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Review

The essential role of light-induced autophagy in the inner choroid/outer retinal neurovascular unit in baseline conditions and degeneration

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Abstract: In age-related macular degeneration (AMD) autophagy dysfunction occurs in the outer retinal segments and inner choroid to induce cell loss and extracellular aggregates. Therefore, a detailed analysis of the autophagy status encompassing the choriocapillaris (CC) and the retinal pigment epithelium (RPE) including the interposed Bruch's membrane (BM) is key to understand the fine anatomy and altered biochemistry, which underlie the onset and progression of AMD. The present article discusses the role of altered autophagy both within inner choroid (including endothelial cells and pericytes of CC), BM, and outer retina (RPE and distal segment of photoreceptors). Here autophagy is needed to maintain the metabolic requirements and to provide the specific physiological activity sub-serving the process of vision. Activation of autophagy within RPE strongly depends on light exposure and it is concomitant with activation of outer segment of photoreceptors and CC, which allows increased blood flow and metabolic support. Thus, it is not surprising that the inner choroid and outer retina are mutually dependent and their activity is orchestrated by light exposure to cope with metabolic demand. This is tuned by the autophagy status which works as a sort of pivot in the cross-talk within the inner choroid/outer retina neurovascular unit.

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1. Introduction

The autophagy machinery is fundamental to preserve the integrity of the outer retinal segments and inner choroid and to promote visual processing. This is mainly due to biochemical cascades placed downstream to autophagy activation, which consist of various pathways mostly placed within retinal pigment epithelium (RPE). Nonetheless, other

structures placed at the choroid-retinal border are influenced by the stimulation of autophagy machinery. In detail, autophagy is abundant at this level, where actual phototransduction takes place, which is in need of trophic support, and protein/organelle turnover to constitute a sort of morpho-functional unit, which may be defined as inner choroid/outer retina neurovascular unit (red bracket of Figure 1).

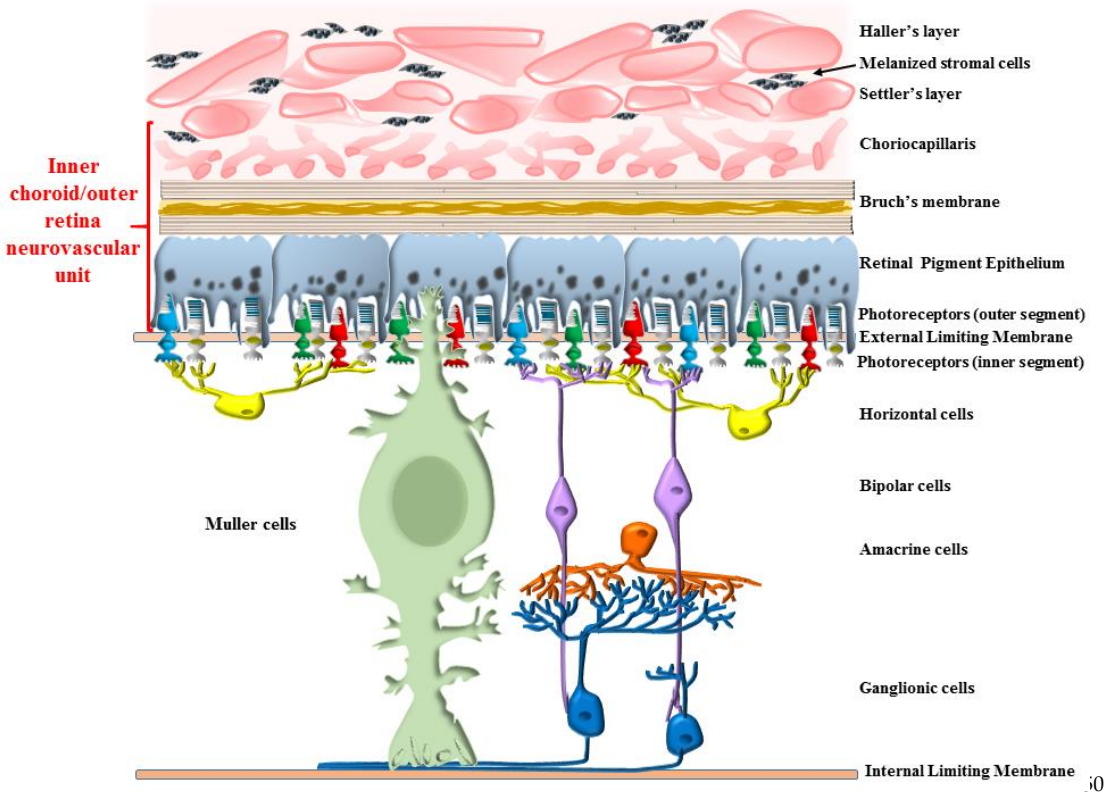

Figure 1. Anatomical placement of the inner choroid/outer retina segment. The cartoon focuses on structures placed at the interface between choroid and retina. These are anatomically connected and functionally bond together to cluster a metabolic unit and are named inner choroid/outer retina neurovascular unit, which is shown in red bracket. These structures include the choriocapillaris (CC), where pericytes and endothelial cells occur being separated from RPE by the Bruch's membrane (BM). The retinal side features RPE cells which embrace the distal segment of photoreceptors. The metabolic demand of these connected compartments is driven by the amount of light, since this promotes photo-transduction in the photoreceptors, protein and organelle turn-over in the RPE cells, blood supply and trophic support from the CC. All these activities, which are unique for each cell type, seem to be orchestrated synergistically by the tuning of autophagy flux. The main scopus of the review is to analyze in depth autophagy-dependent interactions in this retinal area.

In fact, in the recent elegant study by Ramachandra Rao and Fliesler [1] ongoing autophagy in the whole retina was analyzed including neurons, glia and endothelial cells. In this study, the amount of autophagy prevails at large in the outer retinal segment (Figure 2). This corresponds to the site, where the turn-over of autophagy-related substrates need to be stronger and faster.

