Journal of
Clinical Medicine

Biomarkers for Diagnosis, Complications and Therapy Effects in Civilization Diseases Management

Edited by
Katarzyna Komosinska-Vassev and Pawel Olczyk

Printed Edition of the Special Issue Published in
Journal of Clinical Medicine

www.mdpi.com/journal/jcm

MDPI

Biomarkers for Diagnosis, Complications and Therapy Effects in Civilization Diseases Management

Biomarkers for Diagnosis, Complications and Therapy Effects in Civilization Diseases Management

Editors

Katarzyna Komosinska-Vassev
Pawel Olczyk

MDPI • Basel • Beijing • Wuhan • Barcelona • Belgrade • Manchester • Tokyo • Cluj • Tianjin

Editors

Katarzyna Komosinska-Vassev
Clinical Chemistry and
Laboratory Medicine
Medical University of Silesia
Sosnowiec
Poland

Pawel Olczyk
Department of Community
Pharmacy
Medical University of Silesia in
Katowice
Sosnowiec
Poland

Editorial Office
MDPI
St. Alban-Anlage 66
4052 Basel, Switzerland

This is a reprint of articles from the Special Issue published online in the open access journal *Journal of Clinical Medicine* (ISSN 2077-0383) (available at: www.mdpi.com/journal/jcm/special_issues/civilization_diseases_management).

For citation purposes, cite each article independently as indicated on the article page online and as indicated below:

LastName, A.A.; LastName, B.B.; LastName, C.C. Article Title. *Journal Name* **Year**, *Volume Number*, Page Range.

ISBN 978-3-0365-3746-7 (Hbk)
ISBN 978-3-0365-3745-0 (PDF)

Contents

About the Editors

Katarzyna Komosinska-Vassev

Prof. Katarzyna Komosinska-Vassev, Head of the Department of Clinical Chemistry and Laboratory Diagnostics, Faculty of Pharmaceutical Sciences in Sosnowiec, Medical University of Silesia in Katowice, Poland.

Education background: Master of medical analytics, Silesian Medical Academy (1993); Ph.D. in the biological sciences, Silesian Medical Academy (1998); Habilitation in the discipline of medical biology, Medical University of Silesia in Katowice (2013), Specialist diploma in Medical laboratory diagnostics (2010, scientific title of professor (2020).

The scientific research activity is focused on assessment of the extracellular matrix components metabolism and free radical and antioxidant activity during physiological conditions and selected pathologies, including connective tissue diseases, diabetes type 2, obesity, and inflammatory bowel diseases. The scientific research activity is focused on the analyses of glycosaminoglycans structure and content, as well as extracellular matrix proteins during tissue repair process in experimental burn wounds healing and the implementation of the pluripotent natural raw material, for the improvement of the efficiency of extracellular matrix remodeling, in the course of experimental burn wounds healing. Additional research is related with the assement of the role of adipocytokines in the development of the obesity and type 2 diabetes mellitus.

Membership in scientific societies: International Federation of Clinical Chemistry, College of Laboratory Medicine in Poland, Member of the Management Board in 2013-2014; Chairperson of the Workgroup: Clinical biochemistry/Laboratory diagnostics from 2015-2022.

Scientific awards: The award from the Minister of Agriculture and Natural Resources, several awards of the Rector of the Medical University of Silesia, top 2% of the most-cited scientists in the word according to the Stanford University (2021).

Pawel Olczyk

Paweł Olczyk professor of medical sciences and health sciences in the discipline of pharmaceutical sciences.

Professor Paweł Olczyk - Head of the Department of Pharmacy Pharmacy, Faculty of Pharmaceutical Sciences in Sosnowiec of the Medical University of Silesia in Katowice, Deputy Chairperson of the Pharmaceutical Sciences Discipline Council of the Medical University of Silesia (since 2019), Member of the Council of the Silesian Pharmaceutical Chamber (since 2017), Expert of the Regional Operational Program of the Silesian Voivodeship. As a representative of the Polish Pharmaceutical Society, he is also the CMKP Expert for developing continuous education course programs (since 2020). He was elected a member of the Drug Therapy and Sciences Committee of the Polish Academy of Sciences for the 2020-2023 term.

Scientific and research activity of professor Paweł Olczyk covers the biochemistry and pathobiochemistry of connective tissue in relation to the repair of tissue damage and metabolic disorders.

Preface to "Biomarkers for Diagnosis, Complications and Therapy Effects in Civilization Diseases Management"

It is a great pleasure to introduce the "Biomarkers for Diagnosis, Complications and Therapy Effects in Civilization Diseases Management" book.

The aim is to offer readers the best overview of the current state of knowledge of biomolecular biomarkers useful in diagnosis and therapy of civilization illness. The prevalence of civilization diseases, such as cancer, diabetes, obesity, and autoimmune diseases, has grown rapidly around the world in recent years. The development of molecular, biotechnological, and genetic tools has provided clinical practice with valuable diagnostic tools based on new molecules, proteins, as well as genes that not only participate in the pathogenesis of civilization diseases, but also serve as markers to assess the effectiveness of therapy or to predict the development of complications. Today, the variety of diagnostic tools available has helped health-care professionals change the approach to diseases of affluence towards the concept of personalized medicine. In this Special Issue, researchers present original research papers or review articles focusing on the role of new proteins, molecular, and genetic markers that can be used in the diagnosis and monitoring of the progress of a number of complications related to civilization diseases, as well as biomarkers useful in monitoring the effects of implemented treatment. Additionally, valuable are the review publications whose main topic is the participation of extracellular matrix components in the pathogenesis of civilization diseases, including cancer and autoimmune diseases.

Katarzyna Komosinska-Vassev and Pawel Olczyk
Editors

Journal of
Clinical Medicine

MDPI

Review

The Role of Extracellular Matrix Proteins in Breast Cancer

Arkadiusz Lepucki [1], Kinga Orlińska [1], Aleksandra Mielczarek-Palacz [2], Jacek Kabut [2], Pawel Olczyk [1,*] and Katarzyna Komosińska-Vassev [3]

[1] Department of Community Pharmacy, Faculty of Pharmaceutical Sciences in Sosnowiec, Medical University of Silesia in Katowice, 41-200 Sosnowiec, Poland; alepucki@interia.pl (A.L.); kinga.orlinska@sum.edu.pl (K.O.)
[2] Department of Immunology and Serology, Faculty of Pharmaceutical Sciences in Sosnowiec, Medical University of Silesia, 41-200 Sosnowiec, Poland; apalacz@sum.edu.pl (A.M.-P.); jacekkabut@poczta.onet.pl (J.K.)
[3] Department of Clinical Chemistry and Laboratory Diagnostics, Faculty of Pharmaceutical Sciences in Sosnowiec, Medical University of Silesia, 41-200 Sosnowiec, Poland; kvassev@sum.edu.pl
* Correspondence: polczyk@sum.edu.pl

Abstract: The extracellular matrix is a structure composed of many molecules, including fibrillar (types I, II, III, V, XI, XXIV, XXVII) and non-fibrillar collagens (mainly basement membrane collagens: types IV, VIII, X), non-collagenous glycoproteins (elastin, laminin, fibronectin, thrombospondin, tenascin, osteopontin, osteonectin, entactin, periostin) embedded in a gel of negatively charged water-retaining glycosaminoglycans (GAGs) such as non-sulfated hyaluronic acid (HA) and sulfated GAGs which are linked to a core protein to form proteoglycans (PGs). This highly dynamic molecular network provides critical biochemical and biomechanical cues that mediate the cell–cell and cell–matrix interactions, influence cell growth, migration and differentiation and serve as a reservoir of cytokines and growth factors' action. The breakdown of normal ECM and its replacement with tumor ECM modulate the tumor microenvironment (TME) composition and is an essential part of tumorigenesis and metastasis, acting as key driver for malignant progression. Abnormal ECM also deregulate behavior of stromal cells as well as facilitating tumor-associated angiogenesis and inflammation. Thus, the tumor matrix modulates each of the classically defined hallmarks of cancer promoting the growth, survival and invasion of the cancer. Moreover, various ECM-derived components modulate the immune response affecting T cells, tumor-associated macrophages (TAM), dendritic cells and cancer-associated fibroblasts (CAF). This review article considers the role that extracellular matrix play in breast cancer. Determining the detailed connections between the ECM and cellular processes has helped to identify novel disease markers and therapeutic targets.

Keywords: breast cancer; extracellular matrix; proteins; tumorigenesis; tumor microenvironment

Citation: Lepucki, A.; Orlińska, K.; Mielczarek-Palacz, A.; Kabut, J.; Olczyk, P.; Komosińska-Vassev, K. The Role of Extracellular Matrix Proteins in Breast Cancer. *J. Clin. Med.* **2022**, *11*, 1250. https://doi.org/10.3390/jcm11051250

Academic Editors: Nicola Avenia and Franco Bassetto

Received: 28 November 2021
Accepted: 22 February 2022
Published: 25 February 2022

1. Introduction

Female breast cancer is the leading cause of global cancer incidence, with an estimated 2.3 million women diagnosed with breast cancer and 685,000 deaths globally. In the end of 2020, there were 7.8 million women who had been diagnosed with breast cancer in the last 5 years, making it the most common cancer in the world. Breast cancer mainly involves the inner layer of the milk glands or lobules and ducts (small tubes that carry milk) [1–3]. Adipose tissue is present in the mammary gland (both female and male). The amount of fat determines the size of the breast [2,4]. There are some differences in the microarchitecture of the female and male mammary gland. In men, less glandular tissue is found in the glands than in women [2,5]. The female breast generally contains 12–20 lobes. These, in turn, are divided into smaller elements called lobules [2,6]. These lobes and lobules are connected via milk ducts. The adipose tissue of the breast is supplied by a network of lymph vessels, lymph nodes, blood vessels and nerves. The breast is also composed of fibrous connective tissue and ligaments which ensure its proper shape [2,7].

Hormonal changes that occur in the female body during the menstrual cycle, pregnancy and puerperium have a significant impact on the structure and function of the mammary gland. One of the hormonal effects of nipple stimulation is secretion of prolactin, which is produced by acidophilic cells (called lactotrophs or mammotrophs) in the anterior lobe of the pituitary gland [2,8]. The epidermis of the areola and nipple is characterized by moderate wrinkling as well as marked pigmentation. The nipple skin contains somewhat small hair and several apocrine and sebaceous sweat glands [2]. Milk sinuses are formed at the base of the nipple by milk ducts (usually 15–25 in number). These ducts transport milk towards the nipples [2,9]. Slightly under the surface of nipple, these sinuses end in coneshaped ampullae. The spherical areola is present around the nipple and is 15–60 mm in diameter [2]. Deep in the areola and nipple, several smooth muscle fibers are set radially and circularly in the dense connective tissue and longitudinally alongside the lactiferous ducts that lengthen up into the nipple. The muscle fibers mentioned above are involved in nipple erection, contraction of areola as well as emptying of milk sinuses [2].

2. ECM

The extracellular matrix (ECM), which is a perfectly organized and efficiently managed structure, is formed from a great variety of macromolecules, forming a multitude of combinations, depending on the tissue in which this structure occurs. It can be regarded as a physical scaffold for cellular components, although the range of functions it performs is much broader, and many of them are not, as yet, believed to be known and described. The proper combination of its components not only ensures appropriate stability and durability of the ECM, but most importantly determines the mechanical properties of a given tissue and serves as a bioreservoir for molecules such as growth factors. The role of ECM in many processes essential for cell homeostasis has been documented, including: adhesion, apoptosis, proliferation, differentiation, and migration. Genetically determined disorders of ECM structure or function have been shown to disrupt tissue and systemic homeostasis followed by various diseases. The composition of the ECM in a given tissue is determined during its development by a biochemical dialogue between the cells and the environment. This composition is an expression of its adaptation to the function performed in the body [10–15]. This section focuses on describing the general properties of ECM proteins, while their role in breast cancer will be discussed in detail in Section 3.4.

2.1. Collagen

Collagen accounts for nearly 30% of the total protein mass found in animals [12,15–18]. In humans, it makes up about 75% of the dry weight of the skin and is the most common component of the ECM [8]. Its essential functions include: maintaining the structural integrity of tissues, participating in wound healing, regulating cell adhesion, enhancing chemotaxis, promoting migration, providing tensile strength to tissues, and in addition, overseeing the proper course of their development and differentiation [12,16].

Collagen is formed from three left-handed polypeptide α chains, organized into a triple helical structure that is right-handed [10,12,17–19]. The described filamentous protein can be either a homotrimer or a heterotrimer [18–21]. In vertebrate animal organisms, 46 chains have been identified that can organize into 28 different collagen types [10,17–21]. These include: fiber-forming collagens (e.g., types I, II, and III), network-forming collagens (e.g., type IV basement membrane collagen), collagens associated with fibrils with breaks in their triple helixes (e.g., types IX and XII), and others (e.g., type VI) [10,17,18,21]. The tight packing of the trihelical structure is possible due to the presence of the characteristic Gly-X-Y motif (Gly—glycine), an amino acid sequence that repeats multiple times in the helix-forming polypeptide chains. Glycine is crucial for the stability of this structure because it is the only one of all the amino acids that is small enough to fit into the central part of the core of the helix described above, into which every third of the amino acid residues building each of the polypeptide α chains enters. The role of electrostatic interactions,

inter-chain hydrogen bonds, and high proline and hydroxyproline content in maintaining the stability of the collagen triple helix is also emphasized [10,17–22].

The process of collagen biosynthesis has been most thoroughly studied and described for fiber-forming collagens [21]. They are synthesized in the form of precollagens, which contains a signal sequence that is cleaved after synthesis in the endoplasmic reticulum. After that, the resulting procollagen molecules contain: an amino-terminal propeptide followed by a short, non-helical N-telopeptide, a central triple helix, a C-telopeptide and a carboxyl propeptide. Individual procollagen molecules can be post-translationally modified in various ways. This is done by: hydroxylation of some proline and lysine residues, glycosylation of some hydroxylysine residues as well as sulphation of tyrosine residues [21,23]. The adoption of the final, stable conformation by collagen depends on the proper attachment to procollagen of a specific molecular chaperone—heat shock protein 47 (Hsp47)—in the endoplasmic reticulum [21,24]. It has been established that for procollagen to be adequately stable at human body temperature, more than 20 Hsp47 molecules per triple helix must be attached [21,25]. Hsp47 is believed to protect procollagen from random unfolding and uncontrolled aggregation. It is also responsible for proteostasis regulation (folding, quality control, secretion) [18,26]. Intracellular Secreted Protein Acidic and Rich in Cysteine (SPARC) also pretends to be a chaperone of procollagen. It has been observed that the lack of this protein or its dysfunction result in defective deposition of collagen in tissues. Its ability to bind to the triple-helical structure of procollagen has also been demonstrated [21,27]. Both propeptides that make up procollagen require removal in a process called maturation [21,28]. The N-propeptide is cleaved by procollagen N-proteinases belonging to the A Disintegrin and Metalloproteinase with the Thrombospondin Motifs (ADAMTS) family, except the N-propeptide of the proα1(V) chain that is cleaved by the procollagen C-proteinase also termed Bone Morphogenetic Protein-1 (BMP-1) [21,29]. BMP-1 cleaves the carboxy-terminal propeptide of procollagens, except the carboxy-terminal propeptide of the proα1(V) chain, that is processed by furin. The telopeptides contain the sites where cross-linking occurs. The mentioned linkage formation is initiated by the oxidative deamination of lysyl and hydroxylysyl residues catalyzed by the enzymes of the lysyl oxidase (LOX) family [21,30].

It is widely believed that the unique mechanical properties of fiber-forming collagens are determined by covalent crosslinks. A special role in this regard is attributed to reducible and mature crosslinks produced via the LOX pathway. Cross-linking is viewed as tissue-specific rather than collagen-specific. Maturation of cross-links has been shown to provide additional resistance to shear stress [21].

Collagen degradation occurs essentially through the catalytic activity of matrix metalloproteinases (MMPs). These are zinc-dependent endopeptidases. They belong to the metzincin superfamily. They may take part both in physiological processes (e.g., development of tissues and their repair after damage) and in pathological processes (e.g., metastasis of neoplastic cells). Fiber-forming collagens (i.e., types I, II, and III) are substrates for: MMP-1 (also known as interstitial collagenase), MMP-8 (whose other name is neutrophil collagenase), MMP-13 (also referred to as collagenase 3), and MMP-14 (membrane-anchored). As far as collagen type I is concerned, it should be mentioned that it can also be degraded by MMP-2. The preferential substrates for particular zinc-dependent endopeptidases are collagen types I and III (for MMP-1 and MMP-8), and collagen type II for MMP-13 [21,31]. MMP-2 and MMP-9 are involved in the degradation of collagen type IV as well as denatured collagen (other names of these enzymes are 72 kDa-gelatinase and 92-kDa-gelatinase, respectively). It is noteworthy that the [α1(I)]3 homotrimer of collagen type I, in contrast to the [α1(I)]2 α2(I) heterotrimer of the same collagen, is not a substrate for mammalian MMPs, which is explained by the resistance of the homotrimer to local triple helix unwinding by MMP-1 due to the higher triple helix stability near the MMP cleavage site [21,32]. MMPs also play a key role in releasing of bioactive fragments or matricryptins (e.g., tumstatin or endostatin—two inhibitors of angiogenesis) from full-length collagens. Another group of

enzymes releases the ectodomain of membrane collagens as soluble forms. These enzymes are collectively called sheddases [21,23,33–35].

It has been established that the effect of collagens on cell–matrix interactions is mediated by receptors. Several families of them have been identified [21,36–39]. These receptors are ligands for integrins, cell-adhesion receptors that lack intrinsic kinase activities. The ability to bind collagen is demonstrated by integrins that have a β1 subunit connected with one of four subunits (i.e., α1, α2, α10 or α11) characterized by the presence of a domain known as αA. The discussed linkage is made by GFOGERlike (GFOGER—glycine–phenylalanine–hydroxyproline–glycine–glutamic acid-arginine) sequences [21,36,38]. Recognition sequences other than the mentioned one (e.g., KGD—lysine–glycine–aspartic acid) have also been identified in some collagens. Integrins αvβ1 and α5β1 have the ability to bind collagen type XVII, which exposes the KGD sequence in its ectodomain [21,36]. Not only can collagens be ligands for integrins, but also their proteolysis products, as confirmed for the following integrins: α3β1, α5β1, αvβ3 and αvβ5 [21,33]. Dimeric discoidin receptors (DDR1 and DDR2) that possess tyrosine kinase activities can also bind collagens. This has been observed for collagen types I, II and III [21,39]. DDR1 is widely expressed in epithelial cells, while DDR2 is mainly found in mesenchymal cells. The major DDR2-binding site in collagens I-III is a GVMGFO (glycine–valine–methionine–glycine–phenylalanine–hydroxyproline) motif [21,37]. It is assumed that collagen binding triggers structural reorganization of DDR2 surface loops, which leads to an activation of discoidin domains, and it is worth highlighting that mentioned domains can independently bind to collagen or simultaneous binding of two domains to the protein triple helix can occur [21,40]. Soluble extracellular domains (DDR1 and DDR2) also promote collagen deposition in the ECM by blocking fibrillogenesis, and in addition DDR2 determines the mechanical properties of collagen type I fibrils [21,40–42]. Collagen binding on platelets is mediated by glycoprotein VI (GPVI). It is a member of the paired immunoglobulin-like receptor [21,36]. Collagen can also bind to the inhibitory leukocyte-associated immunoglobulin-like receptor-1 (LAIR-1) [21,43]. Both GPVI and LAIR-1 are capable of recognizing the GPO (glycine–proline–hydroxyproline) motif in collagens. Membrane collagens (XIII, XVII, XXIII) and fibrillar collagens (I, II, III) act as ligands for LAIR-1, and it is worth noting that type I and III collagens are so-called functional ligands and block the activation of immune cells in vitro. LAIR-1 has multiple binding sites on collagen types II and III. GPVI has lower affinity for collagens type I and III than LAIR-1 [12,43–45]. Three LAIR-1 amino acids central to collagen binding are conserved in GPVI [12,46]. Fibril-forming collagens and collagen type IV are also ligands of Endo180 (urokinase-type plasminogen activator associated protein), a member of the macrophage mannose-receptor family that mediates collagen internalization [21,37,39].

2.2. Elastin

In mammals, elastin is encoded by a single gene and secreted as a 60–70 kDa tropoelastin monomer. Its primary role is to provide elasticity and resilience to tissues that are repeatedly stretched. It is found in tendons, stretchers, ligaments, walls of major blood vessels (e.g., abdominal aorta) and lung tissue [10,13,17,47]. Importantly, elastin stretch is crucially limited by tight association with collagen fibrils [17,48]. Fibulins enable tropoelastin to associate with microfibrils. In this way, elastic fibers are formed. A characteristic feature of all tropoelastin structures is the presence of hydrophobic sequences alternating with lysine-containing cross-linking motifs. Fibrillins and microfibril-associated glycoprotein-1 play important roles in the nucleation and assembly of elastin. An essential role in providing and maintaining the characteristic mechanical properties of elastin is attributed to the extensive cross-linking of tropoelastin, which is catalyzed by LOX [10,13,47,49]. This enzyme oxidizes selective lysine moieties in peptide bonds to allysine. There are two bifunctional cross-links in elastin: dehydrolysinonorleucine and allysine aldol. The former one is formed through the condensation of one residue of allysine and one of lysine. The latter one is formed through the association of two allysine residues. These two cross-links

can further condense with each other or with other intermediates to form desmosine or isodesmosine, being the major cross-links of the mature elastic protein. It has been documented that tropoelastin manifests the ability to form globular structures (aggregates) on the cell surface in a process called microassembly. Cross-linking implies the loss of positive charges on the molecule, which promotes the release of tropoelastin from cells, as well as facilitates global fusion in the presence of microfibrils (macroassembly). Fibulin-4 plays a key role in early stages of elastin assembly, whereas fibulin-5 acts to bridge elastin between the matrix and cells [10,13,47,49].

2.3. Laminin

Laminins form a family of heterotrimeric (one α chain, one β chain, and one γ chain) glycoproteins that includes nearly 20 members. Laminins are assembled into a cross-linked web. In basement membranes, this web is intertwined with a network that is composed of collagen fibrils. The mass of heterotrimers oscillates in the range of 400–800 kDa. It was found that in vertebrate animals there are five α chains and three β and γ chains each [10,50–52]. They are essential for normal organogenesis. They are also involved in early embryonic development [53,54]. For many of the known laminins, the ability to form networks spontaneously through appropriate connections has been documented. Such structures are able to interact with receptors located on cell surfaces [10,50–58].

2.4. Fibronectin

Fibronectin acts as a biological glue, participating in the management of the functions and structure of the interstitial ECM and being involved in facilitating target attachment and promoting cell migration [10,12]. The building units of monomers of this protein are subunits that contain three types of repeats (I, II and III). The average mass of such a monomer is 250 kDa. Fibronectin is secreted as sulfide-linked dimers. It has binding sites to other fibronectin dimers, cell surface receptors, heparin and collagen [10]. Cellular traction forces can stretch fibronectin several times over its resting length. This favors the exposure of cryptic integrin binding sites within the molecule, resulting in pleiotropic changes in cellular behavior. For this reason, fibronectin has been implicated as an extracellular mechanoregulator [12,59]. The fibronectin dimers can form multimers. Further fibronectin deposition is accompanied by structural changes (thickening and elongation) of fibrils. Fibronectin fibrils can be further processed into a deoxycholate-insoluble matrix [10,60]. Fibronectin plays a significant role in cell migration (both during embryonic development and during wound healing) [12,61–63].

2.5. Proteoglycans

Proteoglycans (PGs) are formed from a protein core and laterally attached glycosaminoglycan (GAG) chains. GAGs (hyaluronic acid, heparan sulfate/heparin, dermatan sulfate, chondroitin sulfate, keratan sulfate) are a heterogeneous group of anionic polysaccharides with characteristic disaccharide units (amino sugar, uronic acid or galactose) that are repeated many times in their structure. All except hyaluronic acid are sulfated [10,12,15,64]. It is believed that the negatively charged structure of GAG chains is crucial for the ability of PGs to sequester divalent cations and water. It is on this sequestration that a wide range of functions performed by PGs in tissues (e.g., lubrication functions and conferring space-filling) are commonly believed to depend. Based on the structure of core proteins, localization and composition of GAGs, PGs have been divided into three most important groups: cell-surface PGs, modular PGs and small leucine-rich PGs [10,12,15]. The functional diversity of PGs is based on their molecular diversity. It is worth emphasizing that small leucine-rich PGs participate in multiple signaling pathways (including binding to and activation of low-density lipoprotein-receptor-related protein 1, insulin-like growth factor 1 receptor and epidermal growth factor receptor). They are also involved in the inflammatory response reaction due to their ability to bind and activate transforming growth factor β [10,12,15,65–67]. Cell-surface PGs (glypicans and syndecans) improve the course

of ligand–receptor interactions, so they are attributed to the role of co-receptors [12,68]. Basement membrane modular PGs (agrin and perlecan) can act as both pro- and antiangiogenic molecules [10,12,15]. Modular PGs co-supervise cell proliferation, migration and adhesion [12,15]. The importance of PGs in collagen fibril assembly is also emphasized [10].

2.6. Thrombospondin

In vertebrate animals, thrombospondins are encoded by a THBS gene family consisting of five members. The function of thrombospondin 1 (THBS1) is best understood (under both physiological and pathological conditions). The precursor of this protein is composed of 1170 amino acids, whereas the mature protein, which is devoid of the N-terminal signal peptide compared to the aforementioned precursor, undergoes homotrimerization after secretion. THBS1 comprises roughly twelve asparagine-linked mono-, bi- tri-, and tetraantennary complex oligosaccharides and variable numbers of C-mannosylated tryptophan residues in the type 1 repeats. The protein in question was also found to be O-fucosylated [69–74]. During prenatal development, THBS1 is expressed in many tissues, whereas in adults who do not suffer from cancer, the expression of this protein is low. It has been pointed out that there is a positive correlation between age and plasma THBS1 levels. Moreover, the association of elevated plasma concentrations of this protein with diseases typically associated with old age, such as cardiovascular disease and type 2 diabetes, is emphasized. It is worth noting that THBS1 is the most abundant thrombocyte alpha granule protein. Its plasma concentrations in healthy people are low. There is evidence that stimuli such as: ischemia, tissue remodeling, injury or reperfusion are able to induce THBS1 expression in many locations in the body. Its high plasma concentrations have been reported in patients with rheumatoid synovitis, atherosclerosis and glomerulonephritis. Moreover, high plasma concentrations of lipids and glucose have been found to trigger THBS1 expression. It should be emphasized that increased expression of this protein occurs in the stroma of many cancers. The presence of THBS1 in ECM is transient owing to the fact that endotheliocytes and fibroblasts have the ability to efficiently internalize and degrade this protein. In the subendothelial matrix of some blood vessels as well as at the dermal–epidermal boundary in the skin, THBS1 expression is constitutive [69,75]. From a practical standpoint, the most important molecules that can bind THBS1 are: cathepsin G, fibronectin, some MMPs, fibrinogen, some collagens, active and latent transforming growth factor β1, plasmin, neutrophil elastase, tissue factor pathway inhibitor and heparin [69,76]. In a cell-specific as well as context-dependent manner, THBS1 can stimulate or inhibit proliferation, adhesion, motility, and survival of the cells. This protein is currently recognized as a potent inhibitor of angiogenesis. Nevertheless, the N-terminal proteolytic and recombinant parts of THBS1 have been found to stimulate angiogenesis, with integrin β1 mediating this effect. It has been demonstrated that THBS1 can block the enzymatic activity of proteases such as: neutrophil elastase, cathepsin G, and plasmin. Latent transforming growth factor β1 is in turn stimulated by THBS1. It is also worth highlighting that THBS1 has the ability to block stem cell self-renewal [69,77,78].

2.7. Osteopontin

Osteopontin (OPN) is a glycosylated extracellular matrix phosphoprotein produced by cells such as: osteoblasts, osteoclasts, epitheliocytes, endotheliocytes and immune cells. Depending on the tissue in which it is found, this protein can exhibit both structural and functional heterogeneity. The mass of OPN oscillates between 41 and 75 kDa, which is mainly due to differences in its post-translational modifications [79–86]. Bone remodeling, vascularization, inflammation as well as immune-regulation are processes in which OPN plays an important role. At this point it is worth emphasizing that this protein is also relevant in the course of tumorigenesis [79,87–91]. Data collected so far indicate that OPN together with some integrins, when stimulated by vascular endothelial growth factor (VEGF), may enhance angiogenesis. It is assumed that OPN promotes proliferation and migration of endothelial cells [79,92,93]. Many of the functions that OPN performs in the

body are linked to its interactions with CD44 and integrins. Due to these interactions, OPN has been classified, along with dentin matrix protein 1 (DMP1), dentin sialophosphoprotein (DSPP), bone sialoprotein (BSP) and matrix extracellular phosphoglycoprotein (MEPE), into a group of molecules referred to as small integrin-binding ligand N-linked glycoproteins (SIBLINGs). Arginine–glycine–aspartic acid (RGD) and serine–valine–valine–tyrosine–glutamate–leucine–arginine (SVVYGLR) are two sequences that are necessary for the integrin-binding ability manifested by OPN. The former sequence enables OPN to bind to the following integrins: αvβ1, αvβ3 and αvβ5, while the latter one conditions OPN to bind to the following integrins: α4β1, α4β7, and α9β1 [79,94–97]. The binding of OPN to CD44 and integrins triggers a downstream signaling cascade via the PI3K/AKT signaling pathway leading to cell proliferation and survival which is mediated by NF-κB. Furthermore, via the Ras/Raf/MEK/ERK signaling pathway, an OPN–integrin complex confers a metastatic phenotype on some cancer cell types which appears to be dependent on the induction of activator protein 1-dependent gene expression [79,98–103].

2.8. Osteonectin

Osteonectin (ON) is a 32 kDa calcium-binding matricellular protein. It has been proven that this protein can be expressed in both mineralized and non-mineralized tissues, although initially many researchers believed that ON expression did not occur in the latter. As a rule, osteopontin expression accompanies the expression of fiber-forming collagens (e.g., collagen type I). It is widely believed that the role of ON in osteoid consists of the release of calcium cations on the one hand and the binding of both collagen and hydroxyapatite on the other. This constellation of ON properties is an assumption for its recognition as a promoter of bone mineralization. It is assumed that spatial separation of two domains of this protein, i.e., the hydroxyapatite-binding domain and collagen-binding domain, is essential for ON to properly perform this function [104–107]. ON is encoded by a single gene. This protein has four domains: an N-terminal low-affinity, high capacity, calcium-binding domain that contains the mineral binding region, a cysteine-rich domain, a hydrophilic region, and an extracellular Ca^{2+} (EC) domain with an E-F hand motif at the C-terminus that encompasses the collagen binding domain. ON undergoes differential glycosylation, which depends mainly on its tissue-specific expression. It is worth highlighting that the glycosylated form of this protein is expressed in bone and has a higher affinity for collagen than the form found in thrombocytes [104,108]. It has been discovered that ON can also be produced by fibroblasts and endotheliocytes. Moreover, this protein is found in platelet granules during injuries [104,109–111]. ON facilitates procollagen processing by limiting procollagen association with cell surface receptors, while noting that the ON-binding site on collagen overlaps with that of the collagen receptors called DDR1 and DDR2. It has been suggested that, by binding to collagen type I, ON may block signaling pathways mediated by DDR2. It is speculated that, in the absence of ON, streamlined interactions between soluble collagen and DDR2 entail increased turnover of collagen within the cell surface, resulting in changes in its deposition in the ECM. In addition, ON affects the content and diameter of collagen fibrils in mineralized tissues by regulating the activity of the enzyme called transglutaminase [21,37,39–42,104,112–115].

2.9. Periostin

Periostin (POSTN), which was originally isolated from a mouse osteoblast cell line as osteoblast-specific factor 2, belongs to the matricellular protein family. In humans, POSTN is encoded by the POSTN gene, whose expression can be increased by interleukins (4 and 13), and by transforming growth factor β. The components of the POSTN molecule are: a cysteine-rich domain within the N-terminal region, four fasciclin I domains, and an alternative splicing domain within the C-terminal region. It is worth adding that up to nine splice variants have been identified, but the full-length transcript encodes an approximately 90 kDa secreted protein that includes all exons. It is still unclear what functional significance these variants may have. It is conjectured that they might condition the differential expres-

sion of POSTN in different tissues and diseases [116–121]. POSTN binds both fibronectin and collagen type I and participates in collagen fibrillogenesis [116,122]. It has been proven that such known actions of POSTN as: promotion of adhesion, stimulation of proliferation, enhancement of angiogenesis, facilitation of metastasis or acceleration of cell migration are dependent on its binding to appropriate integrin receptors [116–118,123]. It has been reported that POSTN is engaged in epithelial–mesenchymal transition (EMT) and heart morphogenesis. The described protein is also relevant in such processes as Th2-dependent immune response and inflammation [124].

2.10. Tenascin C

Hexameric tenascin C (TNC) is a member of the tenascin gene family of proteins. It was originally attributed to function as a supervisor of cell adhesion. Nowadays it is known that the range of functions performed by this protein is much wider and includes, e.g., regulation of signaling between cells, modulation of expression of specific genes or participation in maintenance of proper biochemical conditions within the cellular microenvironment [125,126]. Due to differences in post-translational modifications, the described protein, which in vertebrates is characterized by a highly conserved amino acid sequence, is found in different molecular forms. Wide distribution of TNC occurs in embryonic tissues, while in postnatal development its expression is much lower and its synthesis is tightly regulated by many factors. However, there are some situations in which TNC synthesis is enhanced postnatally, e.g., during tissue healing after damage, during carcinogenesis (especially in the stroma of solid tumors), and during inflammation (especially chronic) [125–129]. The high prenatal expression of TNC is suspected to be related to the experimentally demonstrated ability of TNC to influence cell phenotype by interacting with appropriate receptors on the cell surface. Several glycosylation sites within the TNC molecule have been identified. It is suggested that TNC acquires protease resistance due to this modification. There are also reports that glycosylated TNC finds it more difficult to form hexamers. It has been also documented that glycosylation of TNC results in its ability to promote neuronal stem cell proliferation. Another post-translational modification that TNC may undergo is citrullination, which is attributed to the ability to increase the immunogenicity of C-terminal residues of this protein, which in turn leads to the formation of autoantibodies, as described in the case of rheumatoid arthritis [130–132]. TNC can be cleaved by gingipain cysteine proteases and MMPs. This degradation not only regulates TNC turnover in tissues. Indeed, it has been shown that the resulting molecules (soluble fragments) also have specific biological activities, usually different from the parent molecule. For instance, cleavage uncovers cryptic pro-apoptotic activity, hidden fibronectin-binding sites and concealed heparin–sulphate-binding sites that promote cell spreading [125,126].

2.11. Entactin

Entactin is a sulfated, multidomain glycoprotein that is found in many basement membranes. This protein is composed of 1217 amino acids that form two globular domains, linked by a rod-like structure whose essential fragments are four EGF- and one thyroglobulin-like cysteine-rich homology repeats. Entactin has the ability to bind to the following molecules: fibronectin, laminin, fibrinogen and collagen type IV. The described protein plays an important role in endowing the ECM with its proper characteristics. Entactin can promote both phagocytosis and chemotaxis. It is worth noting that these actions are dependent on interactions with integrin receptors. Entactin is also engaged in regulating wound healing and hemostasis by binding to fibrinogen. This binding is not dependent on metal cations. In addition, it has been shown that entactin is involved in controlling cell adhesion, which may be important for its role in tumorigenesis [133,134].

2.12. ECM in the Breast

In a resting adult mammary gland, the basement membrane encapsulates the gland and is the principal ECM that interacts with both the myoepithelium and the luminal epithelium [135]. Its essential components are: collagen type IV, PGs, laminins (111 and 332), entactin and epiligrin. ECM is responsible for maintaining the proper polarity by the epithelial cells, and it should be mentioned that this function is realized mainly due to the presence of the aforementioned laminin 111. The appropriate biochemical dialogue between ECM components and lactogenic hormones is required for full differentiation of mammary epithelial cells [135–139]. Signal transducer and activator of transcription 5 (STAT5) participates in this differentiation and in the process of milk secretion from the breast [135,140]. Inhibition of control of mammary epithelial differentiation in response to prolactin and impaired milk secretion occur when the epithelial cells are placed on the interstitial matrix that is rich in fibrillar collagen (type I and III), PGs, hyaluronan and various glycoproteins [135,141]. The presence of laminin 111 allows this control to be regained, but only if integrin β1 (receptor for laminin 111) is present and has no defects in structure or function. In its absence, STAT5 signaling is impaired and epithelial cells detach from the basement membrane. This integrin is also required for mammary ductal cells to proliferate. Extensive reorganization of the ECM in the mammary gland is observed during pregnancy and lactation. The post-lactational involution occurs with a significant increase in fibrillar collagen and fibrillin content. Increased proteolysis is also observed. Laminin, collagen type IV and entactin are degraded [135,142–146].

3. Breast Cancer

Breast cancer is one of the most common cancers affecting women worldwide, and its incidence continues to increase [147–158]. It is also one of the leading causes of cancer deaths among women [147]. As a metastatic cancer, it can exhibit the ability to spread to multiple organs (e.g., brain, lungs, kidneys, bones, and liver), which significantly worsens the prognosis [148,155]. In the absence of any metastases, the cure rate of patients sometimes reaches 90%, whereas, in the metastatic setting, the cure is not achievable for now. It is believed that in such cases the long-term survival depends mainly on the organs to which the metastases occur, as well as the extent and speed with which it occurs [157]. Early diagnosis facilitates treatment and is associated with a better prognosis [148]. Appropriate prophylaxis is also not without importance [154]. Many risk factors have been identified, including: age, gender, genetics, cigarette smoking, personal and family history, breast pathology (especially proliferative breast disease), reproductive factors, as well as dietary habits and estrogen metabolism disorders [147–158]. At this point, it is worth noting that mutations in two genes (BRCA1 and BRCA2) are the most significant causes of genetically determined breast cancer [149].

3.1. Molecular Subtypes of Breast Cancer

Based on the expression profile of specific genes, supported by immunohistochemical assays, the following molecular subtypes of breast cancer were identified: luminal A, luminal B, luminal HER2, enriched HER2 and triple-negative [2–4,135,137].

Luminal A subtype is the most common (accounting for nearly 50% of all newly diagnosed breast cancer cases) subtype and also the least aggressive [2]. It shares some features with luminal breast epithelial cells, namely, it manifests high expression of cytokeratins (7, 8, 18 and 19). Moreover, the expression of proliferation-stimulating genes is low in this subtype (low Ki-67 index), while the prognosis is very good. Lymph node involvement is rare, and the clinical course is relatively benign. It expresses estrogen receptor (ER) and progesterone receptor (PR), however the expression of human epidermal growth factor receptor 2 (HER2) is very low in this subtype. This constellation of properties makes luminal A subtype susceptible to hormonal therapy (using aromatase inhibitors or selective estrogen receptor modulators) [2–4,135,137].

Luminal subtype B accounts for 20–30% of invasive breast cancer cases and it is worth noting that most breast cancers genetically determined by BRCA2 gene mutation belong to this subtype [149,150]. This subtype is characterized by: high expression of cytokeratins, intermediate prognosis, higher risk of local recurrence after treatment (than in the case of subtype A). It is assumed that higher (than in subtype A) expression of Ki67, cyclin E1 and nuclease sensitive element binding protein 1, indicating increased proliferation, implies worse prognosis than in subtype A. It seems that increased signaling via pathways involving Src and PI3K kinases is also significant in this regard. This subtype is usually treated as the most aggressive form of hormone-dependent breast cancer. In addition to hormone therapy, this subtype usually requires additional treatment options: targeted therapy (if the cancer cells are HER2+) or chemotherapy [147,149,151].

HER2-positive (HER2+) breast cancers are characterized by positive expression of a molecule called HER2, which is classified as a protooncogene and encoded by a gene located at the long arm of human chromosome 17. It is noteworthy that increased expression of HER2 is found in many epithelial tumors. Due to the fact that HER2 belongs to the family of plasma membrane-bound receptor tyrosine kinases, this overexpression results in increased activity of this tyrosine kinase. It is active even in the absence of ligand, which is manifested, among others, by increased signaling promoting uncontrolled cell proliferation. HER2-positive cancers account for 15–20% of all breast cancers [152,153]. The presence of extra copies of the gene encoding HER2 often coexists with alterations in genes responsible for encoding proteins involved in proteolysis or angiogenesis. The repercussions of HER2 gene amplification include: higher risk of metastasis, worse clinical prognosis as well as shorter disease-free survival. Luminal HER2 and enriched HER2 were distinguished. The former (also called triple-positive) has expression of HER2, ER and PR, while its Ki-67 index has an intermediate value, which determines moderate proliferation. The latter has HER2 but no ER and PR, so no hormonal therapy is used. Despite the presence of the HER2 molecule, monoclonal antibody therapy is unsuccessful in almost 50% of patients, which, given the high value of the Ki-67 index noted in this cancer, and which indicates increased proliferation, implies a poor prognosis [154–156].

Triple-negative breast cancers, which account for about 15% of all breast cancers, owe their name to the fact that their cells lack ER, PR and HER2. They have a high Ki-67 index, which is evidence of increased proliferation. It has been proven that people with BRCA1 gene mutation have higher incidence of these cancers. Furthermore, they are found more often in women at a young age. This extremely aggressive (especially in African American women) and heterogeneous subtype of breast cancer is characterized by a very high risk of recurrence (local and systemic), which should be taken into consideration when introducing appropriate therapy. High incidence of early metastasis and recurrence determines a poorer prognosis [155–157].

3.2. Tumor Microenvironment

In recent years, our knowledge of the molecular basis of tumorigenesis in the breast has greatly expanded. It has been proven that cancer of this organ is accompanied by significant changes in the surrounding stroma. Many components of the so-called tumor microenvironment have been identified, as well as tumor-induced changes in the morphology and function of the ECM, immune cells, cytokines and growth factors and their receptors. Some of these changes are thought to facilitate tumor progression. However, alterations in the tumor microenvironment that inhibit tumor progression have also been identified. For example, the enrichment of cytotoxic T cells in the tumor microenvironment can be regarded as a tumor-induced modification while being anti-tumoral. Stromal cells in the breast cancer microenvironment are characterized by aberrant signaling pathways as well as molecular alterations that have prognostic significance for clinicians [159]. Breast cancer is now recognized as a highly heterogeneous (histologically and at the molecular level), genetically determined disease [148,151,152,154–157,160–164]. Both somatic and germline mutations are causative factors in tumorigenesis. It should be emphasized that

certain mutations cause so-called hereditary tumor syndromes in patients, while repercussions of other mutations are certain morphological stages [160–164]. Links between genetic variation and pathological subtypes of breast cancer are the subject of research [164].

The breast cancer microenvironment can be considered at three main levels: local (intratumor), regional (in the breast) and distant (metastatic). Each of these levels contains: different cell types (leukocytes, fibroblasts, epithelial cells, adipocytes and myoepithelial cells), soluble factors (enzymes, growth factors, hormones and cytokines) as well as ECM with specific characteristics. Additionally, each has a different ion concentration (Ca^{2+} and H+) and oxygen content. The occurrence of interplay between components of the tumor microenvironment and breast cancer cells has been repeatedly studied and confirmed [159,165]. Some of the most important molecular players in the tumor microenvironment are T cells, which are the most abundant tumor infiltrating lymphocytes, as shown by experimental data. Recently, it has been demonstrated that T reg cells can promote breast cancer metastasis to bone by synthesizing and secreting receptor activator for nuclear factor kappa B ligand. Therefore, it has been suggested that the finding of multiple T reg cells in the tumor microenvironment worsens prognosis. The idea has been put forward that recruitment of these lymphocytes occurs as a result of prostaglandin E2 secretion by tumor cells. It has been reported that this effect is modulated by transforming growth factor β. The function of effector cells may be suppressed by the tumor via secretion of interleukin 10. The events described above contribute to the formation of the so-called immunosuppressive microenvironment, which is one of the key elements of the process referred to as immunoediting [159,166–172]. Tumor-associated macrophages (TAM), which originate in blood monocytes recruited at the tumor site via factors secreted by both neoplastic and stromal cells, also represent an important cell population in the breast cancer microenvironment. TAM exhibit a characteristic phenotype directed at promoting tumor growth, facilitating both angiogenesis (through producing VEGF) and tissue remodeling as well as suppressing adaptive immunity. Data collected so far indicate that high levels of TAM are associated with poor prognosis in breast cancer [159,173–177]. Tumor-associated stroma shows an abundance of immature dendritic cells (DC) with impaired capacity to stimulate antitumor immunity. These DC have the ability to promote tumor growth by enhancing endothelial cell migration and stimulating the production of proangiogenic factors. The cited DC activities disappear when these cells become mature. Moreover, infiltration of mature DC into primary tumor sites has been shown to reduce metastatic capacity, resulting in a better clinical outcome [159,178–182]. It is also worth highlighting that the role played by cancer-associated fibroblasts (CAF) in the breast cancer microenvironment, which are the source of many soluble factors (e.g., chemokines and growth factors), is not without significance. They are considered to be capable of enhancing tumor aggressiveness and facilitating metastasis. Compared to fibroblasts located in noncancerous tissues, CAF are characterized by significantly higher expression of genes related to morphogenesis and development. Furthermore, there are premises indicating that CAF might affect the transcriptional profile of breast cancer cells. These interactions may promote the formation and maintenance of a specific genetic–biochemical partnership to manage the microenvironment in such a way as to mutually facilitate access to nutrients. It is possible that the source of CAF is the bone marrow and their recruitment to the tumor microenvironment is accomplished by sending appropriate signals from tumor cells that are already present in this microenvironment, although it should be noted here that other concepts as to the provenance of CAF are also considered. While metalloproteinases produced by CAF appear to promote tumor invasion, other factors produced by these cells, such as caveolin-1 and podoplanin, which are associated with wound responses, have been linked with fewer nodal metastases [159,183–190]. It is worth mentioning that the heterogeneity of CAF has been recognized and the importance of four subsets (CAF-S1, CAF-S2, CAF-S3 and CAF-S4) of these cells, whose expression patterns in non-tumorigenic tissues and in breast cancer are different, has been described. CAF-S1 have been shown to be key immunosuppressive factors. They exhibit the ability to attract T lymphocytes and, moreover, to increase the

survival of CD4+CD25+ T lymphocytes. Additionally, they facilitate the differentiation of these lymphocytes into CD25+FOXP3+ cells and stimulate T reg cells to block the proliferation of effector T cells [16,17]. The role of adipocytes in the tumor microenvironment is also important. In a healthy breast there are the following groups of adipocytes: adipose-derived stem cells, preadipocytes and mature adipocytes. Data collected so far indicate that in breast cancer tissue there are adipocytes with different characteristics (enhanced expression of adipokines and inflammatory factors, higher activity of matrix metalloproteinase, smaller size, increased expression of type VI collagen and decreased lipid content) from those found in the non-neoplastic tissue, therefore they are called cancer-associated adipocytes (CAA). CAA exhibit fibroblast-like phenotypes and possess senescent features (especially in obese people). They are located in the vicinity of tumor-transformed cells, with which, as it is presently assumed, they communicate chemically, inducing functional and phenotypic changes favoring tumor progression. Moreover, increased secretion by CAA of molecules, whose activity implies enhanced metastasis and tumor invasiveness, has been reported. The most important of these molecules are: interleukins (1β and 6), leptin, tumor necrosis factor α, parathyroid hormone-related protein, vascular endothelial growth factor and chemokine (C-C motif) ligands (2 and 5). The aforementioned communication at the CAA-tumor cell line also determines the metabolic reprogramming of CAA, which triggers their tumor-promoting potential. It has been discovered that exosomes can act as molecular linkers between CAA and breast cancer cells in enhancing tumorigenesis. Within the tumor microenvironment, exosomes carry onco-miRNA (miRNA-126, miRNA-144 and miRNA-155) from breast cells to adipocytes, leading to the conversion of the latter into CAA [18–24].

3.3. Essential Changes in Breast ECM during Carcinogenesis

These changes occur at every stage of carcinogenesis. In the non-tumorigenic breast, tissue microarchitecture is under precise multifactorial control [140,191].

Signaling for epithelial polarity is one key to the ECM role in tumor suppression. Underlying the disclosure of the tumor phenotype is the loss of this polarity, triggered by disruptions in cell–cell and cell–ECM interactions. If this polarity is restored, the process of carcinogenesis is inhibited [140,191–194]. The basement membrane can arrest nascent in situ carcinomas within its boundaries [140,195–197]. Its crossing by tumor-transformed cells is possible, among other reasons, because these cells are capable of disorganizing cell-to-cell and cell-to-ECM signaling pathways and can disrupt adhesion and migration. The role of cancer cell synthesis and secretion of enzymes that degrade ECM components is also emphasized [140,198–203]. Invasion of the basement membrane is usually temporally and spatially coordinated with increased protease synthesis, enhanced proteolysis, and abnormal turnover of matrix components via, among other things, endocytosis (e.g., laminin and its receptors) [140,204–208].

3.4. ECM Proteins in Breast Cancer

ECM proteins are mainly produced by myoepithelial cells. Therefore, changes in the synthesis of these proteins accompanying carcinogenesis are clearly visible in the mentioned cells. For instance, the loss of the ability of myoepithelial cells to synthesize laminin 111 and the inability of these cells to produce inhibitors of matrix-degrading proteases (such as maspin) have been observed. Conversion of carcinoma in situ to invasive breast cancer appears to be dependent on myoepithelial cell dysfunction [140,209–212]. Invasion and metastasis are preceded by an increase in collagen biosynthesis [140,213,214]. Upregulation of LOX enhances collagen cross-linking. The resulting stiffening of its structure is considered as one of the factors promoting metastasis [90,215–218]. Increased LOX activity has been shown to be induced by transforming growth factor β and hypoxia inducible factor [121,219,220]. Another enzyme involved in collagen metabolism (called prolyl hydroxylase) is also highly expressed in breast cancer tissues, which correlates with poor clinical outcomes. The subsequent reduction of collagen deposition due to silencing

of the mentioned enzyme favors the reduction in metastasis (e.g., to lungs and lymph nodes), as well as decreasing the invasiveness of cancer cells [121,220–222]. There is speculation that increased breast density, which is generally associated with poor prognosis, is a consequence of increased collagen deposition and stiffness of the stromal matrix. This stiffness may further account for integrin clustering and increased activity of signaling pathways involving extracellular signal-regulated kinases (ERK). Due to degradation of the basement membrane, collagen type IV is decreased in breast cancer, while the number of fiber-forming collagens (types I, III, and V) is increased, which has been linked to a higher risk of invasion and malignancy. A collagen scaffold can be used by cancer cells during migration in order to facilitate this process [140,223,224].

It is now thought that many of the ECM proteins (e.g., periostin and tenascin C) are important components of the so-called pre-metastatic niche [135,215]. Periostin is produced by fibroblasts in tumor stroma [215,225,226]. It is important for normal skeletal and myocardial development and is also found in healthy tissues [135,227]. Its increased expression in tumors is usually associated with tissue stiffness-dependent facilitation of disease progression [135,217]. The mentioned increase in tissue stiffness is caused by an increase in LOX activity, which in turn results from an interaction between periostin and BMP-1. It is worth mentioning that LOX accumulates in the pre-metastatic niche and promotes recruitment of MMP-2 producing myeloid cells [135,228]. Moreover, periostin induces Wnt signaling by promoting recruitment of Wnt ligands, which also promotes metastasis formation [215,229]. Induction of periostin by transforming growth factor β3 facilitates breast cancer metastasis to the lung and survival of cancer cells in this organ, and increased plasma levels of this molecule have been linked to a higher risk of secondary breast cancer foci in the bone [135,229,230].

Tenascin C (TNC) assembles into a hexameric structure and is highly upregulated during tissue regeneration, because it participates in the formation and function of the provisional wound matrix [135,231]. TNC has been detected in both primary breast cancer and the invasive front of lung metastasis nodules. Both stromal and cancer cells express a significant amount of TNC. It is highly upregulated especially at invasive fronts [135,215,232]. TNC has the ability to modulate cancer stem cell signaling by enhancing expression of key regulators of the Wnt and Notch pathways, namely leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5) and musashi homolog 1 (MSI1), respectively, which has been associated with an increased risk of recurrence (local and distant) [135,215,233]. TNC is one of six genes in a signature regulated by microRNA 335 in metastatic breast cancer [135,233]. At sites where tissues undergo remodeling, TNC typically coimmunoprecipitates with MMPs. Two of them (MMP-9 and MMP-13) are activated by TNC, which enhances breast cancer invasiveness. It has been reported that TNC expression limited only to the stroma is associated with better prognosis than its expression in both stromal and tumor cells. There is also evidence to suggest that TNC expression predicts poor 5-year survival in patients with breast cancer [135,234–238].

Osteonectin (ON) is a matricellular ECM protein that is nearly absent in normal mammary, however it is highly expressed in breast cancer [135,239]. This increased expression is mediated by β4 integrin, leading to increased invasiveness. ON is associated with basal, HER2+ and luminal B breast cancer subtypes while the luminal A subtype does not express this protein. ON regulates MMP-2 activity and facilitates metastasis to lung tissue [135,240–242]. There is some evidence suggesting an inverse correlation between ON and the estrogen receptor. The expression of ON in breast cancer is associated with poor metastasis-free survival as well as overall survival [135,243–246].

Thrombospondin 1 (THBS1) was originally detected in thrombocytes, but it also shows expression in osteoblasts, macrophages, fibroblasts, and tumor cells [135,247]. On the one hand, this molecule has been proven to inhibit the growth of primary tumors and block angiogenesis but, on the other hand, it has been noted to promote breast cancer metastasis to the lungs, which is most likely accomplished via activation of transforming growth factor β and stimulation of urokinase plasminogen activator. The expression of THBS1

in breast cancer associates with poor metastasis-free survival [135,248–251]. In the case of tumors that show neither estrogen receptor nor progesterone receptor expression, the increase in plasma THBS1 levels in diseased compared to healthy individuals may be of great clinical value owing to the fact that it can be considered as one of the markers of aggressiveness, since it has been noted that lymph node metastasis is much more frequent under the described conditions [135,252–254].

Osteopontin (OPN) is a phosphorylated glycoprotein that interacts with surface receptors including CD44 and several integrins, of which particular importance is commonly attributed to $\alpha v\beta 3$ integrin on account of its participation in cell survival signaling. OPN occurs in bones and has a thrombin cleavage site. After cleavage, both fragments are recognized by integrin receptors. Thrombin cleavage of this molecule has been suggested to lead to an increase in OPN activity [135,255,256]. Overexpression of OPN results in increased tumor size, increased invasiveness, and promotes metastasis. Cancer cells with such an overexpression also have increased expression of urokinase plasminogen activator [135,257,258]. Most often overexpression of OPN occurs in stromal cells (lymphocytes and infiltrating macrophages), nevertheless this protein is also expressed by cancer cells directly and exists both as an immobilized part of the ECM and as a soluble factor circulating in the blood. OPN is expressed in node negative breast cancer. Its presence both in plasma and in tumor tissue may be a prognostic indicator of tumor aggressiveness. Indeed, low levels of this protein in blood plasma are associated with decreased metastatic spread and better overall survival [135,259–262]. Expression of OPN by orthotopically injected breast cancer cells is a necessary factor for the occurrence of bone marrow-derived stem cell mobilization, raising the possibility that this protein is not merely a passive biomarker [135,263].

Increased synthesis and enhanced deposition of fibronectin in tumor-affected tissues have also been found in human breast cancer [213,215,264]. Fibronectin has been detected in the stem cell niche. This molecule is considered as one of the indicators of EMT. For instance, it can promote EMT induced by transforming growth factor β. The effects of fibronectin on metastasis formation and EMT are mediated via the ERK/MAP kinase and Src kinase pathways [215,265,266]. It has been reported that ERK participates in one of the critical pathways in breast cancer progression. Studies to date suggest that binding of collagen type I to DDR stabilizes SNAIL1 (a transcription factor that promotes the repression of the adhesion molecule E-cadherin in order to regulate EMT) by stimulating ERK2 activity. Activated ERK2 can phosphorylate SNAIL. If this reaction occurs, SNAIL1 accumulates in the cell nucleus and subsequently promotes breast cancer invasion and enhances metastasis [215,267]. Abnormalities in the distribution of receptors for fibronectin on the surface of tumor cells were also highlighted. In general, fibronectin expression in breast cancer is associated with adverse clinical outcomes [268–275].

Associations of several laminin subtypes (111, 332, and 511) with tumorigenesis in the mammary gland have been established. Abnormal expression of laminin 111 or its loss, which are usually observed in the breast undergoing tumorigenesis, result in disturbed cell polarity. In view of the role of this laminin in the regulation of cell–cell adhesion, it is speculated that it has the ability to limit the spread of tumor cells [270,276–279]. Some studies have provided evidence that other laminins containing $\alpha 4$ subunits (such as laminin 332 and laminin 511) enhance cancer progression. Expression of laminin 332 accompanies aggressive breast cancer phenotype, whereas tumor-derived laminin 332 promotes anchorage-independent survival via interaction with integrin $\alpha 6\beta 4$ receptors [270,280]. Interactions of laminin 332 with integrin $\alpha 3$ result in increased migration and invasion of tumor-transformed cells. Regarding laminin 511, it has been shown to have the ability to increase breast cancer invasiveness by promoting adhesion and migration of tumor cells. In a subpopulation of cells capable of self-renewal and tumor initiation, this laminin interacts with integrin $\alpha 6\beta 1$ [270,281].

Regarding elastin, it is worth mentioning that elastosis, which results from an abnormal increase in expression of the components of elastin fibers and excessive degradation

of normal elastic fibers, is a common feature in breast cancer. Elastosis increases with tumor progression. Ductal elastosis is particularly common in invasive cancer. Elastosis is recognized as a complex phenomenon resulting in both deposition of elastotic masses and local production of elastin fragments. These two manifestations must be distinguished within the matrix [270,282]. Elastin-derived peptides affect tumor cells and surrounding stroma. They promote invasion of this stroma and migration of cancer cells. These peptides also upregulate the expression of MMPs as well as facilitate chemotaxis, angiogenesis and elastase release. Moreover, they can prevent apoptosis [270,283,284]. The roles of the selected ECM molecules in tumor microenvironment were shown in Figure 1.

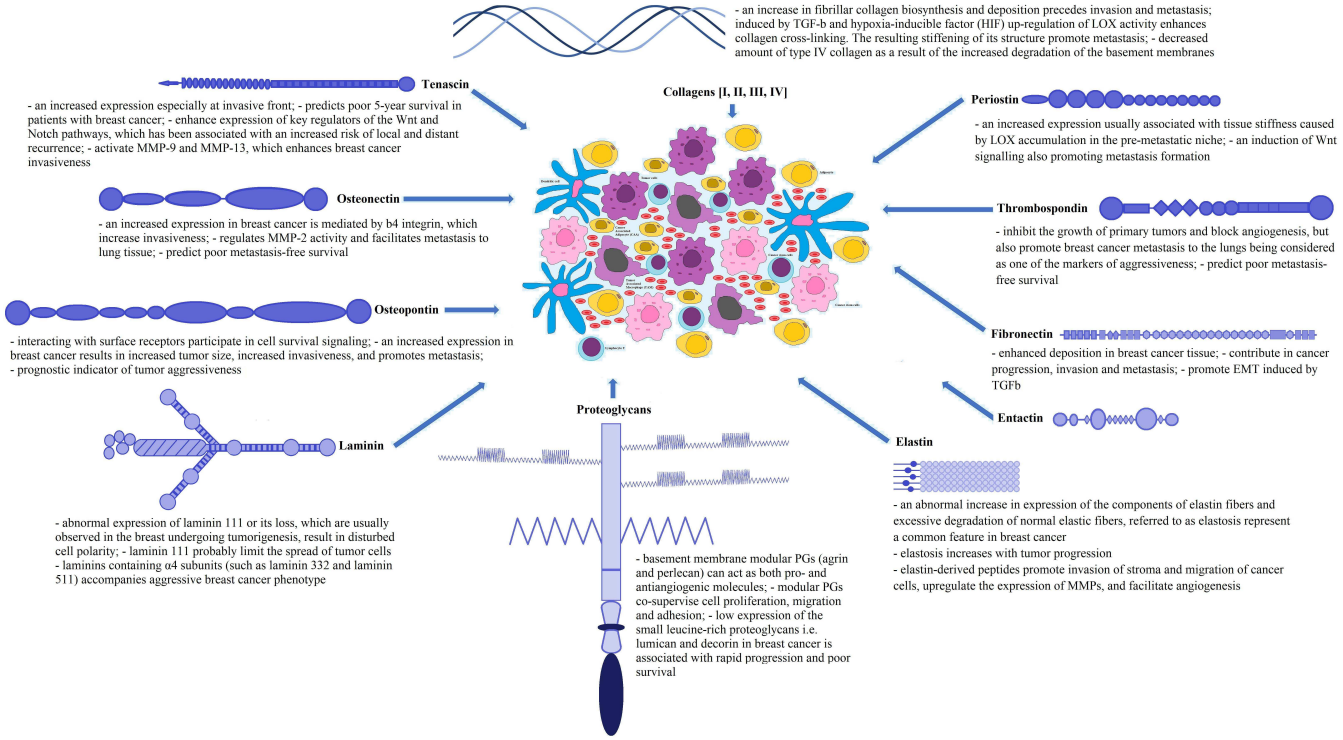

Figure 1. Chosen ECM molecules role in tumor microenvironment [10,12,15,65,66,90,121,135,140,213–220,225,226,228,229,232,234–238,240–246,248–251,255,256,264–267,270,276–279,281–284] modified.

3.5. Clinical Considerations

It seems obvious that clinical aspects of the discussed issues (e.g., the possibility of using ECM proteins as diagnostic markers in predicting the clinical course of the disease or the influence of specific anticancer therapies on the expression and function of the mentioned proteins in different subtypes of breast cancer) are of special interest for researchers dealing with breast cancer. In this chapter this topic will be discussed.

Data collected so far indicate that there is an association between the expression profiles of genes encoding specific ECM proteins and resistance of breast cancer cells (including metastatic ones) to chemotherapeutics. Increased expression of ON, POSTN, fibulin-1 and THBS2 has been shown to predispose stromal cells to show resistance to drugs such as cyclophosphamide (CPH), 5-fluorouracil (5-FU) and doxorubicin (DOXO) [285]. Moreover, it has been proven that THBS1 overexpression results in resistance to DOXO-mediated apoptosis of breast cancer cells (type I collagen seems to be involved in this effect), whereas OPN overexpression results in resistance to CPH-dependent apoptosis of these cells [286,287]. It has been suggested that, at least in some cases, the mere presence of a chemotherapeutic agent in the TME implies enhanced synthesis of specific ECM proteins. Interestingly, the present hypothesis has been confirmed in relation to DOXO, because in both in vitro and in vivo models it has been found that its presence in the breast cancer microenvironment

induces an increase in the expression of laminin 111 and fibulin-1 [288,289]. Taking into account the above-described relationship between fibulin-1 and resistance of cancer cells to chemotherapeutic drugs, it can be speculated that this protein is a mediator of resistance induced by the presence of DOXO. It is worth noting that the effect of expression profiles of ECM proteins has been studied not only on the course of chemotherapy, but also on radiotherapy and hormone therapy. With regard to the last of the therapies mentioned in the previous sentence, it should be noted that the studies, which concerned the influence of ECM protein overexpression on tamoxifen therapy, provided the conclusions that increased levels of tenascin C, ON and fibronectin in TME predispose cancer cells to exhibit resistance to therapy with selective estrogen receptor modulators and, moreover, indicate poor prognosis [290]. With regard to laminins, one cannot help but mention the discovery that laminin 332 is responsible for the failure of anti-HER2 therapy in HER2-positive tumors. This effect is mediated by the following molecules: tetraspanin CD151 and two integrins ($\alpha6\beta4$ and $\alpha3\beta1$) [291]. It is also worth emphasizing that TNBC, which display mutant p53 and are characterized by enhanced angiogenesis and poor survival, lack laminins expression. ECM proteins are also important in the context of radiotherapy outcome as has been observed that fibronectin and laminins increase resistance to ionizing radiation in vitro [292]. The relationships described above encourage the search for possible therapeutic interventions, as described in Section 3.6. For some subtypes, correlations between the presence of mRNA of certain ECM proteins and clinical prognosis have been documented. It is important to note here that the presence of a particular protein in high concentration in a particular subtype does not imply at once that it is a diagnostic marker or prognostic indicator in that subtype. For instance, the concentration of mRNA for fibronectin in TNBC and HER2-positive, but it can be considered a prognostic marker only for those tumors that express ER and PR. In contrast, mRNA levels for ON are, admittedly, highest in the luminal A subtype. Nevertheless, it is not a prognostic indicator in luminal cancers, but in HER2-positive and basal subtypes. It follows from the above that caution should be exercised when interpreting the results of studies on concentrations of individual ECM proteins in a given subtype [293–297]. Moreover, the existence of different protein variants as a result of alternative splicing is not without significance. These variants usually undergo a process called isotype switching during tumorigenesis, which may affect the clinical effect of introduced therapies. As far as radiotherapy of breast cancer is concerned, it has been shown that those tumor cells which express higher level of splice form of fibronectin called ED-A, as well as its receptor—integrin $\alpha5\beta1$, are more resistant to it. ED-A is a form that is particularly susceptible to polymerization and is associated with increased angiogenesis within the tumor. TNC also appears in the tumor-bearing breast in such isoforms, which are absent in the healthy body. It has been reported that the presence of these isoforms enhances invasiveness, in which matrix metalloproteinases are most likely involved [298–307]. Collagen type I, an essential determinant of stiffness in both healthy and cancerous breasts, has been proposed to link mammographically detected increased mammary gland density with increased breast cancer risk [308]. This role of collagen type I was confirmed in a mouse model, while in women a statistically significant correlation was observed between overexpression of genes encoding fibrillar collagens and increased degree of breast cancer invasiveness. The degree of collagen cross-linking was also found to influence invasiveness and prognosis. A loose structure of the network formed by this protein increases invasiveness, while a compact one reduces it. Sometimes, dense cross-linking of collagen fibers increases the local tissue density to such an extent that it can be detected by palpation of the breast [309–311]. It has also been observed that the density of chemotherapy-resistant tumors does not decrease after treatment (sometimes it even increases), whereas the density of chemotherapy-susceptible tumors decreases after treatment. It has been shown that collagen type III disorganizes the dense structure of collagen type I and furthermore impedes its formation, which is associated with a decrease in tumor aggressiveness. On the other hand, a decrease in collagen type III implies an increase in tumor invasiveness. A dense network of collagen fibers perpendicular to tumor

border predicts invasiveness and poorer overall survival. Inhibition of lysine oxidase and blockade of transforming growth factor β result in a reduction in the stiffness and density of the collagen fiber network in the mammary gland, indicating this network as a potential therapeutic target for breast cancer treatment. In the context of the effectiveness of anticancer therapies, it is worth highlighting that a reduction (regardless of how this effect is achieved) in the density and stiffness of the collagen scaffold facilitates drug penetration. It has been observed that this is accompanied by a local reduction in fibrinogen accumulation and a decrease in the resistance of cancer cells to drug-induced apoptosis [312–315].

3.6. ECM Proteins as Targets for Anticancer Therapies

With regard to periostin (POSTN), it has been proven that neutralizing it with appropriate antibodies entailed a reduction in breast cancer metastasis to lung tissue. This is a promising result for future work in this area [135,316]. Furthermore, a POSTN-binding DNA aptamer has been shown to inhibit breast cancer growth and metastasis. It has been suggested that the use of such or similar aptamers may serve as a future therapeutic tool against those breast cancers that overexpress POSTN [317]. There are also high hopes for the effects of POSTN in the context of combating resistance to chemotherapeutics currently used to treat breast cancer. POSTN inhibition has been reported to overcome chemoresistance via reducing the expansion of mesenchymal tumor subpopulations in breast cancer. Knockdown of POSTN inhibited growth and invasion of mesenchymal tumor cells upon chemotherapy. Furthermore, chemotherapy upregulated cancer-specific variants of POSTN and application of a blocking antibody specifically targeting those variants overcame chemoresistance as well as halted disease progression in the absence of toxic effects [318].

Endostatin (an antiangiogenic factor that is a C-terminal fragment derived from collagen type XVIII) also appears to be a promising target for anticancer therapy. Endostatin has been shown to induce RAW264.7 phenotype polarization to M1 in vitro. There have been suggestions that it may inhibit breast cancer growth in mice in vivo via the regulation of polarization of TAM. Macrophage polarization is the process of differentiation of M0 macrophages into M1 or M2, in which these cells, due to the expression of various surface markers, show different functions in response to activating factors from the microenvironment. Macrophages with the M1 phenotype are pro-inflammatory cells with anti-tumor functions, and M2 macrophages have a tumor-promoting effect. It is suspected that this occurs by shifting the polarity of TAM from the M2-like to M1-like functional phenotype or by increasing the M1-like TAM via specific inhibition of M2 polarity. In addition, data collected so far indicate that the combination of chemotherapy with endostatin administration is characterized by higher efficacy than the implementation of chemotherapy alone. Based on these reports, it can be assumed that in the future the above-mentioned combination therapy may be a valuable option in the treatment of breast cancer. It seems, however, that further research is needed in this area owing to the fact that endostatin gene variation may be relevant in this regard [319–329].

Currently, in breast cancer research, three-dimensional (3D) in vitro models are used, in which it is possible to recreate the interactions between cancer cells and the extracellular matrix, as well as the relationship between cancer cells and stromal cells [330].

Interesting observations were provided by the studies conducted by Berger et al. [331], who analyzed the mechanism by which the stiffness of the substrate may influence the invasive behavior of breast cancer cells. Increasing stiffness from low to high (2 to 12 kPa) led to a switch from proteolytically independent invasion to a proteolytically dependent phenotype. The authors stated that cells in high stiffness had increased expression of Mena, an invadopodia protein associated with metastasis in breast cancer, as a result of EGFR and PLCγ1 activation. The results obtained provide important insight into the role of matrix stiffness, composition and organization in promoting cancer invasion [331]. The research conducted by Han et al. [332] showed an important role of spatiotemporal coordination of cellular physical properties in tissue organization and disease progression. According to

the authors, using the multicellular model of the breast cancer organoid, we map the spatial and temporal evolution of the positions, movements and physical features of individual cells in three dimensions [332].

4. Conclusions

The relationships between ECM molecules and cancer development presented in this article show a significant relationship between the structure and function of the breast ECM and the interaction of many molecules both in physiological and pathological conditions. It is clear that any ECM reorganization in the breast must be under the strict and coordinated control of the organism. Disruption of cell–cell and cell–ECM interactions may lead to the development of a neoplastic process.

Moreover, the multitude and variety of interrelationships between the molecules that make up the tumor microenvironment makes it an important element, without understanding of which modern oncology will not be able to cope with many clinical challenges. Last but not least, it seems evident that as the understanding of the role of ECM proteins in breast cancer advances, there is a growing desire to put this knowledge into practice in the development and implementation of less toxic and more effective anti-cancer therapies.

Author Contributions: Conceptualization, A.L., K.O. and P.O. provided the idea of an article, conducted and wrote the entire manuscript. J.K., A.M.-P., K.K.-V. and P.O. searched the literature and edited the whole manuscript. A.M.-P., K.K.-V. and P.O. supervised the manuscript thoroughly and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Conflicts of Interest: Authors declare no conflict of interest.

References

1. World Health Organisation. Breast Cancer. Available online: https://www.who.int/news-room/fact-sheets/detail/breast-cancer (accessed on 26 March 2021).
2. Ataollahi, M.R.; Sharifi, J.; Paknahad, M.R.; Paknahad, A. Breast cancer and associated factors: A review. *J. Med. Life.* **2015**, *8*, 6–11.
3. Akram, M.; Iqbal, M.; Daniyal, M.; Khan, A.U. Awareness and current knowledge of breast cancer. *Biol. Res.* **2017**, *50*, 33. [CrossRef] [PubMed]
4. Aronson, K.J.; Miller, A.B.; Woolcott, C.G.; Sterns, E.E.; McCready, D.R.; Lickley, L.A.; Fish, E.B.; Hiraki, G.Y.; Holloway, C.; Ross, T.; et al. Breast adipose tissue concentrations of polychlorinated biphenyls and other organochlorines and breast cancer risk. *Cancer Epidemiol. Biomark. Prev.* **2000**, *9*, 55–63. [PubMed]
5. Stark, G.B.; Grandel, S.; Spilker, G. Tissue suction of the male and female breast. *Aesthetic Plast. Surg.* **1992**, *16*, 317–324. [CrossRef] [PubMed]
6. Tanis, P.J.; Nieweg, O.E.; Valdés Olmos, R.A.; Kroon, B.B. Anatomy and physiology of lymphatic drainage of the breast from the perspective of sentinel node biopsy. *J. Am. Coll. Surg.* **2001**, *192*, 399–409. [CrossRef]
7. Thomsen, S.; Tatman, D. Physiological and pathological factors of human breast disease that can influence optical diagnosis. *Ann. N. Y. Acad. Sci.* **1998**, *838*, 171–193. [CrossRef] [PubMed]
8. Jagannathan, N.R.; Sharma, U. Breast Tissue Metabolism by Magnetic Resonance Spectroscopy. *Metabolites* **2017**, *7*, 25. [CrossRef] [PubMed]
9. Hassiotou, F.; Geddes, D. Anatomy of the human mammary gland: Current status of knowledge. *Clin. Anat.* **2013**, *26*, 29–48. [CrossRef]
10. Yue, B. Biology of the extracellular matrix: An overview. *J. Glaucoma* **2014**, *23*, 20–23. [CrossRef]
11. Lu, P.; Takai, K.; Weaver, V.M.; Werb, Z. Extracellular matrix degradation and remodeling in development and disease. *Cold Spring Harb. Perspect. Biol.* **2011**, *3*, a005058. [CrossRef]
12. Frantz, C.; Stewart, K.M.; Weaver, V.M. The extracellular matrix at a glance. *J. Cell Sci.* **2010**, *123*, 4195–4200. [CrossRef]
13. Mecham, R.O. Overview of extracellular matrix. *Curr. Protoc. Cell Biol.* **2012**, *57*, 10.1.1–10.1.16. [CrossRef]
14. Järveläinen, H.; Sainio, A.; Koulu, M.; Wight, T.N.; Penttinen, R. Extracellular matrix molecules: Potential targets in pharmacotherapy. *Pharmacol. Rev.* **2009**, *61*, 198–223. [CrossRef]

15. Schaefer, L.; Schaefer, R.M. Proteoglycans: From structural compounds to signaling molecules. *Cell Tissue Res.* **2010**, *339*, 237–246. [CrossRef]
16. Rozario, T.; DeSimone, D.W. The extracellular matrix in development and morphogenesis: A dynamic view. *Dev. Biol.* **2010**, *341*, 126–140. [CrossRef]
17. Shoulders, M.D.; Raines, R.T. Collagen structure and stability. *Annu. Rev. Biochem.* **2009**, *78*, 929–958. [CrossRef]
18. Karamanos, N.K.; Theocharis, A.D.; Piperigkou, Z.; Manou, D.; Passi, A.; Skandalis, S.S.; Vynios, D.H.; Orian-Rousseau, V.; Ricard-Blum, S.; Schmelzer, C.E.H.; et al. A guide to the composition and functions of the extracellular matrix. *FEBS J.* **2021**, *288*, 6850–6912. [CrossRef]
19. Boot-Handford, R.P.; Tuckwell, D.S. Fibrillar collagen: The key to vertebrate evolution? A tale of molecular incest. *Bioessays* **2003**, *25*, 142–151. [CrossRef]
20. Egeblad, M.; Rasch, M.G.; Weaver, V.M. Dynamic interplay between the collagen scaffold and tumor evolution. *Curr. Opin. Cell Biol.* **2010**, *22*, 697–706. [CrossRef]
21. Ricard-Blum, S. The collagen family. *Cold Spring Harb. Perspect. Biol.* **2011**, *3*, a004978. [CrossRef]
22. Persikov, A.V.; Ramshaw, J.A.; Kirkpatrick, A.; Brodsky, B. Electrostatic interactions involving lysine make major contributions to collagen triple-helix stability. *Biochemistry* **2005**, *44*, 1414–1422. [CrossRef] [PubMed]
23. Myllyharju, J.; Kivirikko, K.I. Collagens, modifying enzymes and their mutations in humans, flies and worms. *Trends Genet.* **2004**, *20*, 33–43. [CrossRef] [PubMed]
24. Sauk, J.J.; Nikitakis, N.; Siavash, H. Hsp47 a novel collagen binding serpin chaperone, autoantigen and therapeutic target. *Front. Biosci.* **2005**, *10*, 107–118. [CrossRef] [PubMed]
25. Makareeva, E.; Leikin, S. Procollagen triple helix assembly: An unconventional chaperone-assisted folding paradigm. *PLoS ONE* **2007**, *2*, e1029. [CrossRef] [PubMed]
26. DiChiara, A.S.; Taylor, R.J.; Wong, M.Y.; Doan, N.D.; Rosario, A.M.; Shoulders, M.D. Mapping and Exploring the Collagen-I Proteostasis Network. *ACS Chem. Biol.* **2016**, *11*, 1408–1421. [CrossRef] [PubMed]
27. Martinek, N.; Shahab, J.; Sodek, J.; Ringuette, M. Is SPARC an evolutionarily conserved collagen chaperone? *J. Dent. Res.* **2007**, *86*, 296–305. [CrossRef] [PubMed]
28. Greenspan, D.S. Biosynthetic processing of collagen molecules. *Top. Curr. Chem.* **2005**, *247*, 149–183.
29. Hopkins, D.R.; Keles, S.; Greenspan, D.S. The bone morphogenetic protein 1/Tolloid-like metalloproteinases. *Matrix Biol.* **2007**, *26*, 508–523. [CrossRef]
30. Mäki, J.M. Lysyl oxidases in mammalian development and certain pathological conditions. *Histol. Histopathol.* **2009**, *24*, 651–660.
31. Klein, T.; Bischoff, R. Physiology and pathophysiology of matrix metalloproteases. *Amino Acids* **2011**, *41*, 271–290. [CrossRef]
32. Han, S.; Makareeva, E.; Kuznetsova, N.V.; DeRidder, A.M.; Sutter, M.B.; Losert, W.; Phillips, C.L.; Visse, R.; Nagase, H.; Leikin, S. Molecular mechanism of type I collagen homotrimer resistance to mammalian collagenases. *J. Biol. Chem.* **2010**, *285*, 22276–22281. [CrossRef]
33. Ricard-Blum, S.; Ballut, L. Matricryptins derived from collagens and proteoglycans. *Front. Biosci. Landmark Ed.* **2011**, *16*, 674–697. [CrossRef]
34. Murphy, G. Regulation of the proteolytic disintegrin metalloproteinases, the 'Sheddases'. *Semin. Cell Dev. Biol.* **2009**, *20*, 138–145. [CrossRef]
35. Mundel, T.M.; Kalluri, R. Type IV collagen-derived angiogenesis inhibitors. *Microvasc. Res.* **2007**, *74*, 85–89. [CrossRef]
36. Heino, J. The collagen family members as cell adhesion proteins. *Bioessays* **2007**, *29*, 1001–1010. [CrossRef]
37. Heino, J.; Huhtala, M.; Käpylä, J.; Johnson, M.S. Evolution of collagen-based adhesion systems. *Int. J. Biochem. Cell Biol.* **2009**, *41*, 341–348. [CrossRef]
38. Humphries, J.D.; Byron, A.; Humphries, M.J. Integrin ligands at a glance. *J. Cell Sci.* **2006**, *119*, 3901–3903. [CrossRef]
39. Leitinger, B.; Hohenester, E. Mammalian collagen receptors. *Matrix Biol.* **2007**, *26*, 146–155. [CrossRef]
40. Carafoli, F.; Bihan, D.; Stathopoulos, S.; Konitsiotis, A.D.; Kvansakul, M.; Farndale, R.W.; Leitinger, B.; Hohenester, E. Crystallographic insight into collagen recognition by discoidin domain receptor 2. *Structure* **2009**, *17*, 1573–1581. [CrossRef]
41. Flynn, L.A.; Blissett, A.R.; Calomeni, E.P.; Agarwal, G. Inhibition of collagen fibrillogenesis by cells expressing soluble extracellular domains of DDR1 and DDR2. *J. Mol. Biol.* **2010**, *395*, 533–543. [CrossRef]
42. Sivakumar, L.; Agarwal, G. The influence of discoidin domain receptor 2 on the persistence length of collagen type I fibers. *Biomaterials* **2010**, *31*, 4802–4808. [CrossRef]
43. Lebbink, R.J.; de Ruiter, T.; Adelmeijer, J.; Brenkman, A.B.; van Helvoort, J.M.; Koch, M.; Farndale, R.W.; Lisman, T.; Sonnenberg, A.; Lenting, P.J.; et al. Collagens are functional, high affinity ligands for the inhibitory immune receptor LAIR-1. *J. Exp. Med.* **2006**, *203*, 1419–1425. [CrossRef]
44. Lebbink, R.J.; Raynal, N.; de Ruiter, T.; Bihan, D.G.; Farndale, R.W.; Meyaard, L. Identification of multiple potent binding sites for human leukocyte associated Ig-like receptor LAIR on collagens II and III. *Matrix Biol.* **2009**, *28*, 202–210. [CrossRef]
45. Lebbink, R.J.; van den Berg, M.C.; de Ruiter, T.; Raynal, N.; van Roon, J.A.; Lenting, P.J.; Jin, B.; Meyaard, L. The soluble leukocyte-associated Ig-like receptor (LAIR)-2 antagonizes the collagen/LAIR-1 inhibitory immune interaction. *J. Immunol.* **2008**, *180*, 1662–1669. [CrossRef]

46. Brondijk, T.H.; de Ruiter, T.; Ballering, J.; Wienk, H.; Lebbink, R.J.; van Ingen, H.; Boelens, R.; Farndale, R.W.; Meyaard, L.; Huizinga, E.G. Crystal structure and collagen-binding site of immune inhibitory receptor LAIR-1: Unexpected implications for collagen binding by platelet receptor GPVI. *Blood* **2010**, *115*, 1364–1373. [CrossRef]
47. Kozel, B.A.; Mecham, R.P.; Rosenbloom, J. Elastin. In *The Extracellular Matrix: An Overview*; Mecham, R.P., Ed.; Springer: Berlin/Heidelberg, Germany, 2011; pp. 267–299.
48. Wise, S.G.; Weiss, A.S. Tropoelastin. *Int. J. Biochem. Cell Biol.* **2009**, *41*, 494–497. [CrossRef]
49. Lucero, H.A.; Kagan, H.M. Lysyl oxidase: An oxidative enzyme and effector of cell function. *Cell. Mol. Life Sci.* **2006**, *63*, 2304–2316. [CrossRef] [PubMed]
50. Durbeej, M. Laminins. *Cell Tissue Res.* **2010**, *339*, 259–268. [CrossRef] [PubMed]
51. Aumailley, M.; Bruckner-Tuderman, L.; Carter, W.G.; Deutzmann, R.; Edgar, D.; Ekblom, P.; Engel, J.; Engvall, E.; Hohenester, E.; Jones, J.C.; et al. A simplified laminin nomenclature. *Matrix Biol.* **2005**, *24*, 326–332. [CrossRef] [PubMed]
52. Colognato, H.; Yurchenco, P.D. Form and function: The laminin family of heterotrimers. *Dev. Dyn.* **2000**, *218*, 213–234. [CrossRef]
53. Hallmann, R.; Horn, N.; Selg, M.; Wendler, O.; Pausch, F.; Sorokin, L.M. Expression and function of laminins in the embryonic and mature vasculature. *Physiol. Rev.* **2005**, *85*, 979–1000. [CrossRef] [PubMed]
54. Miner, J.H.; Cunningham, J.; Sanes, J.R. Roles for laminin in embryogenesis: Exencephaly, syndactyly, and placentopathy in mice lacking the laminin alpha5 chain. *J. Cell Biol.* **1998**, *143*, 1713–1723. [CrossRef] [PubMed]
55. McKee, K.K.; Harrison, D.; Capizzi, S.; Yurchenco, P.D. Role of laminin terminal globular domains in basement membrane assembly. *J. Biol. Chem.* **2007**, *282*, 21437–21447. [CrossRef]
56. Schéele, S.; Nyström, A.; Durbeej, M.; Talts, J.F.; Ekblom, M.; Ekblom, P. Laminin isoforms in development and disease. *J. Mol. Med.* **2007**, *85*, 825–836. [CrossRef] [PubMed]
57. Timpl, R.; Sasaki, T.; Kostka, G.; Chu, M.L. Fibulins: A versatile family of extracellular matrix proteins. *Nat. Rev. Mol. Cell Biol.* **2003**, *4*, 479–489. [CrossRef] [PubMed]
58. Tzu, J.; Marinkovich, M.P. Bridging structure with function: Structural, regulatory, and developmental role of laminins. *Int. J. Biochem. Cell Biol.* **2008**, *40*, 199–214. [CrossRef] [PubMed]
59. Smith, M.L.; Gourdon, D.; Little, W.C.; Kubow, K.E.; Eguiluz, R.A.; Luna-Morris, S.; Vogel, V. Force-induced unfolding of fibronectin in the extracellular matrix of living cells. *PLoS Biol.* **2007**, *5*, e268. [CrossRef]
60. Schwarzbauer, J.E.; DeSimone, D.W. Fibronectins, their fibrillogenesis, and in vivo functions. *Cold Spring Harb. Perspect. Biol.* **2011**, *3*, a005041. [CrossRef]
61. Patten, J.; Wang, K. Fibronectin in development and wound healing. *Adv. Drug Deliv. Rev.* **2021**, *170*, 353–368. [CrossRef]
62. Tsang, K.Y.; Cheung, M.C.; Chan, D.; Cheah, K.S. The developmental roles of the extracellular matrix: Beyond structure to regulation. *Cell Tissue Res.* **2010**, *339*, 93–110. [CrossRef]
63. Briggs, S.L. The role of fibronectin in fibroblast migration during tissue repair. *J. Wound Care* **2005**, *14*, 284–287. [CrossRef]
64. Iozzo, R.V.; Murdoch, A.D. Proteoglycans of the extracellular environment: Clues from the gene and protein side offer novel perspectives in molecular diversity and function. *FASEB J.* **1996**, *10*, 598–614. [CrossRef]
65. Goldoni, S.; Iozzo, R.V. Tumor microenvironment: Modulation by decorin and related molecules harboring leucine-rich tandem motifs. *Int. J. Cancer* **2008**, *123*, 2473–2479. [CrossRef]
66. Schaefer, L.; Iozzo, R.V. Biological functions of the small leucine-rich proteoglycans: From genetics to signal transduction. *J. Biol. Chem.* **2008**, *283*, 21305–21309. [CrossRef]
67. Esko, J.D.; Kimata, K.; Lindahl, U. Proteoglycans and Sulfated Glycosaminoglycans. In *Essentials of Glycobiology*, 2nd ed.; Varki, A., Cummings, R.D., Esko, J.D., Freeze, H.H., Stanley, P., Bertozzi, C.R., Hart, G.W., Etzler, M.E., Eds.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, USA, 2009; Chapter 16, pp. 1–17.
68. Iozzo, R.V.; Zoeller, J.J.; Nyström, A. Basement membrane proteoglycans: Modulators Par Excellence of cancer growth and angiogenesis. *Mol. Cells* **2009**, *27*, 503–513. [CrossRef]
69. Isenberg, J.S.; Roberts, D.D. THBS1 (thrombospondin-1). *Atlas Genet. Cytogenet. Oncol. Haematol.* **2020**, *24*, 291–299. [CrossRef]
70. Leonhard-Melief, C.; Haltiwanger, R.S. O-fucosylation of thrombospondin type 1 repeats. *Methods Enzymol.* **2010**, *480*, 401–416.
71. De Peredo, A.G.; Klein, D.; Macek, B.; Hess, D.; Peter-Katalinic, J.; Hofsteenge, J. C-mannosylation and o-fucosylation of thrombospondin type 1 repeats. *Mol. Cell. Proteom.* **2002**, *1*, 11–18.
72. Hofsteenge, J.; Huwiler, K.G.; Macek, B.; Hess, D.; Lawler, J.; Mosher, D.F.; Peter-Katalinic, J. C-mannosylation and O-fucosylation of the thrombospondin type 1 module. *J. Biol. Chem.* **2001**, *276*, 6485–6498. [CrossRef]
73. Wang, L.W.; Leonhard-Melief, C.; Haltiwanger, R.S.; Apte, S.S. Post-translational modification of thrombospondin type-1 repeats in ADAMTS-like 1/punctin-1 by C-mannosylation of tryptophan. *J. Biol. Chem.* **2009**, *284*, 30004–30015. [CrossRef]
74. Furukawa, K.; Roberts, D.D.; Endo, T.; Kobata, A. Structural study of the sugar chains of human platelet thrombospondin. *Arch. Biochem. Biophys.* **1989**, *270*, 302–312. [CrossRef]
75. Isenberg, J.S.; Martin-Manso, G.; Maxhimer, J.B.; Roberts, D.D. Regulation of nitric oxide signalling by thrombospondin 1: Implications for anti-angiogenic therapies. *Nat. Rev. Cancer* **2009**, *9*, 182–194. [CrossRef]
76. Resovi, A.; Pinessi, D.; Chiorino, G.; Taraboletti, G. Current understanding of the thrombospondin-1 interactome. *Matrix Biol.* **2014**, *37*, 83–91. [CrossRef]
77. Murphy-Ullrich, J.E.; Suto, M.J. Thrombospondin-1 regulation of latent TGF-β activation: A therapeutic target for fibrotic disease. *Matrix Biol.* **2018**, *68–69*, 28–43. [CrossRef]

78. Kaur, S.; Soto-Pantoja, D.R.; Stein, E.V.; Liu, C.; Elkahloun, A.G.; Pendrak, M.L.; Nicolae, A.; Singh, S.P.; Nie, Z.; Levens, D.; et al. Thrombospondin-1 signaling through CD47 inhibits self-renewal by regulating c-Myc and other stem cell transcription factors. *Sci. Rep.* **2013**, *3*, 1673. [CrossRef]
79. Hao, C.; Cui, Y.; Owen, S.; Li, W.; Cheng, S.; Jiang, W.G. Human osteopontin: Potential clinical applications in cancer (Review). *Int. J. Mol. Med.* **2017**, *39*, 1327–1337. [CrossRef]
80. Weber, G.F.; Cantor, H. The immunology of Eta-1/osteopontin. *Cytokine Growth Factor Rev.* **1996**, *7*, 241–248. [CrossRef]
81. Denhardt, D.T.; Noda, M. Osteopontin expression and function: Role in bone remodeling. *J. Cell Biochem.* **1998**, *72*, 92–102. [CrossRef]
82. Giachelli, C.M.; Liaw, L.; Murry, C.E.; Schwartz, S.M.; Almeida, M. Osteopontin expression in cardiovascular diseases. *Ann. N. Y. Acad. Sci.* **1995**, *760*, 109–126. [CrossRef]
83. Christensen, B.; Petersen, T.E.; Sørensen, E.S. Post-translational modification and proteolytic processing of urinary osteopontin. *Biochem. J.* **2008**, *411*, 53–61. [CrossRef]
84. Christensen, B.; Nielsen, M.S.; Haselmann, K.F.; Petersen, T.E.; Sørensen, E.S. Post-translationally modified residues of native human osteopontin are located in clusters: Identification of 36 phosphorylation and five O-glycosylation sites and their biological implications. *Biochem. J.* **2005**, *390*, 285–292. [CrossRef] [PubMed]
85. Christensen, B.; Kazanecki, C.C.; Petersen, T.E.; Rittling, S.R.; Denhardt, D.T.; Sørensen, E.S. Cell type-specific post-translational modifications of mouse osteopontin are associated with different adhesive properties. *J. Biol. Chem.* **2007**, *282*, 19463–19472. [CrossRef] [PubMed]
86. Sodek, J.; Ganss, B.; McKee, M.D. Osteopontin. *Crit. Rev. Oral Biol. Med.* **2000**, *11*, 279–303. [CrossRef] [PubMed]
87. Cho, H.J.; Cho, H.J.; Kim, H.S. Osteopontin: A multifunctional protein at the crossroads of inflammation, atherosclerosis, and vascular calcification. *Curr. Atheroscler. Rep.* **2009**, *11*, 206–213. [CrossRef]
88. Denhardt, D.T.; Guo, X. Osteopontin: A protein with diverse functions. *FASEB J.* **1993**, *7*, 1475–1482. [CrossRef]
89. El-Tanani, M.K.; Campbell, F.C.; Kurisetty, V.; Jin, D.; McCann, M.; Rudland, P.S. The regulation and role of osteopontin in malignant transformation and cancer. *Cytokine Growth Factor Rev.* **2006**, *17*, 463–474. [CrossRef]
90. Coppola, D.; Szabo, M.; Boulware, D.; Muraca, P.; Alsarraj, M.; Chambers, A.F.; Yeatman, T.J. Correlation of osteopontin protein expression and pathological stage across a wide variety of tumor histologies. *Clin. Cancer Res.* **2004**, *10*, 184–190. [CrossRef]
91. Rittling, S.R.; Chambers, A.F. Role of osteopontin in tumour progression. *Br. J. Cancer* **2004**, *90*, 1877–1881. [CrossRef]
92. Babarović, E.; Valković, T.; Budisavljević, I.; Balen, I.; Štifter, S.; Duletić-Načinović, A.; Lučin, K.; Jonjić, N. The expression of osteopontin and vascular endothelial growth factor in correlation with angiogenesis in monoclonal gammopathy of undetermined significance and multiple myeloma. *Pathol. Res. Pract.* **2016**, *212*, 509–516. [CrossRef]
93. Kerenidi, T.; Kazakou, A.P.; Lada, M.; Tsilioni, I.; Daniil, Z.; Gourgoulianis, K.I. Clinical Significance of Circulating Osteopontin Levels in Patients With Lung Cancer and Correlation With VEGF and MMP-9. *Cancer Investig.* **2016**, *34*, 385–392. [CrossRef]
94. Scatena, M.; Liaw, L.; Giachelli, C.M. Osteopontin: A multifunctional molecule regulating chronic inflammation and vascular disease. *Arterioscler. Thromb. Vasc. Biol.* **2007**, *27*, 2302–2309. [CrossRef]
95. Smith, L.L.; Cheung, H.K.; Ling, L.E.; Chen, J.; Sheppard, D.; Pytela, R.; Giachelli, C.M. Osteopontin N-terminal domain contains a cryptic adhesive sequence recognized by alpha9beta1 integrin. *J. Biol. Chem.* **1996**, *271*, 28485–28491. [CrossRef]
96. Yokosaki, Y.; Matsuura, N.; Sasaki, T.; Murakami, I.; Schneider, H.; Higashiyama, S.; Saitoh, Y.; Yamakido, M.; Taooka, Y.; Sheppard, D. The integrin alpha(9)beta(1) binds to a novel recognition sequence (SVVYGLR) in the thrombin-cleaved amino-terminal fragment of osteopontin. *J. Biol. Chem.* **1999**, *274*, 36328–36334. [CrossRef]
97. Bellahcène, A.; Castronovo, V.; Ogbureke, K.U.; Fisher, L.W.; Fedarko, N.S. Small integrin-binding ligand N-linked glycoproteins (SIBLINGs): Multifunctional proteins in cancer. *Nat. Rev. Cancer* **2008**, *8*, 212–226. [CrossRef]
98. Mi, Z.; Guo, H.; Wai, P.Y.; Gao, C.; Kuo, P.C. Integrin-linked kinase regulates osteopontin-dependent MMP-2 and uPA expression to convey metastatic function in murine mammary epithelial cancer cells. *Carcinogenesis* **2006**, *27*, 1134–1145. [CrossRef]
99. Chen, R.X.; Xia, Y.H.; Xue, T.C.; Zhang, H.; Ye, S.L. Down-regulation of osteopontin inhibits metastasis of hepatocellular carcinoma cells via a mechanism involving MMP-2 and uPA. *Oncol. Rep.* **2011**, *25*, 803–808.
100. Tuck, A.B.; Hota, C.; Chambers, A.F. Osteopontin(OPN)-induced increase in human mammary epithelial cell invasiveness is urokinase (uPA)-dependent. *Breast Cancer Res. Treat.* **2001**, *70*, 197–204. [CrossRef]
101. Wang, Y.; Yan, W.; Lu, X.; Qian, C.; Zhang, J.; Li, P.; Shi, L.; Zhao, P.; Fu, Z.; Pu, P.; et al. Overexpression of osteopontin induces angiogenesis of endothelial progenitor cells via the avβ3/PI3K/AKT/eNOS/NO signaling pathway in glioma cells. *Eur. J. Cell Biol.* **2011**, *90*, 642–648. [CrossRef]
102. Wu, C.M.; Chen, P.C.; Li, T.M.; Fong, Y.C.; Tang, C.H. Si-Wu-tang extract stimulates bone formation through PI3K/Akt/NF-κB signaling pathways in osteoblasts. *BMC Complement. Altern. Med.* **2013**, *13*, 277. [CrossRef]
103. Ogata, T.; Ueyama, T.; Nomura, T.; Asada, S.; Tagawa, M.; Nakamura, T.; Takahashi, T.; Matsubara, H.; Oh, H. Osteopontin is a myosphere-derived secretory molecule that promotes angiogenic progenitor cell proliferation through the phosphoinositide 3-kinase/Akt pathway. *Biochem. Biophys. Res. Commun.* **2007**, *359*, 341–347. [CrossRef]
104. Rosset, E.M.; Bradshaw, A.D. SPARC/osteonectin in mineralized tissue. *Matrix Biol.* **2016**, *52–54*, 78–87. [CrossRef] [PubMed]
105. Murphy-Ullrich, J.E.; Sage, E.H. Revisiting the matricellular concept. *Matrix Biol.* **2014**, *37*, 1–14. [CrossRef] [PubMed]
106. Bradshaw, A.D. Diverse biological functions of the SPARC family of proteins. *Int. J. Biochem. Cell Biol.* **2012**, *44*, 480–488. [CrossRef] [PubMed]

107. Pataquiva-Mateus, A.Y.; Wu, H.C.; Lucchesi, C.; Ferraz, M.P.; Monteiro, F.J.; Spector, M. Supplementation of collagen scaffolds with SPARC to facilitate mineralization. *J. Biomed. Mater. Res. B Appl. Biomater.* **2012**, *100*, 862–870. [CrossRef]
108. Kelm, R.J., Jr.; Mann, K.G. The collagen binding specificity of bone and platelet osteonectin is related to differences in glycosylation. *J. Biol. Chem.* **1991**, *266*, 9632–9639. [CrossRef]
109. Reed, M.J.; Puolakkainen, P.; Lane, T.F.; Dickerson, D.; Bornstein, P.; Sage, E.H. Differential expression of SPARC and thrombospondin 1 in wound repair: Immunolocalization and in situ hybridization. *J. Histochem. Cytochem.* **1993**, *41*, 1467–1477. [CrossRef]
110. Brekken, R.A.; Sage, E.H. SPARC, a matricellular protein: At the crossroads of cell-matrix communication. *Matrix Biol.* **2001**, *19*, 816–827. [CrossRef]
111. Breton-Gorius, J.; Clezardin, P.; Guichard, J.; Debili, N.; Malaval, L.; Vainchenker, W.; Cramer, E.M.; Delmas, P.D. Localization of platelet osteonectin at the internal face of the alpha-granule membranes in platelets and megakaryocytes. *Blood* **1992**, *79*, 936–941. [CrossRef]
112. Trombetta-eSilva, J.; Rosset, E.A.; Hepfer, R.G.; Wright, G.J.; Baicu, C.; Yao, H.; Bradshaw, A.D. Decreased Mechanical Strength and Collagen Content in SPARC-Null Periodontal Ligament Is Reversed by Inhibition of Transglutaminase Activity. *J. Bone Miner. Res.* **2015**, *30*, 1914–1924. [CrossRef]
113. Rentz, T.J.; Poobalarahi, F.; Bornstein, P.; Sage, E.H.; Bradshaw, A.D. SPARC regulates processing of procollagen I and collagen fibrillogenesis in dermal fibroblasts. *J. Biol. Chem.* **2007**, *282*, 22062–22071. [CrossRef]
114. Harris, B.S.; Zhang, Y.; Card, L.; Rivera, L.B.; Brekken, R.A.; Bradshaw, A.D. SPARC regulates collagen interaction with cardiac fibroblast cell surfaces. *Am. J. Physiol. Heart Circ. Physiol.* **2011**, *301*, H841–H847. [CrossRef]
115. Zhu, Y.S.; Zhang, J.N.; Mo, T.T.; Jiang, C.; Ma, R.C.; Chen, L. Discoidin domain receptor 2 activation of p38 mitogen-activated protein kinase as an important pathway for osteonectin-regulating osteoblast mineralization. *J. Orthop. Surg. Res.* **2021**, *7*, 16711. [CrossRef]
116. O'Dwyer, D.N.; Moore, B.B. The role of periostin in lung fibrosis and airway remodeling. *Cell. Mol. Life Sci.* **2017**, *74*, 4305–4314. [CrossRef]
117. Izuhara, K.; Conway, S.J.; Moore, B.B.; Matsumoto, H.; Holweg, C.T.; Matthews, J.G.; Arron, J.R. Roles of Periostin in Respiratory Disorders. *Am. J. Respir. Crit. Care Med.* **2016**, *193*, 949–956. [CrossRef]
118. Horiuchi, K.; Amizuka, N.; Takeshita, S.; Takamatsu, H.; Katsuura, M.; Ozawa, H.; Toyama, Y.; Bonewald, L.F.; Kudo, A. Identification and characterization of a novel protein, periostin, with restricted expression to periosteum and periodontal ligament and increased expression by transforming growth factor beta. *J. Bone Miner. Res.* **1999**, *14*, 1239–1249. [CrossRef]
119. Ashley, S.L.; Wilke, C.A.; Kim, K.K.; Moore, B.B. Periostin regulates fibrocyte function to promote myofibroblast differentiation and lung fibrosis. *Mucosal Immunol.* **2017**, *10*, 341–351. [CrossRef]
120. Takayama, G.; Arima, K.; Kanaji, T.; Toda, S.; Tanaka, H.; Shoji, S.; McKenzie, A.N.; Nagai, H.; Hotokebuchi, T.; Izuhara, K. Periostin: A novel component of subepithelial fibrosis of bronchial asthma downstream of IL-4 and IL-13 signals. *J. Allergy Clin. Immunol.* **2006**, *118*, 98–104. [CrossRef]
121. Morra, L.; Rechsteiner, M.; Casagrande, S.; von Teichman, A.; Schraml, P.; Moch, H.; Soltermann, A. Characterization of periostin isoform pattern in non-small cell lung cancer. *Lung Cancer* **2012**, *76*, 183–190. [CrossRef]
122. Kudo, A. Periostin in fibrillogenesis for tissue regeneration: Periostin actions inside and outside the cell. *Cell Mol. Life Sci.* **2011**, *68*, 3201–3207. [CrossRef]
123. Ruan, K.; Bao, S.; Ouyang, G. The multifaceted role of periostin in tumorigenesis. *Cell Mol. Life Sci.* **2009**, *66*, 2219–2230. [CrossRef]
124. Yu, L.; Wang, J.; Liu, K. Role of periostin in ECRS. *Eur. Arch. Otorhinolaryngol.* **2021**, *278*, 2665–2672. [CrossRef] [PubMed]
125. Midwood, K.S.; Chiquet, M.; Tucker, R.P.; Orend, G. Tenascin-C at a glance. *J. Cell Sci.* **2016**, *129*, 4321–4327. [CrossRef] [PubMed]
126. Giblin, S.P.; Midwood, K.S. Tenascin-C: Form versus function. *Cell Adh. Migr.* **2015**, *9*, 48–82. [CrossRef] [PubMed]
127. Brösicke, N.; Faissner, A. Role of tenascins in the ECM of gliomas. *Cell Adh. Migr.* **2015**, *9*, 131–140. [CrossRef]
128. Lowy, C.M.; Oskarsson, T. Tenascin C in metastasis: A view from the invasive front. *Cell Adh. Migr.* **2015**, *9*, 112–124. [CrossRef]
129. Midwood, K.S.; Hussenet, T.; Langlois, B.; Orend, G. Advances in tenascin-C biology. *Cell. Mol. Life Sci.* **2011**, *68*, 3175–3199. [CrossRef]
130. Schwenzer, A.; Jiang, X.; Mikuls, T.R.; Payne, J.B.; Sayles, H.R.; Quirke, A.M.; Kessler, B.M.; Fischer, R.; Venables, P.J.; Lundberg, K.; et al. Identification of an immunodominant peptide from citrullinated tenascin-C as a major target for autoantibodies in rheumatoid arthritis. *Ann. Rheum. Dis.* **2016**, *75*, 1876–1883. [CrossRef]
131. Yagi, H.; Yanagisawa, M.; Suzuki, Y.; Nakatani, Y.; Ariga, T.; Kato, K.; Yu, R.K. HNK-1 epitope-carrying tenascin-C spliced variant regulates the proliferation of mouse embryonic neural stem cells. *J. Biol. Chem.* **2010**, *285*, 37293–37301. [CrossRef]
132. Song, J.; Schwenzer, A.; Wong, A.; Turcinov, S.; Rims, C.; Martinez, L.R.; Arribas-Layton, D.; Gerstner, C.; Muir, V.S.; Midwood, K.S.; et al. Shared recognition of citrullinated tenascin-C peptides by T and B cells in rheumatoid arthritis. *JCI Insight* **2021**, *6*, e145217. [CrossRef]
133. Chung, A.E.; Dong, L.J.; Wu, C.; Durkin, M.E. Biological functions of entactin. *Kidney Int.* **1993**, *43*, 13–19. [CrossRef]
134. Jagroop, R.; Martin, C.J.; Moorehead, R.A. Nidogen 1 regulates proliferation and migration/invasion in murine claudin-low mammary tumor cells. *Oncol. Lett.* **2021**, *21*, 52. [CrossRef] [PubMed]
135. Oskarsson, T. Extracellular matrix components in breast cancer progression and metastasis. *Breast* **2013**, *22*, 66–72. [CrossRef] [PubMed]

