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Lipofuscin Accumulation into and Clearance from Retinal Pigment Epithelium Lysosomes: Physiopathology and Emerging Therapeutics

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Abstract

Photoreceptors undergo a constant renewal of their light sensitive outer segments (POSs). In the renewal process, 10% of the POS mass is daily phagocytized by the adjacent retinal pigment epithelium (RPE). POS contain vast amounts of 11-cis retinal and all-trans-retinal, two highly reactive vitamin A aldehydes that spontaneously dimerize into lipid bisretinoids (LBs) and accumulate into RPE lysosomes during phagocytosis. As LBs are refractory to lysosomal hydrolases and RPE cells do not divide, this accumulation is irreversible and results in the formation of lipofuscin granules. Lipofuscin accumulation is toxic for RPE cells through a variety of light-dependent and light-independent mechanisms. Beyond a threshold, RPE cells die resulting in secondary loss of overlying photoreceptors. Currently, there are no effective treatments for retinal disorders associated with genetic or age-associated LB accumulation, such as Stargardt disease and age-related macular degeneration (AMD). Thus, there is a great need for medical interventions. Here, we discuss the current understanding of lipofuscin's pathogenicity and the status of different strategies under development to promote LB elimination from RPE lysosomes.

Keywords: lipofuscin, Stargardt, age-related macular degeneration (AMD), bisretinoids, retinal pigment epithelium (RPE), cyclodextrins, cellular clearance, TFEB, lysosome

1. Introduction

To understand the origin and consequences of the lysosomal accumulation of lipofuscin in the eye, a basic knowledge of retinal function and organization is required.

1.1. The retinal pigment epithelium (RPE) in vertebrate's eyes

Light entering the eye gets refracted by the cornea and lens on the neural retina, where photoreceptors (PR) convert photons into a cascade of chemical and electrical events that propagate to second-order (horizontal, bipolar, and amacrine cells) and third-order (ganglion cells) retinal neurons, which distribute this information to various visual centers of the brain through the fibers of the optic nerve. The bodies of PR cells, rods and cones, display three sectors (Figure 1): the *outer segment*, filled with stacks of disks densely packed with light-sensitive photopigment; the *inner segment*, filled with genetic, biosynthetic, and metabolic organelles

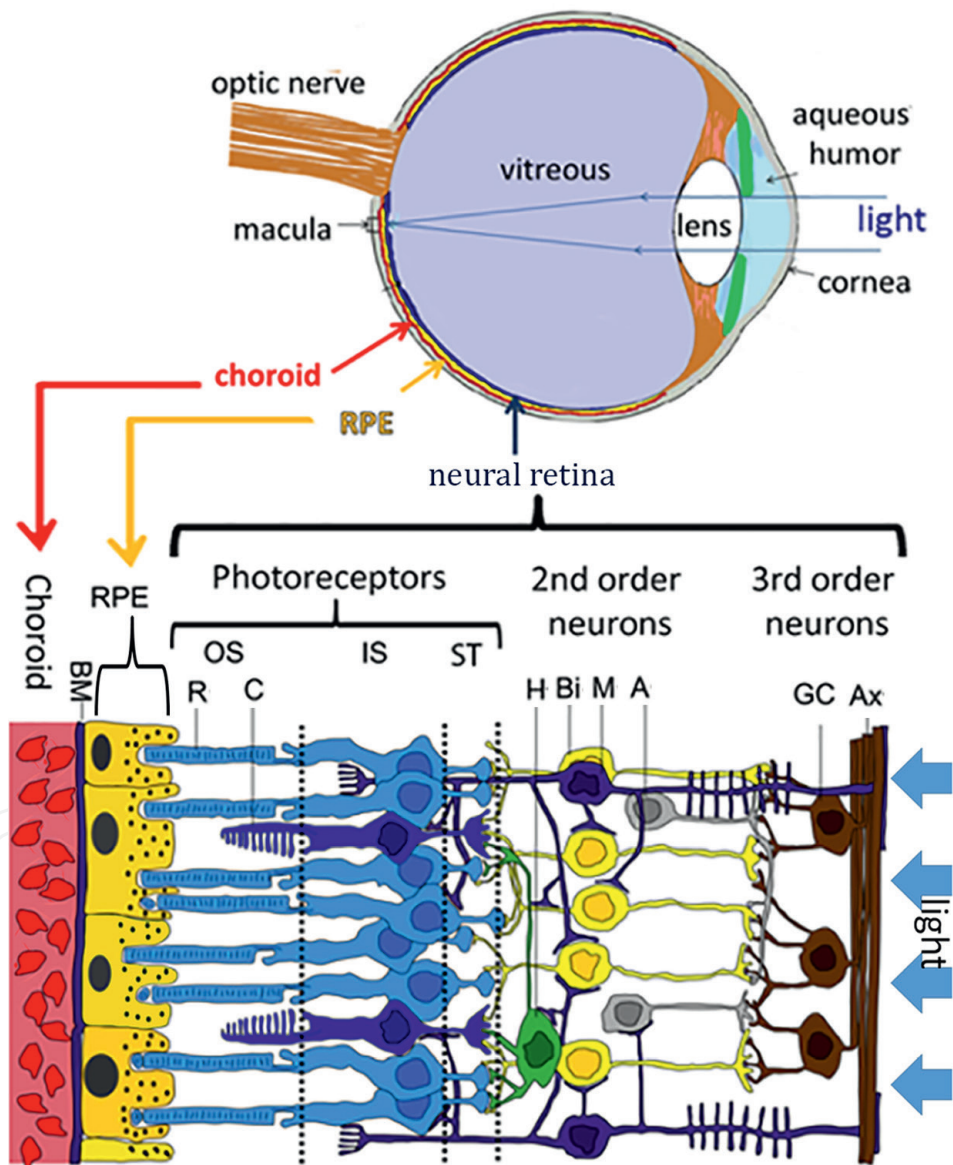


Figure 1. Basic structure of retina's inverted architecture. Light travels through ganglion cells (GC) as well as second-order neurons comprising bipolar (Bi), amacrine (A), horizontal (H), and Muller (M) cells, the retina. RPE localizes between the choroid capillaries and the cone (C) and rod (R) photoreceptors. Photoreceptors outer segments (OS), inner segments (IN), and synaptic terminals (ST).