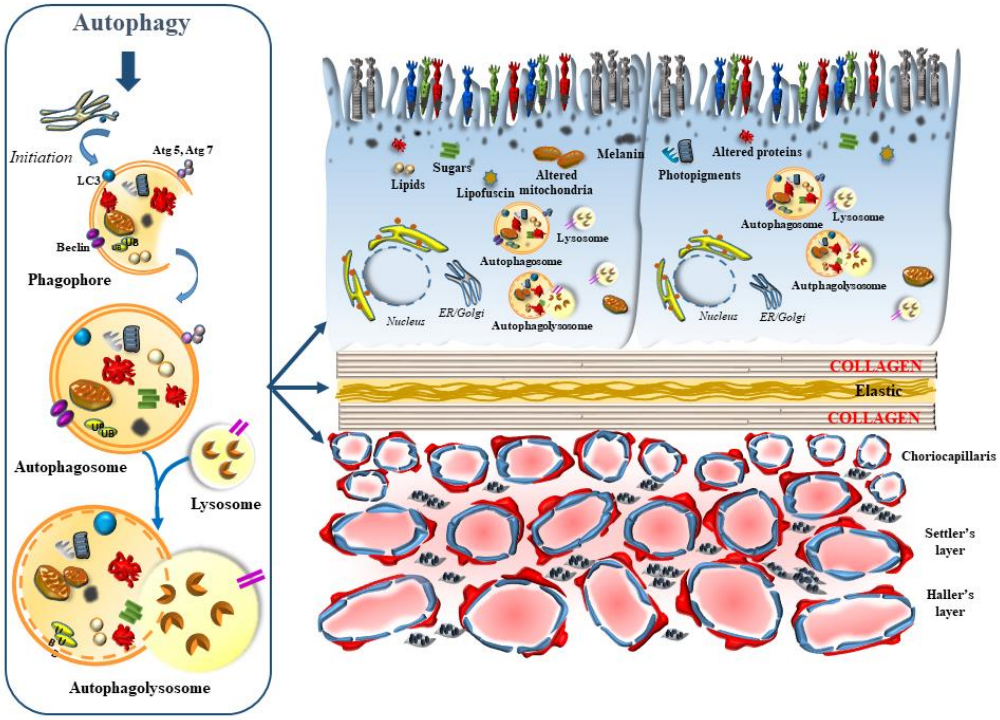

Figure 2. The autophagy pathway sustains the integrity of the inner choroid/outer retina segment. In the retina the strongest autophagy activity takes place in the outer regions and mostly within RPE cells. These cells feature the highest amount of autophagy-related organelles and the highest autophagy turn-over. In fact, the staining for autophagy-related proteins such as LC3 and the amount of small lysosomes, autophagosomes and the merging between these organelles to form autophagolysosomes is elevated. These autophagy-related structures are responsible for clearing the cells from altered mitochondria, sugars, lipid droplets, misfolded proteins, lipofuscin, melanin, and photo-pigment along with aged outer segment of photoreceptors. Similarly autophagy is key in the choriocapillaris and produces some effects at the level of the Bruch's membrane.

If one analyzes autophagy structures at this level, autophagosomes and lysosomes are small and abundant to cope with the need to provide a quick cycling of these organelles intensely involved in the clearance of altered mitochondria, protein lipids and sugars along with melanin and photopigments. Thus, it is not surprising that, among all retinal layers the RPE possesses the highest autophagy rate [1]. This is fundamental in keeping steady retinal integrity during baseline conditions and mostly in counteracting retinal degeneration. This is also evident in experimental models were specific autophagy-inducing genes such as *ATG 5* and *ATG 7* are knocked out [2]. This is key at the onset and during the course of age-related macular degeneration (AMD). In fact, when knocking down these autophagy-related genes specifically within RPE cells, the occurrence of retinal abnormalities mimicking those described in human AMD is consistently documented [2] (Figure 3). In fact, in these models the genetic suppression of autophagy produces alterations, which are reminiscent of early stages of AMD. These similarities include stagnant vacuoles within altered RPE cells, which modify their shape and size along with the amount of pigmentation, and occurrence of frank RPE cell death (Figure 3).

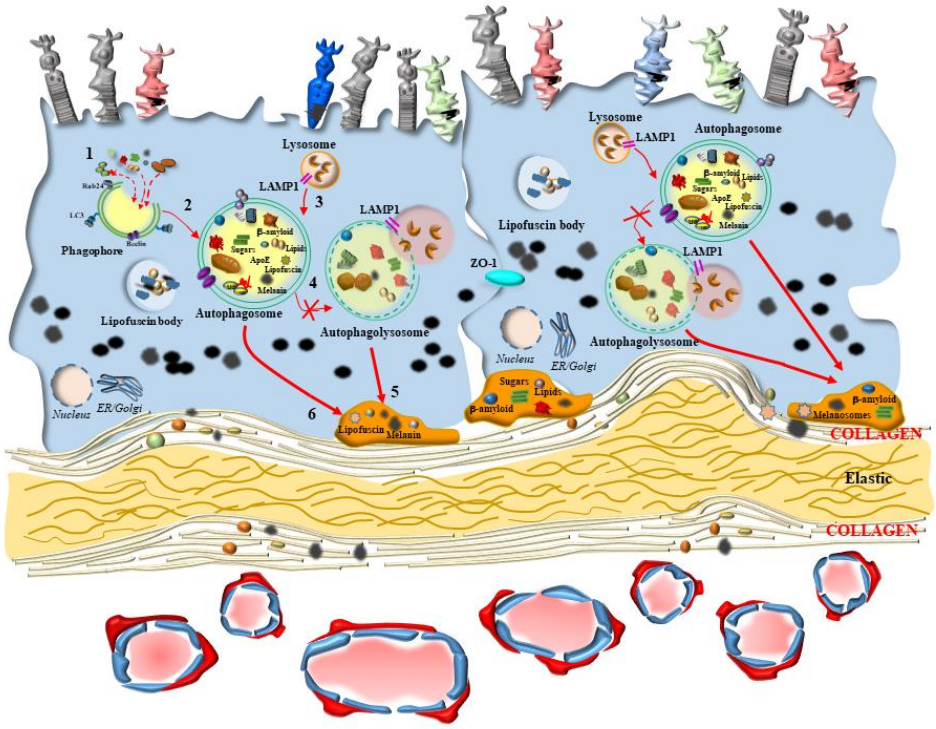

Figure 3. Autophagy impairment in RPE mimics AMD. When autophagy is impaired selectively within RPE cells, a number of pathological effects mimicking the fine neuropathology of AMD are induced. These include stagnant vacuoles within altered RPE cells, which modify their shape and size along with the amount of pigmentation. The autophagy pathway is defective already during early stages when phagophore (1) should starts vacuolization to proceed towards autophagosome formation (2). A defect of merging between autophagosomes (2) and lysosomes (3) leads to a defective generation of autophagolysosomes (4). Nonetheless, the massive defect in lysosome clearance is in excess compared with a decrease in autophagolysosome formation. Therefore, slowed autophagy flux leads to an accumulation of autophagolysosomes. These abundant stagnant vacuoles include lysosomes and autophagosomes, which increase their size, and persist within the cells without any further clearance or they are released towards the Bruch's membrane to accumulate in the process of drusen formation. In fact, altered autophagosomes (5) or lysosomes (6) may be extruded through exocytosis or as myelinosomes. These process (5, 6) is thought to generate extracellular drusen, which feature non-digested substrates of the lysosomes such as melanolipofuscin, lipids, sugars, misfolded proteins, including beta-amyloid, and altered mitochondria. The distal segment of RPE cells reduces cell processes and melanin content is more clustered within the cells and it is no longer abundant between distal segments of photoreceptors. The space between RPE cells, which is normally absent due to the occurrence of abundant tight junctions, becomes evident following autophagy inhibitions due to a loss of specific tight junction proteins such as *zonula occludens* protein 1 (ZO1).

These models were developed in *ATG5* and *ATG7* conditioned knock out, where gene manipulation was specifically carried out within RPE cells. Some alterations, which occur in wet AMD such as neovascularization and inflammation are rarely observed in these models. This suggests that autophagy dysfunction within RPE cells may reproduce most of the features, which characterize dry AMD, although it is likely that additional structures need to be affected within the choroid/retinal border to fully develop the whole pathological hallmarks of AMD, including the wet variant. At this aim, the role of autophagy within the inner choroid deserves specific attention as well.