136. Insua-Rodríguez, J.; Oskarsson, T. The extracellular matrix in breast cancer. *Adv. Drug Deliv. Rev.* **2016**, *97*, 41–55. [CrossRef] [PubMed]
137. Gudjonsson, T.; Rønnov-Jessen, L.; Villadsen, R.; Rank, F.; Bissell, M.J.; Petersen, O.W. Normal and tumor-derived myoepithelial cells differ in their ability to interact with luminal breast epithelial cells for polarity and basement membrane deposition. *J. Cell Sci.* **2002**, *115*, 39–50. [CrossRef] [PubMed]
138. Edwards, G.M.; Wilford, F.H.; Liu, X.; Hennighausen, L.; Djiane, J.; Streuli, C.H. Regulation of mammary differentiation by extracellular matrix involves protein-tyrosine phosphatases. *J. Biol. Chem.* **1998**, *273*, 9495–9500. [CrossRef] [PubMed]
139. Lee, Y.J.; Hsu, T.C.; Du, J.Y.; Valentijn, A.J.; Wu, T.Y.; Cheng, C.F.; Yang, Z.; Streuli, C.H. Extracellular matrix controls insulin signaling in mammary epithelial cells through the RhoA/Rok pathway. *J. Cell. Physiol.* **2009**, *220*, 476–484. [CrossRef]
140. Muschler, J.; Streuli, C.H. Cell-matrix interactions in mammary gland development and breast cancer. *Cold Spring Harb. Perspect. Biol.* **2010**, *2*, a003202. [CrossRef]
141. Streuli, C.H.; Schmidhauser, C.; Bailey, N.; Yurchenco, P.; Skubitz, A.P.; Roskelley, C.; Bissell, M.J. Laminin mediates tissue-specific gene expression in mammary epithelia. *J. Cell Biol.* **1995**, *129*, 591–603. [CrossRef]
142. Streuli, C.H.; Edwards, G.M.; Delcommenne, M.; Whitelaw, C.B.; Burdon, T.G.; Schindler, C.; Watson, C.J. Stat5 as a target for regulation by extracellular matrix. *J. Biol. Chem.* **1995**, *270*, 21639–21644. [CrossRef]
143. Naylor, M.J.; Li, N.; Cheung, J.; Lowe, E.T.; Lambert, E.; Marlow, R.; Wang, P.; Schatzmann, F.; Wintermantel, T.; Schüetz, G.; et al. Ablation of beta1 integrin in mammary epithelium reveals a key role for integrin in glandular morphogenesis and differentiation. *J. Cell Biol.* **2005**, *171*, 717–728. [CrossRef]
144. Schedin, P.; O'Brien, J.; Rudolph, M.; Stein, T.; Borges, V. Microenvironment of the involuting mammary gland mediates mammary cancer progression. *J. Mammary Gland Biol. Neoplasia* **2007**, *12*, 71–82. [CrossRef]
145. O'Brien, J.H.; Vanderlinden, L.A.; Schedin, P.J.; Hansen, K.C. Rat mammary extracellular matrix composition and response to ibuprofen treatment during postpartum involution by differential GeLC-MS/MS analysis. *J. Proteome Res.* **2012**, *11*, 4894–4905. [CrossRef]
146. Klinowska, T.C.; Soriano, J.V.; Edwards, G.M.; Oliver, J.M.; Valentijn, A.J.; Montesano, R.; Streuli, C.H. Laminin and beta1 integrins are crucial for normal mammary gland development in the mouse. *Dev. Biol.* **1999**, *215*, 13–32. [CrossRef]
147. Shah, R.; Rosso, K.; Nathanson, S.D. Pathogenesis, prevention, diagnosis and treatment of breast cancer. *World J. Clin. Oncol.* **2014**, *5*, 283–298. [CrossRef]
148. Sun, Y.S.; Zhao, Z.; Yang, Z.N.; Xu, F.; Lu, H.J.; Zhu, Z.Y.; Shi, W.; Jiang, J.; Yao, P.P.; Zhu, H.P. Risk Factors and Preventions of Breast Cancer. *Int. J. Biol. Sci.* **2017**, *13*, 1387–1397. [CrossRef]
149. Ma, C.; Nepal, M.; Kim, J.H.; Fan, P.; Fei, P. A new look at molecular biology of breast cancer. *Cancer Biol. Ther.* **2019**, *20*, 1–5. [CrossRef]
150. Makarem, N.; Chandran, U.; Bandera, E.V.; Parekh, N. Dietary fat in breast cancer survival. *Annu. Rev. Nutr.* **2013**, *33*, 319–348. [CrossRef]
151. Testa, U.; Castelli, G.; Pelosi, E. Breast Cancer: A Molecularly Heterogenous Disease Needing Subtype-Specific Treatments. *Med. Sci.* **2020**, *8*, 18. [CrossRef]
152. Tao, Z.; Shi, A.; Lu, C.; Song, T.; Zhang, Z.; Zhao, J. Breast Cancer: Epidemiology and Etiology. *Cell Biochem. Biophys.* **2015**, *72*, 333–338. [CrossRef]
153. Cavalieri, E.L.; Rogan, E.G. The etiology and prevention of breast cancer. *Drug Discov. Today Dis. Mech.* **2012**, *9*, e55–e69. [CrossRef]
154. Polyak, K. Breast cancer: Origins and evolution. *J. Clin. Investig.* **2007**, *117*, 3155–3163. [CrossRef] [PubMed]
155. Jin, L.; Han, B.; Siegel, E.; Cui, Y.; Giuliano, A.; Cui, X. Breast cancer lung metastasis: Molecular biology and therapeutic implications. *Cancer Biol. Ther.* **2018**, *19*, 858–868. [CrossRef] [PubMed]
156. Esparza-López, J.; Escobar-Arriaga, E.; Soto-Germes, S.; Ibarra-Sánchez, M.J. Breast Cancer Intra-Tumor Heterogeneity: One Tumor, Different Entities. *Rev. Investig. Clin.* **2017**, *69*, 66–76. [CrossRef] [PubMed]
157. Welch, D.R.; Steeg, P.S.; Rinker-Schaeffer, C.W. Molecular biology of breast cancer metastasis. Genetic regulation of human breast carcinoma metastasis. *Breast Cancer Res.* **2000**, *2*, 408–416. [CrossRef] [PubMed]
158. Sariego, J. Breast cancer in the young patient. *Am. Surg.* **2010**, *76*, 1397–1400. [CrossRef] [PubMed]
159. Soysal, S.D.; Tzankov, A.; Muenst, S.E. Role of the Tumor Microenvironment in Breast Cancer. *Pathobiology* **2015**, *82*, 142–152. [CrossRef] [PubMed]
160. Liu, M.; Xie, F.; Liu, M.; Zhang, Y.; Wang, S. Association between BRCA mutational status and survival in patients with breast cancer: A systematic review and meta-analysis. *Breast Cancer Res. Treat.* **2021**, *186*, 591–605. [CrossRef]
161. Weigelt, B.; Geyer, F.C.; Reis-Filho, J.S. Histological types of breast cancer: How special are they? *Mol. Oncol.* **2010**, *4*, 192–208. [CrossRef]
162. Al-Thoubaity, F.K. Molecular classification of breast cancer: A retrospective cohort study. *Ann. Med. Surg.* **2019**, *49*, 44–48. [CrossRef]
163. Winters, S.; Martin, C.; Murphy, D.; Shokar, N.K. Breast Cancer Epidemiology, Prevention, and Screening. *Prog. Mol. Biol. Transl. Sci.* **2017**, *151*, 1–32.
164. Børresen-Dale, A.L.; Sørlie, T.; Kristensen, V.N. On the molecular biology of breast cancer. *Mol. Oncol.* **2010**, *4*, 171–173. [CrossRef]

165. Coleman, R.E.; Gregory, W.; Marshall, H.; Wilson, C.; Holen, I. The metastatic microenvironment of breast cancer: Clinical implications. *Breast* **2013**, *22*, 50–56. [CrossRef]
166. Tan, W.; Zhang, W.; Strasner, A.; Grivennikov, S.; Cheng, J.Q.; Hoffman, R.M.; Karin, M. Tumour-infiltrating regulatory T cells stimulate mammary cancer metastasis through RANKL-RANK signalling. *Nature* **2011**, *470*, 548–553. [CrossRef]
167. Mahmoud, S.M.; Paish, E.C.; Powe, D.G.; Macmillan, R.D.; Grainge, M.J.; Lee, A.H.; Ellis, I.O.; Green, A.R. Tumor-infiltrating CD8+ lymphocytes predict clinical outcome in breast cancer. *J. Clin. Oncol.* **2011**, *29*, 1949–1955. [CrossRef]
168. Jiang, X. Harnessing the immune system for the treatment of breast cancer. *J. Zhejiang Univ. Sci. B.* **2014**, *15*, 1–15. [CrossRef]
169. Ohara, M.; Yamaguchi, Y.; Matsuura, K.; Murakami, S.; Arihiro, K.; Okada, M. Possible involvement of regulatory T cells in tumor onset and progression in primary breast cancer. *Cancer Immunol. Immunother.* **2009**, *58*, 441–447. [CrossRef]
170. Bohling, S.D.; Allison, K.H. Immunosuppressive regulatory T cells are associated with aggressive breast cancer phenotypes: A potential therapeutic target. *Mod. Pathol.* **2008**, *21*, 1527–1532. [CrossRef]
171. Schreiber, R.D.; Old, L.J.; Smyth, M.J. Cancer immunoediting: Integrating immunity's roles in cancer suppression and promotion. *Science* **2011**, *331*, 1565–1570. [CrossRef]
172. Linehan, D.C.; Goedegebuure, P.S. CD25+ CD4+ regulatory T-cells in cancer. *Immunol. Res.* **2005**, *32*, 155–168. [CrossRef]
173. Solinas, G.; Germano, G.; Mantovani, A.; Allavena, P. Tumor-associated macrophages (TAM) as major players of the cancer-related inflammation. *J. Leukoc. Biol.* **2009**, *86*, 1065–1073. [CrossRef]
174. Mantovani, A.; Allavena, P.; Sica, A.; Balkwill, F. Cancer-related inflammation. *Nature* **2008**, *454*, 436–444. [CrossRef] [PubMed]
175. Allen, M.; Louise Jones, J. Jekyll and Hyde: The role of the microenvironment on the progression of cancer. *J. Pathol.* **2011**, *223*, 162–176. [CrossRef]
176. Tsutsui, S.; Yasuda, K.; Suzuki, K.; Tahara, K.; Higashi, H.; Era, S. Macrophage infiltration and its prognostic implications in breast cancer: The relationship with VEGF expression and microvessel density. *Oncol. Rep.* **2005**, *14*, 425–431. [CrossRef]
177. Zhang, Y.; Cheng, S.; Zhang, M.; Zhen, L.; Pang, D.; Zhang, Q.; Li, Z. High-infiltration of tumor-associated macrophages predicts unfavorable clinical outcome for node-negative breast cancer. *PLoS ONE* **2013**, *8*, e76147. [CrossRef] [PubMed]
178. Melief, C.J. Cancer immunotherapy by dendritic cells. *Immunity* **2008**, *29*, 372–383. [CrossRef] [PubMed]
179. Fainaru, O.; Almog, N.; Yung, C.W.; Nakai, K.; Montoya-Zavala, M.; Abdollahi, A.; D'Amato, R.; Ingber, D.E. Tumor growth and angiogenesis are dependent on the presence of immature dendritic cells. *FASEB J.* **2010**, *24*, 1411–1418. [CrossRef] [PubMed]
180. Korkaya, H.; Liu, S.; Wicha, M.S. Breast cancer stem cells, cytokine networks, and the tumor microenvironment. *J. Clin. Investig.* **2011**, *121*, 3804–3809. [CrossRef] [PubMed]
181. Lotze, M.T. Getting to the source: Dendritic cells as therapeutic reagents for the treatment of patients with cancer. *Ann. Surg.* **1997**, *226*, 1–5. [CrossRef]
182. Liu, Q.; Zhang, C.; Sun, A.; Zheng, Y.; Wang, L.; Cao, X. Tumor-educated CD11bhighIalow regulatory dendritic cells suppress T cell response through arginase I. *J. Immunol.* **2009**, *182*, 6207–6216. [CrossRef]
183. Folgueira, M.A.; Maistro, S.; Katayama, M.L.; Roela, R.A.; Mundim, F.G.; Nanogaki, S.; de Bock, G.H.; Brentani, M.M. Markers of breast cancer stromal fibroblasts in the primary tumour site associated with lymph node metastasis: A systematic review including our case series. *Biosci. Rep.* **2013**, *33*, e00085. [CrossRef]
184. Radisky, E.S.; Radisky, D.C. Stromal induction of breast cancer: Inflammation and invasion. *Rev. Endocr. Metab. Disord.* **2007**, *8*, 279–287. [CrossRef]
185. Martinez-Outschoorn, U.E.; Lisanti, M.P.; Sotgia, F. Catabolic cancer-associated fibroblasts transfer energy and biomass to anabolic cancer cells, fueling tumor growth. *Semin. Cancer Biol.* **2014**, *25*, 47–60. [CrossRef]
186. Kalluri, R.; Zeisberg, M. Fibroblasts in cancer. *Nat. Rev. Cancer* **2006**, *6*, 392–401. [CrossRef]
187. Direkze, N.C.; Jeffery, R.; Hodivala-Dilke, K.; Hunt, T.; Playford, R.J.; Elia, G.; Poulsom, R.; Wright, N.A.; Alison, M.R. Bone marrow-derived stromal cells express lineage-related messenger RNA species. *Cancer Res.* **2006**, *66*, 1265–1269. [CrossRef]
188. Orimo, A.; Gupta, P.B.; Sgroi, D.C.; Arenzana-Seisdedos, F.; Delaunay, T.; Naeem, R.; Carey, V.J.; Richardson, A.L.; Weinberg, R.A. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell* **2005**, *121*, 335–348. [CrossRef]
189. Xing, F.; Saidou, J.; Watabe, K. Cancer associated fibroblasts (CAFs) in tumor microenvironment. *Front. Biosci. Landmark Ed.* **2010**, *15*, 166–179. [CrossRef]
190. Del Valle, P.R.; Milani, C.; Brentani, M.M.; Katayama, M.L.; de Lyra, E.C.; Carraro, D.M.; Brentani, H.; Puga, R.; Lima, L.A.; Rozenchan, P.B.; et al. Transcriptional profile of fibroblasts obtained from the primary site, lymph node and bone marrow of breast cancer patients. *Genet. Mol. Biol.* **2014**, *37*, 480–489. [CrossRef]
191. Bissell, M.J.; Radisky, D. Putting tumours in context. *Nat. Rev. Cancer* **2001**, *1*, 46–54. [CrossRef]
192. Bissell, M.J.; Kenny, P.A.; Radisky, D.C. Microenvironmental regulators of tissue structure and function also regulate tumor induction and progression: The role of extracellular matrix and its degrading enzymes. *Cold Spring Harb. Symp. Quant. Biol.* **2005**, *70*, 343–356. [CrossRef]
193. Zhan, L.; Rosenberg, A.; Bergami, K.C.; Yu, M.; Xuan, Z.; Jaffe, A.B.; Allred, C.; Muthuswamy, S.K. Deregulation of scribble promotes mammary tumorigenesis and reveals a role for cell polarity in carcinoma. *Cell* **2008**, *135*, 865–878. [CrossRef]
194. Weaver, V.M.; Petersen, O.W.; Wang, F.; Larabell, C.A.; Briand, P.; Damsky, C.; Bissell, M.J. Reversion of the malignant phenotype of human breast cells in three-dimensional culture and in vivo by integrin blocking antibodies. *J. Cell Biol.* **1997**, *137*, 231–245. [CrossRef]

195. Hwang, E.S.; DeVries, S.; Chew, K.L.; Moore, D.H., 2nd; Kerlikowske, K.; Thor, A.; Ljung, B.M.; Waldman, F.M. Patterns of chromosomal alterations in breast ductal carcinoma in situ. *Clin. Cancer Res.* **2004**, *10*, 5160–5167. [CrossRef] [PubMed]
196. Allred, D.C.; Wu, Y.; Mao, S.; Nagtegaal, I.D.; Lee, S.; Perou, C.M.; Mohsin, S.K.; O'Connell, P.; Tsimelzon, A.; Medina, D. Ductal carcinoma in situ and the emergence of diversity during breast cancer evolution. *Clin. Cancer Res.* **2008**, *14*, 370–378. [CrossRef]
197. Chin, K.; de Solorzano, C.O.; Knowles, D.; Jones, A.; Chou, W.; Rodriguez, E.G.; Kuo, W.L.; Ljung, B.M.; Chew, K.; Myambo, K.; et al. In situ analyses of genome instability in breast cancer. *Nat. Genet.* **2004**, *36*, 984–988. [CrossRef]
198. Mercurio, A.M.; Bachelder, R.E.; Chung, J.; O'Connor, K.L.; Rabinovitz, I.; Shaw, L.M.; Tani, T. Integrin laminin receptors and breast carcinoma progression. *J. Mammary Gland Biol. Neoplasia* **2001**, *6*, 299–309. [CrossRef] [PubMed]
199. Hood, J.D.; Cheresh, D.A. Role of integrins in cell invasion and migration. *Nat. Rev. Cancer* **2002**, *2*, 91–100. [CrossRef] [PubMed]
200. Orimo, A.; Weinberg, R.A. Stromal fibroblasts in cancer: A novel tumor-promoting cell type. *Cell Cycle* **2006**, *5*, 1597–1601. [CrossRef] [PubMed]
201. Hu, M.; Yao, J.; Carroll, D.K.; Weremowicz, S.; Chen, H.; Carrasco, D.; Richardson, A.; Violette, S.; Nikolskaya, T.; Nikolsky, Y.; et al. Regulation of in situ to invasive breast carcinoma transition. *Cancer Cell* **2008**, *13*, 394–406. [CrossRef] [PubMed]
202. Joyce, J.A.; Pollard, J.W. Microenvironmental regulation of metastasis. *Nat. Rev. Cancer* **2009**, *9*, 239–252. [CrossRef]
203. Butcher, D.T.; Alliston, T.; Weaver, V.M. A tense situation: Forcing tumour progression. *Nat. Rev. Cancer* **2009**, *9*, 108–122. [CrossRef]
204. Rowe, R.G.; Weiss, S.J. Breaching the basement membrane: Who, when and how? *Trends Cell Biol.* **2008**, *18*, 560–574. [CrossRef]
205. Bergamaschi, A.; Tagliabue, E.; Sørlie, T.; Naume, B.; Triulzi, T.; Orlandi, R.; Russnes, H.G.; Nesland, J.M.; Tammi, R.; Auvinen, P.; et al. Extracellular matrix signature identifies breast cancer subgroups with different clinical outcome. *J. Pathol.* **2008**, *214*, 357–367. [CrossRef]
206. Sottile, J.; Chandler, J. Fibronectin matrix turnover occurs through a caveolin-1-dependent process. *Mol. Biol. Cell.* **2005**, *16*, 757–768. [CrossRef]
207. Coopman, P.; Nuydens, R.; Leunissen, J.; De Brabander, M.; Bortier, H.; Foidart, J.M.; Mareel, M. Laminin binding and internalization by human and murine mammary gland cell lines in vitro. *Eur. J. Cell Biol.* **1991**, *56*, 251–259.
208. Weir, M.L.; Oppizzi, M.L.; Henry, M.D.; Onishi, A.; Campbell, K.P.; Bissell, M.J.; Muschler, J.L. Dystroglycan loss disrupts polarity and beta-casein induction in mammary epithelial cells by perturbing laminin anchoring. *J. Cell Sci.* **2006**, *119*, 4047–4058. [CrossRef]
209. Henke, E.; Nandigama, R.; Ergün, S. Extracellular Matrix in the Tumor Microenvironment and Its Impact on Cancer Therapy. *Front. Mol. Biosci.* **2020**, *6*, 160. [CrossRef]
210. Allinen, M.; Beroukhim, R.; Cai, L.; Brennan, C.; Lahti-Domenici, J.; Huang, H.; Porter, D.; Hu, M.; Chin, L.; Richardson, A.; et al. Molecular characterization of the tumor microenvironment in breast cancer. *Cancer Cell* **2004**, *6*, 17–32. [CrossRef]
211. Streuli, C.H. Maspin is a tumour suppressor that inhibits breast cancer tumour metastasis in vivo. *Breast Cancer Res.* **2002**, *4*, 137–140. [CrossRef]
212. Clarke, C.; Sandle, J.; Lakhani, S.R. Myoepithelial cells: Pathology, cell separation and markers of myoepithelial differentiation. *J. Mammary Gland Biol. Neoplasia* **2005**, *10*, 273–280. [CrossRef]
213. Provenzano, P.P.; Inman, D.R.; Eliceiri, K.W.; Knittel, J.G.; Yan, L.; Rueden, C.T.; White, J.G.; Keely, P.J. Collagen density promotes mammary tumor initiation and progression. *BMC Med.* **2008**, *6*, 11. [CrossRef]
214. Itano, N.; Kimata, K. Altered hyaluronan biosynthesis in cancer progression. *Semin. Cancer Biol.* **2008**, *18*, 268–274. [CrossRef]
215. Zhu, J.; Xiong, G.; Trinkle, C.; Xu, R. Integrated extracellular matrix signaling in mammary gland development and breast cancer progression. *Histol. Histopathol.* **2014**, *29*, 1083–1092.
216. Pickup, M.W.; Laklai, H.; Acerbi, I.; Owens, P.; Gorska, A.E.; Chytil, A.; Aakre, M.; Weaver, V.M.; Moses, H.L. Stromally derived lysyl oxidase promotes metastasis of transforming growth factor-β-deficient mouse mammary carcinomas. *Cancer Res.* **2013**, *73*, 5336–5346. [CrossRef]
217. Levental, K.R.; Yu, H.; Kass, L.; Lakins, J.N.; Egeblad, M.; Erler, J.T.; Fong, S.F.; Csiszar, K.; Giaccia, A.; Weninger, W.; et al. Matrix crosslinking forces tumor progression by enhancing integrin signaling. *Cell* **2009**, *139*, 891–906. [CrossRef]
218. Kumar, S.; Weaver, V.M. Mechanics, malignancy, and metastasis: The force journey of a tumor cell. *Cancer Metastasis Rev.* **2009**, *28*, 113–127. [CrossRef]
219. Postovit, L.M.; Abbott, D.E.; Payne, S.L.; Wheaton, W.W.; Margaryan, N.V.; Sullivan, R.; Jansen, M.K.; Csiszar, K.; Hendrix, M.J.; Kirschmann, D.A. Hypoxia/reoxygenation: A dynamic regulator of lysyl oxidase-facilitated breast cancer migration. *J. Cell. Biochem.* **2008**, *103*, 1369–1378. [CrossRef]
220. Gilkes, D.M.; Bajpai, S.; Chaturvedi, P.; Wirtz, D.; Semenza, G.L. Hypoxia-inducible factor 1 (HIF-1) promotes extracellular matrix remodeling under hypoxic conditions by inducing P4HA1, P4HA2, and PLOD2 expression in fibroblasts. *J. Biol. Chem.* **2013**, *288*, 10819–10829. [CrossRef] [PubMed]
221. Gilkes, D.M.; Chaturvedi, P.; Bajpai, S.; Wong, C.C.; Wei, H.; Pitcairn, S.; Hubbi, M.E.; Wirtz, D.; Semenza, G.L. Collagen prolyl hydroxylases are essential for breast cancer metastasis. *Cancer Res.* **2013**, *73*, 3285–3296. [CrossRef]
222. Xiong, G.; Deng, L.; Zhu, J.; Rychahou, P.G.; Xu, R. Prolyl-4-hydroxylase α subunit 2 promotes breast cancer progression and metastasis by regulating collagen deposition. *BMC Cancer* **2014**, *14*, 1. [CrossRef]

223. Li, N.; Zhang, Y.; Naylor, M.J.; Schatzmann, F.; Maurer, F.; Wintermantel, T.; Schuetz, G.; Mueller, U.; Streuli, C.H.; Hynes, N.E. Beta1 integrins regulate mammary gland proliferation and maintain the integrity of mammary alveoli. *EMBO J.* **2005**, *24*, 1942–1953. [CrossRef]
224. Paszek, M.J.; Zahir, N.; Johnson, K.R.; Lakins, J.N.; Rozenberg, G.I.; Gefen, A.; Reinhart-King, C.A.; Margulies, S.S.; Dembo, M.; Boettiger, D.; et al. Tensional homeostasis and the malignant phenotype. *Cancer Cell.* **2005**, *8*, 241–254. [CrossRef] [PubMed]
225. Contié, S.; Voorzanger-Rousselot, N.; Litvin, J.; Clézardin, P.; Garnero, P. Increased expression and serum levels of the stromal cell-secreted protein periostin in breast cancer bone metastases. *Int. J. Cancer* **2011**, *128*, 352–360. [CrossRef] [PubMed]
226. Gillan, L.; Matei, D.; Fishman, D.A.; Gerbin, C.S.; Karlan, B.Y.; Chang, D.D. Periostin secreted by epithelial ovarian carcinoma is a ligand for alpha(V)beta(3) and alpha(V)beta(5) integrins and promotes cell motility. *Cancer Res.* **2002**, *62*, 5358–5364.
227. Morra, L.; Moch, H. Periostin expression and epithelial-mesenchymal transition in cancer: A review and an update. *Virchows Arch.* **2011**, *459*, 465–475. [CrossRef]
228. Maruhashi, T.; Kii, I.; Saito, M.; Kudo, A. Interaction between periostin and BMP-1 promotes proteolytic activation of lysyl oxidase. *J. Biol. Chem.* **2010**, *285*, 13294–13303. [CrossRef]
229. Malanchi, I.; Santamaria-Martínez, A.; Susanto, E.; Peng, H.; Lehr, H.A.; Delaloye, J.F.; Huelsken, J. Interactions between cancer stem cells and their niche govern metastatic colonization. *Nature* **2011**, *481*, 85–89. [CrossRef]
230. Sasaki, H.; Yu, C.Y.; Dai, M.; Tam, C.; Loda, M.; Auclair, D.; Chen, L.B.; Elias, A. Elevated serum periostin levels in patients with bone metastases from breast but not lung cancer. *Breast Cancer Res. Treat.* **2003**, *77*, 245–252. [CrossRef]
231. Orend, G.; Chiquet-Ehrismann, R. Tenascin-C induced signaling in cancer. *Cancer Lett.* **2006**, *244*, 143–163. [CrossRef]
232. Tsunoda, T.; Inada, H.; Kalembeyi, I.; Imanaka-Yoshida, K.; Sakakibara, M.; Okada, R.; Katsuta, K.; Sakakura, T.; Majima, Y.; Yoshida, T. Involvement of large tenascin-C splice variants in breast cancer progression. *Am. J. Pathol.* **2003**, *162*, 1857–1867. [CrossRef]
233. Jahkola, T.; Toivonen, T.; Virtanen, I.; von Smitten, K.; Nordling, S.; von Boguslawski, K.; Haglund, C.; Nevanlinna, H.; Blomqvist, C. Tenascin-C expression in invasion border of early breast cancer: A predictor of local and distant recurrence. *Br. J. Cancer* **1998**, *78*, 1507–1513. [CrossRef]
234. Ilunga, K.; Nishiura, R.; Inada, H.; El-Karef, A.; Imanaka-Yoshida, K.; Sakakura, T.; Yoshida, T. Co-stimulation of human breast cancer cells with transforming growth factor-beta and tenascin-C enhances matrix metalloproteinase-9 expression and cancer cell invasion. *Int. J. Exp. Pathol.* **2004**, *85*, 373–379. [CrossRef]
235. Troup, S.; Njue, C.; Kliewer, E.V.; Parisien, M.; Roskelley, C.; Chakravarti, S.; Roughley, P.J.; Murphy, L.C.; Watson, P.H. Reduced expression of the small leucine-rich proteoglycans, lumican, and decorin is associated with poor outcome in node-negative invasive breast cancer. *Clin. Cancer Res.* **2003**, *9*, 207–214.
236. Hancox, R.A.; Allen, M.D.; Holliday, D.L.; Edwards, D.R.; Pennington, C.J.; Guttery, D.S.; Shaw, J.A.; Walker, R.A.; Pringle, J.H.; Jones, J.L. Tumour-associated tenascin-C isoforms promote breast cancer cell invasion and growth by matrix metalloproteinase-dependent and independent mechanisms. *Breast Cancer Res.* **2009**, *11*, R24. [CrossRef]
237. Ishihara, A.; Yoshida, T.; Tamaki, H.; Sakakura, T. Tenascin expression in cancer cells and stroma of human breast cancer and its prognostic significance. *Clin. Cancer Res.* **1995**, *1*, 1035–1041. [PubMed]
238. Yoshida, T.; Ishihara, A.; Hirokawa, Y.; Kusakabe, M.; Sakakura, T. Tenascin in breast cancer development—is epithelial tenascin a marker for poor prognosis? *Cancer Lett.* **1995**, *90*, 65–73. [CrossRef]
239. Barth, P.J.; Moll, R.; Ramaswamy, A. Stromal remodeling and SPARC (secreted protein acid rich in cysteine) expression in invasive ductal carcinomas of the breast. *Virchows Arch.* **2005**, *446*, 532–536. [CrossRef]
240. Gilles, C.; Bassuk, J.A.; Pulyaeva, H.; Sage, E.H.; Foidart, J.M.; Thompson, E.W. SPARC/osteonectin induces matrix metalloproteinase 2 activation in human breast cancer cell lines. *Cancer Res.* **1998**, *58*, 5529–5536.
241. Minn, A.J.; Gupta, G.P.; Siegel, P.M.; Bos, P.D.; Shu, W.; Giri, D.D.; Viale, A.; Olshen, A.B.; Gerald, W.L.; Massagué, J. Genes that mediate breast cancer metastasis to lung. *Nature* **2005**, *436*, 518–524. [CrossRef]
242. Park, S.Y.; Lee, H.E.; Li, H.; Shipitsin, M.; Gelman, R.; Polyak, K. Heterogeneity for stem cell-related markers according to tumor subtype and histologic stage in breast cancer. *Clin. Cancer Res.* **2010**, *16*, 876–887. [CrossRef]
243. Graham, J.D.; Balleine, R.L.; Milliken, J.S.; Bilous, A.M.; Clarke, C.L. Expression of osteonectin mRNA in human breast tumours is inversely correlated with oestrogen receptor content. *Eur. J. Cancer* **1997**, *33*, 1654–1660. [CrossRef]
244. Witkiewicz, A.K.; Freydin, B.; Chervoneva, I.; Potoczek, M.; Rizzo, W.; Rui, H.; Brody, J.R.; Schwartz, G.F.; Lisanti, M.P. Stromal CD10 and SPARC expression in ductal carcinoma in situ (DCIS) patients predicts disease recurrence. *Cancer Biol. Ther.* **2010**, *10*, 391–396. [CrossRef]
245. Hsiao, Y.H.; Lien, H.C.; Hwa, H.L.; Kuo, W.H.; Chang, K.J.; Hsieh, F.J. SPARC (osteonectin) in breast tumors of different histologic types and its role in the outcome of invasive ductal carcinoma. *Breast J.* **2010**, *16*, 305–308. [CrossRef] [PubMed]
246. Helleman, J.; Jansen, M.P.; Ruigrok-Ritstier, K.; van Staveren, I.L.; Look, M.P.; Meijer-van Gelder, M.E.; Sieuwerts, A.M.; Klijn, J.G.; Sleijfer, S.; Foekens, J.A.; et al. Association of an extracellular matrix gene cluster with breast cancer prognosis and endocrine therapy response. *Clin. Cancer Res.* **2008**, *14*, 5555–5564. [CrossRef] [PubMed]
247. Lopez-Dee, Z.; Pidcock, K.; Gutierrez, L.S. Thrombospondin-1: Multiple paths to inflammation. *Mediat. Inflamm.* **2011**, *2011*, 296069. [CrossRef]
248. Yee, K.O.; Connolly, C.M.; Duquette, M.; Kazerounian, S.; Washington, R.; Lawler, J. The effect of thrombospondin-1 on breast cancer metastasis. *Breast Cancer Res. Treat.* **2009**, *114*, 85–96. [CrossRef]

249. Albo, D.; Berger, D.H.; Wang, T.N.; Hu, X.; Rothman, V.; Tuszynski, G.P. Thrombospondin-1 and transforming growth factor-beta l promote breast tumor cell invasion through up-regulation of the plasminogen/plasmin system. *Surgery* **1997**, *122*, 493–500. [CrossRef]
250. Martin-Manso, G.; Calzada, M.J.; Chuman, Y.; Sipes, J.M.; Xavier, C.P.; Wolf, V.; Kuznetsova, S.A.; Rubin, J.S.; Roberts, D.D. sFRP-1 binds via its netrin-related motif to the N-module of thrombospondin-1 and blocks thrombospondin-1 stimulation of MDA-MB-231 breast carcinoma cell adhesion and migration. *Arch. Biochem. Biophys.* **2011**, *509*, 147–156. [CrossRef] [PubMed]
251. Fontana, A.; Filleur, S.; Guglielmi, J.; Frappart, L.; Bruno-Bossio, G.; Boissier, S.; Cabon, F.; Clézardin, P. Human breast tumors override the antiangiogenic effect of stromal thrombospondin-1 in vivo. *Int. J. Cancer* **2005**, *116*, 686–691. [CrossRef]
252. Suh, E.J.; Kabir, M.H.; Kang, U.B.; Lee, J.W.; Yu, J.; Noh, D.Y.; Lee, C. Comparative profiling of plasma proteome from breast cancer patients reveals thrombospondin-1 and BRWD3 as serological biomarkers. *Exp. Mol. Med.* **2012**, *44*, 36–44. [CrossRef]
253. Byrne, G.J.; Hayden, K.E.; McDowell, G.; Lang, H.; Kirwan, C.C.; Tetlow, L.; Kumar, S.; Bundred, N.J. Angiogenic characteristics of circulating and tumoural thrombospondin-1 in breast cancer. *Int. J. Oncol.* **2007**, *31*, 1127–1132.
254. Nathan, F.E.; Hernandez, E.; Dunton, C.J.; Treat, J.; Switalska, H.I.; Joseph, R.R.; Tuszynski, G.P. Plasma thrombospondin levels in patients with gynecologic malignancies. *Cancer* **1994**, *73*, 2853–2858. [CrossRef]
255. Noti, J.D. Adherence to osteopontin via alphavbeta3 suppresses phorbol ester-mediated apoptosis in MCF-7 breast cancer cells that overexpress protein kinase C-alpha. *Int. J. Oncol.* **2000**, *17*, 1237–1243. [CrossRef]
256. Shevde, L.A.; Das, S.; Clark, D.W.; Samant, R.S. Osteopontin: An effector and an effect of tumor metastasis. *Curr. Mol. Med.* **2010**, *10*, 71–81. [CrossRef]
257. Oates, A.J.; Barraclough, R.; Rudland, P.S. The identification of osteopontin as a metastasis-related gene product in a rodent mammary tumour model. *Oncogene* **1996**, *13*, 97–104. [PubMed]
258. Tuck, A.B.; Arsenault, D.M.; O'Malley, F.P.; Hota, C.; Ling, M.C.; Wilson, S.M.; Chambers, A.F. Osteopontin induces increased invasiveness and plasminogen activator expression of human mammary epithelial cells. *Oncogene* **1999**, *18*, 4237–4246. [CrossRef]
259. Tuck, A.B.; O'Malley, F.P.; Singhal, H.; Harris, J.F.; Tonkin, K.S.; Kerkvliet, N.; Saad, Z.; Doig, G.S.; Chambers, A.F. Osteopontin expression in a group of lymph node negative breast cancer patients. *Int. J. Cancer* **1998**, *79*, 502–508. [CrossRef]
260. Tuck, A.B.; O'Malley, F.P.; Singhal, H.; Tonkin, K.S.; Harris, J.F.; Bautista, D.; Chambers, A.F. Osteopontin and p53 expression are associated with tumor progression in a case of synchronous, bilateral, invasive mammary carcinomas. *Arch. Pathol. Lab. Med.* **1997**, *121*, 578–584. [PubMed]
261. Singhal, H.; Bautista, D.S.; Tonkin, K.S.; O'Malley, F.P.; Tuck, A.B.; Chambers, A.F.; Harris, J.F. Elevated plasma osteopontin in metastatic breast cancer associated with increased tumor burden and decreased survival. *Clin. Cancer Res.* **1997**, *3*, 605–611.
262. Bramwell, V.H.; Doig, G.S.; Tuck, A.B.; Wilson, S.M.; Tonkin, K.S.; Tomiak, A.; Perera, F.; Vandenberg, T.A.; Chambers, A.F. Serial plasma osteopontin levels have prognostic value in metastatic breast cancer. *Clin. Cancer Res.* **2006**, *12*, 3337–3343. [CrossRef]
263. McAllister, S.S.; Gifford, A.M.; Greiner, A.L.; Kelleher, S.P.; Saelzler, M.P.; Ince, T.A.; Reinhardt, F.; Harris, L.N.; Hylander, B.L.; Repasky, E.A.; et al. Systemic endocrine instigation of indolent tumor growth requires osteopontin. *Cell* **2008**, *133*, 994–1005. [CrossRef]
264. Christensen, L. The distribution of fibronectin, laminin and tetranectin in human breast cancer with special attention to the extracellular matrix. *APMIS Suppl.* **1992**, *26*, 1–39.
265. Saad, S.; Gottlieb, D.J.; Bradstock, K.F.; Overall, C.M.; Bendall, L.J. Cancer cell-associated fibronectin induces release of matrix metalloproteinase-2 from normal fibroblasts. *Cancer Res.* **2002**, *62*, 283–289.
266. Park, J.; Schwarzbauer, J.E. Mammary epithelial cell interactions with fibronectin stimulate epithelial-mesenchymal transition. *Oncogene* **2014**, *33*, 1649–1657. [CrossRef]
267. Zhang, K.; Corsa, C.A.; Ponik, S.M.; Prior, J.L.; Piwnica-Worms, D.; Eliceiri, K.W.; Keely, P.J.; Longmore, G.D. The collagen receptor discoidin domain receptor 2 stabilizes SNAIL1 to facilitate breast cancer metastasis. *Nat. Cell Biol.* **2013**, *15*, 677–687. [CrossRef]
268. Bae, Y.K.; Kim, A.; Kim, M.K.; Choi, J.E.; Kang, S.H.; Lee, S.J. Fibronectin expression in carcinoma cells correlates with tumor aggressiveness and poor clinical outcome in patients with invasive breast cancer. *Hum. Pathol.* **2013**, *44*, 2028–2037. [CrossRef]
269. Davidson, L.A.; Keller, R.; DeSimone, D.W. Assembly and remodeling of the fibrillar fibronectin extracellular matrix during gastrulation and neurulation in *Xenopus laevis*. *Dev. Dyn.* **2004**, *231*, 888–895. [CrossRef]
270. Sevilla, C.A.; Dalecki, D.; Hocking, D.C. Extracellular matrix fibronectin stimulates the self-assembly of microtissues on native collagen gels. *Tissue Eng. Part A* **2010**, *16*, 3805–3819. [CrossRef]
271. Li, K.; Kang, H.; Wang, Y.; Hai, T.; Rong, G.; Sun, H. Letrozole-induced functional changes in carcinoma-associated fibroblasts and their influence on breast cancer cell biology. *Med. Oncol.* **2016**, *33*, 64. [CrossRef]
272. Luo, H.; Tu, G.; Liu, Z.; Liu, M. Cancer-associated fibroblasts: A multifaceted driver of breast cancer progression. *Cancer Lett.* **2015**, *361*, 155–163. [CrossRef]
273. Erdogan, B.; Ao, M.; White, L.M.; Means, A.L.; Brewer, B.M.; Yang, L.; Washington, M.K.; Shi, C.; Franco, O.E.; Weaver, A.M.; et al. Cancer-associated fibroblasts promote directional cancer cell migration by aligning fibronectin. *J. Cell Biol.* **2017**, *216*, 3799–3816. [CrossRef]
274. Nolan, J.; Mahdi, A.F.; Dunne, C.P.; Kiely, P.A. Collagen and fibronectin promote an aggressive cancer phenotype in breast cancer cells but drive autonomous gene expression patterns. *Gene* **2020**, *761*, 145024. [CrossRef]
275. Ren, J.; Smid, M.; Iaria, J.; Salvatori, D.C.F.; van Dam, H.; Zhu, H.J.; Martens, J.W.M.; Ten Dijke, P. Cancer-associated fibroblast-derived Gremlin 1 promotes breast cancer progression. *Breast Cancer Res.* **2019**, *21*, 109. [CrossRef]

276. Chang, C.; Goel, H.L.; Gao, H.; Pursell, B.; Shultz, L.D.; Greiner, D.L.; Ingerpuu, S.; Patarroyo, M.; Cao, S.; Lim, E.; et al. A laminin 511 matrix is regulated by TAZ and functions as the ligand for the α6Bβ1 integrin to sustain breast cancer stem cells. *Genes Dev.* **2015**, *29*, 1–6. [CrossRef]
277. Simonova, O.A.; Kuznetsova, E.B.; Poddubskaya, E.V.; Kekeeva, T.V.; Kerimov, R.A.; Trotsenko, I.D.; Tanas, A.S.; Rudenko, V.V.; Alekseeva, E.A.; Zaletayev, D.V.; et al. DNA methylation in the promoter regions of the laminin family genes in normal and breast carcinoma tissues. *Mol. Biol.* **2015**, *49*, 667–677. [CrossRef]
278. Carpenter, P.M.; Ziogas, A.; Markham, E.M.; Cantillep, A.S.; Yan, R.; Anton-Culver, H. Laminin 332 expression and prognosis in breast cancer. *Hum. Pathol.* **2018**, *82*, 289–296. [CrossRef]
279. Fiore, A.P.Z.P.; Spencer, V.A.; Mori, H.; Carvalho, H.F.; Bissell, M.J.; Bruni-Cardoso, A. Laminin-111 and the Level of Nuclear Actin Regulate Epithelial Quiescence via Exportin-6. *Cell Rep.* **2017**, *19*, 2102–2115. [CrossRef]
280. Kim, B.G.; Gao, M.Q.; Choi, Y.P.; Kang, S.; Park, H.R.; Kang, K.S.; Cho, N.H. Invasive breast cancer induces laminin-332 upregulation and integrin β4 neoexpression in myofibroblasts to confer an anoikis-resistant phenotype during tissue remodeling. *Breast Cancer Res.* **2012**, *14*, R88. [CrossRef]
281. Kusuma, N.; Denoyer, D.; Eble, J.A.; Redvers, R.P.; Parker, B.S.; Pelzer, R.; Anderson, R.L.; Pouliot, N. Integrin-dependent response to laminin-511 regulates breast tumor cell invasion and metastasis. *Int. J. Cancer* **2012**, *130*, 555–566. [CrossRef] [PubMed]
282. Salesse, S.; Odoul, L.; Chazée, L.; Garbar, C.; Duca, L.; Martiny, L.; Mahmoudi, R.; Debelle, L. Elastin molecular aging promotes MDA-MB-231 breast cancer cell invasiveness. *FEBS Open Bio* **2018**, *8*, 1395–1404. [CrossRef] [PubMed]
283. Zakout, Y.M.; Abdullah, S.M.; Ali, M.A. Assessment of elastosis in invasive ductal carcinoma of the breast compared to fibroadenoma among Sudanese patients using conventional histochemical methods. *Biotechnol. Histochem.* **2012**, *87*, 122–125. [CrossRef] [PubMed]
284. Wang, Y.; Song, E.C.; Resnick, M.B. Elastin in the Tumor Microenvironment. *Adv. Exp. Med. Biol.* **2020**, *1272*, 1–16.
285. Farmer, P.; Bonnefoi, H.; Anderle, P.; Cameron, D.; Wirapati, P.; Becette, V.; André, S.; Piccart, M.; Campone, M.; Brain, E.; et al. A stroma-related gene signature predicts resistance to neoadjuvant chemotherapy in breast cancer. *Nat. Med.* **2009**, *15*, 68–74. [CrossRef]
286. Pang, H.; Cai, L.; Yang, Y.; Chen, X.; Sui, G.; Zhao, C. Knockdown of osteopontin chemosensitizes MDA-MB-231 cells to cyclophosphamide by enhancing apoptosis through activating p38 MAPK pathway. *Cancer Biother. Radiopharm.* **2011**, *26*, 165–173. [CrossRef]
287. Yang, L.; Wei, L.; Zhao, W.; Wang, X.; Zheng, G.; Zheng, M.; Song, X.; Zuo, W. Down-regulation of osteopontin expression by RNA interference affects cell proliferation and chemotherapy sensitivity of breast cancer MDA-MB-231 cells. *Mol. Med. Rep.* **2012**, *5*, 373–376.
288. Said, G.; Guilbert, M.; Morjani, H.; Garnotel, R.; Jeannesson, P.; El Btaouri, H. Extracellular matrix proteins modulate antimigratory and apoptotic effects of Doxorubicin. *Chemother. Res. Pract.* **2012**, *2012*, 268681. [CrossRef]
289. Pupa, S.M.; Giuffré, S.; Castiglioni, F.; Bertola, L.; Cantú, M.; Bongarzone, I.; Baldassari, P.; Mortarini, R.; Argraves, W.S.; Anichini, A.; et al. Regulation of breast cancer response to chemotherapy by fibulin-1. *Cancer Res.* **2007**, *67*, 4271–4277. [CrossRef]
290. Tzanakakis, G.; Giatagana, E.M.; Kuskov, A.; Berdiaki, A.; Tsatsakis, A.M.; Neagu, M.; Nikitovic, D. Proteoglycans in the Pathogenesis of Hormone-Dependent Cancers: Mediators and Effectors. *Cancers* **2020**, *12*, 2401. [CrossRef]
291. Yang, X.H.; Flores, L.M.; Li, Q.; Zhou, P.; Xu, F.; Krop, I.E.; Hemler, M.E. Disruption of laminin-integrin-CD151-focal adhesion kinase axis sensitizes breast cancer cells to ErbB2 antagonists. *Cancer Res.* **2010**, *70*, 2256–2263. [CrossRef]
292. Cordes, N.; Blaese, M.A.; Plasswilm, L.; Rodemann, H.P.; Van Beuningen, D. Fibronectin and laminin increase resistance to ionizing radiation and the cytotoxic drug Ukrain in human tumour and normal cells in vitro. *Int. J. Radiat. Biol.* **2003**, *79*, 709–720. [CrossRef]
293. García-Teijido, P.; Cabal, M.L.; Fernández, I.P.; Pérez, Y.F. Tumor-Infiltrating Lymphocytes in Triple Negative Breast Cancer: The Future of Immune Targeting. *Clin. Med. Insights Oncol.* **2016**, *10*, 31–39. [CrossRef]
294. Kosok, M.; Alli-Shaik, A.; Bay, B.H.; Gunaratne, J. Comprehensive Proteomic Characterization Reveals Subclass-Specific Molecular Aberrations within Triple-negative Breast Cancer. *iScience* **2020**, *23*, 100868. [CrossRef]
295. Novaro, V.; Roskelley, C.D.; Bissell, M.J. Collagen-IV and laminin-1 regulate estrogen receptor alpha expression and function in mouse mammary epithelial cells. *J. Cell Sci.* **2003**, *116*, 2975–2986. [CrossRef]
296. Azim, H.A., Jr.; Singhal, S.; Ignatiadis, M.; Desmedt, C.; Fumagalli, D.; Veys, I.; Larsimont, D.; Piccart, M.; Michiels, S.; Sotiriou, C. Association between SPARC mRNA expression, prognosis and response to neoadjuvant chemotherapy in early breast cancer: A pooled in-silico analysis. *PLoS ONE* **2013**, *8*, e62451. [CrossRef]
297. Dent, R.; Trudeau, M.; Pritchard, K.I.; Hanna, W.M.; Kahn, H.K.; Sawka, C.A.; Lickley, L.A.; Rawlinson, E.; Sun, P.; Narod, S.A. Triple-negative breast cancer: Clinical features and patterns of recurrence. *Clin. Cancer Res.* **2007**, *13*, 4429–4434. [CrossRef] [PubMed]
298. Venables, J.P. Aberrant and alternative splicing in cancer. *Cancer Res.* **2004**, *64*, 7647–7654. [CrossRef] [PubMed]
299. Wawrzyniak, D.; Grabowska, M.; Głodowicz, P.; Kuczyński, K.; Kuczyńska, B.; Fedoruk-Wyszomirska, A.; Rolle, K. Down-regulation of tenascin-C inhibits breast cancer cells development by cell growth, migration, and adhesion impairment. *PLoS ONE* **2020**, *15*, e0237889. [CrossRef] [PubMed]
300. Brown, L.F.; Guidi, A.J.; Schnitt, S.J.; Van De Water, L.; Iruela-Arispe, M.L.; Yeo, T.K.; Tognazzi, K.; Dvorak, H.F. Vascular stroma formation in carcinoma in situ, invasive carcinoma, and metastatic carcinoma of the breast. *Clin. Cancer Res.* **1999**, *5*, 1041–1056.

301. Di Modugno, F.; Iapicca, P.; Boudreau, A.; Mottolese, M.; Terrenato, I.; Perracchio, L.; Carstens, R.P.; Santoni, A.; Bissell, M.J.; Nisticò, P. Splicing program of human MENA produces a previously undescribed isoform associated with invasive, mesenchymal-like breast tumors. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 19280–19285. [CrossRef]
302. Ghigna, C.; Valacca, C.; Biamonti, G. Alternative splicing and tumor progression. *Curr. Genom.* **2008**, *9*, 556–570. [CrossRef]
303. Schiefner, A.; Gebauer, M.; Skerra, A. Extra-domain B in oncofetal fibronectin structurally promotes fibrillar head-to-tail dimerization of extracellular matrix protein. *J. Biol. Chem.* **2012**, *287*, 17578–17588. [CrossRef]
304. Adams, M.; Jones, J.L.; Walker, R.A.; Pringle, J.H.; Bell, S.C. Changes in tenascin-C isoform expression in invasive and preinvasive breast disease. *Cancer Res.* **2002**, *62*, 3289–3297. [PubMed]
305. Fukuda, T.; Yoshida, N.; Kataoka, Y.; Manabe, R.; Mizuno-Horikawa, Y.; Sato, M.; Kuriyama, K.; Yasui, N.; Sekiguchi, K. Mice lacking the EDB segment of fibronectin develop normally but exhibit reduced cell growth and fibronectin matrix assembly in vitro. *Cancer Res.* **2002**, *62*, 5603–5610. [PubMed]
306. Nam, J.M.; Onodera, Y.; Bissell, M.J.; Park, C.C. Breast cancer cells in three-dimensional culture display an enhanced radioresponse after coordinate targeting of integrin alpha5beta1 and fibronectin. *Cancer Res.* **2010**, *70*, 5238–5248. [CrossRef] [PubMed]
307. Mao, Y.; Schwarzbauer, J.E. Fibronectin fibrillogenesis, a cell-mediated matrix assembly process. *Matrix Biol.* **2005**, *24*, 389–399. [CrossRef]
308. Chang, J.M.; Park, I.A.; Lee, S.H.; Kim, W.H.; Bae, M.S.; Koo, H.R.; Yi, A.; Kim, S.J.; Cho, N.; Moon, W.K. Stiffness of tumours measured by shear-wave elastography correlated with subtypes of breast cancer. *Eur. Radiol.* **2013**, *23*, 2450–2458. [CrossRef]
309. Barton, M.B.; Harris, R.; Fletcher, S.W. The rational clinical examination. Does this patient have breast cancer? The screening clinical breast examination: Should it be done? How? *JAMA* **1999**, *282*, 1270–1280. [CrossRef]
310. Falou, O.; Sadeghi-Naini, A.; Prematilake, S.; Sofroni, E.; Papanicolau, N.; Iradji, S.; Jahedmotlagh, Z.; Lemon-Wong, S.; Pignol, J.P.; Rakovitch, E.; et al. Evaluation of neoadjuvant chemotherapy response in women with locally advanced breast cancer using ultrasound elastography. *Transl. Oncol.* **2013**, *6*, 17–24. [CrossRef]
311. Brisson, B.K.; Mauldin, E.A.; Lei, W.; Vogel, L.K.; Power, A.M.; Lo, A.; Dopkin, D.; Khanna, C.; Wells, R.G.; Puré, E.; et al. Type III Collagen Directs Stromal Organization and Limits Metastasis in a Murine Model of Breast Cancer. *Am. J. Pathol.* **2015**, *185*, 1471–1486. [CrossRef]
312. Abba, M.C.; Drake, J.A.; Hawkins, K.A.; Hu, Y.; Sun, H.; Notcovich, C.; Gaddis, S.; Sahin, A.; Baggerly, K.; Aldaz, C.M. Transcriptomic changes in human breast cancer progression as determined by serial analysis of gene expression. *Breast Cancer Res.* **2004**, *6*, R499–R513. [CrossRef]
313. Liu, J.; Liao, S.; Diop-Frimpong, B.; Chen, W.; Goel, S.; Naxerova, K.; Ancukiewicz, M.; Boucher, Y.; Jain, R.K.; Xu, L. TGF-β blockade improves the distribution and efficacy of therapeutics in breast carcinoma by normalizing the tumor stroma. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 16618–16623. [CrossRef]
314. Provenzano, P.P.; Eliceiri, K.W.; Campbell, J.M.; Inman, D.R.; White, J.G.; Keely, P.J. Collagen reorganization at the tumor-stromal interface facilitates local invasion. *BMC Med.* **2006**, *4*, 38. [CrossRef]
315. Conklin, M.W.; Eickhoff, J.C.; Riching, K.M.; Pehlke, C.A.; Eliceiri, K.W.; Provenzano, P.P.; Friedl, A.; Keely, P.J. Aligned collagen is a prognostic signature for survival in human breast carcinoma. *Am. J. Pathol.* **2011**, *178*, 1221–1232. [CrossRef]
316. Kyutoku, M.; Taniyama, Y.; Katsuragi, N.; Shimizu, H.; Kunugiza, Y.; Iekushi, K.; Koibuchi, N.; Sanada, F.; Oshita, Y.; Morishita, R. Role of periostin in cancer progression and metastasis: Inhibition of breast cancer progression and metastasis by anti-periostin antibody in a murine model. *Int. J. Mol. Med.* **2011**, *28*, 181–186.
317. Lee, Y.J.; Kim, I.S.; Park, S.A.; Kim, Y.; Lee, J.E.; Noh, D.Y.; Kim, K.T.; Ryu, S.H.; Suh, P.G. Periostin-binding DNA aptamer inhibits breast cancer growth and metastasis. *Mol. Ther.* **2013**, *21*, 1004–1013. [CrossRef]
318. Nakazawa, Y.; Taniyama, Y.; Sanada, F.; Morishita, R.; Nakamori, S.; Morimoto, K.; Yeung, K.T.; Yang, J. Periostin blockade overcomes chemoresistance via restricting the expansion of mesenchymal tumor subpopulations in breast cancer. *Sci. Rep.* **2018**, *8*, 4013. [CrossRef]
319. Li, K.; Shi, M.; Qin, S. Current Status and Study Progress of Recombinant Human Endostatin in Cancer Treatment. *Oncol. Ther.* **2018**, *6*, 21–43. [CrossRef]
320. Chen, J.; Yao, Q.; Huang, M.; Wang, B.; Zhang, J.; Wang, T.; Ming, Y.; Zhou, X.; Jia, Q.; Huan, Y.; et al. A randomized Phase III trial of neoadjuvant recombinant human endostatin, docetaxel and epirubicin as first-line therapy for patients with breast cancer (CBCRT01). *Int. J. Cancer* **2018**, *142*, 2130–2138. [CrossRef]
321. Guo, H.; Liu, Y.; Gu, J.; Wang, Y.; Liu, L.; Zhang, P.; Li, Y. Endostatin inhibits the growth and migration of 4T1 mouse breast cancer cells by skewing macrophage polarity toward the M1 phenotype. *Cancer Immunol. Immunother.* **2016**, *65*, 677–688. [CrossRef]
322. Zhao, Y.; Zheng, X.; Zheng, Y.; Chen, Y.; Fei, W.; Wang, F.; Zheng, C. Extracellular Matrix: Emerging Roles and Potential Therapeutic Targets for Breast Cancer. *Front. Oncol.* **2021**, *11*, 650453. [CrossRef]
323. Walia, A.; Yang, J.F.; Huang, Y.H.; Rosenblatt, M.I.; Chang, J.H.; Azar, D.T. Endostatin's emerging roles in angiogenesis, lymphangiogenesis, disease, and clinical applications. *Biochim. Biophys. Acta* **2015**, *1850*, 2422–2438. [CrossRef]
324. Zatterstrom, U.K.; Felbor, U.; Fukai, N.; Olsen, B.R. Collagen XVIII/endostatin structure and functional role in angiogenesis. *Cell Struct. Funct.* **2000**, *25*, 97–101. [CrossRef] [PubMed]
325. Marneros, A.G.; Olsen, B.R. Physiological role of collagen XVIII and endostatin. *FASEB J.* **2005**, *19*, 716–728. [CrossRef] [PubMed]
326. Cao, Y. Endogenous angiogenesis inhibitors: Angiostatin, endostatin, and other proteolytic fragments. *Prog. Mol. Subcell. Biol.* **1998**, *20*, 161–176. [PubMed]

327. Sim, B.K.; MacDonald, N.J.; Gubish, E.R. Angiostatin and endostatin: Endogenous inhibitors of tumor growth. *Cancer Metastasis Rev.* **2000**, *19*, 181–190. [CrossRef]
328. Bachelot, T.; Ratel, D.; Menetrier-Caux, C.; Wion, D.; Blay, J.Y.; Berger, F. Autoantibodies to endostatin in patients with breast cancer: Correlation to endostatin levels and clinical outcome. *Br. J. Cancer* **2006**, *94*, 1066–1070. [CrossRef]
329. Balasubramanian, S.P.; Cross, S.S.; Globe, J.; Cox, A.; Brown, N.J.; Reed, M.W. Endostatin gene variation and protein levels in breast cancer susceptibility and severity. *BMC Cancer* **2007**, *7*, 107. [CrossRef]
330. Huerta-Reyes, M.; Aguilar-Rojas, A. Three dimensional models to study breast cancer. *Int. J. Oncol.* **2021**, *58*, 331–343.
331. Berger, A.J.; Renner, C.M.; Hale, I.; Yang, X.; Ponik, S.M.; Weisman, P.S.; Masters, K.S.; Kreeger, P.K. Scaffold stiffness influences breast cancer cell invasion via EGFR-linked Mena upregulation and matrix remodeling. *Matrix Biol.* **2020**, *85–86*, 80–93. [CrossRef]
332. Han, Y.L.; Pegoraro, A.F.; Li, H.; Li, K.; Yuan, Y.; Xu, G.; Gu, Z.; Sun, J.; Hao, Y.; Gupta, S.K.; et al. Cell swelling, softening and invasion in a three-dimensional breast cancer model. *Nat. Phys.* **2020**, *16*, 101–108. [CrossRef]

Journal of
Clinical Medicine

MDPI

Review

The Role of Extracellular Matrix Components in Inflammatory Bowel Diseases

Alicja Derkacz [1], Paweł Olczyk [2], Krystyna Olczyk [1] and Katarzyna Komosinska-Vassev [1,*]

[1] Department of Clinical Chemistry and Laboratory Diagnostics, Faculty of Pharmaceutical Sciences in Sosnowiec, Medical University of Silesia in Katowice, 41-200 Sosnowiec, Poland; derkacz.alicja@gmail.com (A.D.); olczyk@sum.edu.pl (K.O.)
[2] Department of Community Pharmacy, Faculty of Pharmaceutical Sciences in Sosnowiec, Medical University of Silesia in Katowice, 41-200 Sosnowiec, Poland; polczyk@sum.edu.pl
* Correspondence: kvassev@sum.edu.pl; Tel.: +48-32364-1150

Abstract: The remodeling of extracellular matrix (ECM) within the intestine tissues, which simultaneously involves an increased degradation of ECM components and excessive intestinal fibrosis, is a defining trait of the progression of inflammatory bowel diseases (IBDs), which include ulcerative colitis (UC) and Crohn's disease (CD). The increased activity of proteases, especially matrix metalloproteinases (MMPs), leads to excessive degradation of the extracellular matrix and the release of protein and glycoprotein fragments, previously joined with the extracellular matrix, into the circulation. MMPs participate in regulating the functions of the epithelial barrier, the immunological response, and the process of wound healing or intestinal fibrosis. At a later stage of fibrosis during IBD, excessive formation and deposition of the matrix is observed. To assess changes in the extracellular matrix, quantitative measurement of the concentration in the blood of markers dependent on the activity of proteases, involved in the breakdown of extracellular matrix proteins as well as markers indicating the formation of a new ECM, has recently been proposed. This paper describes attempts to use the quantification of ECM components as markers to predict intestinal fibrosis and evaluate the healing process of the gut. The markers which reflect increased ECM degradation, together with the ones which show the process of creating a new matrix during IBD, allow the attainment of important information regarding the changes in the intestinal tissue, epithelial integrity and extracellular matrix remodeling. This paper contains evidence confirming that ECM remodeling is an integral part of directional cell signaling in the progression of IBD, and not only a basis for the ongoing processes.

Keywords: inflammatory bowel disease; ulcerative colitis; Crohn's disease; extracellular matrix components; hyaluronan

Citation: Derkacz, A.; Olczyk, P.; Olczyk, K.; Komosinska-Vassev, K. The Role of Extracellular Matrix Components in Inflammatory Bowel Diseases. *J. Clin. Med.* **2021**, *10*, 1122. https:/doi.org/10.3390/jcm10051122

Academic Editor: Emmanuel Andrès

Received: 16 February 2021
Accepted: 2 March 2021
Published: 8 March 2021

1. Introduction

Inflammatory bowel diseases (IBD), including two main conditions: Crohn's disease (CD) and ulcerative colitis (UC) are chronic and relapsing inflammatory diseases of the gastrointestinal tract, which have become a global healthcare burden in the 21st century. In CD, the inflammation may occur in any part of the gastrointestinal tract; however, it is usually located in the last region of the ileum and the colon, and the lesions usually stay next to healthy regions of the intestine [1]. In UC, on the other hand, the highest severity of the inflammation and pathological changes is observed in the rectum and decreases from the distal part of the bowel, until an abrupt switch to a healthy tissue. The inflammation in CD may affect the intestinal wall along its whole depth (from mucosa to serosa); in UC, it is usually restricted to the mucosa and submucosa [2]. One of the defining characteristics of inflammatory bowel diseases is the increased remodeling of the extracellular matrix. An excessive and prolonged inflammatory response occurring in the intestinal tissue is a cause of progressive changes to the structure and functioning of the intestinal tissue extracellular matrix [1,2].

The extracellular matrix is a highly dynamic structure present in all tissues, which undergoes controlled remodeling. During this process, both quantitative and qualitative changes of its components take place in order to control homeostasis and tissue architecture [3]. Matrix components include: (1) collagen proteins: type I collagen, basement membrane collagens (type IV, VIII and X), type VI microfibrillar collagens, (fibril-associated collagens with interrupted triple helices (FACIT); (2) non-collagen ECM proteins (elastin, fibronectin, laminin, thrombospondin or tenascin; (3) proteoglycans (PG) and glycosaminoglycans (GAG), and (4) growth factors, enzymes, including matrix metalloproteinases. The extracellular matrix is not only a scaffold for cells within the tissues, but also a dynamic component of the tissue, involved in multiple molecular pathways and processes, such as cellular proliferation, migration and adhesion [1,2]. The structure of ECM undergoes constant deposition, degradation or modification. The changes in the extracellular matrix, or so-called ECM remodeling, are caused by specific enzymes responsible for its degradation and rebuilding, such as: matrix metalloproteinases (MMPs), neutrophil elastases (NE) and meprins. MMPs are the main enzymes involved in ECM degradation. Normally, their activity is low, and its increase can be observed during repair or remodeling processes, or during inflammation. The progression of IBD involves a disturbance to the proteolytic–antiproteolytic balance, which leads to increased degradation of ECM components, in which MMPs take part [3]. Apart from increased proteolytic activity, ECM remodeling during IBDs is also related to wound healing and involves the creation of a new ECM, which involves a particularly large increase in the synthesis of fiber-forming collagens (type I, III and V collagens), and FACIT-type collagens (type XII, XVI and XIX). Excessive ECM formation leads to a progressive intestinal fibrosis, which in turn causes intestinal lumen narrowing [1–3]. Fibrosis occurs through the activation of fibroblasts and the secretion of ECM components, which bind through the activity of lysyl oxidase (LOX). The increased stiffness of the fibrotic tissue, on the other hand, leads to further fibrogenesis. Progressive tissue damage and IBD-related complications—resulting from unbalanced and deregulated ECM remodeling—cause the disappearance of barriers between the epithelial and the endothelial tissue and contribute to the formation of fistulas, eventually leading to intestinal perforation. The remodeling of extracellular matrix components within the intestines, which involves an increased degradation of ECM components and excessive intestinal fibrosis, is a characteristic feature of the progression of inflammatory bowel diseases. The changes within the extracellular matrix, through various molecular mechanisms, contribute to tissue damage over the course of IBD [4].

2. The Role of MMPs in Extracellular Matrix Remodeling in the Progression of Inflammatory Bowel Diseases

Metalloproteinases are a group of zinc-dependent endopeptidases, whose main function is remodeling extracellular matrix components. These enzymes are synthesized by fibroblasts and other types of connective tissues cells, as well as by leukocytes, monocytes, macrophages and endothelial cells, and then released into the extracellular space in an inactive form (proMMP). The enzyme is activated by proteolytic cleavage in the propeptide region. The activity of metalloproteinases is precisely regulated at the transcription and translation level, and by endogenous inhibitors, such as α2-macroglobulin and tissue inhibitors of metalloproteinases (TIMPs) [4–7]. In physiological conditions, MMPs participate in processes such as embryogenesis and angiogenesis, allow the migration of inflammatory response cells to the damaged tissues, release cytokines and their receptors from cell membranes, and regulate the activity of blood platelets. Changes in MMP activity have been observed during many pathological states involving an increased intensity of inflammatory processes [8–11]. Metalloproteinases are also significant factors during IBD pathogenesis, since they promote inflammation and the degradation of ECM components, causing excessive damage to intestinal tissues. The research conducted so far has pointed to an increased expression of metalloproteases responsible for matrix remodeling in IBD patients [4–6].

The substrates for metalloproteinases are extracellular matrix proteins, including collagens, fibronectin, laminin, vitronectin or tenascin. The increased MMP activity in IBD patients promotes ECM degradation and causes an increase in the amount of its components released into circulation. In response to proinflammatory cytokines and interactions between cells—or between cells and the extracellular matrix—MMPs are regulated at the transcription level. The most investigated MMPs in IBD are collagenases (MMP-1, MMP-8, MMP-13), gelatinases (MMP-2, MMP-9), stromelysins (MMP-3, MMP-10), matrilysin (MMP-7) and macrophage elastase (MMP-12). The role of individual metalloproteinases in modulating the progression of IBD is summarized in Table 1 [5–13].

Table 1. The role of matrix metalloproteases in inflammatory bowel diseases.

MMP	Group	Common Name	Role in IBD
MMP-1 MMP-8 MMP-13	collagenases	collagenase-1 collagenase-2 collagenase-3	• prevents intestinal fibrosis • neutrophil infiltration into the intestinal tissue • activates TNF-α and endostatin [5–7,14–16]
MMP-2	gelatinases	gelatinase A	• prevents intestinal fibrosis • produces antiangiogenic factors • supporting epithelial tissue [17]
MMP-9		gelatinase B	• neutrophil infiltration into the intestinal tissue, • chemokine expression • produces antiangiogenic factors • prevents intestinal fibrosis [6–9,14]
MMP-3 MMP-10	stromelysins	stromelysin-1 stromelysin-2	• activating endostatin [7,18] • involved in wound healing [10]
MMP-7	matrilysins	matrilysin-1	• activating A-defensins • chemokine expression • endostatin production [7,11,12,18,19]
MMP-12	other metallo-proteases	macrophage metalloelastase	• degrades intestinal epithelium [13–15]

A group of **collagenases** (MMP-1, MMP-8, MMP-13) plays an important role in IBD progression, and they only occur in inflamed tissues [14]. MMP-1 prevents intestinal fibrosis, which occurs when the synthesis of the extracellular matrix exceeds its degradation. MMP-1cleaves mature collagen fibers, promoting fibrin degradation [5,15]. MMP-8 also has this property; it also participates in neutrophil infiltration into intestinal tissues. Neutrophils release MMP-8 into the inflammatory environment, where it further modulate proinflammatory cytokines and chemokines, enabling the recruitment of further leukocytes [14].

In turn, the proinflammatory role of MMP-13 is due to its ability to activate endostatin and release TNF-α from the membranes, which affects the integrity of the intestinal epithelial barrier [16].

Gelatinases (MMP-2, MMP-9) are responsible for most processes throughout IBD progression, including collagen breakdown, similar to collagenases. This action prevents intestinal fibrosis and neutrophil infiltration into the intestinal tissue. MMP-2 participates in remodeling of collagen structures [5]. In IBD pathogenesis, MMP-2 deficits can lead to a deregulation of the intestinal barrier functions and to fibrosis; in case of increased expression, an excess of MMP-2 may induce the formation of fistulas, as seen in Crohn's disease [14]. MMP-9 plays a similar role to MMP-2 in terms of fistula creation, but MMP-9 may also promote tissue damage by activating neutrophils in regions subject to acute inflammation and by accelerating the proteolysis of matrix proteins partially degraded by other MMPs. MMP-9 participates in the inflammatory response, slows down epithelial repair process, hinders wound healing, increases endothelium permeability and activates cytokines and chemokines, including interleukin IL-1β, IL-8, and TGF-β [7,14].

Stromelysins (MMP-3 and MMP-10) play an important role in wound healing, as they cause ECM protein degradation, which allows the endothelial cells, epithelial cells and fibroblasts to detach and reconnect to the ECM in a controlled manner [14].

They also facilitate cell migration through the damaged tissue, which promotes angiogenesis, re-epithelialization and granulation of wounds. Furthermore, MMP-3 causes the release of endostatin from collagen particles; it strongly inhibits the proliferation of endothelial cells, migration and angiogenesis [19]. MMP-7 takes part in endostatin production; furthermore, this matrilysin has an indirect antibacterial effect, as it activates defensins and induces their release from Paneth cells into the intestinal lumen [14,18]. Among other metalloelastases, MMP-12 is also noteworthy, since an increase in its expression has a relation to IBD, as it participates in the immunological response of lamina propria macrophages. MMP-12 is necessary for the migration of macrophage, which degrade various proteins, causing the degradation of intestinal epithelium [14,15].

During inflammation or healing processes within the intestine, an overexpression of a number of MMPs, undetectable in the healthy gut (MMP-1, MMP-8, MMP-9, MMP-10, MMP-12, MMP-13) is observed. It has been shown that increased MMP-1 expression can be seen in the mucous membrane of the colon in IBD patients and it correlates with an exacerbation of the inflammation [17,20]. The intestinal stromal cells, including fibroblasts, myofibroblasts, pericytes, endothelial cells, and smooth muscle cells, have been shown in human colonic myofibroblasts and in IBD patients to secrete MMP-2 during the activation of inflammatory processes and after a stimulation by MMP-1, MMP-3 and MMP-9 [21–23]. It has been shown that MMP-3 and MMP-9 are two key enzymes involved in the degradation of intestinal tissue during CD and UC. A significant increase in MMP-3 expression has been noted in the inflamed regions of the colon in IBD patients, compared to the non-inflamed regions [24]. The cleavage of collagen XVIII by MMP-3 leads to increased generation of biologically active endostatin fragments, which are antiangiogenic factors. The significant role of metalloproteases in ECM remodeling has also been confirmed during subsequent investigations, which have shown that the epithelium of the colon in IBD patients produced increased quantities of MMP-1, MMP-3, MMP-7, MMP-9, MMP-10 and MMP-12 [25]. Other studies in the intestinal tissue from IBD patients and mice with dextran sodium sulfate-induced colitis have shown that macrophages are the main source of MMP-8, MMP-9 and MMP-10 during IBD, which suggests a significant role of neutrophils in intestinal tissue infiltration [6,10]. It has been shown that MMP-8 and MMP-9 are stored in neutrophil granules and released into the inflammatory environment, where they modulate the action of pro-inflammatory cytokines and chemokines, which enables the recruitment of further neutrophils to the site of injury. Studies so far have found that serum MMP-9 levels were higher in both UC and CD patients compared to controls and correlated well with the disease activity [26–28]. Increased MMP-9 expression and activity promote ECM degradation, which can enhance inflammatory cell infiltration. MMP-10 and MMP-7 matrilysin play a role in intestinal wound healing. The expression of MMP-12 was also increased in IBD patients, which lead to increased degradation of intestinal epithelial cells [13]. It has also been shown that MMP-13 occurred in the inflamed regions of the colon in IBD patients, while it was not present in non-inflamed intestines or during acute diverticulitis, and its expression correlated with the degree of inflammation visible in the histological image of the tissues [29]. Although the main role of metalloproteinases involves the degradation of ECM proteins and basal membrane proteins, these enzymes—including MMP-3, MMP-8, MMP-9 and MMP-7—also participate in inflammatory processes. In addition, the activity and expression of MMPs altered in the course of IBD affects cell adhesion, migration of immune cells, cytokine synthesis and wound healing [6–12].

Apart from MMPs, the process of extracellular matrix remodeling throughout IBD also involves neutrophil elastases and meprins. Neutrophil elastase is a serine protease, produced mainly by neutrophil granulocytes. Its activity is higher during inflammation, which causes increased elastin degradation and promotes the inflammatory process even more. The third discussed group of enzymes—meprins—are zinc-dependent proteases necessary for the homeostasis of gut microbiome. They are responsible for the breakdown of ECM proteins, such as type IV collagen, laminin and nidogen. On one hand, proper intestinal tissue metabolism requires proper functioning of cells responsible for the synthesis

of ECM components; on the other hand, it depends on the activity of specific proteases which degrade them. In a properly functioning tissue, the activity of preteolytic enzymes is subject to complex control processes. MMP expression and the balance between their levels and the levels of tissue metalloproteinases inhibitors is crucial for proper homeostasis of the extracellular matrix in intestinal tissues. Disturbing this balance may lead to intestinal fibrosis. Intestinal fibrosis is a common complication in patients with long-term IBD and interleukin-13 is a potent inducer of tissue fibrosis in IBD patients [29]. Various studies indicate a pathogenic role of MMP-9 in animal models of intestinal inflammation. Of the matrix metalloproteinases, especially MMP-9 has been shown to be consistently increased in different animal models of colitis, such as T cell–mediated colitis, colonic damage induced by dextran sodium sulfate (DSS) treatment, trinitrobenzene sulfonic acid-induced colitis, and human colitis as well [24,30–32]. These data confirm that MMP-9 is an important marker of inflammation in IBD [30,31]. It has been also found that MMP-9 knockout mice are resistant to the development of experimental colitis [28]. Increased expression of MMP-1, -3, -7, -9, -10 and -12 has been demonstrated in human colon epithelium in the intestinal tissue from IBD patients [10,14,18]. Mucosal biopsies of UC patients showed the presence of vascular endothelial cells and infiltrating leukocytes as the main sources of MMP-7 and -13 [32]

3. The Role of MMP in the Immunological Response

The processes catalyzed by the enzymes which participate in rebuilding the ECM are irreversible, and the activity of enzymes has to be regulated at the level of transcription, translation, secretion, and activation; it is limited by metalloprotease inhibitors within the tissues. This introduces a higher level of control, mainly in the regulation of the immune system. The proteolysis of ECM and basal membrane proteins, which involves ECM proteases, makes it easier for immune cells to migrate up to the mucous membrane of the intestine [24,25]. The proteolysis-related change in the structure of ECM components also affects their functioning. The ECM fragments released via degradation processes influence the expansion of the cells which bind them—macrophages and lymphocytes—making it easier to remove them from circulation and migrate to the inflamed regions. Prolonged deposition of these activated cells increases the intensity of the inflammation. Due to the great number of ligands capable of interacting with ECM particles, it is believed that changes to their structure may cause disturbance to the microenvironment and the dynamics of immune response, affecting proper immune processes and leading to the development of IBD. Damage to ECM structure allows the interaction of microbial antigens and the immune system, exacerbating the chronic inflammation related to IBD. Furthermore, some ECM proteins act as inflammatory factors. It has been proven that increased gelatinase B activity, through its involvement in ECM proteolysis and the promotion of immunological signaling, supports IBD development. It works due to the proinflammatory cycle in the intestines. The activity of gelatinase B induces the formation of a product of collagen breakdown: proline–glycine–proline (PGP). It participates in neutrophil chemotaxis and induces the expression of gelatinase B [28]. The increased gelatinase B concentration in the extracellular space leads to the exacerbation of the inflammation in the intestine [33]. Another vital mechanism suggesting a regulatory function of ECM integrity in IBD pathogenesis is the signaling which occurs through hyaluronan (HA), which controls the recruitment of immune system cells, release proinflammatory cytokines and participates in hemostasis.

Research indicates that single nucleotide polymorphism (SNP) within the promoters of genes encoding metalloproteinases influences the expression of these enzymes and therefore the development of IBD [34–38]. Phenotypic analysis of the SNPs revealed associations with various measures of disease severity such as: age at first onset, location of pathological changes, extra intestinal manifestations and the requirement for bowel resection [34,37]. Studies have shown several SNPs in MMP-10 gene significantly associated with UC, for example Rs4431992 polymorphism was associated with extra-intestinal manifestations and Rs12290253 was associated with the female gender, age at first diagnosis above 40 years

and pancolitis [34]. Moreover research revealed SNPs in MMP-8 gene which were also significantly associated with UC. Other studies suggest that the MMP-3 SNP can also be associated with increased risk of stenosing behavior in CD [35]. Although further studies are needed to determine the robustness of these observations.

Researchers also bring attention to the MMP inhibitors that show therapeutic potential in the treatment of IBD. The tested inhibitors are expected to reduce the expression of MMPs and therefore positively affect the course of the disease [37,39,40]. For example, administration of mesalamine may result in reduced MMP-2 expression and inflammation in the course of UC [39]. Moreover, treatment of CD patients with immunosuppressives such as methotrexate, corticosteroids or anti-TNF therapeutics resulted in a decrease in epithelial MMP-7 and stromal MMP-9 and -26 which also led to improvement of patients [40]. The therapeutic properties of MMP inhibitors prove that MMPs greatly determine the onset and course of IBD.

4. The Role of Hyaluronan in the Progression of Inflammatory Bowel Diseases

The regulatory role in maintaining ECM integrity during IBDs is played by molecular mechanisms based on signaling pathways which involve hyaluronan (HA). Hyaluronan is an unbranched, unsulfated glycosaminoglycan, whose polymerization level reflects the integrity of the HA matrix; the presence of fragmented HA parts with a low molecular weight, on the other hand, reflects its degradation. HA participates in wound healing, proliferation and migration of cells and modulating the inflammatory process. This unsulfated GAG which does not form covalent bonds with proteins, can also bind water in its polysaccharide net, providing proper hydration to the matrix, which allows cell migration and substance diffusion. Hyaluronan actively participates in many diseases, including IBD, and its level increases in the inflamed regions. During excessive inflammation, these polymers are cleaved to fragments of lower molecular weight that take on signaling roles [41]. The regulatory role of HA in chronic inflammatory conditions associated with IBD, has been presented in Figure 1.

As an ECM component, hyaluronan has shown minimal adhesive properties towards leukocytes in proper conditions; throughout IBD, not only is there an increase in its synthesis, but it also forms complexes with trypsin inter-α inhibitor, which are highly adhesive towards leukocytes and exacerbate the inflammation.

The deposition of hyaluronan fragments in the intestine is related to the inflammatory state during IBD [41–43]. Certain cell types relevant to the pathobiology of IBD, such as human smooth muscle and intestinal vascular endothelial cells, and porcine intestinal epithelial cells, have been observed to positively influence monocyte adhesion by producing HA chains in response to inflammatory stimuli [44,45]. Therefore, the proinflammatory signals induced by e.g., TNF-α lead to increased synthesis of hyaluronan, forming an HA matrix with strong adhesive properties [46,47]. The biosynthesis of hyaluronan matrix is made possible by tumor necrosis factor-stimulated gene 6 (TSG-6), which is a protein weighing 35 kDa. It catalyzes the movement of heavy chains (HC) from a serum proteoglycan complex, inter-alpha-inhibitor, to a hyaluronan particle, which leads to the creation of an HA–HC matrix [48,49]. TSG-6 expression is induced in the presence of inflammation [4]. Hyaluronan—in the form of an HA–HC matrix, as opposed to an HA matrix—which shows adhesive properties towards blood platelets and leukocytes [49–51].

Thus, the deposition of hyaluronan fragments and HA–HC matrix with strong adhesive properties induces the infiltration of leukocytes towards the intestinal lumen, which leads to the activation of an inflammatory response [42,43]. On the other hand, the accumulation of HA fragments with a low molecular mass also influences wound healing by inducing fibroblast proliferation and myoblast differentiation, which in turn influences the fibrosis processes during IBD. The accumulation of HA fragments confirms the active role of the ECM in commonly known pathogenic pathways responsible for initiating intestinal pathology and IBD progression [52,53].

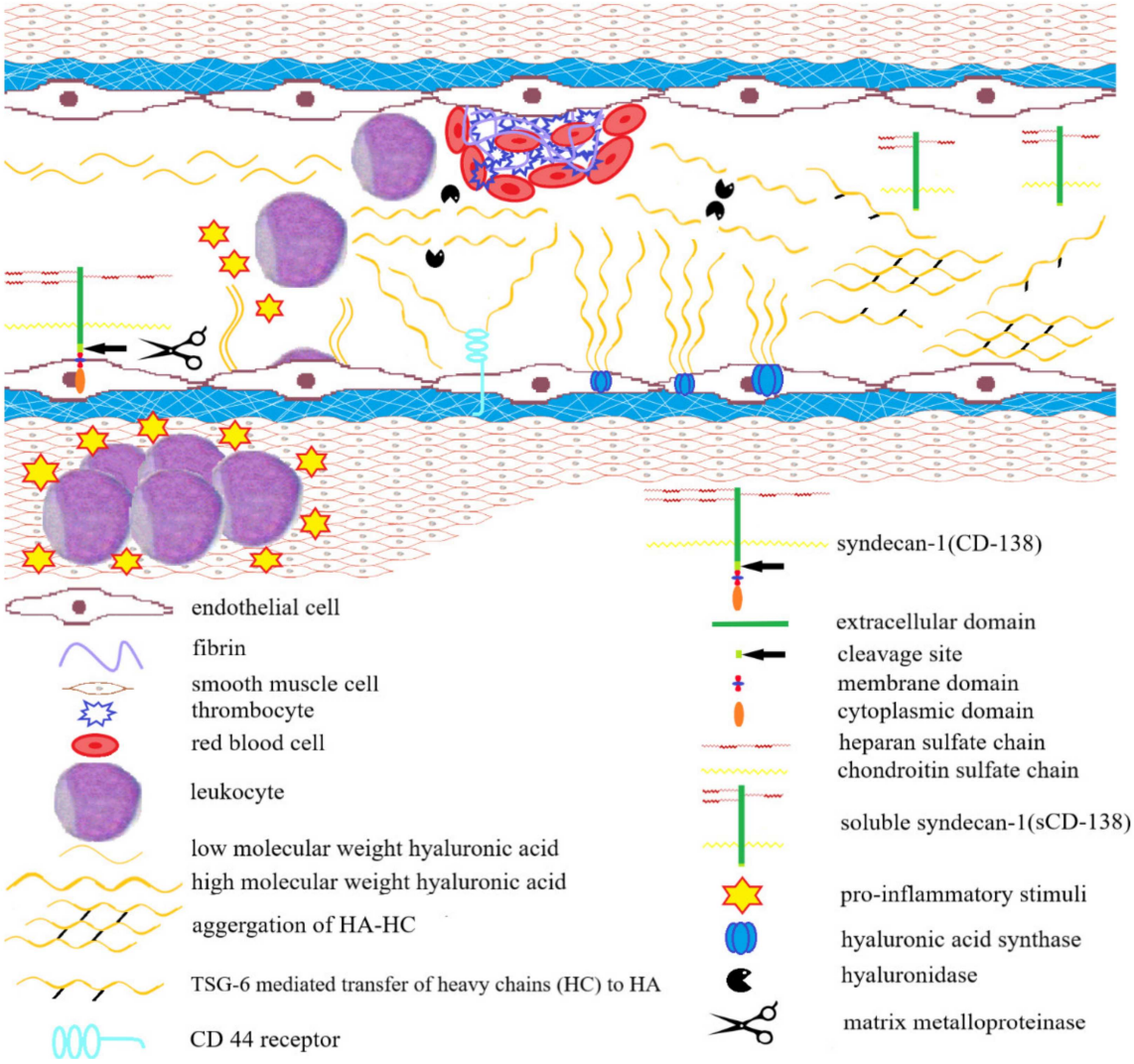

Figure 1. The regulatory role of hyaluronan in the progression of inflammatory bowel diseases. While induced by inflammatory stimuli, synthesis of hyaluronan (HA), an abundant component of the extracellular matrix, increases. On the other hand, in chronic inflammatory conditions such as inflammatory bowel diseases (IBD), high molecular weight HA (HMW-HA) is degraded into fragments that are capable of induces signaling inflammatory responses via specific receptors, induces angiogenesis and inhibits fibroblasts differentiation. Moreover, throughout IBD, HA forms complexes with trypsin inter-α inhibitor (HA–HC matrix), which are highly adhesive towards leukocytes and exacerbate the inflammation.

Furthermore, studies on mice and rats have shown that HA synthesis precedes the infiltration of inflammatory cells and therefore promotes inflammation. For this reason, controlling HA levels on the surface of cells may play a regulatory role during IBD [47]. The chronic inflammation typical of IBD results in a significant deposition of HA in the affected tissues, which both precedes and promotes the infiltration of immune cells, tissue destruction and blood coagulation. Perhaps the balance between HA synthesis and degradation and the interactions between this matrix component and binding proteins are the controlling factors in the recruitment of inflammatory cells by the HA–HC matrix [47–49].

The role of hyaluronan in IBD pathophysiology is also related to the fact that it possesses the ability to regulate the function of intestinal epithelium, whose integrity is vital for preventing the formation of blood clots [52,54–57]. In IBD patients, as opposed to healthy people, microvascular closure in the mucous membrane is observed [58]. The characteristic features of IBD are: fluctuations in the platelet reactivity, increased coagulation and an increased risk of arterial and venous thrombosis [58,59]. The risk of blood clots becomes apparent during the remission phase and increases with the activity of IBD. Inflammation

mediators TNF-α and IL-6 have the ability to initiate coagulation and activate HA synthesis [60,61]. Fibrinogen, a protein which participate in the final part of the clotting process and is converted to fibrin, also has the ability to bind HA [62–64]. As a result, it leads to the formation of hydrated matrices and an increased influx of inflammatory cells into the clot [65,66].

It has also been shown that thrombin, which participates in the coagulation and is necessary for converting fibrinogen to fibrin, can also interact with HA–HC fragments [67]. Furthermore, the increased amount of HA in IBD patients leads to inhibited synthesis of antitrombin—a protein belonging to serine protease inhibitors, whose function involves inhibiting coagulation factors such as thrombin, coagulation factors IXa, XIa, XIIa, or kallikrein [64–66]. On the other hand, in vitro studies on blood platelet functions have proven that purified hyaluronan has the ability to inhibit adhesion and aggregation of thrombocytes, which prevents blood clot formation [54,65–67]. Due to its engagement in multiple molecular mechanisms, hyaluronan may promote blood coagulation and the recruitment of leukocytes during IBD progression [43].

In summary, hyaluronan participates in inducing and maintaining the inflammation, in the coagulation process, and in modulating the remission and activity of the disease. Inflammation and coagulation are two interconnected processes which reinforce and support each other, finally leading to the inhibition of damage progression. Molecules which mediate inflammation and clotting, such as HA, may contribute to both weakening and aggravating disease, depending on the structure. The HA–HC also supports the recruitment of inflammatory cells. A prolonged inflammation leads to impaired epithelial function, increased thrombus formation, tissue damage and an increased disease activity [43,60–63].

5. Other Extracellular Matrix Components Involved in the Inflammatory Process during IBD

5.1. Laminin (LN)

In order to detect changes in the extracellular matrix in the course of IBD, attention was also paid to the potential diagnostic and/or prognostic utility of determining other components of the extracellular matrix in the blood. One of the new diagnostic markers is the concentration of **laminin (LN)** determined in the serum of IBD patients [68–71]. Laminin is synthesized by epithelial cells and stromal cells of the intestines. This protein allows maintenance of the structure and function of the basement membrane [71]. It was found that the concentration of LN in the blood serum of patients with CD is higher than in the control group and is related to the disease activity. During inflammatory bowel diseases, one can observe an overexpression of laminin Lm-α1 and Lm-α5 cells in colon tissue. In patients with Crohn's disease, increased expression of the Lm-α1 chain was found in the crypts of the small intestine, both in inflammatory and non-inflamed sites, while increased expression of the Lm-α3 chain appeared only in the crypts of the small intestine that were inflamed [68–70].

5.2. Heparan Sulfate Proteoglycans (HSPG)

One of the most investigated ECM components of the intestinal tissues are also heparan sulfate proteoglycans (HSPG). It has been shown that HSPGs promote intestinal regeneration, which suggests that they play a role in the homeostasis of intestinal stem cells (ISCs). The HSPGs, whose presence has been noticed on the basal surface of epithelial cells and which play a vital role in maintaining proper functions of the intestinal barrier, include syndecans [72–74]. The biological functions of syndecans depend on the presence of glycosaminglycans and include the activation and binding of growth factors, as well as participation in cell adhesion. Syndecans contain from three to five heparan sulfate chains and chondroitin sulfate chains, which allow them to interact with many different ligands, including the fibroblast growth factor, vascular endothelium growth factor, fibronectin and antitrombin-1 [75]. The cytoplasmatic domain of syndecan enables the creation of connections between the cell and the extracellular matrix. Syndecan-1 (CD138) acts as a

co-receptor of multiple extracellular ligands, including proinflammatory cytokines and growth factors. Due to these properties, it plays a vital role in many metabolic processes, such as: remodeling the matrix, repairing tissues, regulating the immune system and the progression of inflammatory processes. Within the intestines, syndecan-1 is also responsible for maintaining epithelial integrity and permeability, and protecting enterocytes during their interactions with bacteria. An extracellular fragment of syndecan-1 (sCD138) is released from the surface of cells in a proteolytic, continuous manner, in a process known as ectodomain shedding. The process is activated in response to inflammation, pathogen infection, and wound healing. The presence of the extracellular domain of syndecan-1 in blood might therefore be a precious marker of the activity of inflammatory processes. Pioneering studies have shown [76] that sCD138 concentration in serum during Crohn's disease is higher than in the healthy population and correlated with the intensiveness of inflammation, which makes it a reliable marker of disease activity. Increased sCD138 concentrations have also been noticed in UC patients, accompanied by an increased level of tumor necrosis factor alpha (TNF-α) and neutrophil amount. It is suggested that releasing the extracellular domain of syndecan-1 from the intestinal epithelial cells may reduce the intensity of inflammatory bowel diseases and the transmigration of neutrophils by deactivating key inflammatory mediators and reducing the expression of proinflammatory cytokines. The protective effect of the syndecan-1 extracellular domain circulating in blood has been confirmed during research on mice lacking sCD138; when subjected to an experimental colitis, they have exhibited significantly increased mortality, impaired mucous membrane regeneration and extended inflammatory cell recruitment. Furthermore, treating animals with a functional analog of the sCD138 ectodomain significantly affected the symptoms of the inflammation [72–77].

5.3. Fibronectin (FN)

Other proposed biomarkers of intestinal fibrosis include the level of extracellular matrix proteins in serum, including fibronectin. Fibronectin (FN) is an extracellular glycoprotein which binds with various matrix components [68]. Throughout IBD progression, this glycoprotein participates in interactions between the cell and the matrix. It is responsible for fibroblast migration and proliferation through regulating the bioavailability of TGFβ. FN also binds with TNFα, influencing chemotaxis and MMP-9 expression in monocytes. The concentration of fibronectin in the blood was assessed in patients with inflammatory bowel disease. It has been shown that fibronectin concentration in the blood of CD and UC patients differs from those of the healthy population. In CD patients, this marker was significantly lower during active inflammation than in remission [78–81].

5.4. Sulfated GAGs

Characteristic for inflammatory bowel diseases, the process of intensified and abnormal remodeling of ECM components is associated with an excessive and chronic inflammatory response in the intestinal tissue. In turn, the degree and depth of intestinal lesions can be assessed by quantifying the concentration of blood markers that have been released as a result of increased protease activity [82,83] Keratan and dermatan sulfates (KS and DS) belong to the group of sulfated GAGs of the intestinal epithelium. Their amount, released due to excessive ECM degradation during IBD, may be assessed in body fluids and serve as a biomarker for evaluating ECM function with regards to regulating epithelium permeability. Sulfated GAGs participate in the regulation of inflammatory processes and are involved in regulating the permeability of the intestinal epithelium. A significant loss of sulfated GAGs has been confirmed in the subepithelial basal plate of the tissue samples obtained from IBD (both CD and UC) patients. Another possible pathogenetic mechanism, which affects ECM remodeling observed in IBD patients may be the disturbance of GAG structure and function in the connective tissue, related to the ongoing inflammation [83].

6. Extracellular Matrix Components as Biomarkers in IBD Diagnosis

So far, there are no known markers which would allow diagnosis and differential diagnosis between UC and CD and possession of the required sensitivity and specificity. Extending the research on ECM may help to identify a component of the extracellular matrix that could supplement or even replace the currently used invasive, time-consuming and expensive colonoscopy and histopathological techniques. Research has shown that fecal MMP-9 concentration reflects the activity of bowel inflammation and mucosal healing in UC [84]. The research by Farkas et al. has shown a correlation between MMP-9 and the clinical symptoms of inflammation and its activity during ulcerative colitis [85]. An overexpression of MMP-2 in the mucous membrane of the colon in UC patients has also been observed [86]. MMP-9 is connected to disease activity in both UC and CD, although higher MMP-9 levels have been observed for UC. Metalloproteinases have therefore been considered potential biomarkers of disease activity during IBD [87].

Other research involved evaluating the potential diagnostic value of measuring TIMP levels, in tissue samples obtained from intestinal tissues, as markers of inflammation severity during IBD [88–91]. It is commonly known that UC involves increased concentrations of MMP-1 and TIMP-1 and that their expression in colon walls is correlated with the severity of the disease, while the concentrations of these proteins in serum correlated with their expression in the mucous membrane [78]. MMP and TIMP levels in CD patients have been measured before and after immunosuppressive treatment; it has been observed that the histological result was positively correlated with neutrophil MMP-9 and MMP-26, and macrophage TIMP-1 [88]. Furthermore, gelatinase B activity in the intestinal tissues in IBD patients has shown correlation with the clinical improvement and healing of the intestinal mucous membrane [89–91]. In many investigations, gelatinase B has been recently considered a serological, urinal and fecal IBD biomarker, suitable as a tool for diagnosing and monitoring IBD. It has been shown that its levels in the body fluids of patients are positively correlated with other known markers of IBD activity and are influenced by immunosuppressive treatment [92,93].

Raised considerable interest can be observed in relation to glycosaminoglycan/proteoglycan-based diagnostic tools. The most important extracellular matrix component evaluated as a potential IBD biomarker is hyaluronan. This glycan, along with the enzymes and binding proteins responsible for its synthesis and degradation, may directly modulate the progression of the disease by controlling the recruitment of immune cells and releasing proinflammatory cytokines. Hyaluronan particles may therefore modulate both the progression and the remission of the disease. HA particles or the products of its degradation, which stimulate the expression of genes regulating the inflammatory processes, may be a marker of disease progression. Research has shown that the presence of HA–HC complexes may exacerbate the disease. Further research—aimed at evaluating disturbed interactions between immunological cells and HA–HC, direct blocking of the HA–HC matrix synthesis and a selective degradation of HA–HC in experimental models of colitis—are necessary for isolating the molecular paths which allow this polymer to regulate the inflammation and may lead to designating HA–HC a marker of inflammation in IBD [46,47,53,54].

7. Conclusions

Inflammatory bowel disease leads to the degradation and fibrosis of tissues. Disturbed ECM remodeling during IBD is closely related to an increased activity of MMPs. This leads to excessive degradation of extracellular matrix components, the release of proteins and glycoproteins that build the extracellular matrix into the local tissue and the circulation. Additionally, ECM remodeling is a key mechanism that contributes to the development of IBD and is associated with the accumulation of HA fragments that enable wound healing and contribute to intestinal fibrosis. Any change involving the ECM affects cellular processes, and since ECM also acts as a reservoir for signaling molecules, such a change also induces signaling pathways, generating major switches in the regulation of immune system and

other physiological processes. Further research into the molecular mechanisms underlying the remodeling and degradation of ECM would create the basis for the discovery of a component of ECM that would be useful both for the recognition of CD and UC, and for differential diagnosis.