(nucleus, endoplasmic reticulum, Golgi complex, ribosomes, mitochondria); and the *synaptic terminal* that connects with bipolar neurons of the retina. In vertebrates, the retina is inverted in the sense that light passes through secondary and tertiary neuronal layers in the inner retina before reaching the rods and cones in the outer retina (**Figure 1**). Photoreceptors are metabolically very active cells that require large amounts of nutrients and generate massive amounts of waste. Provision of nutrients and removal of waste are critical support tasks performed by the retinal pigment epithelium (RPE), a monolayer of cuboidal cells tightly opposed to the photoreceptors through a narrow *subretinal space*. The multifunctional RPE cells constitute the blood-outer retina barrier that controls the movement of nutrients, ions, water, gases, and wastes between the photoreceptors and underlying choroidal blood vessels [1], absorbs excess light through their melanin granules, performs segments of the visual cycle that regenerates the visual pigment, performs daily phagocytosis of photoreceptor outer segments, and produces trophic factors necessary for photoreceptor survival [2]. It may be rationalized that a fundamental objective of the inverted retina design is to bring photoreceptors in close contact with RPE, a key relationship for the integrity of the retina.

1.2. Role of RPE in visual-pigment regeneration

In 1967, George Wald was awarded the Nobel Prize for revealing the essential role of Vitamin A for vision [3]. Vitamin A entirely is derived from the diet. A critical function of RPE cells is to supply the vitamin A-derived chromophore, 11-cis retinal, required for the light-sensing function of visual pigments to photoreceptor cells. Visual pigments are G-protein receptors (opsins) covalently linked to 11-cis-retinal. Opsins cannot respond to light by themselves but need their prosthetic group, 11-cis-retinal that undergoes cis-trans isomerization upon illumination. The absorption characteristics of 11-cis retinals, in different pigments, are determined by the opsins. Humans have four types of visual pigments: *rhodopsin*, expressed by rod photoreceptors, which is sensitive to dim light and provides black-and-white vision; *L-opsin*, *M-opsin*, and *S-opsin* expressed by cone photoreceptors sensitive to red, green, and blue color lights, respectively [4]. When light strikes the visual pigments, it promotes isomerization of 11-cis retinal into all-trans retinal (ATR), which in turn promotes the structural rearrangement of opsin into an active conformation that initiates phototransduction (**Figure 2**).

To recover photosensitivity, opsin must be regenerated by releasing all-trans retinal and binding new 11-cis retinal. Released all-trans retinal is pumped out of the disks into the cytosol by a photoreceptor specific ATP-binding transporter (ABCA4) and reduced to all-trans-retinol by all-trans-retinal dehydrogenases (RDH8 and RDH12). All-trans-retinol diffuses into the RPE where it is esterified by lecithin:retinol acyltransferase (LRAT) to all-trans-retinyl esters, which are stored in retinosomes. All-trans-retinyl esters are isomerized by 65-kDa RPE-specific protein (RPE65) to 11-cis retinol, which is oxidized to 11-cis retinal before traveling back to the photoreceptors outer segment where it is again conjugated to an opsin to form new, functional visual pigment. These series of metabolic steps, by which all-trans-retinal is converted to regenerate the visual pigments, constitute the classical visual cycle [5]. As described above, the RPE performs a number of critical steps in the classical visual cycle that provides 11-cis retinal to rods and cones. There is also a cone-specific visual cycle [6], which

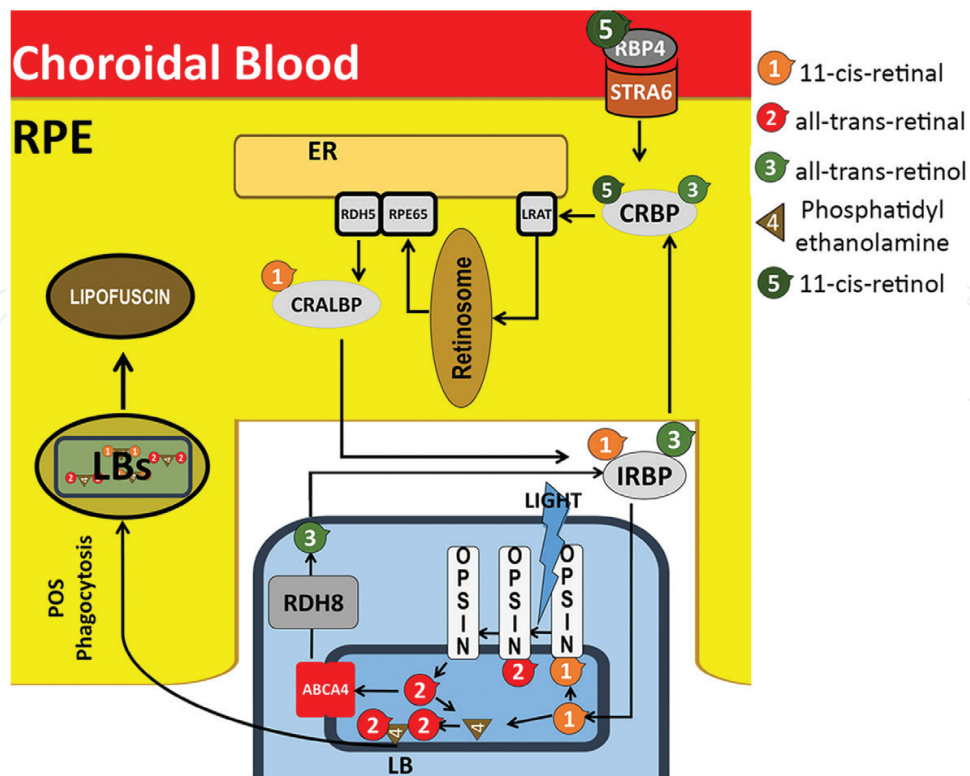


Figure 2. Classical visual cycle. Light induces cis-trans isomerization of retinal in visual pigments. All-trans retinal is released from opsins and pumped to the cytosol by ABCA4 where it is reduced to all-trans-retinol by dehydrogenases (RDH8). All-trans-retinol diffuses into RPE via a series of Interphotoreceptor (IRBP) and cellular (CRBP and CRALBP) retinoid-binding proteins. In RPE all-trans-retinol is esterified by lecithin:retinol acyltransferase (LRAT) and is stored as retinyl ester lipid droplets called retinosomes. When mobilized, all-trans-retinyl esters are isomerized to 11-cis-retinol by RPE65 and oxidized to 11-cis-retinal by RDH5 before traveling back to the photoreceptor disks where it is again conjugated to an opsin to form new, functional visual pigment. Circulating retinol-binding protein 4 (RBP4) and its membrane receptor STRA6 coordinate 11-cis-retinol uptake into cells with high demand of Vitamin-A.

is RPE independent, that will not be discussed here. The RPE can also generate 11-cis retinal from vitamin A captured via its transmembrane transporter Stra6 from the choroidal circulation. Another important source of 11-cis retinal is the retinosomes, i.e., intracellular lipid droplet deposits of all-trans-retinyl esters in the cytoplasm of RPE cells (Figure 2).

