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Lipids, Oxidized Lipids, Oxidation-specific Epitopes, and Age-related Macular Degeneration

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Abstract

Age-related Macular Degeneration (AMD) is the leading cause of blindness among the elderly in western societies. While antioxidant micronutrient treatment is available for intermediate non-neovascular disease, and effective anti-vascular endothelial growth factor treatment is available for neovascular disease, treatment for early AMD is lacking due to an incomplete understanding of the early molecular events. The role of lipids, which accumulate in the macula, and their oxidation, has emerged as an important factor in disease development. These oxidized lipids can either directly contribute to tissue injury or react with amine on proteins to form oxidation-specific epitopes, which can induce an innate immune response. If inadequately neutralized, the inflammatory response from these epitopes can incite tissue injury during disease development. This review explores how the accumulation of lipids, their oxidation, and the ensuing inflammatory response might contribute to the pathogenesis of AMD.

Keywords

Age-related macular degeneration; Bruch's membrane; basal deposits; drusen; low-density lipoprotein (LDL); oxidation-specific epitopes; retinal pigmented epithelium (RPE)

Introduction

Age-related macular degeneration (AMD) is the leading cause of vision loss among the elderly in western societies[1]. Today, over 1.75 million people in the United States have advanced AMD, and as the "baby boomers" age, it is estimated that 3 million people will be affected by 2020[2]. While generally afflicting people over 60 years old[3], 7 million people are currently at risk of developing advanced AMD, and 1 in 3 people over 70 years old with early AMD will develop advanced disease over the next decade[3, 4]. AMD is a major public health problem that costs $30 billion annually in the United States[5], and is predicted to rise to $59 billion over the next 20 years[6]. The impact of AMD on an individual's

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quality of life is unexpectedly high. The decrease in quality of life from early AMD is similar to a person with symptomatic HIV, and with advanced AMD, to one with metastatic prostate cancer having uncontrollable pain[7]. With vision loss, a person with AMD is less active[8] and is at higher risk of depression[9, 10] and anxiety[11] than unaffected elderly people. Because there is no therapy that restores vision, individuals with early AMD are as anxious over losing vision as patients who are actually blind[11].

AMD is a multifactorial, chronic, age-related disease that is influenced by both environmental and genetic factors. Oxidative stress has long been considered a major influence on AMD pathophysiology. A decade ago, the discovery of polymorphisms in complement factor H (CFH) with AMD risk introduced innate immunity as a pathophysiologic factor. Since this discovery, multiple other components of innate immunity have been implicated in AMD pathophysiology. One consequence of oxidative stress is the formation of oxidized lipids, which can elicit an immune response. Inadequate neutralization of the oxidized lipids can transform this initially protective response into a pathologic reaction that induces tissue injury. In this review, we will describe how oxidized lipids and innate immune dysfunction contribute to AMD pathophysiology.

What is the Macula and what is AMD?

The macula is the central retina that is designed for high acuity and color vision. It is composed of the neurosensory retina, the retinal pigmented epithelium (RPE), Bruch's membrane, and choroid. While the clinician defines the macula as the central 6 mm diameter area located between the major retinal vascular arcades (Figure 1A), the anatomist defines the macula as the retina that contains more than one layer of ganglion cell nuclei (Figure 1B) [12]. The neurosensory retina is composed of the internal limiting membrane, nerve fiber layer, ganglion cell layer, inner plexiform layer, inner nuclear layer, outer plexiform layer, and photoreceptors. In communication with the photoreceptors, the RPE is a highly specialized, polarized cell that maintains the health of the photoreceptors. The RPE has multiple essential functions including the daily phagocytosis of photoreceptor outer segments, light absorption, heat exchange, vitamin A metabolism, outer blood retinal barrier, and maintenance of the choriocapillaris[13]. The RPE is adherent to Bruch's membrane (BrM), a specialized, pentalaminar extracellular matrix composed of the RPE basement membrane, inner collagenous layer, elastic layer, outer collagenous layer, and the choriocapillaris basement membrane[14].

AMD is categorized into two forms, a non-neovascular, "dry" form and a neovascular, "wet" form. In wet AMD, neovascular tufts grow in the subretinal space, within Bruch's membrane, and/or the sub-RPE space. These vessels can hemorrhage or leak fluid, or develop into fibrovascular complexes, and cause rapid decrease in vision. With the dry form, vision loss is typically gradual. To the clinician, the hallmark signs are drusen, or yellowish subRPE deposits, and RPE hyper- or hypopigmentary abnormalities (Figure 2). The pigmentary abnormalities are the clinical manifestation of RPE degeneration, which ultimately culminates in cell death[15–17]. Confluent patches of RPE cell loss results in areas of "geographic atrophy", a late form of dry AMD. When the geographic atrophy involves the foveola, vision loss is severe.

Besides drusen, deposits that form in the subretinal space between the photoreceptors and RPE are an integral component of the AMD pathology. Identified with little recognition by Mimoun et al. in 1990[18], reticular pseudodrusen were first clinically observed in AMD. Zweifel et al. showed that these deposits, which Mimoun et al. had hypothesized were derived from the choroid, were instead located in the subretinal space using optical coherence tomography (OCT)[19]. This clinical observation was confirmed by histopathological analysis showing subretinal deposits that contained lipoprotein particles and debris in autopsy eyes[20, 21]. These deposits have long been ignored, in part, because they can be challenging to visualize on clinical exam. These lesions are now within the mainstream assessment of clinicians due to improvements in imaging modalities such as OCT and fundus autofluorescence, and heightened awareness that these lesions are a marker for disease advancement[19, 22–25].

Histopathologically, the cardinal changes of AMD are RPE degeneration and extracellular deposits on either side of the RPE. The normal cuboidal RPE morphology becomes irregularly shaped, then flattened or atrophic, and finally dies[26–28]. These changes are reminiscent of epithelial-mesenchymal transition (EMT) where a cell dedifferentiates to survive a stressful microenvironment at the expense of losing function. In AMD, the RPE displays key features of “type 2” EMT, which is associated with tissue regeneration or repair[29], and includes: **i)** initiation by tissue injury or inflammation; **ii)** loss of cell polarity; **iii)** loss of cell-cell adhesion; and **iv)** a gain of cell migration. In particular, the RPE degenerates from a single nucleated hexagonal cell to a multinucleated, larger hexagonal cell, and then to a flattened cell that either “subducts into the sub-RPE or migrates to the subretinal space[17, 30, 31].

