• Review Article •

Lipid accumulation and protein modifications of Bruch's membrane in age-related macular degeneration

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Received: 2020-04-23 Accepted: 2021-02-26

Abstract

● Age-related macular degeneration (AMD) is a progressive retinal disease, which is the leading cause of blindness in western countries. There is an urgency to establish new therapeutic strategies that could prevent or delay the progression of AMD more efficiently. Until now, the pathogenesis of AMD has remained unclear, limiting the development of the novel therapy. Bruch's membrane (BM) goes through remarkable changes in AMD, playing a significant role during the disease course. The main aim of this review is to present the crucial processes that occur at the level of BM, with special consideration of the lipid accumulation and protein modifications. Besides, some therapies targeted at these molecules and the construction of BM in tissue engineering of retinal pigment epithelium (RPE) cells transplantation were listed. Hopefully, this review may provide a reference for researchers engaged in pathogenesis or management on AMD.

● **KEYWORDS:** Bruch's membrane; lipid accumulation; protein modifications; therapy

DOI:10.18240/ijo.2021.05.19

Citation: Meng LH, Chen YX. Lipid accumulation and protein modifications of Bruch's membrane in age-related macular degeneration. *Int J Ophthalmol* 2021;14(5):766-773

INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of severe and irreversible blindness in developed countries, and it has no cure[1]. Also, it contributes an increasingly substantial burden in China as a consequence of exponential population ageing[2]. Although it is prevalent all over the world, the initiation and pathogenesis have been not well understood yet, thus limiting its success of treatment. To date, it has been acknowledged that AMD pathology is tightly related to the dysfunction of retinal pigment epithelium (RPE) cells and dynamic changes of Bruch's membrane (BM). Clinically, AMD is categorized into two groups. One is the early stage and the other is the late stage (neovascular and atrophic type). BM exhibits most significant symptom of AMD, especially in the early stage. Therefore, a thorough understanding of BM pathophysiology may help us find more opportunities to treat AMD. Herein, we focus on the lipid accumulation, protein modifications to BM, as well as some potential novel therapy that targets at these molecules. And we also summarize the new achievements about reconstruction of BM for RPE transplantation. Hopefully, this review could provide some clues for ophthalmologists on AMD pathogenesis and management.

BM is an acellular layer of extracellular matrix (ECM) that located between the RPE cells and choroid. It is 2-4 μm thick, and consists of five layers as follows (from the RPE to the choroid): the basement membrane of the RPE (RPE-BL), the inner collagenous layer (ICL), the elastin layer (EL), the outer collagenous layer (OCL) and finally, the basement membrane of the choriocapillaris (CHC-BL)[3]. This pentalaminar sheet plays a crucial role in the diffusion of oxygen, biomolecules, nutrients and waste products between choroidal vasculature and RPE. Besides, it physically supports RPE layer, providing a division barrier and restricting the cellular migration of the choroidal and retinal layers[4]. It has been clear that structural or compositional changes occurring in BM with aging have significant effects on the onset and progression of AMD by destroying its normal function[5]. Mostly, molecular changes such as iron or zinc depositions, lipids accumulation, and turnover of proteoglycans, and structural deformation such as collagen linkage and thickness increasing occur simultaneously, and could not separate from each other but do effects mutually.

LIPID ACCUMULATION

There are different kinds of lipids accumulating with aging

in BM, including the phospholipids, triglycerides, fatty acids, and free cholesterols. This process is tightly associated with the function of RPE, such as the gradual loss of RPE melanin and the obvious increase of lipofuscin, an intracellular auto-fluorescent pigment mainly from the phagocytosed outer segments of photoreceptors (Figure 1)[6]. In young eyes, lipid inclusions have associations with fine elastin and the collagen filaments in ICL, EL, and OCL. Once the EL and OCL are filled with particles, lipid-like particles (LLP) will continue to accumulate near the RPE layer, but will not increase in the OCL anymore. Finally, the lipid inclusions fill the interfibrillary spaces in the EL and accumulate in the ICL, thus forming the so-called lipid wall[7]. This is a normal phenomenon that occurs in BM with human aging. One interesting sign is that lipid wall thickness differs per individual. Even the content or the phospholipids to neutral fats ratio varies per individual, perhaps in part due to diet. Basal deposits and drusen, which represent small and large extracellular deposits, appear in BM with aging gradually. These depositions are composed of abundant (un-) esterified cholesterol (UC, EC), oxy-cholesterols, and other biomolecules that contained lipid.

