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CHAPTER 7

The Dynamic Nature of Bruch's Membrane

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Abstract

Bruch's membrane (BM) is a unique pentalaminar structure, which is strategically located between the retinal pigment epithelium (RPE) and the fenestrated choroidal capillaries of the eye. BM is an elastin- and collagen-rich extracellular matrix that acts as a molecular sieve. BM partly regulates the reciprocal exchange of biomolecules, nutrients, oxygen, fluids and metabolic waste products between the retina and the general circulation. Accumulating evidence suggests that the molecular, structural and functional properties of BM are dependent on age, genetic constitution, environmental factors, retinal location and disease state. As a result, part of the properties of BM are unique to each human individual at a given age, and therefore uniquely affect the development of normal vision and ocular disease.

The changes occurring in BM with age include increased calcification of elastic fibres, increased cross-linkage of collagen fibres and increased turnover of glycosaminoglycans. In addition, advanced glycation end products (AGEs) and fat accumulate in BM.

These age-related changes may not only influence the normal age-related health of photoreceptor cells, but also the onset and progression of diseases like retinitis pigmentosa (RP) and age-related macular degeneration (AMD). Undoubtedly, BM is the site of drusen development. Confluent drusen and uncontrolled activation of the complement cascade are most likely the first signs of AMD. Furthermore, the nature of adhesive interactions between the RPE and BM are instrumental in the development of retinal detachments and proliferative retinal disease. Finally, BM is passively or actively involved in a range of other retinal disorders such as Pseudoxanthoma elasticum (PXE), Sorsby's Fundus Dystrophy and Malattia Leventinese.

Here, we review the dynamic nature of Bruch's membrane, from molecule to man, during development, aging and disease. We propose a simple and straightforward nomenclature for BM deposits. Finally, we attempt to correlate recently published mRNA expression profiles of the RPE and choroid with molecular, structural and functional properties of BM. Our review may shed light on the complex involvement of BM in retinal pathology, notably age-related macular degeneration.

Introduction

In the past, many investigators considered Bruch's membrane (BM) a relatively boring and simple sheet of extracellular matrix, merely occupying space between the retinal pigment epithelium (RPE) and the choroid. Recently, however, interest in BM has increased exponentially; and understandably so, given its strategic location between the retina and the general circulation, and its crucial role in retinal function, aging and ocular disease. The pentalaminar BM structure forms a single functional unit with the RPE and choriocapillaris. It is involved in the essential exchange of numerous biomolecules, oxygen, nutrients and waste products in between these tissues. Given its unique location and structure, BM plays a crucial role in cell-cell communication, cellular differentiation, proliferation or migration, tissue remodelling and in shaping pathologic processes [1-4]. The nature of BM is highly dynamic, and depends on genetic factors, environmental burden, the topographic position in the retina, age and disease [5-11]. It has also become clear that BM is the focal point of local and systemic risk factors for initial stages of the most frequent untreatable blinding disorder known to man: age-related macular degeneration (AMD). In addition, BM is primarily or secondarily involved in a number of additional genetically determined ophthalmic disorders such as proliferative vitreoretinopathy (PVR)[12,13], pseudoxanthoma elasticum (PXE) [14,15], Marfan syndrome [16-18], Sorsby's fundus dystrophy [19-21] and others.

Here, we review the existing knowledge on BM, with a focus on its normal structure and function and the role of BM in normal aging and early AMD pathology. Finally, we provide an outlook on possible AMD disease prevention or treatment.

Bruch's Membrane: molecular composition, structure and normal physiology

Development of BM

The retina is a derivative of the neuroectoderm of the diencephalon. Around the 4th week of gestation, a secondary eye bubble, surrounded by ectoderm and mesenchyme, develops, which forms the future optic cup. Upon invagination of the optic cup in the sixth week of development, the future RPE and the undifferentiated neural retina can be distinguished. In the next stage, neural crest-derived mesenchyme, which will form the future choroid, starts to condensate around the optic cup. At the same time, the primitive retina, by now consisting of an RPE cell layer, an outer nuclear zone and an acellular marginal zone flanked by basal membranes, continues to differentiate.

The outer RPE basal membrane becomes incorporated in the future BM. Analysis of the developing chick retina showed that, at the tenth week of gestation, collagen fibrils are deposited beneath the basal RPE lamina. The elastic fibre layer can be detected 3-4 weeks later. Full differentiation of the elastic fibre layer to a perforated sheet is achieved by mid-term. In chicken, BM most likely continues to mature over the next weeks, months or even years [2,22]. In normal mice, initially the basal membranes of the RPE and choroid develop, followed by the development of the collagen layers, and, finally the central elastin layer [23]. Relatively little is known about the molecular and cellular events that regulate the early developmental phases of BM in man. However, it is not hard to imagine that deposition of collagen and elastin proteins is preceded by upregulation of the expression of corresponding genes in the adjacent tissues. Once BM formation has started, the ECM layers may affect cell-cell communication directly, thereby possibly creating the opportunity to further direct their own formation and differentiation. Although it is not exactly clear how BM is formed, gene expression data on adult RPE and choroid from our own lab (**Figure 1)** [11, 24], indicate that both the choroid and RPE cells are, in principle, capable of synthesizing the major components of BM. In conclusion, available evidence suggests that BM is (ultimately) formed or maintained from both the RPE and choroidal side, perhaps in a coordinated or stochastic fashion.

Ultrastructure and protein content of BM layers

According to the classification of Hogan in the early 1960’s [25], BM consists of five layers (**Figure 1**). From the RPE toward the choroid, the following layers can be distinguished histologically: the basement membrane of the RPE, the inner collagenous layer (ICL), the elastin layer (EL), the outer collagenous layer (OCL) and, finally, the basement membrane of the choriocapillaris (**Figure 1**).

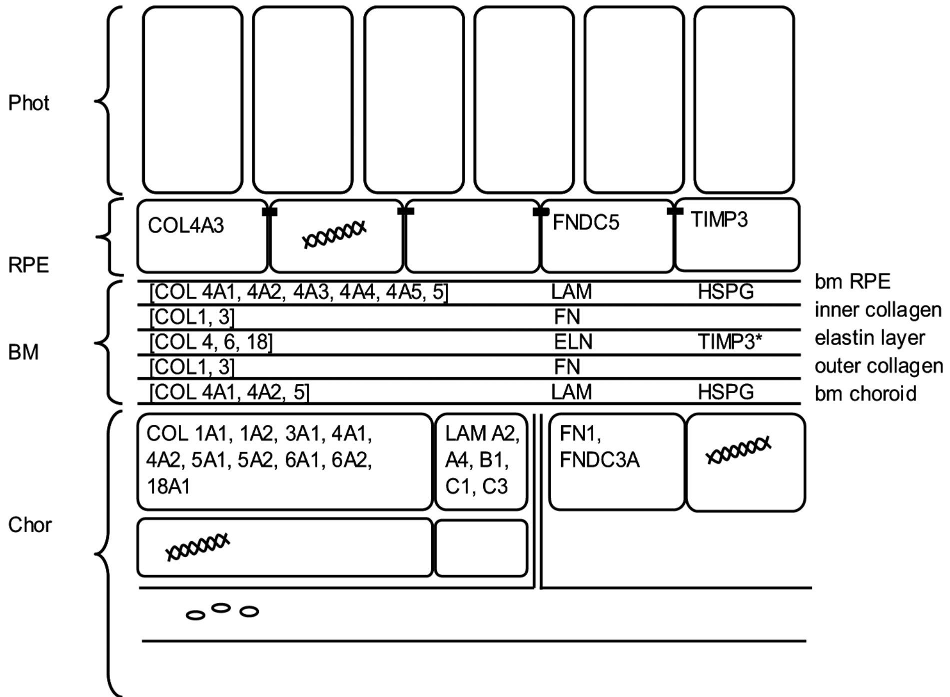

Figure 1. Schematic Drawing of Proteins Present in Bruch's Membrane and the Corresponding Genes Expressed in Adjacent Cells.

We identified three genes (*COL4A3, FNDC5, TIMP3*) with higher expression levels in the RPE than in the choroid, and 17 genes (remainder) with higher expression levels in the choroid than in the RPE. A qualitative impression of the gene expression is given. Gene expression levels were determined by RNA microarray study comparing gene expression levels from two adjacent tissue types from the same donor. Experiments were performed in triplicate (on three different healthy older human donor eyes)[24]. bm RPE: basement membrane of the RPE, bm choroid: basement membrane of the choroid. The abbreviation for basement membrane is not used in the accompanying text. A color version of this figure can be found in the color figures section.

The basement membrane of the RPE

The basement membrane of the RPE is a continuous BM layer approximately 0.14-0.15 µm in thickness in the young [26]. It resembles in many aspects other basement membranes in the body [27,28]. The RPE basement membrane contains many components similar to the basement membrane of the choriocapillaris: collagens type IV [29], laminin [30], fibronectin [31], heparan sulphate and chondroitin/dermatan sulphate [32] (**Figure 1**). In contrast, collagen type VI is not present in the basement membrane of the RPE.

The inner collagenous layer (ICL)

The inner collagenous layer is approximately 1.4 µm in diameter. The ICL consists of 60 nm thick striated fibres of collagen type I, III, and V, organized in a multilayered grid-like structure [33]. The collagen grid is embedded in a mass of interacting biomolecules, such as glycosaminoglycans (chondroitin sulphate, dermatan sulphate and hyaluronic acid) [32] and components of the coagulation and complement system.

