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Structure, function, and pathology of Bruch’s membrane

Introduction, history, embryology

Bruch’s membrane is a thin (2-4 μm), acellular, 5-layered extracellular matrix located between the retina and choroid (1, 2). It extends anteriorly to the ora serrata, interrupted only by the optic nerve. Tissue resembling Bruch’s membrane is visible anterior to the ora serrata extending forward to the pigmented epithelium of the ciliary body. Bruch’s membrane lies between the metabolically active retinal pigment epithelium (RPE) and a capillary bed (choriocapillaris) and thus serves two major functions as the substratum of the RPE and a vessel wall. It has major clinical significance because of its involvement in age-related macular degeneration (AMD) and other chorioretinal diseases.

Early history

Carl Ludwig Wilhelm Bruch first isolated the “lamina vitrea” that we now know as Bruch’s membrane, and described it in his 1844 doctoral thesis (3, 4) where he also first described the tapetum found in many mammals. By light microscopy, Bruch’s membrane appeared transparent with little internal structure. Later studies by A.E. Smirnow (5) divided this membrane into an outer elastic layer (first described by Sattler in 1877) and an inner cuticular layer, separated by a dense plexus of very fine elastic fibers. (6, 7)

Development of Bruch's Membrane

The bipartite character of Bruch’s membrane arises from the embryology of its tissue. When the optic cup invaginates and folds, its inner layer forms the neural retina, and its outer layer, the RPE. The RPE lies in contact with mesenchyme. At this apposition, Bruch’s membrane forms by 6-7 weeks gestation. Thus, its inner layer is composed of ectodermal
tissue and its outer, mesodermal. At the border of two layers, the elastic layer forms last, becoming histologically visible by 11-12 weeks. (8-10)

The collagen that fills the extracellular space, and the later appearing elastin, appear to be made by invading fibroblasts and the filopodia of endothelial cells lining the adjacent choriocapillaris. The two basal laminae are produced by their associated cell layers. (11). In addition to Col IV subunits specific to specialized basal lamina, RPE expresses genes for structural collagen III and angiostatic collagen XVIII in a developmentally regulated manner linked to photoreceptor maturation (12).

By week 13, fenestrations are apparent in the endothelium facing Bruch’s membrane, (10) indicating that at this stage, transport across this tissue may be functional. Choroidal endothelial cells originate from para-ocular mesenchyme. Development of the choroidal vasculature, and Bruch’s as part of it, depends on differentiated RPE and its production of inductive signals, including bFGF and VEGF (13).

Structure of Bruch's Membrane in the young adult eye

Hogan’s 5-layer nomenclature for Bruch’s membrane (14) is commonly used. Gass proposed a 3-layer system that did not include the cellular basal laminae as part of Bruch’s proper (15). These layers are shown in Figure 1 and their constituents in Table 1.

RPE basal lamina (RPE-BL)

This ~ 0.15 μm thick layer is a meshwork of fine fibers like other basal laminae in the body (16, 17). The RPE-BL resembles that of the choriocapillaris endothelium but does not contain collagen VI. The RPE-BL contains collagen IV α3-5 (18), like that of kidney glomerulus, another organ with specialized filtration and transport functions. The RPE
The ICL is ~1.4 μm thick and contains 70 nm-diameter fibers of collagens I, III, and V in a multi-layered crisscross, parallel to the plane of Bruch’s membrane (1). The collagen grid is associated with interacting molecules, particularly the negatively charged proteoglycans chondroitin sulfate and dermatan sulfate (20) (21).

The EL consists of stacked layers of linear elastin fibers, crisscrossing to form a 0.8 μm thick sheet with inter-fibrillary spaces of ~1 μm. This sheet extends from the edge of the optic nerve to the ciliary body pars plana (1). In addition to elastin fibers, the EL contains collagen VI, fibronectin, and other proteins, and collagen fibers from the ICL and OCL can cross the EL. Some EL elastin fibers are said to cross the tissue space between the choriocapillaris and join bundles of choroidal elastic tissue (22). The EL confers biomechanical properties, vascular compliance, and anti-angiogenic barrier functions. It is more discontinuous in the macula, perhaps explaining why choroidal neovascularization is more prominent there (23). This concept is supported by the extensive laser-induced neovascularization in mice deficient in lysyl oxidase-like 1, an enzyme required for elastin polymerization (24).

The OCL contains many of the same molecular components as the ICL, and the collagen fibrils running parallel to the choriocapillaris additionally form prominent bundles.

This layer, unlike the ICL, has periodic outward extensions between individual
choriocapillary lumens called intercapillary pillars, where thickness cannot be determined
due to the lack of a boundary. Between pillars, OCL thickness can range from 1-5 μm (25).

Choriocapillaris basal lamina (ChC-BL)

This 0.07 μm-thick layer is discontinuous with respect to Bruch’s membrane due to
the interruptions of the intercapillary pillars of the choroid. It is continuous with respect to
the complex network of spaces defined by the choriocapillary lumens because the basal
lamina envelops the complete circumference of the endothelium. A remarkable structural
feature of the adjacent choriocapillary endothelium is fenestrations that are permeable to
macromolecules (Figure 2) (26). This basal lamina may inhibit endothelial cell migration
into Bruch’s membrane, as do basal laminas associated with retinal capillaries (27).

Bruch’s membrane in an aged eye

Aging is the largest risk factor for developing AMD (28), and Bruch’s membrane
undergoes significant age-related changes. Identification of factors predisposing to disease
progression is a priority. This task has been challenged by difficulty imposed by the thinness
of the tissue, and the closely integrated functions of RPE, Bruch’s, and choriocapillaris.

Current opinion holds that RPE and Bruch’s membrane age in concert, and normal Bruch’s
membrane aging transforms insidiously into AMD pathology (1, 16, 17, 29). This section
covers aging, to inform the following section on function.

Lipid accumulation: Bruch’s membrane lipoproteins

Early electron microscopists described aged Bruch’s membrane as being filled with
debris, including amorphous electron dense material, membrane fragments, vesicles, and
calcification (1, 25). Debris deposition in ICL and OCL begins in the second decade in the
macula and is delayed in equatorial regions, a regional lag also reported for individual
components (30). Identifying this material has been a fruitful approach to understanding antecedents of disease.

Most prominent among the changes in Bruch's membrane is a profound accumulation of lipids. Clinical observations on fluid-filled RPE detachments in older adults led to Bird and Marshall’s hypothesis that a lipophilic barrier in Bruch’s blocked a normal, outwardly-directed fluid efflux from the RPE (31) (as opposed to leakage from choroidal neovascularization). This hypothesis motivated a seminal histochemical study by Pauliekhoff (32) that demonstrated oil red O-binding material (EC, esterified cholesterol; TG, triglyceride; FA, fatty acid) localized exclusively to Bruch’s membrane, unlike other stains. This lipid was absent <30 years, variably present at 31-60 years, and abundant at ≥61 years (33, 34). A specific fluorescent marker, filipin, which binds the 3-β-hydroxy group of sterols to reveal unesterified (free) cholesterol (UC) or EC depending on tissue pre-treatment (35), indicated that EC is a prominent component of the oil red O binding deposition. (35) Macular EC rose linearly from near zero at age 22 yr to reach high and variable levels in aged donors. EC was detectable in periphery at ~1/7 macular levels and increased significantly with age. Hot stage polarizing microscopy (34) similarly demonstrated prominent age-related increases in EC in Bruch’s membrane, manifest as liquid crystals (“Maltese crosses”) when examined through a polarizing filter. Few birefringent crystals signifying the neutral lipid TG were found.