The role for lipids in AMD is a matter of great concern. In AMD, it is noticeable that the elderly macula contains much more cholesterol esters, seven times over the retinal periphery[8]. The metabolism of different lipids and lipoproteins is tightly associated with disease pathogenesis in multiple ways. Drusen, a kind of extracellular deposits of bio-materials which contain different classes of lipids, polysaccharides, glycosaminoglycans and minerals, are considered clinical hallmarks for AMD. Its location is between the RPE-BL and ICL of BM[9]. Generally, drusen is characterized by focal clumps or dispense spots of hyperpigmentation on color photographs and slight elevations of the RPE with different reflectivity internally that depends on its histopathologic types using optical coherence tomography (OCT) imaging (Figure 2)[10]. Eyes with soft and confluent drusen in macula have great risk for AMD development[11]. If drusen becomes visible at ophthalmoscopic examination, it means that the progression to AMD pathology of normal aging have insidiously occurred. Drusen consists of acute phase proteins, C-reactive protein, complements, complement inhibitors, apolipoproteins, lipids and lipoproteins[12]. The fat and cholesterol amount of drusens are varied, but they have a stable ratio of EC to UC. Drusen develops more commonly in macula, which may be due to the structural, molecular and functional properties of this area. However, the specific mechanisms have remained unclear. Researchers proposed that the extremely high density of photoreceptors, thinner elastic layer and local functional macular RPE properties may play a role in this feature. In addition to drusen, which are focal forms of deposits, basal linear deposits (BlinD) also appear in this space as diffuse forms (Figure 3).

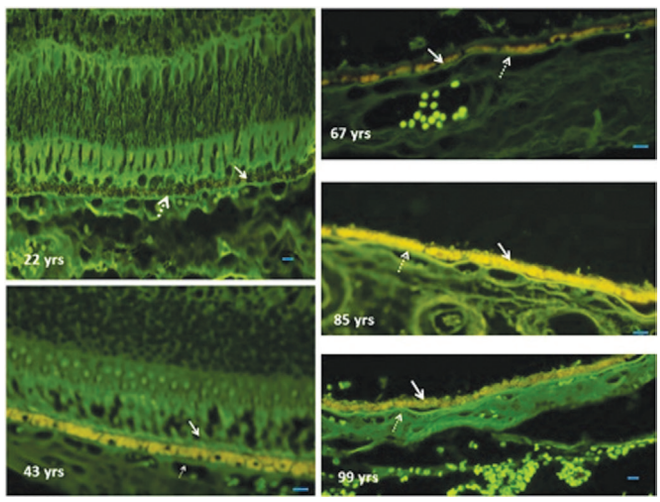

Figure 1 Age-related changes of retina by hematoxylin and eosin staining under the fluorescent microscope It suggested a gradual decrease of the melanin content (black nonfluorescent granules) and an obvious increase of the lipofuscin content (yellow fluorescence dots) with aging. RPE (solid arrows) and BM (dotted arrows) could be appreciated. BM: Bruch's membrane; RPE: Retinal pigment epithelium.

The lipid content in BM is highly attractive for many years. It has been proposed that Bruch's membrane lipoprotein-like particles (BM-LLPs) represent a major portion of lipids in BM. RPE is considered to be the major origin of these lipids. However, the composition of LLPs resembles plasma LDL more than that of photoreceptor membrane, despite the differences in density profile, cholesterol distribution, or morphology between BM-LLPs and plasma lipoproteins[13]. Trafficking of lipids are highly active in BM, connecting the communication between the choroidal capillaries and RPE cells. Lipids from plasma are transported through the BM to RPE cells. Meanwhile, the RPE secretes LLPs back to circulation through BM. As for density profile, BM-LLP contains largely EC, which was approximately 10-fold more than triglyceride[14]. The accumulation of EC could act as a transport barrier in aged people[15]. Over time, the abundance of EC could negatively impact BM's permeability and inversely exacerbate the accumulation. As a result, the critical threshold will be reached and deposits will start formation subsequently.

