Special Issue

Soft Drusen in Age-Related Macular Degeneration: Biology and Targeting Via the Oil Spill Strategies

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AMD is a major cause of legal blindness in older adults approachable through multidisciplinary research involving human tissues and patients. AMD is a vascular-metabolic-inflammatory disease, in which two sets of extracellular deposits, soft drusen/basal linear deposit (BLinD) and subretinal drusenoid deposit (SDD), confer risk for end-stages of atrophy and neovascularization. Understanding how deposits form can lead to insights for new preventions and therapy. The topographic correspondence of BLinD and SDD with cones and rods, respectively, suggest newly realized exchange pathways among outer retinal cells and across Bruch's membrane and the subretinal space, in service of highly evolved, eye-specific physiology. This review focuses on soft drusen/BLinD, summarizing evidence that a major ultrastructural component is large apolipoprotein B,E-containing, cholesterol-rich lipoproteins secreted by the retinal pigment epithelium (RPE) that offload unneeded lipids of dietary and outer segment origin to create an atherosclerosis-like progression in the subRPE-basal lamina space. Clinical observations and an RPE cell culture system combine to suggest that soft drusen/BLinD form when secretions of functional RPE back up in the subRPE-basal lamina space by impaired egress across aged Bruch's membrane-choriocapillary endothelium. The soft drusen lifecycle includes growth, anterior migration of RPE atop drusen, then collapse, and atrophy. Proof-of-concept studies in humans and animal models suggest that targeting the "Oil Spill in Bruch's membrane" offers promise of treating a process in early AMD that underlies progression to both end-stages. A companion article addresses the antecedents of soft drusen within the biology of the macula.

Keywords: age-related macular degeneration, drusen, atrophy, lipoproteins, cholesterol, retinal pigment epithelium, Bruch's membrane, apolipoprotein mimetic, statin, non-human primate, mouse models

Introduction and Synopsis

AMD is a major cause of legal blindness in older adults approachable through multidisciplinary research involving human tissues and patients via clinical imaging and genetics. The central theses of this review are as follows:

1. Soft drusen and basal linear deposit (BLinD) are two forms of the same extracellular lipid rich material that together make up an Oil Spill on Bruch's membrane (BrM). Drusen are defined in reference to a three-layer BrM and in distinction to other entities that are not drusen;
2. AMD is a vascular-metabolic-inflammatory disease in which soft drusen/BLinD and subretinal drusenoid deposit (SDD; also called reticular pseudodrusen) are major risk factors for progression to end-stages of atrophy and neovascularization that involve substantial loss of retinal pigment epithelium (RPE) and photoreceptors[1];
3. The topographic relation of soft drusen/BLinD to cones and SDD to rods strongly suggests that deposit biogenesis reflects newly realized exchange pathways among cones, rods, RPE, Müller cells, and choriocapillary endothelium, across BrM and the subretinal space, in service of highly evolved, eye-specific physiology;
4. A major component of soft drusen/BLinD is lipoprotein particles containing apolipoproteins B and E, secreted by RPE in a physiologic lipid-recycling program. The composition suggests a dual origin of lipids (fatty acids from diet, cholesterol from diet and photoreceptor outer segments);
5. Clinical imaging and an RPE cell culture system together define a druse lifecycle to which RPE demise can be linked. Soft drusen/BLinD form when secretions of functional RPE back up in the subRPE-basal lamina space, because egress across aged BrM-choriocapillary endothelium is impaired. Drusen can expand in volume, RPE migrate off the top into the retina, leading to disintegration of the RPE layer, druse collapse, and atrophy;
6. The Oil Spill strategies for druse abatement to forestall type 1 neovascularization and geographic atrophy have supportive preclinical and clinical data; and
7. Understanding outer retinal physiology driving lipoprotein production has potential to advance treatments as impactful for AMD as statins have been for atherosclerotic cardiovascular disease; relevant model systems exist.

This conceptual framework directs attention to understanding the formation and clearing of drusen as a basis for targeting precursors pharmacologically to delay end-stages. The overall

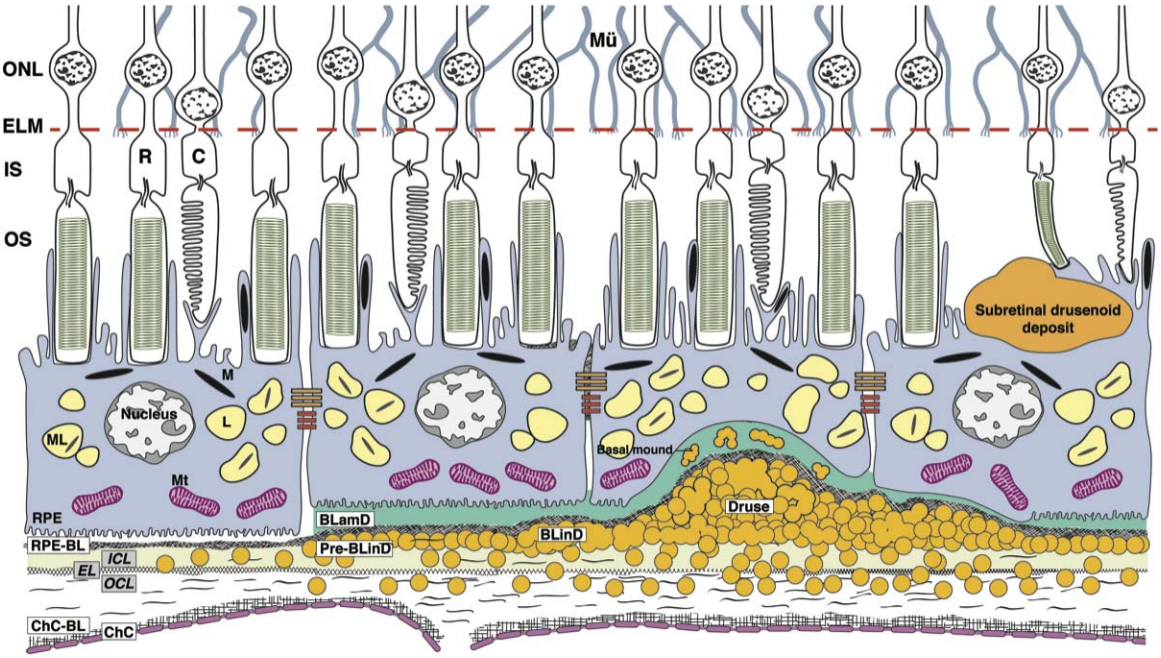

Figure 1. AMD by the layers. BrM consists of the ICL, EL, and OCL. Soft drusen and BLinD are two forms (lump and layer) of the same AMD-specific extracellular deposit. BLamD is a thickening of the RPE-BL. Basal mound is soft druse material within BLamD. Subretinal drusenoid deposit localizes to the subretinal space (between photoreceptors and RPE). RPE cells contain melanosomes, lipofuscin and melanolipofuscin, and mitochondria that provide signals for color fundus photography, fundus autofluorescence, and OCTs. Abbreviations from inner to outer: ONL, outer nuclear layer; ELM, external limiting membrane; IS, inner segments of photoreceptors; OS, outer segments of photoreceptors; R, rods; C, cones; L, lipofuscin; M, melanosome; ML, melanolipofuscin; Mt, mitochondria; Mu, Müller glia; *circles*, lipoprotein particles.

hypothesis is limited to discussion of soft drusen (and their differential diagnoses) and should be contextualized among other known contributors to AMD pathobiology. These include aging in the choroidal vasculature,[2] inflammation, and activity of resident/transient immune cells,[3] among others. Many mechanisms operating simultaneously give rise to AMD's complexity. Validated multimodal clinical imaging offers bright prospects for connecting disparate pieces in a coherent timeline to clarify therapeutic strategies. Despite knowledge gaps, enough is known about soft drusen/BLinD biology to launch new approaches. A companion article considers what aspects of macular biology drive soft drusen biogenesis.[4]

Neurobiology and Aging of the Macula

A neurovascular unit[5,6] comprises microvessels, neurons, glia, pericytes, and extracellular matrix that link blood flow to the metabolic demands of neurons. The cells and tissues most prominently affected by AMD pathology are those of the outer retinal neurovascular unit[7] (i.e., photoreceptors, RPE, Müller cells [in neurosensory retina], and the choriocapillaris [ChC] endothelium [in the choroidal vasculature]). The choroid has the highest blood flow in the body, and the choriocapillaris is sinusoidal and fenestrated. Between RPE and ChC is a laminated subendothelial extracellular matrix called Bruch's membrane (BrM), which functions as a vessel wall laid out flat, paralleling vascular lumens.[8] The RPE is a monolayer of cuboidal polygonal cells embedded between photoreceptors and BrM. Strong apical to basolateral polarization makes the RPE a key player in the homeostasis of photoreceptors and the pathology of SDD apically and choriocapillaris and the pathology of drusen basally. The macular neurosensory retina consists of a 0.8-mm diameter all-cone fovea surrounded by a rod-dominated annulus of 6-mm outer diameter. The Henle fiber layer contains inner fibers of photoreceptors and Müller glia that form junctions at the external limiting membrane. Among numerous Müller cell functions[9] are recently recognized roles in delivering to cones for phototransduction vitamin A derivatives of dietary origin.[10,11] Xanthophyll pigments lutein and zeaxanthin are prominent in the foveal center, and lutein, in the Henle fiber and inner plexiform layers.[12,13] A hypothesis that Müller cells are major xanthophyll reservoirs is explored separately.[4]

Of major age-related tissues changes detailed separately,[4] we focus on BrM, where AMD pathology is prominent, including cross-linking,[14] thickening,[15] and lipidization,[16–18] and loss of ChC density and apposition to BrM.[19] The lipidization of BrM provides a straightforward path to lipids in soft drusen, arguably the first druse component described.[20–24] Lipid accumulation in vessel walls connects to both the pathophysiology of atherosclerotic cardiovascular disease[25] and the clinical success in reducing its public health burden.[26]