Histochemical, ultrastructural, biochemical, gene expression, and cell biological evidence now indicate that the EC-rich material accumulating with age in Bruch's membrane is a lipoprotein containing apolipoprotein B, assembled by the RPE (36). This process, ongoing throughout life yet first revealed by aging, has implications for formation of AMD-specific lesions, intraocular transport, RPE physiology, nutrition of outer retina, and
maintenance of photoreceptor health. In 1926, Verhoeff speculated that lipid deposition might precede Bruch’s membrane basophilia and fragmentation, common in older eyes due to “lime salts {calcification} in the elastic layer” (37).

Ultrastructural studies described in Bruch’s membrane of older eyes (36) numerous small (<100 nm), round electron-lucent vesicular profiles, implying aqueous interiors. Lipid-preserving preparation techniques together with extraction studies show that these so-called vesicles are actually solid, lipid-containing particles, now considered lipoproteins (Figure 3B). These methods include post-fixation in osmium paraphenylenediamine (OTAP) (35) and most strikingly, quick-freeze/deep-etch (QFDE), a freeze fracture method with an etching step to remove frozen water (38-40). Particles vary in size from 60-100 nm but could be as large as 300 nm, occasionally appearing to coalesce (Figure 3).

Lipoprotein particles are first seen among fibrils of the elastic layer in early adulthood, extending inward to ultimately fill most of the open space of the ICL by the seventh decade of life (40). Most fatefully, a new layer, the Lipid Wall (38), then forms with solid particles stack 3-4 deep occupying nearly 100% of a space between RPE basal lamina and OCL of many older eyes. The Lipid Wall displaces ICL collagen fibrils that anchor the RPE basal lamina (Figure 3). It is a considered a precursor to basal linear deposits, a specific lesion of AMD (see below).

Lipoprotein composition can provide clues to sources of its components (41). When isolated (Figure 4A), Bruch’s membrane lipoproteins are found to be EC-enriched (EC/total cholesterol = 0.56; EC/TG = 4-11; Figure 4B). For comparison, hepatic VLDL, of similar diameter, is TG-rich. An early report of TG-enriched Bruch’s membrane neutral lipid (42) was not replicated. Abundant EC points to the only mechanism by which neutral lipids are released directly from cells, an apoB-containing lipoprotein, like hepatic VLDL or intestinal
chylomicrons. Significantly, RPE expresses the apoB gene and protein, along with microsomal triglyceride transfer protein (MTP), required for apoB lipidation and secretion. Lack of functional MTP is the basis of abetalipoproteinemia, a rare inherited disorder that includes a pigmentary retinopathy. (43, 44) The combination of apoB and MTP within native RPE marks these cells as a constitutive lipoprotein secretors (45). Secretion of full-length apoB has been demonstrated in rat-derived and human-derived RPE cell lines. (46, 47) Consistent with an RPE origin, particles first appear in the elastic layer of Bruch’s membranes and fill in towards the RPE. (39)

Indirect evidence that Bruch’s membrane lipoproteins are of intraocular origin also emerges from the epidemiologic literature. If the EC deposition in Bruch’s membrane and AMD-associated lesions were a manifestation of systemic perifibrous lipid and atherosclerosis, then a strong positive correlation between disease status and plasma lipoprotein levels, like that documented for coronary artery disease (48), might be expected but such has not emerged (49).

Identifying the upstream sources of Bruch’s membrane lipoprotein constituents is essential for understanding the biological purpose of this pathway and the prospects for eventual clinical exploitation. Studies using isolated lipoproteins from Bruch's membrane (50) and Bruch’s membrane-choroid EC (51) report a high mole percentage of linoleate (>40%) and low docosahexaenoate (<1%), for all lipid classes (52). This composition strongly points away from photoreceptor outer segments (35% docosahexaenoate in membrane phospholipids) as an upstream source, as long postulated (53) (54), and towards plasma lipoproteins (45-55% linoleate in all lipid classes). These data have been interpreted to signify that plasma lipoproteins are major contributors upstream to an apoB lipoprotein of
RPE origin. In contrast, the source(s) of UC in Bruch’s lipoproteins are not yet known and could be outer segments, plasma lipoproteins, endogenous synthesis, or a combination.

Lipoproteins may thus be assembled from several sources, including outer segments, remnant components from the photoreceptor nutrient supply system, and endogenous synthesis. According to this model (52), plasma lipoproteins serve as vehicles for delivery of lipophilic nutrients (carotenoids (55), vitamin E, and cholesterol (56)) to photoreceptors by RPE, which has functional receptors for LDL and HDL (57, 58). Nutrients are stripped from these lipoproteins by the RPE for delivery to the photoreceptors, and the remnants are repackaged for secretion into Bruch’s membrane as part of apoB-containing lipoproteins, where they begin to accumulate during age and become toxically modified to instigate inflammation in AMD.

Other aging changes

Bruch’s membrane thickens throughout adulthood (20-100 yr), 2-3-fold under the macula and becoming more variable between individuals at older ages (25, 59, 60). Equatorial Bruch’s membrane changes little while Bruch’s membrane near the ora serrata increases 2-fold during this time (25). In the macula, the OCL thickens more prominently than the ICL (61). A large ultrastructural study of 121 human donor eyes demonstrated that the macular EL is 3-6 times thinner than peripheral EL (23) at all ages.

Unbalanced regulation of extracellular matrix molecules and their modulators matrix are thought to result in Bruch's membrane thickening. Increased histochemical reactivity for glycoconjugates, glycosaminoglycans, collagen and elastin is seen in the macula relative to equator and near the ora serrata (25). Collagen solubility declines with age (62). Metalloproteinases MMP-2 and MMP-3 increase with age as does a potent inhibitor of metalloproteinases, TIMP-3. TIMP-3 immunoreactivity reaches adult levels at 30 yr of age.
near vasculature in lung, kidney, and in Bruch’s membrane, signifying the end of developmental organogenesis (63). The reduction or absence of TIMP-3 is pro-angiogenic, as this protein not only regulates metalloproteinases during the normal turnover of Bruch’s membrane matrix components but it also binds to VEG-F (64, 65).

The EL thickens with age but decreases relative to overall thickening of Bruch’s membrane (23). Thus elastin referenced to other Bruch’s constituents, as detected by Raman spectroscopy, decreases with age (66). Similar arguments can be made for collagen III and IV. A prominent age-change (67), noted early (37), is calcification and ensuing brittleness. This process involves fine deposition of electron-dense particulate matter (14) confirmed as calcium phosphate (68) on individual elastin fibrils.

Long-lived proteins like collagens are modified in vivo by non-enzymatic Maillard and free radical reactions to yield advanced glycation end products (AGEs) and the formation of lipid-derived reactive carbonyl species like malondialdehyde (MDA), and 4-hydroxyhexenal (HHE), collectively called age-related lipo-peroxidation end products (ALEs). Accumulation of AGEs and ALEs, characteristic of diabetes and atherosclerosis, also occurs in aging Bruch’s membrane (Table 1). Finally, other components more prominent in aged eyes include complement components C3d, C5b-9, and pentraxin-3, a homolog of the acute phase respondent C-reactive protein. Thus, at the molecular level, aging Bruch’s membrane contains evidence of many biological activities including remodeling, oxidative damage, and inflammation, in addition to lipoprotein accumulation.

Function of Bruch’s membrane

As a vessel wall of the choroid, Bruch’s membrane primary function is structural, like other vessel walls. Its architecture is similar to vascular intima, with a sub-endothelial
extracellular matrix and elastic layer corresponding to the internal elastic lamina. The
abluminal surface of Bruch’s differs from other vessel walls in that it abuts a basal lamina,
that of the RPE. The luminal surface faces a fenestrated vascular endothelium and basal
lamina, making Bruch’s membrane structurally analogous to the renal glomerulus and
providing a basis for commonality between retinal and kidney disease (69-71). The
importance of fluid and macromolecular transport across the renal glomerulus is well known
(72). Transport is a second important function of Bruch’s membrane.