The seminal role of autophagy in sustaining retinal integrity during AMD is likely to be naturally induced by the beneficial effects of light pulses and light-sensitive phytochemicals, which are reported to maintain retinal structure and visual acuity as well as to counteract retinal degeneration [3-18]. The amount of chemical species which undergo conformational changes and the number of organelles, which are affected during high metabolic turn-over of this part of the retina require a powerful and fast replacement, which is based on their effective removal by a powerful autophagy pathway [19]. This applies to proteins, lipids and sugars and extends to specific autophagy-dependent organelles such as mitochondria. All these structures may represent a target of metabolic dysfunctions which may produce free radicals, altering DNA integrity, disrupting tissue structure and sustaining retinal degeneration [20-23]. In the retina, as well as in the central nervous system [24,25], autophagy activation significantly depends on the regulation of the molecular complex named mechanistic target of rapamycin (mTOR).

In fact, mTOR is altered within RPE and inner choroid in the course of AMD [26]. In detail, the regulation of autophagy activity by mTOR appears to be orchestrated in synergism within the choroid domain (CC) and retinal border (RPE) [26]. In this way, the autophagy status would produce simultaneous and synergistic effects on both sides of the Bruch's membrane (BM) under the modulation of mTOR. When autophagy-mediated enzymatic clearance of altered organelles and chemical species is engulfed by an excess of substrates, RPE cells may use an alternative pathway to clear non-digested material by promoting cellular extrusion (Figure 3). In this way, polymorphous inert aggregates within RPE may be released as myelinosomes, recently described by Yefimova [27]. In this way, myelinosomes add on the high intercellular trafficking of altered structures at the retinal choroid border in the course of AMD, consisting of engulfed lysosomes, autophagosomes, and exosomes. These structures operate in the RPE of the retina in order to provide proteostasis and clearance of damaged organelles, which is seminal to keep retinal integrity, as described by Ferrington et al. [28]. In most cases, the metabolic polarization of RPE cells extrudes material towards the BM, which separates the outer retina from the inner choroid. This alters the permeability and morphology of the BM. In fact, during AMD as well as experimental models developing AMD a thickening of the BM is described [29]. These Authors demonstrated that thickening of BM in a model of AMD is concomitant with a decrease in autophagy activity within RPE cells as shown by alterations of microtubule-associated protein 1A/1B Light Chain 3 (LC3) and (Lysosomal-Associated Membrane Protein 1) LAMP1 labeling. These cells develop an altered LC3-II/LC3-I ratio as an index of an impaired autophagy flux, which is concomitant with altered amount of specific autophagy-related proteins [29]. One may consider the alterations of the BM as the mere consequence of altered autophagy within RPE cells. However, in persons affected by AMD, alterations of the BM are associated also with a defective autophagy in contiguous inner choroid at the level of pericytes and endothelial cells within CC [30]. Pericytes alterations are presently underscored since recent evidence shows that it may anticipate alterations within endothelial cells of the CC. Degenerating inner choroid produces massive vesicular and fibrillary aggregated materials moving towards the BM [31,32]. Thus, it appears that the thickening of the BM in the course of AMD may be indeed the consequence of both an external perturbation coming from the inner choroid (CC) and an internal alteration coming from the outer retina (RPE) (Figure 4).

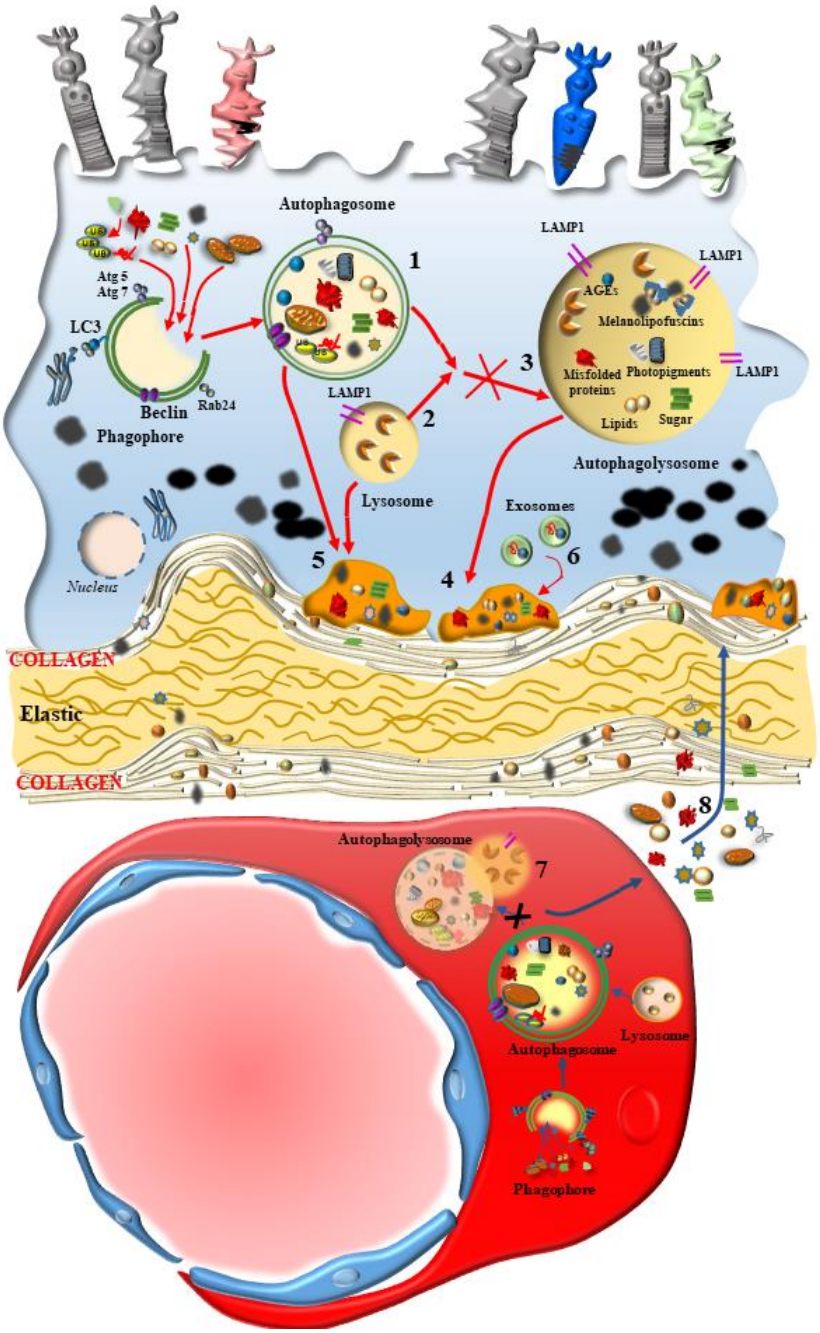

Figure 4. Bipolar alterations converge on altering the Bruch's membrane (BM).The occurrence of alterations in the BM in the course of AMD may depend on converging pathological factors coming either from RPE cells and/or from the CC. In detail, the disruption of the thin collagen and elastic layers of BM may be concomitantly generated by the drusen components (described in Figure 3), which contact and disrupt BM. Namely, altered and stagnant autophagosomes (1) and lysosomes (2) formed in the RPE may produce stagnant and persistent intracellular RPE autophagolysosome vacuoles (3) and being extruded towards the BM both as authentic organelles (4; 5) or upon commitment towards the exosome compartment (6) to deliver non-digested toxic species, which may potentially alter the structure of BM. Concomitantly, similar effects occur within cells of CC. In fact, altered autophagy in the CC generates again stagnant autophagolysosome vacuoles (7), which may be extruded as well towards the BM (8). In this way, converging bipolar mechanisms concur to produce BM alterations. The relevance of those factors coming from an altered autophagy within RPE compared with those produced by an altered autophagy within CC is likely to depend on specific isotype of AMD, where systemic risk factors are more effective in altering the autophagy status in the CC, whereas an intrinsic retinal defect is likely to be more relevant in altering RPE cells. In

most cases, both sides are engaged since systemic risk factors add on the frail autophagy equilibrium of RPE cells which progressively deteriorates during age.