Defining the Layers of AMD

A cellular- and molecular-level understanding of drusen begins with delicate tissue layers in the RPE-BrM-ChC complex and adjoining potential spaces (Fig. 1). The anatomic definition of BrM[27] is five layers (from inner to outer), RPE-basal lamina (BL) and inner collagenous, elastic, and outer collagenous layers (inner collagenous layer [ICL], elastic layer [EL], outer collagenous layer [OCL]). Pathology may be best understood with the Sarks-Gass concept of a three-layer BrM (ICL+EL+OCL) that does not include the RPE and ChC basal laminas, thereby defining the subRPE-BL space between the RPE-BL and the ICL. Drusen are focal deposits located between the RPE-BL and the ICL of BrM, in the subRPE-BL space. BLinD is a thin layer of soft druse material, in the same compartment. This framework facilitates explaining the participation of basal laminar deposit (BLamD) in clinical AMD, the trajectory of type 1 (subRPE-BL)

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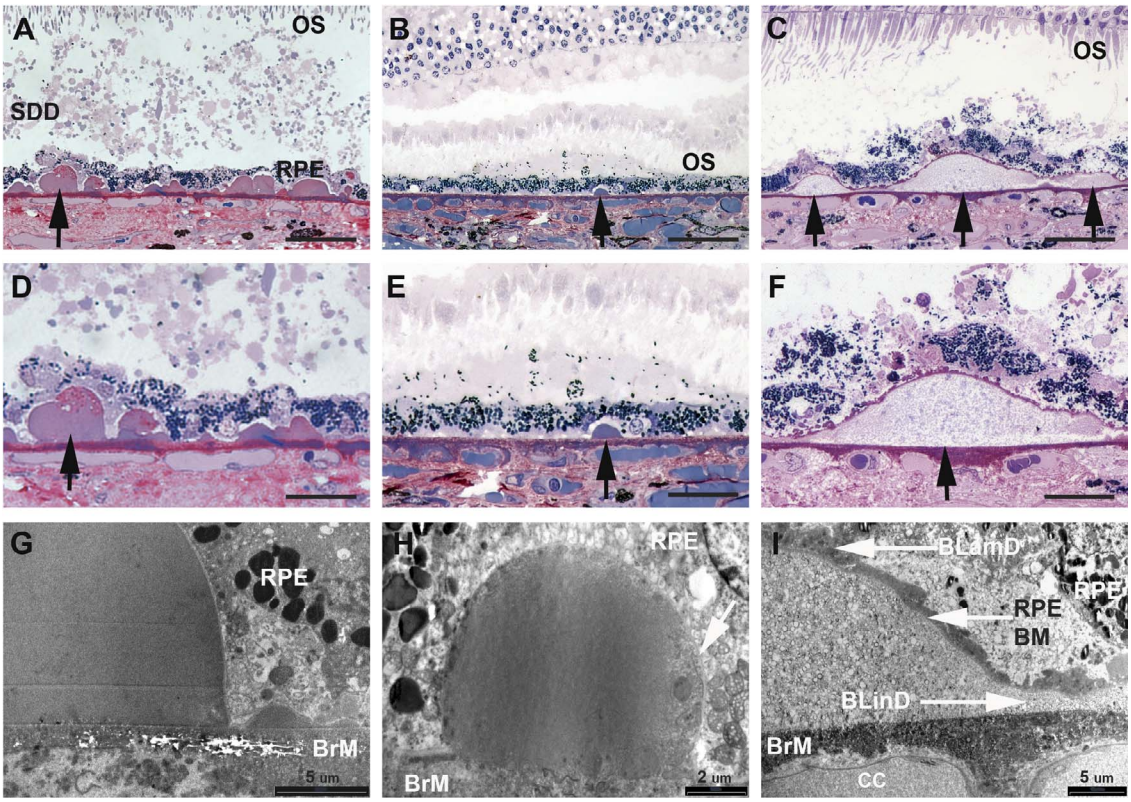

Figure 2. Drusen types in AMD macula have distinct geometry and ultrastructure. Cuticular, small hard and soft drusen in (**A**–**F**) high-resolution light microscopy and (**G**–**I**) transmission electron microscopy. (**A**–**C**) Drusen are found between the RPE-BL and the inner collagenous layer of BrM). Hard drusen and cuticular drusen are small with steep sides and contain dense hyalinized contents; cuticular drusen are numerous. Soft drusen are large and have sloping sides. BLamD and BLinD associate only with soft drusen (**F**) and not (**D**) cuticular drusen or (**E**) small, hard drusen. (**G**, **H**) Cuticular and small hard drusen are homogenous and electron dense, with small vacuoles attributed to extracted lipids distributed throughout. (**I**) Soft drusen are packed with 'membranous debris' (considered partially preserved lipoproteins) and are continuous with BLinD, giving rise to a 'soft' appearance in the fundus. Detachment of the retina from RPE is a postmortem artifact. *Black arrows* denote individual drusen. Images (**A**, **D**, **G**) taken from macular sections of the left eye of the patient with cuticular drusen. *Scale bars*: (**A**–**C**) 60, (**D**–**F**) 30, (**G**) 5, (**H**) 2, and (**I**) 5 μm. BM, basement membrane; CC, choriocapillaris. Reprinted with permission from Balaratnasingam C, Cherepanoff S, Dolz-Marco R, et al. Cuticular drusen: Clinical phenotypes and natural history defined using multimodal imaging. *Ophthalmology.* 2018;125:100–118. © 2017 by the American Academy of Ophthalmology.

neovascularization, the differing embryologic origins of RPE-BL versus ICL+EL+OCL, and Mendelian disorders preferentially affecting the RPE-BL[28–30] versus structural elastin and collagen.[31,32] By this definition, the Oil Spill *in* aging BrM[33] becomes the Oil Spill *on* BrM.

Drusen are focal and can be recognized clinically. In contrast BLinD is thin and diffusely distributed, poorly visible in paraffin histology, and invisible clinically, leading to a common misperception that BrM thickens in AMD when in fact new layers are interposed (Fig. 1).

Imaging, Epidemiology, and the Expanding Spectrum of Drusen

Drusen are the major intraocular risk factor for progression, and how they are detected clinically impacts theories of their formation and significance.[34] Drusen were linked to end-stages of geographic atrophy (GA) and neovascularization on a time course of years by Gass using color fundus photography (CFP) and fluorescein angiography,[35] as repeated in large samples.[36,37] Major epidemiologic studies of European-derived populations since 1991 are based on standardized CFP-based grading systems.[38] Soft drusen are yellow-white elevations ranging from 30 μm to more than 1000 μm in diameter with an indistinct border due to sloping sides (Figs. 2C, 2F, 2I).[39] Numerous hard drusen (Figs. 2B, 2E, 2H) and cuticular drusen (originally called basal laminar drusen [Figs. 2A, 2D, 2G]) increase risk in the aggregate and over the long term (15 years), in part by increasing risk for soft drusen.[40–42] East and South Asian populations have low prevalence of typical drusen but progress to neovascularization.[43,44]

Spectral-domain optical coherence tomography (OCT), commercialized in 2007, is an interferometry technique using low-coherence light to achieve depth-resolved, comprehensive, and noninvasive cross-sectional views of chorioretinal structure. Advancements, such as eye tracking and signal averaging, combine to make cross-sectional structural OCT the base modality for AMD clinical trials going forward.[45] By OCT, soft drusen are dome-shaped RPE elevations with homogenous and moderately reflective "ground-glass" interiors internal to BrM, which appears at the druse base as a fine reflective line. In small cohorts examined so far, soft drusen are the most common among macular druse types.[46] Internal structure in soft drusen visible on OCT signify risk for progression,[47–51] and approximately 10% of soft drusen may have subclinical (nonexudative) neovascularization.[52] A spectrum of RPE elevations now exists.[53–59] By the gold standard of histology of clinically documented cases (Fig. 2),[40,60–63] hard and cuticular drusen are ultrastructurally similar, small, globular

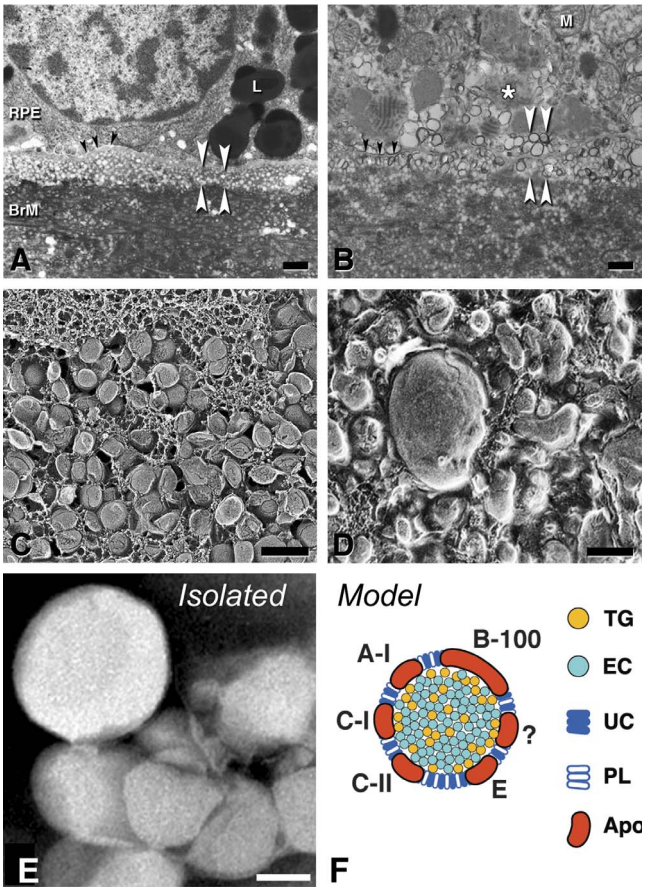

Figure 3. A major component of BLinD and pre-BLinD is lipoproteins. *Black arrowheads*, RPE-BL; *white arrowhead*, subRPE-basal laminar space. (**A**, **B**) Thin-section transmission electron microscopy; osmium postfixation, vertical cross-section, *scale bars*: 1 μm. (**C**, **D**) Quick freeze deep etch microscopy; en face fracture plane of subRPE-basal laminar space; *scale bars*: 200 nm. (**A**, **C**) Pre-BLinD is a layer of lipoproteins 3 to 4 deep in the subRPE-BL space of many older eyes. Lipoproteins are spherical particles of uniform diameter with surface-and-core morphology. They were originally described as vesicles in osmium postfixed specimens (**A**). RPE, (**B**, **D**) BLinD in an eye with geographic atrophy is a mixture of native and fused lipoprotein particles, with lipid pools, in the same plane, originally described as membranous debris in osmium postfixed specimens (**B**). (**E**) Lipoprotein particles isolated from BrM are large and spherical; negative stain,[144] *scale bar*: 50 nm. (**F**) BrM lipoprotein composition inferred from direct assay,[97,144] druse composition, and RPE gene expression.[135,358] Apo, apolipoproteins. ?, as-yet-unknown apolipoproteins.

