressing hepatic lipogenesis.

The enzymes DGAT1 and DGAT2, involved in the final step of triacylglycerol synthesis, also play a role in developing hepatic steatosis. Overexpression of DGAT1 is linked to elevated levels of triacylglycerols incorporated into very-low-density lipoproteins (VLDL), whereas DGAT2 is more closely associated with the synthesis of triacylglycerols stored in the cytosol [117]. Lingonberry supplementation reduced the expression of DGAT1 and DGAT2, which may contribute to low hepatic TG content in high-fat-diet-fed mice. Additionally, lingonberry supplementation inhibited the expression of CD36, a fatty acid uptake receptor, potentially leading to reduced hepatic fatty acid uptake in high-fat-diet-fed mice [32].

Cranberry supplementation also prevented the upregulation of genes involved in lipid metabolism, such as monoacylglycerol O-acyltransferase 1 (Mogat1). Mogat1 plays a role in TG metabolism in the liver, fat absorption in the gastrointestinal tract, and the early development of type 2 diabetes, hepatic steatosis, and obesity. As an enzyme that converts monoacylglycerol to diacylglycerol, Mogat1 expression in the liver notably rises in mouse models fed a high-fat diet and is activated by PPAR- γ [80].

Several studies on NAFLD suggest promising potential for using *Vaccinium* species in antifibrotic therapy. Supplementation with Mirtoselect, cranberry, blueberry, and lingonberry may effectively mitigate disease progression [71,72,77,80]. Mirtoselect significantly reduced liver collagen content and decreased the expression of fibrosis-related genes, such as Col1a1 and Acta2. Cranberry demonstrated similar potential by reducing levels of TGF- β , α -SMA, and hydroxyproline, leading to decreased collagen deposition, while lingonberry supplementation inhibited the upregulation of genes associated with liver inflammation and fibrosis. The antifibrotic potential of extracts from lingonberry, cranberry, and blueberry was confirmed by Sergazy et al. [118]. They demonstrated that in rats treated with these extracts, fibrosis was either absent or had a mild, focal character, suggesting that these extracts may effectively inhibit the development of liver fibrosis in cases of liver damage. The authors hypothesized that this improvement was likely due to the antifibrotic effects of the berry extracts, which might be attributed to a reduction in inflammation and oxidative stress. Sun et al. [119] demonstrated that blueberry anthocyanins might offer protective effects against liver fibrosis induced by CCL4. This effect could be attributed to their ability to reduce ROS generation and associated oxidative damage, lower the impact of proinflammatory cytokines, inhibit hepatic stellate cell activity, and decrease the expression of collagen III and α -SMA, while enhancing the expression of MMP-9.

Clinical research on the use of *Vaccinium* species fruits is limited. The studies reviewed focused exclusively on patients with confirmed NAFLD, excluding those with related conditions. The results suggest that cranberry may have beneficial effects in treating NAFLD through various pharmacological actions, including lowering glycemic response, HOMA-IR, lipid levels, and liver enzymes (AST, ALT, ALP) [34,85]. These clinical studies primarily used non-invasive diagnostic methods like ultrasound. Both trials are considered high quality; however, since the review includes only two clinical trials, it is not easy to

perform a meta-analysis and, thus, it was decided to forgo this approach. Additionally, the studies cited employed groups of varying and relatively small sample sizes, different doses and methods of cranberry extract administration, and varying study durations. Despite these limitations, the findings underscore the potential of cranberry fruits in NAFLD treatment and highlight the need for further in-depth clinical research to understand their efficacy and fully establish standardized treatment protocols.

5. Future Research

Future research on the use of *Vaccinium* plants in the treatment of NAFLD and liver diseases should focus on several key areas. Firstly, it is essential to conduct more clinical studies involving a broad range of patients, including those with comorbid conditions associated with NAFLD, to more accurately assess the effectiveness of these plants in diverse populations. Randomized controlled trials should be designed to assess the efficacy of *Vaccinium* berries and leaves in various forms, such as whole extracts, standardized extracts rich in anthocyanosides, and isolated bioactive compounds. These trials should explore their effects on liver function, lipid profiles, and inflammatory markers over extended periods. Additionally, RCTs should compare different dosages and delivery methods, such as oral supplementation versus dietary inclusion, to determine the most effective approach. Secondly, understanding the molecular mechanisms through which bioactive compounds in *Vaccinium* fruits and leaves influence lipid metabolism and liver inflammation will be crucial. Further studies should also aim to optimize dosages and forms of supplementation to maximize health benefits. An important step is evaluating the long-term effects of these extracts to ensure that no adverse side effects occur and that therapeutic benefits are sustained. It may be interesting to investigate whether high levels of specific anthocyanosides from *Vaccinium* are associated with positive outcomes. However, the focus should be on using modern and standardized methods for the measurement and diagnosis of liver diseases, including NAFLD/NASH. It is necessary to investigate ways to enhance the bioavailability of bioactive compounds from *Vaccinium*. This can be achieved by modifying the delivery form, such as using nanotechnology, microencapsulation, or liposomal formulations, which may improve the absorption and stability of these compounds. Finally, future research should investigate potential interactions between *Vaccinium* supplements and other medications used in NAFLD treatment to ensure safety and efficacy.

In summary, preclinical and clinical studies on extracts, juices, or nutraceuticals derived from *Vaccinium* have demonstrated a favorable safety profile. These findings may be attributed to the small cohort sizes or the use of low doses of *Vaccinium* preparations, which may have fallen below the threshold for biological efficacy. However, it is essential to note that these studies exhibit significant variability in the active compounds used, the matrices administered, the dosages, and the duration of treatment. Nonetheless, the promising results from these studies suggest that more extensive cohort studies could further clarify the efficacy of *Vaccinium*-based interventions in the treatment of liver diseases.

6. Strength and Limitations

This review provides a thorough analysis of *Vaccinium* spp. berries in preventing and treating NAFLD, covering clinical, preclinical, and in vitro studies from the past 14 years. It was carried out according to PRISMA guidelines by three independent researchers who evaluated the internal quality of the selected reports. Additionally, applying the Jadad scale and the methodology of the included animal studies following the Stroke Therapy Academic Industry Roundtable in this review provides an objective framework, minimizing subjective biases. Both trials are regarded as high quality according to the Jadad scale. The quality assessment of preclinical studies, according to STAIR, indicates that the included studies received ratings reflecting the fulfillment of fundamental methodological criteria. Results from studies rated at level 4 may suggest potential therapeutic efficacy but require further research to confirm these observations. It may be advisable to supplement the findings with additional analyses or conduct small confirmatory experiments before advancing to the clinical phase. For in vitro studies, interpreting the collective evidence is

difficult because of variations in cellular models, experimental protocols, and molecular pathways evaluated.

This study has certain limitations. The first limitation of the study is that the findings in this work are based on animal studies (in vivo), and therefore may not be directly applicable to clinical practice. Secondly, the discussed clinical studies are limited and pertain to only two papers analyzing cranberry capsules administered in various doses. Additionally, the sample sizes were small, with the most significant number of participants only 110. As a result, the obtained data may be considered controversial. Third, the short observation periods present another limitation of the identified studies. All of the clinical studies had a duration of 6 months, meaning the long-term impact of cranberries on treating NAFLD and related diseases remains to be discovered. In animal studies, diverse dietary models and various forms of *Vaccinium* extracts have been employed to investigate NAFLD. Another limitation is that the results were based exclusively on two animal species, namely rats and mice, indicating the need for comparative studies with other species. However, due to NAFLD's complex and multifaceted nature, only some animal models fully capture the disease's entirety. Another limitation is that this current review serves as a proof of concept, with the findings in this study laying the groundwork for future clinical trials.

7. Conclusions

NAFLD is a common chronic liver condition where the primary therapeutic approach focuses on lifestyle modifications such as dietary changes and physical activity. Preclinical and preliminary clinical studies suggest that extracts from the fruits and leaves of *Vaccinium* plants may have a beneficial impact on the treatment of NAFLD. These extracts may prevent liver steatosis and its progression to NASH by reducing oxidative stress, regulating apoptosis, and decreasing inflammation. *Vaccinium* extracts' mechanisms include reducing reactive oxygen species production, improving mitochondrial content and membrane potential, and regulating apoptosis-related proteins (Bcl-2, Bax, HO-1). Additionally, these extracts may affect the Nrf-2 pathway, crucial in the antioxidant response. *Vaccinium* extracts exhibit anti-inflammatory effects by lowering levels of proinflammatory cytokines such as IL-6, IL-1 β , and TNF- α . They also reduce NF- κ B activity, which regulates the production of proinflammatory cytokines and affects liver fibrogenesis. Furthermore, *Vaccinium* extracts influence metabolic processes, including de novo lipogenesis, by decreasing the expression of critical enzymes such as FAS, ACC1, DGAT1, and DGAT2 and regulating SREBP-1c and PPAR- α activity, leading to reduced lipid accumulation in the liver. In summary, extracts from *Vaccinium* plants show therapeutic potential in treating NAFLD by offering benefits through their antioxidant, anti-inflammatory, and lipid-metabolism-modulating effects. Evidence from clinical trials, animal studies, and in vitro models regarding the use of *Vaccinium* species in mitigating NAFLD provides a substantial source of supporting positive evidence that should be considered before completely dismissing the potential therapeutic role of these plants. However, further research is needed to assess their efficacy and safety fully.

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Brief Report

Quercetin's Potential in MASLD: Investigating the Role of Autophagy and Key Molecular Pathways in Liver Steatosis and Inflammation

Ioannis Katsaros ^{1,*†}, Maria Sotiropoulou ^{1,†}, Michail Vailas ¹, Emmanouil Ioannis Kapetanakis ², Georgia Valsami ³, Alexandra Tsaroucha ⁴ and Dimitrios Schizas ¹

¹ First Department of Surgery, National and Kapodistrian University of Athens, Laikon General Hospital, 17 AgiouThoma Str., Athens 11527, Greece; marosotiropoulou@gmail.com (M.S.); mike_vailas@yahoo.com (M.V.); schizasad@gmail.com (D.S.)

² Department of Thoracic Surgery, National and Kapodistrian University of Athens, Attikon University Hospital, Athens 12462, Greece; emmanouil.kapetanakis@gmail.com

³ Laboratory of Biopharmaceutics-Pharmacokinetics, Department of Pharmacy, School of Health Sciences, National and Kapodistrian University of Athens, Athens 15774, Greece; valsami@pharm.uoa.gr

⁴ Laboratory of Experimental Surgery, Faculty of Medicine, Democritus University of Thrace, Alexandroupolis 68100, Greece; atsarouc@med.duth.gr

* Correspondence: gikats.md@gmail.com; Tel.: +30-6970801235

† These authors contributed equally to this work.

Abstract: Metabolic dysfunction-associated fatty liver disease (MASLD) is a widespread liver disorder characterized by excessive fat accumulation in the liver, commonly associated with metabolic syndrome components such as obesity, diabetes, and dyslipidemia. With a global prevalence of up to 30%, MASLD is projected to affect over 100 million people in the U.S. and 20 million in Europe by 2030. The disease ranges from Steatotic Liver Disease (SLD) to more severe forms like metabolic dysfunction-associated steatohepatitis (MASH), which can progress to cirrhosis and hepatocellular carcinoma. Autophagy, a cellular process crucial for lipid metabolism and homeostasis, is often impaired in MASLD, leading to increased hepatic lipid accumulation and inflammation. Key autophagy-related proteins, such as Beclin1, LC3A, SQSTM1 (p62), CD36, and Perilipin 3, play significant roles in regulating this process. Disruption in these proteins contributes to the pathogenesis of MASLD. Quercetin, a natural polyphenolic flavonoid with antioxidant and anti-inflammatory properties, has promising results in mitigating MASLD. It may reduce hepatic lipid accumulation, improve mitochondrial function, and enhance autophagy. However, further research is needed to elucidate its mechanisms and validate its therapeutic potential in clinical settings. This underscores the need for continued investigation into autophagy and novel treatments for MASLD.

Keywords: quercetin; NAFLD; MASLD; autophagy; Beclin1; LC3A; SQSTM1; CD36; Perilipin3

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is a major public health disease defined by liver fat accumulation with concomitant cardiometabolic risk factors and the absence of harmful alcohol intake [1]. The European Association for the Study of the Liver (EASL) and the American Association for the Study of Liver Diseases (AASLD) adopted the term metabolic dysfunction-associated fatty liver disease (MASLD) in order to better reflect its pathophysiologic background and alleviate the stigma often associated with it [2,3]. MASLD has a global prevalence of up to 30% and is projected to affect over 100 million people in the United States and 20 million in Europe by 2030 [4,5]. It is a part of the metabolic syndrome and is associated with diabetes mellitus type 2, dyslipidemia, and obesity [6–8]. The severity of MASLD spans from Steatotic Liver Disease (SLD) to metabolic dysfunction-associated steatohepatitis (MASH), with potential progression to liver cirrhosis

and hepatocellular carcinoma (HCC) [1,2,9,10]. Early diagnosis, along with therapeutic interventions and lifestyle modifications, can potentially reverse MASLD in its initial stages, whereas the later stages are marked by irreversible liver damage, often necessitating liver transplantations [11].

The development of MASLD was traditionally understood through the “two-hits” pathophysiological model. In this framework, the first “hit” involves the accumulation of lipids within hepatocytes, leading to liver steatosis. This is largely driven by an imbalance between the input and output of fatty acids within the liver cells, often associated with metabolic disturbances such as central obesity and insulin resistance [12,13]. The hepatic lipid overload creates a vulnerable state, priming the liver for further injury. The “second hit” is thought to occur when additional factors, including inflammatory cytokines, oxidative stress, bacterial endotoxins, and mitochondrial dysfunction, exacerbate liver damage, causing inflammation, hepatocellular injury, and eventually fibrosis [14,15].

However, more recent insights have shifted the paradigm from the “two-hits” model toward a more nuanced understanding known as the “multiple parallel hits” hypothesis. This hypothesis suggests that rather than a sequential process, various factors act simultaneously to contribute to the progression of MASLD. These factors include genetic predispositions, alterations in gut microbiota, increased circulating free fatty acids, and adipokine imbalances, which together lead to insulin resistance, oxidative stress, and chronic inflammation. As a result, MASLD is now recognized as a multifactorial disease, where metabolic, environmental, and genetic elements collectively trigger the development and progression of liver steatosis, inflammation, and fibrosis [16]. This hypothesis better explains the complexity of the disease and the heterogeneity observed in its clinical manifestations, emphasizing that liver damage can occur at varying levels and stages depending on the interplay of these contributing factors.

The development of both experimental and clinical studies is crucial to fully understand the underlying pathophysiological pathways of MASLD. Novel therapeutic approaches and agents targeting these pathways could potentially reverse liver steatosis, inflammation, and hepatocellular injury.

2. The Role of Quercetin in MASLD

Several pharmacological treatments have been proposed against MASLD, but they have shown limited efficacy and long-term results [17]. Natural polyphenols are a heterogeneous class of polyphenolic compounds, are used for the treatment of metabolic disorders, and have been shown to have a hepatoprotective action [18]. This action is mainly a result of both the increased lipid breakdown in the liver and the reduction in fibrosis lipolysis [19]. Thus, oxidative stress and the subsequent hepatocellular damage are reduced [19].

Quercetin (QUE) is a naturally occurring polyphenolic flavonoid widely recognized for its broad range of beneficial biological properties, including anti-inflammatory, antioxidant, anti-apoptotic, and immunoprotective effects [20]. Numerous studies have demonstrated quercetin’s hepatoprotective capabilities, particularly in the context of liver diseases, making it a promising candidate for therapeutic use. However, the exact pathophysiological mechanisms through which quercetin exerts its protective effects on MASLD remain not fully elucidated [20–23]. Some research suggests that quercetin may help reduce hepatic lipid accumulation, improve mitochondrial function, and modulate oxidative stress, all of which play a role in MASLD pathogenesis. Autophagy plays a critical role in maintaining lipid homeostasis in hepatocytes, primarily through the process of lipophagy—the selective degradation of lipid droplets. Impaired autophagy in MASLD contributes to excessive lipid accumulation, which exacerbates inflammation and liver damage. Studies show that downregulated autophagy markers, including Beclin1 and LC3A, correlate with increased hepatic lipid droplet accumulation [24,25]. Additionally, quercetin may attenuate liver inflammation by regulating cytokine production and mitigating the harmful effects of oxidative stress-induced apoptosis within hepatocytes [20–23].

Alongside QUE, several other polyphenols are under investigation for their therapeutic potential in MASLD due to their anti-inflammatory, antioxidant, and lipid-modulating properties [26,27]. Resveratrol, a polyphenol found in grapes, has shown promise in reducing hepatocellular damage, as it activates the AMPK pathway and sirtuin-1 (SIRT1), leading to reduced inflammation and reduced oxidative stress [28,29]. Curcumin, a compound derived from turmeric, exhibits anti-inflammatory and antifibrotic properties. By modulating pathways such as NF- κ B and TGF- β , curcumin reduces hepatic inflammation and fibrosis, making it another potential therapeutic agent in MASLD [26,30]. Epigallocatechin gallate (EGCG), a green tea polyphenol, has also demonstrated hepatoprotective effects. EGCG decreases lipogenesis and oxidative stress through the activation of AMPK and inhibition of lipogenic enzymes like SREBP1 [31]. These polyphenols, similar to QUE, show multi-targeted effects on lipid metabolism, inflammation, and oxidative stress, highlighting the potential of polyphenol-based interventions for managing MASLD. Future studies comparing the efficacy and mechanisms of these compounds could further clarify their roles as natural treatments in MASLD.

Given these properties, quercetin shows potential as a novel therapeutic agent for managing MASLD, offering a natural and multifaceted approach to addressing both liver steatosis and its associated complications. Further research is needed to confirm its efficacy and underlying mechanisms in human models.

3. Current Evidence

Several experimental animal studies have shown the hepatoprotective action of natural polyphenols, including QUE against liver steatosis and MASLD, but their mechanism of action is not yet elucidated. Yang et al. suggested the protective action of QUE in a mice MASLD model by restoring lipid homeostasis through the downregulation of the mTOR/YY1 signaling pathway [32]. He et al. investigated the effect of the famous traditional Chinese shengi pill, whose active compound is QUE, on a MASH rat model and concluded that liver injury and lipid metabolism were significantly improved after its administration by reducing the effect of pro-inflammatory cytokines (IL-6, TNF- α) [33]. Ying et al. reached the same conclusion showing that oral intake of QUE for 14 days in a high-fat-diet-induced MASH rat model significantly reduced liver lipid accumulation by lowering pro-inflammatory cytokines TNF- α and IL-6 [34]. Furthermore, Ma et al. revealed that QUE attenuated steatohepatitis by inhibiting the activated NLRP2 inflammasome through suppression of the expression of HSP90 [35]. An amelioration of MASH was also reported by Macrolin et al., who also showed an attenuation of several pro-fibrotic and pro-inflammatory pathways in mice fed with QUE [36]. Finally, Panchal et al. also reported an improvement in MASLD induced in high-fat fed rats after 8 weeks of QUE by downregulating the NF- κ B pathway, a factor stimulating inflammation, and upregulating Nrf2, a major protective factor against oxidative stress [37].

MASLD progression is influenced by multiple pathways, each of which quercetin may modulate to reduce lipid accumulation, inflammation, and fibrosis (Figure 1). The AMPK pathway plays a central role in energy regulation and induces mitophagy, helping maintain mitochondrial health and reducing oxidative stress. Studies indicate that quercetin activates AMPK, thereby supporting mitophagy and mitigating mitochondrial dysfunction in MASLD [22]. The SREBP1/FAS pathway, which regulates lipid synthesis, is often upregulated in MASLD, contributing to excess lipid accumulation. Quercetin inhibits SREBP1 and FAS expression, decreasing lipogenesis and lipid accumulation [38]. Another key pathway, PI3K/AKT, is involved in cell growth and survival; quercetin modulates this pathway, attenuating hepatic inflammation and fibrosis [32]. Additionally, quercetin affects FXR/TGR signaling, enhancing bile acid metabolism and reducing lipid overload [18]. Finally, the TGF- β 1/Smads pathway, a major driver of fibrosis in MASLD, is downregulated by quercetin, which decreases fibrotic markers and may slow disease progression [35]. Together, these pathways highlight the multifaceted role of quercetin in addressing MASLD's complex pathophysiology.



Figure 1. The effect of Quercetin on MASLD (metabolic dysfunction-associated fatty liver disease).

QUE has been shown to modulate autophagy through the mTOR pathway, influencing lipid metabolism and reducing hepatic inflammation. By activating autophagic processes, quercetin potentially restores lipid homeostasis, alleviating steatosis and inflammation in MASLD [32]. In addition to lipophagy, mitophagy—a selective form of autophagy that removes dysfunctional mitochondria—is relevant to MASLD pathogenesis. Mitochondrial dysfunction contributes to oxidative stress and inflammation in MASLD. Furthermore, QUE activates AMPK-mediated mitophagy and regulates Frataxin-mediated PINK1–Parkin-dependent mitophagy, which can help reduce mitochondrial damage and oxidative stress, thereby potentially mitigating the progression of MASLD [21,39]. Finally, through the TGF-β1/Smads and PI3K/Akt pathway, QUE exerts antifibrotic effects that are critical for slowing or preventing fibrosis progression in MASLD. By modulating this pathway, QUE could serve as an adjunct therapy to reduce liver fibrosis associated with chronic MASLD [36,40].

Clinical studies investigating the effect of QUE on MASLD are limited, but they have reached similar conclusions to experimental studies. Pryszazhnyuk and Voloshyn recruited 41 MASLD patients and revealed a significant decrease in the levels of total cholesterol, triacylglycerol, and liver function tests following two weeks of QUE, showing a potential protective effect of QUE [41]. Pasdar et al. enrolled 90 patients suffering from MASLD and randomly assigned them to either a QUE or placebo group for 12 weeks. Patients receiving QUE had a significant increase in red blood cell count, thus reversing the effect of oxidative stress [42].

4. Autophagy and MASLD

Autophagy is a crucial cellular process responsible for maintaining homeostasis by degrading and recycling damaged organelles and proteins. It plays a vital role in hepatocellular lipid metabolism, which is essential for liver health and has been implicated in the pathogenesis of MASLD [43]. In the setting of MASLD, autophagy is frequently downregulated, contributing to increased lipid accumulation in hepatocytes. Evidence indicates that impaired autophagy in the fatty liver occurs primarily due to the disruption

of autophagosome–lysosome fusion, leading to the accumulation of autophagosomes and ineffective degradation of intracellular lipids [44]. This defective autophagic flux contributes to impaired lipophagy, further promoting hepatic steatosis and the development of MASLD [45].

A critical component of this process involves key autophagy proteins such as Beclin1 and LC3A. Beclin1 plays a major role in initiating autophagy by forming complexes that promote autophagosome formation. LC3A is responsible for the elongation and closure of autophagosomes. Research by Zhao et al. has demonstrated that hepatic steatosis results in the downregulation of both Beclin1 and LC3A, leading to decreased autophagic activity and the accumulation of lipid droplets in hepatocytes [25]. Another critical autophagy-related protein is SQSTM1 (p62), which serves as a marker of impaired autophagy. SQSTM1 binds to ubiquitinated proteins, facilitating their degradation through autophagy. When autophagy is disrupted, SQSTM1 accumulates, signaling defective autophagic processes and contributing to the further progression of MASLD [45,46].

In addition to these key autophagic proteins, other factors such as CD36 and Perilipin 3 also play significant roles in the development of MASLD [47,48]. CD36 is a fatty acid transporter that facilitates the uptake of lipids into hepatocytes. In MASLD, CD36 is often upregulated, contributing to increased lipid accumulation and subsequent liver damage [47]. Perilipin 3, a regulator of lipid droplet dynamics, is essential for the mobilization of lipids within hepatocytes. Dysregulation of Perilipin 3 can impair lipid metabolism, further aggravating hepatic steatosis and inflammation in MASLD [48].

Autophagy serves as a protective mechanism in liver cells, regulating lipid homeostasis and preventing excess lipid buildup. However, in MASLD, its downregulation leads to a cascade of metabolic disturbances that promote the progression from simple steatosis to more severe liver conditions, including metabolic dysfunction-associated steatohepatitis (MASH), fibrosis, and cirrhosis [24,46,49]. Further research is needed to fully elucidate the molecular mechanisms behind autophagy impairment in MASLD, particularly the roles of Beclin1, LC3A, SQSTM1, CD36, and Perilipin 3. A deeper understanding of these processes may lead to novel therapeutic strategies targeting autophagy to reverse hepatic steatosis and prevent disease progression.

5. Conclusions and Implications for Future Research

MASLD is a prevalent and growing liver disease affecting a substantial proportion of the global population. While some progress has been made in understanding its pathophysiology, the mechanisms driving liver steatosis, oxidative stress, impaired autophagy, and mitochondrial dysfunction in hepatocytes remain unclear. Unraveling these pathways is essential for developing therapeutic strategies that can halt disease progression or potentially reverse liver damage.

Flavonoids like quercetin have demonstrated hepatoprotective properties, showing promise as potential treatments for MASLD. Specifically, quercetin's antioxidant and anti-inflammatory effects have been supported by several experimental studies, suggesting it may help reduce liver injury and steatosis in MASLD. Autophagy, a critical cellular process that removes damaged organelles and excess lipids, plays a significant role in maintaining liver homeostasis. Impaired autophagy has been implicated in the progression of MASLD, particularly in the accumulation of lipid droplets and the promotion of liver inflammation. Quercetin's potential to modulate autophagy and promote lipophagy could be a key mechanism in reducing hepatic steatosis and inflammation. However, further investigation is needed to fully understand the pathophysiological mechanisms underlying its effects.

Current research on quercetin's role in autophagy, lipophagy, and the alleviation of hepatic steatosis and inflammation is limited, creating uncertainty about its efficacy against MASLD. Additional experimental and clinical studies are required to clarify the role of quercetin in MASLD.