Thus, an orchestrated defect in clearing mechanisms placed at the choroid-retinal border is likely to produce a deleterious synergism in the course of AMD fostering the accumulation of altered mitochondria, photopigment, melanin, sugars, lipids and proteins to form lipofuscin and their accumulation into drusen. In fact, in the presence of altered clearance, the enhancement of drusen formation between the BM and RPE can be observed [33-35].

The contribution of various clearing pathways within various structures of the choroid-retinal border is analyzed here. This review focuses on the functional interactions which take place at the anatomical border between choroid and retina, where a dysfunctional RPE may perturb CC and *vice-versa*, under the effects of a site-specific defect of autophagy. This pathway working at the border between inner choroid and outer retina defines and regulates a sort of neurovascular unit. This anatomical complex is key for autophagy to maintain anatomical integrity and visual processing, while in AMD it represents the pathological unit driving retinal degeneration.

2. The role of autophagy in the choriocapillaris (CC)

The autophagy occurring within endothelial cells of the CC is seminal to sustain the integrity of the retina (Figure 4). In fact, protein and mostly mitochondrial removal within CC endothelial cells counteracts ongoing injuries. In keeping with autophagy, the removal of altered mitochondria from the CC through mitophagy is critical to prevent and counteract retinal degeneration. This occurs by preventing the fragmentation of stagnant damaged mitochondria, which instead are removed by an effective mitophagy. In this way, the microvascular damage in the retina is prevented [36] (Figure 5). Thus, the inner choroid represented by the BM and the CC contributes to sustain the integrity of the outer retina.

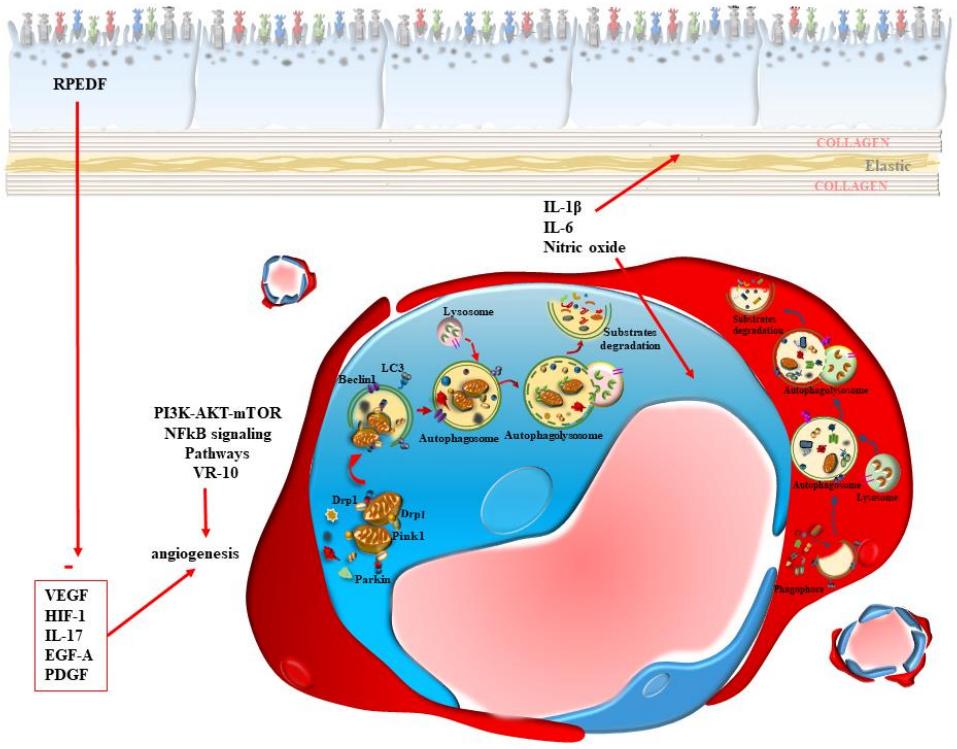

Figure 5. Within CC, autophagy is key in both endothelial cells and pericytes. Within CC the autophagy status is altered leading to accumulation of dysfunctional mitochondria where the mitophagy proteins Dynamin-related protein 1 (Drp1), PTEN-induced putative kinase1 (PINK1) and Parkin no longer interact to promote mitochondrial clearance within nascent autophagosomes. A defect of autophagosomes formation and a failure of their progression towards lysosomes generates a defect in the clearance of a number of organelles and chemical species. Analogous effects involve the pericytes, where these alterations appear to anticipate the dysfunction of endothelial cells. These phenomena, apart from producing a damage to the BM due to the accumulation of non-digested material at the choroid side of the BM itself, produce the synthesis of a number of compounds which exert a powerful angiogenetic effect. This contribution of a defective autophagy in the CC exerts a prominent role in causing AMD in the course of systemic disorders such as diabetes, hypercholesterolemia, metabolic syndrome, and other specific metabolic disorders. Nonetheless, the dysfunctional CC may be induced by primary alteration within RPE cells. In fact, alterations of RPE may produce trophic factors stimulating angiogenesis such as Vascular Endothelial Growth Factor (VEGF) and Platelet-Derived Growth Factor (PDGF) in the course of retinal degeneration by acting on the CC to induce endothelial cells and pericytes proliferation, migration and angiogenesis to shift AMD from a dry to a wet variant. Similarly, a precursor of Epidermal Growth Factor may support angiogenesis via phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)-mTOR and Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathways. Similarly, the expression of VEGF may derive from endothelial cells to stimulate angiogenesis via acting on the AKT/mTOR pathway. It is remarkable that healthy RPE cells may counteract these angiogenetic effects by releasing retinal epithelial pigment-derived factor (REPDF), which inhibits the very same angiogenetic pathway. The cross talk between the CC and RPE components may also act in opposite direction, from the CC to the RPE. In fact, CC cells upon autophagy inhibition may synthesize interleukins such as IL-1β, and IL-6 along with nitric oxide. These molecules may activate the inflammasome focally, within CC although, when AMD shifts towards the wet form, they migrate through the BM to activate macrophages passing through newly generated blood vessels in the disrupted outer retina.

In detail, the physiological role of the CC is bound to the presence of appropriate blood vessels to maintain the viability and the high metabolic rate of RPE cells. The ability to produce these effects is bound to the integrity and permeability of the BM. In this way, the inner choroid and outer retina work in symbiosis to provide anatomical integrity of the retina and sustaining visual function. According to Edwards and Lutty [37], a functional unit can be described, which is composed of the following structures: (i) outer segment of photoreceptors; (ii) RPE cells; (iii) BM; (iv) CC. It appears that these structures, which are orchestrated by specific metabolic pathways need to work as a single physiological unit, which loses its function in the course of retinal damage and mostly in the course of AMD. In fact, during AMD the CC early loses its function and integrity [37]. Cai et al. [38] described how a combined defect of the autophagy stimulator microRNA-29 occurring within RPE and the choroid vessels occurs in AMD. This may lead to autophagy inhibition concomitantly in the inner choroid and outer retina. Autophagy inhibition is known to promote neovascularization starting from CC under the effects of inflammasome activation within macrophages which can be induced by Interleukin-1beta (IL-1β), IL-6 and nitrite oxide [39,40]. This is why in baseline conditions, mTOR dependent autophagy activity in the CC is crucial to modulate the permeability for specific molecules, which may or may not cross the CC to reach the outer retina. In addition, autophagy modulates angiogenesis in the retinal choroid border [41]. Although so far most manuscripts attribute these roles of the CC to endothelial cells, mounting evidence is now demonstrating the seminal role of pericytes and the engagement of this cell type at early stages of AMD as the first cell responsible for the alteration occurring in the CC, when endothelial cells are not involved yet. Each cell type of the CC will be analyzed separately.