Significant changes are also seen in Bruch’s membrane. Sarks categorized AMD severity after thorough clinical and pathological examination on eyes over a wide age range[28]. The analysis focused on basal deposit formation, or the accumulation of heterogeneous debris within Bruch’s membrane. The composition and location define the extent that basal deposits are associated with aging or AMD. With aging, Bruch’s membrane thickens, mainly due to accumulations in the outer collagenous layer, which begin early in life[32]. Basal laminar deposits (BlamD) accumulate between the RPE cell and its basement membrane while basal linear deposits (BlinD) are heterogeneous deposits within the inner collagenous layer (Figure 3). BlamD accumulate with aging, and are not associated with AMD when they are thin and homogeneous in composition. They look identical to the RPE basement membrane on electron microscopy, and consist in part, of normal RPE basement membrane molecules such as collagen IV, laminin, and heparan sulfate proteoglycans[33]. In early AMD, BlamDs are thin and continuous, and associated with RPE pigmentary disturbance. With AMD, BlamD thicken and contain heterogeneous debris including long spacing collagen, inflammatory debris, and lipids. In AMD, Bruch’s membrane also accumulates BlinD and large drusen (>125microns in diameter)[34–36], which constitute different morphologic forms of the same lesion (Figure 3)[34, 36–38].

Genetic risk factors in AMD

Genetic variants in a number of genes are associated with AMD risk, and as a result, they have revolutionized how we think about AMD. The most compelling linkage has been identified at 1q25–32 and 10q26[39–41]. Patients who are homozygous for single nucleotide polymorphisms (SNP) in CFH on 1q32 have a 6.32 odds ratio risk of AMD[42–45]. Genome-wide association studies (GWAS) have also identified the chromosome 10q26 susceptibility locus that contains ARMS2 (age-related maculopathy susceptibility 2) and HTRA1, a serine protease, which carry similar risk as the CFH variant[46]. Just as the discovery of CFH polymorphisms has provided an unexpected role for complement activation into its pathophysiology, genetic links of lipid related genes may provide further insights into how lipid biology influences AMD. GWAS studies have identified risk variants in genes involved in lipid metabolism and the transfer of lipids among lipoproteins, such as ABCA1, LIPC, CETP, and LPL, and apolipoprotein E4 (APOE4), which has a protective effect[47, 48].

Epidemiologic Risk Factors in AMD

The incidence of AMD is approximately 3% per year for patients over 65 years old[49, 50]. A variety of non-genetic or environmental factors contribute to AMD risk[51]. Some risk factors are not modifiable, such as advancing age or gender[51, 52].Chronological age is the strongest risk factor for AMD while cigarette smoking is the second strongest, but modifiable risk factor[53]. Epidemiologic data from several large studies indicate that smoking influences both the onset and progression of dry AMD[53–56]. The smoking “pack-years” strongly correlates with AMD risk while smoking cessation reduces the risk[54]. In the Blue Mountains Eye study, the RPE was found to be a specific target of cigarette smoke as RPE abnormalities were a prominent change in smokers[57]. In the Age-related Eye Disease Study (AREDS), smoking was correlated with geographic atrophy[58]. A number of other factors have been found to influence AMD, including diet, high body mass index, cardiovascular disease, hypertension, nutrition, and sunlight exposure [51, 52, 56].

Epidemiologic studies have also evaluated the role of serum lipids in AMD. Thus far, the data supporting a role for serum lipids with AMD risk have been mixed. Prior studies have shown either no or an inverse association of HDL cholesterol and total cholesterol with AMD[50, 53, 59–69]. Alternatively, the Age, Gender/Environment Susceptibility Study (AGES) and Rotterdam Study found that elevated plasma HDL cholesterol, but not total cholesterol, low-density lipoprotein cholesterol, and triglycerides were associated with incidence of AMD[49, 62], and the Beaver Dam Eye Study found that HDL cholesterol was associated with geographic atrophy[50]. The ambiguous association of serum cholesterol with AMD risk might be explained by the local production of lipoproteins in the eye, which accumulate in Bruch’s membrane rather than a systemic origin, as discussed the next section, “Role for Lipids in AMD”.

As an alternative to serum cholesterol, omega-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFAs), including eicosapentaenoic acid (EPA) and docohexanoic acid (DHA), have

been investigated as biomarkers of AMD. DHA in particular, has important structural and protective functions in the retina, especially in photoreceptors[70]. Since photoreceptor cell death is a fundamental change in AMD, to determine what is released during retinal injury and identify molecular biomarkers for AMD, Orban et al. induced photoreceptor and RPE degeneration using light exposure in ABCA4$^{-/-}$RD8$^{-/-}$ and wild type mice[71]. Serum DHA levels were significantly decreased after light exposure in both experimental groups. This finding prompted an evaluation in patients where serum DHA was also found to be decreased in AMD (n=24) relative to control patients (n=21). The suggestion of omega 3 fatty acids as a biomarker from this small cohort is supported by Merle et al. who showed that reduced red blood cell membrane EPA and serum EPA-DHA, as long-term biomarkers of n-3 dietary PUFA status were associated strongly with neovascular AMD[72]. The role that decreased PUFAs have in AMD pathophysiology requires further study because in two prospective trials, the Nutritional AMD Treatment 2 Study and the Age-related Eye Disease Study 2, dietary PUFAs had no effect on the progression of early to late stage AMD[73, 74].

Oxidized lipids offer a potential biomarker for AMD. In particular, carboxyethylpyrrole (CEP) is an oxidative protein modification generated from DHA-containing phospholipids, which are highly abundant in photoreceptors. As a result, CEP is a unique oxidized lipid originating from photoreceptors that could serve as an AMD biomarker. Indeed, CEP has been identified in photoreceptors, drusen, and choroid from patients with AMD, and is elevated in serum of AMD patients compared to age-matched control patients[75, 76]. The odds ratio for elevated CEP was more than 3-fold greater in AMD than in control patients[77]. When genotype is included in the model, the AMD risk for patients with elevated CEP and risk genotypes, especially ARMS2 and HTRA1, was 2–3-fold greater than the risk based on genotype alone.