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Review

Regular Consumption of Green Tea as an Element of Diet Therapy in Drug-Induced Liver Injury (DILI)

Anna Winiarska-Mieczan ^{1,2,*}, Karolina Jachimowicz-Rogowska ¹, Małgorzata Kwiecień ¹, Marta Borsuk-Stanulewicz ³, Agnieszka Tomczyk-Warunek ⁴, Ewa Stamirowska-Krzaczek ², Cezary Purwin ³, Małgorzata Stryjecka ² and Marzena Tomaszewska ²

¹ Institute of Animal Nutrition and Bromatology, Department of Bromatology and Nutrition Physiology, University of Life Sciences in Lublin, Akademicka 13, 20-950 Lublin, Poland; karolina.jachimowicz@up.lublin.pl (K.J.-R.); malgorzata.kwiecien@up.lublin.pl (M.K.)

² Institute of Human Nutrition and Agriculture, The University College of Applied Sciences in Chełm, Poczłowa 54, 22-100 Chełm, Poland; ekrzaczek@panschem.edu.pl (E.S.-K.); mstryjecka@panschem.edu.pl (M.S.); mtomaszewska@panschem.edu.pl (M.T.)

³ Department of Animal Nutrition and Feed Science, University of Warmia and Mazury in Olsztyn, 10-719 Olsztyn, Poland; marta.borsuk@uwm.edu.pl (M.B.-S.); purwin@uwm.edu.pl (C.P.)

⁴ Laboratory of Locomotor Systems Research, Department of Rehabilitation and Physiotherapy, Medical University of Lublin, Jaczewskiego 8, 20-954 Lublin, Poland; a.tomczykwarunek@gmail.com

* Correspondence: anna.mieczan@up.lublin.pl

Abstract: The liver is a highly metabolically active organ, and one of the causes of its dysfunction is the damage caused by drugs and their metabolites as well as dietary supplements and herbal preparations. A common feature of such damage is drugs, which allows it to be defined as drug-induced liver injury (DILI). In this review, we analysed available research findings in the global literature regarding the effects of green tea and/or its phenolic compounds on liver function in the context of protective action during prolonged exposure to xenobiotics. We focused on the direct detoxifying action of epigallocatechin gallate (EGCG) in the liver, the impact of EGCG on gut microbiota, and the influence of microbiota on liver health. We used 127 scientific research publications published between 2014 and 2024. Improving the effectiveness of DILI detection is essential to enhance the safety of patients at risk of liver damage and to develop methods for assessing the potential hepatotoxicity of a drug during the research phase. Often, drugs cannot be eliminated, but appropriate nutrition can strengthen the body and liver, which may mitigate adverse changes resulting from DILI. Polyphenols are promising owing to their strong antioxidant and anti-inflammatory properties as well as their prebiotic effects. Notably, EGCG is found in green tea. The results of the studies presented by various authors are very promising, although not without uncertainties. Therefore, future research should focus on elucidating the therapeutic and preventive mechanisms of polyphenols in the context of liver health through the functioning of gut microbiota affecting overall health, with particular emphasis on epigenetic pathways.

Keywords: DILI; liver; green tea; catechin; metabolism of drugs; microbiota

1. Introduction

The liver is an important gland of the digestive system and one of the most essential organs for the entire body, as it performs many critical functions necessary for its proper functioning. The liver has several functions, including filtration, storage, detoxification, and metabolism, which complement each other [1]. The detoxification function of the biotransformation of xenobiotics is crucial for maintaining homeostasis in the body. Any substance that is not utilised by the body is excreted quickly. This was facilitated by the biphasic metabolism of xenobiotics. Because the liver performs many critical functions in the body, its damage is the cause of many diseases, sometimes with serious consequences [1–3].

Given that the liver is a highly metabolically active organ, one of the causes of liver dysfunction is damage due to drugs and their metabolites as well as dietary supplements and herbal preparations [4]. A common feature of such damage is its cause, which is, the drug, which allows it to be classified as drug-induced liver injury (DILI). DILI accounts for approximately 10% of all cases of acute hepatitis, causes acute jaundice in 50% of patients with new jaundice, and is responsible for approximately 50% of cases of acute liver failure [5]. The pathogenesis of DILI is highly complex and is not yet fully understood. Various drugs and/or their toxic metabolites can directly affect liver cells, as well as induce inflammation, oxidative stress, and mitochondrial damage [6–10]. Often, drugs cannot be eliminated, but appropriate nutrition can strengthen the body and the liver, which may mitigate adverse changes resulting from DILI. Some dietary components, such as phenolic compounds, exhibit antioxidant and anti-inflammatory effects [11]. These are found in many unprocessed or minimally processed plant-based foods, such as tea [11,12]. Tea can be consumed widely, regardless of age and physiological conditions, making it an excellent candidate as a fundamental element of diet therapy in DILI. In particular, green tea deserves special attention because it contains the highest amount of catechins, primarily epigallocatechin gallate (EGCG), which exhibits strong hepatoprotective effects [13,14].

In this review, we analyse the available global literature on the effects of green tea and/or its phenolic compounds on liver function in the context of protective action during prolonged exposure to xenobiotics. We focused on both the direct detoxifying effect of EGCG in the liver and the impact of EGCG on the gut microbiota and its influence on liver health. The role of the gut microbiota in the metabolism of EGCG and its impact on liver function is a unique aspect of this text. This highlights the complex relationships between gut flora, food components, and their influence on physiological functions. Strengthening liver health can directly translate into mitigating DILI symptoms and reducing the risk of disease in individuals taking high doses of pharmaceutical agents.

2. Methodology of Information Retrieval in Databases

During the preparation of this review, scientific information available in the Scopus, PubMed, Web of Science, and Google Scholar databases was analysed. The analysis was conducted in May 2024. The following keywords were used: “DILI”, “drug-induced liver injury”, “liver”, “polyphenols”, “EGCG”, “catechins”, “green tea”, “gut microbiota”, “oxidative stress”, and “inflammation”, in both Polish and English (Figure 1). Titles and abstracts were screened, and articles not meeting the substantive criteria (Condition 1: articles from 2014 to 2024, and Condition 2: research studies and meta-analyses), specifically those that did not include results from studies conducted on laboratory animals or involving humans, were excluded. Only research studies and meta-analyses have been conducted to date. In the case of meta-analyses, duplicate data were used only once (source texts were referenced).

Articles that met these criteria underwent a more detailed analysis to identify the most significant publications and to exclude duplicates. Additionally, the bibliographies of all selected articles were reviewed to uncover any further potentially relevant texts. The search was restricted to studies published between 2014 and 2024. In total, 1842 publications were analyzed, with 130 being included in the final manuscript. The protocol was developed following the guidelines outlined in the Preferred Reporting Items for Systematic Reviews and Meta-Analysis Protocols (PRISMA-P) 2015 statement.

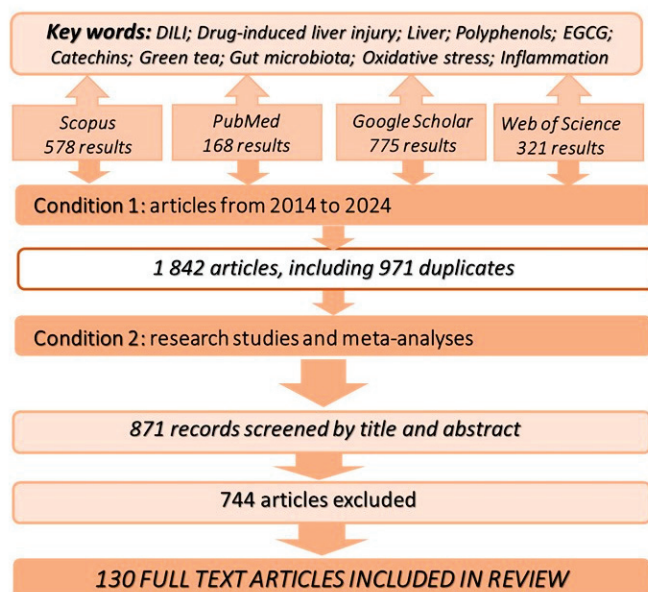


Figure 1. Methodology for reviewing the existing literature.

3. Pathophysiological Mechanisms of DILI

DILI is a complex condition characterised by liver damage caused by the administration of drugs and their metabolites. The pathophysiological mechanisms underlying DILI are multifaceted (Figure 2). However, several key processes have been identified: direct toxic effects of drugs, oxidative stress, inflammation, mitochondrial damage, and immune reactions [2,6,8–10]. The most common cause of DILI is the use of antimicrobial agents, herbal and dietary supplements, and medicinal preparations [15–17]. According to some data, over 50% of acetaminophen overdose cases result in acute liver failure and approximately 20% lead to liver transplantation [18]. The use of high doses of acetaminophen leads to the accumulation of the reactive metabolite *N*-acetyl-*p*-benzoquinone imine (NAPQI), which causes liver cell damage leading to necrosis and resulting in liver failure [18].

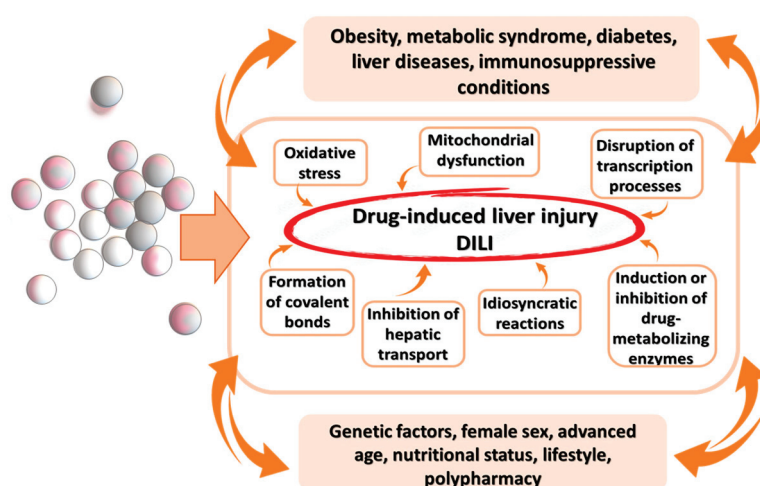


Figure 2. Pathophysiological mechanisms of DILI.

Among the biochemical disturbances accompanying DILI, the most characteristic, although non-specific, indicator of liver parenchymal damage is an increase in alanine aminotransferase (ALT) activity > 200 U/L, aspartate aminotransferase (AST) > 200 U/L, total bilirubin > 3.0 mg/dL, or international normalised ratio (INR > 3). Other indicators include oliguria, creatinine > 200 micromol/L, acidosis (pH < 7.3), and lactate > levels > 3 mmol/L [19,20]. Genetic factors, female sex, advanced age, nutritional status, lifestyle

(smoking, alcohol, and drugs), and polypharmacy (exposure to multiple drugs simultaneously) predispose individuals to DILI [20,21]. Concomitant metabolic diseases such as obesity, metabolic syndrome, diabetes, liver diseases, and immunosuppressive conditions also play significant roles [19,20].

There are three types of DILI: hepatocellular, cholestatic, and mixed [7,22]. The pathogenesis of DILI is highly complex and not yet fully understood. However, recent studies using improved preclinical models have provided better insights into the mechanisms underlying drug-induced liver injury. Various drugs and their toxic metabolites can directly affect liver cells and cause inflammation, oxidative stress, and mitochondrial damage. Drugs can induce liver cell damage through different molecular pathways, including direct hepatotoxicity and immune responses. Consequently, various pathomechanisms of DILI can be distinguished [6–10]: (1) Induction of oxidative stress: this causes the generation of large amounts of reactive oxygen species (ROS), which activate Kupffer cells; (2) mitochondrial dysfunction: resulting from oxidative stress, leading to impaired energy production and increased cell damage; (3) disruption of transcription processes: due to oxidative stress, affecting normal cell functions and survival; (4) formation of covalent bonds: leading to loss of function and/or autoimmune reactions; inhibition of hepatic transport: affecting the normal excretion and processing of substances in the liver; (5) induction or inhibition of drug-metabolising enzymes: affecting how drugs are processed and potentially leading to toxic accumulations; and (6) idiosyncratic reactions: unpredictable reactions that are not dose-dependent and occur only in susceptible individuals. These mechanisms highlight the diverse ways in which drugs can induce liver damage, underscoring the need for a nuanced understanding of the pathogenesis of DILI to develop effective preventive and therapeutic strategies. The metabolism (biotransformation) of drugs in the body involves two phases: (1) Phase I reactions, which modify the chemical structure of the drug through oxidation, reduction, and hydrolysis; and (2) Phase II reactions, which involve conjugating the Phase I metabolite with active acetate CoA, 3'-phosphoadenosine-5'-phosphosulfate, glucuronic acid, and amino acids [23,24].

Lipid peroxidation is a major pathogenic factor that triggers hepatocyte necrosis and inflammatory processes in liver diseases [25]. Mitochondrial oxidative stress alone is insufficient to induce hepatocyte cell death; however, this process is stimulated by mitogen-activated protein kinases (MAPKs), leading to increased expression of the JNK genes JNK1 and JNK2 in the liver [6,26,27]. The combined activities of JNK1 and JNK2 in hepatocytes protect against toxic liver injury, as demonstrated in studies using mice intoxicated with CCl₄ and acetaminophen as well as in vitro studies using human hepatocytes [6].

4. Effect of Gut Microbiota on Liver Function

The gut and liver are closely interconnected and communicate via the portal vein and bile duct system, exposing the liver to bacterial products and metabolites originating from the intestine [28]. Gut microbiota can influence liver function and health through various mechanisms: (1) Production of metabolites: gut microbiota ferment dietary, producing short-chain fatty acids (SCFAs) such as acetic acid, propionic acid, and butyric acid [29]; SCFAs help maintain gut integrity by stimulating mucus production and by forming a protective barrier between the gut lumen and epithelial cells [30]; (2) changes in gut barrier integrity: dysbiosis can lead to dysfunction of the gut barrier, allowing microbial components to translocate to the liver and contributing to liver diseases [29–34]; (3) immunomodulatory effects: microbiota-derived metabolites regulate the function and efficiency of the immune system, and diet plays a key role in shaping the composition and function of the gut–liver axis microbiota [35]; chronic inflammation triggered by microbiota dysbiosis and resulting immune dysregulation affects the development of chronic liver diseases [36–39]; and (4) impact on bile acid metabolism: bile acids are synthesised in the liver from cholesterol and undergo biotransformation by the gut microbiota in the small intestine (Figure 3), involving major gut microbiota groups such as *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria* [40–43].

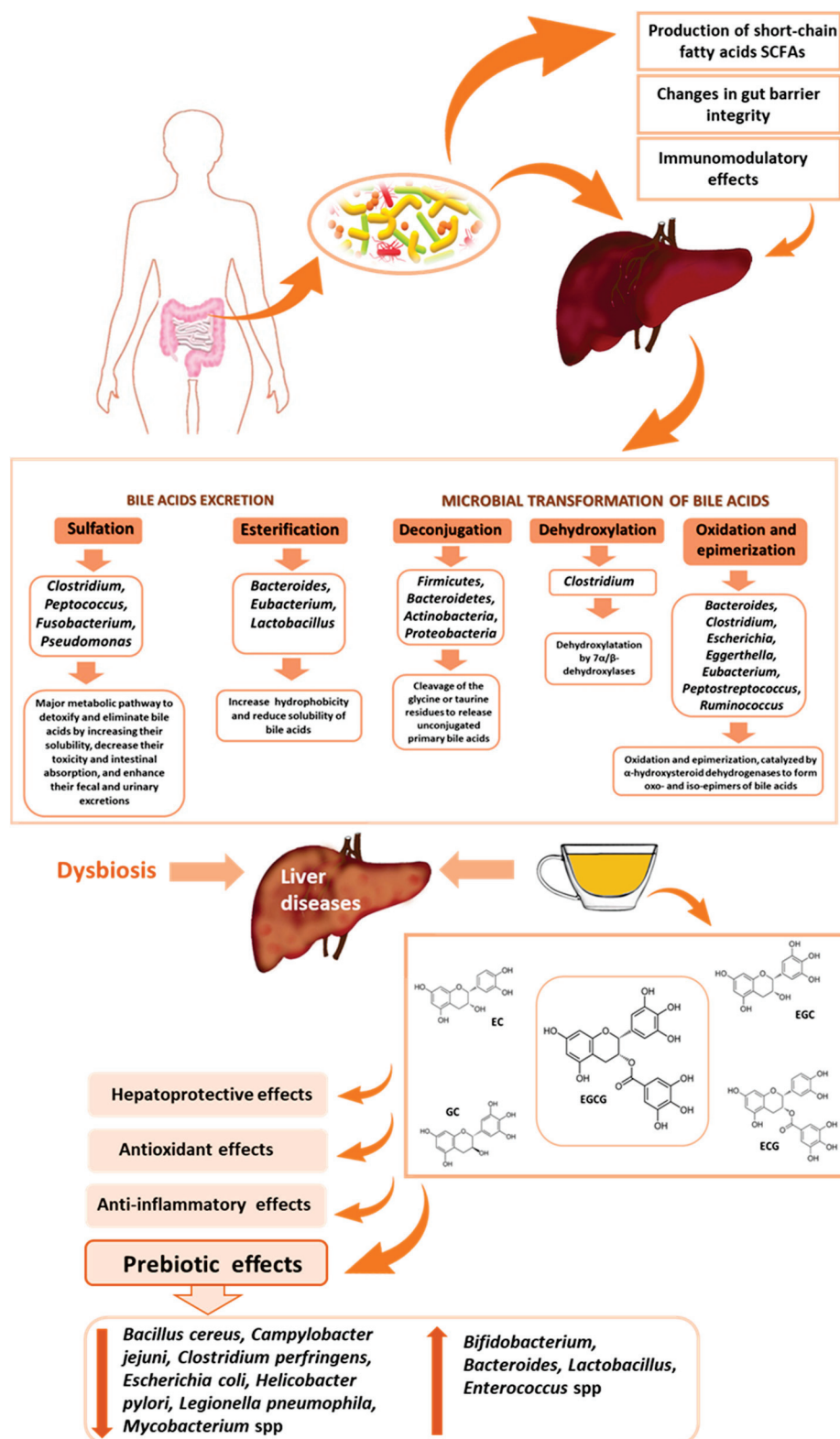


Figure 3. Relationship between gut microbiota, tea, and liver health.

Gut microbiota can influence liver function and health through various mechanisms: production of metabolites, changes in gut barrier integrity, immunomodulatory effects, and impact on bile acid metabolism. Bile acids are synthesised in the liver from cholesterol and undergo biotransformation by the gut microbiota in the small intestine, involving major gut microbiota groups such as *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria*.

Primary bile acids conjugated in the liver are deconjugated by the microbiota, leading to the formation of glycine, taurine, and deconjugated primary bile acids, which are further metabolised into secondary bile acids and excreted from the body. The gut microbiota regulates bile acid metabolism by lowering the levels of tauro- β -muricholic acid, a natural antagonist of the farnesoid X receptor (FXR), and inhibiting bile acid synthesis in the liver by alleviating FXR inhibition in the ileum [40,41]. In turn, bile acids and their metabolites can modulate gut microbiota composition by exerting antimicrobial effects and activating host signalling pathways [42]. Studies have shown that Gram-negative bacteria are more resistant to bile acids than Gram-positive bacteria, although the results have not been entirely consistent [43]. It has also been demonstrated that unconjugated bile acids have stronger antibacterial activities than conjugated bile acids [42,43].

Structural changes in the microbiota led to modifications in metabolite production, possibly contributing to liver disease progression [36–39]. Studies have indicated that dietary factors influence microbiota composition, metabolite production, and gut permeability [44–46]. Therefore, modulating the gut microbiota composition and/or its metabolites through bioactive food components could be considered a potential dietary therapy approach for liver diseases, including DILI.

5. The Potential of Green Tea as a Component of Dietotherapy in DILI

Risk factors for DILI, besides the use of large quantities of pharmaceutical agents, also include several factors influencing the body's condition. Most commonly mentioned are oxidative stress, inflammation, and chronic diseases, with oxidative stress and inflammation being markers for conditions such as obesity, metabolic syndrome, and diabetes [7,47]. Some active substances present in food have properties that can reduce the risk of these factors. Green tea exhibits antioxidant, anti-inflammatory, anti-obesogenic, and antidiabetic properties [11,12,48,49]. Additionally, its hepatoprotective effects have been demonstrated (Tables 1 and 2). The presence of phenolic compounds, notably catechins such as EGCG, contributes to this, as they are major secondary metabolites in tea and frequently serve as indicators of its quality [12,48,50]. Therefore, green tea can be considered as a component of dietotherapy for liver diseases, including DILI. However, it is important to note the controversies associated with consuming excessive amounts of green tea, primarily because of the presence of EGCG.

Table 1. The impact of green tea on antioxidant parameters and liver function parameters—studies involving humans.

Characteristic	Duration of the Study	Disease	Dosage of Green Tea	Antioxidant Parameters	Liver Parameters	Type of Study	Reference
Control n = 40 Experimental n = 40	12 weeks	Non-alcoholic fatty liver disease	500 mg/day of GTE		↓ ALT, ↓ AST	Double-blind, placebo-controlled, randomized clinical trial	[51]
Control n = 40 Experimental n = 80	12 weeks	Non-alcoholic fatty liver disease	1000 mg/day of GTE		↓ ALT, ↓ AST, ↓ hs-CRP	Double-blind, placebo-controlled, randomized clinical trial	[52]
Control n = 20 Experimental n = 20	12 weeks	Moderate hypercholesterolemia	2 × 300 mL catechin-enriched green tea/day	↑ TEAC, ↑ GSH, ↑ SOD, ↑ CAT, ↑ GPx, ↑ GR	reverting mild fatty liver to the normal hepatic condition	Randomized, controlled trial	[53]
Control n = 12 Experimental n = 26	6 months	Non-alcoholic steatohepatitis	600 mg/day catechins		↓ ALT, ↓ AST	Double-blind, placebo-controlled, randomized clinical trial	[54]
Control n = 24 Experimental n = 21	3 months	Non-alcoholic fatty liver disease	550 mg/day green tea tablets		↓ AST	Placebo-controlled, randomized clinical trial	[55]
Control n = 16 Experimental n = 16	4 weeks	Operating room staff chronically exposed to inhalation anesthetics			↓ AST, ↓ ALT, ↓ ALP, ↓ bilirubin	Placebo-controlled, randomized clinical trial	[56]

↑—Increased concentration or activity in comparison to the control (healthy) group; ↓— decreased or inhibited concentration or activity in comparison to the control (healthy) group; ALT—alanine aminotransferase; AST—aspartate aminotransferase; ALP—alkaline phosphatase; CAT—catalase; GTE—green tea extract; GSH—glutathione; GPx—glutathione peroxidase; GR—glutathione reductase; hs-CRP—high-sensitivity C-reactive protein; SOD—superoxide dismutase; and TEAC—trolox equivalent antioxidant capacity.

Table 2. The impact of green tea on antioxidant parameters and liver function parameters—studies involving laboratory animals.

	Animal Species	Duration of Experiment	Treatments	Dosage of Green Tea	Antioxidant Parameters	Liver Parameters	Anti-inflammatory Indices	Tissues	References
Control n = 6 Experimental n = 18	Male Wistar rats	28 days	Intraperitoneal injections of N-nitrosodimethylamine in a dose of 1 mg/100 g body weight on 3 consecutive days of a week	0.2 mg EGCG/100 g body weight	↓ MDA	↓ ALT, ↓ AST		Serum	[57]
Control n = 5 Experimental n = 25	Male and female mice ICR	7 days	Stress-induced liver injury and immunosuppression	40 mg EGCG/kg		↓ ALT, ↓ AST	↓ IL-1β, ↓ IL-2, ↓ IL-6	Serum, liver	[58]
Control n = 6 Experimental n = 22	Female Sprague–Dawley rats	8 weeks	Non-alcoholic fatty liver disease	50 mg EGCG/kg	↓ iNOS, ↓ COX-2, ↓ TNF-α	↓ ALT, ↓ AST ratio, ↓ number of fatty score, necrosis	↓ inflammatory foci	Serum, liver	[59]
Control n = 8 Experimental n = 32	Male C57BL/6 mice	4 weeks	Methionine- and choline-deficient diet-induced non-alcoholic steatohepatitis	25, 50, or 100 mg EGCG/kg		↓ ALT, ↓ AST		Serum	[60]
Control n = 5 Experimental n = 30	Female C57BL/6 mice	4 days	CCl ₄ -induced liver injury	100 mg GTE/kg		↓ ALT, ↓ AST, ↓ liver index		Serum, liver	[61]
Control n = 6 Experimental n = 18	Male ICR mice	7 days	Lipopolysaccharide-induced inflammatory liver injury	100 or 200 mg green tea polyphenols/kg body weight	↓ MDA, ↓ GSH, ↑ SOD	↓ ALT, ↓ AST	↓ IL-1β, ↓ IL-18, ↓ IL-6, ↓ TNF-α	Serum, liver	[62]
Control n = 6 Experimental n = 24	Male Sprague–Dawley rats	2 months	Thioacetamide-induced liver injury	250 mg/kg or 500 mg/kg daily methanolic GTE	↓ MDA, ↑ SOD, ↑ CAT	↓ ALT, ↓ AST, ↓ ALP, ↓ bilirubin		Serum, liver	[63]
Control n = 12 Experimental n = 36	Male C57BL/6 mice	4 weeks	Methionine–choline-deficient diet-induced non-alcoholic steatohepatitis	50 mg/kg EGCG		↓ ALT		Serum	[64]

Table 2. Cont.