deposits 30 to 60 μm in diameter. Cuticular drusen are numerous in generally younger patients, exhibiting imaging signs of RPE attenuation at the apices.[64]

A major limitation to current estimates of progression risk is the recent recognition of extracellular deposits in the subretinal space, between photoreceptors and RPE, first called reticular pseudodrusen[65] and recently, SDD.[66,67] SDD is biologically distinct and not just drusen in the wrong place[34] (see the Subretinal Drusenoid Deposits: Extracellular, Space-Filling, Distinct From Drusen section). SDD were in part misclassified as soft drusen or omitted altogether from five CFP-based grading systems[36–38,68–71] that underlie prevalence estimates, risk models, and genetic associations. Thus, risk attributed to soft drusen in some CFP-based grading systems is aggregate risk of soft drusen plus SDD. All literature must therefore be interpreted anew—do authors mean subretinal or subRPE? Did study eyes have SDD? Consequently, experimental

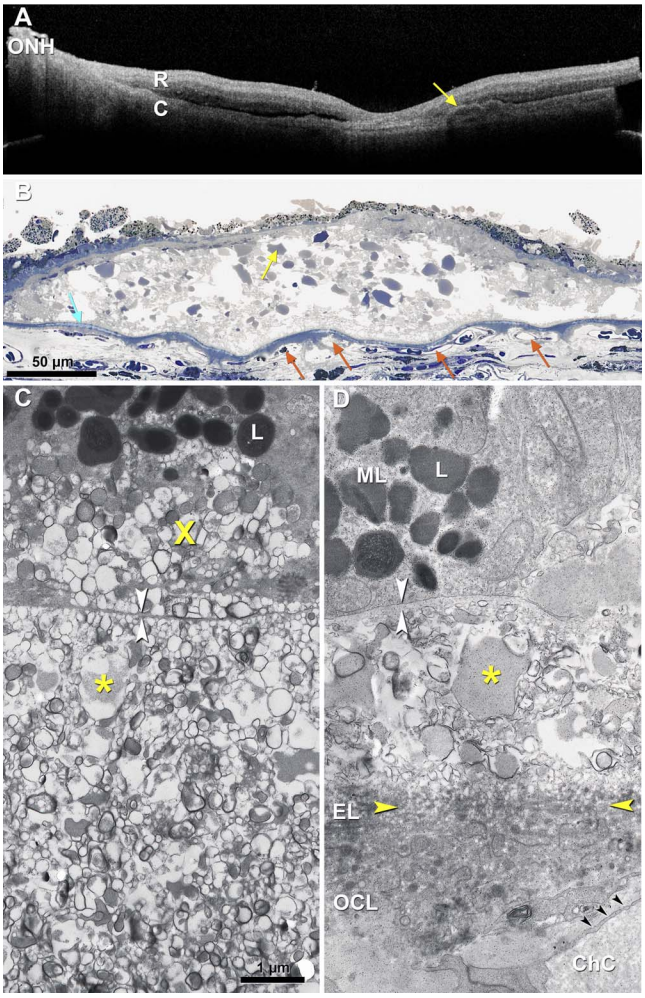

Figure 4. Soft drusen/basal linear deposit: lipid lakes, no structural collagen/elastin. (**A**) Ex vivo OCT imaging of a short postmortem donor eye with large soft drusen (*arrow*, shown in [**B**]) and central GA, 79-year-old male. R, retina, (**C**) choroid; ONH, optic nerve head; (**B**) Large soft druse shown in (**A**) has numerous lipid pools (*arrow*) containing esterified cholesterol (refer to Fig. 3C,[135] overlaid with dysmorphic RPE and BLamD. The underlying BrM has refractile patches of hydroxyapatite (*light blue*). Choriocapillaris endothelium ranges from normal to ghosts. Submicrometer section, osmium tannic acid paraphenylenediamine postfixation, toluidine blue stain. (**C**, **D**) Soft druse (**C**) and BLinD ([**D**] above the *yellow arrowheads*) from different donors[126] contain membranous profiles with electron-dense exteriors and homogeneous and moderately electron-dense interiors, thought to represent partly preserved lipoprotein particles. The same material is found internal (basal mound,[8] X) and external to the RPE-BL. *Scale bar*: 1 μm. Osmium postfixation and transmission electron microscopy. *White arrowheads*, RPE-BL. *Asterisks*, lipid lake.

studies must include highly polarized RPE cells for greatest AMD relevance (see the Model Systems for Mechanistic and Translational Drusen Research section).

Introduction to Cholesterol and Lipoproteins

Because ample multidisciplinary evidence supports lipoprotein particles as a major component of soft drusen, we introduce the chemistry and biology of cholesterol and lipoproteins (Fig. 3); comprehensive reviews are available.[72,73] Cholesterol is a lipid with a hydrophobic four-ring system. A 3β-hydroxyl group binds long-chain fatty acids to form esters. We refer to

unesterified and esterified cholesterol (UC and EC), respectively. EC, accounting for approximately 70% of total cholesterol in humans, is used for storage and transport. UC is essential to all animal cells in roles of membrane integrity, fluidity, and permeability. Membrane UC is intercalated among phospholipids (PL) and concentrated in lipid rafts to influence many cellular activities, include gene transcription, nerve conduction, and synaptogenesis. Three physical forms—oily droplets, lamellar membranes, and monohydrate crystals—differ in the relative proportions of EC, UC, and PL. For transport through plasma and interstitial fluid, UC and EC form with apolipoproteins, PL, and triglycerides (TG) spherical multimolecular complexes called lipoproteins. Plasma lipoprotein classes identified by ultracentrifugation include (from large to small) chylomicrons (CM), very low-density (VLDL), low-density (LDL), and high-density (HDL) lipoproteins. Apolipoprotein B-100 (apoB-100) is the principal protein of LDL and is present with apoE in VLDL of hepatic origin, which is the parent particle of LDL. Apolipoprotein A-I (apoA-I) is the principal protein of plasma HDL. Brain HDL lipoproteins are rich in apoE.[74] Cross talk between plasma lipoproteins and complement components is under investigation.[73]

Cells have many ways to efflux UC, and RPE may be capable of all, because some evidence currently exists for many. These include transfer to circulating HDL,[75] complexing with endogenously synthesized apolipoproteins, conversion to an oxysterol capable of passing through cellular membranes,[76] and release as microvesicles (budding of plasma membrane) or exosomes (trafficked from endosomes).[7] Conversely, lipoprotein particles and milk fat represent the only known ways by which cells release EC.

Genetics and Gene Expression Studies Relevant to Lipids

AMD's major genetic associations are complement factor H (*CFH*)[78] and *ARMS2*, a gene with an uncertain function, now separated statistically from *HTRA1*, also on chromosome 10.[79] Among pathways, lipids are the most highly implicated after complement.[80] Candidate gene studies reported an association with AMD of single nucleotide polymorphisms (SNP) in *APOE*.[81,82] Genome-wide association studies (GWAS) later also identified SNPs associated with advanced AMD in *CETP*, *ABCA1*, and *LIPC*, best known from plasma HDL homeostasis.[83,84] The International Age-related Macular Degeneration Genomics Consortium found associations of these genes with AMD (n = 16,144 cases and 17,832 controls) but with not elevated levels of plasma HDL[85] (see Refs. 86–88). The Consortium dataset was probed via Mendelian randomization,[89] which showed that three variants of genes associated with plasma lipid levels (LIPC, 2; CETP, 1) reached genome-level significance, placing AMD between cardiovascular disease and Alzheimer disease in the strength of lipid gene associations. SNPs in *LIPC* and *ABCA1* are associated with intermediate and large drusen, and *CFH*, *C3*, *C2*, and *ARMS2/HTRA1*, large drusen.[90] A rare *CFH* variant is associated with abundant soft drusen,[91] and two *CFH* SNPs, with greater drusen area in central macula.[92]

These studies and others[72,93] suggest that lipid genes impact AMD risk significantly, yet independent of, or even reverse to, plasma lipoprotein profiles from cardiovascular disease, a paradox likely related to the existence of intraocular regulatory mechanisms. In normal human donor eyes, microarray[93] and comprehensive RNA-sequencing[94] analysis demonstrated that scores of genes controlling all aspects of cholesterol and lipoprotein homeostasis are expressed in both neurosensory retina and RPE. Immunolocalization using validated antibodies and polarized RPE (in vivo or high-fidelity culture, Refs. in 34) include *APOE* (photoreceptor outer segments, RPE, Müller cells, drusen, and SDD); *ABCA1* (diffuse labeling of RPE cell bodies); *CETP* (photoreceptor outer segments and outer plexiform layer (OPL), with some labeling in the choroid); LIPC (all retinal neurons including photoreceptors and ganglion cells plus RPE, and not in Müller cells). Thus, theories of AMD pathogenesis based on genes well studied in liver, intestine, adipose tissue, and brain must also incorporate chorioretinal expression.[94]

