Aging, age-related macular degeneration, and the response-to-retention of apolipoprotein B-containing lipoproteins

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ABSTRACT

The largest risk factor for age-related macular degeneration (ARMD) is advanced age. A prominent age-related change in the human retina is the accumulation of histochemically detectable neutral lipid in normal Bruch's membrane (BrM) throughout adulthood. This change has the potential to have a major impact on physiology of the retinal pigment epithelium (RPE). It occurs in the same compartment as drusen and basal linear deposit, the pathognomonic extracellular, lipid-containing lesions of ARMD. Here we present evidence from light microscopic histochemistry, ultrastructure, lipid profiling of tissues and isolated lipoproteins, and gene expression analysis that this deposition can be accounted for by esterified cholesterol-rich, apolipoprotein B-containing lipoprotein particles constitutively produced by the RPE. This work collectively allows ARMD lesion formation and its aftermath to be conceptualized as a response to the retention of a sub-endothelial apolipoprotein B lipoprotein, similar to a widely accepted model of atherosclerotic coronary artery disease (CAD) (Tabas et al., 2007). This approach provides a wide knowledge base and sophisticated clinical armamentarium that can be readily exploited for the development of new model systems and the future benefit of ARMD patients.

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Contents

1. Statement of scope

Age-related macular degeneration (ARMD) is a major cause of vision loss in the elderly of the industrialized world. The largest risk factor for ARMD is advanced age. One of the most prominent age-related changes to the human retina is the accumulation of histochemically detectable neutral lipid in normal Bruch's membrane (BrM) throughout adulthood (Pauleikhoff et al., 1990). Arguably one the most important observations relevant to ARMD pathobiology, the Pauleikhoff study indicated that a significant, universal, and previously unknown change to BrM occurs with aging. This change, the accumulation of lipid in BrM, has the potential to have a major impact on physiology of the retinal pigment epithelium (RPE), the cell layer that supports photoreceptors. Further, this lipid deposition occurs in the same BrM compartment in which the pathognomonic extracellular, lipid-containing lesions of ARMD later arise.

Our thinking about this phenomenon has been extensively influenced by the vast knowledge base available for atherosclerosis, a condition for which lipid deposition in vessel walls is a well-established causative agent. Others have sought similar connections between ARMD and vascular disease with an emphasis on hemodynamics (Friedman, 2004). Here we review recent work primarily from our laboratories establishing that BrM lipid accumulation can be accounted for by cholesterol-rich lipoproteins of intraocular origin. This work collectively lays a foundation for considering the RPE as a constitutive lipoprotein secretor and ARMD as exhibiting both key similarities and differences with atherosclerosis. After we examine the process of lipoprotein accumulation in BrM, we summarize briefly aspects of lipoprotein biology and atherosclerosis pathobiology. We then review the evidence supporting local production of lipoproteins and consider the possible pathological implications of lipoprotein accumulation in BrM. We conclude by examining implications of this work for future research in basic and clinical areas. Many ARMD-relevant topics not covered herein are reviewed elsewhere (Edwards and Malek, 2007; Grisanti and Tatar, 2008; Jackson et al., 2005; Montezuma et al., 2007).

2. Introduction to outer retina and choroid (Fig. 1)

The 100 million rod and cone photoreceptors located at the outer surface of the retinal sheet are supported by the RPE. This polarized monolayer serves diverse functions essential for optimal photoreceptor health, including daily phagocytosis of photoreceptor outer segment tips, vitamin A metabolism, maintenance of retinal attachment, and coordination of cytokine-mediated immune protection. While inner retinal layers rely on the intrinsic retinal circulation, the photoreceptors and RPE depend on the choroid located external to them (Fig. 1) Branches of the ophthalmic artery enter through the sclera with the optic nerve and ramify to form the choroid and the choriocapillaris, a dense capillary bed. About 200–300 μm thick, the choroid has the highest blood flow per unit tissue perfused in the body, with 7-fold greater flow in the macula relative to the periphery. The innermost 2–4 μm of the choroid (subjacent to the RPE) is BrM (Figs. 1, 2A,D), a pentilaminar vessel wall that is laid out flat along one side of the choriocapillaris rather than wrapped around individual lumens. Unhindered transport across BrM of nutrients to and metabolites from the RPE is essential for normal vision by the photoreceptors (Hillenkamp et al., 2004; Marshall et al., 1998).
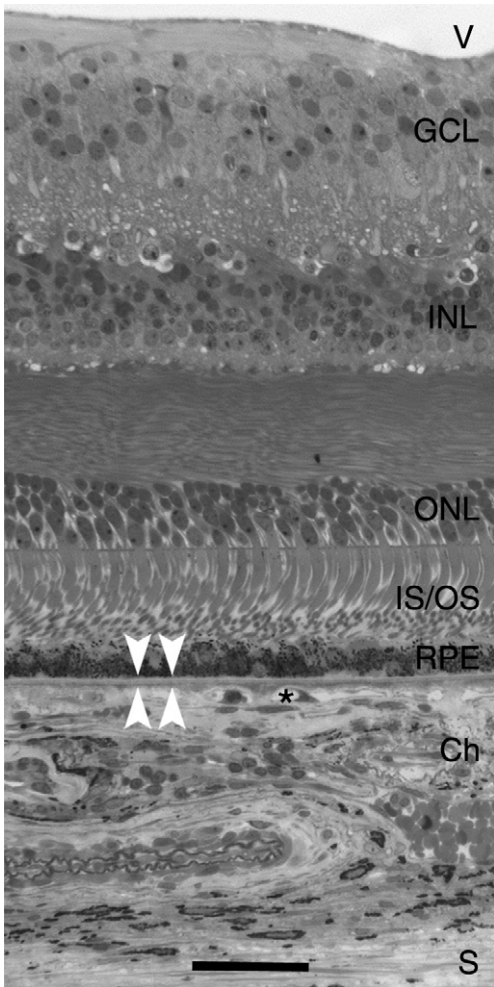
Fig. 1. Chorioretinal anatomy in macula. V, vitreous; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; IS/OS, inner and outer segments of photoreceptors; RPE, retinal pigment epithelium; Ch, choroid; asterisk, choriocapillaris; white arrowheads, BrM; S, sclera. Bar, 50 µm.

3. Introduction to ARMD

3.1. Epidemiology and risk factors

While responsible for high-acuity vision, the macula is also vulnerable to ARMD, the major cause of vision loss among elderly of European derivation living in industrialized societies (Congdon et al., 2004). RPE cell death across the macula (geographic atrophy) is a slow but devastating loss of vision ("dry" ARMD). Some patients at early to intermediate stages of dry ARMD can benefit from supplementation with an anti-oxidant mixture (2001a). Choroidal neovascularization, an invasion of choriocapillaries across BrM and under the RPE or retina (Grossniklaus and Green, 2004), is an urgent and sight-threatening complication ("wet" ARMD) of ARMD's underlying degeneration. Recently, extraordinary progress has culminated in the development of highly specific inhibitors of vascular endothelial growth factor that, when injected intravitreally, not only slow vision loss but also improve vision in many of the 15% of ARMD patients afflicted with choroidal neovascularization (Ciulla and Rosenfeld, 2009). However, the majority of ARMD patients lack neovascularization and cannot be treated in this manner.

Of ARMD risk factors (Klein et al., 2004), advanced age and family history are strongly and consistently related to ARMD across multiple studies. Cigarette smoking, hypertension, and cataract surgery appear to increase the risk of progression to neovascular ARMD in most studies. Other risk factors, like inflammatory disease, obesity, atherosclerotic vascular disease, and hyperopia, have less consistent findings, and associations, if significant, are often weaker. No association exists between ARMD and either diabetes or plasma cholesterol levels (Section 9.2.2). Linkage studies and genome-wide scans have identified polymorphisms in complement factor H, HTRA1, ARMS2, and mitochondrial DNA polymorphism A4917G as risk factors, and complement factor B, C3, and apolipoprotein E4 (apoE4) as protective factors (Canter et al., 2008; Dewan et al., 2006; Gold et al., 2006; Hageman et al., 2005; Kanda et al., 2007; Souied et al., 1998; Yates et al., 2007).

3.2. Histopathology (Fig. 2)

A prominent histopathologic sign of ARMD is the finding of extracellular lesions in the RPE/BrM complex (basal deposits and drusen, Fig. 2B,C) that ultimately impact RPE and photoreceptor health (Green and Enger, 1993; Sarks, 1976). In addition to the lesions, ARMD involves multiple, temporally overlapping biological processes such as cell death, choroidal neovascularization, inflammation, and scar formation subsequent to vessel in-growth. This review focuses on the lesions, as they are pathognomonic for the disease, and they have not yet been faithfully reproduced in laboratory animals. New insights into the pathobiology of early ARMD lesions to inform the development of model systems and new treatments are therefore needed.

Drusen are yellow-white deposits seen in a retinal fundus examination and are associated with ARMD. They are typically classified as "hard" and "soft" at the level of the RPE by the characteristics of their borders, with the latter associated with higher risk for developing advanced disease (Davis et al., 2005; Klein et al., 1991, 2007; Sarks, 1980). Histologically, drusen are defined as focal, dome-shaped lesions between the RPE basal lamina and the inner collagenous layer of BrM. Drusen of 30–300 µm diameter are visible in the fundus by the intrinsic elevation of the dome and loss of pigment from the effaced overlying RPE (Sarks et al., 1999). Pale spots in the fundus have sometimes proven to be other entities by histological analysis (e.g., lipid-filled RPE cells, sub-retinal debris) (Anderson et al., 2006; Bressler et al., 1994; Raoul et al., 2008; Rudolf et al., 2008b). Very common in eyes of older persons, drusen are numerous in peripheral retina, with soft drusen (loosely packed with membranous material) confined primarily to the macula (Rudolf et al., 2008a).

The last decade has witnessed intense study of druse composition, particularly by Hageman, Anderson, and Johnson (Anderson et al., 2002). Many druse components are now identified, including vitronectin, tissue inhibitor of metalloproteinase 3 (TIMP-3), complement factor H, fibrillar and non-fibrillar amyloid, complement component C3, and zinc (Fariss et al., 1997; Hageman et al., 1999, 2005; Johnson et al., 2002; Johnson et al., 2001; Lengyel et al., 2007; Luibl et al., 2006). Cholesterol and apolipoproteins in lesions will be discussed in Section 8.1.

Basal deposits are two diffuse lesions associated with ARMD that have distinctly different size, composition, and significance (Fig. 2; (Sarks et al., 2007)). Basal laminar deposit (BlamD), between the RPE and its basement membrane, forms small pockets in many older eyes or a continuous layer as thick as 15 µm in eyes with ARMD. Ultrastructurally, BlamD resembles basement membrane material and contains laminin, fibronectin, and type IV and VI collagen (Knupp et al., 2000; Löffler and Lee, 1986; Marshall et al., 1992; Reale et al., 2008). Other proteins detected in thick, mature BlamD, such as vitronectin, MMP-7, TIMP-3, C3, and C5b-9 (Lommatzsch et al., 2008) are also found in drusen and may
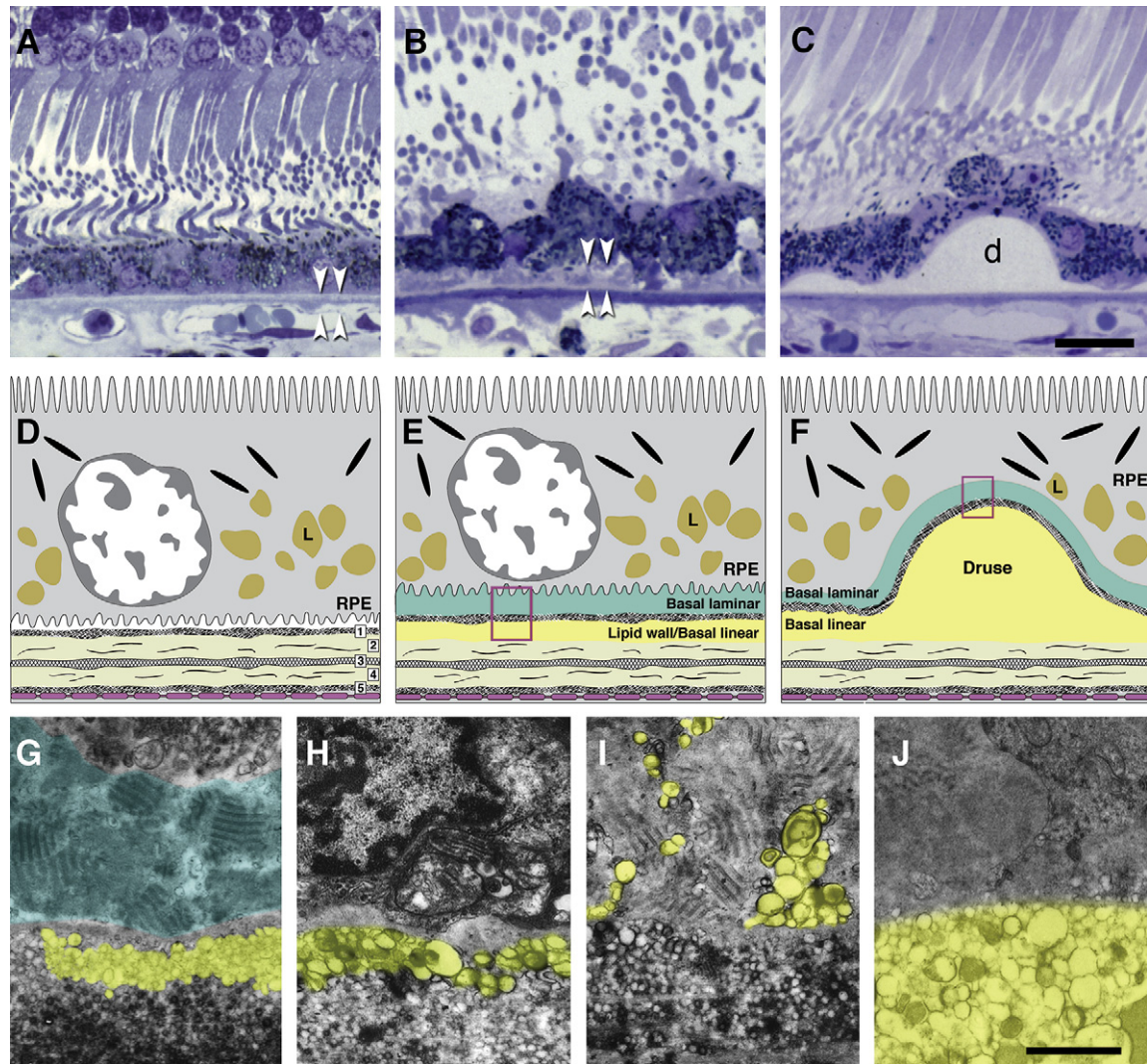
Fig. 2. Bruch's membrane and ARMD lesions. A–C: 1 μm sections, toluidine blue. Bar in C, 20 μm; A. Normal. RPE, BrM (arrowheads). B. Basal deposits (arrowheads) external to disrupted RPE. C: Druse (d). D-F: cartoons of extracellular lesions. D. BrM has 5 layers in a normal eye: 1, basal lamina of the RPE; 2, inner collagenous layer; 3, elastic layer; 4, outer collagenous layer; 5, basal lamina of the choriocapillary endothelium (fenestrated cells, pink). L, lipofuscin. E. An ARMD eye has basal laminar deposit (BlamD) and basal linear deposit (BlinD) and its precursor, the Lipid Wall. Boxed area is shown in panels G–I. F. Drusen, BlinD, and the Lipid Wall occupy the same plane. Boxed area is shown in panel J. G-J: colorized transmission electron micrographs. Aqua, basal laminar deposit (BlamD); yellow, BlinD, membranous debris, and Lipid Wall. Bar in J, 1 μm G. BlamD (aqua) and Lipid Wall (yellow). H. BlinD. I. Membranous debris crosses BlamD. J. Membranous debris within a large soft druse.

represent components in transit from the RPE to BrM. Basal linear deposit (BlinD), between the RPE basement membrane and the inner collagenous layer of BrM, is thin (0.4–2 μm) and contains mostly membrane-like coils and lipid pools. Because BlinD is located in the same plane and contains the same material as soft drusen, these lesions are plausibly considered alternate forms of the same entity by microscopists and clinicians alike (Bressler et al., 1994; Curcio and Millican, 1999).

In 1993, Green and Enger stated that understanding differences between BlamD and BlinD would provide important insight into ARMD pathogenesis (Green and Enger, 1993). Large, confluent, or soft (indistinct edges) drusen and BlinD are universally recognized as the specific lesions of ARMD (Curcio and Millican, 1999; Green and Enger, 1993; Sarks et al., 2007). The presence of BlinD with a continuous layer of BlamD is said to signify threshold ARMD (Sarks et al., 2007). and thick layers of Blam25

D indicate increased risk for ARMD advancement (Sarks, 1976; Spraul and Grossniklaus, 1997). According to Sarks et al, "BlamD is remarkably resilient, persisting even in areas of geographic atrophy

and disciform scars… although not specific for ARMD, it is found in all eyes with disease, making it a …reliable marker for ARMD. In terms of the disease process, however, BlamD appears inert" (Sarks et al., 2007). Thus, understanding the origins and effects of BlinD should now be considered critical. Thin and flat like the RPE layer and BrM adjoining it, BlinD might require fewer formative processes than drusen, which involve three-dimensional expansion and participation of non-RPE cells (Hageman et al., 2001).

3.3. Approaches to new knowledge

Discovery of characteristic lesion components has been a successful means of identifying biological pathways perturbed by coronary artery disease (CAD) (Smith and Slater, 1972), Alzheimer disease (Selkoe et al., 1982), and ARMD (Mullins et al., 2000). We, with others (Handa et al., 1999; Kamei and Hollyfield, 1999), have expanded this approach to include BrM and its age changes, which by virtue of sharing the same compartment as the specific lesions, has great potential for direct involvement in forming them.

Age-related changes in BrM include thickening, accumulation of cross-linked and electron-dense debris, reduced collagen solubility due to cross-linking, and the deposition of neutral lipids, AGEs, and TIMP-3 (Fariss et al., 1997; Feeney-Burns and Ellersieck, 1985; Handa et al., 1999; Karwatowski et al., 1995; Pauleikhoff et al., 1990). We chose to study lipid deposition (Section 4) because of its recognized role in initiating CAD (Section 5). We focused on using normal and ARMD human tissues provided by the Alabama Eye Bank. As ophthalmic records are not required by U.S. eye banks, we developed methods for identifying ARMD eyes via post-mortem fundus appearance (Curcio, 2005; Curcio et al., 1998).

