Review

Recent Advances in Proteomic-based Approaches to Study Age-related Macular Degeneration: A Systematic Review

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Abstract: Age-related macular degeneration (AMD) is a common ocular disease characterized by the degeneration of the central area of the retina in elderly population. Progression and response to treatment is influenced by genetic and non-genetic factors. Proteomics is a powerful tool to study, at the molecular level, the mechanisms underlaying the progression of the diseases, to identify new therapeutical targets and to establish biomarkers to monitor progression and treatment effectiveness. In this work we pursue to systematically review the use of proteomic-based approaches for the study of the molecular mechanisms underlying the development of AMD, as well as the progression of the disease and the on-treatment patient monitoring. The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) reporting guidelines were followed. Proteomic approaches have identified key players on the onset of the disease, such as complement components and proteins involved in lipid metabolism and oxidative stress, but also in the progression to advanced stages, including factors related to extracellular matrix integrity and angiogenesis. Although anti-vascular endothelial growth factor (anti-VEGF)-based therapy has been crucial in the treatment of neovascular AMD it is necessary to get deeper into the underlying disease mechanisms to move forward to next-generation therapies of the later-stage forms of this multifactorial disease.

Keywords: Proteomics; Age-related macular degeneration; inflammation; biomarker; oxidative stress

1. Introduction

AMD is the leading cause of blindness in the elderly population in the Western countries [1], affecting nearly 300 million people worldwide who are visually impaired, either partially or totally. AMD is characterized by progressive degenerative and/or neovascular changes affecting the macula, the highly specialized region of the central retina responsible for fine vision [2]. AMD can be divided into three different stages: early, intermediate and advanced AMD. Regarding advanced AMD, it can be subdivided into the geographic atrophic (GA) or "dry" AMD, which represent around 80% of the cases [3], and the rapidly blinding neovascular form (nAMD), also called "wet" or "exudative" [4]. Whereas GA is characterized by an initial RPE degeneration, in nAMD loss or dysfunction of the choroidal vasculature is the first pathological event [5].

The best available treatment, based on anti-vascular endothelial growth factor (anti-VEGF) intra-vitreal injections, is useful exclusively in patients suffering from nAMD [6, 7]. However, this treatment, in some cases, only delays the progression of the disease [8,

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9]. Furthermore, continuous anti-VEGF treatment has been linked to drug intolerance and occasional development of GA, thus compromising the long-term benefits of the patients [10].

From the etiologic point of view, AMD is a multifactorial disease, determined by genetic and non-genetic factors [11]. Aging appears to be the most critical factor since the prevalence of the disease progressively increases with the older age [1]. A strong association has been described with mutations in genes such as the HTRA1/ARMS2 locus [12] and complement components like complement factor H (CFH) and complement C3 (C3) [13-15]. Finally, several environmental and systemic risk factors such as obesity [16], hypertension [17] and hypercholesterolemia [18, 19] predispose to the development of AMD.

At the molecular level the pathogenesis of the AMD is influenced by the generation of highly reactive free radicals in the macula area of the retina, a zone characterized by a high metabolic rate due in part to high oxygen pressure and redox reactions continuously generated [20]. It is widely believed that the presence of reactive oxygen species (ROS) is strongly linked to the pathogenesis of AMD [21, 22]. The combination of chronic oxidative stress, subsequent impaired autophagy, and inflammation leads to the aging of the Retinal Pigment Epithelial (RPE) cells [23]. In the dry form of AMD, the compromised capacity to neutralize mitochondrial-derived ROS and impaired proteostasis cause a detrimental accumulation of lysosomal lipofuscin, a complex non-degradable polymeric mix of lipid–protein [24] that forms extracellular structures called "drusen" [25], localized between the basal lamina of the RPE and the inner collagenous layer of Bruch's membrane (BrM). The formation of these deposits is one of the hallmarks of aging in the eye and its size and number predicts the progression and the degree of the dry form of the disease [26]. Drusen formation activates the NACHT, LRR and PYD domains-containing protein 3 (NLRP3) inflammasome via the complement C1q (C1Q), a component of drusen [27]. NLRP3 is one of the central molecules involved in pyroptosis, a programmed cell death characterized by swift plasma membrane disruption and subsequent release of cellular content, including typical pro-inflammatory mediators such us IL-1β and IL-18 [28]. Furthermore, changes in the extracellular matrix (ECM) affecting collagen layer structure and elasticity can promote loss of differentiation and epithelial mesenchymal transition (EMT) of healthy RPE cells [29, 30]. It is accepted that interactions between the RPE layer and the fibrous, acellular BrM is critical in the pathogenesis of AMD [31]. Both structures form the blood-retinal barrier, involved in the maintenance of the health of the retina through the exchange of nutrients, oxygen, and waste products with the choroid. On the contrary, in nAMD, retinal pigment epithelial displacement and damage occurs because of choroidal neovascularisation (CNV) through the BrM leading fluid accumulation [32]. This process is driven by angiogenic factors such as VEGF, whose expression is increased in RPE cells in nAMD [2]. The neovascularization process can be classified in three forms using optical coherence tomography (OCT): Type 1 CNV refers to vessels beneath the RPE, whereas type 2 CNV is characterized by vessels expanding into the subretinal space between the neurosensory retina and the RPE [33], and type 3 by retinal angiomatous proliferation [34].

A recent study have revealed that early AMD signs can be detected already in patients under 30 years [35]. Due to the absence of an effective preventive treatment, the number of patients severely disabled by AMD is expected to increase up to 50% in the next decades [36]. The disease not only exerts a tremendous impact on the physical and mental health of the geriatric population and their kindred, but it is also becoming a major public health issue and financial burden. Thus, it is crucial to find strategies to identify patients at high risk of developing AMD and to improve their management.

The term proteomics encompasses all research methodologies developed for the qualitative and quantitative study of the proteome, which are the proteins present in a cell type, tissue or organism at a given stage of development [37]. In the last decade, there has been an exponential increase in the use of proteomics in translational research, due in large part to the progress in state-of-the-art mass spectrometry, an analytical technique,

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developed in the middle of the last century [38] Proteomics has emerged as a powerful tool for biomarker discovery [39], both independently and in combination with complementary, non-MS-based proteomic approaches such us antibody-based multiplex assays, multiplexed enzyme-linked immunosorbent assays (ELISA) and aptamer-based techniques [40, 41]. As regards specificity and reliability of, not only diagnostic tests, but also treatment targets, the source of biomarkers is of paramount importance. Non-invasiveness is a key factor for any diagnostic approach. Tear fluid, for instance, represents a precious source of biomarker panels for disease progression and response to treatment in AMD. First, it is the nearest biological fluid to the pathological spot, the posterior cavity of the eye [42]. Second, it is easily accessible and can be collected by minimally invasive methods. Third, the protein content is relatively high, ranging from 6 to 10 mg/mL [43]. Tear fluid have revealed in the last decades as a source for biomarker discovery, since is an extremely complex biological mixture of proteins, lipids, metabolites, and salts [44]. Up to 1500 different proteins can be identified by quantitative shotgun proteomics [45]. Although tear fluid offers numerous advantages, other fluids and tissues are, at least *a priori*, promising sources for biomarker discovery and for the study the pathogenesis of AMD [46].