	Animal Species	Duration of Experiment	Treatments	Dosage of Green Tea	Antioxidant Parameters	Liver Parameters	Anti-inflammatory Indices	Tissues	References
Control n = 12 Experimental n = 6	Male C57BL/6J mice	14 weeks	High-fat diet-induced non-alcoholic fatty liver disease	EGCG— 50 mg/kg/day	↓ ROS, ↑ GPx, ↑ SOD, ↑ CAT	↓ ALT, ↓ AST		Serum, liver	[65]
Control n = 12 Experimental n = 48	Adult male Wistar rats	6 or 12 weeks	7 mg CdCl ₂ + 50 mg Pb(CH ₃ COO) ₂ per kg of feed	Green tea infusion (contains 111 mg tannic acid) per 1000 mL H ₂ O ₂	↑ SOD, ↑ CAT, ↑ GPx			Liver	[11]
Control n = 20 Experimental n = 23	Male Nrf2-null mice, male C57BL6 WT mice	8 weeks	High-fat diet-induced non-alcoholic steatohepatitis	2% GTE	↓ MDA	↓ ALT	↓ TNF-α	Liver	[66]
Control n = 8 Experimental n = 24	Male Wistar rats	1 week	Halathion 150 mg/kg by gavage	30 mg/kg green tea through intraperitoneal injection	↓ LPO, ↑ TAP, ↑ TTG	↓ ALT, ↓ AST, ↑ ChE		Plasma, liver	[67]
Control n = 20 Experimental n = 40	Adult mice Balb-C strain	12 weeks	High-fat and high-cholesterol diet-induced hepatic steatosis	1% green tea over in food		↓ ALT, ↓ AST, ↓ ALP		Serum	[68]
Control n = 10 Experimental n = 30	Male Kunming mice	12 weeks	D-galactose-induced liver ageing	0.05% green tea polyphenols diet	↑ SOD, ↑ CAT, ↑ GSH, ↑ GST, ↑ TAC, ↓ MDA, ↓ NO	↓ ALT, ↓ AST, ↓ ALP	↓ TNF-α, ↓ TGF-β, ↓ IL-1β, ↓ IL-6		[69]

↑—Increased concentration or activity in comparison to the control (healthy) group; ↓—decreased or inhibited concentration or activity in comparison to the control (healthy) group; ALT—alanine aminotransferase; AST—aspartate aminotransferase; ALP—alkaline phosphatase; CAT—catalase; COX-2—cyclooxygenase-2 inhibitor; GSH—glutathione; GPx—glutathione peroxidase; iNOS—inducible nitric oxide synthase; NO—nitric oxide; ROS—reactive oxygen species; SOD—superoxide dismutase; IL-1β—interleukin-1 beta; IL-2—interleukin 2; IL-6—interleukin 6; IL-18—interleukin 18; MDA—malondialdehyde; LPO—lipid peroxidation; TAP—total antioxidant power; TTG—total thiol group; TAC—total antioxidant capacity; GST—glutathione S-transferase; ChE—cholinesterase activity; Nrf2—nuclear factor erythroid 2-related factor 2; TNF-α—tumour necrosis factor alpha; TGF-β—transforming growth factor-beta; GTE—green tea extract; and EGCG—epigallocatechin gallate.

5.1. Phenolic Compounds in Green Tea

In tea leaves, three primary groups of polyphenols can be distinguished: catechins (EGCG, epicatechin EC, epigallocatechin EGC, epicatechin gallate ECG, gallocatechin GC, and gallate gallate GCG), theaflavins, and thearubigins. The content of polyphenols is influenced by various factors such as tea type, origin, tea-growing region, and the age of the plant; as the plant ages, the content of phenolic compounds in the leaves decreases [12,70]. Green tea infusion contains approximately 0.2–0.5 mg total phenolic content (TPC), 5–8.5 µg flavonoids, and around 0.08–0.1 µg anthocyanins [12]. Tea leaves also contain tannins, which are products of polyphenol oxidation [11]. Phenolic compounds are characterised by low bioavailability in the small intestine [71], and their absorption involves the gut microbiota.

The phenolic content and antioxidant potential of tea infusions are significantly influenced by the brewing method [12,72]. Studies have shown that phenolic compounds are relatively thermostable; at temperatures of 60, 80, and 100 °C, degradation ranges from 15% to 30% after 4 h of exposure [73]. More than half of the polyphenols are extracted into the infusion during the initial 5 min of brewing [72,74]. Research has demonstrated that extending the brewing time of loose-leaf green tea from 5 to 10 min increases the TPC in the infusions by 42% and further by an additional 12% up to 15 min [12]. For green tea in bags, this increase was 35% and 37%, respectively [12]. It is believed that the polyphenols in the tea infusion are responsible for its health benefits. Therefore, longer brewing times are more advantageous for consumers. However, an excessive content of polyphenols, especially tannins, can cause overly bitter infusions, thereby reducing consumer appeal [75,76].

5.2. Hepatoprotective Effects of Green Tea

The hepatoprotective effect of green tea involves preventing cell apoptosis; regulating ALT, AST, and ALP activities; and tumour necrosis factor- α (TNF- α), as well as enhancing antioxidant potential (Tables 1 and 2). Such effects were observed in rats with liver fibrosis induced by thioacetamide (inhibiting liver fibrosis progression, reducing proliferating cell nuclear antigen PCNA, preventing hepatocyte oxidation, increasing superoxide dismutase SOD activity, catalase CAT, decreasing malondialdehyde MDA, and reducing liver cell inflammation) [63] and in rats intoxicated with cyclophosphamide (mildly enlarged portal vein, mild periportal mononuclear cell infiltration, periportal and portal collagen fibre formation) [77]. In mice with acute liver damage induced by carbon tetrachloride CCl₄, green tea (200, 400, and 800 mg/kg, twice per day for 7 days) exhibited hepatoprotective effects by improving the liver antioxidant status and preventing cell apoptosis through caspase-3-dependent signalling pathways [78]. The use of green tea extract in mice subsequently poisoned with CCl₄ confirmed the hepatoprotective action of tea, as evidenced by decreased serum ALT, AST, and ALP activities; inhibition of liver MDA levels; improved hepatosomatic index profile; protection against histological changes; and increased glutathione peroxidase (GPX) and SOD activities compared to mice not receiving tea before CCl₄ exposure [50]. A study conducted by Wang et al. [49] aimed to elucidate the potential anti-fibrogenic role of three abundant tea catechins (ECG, EGC, and EGCG) in CCl₄-induced liver fibrosis in rats and the underlying molecular mechanisms. Catechins effectively improved oxidative state parameters and liver histology as well as alleviated liver fibrosis (reduced desmin expression, α -smooth muscle actin, transforming growth factor-beta (TGF- β), and ERK1/2 and Smad1/2 phosphorylation). The administration of green tea polyphenols (400 mg/kg body weight) to rats poisoned with cadmium sulphate (50 mg CdSO₄ per L) for 30 days led to a sharp decrease in serum ALT and AST activities as well as improved liver architecture, preventing Cd-induced steatosis and hepatocyte necrosis [79]. Oral administration of green tea extract to Pb-poisoned rats for 4 weeks resulted in improved serum ALT, AST, and ALP activity [80]. Furthermore, consuming green tea extract during lactation reduces hepatic lipid accumulation in rats exposed to a high-fat diet from prenatal to adulthood [81]. Providing tea flower extract to mice showed hepatoprotective effects, including protection against *Propionibacterium acnes* and

lipopolysaccharide-induced liver inflammation, reversing histological damage and serum ALT elevation and lowering levels of nitric oxide (NO), TNF- α , and IL-1 β mRNA in mice with immune-mediated liver inflammation [82]. The hepatoprotective potential of green tea in preventing diet-induced fatty liver was observed in studies conducted on Wistar rats [83] and mice, which exhibited reduced liver steatosis, decreased hypertriglyceridemia and hyperglycaemia, and improved insulin resistance [84]. Positive outcomes were achieved through the activation of the sirtuin 1 protein kinase and AMP pathways [84]. The cited studies highlighted that EGCG is responsible for the positive results obtained.

EGCG alleviates hepatocyte damage and dysfunction by mitigating resistance to FGF21 (fibroblast growth factor-21) and enhancing the FGFR/AMPK pathway, thereby alleviating oxidative stress as observed in mice fed a high-fat diet [85]. EGCG effectively mitigated liver damage and mitochondrial dysfunction caused by acetaminophen in laboratory animals [13,14]. The hepatoprotective ability of EGCG stems from its antioxidant properties, which improve membrane potential and respiratory chain complex activity in liver mitochondria, thus preventing mitochondrial dysfunction [13,86]. Studies on rats exposed to CCl₄ have shown that EGCG exerts protective effects on the liver through its antioxidative actions (reducing MDA levels and increasing GSH), anti-inflammatory effects (lowering levels of inflammatory markers: TNF- α , NF- κ B, IL-1 β , and TGF β), and antifibrotic effects (reducing fibrotic markers: *p*-ERK and *p*-Smad1/2 protein expression) [87]. It has been observed that (–)-epigallocatechin 3-O-(3-O-methyl) gallate (EGCG3''Me) effectively mitigates alcohol-induced liver changes in a concentration-dependent manner in mice [88]. Administration of EGCG3''Me at a dose of 100 mg/kg BW per day significantly reduced serum AST and ALT levels and liver MDA levels and restored SOD and GPX activities. In an in vitro study using human liver cells (HL-7702), EGCG reversed Cd-induced reduction in cell viability and apoptosis, likely due to ROS scavenging and maintenance of redox homeostasis [89]. Studies in mice have identified EGCG as a potential inhibitor of ferroptosis by increasing FTH/L (Ferritin H/L), NRF2 (nuclear factor erythroid 2-related factor 2), and GPX4 expression, suggesting that it could be considered a therapeutic agent for liver diseases induced by iron overload [90].

5.3. The Antioxidant Action of Green Tea

The antioxidant effects of tea are largely due to its polyphenolic components, such as catechins, quercetin, theaflavins, thearubigins, and tannic acid, which act through [11,91] (1) the inhibition of oxidative enzyme activity and chelation of trace elements; (2) scavenging of ROS; (3) enhancement of endogenous enzymatic and non-enzymatic antioxidant activities; and (4) ability to neutralise singlet oxygen by donating electrons or hydrogen atoms.

Green and white teas are characterised by the highest antioxidant capacity (total phenolic, flavonoid and caffeine content, ferric-reducing antioxidant power, DPPH radical scavenging capacity) [11,92]; although, the antioxidant activity of tea infusions depends on brewing time, which is 15 min for green tea [12]. It has been shown that green tea infusions strongly inhibit linoleic acid peroxidation in mice [93]. Increased antioxidant potential due to green tea infusion has been observed in the blood serum of rats exposed to Cd (7 mg/kg feed) and Pb (50 mg/kg feed) [11]. Green tea (1.5% aqueous solution) positively affected rats with tamoxifen-induced liver damage (45 mg/kg/day), as evidenced by increased CAT, SOD, and GPX activity [94]. Oral administration of green tea extract to Pb-poisoned rats for 4 weeks significantly improved the antioxidant parameters glutathione S-transferase (GST) and SOD in the liver [80]. Lv et al. [95], through in silico and in vivo studies, demonstrated that green tea can activate the Nrf2 signalling pathway in mouse livers by disrupting the Nrf2-Keap1 protein–protein interaction, explaining green tea's antioxidant action, with EGCG showing the highest activity. The transcription factor Nrf2 controls the expression of genes containing antioxidant response element sequences in their promoters and encodes enzymes such as GST, NAD(P)H quinone dehydrogenase 1, heme oxygenase 1 (HO-1), and γ -glutamylcysteine synthetase (γ -GCS) [96].

5.4. Anti-Inflammatory Action of Green Tea

In vitro studies have demonstrated significant anti-inflammatory effects of aqueous extracts of green tea leaves, which have been attributed to their high flavonoid content [97]. Flavonol glycosides and aglycones from green tea markedly reduced mRNA expression of inflammation-related genes dose-dependently in an in vitro study using mouse macrophages RAW 264.7 [98]. Obese diabetic mice treated with green tea extract showed reduced release of soluble intercellular adhesion molecule-1 (sICAM-1), indicating the tea's anti-inflammatory properties [99]. The anti-inflammatory effect of green tea was also observed in a study involving obese women, where increased adiponectin activity and decreased high-sensitivity C-reactive protein (hs-CRP) levels were noted [100]. Similarly, in obese men, green tea consumption led to a decrease in IL-6 and hs-CRP levels and an increase in adiponectin [101]. The anti-inflammatory properties of green tea have been further confirmed in studies conducted on mice [102] and rats [103].

The impact of tea polyphenols on inflammatory processes involves inhibition of pro-inflammatory cytokine synthesis, IFN- γ , TNF- α , NF- κ B, and chemokines in cells, as well as regulation of mitogen-activated protein kinase (MAPK), inducible nitric oxide synthase (iNOS), HO-1, arachidonic acid, cyclooxygenase-2 (COX-2), and lipoxygenase (LOX) [104–108]. EGCG inhibits NF- κ B and MAPK activation, reduces IFN γ , TNF- α , and IL-1 β expression, stimulates genes related to immunity (e.g., TNF- α , MAPK, NOS), and inhibits apoptosis [109]. EGCG can also inhibit inflammatory leukocyte infiltration and pro-inflammatory IL-8, and studies in mice have shown it decreases NF- κ B and IL-6 inflammatory actions [109–111].

5.5. Anti-Obesogenic and Anti-Diabetic Effects of Green Tea

Green tea contains numerous biologically active substances that facilitate weight loss by [112–114] (1) suppressing appetite by stimulating noradrenaline production and activating the sympathetic nervous system, which induces a feeling of fullness; (2) reduction in the metabolic rate, resulting in an increase in energy expenditure; (3) enhancing lipid metabolism through adrenergic receptor activation; and (4) inhibiting pancreatic lipase production by covalently binding to serine in the enzyme's active site.

Active substances in tea that facilitate weight loss include polyphenols, flavonoids, caffeine, caffeic acid, and chlorogenic acid [115].

Its anti-diabetic properties are primarily exhibited by polyphenols, catechins (especially EGCG), gallic acid, caffeine, theaflavin, and polysaccharides. These compounds regulate blood glucose levels by [116,117] (1) reducing ROS levels; (2) inhibiting α -amylase and α -glucosidase activity, with inhibitory activity dependent on the amount of hydroxyl groups in the compound; and (3) modulating the expression of pro-inflammatory cytokines that may decrease glucose-induced insulin secretion.

Studies in obese diabetic mice have shown that green tea extract has anti-diabetic and anti-adipogenic effects (normalisation of serum glucose, cholesterol, triacylglycerols, non-esterified fatty acids, insulin, adiponectin, and soluble intercellular adhesion molecule-1) [99].

5.6. Impact of Green Tea on Gut Microbiota

The polyphenols in green tea exist in complex oligomeric structures and glycosides. They exhibit low bioavailability in the small intestine [71], and their absorption involves the gut microbiota, primarily *Flavonifractor plautii*, *Slackia equolifaciens*, *Slackia isoflavoniconvertens*, *Adlercreutzia equolifaciens*, *Eubacterium ramulus*, *Eggerthella lenta*, *Lactobacillus* spp., and *Bifidobacterium* spp. [118]. Polyphenol metabolism occurs in three stages: (1) hydrolysis by intestinal enzymes and gut microflora; (2) metabolism in the liver; and (3) absorption of metabolites produced by sulfonate, glucuronide, and catechol transferase activities. Polyphenols influence the growth and metabolism of gut bacteria, thereby demonstrating their prebiotic effects. This prevents damage to the gut barrier and balances the synthesis of pro-inflammatory and anti-inflammatory T lymphocytes [118,119]. In vitro studies using human Caco-2 cells have shown that polyphenols from green tea stimulate the

growth of *Bifidobacterium*, *Lactobacillus*, and *Enterococcus* spp. [112]. Glycans resulting from the breakdown of glycosidic bonds in polyphenols are utilised by bacteria that produce β -glucuronidase, such as *Bacteroides* and *Bifidobacterium* [71,120]. Specific phenolic compounds also influence the composition of the microbiota. Catechins from tea have been shown to inhibit the growth of *Bacillus cereus*, *Campylobacter jejuni*, *Clostridium perfringens*, *Escherichia coli*, *Helicobacter pylori*, *Legionella pneumophila*, and *Mycobacterium* spp. by damaging their cell membranes, particularly binding to peptidoglycans of Gram-positive bacteria, causing their disruption, which does not apply to Gram-negative bacteria [121]. Flavonols positively affect the ability of probiotic *Lactobacillus* to adhere to the intestinal mucosa [122]. Kaempferol improves intestinal barrier integrity and suppresses intestinal inflammation by reducing activation of the TLR4/NF- κ B pathway and counteracts obesity-related dysbiosis [123], which is a risk factor for DILI.

6. Controversies

Some authors have suggested that consuming excessive amounts of green tea may have toxic effects on the liver owing to EGCG. Safe consumption is considered to be 2–3 cups of green tea per day containing about 250 mg of catechins [76], although the EFSA Panel [124] did not confirm that high doses of catechins could adversely affect liver function.

Studies conducted on rats have shown that in the context of clinical acetaminophen (paracetamol) overdose (2 g/kg, orally), administration of green tea extract (8.5 mg/kg, orally) likely exacerbates APAP-induced hepatotoxicity through oxidative stress and caspase 3-dependent apoptosis [24]. In experimental rats after one month, increased liver enzyme levels, necrosis and degeneration of liver cells, congestion, haemorrhage, inflammation, and fibrosis were observed. Other studies in rats have indicated that EGCG may induce hepatotoxicity by exacerbating pre-existing mitochondrial abnormalities, as EGCG can only penetrate mitochondrial membranes when their permeability is increased [13]. Gastric administration of EGCG (500–750 mg/kg) to C57BL/6J mice once daily for three days resulted in liver inflammation, necrosis, and haemorrhage [125]. EGCG-induced hepatotoxicity is associated with increased oxidative stress and decreased levels of SOD and GPX. These mice also exhibited reduced copies of liver mitochondria and decreased mRNA levels of the respiratory chain complex I and III marker genes, sirtuin 3, forkhead box O3a, and peroxisomes. Furthermore, in mice receiving an immortal toxic dose (75 mg/kg) of EGCG, repeated EGCG treatment significantly reduced the levels of exogenous antioxidants in the liver; whereas, a non-toxic dose (45 mg/kg) had no effect on these indicators, and a lethal dose (200 mg/kg) dramatically decreased the main antioxidant defence of the body [10]. Studies in mice have shown that, under selenium deficiency (which exhibits strong antioxidant properties), EGCG activates the hepatic Nrf2 response, leading to increased levels of heme oxygenase 1, NAD(P)H oxidoreductase 1, and thioredoxin activity [126]. This suggests that EGCG is a potent inducer of the Nrf2 system only under selenium-deficient conditions, whereas in mice with optimal selenium levels, thioredoxin and GSH systems serve as the first line of defence against high doses of EGCG-induced stress, sparing Nrf2 system activation [126].

However, results from another study conducted on mice suggested that EGCG may modulate its own bioavailability and that a diet containing EGCG could reduce the toxic impact of high oral bolus doses of EGCG (reducing EGCG levels in the liver by 71% and in serum by 57%) [127]. This finding may partially explain the variability observed in the hepatotoxic responses to dietary supplements containing green tea across different studies. Depending on the dose and biological system involved, EGCG may act either as an antioxidant or an inducer of antioxidant defence through its pro-oxidative action or other unidentified mechanisms.

7. Summary and Perspectives

Improving the effectiveness of DILI detection is crucial for enhancing patient safety and developing methods for assessing potential hepatotoxicity during drug development phases. Understanding the mechanisms underlying DILI will enable the development of strategies to mitigate its effects and minimise risks. Complete elimination of drugs is often not feasible, but supporting the body and liver through appropriate nutrition can help attenuate the adverse effects associated with DILI. Dietary therapy can serve as a simple yet effective approach to complement medical treatment. Polyphenols, particularly EGCG, found in green tea are promising because of their strong antioxidant, anti-inflammatory, and prebiotic properties.

The findings from studies conducted by various researchers are promising, albeit with some uncertainties. Therefore, future research should focus on elucidating the therapeutic and preventive mechanisms of polyphenols in liver health, including their interaction with the gut microbiota and epigenetic pathways, which significantly influence overall health. Studies conducted on obese mice have unequivocally shown the impact of bioactive food components (various parts of the watermelon fruit) on the expression of genes involved in processes such as xenobiotic metabolism in the liver [128]. Understanding the pathophysiological mechanisms of DILI is critical for the development of therapeutic and preventive strategies. Although many aspects of these mechanisms remain to be fully understood, it is widely recognised that factors such as direct toxic effects, oxidative stress, inflammation, mitochondrial damage, and immune reactions all contribute significantly to the development of DILI. Continued research in these areas will help refine our understanding and improve clinical outcomes for patients at risk of liver injury from medication.

One should also not forget about other components of green tea that have a proven protective effect on the liver, as their presence enhances the overall benefits of the tea. In particular, polysaccharides play a significant role in alleviating oxidative damage and inflammatory responses in liver tissues, preventing damage in non-alcoholic fatty liver disease and improving liver metabolism, as demonstrated in studies conducted on mice and rats [129,130].

The analysis of the available literature highlights certain limitations and gaps, particularly concerning clinical studies: (1) the presented studies involve relatively small patient groups, which may hinder the interpretation of the results; (2) the genetic factors of patients, which can influence the outcomes due to varying resistance and tolerance to medications, are often not considered; and (3) the structure of the patients' gut microbiota, which plays a crucial role in overall body function and immunity, is frequently overlooked.

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Article

Effects of Bioactive Dietary Components on Changes in Lipid and Liver Parameters in Women after Bariatric Surgery and Procedures

Edyta Barbara Balejko ^{1,*}, Anna Bogacka ¹, Jarosław Lichota ² and Jan Pawlus ²

¹ Department of Commodity Science, Quality Assessment, Process Engineering and Human Nutrition, West Pomeranian University of Technology in Szczecin, 71-459 Szczecin, Poland

² Unii Lubelskiej 1, Department of General, Minimally Invasive and Gastroenterological Surgery, Independent Public Clinical Hospital No. 1 of Pomeranian Medical University in Szczecin, 71-252 Szczecin, Poland

* Correspondence: edyta.balejko@zut.edu.pl

Abstract: Excess adipose tissue, as well as its distribution, correlates strongly with disorders of lipid and liver parameters and chronic inflammation. The pathophysiology of metabolic diseases caused by obesity is associated with the dysfunction of visceral adipose tissue. Effective and alternative interventions such as the Bioenteric Intragastic Balloon and bariatric surgeries such as the Roux-en-Y gastric bypass. The aim of this study was to assess the effect of modifying the recommended standard weight loss diet after bariatric surgery and procedures on reducing chronic inflammation in overweight patients. In the study, bioactive anti-inflammatory dietary components were used supportively. Changes in the concentrations of lipid parameters, liver parameters, antioxidant enzymes, cytokines, and chemokines were demonstrated. The enrichment of the diet, after bariatric surgery, with the addition of n-3 EFAs (Essential Fatty Acids), bioflavonoids, vitamins, and synbiotics resulted in higher weight losses in the patients in the study with a simultaneous reduction in parameters indicating liver dysfunction.

Keywords: obesity; bariatric surgery; bioactive dietary components; metabolic changes; inflammation; liver dysfunction

1. Introduction

Bariatric surgery is not an ideal form of obesity treatment, but it is often the only one that offers survival and improves the quality of life for patients with morbid obesity. Depending on the BMI (Body Mass Index) value, obese patients are offered bariatric surgery and/or procedures. Among the procedures, the Bioenteric Intragastic Balloon (BIB) has been popular for many years. It is considered the least invasive and reversible and is aimed at patients with grade I obesity (BMI = 30.0–34.9 kg/m²), and in special cases as the first stage of weight reduction for patients with grade II obesity (BMI = 35.0–39.9 kg/m²) preparing for surgery. Laparoscopically performed RYGB gastric bypass, on the other hand, is a popular method of bariatric surgery, aimed at patients with grades II and III obesity (BMI ≥ 40 kg/m²). It involves the simultaneous reduction of the stomach and restriction of absorption by bypassing a significant portion of the intestine. It allows for greater weight reduction, improvement in metabolic parameters including liver and lipids, significant glycemic control, and often a complete remission of type II diabetes [1].

Adipose tissue plays an important metabolic role. Adipocytes are a source of pro-inflammatory biologically active proteins. Excess visceral adipose tissue induces inflammation. In addition, macrophages in obesity-associated inflammation increase the synthesis of pro-inflammatory adipokines. Adipose tissue dysfunction is characterized by decreased release of homeostatic protective factors such as adiponectin, nitric oxide, or protective prostaglandins with the concomitant increased activation of the pathological release of

adipokines such as resistin and visfatin and pro-inflammatory interleukins. Locally and systemically, it causes multiple dysfunctions in glucose and lipid metabolism. Chronic inflammation increases the risk of developing or exacerbating symptoms in the course of hypertension, diabetes, atherosclerosis, and metabolic syndrome, including dyslipidemia. Metabolic, hormonal, and oxidative changes after bariatric surgery initially cause elevations in lipid metabolism parameters and liver enzymes. The aim of this study was to assess the effect of a modified recommended standard weight loss diet after bariatric surgery and procedures on reducing chronic inflammation in overweight patients. It was hypothesized that the enrichment of the diet after bariatric surgery with the addition of n-3 EFAs, bioflavonoids, vitamins, and synbiotics could result in long-term comparable, or greater, weight loss in the subjects with a concomitant reduction in parameters indicative of liver dysfunction.

2. Materials and Methods

The study included 80 women with a mean age of 51 ± 5.4 years, treated at the Sonomed Medical Center and the Department of General and Vascular Surgery of the University Clinical Hospital at Pomeranian Medical University in Szczecin. The observation time was 12 months. Patients were divided into 4 groups: I—patients after BIB using a standard diet (S), II—patients after BIB using an experimental diet (E), III—patients after RYGB with a standard diet (S), and IV—patients after RYGB with the experimental diet (E). The mean BMI of the subjects was 33.79 ± 5.6 kg/m². All patients had made multiple unsuccessful attempts to lose weight with conservative methods, meaning diet. The standard diet complied with the Polish Society for the Treatment of Obesity's requirements for feeding people after surgery and bariatric procedures [2]. The experimental diet is the author's modification of dietary recommendations with increased antioxidant and anti-inflammatory potential [3]. The protein content of the experimental diet (E) was 80–85 g/day, the carbohydrate total was 70 g/day, and the total fat content was 42 g/day. The n-6/n-3 EFA ratio was 1:84. In the experimental diet, shakes, mousses, and purees consisting of products with high antioxidant potential were included. Their antioxidant capacity (TEAC—trolox equivalent antioxidant capacity), ferric reducing antioxidant power (FRAP—ferric reducing antioxidant power), and total polyphenol content from 120.51 mg catechin/100 mL to 250.51 mg catechin/100 mL were determined. [3]. The caloric value of the diets (S, E) in the 1st month was approx. 400 kcal, in the 3rd month it was approx. 600–800 kcal, and in the 12th month it was approx. 1200 kcal. In addition, patients following the experimental diet (groups II and IV) took a synbiotic once a day, every other month during the study, with the following composition: the probiotics *Bifidobacterium lactis*; *Lactobacillus acidophilus*; *Lactobacillus paracasei*; *Lactobacillus plantarum*; *Lactobacillus salivarius*; and *Lactobacillus lactis*; and the prebiotics fructooligosaccharides (FOS) and inulin. The total number of colony-forming units (CFUs) was 1×10^9 CFUs.