4. Neutral lipids accumulate with age in BrM (Fig. 3)

Clinical observations on the natural history of serous (fluid-filled) RPE detachments in older adults led to the hypothesis by Bird and colleagues that a lipophilic barrier in BrM blocked the normal outwardly-directed fluid efflux from the RPE (Bird and Marshall, 1986). Impaired movement of fluid from RPE or from leaky vessels in choroidal neovascular membranes was thought to contribute to the formation of RPE detachments in ARMD patients (Marshall et al., 1998). Lipophilic material in BrM, if unevenly distributed, could explain the non-uniform distribution of water-soluble sodium fluorescein in clinical angiography of RPE detachments.

This hypothesis was tested in a seminal laboratory study by Pauliekhoff and colleagues (Pauleikhoff et al., 1990), who used three histochemical stains to identify lipids in cryosections of eyes from 30 donors (1–95 yr) with grossly normal maculas. Bromine Sudan Black B (BSBB) binds phospholipid (PL), triglyceride (TG), EC, UC, and FA. Bromine-Acetone-Sudan Black B (BASBB) binds only acetone-resistant phospholipid after acetone removes other lipids. Oil red O binds TG, EC, and FA (Adams and Bayliss, 1975; Luna, 1968), which are hereafter called collectively neutral lipids. Of these methods, oil red O-binding material localized to BrM, while the two other dyes additionally labeled cells throughout the choroid. All stains gave similar results with regard to age: no eyes from donors <30 yr exhibited staining, eyes from donors 31–60 yr exhibited variable staining, and eyes from donors ≥61 yr exhibited moderate to intense staining for lipids (Fig. 3A,B). These findings were repeated (44 eyes, 4–88 yr, (Pauleikhoff et al., 2000); 17 eyes,

32 wk to 92 yr (Haimovici et al., 2001)), with less intense staining in peripheral BrM (Haimovici et al., 2001).

This finding had little historical precedent. Verhoeff speculated that lipid deposition might precede basophilia and fragmentation due to "lime salts in the elastic layer" of BrM (Verhoeff and Sisson, 1926). Lauber (Lauber, 1924) (cited by Rones (Rones, 1937)) treated phthisical eyes with a Sudan stain, noted that deposits between the "lamina vitrea" (BrM) and the RPE and granules within the RPE both appeared bright red, and inferred chemically similar substances in both locations and an RPE origin for the deposits. Years later the oil red O staining of retinoids was recognized within lipofuscin, a distinct age-pigment that accumulates markedly in the RPE (Eldred and Lasky, 1993; Pauleikhoff et al., 1990), without evidence that lipofuscin and BrM lipids are chemically related. An early histochemical description of drusen (Wolter and Falls, 1962) can be interpreted as the first evidence for neutral lipid in BrM. Wolter and Falls stated that in two patients 65 and 83 years of age "hyaline bodies {drusen} are in contact with and of similar staining characteristics of {BrM}…they stain reddish with … oil red O," recognizing the common constituents of these two extracellular structures.

5. apoB, MTP, and atherosclerotic cardiovascular disease (Figs. 4,5)

5.1. Cholesterol and its forms (Fig. 4A)

A hydrophobic alcohol built up from condensations of isoprene units, cholesterol is an essential component of all animal cell membranes (Fliesler and Keller, 1997) (Fig. 4A). Cholesterol exists in two chemical forms and three physical forms. The two chemical forms are unesterified (UC) or bound by an ester linkage to a fatty acid at the 3-beta-hydroxy group to produce esterified cholesterol (EC). The three physical forms are differentiated by the relative proportions of UC, EC, and solubilizing PL – oily droplets (EC > UC > PL), membranes (UC and PL), and crystals (UC only) (Small and Shipley, 1974).

Cellular cholesterol levels represent a balance of endogenous synthesis and uptake of exogenous cholesterol delivered via lipoproteins, on one hand, and turnover via release, on the other. Multiple processes for cellular cholesterol release (Cavelier et al., 2006) include
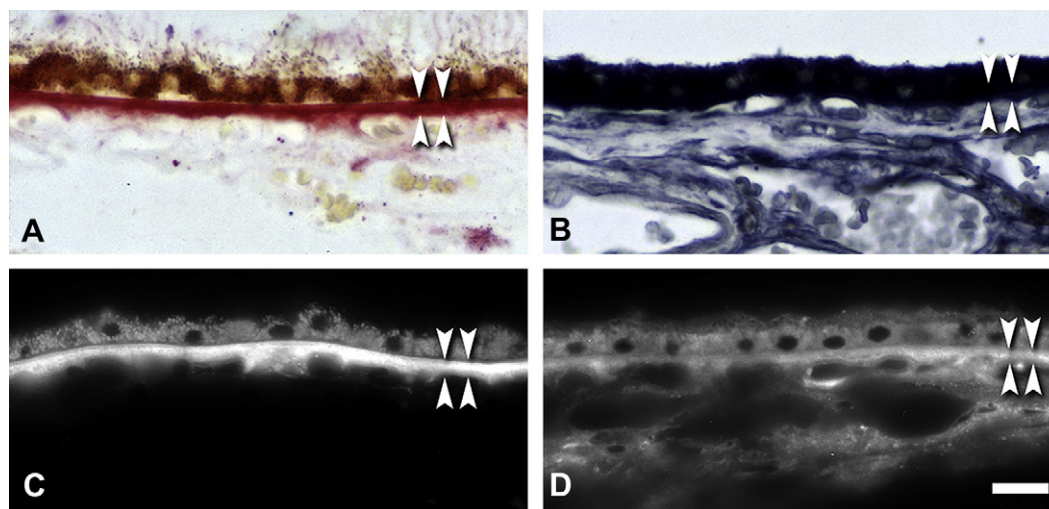
Fig. 3. Lipid histochemistry of BrM and choroid. Normal macula of a 79 yr old donor. RPE is shown at the top, and choroid at the bottom, of each panel. BrM is bracketed by arrowheads. Bar in D, 20 μm. A. Oil red O stains BrM and lipofuscin. B. Sudan Bromine Black B stains BrM, RPE, and cells throughout choroid. C. Filipin for esterified cholesterol stains BrM. RPE lipofuscin autofluorescence is much less intense than filipin, and it is a different color under ultraviolet illumination. D. Filipin for unesterified cholesterol stains BrM, RPE, and choroidal cells.
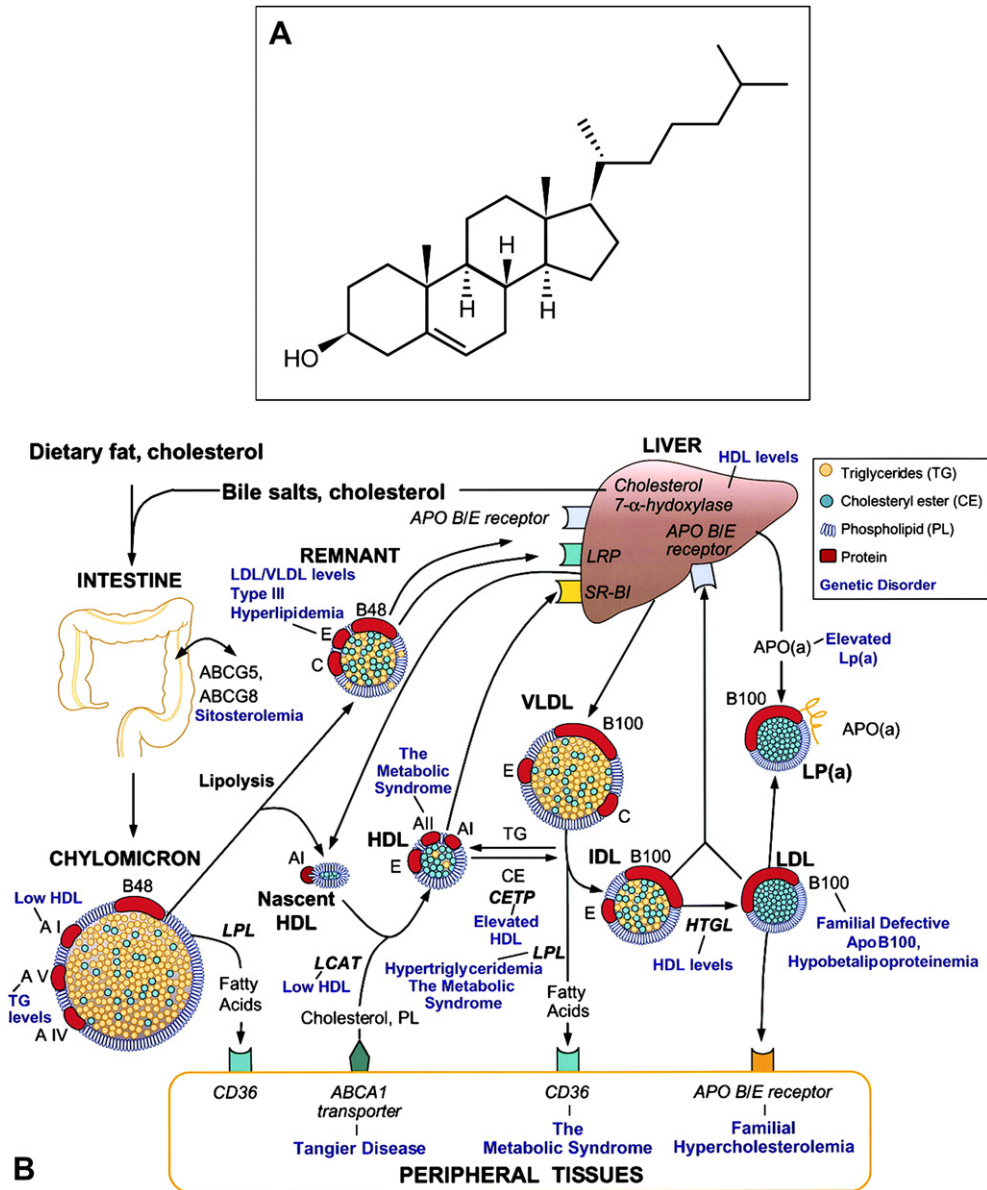
Fig. 4. Cholesterol and lipoprotein basics. A. Cholesterol structure. Cholesterol is esterified to long chain (>16:0) fatty acids at the 3 β-hydroxy group. B. Lipoprotein homeostasis in plasma (with permission (Lusis et al., 2004)). See Section 5.3 for details.

passive diffusion from membranes, release to circulating high density lipoprotein (HDL) via ABCA1 for clearance by the liver as bile salts (reverse cholesterol transport), conversion via cytochrome P450-dependent cholesterol oxidases to either 24S-hydroxycholesterol (by neurons) or 27-hydroxycholesterol (by macrophages) for secretion, and secretion with endogenous apoE (by macrophages and glia). EC, the most highly hydrophobic neutral lipid, is the storage and transport form of cholesterol. It can be passively released from dying cells containing lipid droplets (e.g., (Ball et al., 1995)), and it can be actively secreted from cells as part of apoB-containing lipoprotein particles (e.g., (Temel et al., 2007)).

5.2. Lipoprotein particles (Fig. 4B)

Sometimes called nature's nanoparticles, lipoproteins are multimolecular complexes that solubilize a neutral lipid core (essentially an oil droplet) containing TG and EC, within an

approximately 2 nm thick surface of protein, UC, and PL (Havel and Kane, 2001; Jonas, 2002) (Fig. 4B). The major classes of lipoproteins were initially described and named based on their flotation properties in density gradient ultracentrifugation. Diameters range from 7 nm for HDL to 600 nm for dietary chylomicrons (CM). Low density lipoprotein (LDL) and HDL have the most EC-rich cores and consist of 30–50% protein. LDL (22 nm), very low density lipoprotein (VLDL, 75 nm), and CM have on their surfaces a single molecule of apoB (Section 5.4). In addition to apoB, VLDL additionally has apolipoproteins E and C-III, and CM, apolipoproteins E, C-III, A-I, and A-II, on their respective surfaces. CM and VLDL have highly TG-rich cores and the least protein (1–10%).

5.3. Lipoprotein metabolism in plasma (Fig. 4B)

Cholesterol is transported through the circulation as complexes with various apolipoproteins that sequester the lipids and also act
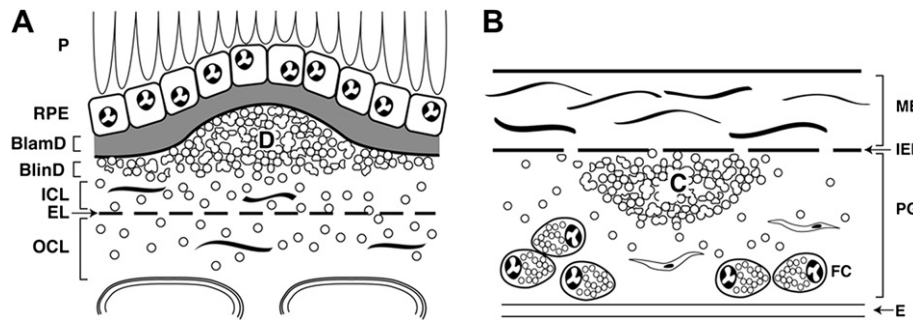
Fig. 5. ARMD lesions vs plaque. Schematic cross-sections of BrM from an eye with ARMD (B) and atherosclerotic arterial intima (B). Endothelium and vascular lumens (choriocapillary, A; artery, B) are at the bottom. Drawings are not at scale. The thickness of normal BrM and intima is 4–6 μm and 100–300 μm, respectively. Small circles in BrM (A) and PG layer (B) indicate EC-rich lipoproteins, native and modified. A. P = photoreceptors, RPE = retinal pigment epithelium, R-BL = RPE basal lamina, BlamD = basal laminar deposit, BlinD = basal linear deposit, D = druse, ICL = inner collagenous layer EL = elastic layer, OCL = outer collagenous layer. B. ME = musculo-elastic layer, IEL = internal elastic layer, C = lipid-rich core, PG = proteoglycan layer, FC = foam cells. Modified from Malek et al., (2003), with permission from the American Society For Investigative Pathology.

as cofactors for enzymes or ligands for uptake by cellular receptors (Crispin, 2002; Havel and Kane, 2001; Jonas, 2002) (Fig. 4B). Dietary lipids are absorbed in the intestine, packaged into CM, and then secreted into lymph. Upon entering the circulation, TG are hydrolyzed through the action of lipoprotein lipase and the resulting remnants taken up by interaction of apoE with the LDL receptor (LDL-R, also called apoB, E receptor) and the LDL-R related protein. During lipolysis, surface PLs and CM proteins slough off to give rise to HDL precursors. Hepatocytes package TG and EC into VLDL particles. Lipoprotein lipase acts on them, hydrolyzing TG to release fatty acids to produce intermediate-density lipoproteins (IDL), which can be taken up at the LDL-R, or further lipolyzed, partly through hepatic lipase action, to produce LDL. Lipids transfer between lipoprotein particles by the activity of cholesteryl ester transfer protein and PL transfer protein.

LDL, the major cholesterol-carrying particle in most individuals, is removed from circulation by the LDL-R. Mature, spherical HDLs are formed largely in the circulation from apoA-I and apoA-II secreted by liver and intestine and from the surface PLs of CM and VLDL during their lipolysis. HDL precursors take up cholesterol from various tissues through interaction with ABCA1 transporter, and this cholesterol is esterified by lecithin: cholesterol acyl-transferase (LCAT). EC is selectively taken up from HDL particles without degradation of apoA-I protein through interaction with the scavenger receptor B-I (SRB-I) at the liver and elsewhere.

5.4. Intracellular production of apoB-lipoproteins

One of the largest plasma proteins, apoB is a secretory glycoprotein with 16 N-linked oligosaccharides related to the egg yolk protein vitellogenin (Hussain et al., 2003; Olofsson and Boren, 2005; Shoulders and Shelness, 2005). One gene encodes 2 forms of apoB by the process of post-transcriptional mRNA editing implemented by apoB editing complex 1 (apoBEC-1). This deoxycytidine deaminase enzyme, present in intestine of all mammals and in liver of rodents, creates a stop-codon at position 2153 that truncates the nascent polypeptide at 48% of its full length. In contrast, the full-length 4536 residue apoB-100, is 512 kDa when fully glycosylated and secreted by liver. The 5 domains in apoB-100 (3 α-helical, 2 β-strand) confer amphipathic properties that promote binding to lipid in the particle core while interacting with plasma at the particle surface. ApoB is insoluble when delipidated, and uniquely among apolipoproteins, cannot transfer from one lipoprotein particle to another in plasma.

Assembly of apoB-lipoproteins requires microsomal triglyceride transfer protein (MTP), a soluble heterodimer in the lumen of

endoplasmic reticulum (Gordon and Jamil, 2000; Wetterau et al., 1997). MTP consists of a widely expressed 58 kDa protein disulfide isomerase and a unique 97 kDa protein that transfers neutral lipid, preferentially TG, to apoB while the apoB transcript is translated (Athar et al., 2004; Jamil et al., 1995). The MTP gene has two splice variants that both encode functional proteins (Dougan et al., 2007; Mohler et al., 2007). MTP-mediated lipid transfer allows apoB to fold correctly and evade intracellular degradation via the ubiquitin-proteosome system and other pathways (Fisher and Ginsberg, 2002; Yao et al., 1997). ApoB and MTP together create a pocket that accommodates an expanding lipid droplet during transit through the endoplasmic reticulum and Golgi (Segrest et al., 1999). Cells expressing apoB without MTP cannot secrete lipoproteins (Gordon et al., 1994; Leiper et al., 1994; Sellers and Shelness, 2001; Wetterau et al., 1992).

ApoB's principal function is delivering exogenous (CM) or endogenous (VLDL) TG and cholesterol to peripheral tissues. CM also deliver lipophilic vitamins A, E, and K. ApoB's role in physiology has been expanded by evidence that it is also expressed in heart, kidney, and placenta (Madsen et al., 2004; Nielsen et al., 1998). The heart secretes lipoproteins to regulate cardiomyocyte TG content and forestall lipotoxicity by releasing fatty acids unconsumed by mitochondrial fatty acid β-oxidation (Björkegren et al., 2001). MTP is even more widely expressed than apoB (cardiomyocytes, kidney, testis, ovary, pancreas, placenta, and adipocytes), highlighting a generalized role in PL transfer (Hussain et al., 2008; Shoulders and Shelness, 2005). In mice, absence of apoB is lethal in utero, and absent or reduced apoB is associated with neural tube defects (Farese et al., 1995; Raabe et al., 1998). Mutations of the MTP gene cause the rare autosomal recessive disorder abetalipoproteinemia (MIM 200100, Bassen-Kornzweig disease) (Berriot-Varoqueaux et al., 2000; Wetterau et al., 1992), and truncation-specifying mutations of the APOB gene cause hypobetalipoproteinemia (MIM 107730), a genetically heterogeneous autosomal trait (Schonfeld, 2003). Both disorders include pigmentary retinopathies historically attributed to reduced delivery of lipophilic vitamins from absent or low apoB lipoproteins in plasma (Grant and Berson, 2001).