In a previous work from 2018 [47], Kersten *et al.* comprehensively reviewed the use of systemic and ocular fluids to identify compounds, including proteins but also metabolites, lipids, auto-antibodies and miRNAs, as potential biomarkers in AMD. However, to the best of our knowledge, there is a lack of a systematic review specifically focused on proteomic studies. Thus, our aim for this review was to systematically compile the most relevant proteomics-based studies on AMD with a special focus on those covering, during the last five years, new biomarkers and therapeutical targets in GA.

2. Methods

2.1. Database retrieval and search strategy

Electronic bibliographic databases including PubMed and Web of Science were used to search published research papers. The design of this study followed the guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses protocols (PRISMA-P) **[48]**. The search filter included the following terms combined with an "AND": "Age-related macular degeneration" and "proteomics". The published language was limited to English and the search results were screened for suitable topics and full articles accessible for systematic review. The workflow is summarized in Figure 3. Only human studies were included, whereas experimental methods and protocols, reviews, systematic reviews, preprinted articles, and conference proceedings and abstracts were excluded.

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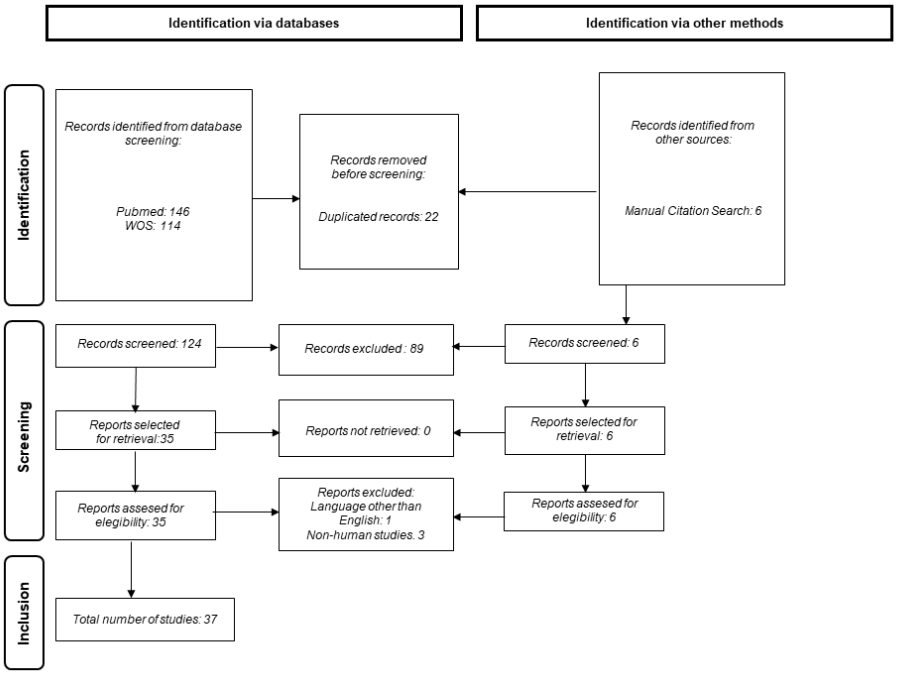

Figure 1. PRISMA workflow followed for the systematic revision of the literature.

2.2. Data extraction

Three independent reviewers extracted data from each eligible study using a standardized data-extraction sheet. Later, results were cross-checked and disagreements between both reviewers regarding the extracted data were resolved by discussion with a fourth reviewer. Thirty-seven published research articles were selected and grouped according to the biological source of biomarkers used for the proteomic study (Tables 1, 2 and 3).

Table 1. Proteomic studies on cells/ocular tissues in AMD.

Study	Biomarker source	Characteristics of the cohort	Proteomic approach(es)	Main findings
Crabb *et al.*, 2002	Drusen and BrM	18 controls 5 donors with AMD	Label-free LC-MS/MS	129 proteins identified. Crystallins are more frequently detected in the diseased group
Alcazar *et al.*, 2009	Exosomes from Hydroquinone-stimulated ARPE-19 cells	N.A.	SDS-PAGE coupled to LC-MS/MS Immunofluorescence	Proteins involved in oxidative phosphorylation, cell junction, focal adhesion, cytoskeleton regulation, and immunogenic processes. Basigin and MMP14 could be involved in progression of dry AMD

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Wang *et al.*, 2009	RPE tissue, drusen and ARPE-19 cells	12 eyes (six donors) with no history of AMD 4 eyes (2 donors) with histories of AMD 8 eyes (8 donors) documented AMD	Immunoblot, ELISA and Luminex	Drusen in AMD donor eyes contain markers for autophagy (atg5) and exosomes (CD63 and LAMP2). Exosome markers are characteristic of drusen from AMD patients and co-localize in the RPE/choroid complex
Yuan *et al.*, 2010	Bruch´s membrane	10 early/mid-stage dry AMD 6 advanced dry AMD, 8 wet AMD 25 normal control post-mortem eyes	iTRAQ (isobaric labelling DDA- LC-MS/MS)	Retinoid processing proteins increased in early/mid dry AMD. Galectin-3 increased in advanced dry AMD.
Biasutto *et al.*, 2013	Exosomes from ARPE-19 under oxidative stress conditions	N.A.	Reverse phase assay	Identification of a subset of phosphorylated proteins including PDGFRβ, VEGFR2 and c-kit that are also detected in the vitreous of AMD patients
Kelly *et al.*, 2020	Bruch´s membrane	3 donors with AMD	Ion mobility-based LC-MS/MS	APOE and APOB over-represented in HDL from BrM vs plasma
Flores-Bellver *et al.*, 2021	RPE monolayers generated from induced pluripotent stem cells (iPSCs) derived from CD34+ cord blood mesenchymal stem cells	N.A.	Label-free LC-MS/MS ELISA Immunoblot	Drusen-associated proteins exhibited distinctive directional secretion mode altered in AMD pathological conditions (e.g., chronic exposure to cigarette smoke)
Cai *et al.*, 2022	RPE cells from donor´s eyes	4 donors with AMD high-risk alleles 2 donors with AMD low-risk alleles	iTRAQ (isobaric labelling DDA- LC-MS/MS)	Exposure of high-risk donors derived RPE cells to the serum from smokers enhance molecular pathways related to development of AMD
Senabouth *et al.*, 2022	iPSCs generated from skin fibroblasts	43 GA 36 Controls	TMT (isobaric labelling	GA patients present mitochondrial dysregulation characterized by an

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			DDA- LC-MS/MS)	increase in Complex I levels and activity
Zauhar *et al.*, 2022	RPE and choroid fibroblasts, pericytes and endothelial cells	N.A.	Label-free LC-MS/MS	Classical complement pathway involvement more robust in retina. New cellular targets for therapies directed at complement

Table 2. Proteomic studies on ocular fluids in AMD.