Role for Lipids in AMD

Lipid particles, predominantly composed of cholesteryl esters, cholesterol, and phosphatidylcholine[78] in apolipoprotein B containing lipoproteins, accumulate in Bruch's membrane prior to the development and in the same location as BlamD, BlinD, and drusen. The most current theory, as described by Christine Curcio, PhD[79], suggests that the RPE synthesizes and secretes very low density-like lipoproteins, which has also been observed by our laboratory[80, 81], rather than originating from the systemic circulation. These lipoproteins transit from the RPE through Bruch's membrane in linear tracks[82] (Figure 3). Due to aging related changes to the elastic layer in Bruch's membrane, lipoproteins first accumulate in the inner collagenous layer adjacent to the elastic layer, and then toward the RPE, eventually forming a "lipid wall". The lipid wall prevents the normal passage of essential nutrients and oxygen as well as potentially toxic metabolites from passing between the RPE and choriocapillaris. In addition, heterogeneous material and inflammatory debris accumulates to thicken the BlamD, and with this accumulation and thickening, BlamD become histopathologically associated with AMD[34].

In addition to lipoprotein secretion, impaired reverse cholesterol transport in macrophages can impact drusen formation. In AMD, macrophages accumulate around drusen[83]. While it unclear whether these macrophages are protective or involved in AMD pathology, the

Apte lab has elegantly shown that the cholesterol efflux regulators ABCA1 and ABCG1 are downregulated in macrophages with aging in both mice and humans, and in neovascular AMD[84]. Since macrophages likely transport cholesterol from drusen to high density lipoproteins (HDL) for removal into the systemic circulation as a protective response, the decline in cholesterol efflux regulators could result in accumulation of cholesterol either in macrophages or drusen. When cholesterol accumulates in macrophages, the macrophages switch from a predominantly pro-inflammatory M1 to pro-angiogenic M2 phenotype, which might help promote the conversion from dry to neovascular AMD. In support of these mechanistic data, ABCA1 variations are related with risk of intermediate AMD, which is characterized by lipid rich soft drusen, and advanced AMD[85]. The combination of apoB100 lipoprotein accumulation in Bruch's membrane along with impaired reverse cholesterol transport from a decline in cholesterol efflux transporters in macrophages results in a net accumulation of drusen.

Although less well characterized, reticular pseudodrusen or subretinal drusenoid deposits (SDD) have been found to contain unesterified cholesterol, apolipoprotein E, vitronectin, and complement[20, 86, 87]. The presence of apoE and cholesterol suggests that HDLs are involved in the SDD process, and that they are distinct from and evolve by a different process than basal deposits and drusen. Recently, Greferath et al. found that SDD extend from the subretinal space through the outer nuclear layer, and even beyond the outer limiting membrane, and contained photoreceptor associated proteins and IBA-1 labeled immune cells. They also found through cellular analysis that SDDs were associated with photoreceptor disruption and loss, localized gliosis, and dysmorphic RPE[88].

Pikuleva and Curcio have proposed a two lesion, two compartment model to explain the formation of basal deposits/drusen and SDDs[82]. The theory relies on the differences in lipid composition and topographical location of cones and rods. While cones are concentrated in the central macula, rods occupy both the perifovea and the peripheral retina[89]. Relative to cones, the apex of rod outer segments are cholesterol poor. In the macula where BlinD develop, in addition to endogenous cholesterol synthesis by the RPE and delivery of lipoproteins from the plasma, the phagocytosis of cones provides an additional source of cholesterol that the RPE must process, and the cholesterol is secreted in very low density like lipoproteins into Bruch's membrane to form the nidus around which BlamD, BlinD, and drusen form. On the other hand, SDDs form in rod-rich areas, such as the perifovea. Rod cell membranes contain a cholesterol gradient with the least cholesterol content in the distal outer segments. The photoreceptors and Muller cells have an active bidirectional transport with the RPE for cholesterol and polyunsaturated fatty acids[90, 91]. Since HDLs cycle between the RPE and photoreceptors in the interphotoreceptor matrix, it is plausible that HDLs accumulate the released cholesterol by rod outer segments in the subretinal space as part of this lipid transport system. With RPE dysfunction, lipid transport system impairment could lead to the accumulation of HDLs as the nidus for SDD formation in the subretinal space. In addition to apoE and unesterified cholesterol, a component of HDLs, SDDs contain CD59, CFH, and vitronectin, which suggests that complement activation plays a role in SDD formation[20, 86, 87]. Thus, it is possible that the materials in SDD are themselves toxic or incite an inflammatory response that appears to include complement activation. With accumulation of SDDs, the normal movement of nutrients

including oxygen from the choriocapillaris to photoreceptor outer segments is compromised, ultimately contributing to outer retinal atrophy.

Role of oxidative stress in AMD

The macula lives in a high oxidative stress environment due to a number of sources of reactive oxygen species (ROS), as reviewed in[92]. Due to the high energy demands of vision, the macula has a high metabolic rate that generates high levels of ROS. To meet these high energy demands, the macula receives some of the highest blood flows in the body, and as a result, the RPE is exposed to high ambient (70–90 mm Hg) oxygen partial pressures[92]. Remarkably, the RPE routinely phagocytizes 30,000 photoreceptor outer segments per day[93]. During this process, intracellular H_2O_2 resulting from NADPH oxidase in the phagosome or β-oxidation of rod outer segment lipids in peroxisomes is generated at levels that are similar to macrophages undergoing phagocytosis[94, 95]. Photo-oxidative stress is a unique, constant source of exogenous oxidative stress. The Chesapeake Bay study on watermen provided epidemiologic evidence of sunlight exposure with AMD risk[96, 97] and the Alienor Study suggested that ultraviolet light exposure is a risk for early AMD [98]. Photo-oxidative stress is linked with oxidative damage to the retina, RPE, and choroid, and multiple other works have substantiated this observation as reviewed in[99].