5.5. Role of apoB lipoproteins in producing atherosclerotic plaques (Fig. 5)

It is instructive to compare BrM to the inner wall of large arteries, which are divided into 3 layers (from inside out): the intima, media, and adventitia (Fig. 5). The intima is located between two diffusion barriers, an endothelial cell layer at the lumen and a dense elastic layer (Stary et al., 1992). Throughout life the intima

of atherosclerosis-prone large arteries thickens adaptively to the mechanical stresses of blood flow and wall tension (Malek et al., 1999). Collagen, elastin, and proteoglycans at sites of intimal thickening specifically interact and bind with lipoprotein particles from plasma (Camejo et al., 1998). The deep intima, near the elastic layer, is the site of extracellular lipid accumulation. Macrophages and smooth muscle cells filled with EC-rich oil droplets (foam cells) accumulate in a region lateral to this lipid-rich core (called the shoulder). Smooth muscle cells also contribute to an overlying fibrous cap.

A widely supported hypothesis of atherogenesis states that CAD is a "Response-to-Retention" of the arterial intima to apoB-containing lipoproteins entering from plasma. This hypothesis, first formulated by Williams and Tabas in 1995 (Tabas, 1999; Williams and Tabas, 1995, 1998, 2005), summarized 8 decades of research, beginning with Anitchkow's cholesterol-fed rabbits in 1913 (Steinberg, 2005). This work established that the earliest and largest cholesterol component in the lipid-rich core of atherosclerotic plaques derives from plasma apoB-containing lipoproteins that directly infuse into the intima (Chao et al., 1990; Guyton and Klemp, 1989; Katz and Small, 1980; Nievelstein et al., 1991; Smith, 1974). These are largely LDL with some partly hydrolyzed CM remnants. Lipoprotein particles enter intima via endothelial cell trancytosis and bind to specific proteoglycans (biglycan, decorin, and versican) (Kovanen and Pentikainen, 1999; O'Brien et al., 2004; Pentikainen et al., 1997). This binding can be enhanced via a link to lipoprotein lipase (Pentikainen et al., 2000, 2002).

Oxidative and non-oxidative processes modify retained lipoproteins and evoke a cascade of downstream events. These events include deposition of cholesterol, differentiation of foam cells from extravasated monocytes-macrophages, proliferation and differentiation of smooth muscle cells, endothelial cell injury, cytokine release, neovascularization, rupture, and hemorrhage. One specific role of the lipid-rich core of a plaque is structural instability (Davies, 1996). Abundant foam cells and thin overlying cap contribute to the vulnerability of a plaque to rupture (Shah, 2007; Varnava et al., 2002).

Evidence supporting the Response-to-Retention hypothesis included the demonstration of early LDL entry through intact endothelium (Schwenke and Carew, 1989a,b), chemical and morphological description of insudated lipoproteins and their successor particles (Chao et al., 1990; Guyton and Klemp, 1989), and the abatement of atherosclerosis by reduced apoB binding to intimal proteoglycans (Skålen et al., 2002). Although recent research has highlighted inflammatory processes (e.g., C-reactive protein levels) as independent risk factors, these may be insufficient to initiate disease in the absence of dyslipidemia (Ridker et al., 2002; Zacho et al., 2008).

With this knowledge about apoB-lipoprotein-instigated disease in arterial intima, the RPE/BrM complex can be interrogated for evidence of physical and chemical forms of cholesterol, lipid-rich and structurally unstable lesions, and lipoprotein particles as a source of extracellular cholesterol. To understand the antecedents of disease in normal physiology, we should also seek a cellular source of lipoprotein particles and biological processes to drive lipoprotein production.

6. Evidence for an intra-ocular apoB-containing lipoprotein

6.1. Cholesterol in aged BrM – histochemical, physicochemical studies (Fig. 6)

Lipids that bind the histochemical stain oil red O increase with age in normal human connective tissues, including the sclera (Broekhuyse, 1972), cornea (Gaynor et al., 1996), intima of large arteries (Smith, 1974), and BrM (Pauleikhoff et al., 1990). In the intima, the oil red O-positive material comprises small (60–200 nm) extracellular droplets highly enriched in EC relative to UC (69% EC, 22% UC, and 9% PL) (Bocan et al., 1986; Chao et al., 1990; Guyton and Klemp, 1988, 1989; Kruth, 1984a). The source of EC in sclera, cornea, and arterial intima is LDL translocated from plasma into connective tissues. EC-enriched particles are thought to arise from smaller LDL particles by extracellular matrix-mediated trapping of LDL, degradation of LDL protein and/or PL components, and fusion of the remaining lipid components (Kruth, 1997). It is thus important to determine whether lipid deposition in BrM is an ocular manifestation of this ongoing systemic process or a phenomenon unique to retina.

Curcio et al (Curcio et al., 2001) examined the EC and UC content of BrM using the fluorescent, polyene antibiotic filipin (Fig. 3C,D). This compound binds specifically to the 3-β-hydroxy group on cholesterol and other sterols, and it can be used to identify cholesterol in tissue sections (Kruth, 1984a,b) and in cultured cells (Lakkaraju et al., 2007). Importantly, EC can also be localized using filipin, by extracting native UC with ethanol, hydrolyzing EC with the enzyme cholesterol esterase, then binding the newly released UC with filipin. This method is an improvement over oil red O, because it is specific for one class of neutral lipids, and because the fluorescence, while labile, is readily quantifiable with digital microscopy. With this method, EC was localized in macula and temporal periphery of 20 normal eyes from age 17–92 years (Curcio et al., 2001) (Fig. 6). In the macula, EC was undetectable before 22 yr and then rose linearly throughout adulthood to reach high and variable levels in aged donors. EC was detectable in periphery at roughly 1/7 the level of macula, but still increased significantly with age. In the same eyes, UC in macular BrM also increased throughout adulthood, although not as steeply as EC, and it did not increase significantly with age in peripheral retina. Extending the original findings of lipids accumulating with age in BrM (Pauleikhoff et al., 1990), this study identified a specific class of molecules (EC) and quantified their increase with age.

Haimovici and colleagues (Haimovici et al., 2001) used hot stage polarizing microscopy, a physicochemical technique (Small, 1988; Waugh and Small, 1984), to examine the birefringence and melting characteristics of lipids in BrM and sclera. EC in tissue sections appears as liquid crystals ("Maltese crosses") when examined through a polarizing filter. When sections are heated and cooled slowly, liquid crystals melt and reform at characteristic temperatures dictated by the saturation level of the major ester. BrM contained Maltese crosses that melted at a higher temperature than those in sclera. This higher melting temperature suggests that BrM EC contains less polyunsaturated fatty acid than scleral EC, which accumulates cholesteryl linoleate (18:2n6)[1] from plasma lipoproteins with age (Smith, 1973). Few birefringent crystals signifying TG were found in either tissue.

6.2. BrM lipoprotein morphology and distribution (Fig. 7)

Ultrastructural studies since the 1960's have illustrated numerous small (<100 nm), round electron-lucent spaces in BrM of older eyes (Bairaiti and Orzalesi, 1963; Feeney-Burns and Ellersieck, 1985; Green and Enger, 1993; Hogan et al., 1971; Killingsworth, 1987; Nakaisumi et al., 1964; Pauleikhoff et al., 1990). The occasional presence of a single electron-dense line at the borders of

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[1] We use these terms for fatty acids. Chemical notation: CXX:Yn-Z, where XX is carbon chain length, Y is the number of double bonds along the carbon chain, and Z is the position of the first double bond, counting from the methyl carbon. Saturated, no double bonds. Unsaturated, one or more double bonds.
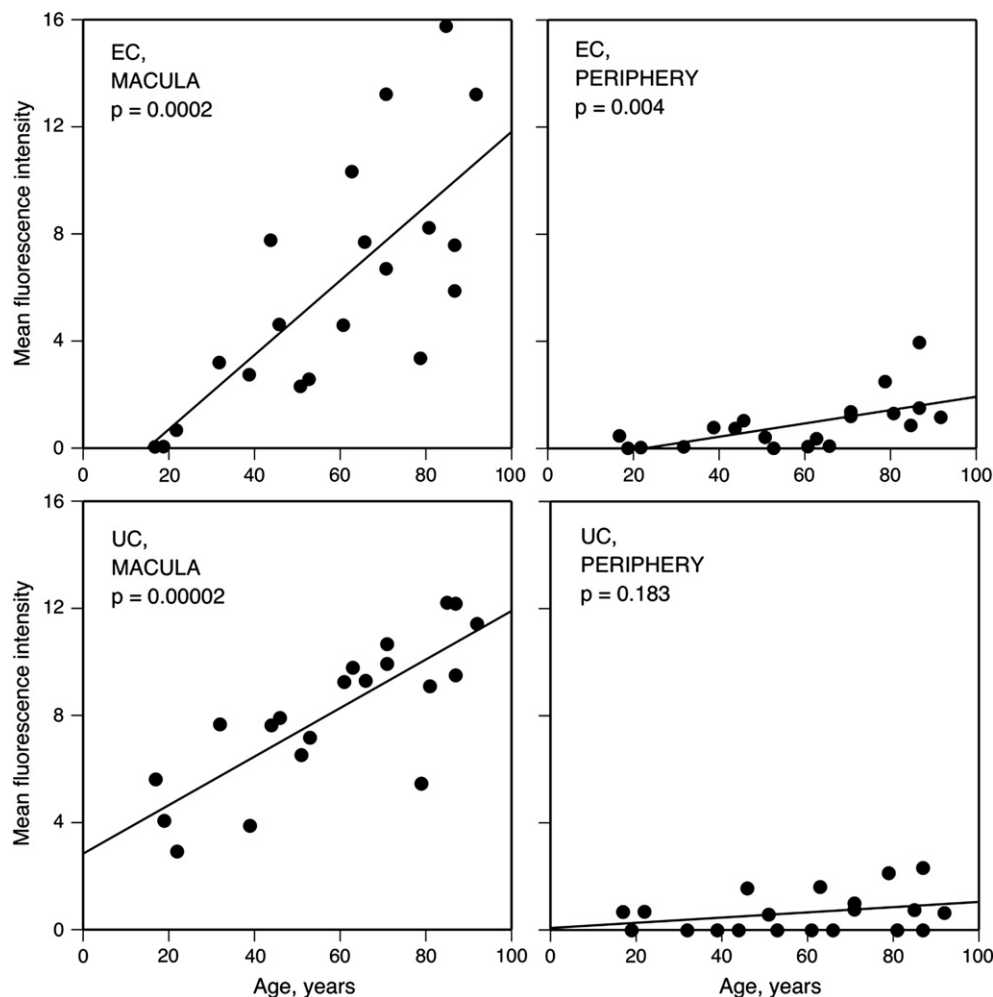
Fig. 6. BrM cholesterol increases with age. Filipin fluorescence due to EC and UC increases with age in normal BrM. For EC in the macula (A) and periphery (B) and UC in the macula (C) and periphery (D), fluorescence intensity (x 10^{-6} arbitrary units) for each eye is corrected for background autofluorescence of BrM. Reprinted from (Curcio et al., 2001); copyright is held by Association for Research in Vision and Ophthalmology.

these spaces presumably accounts for their frequent description as membranous or vesicular (liposomes with aqueous interiors). However, conventional methods of tissue preparation for thin-section transmission electron microscopy extract tissue lipids, resulting in sub-optimal visualization of fine structure. Tissue post-fixation with osmium-tannic acid-paraphenylenediamine (OTAP) preserves neutral lipid (Guyton and Klemp, 1988) and was used to show that BrM vesicles were actually solid and electron dense (Curcio et al., 2001, 2005b). Because biochemical evidence in Sections 6.3–6.4 firmly indicates that these particles are lipoproteins, this name will be used henceforth.

More dramatic views of the particles were made possible by quick-freeze/deep-etch (QFDE), an ultrastructural tissue preparation technique (Shotton and Severs, 1995) that reveals lipids and extracellular matrix with stunning detail. QFDE was used to demonstrate the accumulation of lipoproteins in the aortic wall at the earliest stages of atherosclerosis (Frank and Fogelman, 1989; Nievelstein et al., 1991; Tamminen et al., 1999). Briefly, tissue is rapidly frozen, fractured, and then "etched" by sublimating vitrified water at the fracture surface, leaving only frozen matrix behind. This matrix is then rotary-shadowed with a platinum/carbon mixture, creating a replica that is stabilized with more carbon. The tissue itself is then dissolved away, and the replica is viewed using a transmission electron microscope.

QFDE revealed that the vesicles accumulating in BrM with age were not only solid particles but also distinguished by a surface and core structure (Fig. 7A) (Huang et al., 2007b; Ruberti et al., 2003). Particles typically varied in size from 60 to 100 nm but could be as large as 300 nm. Occasionally particles appeared to coalesce with one another (Fig. 7B,C). Particle appearance in QFDE preparations was consistent with their designation as lipoproteins, in several ways (Huang et al., 2007b). 1) Particles appeared solid and did not etch significantly during the QFDE process, indicating that they contained little water. 2) Particle appearance was similar to that seen previously in QFDE studies of LDL particles accumulating in the aortic intima (Frank and Fogelman, 1989). 3) Particles were found in the same locations in BrM as the lipid-containing particles identified using OTAP (Curcio et al., 2001). 4) Particle cores could be extracted with the Folch reagent for lipid, leaving the surface largely intact (Huang et al., 2007a) (Fig. 7F,G). 5) The age-related accumulation of particles within BrM throughout adulthood (see Section 7.1) was consistent with light micro-scopic studies and biochemical assessment of lipid deposition in this tissue (Huang et al., 2007b, 2008a). Also seen by QFDE was the accumulation in BrM of small granules (<10 nm) of not yet determined identity that formed complexes with the lipo-proteins (Fig. 7D) (Huang et al., 2007b).
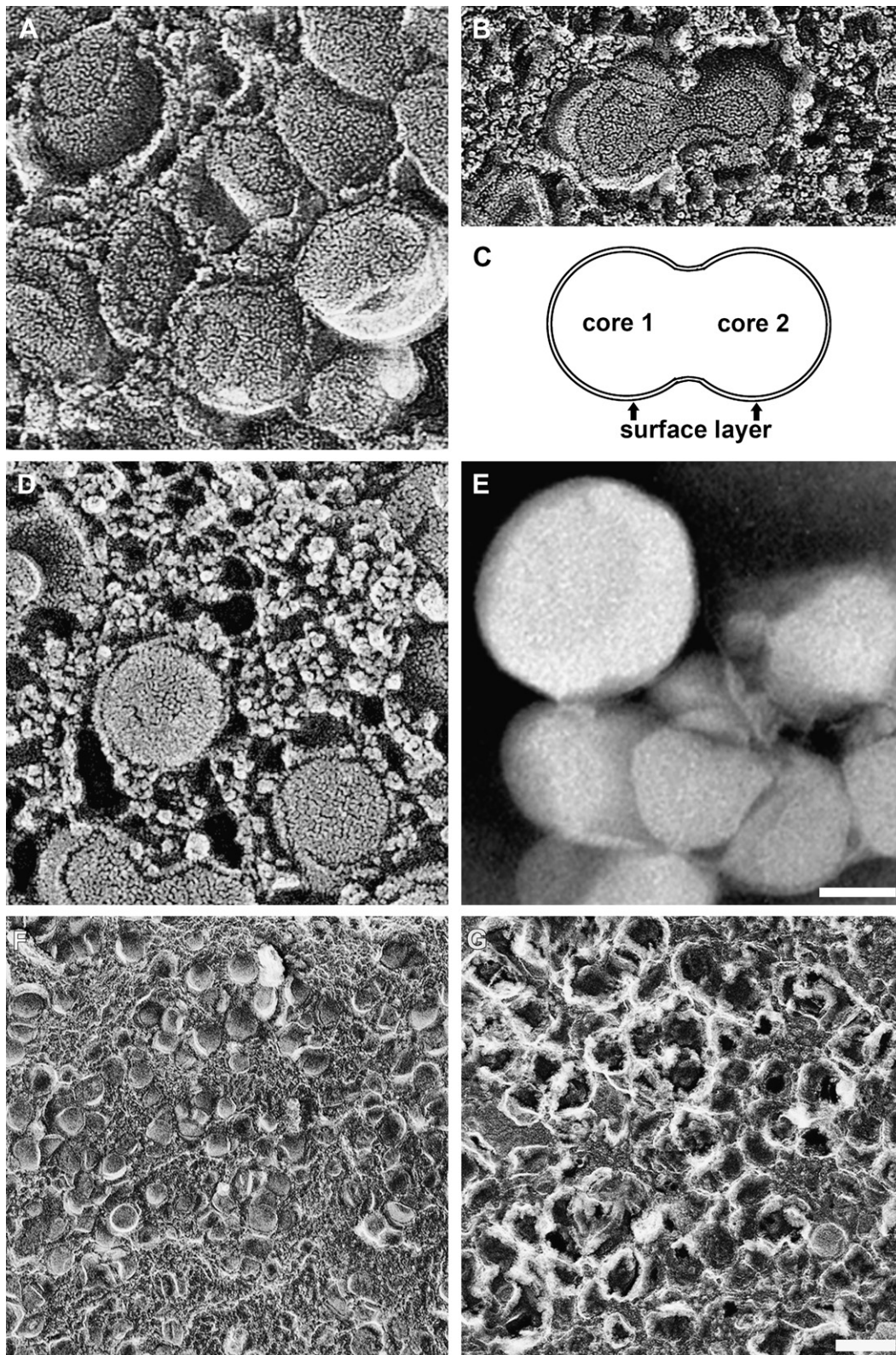
Fig. 7. BrM lipoproteins, in situ and isolated. A-B, D, F-G. QFDE images of normal BrM (Huang, 2007). E. Negative stain electron microscopy (Li et al., 2005a). A. Core and surface of tightly packed lipoproteins. B. Fused lipoproteins. C. Interpretive schematic based on panel B. D. Small granular particles associated with lipoproteins. E. Isolated particles are large, spherical, and electron-lucent. F,G. Lipoproteins in untreated tissue (F) and in tissue treated with chloroform-methanol to remove lipid (G). Only some surface components, presumably apolipoproteins, remain after extraction. Bar in $E = 50$ nm, applies to panels A–E. Bar in $G = 200$ nm, applies to panels F and G.