Author Contributions: K.K.-V. and A.D.—conceptualization, writing—original draft preparation; P.O.—conceptualization, review and editing; K.O.—review and editing, supervision. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Rieder, F.; Fiocchi, C. Intestinal fibrosis in IBD—A dynamic, multifactorial process. *Nat. Rev. Gastroenterol. Hepatol.* **2009**, *6*, 228–235. [CrossRef] [PubMed]
2. Latella, G.; Rogler, G.; Bamias, G.; Breynaert, C.; Florholmen, J.; Pellino, G.; Reif, S.; Speca, S.; Lawrance, I.C. Results of the 4th scientific workshop of the ECCO (I): Pathophysiology of intestinal fibrosis in IBD. *J. Crohn's Colitis* **2014**, *8*, 1147–1165. [CrossRef]
3. Rieder, F.; de Bruyn, J.R.; Pham, B.T.; Katsanos, K.; Annese, V.; Higgins, P.D.; Magro, F.; Dotan, I. Results of the 4th Scientific Workshop of the ECCO (Group II): Markers of intestinal fibrosis in inflammatory bowel disease. *J. Crohn's Colitis* **2014**, *8*, 1166–1178. [CrossRef] [PubMed]
4. Hu, J.; Steen, P.E.V.D.; Sang, Q.-X.A.; Opdenakker, G. Matrix metalloproteinase inhibitors as therapy for inflammatory and vascular diseases. *Nat. Rev. Drug Discov.* **2007**, *6*, 480–498. [CrossRef]
5. Bailey, J.R.; Bland, P.W.; Tarlton, J.F.; Peters, I.; Moorghen, M.; Sylvester, P.A.; Probert, C.S.J.; Whiting, C.V. IL-13 Promotes Collagen Accumulation in Crohn's Disease Fibrosis by Down-Regulation of Fibroblast MMP Synthesis: A Role for Innate Lymphoid Cells? *PLoS ONE* **2012**, *7*, e52332. [CrossRef]
6. Koelink, P.J.; Overbeek, S.A.; Braber, S.; Morgan, M.E.; Henricks, P.A.J.; Roda, M.A.; Verspaget, H.W.; Wolfkamp, S.C.; Velde, A.A.T.; Jones, C.W.; et al. Collagen degradation and neutrophilic infiltration: A vicious circle in inflammatory bowel disease. *Gut* **2013**, *63*, 578–587. [CrossRef] [PubMed]
7. Heljasvaara, R.; Nyberg, P.; Luostarinen, J.; Parikka, M.; Heikkilä, P.; Rehn, M.; Sorsa, T.; Salo, T.; Pihlajaniemi, T. Generation of biologically active endostatin fragments from human collagen XVIII by distinct matrix metalloproteases. *Exp. Cell Res.* **2005**, *307*, 292–304. [CrossRef] [PubMed]
8. Deng, X.; Tolstanova, G.; Khomenko, T.; Chen, L.; Tarnawski, A.; Szabo, S.; Sandor, Z. Mesalamine restores angiogenic balance in experimental ulcerative colitis by reducing expression of endostatin and angiostatin: Novel molecular mechanism for ther-apeutic action of mesalamine. *J. Pharmacol. Exp. Ther.* **2009**, *331*, 1071–1078. [CrossRef]
9. Lee, S.; Jilani, S.M.; Nikolova, G.V.; Carpizo, D.; Iruela-Arispe, M.L. Processing of VEGF-A by matrix metalloproteinases regulates bioavailability and vascular patterning in tumors. *J. Cell Biol.* **2005**, *169*, 681–691. [CrossRef]
10. Koller, F.L.; Dozier, E.A.; Nam, K.T.; Swee, M.; Birkland, T.P.; Parks, W.C.; Fingleton, B. Lack of MMP10 exacerbates experi-mental colitis and promotes development of inflammation-associated colonic dysplasia. *Lab. Investig.* **2012**, *92*, 1749–1759. [CrossRef] [PubMed]
11. Shi, J.; Aono, S.; Lu, W.; Ouellette, A.J.; Hu, X.; Ji, Y.; Wang, L.; Lenz, S.; van Ginkel, F.W.; Liles, M.; et al. A Novel Role for Defensins in Intestinal Homeostasis: Regulation of IL-1β Secretion. *J. Immunol.* **2007**, *179*, 1245–1253. [CrossRef] [PubMed]
12. Swee, M.; Wilson, C.L.; Wang, Y.; McGuire, J.K.; Parks, W.C. Matrix metalloproteinase-7 (matrilysin) controls neutrophil egress by generating chemokine gradients. *J. Leukoc. Biol.* **2008**, *83*, 1404–1412. [CrossRef]
13. Mäkitalo, L.; Piekkala, M.; Ashorn, M.; Pakarinen, M.; Koivusalo, A.; Karikoski, R.; Natunen, J.; Saarialho-Kere, U.; Rintala, R.; Kolho, K.-L. Matrix metalloproteinases in the restorative proctocolectomy pouch of pediatric ulcerative colitis. *World J. Gastroenterol.* **2012**, *18*, 4028–4036. [CrossRef] [PubMed]
14. O'Shea, N.R.; Smith, A.M. Matrix metalloproteases role in bowel inflammation and inflammatory bowel disease: An up to date review. *Inflamm. Bowel Dis.* **2014**, *20*, 2379–2393. [CrossRef]
15. Visse, R.; Nagase, H. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: Structure, function, and biochem-istry. *Circ Res.* **2003**, *92*, 827–839. [CrossRef] [PubMed]
16. Fontani, F.; Domazetovic, V.; Marcucci, T.; Vincenzini, M.T.; Iantomasi, T. MMPs, ADAMs and their natural inhibitors in inflammatory bowel disease: Involvement of oxidative stress. *J. Clin. Gastroenterol. Treat.* **2017**, *3*, 039. [CrossRef]
17. Andoh, A.; Bamba, S.; Brittan, M.; Fujiyama, Y.; Wright, N.A. Role of intestinal subepithelial myofibroblasts in inflammation and regenerative response in the gut. *Pharmacol. Ther.* **2007**, *114*, 94–106. [CrossRef]
18. Gordon, J.N.; Pickard, K.M.; di Sabatino, A.; Prothero, J.D.; Pender, S.L.F.; Goggin, P.M.; MacDonald, T.T. Matrix metallo-proteinase-3 production by gut IgG plasma cells in chronic inflammatory bowel disease. *Inflamm. Bowel Dis.* **2008**, *14*, 195–203. [CrossRef]
19. Wilson, C.L.; Schmidt, A.P.; Pirilä, E.; Valore, E.V.; Ferri, N.; Sorsa, T.; Ganz, T.; Parks, W.C. Differential Processing of α- and β-Defensin Precursors by Matrix Metalloproteinase-7 (MMP-7). *J. Biol. Chem.* **2009**, *284*, 8301–8311. [CrossRef]

20. Wang, Y.-D.; Mao, J.W. Expression of matrix metalloproteinase-1 and tumor necrosis factor- in ulcerative colitis. *World J. Gas-troenterol.* **2007**, *13*, 5926–5932. [CrossRef]
21. Drygiannakis, I.; Valatas, V.; Sfakianaki, O.; Bourikas, L.; Manousou, P.; Kambas, K.; Ritis, K.; Kolios, G.; Kouroumalis, E. Proinflammatory cytokines induce crosstalk between colonic epithelial cells and subepithelial myofibroblasts: Implication in intestinal fibrosis. *J. Crohn's Colitis* **2013**, *7*, 286–300. [CrossRef]
22. Yoo, J.; Perez, C.E.R.; Nie, W.; Sinnett-Smith, J.; Rozengurt, E. Protein kinase D1 mediates synergistic MMP-3 expression induced by TNF-α and bradykinin in human colonic myofibroblasts. *Biochem. Biophys. Res. Commun.* **2011**, *413*, 30–35. [CrossRef] [PubMed]
23. León, A.J.; Gómez, E.; Garrote, J.A.; Bernardo, D.; Barrera, A.; Marcos, J.L.; Fernández-Salazar, L.; Velayos, B.; Blanco-Quirós, A.; Arranz, E. High levels of proinflammatory cytokines, but not markers of tissue injury, in unaffected intestinal areas frompatients with IBD. *Mediat. Inflamm.* **2009**, *2009*, 1–10. [CrossRef]
24. Pedersen, G.; Saermark, T.; Kirkegaard, T.; Brynskov, J. Spontaneous and cytokine induced expression and activity of matrix metalloproteinases in human colonic epithelium. *Clin. Exp. Immunol.* **2009**, *155*, 257–265. [CrossRef] [PubMed]
25. Medina, C.; Santana, A.; Paz-Cabrera, M.C.; Parra-Blanco, A.; Nicolás, D.; Gimeno-García, A.Z.; Quintero, E. Increased Activity and Expression of Gelatinases in Ischemic Colitis. *Dig. Dis. Sci.* **2006**, *51*, 2393–2399. [CrossRef]
26. Lakatos, G.; Hritz, I.; Varga, M.Z.; Juhász, M.; Miheller, P.; Cierny, G.; Tulassay, Z.; Herszényi, L. The impact of matrix metalloproteinases and their tissue inhibitors in inflammatory bowel diseases. *Dig. Dis.* **2012**, *30*, 289–295. [CrossRef]
27. Adhikary, S.; Kocieda, V.P.; Yen, J.-H.; Tuma, R.F.; Ganea, D. Signaling through cannabinoid receptor 2 suppresses murine dendritic cell migration by inhibiting matrix metalloproteinase 9 expression. *Blood* **2012**, *120*, 3741–3749. [CrossRef]
28. Castaneda, F.E.; Walia, B.; Vijay–Kumar, M.; Patel, N.R.; Roser, S.; Kolachala, V.L.; Rojas, M.; Wang, L.; Oprea, G.; Garg, P.; et al. Targeted Deletion of Metalloproteinase 9 Attenuates Experimental Colitis in Mice: Central Role of Epithelial-Derived MMP. *Gastroenterology* **2005**, *129*, 1991–2008. [CrossRef] [PubMed]
29. Reinisch, W.; Panés, J.; Khurana, S.; Toth, G.; Hua, F.; Comer, G.M.; Hinz, M.; Page, K.; O'Toole, M.; Moorehead, T.M.; et al. Anrukinzumab, an anti-interleukin 13 monoclonal antibody, in active UC: Efficacy and safety from a phase IIa randomised multicentre study. *Gut* **2015**, *64*, 894–900. [CrossRef] [PubMed]
30. Marshall, D.C.; Lyman, S.K.; McCauley, S.; Kovalenko, M.; Splangler, R.; Liu, C.; Lee, M.; O'Sullivan, C.; Barry-Hamilton, V.; Ghermazien, H.; et al. Selective allosteric inhibition of MMP9 is efficacious in preclinical models of ulcerative colitis and colo-rectal cancer. *PLoS ONE* **2015**, *10*, e0127063. [CrossRef] [PubMed]
31. De Bruyn, M.; Breynaert, C.; Arijs, I.; de Hertogh, G.; Geboes, K.; Thijs, G.; Matteoli, G.; Hu, J.; Van Damme, J.; Arnold, B.; et al. Inhibition of gelatinase B/MMP-9 does not attenuate colitis in murine models of inflammatory bowel disease. *Nat. Commun.* **2017**, *8*, 15384. [CrossRef]
32. Shang, K.; Bai, Y.-P.; Wang, C.; Wang, Z.; Gu, H.-Y.; Du, X.; Zhou, X.-Y.; Zheng, C.-L.; Chi, Y.-Y.; Mukaida, N.; et al. Crucial Involvement of Tumor-Associated Neutrophils in the Regulation of Chronic Colitis-Associated Carcinogenesis in Mice. *PLoS ONE* **2012**, *7*, e51848. [CrossRef] [PubMed]
33. Milner, C.; Tongsoongnoen, W.; Rugg, M.; Day, A. The molecular basis of inter-α-inhibitor heavy chain transfer on to hyaluronan: Figure 1. *Biochem. Soc. Trans.* **2007**, *35*, 672–676. [CrossRef]
34. Morgan, A.R.; Han, D.-Y.; Lam, W.-J.; Triggs, C.M.; Fraser, A.G.; Barclay, M.; Gearry, R.B.; Meisner, S.; Stokkers, P.; Boeckxstaens, G.E.; et al. Genetic variations in matrix metalloproteinases may be associated with increased risk of ulcerative colitis. *Hum. Immunol.* **2011**, *72*, 1117–1127. [CrossRef] [PubMed]
35. Meijer, M.J.W.; Mieremet- Ooms, M.A.C.; van Hogezand, R.A.; Lamers, C.B.H.W.; Hommes, D.W.; Verspaget, H.W. Role of matrix metalloproteinase, tissue inhibitor of metalloproteinase and tumor necrosis factor-α single nucleotide gene polymor-phisms in inflammatory bowel disease. *World J. Gastroenterol.* **2007**, *13*, 2960–2966. [CrossRef]
36. López-Hernández, R.; Valdés, M.; Campillo, J.A.; Martinez-Garcia, P.; Salama, H.; Bolarin, J.M.; Banaclocha, H.M.; Moya-quiles, M.R.; Puras, A.M.; Sanches-Torres, A.; et al. Pro- and anti-inflammatory cytokine gene single-nucleotide poly-morphisms in inflammatory bowel disease. *Int. J. Immunogenet.* **2015**, *42*, 38–45. [CrossRef]
37. O'Sullivan, S.; Gilmer, J.F.; Medina, C. Matrix Metalloproteinases in Inflammatory Bowel Disease: An Update. *Mediat. Inflamm.* **2015**, *2015*, 1–19. [CrossRef] [PubMed]
38. Kang, E.; Jang, J.; Choi, C.; Kang, S.; Bang, K.; Kim, T.; Seo, G.; Cha, J.; Chun, J.; Jung, Y.; et al. Development of a Clinical and Genetic Prediction Model for Early Intestinal Resection in Patients with Crohn's Disease: Results from the IMPACT Study. *J. Clin. Med.* **2021**, *10*, 633. [CrossRef]
39. Wang, X.; Li, D.; Zhang, Y.; Wu, S.; Tang, F. Costus root granules improve ulcerative colitis through regulation of TGF-β mediation of the PI3K/AKT signaling pathway. *Exp. Ther. Med.* **2018**, *15*, 4477–4484. [CrossRef]
40. Mäkitalo, L.; Sipponen, T.; Kärkkäinen, P.; Kolho, K.-L.; Saarialho-Kere, U. Changes in matrix metalloproteinase (MMP) and tissue inhibitors of metalloproteinases (TIMP) expression profile in Crohn's disease after immunosuppressive treatment correlate with histological score and calprotectin values. *Int. J. Color. Dis.* **2009**, *24*, 1157–1167. [CrossRef] [PubMed]
41. de la Motte, C.A.; Hascall, V.C.; Drazba, J.; Bandyopadhyay, S.K.; Strong, S.A. Mononuclear leukocytes bind to specific hyaluronan structures on colon mucosal smooth muscle cells treated with polyinosinic acid: Polycytidylic acid: Inter-alpha-trypsin inhibitor is crucial to structure and function. *Am. J. Pathol.* **2003**, *163*, 121–133. [CrossRef]

42. Culty, M.; O'Mara, T.E.; Underhill, C.B.; Yeager, H., Jr.; Swartz, R.P. Hyaluronan receptor (CD44) expression and function in human peripheral blood monocytes and alveolar macrophages. *J. Leukoc. Biol.* **1994**, *56*, 605–611. [CrossRef]
43. Kessler, S.; Rho, H.; West, G.; Fiocchi, C.; Drazba, J.; de la Motte, C. Hyaluronan (HA) Deposition Precedes and Promotes Leukocyte Recruitment in Intestinal Inflammation. *Clin. Transl. Sci.* **2008**, *1*, 57–61. [CrossRef] [PubMed]
44. De la Motte, C.; Nigro, J.; Vasanji, A.; Rho, H.; Kessler, S.; Bandyopadhyay, S.; Danese, S.; Fiocchi, C.; Stern, R. Platelet-Derived Hyaluronidase 2 Cleaves Hyaluronan into Fragments that Trigger Monocyte-Mediated Production of Proinflammatory Cytokines. *Am. J. Pathol.* **2009**, *174*, 2254–2264. [CrossRef] [PubMed]
45. Docampo, M.J.; Cabrera, J.; Bassols, A. Hyaluronan mediates the adhesion of porcine peripheral blood mononuclear cells to poly (I:C)-treated intestinal cells and modulates their cytokine production. *Vet. Immunol. Immunopathol.* **2017**, *184*, 8–17. [CrossRef]
46. Kessler, S.P.; Obery, D.R.; de la Motte, C. Hyaluronan Synthase 3 Null Mice Exhibit Decreased Intestinal Inflammation and Tissue Damage in the DSS-Induced Colitis Model. *Int. J. Cell Biol.* **2015**, *2015*, 1–13. [CrossRef] [PubMed]
47. Lauer, M.E.; Glant, T.T.; Mikecz, K.; DeAngelis, P.L.; Haller, F.M.; Husni, M.E.; Hascall, V.C.; Calabro, A. Irreversible Heavy Chain Transfer to Hyaluronan Oligosaccharides by Tumor Necrosis Factor-stimulated Gene-6. *J. Biol. Chem.* **2013**, *288*, 205–214. [CrossRef]
48. Chamouard, P.; Grunebaum, L.; Wiesel, M.L.; Frey, P.L.; Wittersheim, C.; Sapin, R.; Baumann, R.; Cazenave, J.P. Prothrombin fragment 1+2 and thrombin???antithrombin III complex as markers of activation of blood coagulation in inflammatory bowel diseases. *Eur. J. Gastroenterol. Hepatol.* **1995**, *12*, 1183–1188. [CrossRef]
49. Kume, K.; Yamasaki, M.; Tashiro, M.; Yoshikawa, I.; Otsuki, M. Activations of Coagulation and Fibrinolysis Secondary to Bowel Inflammation in Patients with Ulcerative Colitis. *Intern. Med.* **2007**, *46*, 1323–1330. [CrossRef]
50. Slevin, M.; Krupinski, J.; Gaffney, J.; Matou, S.; West, D.; Delisser, H.; Savani, R.C.; Kumar, S. Hyaluronan-mediated angio-genesis in vascular disease: Uncovering RHAMM and CD44 receptor signaling pathways. *Matrix Biol.* **2007**, *26*, 58–68. [CrossRef]
51. Curry, F.E.; Adamson, R.H. Endothelial Glycocalyx: Permeability Barrier and Mechanosensor. *Ann. Biomed. Eng.* **2011**, *40*, 828–839. [CrossRef]
52. van Hinsbergh, V.W. Endothelium–role in regulation of coagulation and inflammation. *Semin. Immunopathol.* **2012**, *34*, 93–106. [CrossRef]
53. Walley, K.R.; Lukacs, N.W.; Standiford, T.J.; Strieter, R.M.; Kunkel, S.L. Balance of inflammatory cytokines related to severity and mortality of murine sepsis. *Infect. Immun.* **1996**, *64*, 4733–4738. [CrossRef]
54. Vigetti, D.; Genasetti, A.; Karousou, E.; Viola, M.; Moretto, P.; Clerici, M.; Deleonibus, S.; de Luca, G.; Hascall, V.C.; Passi, A. Proinflammatory cytokines induce hyaluronan synthesis and monocyte adhesion in human endothelial cells through hyalu-ronan synthase 2 (HAS2) and the nuclear factor- kappaB (NF-kappaB) pathway. *J. Biol. Chem.* **2010**, *285*, 24639–24645. [CrossRef]
55. Kolodgie, F.D.; Burke, A.P.; Wight, T.N.; Virmani, R. The accumulation of specific types of proteoglycans in eroded plaques: A role in coronary thrombosis in the absence of rupture. *Curr. Opin. Lipidol.* **2004**, *15*, 575–582. [CrossRef]
56. Kolodgie, F.D.; Burke, A.P.; Farb, A.; Weber, D.K.; Kutys, R.; Wight, T.N.; Virmani, R. Differential accumulation of proteo-glycans and hyaluronan in culprit lesions: Insights into plaque erosion. *Arterioscler. Thromb. Vasc. Biol.* **2002**, *22*, 1642–1648. [CrossRef] [PubMed]
57. Bergmeier, W.; Hynes, R.O. Extracellular matrix proteins in hemostasis and thrombosis. *Cold Spring Harb. Perspect. Biol.* **2012**, *4*, a005132. [CrossRef]
58. Weigel, P.H.; Fuller, G.M.; Leboeuf, R.D. A model for the role of hyaluronic acid and fibrin in the early events during the inflammatory response and wound healing. *J. Theor. Biol.* **1986**, *119*, 219–234. [CrossRef]
59. Chester, D.; Brown, A.C. The role of biophysical properties of provisional matrix proteins in wound repair. *Matrix Biol.* **2017**, *60*, 124–140. [CrossRef] [PubMed]
60. Wall, C.A.; Hanlon, C.R. Experimental enzymatic dissolution of soft tissue hematomas. I. Streptokinase streptodornase and hyaluronidase. *Surgery* **1957**, *42*, 536–540.
61. Roth, K.L. Inhibition of hyaluronidase activity by factors associated with blood clotting. *Proc. Soc. Exp. Biol. Med.* **1954**, *85*, 533–537. [CrossRef]
62. Crawley, J.T.B.; Zanardelli, S.; Chion, C.K.N.K.; Lane, D.A. The central role of thrombin in hemostasis. *J. Thromb. Haemost.* **2007**, *5*, 95–101. [CrossRef] [PubMed]
63. Petrey, A.C.; de la Motte, C.A. Thrombin Cleavage of Inter-α-inhibitor Heavy Chain 1 Regulates Leukocyte Binding to an Inflammatory Hyaluronan Matrix. *J. Biol. Chem.* **2016**, *291*, 24324–24334. [CrossRef] [PubMed]
64. Yamaguchi, Y.; Noda, H.; Okaniwa, N.; Adachi, K.; Shinmura, T.; Nakagawa, S.; Ebi, M.; Ogasawara, N.; Funaki, Y.; Zhuo, L.; et al. Serum-Derived Hyaluronan-Associated Protein Is a Novel Biomarker for Inflammatory Bowel Diseases. *Digestion* **2017**, *95*, 146–155. [CrossRef]
65. Mitchell, J.D.; Lee, R.; Hodakowski, G.T.; Neya, K.; Harringer, W.; Valeri, C.R.; Vlahakes, G.J. Prevention of postoperative pericardial adhesions with a hyaluronic acid coating solution. *J. Thorac. Cardiovasc. Surg.* **1994**, *107*, 1481–1488. [CrossRef]
66. Verheye, S.; Markou, C.P.; Salame, M.Y.; Wan, B.; Kingiii, S.B.; Robinson, K.A.; Chronos, N.A.F.; Hanson, S.R. Reduced Thrombus Formation by Hyaluronic Acid Coating of Endovascular Devices. *Arter. Thromb. Vasc. Biol.* **2000**, *20*, 1168–1172. [CrossRef]
67. Stone, R.L.; Nick, A.M.; McNeish, I.A.; Balkwill, F.; Han, H.D.; Bottsford-Miller, J.; Rupairmoole, R.; Armaiz-Pena, G.N.; Pecot, C.V.; Coward, J.; et al. Paraneoplastic Thrombocytosis in Ovarian Cancer. *N. Engl. J. Med.* **2012**, *366*, 610–618. [CrossRef] [PubMed]

68. Timpl, R.; Rohole, H.; Robey, R.G.; Rennard, S.I.; Foidart, J.M.; Martin, G.R. Laminin- a glycoprotein from basement mem-branes. *J. Biol. Chem.* **1979**, *254*, 9933–9937. [CrossRef]
69. Schmehl, K.; Florian, S.; Jacobasch, G.; Salomon, A.; Körber, J. Deficiency of epithelial basement membrane laminin in ulcerative colitis affected human colonic mucosa. *Int. J. Color. Dis.* **2000**, *15*, 39–48. [CrossRef]
70. Gelbmann, C.M.; Mestermann, S.; Gross, V.; Kollinger, M.; Scholmerich, J.; Falk, W. Strictures in Crohn's disease are charac-terised by an accumulation of mast cells colocalised with laminin but not with fibronectin or vitronectin. *Gut* **1999**, *45*, 210–217. [CrossRef]
71. Koutroubakis, I.E.; Petinaki, E.; Dimoulios, P.; Vardas, E.; Roussomoustakaki, M.; Maniatis, A.N.; Kouroumalis, E.A. Serum laminin and collagen IV in inflammatory bowel disease. *J. Clin. Pathol.* **2003**, *56*, 817–820. [CrossRef] [PubMed]
72. Yablecovitch, D.; Stein, A.; Shabat-Simon, M.; Naftali, T.; Gabay, G.; Laish, I.; Oren, A.; Konikoff, F.M. Soluble Syndecan-1 Levels Are Elevated in Patients with Inflammatory Bowel Disease. *Dig. Dis. Sci.* **2015**, *60*, 2419–2426. [CrossRef]
73. Bass, M.D.; Morgan, M.R.; Humphries, M.J. Syndecans shed their reputation as inert molecules. *Sci. Signal.* **2009**, *12*, 18–20. [CrossRef]
74. Tkachenko, E.; Rhodes, J.M.; Simons, M. Syndecans: New kids on the signaling block. *Circ. Res.* **2005**, *96*, 488–500. [CrossRef]
75. Bernfield, M.; Kokenyesi, R.; Kato, M.; Hinkes, M.; Spring, J.; Gallo, R.; Lose, E. Biology of the syndecans: A family of trans-membrane heparan sulfate proteoglycans. *Annu. Rev. Cell Biol.* **1992**, *8*, 333–364. [CrossRef]
76. Bertrand, J.; Bollmann, M. Soluble syndecans: Biomarkers for diseases and therapeutic options. *Br. J. Pharmacol.* **2018**, *176*, 67–81. [CrossRef] [PubMed]
77. Palaiologou, M.; Delladetsima, I.; Tiniakos, D. CD138 (syndecan-1) expression in health and disease. *Histol. Histopathol.* **2014**, *29*, 177–189. [PubMed]
78. Lemańska-Perek, A.; Adamik, B. Fibronectin and its soluble EDA-FN isoform as biomarkers for inflammation and sepsis. *Adv. Clin. Exp. Med.* **2019**, *28*, 1561–1567. [CrossRef]
79. Potts, J.R.; Campbell, I.D. Fibronectin structure and assembly. *Curr. Opin. Cell Biol.* **1994**, *6*, 648–655. [CrossRef]
80. Allan, A.; Wyke, J.; Allan, R.N.; Morel, P.; Robinson, M.; Scott, D.L.; Alexander-Williams, J. Plasma fibronectin in Crohn's disease. *Gut* **1989**, *30*, 627–633. [CrossRef]
81. Chen, P.; Zhou, G.; Lin, J.; Li, L.; Zeng, Z.; Chen, M.; Zhang, S. Serum Biomarkers for Inflammatory Bowel Disease. *Front. Med.* **2020**, *7*, 123. [CrossRef]
82. Petrey, A.C.; de la Motte, C.A. The extracellular matrix in IBD: A dynamic mediator of inflammation. *Curr. Opin. Gastroenterol.* **2013**, *33*, 234–238. [CrossRef]
83. Mortensen, J.; Lindholm, M.; Langholm, L.; Kjeldsen, J.; Bay-Jensen, A.; Karsdal, M.; Manon-Jensen, T. The intestinal tissue homeostasis—The role of extracellular matrix remodeling in inflammatory bowel disease. *Expert Rev. Gastroenterol. Hepatol.* **2019**, *13*, 977–993. [CrossRef] [PubMed]
84. Madisch, A.; Hellmig, S.; Schreiber, S.; Bethke, B.; Stolte, M.; Miehlke, S. Allelic variation of the matrix metalloproteinase-9 gene is associated with collagenous colitis. *Inflamm. Bowel Dis.* **2011**, *17*, 2295–2298. [CrossRef]
85. Sutherland, L.; Singleton, J.; Sessions, J.; Hanauer, S.; Krawitt, E.; Rankin, G.; Summers, R.; Mekhjian, H.; Greenberger, N.; Kelly, M. Double blind, placebo controlled trial of metronidazole in Crohn's disease. *Gut* **1991**, *32*, 1071–1075. [CrossRef]
86. Matusiewicz, M.; Neubauer, K.; Mierzchala-Pasierb, M.; Gamian, A.; Krzystek-Korpacka, M. Matrix Metalloproteinase-9: Its Interplay with Angiogenic Factors in Inflammatory Bowel Diseases. *Dis. Markers* **2014**, *2014*, 1–8. [CrossRef] [PubMed]
87. Onderdonk, A.B.; A Hermos, J.; Bartlett, J.G. The role of the intestinal microflora in experimental colitis. *Am. J. Clin. Nutr.* **1977**, *30*, 1819–1825. [CrossRef]
88. Wang, Y.-D.; Tan, X.-Y.; Zhang, K. Correlation of Plasma MMP-1 and TIMP-1 Levels and the Colonic Mucosa Expressions in Patients with Ulcerative Colitis. *Mediat. Inflamm.* **2009**, *2009*, 1–5. [CrossRef]
89. Mizoguchi, E.; Nguyen, D.D.; Low, D. Animal models of ulcerative colitis and their application in drug research. *Drug Des. Dev. Ther.* **2013**, *7*, 1341–1357. [CrossRef] [PubMed]
90. Torrence, A.E.; Brabb, T.; Viney, J.L.; Bielefeldt-Ohmann, H.; Treuting, P.; Seamons, A.; Drivdahl, R.; Zeng, W.; Maggio-Price, L. Serum biomarkers in a mouse model of bacterial-induced inflammatory bowel disease. *Inflamm. Bowel Dis.* **2008**, *14*, 480–490. [CrossRef]
91. Burich, A.; Hershberg, R.; Waggie, K.; Zeng, W.; Brabb, T.; Westrich, G.; Viney, J.L.; Maggio-Price, L. Helicobacter-induced inflammatory bowel disease in IL-10- and T cell-deficient mice. *Am. J. Physiol. Liver Physiol.* **2001**, *281*, G764–G778. [CrossRef] [PubMed]
92. Kirkegaard, T.; Hansen, A.; Bruun, E.; Brynskov, J. Expression and localisation of matrix disease & quot. *Gut* **2004**, *53*, 701–709. [PubMed]
93. Efsen, E.; Saermark, T.; Hansen, A.; Bruun, E.; Brynskov, J. Ramiprilate Inhibits Functional Matrix Metalloproteinase Activity in Crohn's Disease Fistulas. *Basic Clin. Pharmacol. Toxicol.* **2011**, *109*, 208–216. [CrossRef] [PubMed]

Journal of
Clinical Medicine

MDPI

Article

The Diagnostic Usefulness of Circulating Profile of Extracellular Matrix Components: Sulfated Glycosaminoglycans (sGAG), Hyaluronan (HA) and Extracellular Part of Syndecan-1 (sCD138) in Patients with Crohn's Disease and Ulcerative Colitis

Alicja Derkacz [1], Paweł Olczyk [2], Agnieszka Jura-Półtorak [1], Krystyna Olczyk [1] and Katarzyna Komosinska-Vassev [1,*]

[1] Department of Clinical Chemistry and Laboratory Diagnostics, Faculty of Pharmaceutical Sciences in Sosnowiec, Medical University of Silesia in Katowice, 41-200 Sosnowiec, Poland; derkacz.alicja@gmail.com (A.D.); ajura@sum.edu.pl (A.J.-P.); olczyk@sum.edu.pl (K.O.)
[2] Department of Community Pharmacy, Faculty of Pharmaceutical Sciences in Sosnowiec, Medical University of Silesia in Katowice, 41-200 Sosnowiec, Poland; polczyk@sum.edu.pl
* Correspondence: kvassev@sum.edu.pl; Tel.: +48-32-364-1150

Citation: Derkacz, A.; Olczyk, P.; Jura-Półtorak, A.; Olczyk, K.; Komosinska-Vassev, K. The Diagnostic Usefulness of Circulating Profile of Extracellular Matrix Components: Sulfated Glycosaminoglycans (sGAG), Hyaluronan (HA) and Extracellular Part of Syndecan-1 (sCD138) in Patients with Crohn's Disease and Ulcerative Colitis. *J. Clin. Med.* **2021**, *10*, 1722. https://doi.org/10.3390/jcm10081722

Academic Editors: Konstantinos Papamichael and Emmanuel Andrès

Received: 22 March 2021
Accepted: 14 April 2021
Published: 16 April 2021

Abstract: The described research focused on the diagnostic usefulness of sulfated glycosaminoglycans (sGAG), hyaluronan (HA), and extracellular part of syndecan-1 (sCD138) as new markers related to extracellular matrix (ECM) remodeling in the intestine during the two most common forms of inflammatory bowel diseases (IBD), i.e., ulcerative colitis (UC) and Crohn' disease (CD). Inflammatory markers belonging to ECM components were assessed in serum of patients with IBD using an immunoenzymatic method (HA and sCD138) and a method based on the reaction with dimethylmethylene blue (sulfated GAG). Measurements were carried out twice: at baseline and after one year of therapy with prednisone (patients with CD) or adalimumab (patients with UC). No quantitative changes were observed in serum sGAG, HA, and sCD138 concentrations between patients newly diagnosed with CD and the healthy group. In the case of patients with UC, the parameter which significantly differentiated healthy subjects and patients with IBD before biological therapy was HA. Significant correlation between serum HA level and inflammation activity, expressed as Mayo score, was also observed in patients with UC. Moreover, the obtained results have confirmed that steroid therapy with prednisone significantly influenced the circulating profile of all examined ECM components (sGAG, HA, and sCD138), whereas adalimumab therapy in patients with UC led to a significant change in only circulating sGAG levels. Moreover, the significant differences in serum HA levels between patients with UC and CD indicate that quantification of circulating HA may be useful in the differential diagnosis of CD and UC.

Keywords: inflammatory bowel diseases (IBD); Crohn's disease (CD); ulcerative colitis (UC); sulfated glycosaminoglycans (sGAG); hyaluronan (HA); soluble part of syndecan-1 (sCD138)

1. Introduction

In the last decades, global, dynamic growth in the frequency of chronic inflammatory bowel diseases (IBD) has been observed, further highlighting the role of environmental factors in this disease. Despite significant advances in treatment and its increased availability, the diseases still remain a challenge for modern medicine [1]. Among the inflammatory bowel diseases, the two most significant ones from a clinical standpoint are Crohn's disease (CD) and ulcerative colitis (UC). This group of diseases was most likely already recognized in ancient times, which is implied by the preserved descriptions. Thus, it can be assumed that their basis is not determined solely by the characteristics of modern lifestyle and environmental factors [2]. IBD etiology and pathogenesis is not yet completely understood, which leads to a lack of accurate methods for causal treatment. Difficulties in identifying

the factors responsible for provoking and aggravating inflammatory bowel diseases are the result of, among others, their varied genetical basis, multiple environmental factors, including stress and bacterial and viral infections, which often overlap on clinical images [3]. Furthermore, diagnosis IBD is a significant challenge, and the diagnostic process requires a joint analysis of data from medical history, physical examination, and laboratory tests. A key role in diagnosis is played by endoscopic examination along with mucous membrane biopsy followed by a histologic examination of the obtained sample [4,5]. In clinical practice, there is still a lack of non-invasive, reliable, and objective methods supporting diagnostic methods that allow differentiation, assessment of disease activity, and monitoring of treatment and prognosis of the disease course. Among them, biochemical markers related with the extracellular matrix (ECM) represent promising candidates that can support CD and UC diagnostics. Quantitative changes in the composition and structure of ECM components has been described as playing a significant role in the pathogenesis of inflammatory bowel diseases. Thus, determining the concentration of these macromolecules in body fluids, i.e., serum or urine, is finding increasingly more applications during clinical diagnostics, often being a marker of disease activity and correlating with the severity of the disease process. Hence, establishing the relationship between the level of the circulating matrix component and the progression of IBD might be an argument for the implementation of its determination in the assessment of the activity and progression of IBD [6–10].

Among the new markers related to the ECM remodeling in the intestine proteoglycans (PGs) and their sugar components, glycosaminoglycans (GAGs) are of particular interest due to their role in IBD progression. Sulfated GAGs, including chondroitin/dermatan sulfates, heparan sulfates, and keratan sulfates, integrate the structure of the extracellular matrix by interacting with its components [11]. They are also a reservoir for cytokines and growth factors, take part in regulating the progression of inflammatory processes, accelerate wound healing, and take part in morphogenesis [12,13]. The anticoagulative properties of heparan sulfate and heparin are a result of their ability to bind platelet factors and activate lipoprotein lipase. Due to these properties, GAGs play a significant role in the progression of immunological and inflammatory processes, including intestinal inflammation during IBD [10,13]. Apart from GAGs, the process of extracellular matrix remodeling throughout IBD also involves hyaluronan (HA), a non-sulfated glycosaminoglycan which actively participates in wound healing, proliferation, and migration of cells and modulation of the inflammatory process. Due to its ability to bind water within a polysaccharide net, it provides adequate hydration to the matrix, which allows, e.g., cell migration and substance diffusion [14]. During inflammatory conditions such as IBD, HA polymers are cleaved into fragments of low molecular weight that induce signaling of inflammatory responses via specific receptors. Moreover, during IBD, HA also forms complexes with trypsin inter-α-inhibitor, which are highly adhesive towards leukocytes and additionally exacerbate the inflammation [15].

Another significant component of the extracellular matrix, which influences the progression of inflammation processes including those associated with IBD, is syndecans–PGs belonging to the family of type 1 transmembrane heparan sulfate proteoglycans. The biological functions of syndecans, related to their structure, include cell adhesion, activation and binding of growth factors, and interaction with many ligands, including fibroblast growth factor, vascular endothelium growth factor, fibronectin, and antitrombin-1 [16,17]. Four syndecans have been recognized on the basis of their structure and origin: 1, 2, 3, and 4 [17]. The most interesting one in the context of IBD pathogenesis is syndecan-1 (CD138). It is expressed at high rates in vascular endothelium and circulatory cells. It can also be found in immature B cells, where it is regulated by IL-6 and bacterial lipopolysaccharides (LPS). Syndecan-1, as a co-receptor of multiple extracellular ligands including proinflammatory cytokines and growth factors, plays a significant role in inflammatory processes [17,18]. An extracellular part of syndecan-1 (sCD138) is proteolytically released from the cell surface. This process is activated in response to inflammation and pathogen infection [17].

It is therefore clearly implied that the presence of the extracellular domain of syndecan-1 in blood could be a precious marker of the activity of inflammatory processes [19–21]. For this reason, the aim of this article was to evaluate the diagnostic suitability of the serum profile of extracellular matrix components such as sulfated glycosaminoglycans (sGAG), hyaluronan (HA), and soluble syndecan-1 (sCD138) fragments in patients with inflammatory bowel diseases, such as Crohn's disease and ulcerative colitis.

2. Materials and Methods

2.1. Study Population

The investigated biological material consisted of venous blood samples obtained from 87 subjects including 40 healthy individuals and 47 patients with inflammatory bowel diseases, such as Crohn's disease and ulcerative colitis. The diagnosis was made on the basis of clinical symptoms, laboratory test and, in particular, the results of colonoscopy. Additionally, in the case of UC patients, assessment with the Mayo score was used.

2.2. Patients with Ulcerative Colitis (UC)–Inclusion and Exclusion Criteria

UC patients enrolled in this study included a group of 31 patients selected for a clinical research program involving biological treatment with adalimumab (Humira, Abbott GmbH, Wiesbaden, Federal Republic of Germany). Adalimumab bonds specifically with tumor necrosis factor (TNF), restricting its activity by blocking it from binding with p55 and p75 TNF receptors on the cell surface. This antibody also influences the biological response to TNF and impacts the concentrations of intercellular adhesion molecules responsible for leukocyte migration (ELAM-1, VCAM-1, and ICAM-1). In the case of patients with UC, the inclusion criteria included age between 18 and 75 during the control visit, diagnosed ulcerative colitis, diagnosed active form of ulcerative colitis confirmed through colonoscopy with biopsy, active form of ulcerative colitis confirmed through Patient Global Assessment (PGA) 2 or 3, and treatment with corticosteroids or immunosuppressive medicine (azathioprine (Imuran, Aspen Pharma Trading Ltd, Dublin, Ireland)) or 6-mercaptopurine (Mercaptopurinum VIS, Zakłady Chemiczno-Farmaceutyczne „VIS"®Spółka z o.o., Bytom, Poland)) which had not brought the intended results according to the practitioner's opinion.

The criteria for exclusion, in the case of patients with UC, were colectomy with ileorectostomy or colectomy with anastomosis, Kock pouch or ileostomy undertaken in the course or inflammatory bowel disease or before a planned bowel surgery, adalimumab treatment or former participation in clinical research involving adalimumab, exposure to infliximab or any other TNF inhibitor within 56 days from week 0, taking ciclosporin, tacrolimus, or mycophenolate mofetil within 30 days from week 0, intravenous corticosteroids within 14 days from the screening or throughout the screening, diagnosed fulminant colitis and/or toxic colitis, and taking any biological medicine which could potentially influence the disease under investigation. Adalimumab doses delivered to patients with UC were varied, and the patients were randomly assigned to one of two groups: the first received 160 mg of adalimumab in the initial dose, with a reduction to 80 mg and then to 40 mg, and there was a need for a faster response to treatment in these patients; the second group received 160 mg in the initial dose, with a reduction to 40 mg. The mean time from diagnosis was one year. Moreover, in patients with ulcerative colitis who were using prednisone (Encorton, Pabianickie Zakłady Farmaceutyczne Polfa S.A., Pabianice, Poland) at the time of qualifying for biological treatment after introducing biological drugs, the dose of prednisone was gradually reduced. The cigarette smoking status has not been established in patients with UC enrolled in this study.

2.3. Patients with Crohn's Disease (CD)—Inclusion and Exclusion Criteria

The next group of patients with IBD in the investigation included 16 people with CD, treated mainly with prednisone. The dosage of prednisolone was chosen on a case-by-case basis, depending on the response to treatment. Blood samples were obtained from patients at the point of diagnosis and again after a year of treatment. The following inclusion

criteria have been adapted for the volunteers: age above 18 and diagnosed with Crohn's disease (CD). The exclusion criteria were age under 18, diagnosed indeterminate colitis (IC), alcoholism, alcohol-related liver disease or any ongoing liver disease, acute infections of viral, fungal, or bacterial origin, mild or acute myocardial inefficiency, unstable coronary artery disease, chronic respiratory failure, chronic kidney disease, chronic liver disease, demyelinating disease, diabetes, pregnancy or breastfeeding, and diagnosed precancerous condition or cancerous comorbidities. The patients had been ill for twelve months with a period of activity and remission and did not take any additional medications during therapy. The cigarette smoking status has not been established in patients with CD enrolled in this study. None of the patients had a prior ileocolonic resection for CD and perianal fistulizing CD.

All patients were treated at the Department of Gastroenterology of St. Barbara's Regional Specialist Hospital in Sosnowiec. Reference number of the decision by the Bioethics Committee at the Medical University of Silesia in Katowice: KNW/0022/KB/309/15.

2.4. Control Subjects

The reference material for research consisted of blood samples obtained from 40 healthy people in properly sampled age groups. The group qualified for the research involved people who had not been hospitalized during the prior year, had not undergone surgical treatment, and had not been pharmacologically treated. Furthermore, the results of their routine laboratory tests (i.e., complete blood count, erythrocyte sedimentation rate test (ESR test), plasma fasting glucose, fasting lipid profile, creatinine, liver enzymes, rheumatoid factor (RF), and CRP) were within the reference values for their particular age group. Subjects were excluded from the study if they had been taking steroidal or non-steroidal anti-inflammatory drugs. None of the volunteers were cigarette smokers or had any history of drug or alcohol abuse.

2.5. Biological Material for Research

Blood sampled from the elbow vein, contained in test tubes with no added anticoagulant, was centrifuged for 10 min at 1500× g at 4 °C. The serum obtained through centrifugation was subjected to diagnostic examination as requested by the attending physician. The remaining part was frozen and stored at −80 °C until needed for biochemical analysis.

2.6. Assessing the Serum Hyaluronan (HA) Concentration

Hyaluronan concentration was determined using a using enzyme-linked immunosorbent assays test supplied by TECOmedicalGroup TE 1018-2, Sissach, Switzerland. The analytical sensitivity of the method for HA concentrations assessing was 2.7 ng/mL and the intra-run error was 6.4%.

2.7. Assessing the Serum Soluble Part of Syndecan-1 (sCD138) Concentration

The concentration of the extracellular part of syndecan-1 circulating in blood was evaluated with an immunoassay test supplied by Diaclone SAS, BesanconCedex, France. The analytical sensitivity of the method for sCD138 concentrations assessing was 4.94 ng/mL and the intra-run error was 6.2%.

2.8. Assessing the Serum Sulfated Glycosaminoglycans (sGAG) Concentration

Sulfated glycosaminoglycans were assessed using the Wieslab® sGAG quantitative test (GAG 201 RUO, Euro Diagnostica AB, Malmo, Sweden). The principle of the method is based on specific interaction between sulfated glycosaminoglycans (chondroitin sulfates, dermatan sulfates, keratan sulfates, heparan sulfates, and heparin) and the tetravalent cationic Alcian blue. The reagents for this kit, i.e. Alcian Blue stock solution (containing 0.1% H_2SO_4 and 0.4 M GuHCl), 8 M Guanidine-HCl, SAT solution (containing 0.3% H_2SO_4 and 0.75 Triton X-100), DMSO solution (containing 40% dimethylsulphoxide and 0.05 M

Mg_2Cl_2), Gu-Prop solution containing 4 M GuHCl, 33% 1-propanol and 0.25% Triton X-100, calibrators containing 1.1 mL chondroitin sulphate-6 were manufactured by Euro Diagnostica AB from Malmo, Sweden. The assay was performed at pH low enough to neutralize all carboxylic and phosphoric acid groups as well as at ionic strength large enough to eliminate ionic interactions other than those between Alcian blue and sulfated GAGs. Six calibrators containing chondroitin-6-sulfate at concentrations of 12.5, 25, 50, 100, 200, and 400 µg/mL were used. Then, 50 µL of the prepared standard solutions for control and test samples were added to the to the appropriate wells on the microplate. Then, 50 µL of 8 M guanidine-HCl solution (GuHCL) was added, the microplate was covered with sealing tape and incubated for 15 min at room temperature. In the next step, 50 µL of the SAT solution containing 0.3% H_2SO_4 and 0.75% Triton X-100 was added and incubated for 15 min at room temperature. Then, 750 µL of Alcian blue was added and incubated for 15 min at room temperature. The samples were then centrifuged for 15 min at 12,000× *g*. The next step was to remove the supernatant and add 500 µl of DMSO solution containing 40% dimethylsulfoxide and 0.05 M $MgCl_2$ to the pellets. Prepared samples were incubated for 15 min at room temperature, followed by centrifugation for 15 min at 12,000× *g*. After removing the supernatant, 500 µL Gu-Prop solution containing 4 M guanidine-HCl, 33% 1-propanol and 0.25% Triton X-100 was added to the pellets and mixed for 15 min on a shaker. The absorbance of the standard and test samples was measured using a microplate reader at a wavelength of 620 nm.

2.9. Statistical Analysis

The statistical analysis of the obtained results was performed using the STATISTICA software, version 10.0, developed by StatSoft, Krakow, Poland. It involved verifying the normality of distribution of the variable using the Shapiro–Wilk test. The descriptive statistics for non-normally distributed variables included the median (Me) as a measure of position and the interquartile range—between the lower (Q1) and the upper (Q3) quartiles—as a typical measure of variability. To verify the hypotheses related to the influence of treatment on the value of the investigated parameters, the pre- and post-treatment results were compared using the Wilcoxon test. The statistical significance of the difference in results between the affected group and the control group was determined using the Mann–Whitney U test. The pre- and post-treatment clinical parameters have been compared using the Wilcoxon test and the paired *t*-test. A significance level of $p < 0.05$ was used for all tests and statistical analyses.

3. Results

3.1. Research Data

The clinical characteristics of patients with CD and UC before treatment and after a year of treatment has been summarized in Tables 1 and 2.

Table 1. Clinical characteristics of patients with ulcerative colitis (UC).

Parameter	Patients with Ulcerative Colitis		*p*
	before Treatment UC_0	after Treatment UC_1	UC_0 Vs. UC_1
Age (years)	33.38 ± 12.75		
Mayo score	3 (2–3)	2 (1–3)	0.000
CRP (mg/L)	3.37 (0.79–26.44)	2.41 (1.42–7.33)	0.031
Glucose (mmol/L)	4.99 ± 0.72	4.81 ± 0.81	0.331
Cholesterol (mmol/L)	4.91 ± 0.89	4.99 ± 0.86	0.264
Triglycerides (mmol/L)	1.41 ± 0.52	1.14 ± 0.38	0.030
Indirect bilirubin (µmol/L)	5.20 (4.61–8.32)	8.30 (5.50–16.70)	0.000
Direct bilirubin (µmol/L)	3.70 (2.90–4.42)	5.30 (3.51–8.21)	0.010
ALT (U/l)	15.02 (10.04–26.00)	16.01 (10.01–25.03)	0.814
AST (U/L)	19.00 (14.02–21.02)	19.03 (15.02–23.01)	0.100
Total protein (g/L)	73.48 ± 5.43	74.73 ± 5.63	0.235
Albumin (g/L)	42.00 (40.01–46.03)	43.02 (40.03–48.00)	0.264
WBC	7.90 (4.6–13.6)	7.90 (3.9–13.7)	0.61
PLT ($\times 10^9$/L)	372.03 (292.00–457.00)	343.04 (263.02–422.03)	0.050
BMI	24.25 ± 3.59	24.46 ± 4.23	0.455

Comparison of selected parameters before and after treatment. Data are presented as median, interquartile (25th–75th percentile) range or mean ± standard deviation (SD), or percentage (%). Data analyzed using the Wilcoxon test or paired *t*-test. $p < 0.05$, statistically significant. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CRP, C-reactive protein; PLT, platelets; WBC, white blood cells; UC, ulcerative colitis; UC_0, patients with ulcerative colitis before adalimumab therapy; UC_1, patients with ulcerative colitis after 1 year adalimumab therapy.

Table 2. Clinical characteristics of patients with Crohn's disease (CD).

Parameter	Patients with Crohn's Disease		*p*
	before Treatment CD_0	after Treatment CD_1	CD_0 Vs. CD_1
Age (years)	32.10 ± 9.56		
CDAI	299.60 ± 47.93	274.01 ± 50.71	0.323
CRP (mg/L)	15.70 (4.22–39.05)	15.20 (5.30–28.90)	0.665
Glucose (mmol/L)	4.89 (4.72–5.47)	4.95 (4.61–5.17)	0.950
Indirect bilirubin (µmol/L)	5.75 (4.85–7.43)	6.25 (5.2–10.65)	0.073
Direct bilirubin (µmol/L)	3.75 (2.88–4.05)	7.9 (4.5–10.8)	0.020
ALT (U/L)	24 (16.25–29.05)	21.5 (14.5–31)	0.351
AST (U/L)	21.5 (18.5–24.25)	21 (16.75–23.50)	0.373
Total protein (g/L)	72.13 ± 4.90	77.25 ± 5.21	0.004
Albumin (g/L)	43.50 (42–47.25)	43.5 (42–49)	0.531
WBC ($10^3/\mu$L)	7.12 ± 3.20	6.68 ± 2.05	0.421
PLT (x10^9/L)	356.50 (277.50–396.02)	232.20 (134.21–309.11)	0.090
BMI	20.58 ± 3.43	19.84 ± 2.84	0.190

Comparison of selected parameters before and after treatment. Data are presented as median, interquartile (25th–75th percentile) range or mean ± standard deviation (SD), or percentage (%). Data analyzed using the Wilcoxon test or paired *t*-test. $p < 0.05$, statistically significant. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CD, Crohn's disease, CD_0, patients with Crohn's disease before prednisone therapy; CD_1, patients with Crohn's disease after 1 year prednisone therapy; CDAI, The Crohn's disease activity index; CRP, C-reactive protein; PLT, platelets; WBC, white blood cells.

It is interesting to note that the patients with CD had no change in standard markers of inflammation following intervention. The results of circulating levels of serum sulfated glycosaminoglycans (sGAG), hyaluronan (HA), and the soluble part of syndecan-1 (sCD138) in study individuals (control subjects, patients with CD, and patients with UC) are summarized and provided as supplementary material (Table S1).

3.2. Quantitative Changes of Serum sGAG, HA, and sCD138 in Patients with Crohn's Disease

The investigation has shown that sGAG and HA concentration in patients newly diagnosed with Crohn's disease is not significantly different from the concentration of these parameters in the serum of healthy people. A year-long prednisone treatment, on the other hand, caused a significant increase in both sGAG and HA concentrations in the serum of affected patients compared with pre-treatment values. The increase reached 42%

for sGAG and 78% for HA. Furthermore, the differences in the concentration of sulfated GAGs and HA between patients with CD after a year of prednisone treatment and the control group were statistically significant. The increase in concentration reached 53% for sGAG and 41% for HA.

The quantitative evaluation of extracellular sydecan-1 (sCD138) component circulating in blood has shown a decrease in the concentration of this ECM component in the blood of patients newly diagnosed with CD who have not undergone treatment compared with the concentration in the blood of healthy people. The difference, however, was not statistically significant ($p > 0.05$). Moreover, a significant, almost 110% increase in circulating sCD138 was observed in patients with CD after a year-long treatment compared with pre-treatment concentrations.

3.3. Quantitative Changes of Serum sGAG, HA, and sCD138 in Patients with Ulcerative Colitis

In the case of patients with UC before implementing biological therapy, the changes to the serum profile of the analyzed ECM components were not statistically different from the concentrations of these components in healthy people, with the exception of HA. Before adalimumab treatment, patients with UC had significantly increased HA concentrations compared with the control group. A year-long treatment with biological medicine, on the other hand, only affected the circulating profile of sulfated GAGs. A statistically significant increase in sGAG concentration was observed in patients with UC compared with the pre-treatment concentration of these ECM components in the same patients. Moreover, patients with ulcerative colitis before therapy had significantly different HA concentration in the blood than patients with CD before steroid therapy implementation.

The results are shown in Figure 1.

3.4. The Relationship between Serum ECM Components and Inflammatory Processes and Disease Activity

The investigation has also revealed a relationship between disease activity and the circulating profile of ECM components only in the case of patients with UC. In these patients, a correlation between HA concentration in serum and the activity of inflammation, derived from the Mayo score, was observed both before ($r = 0.45$; $p < 0.012$), and after 12-month treatment ($r = 0.42$; $p < 0.039$). A correlation between sCD138 levels in serum and the Mayo score was also observed in patients before biological treatment with adalimumab ($r = 0.44$; $p < 0.014$). Results of the abovementioned correlation analysis are presented as a correlation plot and included in supplementary material (Figure S1).

The relationship between the concentration of the ECM components and a marker of inflammation process commonly used in clinical practice, the CRP protein, was also investigated. The investigation revealed significantly higher CRP concentrations in patients with CD compared to patients diagnosed with UC. A significant effect of 12-month adalimumab treatment inn lowering CRP concentration in the blood of patients with UC was also observed.

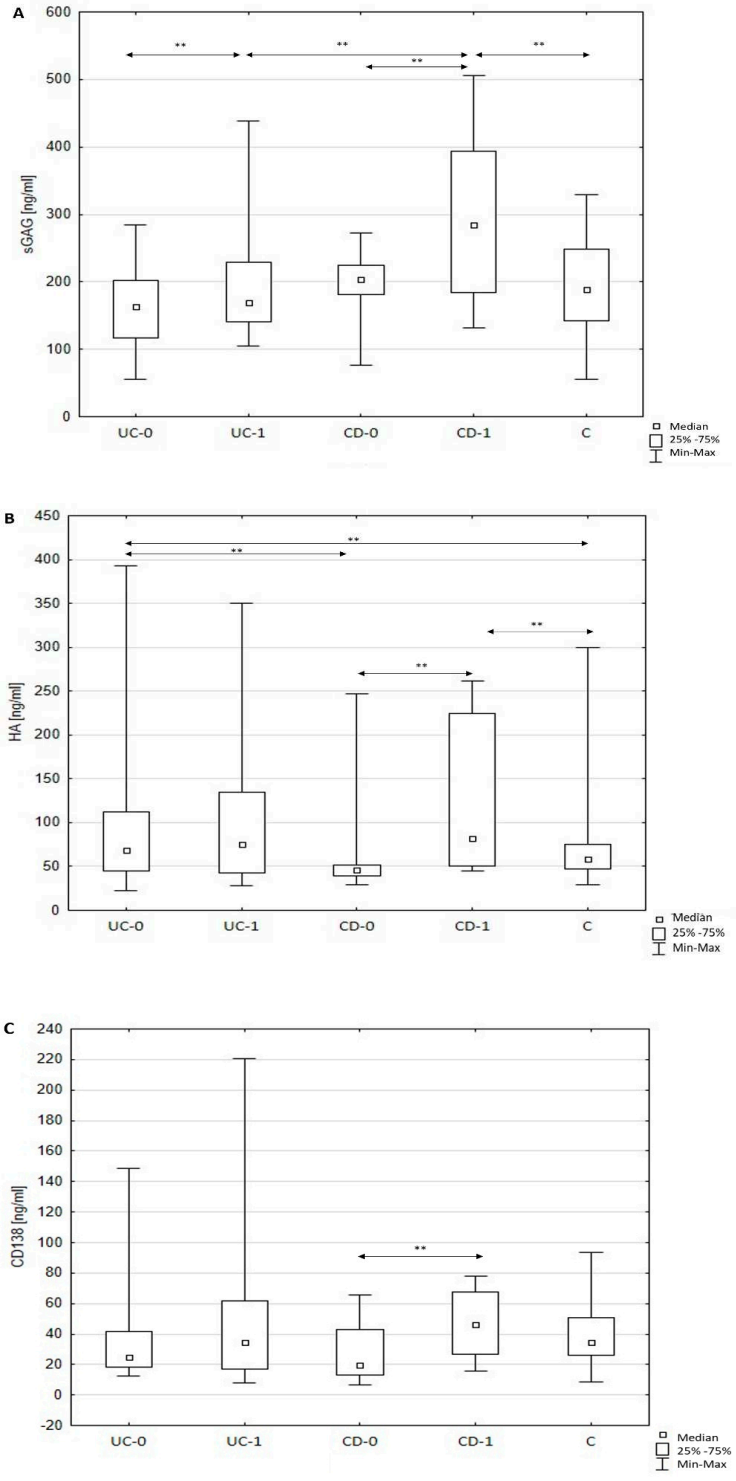

Figure 1. (**A**–**C**) Circulating total sulfated glycosaminoglycans (GAGs), hyaluronic acid (HA), and soluble syndecan-1 (sCD138) levels in healthy subjects and patients with inflammatory bowel diseases (IBD) before and after 12-month therapy. Note: Results are expressed as median, interquartile (25th–75th percentiles) range, and minimum and maximum of data in the groups. C: healthy subjects (control); UC-0: patients with ulcerative colitis before adalimumab therapy; UC-1: patients with ulcerative colitis after 12-month adalimumab therapy; CD-0: patients with Crohn's disease before prednisone therapy; CD-1: patients with Crohn's disease after 12-month prednisone therapy. HA, hyaluronic acid; sGAG, sulfated glycosaminoglycans; sCD138, soluble syndecan-1; ** $p < 0.01$, statistically significant.

4. Discussion

4.1. Quantitative Changes of the ECM Components (sGAG, HA, and sCD138) in Serum of Patients Newly Diagnosed with CD or UC

The constant effect of damaging factors during IBD progression leads to changes in the structure and function of the extracellular matrix [22,23]. Our investigation has shown that disturbances affecting the extracellular matrix in the connective tissue during IBD are reflected in quantitative changes of the composition of matrix components in the serum. In the case of patients newly diagnosed with CD, there was no difference in sGAG, HA, and sCD138 concentrations in their serum compared with that of healthy people. In patients with UC, the parameter which was significantly different for healthy people compared with patients before biological medicine treatment was the concentration of hyaluronan in the serum. During physiological states, hyaluronan plays a significant regulatory role in providing ECM integrity, while the depolymerized, small molecule HA fragments generated in IBD play a significant role as inflammatory factors [15]. Signaling through hyaluronan is a mechanism suggesting the regulatory role of ECM integrity failures in IBD pathogenesis. On the one hand, HA fragments promote wound healing; on the other hand, they also promote fibrotic processes, leading to the proliferation of fibroblasts and differentiating myofibroblasts [14,16,17,24]. The increased accumulation of HA fragments in the intestine is related to the inflammation which affects intestinal tissues during IBD progression. It has also been demonstrated that HA takes part in inducing the infiltration of leukocytes into the bowel and activates the immunological response [16,17]. These mechanisms explain the results of the investigation, which suggest increased concentration of HA in the blood of patients with UC before biological treatment. The research so far suggests that increased hyaluronan concentration in the blood and its accumulation in the intestines facilitates IBD progression by increasing the flow of leukocytes into the intestines [17]. Furthermore, HA binds coagulation factors and increases platelet recruitment, which facilitates and supports inflammation during IBD [10]. The research by Kessler et al. [24] points to a significant increase in the concentration of hyaluronan-associated protein (SHAP-HA) in the serum of patients with UC compared to patients with sustained remission, and this value was positively correlated with endoscopic damage. Importantly, the serum SHAP-HA level in patients with CD was correlated only with TNF-α [24]. A difference in HA turnover between UC and CD patients was also observed during our investigation. The significant difference between HA concentration in the serum of patients newly diagnosed with CD and the concentration of this glycan in the serum of patients with UC before treatment suggests the possibility of measuring HA levels during the differential diagnosis of these two very similar types of bowel inflammation, Crohn's disease and ulcerative colitis. Increased deposition of HA in bowel tissues and its increased transfer into circulation are active participants in the chronic inflammation typical for UC. Quantitative assessment of HA in blood may be an early marker of active disease. Another argument supporting this hypothesis is the fact that our results indicate that HA concentration in the blood of UC patients was correlated with the intensity of the disease as determined from the Mayo score.

4.2. The Influence of Therapy with Prednisone (Patients with CD) or Biological Treatment with Adalimumab (Patients with UC) on the Circulating Profile of Analyzed ECM Components

The performed analyses implied that year-long prednisone treatment applied to patients with CD caused an increase in the concentration of all investigated ECM components in the serum (sGAG, HA, and sCD138) compared with pre-treatment values. In the case of patients with UC, year-long treatment with a TNF-α inhibitor also caused a significant increase in sGAG concentration in serum; however, it did not influence the amount of circulating HA and sCD138. The obtained results confirmed that ECM components are an active part of the progression of the IBD disease process, which is possible to regulate by the type of pharmacological treatment. Sulfated GAGs proved to be the matrix components which undergo significant changes in response to the applied treatment both in CD and

UC patients. The effect of the observed changes, however, was much more noticeable in patients with CD. Moreover, the investigation revealed that sGAG levels in patients with IBD after a year of proper treatment were significantly higher in patients with CD than in patients with UC, which clearly differentiates the two groups of inflammatory bowel diseases. It has also been shown that steroid-based treatment significantly modulated the level of soluble syndecan-1 (sCD138), significantly increasing its levels in the blood. This is in accordance with the results obtained by other authors [21]. It is believed that releasing the extracellular domain of syndecan-1 from the intestinal epithelial cells may reduce the intensity of inflammatory bowel diseases and the transmigration of neutrophils by inactivating key inflammatory mediators and reducing the expression of proinflammatory cytokines. The protective effect of syndecan-1 extracellular domain circulating in the blood has been confirmed during research on mice lacking sCD138; when subjected to an experimental colitis, they have exhibited significantly increased mortality, impaired mucous membrane regeneration, and extended inflammatory cell recruitment. Furthermore, treating animals with a functional analog of the sCD138 ectodomain significantly affected the symptoms of inflammation [21].

4.3. The Influence of Inflammatory Processes and Disease Activity on ECM Components in Patients with IBD

Due to the fact that IBD are diseases involving an active inflammatory process, this article also analyzed the influence of treatment on the grade of inflammation, derived from the concentration of C-reactive protein in blood. The effect of treatment on disease activity, derived from the Mayo score, was also investigated. Even though CRP is not an innate marker of IBD, it may be an indicator of the intensity of the inflammatory process and help in monitoring the state of patients during the stages of remission and aggravation. The research by Mortensen et al. has shown a relationship between CRP and CD activity. The results indicated a significant increase in CRP levels in patients with an active variation of the disease [25]. The research by Chang et al. also revealed a significant correlation between increased CRP concentration and mild or acute clinical form of the disease [26]. In some studies, however, CRP levels were not associated with the disease activity in small intestinal lesions in patients with CD [27,28]. Solem et al. [27], through their analyses, observed that CRP concentration is within the reference range in 75% of patients with CD, in which endoscopic examinations have not confirmed changes to intestinal mucous membrane. In our investigation, pharmacologically treating patients with CD with prednisone also had no influence on changing the clinical activity of the disease, measured using the CDAI score, or on the inflammatory activity, derived from C-reactive protein concentration. On the other hand, significantly higher CRP concentrations have been observed in patients with CD when compared to patients diagnosed with UC; this is consistent with previous studies which showed that patients with CD generally have higher CRP and IL-6 production than patients with UC [29]. However, the mechanisms responsible for this phenomenon have not yet been fully elucidated. One possible mechanism involves the accumulation of mesenteric fat, a major site of IL-6 and TNF-α synthesis, in patients with CD [30]. In our research, the patients with Crohn's disease had no change in markers of inflammation after one year of treatment. This observation may indicate resistance to glucocorticoid treatment or the presence of fistulas. In the case of patients with ulcerative colitis, CRP concentrations observed during our investigation have therefore been—similarly to the results obtained by other authors—significantly lower than in patients with Crohn's disease, which suggests a potential role for CRP markers in differentiating these two diseases of similar symptomatology. We have also observed that 12-month adalimumab treatment applied to patients with UC decreased the activity of inflammatory processes within intestinal walls, which was reflected in lower CRP concentration and lower disease activity according to the Mayo score. This result was connected to reduced symptoms of the disease after 12-month treatment using a biological medicine. These observations are consistent with the results by Tursi et al. [31], who have researched the effectiveness and safety of treating patients with UC with adalimumab. They have noticed a similar decrease in CRP

concentration and reduced disease activity resulting from the biological treatment [31]. The data suggest that a year-long treatment with a biological medicine results in lower activity of the inflammatory process, which is reflected in the results of endoscopic examination. Furthermore, a correlation was observed between hyaluronan concentration in the blood of patients with UC, both before and after 12-month treatment, and the activity of the disease derived from the Mayo score, which supports the hypothesis that hyaluronan is an active participant in the restructuring of the intestinal mucous membrane, related to chronic bowel inflammation. Hyaluronan, along with the enzymes and binding proteins responsible for its synthesis and degradation, may directly modulate the progression of the disease by controlling the recruitment of immune cells and releasing proinflammatory cytokines [19,24]. These attributes of hyaluronan allow its molecules to take part both in the progression and the remission of the disease. It makes HA molecules and, indirectly, the products of its degradation, perfect candidates for the role of UC progression markers.

5. Conclusions

The significant difference in HA concentration in the blood of UC and CD patients suggests the possibility of measuring HA levels during the differential diagnosis of these two very similar types of bowel inflammation. Increased deposition of HA in bowel tissues and its increased transfer into circulation are active participants in the chronic inflammation typical for UC. Quantitative changes of HA in blood may be an early marker of active disease. Moreover, our study confirmed that ECM components are an active part of the progression of the IBD disease process, which is possible to regulate according to the type of pharmacological treatment.

The results obtained during this investigation reveal a variety of mechanisms regulating the activity of the disease and the restructuring of extracellular matrix components as well as the potential usefulness of circulating ECM components as markers for evaluating ECM remodeling during IBD and for monitoring pharmacological treatment. Finding potentially useful diagnostic markers of changes occurring throughout IBD, determined in readily available biological material, may provide an aid to the clinical diagnosis and/or monitoring the progression of treatment. However, further research should be performed on a larger group of patients in order to confirm the obtained results. Further investigation could possibly lead to identifying a component of extracellular matrix that will meet the criteria of an ideal marker and will protect patients from invasive colonoscopy examination.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/jcm10081722/s1, Figure S1: Results of correlation analysis between disease activity expressed as the Mayo score and serum concentration of the hyaluronan (HA) or soluble syndecan-1 (sCD138) level in patients with ulcerative colitis both before, and after 12-month adalimumab therapy; Table S1: Serum concentrations of sulfated glycosaminoglycans (sGAG), hyaluronic acid (HA) and soluble syndecan-1 (sCD138) in patients with Crohn disease (CD) and ulcerative colitis (UC) at baseline and after one year of therapy.

Author Contributions: K.K.-V. and A.D.—conceptualization, investigation, performed the biochemical analysis, writing—original draft preparation; P.O.—conceptualization, investigation, review and editing; K.O.—writing-review and editing, supervision; A.J.-P.—conducting the analysis and contributing to the interpretation of data and the obtained results. All authors have read and agreed to the published version of the manuscript.

Funding: This study was funded by the Medical University of Silesia in Katowice, Poland, grant No. PCN-007/N/0/I.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Bioethics Committee of the Medical University of Silesia in Katowice (protocol code KNW/0022/KB/309/15 and approval date 30 December 2015).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data is contained within the article or supplementary material.

Acknowledgments: The authors would like to thank Maciej Gonciarz from the Department of Gastroenterology, St. Barbara's Specialized Regional Hospital No. 5, Sosnowiec, Poland, for help and participation in the qualification of patients for this study. Our thanks also go to all department members who participated in this effort.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Ng, S.C.; Shi, H.Y.; Hamidi, N.; Underwood, F.E.; Tang, W.; Benchimol, E.I.; Panaccione, R.; Ghosh, S.; Wu, J.C.Y.; Chan, F.K.L.; et al. Worldwide incidence and prevalence of inflammatory bowel disease in the 21st century: A systematic review of population-based studies. *Lancet* **2017**, *390*, 2769–2778. [CrossRef]
2. Actis, G.C.; Pellicano, R.; Fagoonee, S.; Ribaldone, D.G. History of Inflammatory Bowel Diseases. *J. Clin. Med.* **2019**, *8*, 1970. [CrossRef]
3. Ahlawat, S.; Kumar, P.; Mohan, H.; Sandeep Goyal, S.; Kant Sharma, K. Inflammatory bowel disease: Tri-directional relationship between microbiota, immune system and intestinal epithelium. *Crit. Rev. Microbiol.* **2021**, *47*, 254–273. [CrossRef]
4. Gomollón, F.; Dignass, A.; Annese, V.; Tilg, H.; Van Assche, G.; Lindsay, J.O.; Peyrin-Biroulet, L.; Cullen, G.J.; Daperno, M.; Kucharzik, T.; et al. 3rd European evidence-based consensus on the diagnosis and management of Crohn's disease 2016: Part 1: Diagnosis and medical management. *J. Crohn Colitis* **2017**, *11*, 3–25. [CrossRef]
5. Tanaka, M.; Saito, H.; Fukuda, S.; Sasaki, Y.; Munakata, A.; Kudo, H. Simple mucosal biopsy criteria differentiating among Crohn disease, ulcerative colitis, and other forms of colitis: Measurement of validity. *Scand. J. Gastroenterol.* **2000**, *35*, 281–286.
6. Kopylov, U.; Rosenfeld, G.; Bressler, B.; Seidman, E. Clinical utility of fecal biomarkers for the diagnosis and management of inflammatory bowel disease. *Inflamm. Bowel Dis.* **2014**, *20*, 742–756. [CrossRef]
7. Konikoff, M.R.; Denson, L.A. Role of fecal calprotectin as a biomarker of intestinal inflammation in inflammatory bowel disease. *Inflamm. Bowel Dis.* **2006**, *12*, 524–534. [CrossRef]
8. Bennike, T.; Birkelund, S.; Stensballe, A.; Andersen, V. Biomarkers in inflammatory bowel diseases: Current status and proteomics identification strategies. *World J. Gastroenterol.* **2014**, *20*, 3231–3244. [CrossRef] [PubMed]
9. Di Ruscio, M.; Vernia, F.; Ciccone, A.; Frieri, G.; Latella, G. Surrogate fecal biomarkers in inflammatory bowel disease: Rivals or complementary tools of fecal calprotectin? *Inflamm. Bowel Dis.* **2018**, *24*, 78–92. [CrossRef]
10. Petrey, A.C.; De La Motte, C.A. Hyaluronan in inflammatory bowel disease: Cross-linking inflammation and coagulation. *Matrix Biol.* **2019**, *78*, 314–323. [CrossRef] [PubMed]
11. Frantz, C.; Stewart, K.M.; Weaver, V.M. The extracellular matrix at a glance. *J. Cell Sci.* **2010**, *123*, 4195–4200. [CrossRef]
12. Scott, J.E. Supramolecular organization of extracellular matrix glycosaminoglycans, in vitro and in the tissues. *FASEB J.* **1992**, *6*, 2639–2645. [CrossRef]
13. Murch, S.H.; MacDonald, T.T.; Walker-Smith, J.A.; Lionetti, P.; Levin, M.; Klein, N.J. Disruption of sulphated glycosaminoglycans in intestinal inflammation. *Lancet* **1993**, *341*, 711–714. [CrossRef]
14. Toole, B.P. Hyaluronan: From extracellular glue to pericellular cue. *Nat. Rev. Cancer* **2004**, *4*, 528–539. [CrossRef]
15. Derkacz, A.; Olczyk, P.; Olczyk, K.; Komosinska-Vassev, K. The Role of Extracellular Matrix Components in Inflammatory Bowel Diseases. *J. Clin. Med.* **2021**, *10*, 1122. [CrossRef]
16. Grandoch, M.; Bollyky, P.L.; Fischer, J.W. Hyaluronan: A master switch between vascular homeostasis and inflammation. *Circ. Res.* **2018**, *122*, 1341–1343. [CrossRef] [PubMed]
17. Elenius, K.; Vainio, S.; Laato, M.; Salmivirta, M.; Thesleff, I.; Jalkanen, M. Induced expression of syndecan in healing wounds. *J. Cell Biol.* **1991**, *114*, 585–595. [CrossRef] [PubMed]
18. Alexopoulou, A.N.; Multhaupt, H.A.; Couchman, J.R. Syndecans in wound healing, inflammation and vascular biology. *Int. J. Biochem. Cell Biol.* **2007**, *39*, 505–528. [CrossRef]
19. Tkachenko, E.; Rhodes, J.M.; Simons, M. Syndecans: New kids on the signaling block. *Circ. Res.* **2005**, *96*, 488–500. [CrossRef]
20. Yablecovitch, D.; Stein, A.; Shabat-Simon, M.; Naftali, T.; Gabay, G.; Laish, I.; Laish, I.; Oren, A.; Konikoff, F.M. Soluble syndecan-1 levels are elevated in patients with inflammatory bowel disease. *Dig. Dis. Sci.* **2015**, *60*, 2419–2426. [CrossRef]
21. Wang, X.; Li, A.; Li, J.; Lin, S.; Zhou, Y.; Wang, X.; Chen, C.; Liu, S.; Chen, Y. Low molecular weight heparin relieves experimental colitis in mice by downregulating IL-1β and inhibiting syndecan-1 shedding in the intestinal mucosa. *PLoS ONE* **2013**, *8*, e66397. [CrossRef]
22. Page-McCaw, A.; Ewald, A.J.; Werb, Z. Matrix metalloproteinases and the regulation of tissue remodeling. *Nat. Rev. Mol. Cell Biol.* **2007**, *8*, 221–233. [CrossRef]
23. Bosman, F.T.; Stamenkovic, I. Functional structure and composition of the extracellular matrix. *J. Pathol.* **2003**, *200*, 423–428. [CrossRef] [PubMed]
24. Kessler, S.; Rho, H.; West, G.; Fiocchi, C.; Drazba, J.; De La Motte, C. Hyaluronan (HA) deposition precedes and promotes leukocyte recruitment in intestinal inflammation. *Clin. Transl. Sci.* **2008**, *1*, 57–61. [CrossRef] [PubMed]
25. Mortensen, J.H.; Manon-Jensen, T.; Jensen, M.D.; Hagglund, P.M.; Klinge, L.G.; Kjeldsen, J.; Krag, A.; Karsdal, M.A.; Bay-Jensen, A.C. Ulcerative colitis, Crohn's disease, and irritable bowel syndrome have different profiles of extracellular matrix turnover, which also reflects disease activity in Crohn's disease. *PLoS ONE* **2017**, *12*, e0185855. [CrossRef]

26. Chang, S.; Malter, L.; Hudesman, D. Disease monitoring in inflammatory bowel disease. *World J. Gastroenterol.* **2015**, *21*, 11246–11259. [CrossRef]
27. Solem, C.A.; Loftus, E.V., Jr.; Tremaine, W.J.; Harmsen, W.S.; Zinsmeister, A.R.; Sandborn, W.J. Correlation of C-reactive protein with clinical, endoscopic, histologic, and radiographic activity in inflammatory bowel disease. *Inflamm. Bowel Dis.* **2005**, *11*, 707–712. [CrossRef] [PubMed]
28. Florin, T.H.J.; Paterson, E.W.J.; Fowler, E.V.; Radford-Smith, G.L. Clinically active Crohn's disease in the presence of a low C-reactive protein. *Scand. J. Gastroenterol.* **2006**, *41*, 306–311. [CrossRef] [PubMed]
29. Fagan, E.A.; Dyck, R.F.; Maton, P.N.; Hodgson, H.J.; Chadwick, V.S.; Petrie, A.; Pepys, M.B. Serum levels of C-reactive protein in Crohn's disease and ulcerative colitis. *Eur. J. Clin. Investig.* **1982**, *12*, 351–359. [CrossRef]
30. Greenfield, J.R.; Samaras, K.; Jenkins, A.B.; Kelly, P.J.; Spector, T.D.; Gallimore, J.R.; Pepys, M.B.; Campbell, L.V. Obesity is an important determinant of baseline serum C-reactive protein concentration in monozygotic twins, independent of genetic influences. *Circulation* **2004**, *109*, 3022–3028. [CrossRef] [PubMed]
31. Tursi, A.; Elisei, W.; Faggiani, R.; Allegretta, L.; Della Valle, N.; Forti, G.; Franceschi, M.; Ferronato, A.; Gallina, S.; Larussa, T.; et al. Effectiveness and safety of adalimumab to treat outpatient ulcerative colitis. *Medicine* **2018**, *97*, e11897. [CrossRef]

Journal of
Clinical Medicine

MDPI

Review

The Role of Innate and Adaptive Immune Cells in the Pathogenesis and Development of the Inflammatory Response in Ulcerative Colitis

Aleksandra Kałużna [1,*], Paweł Olczyk [2] and Katarzyna Komosińska-Vassev [1]

[1] Department of Clinical Chemistry and Laboratory Diagnostics, Faculty of Pharmaceutical Sciences in Sosnowiec, Medical University of Silesia in Katowice, 41-200 Sosnowiec, Poland; kvassev@sum.edu.pl
[2] Department of Community Pharmacy, Faculty of Pharmaceutical Sciences in Sosnowiec, Medical University of Silesia in Katowice, 41-200 Sosnowiec, Poland; polczyk@sum.edu.pl
* Correspondence: aleksandra.kaluzna@sum.edu.pl; Tel.: +48-32-364-11-55

Abstract: Ulcerative colitis (UC) is a chronic inflammatory disease with an underlying excessive immune response directed against resident microbiota and/or dietary antigens. Both innate and adaptive immune cells play a crucial role in the pathogenesis of UC. In the case of innate immune response cells, neutrophils, dendritic cells, macrophages have a crucial impact on the development of the disease, as well as innate lymphoid cells, which have received a particular attention in recent years. On the other hand, mechanisms of the adaptive immune response involve cells such as: cytotoxic lymphocytes, regulatory lymphocytes Treg, or helper lymphocytes Th–Th2, Th9, Th17, Th22, among which significant discoveries about Th9 and Th17 lymphocytes have been made in recent years. Due to the presence of antibodies directed against resident microbiota or one's own tissues, the influence of B lymphocytes on the development of UC is also highlighted. Additionally, the impact of cytokines on shaping the immune response as well as sustaining inflammation seems to be crucial. This review briefly describes the current state of knowledge about the involvement of the innate and adaptive immune systems in the pathogenesis of UC. The review is based on personal selection of literature that were retrieved by a selective search in PubMed using the terms "ulcerative colitis" and "pathogenesis of ulcerative colitis". It included systematic reviews, meta-analyses and clinical trials. Our knowledge of the involvement of the immune system in the pathophysiology of IBD has advanced rapidly over the last two decades, leading to the development of several immune-targeted treatments with a biological source, known as biologic agents.

Keywords: ulcerative colitis; lymphocytes; macrophages; dendritic cells; innate lymphoid cells

Citation: Kałużna, A.; Olczyk, P.; Komosińska-Vassev, K. The Role of Innate and Adaptive Immune Cells in the Pathogenesis and Development of the Inflammatory Response in Ulcerative Colitis. *J. Clin. Med.* **2022**, *11*, 400. https://doi.org/10.3390/jcm11020400

Academic Editors: Angelo Viscido and Konstantinos Papamichael

Received: 1 December 2021
Accepted: 11 January 2022
Published: 13 January 2022

1. Introduction

Ulcerative colitis (UC) is a chronic inflammatory disease, which belongs with Crohn's disease to the group of the inflammatory bowel diseases (IBDs). The condition is diagnosed mostly between the ages of 20 to 40, however it can occur at every age. Characteristic of UC are alternating periods of clinical relapse and remission. Inflammation of the colon mucosa plays an essential role in pathogenesis of UC, which leads to ulcer formation. Changes observed within the intestinal mucosa are localized in the rectum and spread proximally to the other parts of the colon. The most common clinical symptoms are gastrointestinal disorders such as abdominal pain, diarrhea with mucus and/or blood, nausea and vomiting; nevertheless, general symptoms including fever, weight loss and anemia are also observed with the parenteral symptoms—peripheral arthritis, cholangitis, pyoderma gangrenosum, erythema nodosum and arthropathies [1,2]. Although the disorder is quite common, its exact pathogenesis is not fully understood; however, it is known that UC is connected with the excessive immune response to the environmental factors or resident microbiota among genetically susceptible subjects, and the immunity status plays a crucial role in the

increased intestinal permeability and impaired barrier function, as presented graphically in Figure 1. There are reasonable doubts if the impaired barrier function precedes the onset of the disease, or the inflammation development in the lamina propria of the intestinal mucosa induces the intestinal epithelial disfunction [3].

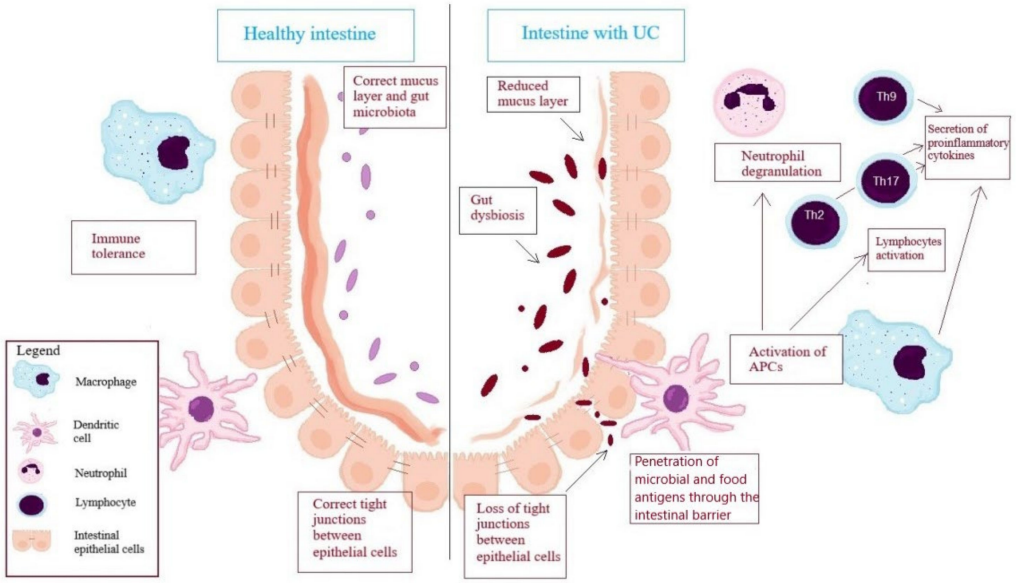

Figure 1. Pathogenesis of ulcerative colitis (UC). APCs—antigen presenting cells; In physiological conditions, a homeostasis between natural microbiota and immune system is maintained. Intestinal mucosa is covered by a thick layer of mucus preventing the penetration of intestinal bacteria to epithelial cells. Integrity of the intestinal barrier is provided by the tight junctions between epithelial cells, and the immune cells present a state of immune tolerance. In UC, a layer of mucus is reduced and the gut microbiota is abnormal, which leads to gut dysbiosis. A reduction of butyrate-producing bacteria (as a result of, e.g., antibiotic treatment) contributes to a decreased amount of butyrate in the intestinal lumen and enhances anaerobic metabolic processes, which generate less energy than the aerobic ones. The resulting oxidative stress damages the mucus-secreting colonocytes. Additionally, enhanced anaerobic processes favor the expansion of facultative anaerobes such as *Salmonella enterica* and *Escherichia coli*. Increased relative abundance of these bacterial species releasing bacterial enterotoxins directly attack and damage the intestinal epithelial cells, which in turn damages the intestinal mucosa and reduces its protective functions. The loss of tight junctions seen in UC allows bacteria and dietary antigens to pass through the intestinal barrier and leads to the activation of antigen-presenting cells. Macrophages and dendritic cells enhance neutrophil migration and degranulation, as well as activating lymphocytes Th2, Th17, Th9. Activation of immune cells results in the secretion of pro-inflammatory cytokines, i.e., TNF-α, IFN-γ, and IL-13, which increases the permeability of the intestinal barrier and thus promotes inflammation in the intestinal mucosa [3–5].

Patients with UC present a decreased number of the goblet cells responsible, among others, for the excretion of biologically active substances that contribute to innate immunity, such as trefoil peptides and peptides binding Fc region of antibodies, as well as for secretion of the mucin, which forms large net-like polymers that lubricate the lumen and form a barrier between sterile intestinal epithelial and gut microbiota [6]. An impaired intestinal barrier and a decreased amount of the mucus leads to increased exposure to food antigens and antigens associated with gut microbiota, which activates the mechanisms of the innate immunity. Innate (passive) immune response is the first line of defense in the body, including cells such as macrophages, innate lymphoid cells, mast cells, and neutrophils to identify and eliminate pathogens in a short time, though its mechanisms are not specific for various antigens [5]. Antigen-presenting cells (APC)—macrophages and dendritic cells (DC)—connect the mechanisms of innate and adaptive (active) immune responses

through presentation of the antigens to certain lymphocytes, which leads to activation of the adaptive immune cells. Presence of a specific cytokine environment results in the activation of transcription factors responsible for polarization of the naive lymphocytes, i.e., Th2, Th17, and Th9, playing crucial roles in the development of the disorder. Moreover, Treg cells, in spite of their increased number in the intestinal mucosa of patients with UC, present reduced immunoregulatory activity. Increased concentration of tumor growth factor β (TGF-β) induces the suppression of Th22 lymphocytes which secrete IL-10 with a protective effect on the intestinal mucosa. The impaired balance between pro- and anti-inflammatory processes leads to the development of the chronic inflammation and impairment of the intestinal barrier function. A key role in the development of the disease is played by cytokines whose functions and influence on inflammation are presented in Table 1 [5].

Table 1. The influence of cytokines taking part in the UC pathogenesis on the development of the immune response [5,7–16].

Cytokine	Expression of Cytokine in UC	Cells Secreting Cytokine	Influence on the Inflammation	Function
IL-1β	Increased	Macrophages	pro-inflammatory	Stimulates T cells to secrete pro-inflammatory cytokines, induces chemotactic factors, influences the proliferation of B cells, systemically causes an increase of temperature and acute phase proteins
IL-4		Th2	anti-inflammatory	Stimulates humoral immune response, inhibits the secretion of pro-inflammatory cytokines (TNF-α, IL-6, IL-1β), activates M2 macrophages, activates transcription factors responsible for differentiation of T CD4+ cells to Th9 cells
IL-5	Increased	Th2		Decreases the activity of Th1 cells and cellular immune response, stimulates the maturation of eosinophils and basophils
IL-6	Increased	Macrophages, dendritic cells	pro-inflammatory	Activates transcription factors responsible for differentiation of T CD4+ cells to Th17 cells, inhibits the secretion of TNF-α
IL-9	Increased	Th9, Th2	pro-inflammatory	Activates mast cells, neutrophils and eosinophils, influences the expression of proteins creating tight junctions in the intestinal barrier
IL-10	Increased	Treg, Th2, Th17, Breg	anti-inflammatory	Inhibits the secretion of tissue metalloproteinases, tissue factor and cyclooxygenase 2, suppresses the expression of transcription factor NF-κB, activates macrophages M2
IL-13	Increased	Th2	anti-inflammatory	Decreases the activity of Th1 cells and cellular immune response, inhibits the secretion of pro-inflammatory cytokines (TNF-α, IL-6, IL-1β), influences the expression of proteins creating tight junctions in the intestinal barrier and the epithelial cells apoptosis, activates macrophages M2

Table 1. *Cont.*

Cytokine	Expression of Cytokine in UC	Cells Secreting Cytokine	Influence on the Inflammation	Function
IL-17	Increased	Th17, monocytes, neutrophils, T CD8+, NK cells	pro-inflammatory	Stimulates monocytes, epithelial and endothelial cells to secrete pro-inflammatory cytokines (IL-1β, TNF-α) and chemokines responsible for leukocytes and neutrophils migration to inflamed tissues, in the absence of IL-23 supports the intestinal barrier through occludin regulation in tight junctions
IL-21		Th17, Th2		Decreases the activity of Th1 cells and thus the cellular immune response, increases the expression of IL-23 receptor, stimulates proliferation and maturation of B, T CD8+ and NK cells
IL-22		Th17, Th22, Th1		Induces the secretion of antimicrobial peptides, IL-10 and mucus, mediates in the tissue repair processes
IL-23	Increased	Macrophages, dendritic cells	pro-inflammatory	Takes part in the differentiation of T CD4+ cells to Th17 cells
IL-25		Th2		Decreases the activity of Th1 cells and cellular immune response
IL-33		Treg, macrophages, dendritic cells, mast cells		Enhances the secretion of IL-4, IL-5, IL-13, increases the accumulation of Th2, stimulates pathogenic Th2 and Th17 response, induces tissue repair through coordination of Treg
IL-35		Treg	anti-inflammatory	Suppress the differentiation of Th17
TGF-β		Treg	anti-inflammatory	Stimulates epithelial repair, decreases expression of IL-33 and Th22, stimulates differentiation of Th17 in the presence of IL-6 and Treg in the absence of IL-6, activates the transcription factors responsible for differentiation of T CD4+ cells to Th9 cells
IFN-γ		Th1, dendritic cells, macrophages		Enhances transcytosis and paracellular transport, activates macrophages
TNF-α	Increased	Th17, macrophages, dendritic cells	pro-inflammatory	Takes part in cell apoptosis, stimulates lymphocytes and activates other immune cells

The altered balance of anti-inflammatory and pro-inflammatory cytokines (mainly towards the secretion of pro-inflammatory cytokines) may lead to the development of an excessive immune response, which is associated with further activation and infiltration of immune cells, increased cell apoptosis, and loss of intestinal barrier integrity [15].

2. Immune Cells in the Pathogenesis of Ulcerative Colitis

2.1. Cells of Innate Immunity

Immune cells play a significant role in the pathogenesis of ulcerative colitis, including cells of innate and adaptive immune response. The cells belonging to the innate immunity are neutrophils, which constitute the main component of the inflammatory infiltrate in an intestinal tissue in UC and are one of the first cells participating in the active phase of the disease. Neutrophils recognize phagocytes and take part in incapacitation of the microorganisms through releasing the neutrophil extracellular traps (NETs) or degranulation of its own grains. NETs are cross-linked structures protruding from the membrane of activated neutrophils, composed of condensed chromatin and DNA. Moreover, they contain some components of the neutrophils' grains, such as neutrophil elastase, myeloperoxidase (MPO), and cathepsin G. NETs generation is a neutrophils' response to the presence of pathogens since the biochemical composition of NETs determines the trapping and upcoming elimination of pathogens. MPO released from the cell catalyzes the HClO synthesis reaction. Further reactions with this acid affect the formation of reactive oxygen species involved in the inactivation of microorganisms, and thus contribute to tissue damage and ulcer formation [7,8,17,18]. The studies conducted so far show that, in patients with UC, the concentration of MPO was several times higher than the expression of the enzyme in the stool of healthy subjects, which suggests an increased activity of neutrophils in these patients. Factors that increase NET release include tumor necrosis factor-α (TNF-α) and bacterial lipopolysaccharides, although this phenomenon is characteristic for neutrophils' activation, not only limited to UC. NETs may also arise when stimulated by other pro-inflammatory cytokines, including IL-8 released by endothelial cells, and by NO or neutrophil autoantibodies characteristic to small blood vessel inflammation and also present in UC. Triggering properties towards releasing of NETs also fulfill protein arginine deiminase 4 (PAD4), which is responsible for histone citrullination—a key process taking place during NETosis. Dinallo et al. [17] proved that epithelial cells and cells of intestinal mucosa exhibited higher PAD4 concentrations in intestinal tissue specimens collected from UC patients, compared to healthy individuals and patients with Crohn's disease. Moreover, comparing the expression of PAD4 in a tissue lesion and in healthy tissue collected from the same patients with UC, it has been noted that in the first case, an expression of PAD4 was significantly higher [7,17,18].

Recently, in pathogenesis of UC, the role of innate lymphoid cells (ILC), which belongs to the family of mononuclear effective cells with common lymphoid progenitor, has been highlighted. The cells take part in the immune response directed towards extra- and intracellular microorganisms, in protection of an intestinal barrier, as well as in tissue repair and remodeling. Taking into account the expression of transcriptional factors and the types of cytokines secreted by the ILC, three types of these cells were distinguished: ILC1, ILC2, ILC3 [5,19,20].

In the Forkel's et al. [19] analysis, an increased number of ILC1 cells correlated with an early stage of Crohn's disease. Additionally, Forkel et al. noted a significant increase in the number of ILC1 cells in the inflamed intestinal mucosa. ILC1 appears as a result of enhanced expression of T-cell-specific T-box transcription factor (T-bet), the expression of which is induced by IL-12. ILC1 is mainly responsible for eradication of the viruses and bacteria. In addition, after pathogens are immobilized by DCs that release IL-12 and IL-18, ILC1 is stimulated to synthesize interferon-γ (IFN-γ). Increased secretion of IFN-γ may contribute to the pathological processes seen in UC; however, the role of ILC1 is more emphasized in the pathogenesis of other type of IBD, such as Crohn's disease, than in UC [5,19,20].

In the chronic stage of UC, the number of ILC1, as well as ILC2 cells, was increased in the intestinal mucosa. Moreover, in biopsies of the inflamed intestinal mucosa collected from the patients with early UC, an increased number of ILC2 cells has also been reported. ILC2 cells appear as a result of enhanced expression of transcription factors such as GATA binding protein 3 (GATA3) and retinoic-related orphan receptor α (RORα). In response to

IL-33 (secreted especially during parasite infection, epithelial cells damage or exposition to allergens), ILC2 releases IL-5, responsible for neutrophil recruitment to inflamed areas, and IL-13 that disrupts the intestinal epithelial barrier function. However, ILC2 is also able to secrete IL-4, -6, -8, -9, granulocyte-macrophage colony-stimulating factor (GM-CSF) and amphiregulin, involved in epithelial repair [19,21].

The third, and at the same time the most diversified, group of innate lymphoid cells are the ILC3 cells, which appears as a result of the action of retinoic-related orphan receptor γt (RORγt). Due to the presence of a natural receptor of cytotoxicity, ILC3 cells can be divided into NKp44+ and NKp44− cells. ILC3 cells are well known for secreting IL-22 and/or IL-17 in response to IL-23 and IL-1β [5,19,21]. Patients with IBD present a reduced number of NKp44+ cells, which negatively correlates with disease activity assessed in endoscopic examination. NKp44+ cells are dominant ILC cells in the healthy mucosa of the ileum, caecum and colon. They are characterized by high expression of IL-22 with a protective role towards intestinal epithelial cells, and low expression of IL-17, which may fulfill both protective and pro-inflammatory functions against the intestinal barrier, depending on the cytokine environment. The reduced number of NKp44+ cells may contribute to a reduction in the expression of the protective IL-22 and thus to the dysfunction of the intestinal barrier. Interestingly, no significant differences were found in the ILC population in the peripheral blood and healthy intestinal mucosa from the IBD patients compared to the ILC population in the peripheral blood and intestinal mucosa from healthy people, which indicates the native nature of these changes [5,19,21].

2.2. Antigen-Presenting Cells (APC)

Antigen-presenting cells, including dendritic cells and macrophages, are cells connecting two types of the immune response. In spite of a different origin, these two types of cells express receptors recognizing molecular patterns such as toll-like receptors (TLR) and nucleotide-binding oligomerization domain-coding protein (NOD). Unlike macrophages, DCs migrate to peripheral lymph nodes when activated, while macrophages locally activate an adaptive immune response. In a healthy organism, intestinal DCs remain immune tolerant because they secrete protective IL-10, while in IBD, DCs shift their activity and the number of pro-inflammatory DCs increases. Hart et al. [22] showed an increased expression of TLR-2 and TLR-4 on the surface of dendritic cells in the biopsy material from patients with UC, while in patients without changes in endoscopic examination, elevated TLR-2 or TLR-4 was found in only a few cases. Activation of these receptors leads to the activation of the nuclear factor kappa-B (NF-κB) and other transcription factors directly influencing the processes related to the development of inflammation [5,20,22].

Macrophages also show significant functional differences depending on the tissue environment. In the absence of inflammatory processes, macrophages perform phagocytic functions, secreting pro-inflammatory cytokines only to a limited extent, and DCs are mainly involved in antigen presentation. During inflammation, the cytokines responsible for macrophages' activation are secreted and, depending on the method of activation, macrophages can be divided into classically activated (M1) or alternatively activated (M2). M1 present pro-inflammatory functions and significant antibacterial activity. They are activated by exposure to interferon-γ (IFN-γ), GM-CSF or LPS and, when stimulated, secrete significant amounts of cytokines (TNF-α, IL-1β, IL-12, IL-18, IL-23), chemokines (CXCl9, CXCL10), reactive oxygen and nitrogen species. M1 take part in the immune response via Th1 and Th17 cells. In contrast, M2 macrophages induced by IL-4, IL-10, and IL-13 exhibit anti-inflammatory functions and take part in tissue healing and fibrosis. M2 are characterized by the significant expression of mannose receptor (CD206) and scavenger-type receptors (CD163 and CD204), and regulate the activation of Th2 cells through the secretion of IL-10 and TGF-β. In addition, via releasing the anti-inflammatory chemokines (CCL-17, CCL-22, CCL-24), M2 promote basophils and eosinophils recruitment. Intestinal macrophages present features characteristic for M1, as well as M2 cells. On the one hand, similar to M1 cells, they present high expression of antigens belonging to the II class of

the major histocompability complex (MHC), along with the expression of TNF-α. On the other hand, like M2 cells, they represent significant phagocytic activity and constitutive expression of IL-10. During IBD, the balance between M1 and M2 is shifted towards the pro-inflammatory type. Intestinal macrophages then secrete pro-inflammatory cytokines, such as IL-6, IL-23 and TNF-α, presenting at the same time increased cytotoxicity and phagocytic activity [1,5].

2.3. Lymphocytes, as an Element of the Adaptive Immune Response in the UC's Pathogenesis

Antigen-presenting cells activate the mechanisms of immune response by antigen presentation to T and B cells. This type of immune response is more time-consuming, although it is more precise than the innate immune response. Depending on the expression of the CD4 and CD8 cell surface molecules, lymphocytes can be divided into T CD8+, mainly cytotoxic cells, and T CD4+ T cells. The first type of cells contributes to the pathological changes observed in UC in the intestine through the production of pro-inflammatory cytokines (i.e., IFN-γ, TNF-α) that increase the inflammation and the secretion of pro-inflammatory cytokines and chemokines that damage epithelial cells. These actions of T CD8+ cells cause the formation of ulcers in the intestine that occurs in ulcerative colitis. The latter type, T CD4+ cells, can be subdivided into helper T cells (Th) and regulatory T cells (Treg). Moreover, Th1, Th2, Th9, Th17 and Th22 were distinguished among Th lymphocytes, based on the cytokine profile. Differentiation from naive cells to the aforementioned helper T cell types depends on the cytokine environment and the activation of individual transcription factors, as shown in Figure 2 [5,23,24]. Inflammation within the intestine tissue is associated with an enhanced activation and maturation of T cells. Lymphocytes isolated from the peripheral blood of UC patients showed increased expression of markers of activation—HLA-DR, β1-integrin and decreased expression of CD62L, characteristic for naïve T cells, compared to lymphocytes isolated from healthy individuals. These results indicate an increased activation of CD4+ and CD8+ cells in patients with UC. Moreover, the phenotype of active cells was correlated with an increased level of systemic and intestinal inflammatory markers in newly diagnosed patients. In the same study group, the percentage of inactive T CD4+ cells expressing CD62L negatively correlated with eosinophil cationic protein and calprotectin in feces considered to be intestinal inflammatory markers. Inflammation promotes lymphocyte activation due to increased APC activity in IBD. Thus, the cytotoxicity of T CD8+ cells can lead to tissue damage, which exacerbates the inflammatory processes [24].

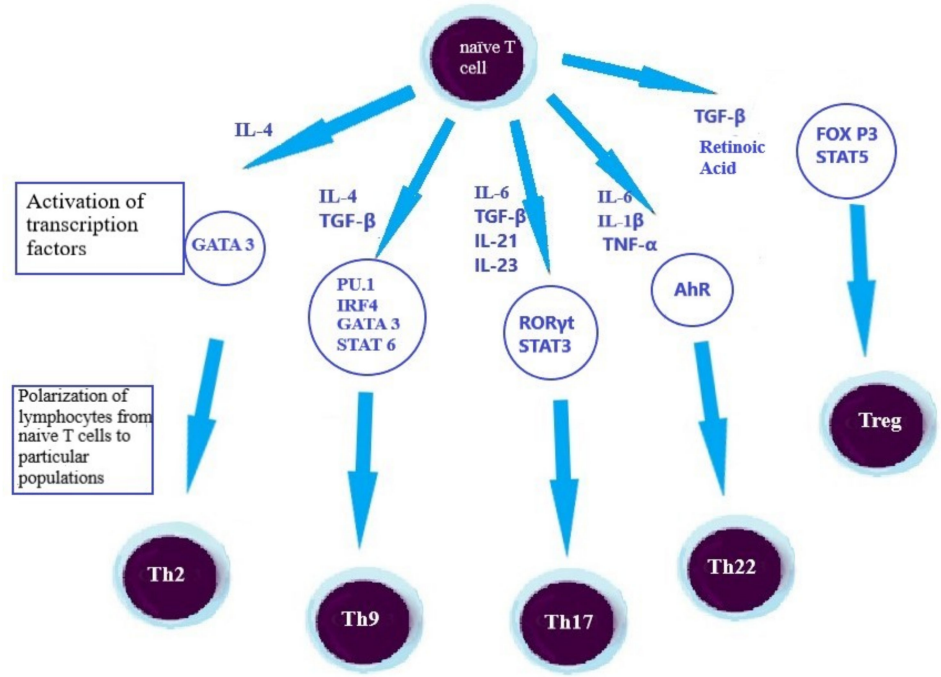

Figure 2. Differentiation of T cells' main population from the naïve T cells [5,9,25]. AhR—aryl hydrocarbon receptor, FOX P3—forkhead box P3, GATA 3—GATA binding protein 3, IRF4—interferon regulatory factor 4, PU.1—Purine-rich Box-1, RORγt—retinoic acid receptor-related orphan receptor γt, STAT3—signal transducer and activator of transcription 3, STAT5—signal transducer and activator of transcription 5, TGF-β—transforming growth factorβ, TNF-α—tumor necrosis factor α.

2.4. Th2 Lymphocytes

Previous research projects have shown that during UC, the expression of Th2 subpopulation of lymphocytes is increased in the inflammatory infiltration of the intestine tissue. Th2 cells are responsible for maintaining the homeostasis of the intestinal mucosa and are also involved in immune response against parasites, pro-inflammatory pathways and tissue repair. Excessive activation of Th2 cells may lead to the development of chronic inflammatory state and progressive tissue fibrosis. Th2 cells mediate the humoral type of immune response, secreting, i.e., IL-4 and IL-10, while by increasing the secretion of IL-5, IL-13, IL-21, and IL-25, they inhibit the activity of Th1 cells involved in cellular immune response [5,26]. Secreted by Th2 cells, IL-13 fulfill a crucial role in the pathogenesis of UC. Through the signal transducer and activator of transcription 6 (STAT-6) and signaling pathways of PI3K kinase, IL-13 influences the integrity of the intestinal barrier, since it increases the expression of claudin-2, creating pores selective for water and small cations. The expression of claudin-2 in the intestinal epithelium is high upon birth, then drops, and the next rise of claudin-2 expression in the intestine epithelium can be observed in inflammatory states including IBD. In addition, IL-13 negatively affects the integrity of the intestinal barrier by inducing apoptosis of intestinal epithelial cells and inhibiting epithelial regeneration. Moreover, TNF-α enhances these effects [24,27,28].

