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Challenges and Opportunities in Systemic Biomarkers for Age-related Macular Degeneration

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ABSTRACT

Blood biomarkers have been widely used in medicine for the prevention, diagnosis, and treatment follow-up of many diseases. Age-related macular degeneration, the main cause of irreversible blindness in old age, does not benefit from these important indicators. In recent decades, technological advances have given us optical coherence tomography. Until a few years ago, this exam was used for the diagnosis, and treatment evaluation of age-related macular degeneration. Currently, as part of multimodal fundus imaging, it has also been used in the prognosis of age-related macular degeneration. However, these exams are still not capable of predicting when individuals may trigger the degenerative macular disease, and consequently adopt preventive measures, such as changes in lifestyle and consumption of antioxidants. In this regard, this article aims to address the various blood biomarkers that may be useful in the early investigation of age-related macular degeneration, even before the appearance of drusen and retinal pigment epithelium changes in the macular region, the first ophthalmoscopic manifestations of age-related macular degeneration. Among these biomarkers analyzed, the blood count, lipid profile, some enzymatic and non-enzymatic antioxidants, as well as the main inflammatory biomarkers, stand out.

Introduction

Studies have correlated age-related macular degeneration (AMD), the main cause of irreversible blindness in old age[1], with an increase in the mortality rate in patients with Covid-19 and acquired immunodeficiency syndrome[2-5]. Diseases such as stroke and cardiovascular diseases (CVDs) also have a worse prognosis in patients with AMD[6-7]. The poor evolution of these systemic diseases may be associated to the increase in systemic inflammatory markers detected in patients with AMD[2-5]. It is known that systemic inflammation is associated with aging and an increased risk of many chronic diseases[8], contributing to the onset and exacerbation of diseases such as obesity, type 2 diabetes, and atherosclerosis[9-11]. As well as systemic diseases, the high systemic levels of cytokines and chemokines may also be involved in the triggering and progression of AMD[12-24]. Consequently, the detection of an increase in these inflammatory markers may allow more effective preventive measures, nullifying or attenuating the progression not only of systemic diseases, but also of AMD[25].

In 2014, Klein and colleagues published the relationships among serum markers of inflammation, oxidative stress and endothelial dysfunction, with the cumulative incidence of early AMD over 20 years. Twenty-three percent of the 975 people who participated in the initial examination in 1988-1990 developed early AMD. This fact was verified in the 1993-1995, 1998-2000, 2003-2005 and 2008-2010 follow-ups. The study revealed modest evidence of relationships between incidence of early AMD and high-sensitivity serum C-reactive protein, tumor necrosis factor-α receptor 2, interleukin-6, and soluble vascular cell adhesion molecule-1[26]. However, recent studies have been suggested that systemic inflammation may contribute to an increased risk of AMD, even knowing that AMD is a local disease with links to local inflammatory events[24,27].

Genetic, nutritional, environmental and cardiovascular factors, among others, are involved in the genesis of AMD[28-29], frequently making it difficult to control. Neovascular age-related macular degeneration (nAMD), one of late forms of AMD, is currently treated with intraocular antiangiogenic injections, which are uncomfortable, periodic, continuous and expensive[30-33]. Furthermore, it does not prevent, in most cases, the progression of the disease and consequent loss of central vision[34]. Additionally, about a third of patients do not obtain the expected effects from anti-vascular endothelial growth factor (VEGF) therapy due to fibrosis or macular atrophy, which makes this disease a poor prognosis[35]. For geographic atrophy (GA), another late form of AMD, Food and Drug Administration (FDA) approved Pegcetacoplan intravitreal injection (complement C3-cleavage inhibitor). It was observed that monthly intravitreal injections of 15 mg Pegcetacoplan for 12 months significantly reduced the growth of lesions in GA[36-37]. In addition to not promoting improved vision, the treatment is also periodic, expensive and not completely free from complications[36]. In order to mitigate this bad evolution, a preventive approach must be initiated as early as possible.