6.3. Assays of BrM/choroid and isolated lipoproteins (Tables 1–3)

Determining the composition of lipids accumulating in BrM is challenging due to the size and marked heterogeneity of the choroid. At 100–300 μm, the choroid is 1–2 orders of magnitude thicker than BrM itself (2–6 μm) (Ramrattan et al., 1994; Spraul et al., 1996). Moreover, the choroid contains numerous blood vessels (arteries, veins, and capillaries) with plasma lipoproteins and blood cells, as well as resident cells (e.g., vascular endothelium, fibroblasts, melanocytes, macrophages, and neurons). Thus it is important that direct assays be validated by results of histochemical studies utilizing tissue sections.

Surprisingly, the potential for contamination from plasma in such assays is probably small. Less than 6% of EC from extracts of partially stripped choroid could be accounted for by plasma lipoproteins retained in vessels, which drain of blood at death (Curcio et al., 1998, 2001). Further, in cryosections only a few choriocapillaris lumens contain immunoreactivity for apoB (presumably on plasma lipoproteins) (Malek et al., 2003). The greater source of contaminating lipids, then, is the extraneous choroidal vessels and stroma left on BrM, as PLs and UC both localize to all choroidal cells. Removal of choroidal tissue is highly operator-dependent and ideally should be monitored with histology.

Table 1 lists 7 studies that assayed BrM lipids from tissue extracts (as opposed to lipoproteins released from BrM, see below). Most studies used fresh tissue, some used formalin-preserved tissue to allow separation of layers or to allow correlative histochemical staining, and some used other parts from the same eyes as positive control or comparison tissues. Studies used vertically oriented cryosections or circular punches of choroid 4–8 mm in diameter, sometimes with major choroidal arteries and veins removed under histological monitoring. Extracted lipids were generally separated into classes by thin layer chromatography or liquid chromatography, followed by derivatization of the fatty acids for analysis by flame ionization detection. Other studies used thin layer chromatography and densitometry for all lipid classes, enzymatic fluorimetry for total and unesterified cholesterol, or electrospray ionization mass spectrometry for EC only. Data were reported as mass (μg or nmoles normalized by dry weight or nominal area of choroidal sample) or as composition (% of total lipids, or % of class).

From these 7 studies, a consensus description of BrM lipid composition (mole percent of the major classes and their fatty acids) was sought. Results obtained with comprehensive assays, results repeated with different assays and/or by different laboratories, and consistency with histochemical findings were given greater weight. There is now agreement on the composition of the neutral lipid that accumulates with age in BrM (Table 2): 1) RPE/BrM/choroid is much more enriched in neutral lipid/EC than neurosensory retina (Bretillon et al., 2008b; Curcio et al., 2001; Gülcan et al., 1993). 2) Within RPE/BrM/choroid, EC is the prominent neutral lipid, exceeding TG by 4–10-fold (Bretillon et al., 2008b; Li et al., 2005a; Wang et al., 2009b), despite an early report to the contrary (Holz et al., 1994). 3) EC represents 50–60% of the cholesterol detected (Bretillon et al., 2008b; Curcio et al., 2001; Wang et al., 2009b). 4) The predominant fatty acids in the neutral lipid/EC fraction are linoleate (18:2, 45.1%), oleate (18:1, 20.3%), palmitate (16:0, 13.9%), arachidonate (20:4n6, 6.8%), and stearate (18:0, 2.5%) (Bretillon et al., 2008b; Li et al., 2005a; Wang et al., 2009b). Together these compounds account for 88–89% of the EC detected. 5) The fatty acid docosahexaenoate (22:n6, 0.5%) is present in minute quantities (Bretillon et al., 2008b; Gülcan et al., 1993; Li et al., 2005a; Spaide et al., 1999; Wang et al., 2009b). 6) Relative to macula, peripheral BrM/choroid has neutral lipid of similar fatty acid composition and less EC relative to UC (Curcio et al., 2001; Gülcan et al., 1993). 7) Neutral lipids increase markedly with age relative to PLs, especially after age 60 yr (Holz et al., 1994; Sheraidah et al., 1993).

Understanding the role of lipoproteins in aging and ARMD has been furthered by direct evidence that particles with lipoprotein characteristics are isolable from BrM/Ch, as they are from arterial intima (Chao et al., 1990; Chung et al., 1994; Rapp et al., 1994). Following a double, high-salt buffer extraction from tissue homogenates (Li et al., 2005a; Wang et al., 2009b), particles released from BrM exhibit a density similar to plasma VLDL (0.95–1.006 g/ml), relative enrichment with EC (59% of total cholesterol), and spherical particle morphology indicative of a neutral lipid core (Fig. 7E). By negative stain electron microscopy, these highly electron-lucent particles have a mean diameter of

Table 1
BrM lipid composition: studies using tissues.

Reference	N	Age, yr {1}	D to P, hr {2}	Fresh/fixed	Region	Tissues	Prep	Assays
(Sheraidah et al., 1993)	27	1.5, 97	n.a.	Fixed 24 h	M	RPE/BrM/Ch, BrM/Ch	Cryosections	TLC; GC, FID
(Holz et al., 1994)	32	3, 97	17.6	Fresh	M, P	BrM/Ch	7 mm	TLC
(Gülcan et al., 1993)	12 pr	4th, 9th decade	<24	Fixed 1 h	M, P	Ret; RPE; BrM/Ch	4 mm	HPLC; saponification & HPLC for FA
(Pauleikhoff et al., 1994)	27	1, 97	n.a.	Fixed 24 h	M	RPE/BrM/Ch	Cryosections	TLC; GC, FID
(Spaide et al., 1999)	13	40, 78	<24	Fresh	M	BrM/Ch	7 mm; vessels removed; histo	Saponified; HPLC for FA
(Curcio et al., 2001)	10	71, 87	<4	Fixed, various times	M (10), P (3)	Ret; BrM/Ch; Ch vessels	8 mm; vessels removed; histo	Enzymatic fluorimetric assay *
(Li et al., 2005a,b)	2	>60	<6	Fresh	All	Ret; BrM	8 mm; vessels removed; histo	ESI/MS
(Bretillon et al., 2008a,b)	27	59, 95	34	Fresh	All	Ret; RPE/Ch	Entire retina, entire RPE/Ch	TLC; GC, FID; saponification & HPLC for FA in retina PL
(Wang et al., 2009a,b)	4	37, 86	<8	Fresh	All	BrM/Ch	Entire BrM, vessels removed	TLC; GC, FID

Notes and abbreviations (see also table at beginning of article).
Age: Minimum and maximum age.
D to P: Death to preparation time.
Fresh/fixed: fixatives were formalin or 4% paraformaldehyde.
Tissues: Ch, choroid.
Region: M, macula; P, periphery.
Prep: Diameter of circular punch given; Histo, quality of dissection monitored by histology.
Assays: ESI/MS, electrospray ionization mass spectrometry; FID, flame ionization detection of fatty acids; GC, gas chromatography; HPLC, high performance liquid chromatography; TLC, thin layer chromatography.
n.a., not available.
*EC mass was originally reported as nmol/g dry weight and should be (nmol × 1000)/g dry weight.

Table 2
Lipid profile of BrM/choroid and isolated particles.

	EC	DG	FA	TG	CL	LYPC	PC	PE	PS	UC	SPM	RE
BrM/choroid												
mole%[a]	29.8	0.7	3.6	3.0	1.5	0.6	15.4	12.6	6.0	26.7	n.a	n.a.
Isolated particles												
mole%[a]	32.4	1.7	6.3	3.3	3.2	1.8	14.2	9.5	4.6	22.9	n.a	n.a.
nmol/eye	270.6	11.7	44.5	23.6	21.4	11.4	109.7	79.4	35.2	184.2	79.5	0.117

EC: esterified cholesterol; DG: diglyceride; FA: non-esterified fatty acid; TG: triglyceride; CL: cardiolipin; LYPC: lysophosphatidylcholine; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PS: phosphatidylserine; UC: unesterified cholesterol; SPM: sphingomyelin; RE: retinyl ester.
n.a., not available.
[a] Calculated relative to the sum of all lipids except SPM and RE.

66 nm. Sub-fractionation produces an additional, higher density peak with less relative EC and a particle appearance consistent with loss of core neutral lipids (Li et al., 2005a). The fragility of larger lipoproteins is well recognized (Anderson et al., 1989; Forte and Nordhausen, 1986), and sub-fractionation is not recommended for future studies of BrM lipoproteins.

These two studies (Li et al., 2005a; Wang et al., 2009b) yielded salient characteristics of BrM lipoproteins that along with large size and enrichment with EC justify appellation of the particles as *bona fide* lipoproteins. The neutral lipid composition (Table 2) was very similar to that determined for native BrM/Ch, suggesting that appropriate lipoprotein isolation techniques can retain most particle properties. The following conclusions can be drawn. 1) EC is the predominant neutral lipid. Most notable was the lack of TG, the major core lipid of plasma apoB lipoproteins in the same size class. EC was 11.6-fold more abundant than TG (moles), even higher than in the tissue studies. 2) The distribution of EC fatty acids as determined with two different methods is as follows: linoleate (18:2, 41.3%), oleate (18:1, 18.9%), palmitate (16:0, 17.4%), arachidonate (20:4n6, 6.5%), stearate (18:0, 3.0%), and docosahexaenoate (22:n6, 0.5%), highly similar to the tissue studies. 3) Phosphatidylcholine is more abundant than phosphatidylethanolamine by a factor of 1.29, and sphingomyelin, which binds tightly to cholesterol, is abundant relative to phosphatidylcholine (0.70). These ratios differ markedly from plasma lipoproteins. 4) Particles contain measurable retinyl ester, the transport form of dietary vitamin A. 5) Lipoprotein fractions contain apoB, apoA-I, and apoE, all of which have been identified in BrM; other apolipoproteins have not yet been sought. 6) The distribution of the major lipid classes in BrM lipoproteins differ importantly from apoB- containing plasma lipoproteins (Table 3), indicating that the particles in BrM are not simply a transudate from the circulation. 7) Within each lipid class, however, BrM lipoprotein fatty acids are overall remarkably similar to those plasma lipoproteins, with the principal exception of ~20% lower linoleate (18:2n6) among all lipid classes. This finding raises the possibility that plasma lipoproteins are a *source* of individual lipid classes in BrM lipoproteins.

6.4. RPE lipid processing

Attempts to deduce the source of BrM lipids from compositional studies like those just described operate under the assumption that composition should reflect the source(s). Conclusions based on this reasoning have been hampered by limited data on composition and/or inadequate attention to whether the lipids discovered were localized only in BrM or throughout the choroid as well (Curcio et al., 2001; Holz et al., 1994; Sheraidah et al., 1993; Spaide et al., 1999). This review has summarized converging and repeatable evidence from light microscopic histochemistry, physical chemistry, ultrastructure, and lipid profiling of tissues and isolated lipoproteins (Sections 6.1–6.4) that establishes EC as the primary lipid accumulating with age in BrM. Further, of the major lipid classes, only EC is exclusively localized to BrM, i.e., not also distributed throughout the choroid.

What does high EC concentration within aged BrM signify about its source? Similar questions were raised decades ago regarding arterial intima, in that the earliest EC in atherosclerotic plaques was thought to consist of numerous oil droplets released from dying foam cells. Multiple lines of evidence indicated a composition consistent with a plasma lipoprotein transudate rather than cells (Chao et al., 1990; Guyton and Klemp, 1989; Kruth, 1997; Smith et al., 1967), a key conclusion that contributed to plasma lipid-lowering therapy via statins for treatment of atherosclerosis.

In the eye, various insults evoke engorgement of RPE cells with large oil red O-binding intracellular droplets (El Baba et al., 1986; Feeney-Burns et al., 1981; Fine and Kwapien, 1978; Majji et al., 2000). Such cells could conceivably contribute to BrM neutral lipid if they die and release their droplets. However, the intracellular droplets are much larger (1–2 μm) than BrM particles (66 nm), they apparently have little EC (Anderson et al., 2006) and abundant retinyl ester (Redmond et al., 1998), few RPE cells exhibit droplets in any one normal eye, lipoidal degeneration is not widely prevalent across eyes, and the rate of age-related RPE cell death (Del Priore et al., 2002) is probably too slow to account for the large and universal age-related accumulation of EC in BrM.

Excluding the possibility that lipids accumulating in BrM with age arise from dying RPE cells leaves the best-documented way to release EC from a healthy cell, namely, within the core of an apoB-containing lipoprotein. What could be the source of such a lipoprotein? Perhaps particles of hepatic or intestinal origin exit plasma in the choriocapillaris and adhere to extracellular matrix in BrM, analogous to events in arterial intima or even arcus (Section 5.5). However, isolated BrM lipoproteins differ from plasma lipoproteins in composition (Section 6.3), and indirect evidence from patients argues against a plasma origin (Section 9.2.2). While it is possible that BrM lipoproteins are modified plasma lipoproteins, available

Table 3
BrM lipoproteins vs plasma apoB-lipoproteins.

Lipoprotein	Diameter, nm	Apolipoproteins	EC/ (EC + UC)	EC/ TG	PC/ PE	SPM/ PC
BrM	66	A-I, B-100, *E*, C-I, -II	0.58	11.32	1.29	0.70
Chylomicron	>70	A-I, B-48, C-I, -II, -III	0.59	0.11	6.09	0.28
VLDL	25–70	B-100, C-I, -II, -II, *E*	0.45	0.13	8.30	0.28
LDL	19–23	B-100	0.69	8.52	14.90	0.37

EC: esterified cholesterol; TG: triglyceride; PC: phosphatidylcholine; PE: phosphatidylethanolamine; UC: unesterified cholesterol; SPM: sphingomyelin.
Values for BrM lipoproteins and plasma lipoprotein lipids from (Li et al., 2005a,b) and (Wang et al., 2009a,b).
Values for plasma lipoprotein diameters and apolipoproteins from (Burtis and Ashwood, 1999).
Values for plasma lipoprotein SPM/PC calculated from (Jonas, 2002).

evidence indicates an important contribution of directly secreted lipoproteins from RPE

6.4.1. Expression of lipoprotein pathway genes of RPE

Determining the genes expressed by native human RPE is challenging even for the highly sensitive reverse transcriptase polymerase chain reaction (RT-PCR). Specific impediments to obtaining high quality RNA are the small amount of starting material (6–11 μg total RNA from 2 eyes), variable post-mortem delay to processing, potential contamination with closely associated blood, vasculature, and neurosensory retina, and the presence of melanin, an RT-PCR inhibitor (Eckhart et al., 2000; Giambernardi et al., 1998). Data interpretation is aided by the use of positive controls like liver and brain. Despite these difficulties, mRNA transcripts for both apoE and apoB were confirmed in human RPE and RPE/choroid preparations (Li et al., 2005b; Malek et al., 2003; Mullins et al., 2000), with apoE expression levels third behind brain and liver (Anderson et al., 2001). RPE contains mRNA transcripts for apos A-I, C-I, and C-II, but not C-III (Li et al., 2006; Malek et al., 2003; Tserentsoodol et al., 2006a).

Full-length apoB protein was localized to native RPE using a specific monoclonal antibody (Li et al., 2005b; Malek et al., 2003). Of significance to the apoB system, apoBEC-1 mRNA is not detectable in human RPE (Li et al., 2005b). However, it is present in rat RPE and the rat-derived RPE-J cell line (L. Wang, unpublished data), along with an apoB-48-like protein immunoprecipitable by anti-rat apoB (Wang et al., 2009b), suggesting that rat RPE expresses both apoB-100 and apoB-48 like liver in this species. Of major importance to the apoB system is the presence of both mRNA and protein for the MTP large subunit within native human RPE. The latter was localized with apoB itself to punctate intracellular bodies, presumably endoplasmic reticulum, within both the RPE and, surprisingly, ganglion cells of the neurosensory retina (Li et al., 2005b). In mouse RPE, both MTP isoforms are expressed (Fujihara et al., 2009). The dual expression of apoB and MTP signifies that RPE has the capability of secreting lipoprotein particles (Section 5.4). Finally, RPE expresses other genes encoding proteins important in cholesterol and lipoprotein metabolism, including ACAT-1,-2,[2] LCAT, ABCA1, fatty acid binding protein 5, LDL-R, scavenger receptors B-I and -I, and CD-36 (Duncan et al., 2002; Li et al., 2005a,b; Ryeom et al., 1996; Tian et al., 2005; Tserentsoodol et al., 2006a; Yamada et al., 2008). Roles of these genes in retinal physiology are only beginning to be understood.

These RPE gene expression data provides a basis for re-designating the pigmentary retinopathies of abetalipoproteinemia and hypobetalipoproteinemia (Section 5.4) as intrinsic retinal degenerations. That the retinopathies associated with these mutations of MTP and APOB genes, respectively, are only partly alleviated by dietary supplementation with lipophilic vitamins carried on plasma apoB-lipoproteins (Chowers et al., 2001) is consistent with this view.

6.4.2. RPE lipid composition and apolipoprotein secretion

Lipid droplets are ultrastructurally detectable in basal cytoplasm of mammalian RPE cells (Hirosawa and Yamada, 1976; Robison and Kuwabara, 1977), including humans (Bairaiti and Orzalesi, 1963), and they increase in number and retinyl ester content after dietary or genetic manipulation of retinoid processing (Hirosawa and Yamada, 1976; Redmond et al., 1998; Robison and Kuwabara, 1977). Lipofuscin, which accumulates with age in RPE cells, contains oil red O-binding lipid in proportions different from outer segments, i.e., less PL, more non-esterified palmitate (16:0), arachidonate (20:4n6), and oleate (18:1n9), and less docosahexaenoate (22:6n3) (Bazan et al., 1990). The lipids in lipofuscin are also distinct from those accumulating with age in BrM (Section 6.3). The major fluorophor of RPE lipofuscin, the retinoid derivative A2E, at concentrations similar to those in vivo, can interfere with cholesterol processing in cultured RPE cells, leading to accumulation of EC, UC, and Nieman-Pick transporter in lysosomes, without affecting other lysosomal functions (Finnemann et al., 2002; Lakkaraju et al., 2007). Thus A2E, prominent in aging eyes, could influence the overall amount of cellular cholesterol available for export from RPE by lipoprotein secretion or other pathways (Section 5.1).