1.3. Lipid-bisretinoid (LB) biogenesis

Vitamin A aldehydes (retinaldehydes) are highly reactive molecules capable of forming adducts with biological amines without the need for a catalyst [7]. In the disks of photoreceptor outer segments (POSs), retinaldehyde concentrations are relatively high, due to the *all-trans-retinal* released by photo-transduction and the *11-cis-retinal* conveyed for the regeneration of visual pigments (Figure 2). Not surprisingly, POS' retinaldehydes tend to covalently react with the amine group of phosphatidyl-ethanolamine (PE) to form N-retinylidene-PE (NRPE), which reacts with the second molecule of retinal to produce *lipid-bisretinoids* (LBs). Thus, LBs are a family of adducts, all structurally related, that derive from the condensation of two retinaldehydes with one PE molecule [8, 9].

1.4. Photoreceptor renewal

Because of their task in vision and proximity to the fast flowing choroidal capillaries, photoreceptors are continually exposed to high doses of radiant energy and oxygen, which makes them prone to photo-oxidative damage. To secure long (decades) of useful life, under these demanding conditions, photoreceptors undergo a daily renewal process wherein the most distal tips of their POS, comprising the ~100 oldest disks, are removed and equivalent number are basally produced to maintain constant outer segment length [10]. This cellular renewal process has a circadian rhythm. The rods shed POS most vigorously in the morning, whereas cones shed more vigorously at the onset of darkness [11]. The enormous amount of waste daily generated by this process is cleared by the adjacent RPE cells. In the mammalian eye, one RPE cell serves approximately 40 photoreceptor cells, each of which sheds ~7% of its mass per day. RPE engulfs and degrades POS fragments via a receptor-mediated phagocytic process similar to that involved in macrophage-mediated removal of apoptotic cells [12, 13]. This is an impressive metabolic task for RPE, since each cell must ingest and digest ~4000 disks before the next phagocytic load. Thus, RPE is one of the most active phagocytic cells in the body. Because RPE cells do not divide, they must completely dispose this daily material to avoid POS components buildup in their lysosomes.

1.5. RPE lipofuscin accumulation

“Lipofuscin” is the generic name given to subcellular material that accumulates with age within the lysosomal compartment of a variety of postmitotic cells and is characterized by its golden-orange autofluorescent emission. Very few compounds of animal origin exhibit fluorescent emissions in the lipofuscin's region of the spectrum [14]. A fairly rigid structure with highly conjugated double bond system is necessary for such fluorescence because, accumulation of lipofuscin is considered an universal biomarker of aging, as it is also referred to as “age pigment”. Lipofuscins are resistant to degradation by lysosomes, proteasomes, and are not evidently exocytosed. Hence, their accumulation appears irreversible in cells that do not divide. Most lipofuscins stain positive for proteins, lipids, and carbohydrates [15]. Their exact composition varies among tissues but most commonly contains a large proportion of incompletely degraded proteins [16]. The RPE is one of the tissues with the largest buildups of lipofuscin. RPE lipofuscin increases with age in all healthy eyes [17, 18]. It localizes in lysosomal bodies of the RPE [19] and can occupy ~ 20% of the cytoplasmic space by 80 years of age [20].

In order to illuminate the cellular processes responsible for the formation of RPE lipofuscin, several groups attempted to analyze RPE-lipofuscin's chemical composition. Eldred and Katz [21] were the first to isolate the fluorescent pigments of the RPE lipofuscin. Spectroscopy and mass spectroscopy analyses of lyophilized chloroform extracts of RPE cells from healthy donors of different ages revealed that the most common fluorophore was a lipid-bisretinoid [22], N-retinylidene-N-retinylethanolamine also called A2E [23]. Protocols for *in vitro* synthesis of A2E as well as its incorporation into lysosomes of cultured RPE were developed, allowing to model RPE lipofuscin accumulation *in vitro* [24, 25]. Further efforts to isolate and characterize the remaining chromophores in the chloroform extracts from RPE lipofuscin granules, yielded additional LBs, including A2-GPE (A2-glycero-phospho-ethanolamine),

A2-DHP-PE (A2-dihydropyridine-phosphatidyl-ethanolamine), all-trans-retinal dimer (ATR-D), and all-trans-retinal dimer phosphatidyl-ethanolamine (ATR-D-PE) (**Figure 3**) as well as several higher molecular weight hydrophobic polymers derived from the reaction between A2E and its oxidation products [26–29].

Of note, the chloroform-insoluble fraction of the RPE lipofuscin, which represents 70% of its dry weight, was not analyzed in these studies. Thus, to fully characterize this fraction, Schutt et al. performed a proteome analysis of sucrose-purified RPE granules [30]. They identified 65 abundant cellular proteins, which included structural, metabolic, mitochondrial, chaperone, transmembrane, and signaling transduction proteins. Many of these proteins were modified by reactive carbonyl compounds (4-hydroxynonenol and malonyldialdehyde) and exhibited advanced glycation end products (AGEs) [31]. A second study by Warburton et al. [32] identified 41 proteins, most of which included phagosomal, lysosomal, and photoreceptor proteins (including rhodopsin) in agreement with the notion that RPE lipofuscin was mainly a buildup of undigested POS material. Surprisingly, only 12 proteins (11%) of Warburton's list were common with the 65 proteins identified by Schutt et al. This discrepancy probably results from variations in the purity of sucrose-isolated granules [33] and from the fact that lipofuscin proteins are microheterogeneous in size due to abundant oxidative modifications while contaminant proteins are intact and therefore, run as well-focused spots. In a third study, Ng et al. [34] analyzed the composition of highly purified RPE-lipofuscin granules devoid of