Currently, an evaluation with retinography, fluorescein angiography, indocyanine green and optical coherence tomography (OCT), alone or combined as in multimodal ocular evaluation (multimodal fundus imaging), are capable to identifying the onset and progression of AMD, providing a more appropriate classification, detecting its activity and serving as a guide to evaluate the treatment effectiveness. Additionally, important AMD biomarkers such as macular pigment optical density (MPOD), drusen volume and pigmentary abnormalities can be measured by those exams[38-39]. Other risk factors for disease progression to advanced stages, such as reticular pseudodrusen, hyperreflective foci and drusen subphenotypes, can be identified by combining those tests[40-41]. Other structural biomarkers such as the reflectivity of the ellipsoid zone and the characteristics of the choriocapillaris flow, analyzed by multimodal fundus imaging, can also contribute to a better understanding of AMD pathogenesis and prognosis[42].

However, as it is a chronic disease, often with decades of evolution, the success of preventive measures in AMD, with the use of antioxidants and lifestyle changes, are not very significant, as they are introduced after the appearance of the initial changes of the disease, such as drusen or pigmentary changes of the retinal pigment epithelium (RPE) in the foveolar region[43-44]. It is worth noting that these initial changes occur in elderly people, often presenting systemic inflammatory components (inflammaging)[45], changes in serum cholesterol levels[46], smokers[47], unhealthy diets[48], obesity[49], sedentary life[50], cardiovascular diseases[51], among other comorbidities[29,52]. As it is a tissue with a very high metabolism, the retina is drastically impacted by such chronic changes, frustrating, in most cases, any preventive and/or treatment measures[53-54]. Therefore, the objective of this review is to define the main blood biomarkers that have the potential to suggest the adoption of preventive measures, even before the appearance of initial changes in AMD.

Blood Biomarkers

BLOOD COUNT

Considering that inflammatory and immunological factors participate in AMD pathogenesis[25,55-59], changes in the blood count may indicate the beginning of systemic imbalance that may induce degenerative macular disease. Even though some studies showed conflicting results regarding white blood cells (monocytes and neutrophils), platelets and mean platelet volume (MPV)[60-62], the increase in the neutrophil-lymphocyte ratio (NLR) associated with neovascular AMD has been demonstrated[63]. Corroborating this finding, another study demonstrated that in addition to the increase in NLR, there was also a significant increase in the platelet/lymphocyte ratio (PLR) in patients with neovascular AMD. This study also demonstrated that NLR and PLR levels were inversely proportional to best corrected visual acuity and directly proportional to central macular thickness[64]. Finally, a cross-sectional study with 7,719 participants detected the association of a higher peripheral monocyte count with a higher prevalence of AMD. Individuals with a monocyte count ≥0.5 × 109/L compared to participants with monocytes of 0.1-0.4 × 109/L had a 1.45-fold increased risk of

any AMD (early, intermediate, and late) and 1.58-fold increase in the risk of intermediate/late AMD[65].

LIPID PROFILE

The lipids are organic compounds with essential functions for the human body which include energy storage, membrane integration and structure (phospholipid bilayer), in the biosynthesis process of substances such as prostaglandins, and as an enzyme cofactor[66,67]. The lipids represent the main target for the reactive oxygen species (ROS), especially the glycolipids, phospholipids, and cholesterol[68]. Cross-sectional and cohort studies have not reported significant associations between serum lipoprotein profiles and AMD[69,70]. Although one study associated higher serum levels of total cholesterol (TC), low-density lipoprotein (LDL), and triglycerides (TG) with a decreased risk of AMD[71], another study demonstrated that male participants who had levels higher high-density lipoprotein (HDL) and lower TG levels were associated with greater odds of having early AMD, whereas female participants who had higher TC and LDL levels were associated with an increased risk of developing early AMD[72]. Another study demonstrated that elevated TC in early middle age may play a role in the early development of AMD[73]. In addition, it was found that alterations in serum lipid profiles, as a reflection of systemic dyslipidemia, have been associated with AMD[74]. Corroborating the association between serum lipoprotein levels and AMD, a systematic review and meta-analysis as well as other population studies identified a significantly increased risk of AMD associated with higher serum HDL levels[46,71,75-80].