The elastin layer (EL)

The elastin layer (EL) consists of several stacked layers of linear elastin fibres of varying shapes and sizes. The fibres form a perforated sheet with interfibrillary spaces of about 1 µm. The sheet is about 0.8 mm thick in the young and extends from the edge of the optic nerve to the pars plana of the ciliary body [33]. In addition to elastin fibres, the EL contains collagen type VI, fibronectin and other protein-associated substances. Recently, Chong and co-workers [34] found, by examining 121 human donor eyes, that the EL is three to six times thinner in the macula than in the periphery in all studied age groups. Collagen fibres from the ICL and OCL frequently cross the EL [33].

The outer collagenous layer (OCL)

The OCL is less thick than the ICL (0.7 µm in the young) but the structure and components are largely identical to those of the ICL (see above) [33].

The basement membrane of the choriocapillaris

The basement membrane of the choriocapillaris is an non-continuous, interrupted BM layer due to the so-called intercapillary columns of the choroid. The basement membrane has an average thickness of 0.14 mm in the young eye and is predominantly composed of laminin, heparan sulphate and collagens type IV, V and VI. Aisenbrey *et al*. (2006) found that the RPE synthesizes laminins that preferentially adhere BM to the RPE through interaction with integrins [30]. Heparan sulphate (HS) is a common glycosaminoglycan in the BM. The (two) HS polysaccharide side chains bind to a variety of protein ligands in BM and regulate a range of biological activities. Roberts and Forrester [22] proposed that collagen type IV in the basement membrane of the choriocapillaris may inhibit endothelial cell migration into the BM. Collagen type V is present in most types of connective tissue, particularly in pericellular spaces and near basement membranes, and plays a role in platelet aggregation, epithelial cell migration and binding of interstitial collagen fibrils [35]. Type VI collagen is the

major structural component of microfibrils, and specific for the choroidal basement membrane. It may be involved in anchoring BM to the capillary endothelial cells of the choroid [36]. Collagen VI possibly interacts with collagen I [22] which is abundant in both the BM-OCL and the choroidal matrix. A remarkable structural feature of the choriocapillaries adjacent to the choroidal basement membrane is the endothelial fenestrations, or pores, that are permeable to macromolecules [37].

Molecular composition of BM considering gene expression of RPE and choroid

Since BM is an acellular layer, it most likely depends on the adjacent RPE and choroidal cells for the production of most of its extracellular matrix constituents [9]. Furthermore, a large number of biomolecules and waste products from the RPE and choroid pass through BM and can get trapped there influencing both the structure and function of BM. Finally, the molecular composition of BM changes with age and there is an extensive turnover of ECM molecules, driven by matrix metallo proteinases (MMPs), during life.

We hypothesized that the RPE and choroidal gene expression profiles are potentially relevant to the molecular composition of BM [9] (**Figure 1**; [24]). In this regard, we observe that 1) both the RPE and choroid are capable of producing BM proteins, such as collagens, fibronectin and heparan sulphate containing proteoglycans; and 2) for those proteins known to be present in BM, the choroidal cells (endothelial cells, fibroblasts, smooth muscle cells) appear to contribute more to BM than the RPE does. In summary, approximately sixteen different proteins (subunits) were previously assigned to BM by immunohistochemistry. Two (COL4A3, TIMP3; 12.5%) of these are predominantly synthesized by the RPE, two (FN, HSP; 12.5%) are synthesized both by the RPE and choroidal cells, and the majority (i.e. numerous collagens, elastin; 75%) of the known BM proteins is mainly synthesized by choroidal cells (**Figure 1**). These data support the hypothesis of Sivaprasad and co-workers who suggested a common origin of BM and the vascular intima [38]. Finally, it is of interest to note that both the RPE and choroidal cells produce mRNA of many more collagen (subunits) and other ECM molecules, that have not (yet) been assigned to BM [9, 24].

Structure and molecular composition of BM in the macula and retinal periphery

Evidence exists that BM is structurally different in the macular area compared to the retinal periphery. Interpretation of the literature in this respect is difficult since investigators use eyes from both human donors and animal models. Also the ages of the studied eyes vary considerably and frequently retinal punches are used

from undefined or various retinal locations. Nevertheless, in sheep, topographical differences in BM thickness were observed as early as 1983 [39].

In human donor eyes of all ages, Chong and colleagues [34] found that the EL of BM in the macular area was three to six times thinner, and two to five times more porous than in the retinal periphery. RPE microarray studies in human donor eyes of age 17-36 years, performed by van Soest *et al*. [9] provided evidence that RPE gene expression of at least 33 structural BM proteins (collagens, laminins, fibronectins and a number of proteoglycans) was lower in the macular area than in the retinal periphery, while two genes showed an inversed expression pattern (COMP and THBS4). For collagen type IV chains, most fibronectin types, as well as elastin, no regional differences in RPE gene expression was observed [9, Bergen and van Soest, unpublished results]. These data suggest that the topographical differences in EL thickness observed by Chong *et al.* [34] may not be due to the higher or lower transcriptional activity of the elastin gene.

Taken together, these data may suggest that region-specific structural and functional properties and/or turnover rates of components of BM exist. With the exception of the genes *ALDH1A3, cKIT, FLJ36353, NADH* dehydrogenase, *RTBND2, TIMM17B,* and *WFDC1* [9,40,41], these gene expression data await further verification from other topographical studies on mRNA and protein level. Confirmation of additional differentially expressed genes will enable definite molecular and bioinformatic modelling of BM, and correlation of observed molecular, structural and functional differences.

BM functions

The three primary functions of BM include 1) regulating the diffusion of (bio-) molecules between choroid and RPE, 2) providing physical support for RPE cell adhesion, migration and perhaps differentiation, and 3) acting as a division barrier, restricting choroidal and retinal cellular migration (**Figure 2**). Obviously, the functional aspects are closely related to the local structure and molecular composition of BM (in the macula or in the periphery).

Diffusion properties

As BM is located between the RPE and choroid, its passive transport function is obvious. BM acts as a semi-permeable filter for the reciprocal exchange of biomolecules between the retina and the choroid. Given the acellular nature of BM, diffusion is primarily regulated by passive processes. Diffusion across BM depends on its molecular composition, which, in turn, is influenced by several factors like

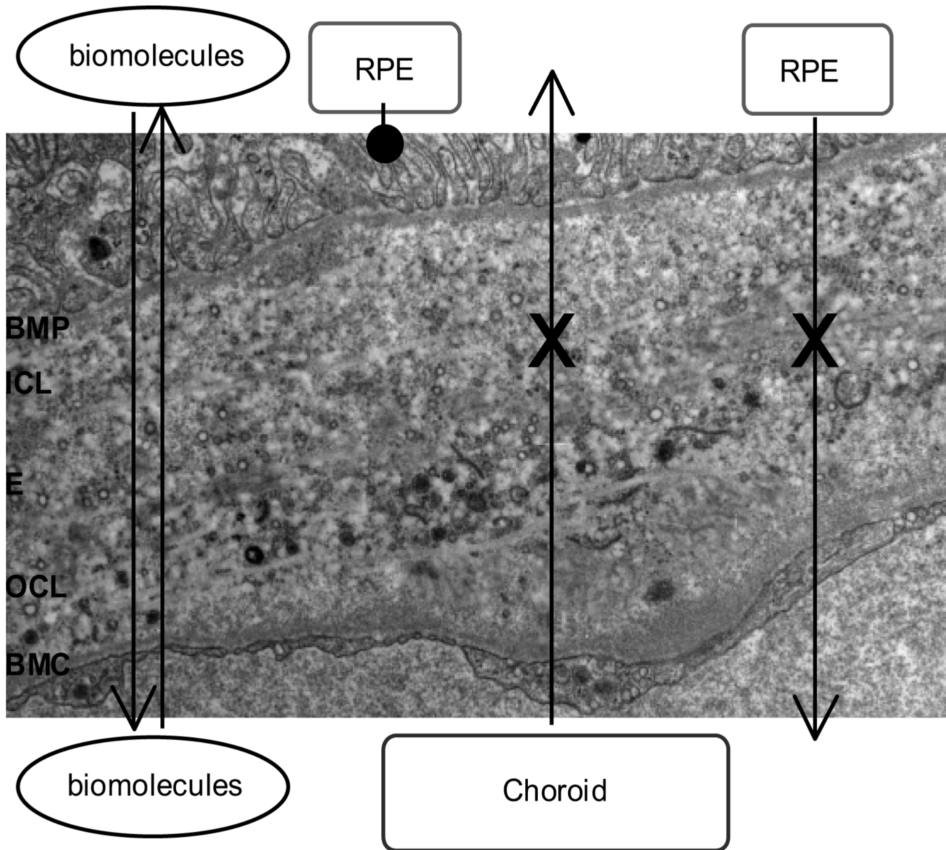

Figure 2. Schematic Drawing Showing the Normal Structure and Functions of Bruch's Membrane. Transmission Electron Microscopic image of BM courtesy of Prof. J. Marshall. BM allows for the transport of biomolecules across the membrane, it attaches RPE cells to the membrane and it acts as a physical barrier to prevent the migration of RPE cells and choroidal cells across the membrane. A color version of this figure can be found in the color figures section.

age and location in the retina. Indeed, Marshall and co-workers found a relationship between BM porosity and water flow: The EL showed the greatest pore size between the more or less randomly organized fibres and the largest water conductivity. The ICL had the smallest pores and the lowest conductivity [33].