2.1. A focus on endothelial cells

Viability of choroid-retinal endothelial cells is profoundly affected by the autophagy status both in baseline conditions and during systemic metabolic disorders such as diabetes [41] or familial hypercholesterolemia which may trigger AMD due to increased sugars and lipoproteins producing a damage to endothelial cells through autophagy inhibition, which in turn can be rescued by autophagy activators [42]. It is not surprising that in the course of these metabolic disorders, within CC the autophagy status is altered within endothelial cells as shown in most vascular districts of the body (Figure 5). Autophagy dysfunction alters the mitochondrial status and the lysosomal activity within endothelial cells [42]. Specifically, endothelial cells of the inner choroid (CC) are rescued by autophagy activation, as shown by Zhang et al. [36]. These authors provided evidence showing that endothelial cells, during retinal degeneration undergo autophagy suppression, which inhibits mitochondrial removal and lipidation of LC3II and p62 degradation. These effects can be replicated by the autophagy inhibitor 3-methyladenine (3-MA), while they are counteracted by the autophagy activator rapamycin [36]. In addition, the specific autophagy inhibitor 3-MA is deleterious for endothelial cells since it worsens the damage induced by high glucose levels, which explains why diabetes by causing an autophagy-dependent damage to the CC may foster the onset of AMD. Consistently, the mTOR inhibitor, autophagy activator rapamycin is able to rescue the endothelial damage in the choroid retinal vessels [36]. The mitochondrial removal is known to be activated by the autophagy-dependent protein PTEN-induced putative kinase1 (PINK1) and parkin. In fact, a strong trigger for mitochondrial removal is produced by the PINK1/parkin interaction at mitochondrial level [43]. Such an interaction is impeded during retinal degeneration. This is why silencing of PINK1 aggravates retinal damage. This is in line with a defective amount of endothelial Dynamin-related protein 1 (Drp1), which promotes mitochondrial fission and mitophagy, during retinal degeneration. In diabetes, Drp1 is detached from mitochondria and it is no longer able to promote mitophagy due to an excess of hexokinase II (HK-II), which removes Drp1 from mitochondria and prevents its interaction with PINK1 to trigger mitophagy [36]. It is remarkable that alterations of newly formed blood vessels, which may be induced in the retina by systemic administration of the diabetes-inducing compound streptozocin mimics those occurring in AMD such as vascular leakage and abnormal newly-formed blood vessels. These alterations can be reversed by the autophagy activator rapamycin [36]. Alterations of the CC may also derive from specific molecules released from neighboring RPE cells. In line with this, it is well-known that trophic factors stimulating angiogenesis such as Vascular Endothelial Growth Factor (VEGF) and Platelet-Derived Growth Factor (PDGF) are produced in the course of retinal degeneration by RPE cells when a shift from dry to wet form of AMD takes place. The release of VEGF and PDGF from RPE cells is inhibited by autophagy activators such as rapalogs, which depress the primary transcript of these proteins, which in turn would induce endothelial cells towards increased proliferation, migration and angiogenesis [44]. This is confirmed by using the natural autophagy activator resveratrol, which inhibits Epidermal Growth Factor precursor homology domain A (EGF-A) via suppression of phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)-mTOR and Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathways. In detail, resveratrol inhibits the phosphorylation of the inhibitor of nuclear factor kappa B (IκB), which inhibits NF-κB [45]. In this way NF-κB cannot migrate any longer to the nucleus, where it stimulates the transcription of VEGF-A [45]. Similarly, the natural autophagy activator berberin inhibits the expression of VEGF from retinal endothelial cells via acting on the AKT/mTOR pathway [46].

2.2. A focus on pericytes

The powerful effects of autophagy in suppressing the proliferation and migration of endothelial cells is backed up by analogous effects on pericytes, which are present in the

CC and proliferate as well in AMD. In fact, the failure of anti-VEGF based therapy is partially due to the residual activity of proliferating pericytes as hypothesized by Asani et al. [47] (Figure 5). Although being missed out for a long time, pericytes are seminal in the course of AMD, mostly considering the wet variant and AMD induced by systemic metabolic disorders. In fact, the mTOR inhibitor, autophagy inducer rapamycin exerts a powerful effect to suppress the amount of pericytes, and inhibits pericytes proliferation in the CC. These mechanisms occlude neovascularization from the choroid to the outer retina, which otherwise occurs in the course of wet AMD (Figure 5). In fact, the activity of mTOR is increased to suppress autophagy in the course of neovascularization within wet AMD [48]. The anti-angiogenic effects of autophagy induction are presently under investigation to design novel compounds, which may be useful in suppressing the excess of angiogenesis occurring in wet AMD. This is the case of the thrombospondin-1 synthetic peptide VR-10, which increases the LC3II/LC3I ratio and degrades p62 being a powerful autophagy inducer [49]. This peptide upregulates the anti-angiogenetic factor Retinal Pigment Epithelium-Derived Factor (RPEDF), while downregulating pro-angiogenetic factors (VEGF, HIF-1 and IL-17) [49]. In line with autophagy-based treatments of abnormal genesis of blood vessels, a number of molecules acting as autophagy inducers are currently under evaluation in the course of choroidal neovascularization [50]. The analysis of CC in relationship with its effects on the autophagy machinery in the course of AMD naturally drives attention to the role of this clearing pathway within endothelial cells. This is naturally due to the development of endothelial cell proliferation in the course of neovascularization, which occurs in the inner choroid to surpass the BM and invading the outer retina, when AMD shifts to the wet variant. However, as previously mentioned, in addition to endothelial cells, pericytes promote neovascularization, through a significant role in the blood vessels of the CC (Figure 5). So far, pericytes were poorly investigated in AMD and they are impaired, even when the damage to endothelial cells is not evident yet. This is why in the present review a special focus is posed on these cells.

Pericytes are placed in the CC to surround externally the fenestrated endothelial cells sharing the same basal lamina (Figure 5). The inherent structure of the CC features a considerable defect in the amount of pericytes since they do not ceil fully the perimeter of the capillaries, while an absence of pericytes is noted externally to endothelial cells. This may be a natural vulnus in the anatomy of the CC blood vessels, which fosters the development of AMD. In fact, drusen deposits apart from aggregating just below the membrane of RPE can be detected across the BM, among inner collagen fibers to form linear deposits described by Curcio and Millican [51] (Figure 4). The occurrence of deposits in the form of vesicles and fibrils is evident in aged inner choroid, which poses the question on whether an extended distribution of extracellular aggregates external to the BM takes place in the genesis of AMD. The role of abnormal perycites was postulated in the progression of this condition. In a recent paper Nag and colleagues described the occurrence of alterations of pericytes in the course of neovascularization [30]. In these conditions, pericytes feature dark mitochondria and cell processes getting in touch with debris at the level of the basal membrane close to BM. At this stage, pericytes lose their basal lamina and intermediate filaments, even when the endothelial cells are still intact. In this way, two domains can be identified at the choroid/retinal border where debris and aggregates can be formed (Figure 4): the classic one, just beneath the RPE, and the other one within the choroid, which is supposed to proceed through the BM in the wide space between outer collagen fibers to disarrange the elastic lamina (Figure 4). It is postulated that these extra-cellular aggregates may be the earliest event in altering the morphology of pericytes within the CC. Alternatively, due to the close space between pericytes and early choroid aggregates, it may be postulated that a metabolic defect at the level of the pericytes may be responsible for extracellular accumulation of vesicles and fibrils in the choroid. In fact, early occurrence of undigested autophagy substrates within pericytes may witness for a defective metabolism of Advanced Glycation End products (AGEs), proteins and lipids (Figure 6). These AGEs are found to be abundant around pericytes within choroid debris.