ANTIOXIDANT ENZYMATIC AND NON-ENZYMATIC SYSTEM

The variation in the activity of antioxidant enzymatic system suggests a response to oxidative stress which is involved in the pathogenesis of AMD[81-82]. The enzymatic system have the function of counterbalancing the damage caused by ROS and reactive nitrogen species (RNS) in the biomolecules of lipids, proteins, and DNA contained in the sensory retina and in the RPE, maintaining the retinal homeostasis and attenuating the installation and AMD progression[83-85].

Glutathione Redox cycle and enzymes

Glutathione, or L-y-glutamyl-L-cysteinyl- glycine, is ubiquitous tripeptide that participates in biological processes such as protein and DNA synthesis, transport, enzymatic activity, metabolism and cellular protection. Glutathione is synthesized intra- cellularly and can be found in the body in its reduced (GSH) and oxidized (GSSG) forms[86]. Free GSH is present mainly in its reduced form, being considered a potent ROS scavenger. Glutathione may be converted into the GSSG by the glutathione peroxidase (GPx) during oxidative stress. The oxidized form (GSSG), in turn, may be reverted into its reduced form by glutathione reductase (GR)[87]. While the concentration of GSH in cells varies from 1 to 10 mM[88], the level of GSH within extracellular fluids and blood plasma reaches only several µM[89]. It is important to point out that a high level of GSH can be explained by an enhanced GSH biosynthesis and a higher conversion of GSSG into GSH by GR. On the other hand, under conditions of marked toxicity or oxidative stress, intracellular GSSG increases substantially[87].

Glutathione

Glutathione, the major water-soluble antioxidant, acts primarily in the cytoplasm and mitochondria and is considered the most important antioxidant in the eye[86,90]. With age, the efficiency of the GSH redox system decreases, predisposing the RPE to increased damage mediated by oxidative stress[90-93]. Some studies have not demonstrated changes in GSH concentrations in the plasma of patients with AMD[81,94]. On the other hand, a study demonstrated higher serum GSH concentrations in AMD patients in relation to control group[95], while most studies showed decreased GSH levels in AMD patients as normal control[96-99]. Consequently, GSH has been considered a non-specific parameter when analyzing the development of AMD[90].

Glutathione peroxidase

Glutathione peroxidase controls hydrogen peroxide and lipid hydroperoxide levels, resulting from an attack of ROS[86]. This enzyme is present in a number of tissues, including the inner layers of the retina and RPE[100 -102]. Its concentration is greatest in the posterior pole, which is constantly exposed to light[101]. Selenium (Se), an essential nutrient, is part of the composition of GPx[100], and Se-deficient animals have markedly decreased GPx activity[103]. In addition to GPx protecting RPE cells in models of oxidative damage-induced retinal degeneration, it plays an important role in the maturation of photoreceptor cells[104]. A population-based, cross-sectional study on cataracts and AMD and their risk factors (2584 participants) showed that the high plasma level of GPx plasma was associated with a nine-fold increase in the prevalence of late AMD[105]. An increase in GPx activity in AMD patients has been associated with the activity of RPE cells, which attempt to eliminate a huge amount of H2O2 produced during the course of the disease[83]. On the other hand, many studies have reported a significant reduction in GPx activity in patients with AMD[106-110].

Oxidized glutathione

Oxidative stress promotes the conversion of GSH to GSSH by GPx enzyme[87]. A report showed that GSSG level is elevated in patients with early AMD as compared to those of healthy control[111].

Glutathione reductase

Although glutathione reductase is not directly an antioxidant, its function is essential for the maintenance of available reduced GSH[87]. This enzyme is highly expressed in the retina and RPE[112-114]. Several studies have demonstrated significantly lower GR activity values in the group of AMD patients as compared those of control group; being associated with a decreasing in GSH levels[85,107,115]. In fact, lower GR activity is associated with weaker antioxidant abilities[85].

Superoxide dismutase (SOD)

Superoxide dismutase plays a key defense role against ROS. By removing superoxide (O2-) and forming O2 and H2O[116], it plays an important role in diseases related to oxidative stress, including aging[117]. In eukaryotic systems, one can find SOD linked to copper and zinc (CuZn)-SOD, present mainly in the cytosol, as well as SOD-2, dependent on manganese (Mn-SOD), and found mainly in mitochondria[118]. Mn-SOD is present in RPE cells and the

inner rod segment[119], and reduced levels are associated with AMD progression[120]. Some studies demonstrated that high erythrocyte SOD activity was not associated with late AMD and early signs of AMD[82,105,108,121]. This finding has been attributed to compensatory mechanisms against oxidative stress[122]. However, other studies report lower values of SOD antioxidant parameters were significantly associated with AMD[85,109,110,115]. Although in vitro studies consistently indicate the role of SOD in responses to oxidative stress, they do not clearly show its association with AMD[83].