Human retina expresses two hallmark genes of hepatic and intestinal lipoprotein secretion, microsomal TG transfer protein (*MTTP*) and apoB (*APOB*) (for expert review see Ref. 95). Localization of both proteins in RPE and in retinal ganglion cells appears consistent with endoplasmic reticulum.[96] Secretion of full-length apoB-100 was demonstrated in rat-[97] and human-derived RPE cell lines[98] and in mouse RPE-choroid explants.[99] *MTTP* is a soluble heterodimer[100,101] that co-translationally transfers lipid to apoB to ensure correct folding.[102,103] Cells expressing apoB without *MTTP* cannot secrete lipoproteins.[104–107] ApoB production is regulated via co- and posttranslational degradation by the ubiquitin-proteasome system, which is in turn regulated by lipid availability.[108] ApoB's classic function is delivering exogenous and endogenous TG, cholesterol, and lipophilic vitamins throughout the body as part of VLDL/LDL and chylomicrons. ApoB is also expressed in kidney, placenta, and heart,[109,110] apparently to regulate TG content and forestall lipotoxicity.[111] In mice, absence of apoB is lethal in utero, and reduced apoB causes neural tube defects.[112,113] Lack of functional *MTTP* and apoB results in abetalipoproteinemia (ABL, OMIM 200100) and hypobetalipoproteinemia (HBL, OMIM 615558), rare Mendelian disorders that include a pigmentary retinopathy and ataxic neuropathy. Attributed to impaired delivery of lipophilic vitamins, ABL/HBL are partly alleviated by long-term dietary supplementation.[114] Intraocular apoB and *MTTP* expression indicates that ABL/HBL are intrinsic degenerations and that lipoprotein assembly and secretion are required for retinal health and good vision. It also means abundant research on hepatic and intestinal lipoproteins are relevant to AMD.

Soft Drusen, BLinD: Lifelong Physiology, Uncovered by Aging

In the 19th century Donders,[115] Wedl,[116] and Müller[117] discovered drusen; Wedl[116] described them as lipid globules. Long-standing theories for druse formation[117] are transformation of the overlying RPE and deposition of materials onto BrM. The latter is now accepted.[118]

S.H. and J.P. Sarks, two ophthalmologists in Australia, together and in collaboration with pathologist M.C. Killingsworth, contributed foundational AMD pathology, including the heterogeneity of drusen within a heterogeneously presenting disease.[23,40,60,65,119–122] Studies using panoramic electron microscopy of affected macular tissue from clinically documented eyes of S.H. Sarks' patients[40,60,119–121,123,124] definitively localized drusen in the subRPE-BL space, distinct from the overlying RPE-BL/BLamD and underlying ICL, and proved that clinical druse phenotypes differed in ultrastructure and thus in composition.[60]

Soft drusen are dome-shaped with sloping sides[125] and filled with 'membranous debris'[60] (Figs. 2, 4), implying lipids, and considered by the Sarks to set the disease course. Soft drusen and BLinD are two physical forms (lump and layer, respectively), often continuous,[126] of the same material; BLinD was also called "diffuse drusen" by paraffin histology.[127] When soft drusen/BLinD are processed for conventional thin-section

electron microscopy using osmium postfixation, biomechanical fragility[23,128,129] and partial extraction of lipid combine to produce curvilinear elements resembling coiled membranes (Figs. 4C, 4D). Thus, the principal soft druse component was initially called membranous debris,[120,123] influencing mechanistic hypotheses and development of model systems exhibiting cellular membrane release.[130,131] Also, descriptions of aging BrM using conventional osmium postfixed tissue mentioned vesicles (i.e., membranous coils with aqueous interiors).[132,133]

Lipid-preserving histochemical and ultrastructural techniques united "vesicles" and "membranous debris" as manifestations of lipoprotein particles at different levels of preservation and disintegrity. Evidence for lipoprotein involvement is best for pre-BLinD (Fig. 3C) and hard drusen, where electron-dense spherical particles are visible, and more than 40% of druse volume is Folch-extractable lipid.[134] Soft drusen/BLinD are biomechanically fragile (called "localized detachments of BLamD")[127] so evidence for their composition rests on consistent ultrastructural and histochemical results across studies. Soft drusen/BLinD exhibit polygonal regions of homogeneously and moderately electron-dense material (Fig. 4), originally called "hard drusen breaking up." When prepared by sterol-specific filipin histochemistry, these shapes are EC-rich lakes.[135,136] Similar processes occur in the lipid-rich cores of atherosclerotic plaques, where plasma LDL insudates[137,138] and binds to extracellular matrix, followed by particle surface degradation, fusion, and pooling of core lipids to create UC-rich liposomes[139,140]; these processes can be mimicked in vitro by physically disrupting LDL.[141]

A natural history of aging BrM (17–92 years) using osmium tannic acid post-fixation showed that "vesicles" were solid, spherical particles approximately 80 nm in diameter. Further, quick-freeze deep-etch analysis of BrM (27–86 years; Fig. 3C)[17,142] revealed that particles had a surface-and-core morphology consistent with lipoproteins.[143,144] Lipoprotein particles also appear in multivesicular bodies in BrM,[17,144–146] and in lines crossing BLamD.[126,127,147,148] Both studies demonstrated three to four rows of densely packed lipoproteins in the subRPE-BL space, logically the direct precursors of BLinD. This formation, first called Lipid Wall, represents preBLinD (Fig. 3C).[143,144] The nondescript fluid phase surrounding particles contain proteins and other components not discernible at these magnifications. We proposed the name "lipoprotein-derived debris"[149] for masses of modified lipoproteins in soft drusen/BLinD (Figs. 3D, 4C, 4D). This debris also appears in basal BLamD (basal mounds)[121,123,150,151] and rarely, within large vacuoles in RPE.[123,126,152] Similar material said to occupy the subretinal space[66,123] is really SDD (see the Subretinal Drusenoid Deposits: Extracellular, Space-Filling, Distinct From Drusen section).

A lipophilic barrier in aged BrM blocking normal, choroid-directed fluid efflux from the RPE was postulated by Bird and Marshall[153] to explain RPE detachments in older adults. A seminal study by Pauleikhoff et al.[16] demonstrated that oil red O binding lipids localized exclusively to BrM of healthy human eyes. This staining was abundant in adults 61 years and older, variably present in midlife adults, and absent in young adults. Direct assay confirmed the age-related increase (although not the initially reported composition).[154,155] Marshall employed BrM explants to explore transport across this tissue.[156–158] Later analysis showed excellent correlation of an age-related increase in resistivity (inverse of hydraulic conductance) with content of hydrophobic EC.[159]

Specific histochemistry and analytic biochemistry combine with gene expression (see the Genetics and Gene Expression Studies Relevant to Lipids section) to support the concept of EC- and linoleate-rich, apoB, apoE-containing, large lipoprotein particles secreted by RPE (Figs. 3E, 3F). The oil red O-binding material is EC, verified by multiple direct assays.[17,97,144,160] EC accumulates markedly in BrM, in 7-fold higher quantities in macula than periphery.[17,18] EC localizes exclusively to BrM whereas UC and PL, also present, additionally localize to nearby cellular membranes.[161] Particles 60 to 80 nm in diameter and with flotation properties and spherical shapes indicating neutral lipid cores are isolable from healthy human BrM.[97,144] In the same fractions are also apolipoproteins B, A-I, and E. BrM lipoproteins are highly EC-enriched relative to TG,[17,97,144,160] unlike hepatic VLDL, of similar diameter. Thus, BrM lipoproteins are large like VLDL and EC-rich like atherogenic LDL. In contrast, the neurosensory retina contains little EC.[17,160]

Lipoproteins are assembled from multiple lipid sources, and fatty acid profiling of EC and other lipid classes in BrM lipoproteins and extracts allowed inferences about the source of this component. Docosahexaenoate (22:n6) is distinctively high in PL of outer segment membranes[162] and neural tissue in general. Yet, high-performance liquid chromatography in two laboratories showed that all lipid classes in BrM are overwhelmingly dominated by the fatty acid linoleate (18:2, most abundant in plasma) with little docosahexaenoate.[97,160] This result suggests that RPE recycles docosahexaenoate back to photoreceptors efficiently, as postulated,[163] and that plasma lipoproteins are the major fatty acid sources to BrM lipids. On the basis of fatty acid composition alone, it is not possible to distinguish BrM lipoproteins from those of plasma origin, in transit to RPE from choriocapillaris. However, BrM lipoprotein composition and gene expression support a local source, because enrichment with EC over TG differs sharply from plasma VLDL, and intracellular gene and protein data (see the Genetics and Gene Expression Studies Relevant to Lipids section) indicate RPE capacity for lipoprotein assembly and secretion.