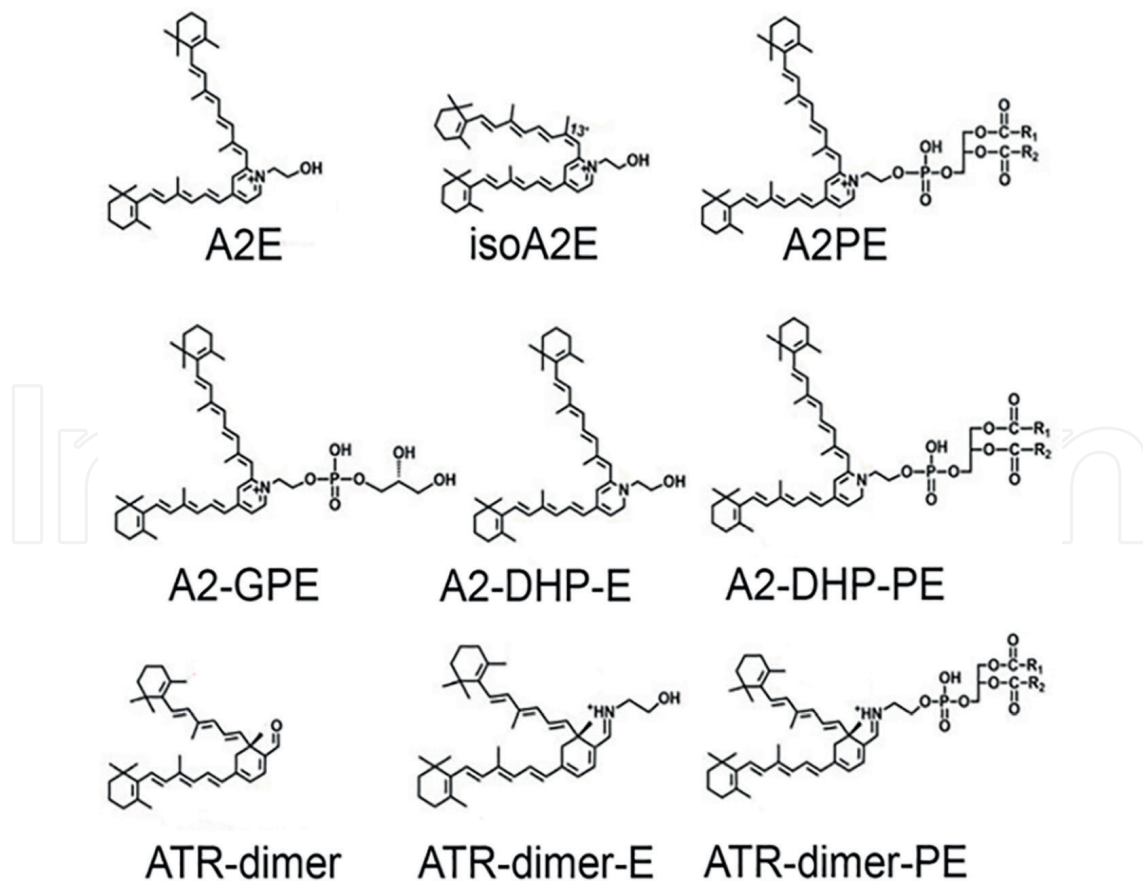


Figure 3. LBs found in RPE lipofuscin. R1, R2 are fatty acids with 14 to 22 carbons and 0 to 6 double bonds.

membranes and reported that the luminal material was 98% lipids, mostly LBs [35]. Taking all this information into account, the current concept is that RPE lipofuscin originates from LBs in photoreceptors and is transferred to RPE lysosomes during POS phagocytosis. This model is supported by animal studies that show that accumulation of lipofuscin in the RPE only occurs if (i) there is a supply of 11-cis-retinal to synthesize visual pigments, as RPE65^{-/-} mice display no lipofuscin [36] and (ii) there is phagocytosis of POS, since no accumulation of lipofuscin is detectable in phagocytosis-defective animals. In healthy individuals, LB formation occurs slowly because the concentrations of retinaldehydes are relatively low, thereby, taking many years to generate significant amounts of LBs. In contrast, in individuals with mutations in ABCA4, the formation of LBs is dramatically accelerated [37].

1.6. Cellular toxicity caused by RPE lipofuscin accumulation

In retinal diseases associated with the accumulation of LBs in RPE lysosomes, vision loss is the result of the death of photoreceptor cells secondary to the functional impairment of RPE. Cell culture experiments have shown that lysosomal accumulation of LBs can cause RPE cell death [38]. However, how exactly lipofuscin accumulation disrupts RPE performance and viability is not fully understood. The variety of LB-elicited toxic mechanisms proposed so far (**Figure 4**) and their investigation as potential pharmacological targets are discussed below.

1.6.1. Phototoxicity

In vitro data with both, whole lipofuscin granules [39] or individual bisretinoids (A2E [40], all-trans-retinal dimer [27] and A2-GPE [41]) loaded into lysosomes have shown that LBs