Catalase

Catalase, a homotetrameric protein, is present in many types of cells, with the highest concentration in erythrocytes and liver[123], and is found mainly in peroxisomes, mitochondria and the nucleus. This enzyme decomposes H_2O_2 into water and molecular oxygen, an extremely important process to prevent the formation of the hydroxyl radical (•OH)[124]. In the retina, catalase is located within RPE peroxisomes, playing its important role in preventing lipid peroxidation and inhibiting lysosomal enzymes through the removal of H2O2 from the phagosome[125,126]. Decreased catalase activity in RPE cells has been reported from the sixth to ninth decade of human life[92]. Corroborating this study, a decrease in catalase immunoreactivity with age was observed in the cytoplasm and lysosomes of macular RPE cells from normal eyes and eyes affected by AMD[127]. Although a study (32 early-AMD patients, 25 late-AMD patients, 50 healthy controls) did not establish a correlation between serum catalase activity and AMD[108], another study (39 early-AMD patients, 100 intermediate-AMD patients, 191 late-AMD patients, and 121 controls) revealed that AMD is associated with lower erythrocyte catalase activity[82]. Other study (240 AMD patients and 270 controls) also reported a reduction in erythrocyte catalase activity in patients with AMD[110].

Total Oxidant Status (TOS) and Total Antioxidant Status (TAS)

Total oxidant status and total antioxidant status are parameters used to evaluate the overall oxidative stress status in the body[128]. An imbalance between TOS and TAS has been proposed to be responsible for the increased lipid, protein and DNA damage observed in AMD patients[129]. Serum (or plasma) concentrations of different oxidant species can be measured in laboratories separately, but the measurements are time-consuming, labor-intensive and costly and require complicated techniques[130].

Total oxidant status may also be named total peroxide (TP), serum oxidation activity, reactive oxygen metabolites or some other synonyms[131]. A study (156 early-AMD patients, 80 wet-late AMD, 72 dry-late AMD and 207 healthy controls) reported that a significantly increased oxidative damage was associated with AMD patients. Both early- and late-AMD patients presented higher TOS levels than healthy controls[109]. Corroborating these findings, other studies also observed a significant increase in TOS levels in the sera of AMD patients when compared to controls[109,129,132-133].

Total antioxidant status, in turn, expresses the free radical scavenging capacity and reflects the residual antioxidant capacity after ROS neutralization[134-135]. Total antioxidant status was shown to be reduced in patients with AMD compared to control[85,115,129]. Another study (32 early-AMD patients, 25 late-AMD patients and 50 healthy subjects) demonstrated that low TAS is associated with AMD and that the combined values of GPx activity and TAS are significant determinants of AMD status[108].

Protein carbonyl (PC)

Protein carbonyls are indicators of the amount of protein that has been oxidized by highly reactive free radicals and are the most studied protein oxidation markers[135,136]. Reactive carbonyl species are important cytotoxic mediators produced from oxidative damage of biomolecules (lipids and sugars), leading to alterations in the cell signaling mechanisms to the nucleus, positively regulating redox-sensitive transcription factors, and inducing irreversible structural modification in important molecules [proteins, peptides (cysteine, lysine, histidine), lipids, DNA][137,138]. Studies have demonstrated that the values of protein carbonyl groups were higher in patients with exudative AMD than in the control group[85,129]. In another study, both patients with late AMD and those with early AMD had higher levels of PC when compared to healthy controls[109].

INFLAMMATORY BIOMARKERS

Pro-inflammatory cytokines and chemokines

Several studies have demonstrated inflammation and dysregulation of inflammatory responses play an important role in the development and progression of AMD to the final stages, which include choroidal neovascularization (CNV) and geographic atrophy (GA)[27,139, 140]. Systemically, studies have demonstrated elevated plasma levels of these inflammatory markers in patients with AMD as compared to those without AMD[13-14, 16-22,141-142]. Considering these facts, inflammatory cytokines and chemokines gain relevance from a physio pathogenesis and therapeutic perspective in AMD.