Th2 lymphocytes arise from the activation of the GATA-3 transcription factor upon stimulation by IL-4 secreted by dendritic cells. Interestingly, Seidelin et al. [29] presented that intestinal IL-33 is able to increase the expression of GATA-3, which indicates that the increased number of Th2 cells might be caused by an increased level of IL-33. Seidelin et al. noted that the expression of IL-33 was higher in intestinal biopsies collected from patients with active UC compared to inactive UC and healthy subjects. IL-33 plays a dichotomous role in the human body. On the one hand, IL-33 has a protective function as it reduces the expression of genes induced by NF-κB pathway; however, on the other hand, acting as an "alarmin", IL-33 is constitutively secreted into the extracellular space and impairs immune homeostasis, leading to the development of inflammation. Its role depends, on the degree

of damage, the presence of pro-inflammatory cytokines, and the stage and localization of the inflammatory process. IL-33 presents an ability to bind DNA; as a result, it can act as a transcription factor. Additionally, IL-33 is also a conventional cytokine, which binds the ST2 receptor on the cell surface [26,29–33]. Bessa et al. [34] examined the influence of compartmentalization of IL-33 on the inflammation development. The researchers used a mouse model with the IL-33 mutation, which lost nucleus location, and noted that mice with the mutation presented a higher expression of IL-33 and developed a multisystem inflammatory response including, e.g., intestine inflammation. At the same time, no inflammation was noted in the mice without a receptor for IL-33 [34]. Physiologically, IL-33 secretion from the cell occurs as a result of cell damage or apoptosis. IL-33 binds the ST2 receptor present on the surface of Th2, ILC2, Treg, and T CD8+ cells. Activation of IL-33/ST2 axis stimulates the secretion of Th2 cytokines, which enhance the activation of Th2 and ILC2. Via TGF-β, IL-33 stimulates the polarization of naïve lymphocytes into Treg cells and also coordinates the functions of Treg and ILC2 during tissue repair. In UC, the concentration of IL-33 and the soluble ST2 receptor in serum is increased compared to healthy individuals and patients with Crohn's disease, and additionally correlates with disease activity, which indicates the contribution of the IL-33/ST2 axis in the initiation and/or maintenance of the inflammation [32,33].

2.5. Th9 Lymphocytes

Other T cells, including Th9, Th17, Th22 and Treg, also play an important role in the pathogenesis of UC. Shohan et al. [10] presented that, in the intestinal biopsies collected from UC patients, a number of Th9 cells was increased compared to the healthy control, which may indicate a crucial role of these cells in UC pathogenesis [10]. Th9 cells appear as a result of polarization of naïve T cells in the presence of IL-4 and TGF-β, thereby acquiring the ability to secrete IL-9. Expression of IL-9 is regulated by various cytokines and transcription factors, such as STAT-6 and GATA-3 (characteristic for Th2 cells), as well as regulatory interferon factor 4 (IRF4) and purine-rich box-1 (PU.1) associated with Th9 cells. In particular, the expression of IL-9 depends on the last of the mentioned factors, since Th9 cells are the main source of IL-9 [25]. IL-9 is involved in the elimination of parasites by activating mast cells and eosinophils. In addition, this interleukin improves mucus synthesis and neutrophil infiltration in an allergic reaction [9]. Moreover, IL-9 also influences the expression of proteins forming the tight junction between the cells, increasing the permeability of the epithelium [10].

In the intestinal mucosa of patients with UC, the expression of mRNA encoding IL-9 was increased compared to the intestinal mucosa of healthy individuals; additionally, mRNA expression correlated with disease activity expressed in the Mayo scale. In order to determine the origin of IL-9, the researchers were looking for cells with PU. 1 expression and discovered that, in UC, the quantity of T CD4+ cells with PU. 1 expression was increased compared to the control group. These results may indicate that Th9 cells play a significant role in the UC's pathogenesis. In the following research, conducted on the mouse model with chronic colitis induced by adoptive T cell transfer, mice with IL-9 deficiency developed less intensified inflammation compared to wild-type mice. Moreover, animals with IL-9 deficiency presented a decreased expression of claudin-2—the protein regulating tight junctions—compared to the wild-type mice, and the rectal administration of recombinant IL-9 increased the expression of claudin-2 in both models of mouse. Thus, IL-9 increases the expression of claudin-2, which, as a selectively permeable pore-forming protein, is one of the factors responsible for the impairment of the intestinal barrier integrity, and promotes the activation of the immune system and increases the progression of colitis. Other factors contributing to intestinal barrier impairment and development of inflammation are the relocation and impaired expression of other proteins, creating tight junctions between epithelial cells (for example, occludin), as well as intensified apoptosis of epithelial cells [25,28,35].

2.6. Th17 Lymphocytes

CD4+ T cells in the presence of IL-6, TGF-β, IL-21 and IL-23 evolve into Th17 cells. IL-6 secreted by macrophages and DCs together with TGF-β mediate the activation of RORγt and signal transducer and activators of transcription 3 (STAT3), which are key transcription factors in the polarization of naive T cells to Th17 cells. IL-21 with features similar to IL-6 enhances the expression of the IL-23 receptor, which, by binding to IL-23, induces the polarization of the naive T cells to Th17 cells (by activating the RORγt-dependent pathway), which also affects cells maturation, phenotype and proliferation. Patients with IBD showed increased expression of IL-23 and interleukins belonging to the IL-17 family, which is related to the increased number of Th17 cells [5,20]. Ivanov et al. [36] noted that the induction of Th17 cells may be caused by the adherence of intestinal bacteria (such as segmented filamentous bacterium—SFB) to intestinal epithelial cells. The resulting activation of dendritic cells may lead to secretion of IL-1β and IL-6 in the intestine. The increased level of IL-23, IL-1β and IL-6 causes a polarization of naïve T cells to Th17 cells [36,37]. Th17 cells secrete cytokines such as IL-21, IL-22, IL-12, and TNF-α, as well as IL-17A and IL-17F belonging to the IL-17 family. IL-17A stimulates monocytes, epithelial and endothelial cells to secrete chemokines (CXCL1, CXCL2, CXCL5, CXCL8) that attract lymphocytes and neutrophils to the inflamed tissue. Moreover, IL-17A stimulates the synthesis of pro-inflammatory IL-1β and TNF-α [5,20]. However, Lee et al. [13] noted that the early and IL-23 independent synthesis of IL-17A plays a protective role as it affects the localization of occludin in tight junctions in a mouse model of experimental colitis induced by dextran sodium sulfate. Occludin is a component of a multiprotein complex creating tight junctions between intestinal epithelial cells; therefore, the lack of this protein or its internalization increases the permeability of the intestinal barrier [12,38]. In addition to IL-17, IL-22 also exerts an activity supporting the intestinal barrier function by inducing the synthesis of mucus and antimicrobial peptides [5,21]. Th17 cells play a dual role in human body; on the one hand they secrete pro-inflammatory cytokines (IL-17, TNF-α), and on the other hand, cytokines that protect the intestinal epithelium (IL-10, IL-22). As a result of the excessive activation, Th17 cells may become the pathogenic cells, contributing to chronic inflammation and the development of UC [5,12,20].

2.7. Th22 Lymphocytes

Despite the similar functions and phenotype of Th17 cells, Th22 cells show lower expression of RORγt, and polarization from naive T cells to Th22 cells occurs as a result of activation of the aryl hydrocarbon receptor (AhR) by IL-6 and TNF-α [5,39]. Leung et al. [39] noticed that, in UC, the percentage of Th22 cells (CD4+, IL-17−, IL-22+) is reduced, while the percentage of Th17 cells (CD4+, IL-17+, IL-22−) increases in mononuclear cells of the lamina propria of the intestinal mucosa taken during a biopsy. These data indicate a disturbed Th17/Th22 balance in patients with UC. Th22 cells are involved in antiviral, antibacterial and antifungal defense, as well as in the tissue repair within the skin and gastrointestinal tract. These lymphocytes mainly secrete cytokines that protect the intestinal barrier, i.e., IL-10 with immunosuppressive properties, and IL-22, which stimulates epithelial cell proliferation and intestinal mucus synthesis. The analysis [39] performed on the experimental murine models additionally showed that TGF-β is able to inhibit the synthesis of IL-22 via the transcription factor c-Maf. This conclusion was further confirmed in cultures of intestinal lamina propria mononuclear cells taken from healthy individuals, which had been exposed to increasing levels of TGF-β in subsequent cultures. The cultures also contained IL-6 and IL-23, which are necessary for lymphocyte polarization from naive T cells to Th17 and Th22, respectively. High expression of TGF-β suppressed CD4+, IL-17−, and IL-22+ cells, and simultaneously increased the number of CD4+, CD25+, and FoxP3+ Treg cells. These results indicate the suppressive role of TGF-β on Th22 polarization, as well as the pleiotropic impact of this cytokine on lymphocyte polarization in different cytokine environments [5,39,40].

2.8. Treg Lymphocytes

The lymphocytes also expressing CD4+ particles are Treg cells, which provide CD4+—expressed lymphocytes that ensure immune tolerance towards intestine microbiota, as well as preventing the development of autoimmune processes since they secrete anti-inflammatory cytokines such as IL-10, IL-35, and TGF-β. The lack of one of these cytokines, IL-10, secreted by the Treg cells, has been shown to lead to the development of spontaneous colitis in a mouse model. Moreover, in the studies by Woźniak-Stolarska [41], an increased expression of IL-10 was observed in patients with UC. Other researchers have additionally observed that increased expression of IL-10 mRNA correlates with disease activity. The anti-inflammatory effect of IL-10 is associated with inhibition of NF-κB, tissue factor, tissue metalloproteinases, and cyclooxygenase 2 [5,11,42]. TGF-β, similarly to IL-10, fulfills the immunosuppressive role. Research conducted on the IL-6 knock-out mouse model presented an elevated level of Treg cells and decreased level of Th17 cells in these mice. TGF-β in the absence of IL-6 suppresses Th17 polarization from naïve T cells and thus, via RORγt, stimulates Treg polarization. In addition, Treg cells may suppress polarization of Th17 cells through IL-10 and IL-35, as well suppressing secretion of cytokines activating Th17 differentiation (IL-6, IL-23) [12]. Treg cells through TGF-β stimulate tissue repair and relieve inflammation by suppressing the expression of IL-33 receptor [13]. Patients with UC, compared to healthy individuals, present an increased number of Treg cells; however, these cells are more likely to undergo apoptosis in inflamed tissue and show a 60% reduced ability to suppress proliferation of other T cells [5].

2.9. B Lymphocytes/Plasmocytes

In UC pathogenesis, B lymphocytes play a crucial role beside T cells. B cells are responsible for the synthesis of antibodies, antigen presentation to T cells and adaptation of the inflammatory response through secretion of IL-2, IL-4, IFN-γ, TGF-β and GM-CSF. Regarding the function of the cell, B cells may be divided into effector cells—secreting antibodies and cytokines—and regulatory cells (Breg), secreting IL-10. Activation of B cells in the intestines takes place in mesenteric lymph nodes and lymph nodules, followed by the migration from nodes and nodules to the intestinal lamina propria and differentiation into plasmocytes, which is associated with the disappearance of the CD19 antigen [43,44].

B cell surface markers have not been clearly identified; however, it is known that CD5 antigen is located on the surface of regulatory lymphocytes. The studies performed showed that Breg cells transformed to plasmocytes can highly express CD24 and CD38 ($CD24^{high}$ $CD38^{high}$). Moreover, the expression of CD95 antigen on the surface of Breg indicates functional exhaustion of B cells, which can be followed by a loss of its functions. Wang et al. [45] used flow cytometry to distinguish the expression of various B-cell surface markers in the peripheral blood of IBD patients. Patients with UC presented a decreased expression of cells with $CD24^{high}$ $CD38^{high}$ and CD5+ phenotypes, compared to healthy individuals. In the course of UC, a reduction of Breg cell population is observed, which leads to a decrease in the concentration of IL-10 in the peripheral blood. Furthermore, the percentage of $CD24^{high}$ $CD38^{high}$ cells negatively correlates with disease activity based on the Mayo scale. The highest percentage of CD95+ cells was found among CD5+ Breg cells in UC patients, compared to healthy subjects, and this percentage also positively correlated with the Mayo scale. The ability of B cells isolated from UC patients to secrete IL-10 as a result of LPS and CD257 stimulation was evaluated, and it was found that the majority of IL-10 secreting cells were those with $CD24^{high}$ $CD38^{high}$ CD5+. However, the reduction in the population of these cells resulted in a decrease in IL-10 levels in UC patients compared with healthy individuals. Thus, patients with UC may present a decreased quantity of B cells, which perform regulatory functions by secreting IL-10 with immunosuppressive properties. At the same time, Breg cells show a reduced functional activity, as evidenced by high expression of the CD95+ antigen. These findings might suggest that the pathogenesis of UC is connected with paucity of regulatory B cells and their decreased immunoregulatory

properties. At the same time, these results may provide a new potential approach to UC treatment [45].

Plasmocytes are the terminal stage of B cell differentiation and are localized mainly in bone marrow and lamina propria. IgA-secreting cells represent the majority of the intestinal plasmocytes, while IgG-secreting cells are related to inflammation. In the UC, the number of plasmocytes is elevated in the intestinal tissue and the balance between these two types of cells is changed, which indicates a significant role of these cells in the pathogenesis of UC. Research conducted by Uo et al. [46] confirms these reports, as the immunohistochemistry examination of UC's mucosal presented a significant influx of plasmocytes to the intestinal mucosa. Moreover, the majority of plasmocytes isolated from the non-IBD controls secrete IgA, while in the UC, plasmocytes secrete mostly IgG [41]. Under physiological conditions, locally secreted antibodies regulate the composition of the microbiota composition and maintain the intestinal barrier homeostasis. In UC, the synthesis of antibodies against one's own microbiota or tissues such as antibodies to flagella or perinuclear antibodies to neutrophils, is frequently observed, and is detected in 2/3 of UC patients. Castro-Dopico et al. [47] assessed the degree of binding of intestinal microbiota by IgG antibodies in the feces. It has been shown that, in UC, the opsonization of gut bacteria by antibodies is increased compared to healthy individuals. Eighty percent of the bacteria isolated from the feces of UC patients were opsonized by antibodies, while in healthy controls less than 10% of the bacteria were found to be opsonized [44,47,48].

As mentioned above, the IgG is mainly secreted in the inflammation and is the main immunoglobulin present in the intestinal tissue in UC. Class G immunoglobulins show high affinity to antigens, the ability to activate the complement system by binding C1q, and also are responsible for cellular memory. By binding to FcγR, IgG eliminates microorganisms, influences migration and maturation of immune cells, and affects the synthesis of inflammatory mediators. FcγR are glycoproteins expressed on the surface of immune cells and can act as activators (FcγRI, FcγRIIA, FcγRIIIA, FcγRIIIB) or inhibitors (FcγRIIB) of the immune response. FcγRs bind mainly to IgG, but can also bind serum amyloid P or C-reactive protein (CRP). In particular, the relationship between CRP and FcγRIIA causes the internalization of autoantigens, microorganisms and damaged cells by neutrophils and monocytes. In UC, the neutrophils state the main component of the intestinal infiltration, which can be associated with the increased expression of genes coding IL-1β, CXCL1 and CXCL8, responsible for recruitment of neutrophils. Expression of IL-1β and CXCL8 correlates most with FcγRIIA expression, which may indicate that IgG antibodies against gut microbiota contribute to the development of intestinal inflammation through FcγRIIA-dependent induction of IL-1β and CXCL8. Among patients with IBD, the cases of incorrect post-translational modification were noted, including defucosylation, enhanced affinity of IgG to FcγRIIA or agalactosylation, and decreased affinity of IgG to FCγRIIB [47,48].

The role of innate cells in the development of ulcerative colitis has been summarized in Figure 3.

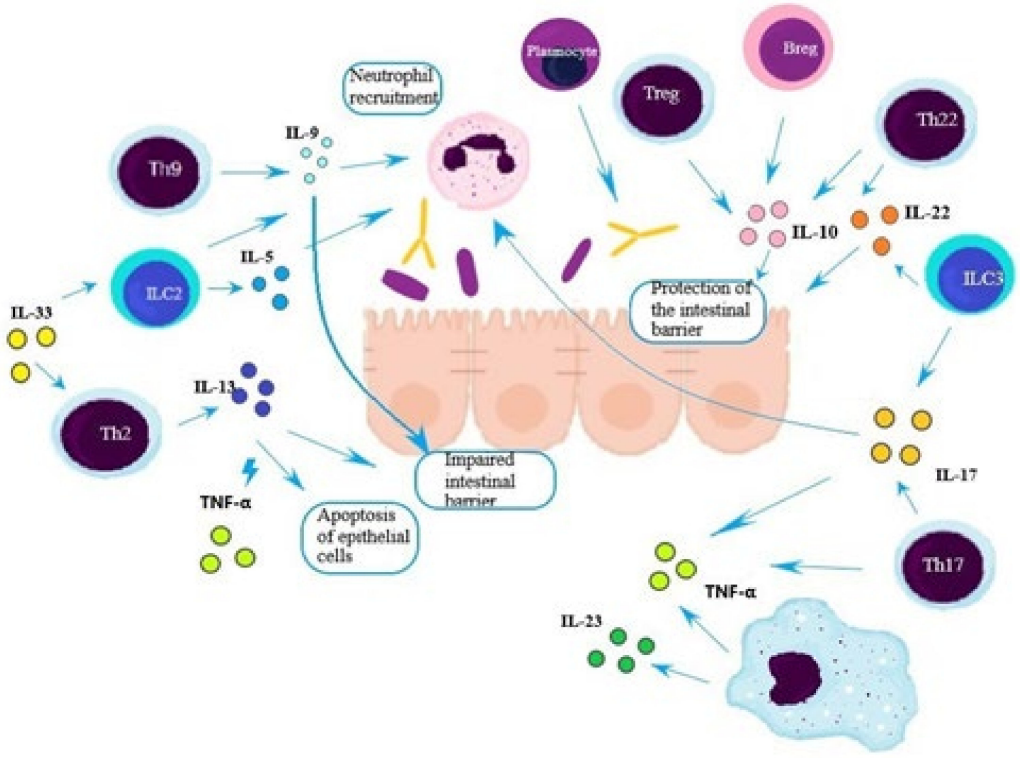

Figure 3. The role of immune cells in the development of ulcerative colitis (UC). Among patients with UC, an increased number of Th2, Th9, and Th17 lymphocytes, as well as ILC2 and ILC3, is observed. The cytokines secreted by these cells contribute to the impairment of the intestinal barrier function through increased expression of claudin-2 and increased apoptosis of epithelial cells. Additionally, cytokines stimulate the migration and degranulation of neutrophils and further activation of immune cells. Activated plasma cells secrete antibodies, including those against gut microbiota inducing chemokines and IL-1β [5,27,33,45]. At the same time, the number and/or activity of Breg, Treg, and Th22 cells is decreased, which leads to reduced protection of the intestinal barrier [5,11,42,45].

3. Conclusions

Ulcerative colitis is associated with chronic inflammation, which possibly results from the abnormal immune response towards intestine microbiota and/or food antigens. The exact mechanisms contributing to the development of UC remain unclear, but significant progress has been made recently, including a better understanding of the innate functions of lymphoid cells and their relationship to changes in disease development. Moreover, Th9 cells were identified among lymphocytes, which by secreting IL-9 influence the integrity of the intestinal barrier and contribute to the degranulation of neutrophils. Among patients with UC, increased activity was noted, as well as the number of pro-inflammatory Th2, Th17, and Th9, with simultaneous inhibition of the activity and number of anti-inflammatory cells—Th22, Treg, and Breg. The activity of immune cells is affected by the cytokines. Cytokines not only contribute to the development and maintenance of inflammation, but also influence the integrity of the intestinal barrier. IL-10 and IL-22 protect the intestinal barrier, while IL-13 and IL-9 disrupt the intestinal barrier by increasing the expression of claudin-2 in tight junctions between epithelial cells and enhancing the apoptosis of the intestinal barrier's epithelial cells. Interestingly, the functions of the cytokines are depended on the cytokine environment; for example, TNF-α enhances the effect of IL-13. The above relationship may be useful in the treatment of UC, because the therapeutic effect of eliminating the influence of one cytokine on the disease process may be canceled out by another type of cytokine.

Author Contributions: A.K.—conceptualization, writing—original draft preparation; P.O.—concep tualization, review and editing; K.K.-V.—conceptualization, review and editing, supervision. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Porter, R.J.; Kalla, R.; Ho, G.-T. Ulcerative colitis: Recent advances in the understanding of disease pathogenesis. *F100 Res.* **2020**, *9*, 294. [CrossRef] [PubMed]
2. Teigen, L.M.; Geng, Z.; Sadowsky, M.J.; Vaughn, B.P.; Hamilton, M.J.; Khortus, A. Dietary factors in sulfur metabolism and pathogenesis of ulcerative colitis. *Nutrients* **2019**, *11*, 931. [CrossRef] [PubMed]
3. Turner, J. Intestinal mucosal barrier function in health and disease. *Nat. Rev. Immunol.* **2009**, *9*, 799–809. [CrossRef]
4. Litvak, Y.; Byndloss, M.X.; Tsolis, R.; Bäumler, A.J. Dysbiotic *Proteobacteria* expansion: A microbial signature of epithelial dysfunction. *Curr. Opin. Microbiol.* **2017**, *39*, 1–6. [CrossRef]
5. Kmieć, Z.; Cyman, M.; Ślebioda, T.J. Cells of the innate and adaptive immunity and their interactions in inflammatory bowel disease. *Adv. Med. Sci.* **2017**, *62*, 1–16. [CrossRef]
6. McCauley, H.A.; Guasch, G. Three cheers for the goblet cell: Maintaing homeostasis in mucosal epithelia. *Trends Mol. Med.* **2015**, *21*, 492–503. [CrossRef] [PubMed]
7. Kaur, A.; Goggolidou, P. Ulcerative colitis: Understanding its cellular pathology could provide insight into novel therapies. *J. Infalmm.* **2020**, *17*, 15. [CrossRef]
8. Wejman, J.; Bartnik, W. *Atlas Kliniczno-Patologiczny Nieswoistych Chorób Zapalnych Jelit*; Wydawnictwa Medyczne Termedia: Warsaw, Poland, 2011; pp. 53–54, 65–73.
9. Shohan, M.; Elahi, S.; Shirzad, H.; Rafieian-Kopaei, M.; Bagheri, N.; Soltani, E. Th9 cells: Probable players in ulcerative colitis pathogenesis. *Int. Rev. Immunol.* **2018**, *37*, 192–205. [CrossRef]
10. Shohan, M.; Sabzevary-Ghahfarokhi, M.; Bagheri, N.; Shirzad, H.; Rahimian, G.; Soltani, A.; Ghatreh-Samani, M.; Deris, F.; Tahmasbi, K.; Shahverdi, E.; et al. Intensified Th9 response is associated with the immunopathogenesis of active ulcerative colitis. *Immunol. Investig.* **2018**, *47*, 700–711. [CrossRef]
11. Polińska, B.; Matowicka-Karna, J.; Kemona, H. Cytokiny w nieswoistych zapalnych chorobach jelit. *Postepy Hig. Med. Dosw.* **2009**, *63*, 389–394.
12. Szulc-Dąbrowska, L.; Gieryńska, M.; Depczyńska, D.; Schollenberger, A.; Toka, F.N. Limfocyty Th17 w zakażeniach bakteryjnych. *Postepy Hig. Med. Dosw.* **2015**, *69*, 398–417. [CrossRef]
13. Lee, S.H.; Kwon, J.E.; Cho, M.-L. Immunological pathogenesis of inflammatory bowel disease. *Intest. Res.* **2018**, *16*, 26–42. [CrossRef]
14. Geremia, A.; Biancheri, P.; Allan, P.; Corazza, G.R.; Di Sabatino, A. Innate and adaptive immunity in inflammatory bowel disease. *Autoimmun. Rev.* **2013**, *13*, 3–10. [CrossRef]
15. Miller, A.M. Role of IL-33 in inflammation and disease. *J. Inflamm.* **2011**, *8*, 22. [CrossRef] [PubMed]
16. Nemeth, Z.H.; Bogdanovski, D.A.; Barratt-Stopper, P.; Paglinco, S.R.; Antonioli, L.; Rolandelli, R.H. Crohn's disease and ulcerative colitis show unique cytokine profiles. *Cureus* **2017**, *9*, 1177. [CrossRef] [PubMed]
17. Dinallo, V.; Marafini, I.; Di Fusco, D.; Laudisi, F.; Franze, E.; Di Grazia, A.; Figliuzzi, M.M.; Caprioli, F.; Stolfi, C.; Monteleone, I.; et al. Neutrophil extracellular traps sustain inflammatory signals in ulcerative colitis. *J. Crohn's Colitis* **2019**, *13*, 772–784. [CrossRef]
18. Matoszka, N.; Działo, J.; Tokarz-Deptuła, B.; Deptuła, W. NET i NEToza—Nowe zjawisko w immunologii. *Postepy Hig. Med. Dosw.* **2012**, *66*, 437–445. [CrossRef]
19. Forkel, M.; van Tol, S.; Höög, C.; Michaëlsson, J.; Almer, S.; Mjösberg, J. Distinct alterations in the composition of mucosal innate lymphoid cells in newly diagnosed and established Crohn's disease and ulcerative colitis. *J. Crohns Colitis* **2019**, *13*, 67–78. [CrossRef]
20. Ordas, I.; Eckmann, L.; Talamini, M.; Baumgart, D.; Sandborn, W.J. Ulcerative Colitis. *Lancet* **2012**, *380*, 1606–1619. [CrossRef]
21. Forkel, M.; Mjösberg, J. Dysregulation of group 3 innate lymphoid cells in the pathogenesis of inflammatory bowel disease. *Curr. Allergy Asthma Rep.* **2016**, *16*, 73. [CrossRef] [PubMed]
22. Hart, A.L.; Al-Hassi, H.O.; Rigby, R.J.; Bell, S.J.; Emmanuel, A.V.; Knight, S.C.; Kamm, M.A.; Stagg, A.J. Characteristic of Intestinal Dendritic Cells in Inflammatory Bowel Diseases. *Gastroenterology* **2005**, *129*, 50–65. [CrossRef]
23. Kappeler, A.; Mueller, C. The role of activated cytotoxic T cells in inflammatory bowel disease. *Histol. Histopathol.* **2000**, *15*, 167–172. [PubMed]
24. Rabe, H.; Malmquist, M.; Barkman, C.; Östman, S.; Gjertsson, I.; Saalman, R.; Wold, A.E. Distinct patterns of naive, activated and memory T and B cells in blood of patients with ulcerative colitis or Crohn's disease. *Clin. Expl. Immunol.* **2019**, *197*, 111–129. [CrossRef] [PubMed]

25. Gerlach, K.; Hwang, Y.; Nikolaev, A.; Atreya, R.; Dornhoff, H.; Steiner, S.; Lehr, H.-A.; Wirtz, S.; Vieth, M.; Waisman, A.; et al. Th9 cells that express the trancription factor PU.1 drive T cel-mediated colitis via IL-9 receptor signaling in intestinal epithelial cells. *Nat. Immunol.* **2014**, *15*, 676–686. [CrossRef] [PubMed]
26. Bamias, G.; Cominelli, F. Role of Th2 immunity in intestinal inflammation. *Curr. Opin. Gastroenterol.* **2015**, *31*, 471–476. [CrossRef]
27. Heller, F.; Fromm, A.; Gitter, A.H.; Mankertz, J.; Schulzke, J.-D. Epithelial apoptosis is a prominent feature of the epithelial barier disturbance in intedtinal inflammation: Effect of pro-inflammatory interlekin-13 on the epithelial cell function. *Mucosal. Immunol.* **2008**, *1*, S58–S61. [CrossRef]
28. Raju, P.; Shashikanth, N.; Tsai, P.-Y.; Pongkorpsakol, P.; Chanez-Paredes, S.; Steinhagen, P.R.; Kuo, W.-T.; Singh, G.; Tsukita, S.; Turner, J.R. Inactivation of paracellular cation-selective claudin-2 channels attenuates immune-mediated experimental colitis in mice. *J. Clin. Investig.* **2020**, *130*, 5197–5208. [CrossRef]
29. Seidelin, J.B.; Coskun, M.; Kvist, P.H.; Holm, T.L.; Holgersen, K.; Nielsen, O.H. IL-33 promotes GATA-3 polarization of gut-derived T cells in experimental and ulcerative colitis. *J. Gastroenterol.* **2015**, *50*, 180–190. [CrossRef]
30. Travers, J.; Rochman, M.; Miracle, C.E.; Habel, J.E.; Brusilovsky, M.; Caldwell, J.M.; Rymer, J.K.; Rothenberg, M.E. Chromatin regulates IL-33 release and extracellular cytokine activity. *Nat. Commun.* **2018**, *9*, 3244. [CrossRef]
31. He, Z.; Chen, L.; Furtado, G.C.; Lira, S.A. Interleukin 33 regulates gene expression in intestinal epithelial independently of its nuclear localization. *Cytokine* **2018**, *111*, 146–153. [CrossRef]
32. Akimoto, M.; Takenaga, K. Role of the IL-33/ST2L axis in colorectal cancer progression. *Cell Immunol.* **2019**, *343*, 103740. [CrossRef] [PubMed]
33. Hodzic, Z.; Schill, E.M.; Bolock, A.M.; Good, M. IL-33 and the intestine: The good, the bad and the inflammatory. *Cytokine* **2017**, *100*, 1–10. [CrossRef]
34. Bessa, J.; Meyer, C.A.; de Vera Mudry, M.C.; Schliht, S.; Smith, S.H.; Iglesias, A.; Cote-Sierra, J. Altered subcellular localization of IL-33 leads to non-resolving lethal inflammation. *J. Autoimmun.* **2014**, *55*, 33–41. [CrossRef] [PubMed]
35. Luettig, J.; Rosenthal, R.; Barmeyer, C.; Schulzke, J.D. Claudin-2 as a mediator of leaky gut barier during intestinal inflammation. *Tissue Barriers* **2015**, *3*, e977176. [CrossRef] [PubMed]
36. Ivanov, I.I.; Atarashi, K.; Manel, N.; Brodie, E.L.; Shima, T.; Karaoz, U.; Wei, D.; Goldfarb, K.; Santee, C.A.; Lynch, S.V.; et al. Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell* **2009**, *139*, 485–498. [CrossRef] [PubMed]
37. Zhao, J.; Lu, Q.; Liu, Y.; Shi, Z.; Hu, L.; Zheng, Z.; Tu, Y.; Xiao, Z.; Xu, Q. Th17 zells in Inflammatory Bowel Disease: Cytokines, plasticity, therapies. *J. Immunol. Res.* **2021**, *2021*, 8816041. [CrossRef] [PubMed]
38. Lee, J.S.; Tato, C.M.; Joyce-Shaikh, B.; Gulan, F.; Cayatte, C.; Chen, Y.; Blumenschein, W.M.; Judo, M.; Chen, K.; Ayanoglu, G.; et al. IL-23 independent IL-17 production regulates intestinal epithelial permeability. *Immunity* **2015**, *43*, 727–738. [CrossRef]
39. Leung, J.M.; Davenport, M.; Wolff, M.J.; Wiens, K.E.; Abidi, W.M.; Poles, M.A.; Cho, I.; Ullman, T.; Mayer, L.; Loke, P. IL-22-producing CD4+ cells are depleted in actively inflamed colitis tissue. *Mucosal. Immunol.* **2014**, *7*, 124–133. [CrossRef] [PubMed]
40. Neurath, M.F. Targeting immune cell circuits and trafficking in inflammatory bowel disease. *Nat. Immunol.* **2019**, *20*, 970–979. [CrossRef] [PubMed]
41. Woźniak-Stolarska, B.; Sajewicz, Z.; Błachut, K. Serum concentration of interleukin-10 in inflammatory bowel disease. *Adv. Clin. Exp. Med.* **2003**, *12*, 717–721.
42. Asselin, C.; Gendron, F.-P. Shuttling of information between the mucosal and luminal environment drives intestinal homeostasis. *FEBS Lett.* **2014**, *588*, 4148–4157. [CrossRef]
43. Maśliński, W.; Kontny, E. *Podstawy Immunologii dla Reumatologów*; Wydawnictwo Narodowego Instytutu Geriatrii, Reumatologii i Rehabilitacji im: Warsaw, Poland, 2015; pp. 77–91.
44. Mizoguchi, A.; Bhan, A.K. Immunobiology of B cells in inflammatory bowel disease. In *Crohn's Disease and Ulcerative Colitis*; Baumgart, D., Ed.; Springer International Publishing: Cham, Switzerland, 2017. [CrossRef]
45. Wang, X.; Zhu, Y.; Zhang, M.; Wang, H.; Jiang, Y.; Gao, P. Ulcerative colitis is chracterized by a decrease in regulatory B cells. *J. Crohns Colitis.* **2016**, *10*, 1212–1223. [CrossRef]
46. Uo, M.; Hisamatsu, T.; Miyoshi, J.; Kaito, D.; Yoneno, K.; Kitazume, M.T.; Mori, M.; Sugita, A.; Koganei, K.; Matsuoka, K.; et al. Mucosal CXCR4+ IgG plasma cells contribute to pathogenesis of human ulcerative colitis through FcγR-mediated CD14 macrophage activation. *Gut* **2013**, *62*, 1734–1744. [CrossRef] [PubMed]
47. Castro-Dopico, T.; Dennison, T.W.; Ferdinand, J.R.; Clare, S.; Parkes, M.; Clatworthy, M.R. Anti-comensal IgG drives intestinal inflammation and type 17 immunity in ulcerative colitis. *Immunity* **2019**, *50*, 1099–1114. [CrossRef] [PubMed]
48. Castro-Dopico, T.; Clatworthy, M.R. IgG and Fcγ receptors in intestinal immunity and inflammation. *Front. Immunol.* **2019**, *10*, 805. [CrossRef] [PubMed]

Journal of
Clinical Medicine

MDPI

Article

The Assessment of IL-21 and IL-22 at the mRNA Level in Tumor Tissue and Protein Concentration in Serum and Peritoneal Fluid in Patients with Ovarian Cancer

Aleksandra Mielczarek-Palacz [1], Celina Kruszniewska-Rajs [2], Marta Smycz-Kubańska [1], Jarosław Strzelczyk [1], Wojciech Szanecki [3], Andrzej Witek [3] and Joanna Magdalena Gola [2,*]

[1] Department of Immunology and Serology, Faculty of Pharmaceutical Sciences in Sosnowiec, Medical University of Silesia, 40-055 Katowice, Poland; apalacz@sum.edu.pl (A.M.-P.); mkubanska@sum.edu.pl (M.S.-K.); jarekstrzelczyk@o2.pl (J.S.)
[2] Department of Molecular Biology, Faculty of Pharmaceutical Sciences in Sosnowiec, Medical University of Silesia, 40-055 Katowice, Poland; ckruszniewska@sum.edu.pl
[3] Department of Gynecology and Obstetrics, Faculty of Medical Sciences in Katowice, Medical University of Silesia, 40-055 Katowice, Poland; wszanecki@sum.edu.pl (W.S.); awitek@sum.edu.pl (A.W.)
* Correspondence: jgola@sum.edu.pl; Tel.: +48-32-364-12-34

Citation: Mielczarek-Palacz, A.; Kruszniewska-Rajs, C.; Smycz-Kubańska, M.; Strzelczyk, J.; Szanecki, W.; Witek, A.; Gola, J.M. The Assessment of IL-21 and IL-22 at the mRNA Level in Tumor Tissue and Protein Concentration in Serum and Peritoneal Fluid in Patients with Ovarian Cancer. *J. Clin. Med.* **2021**, *10*, 3058. https://doi.org/10.3390/jcm10143058

Academic Editors: Katarzyna Komosinska-Vassev and Paola Concolino

Received: 11 June 2021
Accepted: 8 July 2021
Published: 9 July 2021

Abstract: The aim of the analysis was for the first time to assess the expression of genes encoding IL-21 and IL-22 at the mRNA level in ovarian tumor specimens and the concentration of these parameters in serum and peritoneal fluid in patients with ovarian serous cancer. The levels of IL-21 and IL-22 transcripts were evaluated with the use of the real-time RT-qPCR. Enzyme-linked immunosorbent assay (ELISA) was used to determine the concentration of proteins. Quantitative analysis of IL-21 gene mRNA in the tumor tissue showed the highest activity in the G1 degree of histopathological differentiation and was higher in G1 compared to the control group. The concentration of IL-21 and IL-22 in the serum and in the peritoneal fluid of women with ovarian cancer varied depending on the degree of histopathological differentiation of the cancer and showed statistical variability compared to controls. The conducted studies have shown that the local and systemic changes in the immune system involving IL-21 and IL-22 indicate the participation of these parameters in the pathogenesis of ovarian cancer, and modulation in the IL-21/IL-22 system may prove useful in the development of new diagnostic and therapeutic strategies used in patients, which require further research.

Keywords: ovarian cancer; interleukin 21; interleukin 22

1. Introduction

Ovarian cancer is one of the gynecological cancers with the worst prognosis. Both the number of cases and the mortality caused by this disease in the world are constantly increasing [1,2]. In patients, the most important prognostic factor is the stage of clinical advancement, which determines the therapeutic strategy. Unfortunately, this cancer is detected in stage III or IV in more than 70% of women. It is associated with an unfavorable prognosis and a low five-year survival rate. It is caused by asymptomatic tumor growth, lack of characteristic clinical symptoms in the initial stage of the disease and diagnostic tests helpful in early diagnosis [3,4]. Currently conducted research indicates that the protein intercellular mediators—cytokines—play an important role in the formation and development of ovarian cancers. Cytokines interact with cells through characteristic surface receptors that transmit a signal to the interior of the cell. Altered expression of cytokines and their receptors in cancer cells affects the interaction in the tumor microenvironment, which may induce an anti-cancer response, promote tumor growth, participate in invasion and metastasis, and in immunosuppression [5,6].

Recent studies indicate that interleukin 21 (IL-21) and interleukin 22 (IL-22) play an important role in the pathogenesis of cancer and cancer therapy [7–12]. Winkler et al. showed, that Th17 cell sub-populations with expression of IL-21 and/or IL-22 infiltrate the tumor tissue of ovarian cancer [7]. Interleukin-21 is a cytokine secreted mainly by activated CD4 + T cells, including T helper 17 (Th17) and T follicular helper (Tfh) cells and NKT cells [8]. IL-21 is bound to the IL-21 receptor which contains the gamma chain [9]. It has an immunoregulatory function, has the ability to both promote and inhibit the immune response and induce an anti-cancer response, mainly by activating Tc lymphocytes, therefore, attempts are still being made to use it in cancer immunotherapy [10–12]. Studies have shown the effectiveness and safety of the therapy with the use of recombinant IL-21 in advanced melanoma, renal cell carcinoma, and non-Hodgkin's B cell lymphoma [13]. Subcutaneous administration of IL-21 augmented tumor regression and increased tumor infiltration by CD8 + T cells [11]. IL-21 can be also combined with other immunotherapies [14].

Interleukin-22 is a cytokine synthesized by lymphocyte T helper (Th) cells, including their Th17 and Th22 subpopulations as well as NKT lymphocytes, NK cells, and nonspecific lymphoid cells [15]. It is a pro-inflammatory cytokine, it induces the production of acute phase proteins and participates in the pathogenesis of many diseases, including cancer. Interestingly, IL-22 has also been shown to induce multiple cytoprotective mechanisms, participating in tissue regeneration following inflammatory lesions [16]. IL-22 is bound to the heterodimer receptor complex of IL–10R2 and IL-22R1 [17] and, by activating numerous signaling pathways, it influences such processes as cell survival, proliferation, migration, and angiogenesis [17]. These processes are crucial for the development of cancer. Protopsaltis et al. demonstrated that IL-22 directly stimulates angiogenesis through activation of the ERK and Stat3 pathways, and blockade of IL-22 inhibits tumor growth [18]. Interestingly, cancer cells release IL-1, which stimulates IL-22 production by memory T cells [17].

The analyses conducted so far on the evaluation of IL-21 and IL-22 in ovarian cancer have been interesting and induce further research. It has been shown that the determination of IL-21 with other cytokines, for example, IL-17a, may find clinical application as prognostic and therapeutic biomarkers [19]. Interleukin-22 has been shown to be an important factor in the ovarian cancer tumor microenvironment and may find application as a potential therapeutic target and/or biomarker [20]. Its prognostic value in other cancers has also been established, including pancreatic cancer [21] and hepatocellular carcinoma [22].

So far, no studies have been conducted to assess both the expression of IL-21 and IL-22 in tumor tissue, as well as the concentration of these cytokines in the serum and peritoneal fluid in women with ovarian cancer, and considering the fact that understanding the role of these parameters in the pathogenesis of ovarian cancer may be useful in developing new diagnostic and therapeutic regimens in patients with ovarian cancer in the future, the aim of the study was: assessment of IL-21 and IL-22 at the mRNA level in ovarian cancer tissue, analysis of the concentration of IL-21 and IL-22 in the serum and peritoneal fluid of patients with ovarian cancer, and determining whether there is a relationship between the concentration of IL-21 and IL-22 and the degree of histological differentiation of the cancer.

2. Materials and Methods

The study group included 26 women, aged 41 to 82 (mean age: 62.80 $\pm$ 11.21 years) with the diagnosed ovarian cancer with the Cystadenocarcinoma papillare serosum IIIC (7 had G1, 5 had G2, and 14 had G3 staging) Staging employed the criteria recommended by the International Federation of Gynecology and Obstetrics (FIGO). The diagnosis of tumors was done on the basis of clinical symptoms, results of gynecological and histopathological examination and laboratory tests. The women qualified to the studied group were clinically diagnosed with ovarian tumor confirmed with a histopathological examination. Other coexisting disorders of the reproductive organs and autoimmune diseases were excluded. None of the examined women used pharmacological treatment in the last three months. The research was conducted on women hospitalized at the Clinical Department

of Gynecology and Obstetrics of the University Clinical Center in Katowice and the Department of Gynecology and Obstetrics with the Department of Pathology of Pregnancy and Gynecological Oncology of the Provincial Specialist Hospital in Częstochowa. Serum, peritoneal fluid, and tumor tissue were the material examined in all women. In the study group, blood was taken from women after establishing clinical diagnosis, before surgery. Blood was taken in the morning from the cubital vein, to a clot tube, in order to obtain the serum. Thirty minutes after taking the blood, it was centrifuged at 1500× *g* for 15 min. The serum obtained in this manner was kept in small portions at a temperature of −80 °C until the tests. Tumor tissue intended for molecular assessment was collected during the planned surgery and frozen at −80 ° C until the tests were performed. Peritoneal fluid was collected during laparoscopy for bacteriological examination, and then centrifuged at 2000 rpm for 10 min at 4 °C, and the obtained supernatant was partitioned and frozen at −80 °C until the remaining determinations were made.

The control group consisted of six women aged between 40–77 (mean age: 61.83 ± 12.48 years) who have been diagnosed with a benign lesion (Cystadenoma serosum). The concentration of antigen CA125 did not exceed 35 U/m in these patients. In all the women, the blood serum and tissue samples were the research material.

Total RNA was extracted from tissue samples with the use of TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. Each tissue sample was placed in a tube containing TRIzol, and then homogenized using a Polytron® homogenizer (Kinematica AG, Malters, Switzerland). RNA extracts quantitative assessment was performed with the use of nanospectrophotometer MaestroNano MN-913 (MaestroGen Inc., Hsinchu City, Taiwan) and 2100 Bioanalyzer (Agilent Technologies, Inc. Santa Clara, CA, USA) (Figure S1, supplementary materials). The levels of IL-21 and IL-22 transcripts were evaluated with the use of the real-time RT-qPCR. The quantitative analysis was carried out using LightCycler® 480 System (Roche, Basel, Switzerland) and GoTaq® 1-Step RT-qPCR System (Promega, Madison, WI, USA), according to manufacturers' instructions. Amplification was performed using previously described oligonucleotide primers [23,24] and commercially available standards of β-actin cDNA (TaqMan™ DNA Template Reagents; Thermo Fisher Scientific, Waltham, MA, USA). All samples were tested in triplicate. The mRNA copy numbers of the gene examined were recalculated per 1 μg of the total RNA. Melting curve analysis and agarose gel electrophoresis were used to confirm the specificity of amplification and the absence of primer dimers (Figures S2–S4, supplementary materials). Enzyme-linked immunosorbent assay (ELISA) was used to determine the concentration of the studied parameters. The following kits were used for this purpose: IL-21 Human Interleukin-21 ELISA and Human Interleukin-22 (BioVendor–Laboratorni medicina a.s., Brno, Czech Republic). Interleukin-21 Human ELISA, Sandwich ELISA, Biotin-labelled antibody, Calibration Range 78–5000 pg/mL, limit of detection 20.0 pg/mL and Interleukin-22 Human ELISA, Sandwich ELISA, Biotin-labeled antibody, Calibration Range 31.3–2000 pg/mL, limit of detection 5.0 pg/mL.

All the women who participated in the study consented to conducting the research. The approval of the Ethics Committee of the Medical University of Silesia in Katowice was obtained.

The obtained results were statistically analyzed using the Statistica 13.3 software (StatSoft Polska Sp. z o.o., Cracow, Poland). The normality of the distribution of the studied variables was checked using the Shapiro–Wilk test. The median and quartile range were determined for the parameters tested, and the obtained results were compared using the Mann–Whitney test. Correlations were tested by Spearman's rank correlation test and presented as correlation coefficient (*r*).

3. Results

Quantitative assessment of IL21 mRNA in tissue samples showed the highest gene activity in the degree of differentiation G1 (Figure 1). The expression of IL21 gene was higher comparing to control group ($p = 0.002415$), G2 ($p = 0.033161$), and G3 ($p = 0.020397$).

No statistically significant difference was found between G2, G3, and a control group. Moreover, expression of IL21 mRNA did not differ between G2 and G3.

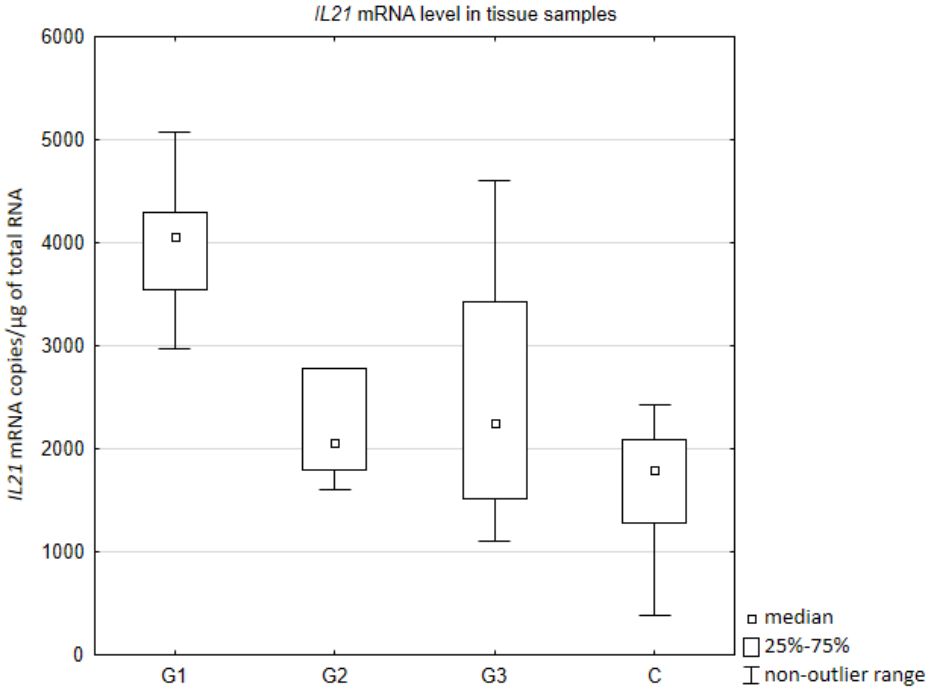

Figure 1. Number of copies of IL21 mRNA/µg of total RNA in tissue samples depending on the degree of ovarian cancer differentiation G1 (well differentiated), G2 (moderately differentiated), G3 (poorly differentiated) compared to the control group (C).

Quantitative assessment of IL22 mRNA in tissue samples did not show statistically significant difference comparing to a control group. Moreover, expression of IL22 mRNA did not differ between degrees of differentiation G1, G2, and G3. Median values of the number of copies of IL21 mRNA/µg of total RNA in tissue samples were as follows: G1: Me = 6660, G2: Me = 5065, G3: Me = 7820, C: Me = 8555.

Il-21/IL-22 mRNA ratio was the highest in the degree of differentiation G1 (Figure 2). In all cancer tissues, regardless of degree of differentiation, the value of Il-21/IL-22 mRNA ratio was higher comparing to a control group (respectively: G1 vs. C $p = 0.003405$; G2 vs. C $p = 0.008114$; G1 vs. C $p = 0.007473$). Moreover, statistically significant difference was found between G1 and G3 ($p = 0.032391$).

The concentration of Il-21 was determined in the serum and peritoneal fluid of women with ovarian cancer. As the obtained values did not correspond to the normal distribution, the results were presented in the form of the median and the lower and upper interquartile range (Q1 and Q3).

In the serum of women with ovarian cancer, Q1 and Q3 were respectively: 314.25 and 736.00 with a median of 408.68 pg/mL. A statistically significantly lower concentration of IL-21 in the serum of women with ovarian cancer was demonstrated compared to the concentration in the control group ($p < 0.001$), where Q1 and Q3 were respectively: 722.19 and 909.60 with a median of 793.59 pg/mL.

In the peritoneal fluid of women with ovarian cancer, Q1 and Q3 were: 113.36 and 356.13, respectively, with a median of 175.63 pg/mL. A statistically significant higher concentration of the examined parameter was found in the serum of women with ovarian cancer as compared to the concentration in the peritoneal fluid ($p < 0.0001$).

Then, the concentration of IL-21 in serum and the peritoneal fluid of women with ovarian cancer depending on the degree of histopathological differentiation of the cancer was analyzed. The analysis of the test results showed a statistical significance between G2 and G3 ($p < 0.001$) and between G1 and G3 ($p < 0.0001$) in the serum of the examined

women. In the peritoneal fluid of women with ovarian cancer, a statistically significant difference was found only between G2 and G3 ($p < 0.05$). The obtained results are presented in Figures 3 and 4.

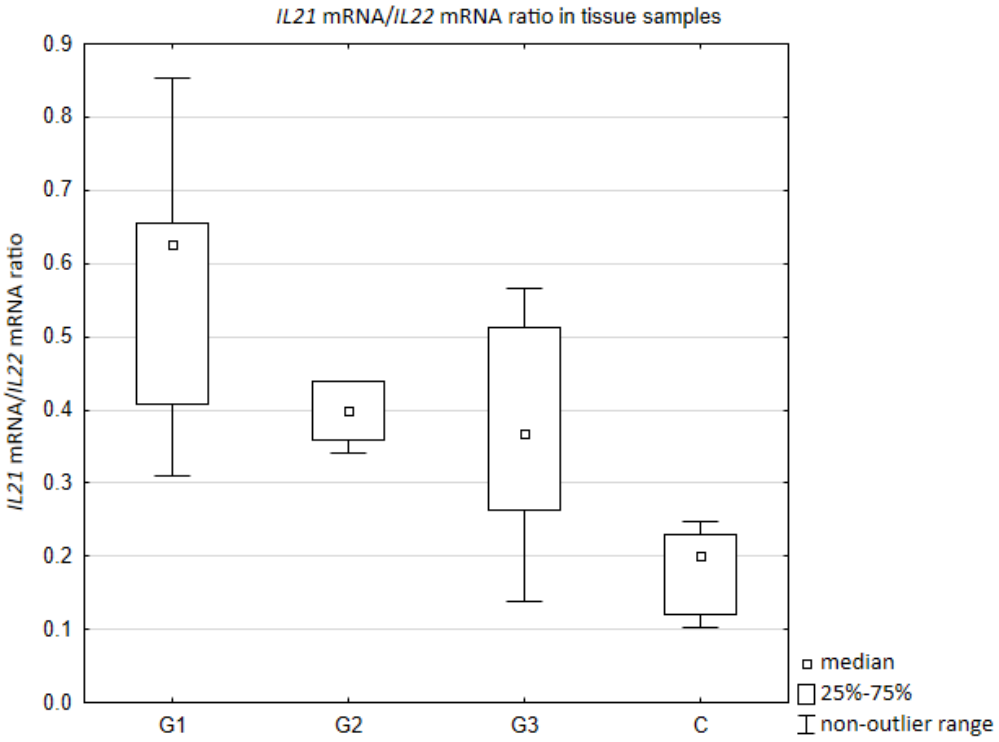

Figure 2. Il-21/IL-22 mRNA ratio in tissue samples depending on the degree of ovarian cancer differentiation G1 (well differentiated), G2 (moderately differentiated), G3 (poorly differentiated) compared to the control group (C). Ratio was calculated based on mRNA copies/µg of total RNA.

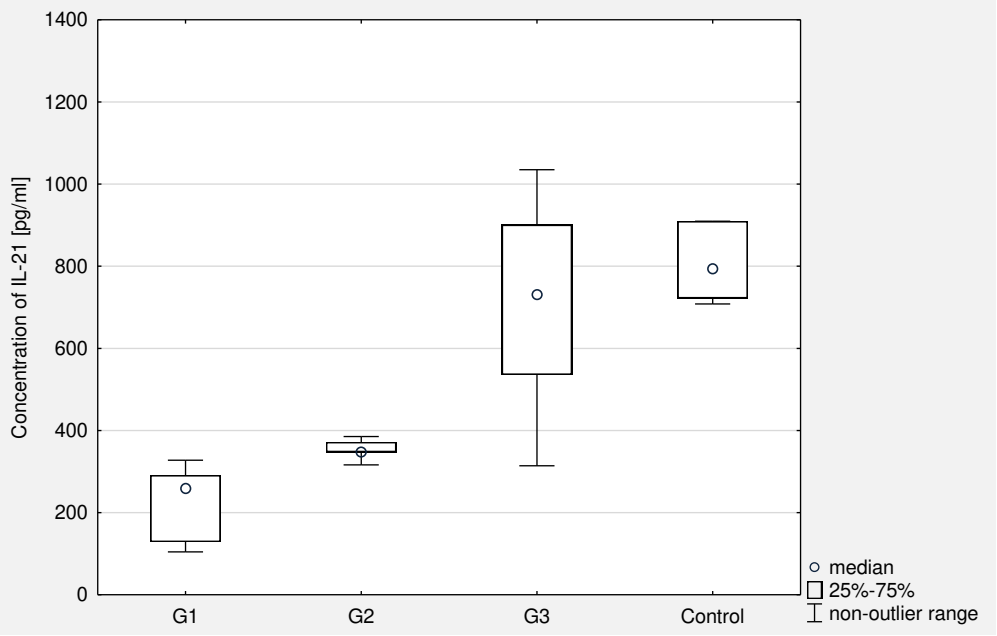

Figure 3. The concentration of IL-21 in the serum of women with ovarian cancer depending on the degree of differentiation G1 (well differentiated), G2 (moderately differentiated), G3 (poorly differentiated) compared to the control group.

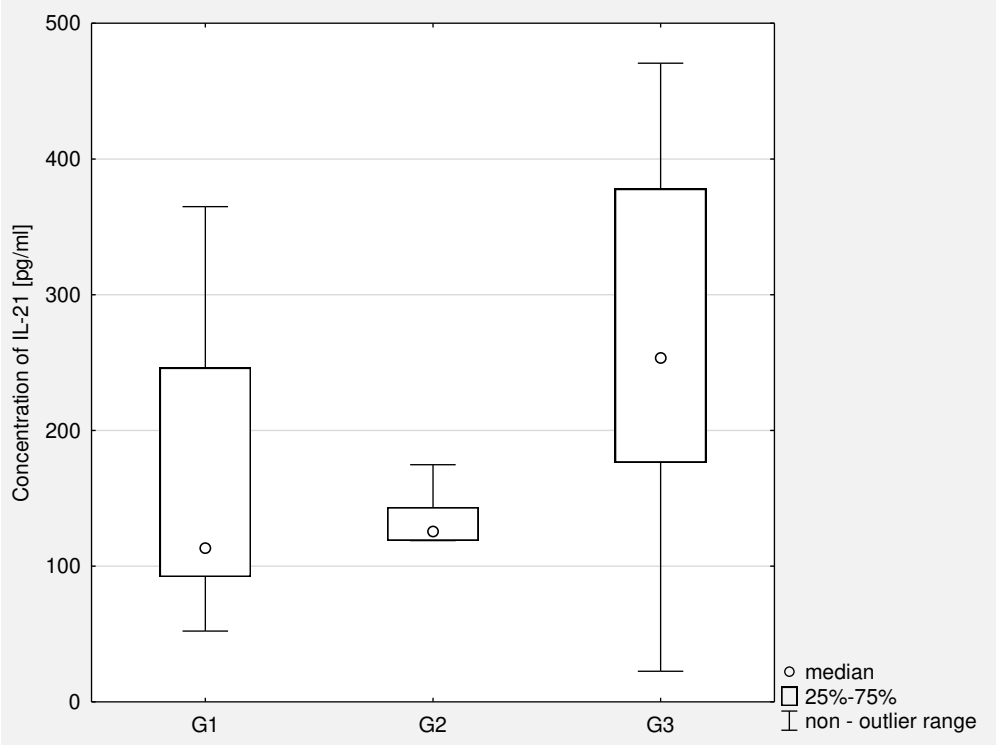

Figure 4. The concentration of IL-21 in the peritoneal fluid in women with ovarian cancer depending on the degree of differentiation G1 (well differentiated), G2 (moderately differentiated), G3 (poorly differentiated).

The results of Il-22 concentration measurement in the serum and peritoneal fluid of women with ovarian cancer were presented in the form of the median and the lower and upper interquartile range (Q1 and Q3).

In the serum of women with ovarian cancer, Q1 and Q3 were respectively: 337.59 and 771.33 with a median of 572.64 pg/mL. A statistically significant higher concentration of IL-22 in the serum of women with ovarian cancer was demonstrated compared to the concentration in the control group ($p < 0.001$), where Q1 and Q3 were respectively: 66.01 and 140.04 with a median of 98.62 pg/mL.

In the peritoneal fluid of women with ovarian cancer, Q1 and Q3 were 132.40 and 275.70, respectively, with a median of 186.01 pg/mL. A statistically significant higher concentration of the examined parameter in the serum of women with ovarian cancer was demonstrated compared to the concentration in the peritoneal fluid ($p < 0.0001$).

Then, the concentration of IL-22 in serum and peritoneal fluid in women with ovarian cancer depending on the degree of histopathological differentiation of the cancer was analyzed. The analysis of the test results showed a statistical significance between G2 and G3 ($p < 0.0001$) and between G1 and G3 ($p < 0.0001$) in the serum of women. In the peritoneal fluid of women with ovarian cancer, no statistically significant differences were found between the degrees of histopathological differentiation of the cancer.

The obtained results are presented in Figures 5 and 6.

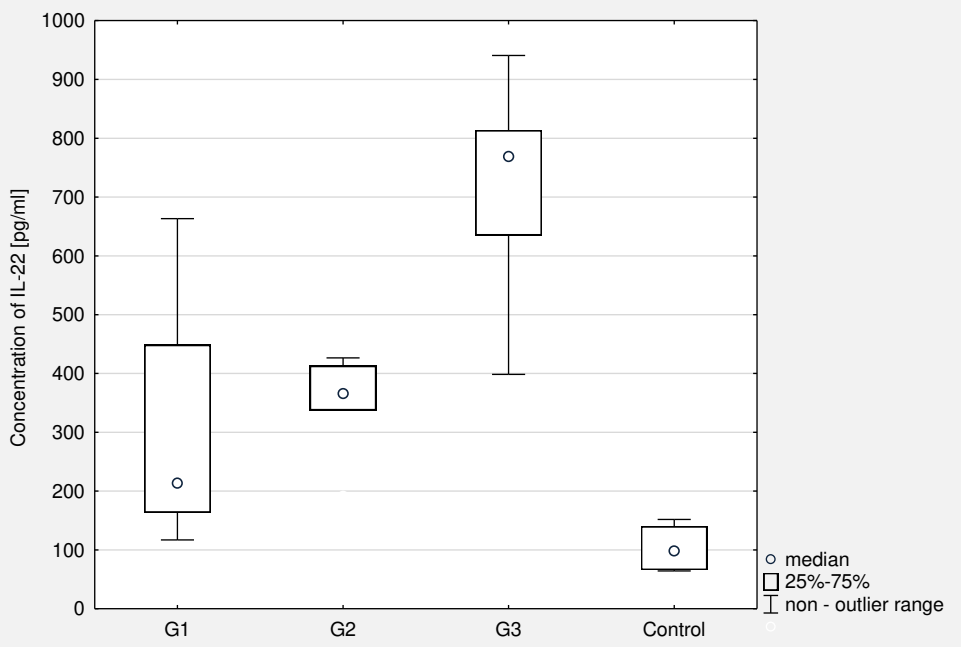

Figure 5. The concentration of IL-22 in the serum of women with ovarian cancer depending on the degree of differentiation G1 (well differentiated), G2 (moderately differentiated), G3 (poorly differentiated) compared to the control group.

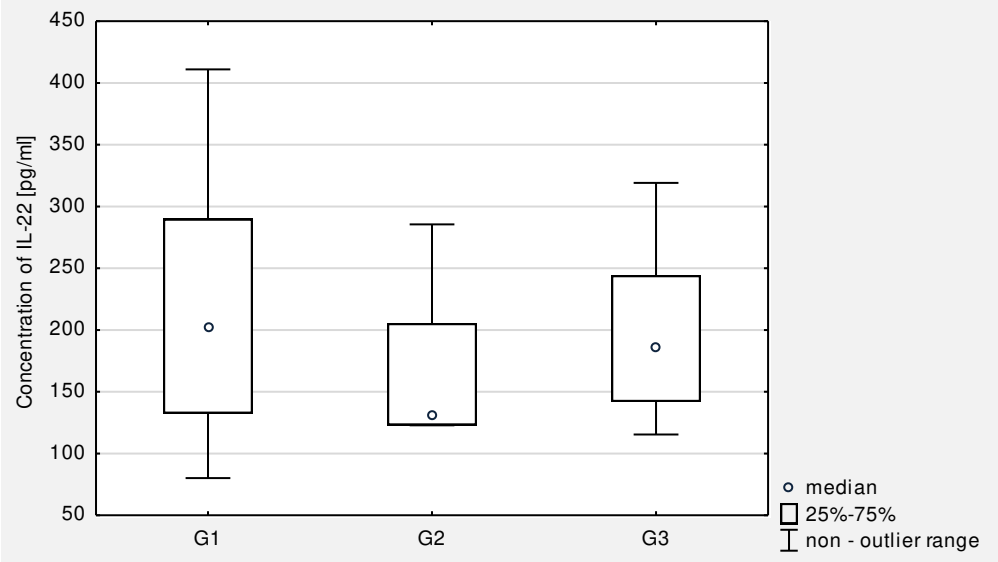

Figure 6. The concentration of IL-22 in the peritoneal fluid in women with ovarian cancer in relation to on the degree of differentiation G1 (well differentiated), G2 (moderately differentiated), G3 (poorly differentiated).

Further analysis concerned the assessment of the relationship between the serum concentration of IL-21 and IL-22 and the peritoneal fluid concentration of IL-21 and IL-22. A statistically significant positive correlation was found between IL-21 and IL-22 in the serum of the examined women, and a positive, but not statistically significant correlation between the concentration of IL-21 and IL-22 in the peritoneal fluid of the examined women. The regression curves are shown in Figures 7 and 8. Ratio of IL-21/IL-22 in serum and peritoneal fluid in patients with ovarian cancer is shown in Figures 9 and 10.

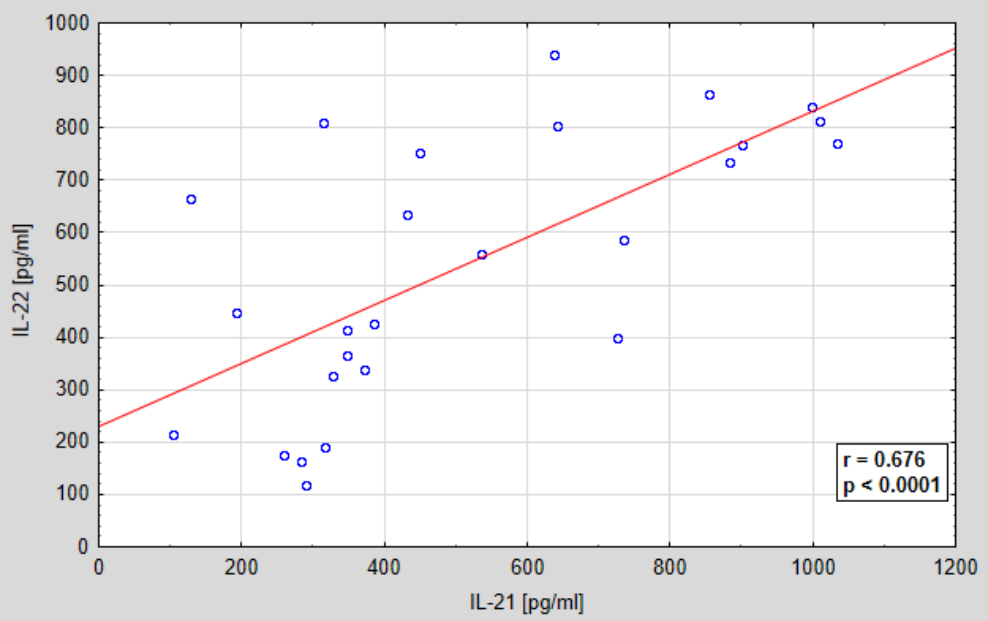

Figure 7. Linear regression curve showing the relationship between IL-21 and IL-22 levels in the serum of women with ovarian cancer; *r*-correlation coefficient, *p*-probability value.

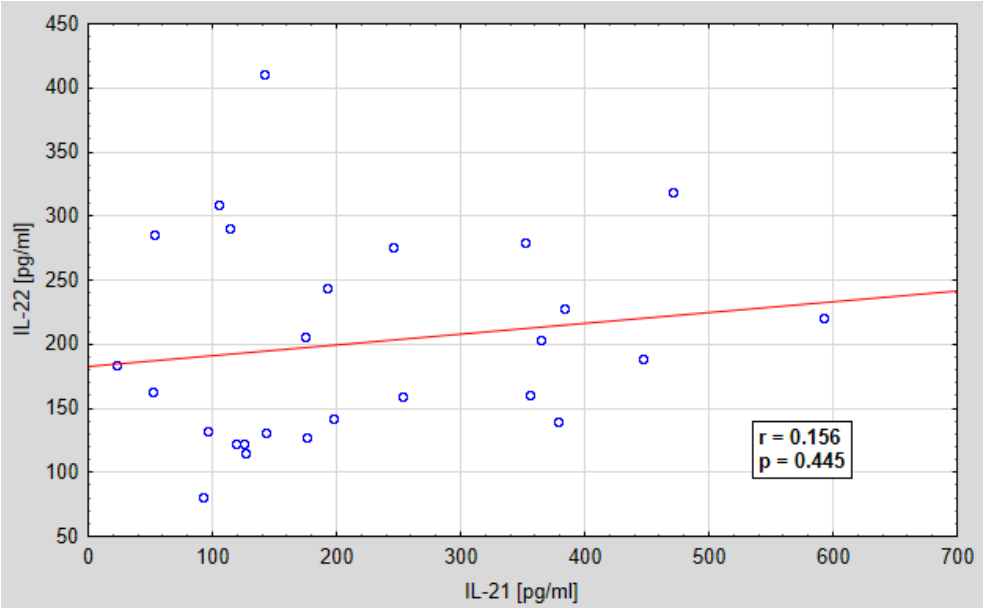

Figure 8. Linear regression curve showing the relationship between the concentration of Il-21 and IL-2 in the peritoneal fluid of women with ovarian cancer; *r*-correlation coefficient, *p*-probability value.

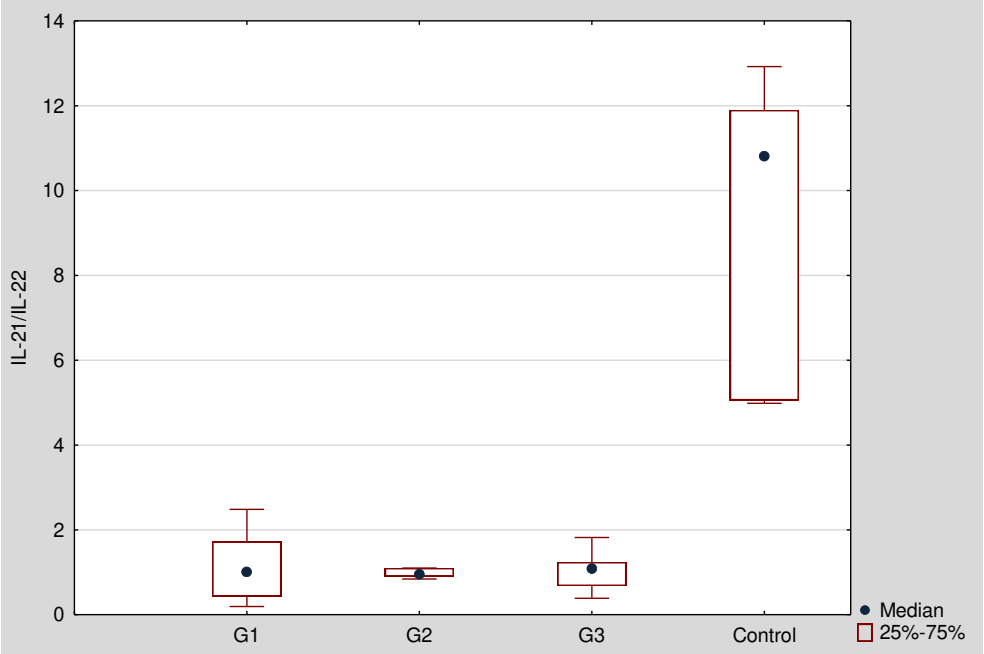

Figure 9. IL-21/IL-22 ratio in the serum of women with ovarian cancer depending on the degree of differentiation G1 (well differentiated), G2 (moderately differentiated), G3 (poorly differentiated) compared to the control group.

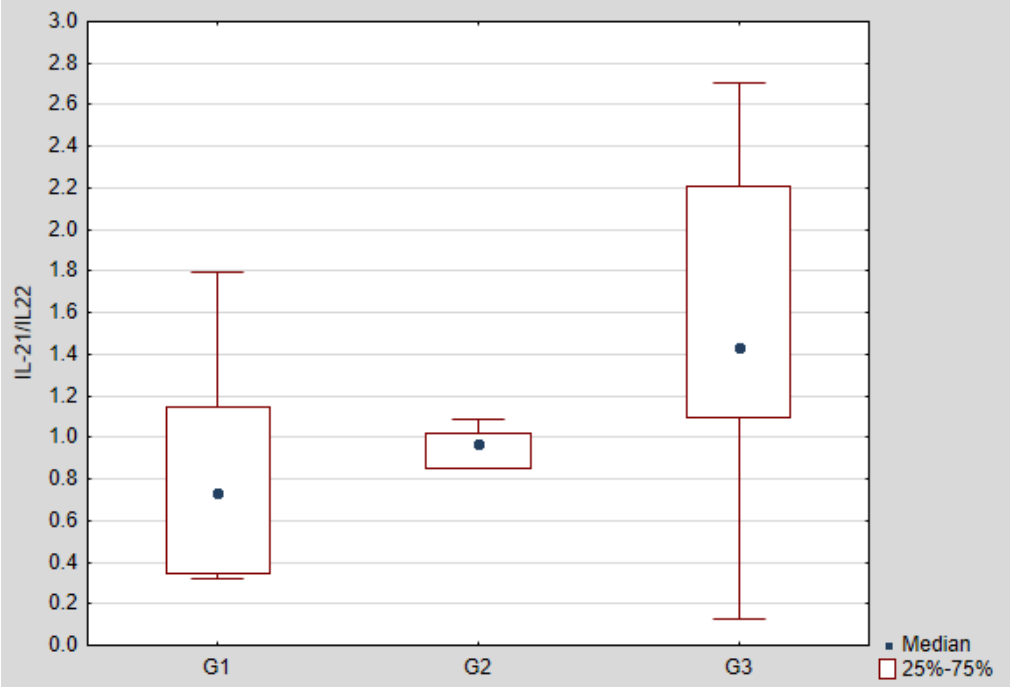

Figure 10. IL-21/IL-22 ratio in the peritoneal fluid of women with ovarian cancer depending on the degree of differentiation G1 (well differentiated), G2 (moderately differentiated), G3 (poorly differentiated).

4. Discussion

For years, the efforts of researchers have been focused on understanding the mechanisms of the formation and development of ovarian cancer in order to identify markers helpful not only in early diagnosis, but also in monitoring therapy. The challenge of contemporary gynecological oncology is to develop an effective targeted therapy that controls the expression of compounds that play a key role in the formation and development of

the cancer. Numerous experimental studies and clinical observations indicate that chronic inflammation plays an important role in the pathogenesis of ovarian cancer, in which cells of the immune system participate, which, through secreted mediators in the tumor's environment, participate in the anti-tumor response and may promote tumor growth, which may be manifested by changes in tissue expression, serum concentrations, and peritoneal fluid.

Recent studies have shown that interleukin 21 and interleukin 22 play an important role in the pathogenesis of cancer, which role in the formation and development of ovarian cancer is still not fully understood. Therefore, the aim of the analysis performed in the study was to assess the expression of IL-21 and IL-22 in the tumor tissue and the concentration of these parameters in the serum and peritoneal fluid in patients with ovarian serous cancer, taking into account the degrees of histological differentiation of the tumor.

For the first time, the expression of genes encoding IL-21 and IL-22 was assessed at the mRNA level in ovarian tumor specimens. The number of mRNA copies of the studied genes was determined by RT-qPCR method (quantitative method real-time). The studies showed that in the samples of the G1 histological differentiation, the number of mRNA copies of IL21 gene was statistically significantly higher, not only compared to the control group, but also compared to the G2 and G3 histological differentiation samples. In the case of IL22, we did not observe any differences in the number of mRNA copies between the cancer specimens and the control group, as well as between the G1, G2, and G3 histologically differentiated groups. The studies by Fagerberg et al. [25] did not show the expression of IL21 and IL22 at the mRNA level in normal ovaries. Therefore, the presence of mRNA of these interleukins in the sections studied by us, both malignant and benign, may be caused by the presence of cells of the immune system. The differences observed by us suggest that the interaction with the cells of the immune system may have a different course in malignant and benign tumors, but this requires further detailed studies.

Our studies showed a statistically significantly lower concentration of IL-21 in the serum of women with ovarian cancer compared to the concentration in the control group, which indicates the participation of the tested cytokine in the immune response against ovarian cancer cells. In addition, statistical significance was demonstrated between the concentration of the tested cytokine and the degrees of histological differentiation in cancer: G2 and G3, and between G1 and G3, which indicates a relationship between IL-21 secretion and histological differentiation in cancer and may prove useful in the future in selecting the optimal therapeutic treatment and in the assessment of prognosis.

Interesting observations were also provided by the analysis of the concentration of IL-21 in the peritoneal fluid of the examined women. The concentration of this parameter was significantly lower in the peritoneal fluid as compared to the serum, which may indicate that the source of the tested cytokine in the serum may be cells of the immune system, which, through secreted mediators, show increased cytotoxic activity against cancer cells. Moreover, it was shown that the concentration of IL-21 in the peritoneal fluid of women with ovarian cancer differed between the grades of G2 and G3, which proves that the secretion of IL-21 is related to the histological differentiation of ovarian cancer also in this biological fluid.

So far, no studies have been conducted to evaluate the expression of IL-21 in tumor tissue and the concentration in serum and peritoneal fluid. However, the potential usefulness of using this cytokine in the diagnosis of ovarian cancer was analyzed. Such research was conducted by Chen YL. et al. [19], who showed the usefulness of the assessment of interleukins IL-17a and IL-21 as prognostic and therapeutic biomarkers. Studies on the role of IL-21 in the anti-tumor response were also conducted by Hermans et al. [26], who pointed to the therapeutic potential of the transient inhibition of LDH during adoptive T-cell immunotherapy with the anti-tumor effect of inhibiting LDH and IL-21. Similar studies were conducted by Dou et al. [27], who showed that SKOV3 cells genetically modified to secrete biologically active IL-21 and GM-CSF were effective in inducing anti-tumor immunity by increasing NK cytotoxicity, promoting the expression of MIC A/B, ICAM-1,

and NKG2D molecules, as well as increasing IFN-γ and TNF-α in the nude mouse model. On the other hand, Bhatt et al. [28] have shown that IL-21 has a strong anti-tumor activity against mantle lymphoma (MCL) cells through direct cytotoxic and indirect immunological effects. According to the authors, in vivo treatment with IL-21 leads to complete regression of FC-muMCL1 tumor in syngeneic mice through NK and T cell dependent mechanisms. Similar studies were also conducted by Li et al. [29], who showed that IL-21 may affect T cells, which are involved in the anti-tumor response, by fusing with an anti-PD-1 antibody. Wang et al. [30] assessed the role of interleukin 21 and its receptor in proliferation, migration, and invasion of breast cancer cells. The conducted research has shown that IL-21 promotes proliferation, invasion and migration of IL-21R + MDA-231 breast cancer cells, it does not show such strong properties against MCF-7 and ZR-75.1 cells, in which IL-21R expression is weak or negative. Moreover, the role of IL-21R in signaling pathways of matrix metalloproteinases, which are necessary for the processes of migration of MDA-231 cells, has been demonstrated. Zhang et al. [31] showed that reduction of the tumor and increased survival is accompanied by an increase of IFN-γ, IL-21, and TNF-α in serum, as well as the cytotoxic activity of the spleen. On the other hand, Gu et al. [32] showed that the co-expression of two members of the γ chain family of the cytokine receptor, IL-21 and IL-7, in anti-cancer vaccines increases anti-tumor immunity in a CD4 + and CD8 + T cell-dependent manner and generates an effective immune memory.

The increase of IL-21 mRNA in tumor tissue at the G1 stage may indicate that there are still efficient mechanisms aimed at limiting its growth, which is also reflected in the IL21/IL22 mRNA ratio value. In the G2 and G3 stages, the expression is reduced in tumor tissue, possibly as a result of disturbed intracellular signaling or epigenetic changes.

On the other hand, the increase in protein concentration, both in the serum and in the peritoneal fluid, suggests the activation of systemic mechanisms and a strong recruitment of the immune system. Due to blocked mechanisms in the tumor tissue itself, the antitumor effect of the immune system is probably ineffective, despite its strong stimulation. However, this hypothesis requires further investigation. It should also be taken into account that gene expression is a multi-step process and its regulation is complex. Strongly activated gene transcription in the tumor tissue does not mean that protein is synthesized and secreted. In our research, the protein source are likely cells of the immune system. If tumor tissue were the main source of interleukins, the protein concentration would be higher in the peritoneal fluid than in the serum.

An interesting observation is the fact that serum protein concentration is significantly decreased by the G1 stage and the concentration increases with the degree of histological differentiation of the cancer. In the G3 stage, the IL21 concentration increases to a level comparable to that in the control. Most likely, in the lower degree of histological differentiation of the cancer, the mechanisms responsible for "masking" the presence of cancer cells are activated. In our research, the study group was not very large, so the results should be interpreted with caution.

Further analysis included the evaluation of interleukin 22 expression at the mRNA level in tumor tissue and serum and peritoneal fluid concentration in women with ovarian cancer. The studies conducted so far have shown that the role of the interleukin IL-22 is complex. On the one hand, this cytokine promotes tumor development, is responsible in particular for the proliferation of malignant neoplasm of epithelial origin, but on the other hand, the intensification of its secretion may constitute a barrier against tumor development by suppressing inflammation due to the functions that protect against tissue damage, promote tissue repair, and prevent inflammation [33,34]. Our studies showed that the concentration of IL-22 in the serum of women with ovarian cancer was statistically significantly higher than that in the control group, which proves the participation of this cytokine in pro-cancer immune response through direct and indirect influence involving the immune system cells participating in the regulation of inflammation. In addition, a statistically significant higher concentration of the parameter in the serum of women with ovarian cancer was demonstrated compared to the concentration in the peritoneal fluid,

which indicates increased secretion of Il-22 in the systemic immune response. Moreover, a relationship has been demonstrated between the concentration of IL-22 and the degrees of histological differentiation of cancer, including: G2 and G3, and between G1 and G3 in the serum of women, which indicates the relationship of this cytokine with tumor development and may in the future prove useful in selecting the optimal therapeutic treatment and in assessing the prognosis.

Further analysis concerned the assessment of the relationship between the concentration of IL-21 and IL-22 in the serum and in the peritoneal fluid. The demonstrated correlation between the concentrations of the tested parameters, both in the serum and in the peritoneal fluid, may indicate the need for further research in order to create complex diagnostic and therapeutic strategies targeting many different mechanisms of the immune response. Due to the fact that the interactions between these parameters may affect the direction of their biological activity, the stoichiometric relationship between the concentration of IL-21 and IL-22 and the number of IL21/IL22 mRNA copies was assessed. The determined IL-21/IL-22 ratio, both at the mRNA level in the tissue and at the protein level in the serum and in the peritoneal fluid in patients with ovarian cancer, showed a variable share of the tested cytokines in the IL-21/IL-22 system, depending on the histological differentiation of the cancer.

Similar studies were conducted by Balint et al. [20], who assessed the expression of IL-22 in human ovarian cancer tissues and in ascites samples. Expression of both IL-22 and the IL-22 receptor was higher in cancer tissues compared to the control tissue, which correlated with poor prognosis. Studies have shown that interleukin-22 is an important factor in the ovarian cancer tumor microenvironment because it stimulates tumor growth by increasing proliferation and serves as a protective factor for ovarian cancer during TNF-induced apoptosis. According to the authors, IL-22 is a potential therapeutic target and/or biomarker in human ovarian cancer. Wang et al. [35] showed an increased expression of Th22 lymphocytes, which produce IL-22 in the tumor microenvironment, which, in their opinion, may stimulate tumor growth and affect the patient's further prognosis. Katara et al. [36] demonstrated the presence of IL-22 at all stages of the breast cancer, and that blocking of IL-22 gene expression resulted in inhibition of tumor invasion and reduction of metastasis. On the other hand, Rui et al. [37] showed an increased expression of the interleukin-22 receptor 1 in breast cancer. IL-22 binds to the IL-10R2 and IL-22R1 receptor complexes, activating the transcription factor STAT3, thereby promoting tumor progression. IL-22R1 is expressed exclusively on the surface of epithelial and tissue cells. Moreover, IL-22 promoted the expression of pro-tumor HOXB-AS5 proteins in breast cancer. Khosravi et al. [38] showed that high expression of IL-22RA1 in KRAS mutated lung adenocarcinoma is associated with a short remission time. Genetic ablation of IL-22 resulted in a significant decrease in tumor mass and a reduction in the number of tumor cells, their proliferation, and the ability to activate STAT3, and it was also associated with a reduction in angiogenesis and the number of inflammatory cells that infiltrated the lung tissue.

5. Conclusions

Local and systemic changes in the immune system with the participation of soluble mediators IL-21 and IL-22 indicate the participation of these parameters in the development of ovarian cancer and may participate in pro and anti-inflammatory activation involving the tested cytokines.

Modulation in the IL-21 and IL-22 system may affect the course of the inflammatory process that accompanies the development of cancer, which may prove useful in the development of new diagnostic and therapeutic strategies used in patients with ovarian cancer, which requires further studies.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/jcm10143058/s1, Figure S1: Quality of total RNA, Bioanalyzer Agilent electrophoresis run, Figure S2: Melting peak analysis showing specificity of RT-qPCR reaction, Figure S3: Agarose

gel electrophoresis of IL22 PCR-amplified product, Figure S4: Agarose gel electrophoresis of IL21 PCR-amplified product.

Author Contributions: A.M.-P.—conceptualization, supervision of immunological research, investigation, writing (original draft preparation, review and editing), funding acquisition; J.M.G.—conceptualization, supervision of molecular research, investigation, writing—review and editing; M.S.-K.—methodology (immunological research), data analysis and interpretation; C.K.-R.—methodology (molecular research), data analysis and interpretation; J.S., W.S., A.W.—clinical research conceptualization and data interpretation. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Medical University of Silesia in Katowice, Poland, grant number PCN-1-100/N/0/O.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of Medical University of Silesia in Katowice, Poland (protocol code KNW/0022/KB1/49/19).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data is contained in the article.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Momenimovahed, Z.; Tiznobaik, A.; Taheri, S.; Salehiniya, H. Ovarian cancer in the world: Epidemiology and risk factors. *Int. J. Women's Health* **2019**, *11*, 287–299. [CrossRef] [PubMed]
2. Bray, F.; Ferlay, J.; Soerjomataram, I.; Siegel, R.L.; Torre, L.A.; Jemal, A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J. Clin.* **2018**, *68*, 394–424. [CrossRef] [PubMed]
3. Kujawa, K.A.; Lisowska, K.M. Ovarian cancer-from biology to clinic. *Postepy Hig. Med. Dosw.* **2015**, *69*, 1275–1290. [CrossRef]
4. Muinao, T.; Boruah, H.P.D.; Pal, M. Diagnostic and Prognostic Biomarkers in ovarian cancer and the potential roles of cancer stem cells—An updated review. *Exp. Cell Res.* **2018**, *362*, 1–10. [CrossRef] [PubMed]
5. Waldmann, T.A. Cytokines in Cancer Immunotherapy. *Cold Spring Harb. Perspect. Biol.* **2017**, *10*, a028472. [CrossRef] [PubMed]
6. Dembic, Z. *The Cytokines of the Immune System*; Elsevier BV: Amsterdam, The Netherlands, 2015; pp. 143–239.
7. Winkler, I.; Woś, J.; Karczmarczyk, A.; Miotla, P.; Gogacz, M.; Skorupska, K.; Rechberger, T.; Tabarkiewicz, J.; Wolińska, E.; Skrzypczak, M. An association of circulating Tregs and Th17 cells producing IL-21 and IL-22 with the ROMA in ovarian cancer patients. *Cytokine* **2020**, *134*, 155194. [CrossRef] [PubMed]
8. Spolski, R.; Leonard, W.J. Interleukin-21: Basic Biology and Implications for Cancer and Autoimmunity. *Annu. Rev. Immunol.* **2008**, *26*, 57–79. [CrossRef]
9. Kang, L.; Bondensgaard, K.; Li, T.; Hartmann, R.; Hjorth, S.A. Rational Design of Interleukin-21 Antagonist through Selective Elimination of the γC Binding Epitope. *J. Biol. Chem.* **2010**, *285*, 12223–12231. [CrossRef]
10. Spolski, R.; Leonard, W.J. Interleukin-21: A double-edged sword with therapeutic potential. *Nat. Rev. Drug Discov.* **2014**, *13*, 379–395. [CrossRef]
11. Davis, M.R.; Zhu, Z.; Hansen, D.M.; Bai, Q.; Fang, Y. The role of IL-21 in immunity and cancer. *Cancer Lett.* **2015**, *358*, 107–114. [CrossRef]
12. Chabab, G.; Bonnefoy, N.; Lafont, V. IL-21 Signaling in the Tumor Microenvironment. *Adv. Exp. Med. Biol.* **2020**, *1240*, 73–82. [CrossRef]
13. Andorsky, D.J.; Timmerman, J.M. Interleukin-21: Biology and application to cancer therapy. *Expert Opin. Biol. Ther.* **2008**, *8*, 1295–1307. [CrossRef] [PubMed]
14. Skak, K.; Kragh, M.; Hausman, D.; Smyth, M.; Sivakumar, P.V. Interleukin 21: Combination strategies for cancer therapy. *Nat. Rev. Drug Discov.* **2008**, *7*, 231–240. [CrossRef] [PubMed]
15. Dudakov, J.A.; Hanash, A.M.; Brink, M.R.V.D. Interleukin-22: Immunobiology and Pathology. *Annu. Rev. Immunol.* **2015**, *33*, 747–785. [CrossRef] [PubMed]
16. Witte, E.; Witte, K.; Warszawska, K.; Sabat, R.; Wolk, K. Interleukin-22: A cytokine produced by T, NK and NKT cell subsets, with importance in the innate immune defense and tissue protection. *Cytokine Growth Factor Rev.* **2010**, *21*, 365–379. [CrossRef] [PubMed]
17. Markota, A.; Endres, S.; Kobold, S. Targeting interleukin-22 for cancer therapy. *Hum. Vaccines Immunother.* **2018**, *14*, 2012–2015. [CrossRef]
18. Protopsaltis, N.J.; Liang, W.; Nudleman, E.; Ferrara, N. Interleukin-22 promotes tumor angiogenesis. *Angiogenesis* **2018**, *22*, 311–323. [CrossRef]
19. Chen, Y.-L.; Chou, C.-Y.; Chang, M.-C.; Lin, H.-W.; Huang, C.-T.; Hsieh, S.-F.; Chen, C.-A.; Cheng, W.-F. IL17a and IL21 combined with surgical status predict the outcome of ovarian cancer patients. *Endocr. Relat. Cancer* **2015**, *22*, 703–711. [CrossRef]

20. Balint, K.; Seedial, T.; Gimotty, P.; Coukos, G.; Facciabene, A. Abstract 1421: Interleukin-22, a protective factor for ovarian cancer during TNFa-induced apoptosis. *Tumor Biol.* **2013**, *73*, 1421. [CrossRef]
21. Wang, Z.; Zhao, H.; Zhao, J.; Liu, Z.; Han, T.; Zhang, X.; Du, J. Prognostic significance of CD4 and interleukin-22 expression in pancreatic cancer. *Int. J. Clin. Exp. Pathol.* **2017**, *10*, 9846–9852.
22. Shi, J.; Wang, Y.; Wang, F.; Zhu, Z.; Gao, Y.; Zhang, Q.; Du, Z. Interleukin 22 is related to development and poor prognosis of hepatocellular carcinoma. *Clin. Res. Hepatol. Gastroenterol.* **2020**, *44*, 855–864. [CrossRef] [PubMed]
23. Bard, J.D.; Gelebart, P.; Anand, M.; Zak, Z.; Hegazy, S.A.; Amin, H.M.; Lai, R. IL-21 Contributes to JAK3/STAT3 Activation and Promotes Cell Growth in ALK-Positive Anaplastic Large Cell Lymphoma. *Am. J. Pathol.* **2009**, *175*, 825–834. [CrossRef] [PubMed]
24. Qin, S.; Chen, M.; Guo, X.; Luo, W.; Wang, J.; Jiang, H. The clinical significance of intrahepatic Th22 cells in liver cirrhosis. *Adv. Clin. Exp. Med.* **2019**, *28*, 765–770. [CrossRef] [PubMed]
25. Fagerberg, L.; Hallström, B.M.; Oksvold, P.; Kampf, C.; Djureinovic, D.; Odeberg, J.; Habuka, M.; Tahmasebpoor, S.; Danielsson, A.; Edlund, K.; et al. Analysis of the Human Tissue-specific Expression by Genome-wide Integration of Transcriptomics and Antibody-based Proteomics. *Mol. Cell. Proteom.* **2014**, *13*, 397–406. [CrossRef] [PubMed]
26. Hermans, D.; Gautam, S.; García-Cañaveras, J.C.; Gromer, D.; Mitra, S.; Spolski, R.; Li, P.; Christensen, S.; Nguyen, R.; Lin, J.-X.; et al. Lactate dehydrogenase inhibition synergizes with IL-21 to promote CD8+T cell stemness and antitumor immunity. *Proc. Natl. Acad. Sci. USA* **2020**, *117*, 6047–6055. [CrossRef] [PubMed]
27. Dou, J.; Wang, Y.; Wang, J.; Zhao, F.; Li, Y.; Cao, M.; Hu, W.; Hu, K.; He, X.F.; Chu, L.; et al. Antitumor efficacy induced by human ovarian cancer cells secreting IL-21 alone or combination with GM-CSF cytokines in nude mice model. *Immunobiology* **2009**, *214*, 483–492. [CrossRef] [PubMed]
28. Bhatt, S.; Matthews, J.; Parvin, S.; Sarosiek, K.A.; Zhao, D.; Jiang, X.; Isik, E.; Letai, A.; Lossos, I.S. Direct and immune-mediated cytotoxicity of interleukin-21 contributes to antitumor effects in mantle cell lymphoma. *Blood* **2015**, *126*, 1555–1564. [CrossRef]
29. Li, Y.; Cong, Y.; Jia, M.; He, Q.; Zhong, H.; Zhao, Y.; Li, H.; Yan, M.; You, J.; Liu, J.; et al. Targeting IL-21 to tumor-reactive T cells enhances memory T cell responses and anti-PD-1 antibody therapy. *Nat. Commun.* **2021**, *12*, 1–13. [CrossRef]
30. Wang, L.N.; Cui, Y.X.; Ruge, F.; Jiang, W.G. Interleukin 21 and Its Receptor Play a Role in Proliferation, Migration and Inva-sion of Breast Cancer Cells. *Cancer Genom. Proteom.* **2015**, *12*, 211–221.
31. Zhang, Y.; Wang, J.; Wu, D.; Li, M.; Zhao, F.; Ren, M.; Cai, Y.; Dou, J. IL-21-secreting hUCMSCs combined with miR-200c inhibit tumor growth and metastasis via repression of Wnt/β-catenin signaling and epithelial-mesenchymal transition in epithelial ovarian cancer. *OncoTargets Ther.* **2018**, *11*, 2037–2050. [CrossRef]
32. Gu, Y.-Z.; Fan, C.-W.; Lu, R.; Shao, B.; Sang, Y.-X.; Huang, Q.-R.; Li, X.; Meng, W.-T.; Mo, X.-M.; Wei, Y.-Q. Forced co-expression of IL-21 and IL-7 in whole-cell cancer vaccines promotes antitumor immunity. *Sci. Rep.* **2016**, *6*, 32351. [CrossRef]
33. Winkler, I.; Wos, J.; Karczmarczyk, A.; Gogacz, M.; Paweł, M.; Rechberger, T.; Tabarkiewicz, J.; Kotarski, J. 306 Treg cells and TH17 cells producing IL-21 and IL-22 in a ROMA relationship of patients affected by ovarian tumours. *ePoster* **2019**, *29*, A127–A128. [CrossRef]
34. Hernandez, P.; Gronke, K.; Diefenbach, A. A catch-22: Interleukin-22 and cancer. *Eur. J. Immunol.* **2018**, *48*, 15–31. [CrossRef] [PubMed]
35. Wang, T.; Zhang, Z.; Xing, H.; Wang, L.; Zhang, G.; Yu, N.; Wang, J.; Guo, W.; Jiang, J. Elevated Th22 cells and related cytokines in patients with epithelial ovarian cancer. *Medicine* **2017**, *96*, e8359. [CrossRef] [PubMed]
36. Katara, G.K.; Kulshrestha, A.; Schneiderman, S.; Riehl, V.; Ibrahim, S.; Beaman, K.D. Interleukin-22 promotes development of malignant lesions in a mouse model of spontaneous breast cancer. *Mol. Oncol.* **2019**, *14*, 211–224. [CrossRef]
37. Rui, J.; Chunming, Z.; Binbin, G.; Na, S.; Shengxi, W.; Wei, S. IL-22 promotes the progression of breast cancer through regulating HOXB-AS5. *Oncotarget* **2017**, *8*, 103601–103612. [CrossRef]
38. Khosravi, N.; Caetano, M.S.; Cumpian, A.M.; Unver, N.; Ramos, C.D.L.G.; Noble, O.; Daliri, S.; Hernandez, B.J.; Gutierrez, B.A.; Evans, S.E.; et al. IL22 Promotes Kras-Mutant Lung Cancer by Induction of a Protumor Immune Response and Protection of Stemness Properties. *Cancer Immunol. Res.* **2018**, *6*, 788–797. [CrossRef] [PubMed]

Journal of
Clinical Medicine

MDPI

Article

Eotaxins and Their Receptor as Biomarkers of Colorectal Cancer

Monika Zajkowska [1,*], Agnieszka Kulczyńska-Przybik [1], Maciej Dulewicz [1], Kamil Safiejko [2], Marcin Juchimiuk [2], Marzena Konopko [2], Leszek Kozłowski [2] and Barbara Mroczko [1,3]

[1] Department of Neurodegeneration Diagnostics, Medical University of Bialystok, 15-269 Bialystok, Poland; agnieszka.kulczynska-przybik@umb.edu.pl (A.K.-P.); maciej.dulewicz@umb.edu.pl (M.D.); mroczko@umb.edu.pl (B.M.)
[2] Maria Sklodowska-Curie Oncology Center, Department of Oncological Surgery with Specialized Cancer Treatment Units, 15-027 Bialystok, Poland; kamil.safiejko@gmail.com (K.S.); jumedica.onkologia@gmail.com (M.J.); marzeniedoc@yahoo.com (M.K.); leszek@kozlowski.pl (L.K.)
[3] Department of Biochemical Diagnostics, Medical University of Bialystok, 15-269 Bialystok, Poland
* Correspondence: monika.zajkowska@umb.edu.pl; Tel.: +48-686-5168; Fax: +48-686-5169

Abstract: Colorectal cancer (CRC) is one of the most common malignancies. Despite the availability of diagnostic tests, an increasing number of new cases is observed. That is why it is very important to search new markers that would show high diagnostic utility. Therefore, we made an attempt to assess the usefulness of eotaxins, as there are few studies that investigate their significance, in patients with CRC. The study included 80 subjects (CRC patients and healthy volunteers). Serum concentrations of all eotaxins were measured using a multiplexing method (Luminex), while CCR3 was measured by immunoenzymatic assay (ELISA). CRP levels were determined by immunoturbidimetry and classical tumor marker levels (CEA and CA 19-9) and were measured using chemiluminescent microparticle immunoassay (CMIA). The highest usefulness among the proteins tested showed CCR3. Its concentrations were significantly higher in the CRC group than in healthy controls. The diagnostic sensitivity, specificity, positive and negative predictive value, and the area under the ROC curve (AUC) of CCR3 were higher than those of CA 19-9. The maximum values for sensitivity, negative predictive value, and AUC were obtained for a combination of CCR3 and CRP. Our findings suggest the potential usefulness of CCR3 in the diagnosis of CRC, especially in combination with CRP or CEA.

Keywords: CCL11; CCL24; CCL26; CCR3; CRC

Citation: Zajkowska, M.; Kulczyńska-Przybik, A.; Dulewicz, M.; Safiejko, K.; Juchimiuk, M.; Konopko, M.; Kozłowski, L.; Mroczko, B. Eotaxins and Their Receptor as Biomarkers of Colorectal Cancer. *J. Clin. Med.* **2021**, *10*, 2675. https://doi.org/10.3390/jcm10122675

Academic Editors: Katarzyna Komosinska-Vassev, Pawel Olczyk and Hidekazu Suzuki

Received: 12 April 2021
Accepted: 14 June 2021
Published: 17 June 2021

1. Introduction

Colorectal cancer (CRC) is one of the most common malignancies not only in Europe, but also around the world. According to the World Health Organization (WHO), in 2020, the global incidence of all cancers was 19.3 million new cases, with approximately 9.9 million deaths. CRC comprises about 10% and 9.4% of all cases, respectively. Unfortunately, the incidence of this cancer is slowly but steadily increasing [1]. Currently, as a part of preventive diagnostics of colorectal cancer, tests such as FOBT (fecal occult blood test), FIT (fecal immunochemical test), colonoscopy, sigmoidoscopy, computed tomographic (CT) colonography, or multi-target stool deoxyribonucleic acid (mt-sDNA) test are used [2]. Also, markers such as CEA (carcinoembryonic antigen) or CA 19-9 (carbohydrate antigen 19-9) are routinely determined in patients with CRC. However, their diagnostic sensitivity and specificity are not satisfactory. That is why establishing new, more accurate markers for CRC detection at its earliest stage is vital [3].

Eotaxins belong to a group of small proteins called chemokines. They were discovered relatively recently—less than 30 years ago in London by Williams et al. [4,5]. Therefore, the number of papers on these parameters is still insufficient to fully understand their potential usefulness in the diagnosis or monitoring of certain diseases. The most widespread and so far the best-known usefulness of these proteins has been found in the course of allergic

diseases. It is closely related to their influence on cells such as eosinophils and basophils, which are the most important cells in the development and course of those diseases. All eotaxins have the ability to bind to CCR3 (C-C chemokine receptor type 3) [6,7].

However, there are only a few studies that would indicate the usefulness of these parameters in the diagnosis, monitoring, or staging of neoplastic diseases, such as colorectal cancer. Extending the availability of information on this topic is quite important due to the discrepancy of the available data. Some of the studies show a decrease in the concentration of CCL11, while others, on the contrary, report an increase in the concentration of this parameter in the course of CRC. On the other hand, for the other parameters (CCL24, CCL26, and CCR3), no work is available on their concentration in patients with CRC. However, there are works that describe the increased expression of eotaxins in neoplastic tissues. Some authors believe that those parameters increase the proliferation of neoplastic cells and their migration, and increases the expression of the receptor for eotaxins (CCR3). Activation of this receptor on endothelial cells leads to increased angiogenesis and tumor development. On the other hand, increased tissue expression of eotaxins can also lead to the recruitment of eosinophils (cells with anti-tumor activity) into the tumor environment [8]. Therefore, we made an attempt to clarify and assess the usefulness of eotaxins in patients with colorectal cancer compared to the healthy control. We have investigated serum levels, diagnostic utility (sensitivity, specificity, predictive values of positive and negative test results), and power (ROC curve analysis) of all eotaxins (CCL11, CCL24, CCL26), their receptor (CCR3), comparative tumor markers (CA 19-9, CEA), and inflammatory parameter such as C-reactive protein (CRP) in colorectal cancer detection. The data obtained in this study may prove the usefulness of the analyzed parameters in the detection of CRC.

2. Results

Table 1 shows the serum levels of CCL11, CCL24, CCL26, CCR3, CA 19-9, CEA, and CRP in patients with colorectal cancer and in the control group. Concentrations of CCR3, CEA, and CRP in the total cancer group were statistically significantly higher when compared to the control group (in all cases $p < 0.05$).

Table 1. Serum levels of tested parameters in cancer and control groups.

Parameter		Colorectal Cancer	Control Group	p *
CCL11 (pg/mL)	Median Min–Max	12.95 5.97–39.30	13.74 5.68–44.42	0.346
CCL24 (pg/mL)	Median Min–Max	1476.36 193.97–4102.92	1341.60 222.75–4189.99	0.458
CCL26 (pg/mL)	Median Min–Max	22.00 6.47–31.92	22.00 10.95–78.18	0.380
CCR3 (ng/mL)	Median Min–Max	0.22 0.10–0.53	0.12 0.10–1.19	**0.012**
CA 19-9 (U/mL)	Median Min–Max	4.14 2.00–8199.90	4.94 2.00–16.81	0.493
CEA (ng/mL)	Median Min–Max	1.90 0.50–1176.50	0.83 0.50–7.82	**<0.001**
CRP (mg/L)	Median Min–Max	5.40 1.00–103.50	1.31 0.20–5.80	**<0.001**

* U Mann–Whitney test.