Cigarette smoking adds to the oxidative stress burden for patients who choose to smoke. Conceptually, it is not difficult to understand why smoking raises oxidative stress because cigarette smoke contains nearly 5000 toxins, many of which are strong oxidants[100, 101], and each puff of cigarette smoke contains 10^{15} free radicals[102]. Which or how many of these oxidants actually reach the eye, or how much oxidative stress is generated from a smoke induced inflammatory response is unknown. However, it is clear that cigarette smoke depletes tissues of ascorbic acid and protein sulfhydryl groups, causing the oxidation of DNA, lipids and proteins[103–105]. Many of these molecular changes such as malondialdehyde (MDA), 4-hydroxynonenal (4-HNE), and advanced glycation endproducts (AGE), have been identified in AMD, and suggest that oxidative damage is an important factor in the mechanism of disease development.

The AREDS enrolled nearly 5000 patients to evaluate the effectiveness of antioxidant micronutrients including β-carotene, vitamin C, vitamin E, and zinc[58]. Patients with intermediate, dry AMD who took this formulation had a 25% reduction in progression to advanced AMD. Moriarty-Craige et al., using the AREDS cohort, showed that patients on the AREDS formulation had improved plasma redox potential with increased cysteine/cystine despite no change in the glutathione/glutathione disulfide [106]. Since cysteine helps to regulate apoptosis and immune function, the benefit of the antioxidant treatment on progression could be partially explained by improved cysteine availability. Collectively, the AREDS data suggest that neutralizing oxidative stress with antioxidant micronutrients have a role in treating AMD.

The impaired antioxidant response in AMD

The accumulation of oxidatively damaged tissue indicates that the antioxidant response is insufficient Cells have evolved multiple strategies to neutralize harmful levels of ROS, and at the same time, allow for physiological ROS signaling. These strategies include restricting oxidant producing enzymes to a specific locale near the intended target or the entry of oxidants, such as H_2O_2, through aquaporin channels[107]. The short half-life of ROS also confines any potential spread of oxidative damage to a specific location.

A number of antioxidant systems are regulated through various transcription factors including Nf-κB, AP1, the FoxO family, or PGC-1α. However, the most comprehensive transcription system is mediated through Nuclear factor erythroid-2 related factor 2 (Nrf2), a basic leucine zipper transcription factor[108]. Nrf2 regulates a coordinated transcriptional program that maintains cellular redox homeostasis and protects the cell from oxidative injury[101, 109, 110]. Nrf2 is normally sequestered in the cytosol by Kelch-like ECH-associated protein 1 (Keap1)[111–114]. In the absence of acute stress, Keap1 constitutively suppresses Nrf2 signaling by both degrading Nrf2 through the ubiquitin-proteasomal degradation, which results in a baseline, low antioxidant gene expression[109]. However, upon an acute rise in ROS, Keap1 undergoes a conformational change after multiple cysteine residues interact with ROS, releasing Nrf2 for translocation to the nucleus where it dimerizes with Maf proteins, and binds to the antioxidant response element (ARE) in the promoters of its target genes to initiate the transcription[115–117]. Nrf2 regulates both an early acute phase through induction of “direct” antioxidant enzymes, such as catalase or SOD, and through maintenance of cellular glutathione and thioredoxin, and xenobiotic metabolism enzymes that produce reducing equivalents[118]. When ROS depletes cellular glutathione, cells can die from oxidatively mediated apoptosis[119–121].

Aging can reduce Nrf2 mRNA or protein, which impairs Nrf2 signaling. Suzuki et al. showed an age-dependent decline in Nrf2 from cigarette smoking[122]. These results have been corroborated in mice, where aging suppressed the ability of Nrf2 and its target genes in alveolar macrophages, in response to cigarette smoking[122]. We previously showed that aging impairs the Nrf2 response to acute oxidative stress in mice[123]. While the RPE of young mice elicits a robust induction of Nrf2 mediated antioxidants after exposure to the chemical oxidant sodium iodate, the RPE of old mice had a blunted antioxidant response with accumulation of MDA. MDA damage could be reversed by genetic rescue of Nrf2 signaling by conditionally knocking down Keap1.

Nrf2 also declines with disease. Nrf2 mRNA and protein, and Nrf2-dependent antioxidants and glutathione levels are reduced, and oxidative stress is increased in human emphysematous compared to normal lungs[122, 124] as well as Nrf2 deficient mice, which develop emphysema after chronic cigarette smoke exposure[101]. We have provided evidence that Nrf2 is decreased in AMD[125]. In AMD maculas, Nrf2 immunolabeling was increased with nuclear labeling in morphologically normal RPE compared to the RPE of non-AMD maculas, which suggests that Nrf2 signaling is induced by the high oxidative stress microenvironment of the AMD macula. However, in dysmorphic RPE cells from AMD maculas, Nrf2 immunolabeling was decreased relative to morphologically normal

RPE cells. This finding suggests that in AMD as in other tissues, when the antioxidant system fails, the delicate balance between physiological oxidative stress and the onset of pathological oxidative stress is upset, resulting in tissue damage, as diagramed in Figure 4.