A long-standing hypothesis[164] states that debris in aging BrM represents outer segment membranes phagocytosed and processed by RPE.[165] Outer segment UC content is notably low[93,166,167] but could be concentrated in bulk phagocytosis by RPE.[150] Figure 5 expands this model by postulating that the fatty acids in this material come largely from diet. BrM lipid deposition (steps 1–2, Fig. 5) is proposed as a recycling system in which plasma lipoproteins delivering dietary essentials are stripped of cargo destined for photoreceptors. Unneeded fatty acids and UC are repackaged with outer segment UC for secretion to BrM and eventual choroidal clearance. One appeal of this model is the specificity for BrM, unlike models involving by-products of other lipids.[168,169] Soft drusen/BLinD form (steps 3–4, Fig. 5) when egress is blocked through aging BrM/ChC, either due to abnormal amounts or types of BrM proteins, loss/dysfunction of ChC, loss of VEGF sustenance to ChC, or all. Aged BrM and subsequent soft drusen/BLinD could act as a transport barrier to large molecular complexes,[170] a source of peroxidizable proinflammatory lipids,[171,172] and part of an increased diffusion distance impeding oxygen exchange.[173]

The Calcific End-Stage of Soft Drusen and Differentiation From Amyloid β

One end-stage of soft drusen is calcification, inferred from glistening fundus appearance[35] and in tissues, refractility,[62,123] von Kossa staining,[128,174,175] and microanalysis.[176] Concentric shells within spherules impart the glistening appearance and a punctate reflectivity on OCT.[175] Spherules less than 1-μm diameter[177] show strong hydroxyapatite signal via microprobe synchrotron x-ray fluorescence and specific dyes,[178,179] and they may enclose other druse components and promote deposit expansion.[179] Nonreflective multilobular nodules

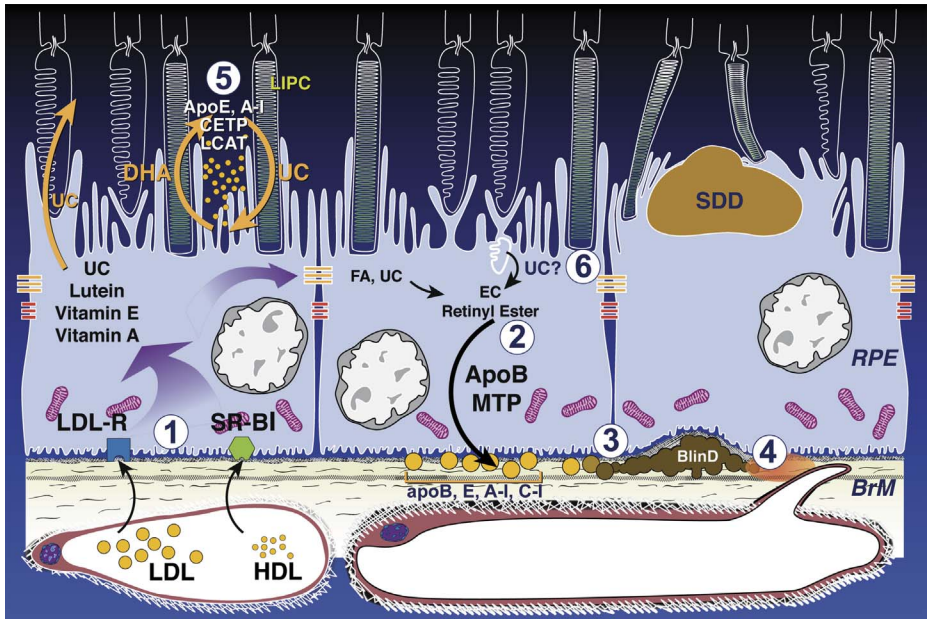

Figure 5. Lipid cycling pathways leading to soft drusen and an atherosclerosis-like progression in the sub-retinal pigment epithelium basal lamina space. BLinD/soft drusen and SDD are localized external and internal to the RPE, respectively. Normal-aging RPE is at the *left* and *center*. AMD is at *right*. Shown are RPE-based lipid recycling pathways for rods and cones that could drive formation of AMD extracellular lesions. (**1**) Plasma LDL and HDL delivering lipophilic essentials, including vitamins E, A, lutein, and cholesterol (UC), enter basolateral RPE via LDL receptor and scavenger receptors BI and BII, respectively. (**2**) ApoB, E lipoproteins secreted basolaterally by RPE (*gold circles*) are assembled from multiple lipid sources. Fatty acids are dominated by linoleate, implicating internalized plasma lipoproteins (from step 1) as a major source, plus UC from all sources esterified to EC. (**3**) Lipoproteins retained by binding to BrM extracellular matrix accumulate throughout adulthood (perhaps in concert with less efficient transport by aged choriocapillaries), creating pre-BLinD between the RPE-BL and the inner collagenous layer of BrM. (**4**) Lipoproteins degrade, fuse, and form lipid pools within BLinD/soft drusen, making them biomechanically fragile, proinflammatory, and cytotoxic. (**5**) Disks in rod OS lose UC and gain docosahexaenoate in transit from OS base to tip (shown as loss of white). OS-derived docosahexaenoate stored as triacylglycerol in RPE after phagocytosis return to OS. The mechanism of transfer is unknown but could be familiar proteins like interphotoreceptor retinoid-binding protein or hypothetical HDL particles cycling between RPE and photoreceptors, especially under rod-rich perifovea, where subretinal drusenoid deposit forms. (**6**) Cone OS maintain high-UC content along their length, because their disks are comb-like projections of plasma membrane. Cone OS UC enters RPE via disk shedding, lysosomal uptake, and acid lipase activity. UC is released for intracellular transfer, esterification, and assembly into basolaterally secreted lipoproteins, especially under cone-rich fovea. Reprinted with permission from Pikuleva IA, Curcio CA. Cholesterol in the retina: the best is yet to come. *Prog Ret Eye Res*. 2014;41:64–89. Copyright © 2014 Elsevier Ltd.

calcific (5–100 μm) within drusen are associated with reduced autofluorescence signal of overlying RPE.[49,177] Hydroxyapatite is also abundant in subRPE deposits created by well-differentiated cultured RPE,[151] emphasizing the importance of basolaterally directed physiologic mineral regulation.

An alternate interpretation stems from the finding of colocalized and spherically distributed activation fragments of complement C3 with amyloid β peptide, a major constituent of Alzheimer disease neuritic plaques[180] in some drusen of some AMD eyes,[181] with staining correlated to overall drusen load per eye.[182] Light and electron microscopy showed concentric shells,[129,182] which were not labeled by antibodies to other amyloids.[183,184] Many proteins bind to hydroxyapatite,[185] which is used in chromatography, raising the possibility that amyloid binding to spherules is nonspecific. Amyloid β peptide was recently found in inner retina of Alzheimer patients, signifying a separate neurodegeneration, distant from drusen.[186]

Other Components of Soft Drusen

Understanding druse composition is considered an important route to discern pathways perturbed in AMD[187] (Supplementary Table S1). ApoE was an early, consistent, and abundant component.[81,135,187,188] Proteomics and immunohistochemistry also revealed vitronectin, complement components, clusterin, ATP synthase subunit beta, scavenger receptor B2, and retinol dehydrogenase.[134,189–191] Oxidatively modified proteins including tissue metalloproteinase inhibitor 3 and vitronectin, and carboxyethyl pyrrole protein adducts also[191] supporting oxidative damage as important in AMD progression.[192]

Many proteins, minerals such as zinc, and carbohydrates can be confidently placed in macular drusen that confer progression risk (Supplementary Table S1). However, it is unclear if these signals are specific to macula, a question of biologic importance.[4] Data comparing macular and peripheral drusen in the same eyes are sparse.[129,193] The macula is 3% of total retinal area,[194] requiring specific measures for its analysis. Many studies assayed peripheral drusen,[134,182] combined macular and peripheral drusen,[191] or did not specify regional source.[189] The apparent synergy of immunohistochemistry with genetic associations implicating complement was largely based on labeling that cannot be definitively placed in the macula. Neither membrane attack complex (terminal element of the complement cascade)[195] nor CD59[196] localized to macular soft drusen. Experimental studies suggest that BrM lipoprotein binding can be modulated by plasma CFH factor H,[197] and genetics implicate a role for *CFH* in soft drusen biogenesis (see the Genetics and Gene Expression Studies Relevant to Lipids section). Continued investigation is warranted.

RPE Lipofuscin — Distinct From Drusen

RPE lipofuscin comprises abundant and long-lasting intracellular inclusion bodies, related to lysosomes, which are rich in

Table 1. Differentiating Soft Drusen From Subretinal Drusenoid Deposit

	Soft Drusen/BLinD	SDD	Reference
Location	Between the RPE-BL and ICL of BrM* (sub-RPE-BL space)	Between RPE and photoreceptors (subretinal space)	66, 122, 123, 225, 226
Proteins	ApoE, vitronectin, CFH; CD59−	ApoE, vitronectin, CFH; CD59+	66, 226
Lipids	Unesterified and esterified cholesterol; oil red O-binding	Unesterified cholesterol; oil red O binding	150, 226, 359
Minerals	Hydroxyapatite	Undetected to date	151, 175, 179, 254
Topography	Follows cones (BLinD)	Follows rods	225, 227, 360
Specificity for AMD	AMD	AMD; inherited diseases of BrM, retinoid transport	60, 123, 126, 361–365
Associated neovascular subtype	Type 1 (subRPE), 2 (subRPE, subretinal)	Type 3 (intraretinal)	228, 366–368

* RPE-BL, basal lamina of the RPE.

bisretinoids (vitamin A derivatives).[198] Appearing in humans in childhood and increasing throughout adulthood, RPE lipofuscin is the principal signal source of fundus autofluorescence imaging. Lipofuscin has been proposed as a source of intermediates in the pathway to age-related glycation products in drusen.[199,200] Evidence included in vitro studies[201] exposing cells to a lipofuscin fluorophore recently found to be less abundant in macula than in periphery.[202–207] Histopathology of human AMD eyes indicate that lipofuscin is present in RPE[40,60,121,123] and rarely in drusen.[129] Further evidence that lipofuscin is not a major source of druse components includes different topographies of lipofuscin (high in perifovea[208,209]) and soft drusen (high in central macula) and different emission spectra of fluorophores in lipofuscin versus soft drusen.[210] Because lipofuscin-attributable autofluorescence is a superb reporter of RPE metabolism that can be combined with OCT for subcellular-level insight in vivo,[211] the biology and role in AMD pathophysiology of RPE lipofuscin remains a research priority.