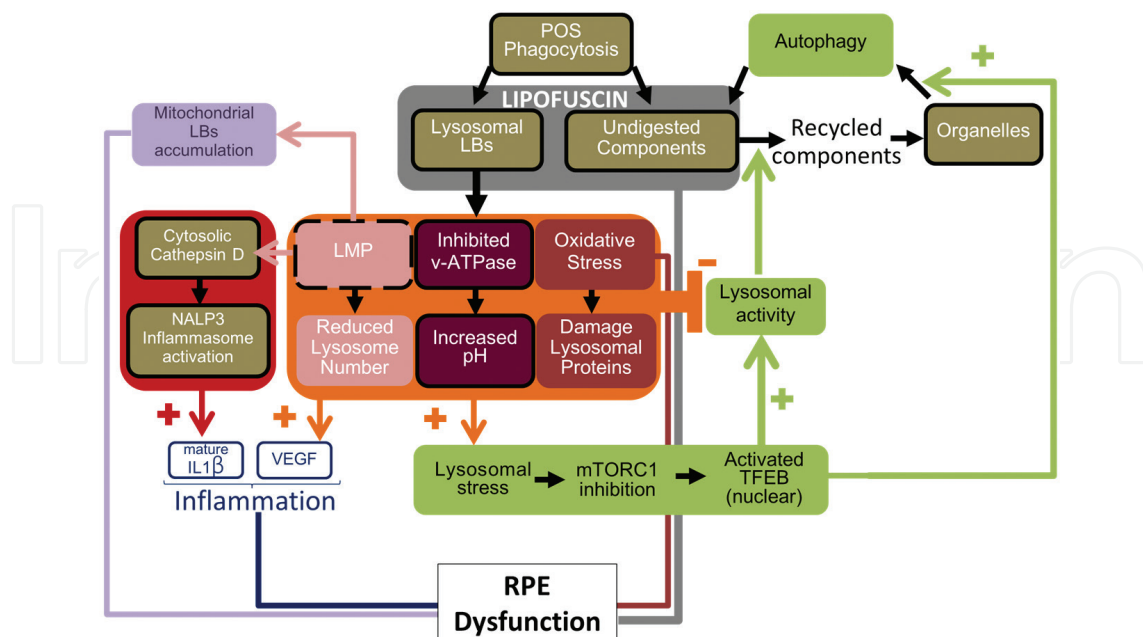


Figure 4. Modeling the effect of LB accumulation on RPE's lysosomal function. LBs cause loss of lysosomal function by inhibition of v-ATPase, oxidative damage, and/or LMP leading to buildup of undigested POS, autophagy material and TFEB-mediated lysosomal stress. TFEB induced activation helps RPE to temporarily cope with the LB burden.

sensitize RPE cells to light exposure. Cellular photosensitivity is proportional to the amount of LBs accumulated [42] and the wavelength, with a maximum at 430 nm (blue light), which coincides with the excitation spectrum of the LBs [43]. The absorption of blue photons by the LBs' extended double bond conjugated system, in the presence of oxygen, leads to the formation of oxidized LB species [44, 45] that after repetitive oxidative attacks become fragmented into far reaching, highly reactive, carbonyl bearing small molecules [43, 44, 46–48]. These fragments promote cell damage by forming Schiff base adducts with free amine groups in lysosomal hydrolases, nucleotides, phospholipids, lipids, proteins [49], DNA [50], proteasomes [51], and molecules in extracellular retinal deposits (drusen), which could trigger local innate and adaptive immune responses [52, 53]. Interestingly, healthy mice immunized with Schiff base adducts found in the AMD lesions, developed AMD-like retinal pathology [54]. There is also *in vivo* evidence indicating that RPE lipofuscin undergoes photodegradation in the eye. Ueda et al [55] showed that ABCA4^{-/-} animals were more susceptible to light damage than WT animals and that in both groups, older animals carrying larger amounts of LBs were also more susceptible. More recently Ref. [56] showed that RPE-lipofuscin photodegradation takes place in mouse eyes under standard ambient illumination. Specifically, they found that WT and ABCA4^{-/-} mice reared in constant darkness contained 45 and 62% more LBs in the RPE than their respective 12-h cyclic light-reared controls. In addition, ABCA4^{-/-} mice who received vitamin E, a potent inhibitor of LB oxidation [44], displayed 54% more LBs than controls. Studies in humans, using fluorescence microscopy for quantifying lipofuscin, and MALDI-IMS (high-resolution matrix assisted laser desorption-ionization imaging mass spectrometry) for detecting A2E showed that lipofuscin fluorescence colocalized with A2E only in the darkest zones of the retina [57]. When the same technology was applied to the eyes of ABCA4^{-/-} mice, lipofuscin fluorescence, and A2E colocalized 100% [58]. Since RPE is exposed to higher levels of illumination in eyes from diurnal than nocturnal species, these data suggest that A2E is much more photooxidized into MALDI-IMS unidentifiable fluorescent derivatives in human eyes than in mice eyes. However, how much LB photooxidation contributes to retinal pathology is an open question. There is a large amount of clinical trial data on the use of antioxidants (lutein, zeaxanthin, and vitamins C and E) supplementation (alone or in combination) to prevent or delay retinal degeneration. A Cochrane meta-analysis performed on four large, high-quality-randomized clinical trials involving a total of 65,250 participants, without signs of AMD at baseline showed no effect of antioxidant therapy for preventing the onset of retinal degeneration *per se* [59]. Another Cochrane review meta-analysis [60] involving data from 13 randomized clinical trials, including two large trials, the AREDS1/2 and the Vitamin E Intervention in Cataract and Age-Related Maculopathy study, and 11 smaller (20–400 participants) randomized trials were performed to decide whether antioxidants can slow progression of retinal damage in patients with established AMD. The AREDS1/2 shows that long-term, high-dose supplementation with vitamin E (400 IU), vitamin C (500 mg), beta-carotene (15 mg), zinc (80 mg), and copper (2 mg) reduced the risk of progression to geographic atrophy AMD by 8% in only a subgroup of patients with intermediate AMD at baseline. The other 11 trials demonstrate little evidence for the effectiveness of antioxidant therapy for preventing either visual loss or AMD progression. In summary, treatments with antioxidants have shown very modest efficacy at preventing or stopping the progression of lipofuscin-associated retinal degenerations. Indeed, patients with mutations

in ABCA4 (Stargardt-1, CRD, and RP) gene are not cured by high-doses of antioxidants [61]. This may indicate that scavenging reactive oxygen species is not the best approach to halt LB-driven damage. Alternatively, LB-photooxidation could be damaging through its propensity to activate the complement system [62]. In support of this idea, there is a histologic evidence of complement deposition in drusen of retinas with AMD [63] and animal studies show that overexpression of inhibitors of complement protects retinas of mice with elevated LB content [64]. Furthermore, genetic polymorphism in genes encoding complement factor H (CFH), CFB component C2, CFI, and complement components 2, 3 and 7 has been associated with elevated risk for LB-driven retina disease. Initially, there was a tremendous excitement to test complement inhibitors in the eye. Out of a dozen tested, only 1 molecule, Lampalizumab (Genentech), has made it into phase 3 clinical trials. In the phase 2 clinical test, Lampalizumab decreased the rate of growth of the geographic atrophy area, especially those with CFI polymorphisms [65]. Two world-wide multi-center prospective phase 3 clinical trials, which are enriched with CFI subjects, are now fully enrolled and results are expected in early 2018.