Now, some new perspectives on lipid research proposed that lipid-related treatment might be potential options for AMD. Many researches have focused on the pathogenesis of AMD, including the dietary or circulating lipids related studies, and the genetic or Mendelian randomization studies. The results indicated that circulating lipoproteins and local lipids trafficking are both crucial in AMD pathology. The next thing for researchers is to devise interventions that target

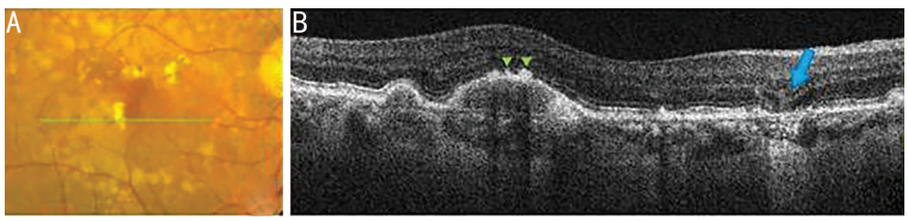

Figure 2 Imaging of AMD with large drusen A: Color fundus photography showed large drusen; B: SD-OCT B-scan at location of green line (A) showed hypperreflective foci (green arrow heads) and absence of RPE. RPE: Retinal pigment epithelium; SD-OCT: Spectral-domain optical coherence tomography.

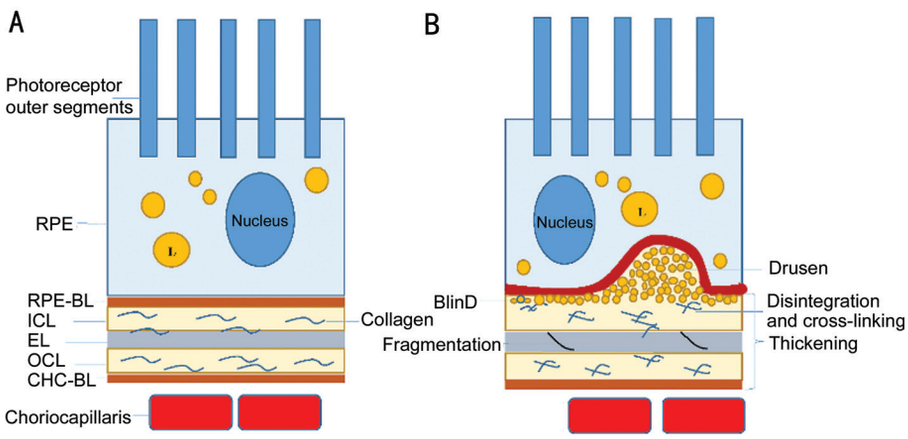

Figure 3 BM and characteristic lesions of AMD A: Five layers of BM in a normal eye: RPE-BL, ICL, EL, OCL and finally, CHC-BL; B: AMD eyes might have drusen, BlinD, fragmentation of EL, dysregulation or cross-linking of collagen fibrils, and thickness increasing in BM. L: Lipofuscin; BM: Bruch's membrane; ICL: Inner collagenous layer; RPE-BL: Basement membrane of the retinal pigment epithelium; EL: Elastin layer; OCL: Outer collagenous layer; CHC-BL: Basement membrane of the choriocapillaris; BlinD: Basal linear deposits.

the systemic and/or local metabolism of lipoproteins for AMD treatment[16]. Recently, statins that have a significant effect in atherosclerosis and hyperlipidemia have gained great attention in AMD. However, conflicting results existed about the statins usage. Several researchers have observed the benefit of statin use in AMD, such as the decreased rates of choroidal neovascularization (CNV), facilitating regression of large drusen, and the findings that patients with AMD were significantly less likely to receive a statin prescription[17-19]. Researchers speculated that the potential mechanisms behind might be related with lowering risk of oxidant-induced damage in RPE cells, decreasing gene expression of vascular endothelial growth factors (VEGF) and reducing the occurrence of empty vacuoles of the RPE, lipid droplets and thickening or fragmentation of EL in BM[20]. Unfortunately, some authors found no effect or even higher risk of statins on the AMD incidence or progression[21-22]. Whether there is an association between the use of statins and AMD has remained controversial. Therefore, more in-depth researches such as randomized clinical trials (RCTs) with large sample size and various experimental models are needed in order to further clarify this issue.

Besides statins, targeting high-density lipoprotein (HDL) metabolism may also be possible for AMD treatment. But considering its risk of cardiovascular disease, whether it can be used broadly and safely still needs much more investigations on its mechanisms in the future[3].