This is concomitant with a loss of intermediate filaments from pericytes and mitochondrial alterations. These phenomena are associated with a defective autophagy machinery. In fact, within pericytes the abundance of intermediate filaments is related to the presence of effective autophagy, which confers protection to this cell type [30]. These findings suggest that even subtle alterations of pericytes, under the effects of a defective autophagy, may promote endothelial alterations, which develop progressively during the disease course. The vulnerability of pericytes in AMD may be also the consequence of degenerating choroid melanized stromal cells (so-called *lamina fusca*), which loses its melanin content during age, making it more deleterious the impact of free radicals formed either by a greater diffusion of ultraviolet quanta during light exposure or from circulating oxidative species. These extracellular factors add on systemic metabolic disorders and specific autophagy-dependent intra-cellular defects of pericytes in the CC. In fact, pericytes in baseline conditions feature a lower amount of ongoing autophagy machinery compared with contiguous endothelial cells [30]. This may explain why, within CC, pericyte degeneration may precede alterations of endothelial cells in the course of AMD. In line with a selective autophagy defect, alterations occur early within CC pericytes during AMD (as shown by representative Figure 6).

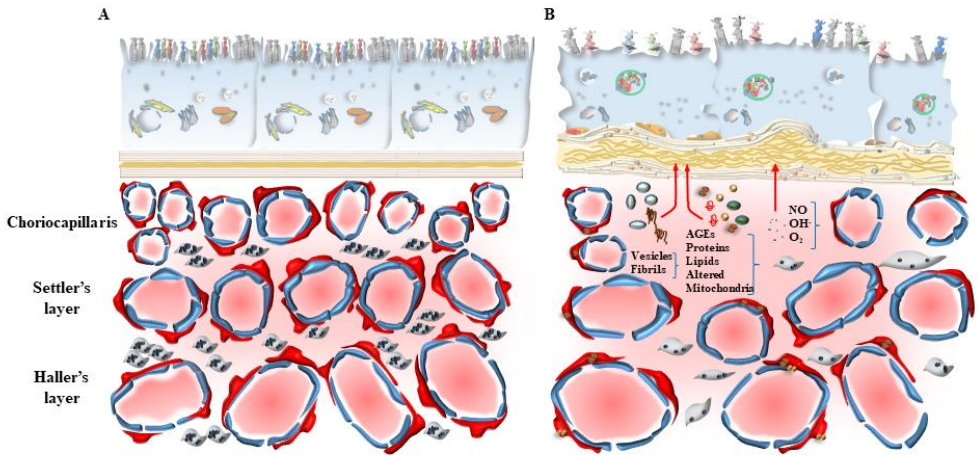

Figure 6. The autophagy pathway in the normal (**A**) and diseased (**B**) choriocapillaris. An autophagy defect in the CC is primarily sensed by pericytes, which feature early occurrence of undigested autophagy substrates witnessing for a defective metabolism of Advanced Glycation End products (AGEs), proteins and lipids. These AGEs are found to be abundant around pericytes within choroid debris. This is concomitant with a loss of intermediate filaments from pericytes and mitochondrial alterations. These phenomena are associated with a defective autophagy machinery. In fact, within pericytes the abundance of intermediate filaments is related to the presence of effective autophagy, which confers protection to this cell type. The vulnerability of pericytes in AMD may be the consequence of degenerating choroid melanized stromal cells (so-called *lamina fusca*), which worsens the impact of light- or systemic-induced free radicals.

3. Dysfunctional effects of impaired CC autophagy

This may explain why polymorphous aggregates may develop on both sides of BM in advanced stages of AMD. In fact, an updated perspective considers drusen beyond

their protein and beta-amyloid content as a complex aggregate of lipid droplets, sugars, melanin, lipofuscin, chromatin vesicles (spheroidal structures that are made of extra-nuclear DNA leaking out of the nucleus), degenerated melanosomes, degenerated mitochondria, aqueous vesicles, and ultrafine cellular fragments. These drusen are expected to disrupt the BM causing collagen degeneration and loss of elastin, which in turn produces a loss of anatomical integrity. This allows drusen content and cellular debris from necrotic RPE cells to move across BM from the retina towards the choroid [52]. The scenario emerging from these data leads to consider the option that, in the course of AMD, extra-cellular material may accumulate *de novo* within the choroid side of the BM. In fact, when considering the etiology of AMD, diabetes and familial hypercholesterolemia are strong predisposing factors. In these conditions, a primary autophagy defect within retinal structures may not be present; however, a defective autophagy may rather depend on extra-retinal compounds which reach up the choroid-retinal border through the blood stream, within the most intimate choroid layer, the CC, which is expected to undergo the primary metabolic dysfunction triggering retinal degeneration. In these conditions, circulating extracellular chemical species may trigger the impairment of autophagy, which initiates just in the CC, which is the place where the blood delivers these compounds. In line with this, the occurrence of Low Density Lipoproteins (LDL) within CC endothelial cells is key in regulating the autophagy pathway. In fact, as shown by Torisu et al. [53] baseline endothelial autophagy is required to maintain vascular lipid homeostasis. In detail, while low levels of LDL produce a stimulation of the autophagy flux, when LDL is in excess, autophagy structures are engulfed. This phenomenon is worsened when endothelial cells own a decreased expression of the autophagy gene *ATG 7*, which witness how important is the autophagy activity within endothelial cells to handle properly an excess of circulating lipids. When this process is impaired, autophagy substrates recruit the neighboring RPE cells [53].

In fact, Chang et al. [54] found that non-saturated lipid chains such as oleic acid trigger specific angiogenic factors such as VEGF and basic Fibroblast Growth Factor (bFGF) along with stimulating AMP-activated protein kinase (AMPK)/mTOR/ Ribosomal protein S6 kinase beta-1 (p70S6K) signaling and inducing hyperphosphorylation of mitogen-activated protein kinase (MAPK) pathway, such as Extracellular signal-Regulated Kinase (ERK), Jun N-terminal Kinase (JNK), and p38 MAPK, as well as NF-κB activation. All these events are implicated in choroid neovascularization, which occurs in wet AMD. Similarly, in this condition other AMD reminiscent abnormalities, such as basal laminar deposits, thickening of BM with drusen-containing deposits, RPE and photoreceptor degeneration, are described following a high fat diet in a mouse model heterozygous for the Peroxisome proliferator-activated receptor Gamma Coactivator 1-alpha (Pgc-1α). These Pgc-1α+/- mice following a high-fat diet undergo autophagy suppression with decreased mitochondrial activity and increased levels of reactive oxygen species and inflammatory response in the retina [55]. Even in this context the interaction of choroid-derived blood constituents with RPE cells is critical. For instance, co-culturing damaged RPE with blood-derived macrophages leads macrophages to release pro-angiogenic factors through the activation of inflammasome [39].