Structural Role of Bruch’s Membrane

Bruch’s membrane encircles more than half the eye and stretches with the corneoscleral
envelope as intraocular pressure (IOP) increases. It therefore withstands this stretch and
return to its original shape when IOP decreases. This tissue also stretches to accommodate
changes in choroidal blood volume. Finally, the choroid (and Bruch’s membrane with it) may
act as a spring that pulls the lens during accommodation. (73, 74) For these reasons, then,
Bruch’s membrane requires elasticity. Marshall and Hussain’s group estimated the modulus
of elasticity in Bruch’s membrane-choroid preparations to be 7-19 MPa). (75) These values
are similar to those of sclera (although sclera is much thicker and thus can support more load)
consistent with the notion that Bruch’s membrane contributes to load bearing. After early
adulthood, the modulus of elasticity of human Bruch’s membrane-choroid complex increases
(p<0.001) at a rate of ~1% per year. Bruch’s membrane stiffness in AMD eyes does not
differ from age-matched normals (76).

Transport Role of Bruch’s Membrane

The choroid services the metabolic needs of the outer retina, facilitated in part by
fenestrated endothelium. Oxygen, electrolytes, nutrients and cytokines destined for the RPE
and photoreceptors pass from the choriocapillaris and through Bruch’s membrane, and waste
products travel back in the opposite direction for elimination. Vitamins, signaling molecules, and other factors needed for photoreceptor function are carried to the RPE by lipoprotein particles passing through Bruch’s membrane, as do the RPE-produced lipoproteins that are eliminated in the opposite direction. The RPE pumps water from the sub-retinal space to counter the swelling of the interphotoreceptor matrix glycosaminoglycans (GAGs). This fluid also flows across Bruch’s membrane to reach the circulation. Thus, many transport processes involve Bruch’s membrane, as reviewed here.

**HYDRAULIC CONDUCTIVITY OF BRUCH’S MEMBRANE**

GAGs are concentrated in the interphotoreceptor matrix (77, 78) and corneal stroma (79). In both locations, these highly charged macromolecules maintain geometric fidelity essential for vision (periodic collagen spacing for corneal transparency, orderly photoreceptor spacing for visual sampling (78, 80, 81)). GAGs generate significant swelling pressure (up to 50 mm Hg in cornea). (82, 83) Without a mechanism to maintain tissue deturgescence, GAGs would imbibe fluid, swell, destroy tissue geometry, and interfere with visual function. Corneal endothelium forestalls swelling by continuously pumping fluid out. This function is accomplished for retina by the RPE, and its failure can lead to retinal detachment. A driving force adequate to overcome the collective flow resistance of RPE, Bruch’s membrane, and choriocapillaris endothelium is provided by a gradient in fluid pressure and oncotic pressure (the osmotic pressure generated by plasma proteins). This balance is embodied by Starling’s Law that characterizes the relationship between fluid flux ($q$: flow per unit area; positive when flow is out of the blood vessel) across a capillary vessel wall and the forces driving this flow:

$$q = L_p \ast (\Delta P - \sigma \Delta II)$$  \hspace{1cm} (1)
$L_p$ is hydraulic conductivity, which characterizes the ease with which fluids flow cross the vessel wall. If the surface area of the blood vessel is $A$, then $1/(L_pA)$ is the flow resistance of the vessel wall. $\Delta P$ is the difference between the fluid pressure within the blood vessel ($P_{cc}$) and the pressure at the basal surface of the RPE ($P_{RPE}$). $\Delta \Pi$ is the difference between the oncotic pressure within the blood vessel ($\Pi_{cc}$) and that at the basal surface of the RPE ($\Pi_{RPE}$). $\sigma$ is the reflection coefficient that characterizes the extent to which the vessel wall rejects the plasma proteins species generating $\Delta \Pi$. $\sigma$ ranges from 0 for a freely permeable species to 1 when a species is completely rejected by the membrane.

We can estimate the magnitude of $\Delta P - \sigma \Delta \Pi$ using measured value of $q$ and $L_p$. The fluid pumping rate by human RPE has been measured as $q=11 \, \mu$L/hr/cm$^2$, similar to that in other animals (Table 2). The hydraulic conductivity of macular Bruch’s membrane/choroid of healthy young humans ranges from $20-100 \times 10^{-10}$ m/s/Pa. Then, using $q=11 \, \mu$L/hr/cm$^2$ and $L_p=50 \times 10^{-10}$ m/s/Pa, we can calculate that the magnitude of $(\Delta P - \sigma \Delta \Pi)$ necessary to drive this flow through Bruch’s membrane is roughly 0.05 mm Hg. (This does not include the flow resistance of choriocapillaris endothelium, which is not measured when $L_p$ of a Bruch’s membrane/choroidal preparations is determined. For this highly fenestrated endothelium, $L_p$ can be estimated as roughly $25 \times 10^{-10}$ m/s/Pa (85), which does not affect our conclusions.)

$\sigma$ can be roughly estimated by assuming that the fluid in the suprachoroidal space is in equilibrium with blood in the choroid. Using measurements of fluid pressure and of the plasma protein concentration (to estimate oncotic pressure) inside and outside of the choriocapillaris (86) (87) (88), equation (1) can be used to find $\sigma \approx 0.5$. 
Allowing that $\Pi_{cc} = 27$ mm Hg, $P_{cc} = IOP + 8$ mmHg, $\Pi_{RPE} = 0$ mm Hg (fluid pumped by the RPE is assumed protein-free), and we take $P_{RPE} = IOP$ (assuming no pressure is generated by the RPE above that necessary for crossing Bruch’s), we find that $\Delta P - \sigma \Delta II$ is approximately $-5.5$ mm Hg pulling fluid into the choroid. Thus, in normal young adults, oncotic pressure within the choroid is more than sufficient to adsorb all the fluid pumped by the RPE. We can also use equation (1) to calculate that the lowest value of $L_p$ that still adsorbs fluid pumped by the RPE without generating an elevated pressure at the RPE basal surface is $L_p > 0.4 \times 10^{-10}$ m/s/Pa.

Experiments using laser ablation of Bruch’s membrane/choroid explants allowed Starita et al. (89) to conclude that the ICL was responsible for most of the flow resistance in Bruch’s membrane. Attempts to further localize the flow resistance using morphometric methods are complicated by (i) stereological issues (90) and (ii) the loss of ultrastructural fidelity from connective tissue conventionally processed for electron microscopy (38). Failure to appreciate the former difficulty can lead to un-physiologically low estimates for tissue porosity and thereby hydraulic conductivity (e.g. (1)).

AGE-RELATED CHANGES IN HYDRAULIC CONDUCTIVITY AND DISEASE

Fisher was the first to measure $L_p$ of human Bruch’s membrane (91), finding that $L_p$ decreased significantly with age. However, his values for $L_p$ of Bruch’s membrane and other tissues are much lower than those found by later investigators (92) (85, 93). Marshall and Hussain carefully revisited these measurements using Bruch’s membrane/choroid with RPE removed, a preparation that was simpler to create. They showed using laser ablation that the flow resistance of these preparations was entirely due to Bruch’s membrane (89). They also found that flow rate increased linearly with driving pressure, indicating that $L_p$ of Bruch’s membrane is relatively insensitive to pressure up to 25 mm Hg.
They reported that $L_p$ of macular Bruch’s membrane exhibited a dramatic, exponential decline throughout life (Figure 5), dropping from $130 \times 10^{-10}$ m/s/Pa in young children to $0.52 \times 10^{-10}$ m/s/Pa in old age. $L_p$ of macular Bruch’s membrane dropped more rapidly with age than did that of the periphery, consistent with an accelerated process occurring in the macula (1, 84, 94, 95). Note that the lowest value measured for $L_p$ of Bruch’s membrane in normal eyes is similar to the calculated minimum value of $L_p$ that allows complete fluid resorption ($0.4 \times 10^{-10}$ m/s/Pa, see above). Marshall and Hussain reached similar conclusions regarding this process (94).