The ocular “response to retention” hypothesis

The accumulation of lipoproteins in Bruch’s membrane is reminiscent of the “response to retention” hypothesis of atherosclerosis, which proposes that plasma lipoproteins accumulate in the arterial subendothelial space, to initiate a cascade of inflammatory events leading to plaque formation[126, 127]. The accumulation of cholesterol and lipids in Bruch’s membrane is a necessary step in basal deposit and drusen formation, but it is not sufficient. Our lab investigated the role of lipoprotein deposition in Bruch’s membrane in a mouse model. A single APOB gene encodes the apoB100 and apoB48 isoforms. In humans, apoB48 is synthesized in the small intestines and is present in chylomicrons while apoB100 is mainly produced by the liver. In contrast, apoB48 is the major apoB form expressed in all tissues of mice. To simulate apoB100 lipoprotein production in humans, “apoB100” mice that have a mutation in codon 2153 corresponding to the apo-B48 editing codon, which reduces the translation of apoB48 so that apoB100 is predominantly produced, have been used for study of atherosclerosis[128]. We showed that these mice accumulate apoB100 lipoproteins in Bruch’s membrane as early as two months of age[81]. However, when aged to 18 months, these mice do not develop basal deposits or drusen, suggesting that the accumulation of lipoproteins is not sufficient for basal deposit formation. Part of the ocular response to retention theory relies on an inciting agent that will generate an innate immune response with the accumulation of inflammatory debris to form BlinD. Oxidized lipids are an obvious choice given the high oxidative stress environment, the reduced antioxidant capability with aging, and that oxidized lipids induce a pro-inflammatory response. Polyunsaturated fatty acids are in particular, vulnerable to oxidation, which results in highly reactive degradative products such as MDA, 4-HNE, and core aldehyde, 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphocholine (POVPC)[129]. Indeed, multiple oxidized lipids have been identified in various layers of the macula with AMD including MDA, 4-HNE, CEP, oxidized phospholipids (OxPL), and 7-ketocholesterol (7KCh). In the macula, CEP localizes to photoreceptor outer segments, the RPE, and Bruch’s membrane[76, 130]. OxPL has also been identified in photoreceptor outer segments[131]. The RPE contains MDA and 4-HNE[132, 133], while Bruch’s membrane, including drusen, contains MDA, CEP, 7KCh and OxPL[75, 130, 134–136].

Consequences of oxidized lipids, oxidation specific epitopes, and activation of innate immunity in AMD

As mentioned above, a trigger for the inflammatory component of the response to retention hypothesis is oxidized lipids. Oxidized lipids can generate oxidative stress themselves, or they can incite an inflammatory response. For example, the oxysterol, 7-ketocholesterol (7KCh), is an abundant oxidized lipid found in oxidized lipoproteins in both atherosclerosis and Bruch’s membrane[136–139]. 7KCh is toxic to a number of cell types including the RPE[140]. In atherosclerosis, oxysterols contribute to the conversion of macrophages into

foam cells[141]. In the RPE, oxysterols are taken up at a high rate, and can induce inflammation and apoptosis[142]. The Rodriguez laboratory has shown that the majority of the pro-inflammatory signaling by 7KCh is mediated through its interaction with Toll-like receptor 4 (TLR-4) both in vitro and in vivo[143]. Interaction of 7KCh with TLR-4 activates NF-kB signaling to induce pro-inflammatory cytokine expression. Given the aging associated accumulation of cholesteryl esters in Bruch's membrane and drusen, and their tendency to become oxidized into oxysterols, the toxic role of 7KCh and/or the failure to neutralize oxysterols may explain in part, how oxidized lipoproteins contribute to AMD pathology[144].

Picard et al. found that in RPE cells, CD36 participates in oxLDL uptake and clearance of subRPE deposits[145]. Furthermore, CD36 deficient mice accumulated oxLDL in Bruch's membrane even when fed a normal chow diet. ApoE knockout mice given a high fat, high cholesterol diet, typically accumulate lipids in Bruch's membrane, but when given a CD36 agonist, had diminished Bruch's membrane thickening and preserved photoreceptor function. These results suggest that CD36, by neutralizing oxLDLs, can protect against basal deposit formation.

The Wang laboratory recently showed that retinal microglia have chemotropism for, and internalize 7KCh[146]. 7KCh was capable of inducing the migration retinal microglia, which were activated into an M1 pro-inflammatory phenotype, into the subretinal space. These activated microglia also expressed pro-angiogenic cytokines with reduced expression of neurotrophic factors. This profile favors retinal degeneration and initiation of choroidal neovascularization, or the advanced, neovascular form of AMD.

The recruitment of microglia to the subretinal space is significant because it may constitute an initiating step in the immune microenvironment during AMD progression. It is possible that resident choroidal macrophages or circulatory monocytes are also recruited with 7KCh accumulation via CCL2 and CXCR2 chemokine signaling[146–148]. In support of this notion, Sennlaub et al. have identified increased intraocular CCL2 and CCR2+circulating monocytes in atrophic AMD[149]. They showed that CX3CR1, which is constitutively expressed in the retina, represses CCL2 expression, and the recruitment of CCR2 pro-inflammatory monocytes. In Cx3Cr1 deficient mice, they found that aging or light exposure induced subretinal inflammation and photoreceptor degeneration, that subretinal monocytes overexpressed CCL2, and that impairment of CCL2 or CCR2 prevented inflammatory monocyte recruitment and photoreceptor degeneration. While these findings in mouse models are suggestive of a role for retinal microglia in the subretinal space, evidence of a role for retinal microglia in early AMD is lacking.

Oxidized lipid products can react from covalent bonds with primary amines of proteins and amino groups of lipids such as phosphatidylethanolamine (PE), and thereby form oxidation-specific epitopes (OSEs), which are recognized by innate immune receptors in a hapten-specific manner[150]. Unless neutralized in a timely manner, OSEs can generate unwanted inflammation and contribute to tissue injury. Whether as a component of oxLDLs or independently accumulating in the RPE/Bruch's membrane/choroid, MDA is an abundant lipid peroxidation degradation product that accumulates in a number of oxidative stress