1.6.2. Inactivation of lysosome-dependent degradative processes

Because RPE cells are the most active postmitotic phagocytes in the body, they heavily rely on the fitness of their degradative machinery to operate. Indeed, a high baseline of autophagic activity level has been detected in the RPE and photoreceptors [66, 67], which were further enhanced during periods of POS phagocytosis [68]. Digestion of rhodopsin is also necessary for adaptation of rods to changes in light intensity [69]. Chemical or genetic inhibition of autophagy in RPE cells increased accumulation of undigested material and reduced cell viability [70]. Deletion of the autophagy inducer gene RB1CC1 in rodent RPE caused severe retinal degeneration, underlining the importance of basal autophagy [71]. Histological examination of retinas from Stargardt and AMD subjects revealed massive accumulation of lysosomal material similar to lipofuscin in the apical regions of RPE cells and of extruded extracellular deposits (drusen and pseudo-drusen) that support the idea of a defect in the recycling of endocytic and autophagic cargoes [72, 73]. *In vitro* experiments in which exogenous A2E was loaded in the lysosomes of cultured RPE cells, as surrogate of lipofuscin accumulation, show also a significant impairment in the digestion of phagocytized POS [74, 75] and autophagocytized proteins [76], implying lysosome-dependent degradative pathways are a primary point of attack by LB accumulation. The mechanism by which LBs mediate these inhibitions is not fully understood yet. Measurements of lysosomal protease, lipase, glycosidase, nucleases, sulfatase, and phosphatase activities in homogenates of RPE revealed that A2E does not inhibit lysosomal activity by direct interaction with the hydrolases [77]. Lysosomes-containing A2E seems to have increased pH [74]. Bergmann et al. [76], working with purified lysosomes, provided evidence that A2E inhibits the vacuolar H(+)-ATPase (v-ATPase). v-ATPase is a transmembrane lysosomal protein in charge of maintaining the acidic environment within the lysosomes. Because acidic conditions are a prerequisite for the activity of lysosomal hydrolases, A2E-induced increase of lysosomal pH would explain, in part, its effect on lysosomal functions and autophagy [78]. Consistently, restoration of acidic pH in RPE lysosomes has shown promising results at improving lysosomal dependent

degradative processes [79]. Furthermore, v-ATPase, together with mTORC1 complex, Rag GTPases, Ragulator, and Rheb, is an essential component of the lysosome nutrient-sensing (LYNUS) complex [80]. Under conditions of plenty of food, the v-ATPase complex senses luminal amino acids [81] and recruits mTORC1 to the lysosomal surface where it gets activated by phosphorylation [82]. Active mTORC1 complex is the main kinase negatively controlling autophagy and lysosomal biogenesis. When v-ATPase is inhibited by starvation, mTORC1 is released from the lysosome, becomes immediately inactive by dephosphorylation, and can no longer inhibit autophagy or TFEB nuclear translocation [83, 84]. The latter, by increasing lysosomal number, trafficking, hydrolase content, initiation of autophagy [85, 86], and lipid catabolism [87], facilitates the rapid degradation of a variety of substrates. How A2E inhibition of v-ATPase affects these cascades is not yet understood. Few studies have characterized the status of endogenous mTORC1 and TFEB in the RPE [88–90] and no enough data are available for LB-loaded RPE. A likely scenario is that TFEB activation by LBs provides a first line of defense that is insufficient to address accumulating autophagosomes containing partially degraded POS. However, in the absence of such lysosomal stress response, the RPE might succumb even faster. This model would explain experimental data showing that A2E induced a concentration- and time-dependent protective autophagic response in RPE cell cultures. [91]. Clinical trials using rapamycin, a mTORC1 specific inhibitor, to treat advanced stages of AMD showed no positive results [92, 93].

1.6.3. Lysosomal membrane permeabilization (LMP)

The A2E molecule contains a central pyridinium ring that houses permanently positive amine nitrogen and two long hydrophobic polyene arms. A similar structure is shared by other LBs, including A2-GPE, A2PE, and their isomers. Instead, A2-DHP-PE, all-trans-retinal (ATRD), all-trans-retinal dimer-E (ATRD-E), and all-trans-retinal dimer-PE (ATRD-PE) have non-charged ring cores, although ATRD-E and ATRD-PE have protonable nitrogens that confer them with amphipathic character at low pH. Amphipathic LBs have the potential to intercalate into membranes [22, 94]. Schutt et al [95] investigated the destabilizing effects of A2E on purified lysosomes by measuring the release of luminal β -hexosaminidase to the supernatant. Concentrations as low as 2 μ M induced leakage, whereas plasma membranes were insensitive to much higher concentrations. In support of the idea that amphipathic LBs cause lysosomal membrane permeabilization (LMP) is the observation that RPE cells loaded with lipofuscin granules or A2E into their lysosomes undergo significant LMP [40]. Multiple mechanisms can be responsible for A2E mediating LMP. A2E can act as a surfactant and cause direct membrane damage. De and Sakmar [94] found that A2E-induced leakage of liposomes at concentrations of 200–300 μ M. LMP could also be the result of A2E crystallization within lysosomes, which might cause inflammation by activating a multimolecular signaling complex of the innate immune system, the NLRP3 inflammasome, resulting in a caspase-1-mediated activation and secretion of mature IL1 β family cytokines [96, 97]. Relevantly, A2E accumulation induces NALP3-mediated secretion of mature IL1 β [98]. Fluorescence staining of lipofuscin revealed a membrane bound autofluorescent granule with the bulk of A2E in the lumen rather than in the membrane. Atomic force microscopy shows the core of the granule comprises of solid mini aggregates [99]. Accordingly, we observed A2E (MW 592 Da) in aqueous media

cannot cross 0.10 micron pore size filters with molecular weight cutoff of 300,000 Da. This retention was due to size exclusion as A2E passed through 3 micron filters of the same material (**Figure 5**).

Toxicity of lipofuscin could also involve other less-studied mechanisms, including *mitochondrial poisoning*, as it has been shown that lysosomal A2E progressively leaks into the mitochondrial compartment [100], where it destabilizes the membrane [101] and inhibits oxidative phosphorylation [102], derail of **cholesterol trafficking** [103], **activation of Retinoid Acid Receptor (RAR)**-dependent VEGF secretion in RPE [104, 105], and **inhibition of RPE-65** isomerohydrolase activity, which limits the RPE supply of 11-cis retinal [106]. In summary, although numerous mechanisms of toxicity elicited by pathologic accumulation of LBs in RPE lysosomes have been proposed, no viable therapeutic options have resulted yet from targeting them. Hence, strategies to reduce LB accumulation from RPE have been further investigated.

2. Strategies to reduce lipofuscin accumulation in RPE cells

Alternative strategies to mitigate the cytotoxic effects of LBs involve preventing their accumulation. Two approaches have been pursued (1) to prevent *de novo* formation of LB and (2) to remove previously accumulated LB.