Determining $L_p$ of Bruch’s membrane in isolated macular samples of AMD eyes is difficult due to scar formation and other changes (94). However, Marshall and Hussein’s group showed that in the periphery, $L_p$ of Bruch’s membrane is decreased in AMD eyes as compared to age-matched normal eyes (Figure 5). (94) Assuming that similar processes occur in macular Bruch’s membrane due to the profound lipid accumulation in this region, then in diseased eyes, the RPE must generate higher pressures at its basal surface to drive fluid into the choriocapillaris, with further pathological consequences (31). Above an unknown threshold level, higher pressure will cause the RPE-EL to separate from the ICL, leading to RPE detachment and fluid accumulation, as seen in 12-20% of AMD patients. (94)

What causes the dramatic age-related decrease in $L_p$ of Bruch’s membrane? It is natural to suspect the age-related lipid accumulation. In fact, McCarty et al. (96) showed that lipid particles trapped in an extracellular matrix can generate very significant flow resistance, more than would be expected based simply on their size and number. However, Marshall and Hussain observed that most of the marked change in $L_p$ occurred before age 40 (Figure 6A) while the increase in Bruch’s membrane lipid content occurred largely after this age. They thus concluded that other age-related changes must be responsible for changes in $L_p$ (1, 84)
A different conclusion can be reached from examining age-effects on flow resistivity, the inverse of $L_p$. Resistivity increases from a low of roughly $R=10^8$ Pa/m/s for young individuals to $R=10^{10}$ Pa/m/s for aged persons. Thus, when hydraulic conductivity $L_p$ drops from roughly $100\times10^{-10}$ m/s/Pa to $25\times10^{-10}$ m/s/Pa between the ages of birth and 40 years of age, 75% of its total possible decrease, resistivity $R$ increases from $1\times10^8$ Pa/m/s to $4\times10^8$ Pa/m/s, only 4% of the ultimate increase. Simply put, hydraulic conductivity drops more rapidly with age at young ages because it value is high to start with. Figure 6B plots resistivity and histochemically detected EC against age for Bruch’s membrane (97). The agreement between the trends and the fits to the data are striking. This is strong evidence that the increasing lipid content and progressively hydrophobic character of Bruch’s membrane are responsible for impairing fluid transfer with age, as postulated (31). The strong correlation between flow resistivity of Bruch’s membrane and lipid content was likewise found by Marshall and Hussain. (1, 84, 95) Laser ablation studies localizing flow resistance to the ICL (89) further supports this conclusion, because lipids accumulate prominently in the ICL with aging (39). Further, more laser pulses were required to abolish flow resistance in the oldest eyes, consistent with presence of a Lipid Wall, requiring prior removal.

Thus, it appears that decreased $L_p$ and increased resistivity of Bruch’s membrane with aging is closely related to the age-related accumulation of lipids, primarily EC. Lipids accumulate more rapidly in the macular Bruch’s membrane than in the periphery. (35, 98) Thus, $L_p$ of the macula decreases more rapidly with age than it does in the periphery.

PERMEABILITY OF BRUCH’S MEMBRANE TO SOLUTE TRANSPORT

Along with bulk fluid flow, there is significant transport of individual molecular species across Bruch’s membrane, including dissolved gases, nutrients, cytokines, and waste products driven by passive diffusion. Flow crossing Bruch’s membrane is too
slow to influence this process. This can be seen through calculation of the Peclet number, the relative magnitude of convection of a species due to bulk flow to that of diffusion (99):

\[ \frac{V L}{D_0} \]  

(3)

where \( V \) is the velocity of the flow, \( L \) is the transport path length, and \( D_0 \) the free diffusion coefficient of the species being transported. (The free diffusion coefficient in saline is used rather than its value in tissue, since the species carried by flow is constrained to the same extent by the tissue as is its diffusion). Using the RPE pumping rate (Table 2) for \( V \), Bruch’s membrane thickness (average of 3 \( \mu \)m (59)) for \( L \), and a range of diffusion coefficients of species crossing Bruch’s membrane (2x10^{-7} cm^2/sec for LDL to 2x10^{-5} cm^2/sec for oxygen; (99, 100)), we find that the Peclet number ranges in value from 5x10^{-5} to 5x10^{-3}. Thus, convection is negligible in transporting species across Bruch’s membrane under physiological conditions.

Diffusion follows Fick’s law whereby the diffusive flux per unit area (\( j \)) is proportional to the diffusion coefficient (\( D \)) of that species in the medium through which it passes and to the concentration difference across the medium (\( \Delta C \)), and inversely proportional to the diffusion length:

\[ j = D \frac{\Delta C}{L} \]  

(4)

The permeability of a tissue to a given species is defined as \( P = j/\Delta C \). We see then that \( P = D/L \). For example, the permeability of Bruch’s membrane to oxygen is \( \sim 0.067 \) cm/sec.

Note that since diffusion moves down a concentration gradient, one species might be
diffusing across Bruch’s membrane toward the RPE (e.g. oxygen) while another species (e.g. carbon dioxide) diffuses simultaneously in the other direction.

With high diffusion coefficient and little interaction with extracellular matrix, small molecules (e.g. oxygen) diffuse quickly across Bruch’s membrane. However, macromolecules have much smaller free solution diffusion coefficients due to their size. Coefficients are further reduced by interactions with extracellular matrix or lipoproteins that accumulate with age, so macromolecule transport across Bruch's membrane is impeded.

The transport of amino acids, (101) serum proteins, (102), drugs, (103) and LDL (104) across Bruch’s membrane has been examined. There are technical challenges to these experiments. First, as indicated in equation (4), diffusional flux depends on the length of the tissue. Since the diffusion coefficient of the transported species is likely different in Bruch’s membrane than in the choroid in a combined preparation, but the path lengths of both tissue components are usually not determined, it is difficult to use the measured values to determine absolute values of permeability. Instead the more easily measured flux rate \(j\) (see equation 4) is usually presented. Second, since larger macromolecules used in diffusion studies are hindered in their passage into the tissue, an oncotic pressure could develop, generating a fluid flow in the opposite direction of transport and thus complicating the results. Nonetheless, useful comparative results can be generated.

The transport rate across human Bruch’s membrane declines linearly with age for all molecules measured. Amino acids exhibited permeabilities of \(0.6 \times 10^{-4}\) cm/sec (phenylalanine) to \(1.2 \times 10^{-4}\) cm/sec (glycine) for young Bruch’s membrane and exhibited a modest decline (2-fold or less) with aging. (101) Serum proteins decrease more markedly, dropping from \(3.5 \times 10^{-6}\) cm/sec in the first decade to \(0.2 \times 10^{-6}\) cm/sec in the ninth decade, a >10-fold decrease (102) In particular, proteins larger than 100 kDa have significantly
decreased flux through Bruch’s membrane of older individuals. Macular Bruch's membrane showed a steeper decrease with age than did the periphery (105). Permeability was reduced in eyes with AMD relative to age-matched normal eyes. (105)

Decreased permeability of Bruch’s membrane to transport is likely due to a decrease in diffusion coefficients, especially for the larger species affected by interaction with extracellular matrix and lipoproteins. As indicated in equation (4), increased path length due to age-related thickening of Bruch’s membrane (59) could also have a significant effect.