related diseases including AMD. Innate immunity has placed substantial natural selection on MDA adducts because 15% of all IgM natural antibodies bind MDA adducts[151]. In a multi-laboratory collaboration headed by the Binder laboratory, our group found that CFH, previously recognized only for its major regulation of the alternative complement pathway, is a novel pattern recognition receptor that specifically binds MDA adducts, but not other oxidized epitopes such as oxidized phosphocholine, CEP, or 4-HNE[152]. This finding is significant because CFH SNPs are highly associated with AMD risk[42–45]. The short consensus repeats SCR7 and SCR20 of CFH that contain various disease-related mutations including the 402H variant in SCR7, bind to MDA. Importantly, plasma from patients who have the 402H CFH variant had impaired MDA binding[153]. CFH also bound to apoptotic debris that contained MDA epitopes, which increased the generation of iC3b inactivation fragments. In contrast, the 402H CFH variant showed impaired generation of iC3b inactivation fragments when incubated with MDA decorated apoptotic particles. Since RPE apoptosis is likely an early event in AMD[15] and iC3b opsonins promote the clearance of apoptotic cells without generating inflammation[154], the reduced iC3b could translate into unwanted inflammation during clearance of apoptotic debris. CFH was also found to block the uptake of MDA-modified proteins and their proinflammatory effects by macrophages and RPE cells in vitro. When mice were given intravitreal injections of MDA-BSA, Il-8 was induced by the RPE/choroid, which was neutralized by co-injection with CFH. Toomey et al. provided interesting insight into the role of CFH in AMD pathogenesis from studying CFH deficient mice[155]. While decreased CFH was associated with subRPE deposit formation, decreased vision was only seen in $CFH^{+/-}$ mice with increased complement activation while $CFH^{-/-}$ mice, which have impaired complement function due to secondary complement depletion, had intact ERG responses. This finding indicates that it is not subRPE deposits, but complement activation that induces RPE dysfunction sufficient to decrease vision. However, given the alternative function of CFH, of binding MDA adducts and neutralizing pro-inflammatory cytokine expression, CFH could regulate visual function by controlling non-complement inflammation, too. Future studies will be needed to determine the relative contribution of complement and complement independent inflammation regulated by CFH.

Another OSE that was recently recognized is OxPL, a prominent consequence of lipid peroxidation of the polyunsaturated fatty acids found in high abundance in photoreceptors. Oxidized phosphatidylcholine appears in the retina and RPE with aging, and increases in AMD[135]. C-reactive protein (CRP) and CD14 are pattern recognition receptors that recognize oxidized phosphatidylcholine, and have been identified in AMD tissue[156, 157]. A unique OxPL species is the oxidation of phosphocholine (PC) containing PL, which is detected with the IgM natural antibody E06 that binds to the PC moiety of OxPL, but not to the PC moiety of unoxidized phospholipids[158, 159]. This OxPL accounts for 85% or more of all phosphocholine (PC) containing OxPL found on lipoproteins in plasma and on apoptotic cells, as determined by the binding of murine monoclonal antibody E06[160, 161].

Lipoprotein (a) [Lp(a)] is a lipoprotein composed of apolipoprotein(a) [apo(a)] covalently bound to apolipoprotein B-100 (apoB) by a disulfide bond between Cys4326 of apoB and Cys4057 of apo(a) on kringle IV type 9 (KIV9)[162–164]. Apo(a) is highly homologous to the plasminogen gene, which contains 5 kringles (K) and a protease domain. However, apo(a) is distinct from plasminogen as it only contains KIV (10 subtypes of which KIV-2 is

present in multiple and variable copies) and KV, and has an inactive protease domain due to a Ser^{561}-Ile^{562} substitution for Arg^{561}-Val^{562} that prevents plasminogen activators from converting apo(a) to plasmin[165]. Unlike plasminogen, apo(a) is present only in humans, non-human primates, and old world monkeys. In humans, Lp(a) has been shown to bind OxPL[160]. While likely a protective effect, it is theorized that Lp(a) can become atherogenic with enhanced binding to arterial intimal proteoglycans, which increases local concentrations of OxPL, inducing an inflammatory response by the tissue. Our laboratory recently found by immunohistochemistry that OxPL, apo(a), and apoB accumulate in maculas including drusen of AMD and age-matched controls[131] (Figure 5). Since immunohistochemistry is not quantitative, to elucidate the impact of Lp(a) in the fundus, we evaluated transgenic Lp(a) mice and mutant Lp(a) mice, which are unable to bind OxPL, that were stressed with a high fat diet. Lp(a) mice developed mild age-related changes in the fundus. In contrast, high fat diet-fed mutant Lp(a) mice that are unable to bind OxPL, developed RPE cell degeneration, basal deposits, and occasional drusen, which were visualized by light and electron microscopy. These morphologic changes were associated with increased oxidized phospholipids, decreased antioxidant proteins, increased complement, and decreased complement regulators. Our data suggest that in the eye, Lp(a), by binding OxPL, neutralizes its potential pro-inflammatory, tissue injuring effect, and may be protective against any contribution of OxPL on AMD pathology.

As mentioned previously, CEP is a distinct and perhaps signature OSE in the macula. In the retina, docosohexanoic acid (DHA) is the most abundant fatty acid in photoreceptor tips, and is also the most oxidizable fatty acid in the body because of its unsaturated structure. In the retina, DHA is oxidatively modified to CEP[76]. Matching the distribution of DHA, CEP adducts immunolocalize in photoreceptor outer segments, the RPE, and drusen in higher abundance than age-matched controls patients[75]. This distribution suggests that CEP is formed in photoreceptor outer segments and is phagocytized by the RPE as the routine regeneration of outer segment processing, and ultimately released by the RPE through Bruch's membrane into the choriocapillaris for removal into the circulation. Indirect proof of this theory is that CEP autoantibodies are found in higher concentration in the plasma of AMD patients than age-matched controls. Hollyfield et al. immunized mice with CEP adducts and found elevated anti-CEP antibodies compared to controls[166]. Upon pathologic examination, photoreceptors were edematous overlying areas of RPE degeneration. RPE atrophy and loss were seen in patches, which was reminiscent of geographic atrophy. Bruch's membrane developed areas of prominent basal laminar deposits. These ultrastructural changes were associated with complement deposition of C3d, a marker for C3 activation, in Bruch's membrane. In this model, since the anti-CEP antibodies are both IgMs and IgGs, it is likely that both the innate (i.e. IgM) and adaptive (i.e. IgG) immune response is involved in the response to CEP adducts.

Final Thoughts

AMD is a highly prevalent, complex aging related disease that occurs within a 6 mm diameter area. While multifactorial in nature, the collision of oxidative stress, from the high ambient oxidative stress environment, the addition of exogenous oxidants, and failure of the anti-oxidant protective systems jeopardizes the integrity of the basic structural and

functional molecules within cells. It is clear that oxidatively modified lipids form in all layers of the macula, and either directly contribute to tissue injury as a source of oxidative stress, or by adduct formation, develop oxidation-specific epitopes. The OSEs initially incite a protective immune response that is designed to neutralize or remove these potentially toxic molecules. However, if inadequately neutralized, the OSEs can convert the protective inflammatory response into a pathologic, chronic tissue injuring reaction. Currently, our understanding of how these OSEs participate in AMD pathogenesis has not been elucidated in part, because of our reliance on mouse models of AMD, which do not develop a complete AMD phenotype. While rejuvenating the antioxidant systems in the macula might reduce the formation of these potentially toxic oxidized lipids, fully categorizing the oxidized lipids that develop and the specific pattern recognition receptors that neutralize them, will offer new insights into disease etiology, and to develop alternative targets that prevent tissue injury in AMD.