Anthropometric measurements were taken using a CEO 123-certified IOI 353 analyzer, which complies with MDD 93/42/EEC medical device directives. The weight and visceral fat area were determined. Most examinations were performed before the start of treatment and diet and at the end of the follow-up period. Patients gave voluntary consent to participate in the study. The study design was approved by the Bioethics Committee of the Regional Chamber of Medicine in Szczecin OIL-Sz/Mf/KB/452/06/05/2015.

The standard panels of liver enzymes aspartate transferase (AST) and alanine transferase (ALT) with total values of CH(Total Cholesterol), HDL(High-density Lipoproteins), LDL(Low-density Lipoproteins), and TG(Triglycerides) via the colorimetric method were determined in all groups of patients from the collected blood. Assays were performed before the start of treatment and at the 3rd and 12th months. Analyses were performed at accredited diagnostic laboratories.

In erythrocytes, the activity of antioxidant enzymes was evaluated via the spectrophotometric method:

- Superoxide dismutase (SOD), using the ability to oxidize adrenaline to adrenochrome [4].

- Catalase (CAT), assessing the enzyme's ability to degrade superoxides [5].
- Glutathione peroxidase (GSH-Px), which reduces hydrogen peroxide to water with reduced glutathione [6].

Plasma malondialdehyde (MDA) concentration was determined via the spectrophotometric method according to Rice-Evans et al. without chromogen extraction [7].

The immunoenzymatic ELISA technique was used to determine the adiponectin, resolvin, and chemokine contents in serum. The absorbance was read at 450 nm $\pm$ 2 nm using a multi-channel ELISA reader, ELx808 (BIO-TEK Instruments, Inc., Winooski, VT, USA). The KC Junior for Windows calculator program from BIO-TEK Instruments, Inc., USA, was used to develop calibration curves and calculate the concentration values of the parameters tested. The following commercial kits were used for the study:

- Adiponectin (catalog No.: EO605h, EIAab Science Co., Wuhan, China; method accuracy was $\pm 1\% \pm 0.010$ absorbance with a range of 0.156–10 ng/mL;
- Resolvin D1 (RvD1) series (catalog No.: 500380, Cayman Chemical Co., Ann Arbor, MI, USA); method accuracy was $\pm 1\% + / - 0.010$ absorbance with a range of 3.3–2000 pg/mL;
- TNF- α (catalog No.: EIA-4641, DRG Diagnostics Int., Inc., Springfield, NJ, USA); method accuracy was $\pm 1\% \pm 0.010$ absorbance with a measurement range of 7–500 pg/mL;
- TGF- $\beta 1$, (catalog No.: EIA-1864, DRG Diagnostics Int., Inc., Springfield, NJ, USA); method accuracy was $\pm 1\% \pm 0.010$ absorbance with a measurement range of 1.9–600 pg/mL;
- IL-1 β , (catalog No.: EIA-4437, DRG Diagnostics Int., Inc., Springfield, NJ, USA); method accuracy was $\pm 1\% \pm 0.010$ absorbance, with a measurement range of 0.35–1200 pg/mL;
- IL-10, (catalog No.: EIA-4699, DRG Diagnostics Int., Inc., Springfield, NJ, USA); method accuracy was $\pm 1\% \pm 0.010$ absorbance, with a measurement range of 1.6–2000 pg/mL;
- IL-6 (catalog No.: EIA-4640, DRG Diagnostics Int., Inc., Springfield, NJ, USA); the accuracy of the method was $\pm 1\% \pm 0.010$ of absorbance, with a measurement range of 1.6–2000 pg/mL.

Statistical Analysis

The statistical analysis of the results of the study was performed using Statistica version 13 software. To show statistically significant differences in concentrations between the groups, the non-parametric Wilcoxon paired rank-order test was used. Statistical significance was set at a level of $p < 0.05$.

3. Results

We compared the effects of weight reduction using the bioelectrical impedance method in the four study groups before surgery and after one year of follow-up (Figures 1 and 2).

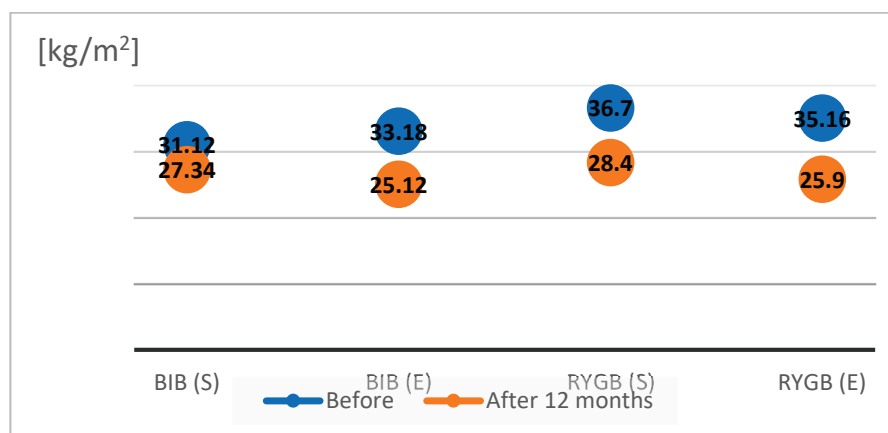


Figure 1. BMI values of patients before and after BIB and RYGB.

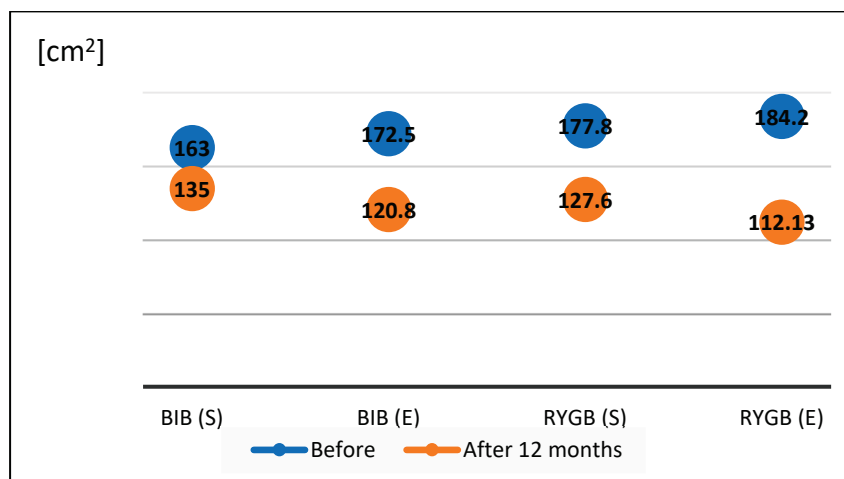


Figure 2. Visceral fat area of patients before and after BIB and RYGB.

The BMI value on the day of the BIB procedure in group I patients following a standard diet averaged 31.12 ± 3.7 kg/m², with an average visceral fat area of 163 ± 12.1 cm². After one year of follow-up, the BMI in this group decreased to 27.34 ± 6.02 kg/m², and the visceral fat volume decreased to 135.6 ± 4 cm². The BMI value on the day of BIB surgery in group II patients on the modified diet averaged 32.18 ± 4.9 kg/m², and the visceral fat area averaged 172.5 ± 27.21 cm². After one year of follow-up, the average BMI decreased to 25.12 ± 4.1 kg/m², and the average visceral fat volume decreased to 120.8 ± 15.7 cm². The BMI of patients in group III on the day of RYGB surgery who followed a standard diet averaged 36.7 ± 3.9 kg/m², and the visceral fat area averaged 177.8 ± 17.5 cm². After one year of follow-up, the BMI average decreased to 28.4 ± 3.5 kg/m² and the average visceral fat volume decreased to 127.6 ± 6.2 cm². The BMI of patients on the experimental diet (group IV) on the day of RYGB surgery averaged 35.16 ± 4.8 kg/m², and the visceral fat area averaged 184.2 ± 12.6 cm². After one year of follow-up, the average BMI decreased to 25.9 ± 3.7 kg/m², and the average visceral fat volume decreased to 112 ± 13.7 cm².

Table 1 shows the mean results and standard deviations of the obtained lipid parameters and liver enzymes in the sera of the four groups of patients. In all patients, abnormally elevated levels of all determined lipid fractions and liver enzymes were noted before the procedures and surgeries. After the 3rd and 12th months of balloon insertion into the stomach, slight decreases in the mean concentrations of all parameters studied were observed in the serum of patients following a standard diet (group I). These were statistically insignificant differences. Only the mean TG concentration, which was initially 155 ± 14.3 and 109.14 ± 10.7 after the 3rd month, increased to 115.12 ± 16.2 [mg/dL] after the 12th month. In patients after BIB following the experimental diet (group II), there was a significant statistical increase in HDL cholesterol levels from 39.8 ± 15.2 to 50.16 ± 5.4 [mg/dL]. Other mean concentrations of lipid fractions and liver enzymes decreased significantly. The analysis of the studied parameters showed, in post-RYGB patients following the standard diet (group III), statistically significant differences in the mean concentrations of total cholesterol and TG. In contrast, there were no significant differences in HDL or LDL cholesterol concentrations over the course of the study. ALT and AST enzyme activities increased after 3 months to decrease after one year of follow-up. In patients after RYGB and following the experimental diet (group IV), the mean values of all parameters were statistically significantly different. In this group of subjects, the activity of ALT and AST enzymes gradually decreased during the 12 months of follow-up. There were also statistically significant differences between the results of groups III and IV due to the differences in the diets.

Table 1. Serum levels of lipid fractions and liver enzymes in patients, before and after the BIB and RYGB after 12 months.

Parameters Measured	BIB (S)	BIB (E)	<i>p</i> Value	Statistical Significance	RYGB (S)	RYGB (E)	<i>p</i> Value	Statistical Significance
	$\bar{x} \pm SD$	$\bar{x} \pm SD$			$\bar{x} \pm SD$	$\bar{x} \pm SD$		
TC [mg/dL]	190.8 $\pm$ 12.11	154 $\pm$ 3.6	0.005	S	159.8 $\pm$ 27.3	152.5 $\pm$ 12.1	0.005	S
HDL [mg/dL]	38.7 $\pm$ 5.12	50.16 $\pm$ 5.4	0.0001	S	40.2 $\pm$ 12.6	52.5 $\pm$ 12.1	0.016	S
LDL [mg/dL]	147.2 $\pm$ 11.3	108.12 $\pm$ 11.3	0.043	S	110.4 $\pm$ 11.7	90.4 $\pm$ 7.8	0.000132	S
TG [mg/dL]	115.12 $\pm$ 16.2	88.17 $\pm$ 10.7	0.000089	S	99.30 $\pm$ 23.7	62.12 $\pm$ 13.7	0.004	S
ALT [U/L]	28.7 $\pm$ 7.6	27.5 $\pm$ 9.7	0.806	NS	29.8 $\pm$ 7.5	22.12 $\pm$ 13.7	0.000103	S
AST [U/L]	24.16 $\pm$ 5.3	22.7 $\pm$ 6.2	0.99	NS	20.5 $\pm$ 6.4	17.54 $\pm$ 8.7	0.0112	S

Statistically significant differences were found between groups I(S) and II(E) of total cholesterol, HDL, LDL, and TG fractions. In addition, statistically significant differences were also found in lipid fractions between groups III(S) and IV(E) HDL, LDL, TG, and enzymes ALT and AST.

We evaluated oxidative stress in obese patients by analyzing the activity of antioxidant enzymes and the degree of lipid peroxidation. Oxidative stress results in lipid peroxidation. Peroxidation is the oxidation of unsaturated fatty acids that make up phospholipids, of which MDA is a marker. Antioxidant enzymes are the first line of defense against reactive oxygen species. In our study, before BIB surgery and RYGB surgery, a significant increase in the severity of oxidative stress was observed in all obese patients. Table 2 shows the results of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) enzyme activities and malondialdehyde (MDA) levels.

Table 2. Antioxidant enzyme activity in patients before and after BIB and RYGB.

Parameters Measured	BIB (S)	BIB (E)	<i>p</i> Value	Statistical Significance	RYGB (S)	RYGB (E)	<i>p</i> Value	Statistical Significance
	$\bar{x} \pm SD$	$\bar{x} \pm SD$			$\bar{x} \pm SD$	$\bar{x} \pm SD$		
CAT [A/g Hb]	183 $\pm$ 15	183 $\pm$ 21	0.806	NS	199 $\pm$ 25	316 $\pm$ 12	0.00036	S
SOD [U/g Hb]	1213.93 $\pm$ 45.7	1778.76 $\pm$ 15.3	0.0034	S	1414.53 $\pm$ 38	2089.64 $\pm$ 21.4	0.0017	S
GSH-Px [U/g Hb]	7.58 $\pm$ 1.5	10.39 $\pm$ 3.3	0.005	S	7.26 $\pm$ 1.3	12.6 $\pm$ 4.9	0.0348	S
MDA [μ mol/L]	7.37 $\pm$ 1.2	5.10 $\pm$ 1.3	0.0026	S	6.25 $\pm$ 1.7	3.49 $\pm$ 2.1	0.0366	S

In patients after BIB and RYGB, a reduction in serum MDA levels was noted in addition to a reduction in body weight and body fat. Dietary components with antioxidant properties may reduce patients' exposure to oxidative stress associated with obesity. In our study, the changing activity of antioxidant enzymes was noted (Table 2).

Statistically significant differences were found between groups I(S) and II(E) of SOD, GSH-Px, and MDA. In addition, statistically significant differences were also found between groups III(S) and IV(E) in CAT, SOD, GSH-Px, and MDA.

The results of the mean serum cytokine concentrations of patients in all groups are shown in Table 3. High concentrations of pro-inflammatory cytokines occurred in all patients before therapy. These results correlated positively with excess body weight and body fat before BIB and RYGB surgery. With the remission of obesity, a decrease in pro-inflammatory cytokines and an increase in anti-inflammatory cytokines were observed in both groups. The selection of appropriate bioactive components of the diet contributed to the normalization of inflammatory factors in obese patients.

Table 3. Serum cytokine levels in patients, in the four groups before and after BIB surgery and RYGB surgery and the diets used.

Parameters Measured	BIB (S)	BIB (E)	<i>p</i> Value	Statistical Significance	RYGB (S)	RYGB (E)	<i>p</i> Value	Statistical Significance
	$\bar{x} \pm SD$	$\bar{x} \pm SD$			$\bar{x} \pm SD$	$\bar{x} \pm SD$		
TNF- α	13.23 $\pm$ 1.7	8.8 $\pm$ 4.5	0.005	S	15.23 $\pm$ 3.7	7.01 $\pm$ 5.3	0.0034	S
TGF- β	3.6 $\pm$ 0.9	3.2 $\pm$ 1.1	0.82	NS	4.5 $\pm$ 1.2	3.1 $\pm$ 1	0.01	S
IL-1 β	36.6 $\pm$ 2.5	32.1 $\pm$ 3.3	0.96	NS	35.6 $\pm$ 6	24.13 $\pm$ 5.3	0.036	S
IL-10	6.2 $\pm$ 3	10.42 $\pm$ 1.3	0.005	S	7.5 $\pm$ 2	15.3 $\pm$ 2.1	0.0011	S
IL-6	18.41 $\pm$ 3.6	12.5 $\pm$ 3.1	0.0005	S	18.4 $\pm$ 2.2	10.31 $\pm$ 1.2	0.012	S

The statistical analysis of cytokine results showed no statistically significant differences only in TGF- β and IL-1 β levels.

Assuming that the diet has anti-inflammatory properties, the serum levels of adiponectin and resolvins were determined in patients (Table 4). An increase in adipokine levels was observed in all obese patients after BIB and RYGB. Statistically significant differences were shown in groups I vs. II and III vs. IV, depending on the diet used. Moreover, it was confirmed that higher adiponectin concentrations correlate with anti-inflammatory IL-10. In addition, resolvins are involved in extinguishing the acute phase of inflammation. The lowest concentrations of resolvins were obtained in all obese patients before BIB and RYGB. In the blood of Group II patients after the observation period, a small, not statistically significant increase in resolvin D concentrations was obtained. Significantly higher concentrations of the tested anti-inflammatory mediator were recorded in group IV after the end of therapy. It is presumed that such an effect was the result of a diet with antioxidant properties containing DHA. Resolvin D has an immunosuppressive effect on the release of pro-inflammatory cytokines [8], so this was also the subject of analysis in our study. The concentrations of both adipokines were inversely proportional to the elevated parameters of existing inflammation.

Table 4. Mean values of serum levels of adiponectin [ng/mL] and resolvins [pg/mL], in patients in the four groups before and after BIB and RYGB and the diets used.

Parameters Measured	BIB (S)	BIB (E)	<i>p</i> Value	Statistical Significance	RYGB (S)	RYGB (E)	<i>p</i> Value	Statistical Significance
	$\bar{x} \pm SD$	$\bar{x} \pm SD$			$\bar{x} \pm SD$	$\bar{x} \pm SD$		
Adiponectin [ng/mL]	10.43 $\pm$ 3.52	16.88 $\pm$ 2.5	0.0017	S	12.2 $\pm$ 3.6	21.2 $\pm$ 4.5	0.000029	S
Resolvins [pg/mL]	35.56 $\pm$ 3	45.55 $\pm$ 5	0.00005	S	37.46 $\pm$ 5	47.34 $\pm$ 5.1	0.000145	S

Statistical analysis of the results of adiponectin and resolvin values in the four groups analyzed showed statistically significant differences between them.

4. Discussion

Based on the possibility of using functional ingredients in supporting the treatment of obesity, an attempt was made to formulate a diet with functional characteristics. Bioactive compounds such as flavonoids exhibit broad antioxidant, anti-inflammatory, and hepato-protective effects. Research indicates that dedicated diets can be used to effectively reduce body fat and inflammation.

It is known that excess body fat, as well as unfavorable fat distribution, induces chronic inflammation [9]. In most studies, authors focus on the results of reducing lipid and/or liver parameters. In our study, we attempted to demonstrate the effect of selected dietary components as a supportive element of bariatric surgery and procedures, on changing inflammatory parameters and hepatic dysfunction. For this purpose, the diet was composed by way of increasing the proportion of bioflavonoids, vitamins, minerals, fatty acids, and probiotics giving it bioactive and immunomodulatory characteristics [3].

Adiposopathy, or adipose tissue dysfunction, is characterized by the production of inflammatory cytokines and chemokines. The cause of organ dysfunction in obesity disease is oxidative stress expressed via the increased synthesis of reactive oxygen species (ROS) [10]. Oxidative stress leads to the development of non-alcoholic steatohepatitis (NASH). Our studies have shown changes in the activity of antioxidant enzymes and lipid peroxidation, indicating a reduction in oxidative stress. The strongest satisfactory effect was obtained after RYGB surgery in patients following the experimental diet. Obese patients have a threefold higher incidence of non-alcoholic steatohepatitis, with a twofold higher complication rate of cirrhosis or bile duct stones with concomitant conditions of severe acute pancreatitis [11,12]. In addition, the long-term adherence to a low-energy diet is a contributing factor to liver fatness.

One of the patient's qualifying factors for surgery is the requirement for a 10% loss in baseline body weight [13]. Often, patients starve themselves for many months to achieve the necessary reduction. For the next three months after surgery, a feeding regimen that is energy-deficient is in effect. The rate of weight loss after surgery is significantly faster than the physiological rate. Therefore, after operations, increasing liver enzyme activity is observed, as well as an increase in liver fatness on ultrasound imaging. This is due to the action of lipase. Rapid weight loss causes the rapid release of a large number of lipid cells from adipose tissue into the bloodstream, resulting in hepatic steatosis. In addition, the thickening of bile and its backlog results in a 35% higher risk of gallstones [14]. In our study, ALT and AST enzyme activities initially increased in patients after RYGB. Upon longer follow-up, satisfactory low levels of lipid parameters and liver enzymes were achieved. After bariatric surgery combined with dietary care, an improvement in the ratio of lipid fractions and a decrease in liver enzymes is achieved [15–17]. The inflammation that accompanies obesity promotes pathological changes in the liver. Dietary care in self-observation had a preventive goal against NASH. In a proper diet, the appropriate ratio of EFA n-6 /n-3 should be 4:1. An excess of n-6 fatty acids in the diet inhibits the metabolism of n-3 fatty acids so that the balance between pro- and anti-inflammatory lipid mediators is disrupted. Giving obese patients increased amounts of n-3 fatty acids in an experimental diet was thought to reduce the inflammatory process. In 2004, Bays defined a new concept, aiming not only to reduce fat mass but also to correct adiposopathy [18]. The high correlation between obesity and the incidence of abnormal metabolic parameters, e.g., lipid profile, blood glucose or liver enzymes, and insulin resistance, is due to the endocrine function of adipose tissue—the secretion of adipokines [19].

Adipocytokines affect all phases of the immune response, regulating the proliferation, differentiation, and activation of B lymphocytes, T cells, NK cells, monocytes/macrophages, and granulocytes. Increased macrophage infiltration and increased levels of the cytokines or chemokines MCP-1, IL-6, IFN- γ , and TNF- α are observed. Buechler et al. described the involvement of TGF- β in liver fibrosis. Blocking TGF- β signaling protects against obesity, insulin resistance, and hepatic steatosis [20]. In our study, TGF- β 1 levels were higher in all patients before BIB and RYBG.

Adipocytokines are involved in glucose and lipid metabolism. Adiponectin is synthesized mainly in adipocytes. Two isoforms of membrane receptors for adiponectin AdipoR1 and AdipoR2 are located in the liver. It is involved in glucose and fatty acid metabolism in the liver and muscle. A reduction in acetyl-CoA (ACC) activity in the skeletal muscle and liver by both AdipoR1 and AdipoR2 receptors stimulates and enhances the β -oxidation of fatty acids, with the direct consequence of lowering serum triglyceride levels [21]. It inhibits free radical production, TNF- α , IL-8 reduction, and LDL cholesterol oxidation. An IL-1 receptor antagonist reduces TNF- α release, as confirmed in our study. At the same time, it increases IL-10 levels, thus exhibiting anti-atherosclerotic and anti-inflammatory functions and increasing insulin resistance [22,23]. Adiponectin protects the liver from steatosis and inflammation by directly suppressing TNF- α and decreases the concentration of free fatty acids [24]. In adipocytes, TNF- α inhibits the esterification capacity of fatty acids.

In our study, adiponectin and resolvin levels increased in the blood of post-RYGB patients following an anti-inflammatory diet. D-resolvins play an important role in the metabolic syndrome associated with obesity [25] and show protective effects in inflammation caused by oxidative stress [26]. Titos demonstrated the immunoregulatory effect of D1 resolvins on IL-10 levels in inflamed adipose tissue. RvD1 inhibited uncontrolled adipose tissue inflammation in obese subjects. According to Titos et al., RvD1 can treat obesity-related insulin resistance and other metabolic complications, reducing adipose tissue inflammation by lowering the concentrations of cytokines and adipokines (TNF- α , IL-6, IL-8, IL-1 β , MCP-1), and reducing inflammation in obesity-induced NASH [27,28]. In patients of both groups after one year of follow-up, higher levels of resolvin were observed. The highest in patients after RYGB. Statistically significant differences were shown in resolvin D concentrations between groups III and IV patients. The experimental diet in the author's modification contained 28% PUFA, where the amount of n-3 acids was 9.87% of rations, and n-6 was 18.20% of rations, achieving a favorable ratio (n-6)/(n-3) of PUFA = 1:84. Changing the profile of the diet and enriching it with n-3 fatty acids, such as eicosapentaenoic acid and docosahexaenoic acid increased the synthesis of resolvin D1. The obtained values of resolvin D correlated with a decrease in total TG and LDL cholesterol and an increase in HDL in group IV. In addition, visceral fat reduction and dietary components regulating adiponectin and resolvins had a cascading effect on the regulation of pro- and anti-inflammatory cytokines, thereby minimizing chronic inflammation. It is noteworthy that the deficiency in resolvins in obesity appears to be a generalized defect in all tissues, as deficiencies are found in blood, liver, and skeletal muscle in addition to adipose tissue [29]. The effects of enriching the diet with n-3 fatty acids in patients with associated inflammation and the beneficial effects of resolvins are pointed out by Moro et al. [30]. Resolvin D is considered an immunomodulator in chronic inflammation caused by hypercholesterolemia. The progression of simple steatosis to NASH is associated with the chronic exposure of hepatocytes to pro-inflammatory cytokines. The main inflammatory cytokines during these lesions are TNF- α , IL-6, and IL-1 β , and this can result in the development of inflammation and fibrosis [31]. Our study showed a statistically significant decrease in the concentrations of pro-inflammatory cytokines, which can contribute to the apoptosis and necrosis of hepatocytes, neutrophil chemotaxis, the activation of stellate cells, and the production of Mallory bodies [32].