Study	Biomarker source	Characteristics of the cohort	Proteomic approach(es)	Main findings
Koss *et al.*, 2014	Vitreous humor	73 naïve patients 15 control samples from patients with idiopathic floaters	CE-MS	Acute phase response and blood coagulation up-regulated in AMD, Alpha-1-antitrypsin among them
Nobl. *et al.*, 2016	Vitreous humor	128 nAMD 24 controls	CE-MS ELISA	Clusterin and PEDF levels are predictive for nAMD
Schori et al., 2018	Vitreous humor	6 patients with dry AMD 10 patients with nAMD 9 patients with proliferative diabetic retinopathy 9 patients with epiretinal membrane	Label-free LC-MS/MS	Oxidative stress and focal adhesion pathways modulated in dry AMD and nAMD, respectively.
Baek *et al.*, 2018	Aqueous humor	13 patients undergoing cataract 11 patients with dry AMD and 2 patients with no retinal diseases	DIA -MS (SWATH) ELISA	8 proteins involved in drusen development including APOA1, CFHR2, and CLUS were accumulated in the AH of dry AMD patients
Winiarczyk *et al.*, 2018	Tear	8 wet AMD, 6 dry AMD 8 controls	2D-LC-MALDI-TOF	Graves disease carrier protein, actin cytoplasmic 1, prolactin-inducible protein 1, and protein S100-A7A were upregulated in the tear film samples isolated from AMD patient

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Coronado *et al.*, 2021	Aqueous humor	Group 1: nAMD patients: good responders to anti-VEGF) Group 2: nAMD patients (poor/non-responsive to anti-VEGF) Group 3: patients without systemic diseases or signs of retinopathy	Label-free LC-MS/MS	39 potential disease effectors, including players of lipid metabolism, oxidative stress, inflammation, and angiogenesis. VEGFR-1 is up-regulated in non-responsive patients which could explain resistance to treatment
Joo *et al.*, 2021	Aqueous humor	13 nAMD patients (type 1: n=8; type 2: n=5) and 10 controls undergoing cataract surgery with no retinal diseases	Multiplexed antibody-based array	VEGF is specifically increased in nAMD patients with type 2 CNV
Rinsky *et al.*, 2021	Aqueous humor	Discovery: 10 nAMD patients and 10 controls Validation: 20 controls, 15 atrophic AMD and 15 nAMD patients	intensity-based label-free quantification (MS1) Multiplex ELISA	Clusterin overrepresented in the aqueous of nAMD patients
Winiarczyk *et al.*, 2021	Tear	15 nAMD patients 15 controls	2D-LC-MALDI-TOF	AIF-1, ABCB1 and annexin-1 are higher in AMD
Cao *et al.*, 2022	Aqueous humor	122 nAMD with anti-VEGF therapy	DIA -MS (SWATH)	APOB100 expression was higher in AMD vs control.
Shahida-tul-Adha *et al.*, 2022	Tear and plasma	36 eAMD 36 lAMD 36 controls	ELISA	Tear VEGF level presents high sensitivity and specificity as a predictor of the severity of the disease
Tsai *et al.*, 2022	Exosomes from Aqueous humor	28 eyes from AMD patients (2 of them followed during Ranibizumab treatment. 25 control eyes from senile cataract patients without other ocular or systemic diseases	Label-free LC-MS/MS	APOA1, clusterin, C3 and opticin significantly accumulated in AMD. Anti-VEGF therapy progressively decrease levels of SERPINA1 and AZGP1

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Valencia *et al.*, 2022	Tear	60 patient cohort: 31 with diagnosed GA-AMD	ELISA	Upregulation of MT1A and S100A6 in GA-AMD patients

Table 3. Proteomic studies on systemic fluids in AMD.

Study	Biomarker source	Characteristics of the cohort used for the proteomic study	Proteomic approach(es)	Main findings
Lip *et al.*, 2001	Plasma	28 "dry" AMD 50 "exudative" AMD 25 "healthy" controls	ELISA	VEGF and VWF significantly increased in AMD
Sivaprasad *et al.*, 2005	Plasma	26 nAMD 30 eAMD **15 controls**	**ELISA**	Elastin-derived peptides elevated in the serum of nAMD patients *vs* eAMD and control subjects
Tsai *et al.*, 2006	Plasma	17 dry AMD 42 wet CNV/AMD 18 scar/AMD **64 non-AMD**	**ELISA**	**VEGF significantly increased in CNV/AMD.**
Wu *et al.*, 2007	Serum	159 eAMD 38 lAMD **433 controls**	ELISA	No consistent pattern of association found between AMD and circulating inflammatory markers
Rudnicka *et al.*, 2010	Serum	81 AMD 77 controls	ELISA	FVIIc and possibly F1.2 were inversely associated with the risk of AMD. No evidence of associations between AMD and systematic markers of arterial thrombosis

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Carneiro *et al.*, 2012	Plasma	43 exudative AMD: 19 ITV ranibizumab 24 ITV bevacizumab 19 age-related controls	ELISA	No basal differences in VGEF between AMD and controls Significant reduction in VEGF levels with intravitreal bevacizumab
Gu *et al.*, 2013	Serum	39 neovascular AMD with single dose ranibizumab 39 healthy controls	ELISA	No basal differences in VGEF between AMD and controls VEGF levels significantly decreased after injection but increased later.
Kim *et al.*, 2014	Plasma	20 exudative AMD 20 healthy control patients Validation: 233 case-controlled samples	LC-MS/MS ELISA WB	Vinculin was identified as a potential plasma biomarker for AMD
Kim *et al.*, 2016	Plasma	90 Healthy controls 49 eAMD 87 exudative AMD	ELISA	MASP1 and, specially, PLPT useful as predictors of AMD progression
Zhang *et al.*, 2017	Plasma	344 adults	Selected Reaction Monitoring	Development of a method to quantify Y402H and I62V AMD-associated variants of Complement Factor H
Lynch *et al.*, 2019	Plasma	10 nAMD 10 GA 10 age-matched cataract controls	Aptamer-based proteomics	Higher levels of vinculin and lower levels of CD177 were found in patients with neovascular AMD compared with controls