Neutral lipids have also been directly assayed in RPE. Frog RPE contains measurable TG and EC (Chen and Anderson, 1992), and outer segment docosahexaenoate is transiently stored as newly synthesized, rapidly hydrolysable TG before being recycled to retina (Bazan et al., 1992; Rodriguez de Turco et al., 1999). Native human RPE and ARPE-19 cells contain UC and EC in lower quantities than the in vitro lipoprotein secretor HepG2 (hepatoma). The EC fatty acid distribution in RPE resembles that of the particles accumulating with age in (i.e., rich in linoleate and poor in docosahexaenoate (Li et al., 2005a,b)).

Recent evidence indicates that the RPE releases apolipoproteins and cholesterol using pathways previously well characterized in other cell types (Sections 5.3–5.4)[3]. Cultured RPE constitutively secrete 37 kDa apoE into high-density fractions ($d = 1.18$–1.35 g/ml); lower density fractions were not examined (Ishida et al., 2004). These investigators also demonstrated a transfer of radiolabeled docosahexaenoate from outer segment membranes to HDL or lipid-free apoA-I in the medium, presumably by ABCA1-mediated mechanisms (Ishida et al., 2006). ARPE-19 cells (from human) and RPE-J cells (from rat) secrete EC into a lipoprotein-containing fraction following a standard fatty acid supplementation paradigm (oleate for ARPE-19, palmitate for RPE-J) (Li et al., 2005b; Wang et al., 2009b). Similar in size (mean, 56 nm) to the particles found in native BrM (Section 6.3), they contain little TG. Importantly, RPE-J cells and medium also contain immunoprecipitable [^{35}S]-methionine-labeled apoB, in a full-length (512 kDa) form and a lower molecular weight band that may be apoB-48 (Wang et al., 2009b). This assay for protein synthesis and secretion, considered definitive, was also used to show apoB secretion from ARPE-19 cells (Fijalkowski et al., 2009).

The apoB-containing lipoproteins secreted by cultured RPE cell lines are unusual in several respects compared to other such lipoproteins: 1) Particles are EC-rich, despite being as large as TG-rich VLDL. 2) Particles are EC-rich when newly secreted, unlike LDL and HDL, whose composition is achieved by enzymatic remodeling in plasma (Section 5.3). 3) Fusion of smaller particles to create larger ones, as postulated for LDL in arterial intima (Kruth, 1997), is not required to achieve the size distribution seen in aged BrM (Huang et al., 2008a).

6.4.3. Source of lipids found in RPE lipoproteins (Table 4)

There has been speculation in older literature that the age-related accumulation of BrM neutral lipid is related to photoreceptor phagocytosis, either by direct deposition of debris (Grindle and Marshall, 1978) or by stress-related basolateral secretion

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[2] Acyl coenzyme A cholesterol acyl transferase 1, 2 is also called sterol-O-acyl-transferase 1, 2 (SOAT).

[3] We omit from this section clusterin, a widely expressed, multifunctional 70 kDa glycoprotein whose strong association with plasma HDL earned it the name of apolipoprotein J (de Silva et al., 1990; Vaisar et al., 2007). Although secreted by RPE and present in drusen, it is not universally considered a true apo (Alaupovic, 2003), so it is not discussed further here.

Table 4
Fatty acids in lipoproteins and outer segments (mole%).

Common name	Molecular name	EC in BrM lipoproteins	EC in plasma lipoproteins*				Phospholipids in outer segments **
			HDL	LDL	VLDL	CM	
Palmitic acid	16:0	15.6	13.2	12.0	11.3	12.9	16.3
Stearic acid	18:0	3.7	1.4	0.7	2.3	3.9	23.9
Oleic acid	18:1n9	18.4	21.1	16.0	19.2	20.4	9.1
Linoleic acid	18:2n6	41.6	54.4	56.7	50.8	47.0	0.9
Arachidonic acid	20:4n6	7.8	6.7	6.9	6.2	5.8	5.6
Docosahexaenoic acid	22:6n3	0.5		0.6	0.6	0.5	33.5

HDL, high density lipoprotein; CM, chylomicron.
* (Iglesias et al., 1996) for HDL (20 normolipemic subjects). (Wang et al., 2009a,b) for LDL, VLDL, CM (4 normolipemic subjects).
** (Rapp et al., 2000), for outer segment phospholipids in human donor eyes.

subsequent to engorgement of RPE with lipofuscin (Young, 1987). The purpose of the BrM lipoprotein is likely similar to lipoproteins elsewhere, i.e., secretion of excess lipid that cells cannot use or process. An initially attractive hypothesis that an apoB-lipoprotein from the RPE could eliminate fatty acids released by lysosomal phospholipases after outer segment phagocytosis (Li et al., 2005b) is now considered unlikely, for several reasons. 1) Neither the neutral lipids nor the PLs of BrM lipoproteins have a fatty acid composition that resembles that of outer segments (Table 4) particularly with regard to docosahexaenoate (22:6n3)(Fliesler and Anderson, 1983; Rapp et al., 2000), which is abundant in outer segments and sparse in BrM lipoproteins (Table 4). 2) BrM lipoproteins and drusen are highly enriched in EC (Sections 6.3, 8.1), which is essentially absent from outer segments (Fliesler and Anderson, 1983). 3) BrM lipoproteins and drusen are more highly enriched in UC (Sections 6.3, 8.1) than outer segments or isolated disk membranes (Boesze-Battaglia et al., 1990; Fliesler and Anderson, 1983). 4) Phagocytosis is not required for secretion of neutral lipids or apolipoproteins in RPE cell lines (Ishida et al., 2004; Li et al., 2005b; Wang et al., 2009b).

Several explanations for the dissimilarity between BrM lipoproteins and outer segments are possible. 1) Outer segment lipids may be catabolized by RPE (Bazan et al., 1990) prior to re-synthesis as particle components. 2) Docosahexaenoate (22:6n3) is efficiently recycled back to the retina (Bazan et al., 1992), leaving little for export. 3) Outer segment lipids may be present in a form (e.g., oxidized (Suzuki et al., 2007)) not recognizable by the assays utilized. 4) Some lipids within BrM lipoproteins may be selectively and extensively hydrolyzed in the extracellular compartment (e.g., by choroid-resident (Casaroli-Marano et al., 1996) or RPE-secreted lipases), an option made less likely by concordant results from whole tissues, isolated particles, and cultured cells. 5) BrM lipoproteins are dominated by other sources, specifically plasma lipoproteins, which they closely resemble with regard to fatty acid composition (Table 4), although BrM lipoproteins differ from plasma in other important respects (Section 6.3). This last explanation is the simplest.

Table 5
Age-related Macular Degeneration vs Coronary Artery Disease.

Factor	Age-related maculopathy	Coronary artery disease
ApoE4 genotype	▼ Risk	▲ Risk
Elevated plasma cholesterol	–	▲ Risk
Elevated plasma HDL	–	▼ Risk
Diabetes (Type 2)	–	▲ Risk
Statin therapy	–	Benefit
Anti-oxidant therapy	Benefit	–

–, No effect.

Plasma lipoproteins LDL and HDL are both taken up by RPE, which expresses functional receptors for both LDL (LDL-R) and HDL (scavenger receptor B-I, SRB-I) (Duncan et al., 2002; During et al., 2008; Elner, 2002; Hayes et al., 1989; Tserentsoodol et al., 2006a). This system is likely in place for re-supply of essential lipophilic nutrients. LDL-derived cholesterol partitions with remarkable speed (within hours) into membranes of the neurosensory retina (Gordiyenko et al., 2004; Tserentsoodol et al., 2006a). The xanthophylls lutein and zeaxanthin, major components of macular pigment, are carried equally on LDL and HDL in humans (Loane et al., 2008). ARPE-19 cells preferentially take up xanthophylls at SRB-I (During et al., 2008), and avian retina uniquely depends on HDL-mediated xanthophyll delivery to maintain high tissue levels *in vivo* (Connor et al., 2007). Vitamin E, essential for outer retinal health (Friedrichson et al., 1995; Yokota et al., 2001), is also delivered by lipoproteins via LDL-R- and SRB-I-dependent pathways (Hacquebard and Carpentier, 2005). Thus, delivery of cholesterol, xanthophylls, and vitamin E are the most plausible reasons for a major plasma lipoprotein input to RPE. With regard to its nutrition, then, retina differs markedly from brain, which relies almost exclusively on endogenous cholesterol synthesis (Björkhem and Meaney, 2004) and contains little lutein or zeaxanthin (Connor et al., 2007).

A less likely reason for lipoprotein-mediated delivery to the RPE is the re-supply of retinal docosahexaenoate, a mechanism postulated but not directly demonstrated two decades ago (Scott and Bazan, 1989). A recent comprehensive analysis of retina, RPE/choroid, and orbital fat lipid composition (Bretillon et al., 2008b) concluded that only a small portion of retinal docosahexaenoate is derived from diet, with intra-retinal biosynthesis and recycling the most likely source, but left open the possibility that RPE/choroid is influenced by diet. It is also possible that retinal docosahexaenoate will prove to be also delivered by albumin, the major plasma carrier of non-esterified fatty acids to body tissues (Goldberg et al., 2009).

Given that the lipids in BrM lipoproteins are not likely to originate in outer segments and that the fatty acid composition of these lipoproteins is markedly similar to that of plasma LDL and HDL, it is tempting to conclude that the BrM lipoprotein is a mechanism to eliminate plasma lipoprotein residues from RPE after specific nutrients (e.g., xanthophylls, cholesterol, vitamin E) are extracted for use by photoreceptors. According to this model, EC-rich lipoproteins from plasma are taken up by RPE, stripped of nutrients, and repackaged for secretion in BrM as large, EC-rich apoB-lipoproteins. Rather than representing a system to dispose of outer segments, then, the BrM lipoprotein may be a by-product of an immense and poorly understood system to deliver specific lipophilic nutrients to retina. Within the polarized RPE, the apically-directed recycling of docosahexaenoate to the retina (Bazan et al., 1992) may be accompanied by an independent, basally-directed recycling of plasma lipoproteins repackaged for egress to the systemic circulation across BrM.

7. BrM, lipids, and transport

7.1. EC-rich barrier in aged BrM; Lipid Wall (Figs. 8–10)

As revealed by QFDE analysis of normal eyes of different ages (Huang et al., 2007b, 2008a; Ruberti et al., 2003), significant lipoprotein accumulation begins during the third or fourth decade of life in or near the elastic layer of macular BrM. This process is reminiscent of the preferential deposition of lipoprotein-derived EC near elastin in arterial intima (Guyton et al., 1985). With advancing age, following the elastic layer becoming filled with these particles and other debris, accumulation was also seen in the inner collagenous layer (Fig. 8), as if particles were "backing up" into this region. The volume fractions occupied by lipoprotein particles in these tissues increased in roughly a linear fashion (Fig. 9) (Huang et al., 2008a). The outer collagenous layer showed a more complicated pattern (Fig. 9), with an early accumulation that decreases later in life, as if their source had diminished. During this time the small (10 nm) granular particles (Section 6.2) also accumulate throughout the layers.

In eyes over 60 years of age, accumulated particles fill most of the inter-fibrillar space in the inner collagenous layer, and groups of particles appear between this layer and the RPE basal lamina. In eyes over 70 years of age, this process culminates in the formation of a new layer in BrM that we have termed the Lipid Wall (Ruberti et al., 2003). This dense band 3–4 particles thick external to the RPE basal lamina was previously illustrated by others without comment (Fig. 10). This tightly packed layer of space-filling particles, too dense for volume fraction measurements, displaces the structural collagen fibers at the same location in younger eyes that help bind BrM to the RPE basal lamina. Interestingly, particle accumulation and Lipid Wall formation occurs not only in macular BrM, but also in the periphery, although less prominent in the latter region, and in somewhat older eyes (Johnson et al., 2007). Taken together, these observations are consistent with an interpretation that lipoprotein accumulation starts in the elastic layer, backs up into the inner collagenous layer, and eventually forms the Lipid Wall. This blocks

the source of lipoproteins to the outer collagenous layer and explains why the concentration of particles in this layer drops in eyes >60 years of age.

The striking spatial correspondence of the Lipid Wall in aged eyes and BlinD in eyes with ARMD makes the Lipid Wall a likely direct antecedent to BlinD (Section 8.2).

7.2. The barrier hypothesis: lipid accumulation and transport through BrM (Figs. 11,12)

Deposition of lipoproteins, and especially EC within their cores, may render BrM increasingly hydrophobic with age and impede transport of hydrophilic moieties between the RPE and choroidal vessels. Over twenty years ago, Bird and Marshall first introduced the hypothesis of a physical barrier in this tissue (Bird and Marshall, 1986), distinct from the physiological blood-retina barrier formed by retinal capillary endothelium and RPE junctional complexes (Kaur et al., 2008). A BrM transport barrier could pre-dispose older individuals to multiple retinal conditions (Bird and Marshall, 1986; Chuang and Bird, 1988; Kuntz et al., 1996). Marshall and colleagues provided substantial support for a BrM transport barrier, demonstrating reduced hydraulic conductivity and permeability to solutes and macromolecules in BrM of aged and ARM eyes (Hussain et al., 2002; Moore and Clover, 2001; Moore et al., 1995; Starita et al., 1996). The role of lipids in this barrier was tested by examining the correlation between the accumulation of BrM lipids and increased BrM hydraulic resistivity, as measured in BrM/choroid explants from eyes of different ages (Moore et al., 1995; Starita et al., 1996, 1997). Marshall's group concluded that since lipid accumulation occurs mostly after age 40, and the majority of decrease in hydraulic conductivity of BrM was observed before this age, these events are unrelated (Marshall et al., 1998).

However, the hydraulic *resistivity* (inverse of hydraulic conductivity) of BrM actually correlates strongly with its lipid content (Marshall et al., 1998). Indeed, the age-related increase in hydraulic resistivity of BrM exactly mirrors that of the age-related increase of histochemically detected EC in BrM (Ethier et al., 2004)
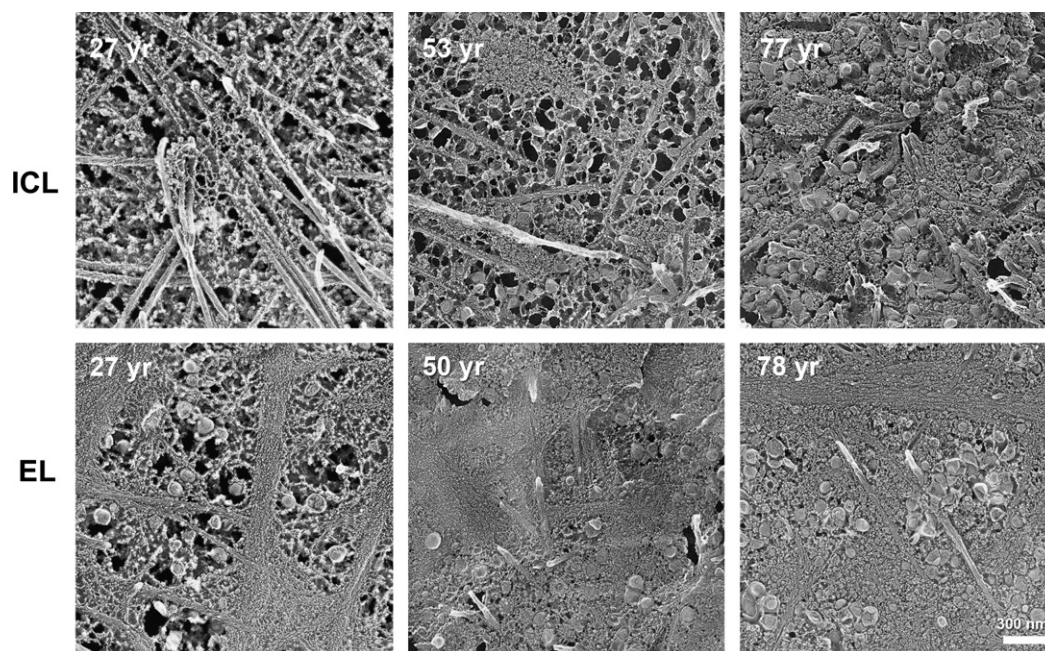

Fig. 8. Lipoprotein deposition in BrM layers throughout adulthood. Apparent lipoprotein particle accumulation appears at earlier ages in elastic layer (EL) than inner collagenous layer (ICL). Oblique views of BrM were obtained from normal maculas prepared using QFDE. Bar is 300 nm. Reprinted from (Huang et al., 2007b) with permission from Elsevier.
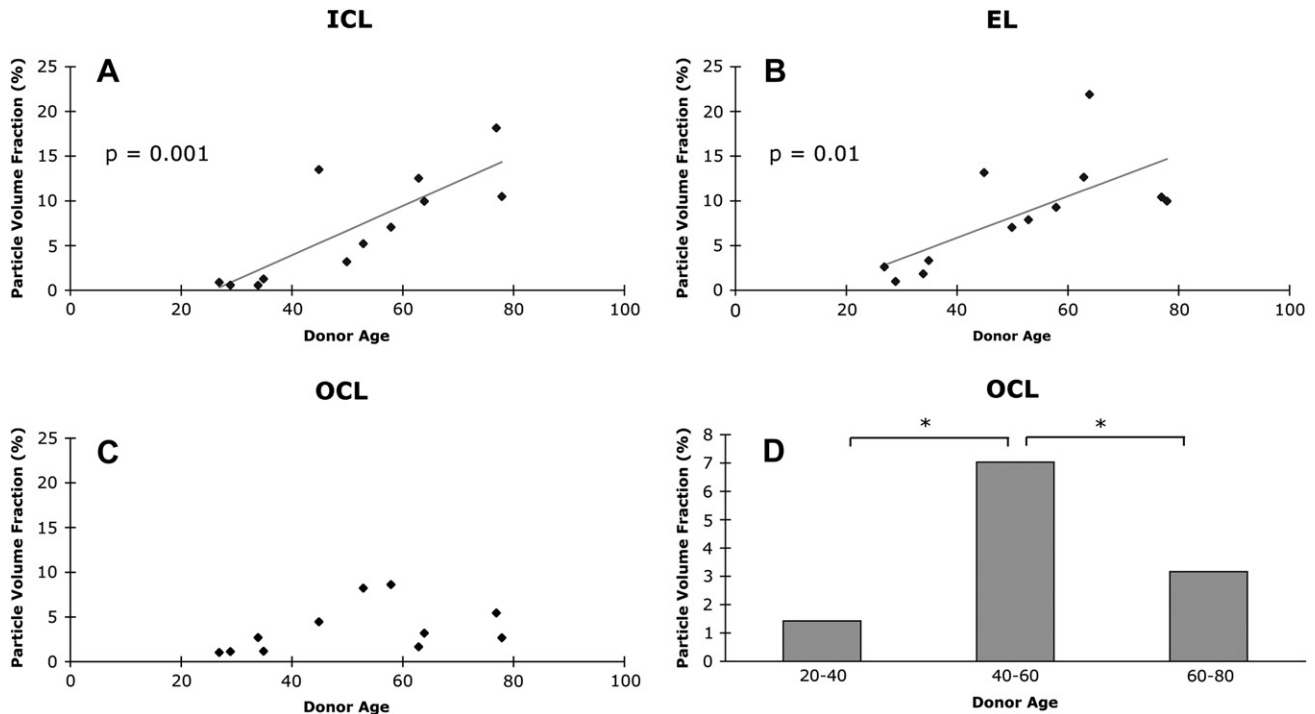
Fig. 9. Volume occupied by lipoproteins in BrM layers. Lipoproteins occupy steadily greater volume with age in inner collagenous and elastic layers (ICL, EL) but increase then decrease in outer collagenous layer (OCL). Asterisk indicates significant difference. Reprinted from (Huang et al., 2008a); copyright is held by Association for Research in Vision and Ophthalmology.