Cytokines are water-soluble, extracellular polypeptides or glycoproteins, ranging from 8 to 30 kDa, generally produced in response to antigenic stimulation, functioning as a chemical messenger to regulate the adaptive and innate immune system. These proteins are produced by all cells involved in antigen response and presentation. They are synthesized when needed or when a cell in the immune system is "activated"[143-144]. Chemokines, in turn, are a large family of small cytokines and their molecular weight varies from 7 to 15kDa. Chemokines play a central role in the physiology of leukocytes and other inflammatory cells, by controlling basal and inflammatory trafficking. There are two major subfamilies of chemokines based on the position of cysteine residues: CXC and CC. As a general rule, members of the CXC chemokine family are chemotactic of neutrophils, and CC chemokines are chemotactic of monocytes and lymphocyte subtypes[144].

Interleukin-6 (IL-6)

slnterleukin-6 is a cytokine that, among its multiple functions, mediates inflammation and the immune response, acting on several cells, including RPE cells[145-147]. IL-6 levels in adults are not expected to exceed 20 pg/mL[148]. Several studies have reported that IL-6 is an

important regulator of CNV and has correlated this cytokine with VEGF expression[149-152]. While one study found no significant association between plasma IL-6 levels and AMD[26], another study considered this interleukin an important marker for the progression of AMD[16]. Recently, a prospective study (42 GA patients, 41 nAMD patients, and 27 healthy controls) demonstrated that plasma IL-6 has predictive capacity for progression and constitutes the first known plasma biomarker of disease activity in GA. These findings highlight its important role of chronic inflammation in the pathogenesis of this disease[17]. Finally, a meta-analysis involving 3,586 individuals (1,865 controls and 1,721 with AMD) suggests that an increase in systemic IL-6 in patients with AMD may be a phenomenon more closely related to late AMD subtypes[153].

Interleukin-1β (IL-1β)

Interleukin-1β, produced primarily by monocytes and macrophages, has been associated with mediating acute and chronic inflammation[154-155]. Interleukin-1β is secreted as an inactive form and requires proteolytic cleavage by the enzyme caspase-1 to be released into an active form[156]. The caspase-1 activation platform, known as the inflammasome, has been associated with the pathophysiology of AMD[157-158]. Addictionally, IL-1β is capable of inducing reactive oxygen species (ROS) in RPE cells[159]. Analysis of plasma inflammation markers in patients with polypoidal choroidal vasculopathy (PCV), patients with neovascular AMD and a healthy control group showed a significant increase in plasma IL-1β in patients with neovascular AMD as compared to those of healthy controls[17]. Furthermore, this chemokine was found to be elevated in patients who progressed from the intermediate to the advanced stage of AMD[24].

C-reactive protein (CRP)

C-reactive protein is predominantly produced in the liver, although, under certain conditions, it can also be secreted by smooth muscle cells and endothelial cells[160,161]. The Centers for Disease Control and Prevention and the American Heart Association have estimated cardiovascular risk in healthy individuals as follows: low-, medium-, and high-risk values defined as < 10, 10 – 30, and > 30 mg/L[162].

CRP is released into circulation upon stimulation by IL-6 and other cytokines[163], and a link with AMD has been suggested[16,115, 164-167]. However, a meta-analysis involving 53 studies with 60,598 participants (10,392 patients and 38,901 controls) revealed that early age-related macular degeneration was not associated with systemic C-reactive protein, whereas late AMD was associated with a small-to-moderate increase in systemic C-reactive protein[168]. On the other hand, another meta-analysis (41,690 participants) showed that high serum CRP levels (> 3 mg/L) were associated with a twofold greater likelihood of late AMD, compared with low levels (< 1 mg/L)[169]. Furthermore, evidence that elevated high-sensitivity C-reactive protein (hsCRP) levels predict future AMD risk was demonstrated in pooled analysis of prospective case-control data[20]. The association of CRP with AMD was also found in a study that analyzed inflammation plasma markers from patients with PCV and neovascular AMD[19], as well as in GA[17].