Diffusion through the BM also depends on hydrostatic pressure on both sides of BM and on rescue and concentration of specific biomolecules and anorganic ions. Biomolecules trying to pass through the BM from the choroid to the RPE include nutrients, lipids, pigment precursors, vitamins (vitamin A), oxygen, minerals, antioxidant components, trace elements and (other) serum constituents [33,42,43] All these molecules bind to BM, or are taken up by the RPE from the bloodstream via BM, since they are needed for optimal function of the photoreceptor RPE complex

and also the neural retina.
(Bio-)molecules trying to pass BM from RPE to choroid, include CO_2, water, ions, oxidized lipids, oxidized cholesterol, and other waste products cleared by the RPE. The waste products consist of metabolic, visual cycle or electrophysiological waste from the photoreceptors or the RPE, as well as waste products from partly digested, partly oxidized, membranous fragments of shed photoreceptor outer segments (POS) [33,42-44]. Obviously, the filtration properties of BM are closely related to its structure and molecular composition and vary from the macula to the periphery. BM's permeability to water is influenced by age-related collagen cross-linking, and the build up of hydrophobic lipids (lipid wall) and membranous debris in the aging BM [45].
Using Ussing chambers, Moore *et al*. [46], and Statira *et al.* [47,48] measured the movement of water across BM-choroid samples experimentally. They found an exponential decrease in permeability to water with age, measured over time-intervals of 9.5 to 15 years; these findings were later confirmed by Hillenkamp *et al.* [49]. The rate of loss of water permeability was largest in the ICL and larger in the macular area than in the retinal periphery [48]. Interestingly, most of the loss of hydraulic permeability appears to occur in early life, long before BM debris can be visualized [50]. The latter finding suggests that, like many other biological and pathogenic processes, the molecular changes in BM precede the changes that can be visualized histologically [46,51].

RPE cell adhesion and differentiation

In addition to having filtration properties, BM also provides support and acts as an attachment site for the RPE [52,53]. BM may also act in RPE differentiation [54] and wound healing [55,56]. BM from young donors is much more efficient in the attachment of RPE cells than BM from old donors [57]. Not all layers of BM show equally strong adhesion properties. For example, the RPE basal lamina of BM is the layer that RPE cells normally adhere to [52].
RPE-BM adhesion is mediated by integrin cell surface receptors. Integrins are a group of (RPE basal) membrane proteins that is capable of binding to a number of extracellular and BM matrix components such as laminin isoforms and type IV collagen in so-called anchoring plaques [58]. Indeed, cultured RPE cells overexpressing integrins show more adhesion to BM than normal RPE cells [59].
The RPE continues to develop until approximately 6 months after birth. After that, the RPE is generally considered to be post-mitotic. However, in the event of mechanical or light-induced damage to the RPE cell layer, the RPE cells can

proliferate in a manner similar to that of other epithelial tissues. Small defects are corrected by migration of cells from the edges of the wound, in larger defects, there is also proliferation of cells. Wound healing is more efficient in the presence of the basal lamina of the RPE than when this layer of BM is absent or damaged [60]. *In vitro* wound healing is impaired upon inhibition of integrins. It is known that BM thickens and calcifies with age, which may also impede the attachment of the RPE to BM. Finally, the process of wound healing appears to be disturbed in AMD patients, possibly due to alterations in the RPE and/or BM. This is exemplified by the fact that viable RPE cells from AMD patients did not grow well in culture. Moreover, dissection of a choroidal neovascularization (CNV) membrane in AMD patients was not followed by complete restoration of the RPE cell layer [61].

Division barrier for cell migration

The outer blood-retina barrier (oBRB) is formed by RPE cells that are connected to each other by tight junctions. The oBRB prevents transport of molecules larger than 300 kDa into and out of the retina [62]. The barrier function of the RPE is physically supported by BM, that acts as a semi-permeable molecular sieve. The inner blood-retina barrier (iBRB) is comprised of a single layer of non-fenestrated retinal vascular endothelial cells connected by tight junctions [62]. If both leukocytes and endothelial cells are normal, leukocytes do not cross the iBRB [62-64]. Experiments in mice and rats showed that bone marrow-derived cells cannot easily pass the iBRB as these cells could not be detected in the retina one year after injection into the circulation [65,66]. Nonetheless, lymphocytes have been shown to infiltrate the normal retina despite an apparently intact iBRB [62] and may pass BM and the oBRB. If lymphocytes are activated, they are able to initiate a transient breakdown in the BRB, enabling sampling of the retinal environment and possibly further recruitment of inflammatory cells [62].

The aging Bruch's membrane and AMD pathology

Normal aging of BM and AMD pathology

The distinction between normal aging and pathology in, for example, AMD, is not clear cut. Interestingly, aging itself is the strongest risk factor for developing AMD. However, features of aging and disease may overlap, may be different for different cell types involved, and may even raise an almost philosophical discussion. For example, some investigators view aging itself as a pathology, that can ultimately be cured, while others do not.

Here, we consider "normal aging" those changes that occur in the majority of individuals continuously from adulthood to old age, without direct clinical consequences. It is clear, however, that these age-related, non-pathogenic changes may affect the overall fitness of cells and tissues, predisposing them to a pathogenic state. We regard "pathogenic changes" as those changes that cause local loss of function, leading to clinical consequences. Since these categories certainly overlap, it may be useful to illustrate our line of thought with a number of examples.

We consider the continuously decreasing vitality of RPE, photoreceptors and choroidal cells part of normal aging. This decrease in cellular fitness continues or is accelerated when specific pathological processes set in. This is in line with observations of Curcio *et al*. in man [67,68]. Interestingly, the rate of spontaneous photoreceptor cell loss with age may be genetically determined, since different wild type mouse strains have different rates of spontaneous photoreceptor loss [69].

We also consider the formation of lipofuscin in RPE as part of normal aging. However, we classify *excessive* lipofuscin accumulation, leading to local loss of function, cell damage and cell death, as (AMD) pathology [70].

Also, the deposition of lipids in BM can be seen as a normal aging phenomenon, until the point where the build up of the lipid wall starts to affect local RPE function.

We also think that the formation of basal laminar deposits as well as (subclinical) drusen development, and the *controlled* involvement of the complement system to clear BM debris is a normal aging phenomenon. In 90 out of 100 apparently healthy Dutch donor eyes, age between 70 and 80, we observed subclinical macular drusen after histology and PAS staining (Bergen and co-workers, unpublished results). Many investigators consider the appearance of drusen a hallmark of AMD (pathogenicity). However, in our view, pathology only sets in when the involvement of the complement system becomes *uncontrolled*, and abnormal loss of local cellular function, cell damage and cell death occur.

In summary, we support the early views of Marshall and co-workers that, with age, RPE and BM change continuously. Normal BM aging can insidiously change into (AMD) pathology [33]. Consequently, the processes underlying normal aging and AMD pathology are difficult to separate, and are discussed in a comprehensive fashion below. Our views are illustrated in **Figure 3**.

Structural and molecular changes of the aging BM

The pentalaminar structure of BM, identified at birth, undergoes age-related changes throughout the larger part of life. The molecular composition and physiological aspects of BM change dramatically. In general, there is an overall increase in thickness

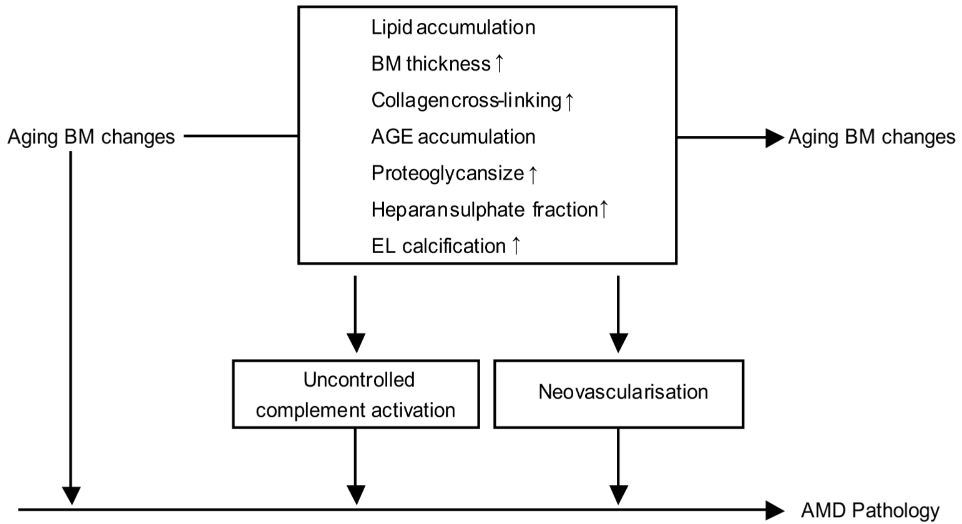

Figure 3. Changes in Bruch's Membrane with Age and its Relation to AMD Pathology.
The upper half of the picture shows the changes that occur in BM with age. The lower half of the picture show how the age related changes can progress into AMD pathology, either through uncontrolled activation of the complement system, through the occurence of neovascularisation or as an indirect result of the aging of BM.

and a reduced filtration capacity due to molecular modification and reconfiguration [50,71,72]. Remodelling of BM with age occurs at the biochemical and histological level, each causing functional changes (see **Figure 4**).

Increased cross-linkage of collagen

With age, increased collagen cross-linking occurs in BM. This has a negative effect on the permeability of BM and changes the nature of the extracellular matrix. Cross-linkage increases the strength and density of the collagen network but it decreases its elasticity, flexibility and perhaps filtration capabilities. The dense collagen network gradually becomes (more) inaccessible for the RPE collagenases which results in a less effective turnover of BM components (see **Figure 4**) [72].