An original proposal of a molecular weight (MW) exclusion limit to Bruch’s membrane macromolecule transport of 66 - 200 kD, (101, 102) has been questioned by more recent work suggesting that if such a limits exists, it is much higher (105). Because of the importance of lipoproteins in transporting lipophilic nutrients to the RPE for ultimate use by the photoreceptors, and also because lipoproteins accumulate with age in Bruch’s membrane, Huang et al. (104) (104) specifically examined the reflection coefficient of bovine Bruch’s membrane to plasma LDL. They measured a reflection coefficient of 0.62 (compared to a reflection coefficient of arterial endothelium to LDL of 0.998 and arterial intima to LDL of 0.827 (106)). Thus, while LDL did not pass freely through Bruch’s membrane, it could nonetheless pass. Hussain et al. (105) also concluded that particles as large as LDL could cross Bruch’s membrane. Accordingly, RPE cells have been shown to internalize plasma LDL from the choroid (56, 107, 108).

These considerations are relevant not only to understanding mass transfer between the choriocapillaris and the RPE, but also for transcleral drug delivery strategies including anti-angiogenic agents for treating AMD and steroids for treating diabetic retinopathy (109) (110). Lipophilic solutes are significantly hindered in their transport by Bruch's membrane/choroid (103), while hydrophilic moieties are blocked by the RPE (111).
SUMMARY AND IMPLICATIONS

Bruch’s membrane’s physiological roles are structural and facilitating transport. Transport across Bruch’s membrane is increasingly hindered with age, due at least partly to the marked age-related accumulation of EC-rich lipoproteins in this tissue, impeding pumping of fluid from RPE (94). A ≥90% decrease in transport of some species from the choroid (102, 105) may include lipophilic essentials delivered by lipoproteins. This decline in transport capability is thought to have functional consequences for photoreceptors (112). A well-characterized change occurring through the lifespan of individuals with healthy maculas is slowed dark adaptation (113), attributed to impaired translocation of retinoids across the RPE-Bruch’s interface. This slowing, worse in AMD patients (114, 115), can be partly ameliorated by short-term administration of high-dose vitamin A (116), presumably overcoming the translocation deficit via mass action.

Pathology of Bruch's Membrane

AMD lesions

In aging and AMD, characteristic extracellular lesions accumulate in tissue compartments anterior to the ICL. Known as drusen and basal deposits (29, 117), these lipid-containing aggregations ultimately impact RPE and photoreceptor health by impairing transport, causing inflammation, and predisposing to choroidal neovascularization (Figure 7). Basal linear deposit (BlinD) forms consequent to lipoprotein accumulation in Bruch's membrane and formation of the Lipid Wall, likely involving oxidation of individual lipid classes and local inflammation. Drusen could form by similar mechanisms, plus lipoprotein aggregation and other undefined processes that cause the distinctive dome shape of these lesions. Basal laminar deposit (BlamD) forms in parallel with lipid deposition in Bruch’s and
may indicate RPE stressed by it. We begin by discussing drusen, due to their importance in AMD.

**DRUSEN**

In a fundus view, drusen are 30-300 μm-diameter yellow-white deposits posterior to the RPE. By optical coherence tomography, they appear as variably hypo-reflective spaces in the same location (118). Histologically, drusen are focal, domed lesions between the RPE basal lamina and the ICL, i.e., in the same sub-RPE tissue compartment as the Lipid Wall and BlinD. Found in most older adults (67, 119), drusen are more numerous in peripheral retina than in macula (120-122). Drusen are typically classified as “hard” and “soft” by the appearance of their borders. Soft drusen confer high risk of advanced disease (123-126) and importantly, are found only in the macula (122). Other rare druse types exist and are less well characterized (127).

In separate 1854 publications, Donders (a Dutch ophthalmologist) and Wedl (an Austrian pathologist) described “colloid bodies” (Colloidkugeln) or "hyaline deposits" on the inner surface of the choroid in older or diseased human eyes (128, 129) (3)(translated by Busk). Both authors interpreted the droplets that filled these deposits as “fat-globules.” The term drusen originated with Müller in 1856, from the German word for geode (not to be confused with drüse, meaning gland) (130). The name drusen was adopted by English writers early in the 20th century (131) yet “colloid body” was used by Verhoeff into the 1920’s (132).

The basis of the fatty content emerged slowly. Lauber (133) (cited by (134)) noted that deposits between the lamina vitrea and the RPE were sudanophilic in 1924. Wolter and Falls (135) stated that “hyaline bodies {drusen} …stain reddish with … oil red O” in 1962.
Extant theories for druse formation, extending back to their discovery (130), fit into two general categories: transformation of the overlying RPE and deposition of materials onto Bruch’s membrane. The latter is now accepted (129) (135). The RPE has been implicated as a source of many druse components, via budding of membrane bound-packets of cytoplasm or secretion. The contribution of plasma-derived components, in contrast, has not been well characterized. The existence of druse subregions additionally suggest remodeling in the extracellular compartment, such as cellular invasion and enzymatic activity) (23) (136) (137) (138) and uplifting of the Lipid Wall (139) (35).

Most prominent among druse constituents are lipids, an observation made by their earliest discoverers. All drusen contain EC and UC, in addition to phosphatidylcholine, other phospholipids and ceramides (34, 35, 137, 139-142). Extractable lipids account for ≥40% of hard druse volume (143) and likely more for macular soft drusen (139). This includes large EC-rich lakes in soft drusen (Figure 8A,B), reminiscent of atherosclerotic plaques (144).

Only half of macular drusen take up hydrophilic fluorescein in angiography (145), possibly reflecting differing proportions of polar and neutral lipids in individual lesions (141).

Discrete non-lipid components in some drusen include amyloid assemblies and granules of lipofuscin or melanin indicating cellular origin (online Table 3). Other constituents present in all drusen include vitronectin, TIMP-3, complement factor H, complement components C3 and C8, crystallins, and zinc (23, 136, 138, 146-150).

Apolipoprotein immunoreactivity appears in drusen with high frequency (100%, apoE; >80% apoB; 60%, A-I) (139, 151-154). Plasma-abundant apoC-III is present in fewer drusen than plasma-sparse apoC-I, indicating a specific retention mechanism for plasma-derived apolipoproteins or an intraocular source. Importantly, hard drusen contain many solid, Folch-extractable electron-dense particles of the same diameter as the lipoproteins that
accumulate with age in Bruch’s membrane. These observations together with the appearance of membranous debris in soft drusen (below) make an RPE-secreted apoB-containing lipoprotein particle an efficient mechanism to place multiple lipids and apolipoproteins within lesion compartments.

The principal lipid-containing component of soft drusen and BlinD was called "membranous debris" by the Sarks (123, 155, 156). These lesions are richer in histochemically detectable UC than surrounding cellular membranes (141, 142). By transmission electron microscopy following osmium tetroxide post-fixation, membranous debris appears as variably sized, contiguous coils of uncoated membranes consisting of uni- or multilamellar electron dense lines, denser than cellular membranes, surrounding an electron-lucent center (Figure 1). Since conventional ultrastructural preparation methods can remove lipids, the building blocks of membranous debris are more plausibly the UC-rich exteriors of lipoproteins (native and fused) whose neutral lipid interiors are not well preserved in post-mortem tissue (39, 153, 157). Rather than vesicles, then, membranous debris is likely aggregated or fused particles that could collectively account for the abundant EC in sub-RPE deposits. EC abundance and ultrastructural evidence for solid, non-vesicular particles suggest that the major lipid-containing component of AMD-specific lesions can be called “lipoprotein-derived debris” rather than membranous debris.