In order to carry out a more detailed analysis of the obtained results, we divided the study group into patients with colon cancer and rectal cancer. Performed statistical analysis with use of the Mann–Whitney U test showed similar results. In the case of the results of colon cancer vs. control group patients, statistical significance was demonstrated for CEA and CRP ($p < 0.001$ in both cases) (Supplementary Table S1). For rectal cancer,

statistical significance was demonstrated for CEA, CRP, and CCR3 ($p = 0.003$; $p < 0.001$; $p = 0.012$, respectively) (Supplementary Table S2). We did not perform a statistical analysis for sigmoid cancer patients due to an insufficient number of patients in this subgroup. We also compared the results between the two subgroups (colon cancer vs. rectal cancer), but there were no statistical differences (Supplementary Table S3). Additionally, we performed a more detailed analysis with division into advancement groups. We divided the colorectal cancer group of patients into Early (TNM stages 0+I+II) and Advanced (TNM stages III+IV) CRC. Statistical analysis with use of Mann–Whitney U test in this case also revealed similar results. In analysis of Early CRC vs. control group, statistical significance was observed in the case of CEA and CRP ($p = 0.006$, $p < 0.001$, respectively) (Supplementary Table S4). In analysis of Advanced CRC vs. control group, statistical significance was observed in the case of CCR3, CEA, and CRP ($p = 0.024$, $p = 0.002$, $p = 0.001$, respectively) (Supplementary Table S5). We also tried to compare results between two TNM subgroups (Early vs. Advanced CRC), but none of the results were significant (Supplementary Table S6). Differences in the statistical significance of CCR3 in the case of rectal cancer and its absence in the case of colon cancer may be related to the differences in the biology of these two neoplasms. However, due to the insufficient number of patients with colon cancer, further analysis was carried out on the entire study group of CRC patients. The differences obtained between the two histological types should be repeated in a larger study group, which could significantly influence the development of knowledge about eotaxins, and we assume that at this point the obtained results should be treated as a pilot, preliminary study.

Table 2 shows the sensitivity (SE), specificity (SP), positive predictive value (PPV), and negative predictive value (NPV) of all tested parameters. We indicated that the highest SE from all tested parameters revealed CCR3 (68%) and this value is comparable to SE of C-reactive protein (72%) and higher than SE of commonly used tumor marker CA 19-9 (40%). Only CEA showed higher SE (80%). In the case of SP, the highest value was observed for CCL26 (92.31%). SP for CCR3 (62.07%) was comparable to results of both tumor markers (CA 19-9 and CEA) but lower than SP observed for CRP (79.31%). Positive predictive value was highest in the case of CCL24 and CCR3 (72.97% and 75.56%, respectively). These values were slightly lower than the PPV of CEA and CRP. Interestingly, almost all tested parameters (CCL11, CCL24 and their receptor) showed higher PPV than CA 19-9. The NPV was highest for CCR3 (52.94%), but slightly lower than the NPV of CEA and CRP. What is more, similar to PPV, the NPV of all tested parameters was similar or even higher than the NPV of commonly used tumor marker—CA 19-9.

According to promising results obtained for CCR3, we decided to check whether the simultaneous analysis of two parameters would significantly change the value of the diagnostic criteria. Therefore, we created two panels consisting of CCR3 with a comparative marker—CEA and CCR3 with CRP. Combined analysis of CCR3 with both parameters resulted in an increase of SE (92%; 94%, respectively) and NPV (76.47%; 82.35%, respectively) in both cases. The most favorable combination proved to be CCR3 + CRP, what may indicate the significance of the inflammatory component in the course of this malignancy.

Table 2. Diagnostic criteria of tested parameters in patients with colorectal cancer.

Tested Parameters	Diagnostic Criteria (%)	Colorectal Cancer
CCL11	SE	53.19
	SP	45.83
	PPV	65.79
	NPV	33.33
CCL24	SE	58.70
	SP	58.33
	PPV	72.97
	NPV	42.42
CCL26	SE	4.26
	SP	92.31
	PPV	50.00
	NPV	34.78
CCR3	SE	68.00
	SP	62.07
	PPV	75.56
	NPV	52.94
CA 19-9	SE	40.00
	SP	58.62
	PPV	62.50
	NPV	36.17
CEA	SE	80.00
	SP	65.52
	PPV	80.00
	NPV	65.52
CRP	SE	72.00
	SP	79.31
	PPV	85.71
	NPV	62.16
CCR3+CEA	SE	92.00
	SP	44.83
	PPV	74.19
	NPV	76.47
CCR3+CRP	SE	94.00
	SP	48.28
	PPV	75.81
	NPV	82.35

SE—sensitivity; SP—specificity; PPV—positive predictive value; NPV—negative predictive value.

The ROC curve is an illustrated relationship between the diagnostic SE and SP. The area under the ROC curve (AUC) indicates the clinical usefulness of a tumor marker and its diagnostic power. All data related to AUC has been shown in Table 3. We noticed that the CCR3 area under the ROC curve (0.683) in the total group of colorectal cancer was the highest from all the tested parameters but lower than the AUC for CEA and CRP. Moreover, similarly to previously mentioned statistical parameters, in the case of all eotaxins, the AUC was similar or even higher than the AUC of CA 19-9. Likewise, in the case of diagnostic criteria, the AUC for the simultaneous analysis of CCR3 with CEA or CRP showed a marked increase in the area under the ROC curve value (0.779; 0.846, respectively). Graphical versions of all significant ROC analysis results are shown in Figure 1. The AUCs for the tested parameters, similar to commonly used tumor markers and combined analysis, were statistically significantly larger in comparison to AUC = 0.5 (borderline of the diagnostic usefulness of the test) ($p < 0.05$ in all cases).

Table 3. AUC of tested parameters in patients with colorectal cancer.

Tested Parameters	ROC Criteria in Total Colorectal Cancer Group			
	AUC	SE	95% C.I. (AUC)	*p* (AUC = 0.5)
CCL11	0.430	0.073	0.283–0.5787	0.173
CCL24	0.555	0.070	0.4086–0.701	0.228
CCL26	0.441	0.072	0.3081–0.5744	0.813
CCR3	0.683	0.060	0.5646–0.803	**0.007**
CA 19-9	0.546	0.066	0.4123–0.6808	0.756
CEA	0.758	0.054	0.6487–0.8665	**<0.001**
CRP	0.830	0.045	0.7425–0.9175	**<0.001**
CCR3+CEA	0.779	0.057	0.6772–0.8814	**<0.001**
CCR3+CRP	0.846	0.049	0.7631–0.9294	**<0.001**

p—statistically significantly larger AUC's compared to AUC = 0.5. AUC—area under curve; SE—standard error; C.I.—confidence interval.

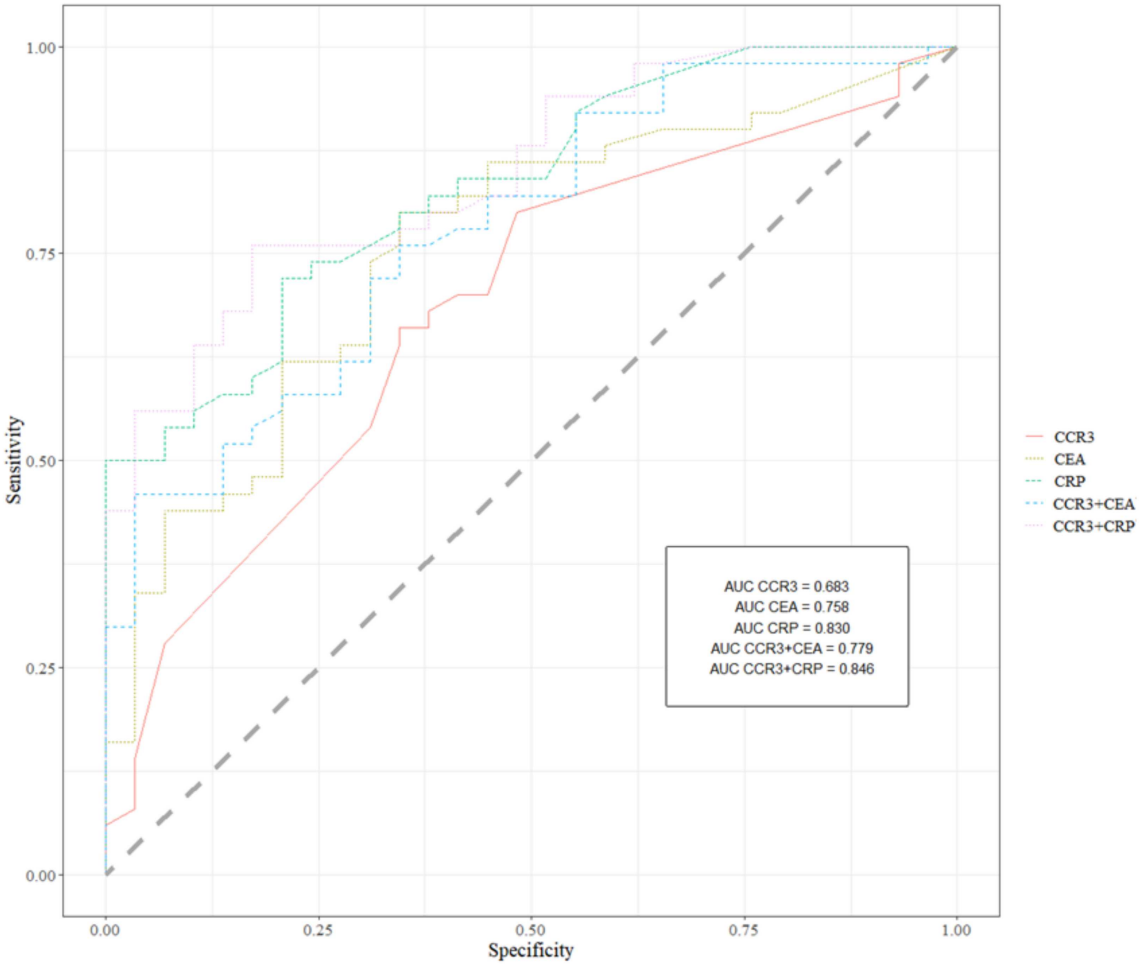

Figure 1. Receiver operating characteristics for all significant ROC analysis results.

3. Discussion

Currently, little is known about the concentration and diagnostic usefulness of eotaxins and their receptor in the course of CRC. Available literature describes results that are fragmentary, characterized by a high discrepancy, or, in some cases, not entirely clear. Therefore, we decided to conduct a confirmation study on whether the examined parameters may be useful in the detection, screening, or prognosis and monitoring of the applied treatment.

Eotaxins are closely related to cells such as eosinophils and basophils. These cells are mainly involved in allergic reactions, which can be included in the group of inflammatory reactions. Neoplastic changes also begin as a result of inflammatory reactions leading to the formation of cancer. Therefore, it can be assumed that similar cytokines may appear and change their concentration as a result of both of these phenomena (allergic reactions and onset of neoplastic changes). The first references to the relationship between eosinophils and neoplastic changes were described over 100 years ago, but so far, their role in the carcinogenesis [7–9] has not been precisely explained. There are suspicions that the involvement of eotaxins in CRC may be related to the large number of circulating eosinophils that appear and accumulate in neoplastic tissues. TATE (tumor-associated tissue eosinophilia) has also been connected with improved prognosis in CRC and some other types of cancer such as esophageal, oral squamous cell, bladder, and prostate cancer [10–16]. Moreover, there are some studies that clearly indicate that tissue eosinophilia in the course of neoplastic changes may be closely related to the factors secreted directly by tumor cells. These factors can certainly include eotaxins. That is why it is so important to extend the research carried out so far on these parameters not only in the course of CRC but also in other cancers [17,18].

We indicated that serum concentrations of CCL11 in the tested group (CRC) were lower than in the control group. Despite the lack of statistical significance, similar results were obtained in the course of other experiments carried out by Wagsater et al. [19]. These researchers obtained significantly lower concentrations of CCL11 in CRC patients (which can be connected with a larger test group). In addition, they also performed IHC (immunohistochemistry) staining and examined the concentration of CCL11 in tissue homogenates. This revealed that CCL11 may accumulate in tissues, which is why its concentration can be lowered in the serum of CRC patients. By contrast, Mir et al. [20], Yamaguchi et al. [21], and Komura et al. [22] reported that CCL11 levels are higher in CRC and inflammatory bowel disease patients than in control groups. This discrepancy might be correlated with some differences between the composition of study groups, i.e., receiving corticosteroids in the case of the Mir et al. [20] study and different ethnicity [21] or smaller study group in the case of the last paper [22].

The concentration of CCL24 (Eotaxin-2) in the CRC group was higher than in the control group, but the results were not significant. It may also be connected with the small size of the tested group. Unfortunately, there were no papers that could confirm or contradict the obtained results. In the case of tissue expression of CCL24, the results of available papers were similar to those concerning CCL11. Cheadle et al. [23] revealed that in biopsy samples of CRC and samples of adjacent liver metastases (with CRC origin), the levels of CCL24 were elevated.

In the case of CCL26 (Eotaxin-3), the medians did not differ between the groups which may indicate that this parameter is not related to the ongoing neoplastic changes. However, some authors [24] indicated that, similar to CCL11 and CCL24, tissue expression of Eotaxin-3 was higher in CRC patients. What is more, its expression increased among those with TNM stage and strongly correlated with lymph node metastasis. Perhaps the small number of patients with distant metastasis (stage IV of TNM classification) in our tested group was too low to affect the entire CRC group and demonstrate statistical significance. It can be associated with a generally small number of patients at this stage. In our department, screening diagnostics for CRC are well developed. In addition, the available research methods are very sensitive, which is why cancer detection occurs at a relatively early stage. Even if a patient arrives already in stage IV, when distant metastases are detected, not every patient can be included in the study group (high BMI, no consent to research, palliative treatment). Therefore, we believe that further studies performed on a larger study group (additional patients with distant metastasis) are vital, and research on the concentrations of this eotaxin should not be discontinued.

The most interesting results were obtained for CCR3, which showed a significantly higher concentration in the serum of CRC patients when compared to the control group

(p = 0.012). Unfortunately, we have not found any studies that would concern the concentrations of this parameter in the course of CRC. However, there are several studies that clearly indicate the usefulness of this parameter. Lan et al. [24], Cheadle et al. [23], and Cho et al. [25] unequivocally showed that the tissue expression of CCR3 in the course of this tumor is much higher when compared to healthy tissues. Interestingly, Devaud et al. [26] demonstrated that CCR3 has an anti-tumor effect correlated with the delayed growth of tumor cells. This information may be very important when we compare this study with the study by Steegenga et al. [27] showing that the tissue expression of this receptor was higher in female than in male mice. This could explain the differences in morbidity and mortality between the sexes for this type of cancer. That is why, further studies on CCR3 concentration and tissue expression should be performed.

We also measured the commonly used tumor markers (CEA, CA 19-9) and C-reactive protein concentrations in CRC patients and in the control group. CEA and CRP revealed significantly higher concentrations in the tested group when compared to healthy subjects. In the case of CA 19-9, we did not observe any significance. This is in accordance with different results obtained in the course of other experiments concerning CRC [28,29].

According to our knowledge, the present study is the first that assesses the diagnostic significance of serum CCL11, CCL24, CCL26, and CCR3 in CRC patients. We have found only one paper concerning diagnostic SE, SP (without PPV and NPV), and AUC for CCL11. In this paper, Yamaguchi et al. [20] reported that diagnostic sensitivity for CCL11 was 75.80%, specificity 66.70%, and AUC 0.714. These values were higher than ours, but the discrepancy might be connected with the size of the tested groups and their ethnicity (Japanese patients). Almost all tested parameters (CCL11, CCL24, and CCR3) showed higher SE than commonly used tumor marker CA 19-9. In the case of CEA and CRP, none of the newly tested parameters showed higher SE. In the case of SP, CCL26 showed the highest value. From the rest of tested parameters, CCR3 and CCL24 showed similar values to CEA and CA 19-9, but lower than the value for CRP. The PPV similar to SE, was higher in the case of almost all tested parameters than the PPV of CA 19-9, but lower than the PPV of CEA and CRP. The NPV, similar to SP, was highest for CCR3 but slightly lower than the NPV of CEA and CRP. What is more, similar to PPV, the NPV of all tested parameters was similar or even higher than the NPV of CA 19-9. In addition, simultaneous analysis of CCR3 + CEA and CCR3 + CRP revealed a marked increase in diagnostic SE and NPV. We noticed that the CCR3 area under the ROC curve in CRC was highest from all tested parameters but lower than AUC for CEA and CRP. Moreover, similar to previously mentioned statistical parameters, in the case of all tested proteins, AUC was comparable or even higher than AUC for CA 19-9. Similar to the diagnostic criteria (SE, SP, PPV, NPV), we observed a higher AUC value for simultaneous analysis of CCR3 with CEA and CRP. Due to the absence of appropriate data, it is impossible to discuss the above-described results.

The observed diagnostic usefulness of the parameters tested indicates that CCR3 could potentially prove to be the best of all tested proteins, especially with the combined analysis with CEA or CRP as a diagnostic panel. As CCR3 is present on the surface of endothelial cells and eosinophils, a significant increase in its concentration and tissue expression may contribute to both the intensification of angiogenesis and the influx of eosinophils, which can lead to tissue eosinophilia (important in the development of neoplastic changes). Considering the current results, it would be necessary in the future not only to expand the study group to a larger number of patients, but also to compare the obtained results with the number of circulating eosinophils, their expression in tissues, and the concentration of other parameters involved in the angiogenesis process, i.e., VEGF (vascular-endothelial growth factor).

4. Materials and Methods

4.1. Patients

The study included 50 colorectal cancer patients (CRC) diagnosed by the oncology group (Table 4). The patients were treated in the Department of Oncological Surgery

with Specialized Cancer Treatment Units, Maria Sklodowska-Curie Oncology Center, Bialystok, Poland. Tumor classification and staging were conducted in accordance with the International Union Against Cancer Tumor-Node-Metastasis (UICC-TNM) classification. Colorectal cancer histopathology was based on the microscopic examination of tissue samples. Moreover, all patients were grouped according to not only tumor stage (TNM), but also depth of tumor invasion (T factor), the presence of lymph node (N factor), and distant metastases (M factor) as well as the histological grade (G factor) of the tumor. The pretreatment staging procedures included physical and blood examinations, computed tomography (CT), and, in case of patients with rectal cancer, magnetic resonance imaging (MRI) of the small pelvis. Additionally, all patients were assessed according to the Eastern Cooperative Oncology Group (ECOG) score. The blood was collected the day before the treatment (surgery, radio, or chemotherapy). The control group included 30 healthy volunteers. For each of the patients qualified for the control group, the following exclusion criteria were applied: active infections and symptoms of an infection (both bacterial and viral), other comorbidities that can affect cytokine concentrations (respiratory diseases, digestive tract diseases) or systemic diseases such as lupus, rheumatoid arthritis, or collagenosis. In addition, none of the patients included in the control group abused alcohol, smoked, or had a personal or familial history of cancer. None of the patients included (both in the study and control group) had a BMI > 35 to fully exclude the influence of an increase in obesity-related inflammatory factors.

Table 4. Characteristics of colorectal cancer and healthy patient groups.

Study Group		No. of Patients
Colorectal Cancer		50
	Gender:	
	Female	18
	Male	32
	Type:	
	Colon Cancer	16
	Rectal Cancer	32
	Sigmoid Cancer	2
	TNM Stage:	
	0	1
	I	15
	II	13
	III	19
	IV	2
	Depth of tumor invasion:	
	In situ	1
	T1	2
	T2	19
	T3	24
	T4	4
	Nodal involvement:	
	N0	33
	N1	10
	N2	7
	Distant metastasis:	
	M0	48
	M1	2
	Age:	33–79
Control Group		30
	Gender:	
	Female	8
	Male	22
	Age:	34–80

TNM—Tumor Node Metastasis classification.

4.2. Biochemical Analyses

Venous blood samples were collected from each patient into a tube with clot activator (S-Monovette, SARSTEDT, Numbrecht, Germany), centrifuged to obtain serum samples, and stored at −80 °C until assayed. The tested chemokines were measured with a multiplexing method (Luminex Human Discovery Assay (3-Plex), R&D Systems, Abingdon, UK). The CCR3 receptor was measured with the enzyme-linked immunosorbent assay (ELISA) (Aviva Systems Biology Corp., San Diego, CA, USA). Serum levels of classical tumor markers were measured with chemiluminescent microparticle immunoassay (CMIA) (Abbott, Chicago, IL, USA), and for the analysis of CRP concentration, immunoturbidimetric method (Abbott, Chicago, IL, USA) was used according to the manufacturer's protocols. In Luminex and ELISA, according to the manufacturer's protocols, duplicate samples were assessed for each standard, control, and sample.

4.3. Statistical Analysis

Statistical analysis was performed by RStudio (Boston, MA, USA). The preliminary statistical analysis (using the Shapiro–Wilk test) revealed that the tested parameters and tumor marker levels did not follow normal distribution. Consequently, statistical analysis between the groups was performed by using the U-Mann–Whitney test, the Kruskal–Wallis test, and a multivariate analysis of various data by the post-hoc Dwass–Steele–Crichlow–Flinger test. The data were presented as a median and a range. Diagnostic sensitivity (SE), specificity (SP), and the predictive values of positive and negative test results (PPV and NPV, respectively) were calculated by using the cut-off values which were calculated by the Youden's index (as a criterion for selecting the optimum cut-off point) from the control group, and for each of the tested parameters were as follows: CCL11—12.75 pg/mL, CCL24—1401.83 pg/mL, CCL26—29.15 pg/mL, CCR3—0.17 ng/mL, CA 19-9—5.44 U/mL, CEA—1.11 ng/mL, CRP—2.5 mg/L. We also defined the receiver-operating characteristics (ROC) curve for all the tested parameters, tumor markers, and CRP to evaluate the diagnostic accuracy. Statistically significant differences were defined as comparisons resulting in $p < 0.05$.

5. Conclusions

According to our knowledge, the present study is the first that compares the diagnostic characteristics of all eotaxins and their receptor with the well-established tumor markers (CEA and CA 19-9) and the marker of inflammation (CRP) in CRC patients. In addition, due to a limited number of papers, it is extremely difficult to determine the direction in which changes in their concentrations are progressing in the course of colorectal cancer, especially if the available literature is limited to the concentrations of only one of the tested parameters. It is certain that further studies on the concentrations of eotaxins in the course of CRC are necessary to confirm and clarify their diagnostic usefulness and clinical application as potential tumor markers of CRC. However, the most promising factor seems to be CCR3, especially in combined use with CRP or CEA.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/jcm10122675/s1, Table S1: Serum levels of tested parameters in colon cancer subtype and control, Table S2: Serum levels of tested parameters in rectal cancer subtype and control, Table S3: Serum levels of tested parameters in cancer subtype groups, Table S4: Serum levels of tested parameters in Early TNM and control groups, Table S5: Serum levels of tested parameters in Advanced TNM and control groups, Table S6: Serum levels of tested parameters in different TNM stages of CRC.

Author Contributions: Conceptualization, M.Z. and B.M.; methodology, M.Z. and A.K.-P.; formal analysis, M.Z., A.K.-P. and M.D.; investigation, M.Z., K.S., M.J., L.K., M.K. and A.K.-P.; resources, M.Z.; data curation, M.Z. and M.D.; writing—original draft preparation, M.Z.; writing—review and editing, M.Z. and B.M.; supervision, B.M.; project administration, M.Z. and B.M.; funding acquisition, M.Z. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Medical University of Bialystok, Poland, grant numbers: SUB/1/DN/20/001/1198 and SUB/1/DN/21/004/1198.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of Medical University of Bialystok (R-I-002/564/2019; 28 November 2019).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. Key data are stated in the text.

Acknowledgments: This study was conducted with the use of equipment purchased by Medical University of Bialystok as part of the RPOWP 2007–2013 funding, Priority I, Axis 1.1, contract No. UDA-RPPD.01.01.00-20-001/15-00 dated 26 June 2015. B.M. has received consultation and/or lecture honoraria from Abbott, Wiener, Roche, Cormay and Biameditek. M.Z. has received lecture honoraria from Roche.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Sung, H.; Ferlay, J.; Siegel, R.L.; Laversanne, M.; Soerjomataram, I.; Jemal, A.; Bray, F. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J. Clin.* **2021**. [CrossRef] [PubMed]
2. Simon, K. Colorectal cancer development and advances in screening. *Clin. Interv. Aging* **2016**, *11*, 967–976. [PubMed]
3. Das, V.; Kalita, J.; Pal, M. Predictive and prognostic biomarkers in colorectal cancer: A systematic review of recent advances and challenges. *Biomed. Pharmacother.* **2017**, *87*, 8–19. [CrossRef]
4. Williams, T.J. Eotaxin-1 (CCL11). *Front Immunol.* **2015**, *6*, 84. [CrossRef] [PubMed]
5. Teixeira, M.M.; Wells, T.N.; Lukacs, N.W.; Proudfoot, E.A.; Kunkel, S.L.; Williams, T.J.; Hellewell, P.G. Chemokine-induced eosinophil recruitment. Evidence of a role for endogenous eotaxin in an in vivo allergy model in mouse skin. *J. Clin. Investig.* **1997**, *100*, 1657–1666. [CrossRef] [PubMed]
6. Zajkowska, M.; Mroczko, B. From Allergy to Cancer-Clinical Usefulness of Eotaxins. *Cancers* **2021**, *13*, 128. [CrossRef]
7. Zajkowska, M.; Mroczko, B. Eotaxins and Their Receptor in Colorectal Cancer-A Literature Review. *Cancers* **2020**, *12*, 1383. [CrossRef]
8. Korbecki, J.; Grochans, S.; Gutowska, I.; Barczak, K.; Baranowska-Bosiacka, I. CC Chemokines in a Tumor: A Review of Pro-Cancer and Anti-Cancer Properties of the Ligands of Receptors CCR1, CCR2, CCR3, and CCR. *Int. J. Mol. Sci.* **2020**, *21*, 8412. [CrossRef]
9. Simon, S.C.S.; Utikal, J.; Umansky, V. Opposing roles of eosinophils in cancer. *Cancer Immunol. Immunother.* **2019**, *68*, 823–833. [CrossRef]
10. Lotfi, R.; Spada, N.; Lotze, M.T. Eosinophils and Cancer. In *Eosinophils in Health and Disease*; Lee, J.J., Rosenberg, H.F., Eds.; Elsevier B.V.: Oxford, UK, 2013; pp. 431–536.
11. Davis, B.P.; Rothenberg, M.E. Eosinophils and cancer. *Cancer Immunol. Res.* **2014**, *2*, 1–8. [CrossRef]
12. Fernández-Aceñero, M.J.; Galindo-Gallego, M.; Sanz, J.; Aljama, A. Prognostic influence of tumor-associated eosinophilic infiltrate in colorectal carcinoma. *Cancer* **2000**, *88*, 1544–1548. [CrossRef]
13. Dorta, R.G.; Landman, G.; Kowalski, L.P.; Lauris, J.R.; Latorre, M.R.; Oliveira, D.T. Tumour-associated tissue eosinophilia as a prognostic factor in oral squamous cell carcinomas. *Histopathology* **2002**, *41*, 152–157. [CrossRef]
14. Ishibashi, S.; Ohashi, Y.; Suzuki, T.; Miyazaki, S.; Moriya, T.; Satomi, S.; Sasano, H. Tumor-associated tissue eosinophilia in human esophageal squamous cell carcinoma. *Anticancer Res.* **2006**, *26*, 1419–1424.
15. Costello, R.; O'Callaghan, T.; Sébahoun, G. Eosinophils and antitumour response. *Rev. Med. Interne* **2005**, *26*, 479–484. [CrossRef]
16. Luna-Moré, S.; Florez, P.; Ayala, A.; Diaz, F.; Santos, A. Neutral and acid mucins and eosinophil and argyrophil crystalloids in carcinoma and atypical adenomatous hyperplasia of the prostate. *Pathol. Res. Pract.* **1997**, *193*, 291–298. [CrossRef]
17. Lotfi, R.; Lee, J.J.; Lotze, M.T. Eosinophilic granulocytes and damage-associated molecular pattern molecules (DAMPs): Role in the inflammatory response within tumors. *J. Immunother.* **2007**, *30*, 16–28. [CrossRef]
18. Ito, N.; Demarco, R.A.; Mailliard, R.B.; Han, J.; Rabinowich, H.; Kalinski, P.; Stolz, N.B.; Zeh, H.J.; Lotze, M.T. Cytolytic cells induce HMGB1 release from melanoma cell lines. *J. Leukoc. Biol.* **2007**, *81*, 75–83. [CrossRef]
19. Wågsäter, D.; Löfgren, S.; Hugander, A.; Dienus, O.; Dimberg, J. Analysis of single nucleotide polymorphism in the promoter and protein expression of the chemokine eotaxin-1 in colorectal cancer patients. *World J. Surg. Oncol.* **2007**, *5*, 84. [CrossRef]
20. Mir, A.; Mínguez, M.; Tatay, J.; Pascual, I.; Peña, A.; Sanchiz, V.; Almela, P.; Mora, F.; Benages, A. Elevated serum eotaxin levels in patients with inflammatory bowel disease. *Am. J. Gastroenterol.* **2002**, *97*, 1452–1457. [CrossRef]
21. Yamaguchi, M.; Okamura, S.; Yamaji, T.; Iwasaki, M.; Tsugane, S.; Shetty, V.; Koizumi, T. Plasma cytokine levels and the presence of colorectal cancer. *PLoS ONE* **2019**, *14*, e0213602. [CrossRef] [PubMed]

22. Komura, T.; Yano, M.; Miyake, A.; Takabatake, H.; Miyazawa, M.; Ogawa, N.; Seki, A.; Honda, M.; Wada, T.; Matsui, S.; et al. Immune Condition of Colorectal Cancer Patients Featured by Serum Chemokines and Gene Expressions of CD4+ Cells in Blood. *Can. J. Gastroenterol. Hepatol.* **2018**, 7436205. [CrossRef] [PubMed]
23. Cheadle, E.J.; Riyad, K.; Subar, D.; Rothwell, D.G.; Ashton, G.; Batha, H.; Sherlock, D.J.; Hawkins, R.E.; Gilham, D.E. Eotaxin-2 and colorectal cancer: A potential target for immune therapy. *Clin. Cancer Res.* **2007**, *13*, 5719–5728. [CrossRef] [PubMed]
24. Lan, Q.; Lai, W.; Zeng, Y.; Liu, L.; Li, S.; Jin, S.; Zhang, Y.; Luo, X.; Xu, H.; Lin, X.; et al. CCL26 Participates in the PRL-3-Induced Promotion of Colorectal Cancer Invasion by Stimulating Tumor-Associated Macrophage Infiltration. *Mol. Cancer Ther.* **2018**, *17*, 276–289. [CrossRef] [PubMed]
25. Cho, Y.B.; Lee, W.Y.; Choi, S.-J.; Kim, J.; Hong, H.K.; Kim, S.-H.; Choi, Y.-L.; Kim, H.C.; Yun, S.H.; Chun, H.-K.; et al. CC chemokine ligand 7 expression in liver metastasis of colorectal cancer. *Oncol. Rep.* **2012**, *28*, 689–694. [CrossRef]
26. Devaud, C.; Bilhere, E.; Loizon, S.; Pitard, V.; Behr, C.; Moreau, J.-F.; Dechanet-Merville, J.; Capone, M. Antitumor activity of gammadelta T cells reactive against cytomegalovirus-infected cells in a mouse xenograft tumor model. *Cancer Res.* **2009**, *69*, 3971–3978. [CrossRef]
27. Steegenga, W.T.; Mischke, M.; Lute, C.; Boekschoten, M.V.; Pruis, M.G.; Lendvai, A.; Verkade, H.J.; Boekhorst, J.; Timmerman, H.M.; Plösch, T.; et al. Sexually dimorphic characteristics of the small intestine and colon of prepubescent C57BL/6 mice. *Biol. Sex Differ.* **2014**, *5*, 11. [CrossRef]
28. Pączek, S.; Łukaszewicz-Zając, M.; Gryko, M.; Mroczko, P.; Kulczyńska-Przybik, A.; Mroczko, B. CXCL-8 in Preoperative Colorectal Cancer Patients: Significance for Diagnosis and Cancer Progression. *Int. J. Mol. Sci.* **2020**, *21*, 2040. [CrossRef]
29. Mroczko, B.; Groblewska, M.; Okulczyk, B.; Kedra, B.; Szmitkowski, M. The diagnostic value of matrix metalloproteinase 9 (MMP-9) and tissue inhibitor of matrix metalloproteinases 1 (TIMP-1) determination in the sera of colorectal adenoma and cancer patients. *Int. J. Colorectal Dis.* **2010**, *25*, 1177–1184. [CrossRef]

Journal of
Clinical Medicine

MDPI

Article

Bone Metabolism and RANKL/OPG Ratio in Rheumatoid Arthritis Women Treated with TNF-α Inhibitors

Agnieszka Jura-Półtorak [1,*], Anna Szeremeta [1], Krystyna Olczyk [1], Aleksandra Zoń-Giebel [2] and Katarzyna Komosińska-Vassev [1]

[1] Department of Clinical Chemistry and Laboratory Diagnostics, Faculty of Pharmaceutical Sciences in Sosnowiec, Medical University of Silesia, Katowice, 41-200 Sosnowiec, Poland; aszeremeta@sum.edu.pl (A.S.); olczyk@sum.edu.pl (K.O.); kvassev@sum.edu.pl (K.K.-V.)
[2] Silesian Center of Rheumatology, Rehabilitation and Prevention of Disability of Gen. Jerzy Ziętek in Ustroń, 43-450 Ustroń, Poland; azongiebel@gmail.com
* Correspondence: ajura@sum.edu.pl; Tel.: +48-32-364-11-50

Abstract: The aim of this study was to evaluate the effect of anti-tumor necrosis factor α (anti-TNF-α) therapy in combination with methotrexate on bone remodeling and osteoclastogenesis in female patients with RA. Serum levels of bone turnover markers (i.e., C- and N-terminal propeptides of type I procollagen (PICP and PINP), C- and N-terminal cross-linking telopeptides of type I collagen (CTX-I and NTX-I), and soluble receptor activator of nuclear factor κB ligand (sRANKL) and osteoprotegerin (OPG)) were determined by immunoassay at baseline and 15 months after initiation of treatment. Bone mineral density was measured by dual-energy x-ray absorptiometry. We found a significant decrease in serum PINP levels, a biomarker of bone formation, and higher levels of CTX-I and sRANKL indicative of increased bone resorption in RA patients prior to TNFαI treatment compared to the controls. Anti-TNF-α therapy was effective in improving bone metabolism in RA patients as reflected in a decrease in CTX-I (at least partially due to the RANKL/OPG reduction) and a concomitant increase in PINP levels. The bone metabolism changes were independent of the type of TNFαI used. PINP and CTX-I were found to be useful markers of bone metabolism, which may prove the effectiveness of TNF-α therapy earlier than the bone density assessment.

Keywords: rheumatoid arthritis; TNF-α inhibitors; bone turnover markers; PINP; PICP; NTX-I; CTX-I; RANKL/OPG

Citation: Jura-Półtorak, A.; Szeremeta, A.; Olczyk, K.; Zoń-Giebel, A.; Komosińska-Vassev, K. Bone Metabolism and RANKL/OPG Ratio in Rheumatoid Arthritis Women Treated with TNF-α Inhibitors. *J. Clin. Med.* **2021**, *10*, 2905. https://doi.org/10.3390/jcm10132905

Academic Editor: Mitsuhiro Takeno

Received: 26 May 2021
Accepted: 28 June 2021
Published: 29 June 2021

1. Introduction

Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease of connective tissue characterized by symmetrical synovitis, eventually leading to bone erosion and cartilage damage. It affects approximately 0.5–1.5% of the world's population and is about 2–3 times more common in women than in men, with an increased incidence in the age of 40–50 years. In the course of the disease, there are numerous extra-articular manifestations of the organs, which are the major causes of rapidly progressing disability, reduced quality of life, and increased mortality [1–3]. RA patients have more skeletal damage and a higher risk of fractures compared to the general population. The bone complications of RA include focal erosion of the marginal and subchondral bone, periarticular osteopenia, and generalized osteoporosis with reduced bone mass. High disease activity (persistent inflammation), long-term glucocorticoid therapy (>3 months), and the physical disability and immobility are the main factors that increase the risk of bone loss in patients with RA [3–5].

Bone is a highly dynamic tissue, undergoing continuous remodeling to maintain a healthy skeleton. Normal bone remodeling is an ongoing process in which osteoclast-mediated bone resorption is tightly coupled both temporally and spatially with osteoblast-mediated bone formation [6,7]. The coupling of these processes becomes disrupted in

RA, resulting in the rapid breakdown of mineralized matrix and deterioration of bone microarchitecture [5,8–10].

Although the cellular mechanism of bone and cartilage destruction in RA is still not fully understood, both experimental and clinical findings indicate that pro-inflammatory mediators including tumor necrosis factor α (TNF-α) play a key role. TNF-α promotes osteoclastogenesis by inducing the expression of the essential osteoclast differentiation factor, the receptor activator of nuclear factor κB (NF-κB) ligand (RANKL), and/or its soluble receptor, osteoprotegerin (OPG), by bone marrow stromal cells of the osteoblast lineage or directly by enhancing the activity of cells in the osteoclast lineage [8–14].

The interaction between RANKL and its receptor-activator of nuclear factor κB (RANK) promotes the differentiation, maturation, activation, and survival of osteoclasts, leading to enhanced bone resorption and bone loss. RANKL/RANK signaling is controlled by a decoy receptor called osteoprotegerin, which competes with RANK for RANKL binding. The biological effects of OPG are opposite to those mediated by RANKL and include inhibition of end stages of osteoclast differentiation, activation of matrix osteoclast suppression, and accelerating osteoclast apoptosis [10,12,14,15]. Overall, the RANKL/OPG ratio determines the physiological balance of bone formation and turnover, with a higher ratio promoting increased bone resorption. It was found that the high RANKL/OPG ratio is associated with increased radiographic damage in RA patients [16]. Thus, the signaling and regulation of RANKL and OPG activity may play a critical role in bone loss associated with RA.

Anti-TNF-α therapies are effective in reducing inflammation and the progression of radiologic damage in RA patients and in murine models of arthritis [17–19], however, the mechanism by which TNF-α inhibitors (TNFαI) prevent the progression of bone destruction is still unclear. Therefore, the aim of this study was to investigate the effect of 15-month anti-TNF-α therapy on bone turnover markers and RANKL/RANK/OPG pathway in women with RA. We assessed the serum biomarkers indicative of bone remodeling—C-terminal propeptides of type I procollagen (PICP) and N-terminal propeptides of type I procollagen (PINP), which are markers of bone formation, and C-terminal crosslinking telopeptides of type I collagen (CTX-I) and N-terminal cross-linking telopeptides of type I collagen (NTX-I), which are markers of bone resorption—in female RA patients treated with TNFαI. However, according to the recommendation of the International Osteoporosis Foundation (IOF)–International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) Working Group for Standardization of Bone Marker Assay (WG-BMA), only s-PINP and s-CTX-I are the reference bone turnover markers [20].

The results obtained in our research will supplement the knowledge of molecular mechanisms of action of selected TNF-α inhibitors used in the treatment of RA. Most of the studies conducted thus far have mainly assessed the effect of infliximab on bone remodeling in RA [21–23]. Only a few studies have included the assessment of the impact of other TNFα inhibitors analyzed in this study including etanercept, adalimumab, certolizumab pegol and golimumab on bone turnover biomarkers and osteoclast regulatory proteins [17,24,25]. Moreover, they included a short follow-up time, less than our number of patients as well as the absence of a control group. Thus, the aim of this study was to evaluate the usefulness of the quantitative serum assessment of individual markers of bone turnover as indicators of the clinical efficacy of anti-TNF-α therapy with etanercept and adalimumab in women with rheumatoid arthritis.

2. Materials and Methods

2.1. Patients and Samples

Fifty female patients that fulfilled the 1987 or 2010 American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) classification criteria for RA [26,27] were enrolled in the study. Baseline patient characteristics are presented in Table 1. All subjects had active RA with a 28 joint count disease activity score (DAS28) > 5.1 at baseline, despite taking at least two synthetic disease-modifying antirheumatic drugs (DMARDs). Exclusion criteria included prior treatment with biologic agents, acute or recent

infection, concomitant diseases affecting bone metabolism, fractures, renal or liver insufficiency, heart failure, chronic alcoholism, pregnancy and breastfeeding. None of the enrolled subjects received bisphosphonates or hormone replacement therapy, which could have interfered with bone metabolism. In addition, none of the patients had smoked cigarettes for at least three months prior to the study. All participants received anti-TNF-α treatment combined with methotrexate (MTX) (25 mg/week) over a 15-month period. Biological agents were administered at recommended doses indicated in RA—for adalimumab (ADA; Humira) 40 mg every other week as subcutaneous (SC) injection, for etanercept (ETA; Enbrel) 50 mg once weekly as SC injection, for certolizumab pegol (CZP; Cimzia) 400 mg at 0, 2, 4 weeks, and then 200 mg every two weeks as SC injection and for golimumab (GLM; Simponi) 50 mg once a month as SC injection. Patients were also given prednisone in a dose of ≤7.5 mg/day and folic acid in the dose of 5 mg/day. The use of calcium (1 g/day) and vitamin D (800–1000 IU/day) supplements were permitted. Concomitant medications remained unchanged for the duration of the study.

Table 1. Demographic and clinical characteristics at baseline of the 50 rheumatoid arthritis patients qualified for the treatment with TNF-α inhibitors.

Parameter	Value
Age (years)	47.52 ± 11.91
Disease duration (years)	6 (3–12)
Height (cm)	163.58 ± 6.78
Weight (kg)	65.52 ± 14.40
BMI (kg/m^2)	24.46 ± 5.17
IgM-RF (+), *n* (%)	44 (88)
Anti-CCP (+), *n* (%)	43 (86)
ESR (mm/h)	17.0 (10.0–29.0)
CRP (mg/L)	6.37 (3.0–10.30)
SJC, *n*	7 (5–10)
TJC, *n*	12 (9–14)
VAS, (0–100 mm)	80 (70–80)
DAS 28-ESR	5.83 ± 0.49
Anti-rheumatic drugs, *n* (%)	
MTX (25 mg/week)	50 (100)
GC (≤7.5 mg/day)	50 (100)
FA (5 mg/day)	50 (100)
Calcium (1 g/day)	50 (100)
25-OH vitamin D (800–1000 IU/day)	50 (100)
TNFαI therapy, *n* (%)	
ETA (Enbrel)	24 (48)
ADA (Humira)	22 (44)
CZP (Cimzia)	2 (4)
GLM (Simponi)	2 (4)

Results are expressed as mean ± SD or median, inter-quartile (25th–75th percentile) range, or percentage (%). ADA, adalimumab; CZP, certolizumab pegol; DAS 28-ESR, disease activity score 28; ESR, erythrocyte sedimentation rate; ETA, etanercept; FA, folic acid; GC; glucocorticosteroid; GLM, golimumab; MTX, methotrexate; SD; standard deviation; SJC, swollen joint count; TJC, tender joint count; TNFαI, tumor necrosis factor α inhibitors; VAS, visual analogue scale.

Presented in Table 1 are the variables of the demographic and clinical data in rheumatoid arthritis patients who qualified for the treatment with TNF-α inhibitors that were obtained in our earlier investigations [28].

As controls, reference values of clinical and bone turnover parameters were obtained from 26 age- and gender-matched healthy volunteers from the Medical University of Silesia in Katowice, Poland. Subjects were selected after obtaining their medical history, clinical examination, and laboratory screening. Women with any medical conditions that interfere with bone metabolism or with surgery in the past three years were excluded. Moreover, all participants included in the study showed normal morphology and biochemical analysis.

Demographic, clinical, and laboratory data of control subjects in the study is presented in Table 2.

Table 2. Demographic and clinical characteristics of the 26 healthy women.

Parameter	Value
Age (years)	46.12 ± 10.91
Height (cm)	166.64 ± 6.23
Weight (kg)	62.64 ± 8.46
BMI (kg/m^2)	22.64 ± 2.26
ESR (mm/h)	9.00 (8.00–12.00)
RBC (10^6/μL)	4.28 ± 0.23
Hb (g/dL)	13.08 ± 0.97
Ht (%)	38.68 ± 3.10
PLT (10^3/μL)	263.88 ± 49.43
WBC (10^3/μL)	8.16 ± 1.63
Glucose (mg/dL)	88.00 (86.00–95.00)
Total cholesterol (mg/dL)	181.08 ± 6.02
HDL-C (mg/dL)	59.51 ± 12.85
LDL-C (mg/dL)	95.44 ± 20.19
Triglycerides (mg/dL)	118,70 (91.90–149.70)
hsCRP (mg/L)	0.61 (0.40–2.81)
Creatinine (mg/dL)	0.86 ± 0.10
Calcium ^{C} (mmol/L)	2.27 ± 0,08
Phosphorus (mmol/L)	1.34 ± 0.25
ALP (U/L)	159.77 ± 25.18
ALT (U/L)	20.61 ± 8.35
ASP (U/L)	20.00 ± 4.55
TSH (mU/L)	2.4 (2.20–2.81)
Uric acid (mg/dL)	4.70 ± 0.70

Results are expressed as mean ± SD or median, inter-quartile (25th–75th percentile) range, or percentage (%). ALP, alkaline phosphatase; ALT, alanine transaminase; ASP, aspartate transaminase; BMI, body mass index; ^{C} albumin-corrected calcium; ESR, erythrocyte sedimentation rate; Hb, hemoglobin; hs-CRP, high sensitive-C-reactive protein; Ht, hematocrit; PLT, platelet; RBC, red blood cell; TSH, thyroid-stimulating hormone; WBC, white blood cell.

None of the volunteers took glucocorticoids or any other pharmaceutics known to affect bone metabolism or smoked cigarettes. We chose women who were able to maintain a healthy weight and had a body mass index (BMI) < 25 kg/m^2. We excluded pregnant women and those who had a bone fracture (within 12 months) or had been operated on in the last three months as well as women with any disease that disturbed bone metabolism.

This study was approved by the Ethical Committee of the Medical University of Silesia in Katowice (KNW/0022/KB/56/I/12/13). All participants gave written informed consent prior to the study, and research was carried out in accordance with the conditions of the Declaration of Helsinki.

2.2. Laboratory Parameters and Evaluation of RA Activity

Measurement of rheumatoid factor (RF; normal value ≤ 15 U/mL) and C reactive protein (CRP; normal value < 5 mg/L) was performed at Konelab Prime 30ISE, bioMérieux, France. The anti-CCP antibodies (normal value ≤ 5 U/mL) were determined by enzyme-linked immunosorbent assay (ELISA) from Euroimmun (Lubeck, Germany). In addition, erythrocyte sedimentation rate (normal range for women: 3–12 mm/h) was determined by the Westergren method (Sediplus® S2000, Sarstedt, Germany). RA activity and response to TNFαI includes three variables with 28 joint counts of tenderness and swelling, and erythrocyte sedimentation rate (ESR) and the patient's assessment of disease activity (three variables). Patients who did not respond well to treatment were excluded from the study. A good response was defined—in accordance with the assumptions of the Polish National Health Fund's Therapeutic Programs—as a reduction in DAS28-ESR by more than 1.2 after the first three months of biological therapy and a further reduction in DAS28-ESR by 1.2

was noted in subsequent medical examinations performed at nine and 15 months after the first dose of TNFαI.

2.3. Measurement of Bone Turnover Markers

Venous blood samples were collected between 7:00 am and 9:00 am after an overnight fast. Serum obtained from healthy subjects and RA patients was separated from whole blood after complete clotting by centrifugation at 3000 rpm for 10 min and immediately stored at −80 °C until analysis. All bone turnover markers were measured at baseline and 15 months after starting TNFαI treatment. Bone formation was analyzed on the basis of serum levels of PICP and PINP using an ELISA method from Cloud-Clone Corp. (Katy, TX, USA). The minimum detectable concentration was <0.067 ng/mL for PICP and <13.2 pg/mL for PINP. Serum levels of bone resorption markers, (i.e., CTX-I and NTX-I) were determined using a competitive enzyme immunoassay from Immunodiagnostic Systems Ltd. (Boldon, UK) and competitive inhibition ELISA using the Osteomark® assay (Alere Scarborough, Inc., Scarborough, ME, USA), respectively. Detection limit for CTX-I was 0.020 ng/mL. The NTX-I serum assay values were expressed in nanomoles bone collagen equivalents per liter (nM BCE). The reference range was between 3.2 and 40.0 nM BCE. The manufacturer's recommended reference values for women ranged from 6.2 to 19 nM BCE. The levels of the markers of osteoclastogenesis, (i.e., total soluble RANKL and OPG) were measured using commercially available ELISA kits from BioVendor R&D (Brno, Czech Republic) according to the manufacturer's instructions. The minimum detectable concentration was estimated to be 0.40 pmol/L for sRANKL and 0.03 pmol/L for OPG. Testing of all samples in duplicate was completed in one day to eliminate the effects of inter-assay variation. The manufacturer's intra-assay coefficients of variation (CVs) were <10% for PICP, PINP and sRANKL, <3% for CTX-I, = 4.6% for NTX-I, and <4.9 for OPG.

2.4. Measurement of Bone Mineral Density

Bone mineral density (BMD) was measured in women with RA at baseline and 15 months after the first TNFαI course by dual-energy x-ray absorptiometry (DXA). DXA examination was performed using the Hologic Horizon Wi (Hologic Inc., Waltham, MA, USA) densitometer by a single technician during the study period. Measurements were taken at the posterior–anterior lumbar spine (region L2–L4) and left proximal neck of femur (femoral neck). In our study, the BMD (g/cm^2), the rate of T-score (density of bone in comparison with young people), and the rate of Z-score (density of bone in comparison with their peers) were studied. The inter-assay coefficient of variation (CV), measured using an anatomical spine phantom daily was less than 1.8.

2.5. Statistical Analysis

Data analyses were performed using TIBCO Software, Inc. (1984–2017); StatSoft Poland Sp. z o. o. 2021 (Palo Alto, CA, USA). The normality of the distribution was verified using the Shapiro–Wilk test. Data not normally distributed were log-transformed before the analyses. Variables are summarized as mean ± SD (for normal distribution) or median and interquartile (25th–75th percentile) range (for abnormal distribution). The homogeneity of variance was assessed by Levene's test. Evaluation of data was carried out using a repeated measures analysis of variance (RM-ANOVA) (normal data distribution) with a sphericity check employing Mauchly's test of sphericity, or using the RM-ANOVA Friedman's test (non-normal data). Post-hoc analyses performed in cases of significant differences between subgroups relied on the Tukey's test (p value < 0.05) or the Mann–Whitney U-test (p value obtained after application of the Bonferroni correction, $p < 0.05$/six possible comparisons). Moreover, the Mann–Whitney U-test was used to determine whether the differences between the values for RA patients, both at the beginning and after 15 months of TNFαI therapy, were significantly different from the control group. Paired Student's t-test (for normal distribution) or Wilcoxon's rank sum test (for abnormal distribution) was used to compare the change in the same parameters in each RA patient before and after 15 months

of anti-TNF-α treatment. p values of less than 0.05 were considered significant. Spearman's rank correlation coefficient was used to evaluate the relationship between selected biomarkers of both bone turnover (PINP, CTX-I and PINP/CTX-I ratio) and osteoclastogenesis (sRANKL, OPG, sRANKL/OPG ratio) in women with RA. The significance in case of multiple comparisons was assessed against a reference p value obtained after applying the Bonferroni correction ($p < 0.05$/six possible comparisons).

3. Results

3.1. Demographic and Clinical Characteristics

A total of 50 female RA patients met the eligibility criteria and were enrolled in this study receiving their first injection of ETA, ADA, CZP, or GLM (Table 1). A total of 31 patients completed the 15 months of anti-TNF-α therapy, and 19 were excluded from the analysis. Among the excluded patients with RA, anti-TNF-α therapy was discontinued for the following reasons: lack of response (five patients), loss of response (three patients), therapy intolerance (three patients), undergoing surgical procedures (four patients), and withdrawal of consent to participation in therapy (four patients). In the end, our study included 31 female patients with RA who continued the TNFαI therapy for 15 months.

During TNFαI therapy, a significant clinical improvement was noted in all RA patients. In line with the EULAR response criteria [29], 31 patients responded well after three months and this effect persisted up to month 15. The clinical parameters such as the number of tender and swollen joints, VAS, and DAS28-ESR score were significantly reduced at three, nine, and 15 months after the initiation of TNFαI therapy compared to the baseline. However, ESR and CRP levels decreased significantly only after nine and 15 months of treatment compared to the baseline (Table 3). This may indicate that in some patients—in spite of good response to the TNFαI therapy applied, as evidenced by reduction in DAS28-ESR—the treatment reduces the inflammation marker values, but they still remain above the normal range, which may entail increased cardiovascular risk in such patients. It has been demonstrated that the inflammation markers (especially ESR) are significantly correlated with the risk of cardiovascular disease (CVD) in rheumatoid arthritis [30]. Additionally, about 45% of women were over 50 years old in which reference values of ESR and CRP were higher than in women under 50 years old.

Presented in Table 3 are the variables of the demographic, clinical, and biochemical variables (except for data related to BMD and outcomes such as: serum levels of calcium, phosphorus, ALP) in rheumatoid arthritis patients during 15-month anti-TNF-α therapy that were obtained in our earlier investigations [28].

3.2. Bone Formation Markers—PINP and PICP

The serum levels of the bone formation marker (i.e., PINP) were lower in women with RA before anti-TNF-α therapy than in healthy women ($p < 0.01$; Figure 1a). In addition, a significant increase in PINP levels in women with RA was found after 15 months of anti-TNF-α therapy compared to the baseline values ($p < 0.001$; Figure 1a). Regarding the second marker of bone formation (i.e., PICP), it was shown that its levels in blood serum did not differ in women with RA before anti-TNF-α therapy compared to the values in healthy subjects ($p = 0.488$; Figure 1b). Fifteen months after starting TNFαI therapy, a statistically significant increase in PICP levels was observed in RA patients compared to the healthy controls ($p < 0.01$; Figure 1b). However, PICP levels did not differ after 15 months of anti-TNF-α treatment compared to the baseline ($p = 0.295$; Figure 1b).

Table 3. The demographic, clinical, biochemical and functional variables during 15-month anti-TNF-α therapy in women with rheumatoid arthritis.

Parameter	Time after Initiation of TNFαI Therapy			
	Baseline (T_0)	3 Months (T_1)	9 Months (T_2)	15 Months (T_3)
Women with RA, *n* (%)	31 (100)			
Premenopausal females, *n* (%)	17 (54.84)			
Postmenopausal females, *n* (%)	14 (45.16)			
Age (years)	45.87 ± 12.28			
Disease duration (years)	5 (3–11)			
Growth (cm)	163.77 ± 6.63			
Weight (kg)	65.89 ± 14.60			
BMI (kg/m^2)	24.62 ± 5.65			
IgM-RF (+), *n* (%)	28 (90.32)			
Anti-CCP (+), *n* (%)	26 (83.87)			
ESR (mm/h)	17.0 (10.0–34.0)	14.0 (9.0–23.0)	13.0 (9.0–18.0) [a]	13.0 (8.0–18.0) [a]
CRP (mg/L)	6.3 (3.08–14.0)	4.0 (2.0–9.0)	4.0 (2.0–4.3) [a]	4.0 (1.5–5.1) [a]
Calcium [C] (mmol/L)	2.30 ± 0.11			2.31 ± 0.11
Phosphorus (mmol/L)	1.36 ± 0.20			1.37 ± 0.21
ALP (U/L)	168.5 (152.5–202)			165.5 (149.5–192)
SJC, *n*	7 (5–10)	2 (0–3) [a,c]	0 (0–0) [a,b]	0 (0–0) [a,b]
TJC, *n*	12 (9–16)	4 (2–7) [a,c]	1 (0–2) [a,b]	0 (0–0) [a,b,c]
VAS, (0–100 mm)	80 (80–80)	40 (30–50) [a,c]	20 (10–30) [a,b]	15 (5–20) [a,b]
DAS 28-ESR	5.78 (5.51–6.24)	3.92 (3.08–4.42) [a,c]	2.75 (2.24–3.13) [a,b]	2.19 (1.75–2.51) [a,b,c]
Disease activity, *n* (%)				
High (>5.1)	31 (100)	2 (6.45)	0 (0)	0 (0)
Moderate (>3.2 and ≤5.1)	0 (0)	20 (64.52)	3 (9.68)	0 (0)
Low (≤3.2 and >2.6)	0 (0)	4 (12.91)	14 (45.16)	5 (16.13)
Remission (≤2.6)	0 (0)	5 (16.13)	14 (45.16)	26 (83.87)
Lumbar L2-L4 BMD (g/cm^3)	0.89 (0.73–1.00)			0.92 (0.79–1.03)
Lumbar L2-L4 T-score	−2.05 (−2.93–0.32)			−1.70 (−2.75—0.65)
Lumbar L2-L4 Z-score	−1.43 (−2.38–0.23)			−1.15 (−2.00–0.15)
Neck femur BMD (g/cm^3)	0.83 (0.69–0.78)			0.85 (0.77–0.85)
Neck femur T-score	−0.30 (−1.30–0.40)			−0.30 (−1.8–0.50)
Neck femur Z-score	−0.10 (−0.90–0.10)			0.00 (−0.70–0.10)
Patients which responded to TNFαI therapy, *n* (%)				
ETA (Enbrel)	16 (51.62)			
ADA (Humira)	13 (41.93)			
CZP (Cimzia)	2 (6.45)			

Results are expressed as mean ± SD or median, inter-quartile (25th–75th percentile) range, or percentage (%). Differences noted for all variables (except for data related to BMD and outcomes such as serum levels of calcium, phosphorus, ALP) considered significant at $p < 0.0083$ by applying Bonferroni correction. [a] significant differences compared to T_0; [b] significant differences compared to T_1; [c] significant differences compared to T_2. ADA, adalimumab; ALP, alkaline phosphatase; BMD, bone mineral density; [C] albumin-corrected calcium; CZP, certolizumab pegol; DAS 28-ESR, disease activity score 28; ETA, etanercept; ESR, erythrocyte sedimentation rate; SJC, swollen joint count; TJC, tender joint count; TNFαI, tumor necrosis factor α inhibitors; VAS, visual analogue scale.

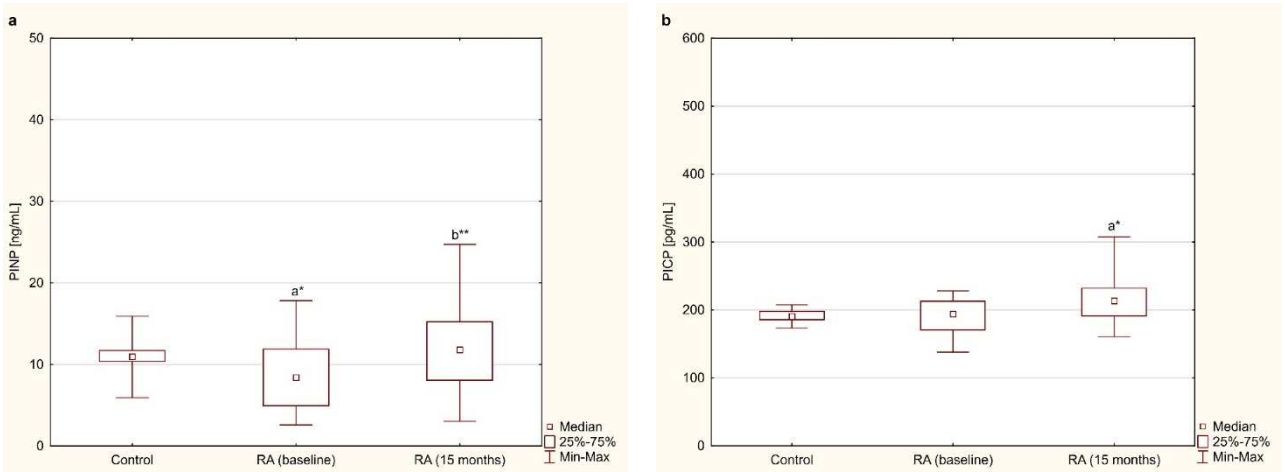

Figure 1. Effect of a 15-month TNFαI therapy on markers of bone formation: PINP (**a**) and PICP (**b**) in women with rheumatoid arthritis, and in healthy women. [a]* $p < 0.01$ compared to the control; [b]** $p < 0.001$ compared to the baseline. PICP, C-terminal propeptides of type I procollagen; PINP, N-terminal propeptides of type I procollagen; RA, rheumatoid arthritis; TNFαI, tumor necrosis factor α inhibitors.

3.3. Bone Resorption Markers—CTX-I and NTX-I

The serum concentrations of CTX-I and NTX-I in women with RA before and after 15 months of anti-TNF-α therapy and in healthy subjects are presented in Figure 2a,b. Before anti-TNF-α treatment, serum levels of CTX-I were significantly higher in women with RA than in healthy subjects ($p < 0.01$; Figure 2a). In addition, in women with RA, CTX-I levels significantly decreased after 15 months of anti-TNF-α therapy compared to the baseline ($p < 0.01$; Figure 2a). Regarding the second marker of bone resorption (i.e., NTX-I), it was shown that its levels in blood serum did not differ in women with RA before and after 15 months of anti-TNF-α therapy compared to the values in healthy subjects ($p = 0.385$ and $p = 0.403$, respectively; Figure 2b). Moreover, the serum levels of NTX-I in RA patients did not show any differences during the 15 months of anti-TNF-α treatment ($p = 0.595$; Figure 2b).

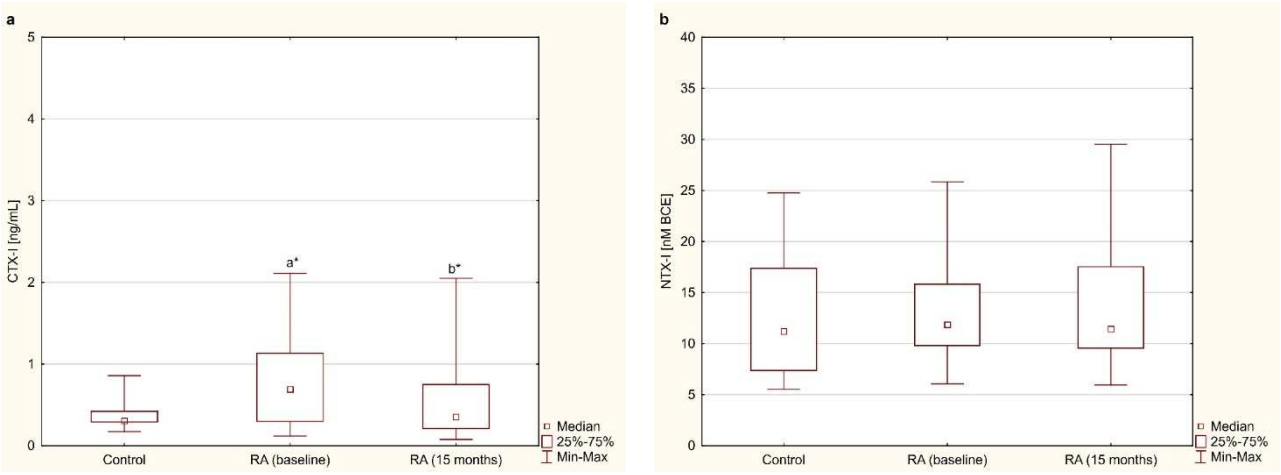

Figure 2. Effect of a 15-month TNFαI therapy on markers of bone resorption: CTX-I (**a**) and NTX-I (**b**) in women with rheumatoid arthritis, and in healthy women. [a]* $p < 0.01$ compared to the control; [b]* $p < 0.01$ compared to the baseline. CTX-I, C-terminal cross-linking telopeptides of type I collagen; NTX, N-terminal cross-linking telopeptides of type I collagen; RA, rheumatoid arthritis; TNFαI, tumor necrosis factor α inhibitors.

3.4. Osteoclastogenesis Markers—sRANKL and OPG

Among the markers of osteoclastogenesis, serum levels of sRANKL were significantly higher in women with RA before and after 15 months of anti-TNF-α therapy than in healthy subjects ($p < 0.001$ for both; Figure 3a). Moreover, with regard to the RANKL pathway, serum levels of sRANKL in RA patients did not show any differences during 15 months of anti-TNF-α therapy ($p = 0.281$; Figure 3a). On the other hand, in the case of OPG, it was shown that the serum levels of OPG in women with RA before treatment with TNF-α inhibitors did not differ from those in healthy subjects ($p = 0.343$; Figure 3b). However, in contrast to sRANKL, 15-month of anti-TNF-α therapy resulted in a significant increase in OPG concentration compared to the baseline level ($p < 0.001$; Figure 3b). Moreover, serum OPG were still significantly higher in RA patients after 15 months of treatment than in healthy individuals ($p < 0.001$; Figure 3b).

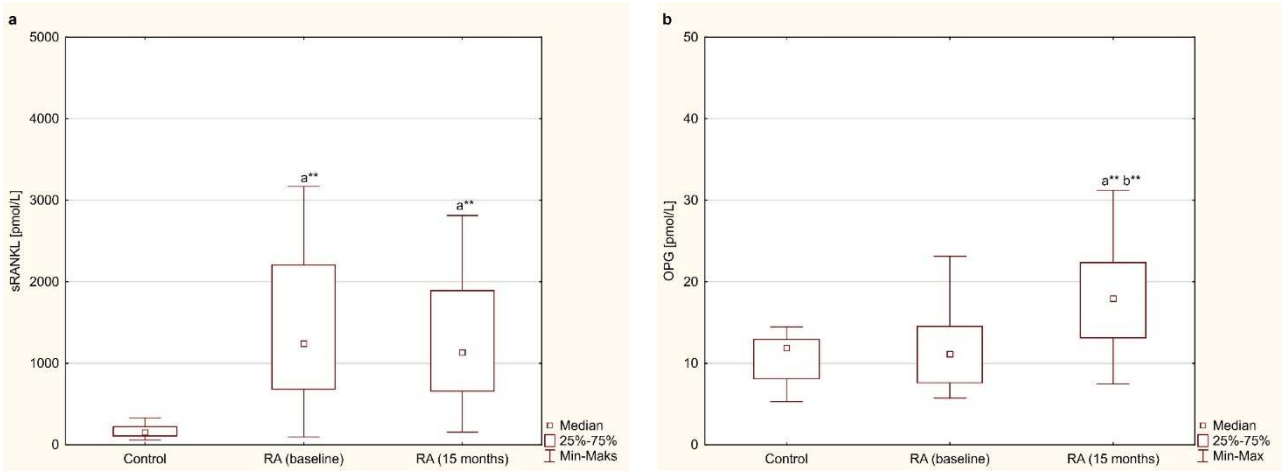

Figure 3. Effect of a 15-month TNFαI therapy on markers of osteoclastogenesis: sRANKL (**a**) and OPG (**b**) in women with rheumatoid arthritis, and in healthy women. [a]** $p < 0.001$ compared to the control, [b]** $p < 0.001$ compared to the baseline. OPG, osteoprotegerin; RA, rheumatoid arthritis; sRANKL, total soluble receptor activator of nuclear factor κB ligand; TNFαI, tumor necrosis factor α inhibitors.

When calculating bone formation/resorption ratios (PINP/CTX-I and sRANKL/OPG), which better reflect bone turnover in RA, the PINP/CTX-I ratio increased significantly after 15 months of treatment compared to the baseline ($p < 0.001$; Table 4), reaching the same value as in healthy people ($p = 0.469$; Table 4). On the other hand, the sRANKL/OPG ratio was significantly higher in women with RA before and after 15 months of anti-TNF-α therapy compared to healthy subjects ($p < 0.001$ for both; Table 4). The applied treatment resulted in a significant decrease in the sRANKL/OPG ratio in women with RA ($p < 0.001$; Table 4), but was still higher than in healthy women ($p < 0.001$; Table 4).

Table 4. The ratios of PINP to CTX-I and sRANKL to OPG in women with rheumatoid arthritis at baseline and after 15 months of anti-TNF-α therapy, and in healthy women.

Parameter	Healthy Subjects	RA Patients ($n = 31$)		p		
		Time after Initiation of TNFαI Therapy				
	A	Baseline (T_0) B	15 Months (T_3) C	A vs. B	A vs. C	B vs. C
PINP/CTX-I ratio	33.79 (25.15–48.31)	12.30 (6.69–20.20)	30.74 (14.51–50.06)	<0.001	NS	<0.001
sRANKL/OPG ratio	14.77 (10.20–23.97)	107.21 (39.20–190.12)	52.87 (34.25–94.28)	<0.001	<0.001	<0.001

Results are expressed as median, inter-quartile (25th–75th percentile) range. CTX-I, C-terminal cross-linking telopeptides of type I collagen; NS, not significant; OPG, osteoprotegerin; PINP, N-terminal propeptides of type I procollagen; RA, rheumatoid arthritis; sRANKL, total soluble receptor activator of nuclear factor κB ligand; TNFαI, tumor necrosis factor α inhibitors.

3.5. Analysis of the Relationship between Bone Turnover Markers, Osteoclastogenesis Markers as Well as Clinical and Laboratory Indicators of Disease Activity

The analysis of the relationship between selected biomarkers of both bone turnover (PINP, CTX-I and PINP/CTX-I ratio) and osteoclastogenesis (sRANKL, OPG, sRANKL/OPG ratio) as well as clinical (DAS 28-ESR) and laboratory (ESR, CRP) indicators of disease activity in RA patients at the beginning and after 15 months of anti-TNF-α therapy are presented in Table 5. However, no significant correlations were found between the biomarkers of bone turnover and osteoclastogenesis with any of the parameters assessed.

Table 5. The relationship between markers of bone turnover and osteoclastogenesis markers as well as PINP/CTX-I, sRANKL/OPG ratios, and clinical and laboratory indicators of disease activity in women with rheumatoid arthritis at the baseline and after 15 months of anti-TNF-α therapy.

Parameter	RA Patients (n = 31) Time after Initiation of TNFαI Therapy					
			Baseline (T_0)			
	PINP	CTX-I	PINP/CTX-I	sRANKL	OPG	sRANKL/OPG
CRP	−0.167 ^{NS}	−0.216 ^{NS}	0.03 ^{NS}	0.238 ^{NS}	−0.05 ^{NS}	0.302 ^{NS}
ESR	0.044 ^{NS}	−0.075 ^{NS}	0.213 ^{NS}	0.247 ^{NS}	0.233 ^{NS}	0.176 ^{NS}
DAS28-ESR	0.012 ^{NS}	−0.216 ^{NS}	0.132 ^{NS}	−0.221 ^{NS}	0.07 ^{NS}	−0.207 ^{NS}
SJC	−0.130 ^{NS}	−0.223 ^{NS}	0.068 ^{NS}	−0.307 ^{NS}	0.001 ^{NS}	−0.305 ^{NS}
TJC	−0.027 ^{NS}	0.0245 ^{NS}	−0.088 ^{NS}	−0.044 ^{NS}	−0.236 ^{NS}	−0.320 ^{NS}
VAS	0.105 ^{NS}	0.146 ^{NS}	−0.097 ^{NS}	−0.047 ^{NS}	0.101 ^{NS}	−0.160 ^{NS}
			15 Months (T_3)			
CRP	−0.08 ^{NS}	0.319 ^{NS}	−0.333 ^{NS}	−0.03 ^{NS}	−0.128 ^{NS}	0.059 ^{NS}
ESR	0.202 ^{NS}	0.067 ^{NS}	0.145 ^{NS}	0.169 ^{NS}	0.285 ^{NS}	0.091 ^{NS}
DAS28-ESR	0.185 ^{NS}	0.112 ^{NS}	0.054 ^{NS}	0.193 ^{NS}	0.255 ^{NS}	0.077 ^{NS}
SJC	0.353 ^{NS}	0.085 ^{NS}	0.097 ^{NS}	0.000 ^{NS}	−0.036 ^{NS}	0.012 ^{NS}
TJC	−0.028 ^{NS}	−0.028 ^{NS}	0.0176 ^{NS}	0.097 ^{NS}	−0.214 ^{NS}	0.051 ^{NS}
VAS	0.082 ^{NS}	0.040 ^{NS}	−0.047 ^{NS}	0.071 ^{NS}	0.322 ^{NS}	−0.091 ^{NS}

Results are expressed as r (correlation coefficient) according to Spearman rank correlation. Correlations were considered significant at: $p < 0.0083$ by applying a Bonferroni correction. DAS 28-ESR, disease activity score 28; CTX-I, C-terminal cross-linking telopeptides of type I collagen; ESR, erythrocyte sedimentation rate; ^{NS}, not significant; OPG, osteoprotegerin; PINP, N-terminal propeptides of type I procollagen; RA, rheumatoid arthritis; SJC, swollen joint count; sRANKL, total soluble receptor activator of nuclear factor κB ligand; TJC, tender joint count; TNFαI, tumor necrosis factor α inhibitors.

3.6. Effect of Anti-TNF-α Treatment on Bone Mineral Density

In the RA patients, anti-TNF-α treatment halted further generalized bone loss over 15 months. During the 15-month anti-TNF-α therapy, no significant difference was found in the BMD of vertebrae (L2–L4) and femoral neck ($p = 0.290$ and $p = 0.513$, respectively; Table 3). Moreover, the rates of T-score and Z-score for L2–L4 vertebral ($p = 0.838$ and $p = 0.510$, respectively; Table 3) as well as femur neck ($p = 0.280$ and $p = 0.09$, respectively; Table 3) did not differ in women with RA before anti-TNF-α treatment compared to the values in 15 months of these therapy.

3.7. Bone Metabolism Markers and PINP/CTX-I, sRANKL/OPG Ratios Depending on the Type of TNF-α Inhibitor Used

In this study, we assessed whether the type of TNF-α inhibitor used had an effect on the levels of bone turnover markers. We compared changes in serum levels of bone turnover markers (PINP, CTX-I, PINP/CTX-I ratio) and osteoclast regulators (sRANKL, OPG, sRANKL/OPG ratio) in females with RA who completed a 15-month anti-TNF-α therapy with ETA or ADA. The effect of certolizumab pegol was not assessed because in this study only two patients were treated with this drug and completed 15 months of treatment with a TNF-α inhibitor (Table 3).

Administration of either ETA or ADA led to a significant increase in serum PINP and OPG levels, and decrease in serum CTX-I levels in women with RA after 15 months of therapy ($p < 0.001$, $p < 0.001$, and $p < 0.01$, respectively; Figure 4a,b,d). Moreover, there were no significantly changes in sRANKL concentration during 15 months anti-TNF-α therapy ($p = 0.326$, Figure 4c). We also demonstrated that therapy with either ETA or ADA significantly increased the PINP/CTX-I ratio ($p < 0.01$; Figure 5a), and decreased the sRANKL/OPG ($p < 0.001$; Figure 5b). There were no significant changes in the serum levels of the assessed bone turnover markers, PINP, CTX-I and PINP/CTX-I ratio ($p = 0.713$, $p = 0.891$ and $p = 0.576$, respectively; Figures 4a,b and 5a) and osteoclast regulators, sRANKL, OPG, and sRANKL/OPG ratio depending on the type of TNF-α inhibitor used ($p = 0.450$, $p = 0.345$, and $p = 0.841$, respectively; Figures 4c,d and 5b).

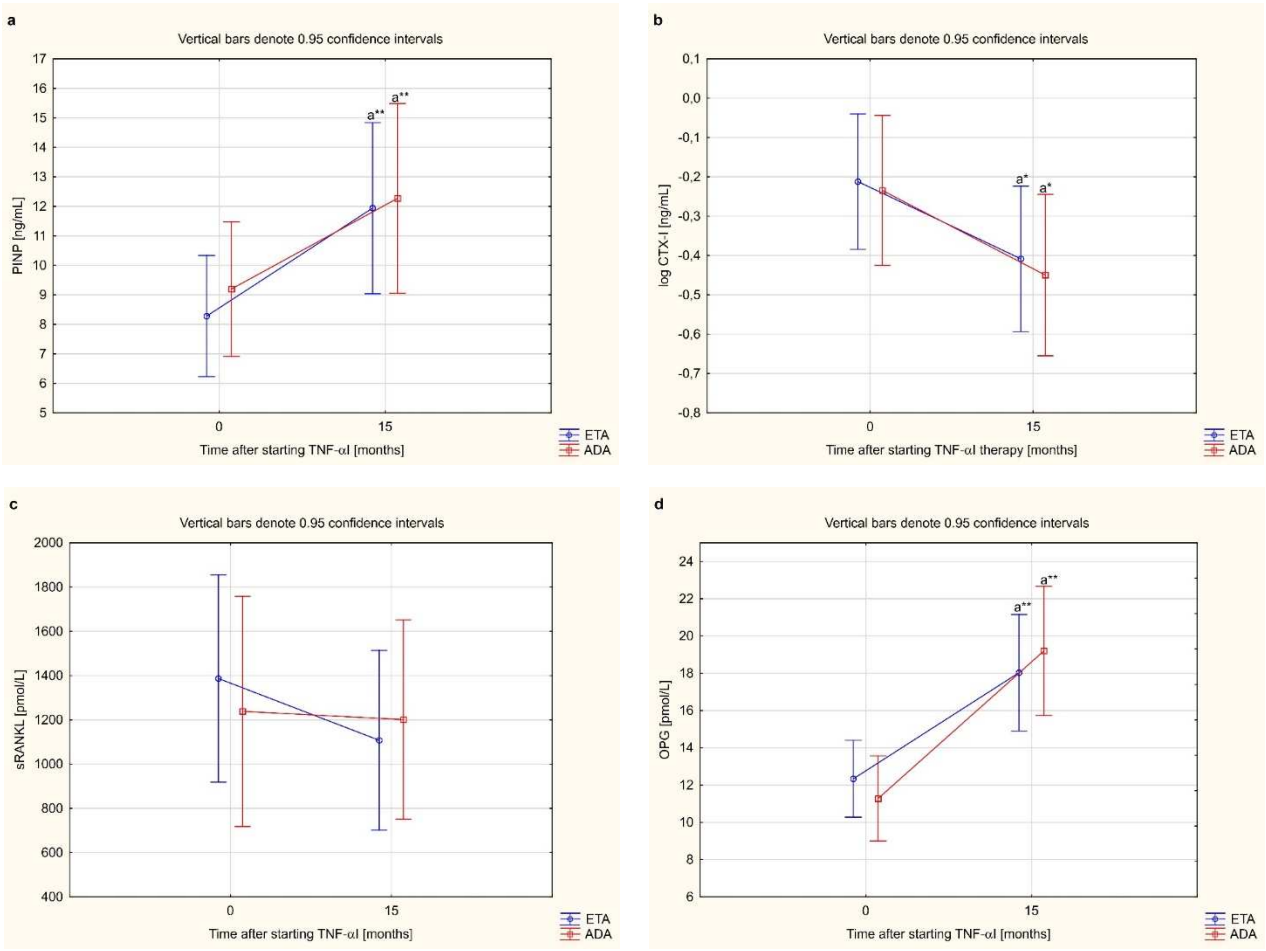

Figure 4. The effect of type TNF-α inhibitor used on serum levels of bone turnover markers, i.e. PINP (**a**), CTX-I (**b**) and osteoclastogenesis markers: sRANKL (**c**) and OPG (**d**) in female rheumatoid arthritis patients during 15-month therapy. ^{a*} $p < 0.01$ or ^{a**} $p < 0.001$ compared to the baseline. ADA, adalimumab; CTX-I, C-terminal cross-linking telopeptides of type I collagen; ETA, etanercept; OPG, osteoprotegerin; PINP, N-terminal propeptides of type I procollagen; RA, rheumatoid arthritis; sRANKL, total soluble receptor activator of nuclear factor κB ligand; TNFαI, tumor necrosis factor α inhibitors.

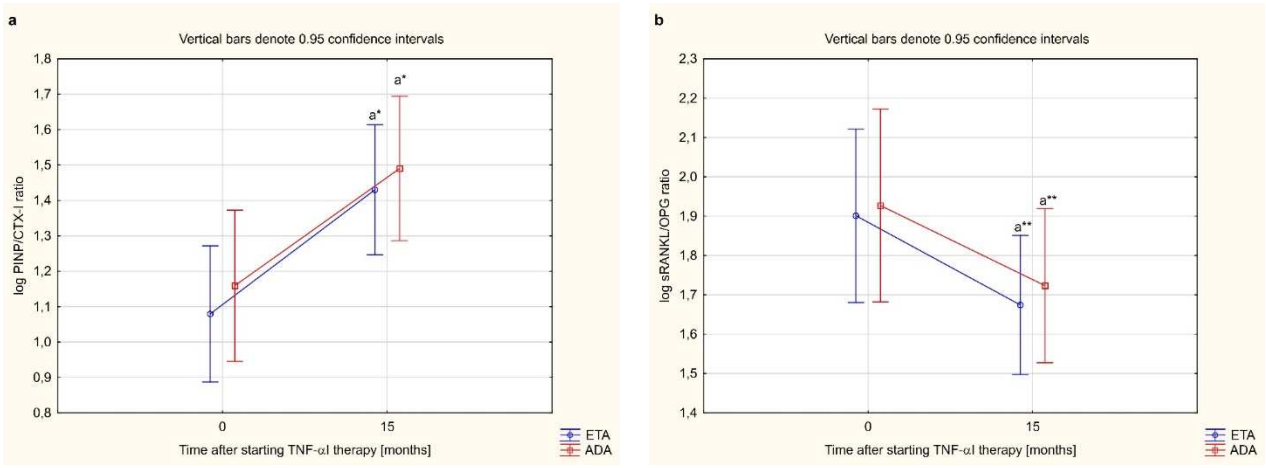

Figure 5. The effect of type TNF-α inhibitor used on PINP/CTX-I (**a**) and sRANKL/OPG ratios (**b**) in female rheumatoid arthritis patients during 15-month therapy. ^{a*} $p < 0.01$ or ^{a**} $p < 0.001$ compared to the baseline. ADA, adalimumab; CTX-I, C-terminal cross-linking telopeptides of type I collagen; ETA, etanercept; OPG, osteoprotegerin; PINP, N-terminal propeptides of type I procollagen; RA, rheumatoid arthritis; sRANKL, total soluble receptor activator of nuclear factor κB ligand; TNFαI, tumor necrosis factor-α inhibitors.

4. Discussion

Bone erosion is one of the radiographic hallmarks of RA. Periarticular bone loss in patients with RA results from excessive local bone resorption and inadequate bone repair [3,5,8,10]. The evaluation of bone turnover markers (BTMs) in body fluids (i.e., synovial fluid, blood, and urine) provides information on the dynamics of bone matrix turnover and reflects the disease activity in bone tissue [31,32]. Approximately 90% of the organic matrix of bone tissue is type I collagen [33]. During type I collagen synthesis, two extension peptides from both ends of the procollagen molecule, C-terminal propeptide of type I procollagen and N-terminal propeptide of type I procollagen, are enzymatically removed from the structure and released into the circulation. Although type I collagen propeptides may also arise from other tissues such as skin, dentin, tendon, and cartilage, these tissues exhibit a slower turnover than bone and make a very small contribution to the pool of circulating propeptide. Thus, PICP and PINP serum concentrations depend mainly on intrinsic bone formation. Meanwhile, C-terminal and N-terminal crosslinking telopeptides of type I collagen are formed during bone collagen breakdown with the enzyme cathepsin K, and their serum levels indicate bone resorption and osteoclast activation [31,32,34]. In the present study, we evaluated the usefulness of BTMs in predicting and monitoring bone metabolism changes during 15-months of anti-TNF-α therapy in female patients with RA. We demonstrated that the effective 15-month anti-inflammatory treatment with TNFαI was associated with improvement in bone metabolism, assessed through serum levels of matrix products released during bone formation (PICP, PINP) and bone resorption (CTX-I, NTX-I). Indeed, higher levels of PINP and lower levels of CTX-I were noted under anti-TNF-α treatment. Thus, circulating PINP and CTX-I turned out to be useful for monitoring bone turnover during TNFαI therapy. Similar results regarding persistent decrease in bone resorption markers and improvement in bone formation markers have been reported in other studies evaluating the effect of therapy with biological agents on bone turnover [21–23,35,36]. Vis et al. [22] described a decrease in the bone resorption marker (i.e., cross-linked C-terminal telopeptides of type I collagen (ICTP)), and an increase in the bone formation markers (i.e., PINP and osteocalcin (OC)) after six weeks of treatment with infliximab in RA patients. Chopin et al. [21] also observed this positive effect in a study with RA patients treated with infliximab for one year. There was an initial decrease in bone resorption marker (i.e., CTX-I) at week 6 and 22, which returned to pretreatment levels at week 54 [21].

In our study, the PINP/CTX-I ratio, which better reflects the bone turnover in RA, was also calculated. We noticed an increase in the PINP/CTX-I ratio in women with RA after 15 months of treatment with TNF-α inhibitors when compared to the baseline. Consistently with our results, Wheater et al. [36] and Chopin et al. [21] demonstrated that effective treatment of RA patients with biologic drugs (rituximab or infliximab) was associated with decreased serum levels of CTX-I and increased PINP/CTX-I ratio, which indicates in favor of bone formation. Moreover, Fassio et al. [37] demonstrated that the effective treatment of RA patients with certolizumab pegol in combination with MTX was associated with an increase in serum levels of PINP and a decrease in serum levels of C-terminal cross-linking telopeptides of type I collagen caused by cathepsin K, suggesting increased synthesis and decreased degradation of the bone matrix components, respectively.

To conclude, we report that the combination of 15-month anti-TNF-α therapy with MTX provides rapid clinical response and, additionally, has a beneficial effect on bone turnover. Thus, increased serum PINP levels accompanied by significantly reduced CTX-I levels under anti-TNF-α treatment indicate a shift of bone turnover toward bone formation, which potentially represents an important mechanism for preventing future bone damage and disability in RA patients. Anti-TNF-α therapy may thus provide the ability, or at least an opportunity, to repair the damaged bone matrix due to effective disease control and inflammation suppression [13,28,38,39]. Previous studies have shown that inhibiting TNF-α in RA reduces local osteoarthritis through a reduction in synovial cell infiltration

and the expression of adhesion molecules, chemokines, and cytokines, coinciding with reduced levels of acute phase reactants such as CRP and interleukin (IL)-6 [28].

We also attempted to evaluate the effect of TNFαI therapy on bone mineral density in female RA patients. In RA patients, BMD was inversely correlated with serum levels of TNF-α [13,39,40]. Thus, treatment with TNF-α inhibitors may modify BMD during this therapy. In our study, we found no significant difference in BMD of vertebrae (L2−L4) and femoral neck during 15-month anti-TNF-α therapy. It can therefore be concluded that alterations in bone marker levels (PINP and CTX-I) in response to TNFαI treatment occur earlier than changes in bone mineral density. This may indicate that anti-TNF-α treatment in female RA patients stabilized bone loss within 15-months. The anti-TNF-α therapy has also been reported to arrest bone loss in the lumbar spine and hip in longitudinal studies [18,22,41]. On the other hand, Hoff et al. [42] reported that adalimumab combined with methotrexate resulted in less hand bone loss than the use of either ADA or MTX monotherapies. Therefore, these last findings suggest that the benefits of anti-TNF-α therapy may not be limited to the control of inflammation, but also include being able to suppress the direct effect of TNF-α on osteoclast activation [41,43].

The changes in serum levels of bone resorption and bone formation markers observed in our study during therapy with TNFαI are most likely mediated by the RANKL/RANK/OPG signaling pathway. In the complex system of bone remodeling that involves the sequential phases of activation, resorption, reversal, formation, and termination, the RANKL/OPG pathway is the connecting factor between bone resorption and bone formation [12,38,44,45]. The balance between RANKL and OPG determines the degree of proliferation and activity of the osteoclasts [44,46]. The RANKL/OPG ratio, known as the regulator of osteoclastogenesis, represents an important determinant of bone resorption. In most of the cases, both RANKL upregulation and OPG downregulation lead to bone loss [45]. Moreover, as previously mentioned, various proinflammatory cytokines regulate expression of RANKL and OPG including TNF-α and IL-1 [46,47]. It was found that the high RANKL/OPG ratio in active RA is associated with increased radiographic damage in active RA [47]. Thus, the signaling and regulation of RANKL and OPG activity may play a critical role in bone loss associated with RA [44,46,47].

We have found that circulating levels of sRANKL were much higher in women with RA before anti-TNF-α treatment than in healthy subjects of the same age. Our finding of increased sRANKL in RA patients was similar to that revealed in many other studies [46,48,49]. Previously, Ziołkowska et al. [48], and Xu et al. [46], also reported elevated sRANKL levels in RA patients when compared to controls. High levels of RANKL were found in synovial fluid of patients with RA [50] in previous studies. Elevated RANKL in RA is probably related to the effect of activity of inflammatory cytokines, such as TNF-α, IL-1, IL-6, and IL-17, with regard to synovial fibroblasts [51]. In addition, patients with early RA who had high levels of sRANKL and low levels of OPG in synovial fluid experienced more rapid progression of the disease towards destruction of joints and bones [12].

We did not find any significant change in sRANKL in female RA patients undergoing treatment with TNFαI. Similar results were reported after anti-TNF-α treatment [52] and by study on treatment with methotrexate only [51]. However, some studies have found normalization or reduction of elevated sRANKL levels after treatment with both TNFαI [48] and DMARDs regimens [53]. These discrepancies can result from methodological differences, especially with regard to the specificity of antibodies used in enzyme immunoassays, capable of recognizing soluble RANKL present in the serum as a free or OPG-bound molecule. Furthermore, since sRANKL may originate from sources other than bone, it is reasonable that the circulating sRANKL levels may not entirely reflect the bone microenvironment in RA patients following the anti-TNF-α treatment [54,55]. In addition, the lack of decrease in sRANKL levels in women undergoing TNFαI therapy that we found during our research may be related to patient age. In the studies of Ziołkowska et al. [48], it was demonstrated that the reduction in sRANKL levels caused by anti-TNF-α treatment was more significant among older RA patients rather than young ones.

As has been mentioned earlier, the balance between bone breakdown and formation is modulated to a large extent by the OPG [56]. The latter one is secreted by osteoblasts, as well as by other cell types, including peripheral blood lymphocytes, endothelial cells (ECs), and vascular smooth muscle cells (SMCs) [54,56,57]. We found that circulating levels of OPG, a member of tumor necrosis factor receptor superfamily 11B (TNFRSF11B) in women with RA before treatment with TNFαI did not differ from those in healthy subjects, while the applied treatment resulted in a significant increase in OPG in women with RA. Our findings differ from those of Ziołkowska et al. [48] and Xu et al. [46], who demonstrated that the levels of OPG were higher in serum of RA patients when compared to healthy controls. On the other hand, Fadda et al. [49] found a decrease in serum OPG and an increase in serum RANKL in RA patients. Such major discrepancies in OPG levels found by different authors may be related, among others, to the impact of various factors on the levels of this marker. Ziołkowska et al. [48] underlined the impact of age on OPG levels. These authors found a significant increase in OPG levels only in RA patients below the age of 48 [48]. Meanwhile, the increased OPG levels in women after 15-month TNFαI therapy that we revealed in our studies may constitute a compensation mechanism limiting bone erosion, as in the case of increased bone turnover disorders, such as osteoporosis [58]. Moreover, previous studies have reported that balanced bone turnover, protects against the progression of vascular calcification and the occurrence of cardiovascular events [59]. It is well known that inflammation which occurs in the RA is one of the main factors responsible for the premature, rapid development of atherosclerosis, as well as for increased morbidity and mortality due to cardiovascular diseases [60,61]. High amounts of OPG can be found in the arterial wall, and the concentration found in human aorta extracts has been at least as high as in the bone extracts and even 1000 times higher in comparison to the concentration in plasma [62]. This may suggest that arterial SMCs might be major contributors to the circulating pool of OPG in RA patients. However, little is known about the functions of OPG in the arterial wall. Experimental data and clinical observations appear rather conflicting [62]. Although several studies have shown that high levels of circulating OPG are associated with cardiovascular mortality in elderly women and cardiovascular disease in the general population, vessel wall-derived OPG was also found to protect from atherosclerosis and vascular calcification in apolipoprotein E-deficient (ApoE-/-) mice [54,57,63–66]. Thus, these studies point to an active role for OPG in the maintenance of cardiovascular homeostasis. However, more evidence is needed to evaluate the predictive and diagnostic value of serum OPG levels for clinical use as a potential marker of CVD risk. To summarize, we can suppose that increased circulating levels of OPG following anti-TNF-α therapy may represent an insufficient compensatory self-defensive mechanism aimed at preventing further vascular damage in patients with RA. This is consistent with the unchanged serum calcium levels found in our study in women with RA after 15 months of anti-TNF-α treatment.

Previous studies have indicated that the RANKL/OPG ratio has a better prognostic value in assessing bone metabolism or the effectiveness of the therapy applied than the results of quantitative analysis of each of these molecules carried out in separation [12,34,50, 67]. As has been demonstrated, the sRANKL/OPG ratio may be a determinant of activation in bone resorption: a high RANKL/OPG ratio is a better indicator of osteoclastogenesis and, therefore, of bone erosion in RA [52]. Moreover, it better reflects the combined effect of the two opposing osteoimmunological mediators (sRANKL and OPG). Several clinical studies have shown that the sRANKL/OPG ratio in serum or synovial fluid may predict the progression of joint and bone destruction in RA patients [16,52]. Furthermore, a recent study demonstrated that the RANKL/OPG ratio independently predicted annual radiological damage over 11 years in early RA [16].

Our study has shown that the sRANKL/OPG ratio was significantly higher in women with RA before and after 15 months of anti-TNF-α therapy when compared to healthy subjects. However, the treatment applied resulted in a significant decrease in the sRANKL/OPG ratio in women with RA. Results similar to ours were found in previous studies [48,50].

Catrina et al. [50] demonstrated a decreased expression of RANKL/OPG in synovial tissue caused by anti-TNF-α therapy through upregulation of OPG. The research so far has shown that systemic TNF-α contributes directly to increased presence of osteoclast precursors in mice, which can be reversed by applying anti-TNF antibody-based drugs [68,69]. Moreover, anti-TNF-α treatment in psoriatic arthritis patients reduced the number of peripheral osteoclast precursors [50,70]. It is plausible, therefore, that the bone-protective effect of TNFαI that we found in our study and that can be seen in women with RA receiving treatment with either ETA or ADA is, at least partially, mediated through the RANKL/OPG pathway.

Regarding the effects of the type of TNF-α inhibitor employed in our study (i.e., etanercept or adalimumab) on the levels of bone turnover markers (PINP, CTX-I) and osteoclast regulators (sRANKL, OPG) in female RA patients, our results have not demonstrated any superiority of ETA therapy in terms of preventing bone damage when compared to ADA. However, a definitive confirmation of our results require further studies in light of the relatively small number of patients in the studied groups.

Our study has several potential limitations. First, the group of women with RA was relatively small but carefully selected according to the Polish National Health Fund Therapeutic Programs that employ TNF-blockers (i.e., B.33: "Treatment of aggressive forms of rheumatoid arthritis (RA) and juvenile idiopathic arthritis (JIA)" (03.0000.333.02) or B.45: "Treatment of an aggressive form of rheumatoid arthritis (03.0000.345.02)"). Moreover, in our study, 19 (38%) female patients discontinued TNFαI treatment due to the following reasons: no response in two patients, loss of response in three patients, intolerance in three patients, surgical procedures in four patients, and withdrawal of consent for participation in the therapy by four patients. The relatively small number limits the potential for detecting smaller changes in variables and although 31 patients responded well to TNFαI treatment, the group was heterogeneous, consisting of pre- and postmenopausal women. Second, PICP as a bone formation marker and NTX-I as a resorption marker have a limited role in the evaluation of bone remodeling for the purposes of assessing the effectiveness of TNFαI therapy. PICP is cleared by the mannose receptor, which is also regulated by thyroid hormone and growth hormone. NTX-I measurements are altered in the cases of liver and renal failure. Another limitation of this study is the fact that both RANKL and OPG levels can be assayed in serum and reflect the production coming from several tissues. Therefore, the circulating levels of osteoclast regulatory proteins may not entirely reflect the bone microenvironment.

5. Conclusions

In conclusion, we report that a 15-month TNFαI therapy combined with MTX not only leads to clinical response, but also provides a beneficial effect on bone turnover. Changes in bone turnover marker levels occur independently of changes in BMD, which may suggest an important role for PINP and CTX-I as short-term tools to monitor bone turnover in RA patients treated with TNFαI. Our study has also demonstrated that TNFαI therapy modulates the RANKL/OPG pathway, a potential mechanism that could explain the improvement of the PINP/CTX-I ratio in RA patients follow treatment with anti-TNF-α inhibitors. Furthermore, the bone metabolism changes in women with RA were independent of the type of TNFαI used.

Author Contributions: Conceptualization, A.J.-P. and A.S.; Methodology, A.J.-P. and A.S.; Formal analysis, A.J.-P., A.S. and K.K.-V.; Investigation, A.J.-P. and A.S.; Data curation, A.J.-P., A.S. and A.Z.-G.; Writing—original draft preparation, A.J.-P. and A.S.; Writing—review and editing, K.K.-V. and K.O.; Supervision, K.K.-V., Project administration, A.S.; Funding acquisition, A.S. and K.O. All authors have read and agreed to the published version of the manuscript.

Funding: This study was funded by the National Science Center, Poland (Grant No. 2013/09/N/NZ5/00815) and by the Medical University of Silesia, Katowice, Poland (Grant No. KNW-2-O19/N/9/K).