2.1. Strategies that prevent *de novo* formation of LBs

Long-term restriction of vitamin A intake has been shown to reduce retinaldehyde levels in RPE but is not a therapeutic option, since it causes night blindness and systemic hypovitaminosis [107]. In 2005, Radu et al. [108] showed that oral administration of a synthetic form of vitamin A (*fenretinide*), already in use against cancer, acne, cystic fibrosis, rheumatoid arthritis, and psoriasis, could **competitively block RBP4 transport of vitamin A from the**

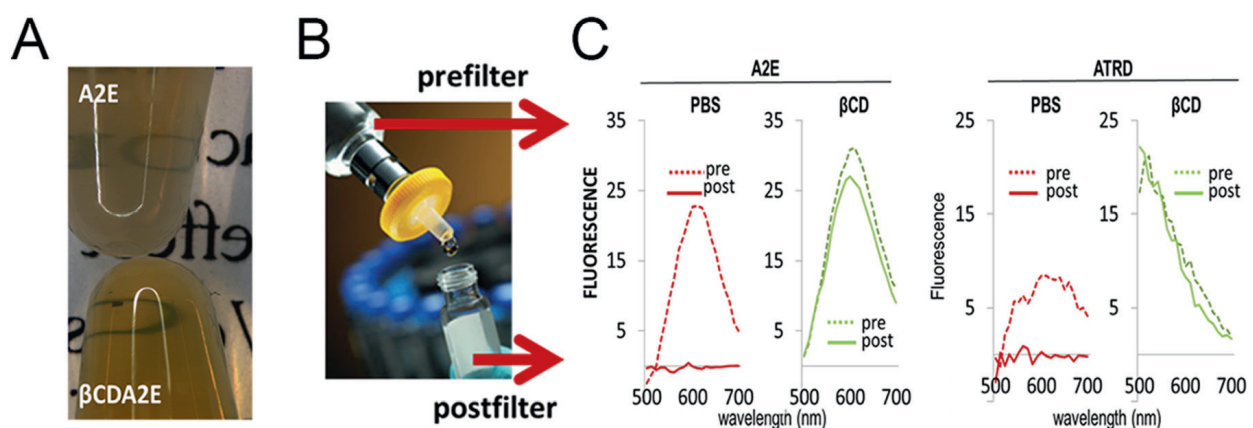


Figure 5. LBs form crystal-like aggregates in aqueous environment. (A) Turbidity of A2E dilutions in water versus 0.1 M β CD. (B) Passage through 0.10 micron pore filters (MW cutoff of 300,000 Da) of LBs in water or β CD solutions. (C) Autofluorescent detection of A2E (MW 592 Da) and ATRD (600 Da) in pre and post filtrates. Retention was due to size exclusion since they crossed 3 micron filters of identical material (not shown).

blood to the RPE. Oral fenretinide produced mild reversible skin dryness and night blindness. However, in 2011, a phase-2 study on 225 AMD patients failed to show beneficial effects. **Oral emixustat hydrochloride** is a synthetic nonretinoid reversible inhibitor of the RPE65 enzyme, which converts all-trans-retinyl to 11-cis-retinal, a rate-limiting reaction of the visual cycle. This drug showed minimal toxicity in phase-1 trials and effectively reduced photoreceptor response to light, consistent with its mechanism of action. However, in May 2016, the results from the phase 2b/3 SEATTLE study did not show any significant difference in retinal degenerative rate or visual acuity changes. **Oral deuterated vitamin A** (ALK-001), is vitamin A modified by replacing hydrogen with deuterium, a safe, nonradioactive isotope. Deuterated vitamin A has lower tendency to spontaneously dimerize into LBs. Long-term, oral administration of ALK-001 to ABCA4^{-/-} reduced the accumulation of lipofuscin and A2E by 70 and 80%, respectively [109]. Assessment of the retina electric response to light signals (electroretinogram) revealed that ALK-001 treatment prevented the gradual loss of visual function observed in the ABCA4^{-/-} mouse. Safety phase-1 clinical trials have been completed but phase-2 is ongoing. It is too early to know whether ALK-001 will be beneficial for Stargardt or AMD patients.

Oral aldehyde traps (VM200, Vision Medicines) constitute of new drugs that react with retinaldehydes forming reversible Schiff bases and thus, reducing the available levels of free aldehydes with cellular amine groups. In preclinical studies, VM200 preserved retinal structure and function of mice retinas in a dose-dependent manner [7]. Safety phase-1 is in progress but there is no effectiveness data in humans.

In summary, none of the visual cycle modulators have made it out of the nearly half a dozen phase 1 and phase 2 clinical trials, so far. They all cause significant night blindness that limit their use. Although they all seem very effective at slowing down the formation of new LBs in animals, they lack effect on previously accumulated LB, which may explain why fenretinide or emixustat did not benefit patients already diagnosed with AMD. Likewise in animal models, where these drugs are given preventively for long periods, humans may need to take them early, i.e., much before the clinical manifestations appear, and for life.

2.2. Strategies for removing previously accumulated LB

Potentially, their main advantage over agents that prevent accumulation of LBs is that they might be administered to patients with Stargardt or AMD who display large buildup of LBs. Most of these strategies are in preclinical stage.

2.2.1. Oral soraprazan (Katairo GmbH)

In 2012, Schraermeyer's group reported successful elimination of lipofuscin from RPE cells in monkey retinas after 1 year of oral administration of the drug. Researchers showed that lipofuscin granules were expelled by RPE cells toward their basolateral side and were cleared by macrophages recruited to the area [110]. Although soraprazan is known to reversibly block the potassium binding site of the gastric H⁺/K⁺ ATPase proton pump, the precise mechanism by which it causes clearance of LB deposits is unknown.

2.2.2. Enzymatic degradation of LBs

Because LBs are refractory to degradation by lysosomal hydrolases, several groups searched for exogenous enzymes with LB destroying activity. Horseradish peroxidase (HRP) was the first one identified [111]. It catalyzes the oxidative cleavage of the polyene-arms of LBs. To test the effectiveness of HRP, cultures of RPE cells preloaded with LBs underwent an enzyme replacement therapy-like treatment with HRP. The efficiency of the clearance was low but the major problem was the considerable amount of highly toxic reactive molecules released as a by-product of the HRP-mediated oxidation of LBs [112]. It was more recently reported in Ref. [113] that neutrophil myeloperoxidase (MPO) catalyzes the *in vitro* degradation of A2E. The authors delivered MPO to lysosomes of RPE cells via mannose-6-phosphate (M6P) receptor. M6P-MOP exhibited a half-life of 10 h in the lysosomes and degraded lysosomal A2E in, but also disrupted lysosomal acidification and triggered lysosomal stress, manifested by the nuclear translocation of TFEB that eventually led to cell death. Thus, the strategy of eliminating LBs with peroxidases seems to be limited by the inherent associated release of detrimental reactive species, which would be equivalent to try to clear LBs by photooxidation.