(Fig. 11). Hydraulic resistances add when flow-limiting regions are in series, while hydraulic conductances add when they are in parallel. Lipid accumulation in BrM adds a new hydraulic resistance in series with an existing resistance. It cannot be bypassed, as the flow must pass through this layer. It is therefore not surprising that hydraulic resistivity shows a better correlation with lipid accumulation than does hydraulic conductivity, which would have an inverse relationship with lipid accumulation in any case.
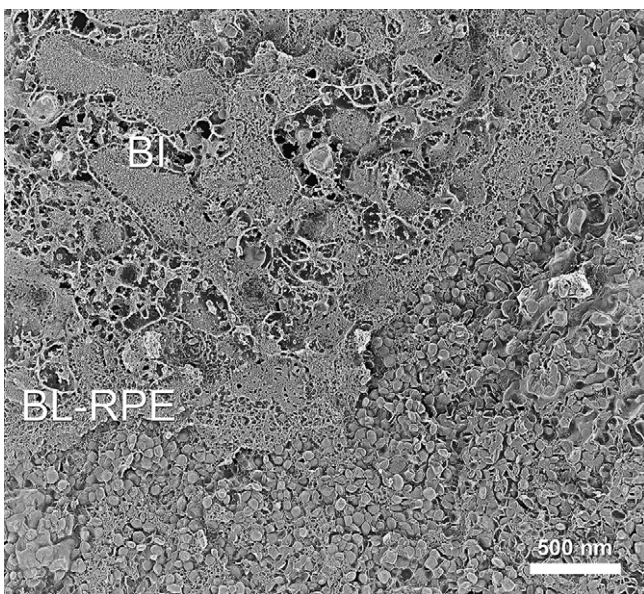

Fig. 10. Lipid Wall. In this oblique view of BrM, lipoproteins are densely packed in the Lipid Wall (lower right corner). Normal macula of an 82-year-old donor, prepared by QFDE. Taken from (Huang et al., 2007b) BI, basal infolding; BL-RPE, basal lamina of the RPE.

Recent evidence (McCarty et al., 2008) indicates that lipid deposition can significantly decrease the hydraulic conductivity of a model extracellular matrix, Matrigel™. In this experimental system, the hydraulic conductivity of Matrigel™ was modified by the addition of LDL-derived lipids[4]. Addition of 5% LDL-derived lipids (by weight) to Matrigel™ lowered its hydraulic conductivity by greater than 50% (Fig. 12). This effect was surprisingly large, much larger than if 5% latex spheres were added to the Matrigel™, and larger than predicted by the Debye–Brinkman equation used to model interstitial fluid movement (McCarty et al., 2008).

8. Cholesterol and apolipoproteins in sub-RPE lesions

Here we consider the cholesterol and apolipoproteins in the extracellular sub-RPE lesions associated with aging and ARMD (Section 3.2 for definitions and descriptions). Lesion composition is best known for drusen, which are amenable both for histochemistry in tissue sections and for isolation and direct assay. The relationship between normal aging and an ARMD-specific lesion may be best appreciated for the Lipid Wall and BlinD. The latter may be more correctly described as containing lipoprotein-derived debris rather than membranous debris, as occurs in many descriptions of this lesion. BlamD lipid content is also discussed.

8.1. Druse histochemistry and immunohistochemistry (Fig. 13)

Studies using sudanophilic dyes or polarizing microscopy identified both neutral lipids and polar lipids in age-related drusen (Haimovici et al., 2001; Malek et al., 2003; Pauleikhoff et al., 1992; Wolter and Falls, 1962). In macular drusen of ARMD eyes and extra-macular drusen of non-ARMD aged eyes (Curcio et al., 2001,

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[4] Hydraulic conductivity is reported rather than resistivity for consistency with previous literature.
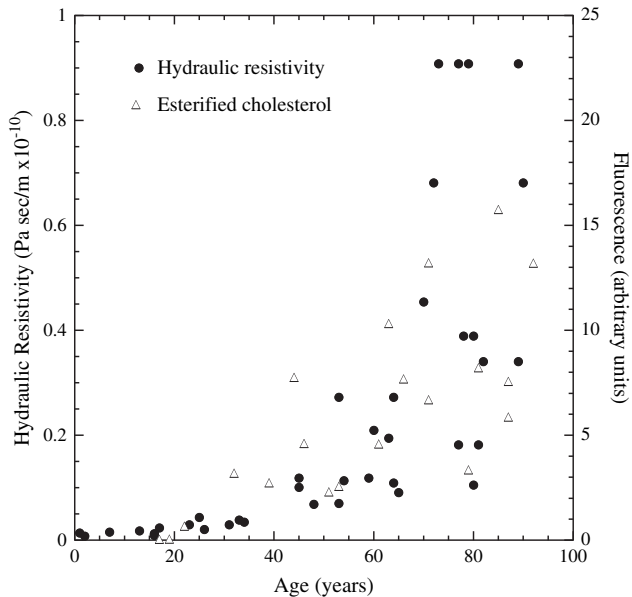
Fig. 11. Hydraulic resistivity and BrM EC in aging. Hydraulic resistivity (Marshall et al., 1998) of excised BrM/choroid (closed symbols) and fluorescence due to histochemically detected esterified cholesterol in sections of normal BrM (Curcio et al., 2001) (open symbols) as a function of age. From (Ethier et al., 2004) Reprinted, with permission, from the Annual Review of Biomedical Engineering, Volume 6 (c)2004 by Annual Reviews www.annualreviews.org.

2005a; Li et al., 2007; Malek et al., 2003) (Fig. 13A–D), virtually every druse, whether hard or soft, has abundant EC and UC as revealed by filipin. Cholesterol in drusen assumes different morphologies reflecting different chemical composition and formative processes. Soft druse contents contain distinctive, loosely packed UC-rich whorls (Fig. 10D). Lakes of EC (Fig. 13A) occur in macular and extra-macular drusen, with larger lakes in the macula. Druse cores are ~15 μm diameter basally located regions (Mullins and Hageman, 1999) that rich in UC (Fig. 13C,D) and in their very centers, poor in EC (Li et al., 2007). This disposition of cholesterol in cores may reflect the activity of invading
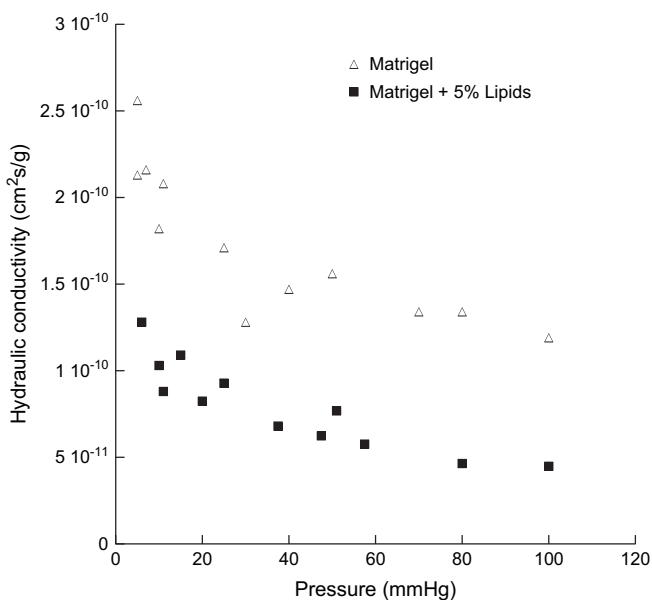

Fig. 12. Lipoprotein-derived lipids reduce fluid transport through an artificial matrix. The hydraulic conductivity, as a function of perfusion pressure, of 1% Matrigel (open triangle) or 1% Matrigel with 5% LDL added (closed squares). Modified from (McCarty et al., 2008).

cellular processes during druse biogenesis (Hageman et al., 2001), with the greater size of UC-rich cores relative to EC-poor cores reflecting a declining gradient of enzymatic activity with increased radial distance from the putative invaders. Finally, extra-macula drusen also exhibited highly fluorescent EC-rich shells subjacent to its cap of RPE (Anderson et al., 2004; Malek et al., 2003). No drusen containing cholesterol crystals have been observed. Nor have there been reports of the distinctive clefts that signify crystals in tissue sections, a distinct difference from atherosclerotic intima or lipid keratopathy (Crispin, 2002; Guyton and Klemp, 1993).

Multiple apolipoproteins are found in drusen, making a lipoprotein particle containing them (Section 6.3) an efficient mechanism to explain the presence of all of them (as speculated (Anderson et al., 2001)). ApoE appears in macular and extramacular drusen, within a tri-laminar distribution in BrM, and it does not localize to lipofuscin within RPE (Anderson et al., 2001; Klaver et al., 1998). Other studies (Li et al., 2006; Malek et al., 2003) found apoE and apoB in 80–100% of peripheral drusen in ARMD and normal eyes and in fewer (55–60%) macular drusen of ARMD eyes, attributed to apolipoprotein dilution by other druse constituents. ApoE but not apoB was found in the drusen proteomics study (Crabb et al., 2002), perhaps due to sample preparation with a chloroform-methanol extraction that de-lipidates and precipitates apoB. ApoA-I immunoreactivity is present in 62% of peripheral drusen (Li et al., 2005a, 2006). Of interest is the relationship between apoCs in plasma and drusen, such that apoC-III, which is abundant in normolipemic plasma, is present in fewer drusen (16.6%) than apoC-I (93.1%), which is sparse. This result suggests either a specific retention mechanism for plasma-derived apolipoproteins with drusen, or a local source.

Immunoreactivity for all apolipoproteins is present throughout each individual druse. This pattern resembles that of abundant druse components like TIMP-3, vitronectin, and CFH (Hageman et al., 1999, 2005; Ruiz et al., 1996) and contrast with non-abundant druse components like amyloid or zinc that preferentially localize to small sub-regions (Johnson et al., 2002; Lengyel et al., 2007; Luibl et al., 2006). Abundant drusen constituents that are also present in normal aged BrM are good candidates for pathways involved at the earliest phases of disease (e.g, EC, UC, apoE, TIMP-3, AGE). Molecules enriched in inner BrM by light microscopy (e.g., apoE, apoB) are likely present in the Lipid Wall and BlinD (Anderson et al., 2001; Malek et al., 2003).

8.2. BlinD – Membranous debris vs lipoprotein-derived debris, and its evolution from the Lipid Wall (Fig. 14)

The morphology of extracellular cholesterol is best described for the lipid-rich core of atherosclerotic plaque in arterial intima. In that location, morphologically distinct components differ in their relative proportions of UC, EC, and PL and the disease stage at which they appear (Curcio et al., 2005b; Guyton and Klemp, 1989, 1996). From incipient to advanced disease, these forms include EC-rich LDL particles (22 nm), small EC-rich droplets (100 nm), multi-lamellar liposomes of UC and PL (40–200 nm), irregular lakes of EC delimited by a PL monolayer (6–20 μm), large EC-rich droplets (1–4 μm) shed from dying foam cells, and crystals of pure UC (4 μm), a sign of plaque maturity.

In ARMD, the principal lipid-containing lesion component has been called membranous debris by Shirley and John Sarks (Sarks et al., 1988, 2007; Sarks, 1980). As viewed by transmission electron microscopy following osmium tetroxide post-fixation, membranous debris appears as variably sized, contiguous coils or whorls of uncoated membranes consisting of uni- or multilamellar electron-dense lines surrounding an electron-lucent center. This material dominates four extracellular lesions – soft drusen, BlinD, basal mounds between the RPE and its basal lamina, and aggregations
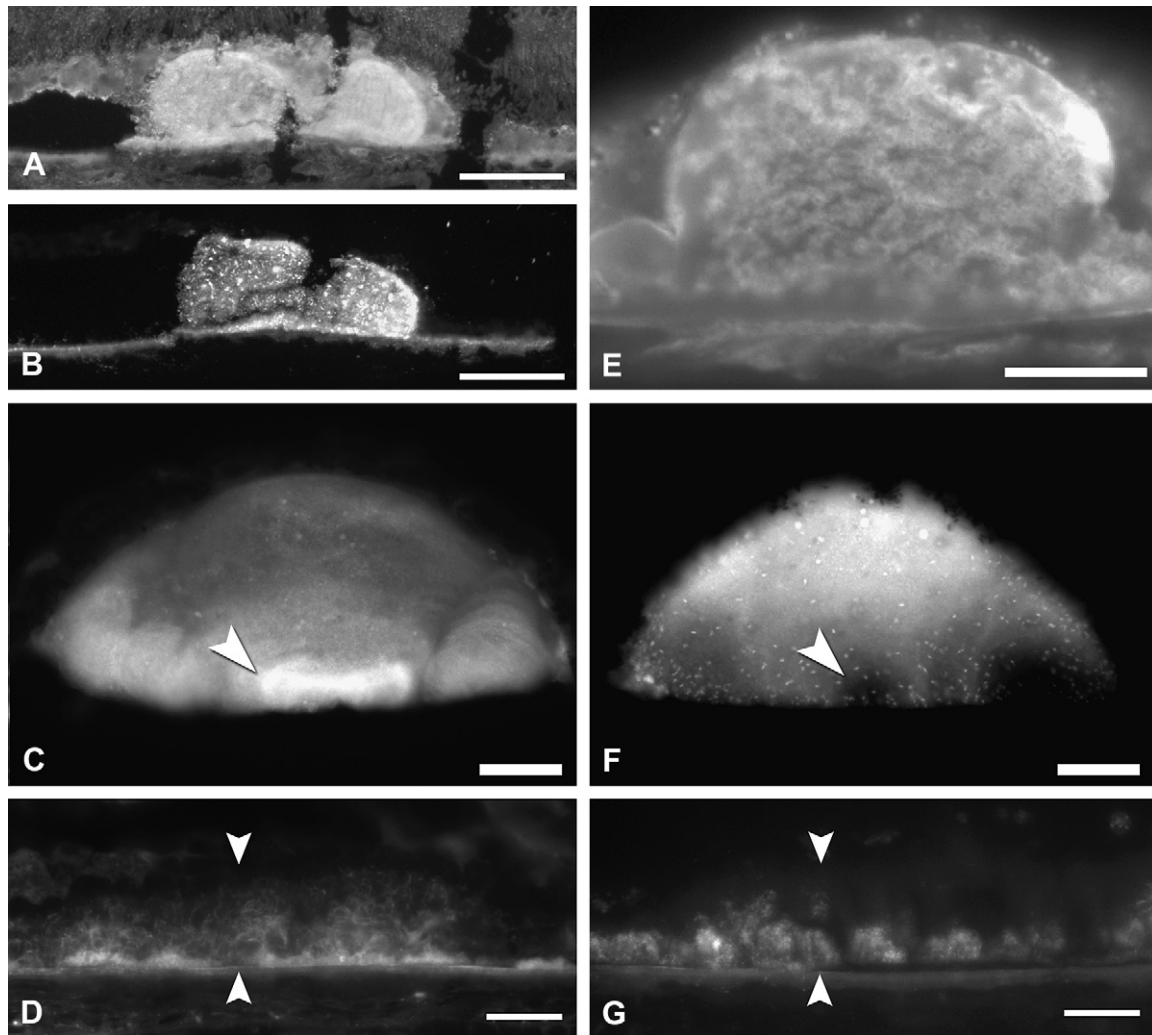
Fig. 13. Cholesterol and apolipoproteins in drusen and deposits. Filipin fluorescence in A–D and F–G. Immunofluorescence in E. Bars in A, B, E 50 μm. Bars in C,D,F,G 20 μm. A. Druse and surrounding chorioretinal tissue contain UC. B. Druse (same as A) and BrM contain EC. Speckles represent lakes of EC. C. A bright UC-rich core (arrowhead) at the base of an isolated, extra-macular druse. D. A thick BlamD (between arrowheads) has bright, delicate fluorescence for UC. E. ApoB immunofluorescence in a druse. F. The core at the base of the same druse as panel C is EC-poor core (arrowhead). This druse also contains EC-rich lakes (speckles). G. A thick BlamD (same as D, between arrowheads) has fluorescence for EC confined to its outer half. Adapted from (Curcio et al., 2005a; Li et al., 2007; Malek et al., 2003).

within the sub-retinal space – and in one intracellular location – intracellular vacuoles within the RPE. By light microscopy, these locations contain histochemically detectable PL and UC, with quantitative evidence indicating much higher UC enrichment than surrounding cells (Curcio et al., 2005a; Pauleikhoff et al., 1992). Accordingly, membranous debris fine structure does not resemble nearby RPE plasma membranes (Curcio et al., 2005a), since it is thick, occasionally multilamellar, and highly electron dense (Fig. 14C,D).

Although membranous debris is frequently interpreted as vesicles with aqueous interiors, recently evidence indicates that it is not vesicular but rather, aggregations of solid particles that could account for the abundant EC in drusen and deposits more plausibly than membranes containing UC and PL (Curcio et al., 2000, 2005a; Malek et al., 2003; Pauleikhoff et al., 1992). The ultrastructural correlate of neutral lipid in lesions is better revealed in tissue post-fixed by the OTAP method, since these lipids are extracted using routine tissue preparation for transmission electron microscopy (Section 6.2). Linear tracks of solid particles cross BlamD in the same configuration as tracks of membranous debris (Fig. 14E,F). Soft drusen and basal mounds contain ovoid-to-spherical bodies with homogeneous, moderately electron-dense interiors and highly electron-dense

exteriors, and pools of neutral lipid (Fig. 14C,D). These images were interpreted to indicate that membranous debris represents the UC-rich exteriors of fused lipoprotein particles whose neutral lipid interiors are not uniformly well preserved in typical preparations of post-mortem tissue (Section 6.2). Lipid-preserving methods can minimize this loss. This interpretation is supported by evidence that when isolated BrM lipoprotein particles lose their neutral lipid cores during extensive sub-fractionation, only UC-enriched surfaces of individual or fused lipoproteins remain (Li et al., 2005a). From this perspective, then, we propose that the major lipid-containing component of ARMD-specific lesions should be called "lipoprotein-derived debris" rather than "membranous debris."