Institutional Review Board Statement: This study was approved by the Ethical Committee of the Medical University of Silesia in Katowice (KNW/0022/KB/56/I/12/13). All participants gave written informed consent prior to the study, and research was carried out in accordance with the conditions of the Declaration of Helsinki.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data are contained within the article.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

1. McInnes, I.B.; Schett, G. The pathogenesis of rheumatoid arthritis. *N. Engl. J. Med.* **2011**, *365*, 2205–2219. [CrossRef] [PubMed]
2. Coras, R.; Murillo-Saich, J.D.; Guma, M. Circulating pro- and anti-inflammatory metabolites and its potential role in rheumatoid arthritis pathogenesis. *Cells* **2020**, *9*, 827. [CrossRef]
3. Heinlen, L.; Humphrey, M.B. Skeletal complications of rheumatoid arthritis. *Osteoporos. Int.* **2017**, *28*, 2801–2812. [CrossRef] [PubMed]
4. Adami, G.; Saag, K.G. Osteoporosis pathophysiology, epidemiology, and screening in rheumatoid arthritis. *Curr. Rheumatol. Rep.* **2019**, *21*, 34. [CrossRef] [PubMed]
5. Goldring, S.R. Differential mechanisms of de-regulated bone formation in rheumatoid arthritis and spondyloarthritis. *Rheumatology* **2016**, *55*, ii56–ii60. [CrossRef]
6. Sims, N.A.; Martin, T.J. Osteoclasts provide coupling signals to osteoblast lineage cells through multiple mechanisms. *Annu. Rev. Physiol.* **2020**, *82*, 507–529. [CrossRef] [PubMed]
7. Kenkre, J.S.; Bassett, J.H.D. The bone remodelling cycle. *Ann. Clin. Biochem.* **2018**, *55*, 308–327. [CrossRef] [PubMed]
8. Schett, G.; Gravallese, E. Bone erosion in rheumatoid arthritis: Mechanisms, diagnosis and treatment. *Nat. Rev. Rheumatol.* **2012**, *8*, 656–664. [CrossRef]
9. Karmakar, S.; Kay, J.; Gravallese, E.M. Bone damage in rheumatoid arthritis–mechanistic insights and approaches to prevention. *Rheum. Dis. Clin. N. Am.* **2010**, *36*, 385–404. [CrossRef]
10. Walsh, N.C.; Crotti, T.N.; Goldring, S.R.; Gravallese, E.M. Rheumatic diseases: The effects of inflammation on bone. *Immunol. Rev.* **2005**, *208*, 228–251. [CrossRef] [PubMed]
11. Zhao, B. TNF and bone remodeling. *Curr. Osteoporos. Rep.* **2017**, *15*, 126–134. [CrossRef] [PubMed]
12. Tobeiha, M.; Moghadasian, M.H.; Amin, N.; Jafarnejad, S. RANKL/RANK/OPG pathway: A mechanism involved in exercise-induced bone remodeling. *BioMed Res. Int.* **2020**, *19*, 6910312. [CrossRef]
13. Zerbini, C.A.F.; Clark, P.; Mendez-Sanchez, L.; Pereira, R.M.R.; Messina, O.D.; Uña, C.R.; Adachi, J.D.; Lems, W.F.; Cooper, C.; Lane, N.E. Biologic therapies and bone loss in rheumatoid arthritis. *Osteoporos. Int.* **2017**, *28*, 429–446. [CrossRef]
14. Tanaka, S. Regulation of bone destruction in rheumatoid arthritis through RANKL-RANK pathways. *World J. Orthop.* **2013**, *4*, 1–6. [CrossRef] [PubMed]
15. Kohli, S.S.; Kohli, V.S. Role of RANKL-RANK/osteoprotegerin molecular complex in bone remodeling and its immunopathologic implications. *Indian J. Endocrinol. Metab.* **2011**, *15*, 175–181. [CrossRef] [PubMed]
16. van Tuyl, L.H.; Voskuyl, A.E.; Boers, M.; Geusens, P.; Landewe, R.B.; Dijkmans, B.A.; Lems, W.F. Baseline RANKL: OPG ratio and markers of bone and cartilage degradation predict annual radiological progression over 11 years in rheumatoid arthritis. *Ann. Rheum. Dis.* **2010**, *69*, 1623–1628. [CrossRef]
17. Gulyás, K.; Horváth, Á.; Végh, E.; Pusztai, A.; Szentpétery, Á.; Pethö, Z.; Váncsa, A.; Bodnár, N.; Csomor, P.; Hamar, A.; et al. Effects of 1-year anti-TNF-α therapies on bone mineral density and bone biomarkers in rheumatoid arthritis and ankylosing spondylitis. *Clin. Rheumatol.* **2020**, *39*, 167–175. [CrossRef]
18. Wijbrandts, C.A.; Klaasen, R.; Dijkgraaf, M.G.W.; Gerlag, D.M.; van Eck-Smit, B.L.F.; Tak, P.P. Bone mineral density in rheumatoid arthritis patients 1 year after adalimumab therapy: Arrest of bone loss. *Ann. Rheum. Dis.* **2009**, *68*, 373–376. [CrossRef] [PubMed]
19. Saidenberg-Kermanac'h, N.; Corrado, A.; Lemeiter, D.; deVernejoul, M.C.; Boissier, M.C.; Cohen-Solal, M.E. TNF-alpha antibodies and osteoprotegerin decrease systemic bone loss associated with inflammation through distinct mechanisms in collagen-induced arthritis. *Bone* **2004**, *35*, 1200–1207. [CrossRef]
20. Morris, H.A.; Eastell, R.; Jorgensen, N.R.; Cavalier, E.; Vasikaran, S.; Chubb, S.A.P.; Kanis, J.A.; Cooper, C.; Makris, K.; IFCC-IOF Working Group for Standardisation of Bone Marker Assays (WG-BMA). Clinical usefulness of bone turnover marker concentrations in osteoporosis. *Clin. Chim. Acta* **2017**, *467*, 34–41. [CrossRef]
21. Chopin, F.; Garnero, P.; le Henanff, A.; Debiais, F.; Daragon, A.; Roux, C.; Sany, J.; Wendling, D.; Zarnitsky, C.; Ravaud, P.T.; et al. Long-term effects of infliximab on bone and cartilage turnover markers in patients with rheumatoid arthritis. *Ann. Rheum. Dis.* **2008**, *67*, 353–357. [CrossRef] [PubMed]

22. Vis, M.; Havaardsholm, E.A.; Haugeberg, G.; Uhlig, T.; Voskuyl, A.E.; van de Stadt, R.J.; Dijkmans, B.A.C.; Woolf, A.D.; Kvien, T.K.; Lems, W.F. Evaluation of bone mineral density, bone metabolism, osteoprotegerin and receptor activator of the NFκB ligand serum levels during treatment with infliximab in patients with rheumatoid arthritis. *Ann. Rheum. Dis.* **2006**, *65*, 1495–1499. [CrossRef]
23. Torikai, E.; Kageyama, Y.; Takahashi, M.; Suzuki, M.; Ichikawa, T.; Nagafusa, T.; Nagano, A. The effect of infliximab on bone metabolism markers in patients with rheumatoid arthritis. *Rheumatology* **2006**, *45*, 761–764. [CrossRef] [PubMed]
24. Perpétuo, I.P.; Caetano-Lopes, J.; Rodrigues, A.M.; Campanilho-Marques, R.; Ponte, C.; Canhão, H.; Ainola, M.; Fonseca, J.E. Effect of tumor necrosis factor inhibitor therapy on osteoclasts precursors in rheumatoid arthritis. *BioMed. Res. Int.* **2017**, 2690402. [CrossRef]
25. Yasunori, K.; Masaaki, T.; Tetsuyuki, N.; Hayato, K.; Akira, N. Reduction of urinary levels of pyridinoline and deoxypyridinoline and serum levels of soluble receptor activator of NF-kappaB ligand by etanercept in patients with rheumatoid arthritis. *Clin. Rheumatol.* **2008**, *27*, 1093–1101. [CrossRef]
26. Arnett, F.C.; Edworthy, S.M.; Bloch, D.A.; McShane, D.J.; Fries, J.F.; Cooper, N.S.; Healey, L.A.; Kaplan, S.R.; Liang, M.H.; Luthra, H.S.; et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum.* **1988**, *31*, 315–324. [CrossRef] [PubMed]
27. Aletaha, D.; Neogi, T.; Silman, A.J.; Funovits, J.; Felson, D.T.; Bingham, C.O., 3rd; Birnbaum, N.S.; Burmester, G.R.; Bykerk, V.P.; Cohen, M.D.; et al. 2010 rheumatoid arthritis classification criteria: An American College of Rheumatology/European League Against Rheuma-tism collaborative initiative. *Ann. Rheum. Dis.* **2010**, *69*, 1580–1588. [CrossRef]
28. Szeremeta, A.; Jura-Półtorak, A.; Zoń-Giebel, A.; Kopeć-Mędrek, M.; Kucharz, J.E.; Olczyk, K. Aggrecan turnover in women with rheumatoid arthritis treated with TNF-α inhibitors. *J. Clin. Med.* **2020**, *9*, 1377. [CrossRef]
29. Fransen, J.; van Riel, P.L. The Disease activity score and the EULAR response criteria. *Rheum. Dis. Clin. N. Am.* **2009**, *35*, 745–757. [CrossRef]
30. Myasoedova, E.; Crowson, C.S.; Kremers, H.M.; Roger, V.L.; Fitz-Gibbon, P.D.; Therneau, T.M.; Gabriel, S.E. Lipid paradox in rheumatoid arthritis: The impact of serum lipid measures and systemic inflammation on the risk of cardiovascular disease. *Ann. Rheum. Dis.* **2011**, *70*, 482–487. [CrossRef]
31. Konukoglu, D. Bone markers. *Int. J. Med. Biochem.* **2019**, *2*, 65–78. [CrossRef]
32. Civitelli, R.; Armamento-Villareal, R.; Napoli, N. Bone turnover markers: Understanding their value in clinical trials and clinical practice. *Osteoporos. Int.* **2009**, *20*, 843–851. [CrossRef] [PubMed]
33. Lin, X.; Patil, S.; Gao, Y.G.; Qian, A. The bone extracellular matrix in bone formation and regeneration. *Front. Pharmacol.* **2020**, *11*, 757. [CrossRef]
34. Fardellone, P.; Séjourné, A.; Paccou, J.; Goëb, V. Bone remodelling markers in rheumatoid arthritis. *Mediat. Inflamm.* **2014**, *2014*, 484280. [CrossRef]
35. Garnero, P.; Landewé, R.; Boers, M.; Verhoeven, A.; Van Der Linden, S.; Christgau, S.; Van Der Heijde, D.; Boonen, A.; Geusens, P. Association of baseline levels of markers of bone and cartilage degradation with long-term progression of joint damage in patients with early rheumatoid arthritis: The COBRA study. *Arthritis Rheum.* **2002**, *46*, 2847–2856. [CrossRef] [PubMed]
36. Wheater, G.; Elshahaly, M.; Naraghi, K.; Tuck, S.P.; Datta, H.K.; van Laar, J.M. Changes in bone density and bone turnover in patients with rheumatoid arthritis treated with rituximab, results from an exploratory, prospective study. *PLoS ONE* **2018**, *13*, e0201527. [CrossRef]
37. Fassio, A.; Adami, G.; Gatti, D.; Orsolini, G.; Giollo, A.; Idolazzi, L.; Benini, C.; Vantaggiato, E.; Rossini, M.; Viapiana, O. Inhibition of tumor necrosis factor-alpha (TNF-alpha) in patients with early rheumatoid arthritis results in acute changes of bone modulators. *Int. Immunopharmacol.* **2019**, *67*, 487–489. [CrossRef]
38. Llorente, I.; García-Castañeda, N.; Valero, C.; González-Álvaro, I.; Castañeda1, S. Osteoporosis in rheumatoid arthritis: Dangerous liaisons. *Front. Med.* **2020**, *7*, 601618. [CrossRef]
39. Marotte, H.; Pallot-Prades, B.; Grange, L.; Gaudin, P.; Alexandre, C.; Miossec, P. A 1-year case-control study in patients with rheumatoid arthritis indicates prevention of loss of bone mineral density in both responders and nonresponders to infliximab. *Arthritis Res. Ther.* **2007**, *9*, R61. [CrossRef]
40. Sponholtz, T.R.; Zhang, X.; Fontes, J.D.; Meigs, J.B.; Cupples, L.A.; Kiel, D.P.; Hannan, M.T.; McLean, R.R. Association between inflammatory biomarkers and bone mineral density in a community-based cohort of men and women. *Arthritis Care Res.* **2014**, *66*, 1233–1240. [CrossRef]
41. Kawai, V.K.; Stein, C.M.; Perrien, D.S.; Griffin, M.R. Effects of anti-tumor necrosis factor α agents on bone. *Curr. Opin. Rheumatol.* **2012**, *24*, 576–585. [CrossRef]
42. Hoff, M.; Kvien, T.K.; Kälvesten, J.; Elden, A.; Haugeberg, G. Adalimumab therapy reduces hand bone loss in early rheumatoid arthritis: Explorative analyses from the PREMIER study. *Ann. Rheum. Dis.* **2009**, *68*, 1171–1176. [CrossRef]
43. Hoff, M.; Kvien, T.K.; Kälvesten, J.; Elden, A.; Kavanaugh, A.; Haugeberg, G. Adalimumab reduces hand bone loss in rheumatoid arthritis independent of clinical response: Subanalysis of the PREMIER study. *BMC Musculoskelet. Disord.* **2011**, *27*, 12–54. [CrossRef]
44. Geusens, P. The role of RANK ligand/osteoprotegerin in rheumatoid arthritis. *Ther. Adv. Musculoskelet. Dis.* **2012**, *4*, 225–233. [CrossRef] [PubMed]

45. Liu, L.N.; Mao, Y.M.; Zhao, C.N.; Wang, H.; Yuan, F.F.; Li, X.M.; Pan, H.F. Circulating levels of osteoprotegerin, osteocalcin and osteopontin in patients with rheumatoid arthritis: A systematic review and meta-analysis. *Immunol. Investig.* **2019**, *48*, 107–120. [CrossRef] [PubMed]
46. Xu, S.; Wang, Y.; Lu, J.; Xu, J. Osteoprotegerin and RANKL in the pathogenesis of rheumatoid arthritis-induced osteoporosis. *Rheumatol. Int.* **2012**, *32*, 3397–3403. [CrossRef]
47. Destiani, D.P.; Naja, S.; Dewi, S.; Rahmadi, A.R.; Sulaiman, S.A.S.; Abdulah, R. Efficacy of methotrexate in reducing the risk of bone erosion in patients with rheumatoid arthritis: A systematic review of randomized controlled trials. *Osteoporos. Int.* **2021**, *32*, 805–816. [CrossRef] [PubMed]
48. Ziolkowska, M.; Kurowska, M.; Radzikowska, A.; Luszczykiewicz, G.; Wiland, P.; Dziewczopolski, W.; Filipowicz-Sosnowska, A.; Pazdur, J.; Szechinski, J.; Kowalczewski, J.; et al. High levels of osteoprotegerin and soluble receptor activator of nuclear factor kappa B ligand in serum of rheumatoid arthritis patients and their normalization after anti-tumor necrosis factor alpha treatment. *Arthritis Rheum.* **2002**, *46*, 1744–1753. [CrossRef]
49. Fadda, S.; Hamdy, A.; Abulkhair, E.; Elsify, H.M.; Mostafa, A. Serum levels of osteoprotegerin and RANKL in patients with rheumatoid arthritis and their relation to bone mineral density and disease activity. *Egypt. Rheumat.* **2015**, *37*, 1–6. [CrossRef]
50. Catrina, A.I.; Klint, E.A.; Ernestam, S.; Catrina, S.B.; Makrygiannakis, D.; Botusan, I.R.; Klareskog, L.; Ulfgren, A.K. Anti-tumor necrosis factor therapy increases synovial osteoprotegerin expression in rheumatoid arthritis. *Arthritis Rheum.* **2006**, *54*, 76–81. [CrossRef]
51. Dhooria, A.; Pandurangan, N.; Mahesh, K.V.; Sachdev, S.; Sharma, A.; Sharma, S.; Gupta, N.; Dhir, V. Circulating levels of osteoprotegerin and sRANKL and the effect of methotrexate in patients with rheumatoid arthritis. *Indian J. Rheumatol.* **2018**, *13*, 90–94.
52. González-Alvaro, I.; Ortiz, A.M.; Tomero, E.G.; Balsa, A.; Orte, J.; Laffon, A.; García-Vicuña, R. Baseline serum RANKL levels may serve to predict remission in rheumatoid arthritis patients treated with TNF antagonists. *Ann. Rheum. Dis.* **2007**, *66*, 1675–1678. [CrossRef]
53. Wechalekar, M.D.; Lester, S.; Nagpal, S.; Cole, S.; Das, A.; Hissaria, P.; Crotti, T.; Spargo, L.; Walker, J.G.; Smith, M.D.; et al. THU0070 RANKL, OPG and OSCAR but not Dkk-1 predict radiographic progression in an inception cohort of seropositive rheumatoid arthritis (RA) treated-to-target with combination conventional DMARD therapy. *Ann. Rheumat. Dis.* **2016**, *75*, 204. [CrossRef]
54. Montagnana, M.; Lippi, G.; Danese, E.; Guidi, G.C. The role of osteoprotegerin in cardiovascular disease. *Ann. Med.* **2013**, *45*, 254–264. [CrossRef] [PubMed]
55. Dovio, A.; Allasino, B.; Palmas, E.; Ventura, M.; Pia, A.; Saba, L.; Aroasio, E.; Terzolo, M.; Angeli, A. Increased osteoprotegerin levels in Cushing's syndrome are associated with an adverse cardiovascular risk profile. *J. Clin. Endocrinol. Metab.* **2007**, *92*, 1803–1808. [CrossRef] [PubMed]
56. Rochette, L.; Meloux, A.; Rigal, E.; Zeller, M.; Cottin, Y.; Vergely, C. The role of osteoprotegerin and its ligands in vascular function. *Int. J. Mol. Sci.* **2019**, *20*, 705. [CrossRef] [PubMed]
57. Sasso, G.R.; Florencio-Silva, R.; Simões, R.S.; Baracat, M.C.; Soares Júnior, J.M.; Baracat, E.C. Elevated serum osteoprotegerin levels in women: Friend or foe? *Rev. Assoc. Med. Bras.* **2015**, *61*, 524–529. [CrossRef] [PubMed]
58. Wu, X.Y.; Peng, Y.Q.; Zhang, H.; Xie, H.; Sheng, Z.F.; Luo, X.H.; Dai, R.C.; Zhou, H.D.; Wu, X.P.; Liao, E.Y. Relationship between serum levels of OPG and TGF-β with decreasing rate of BMD in native Chinese women. *Int. J. Endocrinol.* **2013**, *2013*, 727164. [CrossRef] [PubMed]
59. Cannata-Andia, J.B.; Roman-Garcia, P.; Hruska, K. The connections between vascular calcification and bone health. *Nephrol. Dial. Transpl.* **2011**, *26*, 3429–3436. [CrossRef]
60. Chen, J.; Norling, L.V.; Cooper, D. Cardiac dysfunction in rheumatoid arthritis: The role of inflammation. *Cells* **2021**, *10*, 881. [CrossRef]
61. Hansildaar, R.; Vedder, D.; Baniaamam, M.; Tausche, A.K.; Gerritsen, M.; Nurmohamed, M.T. Cardiovascular risk in inflammatory arthritis: Rheumatoid arthritis and gout. *Lancet Rheumatol.* **2021**, *3*, e58–e70. [CrossRef]
62. Nybo, M.; Rasmussen, L.M. The capability of plasma osteoprotegerin as a predictor of cardiovascular disease: A systematic literature review. *Eur. J. Endocrinol.* **2008**, *159*, 603–608. [CrossRef] [PubMed]
63. Caidahl, K.; Ueland, T.; Aukrust, P. Osteoprotegerin: A biomarker with many faces. *Arterioscler. Thromb. Vasc. Biol.* **2010**, *30*, 1684–1686. [CrossRef] [PubMed]
64. Van Campenhout, A.; Golledge, J. Osteoprotegerin, vascular calcification and atherosclerosis. *Atherosclerosis* **2009**, *204*, 321–329. [CrossRef]
65. Tschiderer, L.; Willeit, J.; Schett, G.; Kiechl, S.; Willeit, P. Osteoprotegerin concentration and risk of cardiovascular outcomes in nine general population studies: Literature-based meta-analysis involving 26,442 participants. *PLoS ONE* **2017**, *12*, e0183910. [CrossRef]
66. Callegari, A.; Coons, M.L.; Ricks, J.L.; Rosenfeld, M.E.; Scatena, M. Increased calcification in osteoprotegerin-deficient smooth muscle cells: Dependence on receptor activator of NF-κB ligand and interleukin 6. *J. Vasc. Res.* **2014**, *51*, 118–131. [CrossRef]
67. Fardellone, P.; Salawati, E.; Le Monnier, L.; Goëb, V. Bone loss, osteoporosis, and fractures in patients with rheumatoid arthritis: A review. *J. Clin. Med.* **2020**, *20*, 3361. [CrossRef]

68. Li, P.; Schwarz, E.M.; O'Keefe, R.J.; Ma, L.; Looney, R.J.; Ritchlin, C.T.; Boyce, B.F.; Xing, L. Systemic tumor necrosis factor α mediates an increase in peripheral CD11bhigh osteoclast precursors in tumor necrosis factor α–transgenic mice. *Arthritis Rheum.* **2004**, *50*, 265–276. [CrossRef]
69. Tanaka, S. Emerging anti-osteoclast therapy for rheumatoid arthritis. *J. Orthop. Sci.* **2018**, *23*, 717–721. [CrossRef]
70. Ritchlin, C.T.; Haas-Smith, S.A.; Li, P.; Hicks, D.G.; Schwarz, E.M. Mechanisms of TNF-α- and RANKL-mediated osteoclastogenesis and bone resorption in psoriatic arthritis. *J. Clin. Investig.* **2003**, *111*, 821–831. [CrossRef]

Journal of
Clinical Medicine

MDPI

Article

Adiponectin Associates with Rheumatoid Arthritis Risk in Overweight and Obesity Independently of Other Adipokines

Yuan Zhang [1,2], Linda Johansson [3], Johanna Andersson-Assarsson [4], Magdalena Taube [4], Markku Peltonen [5,6], Per-Arne Svensson [4,7], Christian Herder [8,9,10], Anna Rudin [1], Lena Carlsson [4], Solbritt Rantapää-Dahlqvist [3,†] and Cristina Maglio [1,2,11,*,†]

1 Department of Rheumatology and Inflammation Research, Sahlgrenska Academy, University of Gothenburg, SE-40530 Gothenburg, Sweden; yuan.zhang@gu.se (Y.Z.); Anna.Rudin@microbio.gu.se (A.R.)
2 Wallenberg Center for Molecular and Translational Medicine, University of Gothenburg, SE-40530 Gothenburg, Sweden
3 Public Health and Clinical Medicine/Rheumatology, Umeå University, SE-90587 Umeå, Sweden; linda.e.johansson@umu.se (L.J.); solbritt.rantapaa.dahlqvist@umu.se (S.R.-D.)
4 Department of Molecular and Clinical Medicine, Sahlgrenska Academy, University of Gothenburg, SE-41345 Gothenburg, Sweden; johanna.andersson@medic.gu.se (J.A.-A.); magdalena.taube@wlab.gu.se (M.T.); per-arne.svensson@medic.gu.se (P.-A.S.); lena.carlsson@medic.gu.se (L.C.)
5 Public Health Promotion Unit, National Institute for Health and Welfare, FI-00271 Helsinki, Finland; markku.peltonen@thl.fi
6 Department of Neurobiology, Care Sciences and Society, Karolinska Institutet, SE-17164 Solna, Sweden
7 Institute of Health and Care Sciences, Sahlgrenska Academy, University of Gothenburg, SE-41346 Gothenburg, Sweden
8 Institute for Clinical Diabetology, German Diabetes Center, Leibniz Center for Diabetes Research at Heinrich-Heine University Duesseldorf, 40225 Duesseldorf, Germany; christian.herder@ddz.de
9 German Center for Diabetes Research (DZD), Partner Düsseldorf, 85764 Neuherberg, Germany
10 Department of Endocrinology and Diabetology, Medical Faculty and University Hospital Düsseldorf, Heinrich-Heine-University Düsseldorf, 40204 Düsseldorf, Germany
11 Rheumatology Clinic, Sahlgrenska University Hospital, Region Västra Götaland, SE-41345 Gothenburg, Sweden
* Correspondence: cristina.maglio@gu.se
† Equal contribution.

Citation: Zhang, Y.; Johansson, L.; Andersson-Assarsson, J.; Taube, M.; Peltonen, M.; Svensson, P.-A.; Herder, C.; Rudin, A.; Carlsson, L.; Rantapää-Dahlqvist, S.; et al. Adiponectin Associates with Rheumatoid Arthritis Risk in Overweight and Obesity Independently of Other Adipokines. *J. Clin. Med.* **2021**, *10*, 2791. https://doi.org/10.3390/jcm10132791

Academic Editor: Katarzyna Komosinska-Vassev

Received: 24 May 2021
Accepted: 22 June 2021
Published: 25 June 2021

Abstract: We recently reported that increased serum adiponectin was associated with rheumatoid arthritis (RA) risk in subjects with obesity. We hereby aim to determine if other adipokines associate with RA risk and if the association between adiponectin and RA is independent of other adipokines. Two nested-case control studies were performed in two different cohorts: 82 participants of the Swedish Obese Subjects (SOS) study who developed RA during follow-up matched with 410 controls, and 88 matched pairs from the Medical Biobank of Northern Sweden. Baseline levels of circulating adipokines were measured using ELISA. In a multivariable analysis in the SOS cohort, higher adiponectin was associated with an increased risk of RA independently of other adipokines (OR for RA risk: 1.06, 95% CI: 1.01–1.12, $p = 0.02$). No association between leptin, resistin, and visfatin levels and the risk of RA was detected. In the cohort from the Medical Biobank of Northern Sweden, higher adiponectin was associated with an increased risk of RA only in participants with overweight/obesity (OR: 1.17, 95% CI: 1.01−1.36, $p = 0.03$), independently of other adipokines. Our results show that in individuals with overweight/obesity, higher circulating levels of adiponectin, but not leptin, resistin, or visfatin, were associated with an increased RA risk.

Keywords: adiponectin; adipokines; rheumatology; obesity; overweight; case control study

1. Introduction

Adipose tissue is the largest endocrine organ in the human body, which produces large amounts of cell-signaling proteins, called adipokines [1]. Adipokines, such as adiponectin, leptin, resistin, and visfatin, are important regulators of metabolism [2]. In obesity, the balance between pro- and anti-inflammatory adipokines shifts to create a pro-inflammatory environment in the adipose tissue contributing to the chronic low-grade inflammation and the metabolic disorders that characterize obesity [2]. However, obesity is not only a risk factor for metabolic disturbances such as type 2 diabetes or metabolic syndrome but also for inflammatory diseases such as gout, psoriasis, and rheumatoid arthritis (RA) [3–7]. Adipokines, by virtue of their regulatory role in the integration between metabolism and systemic inflammation, are candidates to be involved in the pathogenesis of inflammatory diseases [8,9].

Several adipokines have been found elevated in blood from patients with RA, a systemic inflammatory disease affecting mainly the joints [10]. However, it is unknown whether they contribute to the development of the disease or if they are unspecific markers of inflammation [9]. An interesting example is adiponectin, which is the most abundant adipokine circulating in blood [11]. As opposed to other adipokines, circulating adiponectin levels are low in patients with obesity, type 2 diabetes, and metabolic syndrome [12,13]. On the other hand, circulating adiponectin levels are elevated in inflammatory conditions such as RA, and synovial fluids from patients with RA show higher concentrations of adiponectin compared to those from patients with osteoarthritis [9,14].

Other adipokines, such as leptin, resistin, and visfatin, have been associated with RA. Amongst those adipokines, leptin is the one with the most robust association with RA, as high circulating leptin levels correlate with RA disease activity and leptin is also able to stimulate RA fibroblast-like synoviocytes to produce pro-inflammatory cytokines [15,16]. Circulating resistin levels have also been associated with RA, although with inconsistent results [17,18]. However, despite some controversial results, it is generally agreed that resistin is involved in the pathogenesis of RA. Intra-articular injection with resistin causes joint inflammation similar to RA in a murine model, and recombinant resistin is able to induce the production of pro-inflammatory factors from human blood mononuclear cells and fibroblast-like synoviocytes from patients with RA [19,20]. Visfatin is an adipokine mainly produced by visceral adipose tissue and by neutrophils [8]. Patients with RA have elevated levels of visfatin in blood and in inflamed synovial tissue compared to controls, and visfatin is shown to trigger the motility and cytokine production in fibroblast-like synoviocytes [21,22].

Blood samples from patients who later developed RA show signs of inflammation several years before the disease onset. Autoantibodies, cytokines, and biomarkers of inflammation can be elevated up to 10 years before the first signs of RA [23,24]. We have recently shown that serum adiponectin at baseline associated with the future development of RA independently of other risk factors in the Swedish Obese Subjects (SOS) study, a large cohort of patients with obesity followed up for up to 29 years [25].

The objective of the current study was to determine if circulating adipokines other than adiponectin also associate with the future development of RA and if the association between circulating levels of adiponectin and higher risk of RA is independent of other adipokines. To answer these questions, we performed two nested-case control studies, one within the SOS study, which includes only patients with obesity, and the other one at a general population level within the Medical Biobank of Northern Sweden.

2. Results

2.1. Baseline Characteristics of the Nested Case-Control Cohort from the SOS Study

Baseline characteristics of the 82 cases before the development of RA and the 410 matched controls are shown in Table 1. As a result of the matching procedure, the two groups had similar characteristics regarding age, the proportion of men/women, body mass index (BMI), smokers/non-smokers, and patients who underwent bariatric surgery or received

conventional treatment for obesity. Cases had higher levels of C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) than the matched controls. No significant difference was observed in the serum levels of adipokines between the two groups. We performed a Spearman's correlation test, which showed a poor or mild correlation among adipokine levels (Spearman's coefficients < 0.5 for all the combinations).

Table 1. Baseline characteristics of the nested case-control cohort from the SOS study.

Characteristic	Cases (n = 82)	Matched Controls (n = 410)	p-Value
Age, years *	48 ± 6	48 ± 6	0.91
Men, No (%) *	17 (21)	85 (21)	0.99
BMI, kg/m^2 *	42 ± 5	41 ± 4	0.60
Surgery group, No (%) *	43 (52)	218 (53)	0.90
Current or previous smoking, No (%) *	18 (22)	85 (21)	0.80
Diabetes, No (%)	7 (9)	45 (11)	0.50
CRP, mg/L	10 ± 12	7 ± 7	0.001
ESR, mm/h	18 ± 11	15 ± 10	0.048
Adiponectin, ng/mL	9041 ± 4766	8068 ± 4622	0.08
Leptin, ng/mL	45 ± 32	49 ± 29	0.26
Resistin, ng/mL	12 ± 6	12 ± 6	0.84
Visfatin, ng/mL	2.8 ± 2.3	2.6 ± 1.9	0.35

Data are shown as mean ± standard deviation or numbers (percentage). Differences between group means were analyzed using analysis of covariance, whereas proportions were analyzed using a chi-squared test. Adipokines have been measured in serum samples. * Matching variables, plus year of inclusion. Abbreviations: BMI: body mass index; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate.

2.2. Baseline Characteristics of the Nested Case-Control Cohort from the Medical Biobank of Northern Sweden

Table 2 shows baseline characteristics of the cohort from the Medical Biobank of Northern Sweden, both cases and matched controls. The two groups had similar characteristics including age, the proportion of men/women, and BMI. Cases were more frequently smokers than the participants from the control group. No difference in the plasma levels of adipokines was detected between the two groups. Circulating levels of adipokines showed poor correlation (Spearman's coefficients < 0.1 for all the combinations).

Table 2. Baseline characteristics of the nested case-control cohort from the Medical Biobank of Northern Sweden.

Characteristic	Cases (n = 88)	Matched Controls (n = 88)	p-Value
Age, years *	56 ± 10	55 ± 10	0.54
Men, No (%) *	20 (23)	20 (23)	1.00
BMI, kg/m^2	26 ± 4	26 ± 3	0.56
Current or previous smoking, No (%)	43 (49)	28 (32) **	0.03
Adiponectin, ng/mL	7482 ± 4936	6628 ± 4654	0.18
Leptin, ng/mL	21 ± 17	20 ± 19	0.44
Resistin, ng/mL	6.7 ± 3.5	6.9 ± 2.8	0.42

Data are shown as mean ± standard deviation or numbers (percentage). Differences between group means were analyzed using analysis of covariance, whereas proportions were analyzed using a chi-squared test. Adipokines have been measured in plasma samples. * Matching variables, plus date of blood sampling. ** Information about smoking status was missing for one individual. Abbreviations: BMI: body mass index.

2.3. Multivariable Analyses for the Incidence of RA in the Nested Case-Control Cohort from the SOS Study

Table 3 shows the multivariable conditional logistic regression analyses for the risk of RA in the SOS nested case-control cohort. When only adipokines are included in the model, solely the association between adiponectin levels and risk of RA was close to significance

(OR: 1.05, per 1000 ng/mL; 95% CI: 1.00–1.10, $p = 0.05$; Table 3 (A Model 1)). The analysis was not adjusted for age, sex, BMI, or smoking as those variables were used for matching, and they were not different between the two groups (Table 1). As CRP and ESR levels were different between cases and controls (Table 1), we included those two variables in the analysis. As shown in Table 3 (B Model 2), increased serum levels of adiponectin were associated with a higher risk of RA (OR: 1.06; per 1000 ng/mL, 95% CI: 1.01–1.12, $p = 0.02$) independently of other adipokines as well as inflammation markers CRP and ESR. As previously shown in the same cohort [25,26], CRP was also independently associated with a higher risk of RA (OR: 1.67; per 10 mg/L, 95% CI: 1.17–2.42, $p = 0.01$). ESR and serum levels of leptin, resistin, and visfatin did not show any significant association with the risk of RA.

Table 3. Multivariable conditional logistic regression analysis for RA in the nested case-control cohort from the SOS study.

A. Model 1	**OR**	**95% CI**	***p*-Value**
Adiponectin, per 1000 ng/mL	1.05	1.00–1.10	0.05
Leptin, per 10 ng/mL	0.93	0.84–1.03	0.16
Resistin, per 10 ng/mL	0.93	0.59–1.47	0.75
Visfatin, per 1 ng/mL	1.07	0.95–1.21	0.29
B. Model 2	**OR**	**95% CI**	***p*-Value**
Adiponectin, per 1000 ng/mL	1.06	1.01–1.12	0.02
Leptin, per 10 ng/mL	0.92	0.83–1.02	0.13
Resistin, per 10 ng/mL	0.67	0.40–1.15	0.15
Visfatin, per 1 ng/mL	1.04	0.91–1.19	0.57
CRP, per 10 mg/L	1.67	1.17–2.42	0.01
ESR, per 10 mm/h	1.14	0.88–1.48	0.32

Abbreviations: OR: odds ratio; CI: confidence interval; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate.

2.4. Multivariable Analysis for the Incidence of RA in the Nested Case-Control Cohort from the Medical Biobank of Northern Sweden

In the cohort from the Medical Biobank of Northern Sweden, no association between adipokines and risk of RA was detected in a multivariable analysis, including plasma adipokine levels and smoking (Table 4A). Smoking was included in the model as the percentage of current or previous smokers was different at baseline between cases and controls (Table 2). CRP and ESR levels were not available at baseline in the cohort from the Medical Biobank of Northern Sweden and therefore could not be included in the analysis. To mimic the conditions of the SOS cohort characterized by subjects affected by obesity, we then stratified the population according to BMI. Plasma levels of adiponectin were associated with a risk of RA (OR: 1.17, 95% CI: 1.01−1.36, $p = 0.03$) independently of other factors in the subgroup having BMI > 25 kg/m^2 ($n = 109$) but not in the one having BMI $\leq$ 25 kg/m^2 ($n = 67$), as shown in Table 4B,C. These analyses were also adjusted for age, sex, and smoking as cases and controls were not matched for these variables when stratifying for BMI. However, similar results were obtained in the unadjusted analysis (data not shown). The interaction between adiponectin levels and BMI on the risk of RA was not significant ($p = 0.13$). Baseline clinical characteristics of the cohort from the Medical Biobank of Northern Sweden after stratification by BMI are shown in Table 5.

Table 4. Multivariable conditional logistic regression analysis for RA risk (A) and logistic regression analyses after stratifying for BMI (B and C) in the Medical Biobank of Northern Sweden cohort.

Characteristic	OR	95% CI	*p*-Value
A.			
Adiponectin, per 1000 ng/mL	1.05	0.98–1.13	0.19
Leptin, per 10 ng/mL	1.01	0.99–1.03	0.56
Resistin, per 10 ng/mL	0.75	0.85–1.12	0.75
Smoking, yes/no	2.27	1.12–4.62	0.02
B. BMI $\leq$ 25 kg/m^2			
Adiponectin, per 1000 ng/mL	1.02	0.94–1.11	0.64
Leptin, per 10 ng/mL	1.00	0.93–1.07	0.92
Resistin, per 10 ng/mL	1.05	0.85–1.29	0.68
Women, yes/no	1.42	0.25–8.03	0.69
Age, per 1 year	0.99	0.94–1.05	0.79
Smoking, yes/no	2.04	0.72–5.81	0.18
C. BMI > 25 kg/m^2			
Adiponectin, per 1000 ng/mL	1.17	1.01–1.36	0.03
Leptin, per 10 ng/mL	1.00	0.98–1.03	0.94
Resistin, per 10 ng/mL	0.98	0.87–1.09	0.69
Women, yes/no	0.84	0.25–2.78	0.77
Age, per 1 year	1.00	0.97–1.05	0.70
Smoking, yes/no	1.63	0.71–3.72	0.25

Abbreviations: OR: odds ratio; CI: confidence interval; BMI: body mass index.

Table 5. Baseline characteristics of the cohort from the Medical Biobank of Northern Sweden stratified by BMI.

Characteristic	Cases	Matched Controls	*p*-Value
BMI $\leq$ 25	***n* = 30**	***n* = 37**	
Age, years	53 $\pm$ 11	54 $\pm$ 9	0.67
Women, No (%)	26 (87)	32 (87)	0.64
BMI, Kg/m^2	22 $\pm$ 2	23 $\pm$ 1	0.13
Current or previous smoking, No (%)	15 (50)	12 (32)	0.15
Adiponectin, ng/mL	9233 $\pm$ 6575	8708 $\pm$ 5921	0.73
Leptin, ng/mL	13 $\pm$ 8	12 $\pm$ 9	0.79
Resistin, ng/mL	6.9 $\pm$ 2.5	6.8 $\pm$ 2.5	0.99
BMI > 25	***n* = 58**	***n* = 51**	
Age, years	57 $\pm$ 9	57 $\pm$ 10	0.63
Women, No (%)	42 (72)	36 (71)	0.83
BMI, Kg/m^2	28 $\pm$ 3	28 $\pm$ 3	0.76
Current or previous smoking, No (%)	28 (48)	16 (31) *	0.09
Adiponectin, ng/mL	6575 $\pm$ 3570	5118 $\pm$ 2630	0.02
Leptin, ng/mL	25 $\pm$ 18	26 $\pm$ 22	0.80
Resistin, ng/mL	6.7 $\pm$ 3.9	6.9 $\pm$ 2.5	0.74

Data are shown as mean $\pm$ standard deviation or numbers (percentage). Differences between group means were analyzed using analysis of covariance, whereas proportions were analyzed using a Chi-square test or Fisher's Exact test. * Information about smoking status was missing for one individual. Abbreviation: BMI: body mass index.

3. Discussion

By performing two nested case-control studies in cohorts where blood samples and clinical information were available before the diagnosis of RA, we have observed no association between circulating levels of leptin, resistin, and visfatin and the future risk of RA. Only elevated circulating adiponectin levels were associated with an increased risk of developing RA in participants with overweight/obesity independently of other adipokines.

This study confirms what we have shown in a recent publication where increased serum adiponectin levels were associated with the future incidence of RA in participants of the SOS, a longitudinal study including about 4000 subjects with obesity [25]. This association was independent of confounding factors, including bariatric surgery. As circulating levels of other adipokines are known to be elevated in patients with RA compared to controls, we wanted to determine if leptin, resistin, and visfatin levels were also associated with the future development of RA [9]. By performing a nested case-control study within the SOS study based on the same patients with incident RA as in our previous study matched 1:5 with controls without incident RA [25], we did not observe any association between leptin, resistin, and visfatin and the risk of future RA. As expected, serum adiponectin levels were positively associated with an increased risk of developing RA, and this association was independent of other adipokines. As the SOS study only included participants with obesity, we aimed to test our hypothesis at the general population level and therefore measured plasma levels of adiponectin, leptin, and resistin in 88 pre-symptomatic patients before symptom onset of RA and 88 matched controls from the Medical Biobank of Northern Sweden. It was not possible to measure circulating visfatin due to the low amount of available plasma. In this cohort, no adipokine was associated with the development of RA. To mirror the conditions of the SOS study where all the participants are affected by obesity, we decided to stratify the population according to BMI and found that elevated circulating adiponectin levels were associated with an increased risk of RA independently of leptin and resistin only in participants with overweight/obesity. No association of adiponectin, nor other adipokines, and risk of RA was observed in normal-weight participants.

It is unclear why circulating adiponectin was associated with the future risk of RA exclusively in participants with overweight/obesity. A possible explanation is that a chronic pro-inflammatory state as seen in obesity is needed to expose the association between adiponectin levels and RA. Furthermore, as high BMI associates with low circulating adiponectin levels and at the same time with an increased risk of developing RA, it is possible to hypothesize that overweight and obesity uncover the link between adiponectin and risk of RA [12,27]. According to this hypothesis, the association between increased adiponectin levels and the risk of developing future RA is also present at a general population level, but it becomes clearly detectable in subjects with overweight/obesity as they have constitutionally lower circulating levels of adiponectin. However, it is important to point out that this remains a pure hypothesis that we cannot confirm or reject in the present report, as the cohort of the Medical Biobank of Northern Sweden included in this study is not large enough. Moreover, the interaction between adiponectin levels and BMI on the risk of RA was not significant in the same cohort.

The finding that patients with RA have elevated circulating levels of adiponectin compared to controls is known for many years [9,14]. However, as adiponectin has both anti- and pro-inflammatory properties, the increase in adiponectin levels in blood has been hypothesized to be a protective mechanism to counterbalance systemic and local inflammation [28,29]. On the other hand, adiponectin might play an active role in the development of RA as it is able to induce pro-inflammatory responses in cells involved in the pathogenesis of RA, such as fibroblast-like synoviocytes and antibodies against human adiponectin ameliorated rheumatic symptoms in a collagen-induced arthritis mouse model [30,31]. Our previous and current data show that adiponectin levels are associated with the risk of future development of RA in patients with overweight/obesity, thus supporting the hypothesis that this adipokine might play a role in the pathogenesis of RA at least in this group of patients. However, other factors, such as CRP, are known to increase in the blood several years before the onset of RA, and they do not necessarily play a role in the disease development but are unspecific markers of inflammation. Further in vitro studies are needed to determine if adiponectin has a pathogenic role in RA or is a marker of inflammation in the context of RA.

Circulating levels of leptin, resistin, and visfatin have been previously shown to be elevated in patients with RA compared to controls [9,15,17]. Moreover, in vitro studies

have demonstrated that leptin, resistin, and visfatin are able to stimulate the production of pro-inflammatory factors in cells from patients with RA [16,19,22]. However, we have recently reported that, in patients with untreated newly diagnosed RA, leptin and resistin are not associated with markers of disease activity nor pro-inflammatory chemokines [32]. Our present study could not detect any association between circulating levels of leptin, resistin, and visfatin and future risk of RA. Taken together, these results might suggest that those three adipokines are neither involved in the initiation of RA nor in the early pre-clinical phases of RA development. However, future studies in a larger cohort are needed to determine if circulating levels of leptin, resistin, and visfatin are associated with the future risk of RA.

Our study has some limitations. We performed two nested case-control studies from two larger cohorts, and the low number of participants included might have affected the results. Specifically, the nested case-cohort study performed within the Medical Biobank of Northern Sweden is rather small. Based on the obtained ORs, this population does not have enough statistical power to allow detecting an association between circulating adipokines on the risk of developing RA at a general population level. Moreover, the two cohorts are very different in terms of baseline characteristics as well as the proportion of cases/controls. The limited number of participants with obesity in the Medical Biobank of Northern Sweden (10 RA cases and 8 matched controls having BMI > 30) did not allow us to stratify this cohort based on BMI equal to 30, and therefore we decided to use BMI equal to 25 (defining overweight) as a cut-off instead. Another limitation of the study is that it was not possible to measure visfatin in the cohort from the Medical Biobank of Northern Sweden due to the lack of plasma samples. Further studies in larger cohorts are warranted to determine if circulating levels of adipokines are able to predict the future development of RA at a general population level.

4. Materials and Methods

4.1. SOS Study

A nested case-control study was performed within the SOS study. Details about the SOS study, such as recruitment, inclusion criteria, and sample collection and storage, have been previously described [33,34]. Briefly, the ongoing SOS study is a longitudinal non-randomized controlled intervention study investigating the impact of bariatric surgery on mortality and morbidity in patients affected by obesity. Between 1987 and 2001, 4047 individuals with obesity were enrolled in Sweden. Two-thousand and ten patients voluntarily chose bariatric surgery and underwent vertical banded gastroplasty (68%), gastric banding (19%), or gastric bypass (13%). A matched control group including 2037 patients with obesity was created based on 18 different matching variables as previously described [34]. Participants from the control group received conventional treatment for obesity which was provided at their primary health care centers [34,35]. The study was approved by seven regional ethics review boards in Sweden and is registered at https://clinicaltrials.gov/ (accessed on 22 June 2021) with the identifier NCT0147952. All patients gave informed consent to participate in the study.

Diagnoses of RA were retrieved through the Swedish National Patient Register by looking for the following International Classification of Diseases (ICD) codes: 712.38, 712.39 (ICD-8), and 714.0-2 (ICD-9) and M05, M06.0, M06.8, and M06.9 (ICD-10) [26]. SOS study participants were followed up until diagnosis of RA, death, migration, or end of follow-up, which was 31 December 2016. Information on death or migration was obtained from the Cause of Death Register and the Register of the Total Population [36].

Eleven patients had prevalent RA, and 343 had no available serum samples at baseline and were therefore excluded from the analyses (Figure 1A). Among the 3693 participants with available serum and no prevalent RA at baseline, 82 patients developed RA during follow-up (cases). The 82 cases were matched 1:5 with 410 subjects who did not develop RA during follow-up (controls), as depicted in Figure 1A. Matching was performed using the propensity score method based on baseline age, sex, BMI, bariatric surgery yes/no,

year of inclusion, and smoking [37]. The median follow-up time was 21 years, ranging from 0 to 29 years in the entire cohort, whereas the median pre-dating time before the diagnosis of RA was 14 years, ranging from 1 to 27 years.

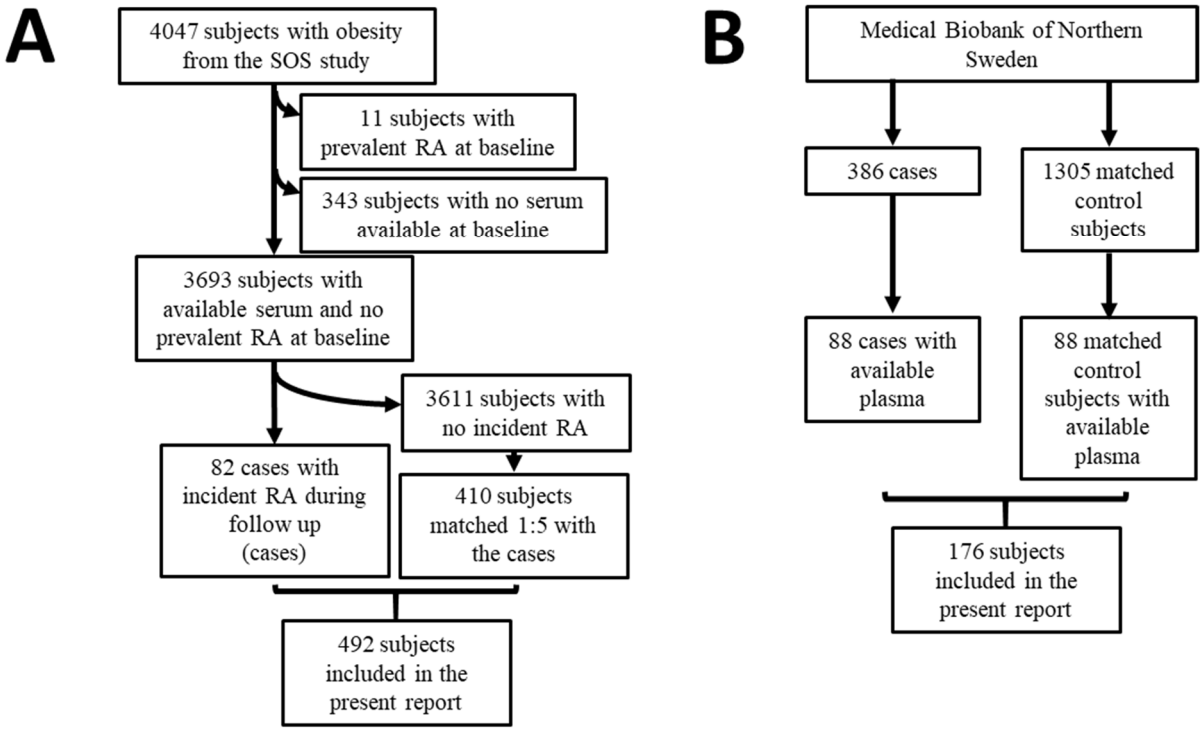

Figure 1. Flow scheme for the present report. (**A**) Nested case-control study from the Swedish Obese Subjects study. (**B**) Nested case-control study within the cohort of the Medical Biobank of Northern Sweden.

4.2. The Cohort from the Medical Biobank of Northern Sweden

A nested case-control study was performed in a group of individuals included in population surveys within the cohort from the Medical Biobank of Northern Sweden. Details about the recruitment, inclusion criteria, and sample collection and storage of the original study have been previously described [24]. Briefly, the register from the Medical Biobank of Northern Sweden was cross-checked with the registers of patients with RA attending the Department of Rheumatology, University Hospital of Umeå, to identify those individuals who had donated blood samples before the symptoms of RA. Diagnosis of RA was based on the 1987 American Rheumatism Association classification criteria [38]. Three-hundred and eighty-six individuals (71 men and 315 women) donating a total of 717 plasma samples were included in the original study (Figure 1B). A total of 1305 controls, matched for age, sex, and date of blood sampling, were randomly identified from the registers of the Medical Biobank of Northern Sweden. The Regional Ethics Committee at the University Hospital, Umeå, Sweden, approved this study (Dnr 2011-168-31M), and all individuals had given their written informed consent to participate in research projects.

Out of the 386 cases who later on developed RA, 88 participants had available plasma and were therefore included in the current report (Figure 1B). The pre-dating time before the diagnosis of RA was 8.5 $\pm$ 5.0 years (mean $\pm$ standard deviation, SD) [23]. The 88 cases were matched 1:1 with 88 available controls. Matched was based on sex, age, and sample year.

4.3. Biochemical Assessments

In the SOS study, ESR was measured at the participants' health care centers at the time of health examination visits. CRP levels at baseline were measured with an ultrasensitive immunoturbidimetric method (Sentinel, Milan, Italy) using the Architect c8200 analyzer

(Abbott Laboratories, Abbott Park, IL, USA) in Helsinki, Finland, between October 2010 and April 2011. Measurement of serum concentrations of adiponectin was performed at the German Diabetes Center, Duesseldorf, Germany, from November 2010 to April 2011 [39]. Total adiponectin was measured using the Human Total Adiponectin/Acrp30 Quantikine ELISA Kit (DRP300, Bio-Techne, Minneapolis, MN, USA, previously R&D Systems, Wiesbaden, Germany). Serum leptin, resistin, and visfatin levels were measured using Human Leptin Quantikine ELISA Kit (DLP00, Bio-Techne, Minneapolis, MN, USA), Human Resistin Quantikine ELISA Kit (DRSN00, Bio-Techne, Minneapolis, MN, USA), and Nampt (Visfatin/PBEF) human ELISA Kit (AG-45A-0006YEK-KI01, AdipoGen Life Sciences, San Diego, CA, USA) respectively, between April and June 2018 at the University of Gothenburg, Sweden. All the ELISA experiments were performed following the manufacturers' instructions. All samples gave values above the limit of detection.

In the cohort from the Medical Biobank of Northern Sweden, plasma adiponectin, leptin, and resistin were measured using the same ELISA kits as for the SOS study. All measurements were performed at the University of Gothenburg (Sweden) in August 2018. Two samples had leptin levels above the detection range, and their values were defined as the upper detection limit. The leptin level of one sample was below the detection range, and its value was defined as 0 ng/mL. Visfatin could not be measured in the cohort from the Medical Biobank of Northern Sweden due to a lack of plasma.

4.4. Statistical Analysis

Data are shown as mean ± SD for continuous variables or number (percentage) for categorical variables. Spearman's test was used to assess the correlation among adipokines. Differences between group means were analyzed using analysis of covariance, whereas proportions were analyzed using a chi-squared or Fisher's Exact test. Multivariable conditional logistic regression analysis was used to calculate adjusted odds ratios (OR) and corresponding 95% confidence intervals (CIs) for the risk of RA in the SOS cohort and in the cohort from the Medical Biobank of Northern Sweden. Odds ratio and corresponding 95% confidence intervals for the risk of RA in the cohort from the Medical Biobank of Northern Sweden after stratifying for BMI were calculated with binary logistic regression after adjustment for preselected risk factors. All p-values were two-sided, and p-values < 0.05 were considered statistically significant. Statistical analyses were performed with the Statistical Package for Social Science (version 24.0; SPSS, Chicago, IL, USA).

5. Conclusions

This study shows that in subjects with overweight/obesity, increased circulating adiponectin levels were associated with a higher risk of developing RA independently of other adipokines. We were not able to detect any association between circulating levels of leptin, resistin, or visfatin and the risk of developing RA, regardless of weight.

Author Contributions: Y.Z. took part in the design of the study, performed the measurements, contributed to the interpretation of the data, and wrote the first draft of the manuscript. L.J. contributed to the data analysis and the interpretation of the results. J.A.-A., M.T. and P.-A.S. contributed to the collection of the samples and interpretation of the data. M.P. contributed to the design of the study and the interpretation of the data. C.H. supervised laboratory measurements and contributed to the interpretation of data. A.R. and L.C. contributed to the design of the study and the interpretation of data. S.R.-D. contributed to the data analysis and the interpretation of the results. C.M. designed the study, contributed to the data analysis and the interpretation of the results. All authors have read and agreed to the published version of the manuscript.

Funding: Funding was provided by the Knut and Alice Wallenberg Foundation and the Wallenberg Centre for Molecular and Translational Medicine at the University of Gothenburg, Sweden, to Cristina Maglio (grant 2016/4839). Funding was also provided by the Sahlgrenska University Hospital Regional Agreement on Medical Education and Research grants to the SOS study and by the Ministry of Culture and Science of the State of North Rhine-Westphalia, the German Federal Ministry of Health, the German Federal Ministry of Education, and Research to the German Center

for Diabetes Research (DZD) to the German Diabetes Centre. This study was also supported by the Swedish Research Council (K2013-52X-20307-07-3 and Dnr:2018-02551), King Gustaf V's 80-Year Fund, King Gustaf V's and Queen Victoria's Fund, the Swedish Rheumatism Association, Umeå University, Umeå.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki. The SOS study was approved by seven regional ethics review boards in Sweden and is registered at https://clinicaltrials.gov/ (accessed on 22 June 2021) with identifier NCT0147952. The cohort from the Medical Biobank of Northern Sweden was approved by the Regional Ethics Committee at the University Hospital, Umeå, Sweden (Dnr 2011-168-31M).

Informed Consent Statement: Informed consent was obtained from all participants.

Data Availability Statement: All relevant data are within the manuscript and its supporting information files.

Acknowledgments: We thank the staff members at 480 primary health care centers and 25 surgical departments in Sweden who participated in the SOS study. Christina Torefalk and Björn Henning are acknowledged for their valuable support. We would like to acknowledge the staff at the Medical Biobank of Northern Sweden for providing samples.

Conflicts of Interest: Y.Z., L.J., J.A.-A., M.T., M.P., P.-A.S., C.H., A.R., S.R.-D. and C.M. declare that they do not have any competing financial interests that may be relevant to the submitted work. L.C. reports receiving consulting fees from Johnson & Johnson. This study was presented at the 2019 European League Against Rheumatism (EULAR) congress (abstract number THU0061).

References

1. Kwon, H.; Pessin, J.E. Adipokines Mediate Inflammation and Insulin Resistance. *Front. Endocrinol.* **2013**, *4*, 71. [CrossRef]
2. Mancuso, P. The role of adipokines in chronic inflammation. *ImmunoTargets Ther.* **2016**, *ume 5*, 47–56. [CrossRef]
3. Must, A.; Spadano, J.; Coakley, E.H.; Field, A.E.; Colditz, G.; Dietz, W.H. The Disease Burden Associated With Overweight and Obesity. *JAMA* **1999**, *282*, 1523–1529. [CrossRef]
4. Choi, H.K.; Atkinson, K.; Karlson, E.W.; Curhan, G. Obesity, weight change, hypertension, diuretic use, and risk of gout in men: The health professionals follow-up study. *Arch. Intern. Med.* **2005**, *165*, 742–748. [CrossRef] [PubMed]
5. I Daïen, C.; Sellam, J. Obesity and inflammatory arthritis: Impact on occurrence, disease characteristics and therapeutic response. *RMD Open* **2015**, *1*, e000012. [CrossRef] [PubMed]
6. Li, W.; Han, J.; Qureshi, A.A. Obesity and risk of incident psoriatic arthritis in US women. *Ann. Rheum. Dis.* **2012**, *71*, 1267–1272. [CrossRef] [PubMed]
7. Feng, J.; Chen, Q.; Yu, F.; Wang, Z.; Chen, S.; Jin, Z.; Cai, Q.; Liu, Y.; He, J. Body Mass Index and Risk of Rheumatoid Arthritis: A Meta-Analysis of Observational Studies. *Medicine* **2016**, *95*, e2859. [CrossRef]
8. Ouchi, N.; Parker, J.L.; Lugus, J.J.; Walsh, K. Adipokines in inflammation and metabolic disease. *Nat. Rev. Immunol.* **2011**, *11*, 85–97. [CrossRef] [PubMed]
9. Neumann, E.; Hasseli, R.; Ohl, S.; Lange, U.; Frommer, K.; Müller-Ladner, U. Adipokines and Autoimmunity in Inflammatory Arthritis. *Cells* **2021**, *10*, 216. [CrossRef] [PubMed]
10. Otero, M.; Lago, R.; Gomez, R.; Lago, F.; Dieguez, C.; Gomez-Reino, J.J.; Gualillo, O. Changes in plasma levels of fat-derived hormones adiponectin, leptin, resistin and visfatin in patients with rheumatoid arthritis. *Ann. Rheum. Dis.* **2006**, *65*, 1198–1201. [CrossRef]
11. Achari, A.E.; Jain, S.K. Adiponectin, a Therapeutic Target for Obesity, Diabetes, and Endothelial Dysfunction. *Int. J. Mol. Sci.* **2017**, *18*, 1321. [CrossRef] [PubMed]
12. Aritaa, Y.; Kiharaa, S.; Ouchia, N.; Takahashia, M.; Maedaa, K.; Ichiromiyagawaa, J.-; Hotta, K.; Shimomuraa, I.; Nakamuraa, T.; Miyaokaa, K.; et al. Paradoxical Decrease of an Adipose-Specific Protein, Adiponectin, in Obesity. *Biochem. Biophys. Res. Commun.* **1999**, *257*, 79–83. [CrossRef] [PubMed]
13. Kadowaki, T.; Yamauchi, T.; Kubota, N.; Hara, K.; Ueki, K.; Tobe, K. Adiponectin and adiponectin receptors in insulin resistance, diabetes, and the metabolic syndrome. *J. Clin. Investig.* **2006**, *116*, 1784–1792. [CrossRef] [PubMed]
14. Lee, Y.H.; Bae, S.-C. Circulating adiponectin and visfatin levels in rheumatoid arthritis and their correlation with disease activity: A meta-analysis. *Int. J. Rheum. Dis.* **2018**, *21*, 664–672. [CrossRef] [PubMed]
15. Lee, S.-W.; Park, M.-C.; Park, Y.-B.; Lee, S.-K. Measurement of the serum leptin level could assist disease activity monitoring in rheumatoid arthritis. *Rheumatol. Int.* **2007**, *27*, 537–540. [CrossRef]
16. Van Laar, J.; O'Reilly, S.; Muraoka, S.; Kusunoki, N.; Takahashi, H.; Tsuchiya, K.; Kawai, S. Faculty of 1000 evaluation for Leptin stimulates interleukin-6 production via janus kinase 2/signal transducer and activator of transcription 3 in rheumatoid synovial fibroblasts. *F1000 Post-Publ. Peer Rev. Biomed. Lit.* **2013**, *31*, 589–595. [CrossRef]

17. Senolt, L.; Housa, D.; Vernerova, Z.; Jirasek, T.; Svobodova, R.; Veigl, D.; Anderlova, K.; Muller-Ladner, U.; Pavelka, K.; Haluzik, M. Resistin in rheumatoid arthritis synovial tissue, synovial fluid and serum. *Ann. Rheum. Dis.* **2006**, *66*, 458–463. [CrossRef]
18. Rho, Y.H.; Solus, J.; Sokka, T.; Oeser, A.; Chung, C.P.; Gebretsadik, T.; Shintani, A.; Pincus, T.; Stein, C.M. Adipocytokines are associated with radiographic joint damage in rheumatoid arthritis. *Arthritis Rheum.* **2009**, *60*, 1906–1914. [CrossRef]
19. Sato, H.; Muraoka, S.; Kusunoki, N.; Masuoka, S.; Yamada, S.; Ogasawara, H.; Imai, T.; Akasaka, Y.; Tochigi, N.; Takahashi, H.; et al. Resistin upregulates chemokine production by fibroblast-like synoviocytes from patients with rheumatoid arthritis. *Arthritis Res.* **2017**, *19*, 1–10. [CrossRef]
20. Bokarewa, M.; Nagaev, I.; Dahlberg, L.; Smith, U.; Tarkowski, A. Resistin, an Adipokine with Potent Proinflammatory Properties. *J. Immunol.* **2005**, *174*, 5789–5795. [CrossRef]
21. Del Prete, A.; Salvi, V.; Sozzani, S. Adipokines as Potential Biomarkers in Rheumatoid Arthritis. *Mediat. Inflamm.* **2014**, *2014*, 1–11. [CrossRef]
22. Meier, F.M.P.; Frommer, K.W.; Peters, M.A.; Brentano, F.; Lefèvre, S.; Schröder, D.; Kyburz, D.; Steinmeyer, J.; Rehart, S.; Gay, S.; et al. Visfatin/Pre-B-cell Colony-enhancing Factor (PBEF), a Proinflammatory and Cell Motility-changing Factor in Rheumatoid Arthritis. *J. Biol. Chem.* **2012**, *287*, 28378–28385. [CrossRef]
23. Johansson, L.; Pratesi, F.; Brink, M.; Ärlestig, L.; D'Amato, C.; Bartaloni, D.; Migliorini, P.; Rantapää-Dahlqvist, S. Antibodies directed against endogenous and exogenous citrullinated antigens pre-date the onset of rheumatoid arthritis. *Arthritis Res.* **2016**, *18*, 1–11. [CrossRef]
24. Rantapää-Dahlqvist, S.; De Jong, B.A.W.; Berglin, E.; Hallmans, G.; Wadell, G.; Stenlund, H.; Sundin, U.; Van Venrooij, W.J. Antibodies against cyclic citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis. *Arthritis Rheum.* **2003**, *48*, 2741–2749. [CrossRef]
25. Zhang, Y.; Peltonen, M.; Andersson-Assarsson, J.; Svensson, P.-A.; Herder, C.; Rudin, A.; Carlsson, L.; Maglio, C. Elevated adiponectin predicts the development of rheumatoid arthritis in subjects with obesity. *Scand. J. Rheumatol.* **2020**, *49*, 452–460. [CrossRef] [PubMed]
26. Maglio, C.; Zhang, Y.; Peltonen, M.; Andersson-Assarsson, J.; Svensson, P.-A.; Herder, C.; Rudin, A.; Carlsson, L. Bariatric surgery and the incidence of rheumatoid arthritis—A Swedish Obese Subjects study. *Rheumatology* **2020**, *59*, 303–309. [CrossRef] [PubMed]
27. Linauskas, A.; Overvad, K.; Symmons, D.; Johansen, M.B.; Stengaard-Pedersen, K.; de Thurah, A. Body Fat Percentage, Waist Circumference, and Obesity As Risk Factors for Rheumatoid Arthritis: A Danish Cohort Study. *Arthritis Rheum.* **2018**, *71*, 777–786. [CrossRef]
28. Folco, E.J.; Rocha, V.Z.; López-Ilasaca, M.; Libby, P. Adiponectin Inhibits Pro-inflammatory Signaling in Human Macrophages Independent of Interleukin-10. *J. Biol. Chem.* **2009**, *284*, 25569–25575. [CrossRef]
29. Senolt, L.; Pavelka, K.; Housa, D.; Haluzik, M. Increased adiponectin is negatively linked to the local inflammatory process in patients with rheumatoid arthritis. *Cytokine* **2006**, *35*, 247–252. [CrossRef]
30. Lee, Y.-A.; Hahm, D.-H.; Kim, J.Y.; Sur, B.; Lee, H.M.; Ryu, C.J.; Yang, H.-I.; Kim, K.S. Potential therapeutic antibodies targeting specific adiponectin isoforms in rheumatoid arthritis. *Arthritis Res.* **2018**, *20*, 245. [CrossRef]
31. Zhang, Y.; Aldridge, J.; Vasileiadis, G.K.; Edebo, H.; Ekwall, A.-K.H.; Lundell, A.-C.; Rudin, A.; Maglio, C. Recombinant Adiponectin Induces the Production of Pro-Inflammatory Chemokines and Cytokines in Circulating Mononuclear Cells and Fibroblast-Like Synoviocytes From Non-Inflamed Subjects. *Front. Immunol.* **2021**, *11*, 569883. [CrossRef]
32. Vasileiadis, G.; Lundell, A.-C.; Zhang, Y.; Andersson, K.; Gjertsson, I.; Rudin, A.; Maglio, C. Adipocytokines in Untreated Newly Diagnosed Rheumatoid Arthritis: Association with Circulating Chemokines and Markers of Inflammation. *Biomolecules* **2021**, *11*, 325. [CrossRef] [PubMed]
33. Sjöström, L.; Narbro, K.; Sjöström, C.D.; Karason, K.; Larsson, B.; Wedel, H.; Lystig, T.; Sullivan, M.; Bouchard, C.; Carlsson, B.; et al. Effects of Bariatric Surgery on Mortality in Swedish Obese Subjects. *N. Engl. J. Med.* **2007**, *357*, 741–752. [CrossRef]
34. Sjöström, L.; Larsson, B.; Backman, L.; Bengtsson, C.; Bouchard, C.; Dahlgren, S.; Hallgren, P.; Jonsson, E.; Karlsson, J.; Lapidus, L. Swedish obese subjects (SOS). Recruitment for an intervention study and a selected description of the obese state. *Int. J. Obes. Relat. Metab. Disord. J. Int. Assoc. Study Obes.* **1992**, *16*, 465–479.
35. Zenténius, E.; Andersson-Assarsson, J.; Carlsson, L.M.; Svensson, P.-A.; Larsson, I. Self-Reported Weight-Loss Methods and Weight Change: Ten-Year Analysis in the Swedish Obese Subjects Study Control Group. *Obesity* **2018**, *26*, 1137–1143. [CrossRef] [PubMed]
36. Ludvigsson, J.F.; Almqvist, C.; Bonamy, A.-K.E.; Ljung, R.; Michaëlsson, K.; Neovius, M.; Stephansson, O.; Ye, W. Registers of the Swedish total population and their use in medical research. *Eur. J. Epidemiol.* **2016**, *31*, 125–136. [CrossRef] [PubMed]
37. Andrade, C. Propensity Score Matching in Nonrandomized Studies: A Concept Simply Explained Using Antidepressant Treatment During Pregnancy as an Example. *J. Clin. Psychiatry* **2017**, *78*, e162–e165. [CrossRef] [PubMed]
38. Arnett, F.C.; Edworthy, S.M.; Bloch, D.A.; McShane, D.J.; Fries, J.F.; Cooper, N.S.; Healey, L.A.; Kaplan, S.R.; Liang, M.H.; Luthra, H.S.; et al. The american rheumatism association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum.* **1988**, *31*, 315–324. [CrossRef]
39. Herder, C.; Peltonen, M.; Svensson, P.-A.; Carstensen, M.; Jacobson, P.; Roden, M.; Sjöström, L.; Carlsson, L. Adiponectin and Bariatric Surgery: Associations With Diabetes and Cardiovascular Disease in the Swedish Obese Subjects Study. *Diabetes Care* **2014**, *37*, 1401–1409. [CrossRef] [PubMed]

Journal of
Clinical Medicine

MDPI

Article

MiR-126 Is an Independent Predictor of Long-Term All-Cause Mortality in Patients with Type 2 Diabetes Mellitus

Justyna Pordzik [1,†], Ceren Eyileten-Postuła [1,†], Daniel Jakubik [1], Pamela Czajka [1], Anna Nowak [1], Salvatore De Rosa [2], Aleksandra Gąsecka [3], Agnieszka Cieślicka-Kapłon [3], Piotr Sulikowski [4], Krzysztof J. Filipiak [3], Dagmara Mirowska-Guzel [1], Jolanta M. Siller-Matula [1,5] and Marek Postuła [1,*]

1 Department of Experimental and Clinical Pharmacology, Centre for Preclinical Research and Technology (CEPT), Medical University of Warsaw, Banacha 1B, 02-097 Warsaw, Poland; justyna.pordzik@ouh.nhs.uk (J.P.); cereneyileten@gmail.com (C.E.-P.); dr.jakubik@gmail.com (D.J.); czajka.pamela@gmail.com (P.C.); nowania.a@gmail.com (A.N.); dmirowska@wum.edu.pl (D.M.-G.); jolanta.siller-matula@meduniwien.ac.at (J.M.S.-M.)
2 Division of Cardiology, Department of Medical and Surgical Sciences, "Magna Graecia" University, 88100 Catanzaro, Italy; saderosa@unicz.it
3 Department of Cardiology, Medical University of Warsaw, 02-097 Warsaw, Poland; aleksandra.gasecka@wum.edu.pl (A.G.); agnieszka.kaplon@gmail.com (A.C.-K.); krzysztof.filipiak@wum.edu.pl (K.J.F.)
4 Department of Computer Science and Information Technology, West Pomeranian University of Technology, 71-210 Szczecin, Poland; psulikowski@wi.zut.edu.pl
5 Department of Internal Medicine II, Division of Cardiology, Medical University of Vienna, 1090 Vienna, Austria
* Correspondence: mpostula@wum.edu.pl; Tel.: +48-221166160
† Authors contributed equally to this work.

Citation: Pordzik, J.; Eyileten-Postuła, C.; Jakubik, D.; Czajka, P.; Nowak, A.; De Rosa, S.; Gąsecka, A.; Cieślicka-Kapłon, A.; Sulikowski, P.; Filipiak, K.J.; et al. MiR-126 Is an Independent Predictor of Long-Term All-Cause Mortality in Patients with Type 2 Diabetes Mellitus. *J. Clin. Med.* **2021**, *10*, 2371. https://doi.org/10.3390/jcm10112371

Academic Editors: Katarzyna Komosinska-Vassev and Pawel Olczyk

Received: 29 April 2021
Accepted: 26 May 2021
Published: 28 May 2021

Abstract: MicroRNAs are endogenous non-coding RNAs that are involved in numerous biological processes through regulation of gene expression. The aim of our study was to determine the ability of several miRNAs to predict mortality and response to antiplatelet treatment among T2DM patients. Two hundred fifty-two patients with diabetes were enrolled in the study. Among the patients included, 26 (10.3%) patients died within a median observation time of 5.9 years. The patients were receiving either acetylsalicylic acid (ASA) 75 mg (65%), ASA 150 mg (15%) or clopidogrel (19%). Plasma miR-126, miR-223, miR-125a-3p and Let-7e expressions were assessed by quantitative real time PCR and compared between the patients who survived and those who died. Adjusted Cox-regression analysis was used for prediction of mortality. Differential miRNA expression due to different antiplatelet treatment was analyzed. After including all miRNAs into one multivariate Cox regression model, only miR-126 was predictive of future occurrence of long-term all-cause death (HR = 5.82, 95% CI: 1.3–24.9; $p = 0.024$). Furthermore, miR-126, Let-7e and miR-223 expressions in the clopidogrel group were significantly higher than in the ASA group ($p = 0.014$; $p = 0.013$; $p = 0.028$, respectively). To conclude, miR-126 expression is a strong and independent predictor of long-term all-cause mortality among patients with T2DM. Moreover, miR-223, miR-126 and Let-7e present significant interactions with antiplatelet treatment regimens and clinical outcomes.

Keywords: microRNA; cardiovascular disease; diabetes; mortality; antiplatelet therapy; platelet reactivity; Let-7e; miR-126; miR-223; miR-125a-3p

1. Introduction

Type 2 diabetes mellitus (T2DM) constitutes the most prevalent chronic metabolic disorder, and its prevalence is progressively increasing. Notably, mortality rates in T2DM patients are known to be up to four-times as high in contrast to subjects without T2DM and predominantly associated with an increased hazard of cardiovascular disease (CVD) [1].

In the context of T2DM, platelets emerge as triggers of vascular injury induced by metabolic disturbances. The hypercoagulable state, which is often observed in T2DM, stems

from disrupted platelet activation, aggregation, alterations in the coagulation processes, as well as endothelial dysfunction due to dysregulation of several signaling pathways [2]. In line with our previous reports, several genetic polymorphisms may contribute to differential platelet reactivity and outcomes in T2DM patients on antiplatelet treatment [3–5]. As we observe, platelets play a central role in the intertwined pathology linking T2DM, progression of atherosclerosis and occurrence of cardiovascular (CV) complications [6–9]. Since platelets are involved in atherogenesis and its complications, the blockade of one or numerous pathways regulating platelet activation and aggregation processes is essential in diminishing atherothrombotic risk in T2DM subjects.

While various mechanisms mediating CVD in diabetes have been determined, the need for identification of biomarkers is as important as ever. Over the last few years, platelets have been found to be a major source of miRNAs, which are highly conserved endogenous, non-coding RNAs that regulate gene expression at a post-transcriptional level through targeting messenger RNAs (mRNAs) [10,11]. Although miRNAs are found mostly intracellularly, a substantial amount is present in the extracellular space, such as the blood [12]. The extracellular miRNAs, which function as chemical messengers and mediators of cell-cell communication, offer exciting opportunities to become biomarkers [13]. Studies showed that platelets release specific miRNAs that can be predictive factors of morbidity and mortality across various diseases, such as coronary artery disease (CAD) and heart failure (HF) and provide information on platelet function itself [14,15]. Recently, miRNAs have been suggested as a potential tool to predict and monitor disease progression and therapeutic effectiveness. Several studies showed that miRNA expression can be regulated by antiplatelet treatment often utilized in higher risk populations [16–19]. Hence, miRNA signature profiles could be used to help modify antiplatelet treatment for the best prognostic outcomes in this cohort.

We aimed to determine the prognostic performance of selected circulating miRNAs for all-cause mortality and their potential usefulness as biomarkers in T2DM patients on antiplatelet treatment. Several studies showed the role of miR-126 and miR-223 in the context of antiplatelet treatment in many different cohorts [16,19–23]. Changes in plasma miRNAs in response to antiplatelet therapy were investigated in a small cohort of healthy volunteers [16,19–23]. Yu et al. analyzed the correlation between plasma miR-126 and miR-223, among others, and the risk of major adverse cardiovascular events in patients on dual antiplatelet therapy (DAPT) after percutaneous coronary intervention [16,19–23]. The association between miRNAs and platelet reactivity was also studied in patients with acute coronary syndrome (ACS) on various antiplatelet therapies [16,19–23]. In a similar study Carino et al. assessed miR-126 and miR-223, among other miRNAs, in patients with ACS before and after the therapeutic switch from DAPT to ticagrelor [16,19–23]. More recently the association of platelet-related miRNAs was studied in ACS patients on DAPT and pathophysiological processes in the TDM population [16,19–23]. Among others, Let-7e and miR-125a-3p are molecules that have been formerly linked to inflammation, platelet reactivity, and mechanisms leading to diabetes; however, the impact of antiplatelet therapy on Let-7e and miR-125a-3p and their prognostic potential has not been studied so far [24–26]. Therefore, our aim was to analyze the impact of antiplatelet treatment on Let-7e and miR-125a-3p expressions and confirm the previous observations for miR-126 and miR-223 expressions, as well as investigate their usefulness as predictive biomarkers specifically in the T2DM cohort.

2. Materials and Methods

2.1. Study Design

The ethics committee of the Medical University of Warsaw approved both the study protocol and the informed consent form. The study was conducted in accordance with the current version of the Declaration of Helsinki at the time when the study was designed, and informed written consent was obtained. Consecutive patients with T2DM presented at the outpatient clinic of the Central Teaching Hospital of the Medical University of Warsaw

and recruited into the multi-center, prospective, randomized and open-label AVOCADO (Aspirin vs./Or Clopidogrel in Aspirin resistant Diabetes inflammation Outcomes) study were included in the present analysis. The full characterization of the study population, including the inclusion and exclusion criteria, was published previously [5]. Shortly, the AVOCADO study included patients aged between 30 and 80 years, with T2DM, irrespective of the type of antidiabetic treatment (with the exception of patients treated only with diet), burdened with at least two additional cardiovascular (CV) risk factors and receiving 75 mg of acetylsalicylic acid (ASA) daily. All inclusion criteria for the study included: age from 30 to 80 years of age, type 2 diabetes diagnosed at least 6 months before recruitment to the study requiring treatment with oral hypoglycemic drugs and/or insulin, history of cardiovascular events (i.e., myocardial infarction or ischemic stroke) or high risk of cardiovascular events defined as presence of at least 2 of the cardiovascular risk factors listed below: overweight or obesity diagnosed on the basis of the BMI (Body Mass Index) $\geq$ 25 kg/m^2 (overweight), $\geq$ 30 kg/m^2 (obesity), history of hypertension (SBP > 130 mmHg, DBP > 80 mmHg or pharmacological treatment), history of dyslipidemia (total cholesterol > 175 mg/dL, HDL-cholesterol: men < 40 mg/dL, women < 50 mg/dL, LDL-cholesterol > 100 mg/dL, or > 70 mg/dL (with concomitant disease ischemic heart disease), triglycerides > 150 mg/dL or pharmacological treatment); documented coronary artery disease (diagnosis previously made on the basis of objective clinical studies), documented cerebral vascular disease (diagnosis previously based on objective clinical studies), documented peripheral vascular disease (diagnosis previously made on the basis of objective clinical studies or a clinical event requiring hospitalization), positive family history of cardiovascular diseases and sudden cardiac death at an early age (female relatives of the 1st degree <55 years of age) 1st degree male relatives <65 years of age), history of smoking [5]. The exclusion criteria included: bleeding diathesis; history of gastrointestinal bleeding; platelet count < 150,000 per mm^3; hemoglobin concentration < 10 g/dL; hematocrit < 30%; end-stage chronic renal disease requiring dialysis; anticoagulants (i.e., low-molecular—weight heparin, warfarin or acenocoumarol) or an alternative antiplatelet therapy other than ASA (i.e., ticlopidine, clopidogrel, prasugrel or dipyridamole); self-reported use of non-prescription non-steroidal anti-inflammatory drugs or drugs containing ASA within 10 days of enrollment; a recent history (within 12 months) of MI, unstable angina, coronary angioplasty or coronary artery bypass grafting; and a major surgical procedure within the previous 8 weeks [5]. The aim of the AVOCADO trial was to assess the effect of an eight-week course of clopidogrel or an increased ASA dose (150 mg) in patients with T2DM and high platelet reactivity on lower ASA dose (75 mg) as previously described [5]. Patients who did not exhibit high platelet reactivity on ASA 75 mg daily continued therapy, and patients with high platelet reactivity were randomized to ASA 150 mg or clopidogrel 75 mg in 1:2 ratio. Briefly, 197 patients were taking 75 mg ASA, 41 patients were taking 150 mg ASA, and 58 patients were taking clopidogrel. For the current analysis, we used only available blood samples taken 8 weeks after randomization according to the initial study design. Briefly, blood samples were taken in the morning 2–3 h after ingestion of antiplatelet drugs, as previously described in more detail [3,5,27–29]. Blood was kept at room temperature for 30 min before centrifugation at 1500 *g* for 15 min at 18–25 °C. Plasma was pipetted into 500 µL aliquots on ice and transferred to a −80 °C freezer for storage. For the purpose of this study, existing samples from patients already randomized to antiplatelet therapy based on study protocol were used, i.e., up to 6 weeks on therapy based on platelet reactivity measured at baseline, as previously described [5,28].

2.2. Study Endpoints

The primary endpoint was defined as all-cause death during the follow-up. The secondary endpoint was a composite of death, myocardial infarction (MI), unstable angina, and stroke or transient ischemic attack (TIA) at follow-up [30]. The composite endpoint was defined in accordance with the current universal criteria [31,32].

2.3. RNA Preparation and Detection and Quantification of miRNAs by Quantitative PCR

Plasma RNA was extracted by miVANA PARIS Kit. Total RNA was obtained as outlined above and diluted 1:10. Diluted RNA (5 µL) was reverse transcribed using the TaqMan miRNA Reverse Transcription kit (ABI) according to the instructions of the manufacturer. Subsequently, 3 µL of the product was used for detecting miRNA expression by quantitative polymerase chain reaction using TaqMan miRNA Assay kits (ABI) for the corresponding miRs on a The CFX384 Touch Real-Time PCR Detection System (BioRad Inc., Hercules, CA, USA). cel-miR-39 was added as a spike-in control. Reactions were run in triplicate, and the mean value was used for all analyses, to control variability associated with methodological reasons [33,34].

2.4. Statistical Analysis

Risk factors, clinical data and categorical variables are presented as percentages of patients and were compared using $\chi 2$ or Fisher's exact tests, as appropriate. Continuous data are expressed as median and interquartile range and compared using Mann–Whitney U test for two independent samples. The distribution of data was checked with the Kolmogorov–Smirnov test. The Kaplan–Meier method was utilized for construction of survival curves. The log-rank test was applied to evaluate differences between groups. Proportional Cox-regression analysis was used to adjust for confounding factors. The following variables (age, gender, history of previous stroke, history of smoking, eGFR < 30) were entered into the Cox model on the basis of known clinical relevance or significant association observed at univariate analysis. We performed Cox regression analyses for each single miRNA and the clinical variables and also a final model in which all four miRNA were included in addition to the clinical variables. Effect estimates were presented as adj. hazard ratios (HR) and 95% CI. All tests were two-sided, and p-value < 0.05 was considered statistically significant. Calculations were performed using SPSS version 22.0 (IBM Corporation, Chicago, IL, USA). Based on 19% long-term mortality in the high-level miR-126 group as compared to 4% in the low-level miR-126 group, we calculated that with at least 125 patients per miR-126 group (1:1 sampling ratio, overall n = 250), our analysis had 96% power to detect differences in the risk of mortality between the miR-126 groups with a two-sided alpha value of <0.05.

3. Results

3.1. Patient Demographics

Patient demographics, T2DM data, concomitant diseases, medication and laboratory results are summarized in Table 1 and Supplemental Table S1. Out of 303 patients included, blood samples for analyses presented in this study were unavailable for 51 individuals. In fact, 48 patients did not present within the given time window for blood sampling, and in 3 remaining patients, the available blood volume was insufficient for miRNAs measurements. Among the 252 patients included, 26 (10.3%) patients died within a median observation time of 71 months (5.9 years). CV risk factors, such as hypertension (92%), dyslipidemia (85%) history of smoking (57%) and CAD (55%) were common in the majority of patients. Patient characteristics according to selected miRNAs are included in Supplemental Table S1.

Table 1. Demographic and clinical characteristics of the study patients.

Patient Demographics	Overall 252 (100%)	Patients Who Died * (N = 26)	Patients Who Survived * (N = 226)	*p*
Age (years) mean ± SD	67.1 ± 8.5	70.52 ± 1.80	66.61 ± 0.60	0.019
Sex (female) n (%)	117 (46%)	111 (49%)	6 (23%)	0.012
Body mass index (BMI)	31.5 ± 12.7	30.21 ± 0.80	31.81 ± 0.99	0.587
Hypertension	228 (92%)	24 (92%)	204 (92%)	1
Dyslipidemia	213 (85%)	20 (77%)	193 (86%)	0.209
HF	91 (37%)	12 (46%)	79 (35%)	0.282
Current smoking	25 (10%)	2 (8%)	23 (11%)	0.745
CAD	136 (55%)	16 (62%)	120 (54%)	0.468
Prior MI	75 (30%)	12 (46%)	63 (28%)	0.058
History of smoking	143 (57%)	20 (77%)	123 (55%)	0.032
Prior ischemic stroke	22 (9%)	5 (19%)	17 (8%)	0.048
Prior TIA	7 (3%)	1 (4%)	6 (3%)	0.708
Laboratory data (mean ± SD)				
White blood cell count (x109/L)	6.9 ± 1.9	6.46 ± 0.40	6.95 ± 0.13	0.222
Platelets (x109/L)	222.6 ± 61.9	204.08 ± 9.62	225.42 ± 4.47	0.113
Hemoglobin (g/dL)	13.7 ± 1.46	13.72 ± 2.67	13.76 ± 0.99	0.969
High-sensitivity C-reactive Protein (mg/dL)	2 [0.3–25.8]	1.9 [1.05–4.0]	2.05 [1.05–4.1]	0.726
Fibrinogen (mg/dL)	402.6 ± 104	407 ± 19.78	404 ± 7.72	0.994
Creatinine (mg/dL)	1 ± 0.31	1.16 ± 0.06	0.97 ± 0.02	0.001
HbA1c	6.5 [6.0–7.4]	6.4 [5.9–7.1]	6.9 [6.3–7.8]	0.060
Tch	158.6 ± 36.5	153.63 ± 6.15	159.34 ± 2.69	0.628
TG	132.4 ± 61.5	123.46 ± 7.13	125.87 ± 3.12	0.990
HDL	50 ± 30.3	47.50 ± 9.31	51.45 ± 2.44	0.634
LDL	83.8 ± 28.3	81.42 ± 5.35	85.36 ± 2.23	0.670
Failure to achieve lipid control				
LDL, %; n **	108 (49%)	13 (54%)	95 (48%)	0.567
HDL, %; n **	100 (44%)	9 (39%)	91 (45%)	0.413
Triglycerides, %; n **	68 (30%)	8 (32%)	60 (29%)	0.778
Concomitant medications n (%)				
ß-blockers	178 (72%)	22 (88%)	156 (70%)	0.054
ACE inhibitors	165 (66%)	14 (56%)	151 (67%)	0.252
Statins	179 (72%)	19 (76%)	160 (71%)	0.630
Calcium channel-blockers	94 (38%)	8 (32%)	86 (38%)	0.532
Proton pump Inhibitors	49 (24%)	8 (31%)	41 (23%)	0.407
Randomization group in the AVOCADO study, %; n				
ASA (75 mg) ***	167 (66%)	11 (42%)	156 (69%)	0.006
ASA (150 mg) ***	32 (13%)	6 (23%)	26 (12%)	0.093
Clopidogrel ***	53 (21%)	9 (35%)	44 (20%)	0.073
ASA total (75 mg + 150 mg) ***	199 (79%)	17 (65%)	182 (81%)	0.073
miRNAs				
miR-126	0.84 [0.16–8.54]	7.23 [1.93–47.3]	0.46 [0.13–6.39]	0.000032
Let-7e	0.64 [0.09–4.10]	3.78 [0.85–25.70]	0.51 [0.08–3.39]	0.000216
miR-223	8.91 [2.09–69.81]	19.98 [6.27–94.85]	8.11 [1.76–67.88]	0.089
miR-125a-3p	0.009 [0.001–0.05]	0.054 [0.006–1.05]	0.0082 [0.001–0.041]	0.0006

* Median follow-up: 5.9 years. ** Failure to achieve lipid control defined as. LDL > 70 mg/dL in patients with a history of coronary artery disease, myocardial infarction, previous stroke or TIA, current smoking or with eGFR < 30 mL/min/1.73 m^2, and LDL > 100 mg/dL in remaining patients, HDL < 40 mg/dL in men, and < 50 mg/dL in women, triglycerides > 150 mg/dL. *** Study medication (treatment duration: 8 weeks). Data are reported as mean ± standard deviation (SD) and median [interquartile range]. Abbreviations: ACE, angiotensin converting enzyme; ACS, acute coronary syndrome; ASA, acetylsalicylic acid; BMI, body mass index; CAD, coronary artery disease; HDL, high-density lipoprotein; HF, heart failure; LDL, low-density lipoprotein; MI, myocardial infarction; TG, triglycerides.

3.2. Circulating miRNA Levels Predict Long-Term All-Cause Death

Patients who died had a 7.5-, 3- and 14-fold higher expression of miR-126, Let-7e and miR-125a-3p as compared with patients who survived, respectively ($p < 0.001$ for all miRNAs; Figure 1a,b,d). The difference in the expression for miR-223 did not differ statistically between those who died or survived ($p = 0.089$; Figure 1c). Since RNA was extracted from blood plasma for the purpose of this investigation, we refer to the expression of miRNA from extracellular space across this manuscript.

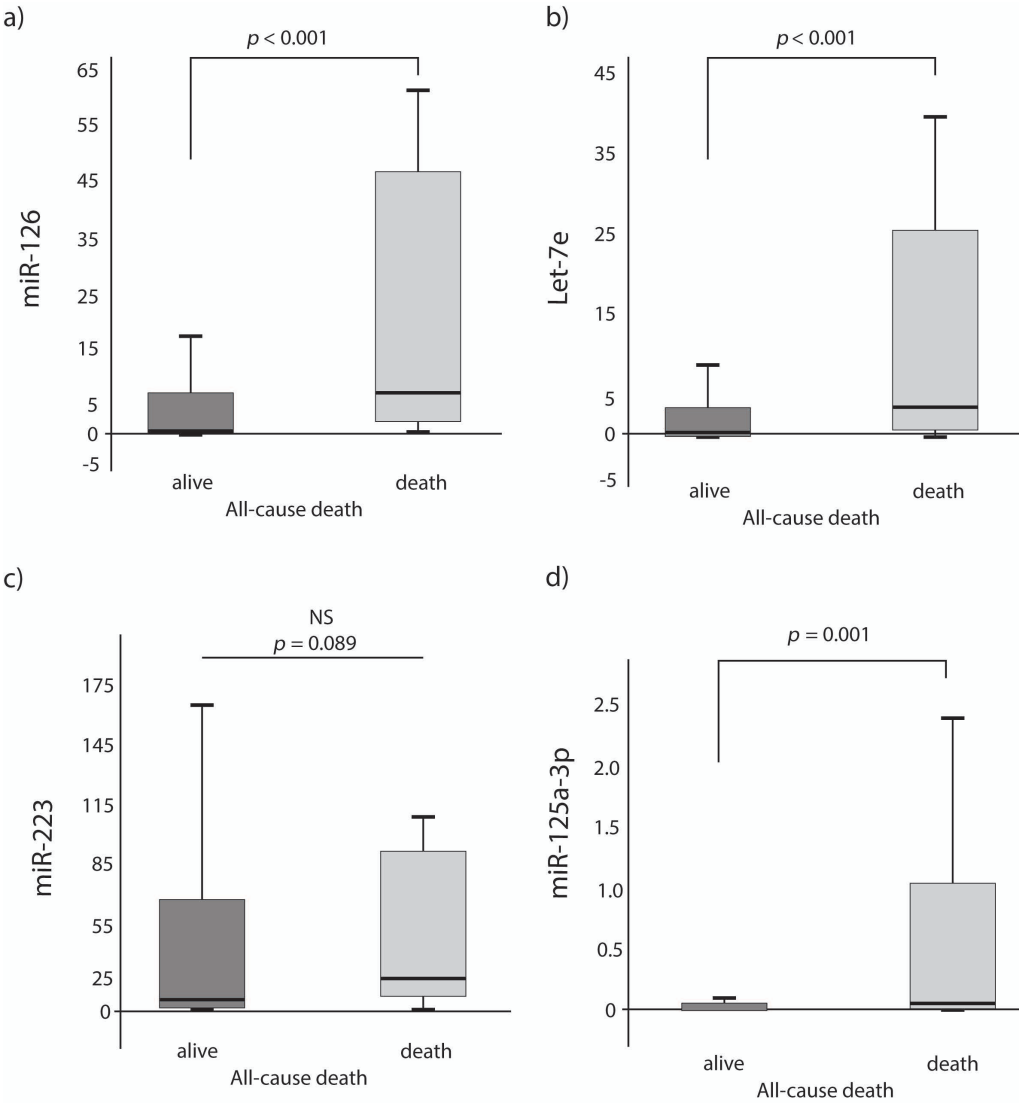

Figure 1. miRNAs expression levels according to survival during the long-term follow-up (**a**) miR-126; (**b**) Let-7e; (**c**) miR-223; (**d**) miR-125a-3p.

3.3. Predictive Value of miR-126, Let-7e, miR-223 and miR-125a-3p of Long-Term All-Cause Mortality

The study population was divided into two subgroups by using ROC curve analysis for each miRNA, i.e., low- or high value of single miRNAs (see Table 2 and Figure 2). The cut-off value of $\geq$2.08, labelled as high miR-126 level (40.5% of the population), the cut-off value of $\geq$0.82, labelled as high Let-7e level (47.6% of the population), the cut-off value of $\geq$6.62, labelled as high miR-223 level (54% of the population) and the cut-off value of $\geq$0.0017, labelled as high miR-125a-3p level (40.9% of the population) provided the prediction of all-cause mortality.

Table 2. Statistical estimates for prediction of all-cause mortality by miR-126, Let-7, miR-223 and miR-125a-3p.

miRNA	c-Index-AUC (95% CI)	*p*	Cut-Off	Sensitivity, %	Specificity, %	Positive Predictive Value, %	Negative Predictive Value, %	Positive Likelihood Ratio
miR-126	0.75 (0.66–0.84)	<0.001	2.078	77%	63%	19%	96%	2.07
Let-7e	0.76 (0.63–0.82)	<0.001	0.8201	81%	66%	18%	96%	1.84
miR-223	0.60 (0.50–0.70)	0.094	6.617	77%	49%	15%	95%	1.49
miR-125a-3p	0.71 (0.60–0.82)	0.001	0.0017	65%	61%	17%	94%	1.78

Abbreviations: AUC—area under the curve; 95% CI—95% confidence interval.

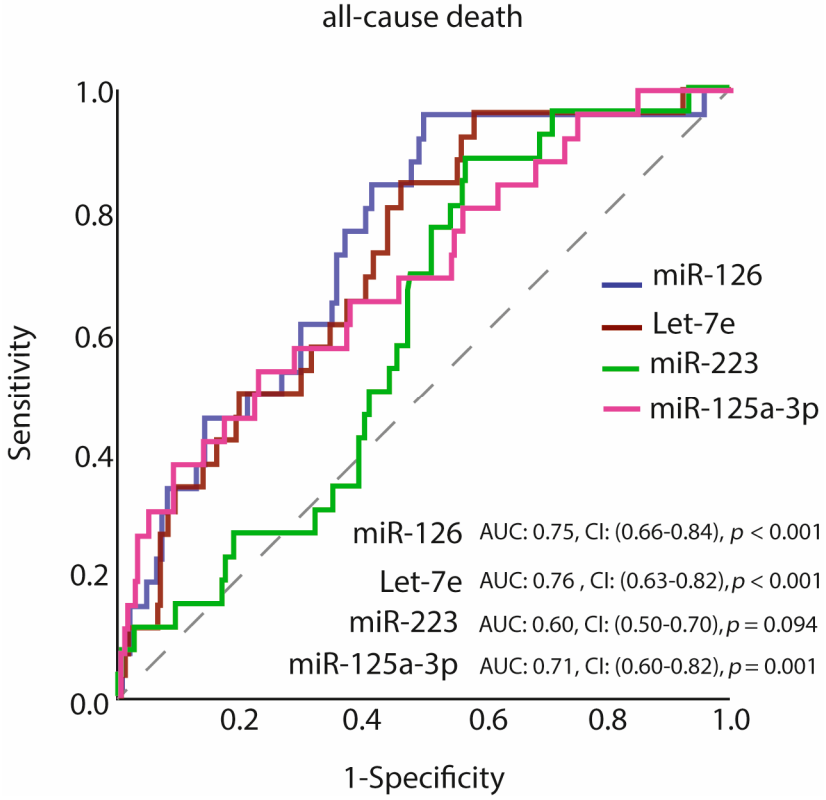

Figure 2. Receiver operating characteristic (ROC) curves of miR-126, Let-7e, miR-223, miR-125a-3p for prediction of all-cause death. Abbreviations: AUC, area under the curve; CI, 95% confidence interval.

3.4. Expression of miRNAs According to the Allocation to the Antiplatelet Treatment Strategy

The expression of miR-126, Let-7e and miR-223 was significantly higher in the clopidogrel group as compared to the ASA 75 mg group ($p = 0.015$; $p = 0.014$; $p = 0.024$, respectively). This difference was not seen when comparing clopidogrel and ASA 150 mg groups for miR-125a-3p (Figure 3a–c; Supplemental Table S2). However, when comparing miRNAs expression between clopidogrel and the whole ASA groups (i.e., 75 mg +150 mg), miR-126, Let-7e and miR-223 expressions remained significantly higher in the clopidogrel subgroup ($p = 0.014$; $p = 0.013$; $p = 0.028$, respectively) (Figure 4).

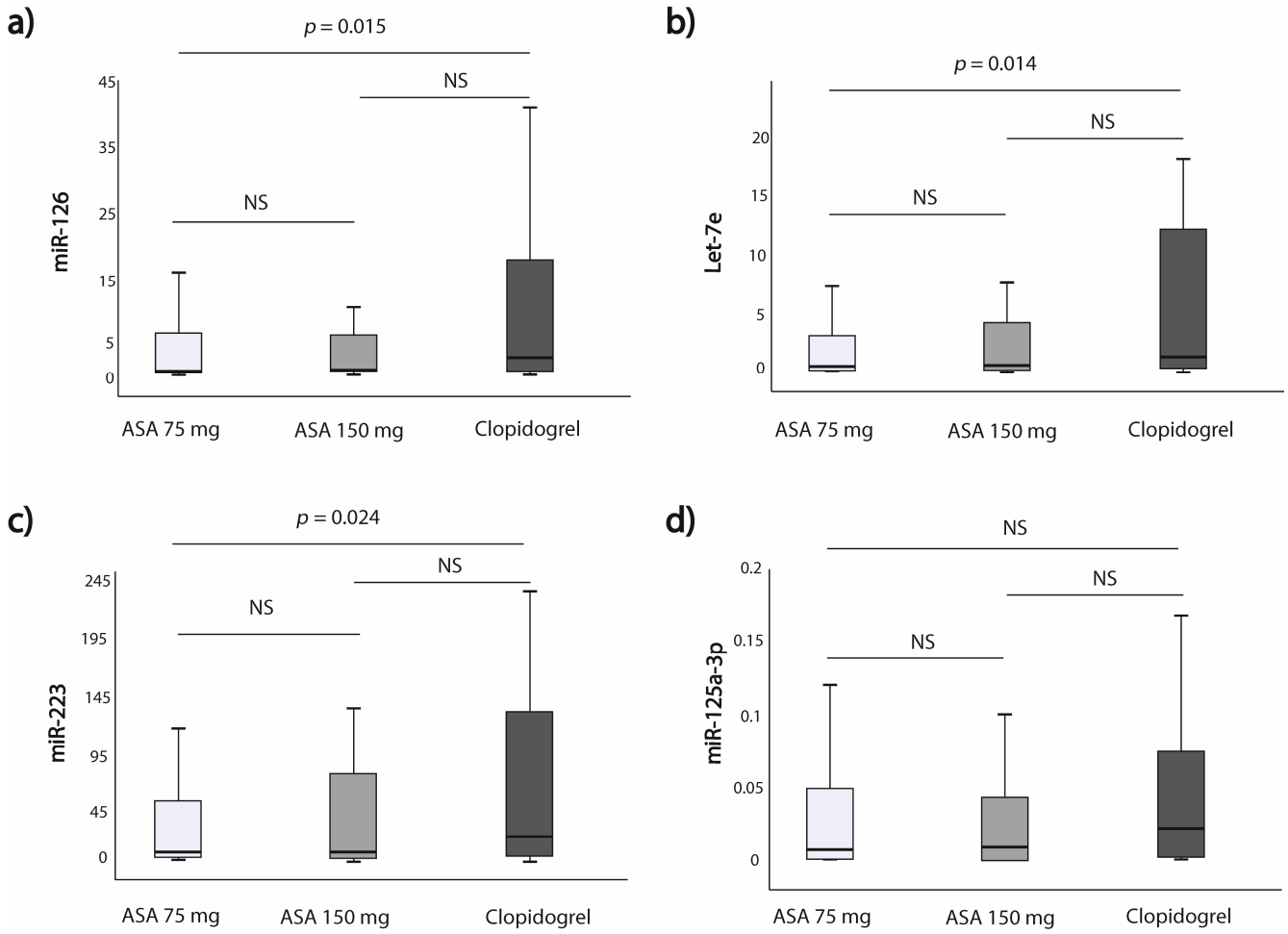

Figure 3. MiRNAs expressions in regard to the type of antiplatelet treatment based on randomization (ASA 75 mg vs. ASA 150 mg vs. Clopidogrel); (**a**) miR-126; (**b**) Let-7e; (**c**) miR-223; (**d**) miR-125a-3p.

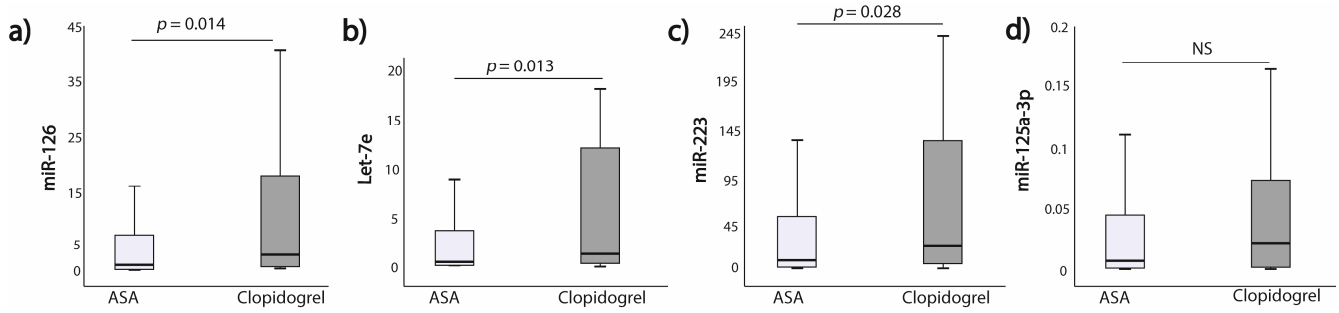

Figure 4. MiRNAs expressions in regard to antiplatelet treatment (ASA vs. Clopidogrel) (**a**) miR-126; (**b**) Let-7e; (**c**) miR-223; (**d**) miR-125a-3p.

3.5. Survival Analysis According to miRNAs Expression

The primary endpoint occurred in 26 (10.3%) out of 252 patients, for whom miR-126, Let-7e, miR-223 and miR-125a-3p levels were available. Six of the patients who died (4%) had low miR-126 values, and 20 patients (19%) had high miR-126 values, whereas for Let-7e, 5 patients (4%) had low Let-7e values, and 21 patients (17.5%) had high Let-7e values. Moreover, six of these patients who died (5%) had low miR-223 values, and 20 patients (14.5%) had high miR-223 values, whereas for miR-125a-3p, 9 patients (6%) had low miR-125a-3p values, and 17 patients (17%) had high miR-125a-3p values.

The primary endpoint in long-term follow-up was significantly more common in patients with high miRNA levels vs. low miRNA levels, for all analyzed miRNAs, i.e., miR-126, Let-7e, miR-223 and miR-125a-3p ($p < 0.001$; $p < 0.001$; $p = 0.016$; $p = 0.005$, respectively).

Adjusted time to event analyses have shown that heightened expression of miRNAs was associated with survival, when the models included one single miRNA and other covariates: miR-126 (HR = 7.31, 95% CI: 2.63–20.28; $p < 0.001$), Let-7e: (HR = 5.85, 95% CI: 2.08–16.46; $p = 0.001$), miR-223: (HR = 3.07, 95% CI: 1.17–8.07; $p = 0.023$), miR-125a-3p (HR = 2.93, 95% CI: 1.27–6.83; $p = 0.013$) (Figure 5; Table 3 and Supplemental Table S3). After inclusion of all 4 miRNAs into one multivariate Cox regression model, increased expression of miR-126 was associated with a 5.8-fold higher risk for long-term all-cause mortality (HR = 5.8, 95% CI: 1.2–24.9; $p = 0.024$), whereas the other miRNAs did not reach the statistical significance. Additionally, age (HR = 1.1, 95% CI: 1.01–1.12; $p = 0.009$) and male gender (HR = 4.06; 95% CI: 1.24–13.34; $p = 0.027$) were found independently associated with long-term all-cause mortality (Table 4).

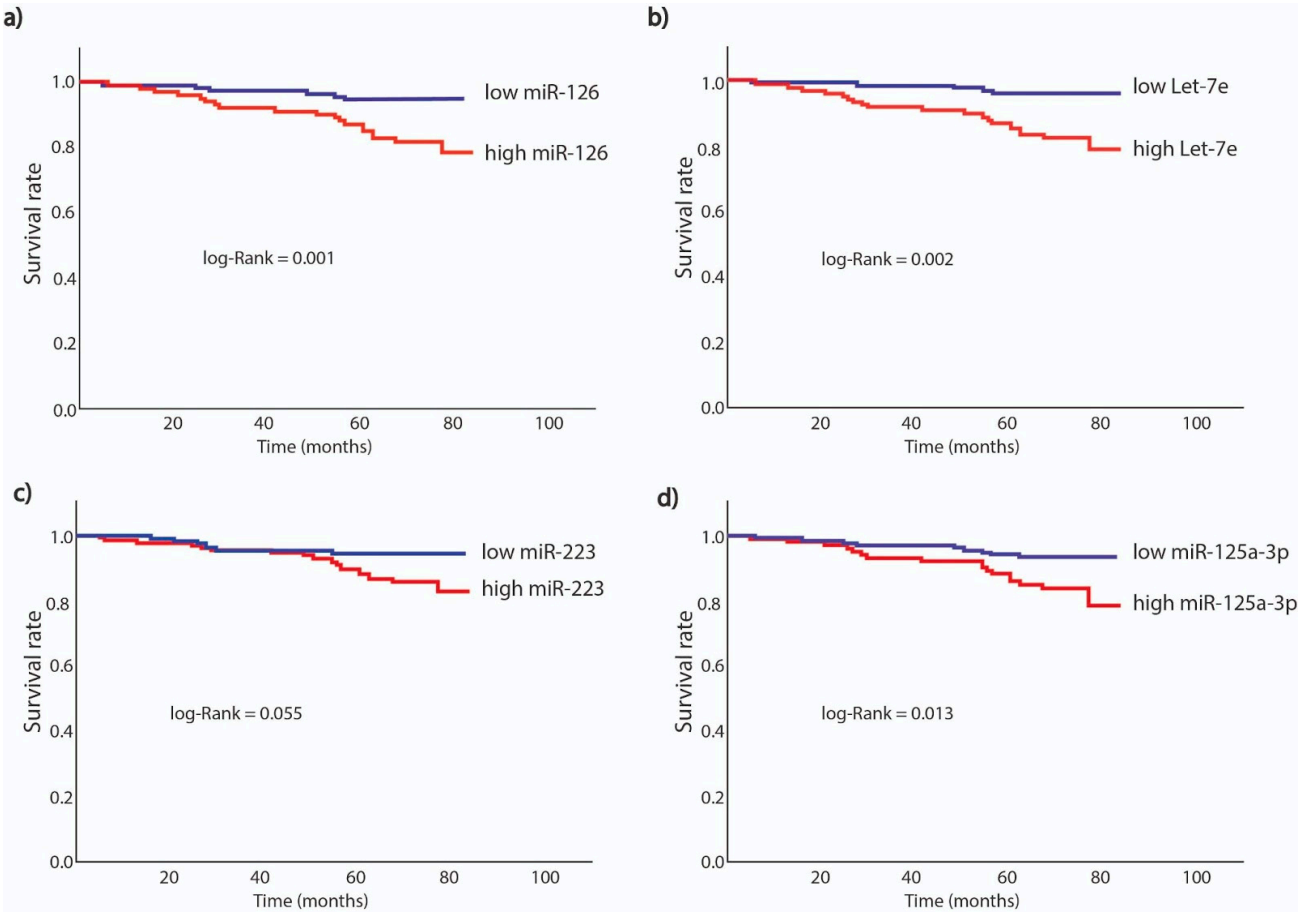

Figure 5. Kaplan–Meier survival analysis for all-cause death. HR are adjusted for clinical variables in a multivariate model with a single miRNA as a covariate (**a**) high vs. low miR-126; (**b**) high vs. low Let-7e; (**c**) high vs. low miR-223; (**d**) high vs. low miR-125a-3p.