Turnover of BM proteoglycans

Proteoglycans (PG) are heavily glycosylated glycoproteins that are "the glue" of extracellular matrices such as BM. PG contain a core protein covalently linked to glycosaminoglycan (GAG) chains. Individual functions of proteoglycans are determined by both the type of core protein and the type of GAG chains. Evidence from RPE cell culture experiments [32] and immuno-electron microscopy stainings [73] suggest that the RPE predominantly synthesizes heparan sulphate, which

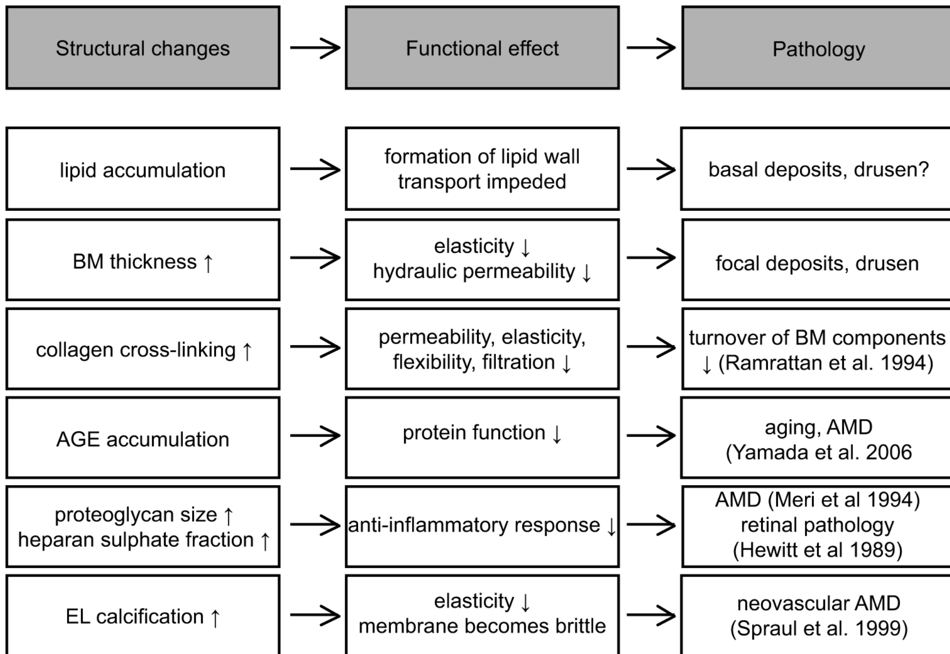

Figure 4. Schematic Overview of the Changes Occuring in the Aging BM, the Functional Consequences and the Subsequent Pathology.

is incorporated into BM. Indeed, 58% of the PGs in BM are of the heparan sulfate type, which is primary located at the basal lamina of the RPE and choroid. Forty-two percent of BM PGs are chondroitin sulphate or dermatan sulphate, which are uniquely associated with collagen fibrils [32,74].

Interestingly, *newly synthesized* PG's consist of 25% heparan sulphate and for 75% chondroitin/dermatan sulphate [32]. Since the ratio between heparan sulphate and chondroitin/dermatan sulphate in BM remains unchanged during life, these data suggested that the turnover of heparan sulphate is slower than that of chondroitin sulphate/dermatan sulphate [32,74]. RPE gene expression data of several individuals suggested that proteoglycan turnover rate is tightly controlled [11]. Nonetheless, after the age of 70 years, there is a slight shift toward larger PG's, indicating the inability of cells to normally digest the PG core protein [32]. Eyes from a limited number of donors with retinitis pigmentosa and diabetic retinopathy revealed relatively high heparan sulphate levels (55% - 64%) in BM compared with healthy controls (23%) [32,75].

PGs have important structural and filtration properties in BM, and may play a role in the anti-inflammatory response. PG molecules form structural networks through

interaction with their side chains. These interactions occur not only among the different types of PGs, but also with collagen fibres in the OCL and ICL, and, most likely, with hyaluronic acid in the interphotoreceptor space.
In terms of filtration properties, the negatively charged PG side chains cause PGs to bind water and positively charged cations such as sodium, potassium and calcium. In addition, they cause PGs to form a barrier for the passage of negatively charged macromolecules [32,76]. Thus, by physical, electrostatic and biochemical means, PGs regulate the movement of molecules through the BM matrix. Evidence also reveals that PGs can affect the activity and stability of proteins and signalling molecules within the matrix [76]. Finally, PGs, and especially heparan sulphate, may have anti-inflammatory properties. Meri *et al*. [77] showed that heparan sulphate interacts with complement factor H, an important regulator of the complement cascade, and regulator of the local immune response.
Conversely, Kelly and co-workers [78] showed that BM-associated heparan sulphate can modulate complement activity, by inhibition of the cleavage of complement factors B and C3 to, respectively Bb and C3b. Given the major involvement of the complement system and the innate immune system in the development of AMD [79], the interaction between heparan sulphate PG(s) and CFH may be one of the key molecular switches that turn normal RPE aging into AMD pathology.

Mineralization of BM

Calcification of BM

As in other soft tissues in the body, calcium can be deposited in the connective tissue of BM. In post mortem eyes, the deposits can readily be demonstrated, for example with the von Kossa staining method. Van der Schaft *et al.* (1992) studied 182 human maculae older than 33 years of age, and found calcium deposition in BM in 59% of the samples [71]. The presence and extent of the calcification was positively correlated with age, but not with AMD. However, a later study by Spraul *et al.* (1999) did demonstrate a significant correlation between BM calcification and AMD [80]. The potential correlation with neovascular AMD agrees well with the notion that calcification of BM renders the membrane more brittle and more susceptible to breaks, allowing faster neovascularization.
This finding was corroborated by the pathology observed in PXE, an autosomal recessive disease characterized by soft connective tissue calcification [81,82]. For reasons yet unknown, BM is a preferred site for these ectopic calcifications and PXE patients often also develop eye pathology. In these patients, extensive calcification of the elastic fibres of BM makes the membrane brittle, and prone to breaks. The

breaks are visible upon funduscopy as angioid streaks that radiate from the macula toward the periphery [33]. Ultimately, the breaks in BM lead to neovascularisation in these patients resulting in loss of visual acuity [83].
The mechanism of soft tissue calcification is currently the focus of intense scientific interest, especially in the cardiovascular system. These studies reveal a multitude of molecules and processes that can influence this process. It has become increasingly clear that control of calcification involves a delicate balance between pro-calcific and anti-calcific mediators [84]. Anti-calcific factors include molecules such as pyrophosphate, and several proteins such as fetuin A, Matrix Gla Protein (MGP) and vitamin K. Pro-calcific factors include high phosphate levels, damaged extracellular matrix and cell death. Many different environmental and genetic factors may be involved in BM calcification. For PXE, a mouse model was made by disrupting the causative *Abcc6* gene. Among other things, the PXE mice develop ectopic calcification in BM [85]. Further elucidation of the calcification process holds the promise that soft tissue and BM calcification can perhaps be influenced by drugs or by dietary means [86] (own unpublished observations in PXE mice, dietary influences in DCC mice [87]).

Iron depositions

It is well known that (ab-)normal levels of iron ions contribute to various disease of aging, including atherosclerosis, Alzheimer's disease, Parkinson's disease and retinal degeneration [88]. The subject of iron homeostasis and toxicity in retinal degeneration was recently and excellently reviewed elsewhere [89]. In summary, iron is essential for many metabolic processes, but can also cause damage through inducing (local) oxidative stress. An entire network of molecules, including metal receptors/transporters and ceruloplasmin, try to maintain (local) iron homeostasis: the balance between benefit and damage. However, with age, iron accumulates in the body. The resulting iron overload in the retina and RPE can cause retinal degeneration. Within the RPE cell, iron and related ferruginous compounds play an important role in the lysosome mediated build up of lipofuscin, and in general cellular oxidative stress, aging and apoptosis [90,91]. At the surface of BM, iron and other metal ions may play a role in the oligomerization of CFH molecules, thereby indirectly affecting the inhibition the complement cascade [92].

Zinc depositions

In 1988, Newsome and co-workers [93] found a beneficial effect for oral zinc supplementation in AMD. These findings were corroborated by a subsequent 6.5 year follow-up study of the AREDS population [94]. In parallel, millimolar amounts of zinc were found in sub-RPE deposits and BM [95,96]. While the true relationship between the presence of zinc and AMD is undoubtedly very complex, Nan and co-workers [92] suggested, on the basis of a series of elegant experiments, that Zn is involved in the oligomerization of CFH, and consequently in AMD related complement regulation.

Advanced glycation end products (AGEs) in BM

Advanced glycation end products (AGEs) are chemically modified glycosylated or oxidized fats and proteins. Outside the body, they are produced by smoking or cooking. The dietary intake of exogenous AGEs may be related to the AGE serum levels, AGE accumulation in tissues, and ultimately total body damage by (per-) oxidative stress. Inside the body, AGEs can be produced by the combined metabolism of fat, proteins and sugar [97,98].