4. What about a specific role for autophagy in BM?

In summary, the relevance of autophagy in the inner choroid in regulating the integrity and pathology of retinal degeneration remains to be largely investigated. Even the BM is affected by autophagy impairment since upon autophagy inhibition the BM undergoes progressive thickening [29]. However, it remains non-explored whether such an effect is indirect, due to a leakage of substrates from the CC and/or the RPE (Figure 4), or the structure of the BM is specifically and directly regulated by the autophagy machinery.

The BM alterations which are typical of AMD are mostly related to the impaired autophagy in the CC at the level of both pericytes and endothelial cells [30]. This is confirmed by the onset of a specific disorder of the CC where increased angiogenesis occurs along with altered structure of the BM [56].

Despite an increasing number of investigations, the regulation of autophagy in the inner choroid remains largely non-explored and deserves further studies. When considering the most internal membrane classified as part of the choroid, the BM, evidence is provided that autophagy reverts the thickening of the BM [29]. During retinal degeneration the dysfunction of the BM appears to be closely related to an autophagy-dependent alteration within pericytes and endothelial cells of CC [30]. In fact, a rare disorder of the CC, with increased angiogenesis and bleeding, produces macular drusen deposition [56]. The data produced so far concerning the impairment of the BM during autophagy inhibition are still in need of further investigation [57]. In fact, to our knowledge no specific experimental effort has been carried out to measure how autophagy may alter the synthesis and release of collagen fibers from fibroblasts involved in the integrity of BM. Indirect evidence suggests that being autophagy a pathway which counteracts fibrosis, a hypothesis to be tested consists in a specific anti-collagenogenic effects of autophagy activation [58,59], which would further clarify why in the course of AMD and/or autophagy inhibition a thickening of the BM takes place where the elastic component is reduced compared to altered collagen structure which is increased. In fact it is demonstrated that during autophagy inhibition, collagen structure is stimulated while the elastic fibers are suppressed [60]. This is evident in the skin [58] upon blue and UV light exposure. The cells where autophagy modulates the synthesis of collagen and elastic fibers correspond to fibroblasts [58,60]. These fibroblasts occur in the retinal choroid border and are known as choroidal mesenchymal fibroblasts, which are connected with RPE cells through a paracrine signaling [61].

5. The loss of autophagy within RPE cells

Despite already evidenced in the introduction, a specific paragraph need to be dedicated to the delicate role of autophagy which is massively engaged, even in baseline conditions within RPE cells. In fact, the classic hallmark of AMD consists of altered RPE cells, where an early and severe defect in the autophagy machinery is well-documented. In fact, in baseline conditions, RPE cells undergo an excess of metabolic activity due to their multiple functions. Namely, most of the autophagy machinery in the RPE is dedicated to the removal of the outer segment of photoreceptors [62]. The engulfment of the autophagy process in the RPE occurs when autophagy is inhibited. This occurs when lysosomes cannot degrade metabolic by-products. This condition is typical of AMD at early stages, where stagnant lysosomes are present in excess containing non-digested material in the form of lipofuscins or melanolipofuscin [57]. In fact, lipofuscin blocks lysosomal enzymes constraining these organelles to release their content below the plasma membrane, where this material aggregates to form drusen. Concrete evidence about a dysfunction of autophagy in AMD is obtained through the isolation of RPE cells from donor patients affected by AMD compared with RPE cells from controls. When compared with controls, RPE cells from AMD patients possess classic alterations [57], which can be related to the impairment of autophagy. The autophagy flux is impaired indeed into AMD-RPE cells compared with controls at least concerning lysosomes, which are stagnant and dilated as shown by large LAMP1 positive spots present in high amount compared with small LAMP1 positive puncta detected in control RPE cells. In detail, as reported by Golestaneh et al. [63], immunostaining for LAMP1 shows enlarged LAMP1-positive structures in AMD-RPE compared with small discrete puncta observed in normal RPE. This is concomitant with depressed autophagy as measured by the ratio of LC3II/LC3I. The slowed au-

tophagy flux in turn, occurs along with a relented decrease of p62 even following autophagy stimulation (through starvation). This slowed autophagy flux may be responsible for accumulation of lipid droplets and glycogen granules within AMD-RPE cells, which in turn may lead to drusen formation. In fact, in a way which is reminiscent of inclusions occurring in autophagy-dependent neurodegenerative disorders, drusen structure in AMD is composed of several autophagy-dependent proteins and organelles [64].

The occurrence of autophagy dysfunction within RPE from AMD patients emerges in several studies carried out in the last few years. The accumulation of autophagy proteins along with autophagy substrates and exosomes within drusen suggests a potential extrusion of altered organelles, lipids, sugars and proteins. In fact, autophagy in the RPE is key in removing these chemical species and subcellular structures. Most protein and lipid clearance is autophagy-dependent [19,21,65]. This is confirmed by the fact that autophagy defects lead to accumulation of misfolded proteins and organelles within RPE [21,66-68]. The loss of autophagy progression leads to abnormal accumulation of misfolded proteins within lysosomes, where they can be found as AGEs along with lipid material within lipofuscin. These molecules may be found already as cellular debris within stagnant lysosomes to represent precursors of drusen following their eventual release from the cells [69]. In AMD drusen occur mostly between RPE cells and the BM. The origin of drusen's structure from RPE can be inferred by their specific composition since they possess RPE-derived endoplasmic reticulum and the outer segment of photoreceptors, which is phagocytosed by RPE [70]. In keeping with this, drusen also feature typical RPE structures such as melanosomes [19]. The structure of lipofuscin, which occurs abundantly within AMD-RPE cells and within drusen in the course of AMD is consistent with their main origin from RPE cells. In fact, they contain a number of lipids, sugars, and proteins along with photopigments residues and altered cell organelles. The fate of lipofuscin, which lacks appropriate digestion is to engulf persistently giant lysosomes within AMD-RPE cells. Therefore, most of them are released vie exosomes to be hosted in the sub-choroidal space and being recruited to form drusen [33,71,72].

An autophagy defect within RPE may lead to a number of downstream deleterious events. In fact, altered organelles and misfolded proteins, sugars and lipids are expected to trigger direct toxicity to the cell. This is likely to occur at short time intervals. The late fate of these aggregates is more questionable since a number of reports range from a detrimental mechanical effect to an inert role as innocent bystanders, simply witnessing for a previous dynamic of the disease.