Table 3. Univariate and multivariate Cox regression model for prediction of long-term all-cause of mortality for miR-126, Let-7e, miR-223 and miR-125a-3p.

Variable	HR	95% CI		*p*-Value
		Lower	Upper	
High miR-126				
Univariate	4.377	1.749	10.956	0.002
Multivariate *	7.310	2.634	20.284	<0.001
High Let-7e				
Univariate	4.208	1.580	11.206	0.004
Multivariate *	5.845	2.076	16.460	0.001
High miR-223				
Univariate	2.389	0.952	5.977	0.063
Multivariate *	3.073	1.170	8.071	0.023
High miR-125a-3p				
Univariate	2.692	1.198	6.052	0.017
Multivariate *	2.929	1.256	6.828	0.013

* After adjustment for age, gender (male), history of smoking, prior ischemic stroke and eGFR < 30 (mL/min/1.73 m^2).

Table 4. Multivariate Cox regression model including high levels of miRNAs and clinical data.

Variable	HR	95% CI		*p*-Value
		Lower	Upper	
High miR-126	5.821	1.259	24.927	0.024
High Let-7e	3.449	0.578	21.176	0.173
High miR-223	0.367	0.080	1.679	0.196
High miR-125a-3p	1.115	0.408	3.050	0.832
Age	1.068	1.016	1.122	0.009
Gender (male)	4.059	1.235	13.344	0.027
History of smoking	1.656	0.519	5.289	0.395
Prior IS	4.041	1.242	12.646	0.016
eGFR<30	5.879	0.841	41.100	0.074

Abbreviations: HR, hazard ratio; MI, myocardial infarction, 95%CI, 95% confidence interval.

4. Discussion

The present study determined the association of miR-126 with long-term all-cause mortality and antiplatelet treatment in patients with T2DM. It demonstrates two major findings. Namely, we revealed that high expression of plasma miR-126 may independently predict the risk of long-term all-cause mortality in the T2DM population. We also found that patients on clopidogrel treatment had higher miR-126, Let-7e and miR-223 expression compared to patients treated with ASA. It can be concluded with some caution that clopidogrel may be less beneficial than ASA 150 mg in T2DM with increased platelet reactivity; however, further studies on larger cohorts should be designed to elucidate the underlying mechanisms of this phenomenon. In a recent report by Angiolillo et al., DM was deemed to be one of the risk factors for high platelet reactivity, clopidogrel non-responsiveness and subsequent increased risk for adverse ischemic events [35].

MiR-126 belongs to the most abundantly expressed miRNAs in endothelial cells and is responsible for vascular development, integrity and response to hemodynamic stress [36]. miRNAs (miR-1, miR-133a and miR-19a) were included into one regression model; miR-126 and miR-223 were not predictive of death [37]. Moreover, Schulte et al. did not find a significant predictive value of miR-126 in CV mortality in CAD patients. Nevertheless, they reported that high miR-223 was predictive of future CV mortality, with 2.1% of the investigated cohort experiencing CV death over a median follow-up time

of 4 years [38]. In our analysis, we showed that patients with high miR-126 expression had a 5.8-fold increased risk to experience death from any reason in long term follow-up. The exact mechanism of miR-126 is yet to be fully elucidated; however, its role in diabetes and vascular inflammation was reported. Even though miR-126 has been linked to angiogenesis and to the development of CVD, its role in platelet activation was also demonstrated [36,39,40]. Inconsistent findings have been reported on the predictive potential of miR-126 in high-risk populations, including patients with diabetes. Previous studies showed a correlation between low miR-126 and miR-223 expressions and increased all-cause and CV mortality, principally in patients with overt HF, as well as in individuals with diabetes. Witkowski et al. demonstrated that vascular tissue factor (TF), which is prompted by hyperglycemia and triggers pro-thrombotic conditions in diabetes, is controlled on the post-transcriptional level by miR-126, among others [41,42]. As reported by the team, coexpression of miR-126 with miR-19a results in control of vascular inflammation and amplifies the post-transcriptional regulation of vascular TF by exhibiting a cooperative suppression of the TF transcript in a luciferase reporter assay. However, it is worth noticing that the study was performed in a relatively small cohort of 44 patients with diabetes who received different hypoglycemic agents, and hence, the results could be biased [41].

The let-7 family are amongst the most highly expressed miRNAs in platelets and are actively secreted in microvesicles [43]. Moreover, Let-7e has a potential role in platelet reactivity, platelet regulatory pathways and apoptosis and is linked to inflammatory response in vascular endothelial cells [44,45]. To date, the influence of Let-7e on antiplatelet treatment has not been studied. In the present analysis, we demonstrated for the first time that Let-7e expression can be altered by different antiplatelet treatment regimens. Let-7e expression was found to be lower in patients taking ASA compared to clopidogrel group. This difference can be due to the anti-inflammatory effect of ASA. Importantly, Let-7e has a significant predictive power and correlates significantly with platelet function itself. A further possible explanation is the fact that antiplatelet treatment itself may exert an impact on circulating levels of miR-223 and miR-126, thus attenuating their prognostic value [46]. Previous studies reported modulation of circulating miRNAs in response to different antiplatelet treatments and in relation to platelet function. Nevertheless, varying—sometimes contrasting—results have been reported in multiple studies [16,21,23,47]. In our study, despite different doses of ASA, no significant impact was observed on circulating levels of miR-126, miR-223, miR-125a-3p and Let-7, whereas a significant difference in the expression of miR-126, miR-223 and Let-7 was observed in patients treated with the purinergic receptor ($P2Y_{12}$) antagonist clopidogrel. The scarce data should be supported by further studies on larger cohorts in order to explain the influence of antiplatelet treatment on miRNAs expression.

In line with our results, miR-223 expression was shown to be differentially expressed in patients on antiplatelet treatment owing to the presence of binding site for miR-223 in natural 3′UTR in $P2Y_{12}$ mRNA. This finding supports the hypothesis that $P2Y_{12}$ expression could be modulated by miR-223 in platelets [17]. Reduced levels of plasma miR-223, primarily of platelet origin, were suggested to be a marker of efficacy of antiplatelet therapy [16,17,21,23,48,49]. However, Chyrchel et al. reported that decreased plasma miR-223 is not a marker of platelet responsiveness to dual antiplatelet therapy (DAPT). Instead, more potent platelet inhibition linked to newer $P2Y_{12}$ antagonists seems to correspond with higher miR-223 as compared to the patients with attenuated responsiveness to DAPT [50]. Interestingly, miR-223-3p in peripheral leukocytes does not correlate with the altered platelet responses in patients treated with clopidogrel [51].

Some reports suggested that miR-125a may have the influence on the development of atherosclerosis [52,53]. Although little is known about its function in the cardiovascular system, it was recently revealed that miR-125a-5p correlates with the number of platelets [54].

As we know, platelet function per se is weaving to such an extent that it makes its usefulness as a disease biomarker for a chronic condition rather weak, as reported in available

literature [55]. On the other hand, our findings demonstrate that certain circulating miRNAs are able to reflect the current clinical status of platelet inhibition (significant difference among ASA and clopidogrel) and prognostic power in our study population. Multiple mechanisms influence the outcome of antiplatelet therapy. Clopidogrel is extensively metabolized, and patients' response to the drug depends on multiple non-genetic and genetic factors [56,57]. The pharmacodynamic response is variable, with up to 40% of patients classed as nonresponders, poor responders or resistant to clopidogrel treatment mainly due to low inhibition of ADP-induced platelet aggregation or activation [58]. This phenomenon should be taken into account while interpreting the data. Our results extend previous findings on the prognostic potential of platelet-derived miRNAs in diabetes [40,46]. The superiority of $P2Y_{12}$ antagonists compared to low-dose ASA deserves further investigation and is in line with recent clinical studies on early ASA interruption in patients on DAPT to prevent bleeding complications [59–63]. Larger studies should be designed to verify the use of miRNAs as potential epigenetic biomarkers with the ability of depicting both genetic and non-genetic risk components.

5. Conclusions

In the current study, we demonstrate that in patients with T2DM, higher miR-126 expression is associated with reduced survival in long-term follow-up. Based on the results of the multivariate Cox regression, after correcting for clinical factors, one can conclude that the impact of increased miR-126 expression is unrelated to well-known risk factors for atherothrombotic disease, which further increases the strength of the results. Our findings demonstrate significantly higher expression of miR-126, miR-223 and Let-7e in patients treated with the $P2Y_{12}$ antagonist clopidogrel compared to patients on ASA treatment. Future studies are needed to warrant the clinical usefulness of miR-126 as a mortality risk predictor and to further investigate whether these miRNAs represent only a marker of disease severity or rather a distinct biomarker, which can be modified by pharmacological or lifestyle interventions. Exciting opportunities exist to further pursue platelet miRNAs miR-126, miR-223 and Let-7 to monitor and optimize antiplatelet treatment.

6. Study Limitations

The major limitation of the study is that only a fraction of all known miRNAs that are related to platelet reactivity and atherosclerosis pathogenesis were analyzed in this study. The miRNAs analyzed in the present study were chosen a priori based on literature and due to their roles in potential roles in processes related with platelet biology. A more comprehensive genome-wide analysis of miRNAs related to platelet function in T2DM is also warranted. It is therefore possible that other miRNAs might also alter primary or secondary endpoints. In AVOCADO study, three point-of-care tests (CEPI-CT and CADP-CT by PFA-100 and ARU by VerifyNow Aspirin Assay) were applied to evaluate platelet reactivity. Light transmission aggregometry (LTA), which is regarded as the gold standard for platelet reactivity assessment, was not used due to significant time consumption and increased cost of research, which were not included in the original study design [64]. Another limitation is the observational study design with a lack of control group without T2DM; therefore, it is impossible to account for all possible confounding influences. Moreover, due to the limited number of observed clinical endpoints, bias cannot be excluded despite efforts to adjust for baseline differences using multivariate Cox regression analysis. The method of collecting and scrutinizing the follow-up may be to blame for this limitation. Due to the long observational period, personal contact and examination of the patient was difficult. Since it is an observational study that was performed on a population with specific study entry criteria, a small number of events were included in primary or secondary endpoint, and not all clinical data or causes of mortality could be incorporated into the analysis. Because insufficient information is included in the Polish Statistical Registries, we were unable to obtain explicit details on the cause

of death of the subject and defined primary end point as all-cause mortality instead of cardiovascular death.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/jcm10112371/s1. Table S1: Patient's characteristics according to miR-126, Let-7e, miR-223 and miR-125a-3p expression subgroup. Comparison of the study groups. Table S2: MiRNAs expression interaction with antiplatelet therapy. Table S3: Multivariate Cox regression model for prediction of long-term all-cause of mortality for miR-126, Let-7e, miR-223 and miR-125a-3p.

Author Contributions: J.P.: substantial contribution to concept and design, RNA extraction, performance of reverse transcription and polymerase chain reaction, analysis and interpretation of data, critical writing or revising the intellectual content and final approval of the version to be published. C.E.-P.: substantial contribution to concept and design, RNA extraction, performance of reverse transcription and polymerase chain reaction, analysis and interpretation of data, statistical analysis, critical writing or revising the intellectual content and final approval of the version to be published. D.J.: RNA extraction, performance of reverse transcription and polymerase chain reaction, analysis and interpretation of data, critical writing or revising the intellectual content and final approval of the version to be published. P.C.: RNA extraction, performance of reverse transcription and polymerase chain reaction, analysis and interpretation of data and final approval of the version to be published. A.N.: critical writing or revising the intellectual content and final approval of the version to be published. S.D.R.: substantial contribution to concept and design, analysis and interpretation of data, critical writing or revising the intellectual content and final approval of the version to be published. A.G.: analysis and interpretation of data and final approval of the version to be published. A.C.-K.: analysis and interpretation of data and final approval of the version to be published. P.S.: analysis and interpretation of data and final approval of the version to be published. K.J.F.: analysis and interpretation of data and final approval of the version to be published. D.M.-G.: analysis and interpretation of data and final approval of the version to be published. J.M.S.-M.: substantial contribution to concept and design, analysis and interpretation of data, critical writing or revising the intellectual content and final approval of the version to be published. M.P.: substantial contribution to concept and design, analysis and interpretation of data, critical writing or revising the intellectual content and final approval of the version to be published. All authors have read and agreed to the published version of the manuscript.

Funding: The study was supported financially as part of the research grant 'The Diamond Grant 2016' from the Polish Ministry of Science and Higher Education (grant number 0072/DIA/2017/46) and internal funding of the Department of Experimental and Clinical Pharmacology, Medical University of Warsaw, Centre for Preclinical Research and Technology CEPT, Warsaw, Poland.

Institutional Review Board Statement: The ethics committee of the Medical University of Warsaw approved both the study protocol and the informed consent form (ethical approval number KB/178/2006). The study was conducted in accordance with the current version of the Declaration of Helsinki at the time when the study was designed.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study. The ethics committee of the Medical University of Warsaw approved the informed consent form.

Acknowledgments: The AVOCADO study was supported financially as part of the research grant from the Polish Pharmaceutical Company ADAMED for a Young Scientist 2007 Award (Grant Number: 1WR DAR1/2007). Research subject implemented with CEPT infrastructure financed by the European Union—the European Regional Development Fund within the Operational Program "Innovative economy" for 2007–2013. This paper was written as a part of cooperation of the international scientific group I-COMET (Intercontinental Cardiovascular and Cardiometabolic Research Team, www.icomet.science). The late accesses date was 29/04/2021. Authors would like to thank Aleksandra Soplinska for her qRT-PCR validations.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Vaidya, V.; Gangan, N.; Sheehan, J. Impact of Cardiovascular Complications among Patients with Type 2 Diabetes Mellitus: A Systematic Review. *Expert Rev. Pharm. Outcomes Res.* **2015**, *15*, 487–497. [CrossRef]
2. Pretorius, L.; Thomson, G.J.A.; Adams, R.C.M.; Nell, T.A.; Laubscher, W.A.; Pretorius, E. Platelet Activity and Hypercoagulation in Type 2 Diabetes. *Cardiovasc. Diabetol.* **2018**, *17*, 141. [CrossRef]
3. Postula, M.; Janicki, P.K.; Eyileten, C.; Rosiak, M.; Kaplon-Cieslicka, A.; Sugino, S.; Wilimski, R.; Kosior, D.A.; Opolski, G.; Filipiak, K.J.; et al. Next-Generation Re-Sequencing of Genes Involved in Increased Platelet Reactivity in Diabetic Patients on Acetylsalicylic Acid. *Platelets* **2016**, *27*, 357–364. [CrossRef] [PubMed]
4. Postula, M.; Janicki, P.K.; Rosiak, M.; Kaplon-Cieslicka, A.; Trzepla, E.; Filipiak, K.J.; Kosior, D.A.; Czlonkowski, A.; Opolski, G. New Single Nucleotide Polymorphisms Associated with Differences in Platelets Reactivity in Patients with Type 2 Diabetes Treated with Acetylsalicylic Acid: Genome-Wide Association Approach and Pooled DNA Strategy. *J. Thromb. Thrombolysis* **2013**, *36*, 65–73. [CrossRef] [PubMed]
5. Postula, M.; Kaplon-Cieslicka, A.; Rosiak, M.; Kondracka, A.; Serafin, A.; Filipiak, K.J.; Czlonkowski, A.; Opolski, G.; Janicki, P.K. Genetic Determinants of Platelet Reactivity during Acetylsalicylic Acid Therapy in Diabetic Patients: Evaluation of 27 Polymorphisms within Candidate Genes. *J. Thromb. Haemost.* **2011**, *9*, 2291–2301. [CrossRef]
6. Beckman, J.A.; Creager, M.A.; Libby, P. Diabetes and Atherosclerosis: Epidemiology, Pathophysiology, and Management. *JAMA* **2002**, *287*, 2570–2581. [CrossRef] [PubMed]
7. Pordzik, J.; Jakubik, D.; Jarosz-Popek, J.; Wicik, Z.; Eyileten, C.; De Rosa, S.; Indolfi, C.; Siller-Matula, J.M.; Czajka, P.; Postula, M. Significance of Circulating microRNAs in Diabetes Mellitus Type 2 and Platelet Reactivity: Bioinformatic Analysis and Review. *Cardiovasc. Diabetol.* **2019**, *18*, 113. [CrossRef] [PubMed]
8. Natarajan, A.; Zaman, A.G.; Marshall, S.M. Platelet Hyperactivity in Type 2 Diabetes: Role of Antiplatelet Agents. *Diab. Vasc. Dis. Res.* **2008**, *5*, 138–144. [CrossRef] [PubMed]
9. Xia, C.; Rao, X.; Zhong, J. Role of T Lymphocytes in Type 2 Diabetes and Diabetes-Associated Inflammation. *J. Diabetes Res.* **2017**, *2017*, 6494795. [CrossRef]
10. Dangwal, S.; Thum, T. MicroRNAs in Platelet Biogenesis and Function. *Thromb. Haemost.* **2012**, *108*, 599–604. [CrossRef]
11. De Rosa, S.; Indolfi, C. Circulating microRNAs as Biomarkers in Cardiovascular Diseases. *Exp. Suppl.* **2015**, *106*, 139–149. [CrossRef] [PubMed]
12. Weber, J.A.; Baxter, D.H.; Zhang, S.; Huang, D.Y.; Huang, K.H.; Lee, M.J.; Galas, D.J.; Wang, K. The microRNA Spectrum in 12 Body Fluids. *Clin. Chem.* **2010**, *56*, 1733–1741. [CrossRef] [PubMed]
13. Mori, M.A.; Ludwig, R.G.; Garcia-Martin, R.; Brandão, B.B.; Kahn, C.R. Extracellular miRNAs: From Biomarkers to Mediators of Physiology and Disease. *Cell Metab.* **2019**, *30*, 656–673. [CrossRef] [PubMed]
14. Fish, J.E.; Santoro, M.M.; Morton, S.U.; Yu, S.; Yeh, R.-F.; Wythe, J.D.; Ivey, K.N.; Bruneau, B.G.; Stainier, D.Y.R.; Srivastava, D. miR-126 Regulates Angiogenic Signaling and Vascular Integrity. *Dev. Cell* **2008**, *15*, 272–284. [CrossRef] [PubMed]
15. Bayés-Genis, A.; Lanfear, D.E.; de Ronde, M.W.J.; Lupón, J.; Leenders, J.J.; Liu, Z.; Zuithoff, N.P.A.; Eijkemans, M.J.C.; Zamora, E.; De Antonio, M.; et al. Prognostic Value of Circulating microRNAs on Heart Failure-Related Morbidity and Mortality in Two Large Diverse Cohorts of General Heart Failure Patients. *Eur. J. Heart Fail.* **2018**, *20*, 67–75. [CrossRef]
16. Willeit, P.; Zampetaki, A.; Dudek, K.; Kaudewitz, D.; King, A.; Kirkby, N.S.; Crosby-Nwaobi, R.; Prokopi, M.; Drozdov, I.; Langley, S.R.; et al. Circulating microRNAs as Novel Biomarkers for Platelet Activation. *Circ. Res.* **2013**, *112*, 595–600. [CrossRef]
17. Shi, R.; Zhou, X.; Ji, W.-J.; Zhang, Y.-Y.; Ma, Y.-Q.; Zhang, J.-Q.; Li, Y.-M. The Emerging Role of miR-223 in Platelet Reactivity: Implications in Antiplatelet Therapy. *Biomed. Res. Int.* **2015**, *2015*, 981841. [CrossRef]
18. Chen, Y.-C.; Lin, F.-Y.; Lin, Y.-W.; Cheng, S.-M.; Chang, C.-C.; Lin, R.-H.; Chuang, C.-L.; Sheu, J.-S.; Chen, S.-M.; Tsai, C.-S. Platelet MicroRNA 365-3p Expression Correlates with High On-Treatment Platelet Reactivity in Coronary Artery Disease Patients. Cardiovasc. *Drugs Ther.* **2019**, *33*, 129–137. [CrossRef]
19. Yu, X.-Y.; Chen, J.-Y.; Zheng, Z.-W.; Wu, H.; Li, L.-W.; Zhang, Z.-W.; Chen, Z.-H.; Lin, Q.-X.; Han, Y.-L.; Zhong, S.-L. Plasma miR-126 as a Potential Marker Predicting Major Adverse Cardiac Events in Dual Antiplatelet-Treated Patients after Percutaneous Coronary Intervention. *EuroIntervention* **2013**, *9*, 546–554. [CrossRef]
20. Stojkovic, S.; Wadowski, P.P.; Haider, P.; Weikert, C.; Pultar, J.; Lee, S.; Eichelberger, B.; Hengstenberg, C.; Wojta, J.; Panzer, S.; et al. Circulating MicroRNAs and Monocyte–Platelet Aggregate Formation in Acute Coronary Syndrome. *Thromb. Haemost.* **2021**. [CrossRef]
21. Kaudewitz, D.; Skroblin, P.; Bender, L.H.; Barwari, T.; Willeit, P.; Pechlaner, R.; Sunderland, N.P.; Willeit, K.; Morton, A.C.; Armstrong, P.C.; et al. Association of MicroRNAs and YRNAs With Platelet Function. *Circ. Res.* **2016**, *118*, 420–432. [CrossRef]
22. Wang, Z.; Xia, L.; Xu, Q.; Ji, Q.; Yao, Z.; Lv, Q. MiR-223 Levels Predicting Perioperative Bleeding in off-Pump Coronary Artery Bypass Grafting. *Ann. Transl. Med.* **2020**, *8*, 1341. [CrossRef]
23. Carino, A.; De Rosa, S.; Sorrentino, S.; Polimeni, A.; Sabatino, J.; Caiazzo, G.; Torella, D.; Spaccarotella, C.; Mongiardo, A.; Strangio, A.; et al. Modulation of Circulating MicroRNAs Levels during the Switch from Clopidogrel to Ticagrelor. *Biomed Res. Int.* **2016**, *2016*, 3968206. [CrossRef] [PubMed]
24. Benoit, C.; Ould-Hamouda, H.; Crepin, D.; Gertler, A.; Amar, L.; Taouis, M. Early Leptin Blockade Predisposes Fat-Fed Rats to Overweight and Modifies Hypothalamic microRNAs. *J. Endocrinol.* **2013**, *218*, 35–47. [CrossRef] [PubMed]

25. Wang, Y.; Bai, Y.; Liu, Y.; Wilfried Noel, S.; Yan, Q.; Pham Thi, H.; Sun, X.; Wei, W.; Ma, J.; Zheng, F. Plasma Exosomal miRNAs Involved in Endothelial Injury in Microscopic Polyangiitis Patients. *FASEB J.* **2020**, *34*, 6215–6228. [CrossRef]
26. Deiuliis, J.A. MicroRNAs as Regulators of Metabolic Disease: Pathophysiologic Significance and Emerging Role as Biomarkers and Therapeutics. *Int. J. Obes.* **2016**, *40*, 88–101. [CrossRef] [PubMed]
27. Ostrowska, M.; Kubica, J.; Adamski, P.; Kubica, A.; Eyileten, C.; Postula, M.; Toma, A.; Hengstenberg, C.; Siller-Matula, J.M. Stratified Approaches to Antiplatelet Therapies Based on Platelet Reactivity Testing. *Front. Cardiovasc. Med.* **2019**, *6*, 176. [CrossRef]
28. Rosiak, M.; Postula, M.; Kaplon-Cieslicka, A.; Kondracka, A.; Trzepla, E.; Czlonkowski, A.; Janicki, P.K.; Filipiak, K.J.; Opolski, G. Effect of ASA Dose Doubling versus Switching to Clopidogrel on Plasma Inflammatory Markers Concentration in Patients with Type 2 Diabetes and High Platelet Reactivity: The AVOCADO Study. *Cardiol. J.* **2013**, *20*, 545–551. [CrossRef]
29. Eyileten, C.; Zaremba, M.; Janicki, P.K.; Rosiak, M.; Cudna, A.; Kapłon-Cieślicka, A.; Opolski, G.; Filipiak, K.J.; Kosior, D.A.; Mirowska-Guzel, D.; et al. Serum Brain-Derived Neurotrophic Factor Is Related to Platelet Reactivity but Not to Genetic Polymorphisms within BDNF Encoding Gene in Patients with Type 2 Diabetes. *Med. Sci. Monit.* **2016**, *22*, 69–76. [CrossRef]
30. Kapłon-Cieślicka, A.; Tymińska, A.; Rosiak, M.; Ozierański, K.; Peller, M.; Eyileten, C.; Kondracka, A.; Pordzik, J.; Mirowska-Guzel, D.; Opolski, G.; et al. Resistin Is a Prognostic Factor for Death in Type 2 Diabetes. Diabetes. *Metab. Res. Rev.* **2019**, *35*, e3098. [CrossRef] [PubMed]
31. Thygesen, K.; Alpert, J.S.; Jaffe, A.S.; Chaitman, B.R.; Bax, J.J.; Morrow, D.A.; White, H.D. Fourth Universal Definition of Myocardial Infarction (2018). *Glob. Heart* **2018**, *13*, 305–338. [CrossRef] [PubMed]
32. Hicks, K.A.; Mahaffey, K.W.; Mehran, R.; Nissen, S.E.; Wiviott, S.D.; Dunn, B.; Solomon, S.D.; Marler, J.R.; Teerlink, J.R.; Farb, A.; et al. 2017 Cardiovascular and Stroke Endpoint Definitions for Clinical Trials. *Circulation* **2018**, *137*, 961–972. [CrossRef] [PubMed]
33. De Rosa, S.; Eposito, F.; Carella, C.; Strangio, A.; Ammirati, G.; Sabatino, J.; Abbate, F.G.; Iaconetti, C.; Liguori, V.; Pergola, V.; et al. Transcoronary Concentration Gradients of Circulating microRNAs in Heart Failure. *Eur. J. Heart Fail.* **2018**, *20*, 1000–1010. [CrossRef] [PubMed]
34. De Rosa, R.; De Rosa, S.; Leistner, D.; Boeckel, J.-N.; Keller, T.; Fichtlscherer, S.; Dimmeler, S.; Zeiher, A.M. Transcoronary Concentration Gradient of microRNA-133a and Outcome in Patients With Coronary Artery Disease. *Am. J. Cardiol.* **2017**, *120*, 15–24. [CrossRef]
35. Angiolillo, D.J.; Capodanno, D.; Danchin, N.; Simon, T.; Bergmeijer, T.O.; Ten Berg, J.M.; Sibbing, D.; Price, M.J. Derivation, Validation, and Prognostic Utility of a Prediction Rule for Nonresponse to Clopidogrel: The ABCD-GENE Score. *JACC Cardiovasc. Interv.* **2020**, *13*, 606–617. [CrossRef]
36. Zampetaki, A.; Kiechl, S.; Drozdov, I.; Willeit, P.; Mayr, U.; Prokopi, M.; Mayr, A.; Weger, S.; Oberhollenzer, F.; Bonora, E.; et al. Plasma microRNA Profiling Reveals Loss of Endothelial miR-126 and Other microRNAs in Type 2 Diabetes. *Circ. Res.* **2010**, *107*, 810–817. [CrossRef]
37. Mayer, O., Jr.; Seidlerová, J.; Černá, V.; Kučerová, A.; Vaněk, J.; Karnosová, P.; Bruthans, J.; Wohlfahrt, P.; Cífková, R.; Pešta, M.; et al. The Low Expression of Circulating microRNA-19a Represents an Additional Mortality Risk in Stable Patients with Vascular Disease. *Int. J. Cardiol.* **2019**, *289*, 101–106. [CrossRef]
38. Schulte, C.; Molz, S.; Appelbaum, S.; Karakas, M.; Ojeda, F.; Lau, D.M.; Hartmann, T.; Lackner, K.J.; Westermann, D.; Schnabel, R.B.; et al. miRNA-197 and miRNA-223 Predict Cardiovascular Death in a Cohort of Patients with Symptomatic Coronary Artery Disease. *PLoS ONE* **2015**, *10*, e0145930. [CrossRef] [PubMed]
39. Wang, K.; Yuan, Y.; Cho, J.-H.; McClarty, S.; Baxter, D.; Galas, D.J. Comparing the MicroRNA Spectrum between Serum and Plasma. *PLoS ONE* **2012**, *7*, e41561. [CrossRef] [PubMed]
40. de Boer, H.C.; van Solingen, C.; Prins, J.; Duijs, J.M.G.J.; Huisman, M.V.; Rabelink, T.J.; van Zonneveld, A.J. Aspirin Treatment Hampers the Use of Plasma microRNA-126 as a Biomarker for the Progression of Vascular Disease. *Eur. Heart J.* **2013**, *34*, 3451–3457. [CrossRef]
41. Witkowski, M.; Tabaraie, T.; Steffens, D.; Friebel, J.; Dörner, A.; Skurk, C.; Witkowski, M.; Stratmann, B.; Tschoepe, D.; Landmesser, U.; et al. MicroRNA-19a Contributes to the Epigenetic Regulation of Tissue Factor in Diabetes. *Cardiovasc. Diabetol.* **2018**, *17*, 34. [CrossRef]
42. Witkowski, M.; Weithauser, A.; Tabaraie, T.; Steffens, D.; Kränkel, N.; Witkowski, M.; Stratmann, B.; Tschoepe, D.; Landmesser, U.; Rauch-Kroehnert, U. Micro-RNA-126 Reduces the Blood Thrombogenicity in Diabetes Mellitus via Targeting of Tissue Factor. Arterioscler. *Thromb. Vasc. Biol.* **2016**, *36*, 1263–1271. [CrossRef] [PubMed]
43. Plé, H.; Landry, P.; Benham, A.; Coarfa, C.; Gunaratne, P.H.; Provost, P. The Repertoire and Features of Human Platelet microRNAs. *PLoS ONE* **2012**, *7*, e50746. [CrossRef] [PubMed]
44. Lin, Z.; Ge, J.; Wang, Z.; Ren, J.; Wang, X.; Xiong, H.; Gao, J.; Zhang, Y.; Zhang, Q. Let-7e Modulates the Inflammatory Response in Vascular Endothelial Cells through ceRNA Crosstalk. *Sci. Rep.* **2017**, *7*, 42498. [CrossRef]
45. Maués, J.H.D.S.; Aquino Moreira-Nunes, C.D.F.; Rodriguez Burbano, R.M. MicroRNAs as a Potential Quality Measurement Tool of Platelet Concentrate Stored in Blood Banks—A Review. *Cells* **2019**, *8*, 1256. [CrossRef] [PubMed]
46. Cavarretta, E.; Chiariello, G.A.; Condorelli, G. Platelets, Endothelium, and Circulating microRNA-126 as a Prognostic Biomarker in Cardiovascular Diseases: Per Aspirin Ad Astra. *Eur. Heart J.* **2013**, *34*, 3400–3402. [CrossRef] [PubMed]
47. De Rosa, S.; Fichtlscherer, S.; Lehmann, R.; Assmus, B.; Dimmeler, S.; Zeiher, A.M. Transcoronary Concentration Gradients of Circulating microRNAs. *Circulation* **2011**, *124*, 1936–1944. [CrossRef] [PubMed]

48. Peng, L.; Liu, J.; Qin, L.; Liu, J.; Xi, S.; Lu, C.; Yin, T. Interaction between Platelet-Derived microRNAs and CYP2C19*2 Genotype on Clopidogrel Antiplatelet Responsiveness in Patients with ACS. *Thromb. Res.* **2017**, *157*, 97–102. [CrossRef]
49. Parker, W.A.E.; Schulte, C.; Barwari, T.; Phoenix, F.; Pearson, S.M.; Mayr, M.; Grant, P.J.; Storey, R.F.; Ajjan, R.A. Aspirin, Clopidogrel and Prasugrel Monotherapy in Patients with Type 2 Diabetes Mellitus: A Double-Blind Randomised Controlled Trial of the Effects on Thrombotic Markers and microRNA Levels. *Cardiovasc. Diabetol.* **2020**, *19*, 3. [CrossRef]
50. Chyrchel, B.; Totoń-Żurańska, J.; Kruszelnicka, O.; Chyrchel, M.; Mielecki, W.; Kołton-Wróż, M.; Wołkow, P.; Surdacki, A. Association of Plasma miR-223 and Platelet Reactivity in Patients with Coronary Artery Disease on Dual Antiplatelet Therapy: A Preliminary Report. *Platelets* **2015**, *26*, 593–597. [CrossRef]
51. Xie, W.; Yin, Q.; Zhang, M.; Li, S.; Chen, S. Leukocyte miR-223-3p Is Not Associated with Altered Platelet Responses to Clopidogrel in Patients with Coronary Artery Disease. Zhong Nan Da Xue Xue Bao Yi Xue Ban. *J. Cent. South Univ. Med. Sci.* **2018**, *43*, 421–427. [CrossRef]
52. Maitrias, P.; Metzinger-Le Meuth, V.; Massy, Z.A.; M'Baya-Moutoula, E.; Reix, T.; Caus, T.; Metzinger, L. MicroRNA Deregulation in Symptomatic Carotid Plaque. *J. Vasc. Surg.* **2015**, *62*, 1245–1250. [CrossRef]
53. Hijmans, J.G.; Levy, M.A.; Garcia, V.; Lincenberg, G.M.; Diehl, K.J.; Greiner, J.J.; Stauffer, B.L.; DeSouza, C.A. Insufficient Sleep Is Associated with a pro-Atherogenic Circulating microRNA Signature. *Exp. Physiol.* **2019**, *104*, 975–982. [CrossRef] [PubMed]
54. Bhatlekar, S.; Manne, B.K.; Basak, I.; Edelstein, L.C.; Tugolukova, E.; Stoller, M.L.; Cody, M.J.; Morley, S.C.; Nagalla, S.; Weyrich, A.S.; et al. miR-125a-5p Regulates Megakaryocyte Proplatelet Formation Via the Actin Bundling Protein L-Plastin. *Blood* **2020**. [CrossRef] [PubMed]
55. Villmann, J.-M.; Burkhardt, R.; Teren, A.; Villmann, T.; Thiery, J.; Drogies, T. Atherosclerosis, Myocardial Infarction and Primary Hemostasis: Impact of Platelets, von Willebrand Factor and Soluble Glycoprotein VI. *Thromb. Res.* **2019**, *180*, 98–104. [CrossRef] [PubMed]
56. Sangkuhl, K.; Klein, T.E.; Altman, R.B. Clopidogrel Pathway. Pharmacogenet. *Genomics* **2010**, *20*, 463–465. [CrossRef]
57. Pereira, N.L.; Rihal, C.S.; So, D.Y.F.; Rosenberg, Y.; Lennon, R.J.; Mathew, V.; Goodman, S.G.; Weinshilboum, R.M.; Wang, L.; Baudhuin, L.M.; et al. Clopidogrel Pharmacogenetics. *Circ. Cardiovasc. Interv.* **2019**, *12*, e007811. [CrossRef] [PubMed]
58. Brandt, J.T.; Close, S.L.; Iturria, S.J.; Payne, C.D.; Farid, N.A.; Ernest, C.S., 2nd; Lachno, D.R.; Salazar, D.; Winters, K.J. Common Polymorphisms of CYP2C19 and CYP2C9 Affect the Pharmacokinetic and Pharmacodynamic Response to Clopidogrel but Not Prasugrel. *J. Thromb. Haemost.* **2007**, *5*, 2429–2436. [CrossRef]
59. Grines, C.L.; Marshall, J.J. Short DAPT after DES: P2Y12 Monotherapy Is In, Aspirin Is Out! *Eur. Heart J.* **2019**, *40*, 2605–2606. [CrossRef]
60. Vranckx, P.; Valgimigli, M.; Jüni, P.; Hamm, C.; Steg, P.G.; Heg, D.; van Es, G.A.; McFadden, E.P.; Onuma, Y.; van Meijeren, C.; et al. Ticagrelor plus Aspirin for 1 Month, Followed by Ticagrelor Monotherapy for 23 Months vs Aspirin plus Clopidogrel or Ticagrelor for 12 Months, Followed by Aspirin Monotherapy for 12 Months after Implantation of a Drug-Eluting Stent: A Multicentre, Open-Label, Randomised Superiority Trial. *Lancet* **2018**, *392*, 940–949. [CrossRef]
61. Hahn, J.-Y.; Song, Y.B.; Oh, J.-H.; Chun, W.J.; Park, Y.H.; Jang, W.J.; Im, E.-S.; Jeong, J.-O.; Cho, B.R.; Oh, S.K.; et al. Effect of P2Y12 Inhibitor Monotherapy vs Dual Antiplatelet Therapy on Cardiovascular Events in Patients Undergoing Percutaneous Coronary Intervention: The SMART-CHOICE Randomized Clinical Trial. *JAMA* **2019**, *321*, 2428–2437. [CrossRef] [PubMed]
62. Mehran, R.; Baber, U.; Sharma, S.K.; Cohen, D.J.; Angiolillo, D.J.; Briguori, C.; Cha, J.Y.; Collier, T.; Dangas, G.; Dudek, D.; et al. Ticagrelor with or without Aspirin in High-Risk Patients after PCI. *N. Engl. J. Med.* **2019**, *381*, 2032–2042. [CrossRef] [PubMed]
63. Guedeney, P.; Mesnier, J.; Sorrentino, S.; Abcha, F.; Zeitouni, M.; Lattuca, B.; Silvain, J.; De Rosa, S.; Indolfi, C.; Collet, J.-P.; et al. Early Aspirin Discontinuation Following Acute Coronary Syndrome or Percutaneous Coronary Intervention: A Systematic Review and Meta-Analysis of Randomized Controlled Trials. *J. Clin. Med. Res.* **2020**, *9*, 680. [CrossRef]
64. Michelson, A.D. Methods for the Measurement of Platelet Function. *Am. J. Cardiol.* **2009**, *103*, 20A–26A. [CrossRef] [PubMed]

Journal of
Clinical Medicine

MDPI

Article

The Serum Concentrations of Hedgehog-Interacting Protein, a Novel Biomarker, Were Decreased in Overweight or Obese Subjects

Hsuan-Wen Chou [1], Hao-Chang Hung [1], Ching-Han Lin [1], An-Chi Lin [1], Ye-Fong Du [1], Kai-Pi Cheng [1], Chung-Hao Li [2], Chih-Jen Chang [3], Hung-Tsung Wu [4,*] and Horng-Yih Ou [1,*]

1 Division of Endocrinology and Metabolism, Department of Internal Medicine, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, Tainan 70403, Taiwan; coolpikachu2007@gmail.com (H.-W.C.); haochang.hung@gmail.com (H.-C.H.); cyclops0113@yahoo.com.tw (C.-H.L.); fiyonlin@gmail.com (A.-C.L.); jamesdu.du@gmail.com (Y.-F.D.); supercabyhome@yahoo.com.tw (K.-P.C.)
2 Department of Health Management Center, National Cheng Kung University Hospital, National Cheng Kung University, Tainan 70403, Taiwan; smallhear@gmail.com
3 Department of Family Medicine, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, Tainan 70403, Taiwan; changcj.ncku@gmail.com
4 Graduate Institute of Metabolism and Obesity Sciences, College of Nutrition, Taipei Medical University, Taipei 11031, Taiwan
* Correspondence: wuht0716@tmu.edu.tw (H.-T.W.); wahoryi@mail.ncku.edu.tw (H.-Y.O.); Tel.: +886-2-26972035 (ext. 135) (H.-T.W.); +886-6-2353535 (ext. 4577) (H.-Y.O.); Fax: +886-2-26972133 (H.-T.W.); +886-6-3028130 (H.-Y.O.)

Citation: Chou, H.-W.; Hung, H.-C.; Lin, C.-H.; Lin, A.-C.; Du, Y.-F.; Cheng, K.-P.; Li, C.-H.; Chang, C.-J.; Wu, H.-T.; Ou, H.-Y. The Serum Concentrations of Hedgehog-Interacting Protein, a Novel Biomarker, Were Decreased in Overweight or Obese Subjects. *J. Clin. Med.* **2021**, *10*, 742. https://doi.org/10.3390/jcm10040742

Academic Editor: Katarzyna Komosinska-Vassev
Received: 22 January 2021
Accepted: 7 February 2021
Published: 12 February 2021

Abstract: Although it was known that obesity is an independent risk factor for metabolic disorders including diabetes, the factors that link these diseases were obscure. The Hedgehog-interacting protein (Hhip) is a negative regulator in tissue remodeling, and inhibits the proliferation of adipocytes, and promotes their differentiation. In addition, Hhip was positively associated with diabetes. However, the relationship between Hhip and obesity in the human body remains unclear. An analysis of the relationship between Hhip and normal weight, overweight, and obesity levels. Participants receiving a physical checkup were recruited. Anthropometric and biochemical data were collected. Serum Hhip levels were determined by enzyme-linked immunosorbent assay (ELISA). Subjects were classified into normal-weight, overweight, and obese groups based on their body mass index (BMI). The association between Hhip and obesity was examined by multivariate linear regression analysis. In total, 294 subjects who were either of a normal weight (n = 166), overweight (n = 90), or obese (n = 38) were enrolled. Hhip concentrations were 6.51 ± 4.86 ng/mL, 5.79 ± 4.33 ng/mL, and 3.97 ± 3.4 ng/mL in normal-weight, overweight, and obese groups, respectively (p for trend = 0.032). Moreover, the regression analysis showed that BMI (β = −0.144, 95% confidence interval (CI) = −0.397−0.046, p = 0.013) was negatively associated with Hhip concentrations after adjusting for sex and age. Being overweight (β = −0.181, 95% CI = −3.311−0.400, p = 0.013) and obese (β = −0.311, 95% CI = −6.393−2.384, p < 0.001) were independently associated with Hhip concentrations after adjusting for sex, age, fasting plasma glucose, the insulin level, and other cardiometabolic risk factors. Our results showed that overweight and obese subjects had lower Hhip concentrations than those of normal weight. Being overweight and obese were negatively associated with Hhip concentrations. Hhip might be a link between obesity and diabetes.

Keywords: hedgehog-interacting protein; impaired fasting glucose; impaired glucose tolerance; newly diagnosed diabetes; normal glucose tolerance

1. Introduction

Obesity is recognized as an independent risk factor for the development of many diseases, such as diabetes mellitus, cardiovascular diseases, and even cancer [1–3]. The

World Health Organization defines obesity as abnormal or excessive fat accumulation that may impair health [4]. In clinical practice, the body mass index (BMI) has been used for diagnosing obesity and being overweight [5]. An energy imbalance of more calories being consumed than expended is the most important cause of obesity, and the consequence is the storage of excess energy in adipose tissues that increase in size by hypertrophy and hyperplasia [6,7].

The Hedgehog (Hh) signaling pathway is known to be an important pathway for the growth, development, and homeostasis of many tissues in animals, especially during embryonic development [8]. Recently, Hh signaling was proven to be related to adipose tissue differentiation [9–12]. Activation of Hh signaling inhibits adipocyte differentiation in vitro [9]. Targeted activation of Hh signaling suppresses high-fat-diet-induced obesity and improves whole-body glucose tolerance and insulin sensitivity in vivo [10]. Because the Hh signaling pathway was reported to be involved in adipogenesis, it was proposed as a potential therapeutic target for metabolic diseases such as type 2 diabetes and obesity [11,13].

The Hh-interacting protein (Hhip), a membrane glycoprotein, is a negative regulator that attenuates Hh signaling by binding to its ligands [14,15]. During 8-day adipocyte differentiation, Hhip messenger RNA and protein expressions peaked at day 6 in 3T3-L1 cells [13]. In addition, Hhip messenger RNA expression in adipose tissues was higher in 3-day-old than in 180-day-old pigs [13]. Recombinant Hhip treatment promoted 3T3-L1 cell differentiation by upregulating the expression of peroxisome proliferator-activated receptor γ and glucose transporter 4 and downregulating the expression of the Hh signaling transcription factor, Gli1 [13]. We previously reported that the Hhip was positively associated with prediabetes and type 2 diabetes [16]. Because obesity is closely associated with dysglycemia [1], we explored the relationship between Hhip levels and being overweight/obese in humans in this study.

2. Materials/Subjects and Methods

2.1. Participants

This study was approved by the Institutional Review Board of National Cheng Kung University Hospital (ER-104-204) (Tainan, Taiwan), and all participants signed an informed consent form before joining the study. All participants in the study were recruited between January 2016 and December 2016 from the Health Examination Center of National Cheng Kung University Hospital.

Blood was sampled at 9:00 from all participants after they had fasted for 12 h overnight. Subjects without a history of diabetes received an oral glucose tolerance test. After fasting blood sampling, subjects were instructed to drink 75 g glucose in 300 mL water within 5 min. Two hours after drinking glucose solution (11 am), a blood sample was collected again to measure blood glucose level. Those who (1) had an acute or chronic inflammatory disease as determined by a leukocyte count of >10,000/mm^3 or clinical signs of infection; (2) had any other major diseases, including generalized inflammation or advanced malignant diseases contraindicating this study; (3) were pregnant; (4) had a history of diabetes and were receiving insulin therapy, glucagon like-peptide-1, or oral antidiabetic drugs; (5) were taking drugs that affect glucose homeostasis, such as corticosteroids, thiazides, etc.; (6) had experienced an acute coronary syndrome, cerebrovascular accident, or pancreatitis during the past three months; or (7) were taking lipid-lowering medications or antihypertensive drugs were excluded.

We grouped all participants into one of three groups according to the recommendations of the Health Promotion Administration of Taiwan based on their BMI—normal weight (18.5 kg/m^2 < BMI < 24 kg/m^2), overweight (BMI $\geq$ 24 kg/m^2), and obese (BMI $\geq$ 27 kg/m^2) [17].

2.2. Data Collection

We measured every subject's body height and waist circumference to the nearest 0.1 cm and body weight (BW) to the nearest 0.1 kg. The BMI was defined as the BW (kg) divided by the body height (m) squared. We asked participants to rest in the supine position in a quiet place to measure the blood pressure between 08:00 and 10:00 while in a fasted status. An appropriate-sized cuff was used for the right upper arm, and the pressure was checked twice at an interval of at least 5 min using a DINAMAP vital signs monitor (model 1846SX; Critikon, Irvine, CA, USA). The hexokinase method (Roche Diagnostic, Mannheim, Germany) was used to measure the blood glucose. An enzyme-linked immunosorbent assay (ELISA) (Mercodia AB, Uppsala, Sweden) was used to measure serum insulin levels. A highly sensitive ELISA kit (Immunology Consultants Laboratory, Newberg, OR, USA) was used to determine high-sensitivity C-reactive protein. A human Hhip ELISA kit (MyBioSource, San Diego, CA, USA) was used for determining serum Hhip concentrations. The intra-assay coefficient of variation of the ELISA was 5.52% and the inter-assay coefficient of variation of it was 4.9%. An autoanalyzer (Hitachi 747E; Tokyo, Japan) in the central laboratory of National Cheng Kung University was employed to obtain serum alanine aminotransferase, aspartate aminotransferase, total cholesterol, triglycerides, high-density lipoprotein cholesterol, and low-density lipoprotein cholesterol. A high-performance liquid chromatographic method (Tosoh Automated Glycohemoglobin Analyzer; Tokyo, Japan) was used to measure glycated hemoglobin (HbA1c). The estimated glomerular filtration rate (eGFR) was calculated by the modification of the diet in a renal disease equation. The homeostasis model assessment of insulin resistance was defined by the formula that is equal to fasting insulin (mU/L) multiplied by fasting plasma glucose (mg/dl) divided by 405 to investigate insulin resistance [18].

2.3. Statistical Analyses

Data were analyzed using SPSS software (vers. 24.0; SPSS, Chicago, IL, USA). Baseline characteristics are expressed as the mean ± standard deviation (SD) for continuous variables or as a percentage for categorical variables. A one-way analysis of variance (ANOVA) was used to determine any difference in variables among the groups. Chi-square tests were used to analyze differences in categorical variables among the groups. The Bonferroni correction was used for a post hoc study to see if serum Hhip concentrations differed among the groups. A multivariate linear regression analysis was performed to identify independent variables related to serum Hhip concentrations. The criterion for statistical significance was a p-value of <0.05.

3. Results

Overall, 294 subjects were enrolled and classified into the normal-weight (n = 166), overweight (n = 90), and obese (n = 38) groups. The average age of them was 61.34 ± 11.87-year-old. Comparisons of baseline characteristics of these participants are shown in Table 1. There were significant differences in the BW ($p < 0.001$), waist circumference ($p < 0.001$), BMI ($p < 0.001$), diastolic blood pressure ($p = 0.022$), HbA1c ($p = 0.026$), high-density lipoprotein cholesterol ($p = 0.008$), triglycerides ($p = 0.008$), homeostasis model assessment of insulin resistance ($p < 0.001$), and insulin levels ($p < 0.001$) among the three groups. Hhip concentrations were 6.51 ± 4.86 ng/mL, 5.79 ± 4.33 ng/mL, and 3.97 ± 3.4 ng/mL in the normal-weight, overweight, and obese groups, respectively (Figure 1, trend test $p = 0.032$). In the post hoc analysis, serum Hhip concentrations were significantly lower in the obese group ($p = 0.006$), compared to the normal-weight group. Serum Hhip concentrations were not different between the overweight group and the normal-weight group ($p = 0.667$). Moreover, after exclusion of the study subjects with diabetes, the serum Hhip concentrations were 5.99 ± 4.86 ng/mL, 5.28 ± 3.91 ng/mL, and 4.1 ± 3.55 ng/mL in the normal-weight, overweight, and obese groups. We still observed that serum Hhip concentrations significantly decreased among groups using post hoc analysis (p for trend test was 0.044).

Table 1. Comparisons of clinical parameters among normal-weight, overweight, and obese subjects.

Clinical Parameters	Normal Weight	Overweight	Obese	*p*
n	166	90	38	
Female (%)	42.8	31.1	44.7	0.147
Hypertension (%)	17.1	23.3	27	0.272
Diabetes (%)	24.7	31.1	31.6	0.459
Age (years)	61.7 ± 11.9	60.5 ± 11.8	61.4 ± 12.3	0.736
Body weight (kg)	56.4 ± 7	67.59 ± 7.47	75.79 ± 10.8	<0.001
Waist circumference (cm)	78.37 ± 6.82	87.51 ± 5.77	94.43 ± 8.44	<0.001
Body-mass index (kg/m^2)	21.65 ± 1.87	25.44 ± 0.88	28.49 ± 1.75	<0.001
SBP (mmHg)	125.3 ± 18.1	128.5 ± 16.1	131.5 ± 16.7	0.098
DBP (mmHg)	72.2 ± 10.4	74.9 ± 9.7	76.8 ± 12.0	0.022
FPG (mg/dL)	101.78 ± 43.28	107.36 ± 35.33	116.47 ± 49.68	0.131
Post-load 2-h glucose (mg/dL)	155.34 ± 80.21	147.54 ± 68.90	175.41 ± 97.48	0.234
HbA1c (%)	6.13 ± 1.28	6.24 ± 1.05	6.79 ± 2.09	0.026
ALT (U/L)	25.51 ± 19.50	35.19 ± 53.50	24.89 ± 10.43	0.068
AST (U/L)	27.23 ± 13.70	30.56 ± 41.84	24.68 ± 25.45	0.429
Creatinine (mg/dL)	0.87 ± 0.20	0.87 ± 0.18	0.88 ± 0.20	0.886
eGFR	90.63 ± 19.76	91.72 ± 15.65	88.22 ± 19.24	0.62
hsCRP (mg/L)	3.68 ± 7.52	3.73 ± 6.27	4.48 ± 4.71	0.809
HDL-C (mg/dL)	55.73 ± 15.39	49.98 ± 13.55	51.74 ± 12.49	0.008
LDL-C (mg/dL)	126.33 ± 33.89	129.02 ± 35.91	131.28 ± 44.65	0.694
Triglycerides (mg/dL)	110.47 ± 68.96	127.56 ± 60.71	144.94 ± 70.84	0.008
Triglycerides (mg/dL) *	1.99 ± 0.21	2.06 ± 0.20	2.12 ± 0.20	<0.001
Cholesterol (mg/dL)	204.16 ± 39.555	204.51 ± 41.545	212.00 ± 47.594	0.56
HOMA-IR	0.50 ± 0.47	0.97 ± 1.50	1.26 ± 1.011	<0.001

Data are expressed as the mean ± standard deviation or as a percentage. * Values were log-transformed before analysis. Hhip, Hedgehog-interacting protein; SBP, systolic blood pressure; DBP, diastolic blood pressure; FPG, fasting plasma glucose; HbA1c, glycated hemoglobin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; eGFR, estimated glomeruli filtration rate; hsCRP, high-sensitivity C-reactive protein; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; HOMA-IR, homeostasis model assessment of insulin resistance.

In the multivariate linear regression analysis (Table 2), the BMI (β = −0.144, 95% CI = −0.397−0.046, $p = 0.013$) was negatively associated with Hhip concentrations after adjusting for sex and age (model 1). After adding the fasting glucose and insulin levels as confounding factors into model 1, the BMI (β = −0.147, 95% CI = −0.411−0.041, $p = 0.017$) was still independently associated with Hhip concentrations (model 2). To evaluate if the BMI status made a difference in Hhip concentrations, the normal-weight group was used as a reference to compare with the overweight and obese groups. We found that being overweight (β = −0.181, 95% CI = −3.311−0.400, $p = 0.013$) and obese (β = −0.311, 95% CI = −6.393~−2.384, $p < 0.001$) were independently negatively associated with Hhip concentrations after adjusting for sex, age, fasting plasma glucose, insulin level, high-sensitivity C-reactive protein, systolic blood pressure, the estimated glomeruli filtration rate (eGFR), alanine aminotransferase, cholesterol, triglycerides, high-density lipoprotein cholesterol, and low-density lipoprotein cholesterol (model 3).

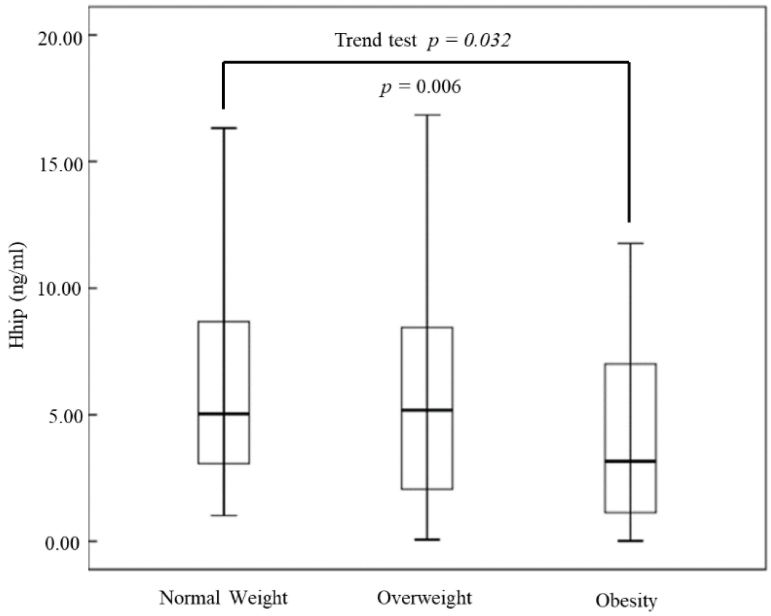

Figure 1. Comparisons of serum concentrations of the Hedgehog-interacting protein (Hhip) in normal-weight, overweight, and obese subjects. Box and whisker plot of serum Hhip concentrations in participants with normal-weight (n = 166), overweight (n = 90), and obese subjects (n = 38). The line inside the box represents the median of the distribution, the box top, and bottom values are defined by the 25th and 75th percentiles, and the whiskers are minimum and maximum values.

Table 2. Results of multivariate linear regression analysis between the Hedgehog-interacting protein (Hhip) and clinical variables.

Variable	Model 1 β (95% CI)	p	Model 2 β (95% CI)	p	Model 3 β (95% CI)	p
Age (years)	−0.029 (−0.056~0.033)	0.612	−0.034 (−0.058~0.032)	0.565	−0.006 (−0.062~0.057)	0.938
Sex	0.143 (0.278~2.412)	0.014	0.134 (0.182~2.350)	0.022	0.158 (0.204~2.857)	0.024
Body-mass index	−0.144 (−0.397~−0.046)	0.013	−0.147 (−0.411~−0.041)	0.017		
OW vs. NW					−0.181 (−3.311~−0.400)	0.013
OB vs. NW					−0.311 (−6.393~−2.384)	<0.001
Fasting glucose (mg/dL)			0.024 (−0.010~0.015)	0.694	0.007 (−0.015~0.016)	0.925
Insulin (mIU/L)			−0.011 (−0.210~0.175)	0.859	0.049 (−0.175~0.354)	0.503
hsCRP (mg/L)					0.016 (−0.078~0.100)	0.809
SBP (mmHg)					0.06 (−0.021~0.054)	0.397
eGFR					−0.026 (−0.042~0.029)	0.715
ALT (U/L)					−0.07 (−0.027~0.008)	0.296
CHOL (mg/dL)					−0.063 (−0.147~0.133)	0.923
TGs (mg/dL) *					0.029 (−8.388~9.701)	0.886
HDL-C (mg/dL)					−0.101 (−0.182~0.116)	0.663
LDL-C (mg/dL)					0.129 (−0.126~0.158)	0.821

* Values were log-transformed before analysis. OW, overweight; NW, normal weight; OB, obese; hsCRP, high-sensitivity C-reactive protein; SBP, systolic blood pressure; ALT, alanine aminotransferase; eGFR, estimated glomerular filtration rate; CHOL, cholesterol; TGs, triglycerides; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

4. Discussion

To the best of our knowledge, this is the first human study to explore the relationship between obesity and the Hhip. We found that Hhip levels progressively decreased from the normal-weight and overweight groups to the obese group. In addition, the BMI was negatively associated with serum Hhip concentrations. Moreover, being overweight and obese were negatively associated with serum Hhip concentrations.

According to our previous study, the presence of prediabetes and type 2 diabetes was positively associated with serum Hhip concentrations, while the BMI was not [16]. However, the average BMI in the previous study was similar among subjects with normal glucose tolerance (BMI = 22.3 kg/m^2), impaired fasting glucose (BMI = 23.6 kg/m^2), impaired glucose tolerance (BMI = 23.4 kg/m^2), and newly diagnosed diabetes (BMI = 23.3 kg/m^2), although the difference reached borderline statistical significance (p = 0.049), which may be at risk of a type 1 error, and subjects with obesity might not have been included. It is therefore unknown whether or not being overweight/obese is associated with plasma Hhip concentrations. Wei et al. reported that recombinant Hhip can increase adipocyte differentiation, which results in increased accumulation of lipid droplets in adipocytes by inhibiting the Hh signaling pathway in 3T3-L1 cells, and Hhip messenger RNA expression in adipose tissues was lower in 180-day-old than in 3-day-old pigs [13]. It was suggested that serum Hhip concentrations may be negatively regulated by differentiated adipose tissues. Once one becomes obese, the production of Hhip should decrease to prevent further adipocyte differentiation. However, the mechanism as to how adipose tissues influence serum Hhip concentrations remains unclear. To address this hypothesis, further human studies are required. In our cohort, obese subjects had a lower level of Hhip protein, which can be viewed as a mechanism of negative feedback, in order to promote the Hh pathway and inhibit fat formation. We still found that serum Hhip concentrations significantly decreased in the obese group after excluding subjects with diabetes. Moreover, in our previous study, subjects with prediabetes or type 2 diabetes have a higher level of Hhip concentrations. We, therefore, speculated that elevated Hhip concentrations might be a hint that these overweight or obese subjects may have a risk to progress into diabetes compared with those who have lower Hhip concentrations. Hh signaling plays an important role in inhibiting fat formation [11]. A previous animal study showed the activation of Hh signaling decreased obesity induced by a high-fat diet in adult mice [10], and a deficiency of Hh signaling in myeloid cells increased the BW of mice [19]. In obese subjects, the circulating leptin level increases. Wang et al. reported that leptin decreased the weight of obese mice induced by a high-fat diet and inhibited Gli1 expression [20]. In a human study, expression of the Hh signaling transcription factor, Gli1, significantly decreased in adipose tissues of insulin-sensitive obese subjects compared to lean subjects, which may indicate that Hh signaling decreases in obese humans [21]. Circulating Hh ligands and expressions of Hh ligands in adipose tissues increased in obese mice. However, serum Hh ligand levels significantly decreased in morbidly obese (BMI > 40 kg/m^2) people, even in those with HbA1c > 7%, possibly due to the inhibitory effect of metformin on Hh ligand expression in adipose tissues [19]. As leptin negatively regulates Hh signaling by decreasing Gli1 expression and Hhip is a negative regulator that attenuates Hh signaling by binding to Hh ligands, we may speculate that decreased serum Hhip concentrations in obese subjects are a compensatory mechanism of decreasing Gli1 expression. However, further study is needed to clarify the regulatory architecture. Cholesterol has been shown to be an endogenous Smoothened activator, which is a second messenger activating the Hedgehog signaling pathway [22]. Exogenously added cholesterol would activate Hh signaling pathway in vitro [23]. Cholesterol is not just necessary but also sufficient to activate signaling by the Hh pathway [24]. Hh signaling plays an important role in inhibiting fat formation [11], which means that elevated cholesterol levels might activate Hh signaling to minimize fat formation at the same time that Hhip should be downregulated to avoid fat formation. However, there has been no human study to discuss the relationship between cholesterol and Hhip so far. In our study, we found no difference in cholesterol levels

among the three groups. Moreover, in multivariate linear regression analysis, cholesterol was not an independent factor of serum Hhip concentrations. The relationship among cholesterol level, Hh signaling pathway, and serum Hhip concentrations needs to be evaluated in a human study. There were some limitations in this study. First, this study was designed as a cross-sectional study which did not allow for causal inferences between serum Hhip concentrations and BMI or obesity. Second, although one study revealed that the Hhip was associated with moderate to severe chronic obstructive pulmonary disease, all of our participants were apparently healthy with no airway symptoms [25]. Third, we could not directly measure Hhip expression by adipose tissues. Therefore, we could not be sure whether serum Hhip concentrations were representative of those in adipose tissues. Finally, all study subjects were Taiwanese, and thus our findings might not be applicable to other ethnicities.

5. Conclusions

Our results demonstrated that serum Hhip concentrations were negatively associated with BMI, and obese subjects had lower serum Hhip concentrations than normal-weight subjects. Further research is needed to explore the pathophysiological roles and clinical implications of the Hhip in obesity.

Author Contributions: Conceptualization, H.-W.C., H.-C.H., and A.-C.L.; methodology, H.-W.C., H.-C.H., and A.-C.L.; software, C.-H.L. (Ching-Han Lin), Y.-F.D., and K.-P.C.; validation, C.-H.L. (Ching-Han Lin), Y.-F.D., and K.-P.C.; formal analysis, H.-W.C., H.-C.H., and A.-C.L.; investigation, C.-H.L. (Ching-Han Lin), Y.-F.D., and K.-P.C.; resources, C.-H.L. (Chung-Hao Li), Y.-F.D., and K.-P.C.; data curation, C.-H.L. (Chung-Hao Li), Y.-F.D., and K.-P.C.; writing—original draft preparation, H.-W.C., H.-C.H., and A.-C.L.; writing—review and editing, C.-H.L. (Ching-Han Lin) and C.-J.C.; visualization, C.-H.L. (Ching-Han Lin) and C.-J.C.; supervision, H.-T.W. and H.-Y.O.; project administration, H.-T.W. and H.-Y.O.; funding acquisition, H.-T.W. and H.-Y.O. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by Grants 109-2314-B-038 -060, 109-2314-B-038-103, and 108-2314-B-006-032-MY3 from the Ministry of Science and Technology of the Republic of China and an unrestricted research grant from the Chen Jie Chen Scholarship Foundation (to H.-Y.O.).

Institutional Review Board Statement: This study was approved by the Institutional Review Board of National Cheng Kung University Hospital (ER-104-204) (Tainan, Taiwan).

Informed Consent Statement: All participants signed an informed consent form before joining the study.

Data Availability Statement: Part of the data generated in this study is included in the article. Patient and clinicopathological data in this cannot be made publicly available due to their content of identifiable human data. Requests to access the datasets should be directed to Horng-Yih Ou.

Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Abbreviations

BMI	body mass index
ALT	alanine aminotransferase
AST	aspartate aminotransferase
DBP	diastolic blood pressure
eGFR	estimated glomeruli filtration rate
FPG	fasting plasma glucose
HbA1c	glycated hemoglobin
HDL-C	high-density lipoprotein cholesterol
Hhip	Hedgehog-interacting protein
HOMA-IR	homeostasis model assessment of insulin resistance
hsCRP	high-sensitivity C-reactive protein
LDL-C	low-density lipoprotein cholesterol
SBP	systolic blood pressure

References

1. Al-Goblan, A.S.; Al-Alfi, M.A.; Khan, M.Z. Mechanism linking diabetes mellitus and obesity. *Diabetes Metab. Syndr. Obes.* **2014**, *7*, 587–591. [CrossRef] [PubMed]
2. Lavie, C.J.; Milani, R.V.; Ventura, H.O. Obesity and Cardiovascular Disease: Risk Factor, Paradox, and Impact of Weight Loss. *J. Am. Coll Cardiol.* **2009**, *53*, 1925–1932. [CrossRef] [PubMed]
3. CDC Press Releases. Available online: https://www.cdc.gov/media/releases/2017/p1003-vs-cancer-obesity.html (accessed on 25 November 2019).
4. Obesity and Overweight. Available online: https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight (accessed on 25 November 2019).
5. Müller, M.J.; Geisler, C. Defining obesity as a disease. *Eur J. Clin. Nutr.* **2017**, *71*, 1256–1258. [CrossRef]
6. De Ferranti, S.; Mozaffarian, D. The perfect storm: Obesity, adipocyte dysfunction, and metabolic consequences. *Clin. Chem.* **2008**, *54*, 945–955. [CrossRef]
7. Ghaben, A.L.; Scherer, P.E. Adipogenesis and metabolic health. *Nat. Rev. Mol. Cell Biol.* **2019**, *20*, 242–258. [CrossRef] [PubMed]
8. Smelkinson, M.G. The Hedgehog Signaling Pathway Emerges as a Pathogenic Target. *J. Dev. Biol.* **2017**, *5*, 14. [CrossRef]
9. Cousin, W.; Fontaine, C.; Dani, C.; Peraldi, P. Hedgehog and adipogenesis: Fat and fiction. *Biochimie* **2007**, *89*, 1447–1453. [CrossRef] [PubMed]
10. Shi, Y.; Long, F. Hedgehog signaling via Gli2 prevents obesity induced by high-fat diet in adult mice. *eLife* **2017**, *6*, e31649. [CrossRef]
11. Suh, J.M.; Gao, X.; McKay, J.; McKay, R.; Salo, Z.; Graff, J.M. Hedgehog signaling plays a conserved role in inhibiting fat formation. *Cell Metab.* **2006**, *3*, 25–34. [CrossRef]
12. Fontaine, C.; Cousin, W.; Plaisant, M.; Dani, C.; Peraldi, P. Hedgehog Signaling Alters Adipocyte Maturation of Human Mesenchymal Stem Cells. *Stem Cells.* **2008**, *26*, 1037–1046. [CrossRef]
13. Wei, H.; Li, J.; Shi, S.; Zhang, L.; Xiang, A.; Yang, G.; Chu, G. Hhip inhibits proliferation and promotes differentiation of adipocytes through suppressing hedgehog signaling pathway. *Biochem Biophys Res. Commun.* **2019**, *514*, 148–156. [CrossRef]
14. Chuang, P.T.; McMahon, A.P. Vertebrate Hedgehog signalling modulated by induction of a Hedgehog-binding protein. *Nature* **1999**, *397*, 617–621. [CrossRef]
15. Lee, B.N.R.; Son, Y.S.; Lee, D.; Choi, Y.J.; Kwon, S.M.; Chang, H.K.; Kim, P.H.; Cho, J.-Y. Hedgehog-Interacting Protein (HIP) Regulates Apoptosis Evasion and Angiogenic Function of Late Endothelial Progenitor Cells. *Sci Rep.* **2017**, 12449. [CrossRef]
16. Lin, A.C.; Hung, H.C.; Chen, Y.W.; Cheng, K.P.; Li, C.H.; Lin, C.H.; Chang, C.-J.; Wu, H.-T.; Ou, H.Y. Elevated Hedgehog-Interacting Protein Levels in Subjects with Prediabetes and Type 2 Diabetes. *J. Clin. Med.* **2019**, *8*, 1635. [CrossRef]
17. Health Promotion Administration, Ministry of Health and Welfare, Taiwan. Available online: https://www.hpa.gov.tw/Pages/Detail.aspx?nodeid=542&pid=9737 (accessed on 24 August 2020).
18. Matthews, D.R.; Hosker, J.P.; Rudenski, A.S.; Naylor, B.A.; Treacher, D.F.; Turner, R.C. Homeostasis model assessment: Insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* **1985**, *28*, 412–419. [CrossRef] [PubMed]
19. Braune, J.; Weyer, U.; Matz-Soja, M.; Hobusch, C.; Kern, M.; Kunath, A.; Klöting, N.; Kralisch, S.; Blüher, M.; Gebhardt, R.; et al. Hedgehog signalling in myeloid cells impacts on body weight, adipose tissue inflammation and glucose metabolism. *Diabetologia* **2017**, *60*, 889–899. [CrossRef] [PubMed]
20. Wang, J.; Ge, J.; Cao, H.; Zhang, X.; Guo, Y.; Li, X.; Xia, B.; Yang, G.; Shi, X.E. Leptin Promotes White Adipocyte Browning by Inhibiting the Hh Signaling Pathway. *Cells* **2019**, *8*, 372. [CrossRef] [PubMed]
21. Todoric, J.; Strobl, B.; Jais, A.; Boucheron, N.; Bayer, M.; Amann, S.; Lindroos, J.; Teperino, R.; Prager, G.; Bilban, M. Cross-Talk Between Interferon-γ and Hedgehog Signaling Regulates Adipogenesis. *Diabetes* **2011**, *60*, 1668–1676. [CrossRef] [PubMed]

22. Kowatsch, C.; Woolley, R.E.; Kinnebrew, M.; Rohatgi, R.; Siebold, C. Structures of vertebrate Patched and Smoothened reveal intimate links between cholesterol and Hedgehog signalling. *Curr Opin Struct Biol.* **2019**, *57*, 204–214. [CrossRef]
23. Huang, P.; Nedelcu, D.; Watanabe, M.; Jao, C.; Kim, Y.; Liu, J.; Salic, A. Cellular Cholesterol Directly Activates Smoothened in Hedgehog Signaling. *Cell* **2016**, *166*, 1176–1187. [CrossRef]
24. Luchetti, G.; Sircar, R.; Kong, J.H.; Nachtergaele, S.; Sagner, A.; Byrne, E.F.; Covey, D.F.; Siebold, C.; Rohatgi, R. Cholesterol activates the G-protein coupled receptor Smoothened to promote Hedgehog signaling. *eLife* **2016**, *5*, e20304. [CrossRef] [PubMed]
25. Zhou, X.; Baron, R.M.; Hardin, M.; Cho, M.H.; Zielinski, J.; Hawrylkiewicz, I.; Sliwinski, P.; Hersh, C.P.; Mancini, J.D.; Lu, K.; et al. Identification of a chronic obstructive pulmonary disease genetic determinant that regulates HHIP. *Hum. Mol. Genet.* **2012**, *21*, 1325–1335. [CrossRef] [PubMed]

Journal of
Clinical Medicine

MDPI

Systematic Review

Pharmacogenomic Biomarkers of Follicle-Stimulating Hormone Receptor Malfunction in Females with Impaired Ovarian Response—A Genetic Survey

Alireza Tafazoli [1,2], Sławomir Wołczyński [3], Natalia Wawrusiewicz-Kurylonek [4], Seyed-Alireza Esmaeili [5,6] and Wojciech Miltyk [1,*]

1 Department of Analysis and Bioanalysis of Medicines, Faculty of Pharmacy with the Division of Laboratory Medicine, Medical University of Białystok, 15-089 Białystok, Poland; Alireza.Tafazoli@umb.edu.pl
2 Clinical Research Centre, Medical University of Białystok, 15-276 Bialystok, Poland
3 Department of Reproduction and Gynecological Endocrinology, Medical University of Białystok, 15-222 Białystok, Poland; slawek.wolczynski@gmail.com
4 Department of Clinical Genetics, Medical University of Białystok, 15-089 Białystok, Poland; natalia.kurylonek@gmail.com
5 Immunology Department, Faculty of Medicine, Mashhad University of Medical Sciences, 9177948564 Mashhad, Iran; imunoman2009@gmail.com
6 Immunology Research Center, Mashhad University of Medical Sciences, 9177948564 Mashhad, Iran
* Correspondence: Wojciech.Miltyk@umb.edu.pl

Abstract: Follicle-stimulating hormone receptor (FSHR) plays an essential role as one of the most important molecules in response to some of infertility related medications. Impaired ovarian reserve and poor response to such treatments are partially dependent on the FSHR molecule itself. However, the function and drug sensitivity for this receptor may change due to various allele and polymorphisms in the *FSHR* gene. Studies indicated some of the FSHR-mediated treatments utilized in clinical centers display different outcomes in specific populations, which may arise from FSHR altered genotypes in certain patients. To support the increased demands for reaching the personalized drug and hormone therapy in clinics, focusing on actionable variants through Pharmacogenomic analysis of this receptor may be necessary. The current study tries to display a perspective view on genetic assessments for Pharmacogenomic profiling of the *FSHR* gene via providing a systematic and critical overview on the genetics of FSHR and its diverse responses to ligands for infertility treatment in females with impaired ovarian responses and show the potential effects of the patient genetic make-up on related binding substances efficacy. All identified functional drug-related alleles were selected through a comprehensive literature search and analyzed. Advanced technologies for the genetic evaluation of them are also discussed properly.

Keywords: FSHR; pharmacogenomics; personalized drug therapy

Citation: Tafazoli, A.; Wołczyński, S.; Wawrusiewicz-Kurylonek, N.; Esmaeili, S.-A.; Miltyk, W. Pharmacogenomic Biomarkers of Follicle-Stimulating Hormone Receptor Malfunction in Females with Impaired Ovarian Response—A Genetic Survey. *J. Clin. Med.* **2021**, *10*, 170. https://doi.org/10.3390/jcm10020170

Received: 29 November 2020
Accepted: 2 January 2021
Published: 6 January 2021

1. Introduction: FSHR, Related Infertility Medicines, and the Role of Pharmacogenetics

Follicle-stimulating hormone receptor (FSHR) is a rhodopsin-like receptor, which belongs to the G-protein-coupled receptors (GPCR) superfamily, and consists of an intracellular domain and a large leucine-rich repeat extracellular domain and passes seven times through the cellular membrane [1]. The *FSHR* gene consists of 10 exons, of which exon 1 to exon 9 encode the extracellular domain and exon 10 is responsible for a small part of the extracellular domain and transmembrane and intracellular parts. FSHR binds the gonadotropin follicle-stimulating hormone (FSH) and other agonists leading to rapid activation of different cellular cascades, mainly cAMP–adenylyl cyclase–protein kinase A (also known as the adenylyl cyclase), Ras-MAPK (mitogen-activated protein kinase), and β-arrestin signal transduction pathways. Adenylyl cyclase and RAS-MAPK are activated through activation of heterotrimeric Gs protein and adaptor proteins, recruiting

adenylyl cyclase, phospholipase C, and guanine nucleotide exchange factors (GEFs) respectively [2,3]. In addition, other related transcription factors, as well as cAMP regulatory element-binding protein (CREB) and extracellular signal-regulated kinase (ERK), cause the final fertility effects of activated FSHR such as maturation and follicular development in addition to the differentiation of ovaries by targeting the promoter of some genes and inducing up/downregulation of them (i.e., *CYP19A1*) [4].

It has been estimated that globally infertility affects between 8–12% of couples of their reproductive age (over 186 million people). However, males and females contribute differently to overall infertility cases [5]. Many FSHR associated treatments like synthetic or natural derived follicle-stimulating medicines, gonadotropins, and ovarian stimulation drugs such as clomiphene and metformin are used for infertility treatment in females [6]. These drugs are also used in assisted reproductive technologies (ART) to stimulate multiple follicles, which need the medications to collect, to develop, and to maturate eggs with different dosages and usage protocols [7]. The differences are because of the various responders in women who received these treatments during infertility therapy approaches. In addition, molecular biology of the triggered cellular pathways for these medications turned to more attention in clinical investigations. From this point of view, FSH and other related medicines as the specific ligands bind to FSHR, leading to start the signal transduction pathways in target cells (granulosa cells in females). This leads to the growth of follicles in ovaries and final ovulation through changes in expression of particular genes and thus triggering proliferation and differentiation of oocyte in females. Studies on side effects, tolerance, drug safety and efficacy, and treatment preferences by different physicians alongside the demographic characters of patients indicated poor to an increased response to FSHR coupled medicines and sometimes even life-threatening excessive response in some patients [8–10]. Indeed, the relationship between infertility treatments and some types of cancers due to the different drug safety and toxicity is still controversial and needs further investigation and long-term follow-up studies [11]. Poor responders or women with impaired ovarian reserve, however, are those patients who display at least two of Bologna three criteria: a) Advanced maternal age, (b) Previous poor ovarian response after ovarian stimulations, and (c) Abnormal ovarian reserve tests. Women with the age of 40 and retrieval of oocytes $\leq$ three are considered as the cutoff values for discrimination of poor ovarian responders. The European Society of Human Reproduction and Embryology (ESHRE) also included the antral follicle count (AFC) and anti-Müllerian hormone (AMH) tests result with the variable levels of <5–7 follicles or <0.5–1.1 ng/mL, correspondingly [12]. Other developed classifications of infertility patients focused on some specific features as well as women's age and provided a more nuanced picture of poor ovarian response as a guide for physicians in patient management. The latter is named as the patient-oriented strategies encompassing individualized oocyte number criteria (POSEIDON) and used for diagnosis and management of low prognosis patients who received ART in the clinic [13,14]. The above-mentioned criteria help the clinicians to categorized different responders with miscellaneous ovarian stimulation outcomes. Such variability also appeared through FSH and other hormonal therapies in infertility centers. The diverse response of patients to recombinant/urinary ligand as FSH plus other FSHR related treatments also have been reported in different regions (some examples are listed in Section 3).

Pharmacogenetics and genomics (PGx) studies also serve as the main path for achieving the best medication(s) for patients with maximum safety and efficacy. Various methods have been employed to identify the genetic landscape related to pharmacogenes, which are responsible for the metabolism of certain drugs and their targets in the body, lead to different prescription adjustments in patients for finding the optimal dosage. With the emergence of high-throughput sequencing technologies, finding the potential genetic variation(s) that influence drug responses in patients would be fast and cost-effective. The ultimate goal for such evaluations in patients is reflected by the famous quote "choosing the right drug with the right dosage for the right person at the right time", as it is mentioned for a while in the personalized medicine considerations [15,16]. To support the

increased demands for reaching the personalized drug and hormone therapy in infertility clinics, focusing on actionable/potentially actionable variants through PGx analysis of FSH receptor may be necessary. This review provides a critical and systematic analysis of the genetic basis for FSHR and the functional alterations due to various alleles in the genome, resulting in diverse responses to associated ligands during infertility treatment in females with impaired ovarian reserve because of poor response to FSH and FSHR related stimulators. In addition, the related cellular pathway genes are listed, and the potential effects of them on related drugs' efficacy are discussed. The latest identified functional alleles for the *FSHR* gene are analyzed, and advanced technologies for genetic evaluation of them are introduced as well.

2. Methods

A literature search was conducted in bibliographic databases (PubMed, Web of Science and Scopus) and gray literature using keywords: "*infertility and personalized medicine*", "*FSHR drugs*", "*FSHR pharmacogenetics*", "*FSHR polymorphisms and mutations*", and "*pharmacogenetics of infertility*" for studies published after 2000, in order to provide an exhaustive report for all the introduced functional variants so far. Among the 85 yielded results, after removing duplicate/similar reports and rolling out non-English language articles, 40 articles were included for further assessments. In the next step, first, the abstracts were screened if the selected keyword expansion were related to our study context, followed by full-text article assessment for those papers of direct implication on FSHR genetic landscape and PGx analysis of infertility-related drugs for females. Ultimately 25 papers were marked, quality assessment was done, and risk of bias was considered based on PRISMA guideline for constructing this review (Figure 1).

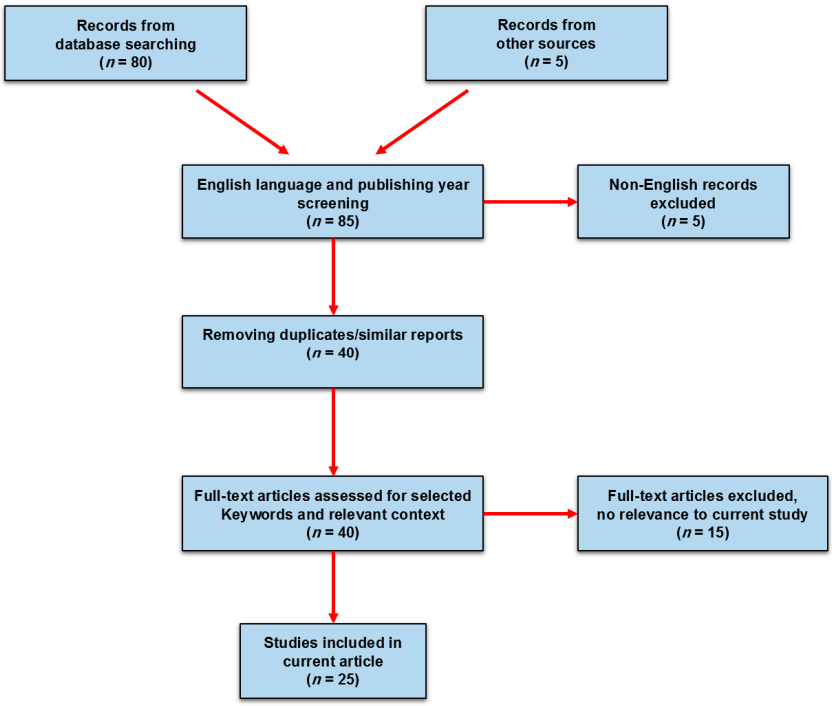

Figure 1. Literature search and inclusion/exclusion criteria for systematic reporting of pharmacogenetics and genomics (PGx) studies in follicle-stimulating hormone receptor (FSHR) malfunction in females with poor ovarian response.

3. A Quick Overview on Pharmacogenomics of *FSHR* in Females with Poor Ovarian Response

Through 40 fully scrutinized publications in this review, 25 of them showed the relevant impact of *FSHR* genotype on infertility treatment outcomes in the clinic. While they are introducing the actionable genetic alterations (mutations and polymorphisms) in

the *FSHR* gene with direct effects on medication applying in females with poor ovarian responses, the dosage adjustment and considerations may be purposed after the genetic testing in such patients. Here, we first have an overview of the different genetic profiles of *FSHR* and their clinical evidence-based, actionable PGx properties in different populations (Figure 2 and Table 1). These are validated genetic variants in the *FSHR* gene, which may need treatment modifications in patients, provided in the form of some examples for such studies. The complete explanation on PGx of *FSHR* and related pharmacovariants are provided in Section 4. In addition, we list some of the diverse responses to FSHR connected treatments in different populations in a detailed Table 2 as examples of such differences in FSHR function because of these genetic profiles in patients. However, due to the lack of similar association between the variants and the drugs in different studies, in which treatment modifications implemented according to the specific type of variants in the *FSHR* gene and also for avoiding any incompatibility through the issue, some information, as well as applied FSH dosages, duration of applying, and so on, are not reported in the current paper. Next, we talk about the latest genetic test methods for finding genetic variations of *FSHR* (next-generation sequencing technologies and advanced single nucleotide polymorphism (SNP)-array analysis). Finally, we provide an overview of the cellular pathway genes, which may show interaction and potential impact on selected drugs' efficacy.

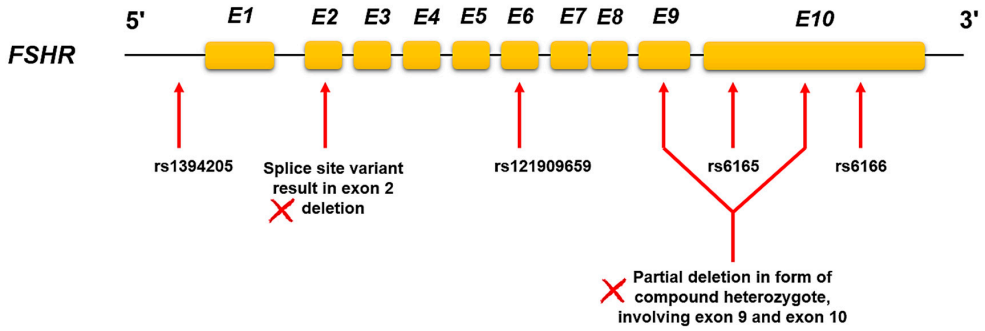

Figure 2. All the actionable and/or functional validated PGx variants in the *FSHR* gene. Pharmacovariants introduced here are based on clinical reports for changing the ovarian responses observed during infertility treatment procedures in females in different populations *(see the text and* Table 1 *for more details)*. FSHR, follicle-stimulating hormone receptor; PGx, pharmacogenomics.

Table 1. *FSHR* gene variations with clinical evidence for pharmacogenetic effects in different populations.

Gene	*Variants with PGx Effects*	*Nucleotide Changes*	*MAF* (%)*	*Variation Effects*	*Reference*
FSHR	rs6166	c.2038 G > A	0.3–0.4	Allele G: poor response to infertility drugs	[17,18]
FSHR	rs6165	c.919 G > A	0.3–0.4	Allele G: poor response to ligands (decreased sensitivity)	[19]
FSHR	rs1394205	-29 G > A	0.2–0.3	Allele A: reduced gene expression level leads to a decreased level of the response to drugs	[20,21]
FSHR	-	exon2 del	NA*	Reduced response of the receptor for infertility treatments due to impaired receptor transferring to the cell membrane	[22,23]
FSHR	-	exon9 and 10 del	NA	Loss of function of receptor lead to FSH resistance	[24]
FSHR	rs121909659	c.479 A > G,T	0.000025	Partial loss of function and impaired cell surface expression of FSHR causes the reduced response in patients (Classified as pathogenic SNP)	[25,26]

MAF*: minor allele frequency, NA*: not available; FSHR, follicle-stimulating hormone receptor; SNP, advanced single nucleotide polymorphism.

Table 2. Examples of diverse responses in different populations to assisted reproductive technologies (ART) treatments due to alterations in patients' *FSHR* genetic make-up.

Country	*Study Population*	*FSHR Evaluated SNP(s)*	*Different Responders to the Treatments Due to FSHR Altered Function*	*Conclusion*	*Reference*
Slovenia	60 women undergoing ovarian stimulation were selected	−29 G > A c.2038 G > A	Poor Responders: 28.3% Normal Responders: 43.4% High Responders: 28.3%	The GG genotype in rs1394205 is associated with poor ovarian response to COH [1], and the related patients may require higher doses of rFSH for ovulation induction.	[27]
Spain	170 women undergoing controlled ovarian stimulation included	c.2038 G > A	Poor responders: 58.4% Normal Responders: - [2] High responders: 27.7%	Discrete set of genes and polymorphisms, including rs6166 in the FSHR gene, may partially explain the poor response to FSH hormone during controlled ovarian stimulation treatments.	[28]
Greece	79 sub-fertile women and 46 normo-ovulatory women with diverse respond to IVF were included	c.2038 G > A	Poor responders: 28% Normal Responders: 36.8% High responders: -	Good (normal) responder group had a statistically significant Asn/Ser heterozygous variant (rs6166) with more follicles and oocytes in patients.	[29]
Ukraine	374 women, including ovary dysfunction patients and healthy individuals with different treatment responses, were selected	c.919 G > A c.2038 G > A	Poor responders: 10.42% Normal responders: 10.7% High responders: - (Study also included control groups: 51.6%)	Combined allelic distribution for rs6165 and rs6166 (Ala307-Ser680/Ala307-Ser680) genotype should have an impact on the delineation of stimulation protocols.	[30]
Germany	161 ovulatory women below the age of 40 years with different FSH stimulation requirements were included	c.919 G > A c.2038 G > A	Poor responders: - Normal Responders: - High responders: -	The Asn/Ser heterozygote genotype for rs6166 was significantly more common in infertile patients with diverse ovarian response. Ovarian response to FSH stimulation depends on the FSHR genotype.	[31]
Germany	93 women (homozygous for Asn/Asn or Ser/Ser) undergoing controlled ovarian hyperstimulation in IVF and ICSI	c.2038 G > A	Poor responders: 25.80% Normal Responders: 74.19% High responders: -	Lower FSH receptor sensitivity due to p.N680S sequence variation in FSHR (rs6166) resulted in lower estradiol levels following FSH stimulation, which cause the patients to need to receive higher FSH doses.	[32]
Sweden and China	Systematic review and meta-analysis on special FSHR variants and altered ovarian response in women undergoing IVF	c.2038 G > A	-	FSHR polymorphism Ser680Asn (rs6166), through the other pharmacogenomics variants, is the most optimal biomarker for implementing in routine clinical practice.	[33,34]
Armenia origin	Case report of a woman with secondary amenorrhea and very high plasma gonadotropin concentrations (especially FSH)	c.479 A > G,T	Poor responders: 100% Normal Responders: - High responders: -	rs121909659 causes partial loss of function, and impaired cell surface expression of FSHR resulted in reduced response in COH. The study reminds us of the population-specific assessments of FSHR.	[25]
United States	35 women undergoing in vitro fertilization included	exon2 del exon6 del exon9 del intron 8 insertion	Poor responders: 8.5% Normal Responders: 68.57%% High responders: 22.85%	FSHR splicing variants, seen in women with a normal menstrual cycle that show an abnormal response to FSH stimulation described. Exon 2 deletion was associated with low ovarian response.	[22]

[1] COH: controlled ovarian hyperstimulation, [2] -: not determined/not applicable, IVF: In vitro fertilization. FSHR, follicle-stimulating hormone receptor; FSH, follicle-stimulating hormone; ICSI, intra-cytoplasmic sperm injection

4. Genetic and Pharmacogenetic Variations in *FSHR* Gene

Different inactivating or hyper-activating genetic alterations such as loss or gain of function mutations and polymorphisms were displayed in coding, regulatory, and splice sites of the *FSHR* gene, leading to receptor malfunction in the form of reduced cell surface expression level, ligand-binding, and total function in target cells with the relative impact on fertility issues in females. This also is part of the reasons for disorders like premature ovarian failure, polycystic ovary syndrome (PCOS), ovarian hyper-stimulation syndrome (OHSS), and also diverse drug responses in some patients [35–37]. Specifically, there are several cases of OHSS due to gain of function mutations of the *FSHR* gene. Activating mutations resulted in a predisposition to OHSS, suggesting that ovarian response may depend on the *FSHR* genotype too. In addition, studies have demonstrated the p.680Asn/Ser polymorphism of the FSHR could be considered as a predictor for the severity of symptoms in patients who develop OHSS [38]. However, the distribution of related alleles is significantly different in various ethnic groups and populations [29,39]. The gain of function mutations was seen mostly in the extracellular domain as an autosomal dominant variant, while the loss of function alterations affects all parts of the receptor in extra- and intracellular in addition to transmembrane sections, mostly as an autosomal recessive or dominant-negative in some cases [40]. While the mutations are rare and the clinical outcomes for them would be expected or predicted easier, the *FSHR* gene polymorphisms as the more common and/or prone to ignore variants in patients with impaired ovarian response were investigated thoroughly for possible impact on reproductive ability. Such studies indicated both types of variations are distributed in different parts of the *FSHR* gene and can cause various effects and outcomes in patients. For example, a splice site variant, which results in exon two deletions in the *FSHR* gene and affects the extracellular domain of FSHR protein and the reduced response of the receptor, was found in women who received infertility treatment. In vitro analysis of this particular variant displayed the formation of functional heterodimers with the wild-type receptor when co-expressed alongside it, which lead to the reduced activity for normal receptor [22,23]. In addition, mutations in the leucine-rich region of the FSHR extra domain result in the reduction of agonist-binding, affecting the drug responses in patients [41,42].

Generally, the mutations and polymorphisms in *FSHR* genes bring about diverse activity of this receptor for infertility medicines and treatment procedures as well as IVF and ICSI (intra-cytoplasmic sperm injection) in females. Two c.919G>A (p.307Thr/Ala) and c.2038G>A (p.680Asn/Ser) single nucleotide variants (SNVs) indicated as well-known functional variants in the *FSHR* gene coding region with different frequencies in populations, showed the associations with FSH and FSH derived infertility drugs response for ovarian stimulation during infertility treatment. Linkage disequilibrium between these two alleles will help to determine the presence of one according to another in individuals. In addition, the association of these two alleles as a distinct diplotype with the ovarian response to FSH stimulation in women who undergo assisted reproduction procedures explored, and the result indicated the significant relationship between the diplotype and ovarian response too [43]. Other characterized functional variants for females' diverse response to ovarian stimulation drugs include g.-29G>A in the 5′ UTR of the *FSHR* gene, which affects the level of gene expression and influences ligand-binding measures in patients [20,31]. These alleles are considered the variations with PGx effects in the *FSHR* gene during infertility treatment approaches, and the consistent data for retrieved oocytes, stimulation duration, FSH consumption, etc., are provided for them adequately. For example, AA homozygous genotype for rs6165 (c.919A>G) indicated more retrieved oocytes and shorter stimulation time compared to the other two genotypes, the AG heterozygotes and GG homozygous in patients who underwent controlled ovarian stimulation protocols. In addition, GG homozygous and AG heterozygotes of *FSHR* rs1394205 (g.-29G>A) showed a significantly lower amount of FSH consumption during the ART procedures [21]. The GG homozygous form for rs6166 (c.2038G>A) showed a higher level of FSH than AA homozygous and AG heterozygotes in PCOS patients [44]. However, such patients are more prone to hyperstim-

ulation during the treatments, instead of a poor ovarian response. Other studies, however, reported the GG homozygous of rs6166 would also be associated with poor response to exogenous FSH and related drugs and the need for a higher amount of them in order to show a good response [17].

Nevertheless, the number of *FSHR* genetic polymorphisms and variations is too much, and not all of them have been investigated or show such function in the female reproduction system [45]. For example, identified mutations with decreased or completely abolished FSH-binding effects in women with primary or secondary amenorrhea are including c.566C>T in exon 7 (p.189Ala/Val), c.1043C>G in exon 10 (p.348Pro/Arg), c.1222G>T in exon 10 (p.408Asp/Tyr), and c.671A>T in exon 7 (p.224Asp/Val) and are found in the extracellular domain, except c.1222G>T which is happening in the transmembrane domain of FSHR protein. Also, they categorized as inactivating mutations in the *FSHR* gene in women and validated through in vitro functional studies [46]. Yet, there is no/not enough clinical evidence for prescription modifications during the infertility treatment of the related patients based on these mutations. Furthermore, a recently identified *FSHR* novel variant (c.1268T>C (p.423Ile/Thr)) in the second transmembrane domain of the protein in a woman with primary ovarian failure was extensively investigated for receptor malfunction and any therapy dosage modifications. Applying in-vitro and in-silico approaches demonstrated the membrane expression level of FSHR was impaired, and the cAMP/PKA signaling pathway would be severely affected while β-arrestin-dependent ERK1/2 phosphorylation received less amount of impact. In conclusion, it has been shown that the mutation will cause FSHR dose-dependent cell signaling functional alterations and attenuated response [47]. Some loss of function mutations are also introduced before, but due to not determined FSH-binding and cell surface expression level of receptor, there was no report for the possible effects or interruption with drug intake and efficacy in patients (i.e., c.662A>T (p.221Val/Gly), c.1253T>G (p.418Ile/Ser), c.1298C>A (p.433Ala/Asp), c.1723C>T (p.575Ala/Val), etc.) [46].

5. Advanced Genetic Screening Methods for FSHR Profiling

Most of the genetic variations for the *FSHR* gene are defined as SNVs, and because of that, many of them could be included in genome-wide array-based genotyping (Infinium BeadChips from Illumina) and/or specified custom SNP array chips as part of infertility genetic profiling for the related patients (Affymetrix Axiom array) [48]. However, such methods are mostly performed just for male infertility cases [49]. Nowadays, traditional orthogonal genetic tests like real-time PCR or multiplex ligation-dependent probe amplification (MLPA) assay replaced by advanced high-throughput sequencing methods in clinical infertility centers. The utilization of such platforms resulted in faster and more accurate genetic screening and identification of possible underlying genetic reasons for FSHR malfunction in infertile patients for a lower price. As the next generation sequencing (NGS) test outcomes proved to be reliable with regards to both clinical validity and utility, the clinical applications for that would be more common in the near future. Today, NGS approaches and mostly targeted sequencing panels are employed by the clinicians and several genetic testing companies for genotyping the known group of infertility related genes or specific variants (including *FSHR*) in order to identify genetic signatures in infertile women [50–52]. These methods have been used successfully for the determination of *FSHR* SNVs and/or other genetic alterations like CNVs in addition to risk assessment in patients. In fact, NGS methods were recently utilized positively in *FSHR* profiling and novel variant identification in numerous studies [46,53–55]. Some examples also have been illustrated in the development of infertility NGS panel for targeted exons and their flanking regions in 75 infertility related genes, including *FSHR* as one of the diagnostic genes which have proven associations with infertility, in order to assess the genetic variations of infertile patients with a custom bioinformatic pipeline for data analysis. By applying the MiSeq platform of Illumina, investigators revealed the underlying genetic cause of infertility in their 25 samples properly [56]. França et al. also reviewed the ability of NGS

methods in the discovery of heterogeneity of some FSHR and other infertility gene-related disorders and concluded the widespread usage of such technologies in the near future for the detection of new players in female reproduction diseases would be recommended [57]. While such results are truly satisfying, but many rare pharmacogenetic variants are heavily population-specific and ethnic background-dependent; more comprehensive sequencing technologies like whole exome and whole genome sequencing (WES and WGS) have also been proposed for profiling, decoding, and revealing any novel genetic variations in *FSHR* and other PGx related genes [58,59]. These techniques will be mostly useful for infertile cases with non-identified genetic variations in selected genes and also without other common encountered reasons in clinics. Here, the huge obtained data and performing correct genotype-phenotype correlations plus labor in vitro validation studies would be considered as the bottleneck for assigning them as the routine approaches in infertility centers. However, the specific bioinformatics and computational prediction tools have been introduced for such PGx data analysis and interpretation [60].

6. Genetic Variations in FSHR Related Cell Signaling Genes as Potential Players for Diverse Infertility Drug Response

Glycosylation of Asn174 or Asn276 in the extracellular domain of FSHR as an important post-translational modification allows the correct folding and conformational changes of the protein, which are required for high-affinity binding of FSH and/or other related ligands. While the binding activity in non-glycosylated mutated receptors will be disrupted, two different forms of glycosylation of FSHR also result in different activities for it, too [61,62]. Some introduced mutations in the *FSHR* gene like c.175C>T, (p.59Arg/Thr), c.573A>T, (p.191Asp/Ile), c.1555C>A, (p.519Pro/Thr), c.1760C>A as a compound heterozygote, (p.587Pro/His), etc. affect the FSH-induced cAMP pathway and were seen in conditions like primary amenorrhea [37]. However, when the FSH hormone or FSHR related drugs and synthetic elements as hydrophilic components bind to the receptor, distinct cell signaling pathways as well as adenylyl cyclase, Ras-MAPK, and β-arrestin will be activated. The core involved enzymes/proteins for these intracellular pathways are Gs alpha subunit, adenylyl cyclase, protein kinase A, cAMP-specific phosphodiesterase, extracellular signal-regulated kinases, and arrestin beta. The related genes are also *GNAS1, ADCY, PKA, PDE4, MAPK1,* and *ARRB1,* respectively [63]. Alongside variants in the *FSHR* gene, mutations in any of the above-mentioned cell signaling core genes can also potentially affect the FSHR intracellular function and cause different responses to treatments in some patients too. Although such mutations may rarely occur in infertile women, they can induce an impaired signaling pathway in a highly complex process of FSHR inducing molecular trafficking [64]. While PGx markers in these genes are introduced for other phenotypes and diseases, so far, there is no infertility annotated variants reported in PGx databases (PharmGKB and CPIC) for the listed genes (pharmgkb.org, cpicpgx.org). However, the consideration of such alleles during *FSHR* genotyping in women with diverse responses to infertility drugs will not be useless. Although the PGx analysis of them is not investigated by the clinical researchers, the potential influences must be taken into account, especially when there is no pathogenic variant identified in the *FSHR* gene, the variants identified only in these genes, and other common possible reasons for the observed negative treatment have been ruled out. Yet, more evidence is required in order to put such variants as part of the new FSHR related modifiers in the field of pharmacogenetics and genomics. Moreover, some of the FSHR medicines like menotropins and choriogonadotropin-alfa also use other cellular receptors as well as LHCGR too. The drug's efficacy assessments based on genetic profiling would be more complicated in such situations and need extra caution and effort. For example, compound heterozygote of allele C in LHCGR-291 and FSHR-29 displayed to result in altered rFSH dosage, and the total amount of mature oocytes in IVF treated Caucasian patients [65]. However, all together, the genetics of FSH responsiveness in infertility treatment can rest not only on potential alterations of the *FSHR* gene via mutations or polymorphisms but also on a host of other core genes for intracellular signaling pathways

too. In addition, epigenetic modifications may be involved in the process as well. The latter may occur due to environmental factors or aging.

7. Discussion

It has been reported that 9–24% of females would be introduced as poor responders after the implementation of ovarian stimulation protocols. Different molecular mechanisms have been suggested for reduced and poor ovarian response [66]. Through various reasons which introduced in different patients, a reduced number of FSHR molecules and impaired signal transduction pathway of that in some patients demonstrated to be directly relative to their genetic make-up (Figure 3).