Tumor Necrosis Factor Alpha (TNF-α)

Tumor necrosis factor alpha is a low molecular weight protein, produced predominantly by activated macrophages, potentially involved in the production and expression of VEGF[170-172]. Interestingly this cytokine also has the potential to inhibit the formation of neovessels[173-174]. This fact may be linked to its receptors, a member of the tumor necrosis factor receptor 1A (Tnfrsf1a) superfamily and a member of the tumor necrosis factor receptor 1B (Tnfrsf1b) superfamily, which act antagonistically by inhibiting endothelial migration or promoting its activation[173-175]. Additionally, it was observed that pre-exposure of TNFα in the primary RPE and ARPE19 induces to increased complement activation and membrane attack complex (MAC) deposition, which may represent an early event in the pathogenesis that leads to the development of AMD[176]. A case-control study showed significantly increased plama levels of soluble receptor for tumor necrosis factor type II (sTNFRII) in patients with early or nAMD and was considered a significant predictor for the prevalence of AMD[177]. Another study that analyzed serum levels of CRP, pro-inflammatory cytokines (TNF-α, IL-1β, IL-2, IL-6 and IL-8) and complement pathway activity in the clinical response to growth factor inhibition vascular endothelial cell in nAMD demonstrated that only lower serum levels of TNF-α were associated with an increase in visual acuity after anti-VEGF therapy[178]. In a prospectively study (42 GA patients, 41 nAMD patients and 27 healthy controls) was observed that patients with GA showed an increase in the pro-inflammatory plasma marker (TNF receptor 2) as compared to those of healthy controls[17]. Investiganting the role of TNF-α on AMD, a recent systematic review (24 studies) showed measurement of systemic and local levels of TNF-α has not produce consistent results on the role of anti-TNF-α agents in the remission of symptoms caused by the disease. That review suggested the role of TNF-α in nAMD is not clear and not all anti-TNF-α agents were considered safe[179]. Corroborating this study, no association was demonstrated between plasma levels of TNF-R2 among patients with PCV, patients with nAMD and a healthy control group[19].

Interleukin-8 (IL-8)

Interleukin-8 or *CXCL8* is a member of the CXC chemokine family originally identified as a chemotactic factor for neutrophils, being released by phagocytes and a wide variety of tissue cells after exposure to inflammatory stimuli[180-181]. In addition to activating neutrophils, IL-8 also increases the expression of adhesion molecules by endothelial cells[182]. Some studies have not demonstrated a correlation between plasma IL-8 levels and nAMD and/or PVC[19,183]. On the other hand, a significant increase in the IL-8 secretion profile of peripheral blood mononuclear cells and serum from patients with nAMD was demonstrated[181,184]. Additionally, this chemokine was found to be elevated in patients who progressed from the intermediate to the advanced stage of AMD[24]. Finally, a recent meta-analysis suggested that the IL-8 +781 C/T polymorphism affects the predisposition to dry AMD and wet AMD. Furthermore, patients with dryAMD and nAMD also have elevated levels of IL-8[185], which could be considered a new genomic biomarker of AMD susceptibility[186].

Monocytic chemotactic protein 1(MCP-1) or Chemokine C-C motif ligand 2 and its receptor CCR2