In general, cellular proteolysis of AGE releases AGE by-products into the serum which can be excreted in the urine. However, extracellular matrix proteins in the body are resistant to proteolysis. Consequently, AGEs accumulate preferentially on structural proteins, like collagens in BM, where they inhibit protein function and cause age related damage [99,100]. High concentrations of AGEs in serum or tissues activate the AGE receptor (RAGE), which is present on multiple cells in the body. Local activation of RAGEs frequently aggravates diseases like atherosclerosis, diabetic nephropathy, and neurodegeneration through inflammatory and other mechanisms [101,102].

AGE accumulations containing pentosidine and carboxymethyllysine (CML) form age-promoting structures in BM, basal deposits, and choroid [103-106]. Indeed, Yamada *et al*. (2006) found that the RPE showed more intense immuno-staining for the AGE receptors RAGE and AGER1 in areas containing basal deposits than in areas of normal BM [105]. In summary, these data suggests that both specific AGEs and AGE receptors are locally present in BM, basal deposits or drusen, and promote aging and/or the development of AMD [105].

Accumulation of lipids in BM

As age increases, there is a progressive accumulation of lipids (phospholipids, triglycerides, fatty acids and free cholesterol) in BM, especially in the macular area [107].

Lipoprotein-like particle (LLPs) composition in BM resembles plasma LDL more than it does photoreceptor membrane composition [108]; moreover, these lipids are mainly derived from the RPE. Only a small proportion is of extracellular choroidal origin [45,109-111].

In young eyes, lipid inclusions, such as LLPs, small granules and membrane-like structures, are associated with fine elastin and collagen filaments in the ICL, EL and OCL. Huang and co-workers (2008) found that, once the EL and ICL were filled with particles, LLP continued to accumulate near the RPE, but did not increase in the OCL anymore [111]. Thus, with age, these lipid inclusions filled the interfibrillary spaces of the EL and accumulated in the ICL, forming the so-called lipid wall [26,45].

Holz *et al*. (1994) observed that the macula of the elderly contained seven times higher concentrations of cholesterol esters than the retinal periphery. However, these findings are not undisputed and perhaps the lipid wall thickness and content may even differ per individual [109]. Interestingly, Holz and others [107,109] also observed that the ratio of phospholipids to neutral fats varies per individual, perhaps in part depending on diet. The accumulation of lipids with increasing age, impairs the capacity of Bruch's membrane to facilitate fluid and macromolecular exchange between the choroid and the RPE or vice versa [50], which is essential for normal retinal function.

With age, small and large extracellular deposits, such as basal deposits and drusen (discussed below) slowly but surely appear in BM. These deposits contain large amounts of (un-) esterified cholesterol, oxy-cholesterols, and many other lipid-based biomolecules.

BM thickening

Throughout life, the 'normal' BM almost doubles in size [71]. In a comparative study of 120 human donor eyes, Ramrattan *et al*. (1994) found that the overall thickness of BM shows a positive linear relationship with age. BM thickness increased from 2 μm in the first decade to 4.7 μm 80 years later [72]. The largest part of BM thickening occurs in the ICL, followed by the OCL. This process starts in the retinal periphery, where RPE gene expression of most structural components of BM appears to be higher than in the macular area [9,112].

In general, BM thickening is caused by increased deposition and cross-linking of (less soluble) collagen fibres and increased deposition of biomolecules, the majority being (oxidized) waste products of RPE metabolism. There is an age-related accumulation of granular, membranous, filamentous and vesicular material eventually resulting in focal deposits and drusen. Obviously, the thickening of BM eventually leads to several functional changes, such as changes in elasticity and hydraulic permeability.

Functional changes of the aging BM

Decreased elasticity and recoil

The elasticity of the BM-choroid complex decreases with age while recoil capacity does not [33]. As discussed above, BM may loose much of its elasticity during life because of increased collagen cross-linking, calcification of the elastic fibres and AGE mediated oxidative stress damage. Overall, the decrease in elasticity and recoil is not exacerbated in AMD [33].

Decreased hydraulic permeability

The normal diffusion properties of BM are discussed above.
Age-related changes in BM, such as accumulation of (neutral) lipids, turnover of proteoglycans, as well as calcification most likely alter the biophysical properties of BM. Indeed, the overall water permeability of BM decreases with age primarily due to the changing properties of the inner half of BM [33].

Basal deposits and drusen

Nomenclature and classification

Basal deposits

Basal deposits are accumulations of waste material between the RPE and BM [113]. Two types of basal deposits exist, basal laminar deposits and basal linear deposits. Basal laminar deposits, located internal to the basement membrane of the RPE cells, contain granular material with collagen type IV, laminin, glycoproteins, glycosaminoglycans (chondroitin- and heparan sulphate), carbohydrates (N-acetyl-galactosamine), cholesterol, and apolipoproteins B and E [33,113-115]. Basal linear deposits are located in the ICL and are electron-dense, containing phospholipid granules [115,116]. Basal linear deposits are stronger markers for progression to drusen and AMD than basal laminar deposits [113].
The nomenclature of basal deposits is confusing and authors have used several different terms for numerous deposits in different layers of BM in the past (reviewed by Marshall in 1998) [33]. The frequently used term "sub-RPE deposit" [117] is also unclear, since it does not clarify the exact location of the deposit below the RPE. In our view, the most straightforward and simple classification is to use the term based on the layer(s) in which, or in between which, the deposits are detected: OCL deposits, ICL deposits, ICL-RPE-basement membrane deposits, etc. If the layer containing a deposit cannot be defined, it seems appropriate to simply use the term BM deposit or basal deposit.

The presence of basal deposits and subclinical drusen have been reported as early as in the third decade [118].

Drusen

Drusen are extracellular deposits that form below the RPE in BM. Drusen initially appear in the macular area, but certainly also occur in the retinal periphery. Several types of drusen exist, they can be defined from a clinical, histological and molecular point of view.

Clinically, drusen are defined according to their location, size and shape: ophthalmologists usually make a distinction between macular and peripheral drusen, small and large drusen, or drusen with defined (hard) and less well defined borders (soft and confluent drusen) [118,119]. The presence of soft, confluent drusen is a major risk factor for AMD [118]. When (confluent) drusen become visible by ophthalmoscopy, normal aging of the RPE and BM insidiously progresses to AMD pathology.

Histologically, drusen can be described by their size, shape and PAS staining [113,114]. They can be seen as small yellow patches, initially in the macular area under the RPE. In the case of well-defined hard drusen, histological staining usually reveals local atrophy of the photoreceptors over clearly defined mounds beneath the RPE, solidly stained by PAS. In the case of less well defined soft drusen, which may become coalescent (confluent drusen), linear granular bands can be observed locally, with a light PAS staining [113]. Lengyel and co-workers suggested that so called auto-fluorescent drusen are strongly associated with the lateral walls of the intercapillary pillars of the choriocapillaris [120].

The molecular classification of drusen is discussed below.

Molecular composition of drusen

Drusen contain acute phase proteins, C-reactive protein, complement components, complement inhibitors, apolipoproteins, lipids and many more proteins [113]. They vary in fat and cholesterol content, with a stable ratio between esterified cholesterol (EC) and unesterified cholesterol (UC). Frequently, drusen proteins are post-translationally modified [119].

Initially, a number of complement proteins were immunolocalized to drusen by Hageman and co-workers [121]. Using a proteomics approach, Crabb *et al*. (2002) subsequently identified 129 different proteins in drusen (**Table 1**) [6]. Sixty-five percent of these proteins were present in drusen from both non-affected and AMD donor eyes. The most common proteins in drusen of non-affected eyes were

tissue metalloproteinase inhibitor 3, clusterin, vitronectin, and serum albumin. The presence of crystallin, and oxidatively modified proteins (TIMP3 and vitronectin) or lipids (docosahexaenoate-containing) in drusen suggested that oxidative stress is critical for drusen formation [6]. Next, a number of additional proteins were assigned to drusen, including the amyloid beta protein [122].

Given their origin, location, and pathobiological involvement, one could possibly consider drusen a mixture between atherosclerotic plaques [123] and Alzheimer (AD) plaques [124,125]. However, comparison of the known molecular constituents of these three extracellular deposits (see **Figure 5**) showed that they only share seven proteins (amyloid (beta, P), APOE, C3, CLU, FGG and VTN). Drusen and AD plaques have 24 known molecular constituents in common. In contrast, drusen share only 10 known molecular components with atherosclerotic plaques. Therefore, we hypothesize that drusen resemble AD plaques more than atherosclerotic plaques.

Table 1. Origin of Drusen Proteins: Gene Expression in the Choroid or the RPE and the Presence of Proteins in Serum.

Primary Sequence Name	Sequence Code	Protein in drusen	Gene expression chor>RPE	Gene expression RPE>chor	Protein in serum
ACTB	NM_001101	+			
ACTG1	NM_001614	+			
ACTN1	NM_001102	+			
ALB	NM_000477	+	+		
ALDH1A1	NM_000689	+	+		+
AMBP	NM_001633	+			
ANXA1	NM_000700	+	+		+
ANXA2	NM_004039	+	+		+
ANXA5	NM_001154	+	+		+
ANXA6	NM_001155	+			
APCS	NM_001639	+			
APOA1	NM_000039	+			
APOA4	NM_000482	+			
APOE	NM_000041	+			
APP	NM_000484	+			+
ATP5A1	NM_004046	+		+	
BFSP1	NM_001195	+			
BFSP2	NM_003571	+			
BGN	NM_001711	+			
C3	NM_000064	+	+		+
C5	NM_001735	+			+
C6	NM_000065	+			
C7	NM_000587	+			
C8B	NM_000066	+			
C9	NM_001737	+			

Table 1 (continued).