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Palestine *et al.*, 2021	Plasma	210 iAMD 102 controls	Multiplex	CCL3 and CCL5 significantly decreased and CCL2 increased in with iAMD compared with controls
Sivagurunathan *et al.*, 2021	Plasma and urine	23 controls 61 AMD	Shotgun LC-MS/MS (TMT) ELISA	SERPINA-1, TIMP-1 APOA-1 Higher in AMD
Emilsson *et al.*, 2022	Serum	Discovery: 1054 eAMD 112 GA pure 160 nAMD 183 GA + nAMD Validation: 15 subjects for each category	Aptamer-based proteomics ELISA	Determination of a set of 28 AMD-associated proteins including CFHR1, TST, DLL3, ST6GAL-NAC1, CFP, and NDUFS4. PRMT3 proposed as predictor for progression to GA

3. Results and discussion

This section is structured in sub-sections according to the different types of human samples used for the proteomic study, starting from the closest structures to the macula and finishing with the systemic fluids. Furthermore, an initial sub-section summarizes the different proteomic approaches found in the literature.

3.1. Recent advances in proteomic approaches for the study of the disease

Old-fashioned proteomic approaches like Peptide Mass Fingerprinting (PMF) or Differential In Gel Electrophoresis (DIGE) followed by Matrix-Assisted Laser Desorption/Ionization-Time-Of Flight mass spectrometry (MALDI-TOF) identification are progressively being replaced by more modern high resolution quantitative techniques allowing a deeper identification and more robust quantitation such as Liquid Chromatography coupled *on line* to mass spectrometry (LC-MS/MS). Historically, most of the quantitative shotgun proteomics-based studies have been done using data dependent acquisition (DDA) methods, where the mass spectrometer settings are adjusted to isolate and fragment peptides based on the intensities observed in MS1 survey scans. Basically, the top (usually 10-20) most intense peptides for each time point along the chromatographic gradient are selected for fragmentation and subsequent MS2 level identification, so the resulting MS/MS spectra is assigned to specific peptides sequences by protein database matching. To avoid redundant acquisition due fragmentation of the same precursor in consecutive time-points, dynamic exclusion can be applied, driving to higher protein coverage and detection of low abundant proteins [49]. Examples of DDA techniques include classical label-free, stable isotope labelling by amino acids in cell culture (SILAC) and chemical-based labelling like Tandem Mass Tags (TMT) or isobaric labelling (iTRAQ) [50-52]. DDA approaches usually lead to the identification of a very complex sets of proteins. However, they have inherent drawbacks related to the stochastic nature of peptide ionization and fragmentation like lack of reproducibility/accuracy in the quantification [53].

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During the last decade the emergence of unbiased data independent acquisition (DIA) methods has revolutionized the field, avoiding the problems derived from the stochastic nature of the peptide ionization. Sequential Windowed Acquisition of All Theoretical Fragment Ion Mass Spectra (SWATH-MS) and Hyper Reaction Monitoring (HMR-MS) are highly robust and reproducible label-free techniques in which the mass spectrometer settings are adjusted to isolate and fragmentate all the precursors detected within slightly overlapping windows, covering the entire working m/z range across the entire chromatographic gradient [54]. In this case, the search is performed not using protein databases, but spectral libraries previously generated by DDA of a pool of the samples instead [55]. Last generation software packages include special algorithms capable of generating those spectral libraries *on the fly* using the same DIA data acquired in the studied samples [56, 57], thus improving data processing speed and reproducibility.

In any case, protein extracts from cultured cells or tissues/biological fluids are highly complex samples that exhibit a wide dynamic range of concentrations [58]. Hence, for quantitative proteomics is generally necessary to quantify the samples, both at the level of total protein and subsequently, prior to injection in the LC-MS system, at the level of peptide. In DDA-based approaches fractionation of the sample is often necessary to reduce its complexity and is therefore highly recommended. On the contrary, fractionation is not recommended for DIA approaches [53].

One of the main limitations of LC-MS-based proteomic techniques is the low sensitivity for identifying scarce proteins like cytokines or growth factors, especially in complex samples or samples with high dynamic range of protein content [59]. Alternatives to overcome this limitation are classical antibody-based technologies like multiplex techniques and ELISA, or more recent aptamer-based approaches, relying on single stranded library DNA-based reagents with high binding specificity and complementarity to target proteins [60]. The reagents are immobilized and incubated with the protein sample to be tested. After washing and removing the unbound fraction, the protein-reagent complexes are again immobilized, and the DNA-based reagent is eluted and quantified using standard techniques.

3.2. Proteomics on Retinal Pigment Epithelial cells and Extracellular Vesicles in AMD

The RPE constitutes a cell monolayer essential to maintain a normal photoreceptor function (Figure 2). RPE not only participates in the visual cycle, but also provides nutrients to the photoreceptors, and is responsible for withdrawing waste debris from their outer segments [8]. Compromised molecular regulation between the RPE layer and the BrM is a hallmark of the early stage of AMD [61].

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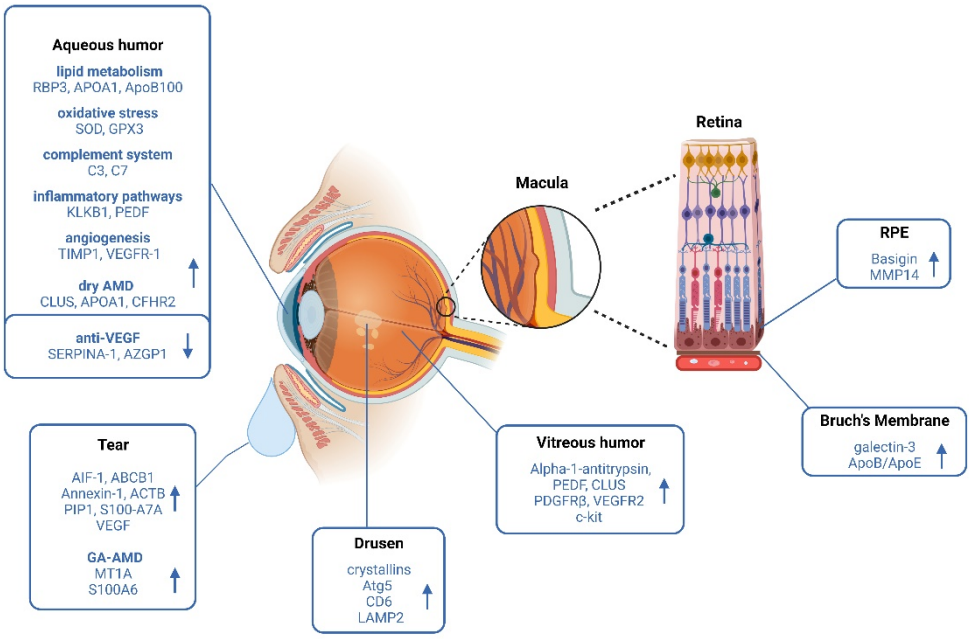

Figure 2. Schematic representation of the main findings regarding biomarker discovery in AMD using ocular tissues/fluids as source. Created with BioRender.com.