The production and release of pro-inflammatory cytokines, both peripherally and locally in the liver, are associated with several factors. The most relevant are lipotoxicity induced via an increase in free fatty acids, insulin resistance, impaired adipose tissue metabolism, and the presence of toxins released from the gut [33]. Numerous studies have shown a correlation between dysbiosis and changes in lipid profiles in obese individuals. Li et al. observed the high efficacy of an applied probiotic supplement in obesity. He showed a decrease in triglycerides and total cholesterol, and an inhibition of serum ALAT and ASPAT activity in probiotic users. Dietary supplementation with *L. acidophilus*, *B. bifidum* BGN4, and *B. longum* BORI reduced hepatocyte hydrops degeneration and decreased hepatic steatosis [34]. Dysbiosis can impair lipid metabolism. It has been shown that the physiological bacterial flora participates in the digestion of dietary macronutrients, absorption of micronutrients, and fermentation of dietary ballast components. In addition, it lowers the concentration of triglycerides; participates in the hydrolysis of cholesterol through supplied enzymes; and prevents obesity, fatty liver, and type II diabetes [35,36]. The beneficial effect of *Lactobacillus* and *Bifidobacterium* lies in the improvement in body function resulting from the reduction in one or more risk factors for disease development. Supplementation with the *Lactobacillus casei* strain *Shirota*, compared to Orlistat, resulted in a greater reduction in fat mass; in addition, it reduced ALAT levels, a marker of liver cell damage [37]. Probiotics in our study had an immunomodulatory effect, stimulating the production of anti-inflammatory cytokines. They are involved in fat mass reduction. In our study, the experimental diet was used, containing probiotics and appropriate proportions of fatty acids, having antioxidant and anti-inflammatory properties, and regulating param-

eters of lipid metabolism, liver enzymes, and inflammation. The result of the appropriate management of patients during the weight loss process was a satisfactory weight reduction resulting from an adaptation to the new dietary regime. Significantly better results were obtained in patients with significant visceral fat loss after RYGB surgery and following the experimental diet.

In the study by Lu et al., the effects of selected bioactive dietary components on obesity were analyzed and the molecular mechanisms were assessed in cellular, animal, and human models. Bioactive compounds from spices such as cinnamon, rosemary, ginger, saffron, and turmeric assisted in reducing body weight. In addition, they reduced lipid accumulation in adipose cells by regulating the expression of transcription factors such as CCAAT-binding protein/enhancer of gene expression for granulocytes (C/EBP) and peroxisome proliferator-activated receptor (PPAR γ). They may also modulate the activities of certain lipogenesis-related enzymes, such as acyl-CoA carboxylase (ACC), fatty acid synthase (FAS), glycerol-3-phosphate dehydrogenase (GPDH), and others. After the oral administration of spice extracts, increased thermogenesis in adipose tissue has been demonstrated, with a concomitant reduction in human body weight [38,39]. Recent results have shown the beneficial actions of the inverse relationship between anthocyanin and flavonoid intake, and the risk of obesity and diabetes has been proposed [39]. Many studies confirm the potential of plant-based bioactive dietary ingredients to reduce adipogenesis and lipid accumulation by interfering with several enzymes and markers involved in lipid formation and breakdown in adipocytes [40].

The study evaluated the effects of a modified weight loss standard diet. Numerous studies worldwide have confirmed the beneficial effects of functional food ingredients on weight loss. Currently, new diets are being sought that produce long-term fat reduction effects. Knowing the pathomechanism of obesity, it is important to consider not only weight reduction but also the reduction in inflammation.

5. Conclusions

The study showed a correlation of lipid, hepatic and inflammatory disorders in patients with excess body fat.

With the reduction of visceral adipose tissue, inflammation decreased. Changes in the concentrations of lipid parameters, liver parameters, antioxidant enzymes, cytokines and chemokines were shown. After enrichment of the diet with n-3 EFAs and antioxidants, there was greater weight loss in the patients studied, with a more effective reduction in parameters indicative of liver dysfunction.

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Review

Nutrition in Gilbert's Syndrome—A Systematic Review of Clinical Trials According to the PRISMA Statement

Zuzanna Goluch ^{1,*}, Aldona Wierzbicka-Rucińska ^{2,*} and Ewelina Książek ³

¹ Department of Food Technology and Nutrition, Wrocław University of Economics and Business, Komandorska 118/120, 53-345 Wrocław, Poland

² Department of Clinical Biochemistry, Radioimmunology and Experimental Medicine, Children's Memorial Health Institute, 04-730 Warsaw, Poland

³ Department of Agricultural Engineering and Quality Analysis, Wrocław University of Economics and Business, Komandorska 118/120, 53-345 Wrocław, Poland; ewelina.ksiazek@ue.wroc.pl

* Correspondence: zuzanna.goluch@ue.wroc.pl (Z.G.); a.wierzbicka-rucinska@ipczd.pl (A.W.-R.)

Abstract: Gilbert syndrome is the most common hyperbilirubinemia, associated with a mutation in the UGT1A1 bilirubin gene, which produces an enzyme that conjugates bilirubin with glucuronic acid. Episodes of jaundice occurring in GS negatively affect patients' quality of life. This systematic review aimed to analyze clinical studies regarding nutrition in people with GS. The study followed the PRISMA guidelines and utilized the Ebsco, Embase, Cochrane, PubMed, Scopus, and Web of Science databases to search clinical trials focused on diet/nutrition in GS (1963–2023 years). The methodological quality of selected studies was assessed using the Jadad scale. As a result, 19 studies met the inclusion criteria. The research mainly focused on the impact of caloric restriction, consumption of various diet variants, and vegetables and fruits on hyperbilirubinemia and metabolic health. A nutritional intervention consisting of not applying excessive calorie restrictions and consuming fats and biologically active compounds in vegetables and fruits (*Cruciferae*, *Apiaceous*, *Rutaceae*) may prevent the occurrence of jaundice episodes. It is justified to conduct further research on detecting such compounds in food, which, by influencing the expression of the UGT liver enzyme gene, could contribute to regulating bilirubin concentration in the blood of people with GS.

Keywords: Gilbert syndrome; Gilbert disease; diet; nutrition; caloric restrictions; fasting; PRISMA

1. Introduction

Gilbert and Lereboullet first described the syndrome in 1901 as “La cholemie simple familiale”. Meulengracht “rediscovered” the syndrome in 1939 in a published paper titled “Icterus intermittens juvenilis” [1,2]. Gilbert's syndrome (GS) is an example of a genetically induced, nutritionally exacerbated metabolic disorder occurring without liver cell damage or hemolysis. GS has long been described as an asymptomatic disorder with no clinical implications. However, studies have shown that jaundice episodes occurring in GS affect patients' quality of life. Kamal et al. [3] demonstrated significant differences in vitality, emotional role, social functioning, worries, and general health status during jaundice episodes in individuals with GS, as measured by the SF-36v2 (Short form-36 Health Survey test, version 2) and CLDQ (Chronic Liver Disease Questionnaire) tests, compared to a control group.

GS is a genetically determined disorder. Because its course can be asymptomatic, the disease may remain undiagnosed until the second decade of life, when a patient may develop mild hyperbilirubinemia with jaundice features [4]. Hyperbilirubinemia typically manifests during puberty, although it is believed that this condition causes some severe jaundice episodes in the neonatal period.

GS is the most commonly described hyperbilirubinemia, a benign disease that occurs worldwide, though its frequency varies depending on population and ethnic background. The prevalence of GS is estimated to be 3–16% in the general population [1,3,5], 2% in

East Asian populations, and 20% in India, South Asia, and the Middle East. Considering ethnic groups, the prevalence of GS in Caucasians is estimated at 2–10% [5]. GS is diagnosed more frequently in men than in women (4:1), with a protective role attributed to hormones-estrogens. Radu and Atsmon [1] also estimated that GS occurs more frequently in men (~12.4%) than in women (~4.8%), which is explained by increased production of unconjugated bilirubin during puberty due to changes in the synthesis of steroid sex hormones (androgens) that inhibit enzymatic glucuronidation [6].

The pathogenesis of GS is linked to the production of bilirubin, a byproduct of hemoglobin catabolism, which is excreted with bile into the gastrointestinal tract. The “bottleneck” in bilirubin elimination is its hepatic conjugation with glucuronic acid, mediated by the enzyme UGT1A1. This reaction converts bilirubin into a bile-soluble compound rapidly excreted into the intestine [7]. GS is likely inherited in an autosomal dominant manner with incomplete penetrance. The pathogenesis of GS is determined by a mutation in the UGT1A1 gene, which encodes UDP-glucuronosyltransferase, reducing its activity by up to 80%. This enzyme is crucial in converting free bilirubin into a form conjugated with glucuronic acid [2,8,9]. A defect in UDP-glucuronosyltransferase hampers the uptake of unconjugated bilirubin at the vascular pole of hepatocytes and its conjugation with uridine diphosphate glucuronic acid. The reduced activity of this enzyme results in decreased conjugation, leading to an increase in the total bilirubin concentration in the blood serum, which prevents its excretion in bile. The mutation in the promoter region of the UGT1A1 gene involves an increased number of thymine–adenine repeats. Most patients (approximately 90%) with GS have a homozygous A(TA)7TAA genotype, meaning a seven-repeat (TA) sequence in both alleles of the UGT1A1 gene. Common polymorphic mutations in GS include a TA insertion mutation in the TATA box [A(TA)7TAA] (UGT1A1*28) and c.211 G>A (p.G71R) in exon 1 (UGT1A1*6). In Caucasian and African populations, almost all GS individuals are homozygous for UGT1A1*28. In Japanese, Chinese, and Korean populations, an additional mutation, UGT1A1*6, exists, which causes GS in the homozygous state [5]. The prevalence of this mutation in the Caucasian population is approximately 16%. The homozygous A(TA)7TAA polymorphism is more frequently reported in Caucasian populations than in Asian populations [10]. However, it is estimated that about 10% of individuals with GS have a heterozygous 6/7 genotype, meaning they possess six TA repeats in one allele and seven TA repeats in the other. These individuals likely have additional mutations in the UGT1A1 gene or other genes that contribute to the manifestation of the disease. Healthy individuals typically have the A(TA)6TAA genotype with six TA repeats, although alleles with five or eight TA repeats are extremely rare [3]. Caucasian wild-type homozygotes UGT1A11 (6/6) or heterozygotes 6/7 have about half the total serum bilirubin concentration compared to homozygotes 7/7 (UGT1A1*28), as confirmed in vivo through enzyme studies on human liver tissue [11]. The formation of bilirubin glucuronide by UGT1A1 was 80% and 50% for genotypes 6/7 and 7/7, respectively, compared to the 6/6 genotype.

The factors most commonly triggering hyperbilirubinemia include being dehydrated, fasting, not getting enough sleep, being stressed, having surgery, hemolytic reactions, febrile illnesses, menstruation, and overexertion. Reducing daily caloric intake by 400 calories can lead to a 2–3 fold increase in blood bilirubin levels within 48 h. A similar increase in bilirubin levels can occur with a normocaloric diet lacking lipid supplementation. However, with a normal diet, bilirubin levels usually return to physiological norms within 12 to 24 h [2]. In the 1990s, it was demonstrated [12,13] that fasting during the perioperative period and the use of pain relievers (acetaminophen) post-surgery exacerbated bilirubinemia in patients with GS. However, a case study [14] suggested that the use of general anesthesia (halothane, thiopental, and ketamine) for surgery worsened bilirubinemia. In contrast, inhalational anesthesia (isoflurane) or general intravenous anesthesia with propofol and remifentanyl did not impair liver function. More recent studies by Kamal et al. [3] indicated that risk factors for the occurrence of clinical jaundice in adult Egyptians with GS included general anesthesia, pregnancy (elevated levels of unconjugated bilirubin were detected between the 10th and 16th weeks of pregnancy and remained high throughout pregnancy and part of

the postpartum period), fasting for more than 12 h, low-calorie weight loss plans (<1200 kcal), systemic infections, and intense physical exertion (such as soccer, tennis, fast swimming, or weightlifting).

GS typically does not cause significant clinical symptoms, aside from episodic hyperbilirubinemia predominantly featuring unconjugated bilirubin (UCB), which usually occurs after exertion or overeating. The first symptom is generally the periodic yellowing of the skin and sclera without skin itching. However, in some patients, the severity of hyperbilirubinemia can be associated with malaise, weakness, loss of appetite, abdominal discomfort, indigestion, flu-like symptoms, nausea, vomiting, concentration difficulties, sweating, anxiety, or palpitations [3,15].

In diagnostic terms, Gilbert's syndrome (GS) may be identified by a mildly elevated total bilirubin (TB) concentration in serum, with normal levels of liver transaminases (ALT, AST, GGTP, ALP), markers of bile duct function, and red blood cell count [5]. A characteristic feature of GS is unconjugated hyperbilirubinemia, typically not exceeding 6 mg/dL (100 µmol/L). Bilirubin concentrations below 5 mg/dL, predominantly consisting of unconjugated bilirubin ("indirect bilirubin") or with conjugated bilirubin ("direct bilirubin") < 0.7 mg/dL (excluding hemolysis), are indicative of GS [16]. The diagnosis of GS is based on repeated measurements of total and indirect bilirubin over 12–24 months. In patients with GS, a characteristic configuration is observed: a persistent increase in total bilirubin concentration with a predominance of the indirect fraction alongside average concentrations of aminotransferases (ALT, AST), GGTP, ALP, and bile acids. Bilirubin levels in individuals with GS often fluctuate and increase under excessive physical exertion and stress (infectious diseases, dietary indiscretions, exam periods, etc.).

Diagnosed GS does not require treatment. Occasionally, attempts have been made to use phenobarbital to induce UDP-glucuronosyltransferase activity and reduce bilirubin levels in the blood; however, the effectiveness of this approach is not always satisfactory. Generally, the prognosis for GS is good as the condition does not lead to liver damage or systemic consequences. Nevertheless, reports indicate that individuals with impaired UDP-glucuronosyltransferase activity may experience exacerbated adverse reactions when using certain medications such as nonsteroidal anti-inflammatory drugs (NSAIDs), antivirals, and anticancer drugs. Therefore, dosages of certain medications, such as paracetamol and ibuprofen, should be individually adjusted in individuals with GS, as standard doses may induce toxic effects. Additionally, adults with GS are significantly more likely to have concurrent schizophrenia compared to the general population. About 50% of GS patients have concurrent hemolytic anemia, increasing their risk of developing gallstones and bile duct stones [17]. Kamal et al. [3] demonstrated among Egyptian adults that the frequency of food intolerance disorders, such as celiac disease and lactose intolerance, was significantly higher in individuals with GS compared to healthy individuals. In contrast, the prevalence of other coexisting conditions (diabetes, rheumatoid arthritis, and hypertension) was similar to the general population.

Therefore, the primary aim of this paper is to provide a systematic review of clinical research on nutrition/diet in Gilbert's disease. Through rigorous selection of relevant data, this study shows the changes that have occurred over 60 years in the approach to nutrition and the resulting health benefits.

2. Materials and Methods

This review was reported following the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) [18].

The study for this systematic review was restricted to clinical trials carried out in humans in the past 60 years (1964–2023). Six databases were selected for literature research: Ebsco, Embase, Cochrane, Pub Med, Scopus, and Web of Science. The search and selection process was performed by three reviewers working independently and in parallel.

Searches included a combination of MeSH terms and keywords, applying quotes and field tags with BOOLEAN operators. Not all databases had the same query options

(for example, selecting a clinical trial), which is why stages and phrases were created. For all databases, five primary exclusion steps were determined: (1) keywords (“Gilbert* Syndrome” OR “Gilbert* Disease” AND diet* OR nutrit* AND clinic*); (2) year (1964–2024); (3) publication type (article); (4) language (English); (5) source type (journal).

For non-medical-only databases, screening was conducted in the following areas: Title, Abstract, Keywords (Scopus–Core Collection), Topic (Web of Science–Medline), and Ebsco (All). The last search was conducted in February 2024 because some publishers delayed publishing articles from 2023. Results from each database were exported to files with the appropriate extension of CSV or Excel, and summaries were created for each database containing publication information, including abstracts. The results files are included in the Repository for Open Data (<https://doi.org/10.18150/RA5ILC>). The initial search resulted in 255 studies. After removing non-English publications and reviews and deleting duplicates, 117 records were selected for abstract review. Among these, 120 studies were excluded according to the exclusion criteria, and the full text of 19 eligible studies was included—only studies involving humans. Articles concerning genetic, pharmacological, or simulation research were excluded from the review. Three independent researchers reviewed the abstracts, and papers that did not meet the criteria for inclusion in the review were excluded through discussion. The literature search process is presented in Figure 1.

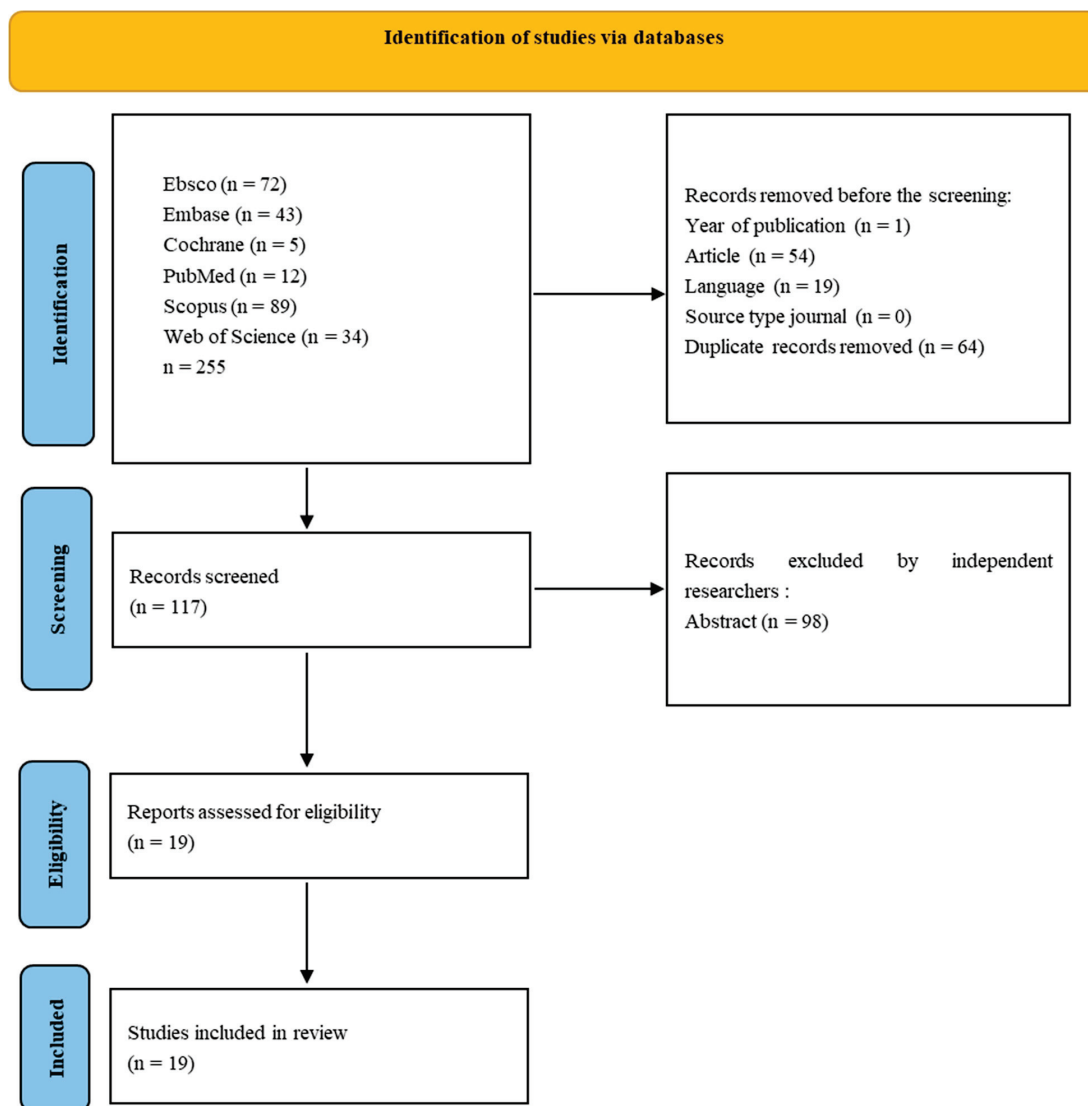


Figure 1. PRISMA flow diagram representing the screening strategy and selection process for research articles. Process of selecting eligible studies.

The Jadad scale was used to assess the quality and reliability of study outcomes in selected clinical trials [19–21]. This scale encompassed three main categories: randomization (0 points for quasi RCTs; 1 point if randomization was declared but without detailed description of random number generation; 2 points if correct random number generation method was described), double blinding (0 points if blinding was not mentioned; 1 point if blinding was mentioned but effectiveness was not described; 2 points if the study employed a robust method such as placebo to ensure double blinding), and withdrawals and dropouts (0 points, if withdrawals were not described despite fewer, analyzed patients compared to recruited ones). Evaluation using the Jadad scale categorized scores of 0–2 as indicative of low-quality clinical trials, 3 as medium-quality, and 4–5 as high-quality clinical trials.

3. Results

3.1. Caloric Restriction in Gilbert's Syndrome

Among the selected publications, 12 focused on caloric restriction (Table 1). These studies were conducted with healthy participants as well as individuals with Gilbert's syndrome, liver diseases, and hemolytic anemia. Participants were divided into groups based on age, gender, and ethnicity. A basic diet (2000–3000 kcal), a restrictive diet (≤ 400 kcal, with varying macronutrient compositions) supplemented or not with nicotinic acid, and a refeeding diet (2300 kcal) were administered. The duration of the restrictive diets and observation periods ranged from 11 h to 1 year.

In the 1970s, Felsher et al. [22] conducted a study involving seven adult patients with Gilbert's syndrome (GS), in which they implemented three dietary phases: baseline, caloric restriction, and refeeding while monitoring changes in serum bilirubin levels. The baseline diet ranged from 2000 to 3000 calories and lasted 48 to 72 h. Subsequently, calorie intake was reduced to fewer than 400 calories (either as a low-calorie carbonated beverage or skim milk or orange juice) for 36 to 72 h (extended to 7 days in 2 studies). After the caloric restriction phase, the diet rapidly increased to baseline levels (refeeding). The study also included six relatives of GS patients and eight healthy individuals as controls. The mean serum bilirubin levels in patients significantly ($p < 0.01$) differed before caloric restriction, during restriction, and refeeding periods (1.7 vs. 4.7 vs. 2.1 mg/100 mL, respectively). A correlation between calorie intake and serum bilirubin levels was observed. A rapid increase in serum unconjugated bilirubin levels occurred within 24 h of initiating short-term fasting. Bilirubin levels markedly decreased within 12 to 48 h after resuming high-calorie intake. Among the eight healthy control subjects, fasting resulted in only a slight increase in serum bilirubin levels. In three out of six relatives of a GS patient, a 48 h fast led to a significant rise in serum bilirubin levels, although they did not have jaundice. The authors of the cited studies demonstrated an association between calorie intake, hyperbilirubinemia, and fluctuations in serum bilirubin levels in GS.

In subsequent studies in the 1970s, Owens and Sherlock [23] aimed to determine whether reducing calorie intake and its effect on blood bilirubin levels could serve as a simple diagnostic test for diagnosing GS compared to healthy individuals or those with other liver diseases and hemolytic anemia. The authors hypothesized that increased blood levels of unconjugated bilirubin (UCB) must be caused by either increased production during hemolysis, decreased hepatic clearance, or a combination of both factors. They conducted a study involving 37 individuals divided into four groups: healthy patients, patients with GS, those with liver diseases, and those with hemolytic anemia. Before starting the diet, they measured the blood's total bilirubin (TB), hemoglobin (HGB), and cortisol levels. Patients received a basic diet for the first two days, then a diet reduced to 400 calories/day for the next three days. Blood samples were taken daily to measure TB, UCB, conjugated bilirubin (CB), haptoglobin, and cortisol levels. Patients were administered indocyanine green intravenously (0.5 mg/kg body weight), and its plasma disappearance was measured after 8 h and a 36 h fast in four healthy individuals and one patient with GS. Cortisol levels were measured in plasma before the diet and 72 h after the low-calorie diet was administered. Additionally, a study was conducted on Sprague-Dawley rats:

the first group ($n = 7$) received a basal diet for three days, while the second group ($n = 7$) was starved. It was found that the average plasma TB concentration in rats on a normal diet was 0.5 mg/100 mL. In comparison, reduced calorie intake increased bilirubin to 0.8 mg/100 mL after 48 h of calorie reduction but did not exceed 1.0 mg/100 mL. A significant ($p < 0.001$) increase in UCB concentration from 0.3 to 0.5 mg/100 mL was observed, but there were no changes in CB levels. TB concentration significantly increased in GS patients with elevated UCB levels in plasma (from 1.8 to 3.5 mg/100 mL) within 24 h of calorie restriction. UCB levels significantly rose in blood after 48 h of starvation (from 1.5 to 3.4 mg/100 mL), with some individuals experiencing an increase of up to 100% compared to basal diet conditions. There were no significant changes in CB concentration in the blood. The average increase in plasma bilirubin concentration after implementing dietary calorie reduction was significantly higher in GS patients (110%) compared to healthy individuals (60%). Meanwhile, TB concentration in plasma averaged 2.3 mg/100 mL in patients with liver diseases under basal diet conditions, and UCB levels were similar to those in GS patients (1.3 mg/100 mL). However, reducing calorie intake in the diet did not significantly decrease TB and UCB concentrations in plasma. Similarly, no significant changes in bilirubin levels were observed in patients with hemolytic anemia after calorie reduction in the diet. None of the patient groups showed significant changes in haptoglobin and cortisol concentrations after implementing the calorie-restricted diet. There were also no significant changes in indocyanine green disappearance time in half of the patients after 36 h of fasting following the marker injection. Moreover, studies on rats have demonstrated that hepatic bilirubin uridine diphosphate glucuronosyltransferases activity (per gram of liver; per gram of nitrogen) was significantly ($p < 0.01$) lower in starved rats compared to those allowed food. According to the authors, individuals with GS can be distinguished from healthy individuals or patients with liver diseases or hemolytic anemia by implementing a 400-calorie reduction in the diet, as they exhibit further significant increases in UCB in serum, but not when they have normal TB levels. The lack of changes in haptoglobin concentration after the reduced-calorie diet excluded the hypothesis of hemolysis as the cause of hyperbilirubinemia. Similarly, the absence of significant changes in indocyanine green clearance did not indicate reduced liver blood flow and reduced hepatic clearance of bilirubin. Starvation in rats led to a significant impairment of uridine diphosphate glucuronosyltransferase activity in the liver, not due to generalized liver protein catabolism. This activity is lower in individuals with GS.