Primary Sequence Name	Sequence Code	Protein in drusen	Gene expression		Protein in serum
			chor>RPE	RPE>chor	
CKB	NM_001823	+		+	
CLU	NM_001831	+	+		
COL1A2	NM_000089	+	+		+
COL6A1	NM_001848	+	+		+
COL6A2	NM_001849	+	+		+
COL8A1	NM_001850	+	+	+	
CRYAA	NM_000394	+			
CRYAB	NM_001885	+	+		+
CRYBA1	NM_005208	+			
CRYBA4	NM_001886	+			
CRYBB1	NM_001887	+			
CRYBB2	NM_000496	+			
CRYGB	NM_005210	+			
CRYGC	NM_020989	+			
CRYGD	NM_006891	+			
CRYGS	NM_017541	+			
CTSD	NM_001909	+	+	+	
DIP2C	NM_014974	+			
EFEMP1	NM_004105	+	+		+
ELN	NM_000501	+			
EPHX2	NM_001979	+			
FBLN5	NM_006329	+	+		+
FGG	NM_021870	+	+		
FN1	NM_054034	+	+		+
FRZB	NM_001463	+	+	+	
GAPDH	NM_002046	+		+	
GPNMB	NM_002510	+	+		
H3F3A	NM_002107	+			
HBA1	NM_000558	+	+		+
HBA2	NM_000517	+	+		+
HIST1H1E	NM_005321	+			
HIST1H2AE	NM_021052	+	+		+
HIST1H2BJ	NM_021058	+			+
HIST1H2BL	NM_003519	+			+
HIST1H4H	NM_003543	+			+
HIST2H2AA3	NM_003516	+	+		+
HIST2H2BE	NM_003528	+			
HIST4H4	BC111093.1	+			
HP	NM_005143	+			
IGHA1	AF067420	+			+
IGHG1	BC037361	+	+		+
IGHG2	AAH62335	+			
IGHG3	AAH33178	+			+
IGHG3	ENST00000319391	+			
IGKC	BC073779.1	+			+
LAMB2	NM_002292	+	+		
LMNA	NM_005572	+	+		+

Table 1 (continued).

Primary Sequence Name	**Sequence code**	**Protein**	**Gene expression**		**Protein**
		in drusen	**chor>RPE**	**RPE>chor**	**in serum**
LTF	NM_002343	+			
LUM	NM_002345	+			+
LYZ	NM_000239	+			
MFAP4	NM_002404	+	+		+
MYH9	NM_002473	+	+		+
MYL6	NM_079425	+			
OGN	NM_033014	+	+		+
ORM1	NM_000607	+			
PLA2G2A	NM_000300	+			+
PLG	NM_000301	+			
PRDX1	NM_002574	+			
PRELP	NM_002725	+			+
PSMB5	NM_002797	+			
RBP3	NM_002900	+		+	
RGR	NM_002921	+	+	+	
RNASE4	NM_002937	+	+		+
RPS14	NM_005617	+			
S100A7	NM_002963	+			
S100A8	NM_002964	+	+		
S100A9	NM_002965	+	+		+
SAA1	NM_000331	+	+		+
SEMA3B	NM_004636	+	+		
SERPINA1	NM_000295	+			
SERPINA3	NM_001085	+	+		+
SERPINF1	NM_002615	+	+	+	
SMC6	NM_024624	+	+		
SPP2	NM_006944	+			
SPTAN1	NM_003127	+			
THBS4	NM_003248	+		+	
TIMP3	NM_000362	+	+	+	
TNC	NM_002160	+			
TUBA1A	NM_006009	+			
TUBA1B	NM_006082	+			
TUBA1C	NM_032704	+			
TUBA3C	NM_006001	+			
TUBB	NM_178014	+			
TUBB2C	NM_006088	+		+	
TUBB3	NM_006086	+		+	
TYRP1	NM_000550	+	+		
UBB	NM_018955	+			
VIM	NM_003380	+	+		+
VTN	NM_000638	+			

Genes corresponding to proteins identified by Crabb *et al.*[6] and confirmed by Ingenuity analysis [126] to have a sequence code (see also Figure 6), '+' = Gene expression levels were found to be at least 2.5-fold higher in the choroid compared to the RPE (chor>RPE) of the same donor eye, in triplicate microarray measurements from three older healthy humans [24]. Gene expression levels at least 2.5-fold higher in the RPE than the choroid are found in the column RPE>chor indicated by a '+' [24]. '+' in the serum indicates genes with expression in serum identified by Ingenuity analysis.

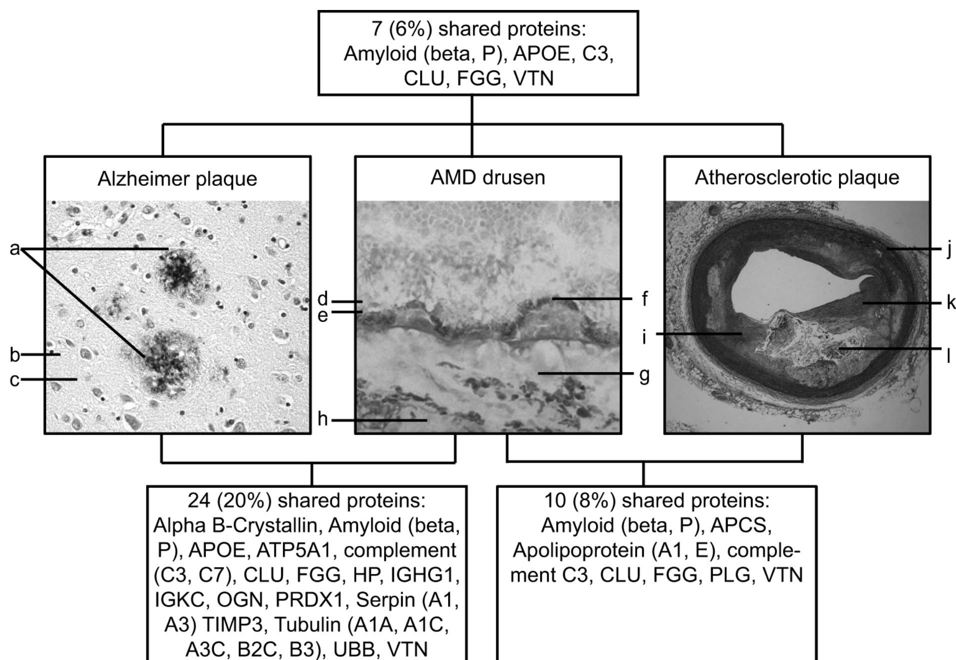

Figure 5. Overlap in Protein Content of Alzheimer Plaques, AMD Drusen, and Atherosclerotic Plaques.
Alzheimer plaque picture courtesy of Dr. I. Huitinga, Netherlands Brain Bank, donor number nhb:2006-060,VU:S06/189. Atheroslcerotic plaque picture courtesy of Dr. P Sampaio Gutierrez.
We analyzed 121 proteins based on the article by Crabb *et al.* [6] and additional literature searches. We obtained NM numbers for 85 of the proteins characterized by Crabb, and an additional 36 proteins were added based on the recent literature [167]. Note that drusen share 20% of their protein content with AD plaques and less than 10% with atherosclerotic plaques.
(**a**). Amyloid deposits, (**b**). Cell nucleus, (**c**). Enthorinal cortex, (**d**). Retinal Pigment Epithelium, (**e**). Bruch's Membrane, (**f**). Druse, (**g**). Choroid, (**h**). Choroidal bloodvessel, (**i**). Intima with atheroslerotic plaque, (**j**). Tunica media, (**k**). Fibrous cap, (**l**). Lipid + necrotic core. A color version of this figure can be found in the color figures section.

Drusen: where do they come from?

A priori, drusen constituents are most likely derived from (modified) fats and proteins produced by the 1) photoreceptor cells, and/or 2) RPE cells, and/or 3) choroidal cells (endothelial cells, fibroblasts, smooth muscle cells), or 4) derived from serum constituents. As discussed above, the majority of lipids in drusen, including (oxidized) cholesterol are derived from the RPE and photoreceptors cells, and only a small part from the serum.

To gain insight into the question: "where do drusen proteins come from?", we compared drusen protein content (modified from Crab *et al.* 2002) [6] with triplicate mRNA expression profiles from human photoreceptor cells, RPE cells and choroidal cells [24] and a proteomics profile from human serum generated by the Ingenuity

knowledge database [126] (Bergen unpublished results) (**Figure 6** and **Table 1**).
By doing so, we can track, within obvious limitations, the main potential origin of the drusen proteins. At least 23 (20%) of 113 proteins identified in drusen are present in serum [6]. Thirty-six (32%) drusen proteins are potentially (also) synthesized by local choroidal cells. Thirteen (12%) drusen proteins are potentially (also) derived from RPE cells and three (3%) (also) from the photoreceptor cells. For the sake of argument, we assumed - and of course this is an oversimplification- that the amount of mRNA expression in cells adjacent to BM is, in general, linear with the amount of protein produced and subsequently transported to drusen. In that case, it is remarkable that the larger part of drusen proteins appear to be derived from the choroidal cells and or serum, and not from the photoreceptors (**Figure 6**).
In summary, and perhaps surprisingly, human drusen consist of 1) *lipids* primarily derived from the photoreceptor cells and serum, and 2) *proteins* apparently primarily derived from choroidal cells and serum. Finally, it must be pointed out that the type and relative amount of molecular constituents of drusen, and patho-biological effects frequently do not have a linear relationship. For example, a minor fraction of serum-derived molecules from the complement cascade can have large functional or pathological consequences.