BLamD - Distinct From Drusen, Important in Druse Biogenesis

BLamD is a distinct deposit meriting its own study (Fig. 1). Continuous subfoveal BLamD is considered diagnostic for AMD, and continuous BLamD in the presence of BLinD is an early AMD threshold.[119,212] BLamD's role besides association with drusen can now be explored in clinical OCT. If RPE is present, BLamD is shadowed and appears hyporeflective.[213] If RPE is absent, BLamD is a moderately reflective line across the atrophic macula.[214,215]

In many older healthy eyes BLamD forms small patches (~5-μm wide) between the basolateral RPE plasma membrane and the RPE-BL. Early (palisade) BLamD is discontinuous, thin, and fibrous. In AMD, continuous BLamD is 15-μm thick or more.[121,150,216] Late BLamD is thick, multilayered, and scalloped on the inner aspect.[123,126,147,214] BLamD ultrastructure resembles basement membrane, containing laminin, fibronectin, type IV, and type VI collagen with 120-nm periodicity,[217–220] as well as vitronectin, matrix metalloproteinase (MMP), metalloproteinase inhibitor 3 (TIMP-3), C3, and C5b-9.[216] Eyes with BLamD also tend to have high drusen loads. BLamD contains lipid-rich particles transiting to BrM[147,150] that aggregate as basal mounds[121] (Fig. 9B[151]; Figs. 3E–H[150]). By retaining lipoproteins en route from RPE to BrM, BLamD may increase exposure time to oxidizing agents that result in proinflammatory, cytotoxic lipids.[221] Some inherited retinopathies exhibit BLamD containing lipid and associate with drusen[30,222] and/or type 1 (subRPE) neovascularization.[147] Other retinopathies lacking drusen also have BLamD[223] suggesting it is a nonspecific RPE stress response with a specific role in AMD.

BLamD and BLinD are often jointly named "basal deposits." This imprecise term (to which this author added[148]) comes from low resolution paraffin and cryosection histology and is unwarranted if epoxy-resin histology or transmission electron microscopy is available. The commendable goal of "determining the origin and pathogenesis of BLamD and BLinD as a route to preventive measures"[127] is best served by high-resolution visualization techniques and precise terminology.

Subretinal Drusenoid Deposits: Extracellular, Space-Filling, Distinct From Drusen

As reviewed,[34] "drusen seen in blue light" reported in 1990[224] were called various names depending on detection technology and patient population,[225] finally settling on reticular pseudodrusen (viewed en face)[65] and SDD (viewed cross-sectionally).[66,67] In 1988 Sarks et al.[123] described by electron microscopy "focal collections of membranous debris"[123] in the subretinal space (see the Soft Drusen, BLinD: Lifelong Physiology, Uncovered by Aging section).[65] In a donor eye, Rudolf et al.[66] described regularly spaced deposits, distinct from photoreceptors and RPE. Definitive histology of clinical cases[122,226] established the presence of extracellular deposits. The association of SDD with atrophy,[227] intraretinal neovascularization,[228] and photoreceptor degeneration[229,230] indicates a place for SDD in the AMD spectrum.[1] Beyond location, SDD differs from soft drusen/BLinD (Table 1) in lipid, protein, and mineral content, specificity for AMD, and association with neovascular subtypes.[34] A histologic survey of AMD donor eyes[225] showing that SDD was thickest in the perifovea, and that soft drusen/BLinD was thickest under the fovea, leading to a novel suggestion that deposits reflect differential physiology of rod and cone photoreceptors, respectively. Hypothesized driving pathways include lipid transport via lipoproteins (Fig. 5) and/or interphotoreceptor retinoid binding protein.[34] A comprehensive understanding of SDD molecular composition is urgently needed.

Proof-of-Concept Via Drusen-in-a-Dish Culture Systems

The BrM lipoproteins that make up soft drusen are thus postulated as dual-source, with fatty acids coming from uptake of plasma lipoproteins and cholesterol coming from outer segments as well (Fig. 5). If diet is an important driver of

constitutive lipid cycling pathways, then cultured RPE cells might generate deposits in vitro with only culture media containing serum (and plasma lipoproteins) and lacking outer segments. Amin et al.[131] demonstrated membranous material between the ARPE-19 cell line and a solid surface in 11 weeks of supplementation with a retinal extract. Recent advances include the use of commercially available culture medium[231] over custom formulations[232] and culture well inserts that allow independent monitoring of apical and basal chambers of polarized cells, essential for parsing druse- and SDD-relevant pathways. In a proof-of-principle study by Johnson et al.,[146] cultured fetal human RPE on 100-μm thick porous supports in a standard medium without retinal supplementation produced particulate deposition of apoE-immunoreactive material within the insert (replicated in Ref. 151).

Using a 10-μm thick polyester membrane that restricted access to the basal compartment to pores crossing the insert, Pilgrim et al.[151] found that beginning at 8-weeks polarized and highly differentiated primary porcine RPE lay down extensive deposits on the insert surface. Deposits were approximately 2-μm thick, extracellular, electron-dense, continuous, and sometime focal, with similar material filling the insert pores. Deposits exhibited histochemical and spectroscopic signatures of soft drusen, including lipid, apoE, and hydroxyapatite. Because cells were polygonal, had good transepithelial resistance, and expressed RPE-specific genes, including MTTP, cells appeared functional. Thus, deposits formed, because egress through the insert was blocked as the pores filled. These data strongly suggest that dietary input is required for druse initiation. Outer segments are not required, although they clearly shape deposit composition in vivo, nor were exogenous stressors. Learning what aspects of culture medium are essential will require selective depletion experiments.

Similarly, Galloway et al.[233] showed that induced pluripotent stem cells (iPS) from patients with inherited retinopathies (see the BLamD - Distinct From Drusen, Important in Druse Biogenesis section) also generate electron-dense deposits between the RPE-BL and culture dish inserts. Deposit was generally sparse and discontinuous and varied according to genotype. Layers of collagen IV and apoE immunoreactivity resembled the RPE-BL and subRPE-BL space in vivo, respectively. Contrary to previous findings,[146] exposure to human serum was not required to initiate deposits but did enrich them with C5b-9 immunoreactivity. Deposit sparseness relative to Pilgrim et al.[151] may be due to withdrawal of fetal bovine serum and/or use of iPS cells at passage 3. The authors concluded that RPE dysfunction leads to deposits in these iPS cells that express mutant genes.[233] However, deposits can be made by wild-type, differentiated porcine RPE.[151]

Culture systems are valuable when functional RPE can be studied in isolation and can demonstrate the minimum requirements for deposit. They offer limited mechanistic insights beyond that, in the absence of choroid and retina. In vivo, the "insert" to which RPE-BL attaches is ICL of BrM. Data suggest that aged BrM/ChC acts as a physical barrier to retain constitutively secreted material that accumulates (as it does in vivo) under the RPE.[142] Neither cell culture studies nor other approaches have addressed whether BrM protein composition, BrM molecular sieving properties, inefficient translocation by aging ChC endothelium, or other factors initiate the binding of lipid in situ.[234–236] Atherosclerosis research may be a source of ideas.[237,238]

How Soft Drusen Lead to Atrophy

AMD natural history is now visualizable at the cellular level with optimized structural eye-tracked OCT imaging.[239] A pathway from soft drusen to subRPE neovascularization, includes gradients of VEGF secretion by stressed RPE, macrophage activity in breaching BrM, invading capillaries that remove or replace friable deposits, and damage to surrounding cells by peroxidized lipids.[192,221,240] Recent data now also strongly implicate drusen as a causative factor in GA, further stimulating interest in targeting drusen to prevent or delay atrophy.

Clinicopathologic correlation,[124] epidemiology,[41,241,242] and clinical observation[243] show that hyperpigmentation is the largest intraocular risk factor for progression after drusen abundance. In OCT, intraretinal hyperreflective foci found overlying drusen[244–247] and appearing frequently in photoreceptor layers[47,248] are correlated with hyperpigmentation on CFP[248,249] and are now attributed to anteriorly migrated RPE.[250] Reflective foci seen by either in vivo or ex vivo OCT could be directly linked to intraretinal RPE by histology[251–256] and distinguished from cells with lipid droplets (presumed microglia or macrophages) in neovascular AMD.[252,255] A high-resolution histology survey of RPE morphology suggested two main pathways of RPE fate.[239] One pathway, apparently apoptotic, comprised the shedding of RPE organelles into underlying BLamD. A second pathway comprised rounding and sloughing of cells into the subretinal space, followed by anterior migration into the neurosensory retina, in coordination with Müller cells and photoreceptors at the external limiting membrane.

Drusen are dynamic, coalescing and disappearing in a manner suggestive of regulated processes.[60,257–260] Over 5 to 7 years, 20% to 34% spontaneously disappear.[261–263] Others disappear after retinal detachment.[91] Drusenoid pigment epithelial detachment (PED; i.e., drusen with >350 μm base diameter) is a defined route to atrophy.[123,264] A PED lifecycle was determined by measuring deposit volume in OCT scans for periods up to 6.6 years.[265] Deposits grew slowly and collapsed quickly, with a legacy of complete RPE and outer retinal atrophy (Fig. 6). Before collapse, the RPE layer thickened at the druse apex, hyperreflective foci appeared vertically above in the retina, and the RPE-BL disintegrated.[254] A similar lifecycle was demonstrated independently for more than 6000 RPE elevations of varying sizes.[266] In some eyes, RPE death/migration leaves a raised line of reflective persistent BLamD across the atrophic area.[215]

These spatiotemporal characteristics together with cell culture studies (see the Proof-of-Concept Via Drusen-in-a-Dish Culture Systems section) clarify how RPE cells die over drusen. If a druse is growing, the RPE is functional enough to secrete druse components, which then back up against the BrM-ChC complex due to slowed clearance.[151] When the druse gets large enough, RPE cells on the apex either migrate or die. Then the druse collapses, because druse component production is discontinued, and clearing processes catch up. It has been thought that as drusen collapse, the RPE dies, but the contrary is true. Further, because drusenoid PED are the largest deposits on a continuum leading to GA, we can judiciously extrapolate to drusen-associated atrophy overall.[267] Interestingly, other clinical studies support this overall model, including drusen over choroidal nevi that compress ChC[59] and diminished ChC flow signal under drusen by OCT angiography.[268]