Bensinger et al. [24], inspired by Gilbert's 1906 hypothesis regarding increased heme turnover during caloric deprivation in individuals with Gilbert's Syndrome (GS), investigated its impact on endogenous carbon monoxide (CO) production. They posited that CO and bilirubin are produced equimolarly during heme catabolism, suggesting that measuring CO levels could reflect this process. The study included 14 participants divided into two groups: a control group ($n = 5$, both sexes) and a GS group ($n = 9$, only men). Both groups underwent a 48 h 400-calorie diet containing approximately 46 g carbohydrates, 16.5 g protein, and 14.5 g fat. Blood samples were collected before and after the diet to measure serum total bilirubin (TB) concentrations and endogenous CO production rates. Significant findings included a notable ($p < 0.07$) 77% increase in TB levels in the control group after caloric restriction (0.44–0.78 mg/100 mL). At the same time, CO production showed no significant change (from 0.34 to 0.45 $\mu\text{mol/h/kg BW}$). In contrast, the GS group exhibited a significant ($p < 0.001$) 138% increase in serum TB levels (from 1.3 to 3.1 mg/100 mL), with no statistically significant change in CO production (from 0.41 to 0.47 $\mu\text{mol/h/kg BW}$). These results led the authors to conclude that the rise in serum bilirubin levels following caloric restriction does not stem from increased heme turnover.

Table 1. Characteristics of clinical studies conducted in people with Gilbert's syndrome regarding the impact of caloric restriction on selected metabolism parameters (n = 12).

Jadad Scale	Participants	Group	Duration	Intervention	Analysis	Results	Total Bilirubin	References
1	Adults, n = 21 Age: no data Sex: no data Ethnicity: no data Country: USA	GS (n = 7) Relatives of people with GS (n = 6) Healthy (n = 8)	2–7 days: Base-line diet 48 to 72 h Fasting diet 36 to 72 h Refeeding diet	Base-line diet 2000–3000 cal Fasting < 400 cal Refeeding 2000–3000 cal	Plasma: TB, CB	The concentration of unconjugated bilirubin (TB) in individuals with Gilbert's syndrome significantly differed before, during, and after caloric restrictions (1.7 vs. 4.7 vs. 2.1 mg/100 mL). A relationship between calorie intake and TB levels in the blood was observed. A sudden increase in conjugated bilirubin (CB) occurred within 24 h of starting short-term fasting, followed by a significant decrease during the 12–48 h of refeeding.	TB > 1.7 mg/dL	Felsher et al. [22]
1	Adults, n = 37 Age: no data Sex: no data Ethnicity: no data Country: UK Rats Sprague-Dawley (n = 14)	Healthy (n = 12) GS (n = 10) Liver disease (n = 12) Hemolytic anemia (n = 3) Rat basal diet (n = 7) Rats starved (n = 7)	5 days	People: 2-day basal diet, reduced to 400 cal/day for 3 days indocyanine green intravenously Rats: first group basal diet, second group 3 days was starved	Venous blood: HGB, UCB, CB Plasma: TB, haptoglobin, cortisol, Hepatic UDPGT	After caloric restriction in the diet, the following changes were observed: - Healthy individuals showed a significant increase in unconjugated bilirubin (UCB) concentration after 48 h. - Individuals with Gilbert's syndrome (GS) exhibited increased total bilirubin (TB) and unconjugated bilirubin (UCB) levels after 24 h. - Patients with liver diseases and hemolytic anemia did not show significant changes in TB and UCB concentrations. - No changes were observed in haptoglobin, cortisol levels, and the half-disappearance time of the indocyanine green injection in any of the studied patient groups. - UDP-glucuronyltransferase (UDPGT) activity was significantly lower in starved rats than those with food access.	TB > 1.8 mg/dL	Owens and Sherlock [23]
2	Adults, n = 14 Age: 19–34 years Sex: 3 women; 11 men Ethnicity: no data Country: USA	Healthy (n = 5): 3 women, 2 men GS (n = 9: men)	2 days	Diet 400 cal/day (46 g of carbohydrates, 16.5 g of protein, and 14.5 g of fat)	LDH, ALP, ALT, ASP, TB, CB, HGB, CO production Liver biopsies	In both control group patients and those with GS, significant increases in serum total bilirubin (TB) levels were observed following caloric restriction, while there were no differences in carbon monoxide (CO) production.	TB > 1.3 mg/dL	Bensinger et al. [24]

Table 1. Cont.

Jadad Scale	Participants	Group	Duration	Intervention	Analysis	Results	Total Bilirubin	References
2	Adults, n = 34 Age: no data Sex: no data Ethnicity: no data Country: USA	GS (n = 11) Hemolysis and unconjugated hyperbilirubinemia (n = 10) Healthy (n = 13)	4–5 days	Diet A 2 or 3-day baseline of 2500–3000 cal/day, the caloric intake was abruptly reduced to approximately 300 cal/day for 2 days	Serum: TB, CB, Hepatic UDPG-T	Forty-eight hour fasting significantly increased TB levels in serum among individuals with Gilbert's syndrome (from 1.5 to 3.5 mg/100 mL) and healthy individuals (from 0.5 to 0.9 mg/100 mL). Serum CB levels remained unchanged in all three patient groups. Reduced hepatic UDPG-T activity to levels observed in GS patients was observed only in two patients with hemolysis.	TB > 1.5 mg/dL	Felsher and Carpio [25]
2	Adults, n = 4 Age: 21–46 years Sex: no data Ethnicity: no data Country: USA	GS (n = 4)	About 2 weeks	Base-line balanced diet for 2 days Tested diet: low carbohydrate; low protein; low fat; isocaloric high fat, low carbohydrate, low protein; isocaloric low carbohydrate diet and a low protein	Anthropometric measurements Plasma: TB, CB	Higher TB levels were observed in individuals with GS after a low-calorie diet compared to a balanced diet. TB levels in individuals on various isocaloric diets with reduced macronutrient content were, on average, 1.1 mg/dL lower than after a low-calorie diet. A low-protein and low-carbohydrate isocaloric diet did not increase TB levels in the blood. None of the diets affected CB levels in the blood. Changes in body weight were observed only after the low-calorie diet.	TB > 1.3 mg/dL	Felsher [26]
2	Youth, adults, n = 12 Age: 14–46 years Sex: 3 women; 9 men Ethnicity: no data Country: Sweden	GS (n = 12)	3 days	Reduced caloric intake (1.7 MJ/24 h) fluid meal (50 g of fat)	Serum: bile acids, UCB	The increase in UCB concentration after caloric restriction was 140%. In fasting, before and after caloric reduction, patients had normal serum bile acid levels. Postprandial concentrations of bile acids in serum were within the normal range before and after caloric restriction.	TB > 1.5 mg/dL	Briheim et al. [27]
3	Adults, n = 28 Age: 19–37 years Sex: 5 women; 25 men Ethnicity: no data Country: Italy	GS (n = 18: 15 men, 3 women) Control (n = 10: 8 men; n = 2 women)	Of at least 2 weeks	Caloric restriction 400 cal: Diet A: 100 g of sucrose/24 h Diet B: 250 g of bran (and 100–250 cm ³ of barium sulfate solution	Plasma: TB	In the control group, there were no differences in TB concentration after diets A and B. TB concentration significantly increased under diet A compared to diet B in individuals with GS. TB concentration did not differ significantly between groups depending on the diet applied.	TB > 1 mg/dL	Ricci and Ricci [28]

Table 1. Cont.

Jadad Scale	Participants	Group	Duration	Intervention	Analysis	Results	Total Bilirubin	References
3	Adolescents/Adults n = 60 Age: 16–48 years Sex: 27 women; 33 men Ethnicity: no data Country: Italy	GS1 (n = 10 men, n = 10 women) with high TB GS2 (n = 13 men, n = 7 women) with normal TB. Control (n = 10 men, n = 10 women)	The 2 tests were performed with an interval of at least 2 weeks.	Test with nicotinic acids (NA) Test fasting/ hyperbilirubinemia (FH) patients 3 days after a control normocaloric diet were given a restricted diet (400 cal/48 h)	Blood: UCB and CB	In the NA test, significant differences were observed between individuals with GS and controls in UCB, maximal increment in AUC, and retention at 240 min (excluding sex). Following calorie restriction, TB levels were higher in GS1 compared to GS2 and the control group. Serum bilirubin increased after 24 h of caloric restriction in GS1, GS2, and normal subjects but not after 48 h. Bilirubin concentrations after 24 and 48 h were higher in males with GS compared to females. The NA test was more accurate in diagnosing GS in both males and females.	TB > 1.35 mg/dL	Gentile et al. [29]
1	Adults, n = 10 Age: no data Sex: no data Ethnicity: no data Country: Turkey	GS (n = 10)	30 days	Compulsory 16 h fasting daily from 400 to 20:00 during Ramadan and consuming 2500 calories (30 g carbohydrates, 20 g protein, and 10 g fat)	Serum: TB, CB, UCB	After the first day of fasting, the concentrations of TB and UCB were significantly higher than before, while CB remained similar. In the following days of fasting (7, 14, 30), TB and UCB concentrations decreased to pre-fasting levels, while CB remained consistent throughout the fasting period.	TB > 1.4 mg/dL	Kapıcıoğlu et al. [30]
3	Adults, n = 146 Age: 20.7 years Sex: 146 women Ethnicity: White Country: Portuguese	Bilirubin concentration ($\mu\text{mol/L}$) I group ≤ 6 (n = 49) II group 6–9 (n = 49) III group ≥ 9 (n = 48)	No data	11 h fasting	Venous blood: hematological data, Plasma: bilirubin, Buffy coat-DNA extraction, screening of TA duplication in the UGT1A1 gene, B/A, International Physical Activity Questionnaire.	Women in the second and third tertiles exhibited higher Hb, Ht, MCH, MCHC concentrations and lower platelet counts than those in the first tertile. In the third tertile, RCB was the highest. There were no differences in total and differential white blood cell counts and non-genetic factors (physical activity, smoking, oral contraception, body fat content, alcohol consumption, and fasting time). The lowest BMI was observed in the third tertile. The genetic allele c-41_-40dupTA, Hb, BMI, and fasting time were identified as the main factors associated with bilirubin concentration in the blood.	TB > 1 mg/dL	Rodrigues et al. [31]

Table 1. Cont.

Jadad Scale	Participants	Group	Duration	Intervention	Analysis	Results	Total Bilirubin	References
4	Adults, n = 120 Age 20–80 years Sex: 40 women, 80 men Ethnicity: no data Country: Austria	Group < 35 years (n = 66: GS and Control: 9 women, 24 men each) Group ≥ 35 years (n = 54: GS and Control: 11 women, 16 men each)	No data	16 h fasting with a diet of 400 cal	Anthropometric measurements, BIA, questionnaire food consumption, Biomarkers of energy, carbohydrate, lipids metabolism	There were no differences between the GS and control groups in the consumption of healthy foods, snacks, red meat, alcohol, physical activity, liver enzymes, iron levels, and AMPKα1 gene expression. Individuals with GS had lower BMI, blood glucose, insulin, C-peptide, and TG levels and were less susceptible to metabolic diseases. In GS individuals, levels of p-AMPK α1/α2, -PPAR α/γ, and Pgc1α were significantly higher, indicating an enhanced metabolic response to fasting.	TB > 1.0 mg/dL	Mölzer et al. [32]
2	Children, young, n = 46; Age: no data Sex: 25 girls, 21 boys Ethnicity: no data Country: Italy	Group OLT (n = 46) Control group (n = 20) non-GS OLT	1 year	1 day reduced caloric intake prolonged (14–16 h) fasting	Venous blood: hematologic parameters Serum: TB, Ch, UCB IXa, monogluconide, sex hormones, liver dysfunction, 2-bp (TA) insertion in the promoter of the gene	Four patients with OLT had hyperbilirubinemia, but CB levels were normal. Tests involving caloric restriction and fasting showed an increase in bilirubin levels. High relative amounts of UCB IXa and a predominance of monogluconide over digluconide were observed. Hematologic parameters and hormone levels in the blood were normal. Adult liver donors had gene mutations related to the 2-bp (TA) insertion.	TB > 1.3 mg/dL	Vajro et al. [33]

Jadad scale—0–5. Explanations are in the Abbreviations section.

Felsher and Carpio [25] conducted a dietary study involving 10 patients with Gilbert's Syndrome (GS), 11 patients with hemolysis and unconjugated hyperbilirubinemia, and 13 normal subjects. Participants underwent a 2-to-3-day baseline diet providing 2500 to 3000 cal/day, followed by a sudden reduction in caloric intake to approximately 300 cal/day (consisting of approximately 30 g carbohydrates, 20 g protein, and 10 g fat) for two days (fasting). Blood samples were collected daily before breakfast to measure serum total bilirubin (TB) concentrations (prefasting level). Significant ($p < 0.01$) increases in serum TB levels were observed in both GS patients and normal subjects following caloric restriction, with a significantly ($p < 0.001$) greater increase noted in GS patients (from 1.5 to 3.5 mg/100 mL) compared to normal subjects (from 0.5 to 0.9 mg/100 mL). Patients with hemolysis also showed an increase in serum TB levels, averaging from 2.8 to 3.5 mg/100 mL, which was significantly ($p < 0.001$) lower than in GS patients and only slightly higher than in normal subjects ($p = 0.04$). However, serum-conjugated bilirubin (CB) levels did not significantly change following a caloric reduction in all three patient groups. No subjects with average postfasting serum bilirubin concentrations exhibited hepatic UDP-glucuronyltransferase (UDPG-T) activity levels reduced to those observed in GS, except in a few cases associated with severe liver fibrosis or fatty infiltration. Under the caloric restriction, UDPG-T activity in two patients with hemolysis was reduced to levels observed in GS. According to the authors, the caloric-induced rise in serum unconjugated bilirubin (UCB) is independent of the prefasting bilirubin level. Furthermore, the dietary test may be diagnostically informative even in patients with GS and normal prefasting serum bilirubin levels.

In subsequent studies, Felsher [26] compared changes in bilirubin concentration in four patients with Gilbert's Syndrome (GS) following a low-calorie and normocaloric diet with significantly reduced carbohydrate, protein, and fat content. For the first two days, patients received a balanced baseline diet (2300 to 2500 cal: 260 g carbohydrates, 90 g protein, 100 g fat). On the third day, the energy value of the diet was reduced to 300 kcal (30 g carbohydrates, 20 g protein, 10 g fat). After 2–4 days of this regimen, the baseline diet was reintroduced for four days. Subsequently, three additional isocaloric diets, each matching the baseline diet in calories and lasting for four days, were sequentially administered to three patients in randomly selected order. These diets were low-carbohydrate (30 g carbohydrates, 169 g protein, 178 g fat), low-protein (22 g protein, 370 g carbohydrates, 93 g fat), or low-fat (10 g fat, 444 g carbohydrates, 118 g protein). Additionally, two patients received a high-fat (220 g), low-carbohydrate (49 g), and low-protein (52 g) isocaloric diet. One patient exclusively received a low-carbohydrate and low-protein isocaloric diet. Each morning before breakfast, blood samples were collected from patients to measure serum total bilirubin (TB) and conjugated bilirubin (CB) concentrations. Significantly higher TB concentrations were observed after the low-calorie diet than the balanced diet (2.8 vs. 1.3 mg/dL). Moreover, TB levels after different isocaloric diet variations with reduced macronutrient content were, on average, 1.1 mg/dL lower than after the low-calorie diet. Low-protein and low-carbohydrate isocaloric diets did not increase serum TB concentration. None of the diets influenced CB concentration in the blood. Additionally, patients' body mass changed only after applying the low-calorie diet. The author concluded that the hyperbilirubinemia in individuals with GS is primarily due to caloric restriction itself, which impairs bilirubin glucuronidation capacity in the liver by reducing UDP-glucuronic acid formation, rather than changes in diet composition, as neither negative nitrogen balance nor reduced lipid content had any impact on bilirubin levels.

Based on the premise that bile acids have superior diagnostic utility to aminotransferases in certain liver diseases, Briheim et al. [27] conducted a study in the 1980s to determine the serum concentrations of these acids in patients with Gilbert's syndrome (GS) ($n = 12$, both genders), depending on their varying bilirubin levels. The serum concentrations of cholic acid, chenodeoxycholic acid, and unconjugated bilirubin (UCB) were measured in patients after overnight fasting. Subsequently, for three days, patients consumed a standardized low-calorie liquid test meal (1.7 MJ/24 h, approximately 406 cal, 50 g fat), and postprandial bile acid levels were measured. Following caloric restriction, a

characteristic increase (average of 140%) in serum UCB levels was observed in GS patients. The 150 min values of cholic and chenodeoxycholic acid were slightly higher after reduced caloric intake ($p < 0.02$ and $p < 0.05$, respectively), but no significant differences were found between values obtained before and after caloric restriction. However, fasting and postprandial bile acid concentrations remained within the reference range before and after the reduced caloric intake. No significant correlation was found between the increase in UCB and postprandial bile acid concentrations. According to the authors, different mechanisms are involved in the hepatic uptake and secretion of bile acids and bilirubin. Therefore, measuring serum bile acid concentrations can assess liver function in GS patients independently of the degree of hyperbilirubinemia. Their normal levels exclude liver function disorders, allowing avoidance of more invasive diagnostic methods such as liver biopsy.

Ricci and Ricci [28] hypothesized that food in the intestines controls serum bilirubin levels. They conducted a study on individuals with Gilbert's syndrome (GS) ($n = 18$, both genders) and a control group ($n = 10$, both genders). In Diet A, caloric restriction involved providing patients with 400 cal/24 h in the form of 100 g sucrose. Conversely, in Diet B (volumetric), patients were given 250 g of wheat bran with 100–250 mL of barium sulfate solution, divided into 2 or 3 meals. Diet B provided 1674 kJ (400 cal: 95% carbohydrates, 0.5% fats, and 4.5% proteins). To enhance palatability, patients were allowed to drink tea (500 mL) and water ad libitum, with permitted artificial flavors and saccharin. Blood samples were taken from the patients before the administration of Diet A, after 24 h, and after a minimum interval of 2 weeks, during which the patients were on Diet B. Serum total bilirubin (TB) levels were measured. In healthy individuals, the increase in plasma TB levels was comparable after both diets (A and B) and was not statistically significant (Diet A from 11.1 to 23.4 $\mu\text{mol/L}$; Diet B from 10.1 to 18.1 $\mu\text{mol/L}$). However, in individuals with GS, TB levels significantly ($p < 0.001$) increased to a greater extent after Diet A compared to the control group (from 34.1 to 81.4 $\mu\text{mol/L}$) than after the volumetric Diet B (from 33.9 to 65.2 $\mu\text{mol/L}$). The serum bilirubin concentration results from a balance between the production rate and the hepatic clearance capacity. The TB levels did not differ significantly between the study groups depending on the diet applied. According to the authors, an intestinal factor, possibly a hormone whose release is dependent on the presence of a large amount of food, plays a role in controlling caloric restriction-associated hyperbilirubinemia.

Gentile et al. [29] compared the diagnostic efficacy of two tests: nicotinic acid (NA) and reduced caloric intake (fasting/hyperbilirubinemia, FH) at 400 calories regarding specificity, sensitivity, and accuracy for optimistic prediction. The authors conducted the tests on 40 patients with Gilbert's syndrome (GS) (both genders) and 20 healthy controls (both genders). Patients with GS were divided into two groups: GS1 (10 males and 10 females) with high serum bilirubin and GS2 (13 males and 7 females) with average values. In the first test, patients received an intravenous injection of 5 $\mu\text{mol/kg}$ BW NA, and blood was drawn at 0, 30, 45, 60, 120, 180, and 240 min post-injection to measure UCB and CB levels. In the second test, after three days of a normocaloric diet, patients were given a restricted diet of 400 calories for 48 h with an allowance for moderate physical activity. Blood samples were collected at 0, 24, and 48 h, and UCB levels were measured within 30 min of collection. It was found that before the tests, the mean serum UCB level in GS1 patients was significantly ($p < 0.001$) higher than in the control group (23.17 vs. 10.55 $\mu\text{mol/L}$). However, no such difference was observed between GS2 and the control group (13.12 vs. 10.55 $\mu\text{mol/L}$). Considering gender, GS1 males had significantly ($p < 0.05$) higher UCB levels than females (28.04 vs. 18.30 $\mu\text{mol/L}$), whereas no gender difference was observed in the control group (10.99 vs. 9.81 $\mu\text{mol/L}$) or GS2 (14.12 vs. 12.78 $\mu\text{mol/L}$). After the NA test, a statistically significant difference ($p < 0.001$) was observed between GS and controls in UCB bilirubin levels, the maximal increment, the AUC, and the retention at 240 min. A significantly higher hyperbilirubinemic effect was also present in GS2 subjects, with no difference observed between GS1 and GS2. There were no gender differences in retention at 240 min. However, significant differences ($p < 0.001$) were noted between males and females with GS in both AUC (6721 vs. 4050 $\mu\text{mol/L/240 min}$) and the maximal

increment ($p < 0.02$) of UCB (60.57 vs. 42.81 $\mu\text{mol/L}$). In the reduced caloric intake test, UCB bilirubin was significantly higher in GS1 than in GS2 and controls, with no difference in controls. Serum UCB rose significantly ($p < 0.001$) in GS1, GS2, and normal subjects after 24 h of caloric restriction, but not after 48 h. Considering sex, UCB concentration after 24 h was significantly ($p < 0.05$) higher in men with GS than in women (61.51 vs. 38.51 $\mu\text{mol/L}$), and ($p < 0.01$) after 48 h (78.31 vs. 47.58 $\mu\text{mol/L}$). In the control group, no gender differences in UCB concentration were found. The authors concluded that in the diagnosis of GS, hyperbilirubinemia induced by NA, particularly its concentration at 240 min post-administration, was more effective for both genders than fasting-induced hyperbilirubinemia.

In the 1990s, Kapıcıoğlu et al. [30], considering that the Muslim community forms about one-third of the world's population, conducted a study on the impact of the mandatory 30-day fast during Ramadan on unconjugated hyperbilirubinemia in adults with Gilbert's syndrome (GS) ($n = 10$). Before the study, the levels of total bilirubin (TB), unconjugated bilirubin (UCB), and conjugated bilirubin (CB) in serum were measured in the patients. During Ramadan, the patients consumed meals at 4:00 AM and 8:00 PM, totaling approximately 2500 calories (approximately 30 g of carbohydrates, 20 g of protein, and 10 g of fat). After measuring bilirubin levels at 4:00 AM, the patients did not eat or drink for 16 h for religious reasons. Throughout the 4-week fasting period, bilirubin levels were measured weekly in the patient's serum. The authors found that after the first day of fasting, all patients with GS showed a significant ($p < 0.013$) increase in TB and UCB levels compared to pre-fasting levels (1.4 and 2.6 vs. 1.0 and 2.1 mg/dL, respectively). In contrast, the CB level remained similar (0.4 mg/dL). Following additional days of fasting (7, 14, and 30 days), the levels of TB and UCB decreased to pre-fasting values and did not change after that. The CB level remained consistent throughout the fasting period. The authors concluded that the results could be significant for diagnosing GS and its clinical features within the Muslim population.

Rodrigues et al. [31] conducted a study to evaluate the effect of Hb concentration and non-genetic factors (smoking, health status, alcohol consumption, physical activity, oral contraceptive therapy, fasting time, and caloric intake) and the genetic contribution of UGT1A1 polymorphisms on bilirubin levels in 146 young (20.7 ± 2.6 years) Portuguese women to eliminate the potential interference of age and sex. Hematological parameters were measured in their blood, and bilirubin levels were measured in plasma. A screening for the TA duplication in the UGT1A1 gene was conducted. Anthropometric measurements were taken, and body fat content was determined using bioelectrical impedance analysis. Data on fasting time, smoking, oral contraception, and energy intake were obtained from questionnaires filled out by the participants. Physical activity was assessed using the Short Form of the International Physical Activity Questionnaire. The participants were divided into three groups according to tertiles of blood bilirubin concentration ($\leq 6 \mu\text{mol/L}$, $n = 49$; $6\text{--}9 \mu\text{mol/L}$, $n = 49$; $\geq 9 \mu\text{mol/L}$, $n = 48$). The hematologic studies showed that subjects in the second and third tertiles had significantly higher Hb concentration, hematocrit (Ht), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) and lower platelet counts, along with an increased frequency of homozygosity for the c.-41_-40dupTA allele, compared with those in the first tertile. No significant differences were found between groups for total and differential white blood cell counts. It was found that women in the third tertile had a significantly higher red blood cell (RBC) count compared to the first tertile. There were no significant differences between groups in physical activity, smoking, oral contraception, body fat, alcohol consumption, and fasting time. A significantly ($p < 0.009$) lower BMI was found in the third tertile compared to the first. Significant positive correlations were observed between total bilirubin (TB) concentration and Hb, Ht, MCH, and MCHC. The c.-41_-40dupTA allele (genetic factor), Hb, BMI, and fasting time were identified as the main factors associated with bilirubin levels in the blood. According to the authors, since the primary source of bilirubin is the degradation of hemoglobin (Hb) in plasma by macrophages from the reticuloendothelial

system, it can serve as a diagnostic marker for Gilbert's syndrome (GS), as an increase in its blood concentration follows an increase in bilirubin levels ($\geq 6 \mu\text{mol/L}$). Additionally, increased red cell mass can be a clinical manifestation of GS or its exacerbation. The hematologic studies showed that subjects in the second and third tertiles had significantly higher Hb concentration, hematocrit (Ht), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) and lower platelet counts, along with an increased frequency of homozygosity for the c.-41_-40dupTA allele, compared with those in the first tertile. No significant differences were found between groups for total and differential white blood cell counts. It was found that women in the third tertile had a significantly higher red blood cell (RBC) count compared to the first tertile. There were no significant differences between groups in physical activity, smoking, oral contraception, body fat, alcohol consumption, and fasting time. A significantly ($p < 0.009$) lower BMI was found in the third tertile compared to the first. Significant positive correlations were observed between total bilirubin (TB) concentration and Hb, Ht, MCH, and MCHC. The c.-41_-40dupTA allele (genetic factor), Hb, BMI, and fasting time were identified as the main factors associated with bilirubin levels in the blood. According to the authors, since the primary source of bilirubin is the degradation of hemoglobin (Hb) in plasma by macrophages from the reticuloendothelial system, it can serve as a diagnostic marker for GS, as an increase in its blood concentration follows an increase in bilirubin levels ($\geq 6 \mu\text{mol/L}$). Additionally, increased red cell mass can be a clinical manifestation of GS or its exacerbation. The authors found that fasting time was an independent variable related to the concentration of TB. In the fasted state, subjects with average bilirubin conjugation capacity might have higher intestinal bilirubin accumulation compared to those with low bilirubin conjugation capacity due to the UGT1A1 polymorphism, which explains the higher bilirubin concentration in women with average TA counts and UGT1A1 repeats and polymorphisms, given the same fasting time. The authors suggest that studies should be conducted to determine the effects of these factors at different ages in both men and women and to investigate whether UGT1A1 changes, other than TA duplication in the promoter region, may affect TB levels.