A combination of transcriptomics and proteomics was followed by Zauhar *et al.* [62], to dissect the role of multiple retinal and choroidal cell types (Müller glia, neurons and RPE/choroid) in determining the complement homeostasis.. The results indicated that this process has a key role on the involvement of RPE cells in the progression to late AMD. Recently, another transcriptome and proteome-based study has identified pathways specifically modulated in GA. Induced pluripotent stem cells (iPSCs) were generated from fibroblasts from a cohort of 43 individuals with GA and 36 controls with genotype data [63]. In this work from Senabouth *et al.* mitochondrial dysfunction, and specifically an increase in Complex I linked to a higher oxygen consumption rate, was identified as a central genetic factor associated with GA.

The extracellular vesicles (EVs) have been revealed as key players in biological processes such as aging, cell homeostasis and disease [64, 65]. In fact, the molecular cargo of the EVs secreted by the RPE cells has important roles in the pathogenesis of AMD [66]. A non-quantitative proteomic approach was used to merely identify the proteins contained into the so-called plasma membrane blebs of ARPE-19 cells incubated with hydroquinone, a major component in cigarette smoke [67]. Glycosylated forms of basigin and MMP-14 were localized in those blebs and the authors proposed an involvement of these proteins in the extracellular matrix remodelling at sites distal to the RPE, potentially contributing to the progression of "dry" AMD. Biasutto *et al.* [68], by using Reversed Phase Protein Assays, identified a subset of phosphorylated proteins that are characteristic of ARPE-19 cells cultured under oxidative stress conditions. Interestingly, some of those phosphorylated isoforms, such as PDGFRβ, VEGFR2 and c-kit, were also detected in the vitreous of AMD patients. Previously, C5b-9 had been identified as part of the coating of the EVs released by stressed RPC cells, suggesting a role of these vesicles in the focalized modulation of complement activation [69].

In a recent study, the pooled serum of both smokers (n=32) and non-smokers (n=35) were collected and used to treat RPE cells obtained from the eyes of four donors harbouring the high-risk ARMS2/HTRA1 alleles for AMD, and the eyes of two donors with low-risk alleles [70]. iTRAQ was used to identify differentially expressed proteins (DEPs). Under the effect of smokers' serum, 464 DEPs were identified in the high-risk group (smokers

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vs. non-smokers). In contrast, in the low-risk group, the number of DEPs decreased to merely 30. Gene ontology analysis showed that smokers' serum enhanced molecular pathways involved in Alzheimer's disease, oxidative phosphorylation and RPE phagocytic function. Interestingly, caveolin-1 and HTRA1 were among the most significantly upregulated proteins in the high-risk group *vs.* the low-risk group after exposure to smokers' serum, strongly supporting a gene-environment interaction between the high-risk alleles ARMS2/HTRA1 and smoking in the occurrence and development of AMD.

Recently, Flores-Bellver *et al*. [71], have taken a step forward in this field. These authors generated RPE cells from CD34+ cord blood mesenchymal stem cells (MSCs)-derived iPSCs. The induced-primary RPE cell monolayers presented hallmarks of cell differentiation and key physiological characteristics of the native RPE tissue like expression of genes involved in essential RPE functions such as functional apical-basal polarization and EV secretion. The proteomic analysis showed that EVs contained proteins involved in AMD pathogenesis and drusen formation and revealed apical-basal directional proteome enrichment. The monolayers were then treated with increasing concentrations of cigarette smoke extract (CSE) to study the effect of both acute and chronic stress. An increase in drusen-related proteins was detected in the cargo of the EVs released under chronic stress conditions.

3.3. Proteomics on Bruch´s Membrane in AMD

BrM is a thin, stratified, extracellular matrix whose main physiological role is structural, but also facilitates transport to help regulate the diffusion of nutrients and waste products between the RPE and the bloodstream [72]. BrM undergoes significant age-related changes, including thickening and decreased permeability, that disrupts normal retinal physiology and contributes to AMD [73]. One of its five differentiated layers is composed mainly of elastin [74]. It has been hypothesized that degradation of elastin at the BrM macula is a key event facilitating CNV [29]. In line with this are the elevated serum levels of elastine-derived peptides (S-EDPs) [75] as well as elastin autoantibodies [76] found in nAMD patients *vs.* eAMD patients and healthy controls.

A quantitative proteomic analysis of BrM using iTRAQ-based chemical labelling was performed on post-mortem collected samples [77]. Nine hundred and one proteins were quantified. Most proteins did not differ in amount between AMD and control samples reflecting the normal proteome of an average 81-year-old individual. A total of 56 proteins were found to be overexpressed and about 60% of these were involved in immune response and host defence, such as α-defensins 1–3, histones and galectin-3, strongly supporting the role of inflammatory processes in the pathology of AMD.

Ion mobility-based LC-MS/MS has been used to study differences on the protein content of the high-density lipoprotein (HDL) fraction isolated from BrM-enriched tissues *vs* plasma in the same individuals [78]. The results showed a striking over-representation of Apolipoprotein B (APOB) and Apolipoprotein E (APOE) in BrM. Since these isoforms bind to glycosaminoglycans, the authors proposed that the deposition of these lipoproteins may play a role in the downstream effects that contribute to RPE dysfunction and destruction, characteristic of AMD. To test whether APOE and APOB could be therapeutic targets for AMD, the anti-inflammatory 5A apolipoprotein A-1 (APOA1) mimetic peptide was used in a mouse model of AMD. The 5A peptide was able to modulate the proteomic profile of circulating HDL and prevent some of the potentially harmful changes in protein composition resulting from the high-fat, high-cholesterol diet in this model.