Further, the application of apolipoprotein A1 (APOA1) mimetic peptide 4F may be a potentially novel therapy. APOA1 is the major protein of plasma HDL, while APOA1 mimetic peptide 4F is a small synthetic peptide that emulates the anti-atherogenic properties of APOA1. Researchers found that intravitreal injections of 4F in $Apoe^{null}$ mice showed a dose-dependent reduction of EC and a restoration of the ultrastructure of BM[23]. Similarly, these substantial pharmacologic changes in aged nonhuman primates have also been reported[24]. Therefore, APOA1 mimetic peptide 4F provides some clues on AMD management by removing lipid deposits from BM.

Studies applying metabolomics, particularly lipidomics help elucidate the lipid homeostasis of the retina. Besides, dietary components could be analyzed with the latest developments in mass spectrometric technology, promoting investigation of their role in AMD[25]. Subsequently, these detailed explanations of lipid metabolism from new perspective may shed light on AMD management.

PROTEIN MODIFICATIONS

As an ECM, BM contains a number of proteins, which undergo significant changes in both concentration and chemical structure in AMD. However, therapy aiming at BM proteins are deficient compared with those that target lipid metabolism. Variation in concentrations of these proteins has been identified using the Raman spectroscopy. Collagen I was reported to have a significant increase with age, whereas collagens III and IV decreased. Other proteins, such as elastin, α-crystallin and the porphyrin heme were also altered[26]. The maintenance of BM's structure attributes largely to the normal function of collagen. However, collagen-linkage in BM increases with age, negatively influencing BM's permeability (Figure 3). On one hand, it could increase the strength of the collagen structure. On the other hand, it could decrease its elasticity, flexibility, filtration capability and might influence the normal function of RPE collagenous. To sum up, these lead to a compromised turnover of BM[3]. BM may lose majority of elasticity during life course due to increased collagen cross-linkage, elastin calcification as well as oxidative stress damage mediated by advanced-glycation end products (AGEs).

Many retinal disorders including AMD are due to the dysregulated accumulation of ECM protein, which is a characteristic process of retinal fibrosis. The majority of BM component is ECM protein[27]. Fibronectin (230-270 kDa glycoprotein), an important constituent in vertebrate ECM, has been identified in each of the five layers of BM. Fibronectin is secreted as a compact dimer. Integrins, such as α5β1 integrin, which are located at cell-surface, are required to engage fibronectin to expose its cryptic self-association domains. These domains are important to form a three-dimensional (3D) matrix[28]. Other proteins incorporated into ECM on the basis of formerly assembled fibronectin matrix. These included collagen types I, III, and IV. Therefore, fibronectin matrix assembly might be crucial for fibrotic disease progression such as AMD. It might be an initiation of pathologic ECM protein accumulation[29].

RPE cells have integrins on the basal lamina, which interact with the ECM protein of BM. Among these, the α5 integrin which could act as a so-called fibronectin receptor together with β1 subunits has been observed in retinal sections of human beings[30]. More fibronectin was spread on RPE than other proteins such as laminin[31]. This reveals again the importance of fibronectin in BM. As described above, drusen, BlinD (within BM) and basal laminar deposits (between the RPE and the innermost layer of BM) occur during thickening of BM in AMD. Evidences have shown that basal depositions contained collagen IV and laminins. The presence of fibronectin was controversial. But *in vitro* studies, researchers have observed that type IV collagen deposition could be prevented by inhibiting the fibronectin assembly. This suggested that collagen IV accumulation relied on the previously-assembled fibronectin matrix[32]. Besides, RPE cells could produce fibronectin by the induction of connective tissue growth factor (CTGF), which could mediate basal deposit formation[33].

Matrix metalloproteinases (MMP) are crucial in BM physiology and pathology. Type IV collagen is buildup due to decrease of MMP-2 and/or MMP-9 activities[3]. In late AMD, neovascularization might happen, mainly due to the increase of MMP activity and shift of fibronectin signaling, making it quite permissive to neovascularization[34-35]. Besides, fibronectin is integrin αvβ3's ligand. The conjugation of them plays a key role in the neovascularization. Animal models have shown success of therapies directed at inhibiting αvβ3 to suppress choroidal neovascularization[36]. These evidences also suggest the important function of fibronectin in the onset and progression of AMD from other aspects. Therefore, therapies related to fibronectin modification may acquire promising outcomes.