Why do drusen develop preferentially in the macular area?

It is currently not known why drusen develop mainly in the macular area. However, a combination of specific structural, molecular and functional properties may predispose the macula to develop drusen. First, the extremely high density of photoreceptors, particularly in the perifoveal ring, may play a role [127]. Local phagocytosis of photoreceptor outer segments (POS) by the RPE causes a highly focussed and localized peak of oxidative stress, and focal build-up of membraneous waste products.
In addition, the specific structural properties of BM in the macular area may also play a role. As discussed above, BM in the macula has a thinner elastic layer and a more open maze compared to the periphery. Initially, in the still healthy eye, the macular RPE and BM may get rid of an excess of oxidized molecules and neutral fats (by transporting them toward the bloodstream rapidly. Most likely, however, additional proteins reach BM from the choroidal side. After oxidative modification, a subset of these proteins may get physically or chemically trapped in BM, thereby initiating the first events of drusen formation in the macular area.
Finally, local functional macular RPE properties, as annotated by gene expression profiles, according to van Soest (2007) and Booij (2009) may also play a role [9,11].

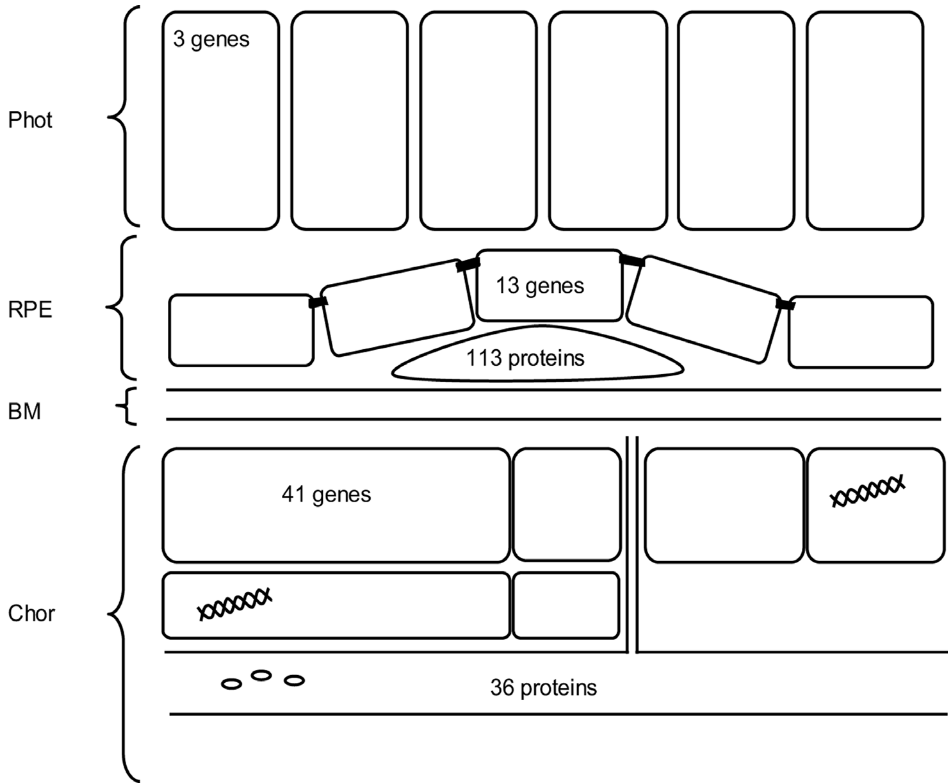

Figure 6. Schematic Drawing of Proteins in Drusen and the Corresponding Genes Expressed in Adjacent Cell Layers.

We could annotate 113 genes with genbank codes (using Ingenuity) which correspond to drusen proteins identified by Crabb *et al.*[6]. Thirty six of these genes had higher expression levels in choroid compared to RPE (chor>RPE), thirteen genes had higher expression levels in RPE than in choroid (RPE>chor) and only three genes had higher expression levels in photoreceptors than RPE (phot>RPE). Gene expression levels were determined by RNA microarray study comparing gene expression levels from two adjacent tissue types from the same donor. Experiments were performed in triplicate (on three different healthy older human donor eyes) [24]. Details of the 113 genes can be found in Table 1. A color version of this figure can be found in the color figures section.

Most importantly, we compared the data of van Soest [9] and Crabb [6]. Van Soest and coworkers identified 438 genes (out of 22.000), that were significantly differentially expressed in macular RPE compared to RPE in the retinal periphery. Crabb *et al.* identified 129 proteins in drusen using a proteomics approach [6]. Interestingly, the overlap between these two datasets consists of 16 genes, while by chance alone this overlap would be no greater than 2.5 genes (Bergen *et al.* manuscript in preparation).

Bruch's membrane and AMD pathology

Oxidative stress in the RPE and its effects on BM

Multiple studies in man, AMD animal models and (RPE) cell lines point toward an important role of oxidative stress in the development of AMD [119].

Epidemiological studies in man demonstrated that smoking is associated with increased risk of AMD. Smoking is a well known source of oxidative stress [118]. A decreased risk of AMD was found with the use of high dietary antioxidants, such as lutein and zeaxanthin or vitamin C and beta-carotene [128,129]. Animal models susceptible to oxidative stress, like *SOD1*-/- [130] and *ERCC6*-/- knock-out mice [131] show remarkable signs of retinal degeneration, if not AMD. Finally, using RPE cell lines, several investigators showed that oxidative stress is implicated in retinal degeneration [132,133].

In the macula, where photoreceptor density is high and incoming light is focussed, the RPE and BM are highly susceptible to high levels of oxidative stress. Sources of local oxidative stress include the high metabolic rate of photoreceptors required to sustain their normal function and structural renewal, the exposure to light, the high local oxygen pressure and the high metabolic rate of the RPE due to processing of photoreceptor outer segments (POS) [79].

The combination of high levels of oxidative stress and segmental POS digestion in the RPE most likely results in the oxidative modification of lipid-related molecules, such as cholesterol [134] and docosahexanoic fatty acid that accumulate in drusen or are exported to the bloodstream through BM. In addition, a vast number of molecular constituents that can neither be digested, nor exported across the plasma membrane, accumulate inside the RPE cell. A well known example is the accumulation of the bisretinoid A2E, an indigestible remnant of POS, and an important constituent of intracellular lipofuscin [135,136]. Finally, the RPE cells attempt to export unneeded or indigestible residual molecules basolaterally, where they accumulate in BM, BM BLDs, or drusen, or diffuse to the bloodstream [33].

Complement activation, inflammation and the immune response

The recent finding of genetic associations between AMD and genes from the complement system (*CFH* [137-140], *C2/FB* and *C3* [79,141,142]) established the long suspected role of the innate immune system in AMD [121,143]. The detailed role of the regulation of the complement system and all complement factors individually has recently been reviewed elsewhere [119,144].

Multiple immune-related cells, including macrophages, fibroblasts, and lymphocytes have been implicated in RPE atrophy, the breakdown of BM, and neovascularization

in AMD [143]. In the healthy and balanced situation (i.e. in the absence of AMD), the (alternative) complement system is activated just sufficiently by foreign antigens to clear up debris in BM, while at the same time invoking relatively little RPE cell cellular damage through the membrane attack complex.
But what is the trigger that activates this pathway? Zhou *et al.* found that intermediates of lipofuscin in the RPE, like the bisretinoid pigment A2E, were recognized as non-self antigens, and activated the complement cascade [145]. Alternatively, Johnson *et al.* co-localized “the Alzheimer protein” amyloid beta (Aβ) which activated complement components in drusen, and suggested that Aβ invokes a local inflammatory response [122]. Recently, the latter data were substantiated by functional studies by Wang and colleagues who showed that Aβ interaction with complement factor I activated the complement cascade [146]. Subsequently, Hollyfield and co-workers showed that oxidative modification of docosahexanoic acid, a polyunsaturated fatty acid abundantly present in the retina, resulted in a unique fragment, carboxyethylpyrrole, that can also invoke a local immune response [147]. To further complicate the issue, Scholl *et al.* reported that not only local factors, but also systemic complement serum factors can activate the alternative complement pathway in the RPE/BM [148]. Complement factors, such as CFH, and C3 are expressed in the RPE and occur in serum, while others only occur in serum [148].
In summary, it is likely that multiple non-self antigen triggers can invoke a local complement/immune response leading to AMD. The local complement response may therefore *initially* be determined by both *the type* and *the amount* of non-self-antigen present. In addition to the type and amount of trigger, the *actual activity* of the complement cascade is regulated by both genetic variation in, and biochemical interaction between a number of regulatory proteins, such as CFH, MCP (CD46), C3 and Factor I. These proteins contain complement control repeats (CCPs) that can bind to other complement factors and/or substances like CRP, heparin and heparan sulphate present at the surface of BM. Through more or less effective binding of these regulatory protein domains, due to DNA sequence variation, metal ion traces, post translational protein modifications or simply by bio-availability of regulatory proteins, the actual activity of the complement system is controlled [144].
It is of interest to note here that BM’s proteoglycan turnover and content appears to change with age (discussed above) and disease. For example, Landers *et al.* found an increased heparan sulphate content in retinas of animal models affected by retinal degeneration [149]. These changes may modify the natural binding characteristics and inhibitory complement capacity of CFH or other complement molecules at the surface of BM with age. This may be one of the factors that determine the rate of

photoreceptor cell loss during aging and neural degeneration. The interaction of local and systemic complement factors, their regulatory binding to extracellular matrix components, growth factors, and other molecules at the BM interface is, so far, poorly understood, and currently subject to thorough investigation [147,150].