Anterior migration of RPE suggests attractants from the retina, repellents in the druse, or both. Oxygen tension is reduced by 30% to 50% at druse apices, depending on height.[173,269] RPE atop drusen are maximally distant from the ChC and may migrate to seek oxygen from retinal capillaries. In intraretinal neovascularization (retinal angiomatous proliferation), ectopic RPE cells positive for VEGF immunoreactivity are found immediately adjacent to capillaries.[255,270,271] Further, druse volume is a strong predictor of which individual deposits

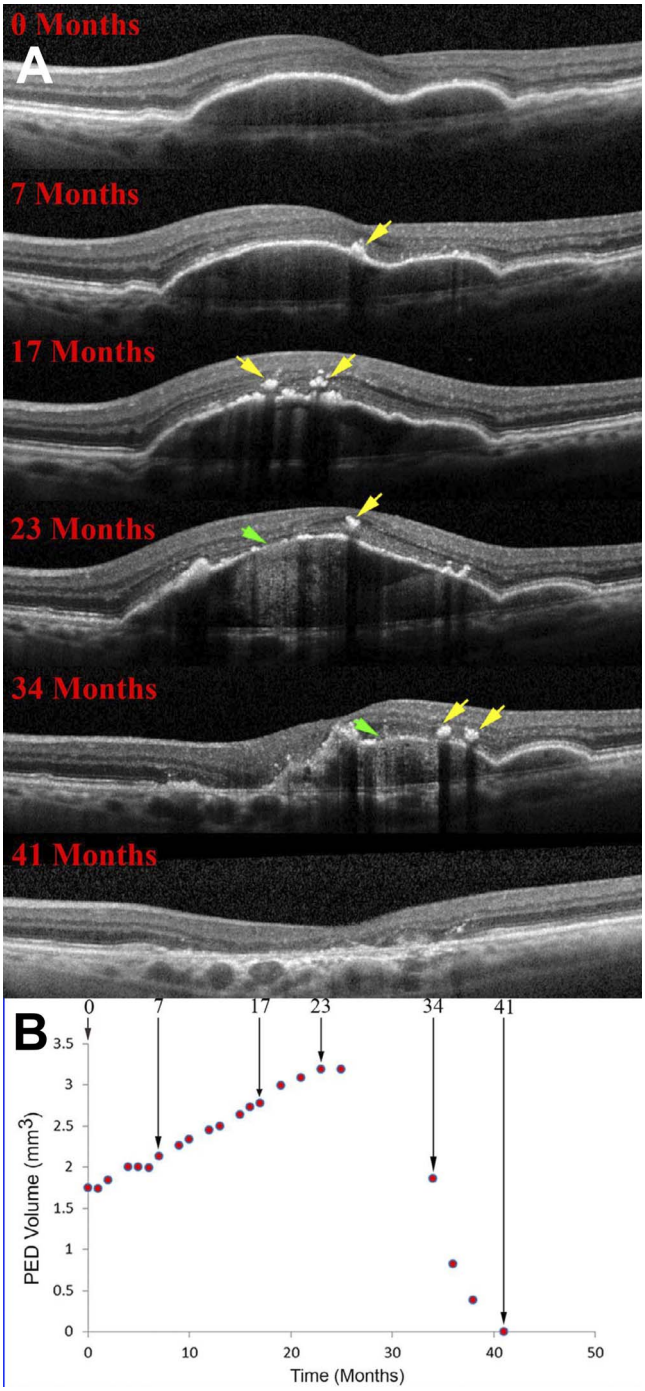

Figure 6. RPE demise linked to the life cycle of drusenoid pigment epithelial detachment (DPED). (**A**) Eye-tracked, spectral-domain OCT, in a 72-year-old patient. Intraretinal hyperreflective foci are first noted at 7 months as localized hyperreflective lesions arising from the RPE-BL band (*yellow arrows*). At 23 months, disruptions to the RPE-BL band (*green arrow*) with increased light transmission (hypertransmission) to the choroid are evident, followed by reduction in DPED volume until 41 months. (**B**) DPED volume increased slowly and declined rapidly in this patient. Modified from Balaratnasingam C, Yannuzzi LA, Curcio CA, et al. Associations between retinal pigment epithelium and drusen volume changes during the lifecycle of large drusenoid pigment epithelial detachments. *Invest Ophthalmol Vis Sci*. 2016;57:5479–5489.

proceed to atrophy.[272] Data can support a model of RPE cell death related to distance from the ChC, in concert with local lipotoxicity,[172] driven by hypoxia, micronutrient deficiency, and bioenergetic failure, with drusen the common underlying mechanism.

Continuing to Learn From Atherosclerotic Cardiovascular Disease

Atherosclerotic cardiovascular disease has been a rich source of molecules, mechanisms, techniques, and inspiration for approaching the biology of soft drusen and new treatments and preventions. Arguably, the several biologic pathways for AMD risk outlined by meta-analyses of GWAS[80,273] align along an atherosclerotic progression.[72]

Table 2 keeps this comparison in perspective by showing that despite many similarities at the level of the vessel wall, from calcific end-stages to extracellular matrix regulation to lipoprotein sources of cholesterol, the top-level biologic risk factors of AMD and cardiovascular disease (e.g., plasma LDL and apoE4 genotype) are dissociated. The evidence that lipid deposition in aging BrM is dictated by needs of outer retinal cells and not an ocular manifestation of systemic perifibrous lipid in human connective tissues and atherosclerosis is compelling. Nevertheless, the commonality of lipoprotein-instigated vascular disease suggests that the many antidyslipidemic agents developed for cardiovascular disease may be intelligently probed for their utility in AMD.

Therapeutic Approaches to Soft Drusen

Our hypotheses motivate the pharmacologic targeting of soft drusen components and antecedent processes to prevent downstream sequelae, a strategy similar to that used for stroke: target vessel walls, so neurons and supporting cells will benefit. We elaborated the Oil Spill strategies (Fig. 7)[33,72]: detoxifying or removing drusen ("Skimmers and Dispersants"), retarding drusen formation by preventing RPE lipoprotein outflow ("Top Kill"), and preventing drusen formation by modulating dietary input ("Bottom Kill").

Statins are widely used inhibitors of 3-hydroxy-3-methylglutaryl-CoA reductase, the rate-limiting enzyme for cholesterol synthesis, that reduce plasma LDL by upregulating LDL receptors throughout the body, especially the liver. Because statins also directly reduce apoB secretion,[274] they are dual-action Top Kill and Bottom Kill, because RPE and RPE-derived cells expresses LDL receptors,[275] secrete apoB, and respond differentially to various statins. [98] Clinical evidence regarding statin efficacy for AMD has been equivocal.[276–278] Retrospective and population-based studies included patients at varying AMD severity levels, used statins of varying lipophilicity, and predated concepts of intraocular cholesterol and lipoprotein homeostasis. Several authors have advocated revisiting statins.[98,279,280]

In a double-masked randomized placebo-controlled proof-of-concept trial by Guymer and associates,[279] 114 normolipemic AMD patients received either simvastatin 40 mg/day or placebo. Patients with bilateral intermediate AMD receiving simvastatin experienced a significant 2-fold decrease in the risk of progression, with no effect seen in unilateral intermediate AMD (advanced AMD in the fellow eye). Further, a single-arm, two-center trial of 80 mg/day atorvastatin reported by Vavvas and associates[281] showed that over 1 year, 10 of 23 patients exhibited marked reduction of large drusen, lack of progression to atrophy and neovascularization, and quiet RPE over druse domes. Three patients dropped out due to side-effects

Investigative Ophthalmology & Visual Science

Table 2. Learning About AMD From Atherosclerotic Cardiovascular Disease

Compare and Contrast* at the Level of the Vessel Wall	
CVD (Arterial Intima, Liver/Intestine)	**AMD (BrM, RPE)**
Calcific, inflammatory, neovascular complications in a vessel wall	Calcification of drusen and BrM, neovascularization types 1-2-3
Toxically modified lipoprotein components	Linoleate hydroperoxide, 7-ketocholesterol
Lipid-rich and biomechanically unstable lesions (necrotic core of plaque)	Soft drusen and basal linear deposit
Stereotypic locations in vasculature	Central macula
Perifibrous lipid – lipoprotein binding to extracellular matrix in sub-endothelial space	Age-related deposition of lipoproteins in BrM
Age-related thickening of sub-endothelial space	Age-related thickening of BrM
Esterified, unesterified, crystalline cholesterol	*Esterified, unesterified cholesterol*
LDL (VLDL remnant) as cholesterol source	*BrM lipoprotein as cholesterol source*
ApoB,E lipoprotein particles	ApoB,E lipoprotein particles
Lipoproteins of hepatocyte, enterocyte origin	*Lipoproteins of RPE origin*
Macrophages are source of foam cells	*Macrophages active in neovascularization, druse clearance (with Müller cells)*
Physiological needs driving lipoprotein production (delivery of fuel, cholesterol, lipophilic vitamins)	Physiological needs driving lipoprotein protein (recycling of unneeded lipids from diet-delivery and outer segment phagocytosis to plasma)
Evolutionary selection of fitness	*Evolutionary selection of acute vision*

Contrast* at the Level of Persons and Populations		
	CVD	AMD
ApoE4 genotype	*Increase risk*	*Decrease risk*
Elevated plasma cholesterol or LDL	*Increase risk*	*Not associated*
Elevated plasma HDL	*Decrease risk*	*± risk*
Diabetes (type 2)	*Increase risk*	*Not associated*
Statin therapy	*Standard of care*	*Under investigation*
Antioxidant therapy	*± effect*	*Standard of care*

Table outlines the limitations of analogizing AMD and CVD.
* Contrast shown in italic.

not uncommon at this dose. Although this study lacked quantification of druse volume and a comparison group to account for the natural history of druse dynamism, results were singular and corroborated the previously seen lack of progression.[279]