In the last decade, Mölzer et al. [32], as part of the BiliHealth project, attempted to elucidate metabolic differences and altered energy regulations between healthy individuals and those with GS in response to fasting. The study focused on the AMPK pathway, which integrates and defines energy and macronutrient metabolism at both the cellular and whole-body levels. The study group consisted of 120 individuals, both healthy and diagnosed with GS (20 men and 40 women each), divided into two age groups: <35 years ($n = 66$, including 33 healthy and 33 with GS) and >35 years ($n = 54$, including 27 healthy and 27 with GS). On the day before the study, all participants underwent a 16 h fast, were given a 400-calorie diet, and had blood drawn to measure UCB levels ($</\geq 17.1 \mu\text{M}$) to confirm the presence of GS. Blood tests included liver enzymes, hormones, lipid profile parameters, and UGT1A1 genotyping. Additionally, anthropometric measurements, body composition analysis, and a questionnaire on the weekly frequency of healthy food consumption, snacking, red meat intake, and alcohol consumption were completed by the participants. Significant ($p < 0.000$) differences were found between the groups in blood UCB levels and the distribution of TA repeats (i.e., UGT1A1*28 genotype). GS patients did not significantly differ in their intake of healthy foods, snacks, red meat, alcohol, or physical activity compared to the control group. No differences were observed in liver health parameters and iron levels (AST, ALT, γ -GT, LDH, albumin, transferrin, and ferritin) between the groups. However, GS patients had significantly lower BMI (22.8 vs. 25.4 kg/m²), blood glucose levels (81.0 vs. 86 mg/dL), insulin (4.1 vs. 5.9 $\mu\text{U/mL}$), C-peptide (1.2 vs. 1.4 ng/mL), and triacylglycerols (73 vs. 85 mg/dL), indicating that they are leaner and healthier and thus less prone to metabolic diseases or premature death. In GS individuals, compared to the control group, levels of p-AMPK $\alpha 1/\alpha 2$, PPAR α/γ , and PgC1 α were significantly ($p < 0.001$) higher (respectively: 178; 400; 1401; 206 vs. 112; 292; 1080; 165 rfU), indicating an increased metabolic response to fasting. However, no difference was observed between

the groups in AMPK α 1 gene expression. The authors provided evidence that GS's energy and macronutrient metabolic response to fasting is markedly increased. Consequently, although all subjects were metabolically healthy and within reference ranges for blood biochemistry parameters, several intergroup differences confirmed the better metabolic health of individuals with GS. Serum UCB increased in response to fasting and was found to be the primary determinant of post-translational activation of AMPK α 1/ α 2 and BMI.

Vajro et al. [33], hypothesizing that GS can be transferred from a donor to recipients of orthotopic liver transplants (OLT), investigated the prevalence of this syndrome in pediatric and adolescent recipients (pre-pubertal), a group in which the influence of hormonal factors on bilirubin UDPGT levels is relatively low and rarely associated with clinical symptoms. The study involved 46 patients in the pediatric ward for a year and had undergone transplantation an average of 3.7 years prior (range 0.5–5 years). The average follow-up time was 5.5 years (range 1.0–9.0 years). The control group consisted of 20 individuals without OLT and GS. Serum bilirubin levels and its fractions, monoglucuronide, and sex hormones were measured in OLT patients. The 2-bp (TA) insertion in the promoter of the gene coding for the enzyme bilirubin glucuronosyltransferase associated with GS was screened. Out of the 46 OLT patients, 4 (2 girls, 2 boys, mean age 3.1 years, range 1.2–7 years, 9% of subjects) showed hyperbilirubinemia, with TB levels ≥ 1.3 mg/dL and normal CB levels (<0.3 mg/dL). In three children, hyperbilirubinemia was detected immediately after OLT, while in one child, it was detected a year later and persisted throughout the observation period (range 2.0–8.1 years). Following several tests involving one-day reduced calorie intake and prolonged overnight fasting, TB levels in these patients' blood samples increased, while CB levels remained unchanged. Returning to a normocaloric diet caused both bilirubin levels to drop to baseline. Significantly lower TB levels (1.2 mg/dL, $p < 0.01$) were observed in OLT recipients without hyperbilirubinemia ($n = 20$) when the same test was conducted. High relative amounts of UCB IXa and a predominance of monoglucuronide over diglucuronide, characteristic of GS, were also observed. DNA analysis of liver donor lymphocytes (four men) showed that they were either homozygous for the TA insertion allele, homozygous for the normal allele, or heterozygous for the TA insertion allele. Hematological parameters and levels of LDH and sex hormones in all patients were within the normal range. The likelihood of developing GS was significantly higher ($p = 0.01$) in pediatric OLT recipients (regardless of sex) who received a liver from an adult donor. According to the authors, the prevalence of GS in pediatric OLT recipients may be higher than expected in the average pre-pubertal population and may present an atypical early form. Results from the HPLC analysis of serum bilirubin fractions, TB levels, and CB ratios after just one dietary test could allow for a rapid diagnosis of GS, while molecular data could confirm this diagnosis.

3.2. Consumption of Various Diet Variants, Vegetables, Fruits, and Alcohol in Gilbert's Syndrome

Among the selected publications, seven focused on consuming various diet variants, vegetables, fruits, and alcohol in Gilbert's Syndrome (Table 2). The studies included different diet variants, namely, standard, restrictive, and hypercaloric, as well as the consumption of six botanical groups of vegetables and fruits, various quantities of vegetable and fruit servings, post-bariatric surgery diet, and alcohol consumption. The studies were conducted in groups of healthy patients stratified by genotype, age, gender, and ethnicity. The duration of the diets ranged from two days to over four years of observation.

Table 2. Characteristics of clinical trials conducted in people with GS regarding the impact of various diet variants, fruit, vegetable, and alcohol consumption on selected metabolic parameters (n = 7).

Jadad Scale	Participants	Group	Duration	Intervention	Analysis	Results	Total Bilirubin	References
4	Young adults, n = 29 Age: 16–43 years Sex: 8 women, 21 men Ethnicity: no data Country: UK		About 10 days: 2 days normal diet Tested diet 1–2 day basal diet	Normal diet Tested diet: Low-energy/ standard Low-energy, standard + IV glucose Low-energy, standard + IV lipid High-carbohydrate, low-lipid (fluid) High-carbohydrate, reduced-lipid Low-energy, high-lipid	Plasma: TB	The highest increase in TB concentration was observed in patients following Diet II (low-energy standard) and Diet III (low-energy, standard + IV glucose). The lowest increase in TB concentration occurred with Diet VI (high-carbohydrate, reduced-lipid). The level of hyperbilirubinemia achieved after oral carbohydrate intake was significantly lower than that observed after intravenous intake or Diet II (low-energy, standard). Diet VII (low-energy, high-lipid) significantly reduced hyperbilirubinemia to a level lower than Diet II-induced (low-energy, 400 cal).	TB > 1.7 mg/dL	Gollan et al. [34]
3	Adults, n = 191 Age: 20–40 years Sex: 112 women, 79 men Ethnicity: no data Country: USA	UGT1A1 genotype: Group 6/6 and 6/7 (n = 169) Group 7/7 (n = 22)		Assessment of the consumption of 6 groups of botanical vegetables and fruits	Demographic survey, health history, 3-D food record Indices: BMI Venous blood: TB, CB, UGT1A1 promoter genotypes.	Men had higher serum levels of TB, CB, and UCB than women. An inverse relationship was demonstrated between all bilirubin levels and the interaction of the UGT1A1*28 genotype with the intake of the botanical group <i>Cruciferae</i> . Individuals with the 7/7 genotype had reduced blood bilirubin levels with increased consumption of cruciferous vegetables.	TB > 1.6 mg/dL	Peterson et al. [35]
4	Adults, n = 70 Age: 20–40 years Sex: 35 women, 35 men Ethnicity: Caucasian, Asian, Other Country: USA	groups of genotypes UGT1A1*1/*1 (n = 29) UGT1A1*1/*28 (n = 36) UGT1A1*28/*28 (n = 5) (1) GSTM1+/GSTT1+ (n = 26) (2) GSTM1-null/GSTT1+ (n = 30) (3) GSTM1-null/GSTT1-null (n = 14)	Each of the four diet periods lasted 14 days, with a 21-day washout period between diet periods	(1) Basal diet without fruit and vegetables; (2) Single-dose cruciferous diet; (3) Double-dose cruciferous diet; (4) Single-dose cruciferous plus apiaceous vegetable diet	GSTM1, GSTT1 genotypes UGT genotyping 3-d food record Plasma: TB, CB Urine: total isothiocyanates Indices: BMI	The decrease in serum bilirubin concentration in response to all three diets compared to the basal diet was particularly pronounced in individuals with the UGT1A1*28/*28 genotype and those consuming a double dose of cruciferous plants, which enhances UGT1A1 activity. Although UGT1A1 activity, measured by serum bilirubin, was higher in individuals with the GSTM1-null genotype than those with GSTM1+, this response was not statistically significant.	TB > 1.2 mg/dL	Navarro et al. [36]

Table 2. Cont.

Jadad Scale	Participants	Group	Duration	Intervention	Analysis	Results	Total Bilirubin	References
3	Adults, n = 239 Age: 20–40 years Sex: 147 women, 146 men Ethnicity: Caucasian, Asian, Other Country: USA	UGT1A1 genotype: Group 6/6 Group 6/7 Group 7/7	No data	Week	Food Frequency Questionnaire 6 botanical families 3-D food record; anthropometric measurements Plasma: TB, UGT genotyping	Gender (men), race (Asian), and the UGT1A1 genotype were associated with serum concentrations of TB, CB, and UCB. There was no association between the consumption of vegetables and fruits (assessed by FFQ) and TB levels. In women, an interaction was found between the UGT1A1 (7/7) genotype and the consumption of citrus fruits (assessed by FFQ) and <i>Rutaceae</i> (assessed by 3DFR), which was related to TB and UCB levels.	TB > 1.0 mg/dL	Saracino et al. [37]
3	Children, n = 29 Age: 8–17 years; Sex: 10 girls, 19 boys Ethnicity: no data Country: Serbia	GS (n = 29)	9 months	24 h of fasting and after 24 h of hypercaloric intake	Questionnaire related to urinary tract Blood: glucose, TB, CB Urine: ketone bodies uroflowmetric test	There was a normal uroflowmetric pattern in 69% of children and after 24 h of hypercaloric diet in all children. Children with an abnormal uroflowmetric pattern had higher CB concentrations after 24 h of fasting than children with a normal pattern. There were no differences in TB and UCB concentrations. In children with normal and abnormal UFC, pre-fasting CB concentration was lower than post-fasting, and glucose concentration was higher. VC in children was normal after 24 h of fasting and after 24 h of hypercaloric intake.	TB > 1.3 mg/dL	Stojanović and Vukavić [38]
3	Adults, n = 30 Age: 18–71 years Sex: 12 women, 18 men Ethnicity: no data Country: Germany	GS (n = 30)	No data	After 2 days of abstinence participants drank 0.1 L of sparkling wine (9 g ethanol)	Anthropometric measurements; Lifestyle and Eating Habits Questionnaire Urine: EtG, EtS, creatinine Venous blood: morphology, ethanol, TB, CB, liver enzymes	In 69% of the children, the uroflowmetric pattern was expected, and after 24 h on a hypercaloric diet, it was typical in all children. Children with an abnormal uroflowmetric pattern had higher CB levels after 24 h of fasting than children with a regular pattern. There were no differences in TB and UCB levels. CB levels were lower pre-fasting than post-fasting in children with normal and abnormal UFC, while glucose levels were higher. Bladder capacity (VC) in children was average after 24 h of fasting and after 24 h of hypercaloric intake.	TB > 0.67 mg/dL	Huppertz et al. [39]

Table 2. Cont.

Jadad Scale	Participants	Group	Duration	Intervention	Analysis	Results	Total Bilirubin	References
2	Obese adults n = 10; Age: 58.4 years Sex: 7 women, 3 men; Ethnicity: no data Country: USA	GS (n = 10) with cirrhosis and nonalcoholic steatohepatitis (NASH).	>4 year (8.7 ± 1.4 year)	Bariatric surgery (laparoscopic sleeve gastrectomy or Roux-en-Y gastric bypass)	Body mass, weight; Venous blood: HbA1c, Plasma: albumin, TB, Indices: TBWL, BMI, INR, MELD-Na	The patient with GS experienced a TBWL of 23.5% and a reduction in BMI of 7.7 kg/m ² . Despite a decrease in HbA1c concentration of 1.3%, insulin treatment was necessary. An increase in TB concentration of 1.4 mg/dL of blood was observed. The MELDNa Score for End-Stage Liver Disease in the patient with GS was 14, indicating a 6% mortality risk.	TB > 0.6 mg/dL	Izzy et al. [40]

Jadad scale—0–5. Explanations are in the Abbreviations section.

In the 1970s, Gollan et al. [34] hypothesized, based on contemporary studies, that dietary factors and fasting could influence the increase in blood bilirubin levels. They conducted a study to determine whether unconjugated hyperbilirubinemia in patients with Gilbert's syndrome arose due to the nature of the diet or the route of administration and to assess the relationship between these factors and fasting-induced hyperbilirubinemia. The experiment was conducted on 29 patients (both genders) aged 14–46 years, who were given a normal diet (I) with an energy value of 10 MJ (2400 cal) before the study, and their plasma total bilirubin (TB) concentration was measured. Patients were then given six test diets: (II, $n = 8$) low-energy, standard (1.6 MJ, 400 cal); (III, $n = 5$) low-energy, standard + IV glucose (10 MJ, 2400 cal); (IV, $n = 5$) low-energy, standard + IV lipid (8.4 MJ, 2000 cal); (V, $n = 7$) high-carbohydrate, low-lipid (fluid) (10 MJ, 2400 cal); (VI, $n = 4$) high-carbohydrate, reduced-lipid (10 MJ, 2400 cal); (VII, $n = 5$) low-energy, high-lipid (1.6 MJ, 400 cal). The fluid diet contained only 2 g of lipids (0.6% of total energy intake) and mainly consisted of glucose polymer, fruit juice, and skim milk. After the test period, patients resumed a normal diet, and their blood TB levels were measured for one or two days. The mean TB concentration in the blood of GS patients before the normal diet was comparable to the TB concentration after 2 days of the diet (34.0 vs. 31.1 $\mu\text{mol/L}$, respectively), with no diurnal variations. The highest increase in TB concentration was observed in patients after diet II (low-energy 400 cal) and diet III (low-energy with IV glucose 2400 cal), with increases of 135% and 127%, respectively. The high-carbohydrate fluid diet resulted in a 76% increase in blood TB levels in patients, whereas the low-energy, high-lipid diet led to a 49% increase. The lowest increase in blood TB concentration was observed with the high-carbohydrate, reduced-lipid diet (8%), which was similar to the normal diet. The level of hyperbilirubinemia achieved after oral carbohydrate administration was significantly ($p < 0.025$) lower than that after intravenous glucose administration or the standard low-energy diet ($p < 0.001$). The low-energy, high-lipid diet (VII) significantly ($p < 0.001$) reduced hyperbilirubinemia to levels lower than those observed with the standard 400-calorie diet. According to the authors, excluding lipids from the diet in some patients receiving parenteral nutrition or during the postoperative period may explain the occurrence of unconjugated hyperbilirubinemia. Furthermore, additional research on neonatal hyperbilirubinemia is warranted, as some initial feeding regimens low in lipids could exacerbate hyperbilirubinemia. Therefore, it is advisable to provide diets with a small amount of lipids to preterm infants/newborns with jaundice.

Peterson et al. [35], in their studies, assumed that UGT1A1 also conjugates 17β -estradiol (the most biologically active estrogen) and estriol, as well as exogenous compounds such as phenols, anthraquinones, and flavones, many of which are found in the diet. The polymorphisms in UGT1A1 may influence bilirubin clearance, endogenous estrogenic load, and exposure to xenobiotics (as well as heterocyclic amines and polycyclic aromatic hydrocarbons). Animal model studies have shown that biologically active vegetable substances (such as sulforaphane, protein and soy isoflavones, dietary monoterpenes, and allyl sulfides) increase UGT activity in the liver. Therefore, the authors evaluated the impact of consuming foods from the botanical families *Cruciferae* (e.g., broccoli), *Rutaceae* (citrus fruits), *Liliaceae* (e.g., onion), and *Leguminosae* (legumes) on UGT activity in humans by measuring blood bilirubin levels, depending on genotype. The recruitment criterion for the study was the consumption of 2.5 or 4.5 servings per day of *Cruciferae* and *Leguminosae*. The study was conducted on three individuals (both sexes, aged 19–40 years) and included demographic data, health history, a 3-day food record, blood TB and CB measurements, and UGT1A1 promoter genotype determination. Vegetable consumption from the four botanical families was assessed according to standardized portions. Participants were divided into genotype groups 6/6 or 6/7 ($n = 169$) and 7/7 ($n = 22$) because previous studies have shown that the greatest difference in UGT1A1 activity is between 7/7 and the presence of one or more 6 alleles; thus, the 6/6 and 6/7 genotypes were combined for the analysis. Total fruit consumption ranged from 0 to 11 servings and vegetables from 0 to 13 servings per day. Consumption of products from the *Cruciferae*, *Rutaceae*,

and *Leguminosae* groups ranged from 0 to 4 servings per day, while *Liliaceae* ranged from 0 to 2. There were significantly ($p < 0.001$) higher TB, CB, and UCB concentrations in men than in women. There was a significant inverse association between BMI and CB ($p < 0.001$). There was no significant difference in the consumption of groups of botanical plants depending on the genotype of the respondents. The authors found no significant associations between TB and intake of carbohydrates, protein, fat, alcohol, total fruit, and vegetables when controlling for UGT1A1 genotype, age, sex, and BMI. Similarly, there was no association between TB, CB, or UCB concentrations in the blood of the subjects and any botanical plant group. However, there was a significant inverse association between TB, CB, and UCB ($p < 0.017$) and the interaction of the UGT1A1*28 genotype with *Cruciferae* intake. There was also a significant inverse association between the UGT1A1-Cruciferae gene interaction and CB ($p < 0.003$) and UCB ($p < 0.020$), but the authors did not present these data. Individuals with the 7/7 genotype had lower blood bilirubin levels with increased consumption of cruciferous vegetables compared to those with 6/6 or 6/7 variants. The authors explained the observed changes by noting that glucosinolates in cruciferous vegetables can be hydrolyzed by the plant enzyme myrosinase to indoles and isothiocyanates when plant cells are damaged (e.g., during cutting or chewing). Indoles bind to AhR (aryl hydrocarbon receptor); sulforaphane induces UGT1A1 by modulating the extracellular signal-regulated kinase mitogen-activated protein kinase pathway in Caco-2 cells. Additionally, although the interaction with cruciferous compounds has not been studied, nuclear receptors, the pregnane X receptor, and the constitutive androstane receptor influence UGT1A1 expression and activity. According to the authors, their study confirmed that individuals with reduced UGT1A1 activity and the 7/7 genotype might, through dietary intervention, have greater potential to reduce the risk of carcinogenesis concerning carcinogens such as heterocyclic amines and polycyclic aromatic hydrocarbons conjugated by this enzyme.

The continuation of the research by Peterson et al. [35] was the study published by Navarro et al. [36]. The authors justified their research by stating that individuals with the UGT1A1*28 polymorphism (characteristic of GS) have reduced transcription compared to the wild type, resulting in decreased enzyme UDP-glucuronosyltransferase (UGT) activity. Such individuals have an increased risk of developing cancer (especially stomach and lung cancer), and chemoprevention is therefore advisable. One group of foods with such properties is cruciferous vegetables (*Brassicaceae*), as the isothiocyanates they contain partially increase the activity of phase II conjugating enzymes, such as UGT. Isothiocyanates, especially GSTM1, are metabolized by glutathione S-transferases, but genetic variants can alter their clearance. In individuals with the GSTM1-null and GSTT1-null genotypes, isothiocyanates are metabolized more slowly, leading to a greater accumulation of isothiocyanates at the tissue level; thus, they are likely to a greater chemoprotective effect. The aims of the study were to determine the effect of cruciferous vegetable consumption and two different doses on bilirubin conjugation as a measure of UGT1A1 activity and to assess the impact of the GSTM1-null and GSTT1-null variant or the UGT1A1*28 polymorphism on this response. A secondary aim of the study was to evaluate whether adding apiaceous vegetables to cruciferous foraging further alters this response. In a study conducted among 70 individuals (aged 20–40 years), the researchers assessed a feeding trial of four diets: (i) a basal diet devoid of fruit and vegetables, (ii) the basal diet supplemented with a prescribed amount of cruciferous vegetables (single-dose cruciferous diet); (iii) the basal diet supplemented with twice this amount of cruciferous vegetables (double-dose cruciferous diet); (iv) and the basal diet supplemented with cruciferous and apiaceous vegetables (single-dose cruciferous plus apiaceous vegetable diet). The single dose of cruciferous vegetables was approximately 7 g/kg body weight, the double dose was approximately 14 g/kg, and the mixed diet included 7 g/kg of crucifers plus 4 g/kg apiaceous vegetables. Diets were first standardized according to servings for a 70 kg individual, determining g food/kg body weight for each plant item. Food was served as meals under the supervision of the research staff or provided for the weekends. Participants kept food diaries, and

plate waste was recorded. Buccal cells, collected before randomization, were isolated, and DNA was extracted to determine GSTM1 and GSTT1 genotypes and participant eligibility. Genotyping of the white blood cell DNA for the UGT1A1 polymorphism was conducted. On days 0, 7, 11, and 14 of each feeding period, blood was drawn in the morning after a 12 h overnight fast to measure TB and CB and calculate UCB. On day 13 of each feeding period, participants collected urine for 24 h to assay urinary total isothiocyanates. Before the experimental diets were implemented, no significant differences in demographic parameters were observed between the groups of individuals depending on the UGT1A1 genotype. However, individuals in the group with the UGT1A1*28/28 genotype had significantly ($p < 0.02$) higher levels of TB and CB compared to the other genotypes (UGT1A11/1 and UGT1A11/*28).

The response to all three diets containing vegetables differed statistically significantly from the basal diet, with lower mean TB levels associated with consuming the test plant diets ($p < 0.02$ for all three), suggesting more excellent UGT1A1 enzyme activity. There was also a dose effect of cruciferous vegetables in the diet, as significantly ($p < 0.03$) lower mean TB levels were found in individuals consuming the double dose of cruciferous vegetables compared to the single dose. The same pattern was observed for UCB, but there were no differences in CB; however, the authors did not present these results. The interaction between diet and gender was not significant. Considering the UGT1A1 genotype variant, significant differences in TB levels were found between individuals with *1/*1 (40%) and *28/*28 (28%) on the basal diet ($p < 0.01$) and single-dose cruciferous vegetable diets ($p < 0.03$). The differences between *1/*1 and *1/*28 were statistically significant only on the single-dose cruciferous diet, with *1/*28 having 10% higher bilirubin concentrations than *1/*1 ($p = 0.02$). Comparing the differences in bilirubin response to diets between groups, individuals with *28/*28 responded slightly better to the single-dose cruciferous diet compared to those with *1/*28 ($p = 0.049$) and the apiaceous diet ($p = 0.03$). Although UGT1A1 activity, measured by serum bilirubin levels, was higher in individuals with GSTM1-null compared to GSTM1+ individuals, it was not statistically significant. There was no statistically significant main effect of the combined GSTM1 and GSTT1 genotypes or the genotype-by-diet interaction. Analysis of urine collected from patients on day 13 of the diet indicated a steep and dose-dependent increase in isothiocyanate excretion compared to the period on the basal diet. The sample collection day had a significant ($p < 0.001$) effect, but the interaction of individual diets was not statistically significant. Dietary supplementation with vegetables resulted in lower serum bilirubin concentrations on days 11 and 14 compared to day 7 ($p < 0.01$), but its concentrations slightly increased from days 11 to 14 ($p = 0.04$). The relationship between CYP1A21F and bilirubin was not statistically significant, and the data were not presented. According to the authors, the increased UGT1A1 enzyme activity and reduced serum bilirubin levels in the studied individuals were contributed to by cruciferous vegetables containing a mixture of isothiocyanates and indoles and apiaceous vegetables containing furanocoumarins. Therefore, these findings may impact the metabolism of carcinogens through dietary intervention, particularly among individuals with UGT1A128/*28.