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Highlights

- Lipoprotein and lipid accumulation in the macula play an essential role in Age-related macular degeneration.
- These lipids become oxidized and can induce tissue injuring inflammation unless neutralized by innate immunity.
- Some oxidized lipids can form adducts to generate oxidation-specific epitopes which can also cause tissue injuring inflammation unless adequately neutralized.

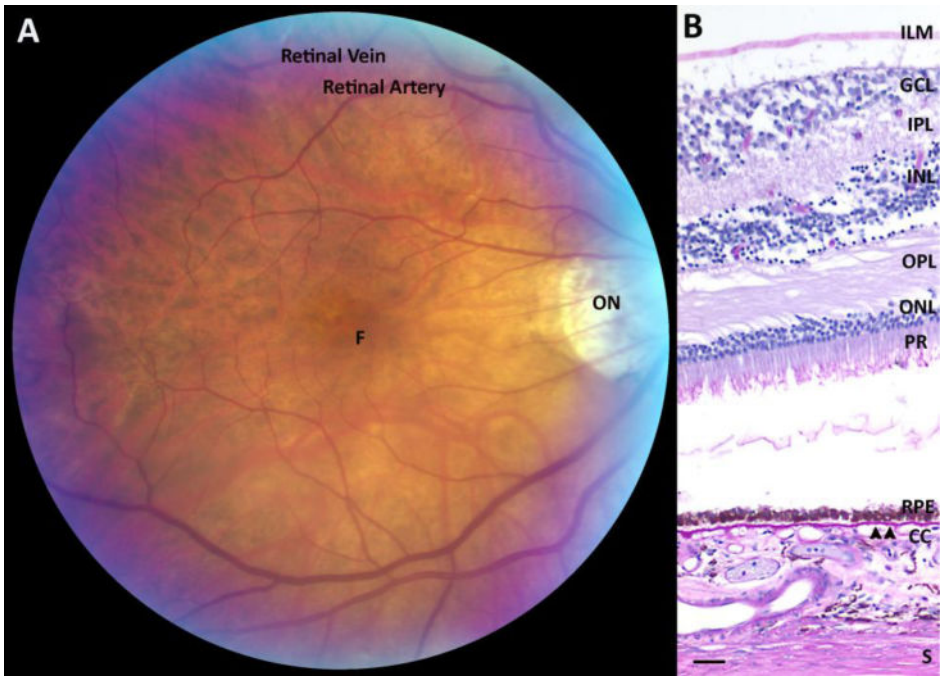

Figure 1.

The clinician and anatomist's view of the macula. A. Fundus photograph of the macula. ON, optic nerve; F, foveola. B. Histological section of the macula with the internal limiting membrane (ILM), multi-layered ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), photoreceptor layer (PR), retinal pigmented epithelium (RPE), Bruch's membrane (arrowheads), choriocapillaris (CC), and sclera (S). Bar=25μm.

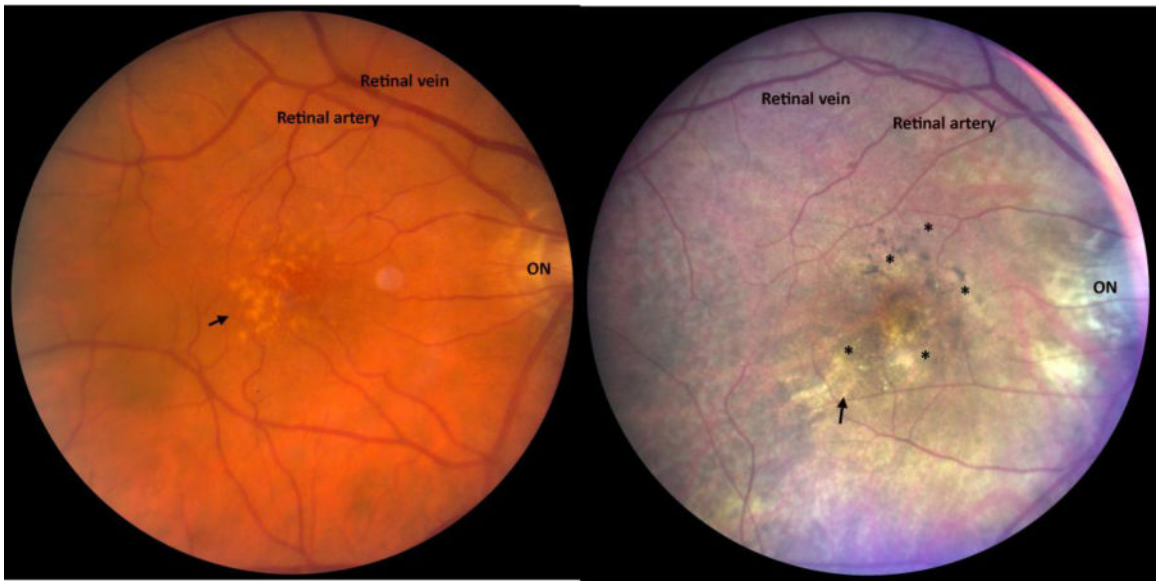

Figure 2.

A. Fundus image of a patient with large drusen (arrow). B. Fundus image of a patient with drusen (arrow) and RPE hypo- and hyper-pigmentary changes (*). ON, optic nerve.