3.4. Proteomics on Drusen in AMD

Drusen are extracellular deposits, composed mainly of lipids, polysaccharides, proteins and glycosaminoglycans that accumulate between the basal side of the RPE and the BrM, and are considered as risk factors for the development of AMD [79]. From a clinical

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point of view, drusen are classified into different types, based on their relative size, shape, imaging characteristics and location.

The protein composition of drusen isolated from eye dissections from AMD patients and controls has been studied using LC-MS/MS [80]. Some proteins like vitronectin, TIMP3 or clusterin were common to both groups, while others such as crystallins were more frequently detected in the disease group. Furthermore, immunoblot analysis showed a higher level of crosslinked species and carboxyethyl pyrrole (CEP) adducts in drusen from patients, which reinforces the importance of oxidative processes in the pathogenesis of AMD. In another study, exosome markers CD63 and LAMP2 were detected in drusen from eyes of AMD donor but not in age-matched controls. Interestingly, CD63 co-localized in these samples with other proteins characteristic of drusen, such us amyloid β, α-B-crystallin, C5b-9 and CFH, suggesting that the release of intracellular proteins via exosomes by the aged RPE may contribute to the formation of drusen [69].

3.5. Proteomics on Vitreous Humour in AMD

Vitreous humour is a colourless, transparent gelatinous substance filling the vitreous cavity, the region between the lens and the retina in the posterior segment of the eye [81]. It is surrounded by a collagen layer called vitreous membrane. Besides helping to maintain the normal shape of the ocular globe, it also acts as a reservoir of metabolites for the surrounding tissues and as a barrier to avoid diffusion of substances between the retina and the anterior segment [82]. Since the vitreous humour is in direct contact with the lens, retina, macula and retinal vessels, the vitreous is, a priori, a promising source of biomarkers for the study of AMD and other ocular pathologies [83]. Furthermore, the vitreous fluid is the target in which intravitreal anti-VEGF injections, the gold standard treatment for nAMD, exert their therapeutic action [84]. However, to date, very few human-based studies on biomarker discovery in vitreous fluid have been published due to the difficulty of sample collection from living specimens [85].

Koss *et al.* used capillary electrophoresis coupled to mass spectrometry (CE-MS) and identified a set of 19 proteins accumulated in the vitreous fluid of AMD patients, most of which were related to acute phase response and blood coagulation [86]. Among them, Alpha-1-antitrypsin was orthogonally validated in an independent set of AMD patients using Western blot analysis. In a subsequent study, the same group used a combination of CE-MS and LC-MS approaches to identify four potential biomarkers of nAMD progression in the vitreous fluid of patients with different degrees of CNV [87]. Validation by ELISA showed the best results for clusterin and PEDF. Clusterin has been related to cytoprotective effect in the retina, reducing apoptosis and ROS levels [88]. It has been hypothesized that clusterin can contribute to AMD pathogenesis through its potential role in modulating the complement system [89], including some of the components with genetic variants considered as risk factors for AMD, such as C3 and CFH [13].

More recently, Schori and colleagues [90] used label-free LC_MS/MS to establish the proteomic landscape in the vitreous of patients with dry AMD, nAMD and diabetic retinal disease (PDR). They identified different clusters of upregulated proteins for each patient group. Interestingly, complement and coagulation cascade appeared to be specially highly modulated in PDR, whereas alteration of oxidative stress and focal adhesion pathways were characteristic of dry AMD and nAMD, respectively.

3.6. Proteomics on Aqueous Humour in AMD

The aqueous humour (AH) is a clear liquid that occupies the anterior and posterior chambers of the eye. Its composition is similar to that of plasma although the protein concentration is much lower. It also contains electrolytes and ascorbate [91]. AH maintains the intraocular pressure, provides nutrients and oxygen to the surrounding eye tissues lacking blood vessels and also removes their waste products [82].

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A DIA quantitative proteomics study has been recently done in patients receiving anti-VEGF therapy [92]. Increased APOB100 levels were detected in *pro re nata* (PRN) treated patients who required less frequent injections. Of interest, APOB100 accumulates within Bruch's membrane as an early component of drusen [93]. Furthermore, APOB100 expression was higher in AMD eyes compared with healthy controls but was lower in eyes developing CNV, consistent with the protective role that has been attributed to this protein. A DIA-based approach was also used by Baek *et al.* [94] to study the proteome of the AH of dry AMD presenting soft drusen and/or reticular pseudodrusen. Eight proteins, APOA1, CFHR2, and CLUS among them, were previously described as major components or regulators of drusen. An additional set of three proteins (SERPINA4 protein, lumican, and keratocan) with no previous link with drusen formation were also increased in AH from dry AMD patients. Specifically, lumican and keratocan are involved in keratan sulphate proteoglycan (PG) biosynthesis and ECM remodelling, which could be partially linked to the ECM degradation that occurs in BrM during AMD development [73].

As previously described for vitreous humour, aqueous clusterin has recently been proposed as a biomarker for AMD progression by Rinsky *et al.*, [95]. Clusterin was first detected as overrepresented in the aqueous humour of nAMD patients *vs* controls (n=10 in both cases) and later validated by ELISA in a larger cohort including nAMD patients (n = 15), aAMD patients (n = 15) and controls (n = 20).

In a pilot study recently conducted by Coronado *et al.* [96], a proteomic analysis of the AH was done to get deep into the molecular pathways driving to choroidal neo-angiogenesis. A small cohort of 15 patients was divided into 3 groups; those with nAMD, who demonstrated a good response to anti-VEGF intravitreal injections during follow-up, those with anti-VEGF-resistant nAMD who demonstrated choroidal neovascularization activity during follow-up and those composed of control patients without systemic diseases or signs of retinopathy. Among the 185 discriminatory proteins, 39 were selected as potential disease effectors, including players of lipid metabolism (RBP3, APOA1), oxidative stress (SOD, GPX3), complement system (C3, C7), inflammatory pathways (KLKB1, PEDF), and angiogenesis (TIMP1, VEGFR-1). Specifically, VEGFR-1 is up-regulated in non-responsive patients. According to the authors, this finding could explain the pathological tolerance that some patients develop to the gold-standard treatment of AMD and the persistence of the disease.

Exosomes isolated from AH collected from 28 AMD and 25 control eyes were lysed and the protein extracted for subsequent DDA label-free LC-MS/MS analysis by Tsai *et al*. [97]. Interestingly, gene ontology analysis showed that the only gene set enriched in AMD *vs* control was lipoprotein metabolic process. APOA1, clusterin, C3 and opticin were among the proteins significantly accumulated in AMD. Furthermore, AH at different time points was collected from only two AMD patients who received continuous anti-VEGF injections of ranibizumab every 12 weeks. LC-MS/MS analysis showed a progressive decrease of SERPINA1 and AZGP1 proteins in both patients. Since SERPINA1 promotes cell migration [98] and AZGP1 could enhance cell proliferation and epithelial-mesenchymal transition (EMT) [99], the authors propose these two proteins as biomarkers for the therapeutic effect of anti-VEGF therapy in AMD.