Breaking the barrier: choroidal neovascularization (CNV)

The whole process of choroidal neovascularization (CNV) has recently been reviewed elsewhere [151]. In summary, CNV is a process whereby new vessels sprout from the choroid and penetrate BM. In many aspects, CNV resembles normal wound healing in the skin [151].

CNV is controlled by local pro- an anti-angiogenic factors. Among these factors is a combination of proteins secreted by the RPE and/or choroidal cells, including well studied growth factors like VEGF-A and PEDF. Another factor is the physical barrier embodied by the RPE/BM complex. Leukocytes, lymphocytes, macrophages and endothelial cells may directly or indirectly degrade BM through the breakdown of collagen, thereby facilitating neovascularization [152]. Nevertheless, new vessels can also penetrate the intact BM [152]. Specific subtypes of macrophages, mononuclear phagocytic series (MPS) cells, have been implicated in the development of new vessels in healthy and AMD eyes [153]. Furthermore, multiple neovascularization studies in normal and genetically modified mouse models support the notion that additional factors, such as the breakdown of BM integrity, are essential for the induction of CNV. To illustrate the complexity of this issue, we present in **Table 2** the genes that are expressed at higher levels in the RPE than in the choroid with the functional annotation 'angiogenesis' [126]. (Bergen and Booij; unpublished results). Analysis of our data showed that at least 23 genes may be involved in this process. If the local balance between pro- an anti-angiogenic factors is disturbed substantially in favour of VEGF, CNV or enhanced fibrosis may occur [154]. For further illustration purposes, **Table 3** shows the genes that are expressed at higher levels in the choroid than in the RPE with the functional annotation 'angiogenesis' [24].

Table 2. The Overlap between Genes Associated with the Term Angiogenesis (Ingenuity) and Genes Identified in Triplicate RNA Microarray Measurements from Older Healthy Human Donor Eyes [24] with Expression Levels Higher in the RPE than the Choroid.

Gene name	Sequence code
BAI1	NM_001702
EGF	NM_001963
EPAS1	NM_001430
EPHA2	NM_004431
FLT1	NM_002019
HGF	BC022308
HMMR	NM_012484
IGF1R	NM_000875
IGHG1	AF035027
IL2	NM_000586
IL18	NM_001562
INSR	NM_000208
MAPK8	AK125150
MFGE8	NM_005928
MMP9	NM_004994
NOS1	NM_000620
NOS3	NM_000603
PLG	NM_000301
PTHLH	M31157
SERPINF1	NM_002615
SOD1	NM_000454
TP73	NM_005427
VEGFA	NM_003376

Table 3. The Overlap between Genes Associated with the Term Angiogenesis (Ingenuity) and Genes Identified in Triplicate RNA Microarray Measurements from Older Healthy Human Donor Eyes [24] with Expression Levels Higher in the Choroid than the RPE.

Gene name	Sequence code
ALOX5	NM_000698
ANPEP	NM_001150
APOE	NM_000041
C3	NM_000064
C5	NM_001735
C3AR1	NM_004054
CCL13	NM_005408
CFB	NM_001710
COL18A1	NM_030582
CSF2	NM_000758
CXCL12	NM_000609
CYR61	NM_001554
EFNA1	NM_004428
HGF	NM_000601
HMMR	BC035392
ICAM1	NM_000201
IGF1	NM_000618
IGF2	NM_000612
IGFBP3	NM_000598
IGHG1	BC037361
IL13	NM_002188
INHBA	NM_002192
ITGB2	NM_000211
LEP	NM_000230
MAPK8	AL137667
MYC	NM_002467
NR3C1	NM_000176
NRP1	NM_003873
PLAU	NM_002658
PTGS2	NM_000963
S100A4	NM_002961
SCYE1	NM_004757
THBS2	NM_003247
TIMP2	NM_003255
TNF	NM_000594
TP53	NM_000546
VCAM1	NM_001078
VEGFC	NM_005429

Outlook and perspectives: Toward rational, genomics-driven, molecular therapies for AMD

Summarizing the events leading up to early AMD

The molecular pathology of AMD has recently been reviewed extensively elsewhere [119]. In summary, Ding *et al*. (2009) reviewed clinical, epidemiological, and genetic aspects of AMD, as well as the use of mouse models for potential AMD therapy. In contrast, we here reviewed the central role of BM in normal retinal aging, in drusen formation and in the early stages of AMD.

Obviously, the normal function and pathology of BM can only be understood in the context of the molecular and cellular events involving the adjacent cell layers (photoreceptor, RPE and choroid) as well as systemic factors (from serum). As discussed, many normal aging processes affect BM, such as thickening of its layers due to fat deposition, calcification of the EL, oxidative stress and drusen formation. Clearly, these normal (subclinical) aging events may predispose BM and the RPE to disease, especially in the macular area. BM is the key acellular tissue involved in the development of age-related macular degeneration. Its extracellular matrix, heavily dominated by heparan sulphate, appears to be the regulatory playground of both local and systemic interactions involving complement activators, proteoglycans, chemokines, cytokines, growth factors and, above all, toxic waste products.

In the healthy, non-AMD, situation these molecular interactions may follow a fixed pattern, which maintains the local homeostasis. This local homeostasis in each individual probably depends on, and is limited by environmental factors, genetic constitution and local anatomy of the neural retina, RPE and BM. However, fuelled by changes due to normal aging, such as prolonged oxidative stress and immune activation, the molecular interactions at the surface of BM change. These changes may be accommodated until local homeostasis cannot be maintained anymore, which ultimately leads to the devastating clinical manifestations of AMD.

Prevention and therapy of AMD; is there a role for BM biology?

So, with the current state-of-the-art knowledge, can we “prevent” or “cure” AMD?

Over the last decades our understanding of environmental risk factors as well as molecular, cellular, and even systemic events underlying AMD has grown tremendously. In summary, environmental risk factors now include smoking, diet and perhaps light exposure [118]. Dietary intake of saturated fats increases the risk of AMD [155]. Intake of anti-oxidants (lutein, zeaxanthin, beta carotene, vitamin C), omega-3 polyunsaturated fatty acids (nuts, fish) and zinc supplements may

be beneficial [94]. Genetic association studies were successfully used to implicate several AMD disease genes (*APOE, CFH*, *C3*, *C2/BF, HTRA1/ARMS2*) [148], or to identify potential candidate disease genes (*CXCR3*, *Il-8*, *ERCC6*) [119]. Genetic and functional studies were instrumental to the discovery of functional pathways in AMD. The most important are, as discussed above, fat metabolism, oxidative stress, complement activation, and *STAT3/VEGF* induced neovascularization. So far, prevention and therapeutic efforts have largely focussed on these four pathways.

By far the best option is, of course, to prevent AMD; that is to aim to postpone its onset or to slow its progression. Most likely, for a large majority of individuals, this can be done by avoiding risk factors for AMD. Although not every individual would benefit equally due to differences in their genetic constitution [156], one should quit smoking, wear sunglasses, and change to a diet that contains sufficient zinc, anti-oxidants and unsaturated fatty acids.

Alternatively, is it possible to influence the progression of AMD by drugs? Several negative and positive developments can be noted. Previously, serum, lipid lowering drugs, like statins, were used to treat AMD, but the outcome of clinical trials were variable [157]. In addition, the obvious idea of local manipulation of the complement system in AMD by complement inhibitors may reduce neovascularization [158], but is not without risk: It may turn an essentially useful chronic inflammation at the RPE-BM interface into a harmful acute inflammation.

On the positive side, a CNTF trial is ongoing that aims to supplement the photoreceptors with small quantities of CNTF over a prolonged period of time [159]. In this case, the photoreceptors remain more viable, which may delay disease onset. In addition, for dry AMD, a drug called fenretinide, which halts the accumulation of retinol-related toxins, thought to be involved in vision loss, shows promise [160,161]. Finally, VEGF based treatments have, of course, been relatively successful in treating the wet form of AMD [162].

These new (experimental) therapies focus on cells (photoreceptors, RPE) or on systemic factors (e.g. statins). However, aside from studies by Del Priore *et al.* [163] and Marshall (unpublished), curiously enough, so far, little attention has been paid to BM, the prime site of AMD development. As discussed, BM not only plays a key role in normal aging of the photoreceptor-RPE-BM-choroid complex, but is also essentially involved in pathogenic effects of fat metabolism, oxidative stress, complement activation, and neovascularization. Even a number of structural BM genes were identified as AMD genes (*Fibl-3* (*EFEMP-1*), *Fibl-5*, *Fibl-6*, *CTRP5*) [79]. For at least two of these genes, a corresponding animal model (*ctpr5* -/-) [164], *Fibl-3* [165] showing AMD like features, is available. At least, in terms of local BM therapy, there

are two options: 1) removal of pathogenic or non-self compounds from BM which slowly accumulate during aging and disease; or 2) "medical bioremediation" [166]: the use of microbial enzymes to augment or restore missing, or failing, metabolic functions. However, both approaches have been largely unsuccessful up till now. Further dissection and definition of the molecular events involved in age-related BM changes is therefore warranted to successfully develop drugs: To end with a sentence from the beginning of this article: For too long many investigators have considered BM to be a relatively boring and simple sheet of extracellular matrix, merely occupying space between the retinal pigment epithelium (RPE) and the choroid. This is about to change.

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