**BASAL LINEAR DEPOSIT**

BlinD is a thin (0.4-2 μm) layer located in the same sub-RPE compartment as soft drusen. BlinD is not visible clinically except as associated with other pathology. By OTAP and QFDE, BlinD is rich in solid lipoprotein particles and lipid pools (Figure 9A,C). BlinD and soft drusen are considered alternate forms (layer and lump) of the same entity (158) and may interchange over time. Soft drusen are oily, difficult to isolate individually, and are
biomechanically more fragile than hard drusen (122), properties applicable to BlinD by
inference. Both lesions could thus be permissive to invading capillaries of type I choroidal
neovascularization (159, 160). ApoE and apoB are present in BlinD and its precursor, the
Lipid Wall (139, 151, 152). Transitional morphologies between Lipid Wall and BlinD have
been reported (161).

Basal laminar deposit (BlamD) forms small pockets between the RPE and the RPE-
BL in many older normal eyes or a continuous layer as thick as 15 μm in AMD eyes (142,
156, 162) (Figure 7). Some authors consider a continuous layer of BlamD a histological
definition of AMD (163). Ultrastructurally, BlamD resembles basement membrane material
(Figure 9B), containing laminin, fibronectin, type IV, and type VI collagen (164-167). The
latter is a distinctive banded material with 120 nm periodicity, called wide- or long-spacing
collagen, which also appears in other ocular locations like epi-retinal membranes. Thick
BlamD, associated with advanced AMD risk (156), contains histochemically detectable lipid
including UC and EC (141, 142) and is a classically described site for membranous debris
(Figure 1C). By lipid-preserving methods, solid particles are seen in BlamD (Figure 9A,B).
Especially enriched in basal mounds (156) (Figure 1C), lipoprotein-derived debris in BlamD
may be considered as retained in transit from the RPE to BlinD and/or drusen (139, 141,
142). Morphologically heterogeneous BlamD also contains vitronectin, MMP-7, TIMP-3,
C3, and C5b-9 (162), EC, and UC (142). Evoked in numerous mouse models of aging, stress,
and genetic manipulation, BlamD is a reliable marker of RPE stress (168).

Subretinal drusenoid debris

Hypotheses of druse formation must eventually also account for subretinal drusenoid
debris (SDD). Located adjacent to RPE in the sub-retinal space, SDD were first described in
AMD eyes by the Sarks (155). Ultrastructurally similar to soft drusen, these deposits are enriched in UC, apoE, vitronectin, and complement factor H, and like drusen, they lack markers for photoreceptors, Müller cells, and RPE apical processes (142, 169). Clinically this material is called reticular drusen in a fundus view (170) and subretinal drusenoid debris in a cross-sectional view (171). Conferring lower risk for advanced AMD than conventional drusen (172), SDD appear in up to 60% of geographic atrophy eyes (172, 173), appearing as focal deposits near the fovea and part of large sheets elsewhere in the macula (174). This coherent morphology suggests a specific formative process, possibly involving microglia resident in that compartment (175, 176).

**Summary**

Levels of significance ascribed to molecules sequestered in drusen (online Table 3), and by inference, BlinD, include toxicity to the overlying RPE, stigmata of formative processes (extrusion of cellular materials, secretion, extracellular enzymatic processing, cellular activity), and markers of a diffusely distributed disease process affecting RPE and Bruch’s membrane. Additional significance can be ascribed to these lesions as physical objects that increase path length between choriocapillaries and retina and provide a biomechanically unstable cleavage plane between RPE-BL and ICL.

*Response-to-retention hypothesis of AMD*

The parallels between the pathology of arterial intima of large arteries and that of Bruch’s membrane are striking. Both diseases feature cholesterol-rich lesions in sub-endothelial compartments within the systemic circulation, involving many of the same molecules and biological processes at multiple steps, as long anticipated (177, 178). According to the Response-to-Retention theory of atherosclerosis, plasma lipoproteins cross the vascular endothelium of large arteries, and bind to extracellular matrix. By itself, this
process is not pathological. However, lipoprotein components become modified via oxidative and non-oxidative processes, and launch numerous downstream deleterious events, including inflammation, macrophage recruitment, and neovascularization leading to disease (179, 180).

Parallel with apoB-lipoprotein-instigated disease in arterial intima, an intraocular Response-to-Retention involving the RPE and Bruch’s membrane in aging and AMD would begin with age-related accumulation of lipoproteins of local origin. Oxidation, perhaps driven by reactive oxygen species from adjacent RPE mitochondria, would then initiate a pathological process resembling that in the vascular system with inflammation-driven downstream events including complement activation and structurally unstable lesions (36).

Neovascular AMD

Choroidal neovascularization (CNV), the major sight-threatening complication of AMD, involves angiogenesis along vertical and horizontal vectors: vertically across Bruch’s membrane, and either laterally external to the RPE (type 1 CNV, (181)), laterally within the subretinal space (type 2 CNV), or further anteriorly into the retina (type 3 CNV) (181) (182) (183). Of 40+ conditions involving CNV, AMD is the most prevalent, followed by ocular histoplasmosis (181), and including angioid streaks (below). CNV is a multifactorial non-specific wound healing response to various specific stimuli, involving VEGF stimulation of choriocapillaris endothelium, compromise to Bruch’s membrane, and participation of macrophages. (181). Impaired transport across Bruch's membrane in AMD increasingly isolates the RPE from its metabolic source in the choriocapillaries and enhances the challenge in waste product disposal. VEGF released by RPE as a stress signal initiates an angiogenic response by the endothelium. However, Bruch’s membrane compromise is essential for CNV to proceed, as evidenced by intrachoroidal neovascularization without CNV in a mouse over-expressing VEGF in the setting of an intact Bruch’s membrane (184)
Bruch’s membrane in a state of compromise can be breached easily by new vessels in AMD. It is notable that the EL is thinner and more interrupted in eyes with neovascular AMD (23). The length of gaps in the EL is greater in eyes with early AMD and any CNV (23). In paired donor eyes with and without CNV secondary to AMD, progressed eyes are distinguished by calcification and breaks in Bruch’s membrane (185). In contrast, calcification in a small number of geographic atrophy eyes is unremarkable (186).

BlinD furthers this process by presenting a horizontal cleavage plane for vessel formation to exploit. The lipid-rich composition, relative lack of structural elements like collagen fibrils, lesion biomechanical instability (122), and pro-inflammatory, pro-angiogenic compounds like 7-ketocholesterol and linoleate hydroperoxide (160, 181, 187) likely promote vessel growth in this plane (160).

Angioid streaks (ABCC6, MTP genes)

Angioid streaks are ruptures in Bruch’s membrane associated with multiple disorders, caused by excess calcification of the elastic layer (188) and often accompanied by CNV. They are prominent ocular manifestation of pseudo-xanthoma elasticum (PXE), a systemic connective tissue disorder. PXE patients harbor mutations of a hepatically expressed lipid transporter ABCC6 (189). Clinical presentation includes, in addition to streaks and CNV, peau d’orange (flat, yellow, drusen-like lesions), optic nerve head drusen, outer retinal tubulations, subretinal fluid, and pigmentary changes (190). PXE clinical manifestations are believed related to ectopic mineralization of non-hepatic tissues, suggesting a defect in the transport of anti-mineralization agents (191).

Angioid streaks are associated with abetalipoproteinemia (192-195), an extremely rare disorder with low plasma apoB-containing lipoproteins, acanthocytosis of erythrocytes, neuropathy, and pigmentary retinopathy. It is historically attributed to lack of lipophilic
vitamins delivered by plasma LDL (44). The RPE is now known to express the
abetalipoproteinemia gene (MTP) (46), which co-translationally lipidates apoB (see above).
How MTP deficiency leads to angioid streaks is unknown. The finding, however, highlights
that *lack* of apoB lipoproteins has negative consequences for Bruch’s membrane health, just
as an *excess* of retained apoB lipoproteins has negative consequences via lesion formation
and impaired transport (see above). Good chorioretinal function thus requires an optimal
balance between these extremes.