Cytokines levels were measured, using multiplex antibody-based arrays, in the AH of nAMD patients and controls [100]. CNV type was determined by fluorescein angiography (FA) pattern. Several members of the C-C motif chemokine family (CCLs 2, 3 and 4) and VEGF were significantly increased in the nAMD group *vs* the control group. When the two nAMD groups were compared separately *vs* the control group, VEGF was found to be specifically increased in type 2 or classic CNV, characterized by increased neovascularization in the subretina and worse disease prognosis than patients with type 1 CNV [101]. Based on these results, the authors suggested that, in patients with type 1 CNV, also known as "occult" CNV, treatment based on VEGF inhibition alone may not be sufficient to achieve clinical benefits.

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3.7. Proteomics on Tear Fluid in AMD

Tear fluid provides a non-invasive and easy source for sensitive proteomics to detect putative biomarkers of ocular surface health [102]. It is produced by lacrimal and accessory glands, as well as by meibomian glands and goblet cells and is mainly composed by lipids, water, and mucin [42]. Tear film is usually collected from the eye onto a Schirmer strip, although there are other alternatives such us the use of glass capillaries [103]. When deciding on the proper approach, it is important to consider that stimulated and non-stimulated tear film do not share all the biochemical properties. It is accepted that the use Schirmer strips triggers more intense tearing, which is helpful for better sample collection, but in turn leads to an underestimation of the actual protein concentration [43].

Historically, lactoferrin (LF), IgE and MMP-9 have been the most common translational biomarkers studied in tear film and their usefulness has been validated in dry eye disease [104], allergic conjunctivitis [105], keratoconus [106] and inflammatory conjunctivitis [107, 108]. As for nAMD, the use of two-dimensional electrophoresis followed by MALDI-TOF/TOF mass spectrometry approach in a recent study [109] has led to the identification of a set of dysregulated tear film proteins including several proteins related to inflammation and neovascularization, like allograft inflammatory factor 1 (AIF1), ATP-dependent translocase (ABCB1) and annexin-1. A previous study from the same group included patients with both "wet" and "dry" AMD, as well as control individuals [110].

To investigate the role of altered metal homeostasis in AMD, a targeted ELISA-based analysis was recently used to measure the levels of a panel of metal-binding proteins of interest in the tear film of 60 patients, including 31 individuals diagnosed with the GA-AMD form [111]. The protein panel consisted of LF, S100 calcium binding protein A6 (S100A6), metallothionein 1A (MT1A), CFH, clusterin and amyloid precursor protein (APP). Results indicated an upregulation of MT1A and S100A6 in GA-AMD patients. The work was complemented with the multi-elemental analysis of the levels of Ca, Mg, P, Na, Zn, Fe and Cu by Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Multivariate logistic regression and machine learning models were applied, and the panel constituted of MT1A, Na, and Mg was found to predict AMD disease in 73% of cases. As a conclusion, the authors proposed a role of metal homeostasis in the progression of AMD.

Also recently, the VEGF level in tear and serum has been simultaneously measured in the same cohort of patients [112]. The cohort was composed by 108 individuals, one third of them belonging to each category (early AMD, late AMD, and controls). The main conclusion of this work was that the tear level of VEGF presented high sensitivity and specificity as a predictor of the severity of the disease. On the contrary, the serum level of VEGF was found to be non-specific and non-predictive. Interestingly, the analysis of the demographic characteristics showed significant differences between controls and late AMD individuals in lifestyle variables, specifically cigarette smoking and alcohol consumption.

3.8. Proteomics on blood in AMD

Novel Aptamer-Based proteomic technologies have been applied to the study of AMD biomarkers in plasma. This approach was used to differentiate the proteomic plasma signature of GA and nAMD patients *vs.* cataract controls [41]. Vinculin levels were significantly higher in nAMD patients, a result that was in concordance with previous mass-spectrometry based studies by Kim *et al.* [113]. Vinculin is a well-known regulator of apoptosis with additional roles in cell growth, migration, differentiation, and survival. On the other hand, the same group validated their LC-MS/MS results by ELISA in two different cohorts of patients including healthy controls and both early AMD and exudative AMD patients [114]. The results showed that two proteins related to inflammation, PLTP and MASP-1, could be useful as candidate biomarkers for AMD progression. ROC and multivariate regression analysis indicated an excellent diagnostic accuracy, especially for PLTP. Using the same technology, the proteogenomic signature of AMD in blood has

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been recently investigated [115] in the "Age, Gene/Environment Susceptibility Reykjavik Study" (AGES-RS) cohort [116]. The authors defined a set of 28 AMD-associated serum proteins. Subsets of these were specifically linked to the distinct stages of the disease and some could be useful to predict disease progression. For instance, serum levels of PRMT3, an arginine methyltransferase controlling ribosomal activity [117], were elevated in early AMD patients who subsequently progressed to GA, but not in those who progressed to nAMD.

Other protein biomarkers have been studied at the systemic level (Figure 3). Given that VEGF is the primary therapeutic target in nAMD, elevated levels of this molecule in the blood of patients could be, *a priori*, expected. However, there are large discrepancies across the different studies so far. VEGF has been found to be increased in blood in the studies by Lip *et al.* [118] and Tsai *et al*. [119], but on the contrary, neither Carneiro *et al.* [120] nor Gu *et al*. [84] have found significant differences between patients and controls. Inconsistent results have been also found for Von Willebrand factor. This factor is released when endothelial cells are damaged, and it has been proposed as an indicator of endothelial damage or dysfunction in subjects with AMD [121]. Whereas one study showed higher levels in AMD compared with controls [118], more recent studies found no such association [122, 123].