Although lipoprotein-derived debris could explain EC, UC, and PL within lesions, actual membranes containing UC, not derived from lipoproteins, are also likely present in these locations. Early and influential micrographs showing membrane-bounded cytoplasmic packets shed from surrounding RPE (Ishibashi et al., 1986a,b) suggested that at least some UC could arrive in extracellular lesions by this route. Recently, proteins of known RPE origin have been detected in BrM and drusen, including RGR (Lin et al., 2007; Nickle and Robinson, 2007) and exosome markers CD63,
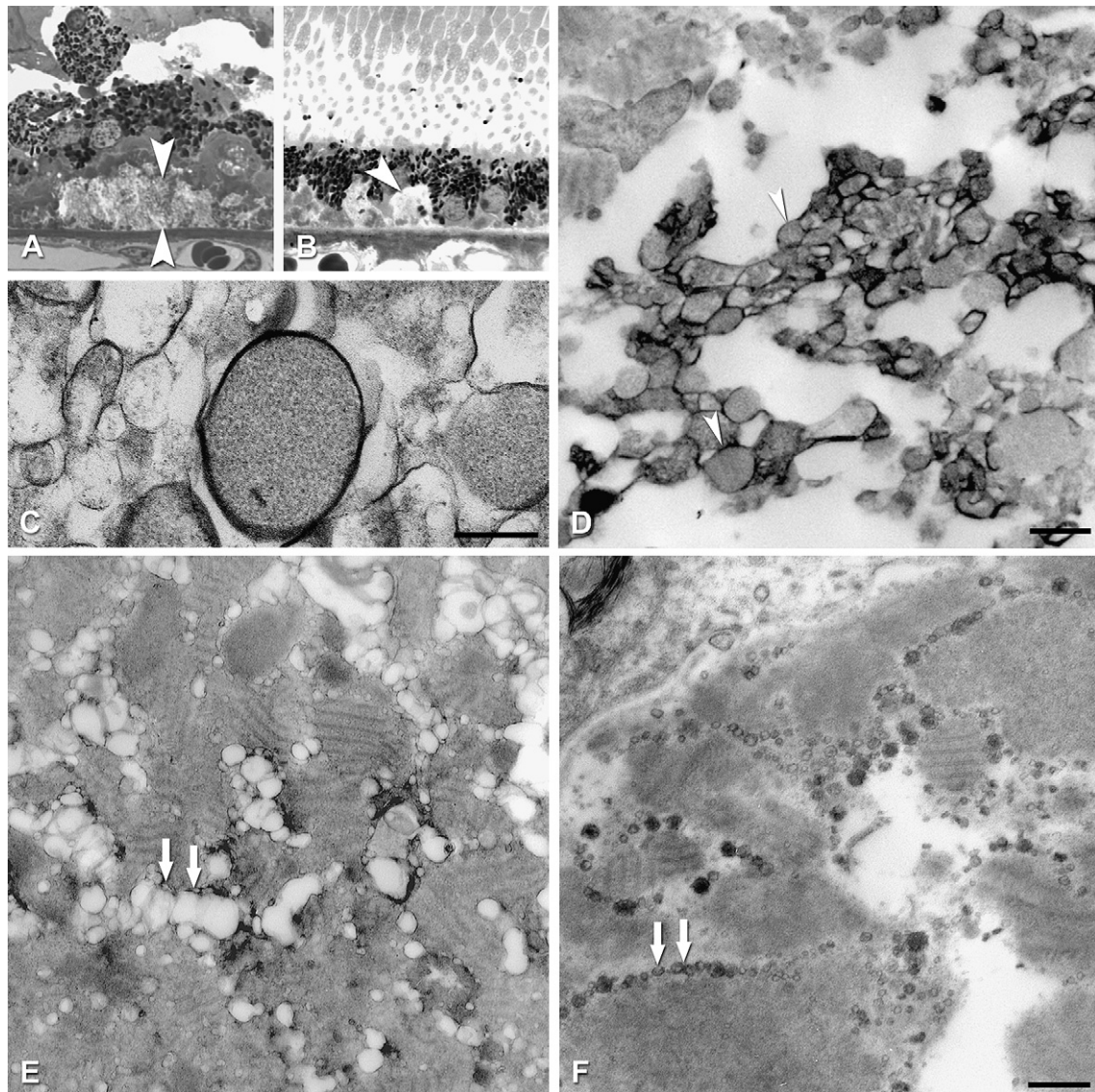
Fig. 14. Ultrastructure of cholesterol-containing components of drusen and deposits. Tissue post-fixed with OTAP, from (Curcio et al., 2005b). A. RPE migrating anteriorly and thick BlamD containing cellular processes, bracketed by large arrowheads. B. BlamD containing basal mounds of membranous debris (arrowhead). Bar, 200 nm C. Membranous debris with contents, interior of a large druse. Bar, 200 nm. D. Individual profiles in the basal mound of panel B are solid and surrounded by an electron-dense band. E,F. Bar is 500 nm. E. Linear tracks of partially extracted material resembling membranous debris (double arrows) in BlamD. F. Linear tracks of solid particles (double arrows) in BlamD of the same eye as E, post-fixed with OTAP.

CD81, and LAMP2 (Lakkaraju and Rodriguez-Boulan, 2008; Wang et al., 2009a). Because these proteins are normally membrane-bound, their presence in lesions is consistent with direct shedding or blebbing of plasma or intracellular membranes by RPE, perhaps secondary to oxidant injury (Strunnikova et al., 2001).

What is the relationship of age-related deposits found in BrM to BlinD in ARMD? The Lipid Wall, located between the inner collagenous layer and RPE basal lamina, is at the same site of BlinD, suggesting that the Lipid Wall might be the precursor of BlinD. Support for this hypothesis comes from ultrastructural examination of eyes at different ARMD stages (Curcio and Millican, 1999; Sarks et al., 2007). We have identified transitional forms between the Lipid Wall and BlinD supporting evolution of BlinD from the Lipid Wall (Messinger et al., 2009). In addition, their correspondence in physical location and similarity in structure support this conclusion. Whereas the Lipid Wall consists of packed lipoproteins of similar size, BlinD in its earliest forms consisted of

material resembling fused lipoproteins of irregular size. These findings suggest that the Lipid Wall is the likely immediate precursor of BlinD, of significance because Lipid Wall composition can be inferred in part from that of the BrM lipoprotein particles (Section 6.4).

Since the Lipid Wall is found in all aged eyes, while BlinD is specifically associated with ARMD, this suggests that the "Response-to-Retention" of lipoproteins in BrM that is key step in the pathogenesis of ARMD lesions. It also suggests that formation of the Lipid Wall is a critical step in this disease process.

Sections 6 and 7 described a model wherein RPE production of apoB lipoproteins for normal physiological transport to the choriocapillaris is gradually blocked by age-related accumulation of these particles in BrM eventually filling this tissue, resulting in a new layer caused by the backwards accumulation of these particles. Once this Lipid Wall begins to form between the inner collagenous layer and the RPE basal lamina, more lipids

preferentially fill this potential space, leading to the formation of BlinD, which is linear because of the geometry of the space containing it. Formation of BlinD may damage RPE cells via decreased transport of nutrients and waste products. Their response to this insult may include production of excessive basal lamina materials. The presence of BlinD and remaining Lipid Wall prevents removal of these materials, contributing to local accumulation of BlamD, which may have already begun due to other RPE stresses. BlamD would have to be internal to BlinD based on this argument.

8.3. BlamD

BlamD, internal to the RPE basal lamina, consists largely of basal lamina-like proteins and glycosaminoglycans (Kliffen et al., 1996; Lommatzsch et al., 2008; Marshall et al., 1992) but also contains lipoprotein-related components. In light microscopic using cryosections and oil red O or filipin, BlamD is easily discernable, and it is stained lightly near the RPE and heavily near BrM (Curcio et al., 2005a; Lommatzsch et al., 2008; Malek et al., 2003; Pauleikhoff et al., 1992). A widespread, thick BlamD associated with mutations of the C1QTNF5 (CTRP5) short-chain collagen gene (Hayward et al., 2003) have abundant EC and UC, apoB immunoreactivity, and linear tracks of contiguous electron-dense solid particles (Milam et al., 2000). These tracks suggest an organized mechanism for tethering and transporting lipoproteins particles to the RPE basal lamina, which they cross in order to reach BrM. These observations are consistent with the idea that the importance of BlamD lies in indicating RPE stress and detaining components *en route* to drusen and BlinD where they will have greater pathogenic significance (Sarks et al., 2007).

9. Response-to-retention of an intra-ocular apoB lipoprotein

Our view of the role of BlinD and soft drusen in ARMD pathogenesis and progression centers around three concepts: 1) a barrier that prevents aqueous phase nutrients in plasma (Pauleikhoff et al., 1990) or other compounds with potential for clearance (e.g., HDL) from gaining access to RPE, thus obstruction the elimination of waste products; 2) a plane that sequesters angiogenic compounds, cytokines, modified lipids, or proteins (Crabb et al., 2002; Spaide

et al., 1999; Yamada et al., 2008) that collectively evoke a local inflammatory response; and 3) a cleavage plane for lateral dissection by opportunistic choriocapillary endothelial cells that have breached BrM (Sarks et al., 1980). The latter is strengthened by observations of pooled neutral lipid in BlinD with little surrounding proteinaceous material to provide cohesion (Curcio et al., 2005b), the detachment of the RPE basal lamina from collagen fibrils of the inner collagenous layer by the Lipid Wall (Section 7.1), and the physical fragility of macular soft drusen (Malek et al., 2003; Rudolf et al., 2008a).

9.1. Statement of model (Fig. 15A,B)

Collectively, the data reviewed in Sections 6 and 7 imply that the RPE constitutively secretes an apoB-containing lipoprotein particle from its basolateral aspect into BrM for eventual clearance into plasma. We envision a large lipoprotein (Fig. 15A) in the VLDL size and density class that contains apoB-100, apoA-I, apoE, apoC-I, apoC-II, and possibly other proteins. It is secreted with an EC-rich neutral lipid core. Plasma lipoproteins taken up by the RPE (Tserentsoodol et al., 2006b) can deliver UC, lipophilic vitamins, and/or xanthophylls for ultimate use by the photoreceptors. However, this process might raise intracellular UC to toxic levels within RPE. Lipoprotein secretion by RPE cells would allow disposal of excess UC in concert with recycling EC and retinoids back to plasma (Qtaishat et al., 2003). This model does not preclude other mechanisms of cholesterol release from RPE (Sections 5.1 and 10.1.1). Although it is possible that smaller lipoproteins secreted by the RPE accumulate in BrM and fuse together to form large particles, the size distribution of particles in aged BrM is inconsistent with a continuously evolving population (Huang et al., 2008a).

We propose that in younger eyes, lipoprotein particles cross BrM for egress to plasma. With advanced age, transit time across BrM increases due to changes in extracellular matrix, particle character, and/or clearance mechanisms, resulting in the accumulation of either native or modified particles, especially at the site of the Lipid Wall. In conjunction with other cellular processes, lipoprotein accumulation and modification eventually causes the formation of BlinD and drusen and contributes to ongoing RPE stress and formation of BLamD. Our model does not indicate that deposit
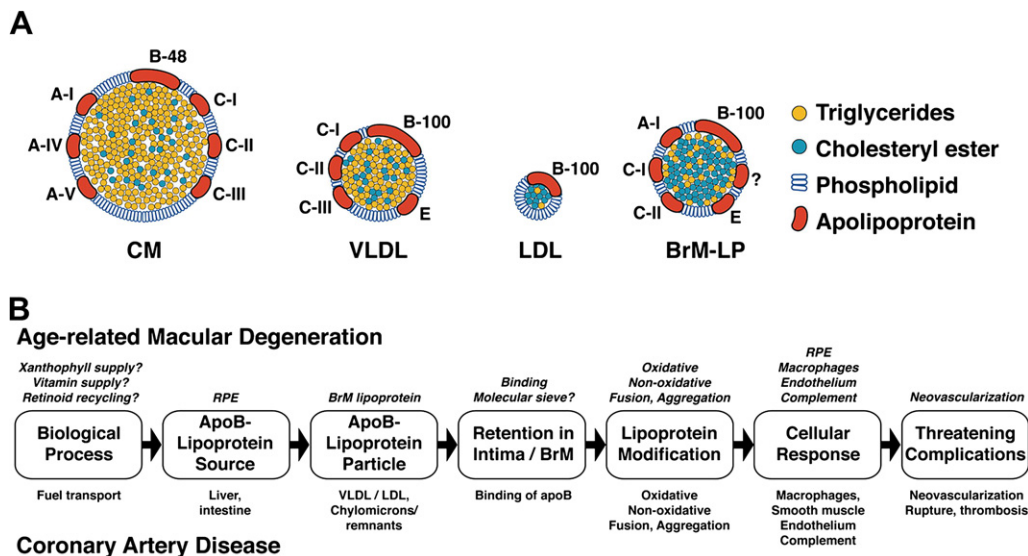
Fig. 15. Response-to-retention: ARMD, CAD. A. BrM lipoprotein (BrM-LP) compared to other apoB-containing lipoproteins well characterized in plasma. Particle diameters are to scale. Not all the proteins on the BrM lipoprotein are known. B. The hypothesized progression of ARMD has many parallels to the Response-to-Retention hypothesis of atherosclerotic coronary artery disease (Tabas et al., 2007), beginning with apoB-lipoprotein deposition in a vessel wall.

formation alone initiates ARMD. Instead, it is likely that the "Response-to-Retention" of lipoproteins (Tabas et al., 2007) in BrM gives rise to, and may even be required for, other key events including complement activation, inflammation, neovascularization, and RPE cell death.

This model allows us to compare ARMD and CAD at many steps (Fig. 15B), importantly, near the beginning where tissue-specific processes are most likely involved. Lipoproteins retained in arterial intima undergo numerous modifications, including oxidation, aggregation, fusion, glycation, immune complex formation, proteoglycan complex formation, and conversion to cholesterol-rich liposomes (Tabas, 1999). Potentially all these processes may also occur in the RPE/choroid complex (Handa, 2007), as oxygen levels are high (Wangsa-Wirawan and Linsenmeier, 2003), high levels of irradiation could promote lipid peroxide formation (Zarbin, 2004), BrM contains proteoglycans and AGE are found to accumulate with age (Handa et al., 1999; Hewitt et al., 1989; Kliffen et al., 1996; Newsome et al., 1987), and the immediately adjacent RPE is a source of secreted enzymes (e.g., sphingomyelinase (Lakkaraju et al., 2007)) that can act on lipoproteins.

The lipid components of BrM lipoproteins are potentially subject to oxidative modification that leads to further deleterious consequences. Antibodies vs copper sulfate-oxidized LDL reveal immunoreactivity in BrM and basal deposits of eyes with ARMD (Yamada et al., 2008). Immunoreactivity for oxidized phosphatidylcholine is present in photoreceptor outer segments, some RPE cells, scattered drusen in eyes with ARMD, and in surgically excised neovascular membranes (Kamei et al., 2007; Suzuki et al., 2007). ARPE-19 cells exposed to oxidized LDL undergo numerous phenotypic shifts, including altered gene expression (Yamada et al., 2008). Oxysterols are metabolites of UC generated *in vivo* primarily by mitochondrial enzymes like CYP27A1 and CYP46A1 (Javitt, 2008), which are present in retina and RPE (Bretillon et al., 2007; Lee et al., 2006). Oxysterols are also studied as *in vitro* as toxic byproducts of LDL oxidation that can contribute to macrophage differentiation into foam cells and induce cell death in RPE cell lines (Joffre et al., 2007; Moreira et al., 2009; Ong et al., 2003; Rodriguez et al., 2004). 7-ketocholesterol is present near BrM in adult monkey eyes and is thought to originate from BrM lipoprotein deposits that oxidized in the high-oxygen choroidal environment (Moreira et al., 2009). These oxidation products can contribute to disease progression, as intravitreal injections of linoleate hydroperoxide can elicit choroidal neovascularization and chorioretinal atrophy (Tamai et al., 2002), and 7-ketocholesterol can up-regulate VEGF expression (Moreira et al., 2009).

The hypothesis that an RPE-derived apoB-lipoprotein retained in a vascular intima evokes downstream consequences (Fig. 15) also provides a new context for the recently recognized roles of inflammatory proteins and regulators in ARMD ((Hageman et al., 2001; Nussenblatt and Ferris, 2007; Richards et al., 2007)). The alternative complement pathway has been implicated by findings of complement components CFH, C5b-9, and others within drusen, and increased risk for ARMD associated with variants in CFH, FB, and C3 genes (Gold et al., 2006; Hageman et al., 2005; Johnson et al., 2000; Yates et al., 2007). For comparison, atherosclerotic plaques contain many of these same molecules, and plaque progression is affected in mice deficient in these proteins (Oksjoki et al., 2007). Within the deep intima, the membrane attack complex C5b-9 co-localizes with modified LDL, appearing shortly after experimental diet-induced hypercholesterolemia (Seifert et al., 1989; Torzewski et al., 1998). Sub-endothelial LDL is thus considered an important activator of complement early in plaque development. That C5b-9 appears in normal BrM and drusen (Johnson et al., 2000; Seth et al., 2008) along with apoB-containing lipoproteins indicates that complement activation could occur by a similar route early in ARMD pathogenesis.

9.2. ARMD ≠ CAD

Like atherosclerotic plaques, drusen and basal deposits feature cholesterol and apoB deposition, and BrM, like arterial intima and other connective tissues, accumulates lipoprotein-derived EC with advanced age. Although knowledge about CAD can provide a powerful conceptual framework for developing new hypotheses about ARMD pathobiology, RPE physiology, and new therapeutic approaches (Curcio et al., 2001), these two complex multi-factorial diseases differ in important and ultimately informative ways, both at the level of the vessel wall and in patient populations.