A "Skimmers and Dispersants" approach is exemplified by a recent preclinical study of a lipid scavenger.[282] Apolipoprotein (apo) A-I mimetics are short (18 amino acids) synthetic amphipathic helical peptides that emulate the antiatherogenic properties of apoA-I (243 amino acids).[283,284] Amphipathicity allows peptides to sequester lipids and travel through an aqueous environment. Peptide 4F has four phenylalanine residues on the nonpolar face of the helix.[283–289] It is anti-inflammatory, avidly binding oxidized phospholipids and fatty acid hydroperoxides[290] and reducing large-artery atherosclerosis in animal models.[283,291–293] In phase II trials for cardiovascular disease, systemic 4F was tolerated,[294,295] but not advanced, due to uneven absorption after oral administration. 4F's small size and the commonality of lipoprotein-instigated vascular disease in atherosclerosis and AMD made it an excellent candidate for targeting drusen and/or druse precursors. A popular model of atherosclerosis, ApoE$^{-/-}$ mice also exhibit BrM disintegrity, thickening, and EC accumulation at 10 to 11 months.[282] One eye was injected with 4F or a scrambled peptide (0.6, 1.2, 2.4 μg), and the fellow eye served as a control. Transmission electron microscopy and perfringolysin-green fluorescent protein histochemistry showed at all doses that BrM ultrastructure improved and EC was reduced.[296] Animals receiving 4F tagged with a fluorescent tracer exhibited fluorescence at 1 day postinjection in BrM, remaining for at least 14 days, while replenished from neurosensory retina. Many questions remain, including effects on plasma inflammatory markers, precise lipids removed, safety profile, and effects on retinal function.[17,143] Despite limitations, this study demonstrated a tolerated and effective pharmacologic reduction of BrM lipids from mice.[126,129] Because soft drusen are extracellular and loosely packed,[126,129] surface-active agents like 4F offer advantages.

Unlike targeting extracellular drusen, other lipid-based approaches involve intracellular RPE lipid. For example, the offloading of cellular cholesterol to circulating HDL via LXR

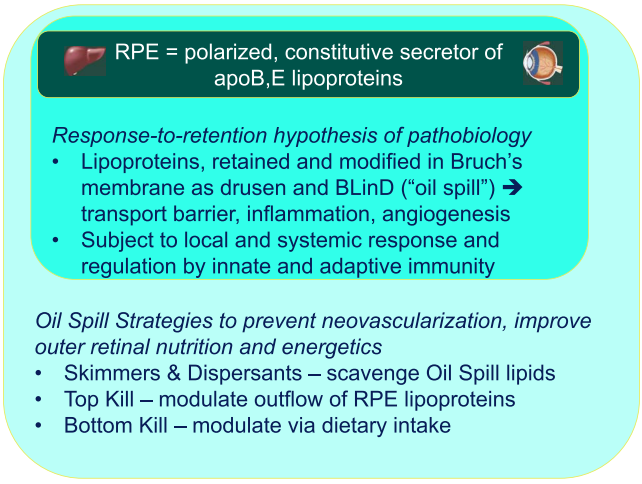

Figure 7. Interlocking Oil Spill strategies for AMD. The RPE is a polarized and constitutive secretor of lipoproteins bearing apolipoproteins B and E (and likely others). As such it fills a role like liver in atherosclerosis, with the role of arterial intima played by BrM.

agonists[297] may reduce substrate available for apoB lipidation and druse biogenesis. Another approach is to stimulating RPE uptake of lipids, presumably to clear drusen (e.g., via the CD36 scavenger receptor).[298] It is instructive to recall that among agents modulating VLDL for cardiovascular disease, the lipid content of source cells was a less fruitful target than impacting the vessel wall through plasma lipid-lowering. Indeed, inhibitors of hepatic microsomal triglyceride transfer protein (MTTP) and acyl cholesterol acyltransferase-1 caused steatosis (fatty liver).[108,299–302] Investigators should thus check for RPE lipoidal degeneration, a steatosis-like intracellular accumulation of lipid droplets associated with depressed electroretinograms.[303–306]

A major roadblock to clinical trials of drugs targeting drusen is nonavailability of approved and appropriate endpoints, although candidates exist. Visual acuity can remain good until late AMD. Rod-mediated dark adaptation and low-luminance visual acuity are sensitive to early disease stages but take time to administer.[307–309] The only imaging endpoint currently approved by regulatory authorities is slowed expansion of GA viewed with fundus autofluorescence, a bar which several agents failed to meet.[310–313] It is possible that GA is too late for intervention, because photoreceptor degeneration and gliosis are already severe.[123,314–318] A earlier-stage surrogate endpoint is druse volume,[261] in the causal pathway to progression and readily calculable from OCT scans.[319] Another potential surrogate is hyperreflective foci over drusen (migrating RPE[254]), a risk factor for atrophy[47,51,320] that can be quantified.[272]

Model Systems for Mechanistic and Translational Drusen Research

Monkeys: Drusen Without Progression

Monkeys in closed colonies have strong matrilines that vary in the degree of AMD pathology.[321] They share with humans AMD susceptibility genes[322] and plasma hyperlipidemias.[323] To date, monkeys have not exhibited neovascularization, GA, BLamD, SDD, migratory RPE, or drusen with internal structure visible on OCT,[306] all typical for human AMD; this possibly reflects a controlled environment and diet. Yet some monkeys appear to have soft drusen and requisite Oil Spill biology. Drusen with "membranous debris" and lipoprotein-like particles in BrM were demonstrated by electron microscopy.[303] BrM exhibits both oil red O and filipin staining for EC.[305] A large study ($n = 60$ eyes, 2- to 26-years old) showed an age-related increase in immunoreactivity for 7-ketocholesterol, an oxidation product of UC,[172] that was selective for RPE-choroid. Like humans, monkey drusen contain apoE[324] and carbohydrates,[325] and BrM has entrapment sites (i.e., upward swellings of the ICL). Unlike humans, monkeys have cellular processes evaginated from RPE.[40,326]

Mouse Models Capture Some Pathways Well

Mice are experimentally advantageous yet exhibit only some AMD-relevant biology. Relative to humans and nonhuman primates, mice differ in the following ways: they are nocturnal; lack an all-cone fovea, foveal pit, centrifugal displacement of photoreceptor terminals, and long Henle fibers; have small cones and densely packed outer segments; naturally lack xanthophyll pigment; exhibit subretinal microglia in aging and in retinal degenerations; have a uniform distribution of bisretinoid A2E in RPE; have panretinal multinucleate RPE (versus in Ref. 327); frequently have vacuolated RPE in retinopathy (versus in Ref. 251); and do not express *CETP* and thus transport cholesterol in plasma HDL rather than in LDL. Nevertheless, components of AMD can be studied to great effect in genetically engineered mice. Several models exhibit BLamD, sometimes containing lipid,[223,328–332] suggesting that mice have apolipoprotein pathways but normally lack a retentive matrix. Other mouse strains have EC in BrM,[99,296,333] activated RPE,[334,335] spontaneous intraretinal neovascularization,[336,337] and xanthophyll accumulation.[338] Because AMD-risk genes like *APOE* are expressed in several outer retinal cell types, technologies for cell-specific knockouts will be especially informative.[339,340]

It is agreed that mice lack drusen (hard or soft).[197,341] Several studies claimed drusen in mice[342–346] but did not meet the Sarks standard of ultrastructurally confirmed focal, extracellular, subRPE-BL material, correlated to fundus appearance. Regularly spaced microglia appear in the subretinal space of several aging mouse strains,[347–349] and correlate to neither human drusen nor SDD.[226,350] Precision in specifying layer and ultrastructural findings in animal models and benchmarking against human pathology will accelerate progress on AMD.

Cell Culture Systems are Standardizing

Several RPE culture systems produce druse-relevant deposits,[146,151,233] with one recreating a continuous deposit.[151] Confluent and polygonal with sharp vertices, healthy RPE in vivo maintains the physiologic blood–retina barrier and exercises distinct roles vis à vis photoreceptors and the choroid. Due to AMD deposits of differing composition in subretinal and subRPE-BL compartments, polarity is more essential than ever for RPE culture systems. Many protocols exist for high-fidelity native and engineered RPE in culture, emphasizing properties of the intact layer, particularly high transepithelial resistance ($\geq$250 MΩ).[77,146,231,232,351–355] Of current interest, cell-based therapies are raising expectations for all RPE culture systems.[356] Studies using nonconfluent cells, cells with low transepithelial resistance, and high-passage cell lines, such as human-derived ARPE-19, should be interpreted cautiously. Ideally cultured RPE should be characterized for polarity, barrier function, cytoskeletal precision, and expression of RPE-specific genes.

Final Thoughts

Soft drusen are a very prominent intraocular risk factor that are seen routinely in vivo. Yet the true impact of soft drusen on AMD progression will be better understood when all the layers in Figure 1 can be followed clinically and their contribution to risk assessed. In less than decade thanks to OCT, SDD went from invisible to a major contributor to retinal dysfunction. Now the participation of BLamD is also becoming known. We anticipate a day when BLinD is visible clinically and its risk assessed along with drusen. In 2007 the Sarks et al.[121] staged eyes by the presence of 'membranous debris'. Arguably some of AMD's infamous heterogeneity is because this specific pathology cannot be directly followed in the clinic. Thus, presentations currently attributed to individual variability may be consequences of invisible BLinD (or other invisible deposits). For example, Asian populations prone to neovascularization without many drusen may have BLinD that escapes detection. Fortunately, imaging technologies with promise of revealing BLinD are emerging.[210,357] There is still much to learn about the biology of soft drusen, in the clinic and in the laboratory. Nevertheless, current knowledge can motivate targeting these deposits and contributory biologic processes, to delay or avoid AMD's sight-robbing late stages.

Limitations to this analysis are sparse experimental confirmation of hypotheses largely generated from human tissues and

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patients. Our hypotheses, while speculative, bring together many evidence lines and do not exclude other major extant hypotheses for AMD biology and may in fact occur in parallel.

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