Elastin is another major components of BM. It provides strong and long-lasting elasticity to BM in combination with other proteins. The EL degrades with age as a normal phenomenon. However, it shows frequently thinning and fragmentation in AMD[37]. The abnormalities of elastin metabolism are not only seen in BM, but also could be observed as a systemic phenomenon in AMD. Patients with neovascular AMD predisposed to have elastolysis of skin and higher levels of serum elastin-derived peptide[38-39]. These results indicate that analyses of elastin degradation products may be potential biomarkers for neovascular AMD[40].

Fibulin 5, a secreted ECM protein with Arg-Gly-Asp (RGD) motif, is involved in cell adhesion *via* interactions with integrins[41]. Normally, it localizes to BM and the intercapillary pillars of choriocapillaris. In AMD eyes, fibulin 5 localizes to pathological sub-RPE deposits[42]. It could downregulate VEGF, strengthen cell adhesions, and control choroidal endothelial cell proliferation. Therefore, the mutation of fibulin 5 gene, leading to misfolded protein may cause subsequent dysfunction, increasing the risk of AMD[43].

Several studies have paid attention to peptide modifications in BM, focusing on the specific amino acids. For example, it has been reported that nitrite, a potent inflammatory byproduct, could modify tyrosine and lysine residues of BM chemically as a beginning step of degenerative changes[44]. Besides, deamination at lysine residues have also been observed[45]. These results coincided with the elevated nitrite concentrations in AMD, implicating the inflammation is a major risk factor. Studies that focused on protein modifications by other pathways besides inflammation process have also been reported, such as lipid metabolism and oxidative stress. Researchers have investigated photochemical modification to

fibronectin by N-retinyl-N-retinylidene ethanolamine (A2E), the major bis-retinoid component of lipofuscin, accumulated normally with age but more pronounced in AMD. They used blue light to generate A2E-derived aldehydes in the mixture of fibronectin and A2E, observing that different A2E-derived aldehyde moieties were attached to the fibronectin peptide at multiple sites, preferentially at lysine and arginine residues[46-47]. These chemical reactions are similar to the Maillard reaction, in which different 2-alkenal species are highly reactive toward lysine and arginine residues because of their nucleophilic nitrogen base, accompanying the AGEs formation[48]. The specific modification sites of fibronectin have been verified. Modifications to laminin, which also preferentially occurred at lysine or arginine residues has been detected in a model using glycolaldehyde and A2E[49]. In another model system for aging of BM, several AGEs were generated after the incubation of a synthetic peptide, consisting of the α5β1 integrin binding region of fibronectin with glycoaldehyde or methylglyoxal. The adducts (aldoamine and Nε-carboxymethyl-lysine) attached preferably at lysine residues when reacting with glycoaldehyde. When mixed with methylglyoxal, Nε-carboxymethyl-lysine adducts were present at lysine residues, whereas hydroimidazolone and tetrapyrimidine adducts were present at arginine residues[50]. As for AGEs, they could do harms on BM's structure and function, such as changing proteins' tertiary structures and impairing their functions by cross-linking. And its accumulation could facilitate BM thickening, impeding the transport of molecules[51-52]. In addition, once ECM becomes glycation by glycoaldehyde, RPE cells could not make attachment and proliferation anymore because of the complete modification of arginine, the tripeptide Arg-Gly-Asp (RGD) binding site[53].

Recently, a new study conducted an exome-wide sequencing about AMD protein-altering variants. It aimed to detect the burden of rare variants in the COL8A1 gene[54]. COL8A1 encodes one of the 2α chains of collagen type VIII, a major component of ocular basement membranes. The localization of COL8A1 in BM has been confirmed, supporting the role of COL8A1 variants in BM changes[55]. Protein-altering variants in COL8A1 might result in changes of BM's structure. This might be responsible for AMD occurrence[56]. This rare protein-altering variants of COL8A1 could affect highly conserved residues in the C-terminal non-collagenous one domain, leading to an aberrantly folded protein, and abnormal assembly of collagen VIII and X. This could impair the transport to BM or alter its integrity or stability, contributing to early AMD developement[54]. Given that ECM protein is of paramount importance in the maintenance of BM structure and function, more protein-altering variants might exist behind AMD pathology.