*Thick basal laminar deposits (TIMP-3, CTRP5, EFEMP1 genes)*

Three autosomal dominant inherited disorders with adult onset – Sorsby fundus
dystrophy, late-onset retinal degeneration (LORD) and Malattia Leventinese - Doyne
honeycomb retinal dystrophy (ML-DH) -- share phenotypic similarities with AMD and
provide mechanistic support for many aspects of Bruch’s membrane physiology and
pathophysiology discussed above. All 3 conditions result from mutations in genes encoding
extracellular matrix proteins or their regulators (Sorsby -TIMP3 (196), LORD - CTRP5
(197), and ML-DH - EFEMP1 (198)). All 3 can progress to choroidal neovascularization to
varying degrees (Sorsby > LORD > ML-DH). All 3 have visual dysfunction, especially rods,
attributed to a nutritional night blindness that is responsive to short-term administration of
high-dose vitamin A in Sorsby and LORD (199-201). Sorsby and LORD are notable for thick
BlamD and areas of RPE atrophy (202) and may involve macula and periphery, while ML-
DL is notable for radially distributed drusen and peripapillary deposits. In Sorsby eyes
mutant TIMP-3 localizes to BlamD. In ML-DL, EFEMP1 localizes to BlamD and not to the
pathognomonic drusen themselves, suggesting an important role of BlamD in druse
formation.
BlamD in Sorsby and LORD, like that in AMD, is notably rich in oil red O-binding lipid (203-205). The significance of these findings were unclear until a model of a Bruch’s membrane lipoprotein was articulated. In LORD eyes (205), deposits contain EC, UC, and apoB, and lipid-preserving ultrastructural methods revealed solid electron-dense particles tracking in intersecting networks across the BlamD. In hindsight, these may represent native lipoproteins in transit from RPE to the choriocapillaris rather than depositions/aggregations of plasma LDL, as originally speculated. Lipid particle disposition within these thick deposits has been replicated in a mouse model expressing the R345W EFEMP1 mutation (168).

Summary

Bruch’s membrane serves essential functions as substrate to the RPE and vessel wall of the outer retina. Its layers and constituent proteins collectively represent a barrier that keeps choroidal vessels at bay, provides a route for water, solutes and macromolecules that transfer between RPE and choroid while supporting the structural integrity of both. It is unusual among human tissues in accumulating a high content of EC-rich neutral lipid over the lifespan. A natural history and biochemical model now suggests this lipid is due to apoB lipoprotein secretion by RPE, which may be part of an outer retinal nutrition system. This deposition can account for the impaired outward movement of fluid from RPE, increasing risk for RPE detachments more common in older persons, and impaired macromolecular transport also leading to RPE stress. Oxidation of these lipid deposits in Bruch’s membrane likely initiates an inflammatory process that leads to lesion formation and choroidal neovascularization in AMD.
<table>
<thead>
<tr>
<th>Layer (common abbreviation)</th>
<th>Component: age change</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal laminar deposit (BlamD)</td>
<td>+ Fibronectin, laminin, IV α4-5, VI, endostatin, EFEMP1</td>
<td>(164) (206) (207) (167) (208) (209)</td>
</tr>
<tr>
<td>RPE-Basal lamina (RPE-BL)</td>
<td>IV α1-5, V, laminins 1, 5, 10, and 11, nidogen-1, heparan sulfate, chondroitin sulfate</td>
<td>(21) (210) (211) (18) (19) (66)</td>
</tr>
<tr>
<td>Lipid Wall/ Basal linear deposit (BlinD)</td>
<td>+ Lipoproteins</td>
<td>(212) (38) (39)</td>
</tr>
<tr>
<td>Elastic layer (EL)</td>
<td>Elastin †, calcium phosphate †</td>
<td>(14) (210) (66) (216) (68) (67)</td>
</tr>
<tr>
<td>Outer collagenous layer (OCL)</td>
<td>I, III, V, fibulin-5, fibronectin, chondroitin sulfate, dermanat sulfate, lipoproteins †, apoE, clusterin</td>
<td>(21) (210) (217) (50) (39) (215) (152)</td>
</tr>
<tr>
<td>ChC-Basal lamina</td>
<td>IV α1,2, V, VI, laminin, heparan sulfate, chondroitin sulfate, endostatin</td>
<td>(210) (211) (18) (218) (208)</td>
</tr>
<tr>
<td>Bruch’s, throughout or layer not specified</td>
<td>†, collagen solubility †, perlecan, MMP-2 †, MMP-9 †, TIMP-2; TIMP-3 †, pentosidine †, CML †, GA-AGE †, RGR-d, apoB, oxidized apoB-100, 7-KCh, MDA, LHP, HHE †, DHP-lys †, C3d †, C5b-9 †, pentraxin-3 †, thrombospondin-1, zinc</td>
<td>(147) (219) (220) (66) (221) (222) (218) (223) (224) (138) (225) (226) (227) (62) (139) (228, 229) (230)</td>
</tr>
</tbody>
</table>

Notes: Table shows definitely localized components. Most determinations were made in macula. Studies showing histochemical/ immunohistochemical verification of biochemistry and ultrastructural validation of structures identified by light microscopy techniques were given greater weight. Localizations were assigned to specific layers if immunogold-electron microscopy or high magnification confocal microscopy images were available. Roman numerals denote collagens. Components are ordered within each layer: structural components, lipoproteins, extracellular matrix and its regulation, modified lipids and proteins, complement/ immunity, cellular response/ activity, metals. Known changes with advancing age are **bold** with an arrow indicating direction of change. New additions with age are shown with a plus (+). Plain text means no change or not tested.

Abbreviations: 7-KCh, 7 keto-cholesterol (229); CML, carboxymethyl-lysine (226); DHP-Lys, dihydropyridine lysine (66); GA-AGE, glycolaldehyde derived AGE (221); HHE, 4-hydroxyhexenal (66); MDA, malondialdehyde (218) (66)
### Table 2: RPE Fluid Pumping Rates

<table>
<thead>
<tr>
<th>Species</th>
<th>Fluid Transport Rate Across RPE $\mu$L/hr/cm$^2$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frog</td>
<td>4.8-7.6</td>
<td>(231, 232)</td>
</tr>
<tr>
<td>Rabbit</td>
<td>12±4</td>
<td>(233, 234)</td>
</tr>
<tr>
<td>Canine</td>
<td>6.4</td>
<td>(235)</td>
</tr>
<tr>
<td>Primate*</td>
<td>14±3</td>
<td>(236, 237)</td>
</tr>
<tr>
<td>Human</td>
<td>11</td>
<td>(238)</td>
</tr>
</tbody>
</table>

Notes: RPE pumping rates were measured by readsoption of subretinal fluid or by direct measurement in culture.