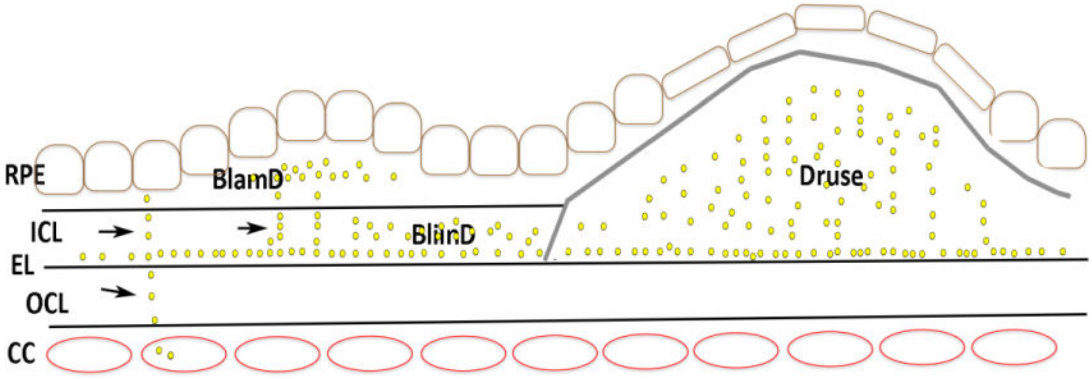

Figure 3.

Cartoon of Bruch's membrane deposits with AMD. Basal laminar deposits (BLamD) develop between the RPE basal lamina (RPE BL) and the RPE cell while basal linear deposits (BLinD) develop in the inner collagenous layer (ICL). A druse occupies the subRPE space and is denoted by the gray line. The yellow dots represent lipoprotein deposits, which can be released from the RPE and transit in linear streaks (arrows) toward the choriocapillaris (CC). With age-related changes to the elastic layer (EL), lipoproteins collect at the inner surface of the elastic layer (EL) and accumulate toward the RPE.

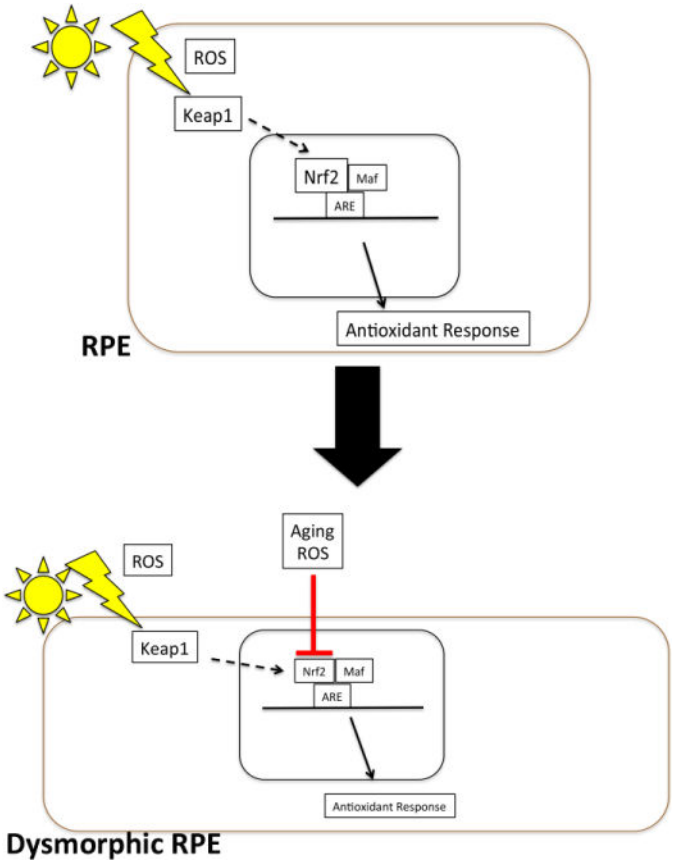

Figure 4.

Impaired Nrf2 signaling in AMD. Oxidative stress with reactive oxygen species (ROS) induce Nrf2 production and release from Keap1 with transit to the nucleus. After binding to antioxidant response elements in the promoters of antioxidant and cytoprotective genes with Maf proteins, antioxidant genes are upregulated. With aging and chronic oxidative stress, Nrf2 production and activity is reduced, and the antioxidant response is impaired, which contributes to RPE dysfunction.

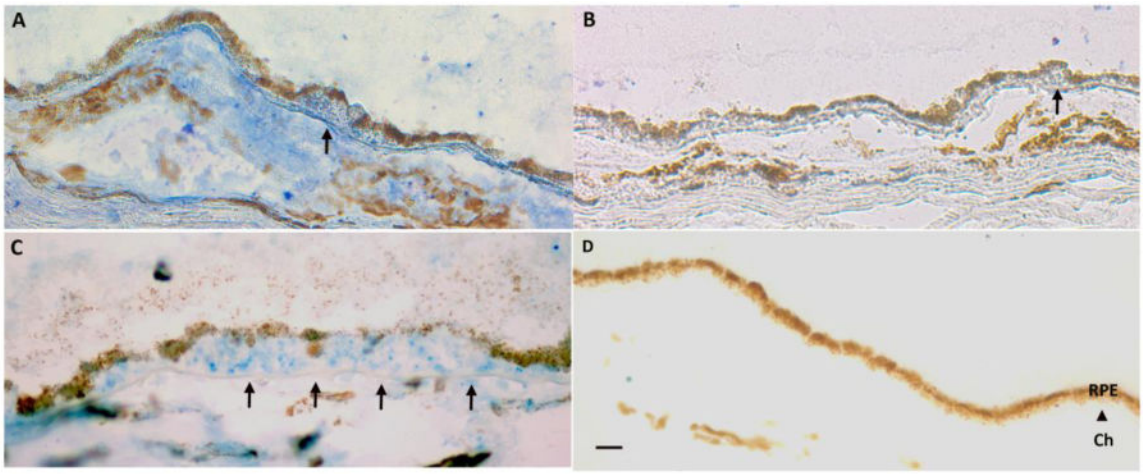

Figure 5.

OxPL and Lp(a) in the macula. A. Immunostaining for OxPL identified with E06 antibody in Bruch's membrane and choroid including a druse (arrow). B. IgM control. C. Immunostaining for apo(a) in Bruch's membrane and choroid including a large druse (arrow). D. IgG control. Ch, choroid. Bar=25μm.