Monocytic chemotactic protein 1, also referred to as chemokine C-C motif ligand 2 (CCL2), is encoded by the CCL2 gene which is located on chromosome 17q11.2[187]. CCL2 is a low molecular weight (810 kDa) chemotactic cytokine that is involved in the recruitment of monocytes to the inflammation site[188- 190]. Chemokine C-C motif ligand 2 can be secreted by numerous cell types, including endothelial cells, activated monocytes, and EPR cells[191-193]. Several studies have correlated CCL2 with AMD. Firstly, oxidized lipids in the retina, arising from phagocytosis of the outer segments of photoreceptor cells, stimulate the expression of chemotactic factors, such as IL-8 and CCL2 by RPE cells, justifying the increase in intraocular CCL2 concentrations in nAMD eyes[194]. Consequently, the recruitment of macrophages promoted by CCL2 would help explain the presence of these cells at sites of CNV[195-198]. Additionaly, macrophages express proangiogenic VEGF, which contributes to CNV formation[199-200]. It was demonstrated that intravitreal injections with a CCR2 antagonist reduce the size of laser-induced CNV in mice, as well as in the number of macrophages infiltrating the choroid and a decrease in VEGF expression[201]. However, CCL2 is not only involved in the pathogenesis of nAMD. Experimental models have shown that CCR2 and its ligand CCL2 are involved in drusen formation and in RPE changes seen in the early stages of AMD[202-203]. Chemokine C-C motif ligand 2 activated monocytes are also involved with apoptosis in the RPE, contributing to the progression of the disease[204]. Furthermore, the level of CCL2 and CCR2 + inflammatory infiltrating monocytes are increased in patients with GA[205]. However, controverse results have been found regarding serum CCL2 levels in patients with AMD. A study involving 150 participants found no association between CCL2 plasma levels and AMD[206]. A cross-sectional study (16 nAMD patients, 18 PCV patients, and 50 controls) did not observe significant differences in CCl-2 in plasma samples between cases and controls, corroborating the previous study[183]. On the other hand, analysis of peripheral blood mononuclear cells, in particular monocytes from patients with nAMD, revealed that higher levels of CCL2 may be associated with the development of CNV[181]. Corroborating with these findings, an increase in serum CCL2 levels in patients with nAMD as compared to those of healthy participants has been observed[207]. Another study demonstrated that plasma CCL2 concentrations are not only elevated in nAMD, but also in PCV[208]. An increase in CCL2 plasma concentrations was also observed in patients with intermediate age-related macular degeneration (iAMD)[209], and early AMD[210]. Similarly, the expression of CCR-2 levels was increased on a sub-type of monocytes in peripheral blood in patients with neovascular AMD[211]. Additionally, it was observed that patients with nAMD had a significantly increased proportion of non-classical CCR2+ monocytes, whereas PCV type 1 was associated with a significant increase in CCR2+ in all monocyte subsets when compared to PCV type 2[212]. A prospective observational study (41 GA patients, 51 nAMD patients, and 30 healthy control) demonstrated that GA was associated with greater monocytic CCR2 expression compared to nAMD[21]. Finally, a significant increase in urinary CCL2 levels was observed in patients with early AMD and GA as compared of those controls. This study concluded that analysis of this urinary biomarker could provide a practical tool for detecting early AMD, monitoring progression and evaluating treatment efficacy[22].

Interferon (IFN)

Interferons are a group of glycoproteins synthesized and secreted by almost all mammalian cells upon stimulation by specific antigens[213-214]. They have antiviral, antiproliferative and immunomodulatory properties that play important roles in host defense mechanisms and in the maintenance of homeostasis[213-214]. The three types of IFN (I, II and III) are classified by their receptor specificity and sequence homology. Type I IFNs include IFN-α, β, ε, κ, and ω. Type II IFNs refer to IFN-γ, and Type III IFNs include IFNλ1, IFNλ2, IFNλ3[215]. The type 1 Interferon exerts antiproliferative and antiangiogenic effects, modulating the activity of various immune cells[216- 219]. On the other hand, type 2 interferon (IFN-γ) is classically considered a pro-inflammatory factor, but in recent years, several studies have found that IFN-γ mediates an immunomodulatory and protective function as well[220-221]. In relation to AMD, IFN-γ can exert important effects on RPE cells, positively regulating the expression of complement factor H (CFH), mediating the positive regulation of VEGF, promoting the activation of the RPE, significantly increasing the expression and secretion of IL-6[147, 222 -223]. In the primary RPE and ARPE19, in addition to promoting increased complement activation, IFN-γ promotes the deposition of the membrane attack complex, which may represent an early event in the development of AMD[176]. Hence, IFN-γ induces pro-inflammatory responses by activating pro-inflammatory cytokines and chemokines, thus recruiting immune cells such as macrophages and T cells[224-226]. Despite the important role of IFN-γ in the pathogenesis of AMD, several studies do not associate the increase in plasma levels of this cytokine with degenerative macular disease[18,177,183,227]. On the other hand, analysis of whole-blood samples collected from humans (27 nAMD patients, 33 PCV patients and 18 healthy individuals) demonstrated that the levels of IFN-γ in the supernatants of cultured peripheral blood mononuclear cells in patients with PCV and nAMD were markedly elevated compared to those of controls. These results suggest that the IFN-γ-related inflammatory pathway may be involved in the pathogenesis of PCV and nAMD[228]. Another study corroborated these results, demonstrating significantly high levels of IFN-γ expression by CD4+ T cells in patients with AMD[13].