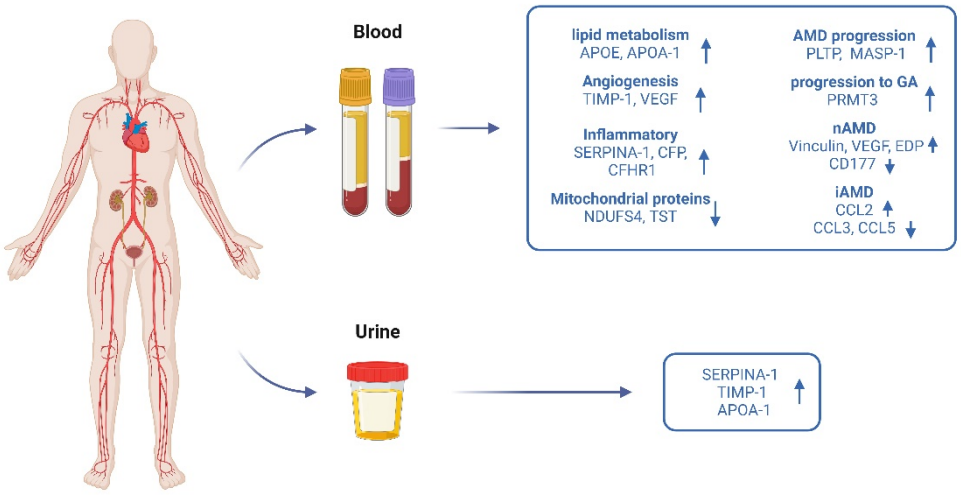

Figure 3. Schematic representation of the main findings regarding biomarker discovery in AMD using systemic fluids as source. Created with BioRender.com.

Regarding cholesterol transport and metabolism, more controversy has been added when studying the role of APOE polymorphisms in the development of AMD. APOE ϵ4 and ϵ2 isoforms decrease and increase risks, respectively, for AMD [124-126]. However, APOE absence in humans and mice does not significantly affect the retina [127], indicating the existence of compensatory mechanisms that minimize the retinal impact of this absence.

A role in the systemic inflammatory processes associated with the development of iAMD has been proposed for plasma C-C chemokines. C-C chemokines are soluble mediators of inflammation-related chemotaxis and features of AMD including drusen-like structures at the level of the RPE. CCL2 concentrations have been reported to be increased

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in patients with iAMD compared with controls, whereas CCL3 and CCL5 have been significantly decreased [128]. Additionally, RPE disruption and photoreceptor degeneration has been previously observed in a CCL2 deficient mice [129].

The strong genetic association of the variants of Complement factor H (CFH) with AMD has also been explored from a proteomic point of view. A targeted, Selected Monitoring Reaction (SRM) assay was developed by Zhang *et al.* to reliably quantify the Y402H and I62V variants [130], a challenging task due to single amino acid substitutions and high sequence homology between complement factor H and complement factor H-related proteins.

3.9. Proteomics on urine in AMD

Several studies have identified common pathogenetic mechanisms underlying renal and retinal diseases [131, 132]. Interestingly, the vascular networks of glomerulus and choroid present similar structure, and the renin–angiotensin–aldosterone hormonal cascade is found in both the kidney and the eye [133]. Increased levels of urinary markers of oxidative stress such us F2-isoprostanes, a marker of lipid peroxidation, and cadmium have been associated with the progression of AMD [134, 135]. Chronic Kidney Disease (CKD) and the main ocular diseases (AMD, diabetic retinopathy, glaucoma, and cataract) share common vascular risk factors including diabetes, hypertension, smoking, and obesity, as excellently reviewed by Wong *et al.* [136].

Based on all this, urine has also been used, in the last years, as a non-invasive easy-to-collect source for biomarker discovery with the aim of identifying not only the metabolomic [137, 138], but also the proteomic signature of the different sub-types of AMD. A tandem mass tagged (TMT) approach identified panels of proteins characteristic of eAMD, GA and nAMD [139]. ELISA validation of some of the candidates showed that SERPINA-1, TIMP-1 and APOA1 were significantly over-expressed in AMD *vs*. controls.

3.10. Therapeutical challenges and future directions

Proteomics-based biomarker discovery for AMD development and progression has identified a set of diverse modulated proteins, summarized in Table 4. Current available therapies, focused on targeting VEGF or inflammation, are an effective approach, but only in the neovascular AMD. So far, translational research in this field has been strongly limited by the difficulties on establishing good experimental models [140], due to anatomical differences of the structure of the eye between rodents and humans [141] or failure to recapitulate the multifactorial characteristics of the disease [142].

Targeting the complement cascade appears to be the more promising therapeutical approach, as has been recently comprehensively reviewed by Patel and colleagues [143] but no drug has been marketed yet.

Currently, ongoing phase III trials include as therapeutical targets complement factors as C3 (APL-2, pegcetacoplan) [144, 145] or C5 (avacincaptad pegol) [146] with promising results. Specifically, therapeutical targeting of C3 using APL-2, a peptide inhibitor that is administered intravitreally, has been very recently showed to be effective even earlier in the progression of AMD prior to the development of GA [148]. On the contrary, a finished phase III trial targeting complement factor D (lampalizumab) showed no difference in the progression of GA compared with placebo [147].

Finally, ocular deliveries innovative solutions based on hydrogels [149] nanocarriers [150] or polymeric micelles [151] will be of paramount importance for maximizing bench-to-bedside transition and to improve patient adherence to the new therapeutical drugs.

Table 4. Main biomarkers of AMD development and progression.

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Process	Protein biomarkers	References
RPE redox maintenance	CCLs	100, 128, 129
	Crystallins	69, 80
Regulation of neovascularization	VEGF	100, 10, 112, 118, 119
	VEGFR	68, 96
	TIMP1	96, 139
	Opticin	97
Metal homeostasis and ECM remodelling	S100A6	111
	CFH, CFHR	80, 94, 111, 130
	TIMP1, TIMP3	96, 139
	Elastin	74, 75, 76
	MMP14	66,67
Lipoprotein metabolism	APOA1	78, 94, 96, 97, 139
	APOB	78, 92, 93
	Clusterin	87, 88, 89, 95, 97, 111
Complement cascade	C3	96, 97
	CFH, CFHR	80, 94, 111, 130
	C5	69, 80
	Clusterin	87, 88, 89, 95, 97, 111

4. Conclusion

AMD is a prevalent condition representing the leading cause of irreversible visual impairment in Western countries in elderly population. Although it is accepted that activation of a cascade of proinflammatory and proangiogenic factors, driven by damage to the choriocapillaris, the RPE and the outer retina, play a key role in the development of the disease, the exact pathogenic mechanisms shared by the different forms of AMD remains elusive and needs to be elucidated to therapeutically address the early stages of the disease. Proteomics has given us, in the last half decade, new clues that will help us in this purpose. Examples of this are the involvement of detoxification pathways, the regulation of the complement by clusterin, the involvement of several members of the C-C motif chemokine family, the role of EVs in the formation of drusen, and the molecular control of processes such us ECM remodeling and EMT as triggering factors for AMD. We

strongly believe that proteomics will be in the next years a fundamental tool to elucidate the precise molecular role of these candidates and to study the clinical progression of patients.

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