Other BM-RELATED THERAPIES As a whole body system, we cannot separate diseases from each other to consider their onset and progresses. Like AMD, increasing studies are focusing on its management by integrating biological systems into a coherent and all-encompassing biological understanding. At the histopathologic level, BM resembles the vascular structure in some aspects and there are obvious similarities between the changes of BM with aging and atherosclerosis. For example, they showed consistency in lipid and lipoprotein deposits, as well as associated parainflammation. The associations between several plasma biomarkers and AMD progression have been discovered as described above. However, large epidemiologic studies did not reveal associations between serum cholesterol levels and the incidence or progression of AMD[57]. Nevertheless, it still provides insights into the management on AMD in reference to atherosclerosis.

Besides finding specific targets as new therapies, the most hopeful method for restoration of the vision loss is RPE cell transplantating. Cell transplants could be used as "rescue therapy" which is characterized by preservation and even restoration of the dead tissues. However, one main obstacle is the survival of RPE cells on BM. As described earlier, BM experiences a variety of abnormal changes in AMD, including thickness increasing, abnormal extracellular debris accumulation, protein crosslinking, non-collagenous protein deposition, lipidization, and increasing of inflammatory mediators. These untoward changes may prevent transplanted RPE cells from survival and differentiation in AMD eyes. Many researchers have made great efforts to resolve this problem, primarily attempting to mimic the properties of the BM for RPE cell culture and transplantation. Methods to produce biomimetic models include breath figure (BF), Langmuir-Blodgett (LB) transfer and combination of breath figure and Langmuir-Schaefer deposition (BF-LS). BF is a simple method to produce semi-permeable, porous, biodegradable and thin films[58]. LB transfer is thought to be powerful in the well-control of molecular organization, such as collagen substrates[59]. BF-LS utilizes semi-permeable microporous films as the substrate for LS deposition, which contains highly organized layers of collagen type I and type IV. The combination of technologies has appeared to produce quite characteristic films similar to native BM as suitable carriers for RPE adhesion, spreading and differentiation[60].

Presently, material fabrication together with tissue engineering (TE) methods have been adopted in order to create an artificial BM scaffold for subretinal replacement with full functions[61-63]. These scaffolds are cell-sheet engineering, decellularised or TE membranes. One key point is that the retinal scaffolds should be made from biocompatible materials that would

not induce foreign body responses to the host. Both natural and synthetic sources are considered. BM as well as human-amniotic membrane, lens capsule has been ever considered as naturally derived membranes for implantation into the subretinal space. However, the consistency of naturally occurring polymers is hard to control, such as collagen, which represents the major component of the BM. Their mechanical attributes and degradation might be tuned *via* various ways[61]. Also, researchers concerned the purity of these polymers and worried about the disease transmission and allergy reaction from animals to human[62]. Synthetic materials scaffolds could be controlled much better, like poly (lactic-co-glycolic) acid (PLGA). These kinds of polymers could be controlled to degrade in some particular conditions depending on its intended conditions[63].

By far, there has been no animal models which could imitate AMD successfully[64]. Instead, most studies use organ culture to analyze complicated RPE-BM interactions. Researchers found that RPE survival on BM in aging people and AMD patients could be improved using chemical therapy. This might increase efficacies of RPE suspended transplants for aging people. These results indicated RPE transplants might have a survival in AMD patients but not use scaffold[65].

Meanwhile, application of precision medicine in AMD has emerged in recent years. It has been reported that several genetic variants, such as SNPs of CFH, ARMS2 and SLC168A, had significantly associations with the risk of AMD[66]. Dysfunction of these genes might trigger alterations of BM structure and permeability, facilitating the damage caused by aging or environmental factors. Most studies regarding to gene therapy are relevant with the anti-VEGF strategies. And There have been clinical trials related to sub-retinal gene therapy in exudative AMD[67-69]. Additionally, gene therapy that targets the complement system in the inflammatory reaction which might be closely related to BM changes is being investigated for geographic atrophy[70].

CONCLUSION

The pathogenesis involved in AMD covers many aspects, including photoreceptors, RPE, BM and choriocapillaries. The pathological characteristics of AMD are triggered by metabolic dysregulation on RPE layers but exhibited significantly within BM. Lipid accumulation and protein modifications in BM are representative phenomena in the development of AMD. Currently, there are some breakthroughs on the analysis of their components and specific mechanisms of changing processes. However, we are still far from the complete understanding of these features and mechanisms behind. Advances on therapy that targeted these molecules and construction of prosthetic BM for RPE transplantation still need a long way to go.

ACKNOWLEDGEMENTS

Conflicts of Interest: Meng LH, None; **Chen YX,** None.

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