Homocysteine (Hcy)

Homocysteine is an amino acid produced from the demethylation of methionine[229]. Mutations in methylene-tetrahydrofolatereductase or cysteine by cystathionine-beta-synthase are related to increased plasma homocysteine (HHcy)[230]. Additionally, deficiency of B complex vitamins, such as folic acid, riboflavin (B2), pyridoxine hydrochloride (B6) and cyanocobalamin (B12) is related to an increase in total Hcy concentration[229, 231-233]. Adult total homocysteine values of 10 µmol/L or less are probably safe, whereas values of 11 µmol/L or above may warrant intervention[234]. Hyperhomocysteinemia has been linked to the

development of Alzheimer's disease[235], diabetic retinopathy[236], as well as an increase in the cardiovascular mortality rate[237-238]. Many experimental studies have suggested an association between HHcy and AMD. Hyperhomocysteinemia caused activation of microglial cells, increased the expression of pro-inflammatory cytokines such as IL-1B and TNFα, as well as downregulated anti-inflammatory cytokines in key cells that constitute the inner outer blood–retinal barriers, human retinal endothelial cells and RPE[239-241]. Blood-retinal barrier dysfunction caused by increased Hcy leads to RPE changes like AMD, including inducing CNV[242-244]. Furthermore, HHcy has been shown to promote the activation of hypoxia-inducible factor (HIF)-1α[244], retinal hypoxia[245], and increase in the expression of VEGF in RPE cells[244]. A meta-analysis indicated weak evidence that increased tHcy may be associated with nAMD; however, this result must be interpreted with caution due to a marked heterogeneity among studies[246]. Another meta-analysis, which evaluated eleven studies (including 1072 cases and 1202 controls), demonstrated that the plasma tHcy level among AMD cases was 2.67 μmol/L higher than controls. Studies involving vitamin B12 and folic acid were also analyzed (including 152 cases and 98 controls) and it was found that the level of vitamin B12 among AMD cases was 64.16 pg/mL lower than controls. Subgroup analyzes showed that the folic acid level was 1.66 ng/mL lower in the wet type. Together, the results demonstrated that AMD is associated with elevated tHcy levels and decreased vitamin B12 levels[247]. In this regard, a prospective study demonstrated that an increase in total vitamin B-12 intake was associated with a 24% reduction in the risk of incidence of any AMD in 10 years[248], just as well as high folate intake is associated with a reduced risk of progression to GA[249]. A randomized, double-blind, placebo-controlled trial including 5442 female health care professionals 40 years or older with CVDs or CVDs risk factors showed that supplementation with folic acid (2.5mg), B12 (1 mg) and B6 (50 mg) led to a 34% reduced risk of any AMD and a 41% reduced risk of visually significant AMD over a 7.3-year period[250].

Conclusion

Several studies point to changes in blood biomarkers in AMD, especially in the late stages of the disease. The increase in the neutrophil-lymphocyte ratio (NLR) and the platelet/lymphocyte ratio (PLR), as well as the higher peripheral monocyte count, suggest the onset of systemic changes that can potentially interfere with the course of AMD. The correlation between AMD and increased HDL, TOS, IL-6, CRP, IL-8, MCP-1 and homocysteine has been observed in many studies, as has a reduction in TAS. Most of the biomarkers analyzed were compared between normal elderly people and elderly people with AMD. There are few long-term studies in the literature analyzing the correlation of these markers with the onset of AMD. Therefore, long-term studies should be encouraged in the search for answers regarding the usefulness of such markers in identifying those people who tend to develop AMD, even before the appearance of any phenotypic changes in the retina, with the aim of predicting the risk, preventing it and monitor progression.

Conflicts of Interest Statement:

The authors have no conflicts of interest to declare.

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