Saracino et al. [37] conducted a study whose aim was to detect the interaction between the genotype and the consumption of citrus fruits, cruciferous vegetables, and soy (and phytochemical components contained in these products), inducing UGT (UDP) activity on the concentration of bilirubin in the blood. Initially, the study was conducted among 293 adults of both sexes (125 women and 146 men aged 20–40 years) living in the Seattle area, but not all completed the study. Participants completed a Food Frequency Questionnaire (FFQ) covering 3 months ($n = 274$) and provided 3-day food records ($n = 289$) to assess fruit and vegetable (F&V) intake, categorized by botanical families (*Cruciferae*, *Rosaceae*, *Solanaceae*, *Leguminosae*, *Rutaceae*, *Umbelliferae*). *Cruciferae* are rich in sulfur-containing glucosinolates, and the *Solanaceae* and *Rutaceae* families contain carotenoids and many flavonoids. Fruit in the *Rosaceae* family are also abundant in flavonoids, and legumes are rich in lignans. Participants ($n = 283$) underwent anthropometric measurements (body mass

and height), and after a 12 h fasting period, blood was drawn to determine TB, CB, and UCB concentrations and to genotype UGT (UGT1A1 genotype 6/6; 6/7; 7/7) in leukocyte DNA. It was found that gender (men vs. women), race (Asian vs. Caucasian), and UGT1A1 genotype were significantly associated with serum concentrations of all three bilirubins. TB and UCB levels were significantly ($p < 0.05$) lower among non-Caucasian races. There was a linear trend of increasing TB, CB, and UCB ($p < 0.001$) with more UGT1A1*28 alleles. Age (<30 or ≥ 30 years) and duration of physical activity (≤ 6 or >6 h/week) were not associated with any form of bilirubin concentration. There was no significant association between total fruit and vegetable intake servings (<4 , 4 – 5 , and >5 per day) estimated from the FFQ and TB levels. However, among women compared to men, an interaction was found between UGT1A1 genotype and citrus fruit intake ($p = 0.006$) and *Rutaceae* ($p = 0.03$) assessed by 3DFR, related to TB and UCB concentrations. Women with the 7/7 genotype who consumed approximately 0.5 daily servings of citrus fruit or *Rutaceae* had lower (approximately 30%) TB, CB, and UCB concentrations than those who consumed less. The authors explain this via the beneficial effect of phytochemicals (such as quercetin) found in citrus fruits, which increase UGT activity by inducing gene expression. The authors also suggest that women consuming more citrus fruits with phytochemicals had lower serum bilirubin levels than men (regardless of UGT1A1 genotype) due to estrogen and/or progesterone-mediated UGT1A1 transcription. The estrogen receptor (ER)-mediated increases in transcriptional activity of the aryl hydrocarbon receptor (AhR) affect UGT1A1. Recent fruit and vegetable intake (estimated by 3DFR) helps detect associations between fruit and vegetable intake and blood bilirubin levels, as changes in UGT expression/activity can be detected within 1 h of phytochemical exposure. The described studies indicated that some components of fruits and vegetables might increase UGT1A1 activity in individuals with the 7/7 genotype variant, which is most common in GS, thereby potentially improving the clearance of certain carcinogens from the body and reducing cancer risk.

Stojanović and Vukavić [38] assumed that high blood bilirubin levels (due to its antioxidant stimulation of nitric oxide production and its relaxing effect) might decrease the baseline tension of bladder and ureter muscles, potentially leading to urinary retention and stasis, predisposing to urinary tract infections (UTIs). They assessed the influence of hyperbilirubinemia in children with Gilbert syndrome (GS) on their lower urinary tract function. The study included 29 children aged 8–17 years (both sexes) who underwent a diagnostic 24 h fasting test (with zero energy intake and water intake ad libitum), followed by a 24 h hypercaloric intake (30% higher energy intake than average for their age). Blood samples were collected from the children at 0, 12, and 24 h fasting and after 24 h of hypercaloric intake to measure glucose concentration, TB, CB, UCB, and urinary ketones. Uroflowmetry was performed after 24 h of fasting (experimental) and after 24 h of a hypercaloric diet (control). According to the survey, 28 children had no history of UTIs, and 29 had no history of constipation. A bell-shaped uroflowmetry pattern was typical in 69% of the children, while 31% had an abnormal pattern. All patients showed a regular uroflowmetric pattern after 24 h of hypercaloric diet intake. Patients with an abnormal uroflowmetric pattern had higher CB concentrations (11.6 vs. 9.35 $\mu\text{mol/L}$) after 24 h of fasting than those with a regular pattern ($p = 0.012$). There were no significant differences in TB and UCB concentrations. In children with a standard urinary flow curve (UFC), CB concentrations were significantly lower pre-fasting compared to post-fasting (7.06 vs. 9.35 $\mu\text{mol/L}$, $p = 0.022$), and glucose levels were significantly higher (4.51 vs. 3.76 mmol/L). Similarly, in children with an abnormal UFC, CB concentrations were significantly lower pre-fasting compared to post-fasting (7.91 vs. 11.60 $\mu\text{mol/L}$, $p = 0.008$), and glucose levels were higher (4.56 vs. 3.29 mmol/L). The children's bladder capacity (VC) was normal after fasting and consuming a hypercaloric diet. However, the authors did not present the results of urinary ketone levels in the urine of the children studied. According to the authors, hyperbilirubinemia, with or without hypoglycemia, may cause intermittent dysfunction of the lower urinary tract in children. The significantly higher CB concentrations in patients

with abnormal UFC than those with normal UFC after 24 h of fasting require confirmation in a larger patient cohort.

In their study, Huppertz et al. [39] aimed to determine the potential impact of Gilbert syndrome (GS) on the formation of ethyl glucuronide (EtG) as a direct marker of ethanol consumption and a product of glucuronidation, both alone and in combination with ethyl sulfate (EtS), used for monitoring abstinence in individuals. The research involved 30 individuals (both genders, aged 18–71 years) with GS, from whom urine samples were collected after at least 2 days of abstinence from alcohol. Participants were instructed to drink 0.1 L of sparkling wine (9 g of ethanol) within 5 min. Anthropometric measurements were taken from the patients, and they were asked to complete a lifestyle questionnaire, including alcohol consumption behaviors. Urine samples were collected at 3, 6, 12, and 24 h post-ethanol consumption to measure EtG, EtS, and creatinine levels. Blood samples from the patients 3 h after wine consumption were analyzed for morphological parameters, ethanol concentration, TB, CB, and liver enzymes. The BMI of the patients ranged from 15.4 to 27.3 kg/m². Ten individuals declared mainly wine consumption (from one glass to two bottles per week), thirteen preferred beer (from two to ten bottles per week), and only one patient regularly consumed larger quantities (≥ 2 drinks per day, more on weekends). No significant abnormalities were found in the urogenital system or blood morphology. AST activity was within the normal range for all patients, with one patient having slightly elevated ALT (above the range of 10–50 U/L), and five patients had increased GGTP levels. Elevated TB concentrations were observed in 21 patients, while UCB levels were normal in 9 patients. EtG and EtS were detected in all patients across a wide range of concentrations (EtG: 3 h sample 0.5–18.43 mg/L and 6 h sample 0.67–13.8 mg/L; EtS: 3 h sample 0.87–6.87 mg/L and 6 h sample 0.29–4.48 mg/L). There was no evidence of impaired EtG formation. Mutations in the UGT1A1 isoform in GS do not affect the overall formation of ethyl glucuronide. Therefore, EtG appears to be a suitable marker of ethanol consumption even in individuals with GS. Gilbert syndrome also does not affect the formation of EtS.

Izzy et al. [40] presented intriguing findings from their study assessing long-term outcomes of bariatric surgery beyond 4 years (mean 8.7 years $\pm$ 1.4 years) in 10 patients (7 women and 3 men, with a mean age of 58.4 years $\pm$ 9.6 years) with compensated liver cirrhosis confirmed by a liver biopsy showing nonalcoholic steatohepatitis (NASH). Among these patients, nine experienced an average total body weight loss (TBWL) of 24 kg (19.2% TBWL before surgery, $p < 0.001$), while one patient had an increase in body weight. Nine patients showed decreased BMI, improved HbA1c levels in those with abnormal glycemia, and reduced total bilirubin (TB) levels. None of these individuals experienced liver decompensation (development of ascites, hepatic encephalopathy, or variceal bleeding), cardiovascular events, or mortality. The MELDNa score (Model for End-Stage Liver Disease incorporating sodium levels) was 9 (± 6) for most patients, indicating a low mortality risk (1.9%). In contrast, in the patient with Gilbert syndrome (GS), long-term follow-up after bariatric surgery showed a TBWL of 23.5%, a decrease in BMI of 7.7 kg/m², and a reduction in HbA1c levels by 1.3%. However, despite the decrease in HbA1c, insulin treatment was still necessary. Additionally, there was a 1.4 mg/dL increase in TB levels. The MELDNa score for this patient was 14, reflecting a 6% mortality risk.

According to the authors, bariatric surgery in patients with compensated liver cirrhosis can lead to beneficial and sustainable weight loss, improvement or stabilization of coexisting metabolic diseases, and stable liver function, even many years post-surgery. The benefits achieved outweigh the surgical risks in such patients.

4. Discussion

Caloric restriction/fasting is a component of human nutrition. However, in the case of Gilbert syndrome, it has primarily been used as a diagnostic tool (provocative tests) for this condition. Studies have shown a correlation between caloric intake and hyperbilirubinemia (impairing the liver's ability to glucuronidated bilirubin by reducing the formation of UDPGA—uridine diphosphoglucuronic acid) and fluctuations in serum bilirubin levels

in individuals with GS [22]. Reducing energy intake to 300–400 cal/day, which led to increased TB and UCB levels in patients, allowed for the differentiation of GS patients from those with other liver diseases [23]. Researchers attempted to understand the physiological mechanism behind these changes. It was found that changes in bilirubin levels were not due to increased heme turnover [24] or bile acid levels [27] as they remained within reference ranges regardless of the degree of hyperbilirubinemia in GS patients. Subsequent studies aimed to determine whether changes in macronutrient composition, aside from caloric restriction, could contribute to hyperbilirubinemia. However, no such effects were observed when considering nitrogen balance and lipid metabolism [26]. However, a low-energy, high-lipid diet was found to reduce hyperbilirubinemia to levels lower than those caused by the standard 400 cal/day diet [34].

Since caloric restriction (400 cal/day) proved to be a critical diagnostic element for GS, it was found that the form of caloric intake affected the degree of hyperbilirubinemia. The increase in TB levels was more significant after sucrose administration compared to a volumetric meal (bran), suggesting that gastrointestinal hormones influenced hepatic clearance in this context [28]. It was essential to establish the duration of caloric restriction necessary to maintain hyperbilirubinemia (measured by TB and UCB levels). It was shown that TB and UCB levels were significantly higher in the first few days of fasting compared to before fasting [25], while CB levels remained similar. Extending the fasting period up to 30 days (16 h/day during Ramadan) did not result in significant changes in bilirubin fractions [30]. In the search for more sensitive tests for GS diagnosis [29], a nicotinic acid test was used in addition to caloric restriction. This test proved to be more effective (in both genders) at inducing hyperbilirubinemia, particularly at 240 min after drug administration, compared to fasting-induced hyperbilirubinemia following caloric restriction (400 cal/day).

Before the introduction of molecular diagnostics for GS, provocative tests were performed, including caloric restriction, nicotine, phenobarbital, rifampicin, or the “inverse starving test” (after a standard European lunch). However, these tests are now historically significant as they are characterized by low sensitivity and specificity [41,42]. Fasting for 24 h and reducing daily caloric intake by 300 calories, as well as the rifampicin test administered after 2 or 4 h, which shows a significant increase in unconjugated bilirubin (UCB), suggest GS-related hyperbilirubinemia [43]. In healthy individuals, total bilirubin concentration increases by about 60% after 48 h of fasting, whereas in those with GS, its concentration increases by about 90% after 24 h, and UCB concentration increases by over 110% after 24 h [3]. Other methods for diagnosing GS include measuring bilirubin kinetics after intravenous injection of cold crystalline pigment or radiolabeled bilirubin or computing via mathematical methods from the survival of ⁵¹Cr-labeled erythrocytes, which provides the most reliable documentation of impaired bilirubin clearance [30].

Additional diagnostics for GS include the invasive procedure of percutaneous liver biopsy with evaluation of the biopsy specimen, although this is not routinely recommended [16]. Histological examination typically reveals pathognomonic changes such as flattening and damage to the microvilli on the vascular pole of hepatocytes, mitochondrial polymorphism, and an increase in endoplasmic reticulum and glycogen. Histopathology typically reveals non-specific lipofuscin pigment within the centrilobular region of the biopsy specimen; otherwise, histology is normal. Currently, the diagnosis of GS is definitively confirmed by molecular testing showing the presence of mutations in the UGT1A1 gene. It should be emphasized that in the case of isolated hyperbilirubinemia, it is essential to exclude hemolytic anemia before making a final diagnosis of liver-origin hyperbilirubinemia [44,45]. Such diagnostic rigor is particularly warranted in cases where GS is familial. Furthermore, other tests such as abdominal ultrasound, hepatobiliary scintigraphy, cholangiography, and/or cholecystography do not reveal abnormalities in individuals with GS compared to healthy individuals [16].

A broader aspect of nutritional status and dietary habits, combined with blood morphological parameters and genetic studies, was first considered in women with GS in the studies by Rodrigues et al. in 2012 [31]. They found that the c.-41_-40dupTA allele (genetic),

HGB, BMI, and fasting duration were the main factors associated with blood bilirubin levels. HGB can be a diagnostic marker in GS, as its increase in the blood follows the rise in bilirubin levels. Interesting findings regarding the metabolic health of individuals with GS were also obtained by Mölzer et al. [32], who demonstrated that people with GS had lower BMI, lower blood glucose, insulin, C-peptide, and triacylglycerol levels compared to healthy controls and were less prone to metabolic diseases or premature death. This positive aspect of metabolic health in GS individuals can be explained by the fact that bilirubin is a physiologically important antioxidant and might assist in neutralizing ROS (reactive oxygen species) and preventing oxidative damage. Wagner et al. [46] demonstrated that in 119 individuals, there were significantly higher serum unconjugated bilirubin (UCB) concentrations, lower BMI, 37% higher antioxidant potential as assessed by iron reduction capacity potential (FRAP), elevated advanced protein oxidation products, and lower levels of apolipoprotein B, hs-C-reactive protein, interleukin 6, and interleukin 1 beta compared to 119 healthy individuals. Unconjugated bilirubin might also have anti-genotoxic potential by preventing oxidative damage to DNA [47]. Interestingly, studies have shown a reduced risk of obesity and its health complications [48], i.e., cardiovascular diseases (CVD) such as ischemic heart disease and hypertension and type 2 diabetes, in individuals with GS, less pronounced pro-inflammatory processes [49–51], and lower levels of blood lipids and lipoproteins [50,52–56]. Serum metabolomics analysis reveals increased lipid catabolism in mildly hyperbilirubinemic GS individuals [46]. The reduced cardiovascular risk and mortality may be due to the lower body mass index (BMI) in individuals with GS [53]. Hyperbilirubinemia is also associated with a lower risk of metabolic syndrome and non-alcoholic fatty liver disease (NAFLD) and may protect against the development of non-alcoholic steatohepatitis (NASH) [57,58].

Moreover, mild unconjugated hyperbilirubinemia may have beneficial effects, such as reducing the risk of endometrial cancer, lung cancer, breast cancer, colorectal cancer, and Hodgkin's lymphoma, as well as decreasing cancer-related mortality, owing to the antioxidant and protective properties of bilirubin [2,12,59–61]. Considering the impact of intestinal microbiota composition on decreased colorectal cancer risk, Zöhrer et al. [62] found no significant differences between 45 individuals with Gilbert's syndrome (GS) and 45 healthy individuals. However, they observed greater bacterial diversity among women. Interestingly, in men with Gilbert syndrome (GS), who chronically have elevated unconjugated bilirubin (UCB) levels in their blood, longer telomeres have been observed, which are potential biomarkers of cellular aging, apoptosis, and age-related diseases [63].

Taking into account the positive impact of mild hyperbilirubinemia on reducing the risk of diseases related to oxidative stress, scientists are conducting studies on inducing "Iatrogenic Gilbert syndrome" using drugs (such as probenecid, rifampicin, valproic acid, osimertinib, atazanavir, biliverdin), as well as nutraceuticals (phycobilins from plants, algae, and cyanobacteria) that reduce the activity of hepatic glucuronidation [63,64].

Research by Vajro et al. [33] also underscores that caloric restriction and genetic testing, such as analysis of the 2-bp (TA) insertion in the promoter of the bilirubin glucuronosyltransferase enzyme gene, can be valuable tools for early detection in children and adolescents before puberty of the risk of transmitting GS from donor to liver transplant recipients. Currently [63], the diagnosis of GS includes the following: isolated, asymptomatic unconjugated hyperbilirubinemia (17–70 $\mu\text{mol/L}$ [1–4 mg/dL]); liver enzyme activities (ALT, AST, ALP, GGT) within physiological range; absence of overt signs of hemolysis (reticulocyte count <1.5%, negative Coombs test); and genetic testing reserved for individuals with higher serum bilirubin concentrations to differentiate GS from Crigler–Najjar syndrome type 2 or when considering treatment with medications that affect UGT1A1 activity.

In other studies, attention was drawn to caloric restrictions and their impact on hyperbilirubinemia, lipid metabolism, and vitamin levels. Kamal et al. [3] conducted a screening survey among 1249 Egyptians (aged 14–45 years) and identified 101 (8.016%) individuals with GS, including 83 with GS and 18 with GS after excluding HCV. All

reported episodes of jaundice lasted, on average, 19–21 days. Among adult Egyptians with GS, provocative tests (fasting 300 cal/day; with rifampin), nutritional status assessments, UGT1A1 genotyping, liver function tests, and measurements of blood levels of vitamins B12, folate, and D3 were conducted. A two-week diet was implemented, and bilirubin levels were measured on days 0, 7, and 14 of the diet. Folate and vitamin D3 levels were lower in individuals with GS compared to the control group, whereas B12 did not differ significantly. Individuals with GS had significantly lower levels of VLDL and IDL. Fasting > 12 h and a diet < 1200 cal significantly influenced the occurrence of jaundice episodes, while a high-fat diet did not.

Kiranmayi et al. [65] presented interesting data on dietary faddism. This term refers to a specific food or food group that is either overly emphasized or omitted in the regular diet for treating a particular condition. The study focused on the impact of dietary trends among 125 patients (both genders) from India diagnosed with GS, characterized by consistently elevated unconjugated bilirubin (UCB) levels with normal liver transaminases, ceruloplasmin, and serum hemoglobin, without liver discomfort or other chronic diseases. Initially, over 6 months (monthly), the study monitored blood levels of vitamin B12 before and after treatment with cyanocobalamin injections. Vitamin B12 deficiency (megaloblastic anemia) was found in 21 patients as a result of dietary restrictions stemming from nutritional myths, leading to ineffective erythropoiesis and prolonged hyperbilirubinemia. The requirement of methylcobalamin explained the occurrence of anemia in individuals with GS as a cofactor in methionine synthase for synthesizing pyrimidines and purines. Folic acid, essential for this process, transfers a methyl group from methyl-tetrahydrofolate to homocysteine, forming methionine and tetrahydrofolate. Disruption of this reaction leads to megaloblastic anemia. Ineffective erythropoiesis exacerbates existing indirect hyperbilirubinemia in GS patients due to hemolysis. Methylmalonyl-CoA mutase converts methylmalonyl-CoA to succinyl-CoA, requiring 5-deoxyadenosylcobalamin as a cofactor. Defects in this reaction lead to the accumulation of methylmalonyl-CoA, believed to be responsible for the neurological consequences of vitamin B12 deficiency. Symptoms observed in GS patients included fatigue and limb paresthesia. Administering vitamin B12 injections for a month improved blood parameters and reduced the occurrence of neurological symptoms.

In the studies included in this systematic review, significant and practical findings were reported by Peterson et al. [35], Navarro et al. [36], and Saracino et al. [37]. These researchers identified the impact of consuming fruits and vegetables containing various biologically active compounds from different botanical groups on serum bilirubin levels and the ability to enhance UDP-glucuronosyltransferase (UGT) enzyme activity in the liver through gene expression induction, taking into account genetic differences among patients. Their results indicate that the consumption of vegetables from the *Cruciferae* and *Apiaceous* groups and fruits from the *Rutaceae* group by individuals with reduced UGT1A1 activity and the 7/7 genotype may increase UGT enzyme activity in the liver. Such dietary interventions may also reduce the risk of carcinogenic factors associated with this enzyme, such as heterocyclic amines and polycyclic aromatic hydrocarbons. However, the observed effect, characterized by a decrease in serum bilirubin levels, depends on the recommended dose of plant consumption, with the double dose of vegetables proving most effective, along with the appropriate timing of consumption.

From the studies included in the review, several weaknesses emerged, such as older studies relying on observation without a control group; inconsistent criteria for inclusion and exclusion; varying durations of dietary interventions; selection bias in patient groups based on gender, age, BMI, and coexisting conditions, thus potentially not producing representative samples; limited groups with the typical GS genotype (UGT1A1*28 7/7); lack of consideration for ethnic groups; discrepancies between reported food intake by patients and actual consumption, with more accurate reflection in 3-day records than in food frequency questionnaires (FFQs); inability to accurately estimate phytochemical intake due to incomplete data in nutrition databases; consumption levels of vegetables from *Cruciferae* and *Apiaceous* plants, e.g., 5–10 servings per day (~300 g–1300 g), generally not

met by residents in various regions worldwide; and the extensive but sometimes conflicting data on the mechanisms of plasma bilirubin response to caloric deprivation.

From the studies included in this systematic review, specific strengths have emerged: newer studies employed a control group and specified criteria for inclusion and exclusion; there was homogeneity among participant groups regarding non-dietary factors influencing UGT enzyme activity such as age, gender, ethnic group, BMI, alcohol and tobacco consumption, dietary supplements and medications, pregnancy or lactation, known allergies/intolerances, weight changes exceeding 4.5 kg in the past year, significant changes in eating habits within the past year (e.g., adoption of a faddish diet), antibiotic use within the past 3 months, food preferences that could affect participation in feeding trials, and other medical conditions; a wide range of fruits and vegetables consumed by participants based on portion size criteria was considered; in some studies, the duration for each tested diet exceeded 2–3 days; blood samples were taken during tested diets, and analysis of multiple blood parameters (morphological, biochemical) and genotypes better illustrated the impact of the applied dietary intervention. Therefore, we agree with Vitek [63] that bilirubin concentrations in both the general population and in people with GS change not only depending on gender, ethnicity, age, smoking, circadian rhythms, seasonal periods, and physical activity but also on nutritional influences.

Therefore, in our opinion, further research should focus on the following: (1) identifying food compounds that could regulate bilirubin levels in individuals with GS by influencing the expression of the hepatic UGT enzyme gene; (2) determining whether UGT1A1 variations other than the TA duplication in the promoter region can affect total bilirubin levels in blood; (3) investigating other types of food that may influence the conjugation of carcinogenic factors in individuals with the UGT1A1*28 7/7 genotype, aiming to reduce the risk of cancer development. However, such studies should be conducted in larger populations or among recruited patients based on the UGT1A1 genotype, encompassing different ages, genders, and ethnic groups. The existence of differences in levels of certain biochemical parameters depending on biogeographical origin underscores the necessity to establish specific reference ranges for each. Therefore, we agree with Vittek's opinion [63] that it would be prudent to create international registries compiling research data that describe the impact of nutritional and non-nutritional factors and the relationships between them on the health-related quality of life of patients with Gilbert's syndrome and/or comorbidities.

5. Strength

This is the initial systematic review of nutrition in Gilbert's syndrome, which spanned up to 60 years and was grounded in clinical studies. This review was conducted following the PRISMA guidelines by three independent researchers who evaluated the internal quality of the selected reports. The findings of this review predominantly include randomized clinical trials, thus boasting excellent reliability and serving as the gold standard for assessing intervention efficacy. Moreover, applying the Jadad scale in this review provides an objective framework, minimizing subjective biases.

Considering the precision and reliability in assessing the methodological quality of the studies, we employed the five-point JADAD scale. Our findings indicate that among the reviewed randomized controlled trials, nine scored 2 or lower, seven were rated as medium quality (scoring 3), and three achieved a score of 4, indicating high-quality clinical trials. None of the studies reached the maximum of 5 points on the JADAD scale. This underscores the imperative for future studies to adopt transparent and well-defined methodologies to ensure the rigorous evaluation of interventions in clinical research. Notably, while children and adults were studied, only two trials specifically addressed individuals aged 60 and above.

6. Conclusions

This systematic review suggests that non-pharmacological therapy, such as dietary intervention, may benefit patients with Gilbert's syndrome. Such interventions should include recommendations to avoid excessive caloric restriction, mono-diets, and nutritional deficiencies (especially vitamins B12, D, and folic acid). Simultaneously, consuming biologically active compounds in vegetables and fruits (*Cruciferae*, *Apiaceous*, *Rutaceae*) could regulate serum bilirubin levels, thereby preventing jaundice episodes. It is also pertinent to emphasize to individuals with GS the importance of adopting a lifestyle that minimizes exposure to triggers of this condition. Furthermore, there remains a scientific challenge to identify food compounds capable of influencing the expression of the liver enzyme UGT, which could contribute to bilirubin regulation in individuals with GS.

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Abbreviations

ALP—Alkaline phosphatase; ALT—alanine transaminase; AMPK—heterotrimeric AMP—activated ser/thr kinase; AMPK1a expr.: AMPK gene expression as relative quantification; AST—aspartate aminotransferase, AST; BIA—bioelectric impedance analysis; BMI—body mass index; BW—body weight; CB—conjugated bilirubin (direct); CLDQ—Chronic Liver Disease Questionnaire; CO—carbon monoxide; CVD—cardiovascular disease; EtG—ethyl glucuronide; EtS—ethyl sulfate; FH—fasting/hyperbilirubinaemia test; GD—Gilbert's disease; GGTP (γ -GT)—gamma-glutamyl trans peptidase; GS—Gilbert's syndrome; GST—glutathione S-transferases; HbA1C—glycated hemoglobin; HCV—hepatitis C virus; HGB—hemoglobin; LDH—lactate dehydrogenase; NA—nicotinic acid; NAFLD—non-alcoholic fatty liver disease; NSAIDs—non-steroidal anti-inflammatory drugs; NASH—non-alcoholic steatohepatitis; OLT—orthotopic liver transplant; p-AMPK α 1/ α 2—phosphorylated 5'-AMP activated kinase; PgC1 α —peroxisome proliferator-activated receptor c coactivator 1; pPar α —phosphorylated peroxisome proliferator activated receptor alpha; pPpar γ —phosphorylated peroxisome proliferator activated receptor gamma; PRISMA—Preferred Reporting Items for Systematic Reviews and Meta-Analysis; ROS—reactive oxygen species; SF-36v2—Short form-36 Health Survey test, version 2; TA—thymidine-adenosine pairs; TB—total bilirubin; TBWL—total body weight loss; TC—total cholesterol; UCB—unconjugated bilirubin (indirect); UDPGA—uridine diphosphoglucuronic acid; UDPG-T—hepatic bilirubin uridine diphosphate glucuronyltransferase; UFV—urinary flow curve; UGT1—UDP-Glucuronosyltransferase 1; UGT1A1—uridine 5-diphosphate-glucuronosyltransferase 1A1 genotype; VC—volume capacity.

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