*Cantrell and Pederson measured a much higher transport rate than that reported here (236), but used fluorescein as a tracer which likely does not track fluid flow due to its high diffusion coefficient.
Table 3 (online): Localized Components of Drusen

<table>
<thead>
<tr>
<th>Component</th>
<th>Phase</th>
<th>Direct assay</th>
<th>Abundance</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipoproteins (EC, UC, phospholipid)</td>
<td>P</td>
<td>✓</td>
<td>All drusen; &gt;40% of hard druse volume; EC pools in soft drusen</td>
<td>(122, 239, 240)</td>
</tr>
<tr>
<td>Apolipoproteins (apoB, A-I, C-I, E)</td>
<td>P</td>
<td>apoE ✓</td>
<td>60-100% of hard drusen; higher rates in periphery than macula</td>
<td>(152, 154, 240)</td>
</tr>
<tr>
<td>Melanin/ lipofuscin granules</td>
<td>P</td>
<td></td>
<td>6% of hard and soft drusen</td>
<td>(122)</td>
</tr>
<tr>
<td>Cells (dendritic, others)</td>
<td>P</td>
<td></td>
<td>3-6% of hard drusen only</td>
<td>(122, 241)</td>
</tr>
<tr>
<td>Amyloid vesicles (0.25 - 10 μm)</td>
<td>P</td>
<td></td>
<td>2% of hard drusen, 40% of compound drusen, frequent in eyes with many drusen, some AMD eyes</td>
<td>(122, 239, 242, 136)</td>
</tr>
<tr>
<td>Calcification</td>
<td>P</td>
<td>✓</td>
<td>43% of macular hard drusen, 1.6% of soft drusen, 2% of peripheral hard drusen</td>
<td>(122)</td>
</tr>
<tr>
<td>Clusterin, TIMP3, vitronectin, apolipoprotein E, complement factor H, complement components 8, 9</td>
<td>D</td>
<td>✓</td>
<td>Reliably detected; abundance inferred</td>
<td>(143, 191)</td>
</tr>
<tr>
<td>Components of classic, lectin, alternative, terminal complement pathways; C3 fragments indicating activation</td>
<td>D</td>
<td>some ✓</td>
<td>Many pathway components evidence key role of complement</td>
<td>(243)</td>
</tr>
<tr>
<td>RGR-d</td>
<td>D</td>
<td></td>
<td>All drusen</td>
<td>(244)</td>
</tr>
<tr>
<td>αA- and αB-crystallin</td>
<td>D</td>
<td>✓</td>
<td>N.A.; higher in BrM, more in AMD drusen</td>
<td>(150, 191)</td>
</tr>
<tr>
<td>Ubiquitin</td>
<td>D</td>
<td></td>
<td>Most drusen in most eyes</td>
<td>(245)</td>
</tr>
<tr>
<td>Exosome markers CD63, CD81, and LAMP2</td>
<td>P</td>
<td></td>
<td>N.A.</td>
<td>(246)</td>
</tr>
<tr>
<td>Bestrophin, membrane-bound</td>
<td>P</td>
<td></td>
<td>N.A.</td>
<td>(247)</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>D</td>
<td></td>
<td>All drusen</td>
<td>(191)</td>
</tr>
<tr>
<td>Zinc</td>
<td>D</td>
<td>✓</td>
<td>Many drusen</td>
<td>(138)</td>
</tr>
</tbody>
</table>

Notes: P, Particulate; D, Dispersed; BrM, Bruch’s membrane; Localization methods: immunohistochemistry, histochemistry, immunogold transmission electron microscopy; Direct assays: proteomics, western blot, microprobe synchrotron X-ray fluorescence for zinc; N.A. not available; Varying estimates of particulate druse components are due to differences in location of samples and druse types examined.
**Figure Captions and Figures**

**Figure 1:** Macular Bruch’s membrane throughout the lifespan. RPE is at the top of all panels. RPE basal lamina (arrowheads) and elastic layer (EL, yellow arrows, discontinuous in macula) are shown. **17 y:** Electron-dense amorphous debris and lipoproteins are absent. Bar, 1 μm. **46 y:** Electron-dense amorphous debris and lipoproteins are present. A coated membrane bounded bound (green arrow) contains lipoproteins. L, lipofuscin. **65 y:** Electron-dense amorphous debris and lipoproteins are abundant. Membranous debris, also called lipoprotein-derived debris (red arrow) has electron-dense exteriors within BlamD (*). Within OCL, banded material is type VI collagen, often found in BlamD.
Figure 2: Surface of the endothelium of the choriocapillaris showing fenestrations with a bicycle-spoke pattern (yellow arrow) and presumed artifactual openings arising from tissue preparation (cyan arrow); QFDE, 64-year-old eye, macula. Bar is 100 nm. (30)

{Permission required}
Figure 3: Lipid Wall, a layer of lipoproteins on the inner surface of Bruch’s membrane.

A. Lipoproteins (spherical vesicles of uniform diameter) accumulate 3-4 deep between the RPE basal lamina (black arrowheads) and Bruch’s membrane ICL (white arrowheads). Thin section transmission electron micrograph following osmium post-fixation. RPE, retinal pigment epithelium; BrM, Bruch’s membrane; L, lipofuscin. Sectioning plane is vertical; bar = 1 μm. B. Quick freeze deep etch shows tightly packed Bruch’s membrane lipoproteins in the Lipid Wall, and that lipoproteins have classic core and surface morphology (39). Fracture plane is oblique, bar = 200 nm.

{Permission required}
Figure 4: Bruch’s membrane lipoprotein composition. A. Lipoprotein particles isolated from Bruch’s membrane are large and spherical; negative stain (153) Bar = 50 nm. B. Bruch’s membrane lipoprotein composition inferred from direct assay (50, 153), druse composition, and RPE gene expression (139, 154) TG, triglyceride; EC, esterified cholesterol; UC, unesterified cholesterol; PL, phospholipid; Apo, apolipoproteins. The question mark signifies that not all apolipoproteins are known. {Permission required}
**Figure 5:** Hydraulic conductivity ($L_p$) of Bruch’s membrane as a function of age. Dotted lines are exponential fits to data from macular and peripheral regions, respectively. Note that all of the data from eyes with AMD (taken only in peripheral region) have lower values of $L_p$ than the best fit to data taken from peripheral Bruch’s membrane of non-diseased eyes. (94)
Figure 6: A. Hydraulic conductivity of human macular Bruch’s membrane/choroidal preparations as a function of age, as compared to lipid accumulation in human macular Bruch’s membrane; lines as exponential fits to the data (modified from (1) [permission necessary]); B. Hydraulic resistivity of human macular Bruch’s membrane/choroidal preparations as a function of age, (1) as compared to esterified cholesterol accumulation in human macular Bruch’s membrane (35); lines are exponential fits to the data (the fits nearly overlaid one another)
**Figure 7:** Bruch’s membrane and characteristic AMD lesions. 

**A.** Bruch’s membrane has 5 layers in a normal eye: 1, basal lamina of the RPE; 2, inner collagenous layer; 3, elastic layer; 4, outer collagenous layer; 5, basal lamina of the choriocapillary endothelium (fenestrated cells, pink). L, lipofuscin. **B.** Older eyes have basal laminar deposit (BlamD) and basal linear deposit (BlinD) and its precursor, the Lipid Wall. Drusen, BlinD, and the Lipid Wall occupy the same tissue compartment. Basal mounds are soft druse material within BlamD. Adapted from (248).
**Figure 8:** Esterified cholesterol (EC) forms lakes in macular soft drusen. **A**, EC lakes in a macular soft druse revealed by filipin fluorescence (arrow). Bar, 25 μm. Adapted from (139).

**B.** Macular soft druse from an AMD eye has lakes of homogeneous electron-dense lipid (arrow) among partially preserved lipoprotein-like material. Basal laminar deposit (asterisk) overlying the druse has similar material, called membranous- or lipoprotein-derived debris (to the right of the asterisk).
Figure 9: Lipoprotein-derived debris and lipid pools in AMD lesions are solid rather than vesicular material. **A.** Above RPE basal lamina (arrowheads) is BlamD with individual particles indicated (arrow). Below RPE basal lamina is numerous solid particles in BlinD. Transmission electron microscopy, OTAP fixation, bar = 500 nm. **B.** BlamD appears as a solid column of basal lamina-like material, with solid particles embedded within (arrow). (Image courtesy of J-D Huang, PhD). **C.** BlinD has lipoproteins of heterogeneous sizes and shapes as well as pooled lipid, consistent with a model of surface degradation and particle fusion. Bar in B, 500 nm; Bar in C, 200 nm.

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