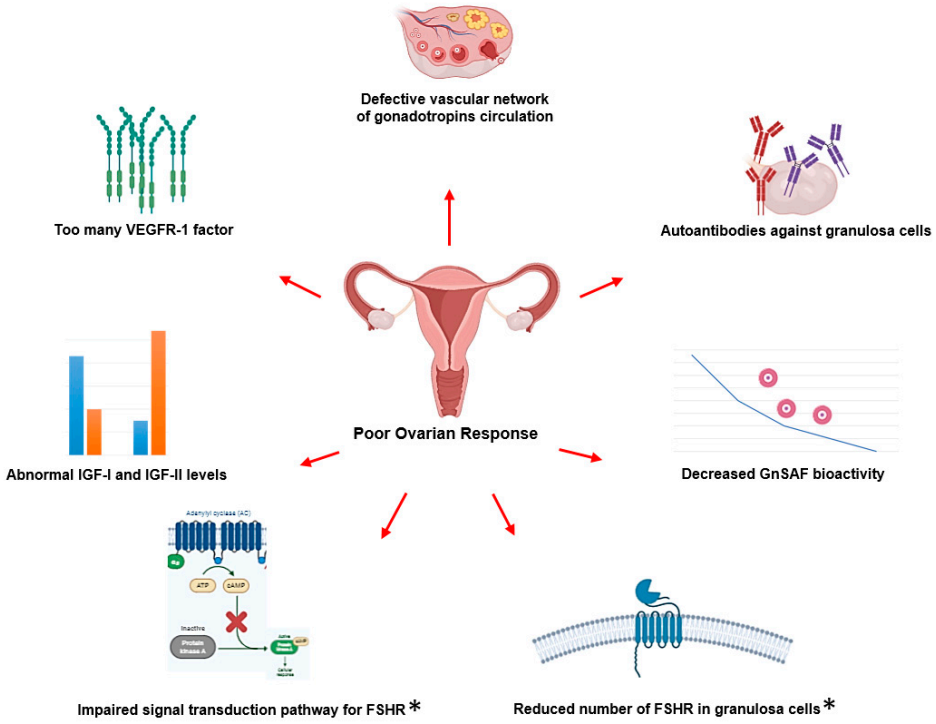

Figure 3. Suggested molecular mechanisms for poor ovarian response in females. The genetic landscape of individuals can potentially affect all of the introduced reasons here. Pharmacogenomic profiling for the reduced number of FSHR and impaired signal transduction pathway of that in patients before the implementation of ovarian stimulation proved to be beneficial in females undergoing infertility treatments. *, PGx implication. VEGFR-1: vascular endothelial growth factor receptor 1; IGF-I, II: insulin-like growth factor 1, 2; GnSAF: gonadotropin surge-attenuating factor

Hence, despite common clinical assessments of AMH and AFC, as the routine tests for prediction of ovarian altered/poor response in females, PGx analysis of the *FSHR* gene is still considered as a matter of worth in special occasions like failure assisted reproductive techniques. As the efficacy of such treatments proved to be related to personalized ovarian stimulation protocols for each patient, PGx assays also may play an essential role in predict the ovarian response before performing stimulation by FSHR related ligands, lead to decreasing the number of ovarian stimulation attempts and the optimization of drug/hormone therapy management [67,68]. However, while the introduced alleles and haplotypes for the *FSHR* gene have been widely studied and well-characterized in different populations, there are some reports which indicated not all of these variants could be traced to specific ethnic groups. For instance, Ilgaz et al. showed there are no significant differences between infertile women and healthy controls for the Asn680Ser variant in the Turkish population, and the genotype landscape is consistent for both groups [36]. In addition, García-Jiménez and colleagues from Mexico indicated a non-significant association between 5′ UTR g.-29G>A and ovary response in IVF-treated women

with controlled ovarian stimulation (COS) [69]. However, most of the investigations indicated lower effects of altered FSHR function on the overall rate of ovarian stimulation and also, the number of studies for ethnic differences in FSHR responses is not high. Still, reports like Turkish and Mexican studies display the necessity of personalized and/or population-specific genotyping approaches for *FSHR* and other infertility related genes through advanced unbiased and untargeted sequencing methods in particular regions (also see Table 1 for other examples) [48].

In addition, the PGx annotation is provided for a few of *FSHR* genetic variations based on clinical findings. Yet, there may not be any dosage guideline or FDA drug label for them, as there is a paucity of evidence. PGx guidelines are usually provided when there is more than one clinical report, which is needed for dosage modifications through different populations or ethnic groups. For *FSHR*-related pharmacovariants, also the number of evidence would be the main criteria, but the fact that *FSHR* gene variants show potential influence on drug response through a change in ligand and exogenous agonist sensitivity still make it necessary to follow the genotyping approaches in order to predict the diverse outcome of the treatment for some special case of infertile women. Moreover, based on major signal transduction activity, most of the core genes in cellular pathways for FSHR drugs, listed here, show the potentially actionable variants too. Hence, PGx annotation for them could be expected to be reached in the near future in personalized infertility treatment approaches. However, the correct decision on dosage specifying for the relevant patients must be considered through comprehensive genetic analysis of both *FSHR* gene and related intracellular pathway genes for related drugs in addition to bearing in mind other main influential factors on ovary reserve in infertile women. The combined analysis of different polymorphisms in *FSHR* at the same time can also be introduced as a valuable tool for investigating and predicting the efficacy of ovulation induction protocols, especially in the group of patients with failed attempts [70]. Moreover, some studies displayed in addition to SNVs, there could be other genetic variants like CNVs for the *FSHR* gene with a direct impact on protein function and drug resistance. The Database of Genomic Variants (DGV—v107) provides such structural variations (SVs) for the *FSHR* gene in detail. Kuechler and her team also reported a 163kb partial deletion in the form of the compound heterozygote, involving exons 9 and 10 of the *FSHR* gene. Conformational and functional studies revealed complete loss of function of FSHR protein and also FSH resistance with clinical manifestations, as the exon 10 encodes all the transmembrane part of the receptor and exon nine engages in making the extracellular domain [24]. Such scenarios cause a specific type of challenges, as most of the sequencing approaches are designed for SNV detection in *FSHR* and other related infertility genes in the clinical practice. However, the above-mentioned cases need the utilization of long-read sequencers like PacBio or Nanopore systems for more accurate genetic profiling of individuals and unraveling the CNV signatures in patients' genes. PGx analysis of CNVs alongside the common SNVs have been implemented for other drug-related genes earlier [71]. The same could be considered for *FSHR* and related genes too. The overall considerations result in the precise genetic scanning of this important gene as an effective and actionable pharmacogene for its related infertility-mediated treatments.

8. Conclusions

While it is in the end, oocyte quality which is the most important parameter and that is no doubt affected by a plethora of factors and resulted in different treatment outcomes, but *FSHR* genotyping is still considered as a promising PGx approach for personalized infertility therapy in women, who receive the related medicines and displayed negative efficiency [72], especially, when there are no other common reasons for non-successful infertility therapy procedures. Various mutations and polymorphisms have been reported for this important pharmacogene with different frequencies in diverse populations. However, not all of them influence the drugs and treatment efficacy. Yet, to avoid any misdiagnosis and/or mismanagement of impaired ovarian response patients,

genetic profiling of *FSHR* should be taken into account in clinical infertility centers too. In addition, the genetic alterations of genes in signal transduction pathways with regard to FSHR function in granulosa cells in females with ovarian failure may contribute to changing the drug response and effects. This is a new PGx area for infertility drugs and needs more investigation and clinical evidence in order to bring new insight into the field of personalized medicine.

Author Contributions: A.T. provided the idea, designed the study, conducted the search for literature, and wrote the entire manuscript. N.W.-K. and S.-A.E. searched for the literature, revised, and edited the manuscript. S.W. and W.M. supervised the study thoroughly. All authors have read and agreed to the published version of the manuscript.

Funding: This article was conducted within the projects which have received funding from the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement No 754432 and the Polish Ministry of Science and Higher Education, from financial resources for science in 2018–2023 grant for the implementation of an international co-financed project.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest during the preparation of this manuscript.

References

1. Ulloa-Aguirre, A.; Zariñán, T.; Pasapera, A.M.; Casas-González, P.; Dias, J.A. Multiple facets of follicle-stimulating hormone receptor function. *Endocrine* **2007**, *32*, 251–263. [CrossRef]
2. Yan, K.; Gao, L.-N.; Cui, Y.-L.; Zhang, Y.; Zhou, X. The cyclic AMP signaling pathway: Exploring targets for successful drug discovery (Review). *Mol. Med. Rep.* **2016**, *13*, 3715–3723. [CrossRef]
3. Thatcher, J.D. The Ras-MAPK Signal Transduction Pathway. *Sci. Signal.* **2010**, *3*. [CrossRef]
4. Conti, M. Specificity of the cyclic adenosine 3′,5′-monophosphate signal in granulosa cell function. *Biol. Reprod.* **2002**, *67*, 4. [CrossRef] [PubMed]
5. Borght, M.V.; Wyns, C. Fertility and infertility: Definition and epidemiology. *Clin. Biochem.* **2018**, *62*, 2–10. [CrossRef]
6. Lindsay, T.J.; Vitrikas, K.R. Evaluation and treatment of infertility. *Am. Fam. Physician* **2015**, *91*, 308–314.
7. Hrometz, S.L.; Gates, V. Review of available infertility treatments. *Drugs Today* **2009**, *45*, 275–291. [CrossRef]
8. Derman, S.G.; Adashi, E.Y. Adverse Effects of Fertility Drugs. *Drug Saf.* **1994**, *11*, 408–421. [CrossRef]
9. Samplaski, M.K.; Nangia, A.K. Adverse effects of common medications on male fertility. *Nat. Rev. Urol.* **2015**, *12*, 401–413. [CrossRef]
10. Conforti, A.; Cariati, F.; Vallone, R.; Alviggi, C.; de Placido, G. Individualization of treatment in controlled ovarian stimulation: Myth or reality. *Biochim. Clin.* **2017**, *41*, 294–305.
11. Momenimovahed, Z.; Taheri, S.; Tiznobaik, A.; Salehiniya, H. Do the Fertility Drugs Increase the Risk of Cancer? A Review Study. *Front. Endocrinol.* **2019**, *10*, 313. [CrossRef]
12. Ferraretti, A.P.; La Marca, A.; Fauser, B.C.J.M.; Tarlatzis, B.; Nargund, G.; Gianaroli, L.; on behalf of the ESHRE working group on Poor Ovarian Response Definition. ESHRE consensus on the definition of 'poor response' to ovarian stimulation for in vitro fertilization: The Bologna criteria. *Hum. Reprod.* **2011**, *26*, 1616–1624. [CrossRef]
13. Esteves, S.C.; Roque, M.; Bedoschi, G.M.; Conforti, A.; Humaidan, P.; Alviggi, C. Defining Low Prognosis Patients Undergoing Assisted Reproductive Technology: POSEIDON Criteria—The Why. *Front. Endocrinol.* **2018**, *9*, 461. [CrossRef]
14. Esteves, S.C.; Alviggi, C.; Humaidan, P.; Fischer, R.; Andersen, C.Y.; Conforti, A.; Bühler, K.; Sunkara, S.K.; Polyzos, N.P.; Galliano, D.; et al. The POSEIDON Criteria and Its Measure of Success through the Eyes of Clinicians and Embryologists. *Front. Endocrinol.* **2019**, *10*, 814. [CrossRef]
15. Abrahams, E. Right drug—right patient—right time: Personalized Medicine Coalition. *Clin. Transl. Sci.* **2008**, *1*, 11. [CrossRef]
16. Lemire, F. The right drug for the right patient: Caring for our patients while minimizing prescription drug misuse. *Can. Fam. Physician* **2013**, *59*, 708.
17. Morón, F.J.; Ruiz, A. Pharmacogenetics of controlled ovarian hyperstimulation: Time to corroborate the clinical utility of FSH receptor genetic markers. *Pharmacogenomics* **2010**, *11*, 1613–1618. [CrossRef]
18. Nenonen, H.; Lindgren, I.A.; Prahl, A.S.; Trzybulska, D.; Kharraziha, I.; Hultén, M.; Giwercman, Y.L.; Henic, E. The N680S variant in the follicle-stimulating hormone receptor gene identifies hyperresponders to controlled ovarian stimulation. *Pharm. Genom.* **2019**, *29*, 114–120. [CrossRef]

19. Achrekar, S.K.; Modi, D.N.; Desai, S.K.; Mangoli, V.S.; Mangoli, R.V.; Mahale, S.D. Poor ovarian response to gonadotrophin stimulation is associated with FSH receptor polymorphism. *Reprod. Biomed. Online* **2009**, *18*, 509–515. [CrossRef]
20. Riccetti, L.; De Pascali, F.; Gilioli, L.; Santi, D.; Brigante, G.; Simoni, M.; Casarini, L. Genetics of gonadotropins and their receptors as markers of ovarian reserve and response in controlled ovarian stimulation. *Best Pr. Res. Clin. Obstet. Gynaecol.* **2017**, *44*, 15–25. [CrossRef]
21. Alviggi, C.; Conforti, A.; Santi, D.; Esteves, S.C.; Andersen, C.Y.; Humaidan, P.; Chiodini, P.; De Placido, G.; Simoni, M. Clinical relevance of genetic variants of gonadotrophins and their receptors in controlled ovarian stimulation: A systematic review and meta-analysis. *Hum. Reprod. Updat.* **2018**, *24*, 599–614. [CrossRef] [PubMed]
22. Gerasimova, T.; Thanasoula, M.N.; Zattas, D.; Seli, E.; Sakkas, D.; Lalioti, M.D. Identification and in vitro characterization of follicle stimulating hormone (FSH) receptor variants associated with abnormal ovarian response to FSH. *J. Clin. Endocrinol. Metab.* **2010**, *95*, 529–536. [CrossRef] [PubMed]
23. Lalioti, M.; Gerasimova, T.; Zattas, D.; Anastasakis, D.; Seli, E.; Sakkas, D. A Deleted Form of FSH Receptor, Found in Women Undergoing Infertility Treatment, Impairs the Function of the Normal Receptor When Co-Expressed In Vitro. *Biol. Reprod.* **2010**, *83*, 193. [CrossRef]
24. Kuechler, A.; Hauffa, B.P.; Köninger, A.; Kleinau, G.; Albrecht, B.; Horsthemke, B.; Gromoll, J. An unbalanced translocation unmasks a recessive mutation in the follicle-stimulating hormone receptor (FSHR) gene and causes FSH resistance. *Eur. J. Hum. Genet.* **2010**, *18*, 656–661. [CrossRef] [PubMed]
25. Beau, I.; Touraine, P.; Meduri, G.; Gougeon, A.; Desroches, A.; Matuchansky, C.; Milgrom, E.; Kuttenn, F.; Misrahi, M. A novel phenotype related to partial loss of function mutations of the follicle stimulating hormone receptor. *J. Clin. Investig.* **1998**, *102*, 1352–1359. [CrossRef]
26. Ramadhan, R.S. Molecular analysis of FSH receptor gene in Iraqi women with PCOS syndrome. *Middle East Fertil. Soc. J.* **2018**, *23*, 404–408. [CrossRef]
27. Čuš, M.; Vlaisavljević, V.; Repnik, K.; Potočnik, U.; Kovačič, B. Could polymorphisms of some hormonal receptor genes, involved in folliculogenesis help in predicting patient response to controlled ovarian stimulation? *J. Assist. Reprod. Genet.* **2019**, *36*, 47–55. [CrossRef]
28. De Castro, F.; Morón, F.J.; Montoro, L.; Galán, J.J.; Hernández, D.P.; Padilla, E.S.-C.; Ramírez-Lorca, R.; Real, L.M.; Ruiz, A. Human controlled ovarian hyperstimulation outcome is a polygenic trait. *Pharmacogenetics* **2004**, *14*, 285–293. [CrossRef]
29. Loutradis, D.; Patsoula, E.; Minas, V.; Koussidis, G.A.; Antsaklis, A.; Michalas, S.; Makrigiannakis, A. FSH receptor gene polymorphisms have a role for different ovarian response to stimulation in patients entering IVF/ICSI-ET programs. *J. Assist. Reprod. Genet.* **2006**, *23*, 177–184. [CrossRef]
30. Livshyts, G.B.; Podlesnaja, S.; Kravchenko, S.; Sudoma, I.; Livshits, L. A distribution of two SNPs in exon 10 of the FSHR gene among the women with a diminished ovarian reserve in Ukraine. *J. Assist. Reprod. Genet.* **2008**, *26*, 29–34. [CrossRef]
31. Mayorga, M.P.; Gromoll, J.; Behre, H.M.; Gassner, C.; Nieschlag, E.; Simoni, M. Ovarian Response to Follicle-Stimulating Hormone (FSH) Stimulation Depends on the FSH Receptor Genotype*. *J. Clin. Endocrinol. Metab.* **2000**, *85*, 3365–3369. [CrossRef]
32. Behre, H.M.; Greb, R.R.; Mempel, A.; Sonntag, B.; Kiesel, L.; Kaltwasser, P.; Seliger, E.; Röpke, F.; Gromoll, J.; Nieschlag, E.; et al. Significance of a common single nucleotide polymorphism in exon 10 of the follicle-stimulating hormone (FSH) receptor gene for the ovarian response to FSH: A pharmacogenetic approach to controlled ovarian hyperstimulation. *Pharmacogenetics Genom.* **2005**, *15*, 451–456. [CrossRef] [PubMed]
33. Yao, Y.; Ma, C.-H.; Tang, H.; Hu, Y.-F. Influence of follicle-stimulating hormone receptor (FSHR) Ser680Asn polymorphism on ovarian function and in-vitro fertilization outcome: A meta-analysis. *Mol. Genet. Metab.* **2011**, *103*, 388–393. [CrossRef] [PubMed]
34. Altmäe, S.; Hovatta, O.; Stavreus-Evers, A.; Salumets, A. Genetic predictors of controlled ovarian hyperstimulation: Where do we stand today? *Hum. Reprod. Updat.* **2011**, *17*, 813–828. [CrossRef] [PubMed]
35. Lussiana, C.; Guani, B.; Mari, C.; Restagno, G.; Massobrio, M.; Revelli, A. Mutations and polymorphisms of the FSH receptor (FSHR) gene: Clinical implications in female fecundity and molecular biology of FSHR protein and gene. *Obstet. Gynecol. Surv.* **2008**, *63*, 785–795. [CrossRef]
36. Ilgaz, N.S.; Aydos, O.S.E.; Karadag, A.; Taspinar, M.; Eryilmaz, O.G.; Sunguroglu, A. Impact of follicle-stimulating hormone receptor variants in female infertility. *J. Assist. Reprod. Genet.* **2015**, *32*, 1659–1668. [CrossRef]
37. Desai, S.S.; Roy, B.S.; Mahale, S.D. Mutations and polymorphisms in FSH receptor: Functional implications in human reproduction. *Reproduction* **2013**, *146*, R235–R248. [CrossRef]
38. Rizk, B. Genetics of ovarian hyperstimulation syndrome. *Reprod. Biomed. Online* **2009**, *19*, 14–27. [CrossRef]
39. Jun, J.K.; Yoon, J.S.; Ku, S.-Y.; Choi, Y.M.; Hwang, K.R.; Park, S.Y.; Lee, G.H.; Lee, W.D.; Kim, S.H.; Kim, J.G.; et al. Follicle-stimulating hormone receptor gene polymorphism and ovarian responses to controlled ovarian hyperstimulation for IVF-ET. *J. Hum. Genet.* **2006**, *51*, 665–670. [CrossRef]
40. Tranchant, T.; Durand, G.; Gauthier, C.; Crépieux, P.; Ulloa-Aguirre, A.; Royère, D.; Reiter, E. Preferential β-arrestin signalling at low receptor density revealed by functional characterization of the human FSH receptor A189 V mutation☆ . *Mol. Cell. Endocrinol.* **2011**, *331*, 109–118. [CrossRef]
41. Bogerd, J. Ligand-selective determinants in gonadotropin receptors. *Mol. Cell. Endocrinol.* **2007**, *260*, 144–152. [CrossRef] [PubMed]

42. Braun, T.; Schofield, P.R.; Sprengel, R. Amino-terminal leucine-rich repeats in gonadotropin receptors determine hormone selectivity. *EMBO J.* **1991**, *10*, 1885–1890. [CrossRef] [PubMed]
43. Lazaros, L.; Hatzi, E.; Xita, N.; Takenaka, A.; Sofikitis, N.; Zikopoulos, K.; Georgiou, I. Influence of FSHR diplotypes on ovarian response to standard gonadotropin stimulation for IVF/ICSI. *J. Reprod. Med.* **2013**, *58*, 395–401. [PubMed]
44. Valkenburg, O. (Olivier); Uitterlinden, A.; Piersma, D.; Hofman, A.; Themmen, A.; De Jong, F.; Fauser, B.; Laven, J. Genetic polymorphisms of GnRH and gonadotrophic hormone receptors affect the phenotype of polycystic ovary syndrome. *Hum. Reprod.* **2009**, *24*, 2014–2022. [CrossRef] [PubMed]
45. Casarini, L.; Santi, D.; Marino, M. Impact of gene polymorphisms of gonadotropins and their receptors on human reproductive success. *Reproduction* **2015**, *150*, R175–R184. [CrossRef] [PubMed]
46. He, W.-B.; Du, J.; Yang, X.-W.; Li, W.; Tang, W.-L.; Dai, C.; Chen, Y.-Z.; Zhang, Y.-X.; Lu, G.-X.; Lin, G.; et al. Novel inactivating mutations in the FSH receptor cause premature ovarian insufficiency with resistant ovary syndrome. *Reprod. Biomed. Online* **2019**, *38*, 397–406. [CrossRef] [PubMed]
47. Zariñán, T.; Mayorga, J.; Jardón-Valadez, E.; Gutiérrez-Sagal, R.; Maravillas-Montero, J.L.; Mejía-Domínguez, N.R.; Martínez-Luis, I.; Yacini-Torres, O.G.; Cravioto, M.-D.-C.; Reiter, E.; et al. A Novel Mutation in the FSH Receptor (I423T) Affecting Receptor Activation and Leading to Primary Ovarian Failure. *J. Clin. Endocrinol. Metab.* **2020**. [CrossRef]
48. Patel, B.; Parets, S.; Akana, M.; Kellogg, G.; Jansen, M.; Chang, C.; Cai, Y.; Fox, R.; Niknazar, M.; Shraga, R.; et al. Comprehensive genetic testing for female and male infertility using next-generation sequencing. *J. Assist. Reprod. Genet.* **2018**, *35*, 1489–1496. [CrossRef]
49. Friemel, C.; Ammerpohl, O.; Gutwein, J.; Schmutzler, A.G.; Caliebe, A.; Kautza, M.; Von Otte, S.; Siebert, R.; Bens, S. Array-based DNA methylation profiling in male infertility reveals allele-specific DNA methylation in PIWIL1 and PIWIL2. *Fertil. Steril.* **2014**, *101*, 1097–1103.e1. [CrossRef]
50. Flugent. Available online: https://www.fulgentgenetics.com/Infertility-Female (accessed on 14 March 2020).
51. Centogene. Available online: https://www.centogene.com/diagnostics/infertility-testing.html (accessed on 14 March 2020).
52. CGCgenetics. Available online: https://www.cgcgenetics.com/en/by-test-a-z/5099 (accessed on 14 March 2020).
53. França, M.M.; Lerário, A.M.; Funari, M.F.; Nishi, M.Y.; Narcizo, A.M.; De Mello, M.P.; Guerra-Junior, G.; Maciel-Guerra, A.T.; De Mendonça, B.B. A Novel Homozygous Missense FSHR Variant Associated with Hypergonadotropic Hypogonadism in Two Siblings from a Brazilian Family. *Sex. Dev.* **2017**, *11*, 137–142. [CrossRef]
54. Liu, H.; Xu, X.; Han, T.; Yan, L.; Cheng, L.; Qin, Y.; Liu, W.; Zhao, S.; Chen, Z.-J. A novel homozygous mutation in the FSHR gene is causative for primary ovarian insufficiency. *Fertil. Steril.* **2017**, *108*, 1050–1055.e2. [CrossRef] [PubMed]
55. Liu, H.; Guo, T.; Gong, Z.; Yu, Y.; Zhang, Y.; Zhao, S.; Qin, Y. Novel FSHR mutations in Han Chinese women with sporadic premature ovarian insufficiency. *Mol. Cell. Endocrinol.* **2019**, *492*, 110446. [CrossRef] [PubMed]
56. Lorenzi, D.; Fernández, C.; Bilinski, M.; Fabbro, M.; Galain, M.; Menazzi, S.; Miguens, M.; Perassi, P.N.; Fulco, M.F.; Kopelman, S.; et al. First custom next-generation sequencing infertility panel in Latin America: Design and first results. *JBRA Assist. Reprod.* **2020**, *24*, 104–114. [CrossRef]
57. França, M.M.; Mendonca, B.B. Genetics of Primary Ovarian Insufficiency in the Next-Generation Sequencing Era. *J. Endocr. Soc.* **2020**, *4*, bvz037. [CrossRef] [PubMed]
58. Reisberg, S.; Krebs, K.; Lepamets, M.; Kals, M.; Mägi, R.; Metsalu, K.; Lauschke, V.M.; Vilo, J.; Milani, L. Translating genotype data of 44,000 biobank participants into clinical pharmacogenetic recommendations: Challenges and solutions. *Genet. Med.* **2019**, *21*, 1345–1354. [CrossRef] [PubMed]
59. Bramble, M.S.; Goldstein, E.H.; Lipson, A.; Ngun, T.; Eskin, A.; Gosschalk, J.E.; Roach, L.; Vashist, N.; Barseghyan, H.; Lee, E.; et al. A novel follicle-stimulating hormone receptor mutation causing primary ovarian failure: A fertility application of whole exome sequencing. *Hum. Reprod.* **2016**, *31*, 905–914. [CrossRef]
60. Zhou, Y.; Fujikura, K.; Mkrtchian, S.; Lauschke, V.M. Computational Methods for the Pharmacogenetic Interpretation of Next Generation Sequencing Data. *Front. Pharmacol.* **2018**, *9*, 1437. [CrossRef] [PubMed]
61. Ay, B.G.B.; May, J.V.; Davis, J.S.; Dias, J.A.; Kumar, T.R. In Vivo and In Vitro Impact of Carbohydrate Variation on Human Follicle-Stimulating Hormone Function. *Front. Endocrinol.* **2018**, *9*, 216. [CrossRef]
62. Davis, D.; Liu, X.; Segaloff, D.L. Identification of the sites of N-linked glycosylation on the follicle-stimulating hormone (FSH) receptor and assessment of their role in FSH receptor function. *Mol. Endocrinol.* **1995**, *9*, 159–170. [CrossRef]
63. Sayers, N.; Hanyaloglu, A.C. Intracellular Follicle-Stimulating Hormone Receptor Trafficking and Signaling. *Front. Endocrinol.* **2018**, *9*, 653. [CrossRef]
64. Landomiel, F.; Gallay, N.; Jégot, G.; Tranchant, T.; Durand, G.; Bourquard, T.; Crépieux, P.; Poupon, A.; Reiter, E. Biased signalling in follicle stimulating hormone action. *Mol. Cell. Endocrinol.* **2014**, *382*, 452–459. [CrossRef]
65. Alviggi, C.; Conforti, A.; Cariati, F.; Alfano, S.; Huhtaniemi, I.; Santi, D.; De Placido, G.; Humaidan, P. Impact of polymorphisms of gonadotropins and their receptors on controlled ovarian stimulation: A prospective observational study. In Proceedings of the 32nd Annual Meeting of ESHRE, Helsinki, Finland, 3–6 July 2016.
66. Badawy, A.; Wageah, A.; El Gharib, M.; Osman, E.E. Prediction and Diagnosis of Poor Ovarian Response: The Dilemma. *J. Reprod. Infertil.* **2011**, *12*, 241–248.
67. Loutradis, D.; Drakakis, P.; Vomvolaki, E.; Antsaklis, A. Different ovarian stimulation protocols for women with diminished ovarian reserve. *J. Assist. Reprod. Genet.* **2007**, *24*, 597–611. [CrossRef]

68. Conforti, A.; Esteves, S.C.; Cimadomo, D.; Vaiarelli, A.; Di Rella, F.; Ubaldi, F.M.; Zullo, F.; De Placido, G.; Alviggi, C. Management of Women With an Unexpected Low Ovarian Response to Gonadotropin. *Front. Endocrinol.* **2019**, *10*, 387. [CrossRef]
69. García-Jiménez, G.; Zariñán, T.; Rodríguez-Valentín, R.; Mejía-Domínguez, N.R.; Gutiérrez-Sagal, R.; Hernández-Montes, G.; Tovar, A.R.; Arechavaleta-Velasco, F.; Canto, P.; Granados, J.; et al. Frequency of the T307A, N680S, and -29G>A single-nucleotide polymorphisms in the follicle-stimulating hormone receptor in Mexican subjects of Hispanic ancestry. *Reprod. Biol. Endocrinol.* **2018**, *16*, 100. [CrossRef]
70. Paschalidou, C.; Anagnostou, E.; Mavrogianni, D.; Raouasnte, R.; Klimis, N.; Drakakis, P.; Loutradis, D. The effects of follicle-stimulating hormone receptor (FSHR) -29 and Ser680Asn polymorphisms in IVF/ICSI. *Horm. Mol. Biol. Clin. Investig.* **2020**. [CrossRef]
71. Ammar, R.; Paton, T.A.; Torti, D.; Shlien, A.; Bader, G.D. Long read nanopore sequencing for detection of HLA and CYP2D6 variants and haplotypes. *F1000Research* **2015**, *4*, 17. [CrossRef]
72. Conforti, A.; Vaiarelli, A.; Cimadomo, D.; Bagnulo, F.; Peluso, S.; Carbone, L.; Di Rella, F.; De Placido, G.; Ubaldi, F.M.; Huhtaniemi, I.; et al. Pharmacogenetics of FSH Action in the Female. *Front. Endocrinol.* **2019**, *10*, 398. [CrossRef]

Journal of
Clinical Medicine

MDPI

Review

Pharmacogenomics, How to Deal with Different Types of Variants in Next Generation Sequencing Data in the Personalized Medicine Area

Alireza Tafazoli [1,2], Natalia Wawrusiewicz-Kurylonek [3], Renata Posmyk [3] and Wojciech Miltyk [1,*]

[1] Department of Analysis and Bioanalysis of Medicines, Faculty of Pharmacy with the Division of Laboratory Medicine, Medical University of Białystok, 15-089 Białystok, Poland; Alireza.Tafazoli@umb.edu.pl
[2] Clinical Research Centre, Medical University of Białystok, 15-276 Bialystok, Poland
[3] Department of Clinical Genetics, Medical University of Białystok, 15-089 Białystok, Poland; natalia.kurylonek@gmail.com (N.W.-K.); rposmyk@gmail.com (R.P.)
* Correspondence: wojciech.miltyk@umb.edu.pl; Tel.: +48-857485845

Abstract: Pharmacogenomics (PGx) is the knowledge of diverse drug responses and effects in people, based on their genomic profiles. Such information is considered as one of the main directions to reach personalized medicine in future clinical practices. Since the start of applying next generation sequencing (NGS) methods in drug related clinical investigations, many common medicines found their genetic data for the related metabolizing/shipping proteins in the human body. Yet, the employing of technology is accompanied by big obtained data, which most of them have no clear guidelines for consideration in routine treatment decisions for patients. This review article talks about different types of NGS derived PGx variants in clinical studies and try to display the current and newly developed approaches to deal with pharmacogenetic data with/without clear guidelines for considering in clinical settings.

Keywords: pharmacogenomics; NGS variants; personalized medicine

Citation: Tafazoli, A.; Wawrusiewicz-Kurylonek, N.; Posmyk, R.; Miltyk, W. Pharmacogenomics, How to Deal with Different Types of Variants in Next Generation Sequencing Data in the Personalized Medicine Area. *J. Clin. Med.* **2021**, *10*, 34. https://dx.doi.org/10.3390/jcm10010034

Received: 30 November 2020
Accepted: 22 December 2020
Published: 24 December 2020

1. Introduction: Pharmacogenomics and High Throughput Sequencing Methods

It has been reported for decades that different drugs show different responses and efficacy in diverse individuals or populations. Investigations proved that part of this diversity (20–30%) is because of genetic background and, more precisely, the inheritance of various alleles and variants in genes for drug-metabolizing and transporting (pharmacogenes) or drug target molecules [1]. Pharmacogenetics is the term for the knowledge of diverse drug responses and effects in people, based on their single genes on the genomic profiles. When a group of genes (multiple genes), or whole genome, and other influential genomic events, such as epigenetics will be addressed at once for such investigations, the phrase would be replaced by pharmacogenomics (PGx). Since the starting of employing high throughput sequencing methods, especially next generation sequencing (NGS) technologies, in addition to some comprehensive orthogonal tests, such as genome-wide single nucleotide polymorphism (SNP) arrays in clinical investigations and practice, numerous genetic variants have been introduced in drug-related genes in the human body. Today, close to 100 variants in each people in more than 900 of such genes are mentioned in literature, and the number is increasing continuously [2,3]. There is no doubt that the NGS methods played a significant role in the identification of PGx variants in a clinical research setting and used in the prediction of the response to or adverse effects of drugs, which result in the calculation or estimation of appropriate drug dosage for patients. According to the patient's responses, the drug outcome could be defined as efficient, inefficient, toxic, and resistant. All of these categories mostly arise from the interaction between the products of many genes in a cellular pathway or between the genes and environmental factors. Hence, genotype-specific therapy could bring huge benefits for drug safety and efficacy in patients

in addition to time and cost reduction of treatment approaches for them [4]. The trends led to the practice of personalized therapy and precision medicine implementation in clinical centers. The explosion of examples in the field of pre-emptive and/or patient genotyping shows the true advantages of high throughput sequencing technologies in the PGx area [5–8]. However, despite the common belief between the physicians and general practitioners in the effects of the genetic landscape on diverse drug responses, if they asked that they order the PGx tests for their patients, less than 15% will answer positively. This is mostly because of the lack of clear guidelines and sufficient clinical evidence for many functional genetic variants (FGVs) in drug-related genes (*FGVs or actionable genetic variants are those alterations in genome, with at least one report for introducing the effects on drug safety and/or efficacy in people. Moreover, the variants found in the research area with strong potential effects on drugs could be considered as FGVs during prescription. However, the latter needs clinical evidence to be influential on treatment decisions by physicians*). Furthermore, the poor knowledge and background of PGx and the different related alleles and variants for many healthcare professionals may directly affect their desire to order the tests.

Yet, several rare and uncommon FGVs can be detected through the PGx tests in both clinical and research areas, especially when comprehensive and high capacity methods, such as NGS, have been utilized [9]. Moreover, it is necessary to distinguish the definition of FGVs and/or uncharacterized variants, such as variants with unknown clinical significance in two distinct genomic medicine areas, PGx, and medical genetics. Although the two concepts are usually mixed and many PGx variants are covered in the medical genetics zone, the first one mostly emphasizes those variants with an impact on pharmacological treatments, while the second group of variants is considered the genetic variations with pathogenicity effects in the human body. For a PGx variant, it might show an interaction with drug dosage modifications or not, but the functional and clinical consequences of a genetic variant may be unknown (does it have pathological consequences?) or well known (it has or not pathological consequences). However, both types of variants will be addressed as the same in NGS primary data analysis steps. To deal with the different genetic variants in PGx profiling of individuals, this review article reviews various NGS derived biomarkers and the possible approaches to use or consider them during the medicine prescription. Those PGx variants with no clear guidelines will be focused on more.

2. Different Types of Variants and Their Classifications in Clinical Pharmacogenomics

Both common and rare alleles are demonstrated as the functional biomarkers in PGx clinical practice. Low frequency and rare variants have been shown by 1–5% and lower than 1% minor allele frequency (MAF), respectively, in populations. Moreover, they proved to be very population-specific and the causative elements for diverse drug responses in alternative ethnic groups [10,11]. NGS methods revolutionized the detection of any type of variants in different aspects of genome analysis and profiling, as well as pharmacogenetics and genomic studies. Such investigations reported that most of the FGVs in the clinical PGx setting are Single Nucleotide Variations (SNVs). However, structural variants (SVs), such as Copy Number Variation (CNVs), small Insertion–Deletions (InDels), tandem-substitutions, and the deletion of entire exons are also identified as effective variants in drug responses [12,13]. In addition to wild-type alleles, the functional outcome for each of these variants may cause the individuals to fall into four main groups of responders including poor, intermediate, extensive, and ultra-rapid metabolizers.

Currently, core web-based resources for clinical PGx annotations include Pharmacogenomics Knowledge Base (PharmGKB), the Clinical Pharmacogenetics Implementation Consortium (CPIC), the Pharmacogenomics Research Network (PGRN), and Dutch Pharmacogenetics Working Group (DPWG). These are considered as reference databases that provide information about how human genetic variations affect response to medications. All of the confirmed data about clinically actionable gene-drug associations and genotype-phenotype relationships are sorted properly and available as a guide for personalized medicine implementation by healthcare professionals. However, other modules, such as

PharmVar, FINDbase, SuperCYP, SEAPharm, etc. could also be applied when a specific type of gene or drug was on the desk. Nevertheless, according to PGx reference organizations (PharmGKB, CPIC-PGRN, and DPWG), all the diagnosed alleles and variants in a gene-drug interaction, based on the number of published studies and clinical evidence, will be classified in various types of level with clear explanations for each of them (Table 1). However, CPIC has also introduced a new categorization system for PGx level in more detail (Table 2). Generally, different levels of clinical relevance for PGx variants and/or gene-drug pairs will be assigned by the reference entities. All of them have their processes to assign the levels and prioritize approaches for providing the related guidelines. Meanwhile, some recommendations are related to each other (CPIC and PharmGKB) and the others go through it independently (DPWG). For example, the clinical pharmacogenetics implementation consortium (CPIC) allocates the levels for a variant in a gene-drug pair, based on three major criteria from PharmGKB clinical annotation levels of evidence and PGx level for Food and Drug Administration (FDA)-approved drug labels and also if it is nominated to CPIC for consideration. Only those gene/drug pairs that have been the subject of guidelines have had sufficient in-depth review of evidence to provide definitive CPIC level assignments. CPIC also use other considerations for assignment of CPIC level through some essential questions, containing the information of prescribing actionability, the severity of the clinical consequences for ignoring the genetic tests, already subjected gene to other CPIC guidelines, availability of genetic test for the gene, high-risk genetic variants, etc. [14,15]. PharmGKB also creates genotype-based summaries describing the phenotypic impact of the variant and provides the PGx levels from 1A to 4 in combination with four instructive labels as "Testing required", "Testing recommended", "Actionable PGx", and "Informative PGx" via literature reviews while considering population size and statistical significance. The labels state different considerations for the drugs, based on gene/protein/chromosomal variants or phenotypes, and conclude the necessity of pre-emptive genetic testing for genotype/phenotype correlation assays and showing the potential changes in efficacy, dosage, metabolism, or toxicity [16,17]. Finally, the Dutch Pharmacogenetics working group (DPWG) uses the drug-gene interaction outcomes to providing the clinical relevance levels, where the AA is the lowest impact and F is the highest one. The impacts are categorized, based on adverse drug events, decreased therapeutic response, and other clinical effects, result in the allocation of specific scores from 1–7 derived from national cancer institute (NCI) common toxicity criteria and 0–4 level of evidence of gene–drug interaction in the literature [18].

Table 1. Different levels of clinical relevance for pharmacogenomics (PGx) variants in reference organizations.

Reference Organization	*PGx Level*	*Summary of Description*	*Reference*
PharmGKB	***1A***	Variants in this level are annotated and have a clear and endorsed guideline while showing a strong role in gene-drug interactions.	[19]
	1B	Annotated variant with strong evidence in the literature. Gene-drug association shows strong effects.	
	2A	The annotated variant is in a VIP *, so functional significance is more likely.	
	2B	Annotated variant but in moderate evidence of an association. There is no reliable replicated study in form of statistical significance or well-designed in size.	
	3	Annotated variant in a single study or multiple studies with no similar associations between the variant and the drug.	
	4	Annotated variant but in a case report and non-significant study or just in an in-vitro assay.	
CPIC	***A***	Variants in this level oblige a change in related drug prescription. Strong clinical evidence and genotype-phenotype correlations exist.	[20]
	B	Evidence is weak for the variant but still genotyping may be useful for alternative prescribing.	
	C	Different levels of evidence are mentioned in various publications for the variant. No prescribing actions are recommended. Mostly suitable for genes that are commonly included in clinical or DTC ** tests.	
	D	Weak evidence and conflicting data are introduced for the variant. Clinical actionability is unclear. No prescribing actions are recommended.	
DPWG	***AA***	Variants with no significant clinical or kinetic effects.	[21]
	A	Variants with minor clinical effects and kinetic effects.	
	B	Variants with mild clinical effects.	
	C	Variants with moderate clinical effects.	
	D	Variants with stronger clinical effects than level C.	
	E	Variants with severe clinical effects as the failure of lifesaving therapy or life-threatening complications.	
	F	Variants with most severe clinical effects, death is anticipated.	
	***		
	4	There are good quality published studies for the variant/gene.	
	3	There are moderate quality published studies for the variant/gene.	
	2	Well documented case reports exist for the variant/gene.	
	1	Published incomplete case reports for the variant/gene.	
	0	Data on file.	
	???	No evidence.	

* VIP: very important pharmacogene, ** DTC: direct to consumer, *** Separate the two different levels definitions of the DPWG.

Table 2. Clinical Pharmacogenetics Implementation Consortium (CPIC) new level of clinical relevance for gene/drug interactions.

Cpic Level	*Clinical Context*	*Level of Evidence*	*Strength of Recommendation*
A	Genetic information should be used to change the prescribing of the affected drug.	The preponderance of the evidence is high or moderate in favor of changing prescribing.	At least one moderate or strong action (change in prescribing) is recommended.
A/B	Preliminary review indicates it is likely that the definitive CPIC level will be either A or B.	Full evidence review is needed to assess the level of evidence, but prescribing actionability is likely.	Full review by expert guideline group to assign strength of recommendation.
B	Genetic information could be used to change prescribing of the affected drug because alternative therapies/dosing are extremely likely to be as effective and as safe as non-genetically based dosing.	The preponderance of the evidence is weak with little conflicting data.	At least one optional action (change in prescribing) is recommended.
B/C	Preliminary review indicates it is likely that the definitive CPIC level will be either B or C.	Prescribing actionability based on genetics is not clear without further evidence review.	Full review by expert guideline group to assess the strength of recommendation.
C	There are published studies at varying levels of evidence, some with mechanistic rationale, but no prescribing actions are recommended because (a) dosing based on genetics makes no convincing difference; (b) alternatives are unclear, possibly less effective, more toxic, or otherwise impractical; or (c) few published studies or mostly weak evidence and clinical actions are unclear. Most important for genes that are subject to other CPIC guidelines or genes that are commonly included in clinical or DTC tests.	Evidence levels can vary.	No prescribing actions are recommended.
C/D	Preliminary review indicates it is likely that the definitive CPIC level will be either C or D.	Evidence levels can vary.	No prescribing actions are recommended.
D	There are few published studies, clinical actions are unclear, little mechanistic basis, mostly weak evidence, or substantial conflicting data. If the genes are not widely tested clinically, evaluations are not needed. Criteria for "widely tested" includes: 1) College of American Pathologists (CAP) proficiency testing is available; 2) gene is in disease-specific panels (e.g., pain, psychiatric, cancer, etc.); or 3) evidence exists for implementation of the gene into clinical practice (CPIC member feedback, publications, etc.).	Evidence levels can vary.	No prescribing actions are recommended.

Adopted from cpicpgx.org/.

Regarding the abovementioned level of classification for the identified variants, the utilization of NGS platforms for clinical PGx tests brings various types of alleles, which after confirmation and validation processes could be categorized as functional/potential effective variants, fall into "five groups of (1) annotated variants with the clear guideline (i.e., rs1057910 in *CYP2C9* and rs9923231 in *VKORC1* genes for Warfarin). (2) Annotated variants with no clinical guideline (i.e., rs6166 in *FSHR* gene for urofollitropin). (3) Variants with annotation or guidelines for other drugs (i.e., rs9322335 in *ESR1* gene for letrozole while the gene is studying and considered as the estrogen receptor and target molecule for

Clomifene). (4) Non-pharmacogenetically annotated variants (i.e., different clinical related variants in *AR* gene as an important target molecule for infertility drugs). And (5) Variants of unknown significance (VUS). The next part will focus on different approaches for such variant interpretation and curation in clinical practice.

3. Approaches to Dealing with Diverse Pharmacogenomics Variants

To finding any clinical relevance for different groups of PGx variants from the sequencing platforms, standard algorithms, and procedures are introduced by the reference sources (Figure 1). These are the recommendations that indicate the approaches for decoding or predicting the variant functions and the related phenotypes as the diverse drug responses in individuals [22]. From the previous section, group 1 is considered as straightforward, actionable variants in gene-drug pairs with direct prescription recommendations for applying in routine clinical practice. Group 2 are the alleles, consisting of the most common types of identified variants during diagnostic procedures for PGx tests. As the PharmGKB included 19,028 variant annotations, most of the identified markers will fall into this group. Here, the number of clinical evidence in addition to statistical signification (i.e., number of patients in cohort studies) and types of the publications, if they are strong genome-wide association study, well designed replicated report, case report, non-significant study, or only an in-vitro study, would be the important factors for clinical consideration and decisions [23]. The other common scenario for the sequencing results of a pharmacogenetic screening test could be found in group 3, which are variants with the recommendations but not for the researchers/clinicians targeted drugs. Generally, if the related gene is introduced as a very important pharmacogene (VIP) in PGx databases, it is mostly well documented so the related cellular pathways must be analyzed thoroughly. Then the caution and consideration before dosage adjustment are suggested for more accurate implementation of personalized medicine in the clinic [24]. If there is a lack of such documents, more confirmation and validation assessments are necessary before any concerns for the patient's prescription. Replicate tests in target drugs in such situations consist of various approaches, from looking for the same result in same/different ethnic groups to implementation of laboratory confirmation tests. However, alternative approaches have also been introduced for PGx findings validation, if replication studies for gene-drug interactions proved to be difficult and costly for some cases [25]. In the end, consulting with gene experts or experienced clinical pharmacologist in the gene–drug interaction field is necessary. So far, reference databases have explained the approaches to deal with variants in group one to three. However, many genetic variations may be classified in group 4, which is introduced as disease-associated biomarkers and placed into the different genomic databases, such as ClinVar, dbGaP, HapMap, gnomAD, COSMIC, etc. (as causative or pathogenic variants), but there is no PGx report for them. This is mostly happening during more comprehensive genomic profiling of individuals for decoding any PGx markers. In such a situation, the first step could be the evaluation of the gene, if it is introduced as drug related in literature and databases before. The positive result may follow the approaches for group 3 as well. If there is any, also clinical assays would help provide evidence in both groups 2 and 3 of variants during the clinical decision making.

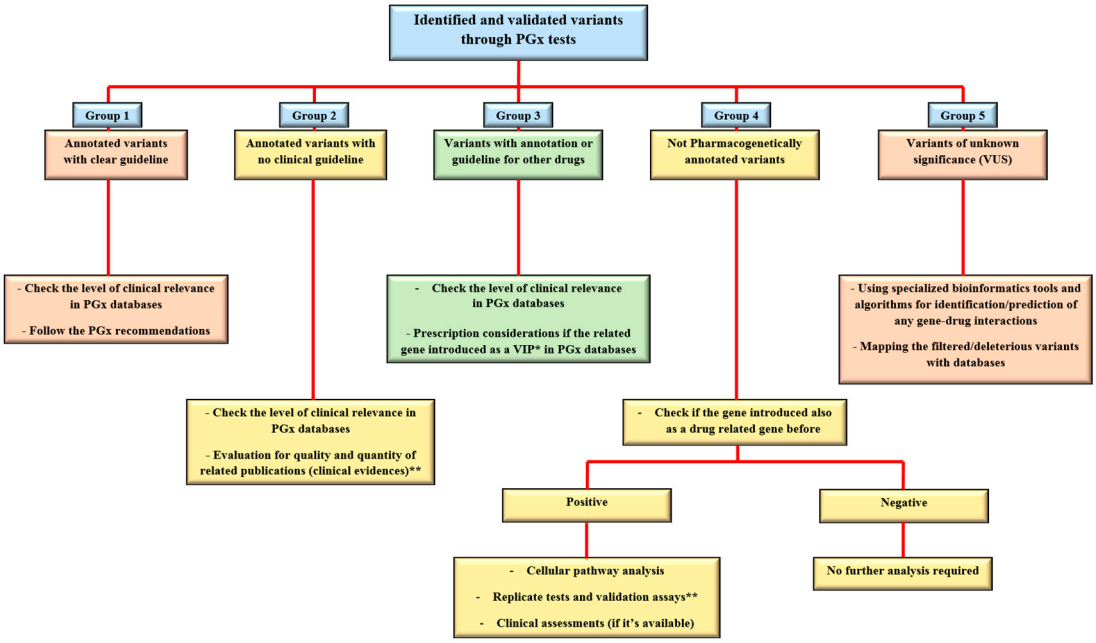

Figure 1. Approaches to deal with different types of PGx variants in clinical centers. After the identification and doing the confirmation tests on a PGx related variant, it could be categorized in one of the main five groups of annotated with PGx guideline, annotated without a guideline, informative for other drugs, not PGx annotated, or variants of unknown clinical significance (VUS). For the annotated variants, checking the level of clinical relevance (Table 1 of the current paper) is the first task to do. Bioinformatics tools are also supporting the analysis of not only VUS but also other types of variants in each group. *Examples for groups 1–5 with explanations are provided in the main text. * VIP: very important pharmacogene. ** see the text for more details.*

The last types of variants (group 5) are the novel and unreported variations in databases (ClinVar, HGMD, PharmGKB), but found in a PGx test mostly through comprehensive methods, such as whole exome or whole genome sequencing (WES and WGS), with no clue for their function in causing a particular phenotype. Moreover, incidental findings (IFs) are the group of known variations, but not related to specifically investigated phenotype, and accidentally revealed during a sequencing test. Both the VUS (novel variants) and IFs will be manageable with higher accuracy by the combined usage of highly specialized bioinformatics pipelines to find any possible interaction with drug responses in patients. IFs are mostly displayed as the annotated functional drug-related variants in pharmacogenes and potentially useful markers if the appropriate genomic analysis and accurate genotype–phenotype correlations are performed subsequently [26]. We will address this topic in detail in the following section.

4. Approaches to Dealing with Novel Pharmacogenomics Variants

As the majority of revealed variants through implementation of broad range high throughput sequencing tests could be categorized in group 4 and 5 (the most challenging groups), the process of identifying clinically relevant PGx variants from complex genomic data mostly concerns about the detection of any potential FGVs in these two categories. The procedures usually start with digging the variant call format (VCF) file for filtration of variants and selection of those alterations, which come from drug-related genes. Based on the employed sequencer machine and the selected platform for PGx data clinical assessment, different types of variants are available in subsequent result analysis (SNVs and/or CNVs from coding and noncoding/regulatory parts of the genome). Routine silico analysis is considered for filtration of NGS derived pharmacovariants data at the first step (including the quality assessments, segregation studies, zygosity mapping, and allele phasing, etc.). Next, the selected variants go for pathogenicity and functional annotation analysis

through the utilization of prediction algorithms in both common (i.e., *SIFT*, *PolyPhen2*, *MutationTaster*) and PGx dedicated tools (i.e., *Stargazer*, *Aldy*, *Astrolabe*) [27–29]. As the final stage, computational and in-vitro confirmation studies can aid in the identification of prediction's sensitivity, specificity, and accuracy level. This is usually implemented via performing the homology modeling, Sanger sequencing, and cell culture modifications. The other approach is the replicate study in an independent validation cohort.

Examples for the generation of clinical recommendations for the variants using in silico analysis of WGS PGx data were done before. The related studies showed the PGx dosage recommendations are heavily influenced by the higher availability of genotyping results, which may lead to more clinical evidence too [30]. Yet, the most important barrier to routine implementation of NGS technologies for PGx tests in clinical centers is the huge amount of uncertain and unknown significant variants in the results (group 5), which need to be confirmed and validated before considered as the influential elements in treatment decision and prescription modification. In addition to some basic problems in using NGS methods, such as poor coverage of the specific parts of the genome, false-positive results in short reads, ignoring many non-coding variants in targeted panels and WES, missing some homopolymer regions, pseudogenes, and GC rich, diverse efficiency for genome capturing due to the utilization of different kits and reagents, etc. [31], any novel or incidental markers still must go through the different validation steps, to be connected to drug-related phenotypes in patients. While looking for previous clinical reports and similar investigations, current approaches in dealing with PGx variants in group 5 are including the computational methods and in-vitro functional analysis of the variants. As the number of altered alleles could be high in NGS data, applying the computational analysis techniques and starting with categorizing, filtering, and functional annotating the variants across the RefSeq and other databases, such as dbSNP or dbNSFP, by special bioinformatics tools, such as *VAT*, *VarAFT*, *ANNOVAR*, etc., is inevitable. Then, the prediction of potentially damaging, deleterious, and/or functionally neutral non-synonymous variants will be performed via the algorithms as mentioned earlier. Currently, the mutual beliefs for PGx data analysis are the combined utilization of 6 to 7 of such prediction tools and choosing those variants, which are commonly introduced as pathogen/likely pathogen in all applied software, according to reliable reference guidelines, such as those given by ACMG, CAP, and CPIC [27]. While there is no universal and widely accepted functional prediction software package, the number of introduced PGx specific analysis tools, such as *Stargazer*, *Astrolabe, PharmCAT*, *PHARMIP*, etc., are increasing rapidly in a fast-developing mode. Hence, integrating them in applied algorithms seems necessary. Table 3 listed some of these special data mining and visualization tools, which are used or considered to be useful in PGx data management. We will talk about the limitations of common analysis facilities later in the discussion section. Next is the pathway mapping of the selected variants against the general and specialized free reference sources, such as PharmGKB, String-db, DAVID, KEGG, etc., to find out about the potential gene–drug and protein–protein interactions. Finally, allele frequency and population derived variant analysis could be achieved through comparing with comprehensive surveys (1000 genome, ExAc, HapMap, ESP, gnomAD, GME) [32]. Moreover, laboratory confirmative assays and characterization could be implemented for just top prioritized functional variants, to roll out any false-positive result and be assured of the real harmful effects on drug response. The final clinical assessments (if it's available) support the necessity for genotype–phenotype correlation procedures too.

Table 3. Special data mining and visualization tools and algorithms, used in PGx data analyzing and phenotype prediction.

Software	*Applications*	*Link*	*Reference*
SIFT	SIFT (Sorting Intolerant From Tolerant) is an online program that predicts whether an amino acid substitution affects protein function based on sequence homology and the physical properties of amino acids.	https://sift.bii.a-star.edu.sg/	[33]
PolyPhen-2	PolyPhen-2 (Polymorphism Phenotyping v2) is a tool that predicts the possible impact of an amino acid substitution on the structure and function of a human protein using straightforward physical and comparative considerations.	http://genetics.bwh.harvard.edu/pph2/	[34]
LOFTEE	Loss-Of-Function Transcript Effect Estimator is a tool to identify LoF (loss-of-function) effects of variations. LOFTEE also makes predictions of another splice (OS) variants that may cause LoF by disrupting normal splicing patterns.	http://www.atgu.mgh.harvard.edu/resources/software/	[35]
VAT	Variant Annotation Tool is a computational framework to functionally annotate variants in personal genomes using a cloud-computing environment.	http://vat.gersteinlab.org/	[36]
VarAFT	Variant Annotation and Filter Tool is for the identification of disease-causing mutations in human genetics. The software improves annotation and filtration steps.	https://varaft.eu/	[37]

Table 3. *Cont.*

Software	*Applications*	*Link*	*Reference*
EV mutation	An online free tool for predicting the mutation effects from sequences.	https://marks.hms.harvard.edu/evmutation/	[38]
UCSF chimera package	UCSF Chimera is a highly extensible program for interactive visualization and analysis of molecular structures and related data, including density maps, supramolecular assemblies, sequence alignments, docking results, trajectories, and conformational ensembles. High-quality images and animations can be generated. The Resource for Biocomputing, Visualization, and Informatics (RBVI) and its precursor, which is interactive software tools and advanced web-based computational resources that provide integrated visualizations and analyses of molecular structures and related non-structural biological information.	https://www.cgl.ucsf.edu/chimera/	[39]
ICM-Molsoft	ICM-Pro empowers a biologist or chemist by providing a high-quality protein structure analysis, modeling, and docking desktop software environment. Main features include: analyze sequences and alignments, inspect protein structure, study pockets, and bound ligands and drugs, create surfaces, calculate electrostatics, make mutations, predict ligand binding sites, predict protein–protein interaction sites, perform small molecule and protein–protein docking, and design ligands.	http://www.molsoft.com/icm_pro.html	-
EVfold	EVfold uses an evolutionary variation to calculate a set of co-evolved residue pairs in a protein family using a global approach called maximum entropy, formally similar to partial correlations.	http://evfold.org/evfold-web/evfold.do	[40,41]

Table 3. *Cont.*

Software	*Applications*	*Link*	*Reference*
xBrowse	xBrowse is a platform for studying rare genetic diseases. It was built to provide genetic researchers and clinical geneticists a collaborative way to search for the causes of genetic disease using exome sequencing data. xBrowse accepts as input a set of variant calls from a whole exome or whole genome sequencing study for further processing and annotation. Currently, the only accepted input format is a VCF file produced by the GATK pipeline.	http://www.atgu.mgh.harvard.edu/resources/software/	.
PLINK	PLINK/SEQ is an open-source C/C++ library for working with human genetic variation data. The specific focus is to provide a platform for analytic tool development for variation data from large-scale resequencing and genotyping projects, particularly whole-exome and whole-genome studies. It is independent of (but designed to be complementary to) the existing PLINK package.	https://atgu.mgh.harvard.edu/plinkseq/	[42]
SKAT	SKAT is a Single Nucleotide Polymorphism (SNP)-set (e.g., a gene or a region) level test for association between a set of rare (or common) variants and dichotomous or quantitative phenotypes, SKAT aggregates individual score test statistics of SNPs in a SNP set and efficiently computes SNP-set level *p*-values, e.g., a gene or a region-level *p*-value, while adjusting for covariates, such as principal components to account for population stratification. SKAT also allows for power/sample size calculations for designing sequence association studies.	www.hsph.harvard.edu/skat	[43]

Table 3. *Cont.*

Software	*Applications*	*Link*	*Reference*
Mutation Assessor	This server predicts the functional impact of amino-acid substitutions in proteins, such as mutations discovered in cancer or missense polymorphisms. The functional impact is assessed based on the evolutionary conservation of the affected amino acid in protein homologs.	http://mutationassessor.org/r3/	[44]
MutationTaster	MutationTaster is a free web-based application to evaluate DNA sequence variants for their disease-causing potential. The software performs a battery of in silico tests to estimate the impact of the variant on the gene product/protein.	http://www.mutationtaster.org/	[45]
PANTHER	The PANTHER (Protein ANalysis THrough Evolutionary Relationships) Classification System was designed to classify proteins (and their genes) to facilitate high-throughput analysis. PANTHER is defined as a method to predict the functional effect of missense variants based on sequence information.	http://www.pantherdb.org/	[46]
PhD-SNP	An SVM-based classifier for the prediction of variant pathogenicity according to sequence profiles.	http://snps.biofold.org/phd-snpg/	[47]
Varscan2	An analysis tool, for the detection of somatic mutations and copy number alterations (CNAs) in exome data from tumor–normal pairs.	http://varscan.sourceforge.net/	[48]
SPLINTER	Detects and quantifies short Insertion–Deletions (InDels) and substitutions in large pools. SPLINTER allows accurate detection and quantification of short insertions, deletions, and substitutions by integrating information from the synthetic DNA library to tune SPLINTER and quantify specificity and sensitivity for every experiment to accurately detect and quantify InDels and substitutions.	https://omictools.com/splinter-tool	[49]

Table 3. *Cont.*

Software	*Applications*	*Link*	*Reference*
GeneSplicer	GeneSplicer is a new, flexible system for detecting splice sites in the genomic DNA of various eukaryotes and predicting the variant effects on the related protein(s).	http://www.cbcb.umd.edu/software/GeneSplicer/gene_spl.shtml	[50]
NMD Classifier	NMD is a tool for systematic classification of nonsense-mediated decay events for either annotated or de novo assembled transcripts.	https://sourceforge.net/projects/transcriptome-analysis/files/NMD_Classifier.tar.gz	[51]
mrSNP	mrSNP provides a web service for researchers working especially with RNA-Seq Data, to predict the impact of an SNP in a 3UTR on miRNA binding.	https://tools4mirs.org/software/mirna_snp_analysis/mrsnp/	[52]
GenoCanyon	GenoCanyon is a whole-genome functional annotation approach based on unsupervised statistical learning. It integrates genomic conservation measures and biochemical annotation data to predict the functional potential at each nucleotide, both in coding, and non-coding regions.	http://genocanyon.med.yale.edu/	[53]
ANNOVAR	ANNOVAR is an efficient software tool to utilize up-to-date information to functionally annotate genetic variants detected from diverse genomes (including human genome hg18, hg19, hg38, as well as mouse, worm, fly, yeast, and many others). Given a list of variants with chromosome, start position, end position, reference nucleotide, and observed nucleotides, ANNOVAR can perform: - Gene-based annotation; - Region-based annotation; - Filter-based annotation, etc.	http://annovar.openbioinformatics.org/en/latest/	[54]

Table 3. *Cont.*

Software	*Applications*	*Link*	*Reference*
CADD	CADD is a tool for scoring the deleteriousness of single nucleotide variants as well as insertion/deletion variants in the human genome. It integrates multiple annotations into one metric by contrasting variants that survived natural selection with simulated mutations. C-scores strongly correlate with allelic diversity, the pathogenicity of both coding and non-coding variants.	https://cadd.gs.washington.edu/	[55,56]
Provean	Provean is a software tool that predicts whether an amino acid substitution or InDel has an impact on the biological function of a protein. It is useful for filtering sequence variants to identify non-synonymous or InDel variants that are predicted to be functionally important.	http://provean.jcvi.org/index.php	[57,58]
ESEfinder	ESEfinder is a web-based resource that facilitates rapid analysis of exon sequences to identify putative exonic splicing enhancers, responsive to the human SR proteins SF2/ASF, SC35, SRp40, and SRp55, and to predict whether exonic mutations disrupt such elements.	http://krainer01.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home	[59]
VarSeq	VarSeq is an intuitive, integrated software solution for tertiary analysis of next generation sequencing (NGS) data. With VarSeq workflows can be automated and analyzing variants for gene panels, exomes, and whole genomes is possible. Moreover, the tool shows the ability to integrate with new resources and databases for advanced and customized variant analysis.	https://www.goldenhelix.com/products/VarSeq/	[60]
FATHMM	A high-throughput web-server capable of predicting the functional consequences of both coding variants, i.e., non-synonymous single nucleotide variants (nsSNVs), and non-coding variants in the human genome.	http://fathmm.biocompute.org.uk/	[61]

Table 3. *Cont.*

Software	*Applications*	*Link*	*Reference*
GERP++	Genomic Evolutionary Rate Profiling (GERP) identifies constrained elements in multiple alignments by quantifying substitution deficits.	http://mendel.stanford.edu/SidowLab/downloads/gerp/	[62]
SiPhy	SiPhy implements rigorous statistical tests to detect bases under selection from multiple alignment data. It takes full advantage of deeply sequenced phylogenies to estimate either unlikely substitution patterns as well as slowdowns or accelerations in mutation rates.	http://portals.broadinstitute.org/genome_bio/siphy/index.html	-
Stargazer	Stargazer is a bioinformatics tool for calling star alleles (haplotypes) in PGx genes using data from NGS or SNP array. Stargazer can accept NGS data from both whole genome sequencing (WGS) and targeted sequencing. Stargazer identifies star alleles by detecting SNVs, InDels, and SVs. Stargazer can detect complex SVs including gene deletions, duplications, and hybrids by calculating paralog-specific copy numbers from read depth.	https://stargazer.gs.washington.edu/stargazerweb/	[63]
PharmCAT	A tool to extract all CPIC guideline gene variants from a genetic dataset (represented as a VCF file), interpret the variant alleles and generate a report.	https://github.com/PharmGKB/PharmCAT	[64,65]
PHARMIP	An in silico method to predict genetics that underpin adverse drug reactions. The tool can be used to reveal genetic risk factors for certain drug ADRs.	http://www.lilab-ecust.cn/pharmmapper/	[66]
PharmVar API	An online source for access to all or selected data of the Pharmacogene Variation Consortium (PharmVar) database.	https://www.pharmvar.org/documentation	-

Table 3. *Cont.*

Software	*Applications*	*Link*	*Reference*
Astrolabe	Astrolabe is software for the translation of whole genome sequence data into pharmacogenetic information that can be used to guide medication selection, dosing, and prescription. It was initially developed under the name Constellation for the *CYP2D6* gene, then extended to *CYP2C9* and *CYP2C19* with additional genes in the process of being validated. Astrolabe is integrated with the PharmVar database	https://childrensmercy.org/genomesoftwareportal/Software/Index/	[67]
Aldy	Aldy performs allelic decomposition of highly polymorphic, multi-copy genes by using whole or targeted genome sequencing data. For a large diverse sequencing data set, Aldy identifies multiple rare and novel alleles for several important pharmacogenes, significantly improving upon the accuracy and utility of current genotyping assays.	http://aldy.csail.mit.edu.	[68]
Cypripi	An algorithm to computationally infer *CYP2D6* genotype at base pair resolution from high throughput sequencing data. It can resolve complex genotypes, including alleles that are the products of duplication, deletion, and fusion events involving *CYP2D6* and its evolutionarily related cousin *CYP2D7*.	http://sfu-compbio.github.io/cypiripi/	[69]

5. Discussion

NGS technologies have been used in several PGx studies in recent years. Based on the employed platforms, the acquired data analyzed through different approaches. Due to the lower amount of identified variants (mostly known alleles), finding the FGVs and phenotype prediction is usually easier when targeted sequencing for a specific set of the gene (panels) is performed as the selected method. WES and WGS, however, show a lot of obstacles when applied for a PGx analysis and this is mainly because of the huge number of functionally unknown and unreported alterations in a patient's genetic profile [70]. Moreover, some intrinsic and substantial complications for PGx tests including the presence of germline mutations with necessary haplotype detection and phase definition in patients, going through specific pharmacogenes with a role in different sophisticated cellular pathways (i.e., *ACE*), following environmental and epigenetic modifications on drug-related genes, working with challenging and problematic variants, in particular drug-related genes (i.e., *CYP2D6* with close pseudogenes and many unknown and novel variants in diverse

populations, different functional tandem repeat variants in the non-coding part of *UGT1A1* gene, etc.), and most important of them the lack of previous knowledge on possible phenotype modifications for many genetic changes (as PGx is a pre-emptive genotyping test in numerous cases) can potentially increase the difficulties in variant analysis and pose the clear effects on changing the drug responses in individuals. Albeit, providing more genotype to phenotype translation methods by reference organizations and guideline developers will result in more consistent genotype interpretation in both clinical and research area [71].

Despite the challenges, the number of publications for NGS derived PGx data analysis are still significant. Gordon et al. successfully identified common, rare, and novel variants in 84 clinically actionable drug-related genes in more than 280 individuals through a targeted resequencing custom panel. They used deep coverage of the known genes to follow both previously recognized and possible novel variants. New potentially deleterious nonsense and missense variants across some VIPs were selected for more genotype-phenotype association studies to find any relation with particular traits (group1, 2, and 5 of the PGx variants). Moreover, actionable plus rare unreported variants in absorption, distribution, metabolism, and excretion (ADME) core genes revealed in 114 drug genes in 376 people by Han and colleagues. The number of variants in each gene (normalized based on gene length), MAF, and novelty assessed and compared to open genotyping datasets (group2, 4, and 5). In silico functional assessments performed by the prediction tools, such as *SIFT*, *PolyPhen2*, and *CAAD,* and deleterious rare-novel variants in some of VIPs evaluated by in-vitro analysis to find impaired functions evidence. Moreover, additional and novel faraway variants (group 5), contributed to the alteration of estrogen receptor binding site and breast cancer risk identified in 400 patients by NGS deep sequencing and functional genomics. As the number of investigated genes was low, any novel PGx variant was confirmed through the laboratory tests, such as chromatin immunoprecipitation (ChIP), gene expression analysis, and protein degradation assays [72–74]. Other utilizations also brought more unprecedented results for clinical PGx investigations. For example variants and haplotype detection of challenging ADME genes were successfully achieved in three core pharmacogenes (*CYP2D6*, *HLA-A,* and *HLA-B*) by applying the long read sequencers (group1 and 2). All the SNVs, CNVs, and InDels were revealed through the utilization of customized long-range PCR and the subsequent NGS machine (MinION nanopore sequencer) [75]. Moreover, 17,733 ADME variants per individual were detected in 231 genes. In addition to known PGx markers, the latter included 1012 novel variants with potential deleterious functions identified in exons, introns, gene promoters, and proximal regulatory regions. The authors reanalyzed WGS provided data to find different PGx markers in close to 500 individuals. In silico analysis used the ANNOVAR tool for annotation and dbSNP137 and Complete Genomics public server for novelty assessments. Functional assays were also predicted via SIFT and Provean algorithms (group1, 2, 4, and 5) [12]. In another effort, whole genome sequencing (WGS) in PGx analysis revealed 227 common and 466 rare population-specific potentially functional SNVs, including 74 novel variants in 437 drug genes (group1, 2, and 5). Variant analysis computational workflow consisted of ANNOVAR and dbSNP138 for variant annotation, *SIFT,* and *PolyPhen2* for functional effect analysis of novel non-synonymous coding SNVs, mapping the deleterious variants with PharmGKB and DrugBank, and finally *PLINK* and *VCFTools* for reaching allele frequencies and validation through 1000 genome and HapMap databases. In the end, a drug pathway map for functionally impaired pharmacogenes displayed, using identified deleterious variants [32]. Even the PGx-specific panel with high accuracy designed and identified clinically relevant variants in 39 genes including *CYP2D6* CNV and *UGT1A1*28* TAA repeats in promoter in addition to allele frequency and homozygosity in 235 patients. Common in-vitro and bioinformatics tools used for both known and novel variant detection rate accuracy and sensitivity (group1, 2, and 5) [76]. Finally, a comprehensive usage for NGS methods can be found in Price and his team effort, which applied exome sequencing for 21,000 human genes and revealed novel genetic loci with a strong association with on-treatment reactivity

and hereditability of platelet and clopidogrel response. Once again, novel loci and related variants in addition to known PGx markers were depicted by common data interpretation pipeline and proved the NGS methods as a powerful approach in unavailing PGx variants in clinical studies [77].

Two important points could be mentioned from the above investigations as well. As the majority of functional prediction tools and algorithms are relying on evolutionary conservation and therefore will not be completely fit with the pharmacogenes (poorly conserved) and show low predictive accuracy as the conventional algorithms (up to 50%), most of the studies emphasize combined utilization of such tools in in silico phenotype prediction for novel variants and introduced various software in each report. This may remind the necessity of the attitude for new PGx data in high throughput sequencing methods, as they are not observable in many cases (pre-emptive genotyping). Recent efforts, however, have been focused on developing new pharmacogene optimized frameworks with more relation to PGx data assessment through the integration of specific algorithms or presenting the allele dedicated for pharmacovariant calling and showed to be more compatible with ADME genes with a higher rate of sensitivity and specificity (90–99%) [27,63]. Other PGx specialized projects are also recently developed a pharmacogenomics clinical annotation tool (*PharmCAT*) and tried to reveal which patients in a clinical dataset include the variants of interest [65].

The second point is the ability of NGS technologies to the detection of any kinds of PGx variants in clinical practice. They have introduced several novel PGx markers successfully and the fact may indicate the faster incorporation of PGx test results into the future precision medicine as well. However, there are still essential issues with high importance in the field, which need to be addressed properly. For example, if the particular novel variant causes a loss of function or gain of function effects on the related protein(s) (making a poor or rapid metabolizer) in tested individuals and also possible misinterpreting of VUS in the result, which may lead to ignore or miss the functional variants in pharmacogenes. Such complexities must be followed by the in-vitro assessments in addition to appropriate pre and post-test counseling for individuals [28,78].

The intricacies are not limited to the detection of variants, but the nature of drug actions according to particular alleles too. Investigations displayed the dual or multiple impacts of some specific pharmacovariants toward the different diseases and/or drugs (Table 4). Furthermore, a certain drug could be the substrate for more than one P450 family and metabolized by different enzymes (i.e., CYP1A2, CYP2C19, and CYP2D6 for antidepressant amitriptyline) [79]. Such scenarios complicate the true functional assessment of pharmacovariants, especially in high throughput sequencing data. Because of that, a comprehensive literature search, replicate studies, and wet lab analysis of the newly identified genetic markers in drug-related genes must be taken into account before any prescription considerations in the clinical setting.

Table 4. Examples of different outcomes for one particular allele/diplotype of *CYP2D6* in different disorders and drugs.

Disease/Disorder	*Drug*	*Gene*	*Diplotype or Allele*	*Decreased Response*	*Increased Response*	*Low Plasma Concentration*	*High Plasma Concentration*	*Toxicity*	*Level of Evidence*	*Reference*
Depressive Disorder Mental Disorders	Paroxetine	*CYP2D6*	*1/*1xN #	-	✓	✓	-	-	1A	[80]
Nausea and Vomiting after Chemotherapy	Ondansetron	*CYP2D6*	*1/*1xN	✓	-	-	✓	?	1A	[81]
Mental Disorders	Desipramine	*CYP2D6*	*1xN	✓	-	-	✓	?	2A	[82]
Alzheimer Disease	Donepezil	*CYP2D6*	*1/*1xN	-	✓	✓	-	-	3	[83]
Pain	Codeine	*CYP2D6*	*1/*1xN	-	✓	✓	-	✓	1A	[84,85]

Gene duplication, which resulted in ultra-rapid metabolizer. * is a standardized nomenclature system used for various haplotypes and alleles in Cytochrome P450 family pharmacogenes. The level of evidence is adopted from PharmGKB [19]. ✓: *Yes*, *?: unknown*, *-: not applicable.*

6. Conclusions

The field of pharmacogenomics faces several challenges throughout the process of the identification of pharmacogenomic variants and their implementation in clinical practices. Many of these challenges arise at the genomics level, including the statistical considerations associated with the design of the clinical trial and genome-wide association studies (GWAS), a large number of candidate variants compared to available samples ($p > n$), the lack of reproducibility in independent studies and determining the functional impact of variants on drug response. In the age of PGx and personalized drug therapy, using the high throughput sequencing approaches will assist the translation of different pharmacovariants into clinical care. As mentioned before, for moving genomic medicine toward personalized drug therapy, there should be a genetic screening test, which fits all ethnicities [12]. NGS, as a time and cost-effective and highly accurate genotyping method, shows the huge benefits for patients PGx clinical assessments. Hence, it would be highly possible for the investigators and clinicians to encounter new and rare population-specific variants during a PGx test. To deal with different NGS derived PGx variants in clinics, all healthcare professionals need to know the classification and interpretation algorithms for such markers properly.

Author Contributions: A.T. provided the idea, designed the study, conducted the literature search, and wrote the entire manuscript. N.W.-K. and R.P. searched for the literature and edited the manuscript. W.M. supervised the study thoroughly and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This article was conducted within the projects, which received funding from the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement No 754432, and the Polish Ministry of Science and Higher Education, from financial resources for science in 2018–2023 granted for the implementation of an international co-financed project.

Conflicts of Interest: The authors declare that there is no conflict of interest during the preparation of this manuscript.

Abbreviations

ACMG	American College of Medical Genetics and Genomics
ADME	Absorption, Distribution, Metabolism, and Excretion
CADD	Combined Annotation-Dependent Depletion
CAP	College of American Pathologists
CNV	Copy Number Variation
CPIC	The Clinical Pharmacogenetics Implementation Consortium
DPWG	Dutch Pharmacogenetics Working Group
FDA	Food and Drug Administration
FGV	Functional Genetic Variation
GWAS	Genome-Wide Association Studies
IF	Incidental Findings
InDel	Insertion–Deletion
MAF	Minor Allele Frequency
NCI	National Cancer Institute
NGS	Next Generation Sequencing
PDG	Pharmacogenomics Dosage Guidelines
PGRN	The Pharmacogenomics Research Network
PGx	Pharmacogenomics
PharmCAT	Pharmacogenomics Clinical Annotation Tool

PharmGKB	Pharmacogenomics Knowledge Base
Provean	Protein Variation Effect Analyzer
SNP	Single Nucleotide Polymorphism
SNV	Single Nucleotide Variation
SV	Structural Variants
VAT	Variant Annotation Tool
VarAFT	Variant Annotation and Filter Tool
VCF	Variant Call Format
VIP	Very Important Pharmacogene
VUS	Variants with Unknown clinical Significance
WES	Whole Exome Sequencing
WGS	Whole Genome Sequencing

References

1. Sim, S.; Kacevska, M.; Ingelman-Sundberg, M. Pharmacogenomics of drug-metabolizing enzymes: A recent update on clinical implications and endogenous effects. *Pharm. J.* **2013**, *13*, 1–11. [CrossRef] [PubMed]
2. Kozyra, M.; Ingelman-sundberg, M.; Lauschke, V.M. Rare genetic variants in cellular transporters, metabolic enzymes, and nuclear receptors can be important determinants of interindividual differences in drug response. *Genet. Med.* **2017**, *19*, 20–29. [CrossRef] [PubMed]
3. Schärfe, C.P.I.; Tremmel, R.; Schwab, M.; Kohlbacher, O.; Marks, D.S. Genetic variation in human drug-related genes. *Genome Med.* **2017**, *9*, 117. [CrossRef]
4. Rabbani, B.; Nakaoka, H.; Akhondzadeh, S.; Tekin, M.; Mahdieh, N. Next generation sequencing: Implications in personalized medicine and pharmacogenomics. *Mol. BioSyst.* **2016**, *12*, 1818–1830. [CrossRef] [PubMed]
5. Walczak, M.; Skrzypczak-Zielinska, M.; Plucinska, M.; Zakerska-Banaszak, O.; Marszalek, D.; Lykowska-Szuber, L.; Stawczyk-Eder, K.; Dobrowolska, A.; Slomski, R. Long-range PCR libraries and next generation sequencing for pharmacogenetic studies of patients treated with anti-TNF drugs. *Pharm. J.* **2019**, *19*, 358–367. [CrossRef]
6. Santos, M.; Niemi, M.; Hiratsuka, M.; Kumondai, M.; Ingelman-Sundberg, M.; Lauschke, V.M.; Rodríguez-Antona, C. Novel copy-number variations in pharmacogenes contribute to interindividual differences in drug pharmacokinetics. *Genet. Med.* **2018**, *20*, 622–629. [CrossRef]
7. Cousin, M.A.; Matey, E.T.; Blackburn, P.R.; Boczek, N.J.; McAllister, T.M.; Kruisselbrink, T.M.; Babovic-Vuksanovic, D.; Lazaridis, K.N.; Klee, E.W. Pharmacogenomic findings from clinical whole exome sequencing of diagnostic odyssey patients. *Mol. Genet. Genome Med.* **2017**, *5*, 269–279. [CrossRef]
8. Klein, K.; Tremmel, R.; Winter, S.; Fehr, S.; Battke, F.; Scheurenbrand, T.; Schaeffeler, E.; Biskup, S.; Schwab, M.; Zanger, U.M. A New Panel-Based Next Generation Sequencing Method for ADME Genes Reveals Novel Associations of Common and Rare Variants With Expression in a Human Liver Cohort. *Front. Genet.* **2019**, *10*, 7. [CrossRef]
9. Hovelson, D.H.; Xue, Z.; Zawistowski, M.; Ehm, M.G.; Harris, E.C.; Stocker, S.L.; Gross, A.S.; Jang, I.-J.; Ieiri, I.; Lee, J.-E.; et al. Characterization of ADME gene variation in 21 populations by exome sequencing. *Pharm. Genome* **2017**, *27*, 89. [CrossRef]
10. Ortega, V.E.; Meyers, D.A. Pharmacogenetics: Implications of race and ethnicity on defining genetic profiles for personalized medicine. *J. Allergy Clin. Immunol.* **2014**, *133*, 16–26. [CrossRef]
11. Ahn, E.; Park, T. Analysis of population-specific pharmacogenomic variants using next generation sequencing data. *Sci. Rep.* **2017**, *7*, 8416. [CrossRef] [PubMed]
12. Mizzi, C.; Peters, B.; Mitropoulou, C.; Mitropoulos, K.; Katsila, T.; Agarwal, M.R.; Van Schaik, R.H.; Drmanac, R.; Borg, J.; Patrinos, G.P. Personalized pharmacogenomics profiling using whole-genome sequencing. *Pharmacogenomics* **2014**, *15*, 1223–1234. [CrossRef] [PubMed]
13. Lalioti, M.; Gerasimova, T.; Zattas, D.; Anastasakis, D.; Seli, E.; Sakkas, D. *A Deleted Form of FSH Receptor, Found in Women Undergoing Infertility Treatment, Impairs the Function of the Normal Receptor When Co-Expressed In Vitro*; Oxford University Press: Oxford, UK, 2010.
14. Hoffman, J.M.; Dunnenberger, H.M.; Hicks, J.K.; Caudle, K.E.; Carrillo, M.W.; Freimuth, R.R.; Williams, M.S.; Klein, T.E.; Peterson, J.F. Developing knowledge resources to support precision medicine: Principles from the Clinical Pharmacogenetics Implementation Consortium (CPIC). *J. Am. Med Inform. Assoc.* **2016**, *23*, 796–801. [CrossRef] [PubMed]
15. Relling, M.; Klein, T. CPIC: Clinical pharmacogenetics implementation consortium of the pharmacogenomics research network. *Clin. Pharmacol. Ther.* **2011**, *89*, 464–467. [CrossRef] [PubMed]
16. Barbarino, J.M.; Whirl-Carrillo, M.; Altman, R.B.; Klein, T.E. PharmGKB: A worldwide resource for pharmacogenomic information. *Wiley Interdiscip. Rev. Syst. Biol. Med.* **2018**, *10*, e1417. [CrossRef]
17. Tilleman, L.; Weymaere, J.; Heindryckx, B.; Deforce, D.; Van Nieuwerburgh, F. Contemporary pharmacogenetic assays in view of the PharmGKB database. *Pharmacogenomics* **2019**, *20*, 261–272. [CrossRef]
18. Swen, J.J.; Nijenhuis, M.; De Boer, A.; Grandia, L.; Der Zee, A.M.-V.; Mulder, H.; Rongen, G.; Van Schaik, R.; Schalekamp, T.; Touw, D.; et al. Pharmacogenetics: From Bench to Byte—An Update of Guidelines. *Clin. Pharmacol. Ther.* **2011**, *89*, 662–673. [CrossRef]
19. PharmGKB. Available online: https://www.pharmgkb.org/page/clinAnnLevels (accessed on 14 April 2020).

20. CPIC. Available online: https://cpicpgx.org/genes-drugs (accessed on 14 April 2020).
21. DPWG. Available online: https://www.pharmgkb.org/page/dpwg (accessed on 14 April 2020).
22. Caudle, K.E.; Gammal, R.S.; Whirl-Carrillo, M.; Hoffman, J.M.; Relling, M.V.; Klein, T.E. Evidence and resources to implement pharmacogenetic knowledge for precision medicine. *Am. J. Health Syst. Pharm.* **2016**, *73*, 1977–1985. [CrossRef]
23. Relling, M.V.; Evans, W.E. Pharmacogenomics in the clinic. *Nature* **2015**, *526*, 343–350. [CrossRef]
24. Whirl-Carrillo, M.; McDonagh, E.M.; Hebert, J.; Gong, L.; Sangkuhl, K.; Thorn, C.; Altman, R.B.; Klein, T.E. Pharmacogenomics Knowledge for Personalized Medicine. *Clin. Pharmacol. Ther.* **2012**, *92*, 414–417. [CrossRef]
25. Aslibekyan, S.; Claas, S.A.; Arnett, D.K. To replicate or not to replicate: The case of pharmacogenetic studies: Establishing validity of pharmacogenomic findings: From replication to triangulation. *Circ. Cardiovasc. Genet.* **2013**, *6*, 409–412. [CrossRef] [PubMed]
26. Lee, E.M.; Xu, K.; Mosbrook, E.; Links, A.; Guzman, J.; Adams, D.R.; Flynn, E.; Valkanas, E.; Toro, C.; Tifft, C.J.; et al. Pharmacogenomic incidental findings in 308 families: The NIH Undiagnosed Diseases Program experience. *Genet. Med.* **2016**, *18*, 1303–1307. [CrossRef] [PubMed]
27. Zhou, Y.; Mkrtchian, S.; Kumondai, M.; Hiratsuka, M.; Lauschke, V.M. An optimized prediction framework to assess the functional impact of pharmacogenetic variants. *Pharm. J.* **2019**, *19*, 115–126. [CrossRef] [PubMed]
28. Devarajan, S.; Moon, I.; Ho, M.-F.; Larson, N.B.; Neavin, D.R.; Moyer, A.M.; Black, J.L.; Bielinski, S.J.; Scherer, S.E.; Wang, L.; et al. Pharmacogenomic Next-Generation DNA Sequencing: Lessons from the Identification and Functional Characterization of Variants of Unknown Significance in CYP2C9 and CYP2C19. *Drug Metab. Dispos.* **2019**, *47*, 425–435. [CrossRef]
29. Twesigomwe, D.; Wright, G.E.; Drögemöller, B.I.; da Rocha, J.; Lombard, Z.; Hazelhurst, S. A systematic comparison of pharmacogene star allele calling bioinformatics algorithms: A focus on CYP2D6 genotyping. *NPJ Genom. Med.* **2020**, *5*, 1–11. [CrossRef]
30. Huser, V.; Cimino, J.J. Providing pharmacogenomics clinical decision support using whole genome sequencing data as input. *AMIA Jt. Summits Transl. Sci. Proc.* **2013**, *2013*, 81.
31. Shigemizu, D.; Fujimoto, A.; Akiyama, S.; Abe, T.; Nakano, K.; Boroevich, K.A.; Yamamoto, Y.; Furuta, M.; Kubo, M.; Nakagawa, H.; et al. A practical method to detect SNVs and indels from whole genome and exome sequencing data. *Sci. Rep.* **2013**, *3*, 2161. [CrossRef]
32. Sivadas, A.; Salleh, M.; Teh, L.; Scaria, V. Genetic epidemiology of pharmacogenetic variants in South East Asian Malays using whole-genome sequences. *Pharm. J.* **2017**, *17*, 461–470. [CrossRef]
33. Pauline, C.N.; Henikoff, S. SIFT: Predicting amino acid changes that affect protein function. *Nucleic Acids Res.* **2003**, *31*, 3812–3814. [CrossRef]
34. Adzhubei, I.; Jordan, D.M.; Sunyaev, S.R. Predicting Functional Effect of Human Missense Mutations Using PolyPhen-2. *Curr. Protoc. Hum. Genet.* **2013**, *76*, 7–20. [CrossRef]
35. MacArthur, D.G.; Balasubramanian, S.; Frankish, A.; Huang, N.; Morris, J.; Walter, K.; Jostins, L.; Habegger, L.; Pickrell, J.K.; Montgomery, S.B.; et al. A Systematic Survey of Loss-of-Function Variants in Human Protein-Coding Genes. *Science* **2012**, *335*, 823–828. [CrossRef] [PubMed]
36. Habegger, L.; Balasubramanian, S.; Chen, D.Z.; Khurana, E.; Sboner, A.; Harmanci, A.; Rozowsky, J.; Clarke, D.; Snyder, M.; Gerstein, M. VAT: A computational framework to functionally annotate variants in personal genomes within a cloud-computing environment. *Bioinformatics* **2012**, *28*, 2267–2269. [CrossRef] [PubMed]
37. Desvignes, J.-P.; Bartoli, M.; Delague, V.; Krahn, M.; Miltgen, M.; Béroud, C.; Salgado, D. VarAFT: A variant annotation and filtration system for human next generation sequencing data. *Nucleic Acids Res.* **2018**, *46*, W545–W553. [CrossRef] [PubMed]
38. Hopf, T.A.; Ingraham, J.B.; Poelwijk, F.J.; Schärfe, C.P.; Springer, M.; Sander, C.; Marks, D.S. Mutation effects predicted from sequence co-variation. *Nat. Biotechnol.* **2017**, *35*, 128–135. [CrossRef]
39. Huang, C.C.; Meng, E.C.; Morris, J.H.; Pettersen, E.F.; Ferrin, T.E. Enhancing UCSF Chimera through web services. *Nucleic Acids Res.* **2014**, *42*, W478–W484. [CrossRef]
40. Marks, D.S.; Colwell, L.J.; Sheridan, R.; Hopf, T.A.; Pagnani, A.; Zecchina, R.; Sander, C. Protein 3D Structure Computed from Evolutionary Sequence Variation. *PLoS ONE* **2011**, *6*, e28766. [CrossRef]
41. Marks, D.S.; Hopf, T.A.; Sander, C. Protein structure prediction from sequence variation. *Nat. Biotechnol.* **2012**, *30*, 1072–1080. [CrossRef]
42. Purcell, S.; Neale, B.; Todd-Brown, K.; Thomas, L.; Ferreira, M.A.R.; Bender, D.; Maller, J.; Sklar, P.; De Bakker, P.I.W.; Daly, M.J.; et al. PLINK: A Tool Set for Whole-Genome Association and Population-Based Linkage Analyses. *Am. J. Hum. Genet.* **2007**, *81*, 559–575. [CrossRef]
43. Ionita-Laza, I.; Lee, S.; Makarov, V.; Buxbaum, J.D.; Lin, X. Sequence Kernel Association Tests for the Combined Effect of Rare and Common Variants. *Am. J. Hum. Genet.* **2013**, *92*, 841–853. [CrossRef]
44. Li, H. Tabix: Fast retrieval of sequence features from generic TAB-delimited files. *Bioinformatics* **2011**, *27*, 718–719. [CrossRef]
45. Schwarz, J.M.; Rödelsperger, C.; Schuelke, M.; Seelow, D. Mutation Taster evaluates diseasecausing potential of sequence alterations. *Nat. Methods* **2010**, *7*, 575–576. [CrossRef] [PubMed]
46. Thomas, P.D.; Muruganujan, A.; Ebert, D.; Huang, X.; Thomas, P.D. PANTHER version 14: More genomes, a new PANTHER GO-slim and improvements in enrichment analysis tools. *Nucleic Acids Res.* **2018**, *7*, D419–D426. [CrossRef]
47. Capriotti, E.; Fariselli, P. PhD-SNPg: A webserver and lightweight tool for scoring single nucleotide variants. *Nucleic Acids Res.* **2017**, *45*, W247–W252. [CrossRef] [PubMed]

48. Koboldt, D.C.; Zhang, Q.; Larson, D.E.; Shen, D.; McLellan, M.D.; Lin, L.; Miller, C.A.; Mardis, E.R.; Ding, L.; Wilson, R.K. VarScan 2: Somatic mutation and copy number alteration discovery in cancer by exome sequencing. *Genome Res.* **2012**, *22*, 568–576. [CrossRef]
49. Vallania, F.; Ramos, E.; Cresci, S.; Mitra, R.D.; Druley, T.E. Detection of Rare Genomic Variants from Pooled Sequencing Using SPLINTER. *J. Vis. Exp.* **2012**, *64*, e3943. [CrossRef] [PubMed]
50. Pertea, M.; Lin, X.; Salzberg, S.L. GeneSplicer: A new computational method for splice site prediction. *Nucleic Acids Res.* **2001**, *29*, 1185–1190. [CrossRef]
51. Hsu, M.-K.; Lin, H.-Y.; Chen, F.-C. NMD Classifier: A reliable and systematic classification tool for nonsense-mediated decay events. *PLoS ONE* **2017**, *12*, e0174798. [CrossRef]
52. Deveci, M.; Çatalyürek, Ü.V.; Toland, A.E. mrSNP: Software to detect SNP effects on microRNA binding. *BMC Bioinform.* **2014**, *15*, 73. [CrossRef]
53. Lu, Q.; Hu, Y.; Sun, J.; Cheng, Y.; Cheung, K.-H.; Zhao, H. A Statistical Framework to Predict Functional Non-Coding Regions in the Human Genome through Integrated Analysis of Annotation Data. *Sci. Rep.* **2015**, *5*, 10576. [CrossRef]
54. Yang, H.; Wang, K. Genomic variant annotation and prioritization with ANNOVAR and wANNOVAR. *Nat. Protoc.* **2015**, *10*, 1556–1566. [CrossRef]
55. Kircher, M.; Witten, D.M.; Jain, P.; O'Roak, B.J.; Cooper, G.M.; Shendure, J. A general framework for estimating the relative pathogenicity of human genetic variants. *Nat. Genet.* **2014**, *46*, 310. [CrossRef] [PubMed]
56. Rentzsch, P.; Witten, D.; Cooper, G.M.; Shendure, J.; Kircher, M. CADD: Predicting the deleteriousness of variants throughout the human genome. *Nucleic Acids Res.* **2019**, *47*, D886–D894. [CrossRef] [PubMed]
57. Choi, Y.; Chan, A.P. PROVEAN web server: A tool to predict the functional effect of amino acid substitutions and indels. *Bioinformatics* **2015**, *31*, 2745–2747. [CrossRef] [PubMed]
58. Choi, Y.; Sims, G.E.; Murphy, S.; Miller, J.R.; Chan, A.P. Predicting the Functional Effect of Amino Acid Substitutions and Indels. *PLoS ONE* **2012**, *7*, e46688. [CrossRef]
59. Cartegni, L.; Wang, J.; Zhu, Z.; Zhang, M.Q.; Krainer, A.R. ESEfinder: A web resource to identify exonic splicing enhancers. *Nucleic Acids Res.* **2003**, *31*, 3568–3571. [CrossRef]
60. VarSeq. VarSeq Brochures. Available online: https://www.goldenhelix.com/products/VarSeq/ (accessed on 12 April 2020).
61. Rogers, M.F.; Shihab, H.A.; Mort, M.; Cooper, D.N.; Gaunt, T.R.; Campbell, C. FATHMM-XF: Accurate prediction of pathogenic point mutations via extended features. *Bioinformatics* **2018**, *34*, 511–513. [CrossRef]
62. Davydov, E.V.; Goode, D.L.; Sirota, M.; Cooper, G.M.; Sidow, A.; Batzoglou, S. Identifying a High Fraction of the Human Genome to be under Selective Constraint Using GERP++. *PLoS Comput. Biol.* **2010**, *6*, e1001025. [CrossRef]
63. Lee, S.-B.; Wheeler, M.M.; Patterson, K.; McGee, S.; Dalton, R.; Woodahl, E.L.; Gaedigk, A.; Thummel, K.E.; Nickerson, D.A. Stargazer: A software tool for calling star alleles from next generation sequencing data using CYP2D6 as a model. *Genet. Med.* **2019**, *21*, 361–372. [CrossRef]
64. Klein, T.E.; Ritchie, M.D. PharmCAT: A Pharmacogenomics Clinical Annotation Tool. *Clin. Pharmacol. Ther.* **2018**, *104*, 19–22. [CrossRef]
65. Sangkuhl, K.; Whirl-Carrillo, M.; Whaley, R.M.; Woon, M.; Lavertu, A.; Altman, R.B.; Carter, L.; Verma, A.; Ritchie, M.D.; Klein, T.E. Pharmacogenomics Clinical Annotation Tool (Pharm CAT). *Clin. Pharmacol. Ther.* **2020**, *107*, 203–210. [CrossRef]
66. Zidan, A.M.; Saad, E.A.; Ibrahim, N.E.; Mahmoud, A.; Hashem, M.H.; Hemeida, A.A. PHARMIP: An insilico method to predict genetics that underpin adverse drug reactions. *MethodsX* **2020**, *7*, 100775. [CrossRef] [PubMed]
67. Twist, G.P.; Gaedigk, A.; Miller, N.A.; Farrow, E.G.; Willig, L.K.; Dinwiddie, D.L.; Petrikin, J.; Soden, S.E.; Herd, S.; Gibson, M.; et al. Constellation: A tool for rapid, automated phenotype assignment of a highly polymorphic pharmacogene, CYP2D6, from whole-genome sequences. *NPJ Genome Med.* **2016**, *1*, 1–10. [CrossRef] [PubMed]
68. Numanagić, I.; Malikić, S.; Ford, M.; Qin, X.; Toji, L.; Radovich, M.; Skaar, T.C.; Pratt, V.M.; Berger, B.; Scherer, S.; et al. Allelic decomposition and exact genotyping of highly polymorphic and structurally variant genes. *Nat. Commun.* **2018**, *9*, 1–11. [CrossRef]
69. Numanagić, I.; Malikić, S.; Pratt, V.M.; Skaar, T.C.; Flockhart, D.A.; Sahinalp, S.C. Cypiripi: Exact genotyping of CYP2D6 using high-throughput sequencing data. *Bioinformatics* **2015**, *31*, i27–i34. [CrossRef] [PubMed]
70. Katsila, T.; Patrinos, G.P. Whole genome sequencing in pharmacogenomics. *Front. Pharmacol.* **2015**, *6*, 61. [CrossRef]
71. Caudle, K.E.; Sangkuhl, K.; Whirl-Carrillo, M.; Swen, J.J.; Haidar, C.E.; Klein, T.E.; Gammal, R.S.; Relling, M.V.; Scott, S.A.; Hertz, D.L.; et al. Standardizing CYP 2D6 Genotype to Phenotype Translation: Consensus Recommendations from the Clinical Pharmacogenetics Implementation Consortium and Dutch Pharmacogenetics Working Group. *Clin. Transl. Sci.* **2020**, *13*, 116–124. [CrossRef]
72. Gordon, A.; Fulton, R.S.; Qin, X.; Mardis, E.R.; Nickerson, D.A.; Scherer, S. PGRNseq: A targeted capture sequencing panel for pharmacogenetic research and implementation. *Pharm. Genome* **2016**, *26*, 161. [CrossRef]
73. Han, S.; Park, J.; Lee, J.; Lee, S.; Kim, H.; Han, H.; Kim, Y.; Yi, S.; Cho, J.Y.; Jang, I.J.; et al. Targeted Next-Generation Sequencing for Comprehensive Genetic Profiling of Pharmacogenes. *Clin. Pharmacol. Ther.* **2017**, *101*, 396–405. [CrossRef]
74. Liu, D.; Ho, M.-F.; Schaid, D.J.; Scherer, S.E.; Kalari, K.; Liu, M.; Biernacka, J.; Yee, V.; Evans, J.; Carlson, E.; et al. Breast cancer chemoprevention pharmacogenomics: Deep sequencing and functional genomics of the ZNF423 and CTSO genes. *NPJ Breast Cancer* **2017**, *3*, 1–9. [CrossRef]

75. Ammar, R.; Paton, T.A.; Torti, D.; Shlien, A.; Bader, G.D. Long read nanopore sequencing for detection of HLA and CYP2D6 variants and haplotypes. *F1000Research* **2015**, *4*, 17. [CrossRef]
76. Gulilat, M.; Lamb, T.; Teft, W.A.; Wang, J.; Dron, J.S.; Robinson, J.F.; Tirona, R.G.; Hegele, R.A.; Kim, R.B.; Schwarz, U.I. Targeted next generation sequencing as a tool for precision medicine. *BMC Med. Genome* **2019**, *12*, 81. [CrossRef] [PubMed]
77. Price, M.J.; Carson, A.R.; Murray, S.S.; Phillips, T.; Janel, L.; Tisch, R.; Topol, E.; Levy, S. First pharmacogenomic analysis using whole exome sequencing to identify novel genetic determinants of clopidogrel response variability: Results of the genotype information and functional testing (gift) exome study. *J. Am. Coll. Cardiol.* **2012**, *59*, E9. [CrossRef]
78. Hoehe, M.R.; Timmermann, B.; Lehrach, H. Human Inter-Individual DNA Sequence Variation in Candidate Genes, Drug Targets, the Importance of Haplotypes and Pharmacogenomics. *Curr. Pharm. Biotechnol.* **2003**, *4*, 351–378. [CrossRef] [PubMed]
79. Strachan, T.; Goodship, J.; Chinnery, P. *Genetics and Genomics in Medicine*; Taylor & Francis: Abingdon, UK, 2014.
80. Gex-Fabry, M.; Eap, C.B.; Oneda, B.; Gervasoni, N.; Aubry, J.-M.; Bondolfi, G.; Bertschy, G. CYP2D6 and ABCB1 Genetic Variability: Influence on Paroxetine Plasma Level and Therapeutic Response. *Ther. Drug Monit.* **2008**, *30*, 474–482. [CrossRef] [PubMed]
81. Kaiser, R.; Sezer, O.; Papies, A.; Bauer, S.; Schelenz, C.; Tremblay, P.-B.; Possinger, K.; Roots, I.; Brockmöller, J. Patient-Tailored Antiemetic Treatment With 5-Hydroxytryptamine Type 3 Receptor Antagonists According to Cytochrome P-450 2D6 Genotypes. *J. Clin. Oncol.* **2002**, *20*, 2805–2811. [CrossRef] [PubMed]
82. Kawanishi, C.; Lundgren, S.; Ågren, H.; Bertilsson, L. Increased incidence of CYP2D6 gene duplication in patients with persistent mood disorders: Ultrarapid metabolism of antidepressants as a cause of nonresponse. A pilot study. *Eur. J. Clin. Pharmacol.* **2004**, *59*, 803–807. [CrossRef] [PubMed]
83. Noetzli, M.; Guidi, M.; Ebbing, K.; Eyer, S.; Wilhelm, L.; Michon, A.; Thomazic, V.; Stancu, I.; Alnawaqil, A.M.; Bula, C. Population pharmacokinetic approach to evaluate the effect of CYP2D6, CYP3A, ABCB1, POR and NR1I2 genotypes on donepezil clearance. *Br. J. Clin. Pharmacol.* **2014**, *78*, 135–144. [CrossRef]
84. Ciszkowski, C.; Madadi, P.; Phillips, M.S.; Lauwers, A.E.; Koren, G. Codeine, Ultrarapid-Metabolism Genotype, and Postoperative Death. *N. Engl. J. Med.* **2009**, *361*, 827–828. [CrossRef]
85. Gasche, Y.; Daali, Y.; Fathi, M.; Chiappe, A.; Cottini, S.; Dayer, P.; Desmeules, J. Codeine Intoxication Associated with Ultrarapid CYP2D6 Metabolism. *N. Engl. J. Med.* **2004**, *351*, 2827–2831. [CrossRef]

MDPI
St. Alban-Anlage 66
4052 Basel
Switzerland
Tel. +41 61 683 77 34
Fax +41 61 302 89 18
www.mdpi.com

Journal of Clinical Medicine Editorial Office
E-mail: jcm@mdpi.com
www.mdpi.com/journal/jcm

MDPI
St. Alban-Anlage 66
4052 Basel
Switzerland

Tel: +41 61 683 77 34
Fax: +41 61 302 89 18

www.mdpi.com

ISBN 978-3-0365-3745-0