2.2.3. Beta-cyclodextrins (β -CDs)

β CDs are membrane-impermeant cyclic sugars made of seven glucose residues. They contain a nonpolar central cavity that is capable of accommodating hydrophobic ligands and a hydrophilic outer surface that makes them soluble in water [114]. Several FDA-approved cyclodextrins are currently used to improve the delivery of lipophilic drugs. We demonstrated that β CDs form soluble complexes with LBs [115] (**Figure 5**). *In silico* modeling predicted 2:1 β CD-A2E complex, where one β CD accommodates per arm of LB. We also observed that β CDs reduced the content of A2E from polarized RPE monolayers on Transwell filter cultures and from RPE in the eyes of mice that accumulate massive amounts of lysosomal LBs [116] indicating that β CD treatment can eliminate not only A2E, but also the complex LB mixes found in RPE lipofuscin. The mechanisms by which β CDs induce clearance of lysosomes' content in RPE is yet to be determined but could potentially be optimized to develop a novel therapeutic approach to clear LB-buildups. The mechanism of cholesterol removal by β CDs is one of the best characterized. Likewise LBs, cholesterol forms soluble inclusion complexes with β CDs. In normal cells, cholesterol is more abundantly present in plasma membrane, common recycling endosome and trans-Golgi complex [117]. Removal of cholesterol from these membranes, requires high concentrations (5–10 mM) and prolonged times because β CDs, which have an ~ 8 Å deep cavity, must form stacked dimers (improbable event) to remove 18 Å long cholesterol out of the lipid bilayer and shelter them from the water [118]. In cells with Niemann Pick Type-C defect, i.e., with inactivating mutations in either NPC1 or NPC2 genes, that code for two intra-lysosomal lipid transporter proteins, cholesterol is, also, aberrantly accumulated within lysosomes. Removal of lysosomal cholesterol buildups requires lower concentrations (0.1–1.0 mM) and shorter incubation times with β CD [119]. Furthermore, sulfo-butyl-ether- β CD (Captisol®), a β CD derivative that cannot form stacked dimers and that therefore cannot solubilize membrane cholesterol, can still reduce lysosomal buildups [120]. The model for β CD-mediated removal of lysosomal cholesterol proposes that

β CD enters lysosomes by endocytosis, where it binds free cholesterol in the lumen and shuttle it to the limiting lysosomal membrane [121, 122]. From there, cholesterol is transferred, by a not fully characterized trafficking machinery, that probably involves points of membrane contact between organelles and cholesterol binding proteins [123], to the ER, plasma membrane, peroxisomes, and mitochondria [124, 125]. In the absence of extracellular cholesterol acceptor molecules, the stoichiometric analysis of β CD clearance provides no evidence of cholesterol release to the media but rather indicates a rapid metabolic processing within the cytosolic compartment [126, 127]. In the case of LBs, if β CDs clearance works similarly, then it would be important to see what putative LB-transport system acts thereafter to ship LBs for degradation [85, 128] or to expel them from the cell. Confirmation of such operating trafficking pathway could represent an important advance to identify pharmacological targets for the elimination of lysosomal LBs.

In a mice model of atherosclerosis [129], β CDs have shown effective removal of cholesterol crystals from macrophage foam cells. The mechanism in this case seems to be mostly mediated by the execution of a LXR (liver-X-receptor) dependent transcriptional program response that enhanced the efflux and degradation of cholesterol and reduced inflammation.

Administration of β CD also lowered the levels of amyloid- β in an animal model of Alzheimer Disease [130] and from drusen deposits in animal models of Stargardt disease [131]. The mechanism in these cases is less clear but it seems to be transcriptionally controlled. Accordingly, it will be important to determine if β CDs trigger a transcriptional program that primes RPE cells to eliminate its lysosomal content, independently on whether they form soluble complex with the wasted material. Similarly, fibroblasts from patients with ceroid-lipofuscinosis, the most common cause of neurodegeneration of children in the United States, and cellular or animal models with misfolded α -synuclein accumulation were cleared by β CDs [132, 133]. The mechanism in these cases seemed to be mediated by TFEB [86, 134]. The pathway responsible for β CD activation of TFEB is not defined and is induced by millimolar doses of cyclodextrins. β CDs appear to induce autophagy [135] and exocytosis of lysosomes [136]. Finally, overexpression of activated TFEB has been demonstrated to ameliorate pathology in late-onset neurodegenerative diseases such as Parkinson, Huntington and Alzheimer, as well as in models of spinal and bulbar muscular atrophy and to clear deposits in lysosomal storage disorders (LSDs) [83, 86, 137–139]. Thus, it will be important to determine whether TFEB is necessary or if it can synergistically contribute to the clearance of RPE lipofuscin.

3. Conclusions

Buildup of lipofuscin in RPE lysosomes often evolves into irreversible damage of overlying photoreceptors. This is a common event in individuals with mutations in ABCA4 gene and is believed to underlie the progression of age-related lesions in AMD people, the most common cause of blindness in the elderly population. Unfortunately, the great majority of patients diagnosed with this problem have no therapeutic options available. Analysis of RPE lipofuscin, identified as major components lipid bisretinoids (LBs), sub-products of the spontaneous dimerization of retinaldehydes produced during the visual cycle. Targeting LBs secondary

complications with oral antioxidants, inhibitors of complement or autophagy inducers provided no or little beneficial effect. The recent failures in clinical trials with visual cycle modulators, which prevent *de novo* formation of LBs may reflect the incapacity of these drugs to stop degeneration once LB-accumulation has been established, which is probably the case for most individuals with clinical symptoms. Hence, the development of novel strategies to permanently remove previously accumulated lipofuscin is an urgent medical need. The characterization of LBs as the core-components of RPE lipofuscin has permitted to rationally develop strategies to remove them from RPE cells in the laboratory. This, combined with an improved understanding of the molecular pathways that govern autophagy and stimulate cellular clearance might allow in the near future, to develop improved therapies for retinal degenerations resulting from genetic or age-related retinal lipofuscin accumulation.

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