9.2.1. At the vessel wall – similarities and differences

BrM and arterial intima have notable similarities (Sivaprasad et al., 2005): 1) similar extracellular matrix components (collagen, elastin, glycosaminoglycans including dermatan sulfate); 2) approximately 2–3 fold thickening with age; 3) decreased collagen solubility with age; 4) involvement of MMP-2 and MMP-9 in new vessel growth; 5) presence of TIMP-3, a regulator of matrix turnover; 6) significant age-related build-up of cellular debris and altered extracellular matrix; 7) age-related accumulation of apoB-containing lipoproteins rich in cholesteryl linoleate; and 8) common proteins accumulating in deposits including C5b-9, C3, C5, clusterin, AGE, apoB, and apoE.

But BrM and arterial intima also have notable differences: 1) BrM and intima likely have different major sources of neutral lipid-bearing lipoproteins (RPE vs liver and intestine via plasma); 2) absolute differences in thickness (2–4 μm for BrM, 100–300 μm for intima); 3) hemodynamics of a capillary bed (choriocapillaris vs muscular artery); 4) BrM, located between an endothelium and epithelium, is influenced by both cell types, whereas intima supports an endothelium only; 5) foam cells congregate distinctly in plaques but macrophages are associated primarily with neovascularization in ARMD; 6) smooth muscle cells, which make the fibrous cap of plaques, are scattered throughout the choroid and have an undefined role in ARMD; 7) fibrous collagens (types I and III) and elastin are major components of incipient lipid-rich plaque core but are absent from drusen and BlinD in ARMD; and 9) cholesterol crystals do not occur in BrM or sub-RPE lesions.

9.2.2. In patients – opposite effects (Table 5)

If the deposition of neutral lipids and the formation of cholesterol-rich lesions in BrM were an ocular manifestation of a systemic process, then elevated plasma cholesterol should be a defining risk factor for ARMD, as it is for CAD (2001b; Verschuren et al., 1995). Yet since 1963, 26 studies measuring plasma cholesterol in ARMD patients provided largely inconclusive results (Dashti et al., 2006). Similar arguments can be made for HDL, considered protective against cardiovascular disease. Plasma apoB and apoA-I concentrations in ARMD patients, a measure now recognized as advantageous for cardiovascular risk analysis (Contois et al., 2009), are unrelated to ARMD stage (Dashti et al., 2006). Consistent with this view are the mixed outcomes of plasma lipid-lowering treatments (e.g., statins) on ARMD (Chuo et al., 2007) and the weak association of ARMD with Type 2 diabetes (Adiels et al., 2008; Klein et al., 2004).

The apoE4 genotype is well established as a modest but consistent risk factor for CAD, conferring decreased longevity and increased mortality (Smith, 2002), yet surprisingly protects against ARMD, reducing risk by 40% (Klaver et al., 1998; Thakkinstian et al., 2006). ApoE in human populations exhibits E2, E3, and E4 isoforms, of which E3 is the most common (Hatters et al., 2006). Isoforms differ from each other by Cys to Arg substitutions at positions 112 and 158, so that *E*2 has a neutral, and E4 a positive, charge. ApoE4 forms partially unfolded structures more readily than apoE3 or apoE2, allowing it to bind lipid particles like lipoproteins or

lysosomal membranes with higher affinities or rates and rendering it more sensitive to proteolysis and degradation. Literature too vast to enumerate here relates effects of apoE isoforms on plasma LDL clearance, hepatic apoB production, and cholesterol efflux from intimal macrophages. The opposite polarity of apoE influence in ARMD and CAD could occur at any or all of these sites within RPE, which shares lipoprotein production and clearance pathways with hepatocytes and macrophages. The following scenario suggests one way in which apoE might have different effects in the two tissues. Cholesterol efflux into the intima prevents macrophages from differentiating into foam cells, and apoE4 hinders this efflux, thus exacerbating atherosclerosis (Cullen et al., 1998). In contrast, cholesterol exported from RPE within lipoproteins accumulates in BrM and promotes lesion formation and neovascularization. Perhaps the opposing effect of cellular cholesterol export in BrM and intima helps explain the opposite direction of apoE4's influence in ARMD and CAD.

10. Future directions

10.1. Directions for laboratory research

The central role of RPE and BrM in ARMD pathogenesis has been recognized for almost 4 decades (Hogan, 1972). The studies reviewed herein have major implications for research in RPE cell biology, as information about the homeostasis of cholesterol and neutral lipid in this polarized epithelium and its supporting basement membrane is surprisingly sparse. While a connection between age-related lipid accumulation in BrM and ARMD was postulated 2 decades ago, many questions remain. Why does lipid start to accumulate in BrM and where does it come from? What is the mechanism of this accumulation? How does this accumulation initiate secondary processes that lead to ARMD? How are waste products from the RPE normally transported away? How can this lipid accumulation and/or its consequences be prevented?

10.1.1. RPE cell biology

The goal of new therapeutic approaches for ARMD may be best served by a comprehensive understanding of the RPE as a polarized, constitutive lipoprotein secretor. Here, ample work on hepatocytes, enterocytes, macrophages, cell lines, and mouse models can provide guidance to the critical questions about the apoB system. Recently developed high fidelity, polarized RPE cultures systems (Maminishkis et al., 2006) and tools permitting tissue-specific modulation of RPE gene expression in vivo (Le et al., 2008; Mori et al., 2002) will be essential.

Some outstanding issues about an RPE apoB system include: 1) Identifying the full range of input lipids to an RPE lipoprotein. Current evidence implicates plasma lipoproteins (Section 6.4.3) but which one(s), how it (they) gain(s) access to RPE, which are quantitatively dominant, and to what purpose, remain to be learned. This knowledge, in turn, would stimulate development of new methods that are both reliable and biologically justified for lipid-loading cultured RPE to elicit secretion. 2) Identifying the full range of RPE lipoprotein outputs. Further study may prove that RPE releases cholesterol by an apoB-dependent mechanism involving a large lipoprotein, apoB-independent mechanisms including ABCA1-mediated release to circulating HDL, and production of its own apoE-containing HDL (Iqbal et al., 2003; Zhang et al., 1996). 3) How an apoB-lipoprotein of RPE origin compares to those from other well-characterized apoB secretors. Specific points of interest are whether B-100 and B-48 is synthesized in RPE of various species, whether other apolipoproteins or proteins are present on secreted particles, what if any tissue-specific promoters drive expression in RPE, how assembly and secretion are regulated

(e.g., via nuclear receptors or hormonal influences), how this pathway interacts with retinoid processing (Qtaishat et al., 2003), and how lipoprotein secretion forestalls lipotoxicity. 4) Defining the relationship of basolaterally directed apoB secretion to the apically-directed lipid transport systems to the neurosensory retina (Bazan et al., 1992; Gonzalez-Fernandez and Ghosh, 2008; Tserentsoodol et al., 2006a).

10.1.2. Animal models

The macula is a highly specialized area unique to humans and other primates, and thus far, other than humans, only macaque monkeys have been shown to accumulate constitutively neutral lipid in BrM (Anderson et al., 2006) and apoE in drusen (Umeda et al., 2005). A short-lived species that also displays these characteristics and permits in vivo tests of disease initiation remains a high priority research goal. Because ARMD and atherosclerosis share several key mechanisms, it is not surprising that eyes of established mouse and rabbit models of atherosclerosis have already been examined in pursuit of that goal.

Several general comments can be made about studies using mice. 1) Mice are naturally resistant to atherosclerosis and differ from humans in key aspects of lipoprotein metabolism. Rodents carry plasma cholesterol principally in HDL rather than LDL, they lack the cholesterol ester transfer protein, and they synthesize apoB-48 in hepatocytes as well as enterocytes (Sparks et al., 1981; Zak et al., 2002). Commonly used models are generated by genetically manipulating apolipoprotein and cholesterol processing pathways (Veniant et al., 2008). 2) The best described models are based on C57BL/6 mice and include knock outs of apoE or LDL-R (Dithmar et al., 2000; Ong et al., 2001; Rudolf et al., 2005; Schmidt-Erfurth et al., 2008) or transgenes of human apoB-100, or apoE (Bretillon et al., 2008a; Kliffen et al., 2000; Malek et al., 2005; Sallo et al., 2009). 3) Interpretation of studies using transgenic mice expressing lipoprotein-related genes are complicated by RPE expression of the same genes (Section 6.4.1), preventing definitive attribution of effects to plasma lipoproteins of hepatic/intestinal origin or to lipoproteins of RPE origin. The ocular changes seen in the animal models described below occur in the setting of hyperlipidemia, which is not associated with ARMD pathogenesis (Section 9.2.2). Future animal models that independently manipulate RPE- and plasma-derived lipoproteins, e.g., by using Cre-loxP technology to effect tissue-specific gene deletion (Le et al., 2008; Mori et al., 2002), will thus be highly informative. 4) Murine BrM is only 250 to ~600 nm thick, presenting a challenge to studies of its morphology and composition. Transmission electron microscopy is often used, using tissue preparation steps that extract lipids. As a result lipids are commonly demonstrated indirectly as empty and round electron-lucent vesicles scattered through BrM (Dithmar et al., 2000; Rudolf et al., 2005; Sallo et al., 2009). One study has used the OTAP technique to neutral lipid-containing particles in inner BrM (Fujihara et al., 2009). Unresolved is whether partially extracted electron-dense material in BlamD is sub-optimally preserved neutral lipid or disintegrating basal RPE infoldings. 5) Histochemical detection of lipids in mouse BrM is also difficult because BrM is extremely thin and can be obscured by surrounding pigmentation, but it is possible to detect neutral lipid with oil red O or filipin (Bretillon et al., 2008a; Malek et al., 2005; Rudolf et al., 2005).

Studies of established atherosclerotic mouse models with hyperlipidemia have reported BrM changes comparable to alterations found in humans. These changes include thickening, loss of laminar architecture, and accumulation of apparent lipid vesicles (Dithmar et al., 2000; Fujihara et al., 2009; Kliffen et al., 2000; Rudolf et al., 2005; Schmidt-Erfurth et al., 2008). In apoE^{-/-} and LDL-R^{-/-} mice (Dithmar et al., 2000; Ong et al., 2001; Rudolf et al.,

2005; Schmidt-Erfurth et al., 2008) or mice expressing human apoB-100 or apoE transgenes (Bretillon et al., 2008a; Fujihara et al., 2009; Kliffen et al., 2000; Malek et al., 2005; Sallo et al., 2009), main factors promoting BrM lipid deposition were advanced age and, in contrast to humans, elevated levels of plasma lipids. High-fat or high cholesterol diets have been used to provoke the desired lesions consistently (Dithmar et al., 2000; Edwards and Malek, 2007; Miceli et al., 2000; Ong et al., 2001; Rudolf et al., 2005). Mice expressing variants of the apoE gene exhibit among other things varying degrees of basal deposits (Dithmar et al., 2000; Kliffen et al., 2000; Malek et al., 2005). Blue light-exposed mice have increased BlamD-like material in the sub-RPE space (Cousins et al., 2002; Espinosa-Heidmann et al., 2004).

A hint as to why lipids get trapped in BrM may come from an apoB-transgenic mouse that also over-expresses biglycans (Sallo et al., 2009). Biglycan expression causes BrM thickening and is suspected of promoting BrM lipid accumulation by interacting with lipoprotein-associated proteins. Older mice expressing human apoE2, E3, or E4 isoforms and consuming a high-fat diet accumulate oil red O-binding material in the sub-RPE space (Malek et al., 2005). Surprisingly, ApoE4, which is protective in humans (Section 9.2.2.), produced the most severe effect of any ApoE allele, showing even spontaneous choroidal neovascularization (Malek et al., 2005).

Not only BrM is affected in these hypercholesterolemic mice, but also the tissues surrounding it. The RPE demonstrates vacuolization, loss of intracellular compartments, reduced cell height, and fewer apical microvilli (Miceli et al., 2000; Rudolf et al., 2005 Schmidt-Erfurth et al., 2008). Further, photoreceptors and the choriocapillary endothelium are compromised as well (Bretillon et al., 2008a; Schmidt-Erfurth et al., 2008). Degenerative ultrastructural changes in aged mice are associated with local oxidative stress measurable by increased presence of nitric oxide metabolites or lipid peroxidation (Sadaba et al., 2008). In these mice, such phenomena can lead to increased expression of ARMD-relevant stress-related growth factors like VEGF, the major stimulus for choroidal neovascularization (Rudolf et al., 2005; Schmidt-Erfurth et al., 2008).

Rabbits are also commonly used in atherosclerosis research, as they demonstrate hypercholesterolemia-inducible aortic lesions resembling human fatty streaks (Moghadasian, 2002). In this merangiotic species, only one vascular system (choroidal) serves the retina, unlike humans. Watanabe heritable hyperlipidemic and New Zealand rabbits after diet-induced hypercholesterolemia exhibit high levels of LDL cholesterol and lesions in the ocular posterior pole (Kouchi et al., 2006; Ramírez et al., 2006; Salazar et al., 2007; Triviño et al., 2006; Walton and Dunkerley, 1974). Principal changes appear in the choroid, with diffuse thickening and accumulation of lipid-rich macrophages resembling foam cells. Of note, the inner collagenous layer of BrM thickens, with electron dense and electron-lucent particles and BlamD, and the RPE acquires lipid inclusions. Changes in choroid, BrM, and RPE were partly reversible after plasma cholesterol levels are normalized (Ramírez et al., 2006; Salazar et al., 2007).

10.1.3. Model systems

Unlike age-related accumulation of cholesterol in other connective tissues, that which occurs in BrM is apparently unique in its potential impact on fluid and nutrient exchange, because of importance of this tissue to transport to and from the RPE. The new biochemical model for BrM lipid accumulation may facilitate the construction of and informed experimentation with *in vitro* model systems to study transport across natural and artificial matrices. Such systems could use readily available LDL as an acceptable surrogate because of its high EC content or VLDL because of its size

similarity to the lipoproteins seen to accumulate in BrM. Use of such systems have already demonstrated that LDL can pass through bovine BrM (Huang et al., 2008b), although slowed in transit, and that LDL deposition significantly decreases the hydraulic conductivity of an extracellular matrix (McCarty et al., 2008). Future studies should focus on determining the mechanism by which lipid accumulates in BrM and impede transport.

There are several changes that might explain why lipoproteins begin to accumulate in BrM. These steps include changes in the extracellular matrix of BrM (e.g. non-enzymatic glycation (Cerami et al., 1986) or increased proteoglycans (Sallo et al., 2009)) can promote lipid accumulation, changes in the amount or character of lipoproteins that transit through BrM (e.g., oxidation (Chang et al., 2001; Wang et al., 2001) such that they are more likely to accumulate, or changes in the mechanism of lipoprotein clearance. Use of natural and artificial matrices would allow investigation of such possibilities (Huang et al., 2008b, 2009; McCarty et al., 2008).

Once trapped or restricted by the extracellular matrix, the process by which lipids continue to accumulate and lead to formation of a transport barrier is also unknown. It is unlikely that the lipid particles simply begin to accumulate in BrM, and this accumulation directly leads to the formation of a transport barrier like the Lipid Wall. Such a process would be expected to be nonlinear, with the initial accumulation being slow and latter stages accelerated as those lipids already trapped impede further transport of lipids. The age-related lipid accumulation rate in BrM is at best only slightly non-linear (Fig. 9A), inconsistent with this two-stage model. This suggests multiple processes (including clearance) that might be best studied by perfusing natural or artificial matrices with lipids and then using imaging techniques (e.g. QFDE, OTAP) to characterize this accumulation process.

10.2. Directions for clinical research

10.2.1. Dietary manipulation

The model presented herein provides a basis for designing dietary enhancement of RPE function via LDL or HDL uptake (Elner, 2002; Tserentsoodol et al., 2006a,b). Such knowledge will be critical for interpreting results emanating from the Age-related Eye Disease Study (http://www.nei.nih.gov/AREDS2), which will test the effects of dietary lutein and docosahexanoate supplementation on ARMD incidence and progression.

10.2.2. Refurbishing BrM for RPE cell replacement

RPE re-populates BrM poorly following surgical extirpation of choroidal neovascular membranes in older patients, unlike younger patients (Tezel et al., 2004). The RPE's difficulty in attaching, growing, and servicing photoreceptors on aged BrM (Sun et al., 2007) complicates the prospects of RPE transplants in ARMD patients, leading to intensive efforts to optimize the substrate for RPE growth by refurbishing aged BrM (Del Priore and Tezel, 1998; Gullapalli et al., 2008; Tezel et al., 2004). The deleterious effect of lipid accumulation on transport across BrM (Section 7.2) is another factor to consider in addition to the adhesive properties of the RPE basal lamina (inner sub-layer of BrM) (Afshari and Fawcett, 2009). Without more information about inputs to and purpose of an RPE apoB-lipoprotein, interfering with its production by RPE (e.g., by MTP inhibitors (Shoulders and Shelness, 2005) or apoB anti-sense (Soutschek et al., 2004) may be premature. However, agents that remove EC or LDL-like particles from BrM should certainly be sought and tested. In particular BrM can serve as a surgical bed for autologous grafts or stem cells to replace damaged RPE and photoreceptors (Binder et al., 2007), and thus refurbishing should include lipid sequestering or solubilizing using detergents

(Tezel et al., 2004), neutral pH cholesterol esterases (Holm and Osterlund, 1999), reconstituted HDL (Chung et al., 2005), apolipoprotein mimetics (White et al., 2009) and bioremediation (Rittmann and Schloendorn, 2007).

10.2.3. Imaging ARMD in patients

Subtle features in druse structure seen in living patients, whether by biomicroscopy or dye angiography, have been attributed to differences in the composition of druse interiors and exteriors (Bird and Marshall, 1986; Pauleikhoff et al., 1992). Neutral lipids identified in drusen (Wolter and Falls, 1962) (Section 8.1) were postulated to account for the variable staining during clinical angiography by the hydrophilic dye fluorescein (Pauleikhoff et al., 1992). Spectral domain optical coherence tomography is fast approaching the ability to reveal druse contents (Khanifar et al., 2008). The ubiquity and abundance of cholesterol in drusen suggests that methods capable of revealing cholesterol forms or lipoproteins in atherosclerotic plaques (e.g., magnetic resonance spectroscopy) (Ruberg et al., 2006; Yuan et al., 1997)) may someday disclose details of druse evolution and provide a biochemical basis for the increased risk indicated by these structures. Finally, the distinctly different compositions of BlinD and BlamD should enable their eventual visualization in living patients.

10.3. Conclusion

We have presented a body of work strongly implicating the RPE as constitutive secretor of EC-rich apoB-lipoproteins which when retained and accumulated in BrM contributes importantly to impeding RPE and photoreceptor function and to forming the specific lesions of ARMD. The conceptual framework, borrowed heavily from decades of atherosclerosis research, provides a wide knowledge base and sophisticated clinical armamentarium that can be readily exploited for the benefit of ARMD patients.

Acknowledgments

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