6. An autophagy defect within RPE may early induce a visual defect rather than later neuropathology

Thus, it is likely that a loss of the primary metabolic function, which is bound to an autophagy defect is expressed in the disease generation more as a dysfunctional molecular defect (visual defect) rather than a morphological aberrancy (neuropathology). In this way, misfolded proteins or dysfunctional mitochondria are likely to exert their toxicity before being encapsulated within lysosomes or drusen, at early stages, when they are still playing a role in the visual processing being free in the cytosol before their aggregation. This hypothesis contradicts the common belief that, an autophagy defect is considered not to be dangerous at early stages but later on when the volume of aggregates of autophagy-dependent structures is noticeable. According to the classic perspective, within the retina this volume effect is commonly expected to alter the planar arrangement of photoreceptors. This was commonly considered to produce visual distortion such as metamorphopsia, which is quite typical in AMD [17,73]. In this way, visual loss was thought to be a secondary event solely generated by the loss of retinal planarity. If this is correct one should expect that the measurement of the drusenoid area, calculated in the retina in the course of AMD should predict the amount of loss in visual acuity and occurrence of visual

distortions. However, when the drusenoid area is calculated in AMD patients a dissociation emerges between the amount of the drusenoid area and the severity of visual symptoms ([73] and representative data of Table 1). These findings lead to hypothesize that an autophagy defect impairs visual function more as a result of a fine molecular deficit impairing the molecular mechanisms of visions, rather than a gross mechanical alteration of retinal planarity due to sub-retinal aggregates. In line with this, recent data indicate that suppression of visual acuity in AMD is the consequence of pure metabolic effects, which may be restored by autophagy stimulators [10,17,40,65,73,74]. These effects are quite independent from the amount of drusenoid deposition [73] (see also Table 1 showing the discrepancy between visual acuity, visual distortion, retinal thickness and drusenoid area).

Table 1. The amount of drusen does not necessarily correlate with visual symptoms

	Patient 1	**Patient 2**
BCVA *	20/32	20/40
Drusenoid area	22.00 mm^2	1.32 mm^2
Central thickness	255 μm a	216 μm
Metamorphopsia	0.4°	0.5°

* BCVA=Best corrected visual acuity

As extensively reported in a previous manuscript [73] and in new Table 1 with representative original data from two patients, the discrepancy between the loss of visual acuity and the amount of the drusenoid area is evident. Table 1 reports opposite conditions. In detail, patient 1 is seriously affected by drusen deposition, which occurs in most of the macular surface (drusenoid area 22.00 mm^2) and surpasses seventeen-fold the drusenoid area measured in patient 2 (1.32 mm^2). Remarkably, such a serious drusenoid involvement in patient 1 occurs along with better visual acuity and less severe metamorphopsia compared with patient 2 affected by a mild drusenoid involvement. The drusenoid area represents the best index to express quantitatively the amount of retina filled with drusen. This index is calculated by multiplying the drusen number and each drusen size. This measurement is more accurate than the simple drusen number or drusen size *per se* or a subjective drusen scoring system as extensively discussed previously [73]. The central thickness obtained at retinal topography provides an indirect measurement of the loss of planar arrangement of the retina. Metamorphopsia (linear distortion) are measured as the degree of loss of linear alignment within horizontal and/or vertical axis.

7. The novel concept of "inner choroid/outer retina neurovascular unit"

The molecular events altering vision may occur also in cell types beyond RPE. In fact, in the first part of the manuscript we discussed the occurrence of autophagy impairment as a wide metabolic dysfunction encompassing the inner choroid and outer retina. This is supposed to affect an anatomical unit, which is coupled to match photoreception with metabolic requests, as originally postulated by Spraul et al. [57]. In this way, the inner choroid/outer retina unit should be encircled as the critical area rather than the sole RPE. This hypothesis makes the inner choroid/outer retina unit similar to the neurovascular

unit recently described in the CNS [75]. In line with this, in the retina the rate of autophagy within rods varies according to light cycle being diurnally regulated [76]. This occurs according to the metabolic requests induced by vision. In this way, it is possible to define a so-called "inner choroid/outer retina" neurovascular unit, where the activity of the outer segment of photoreceptors and RPE cells are coordinated with blood supply through the CC (Figure 7).

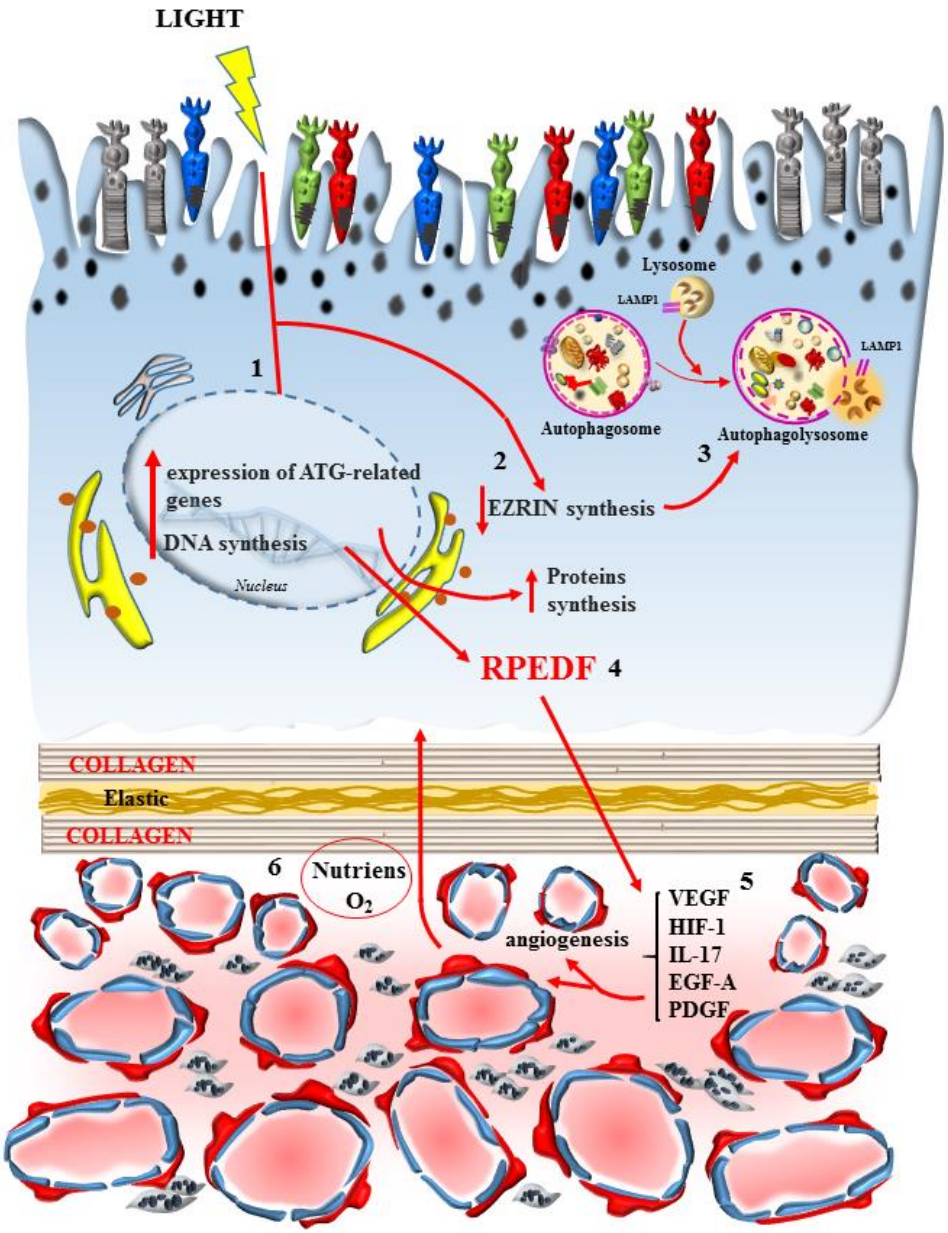

Figure 7. The inner choroid/outer retina neurovascular unit. The inner choroid/outer retina unit is similar to the neurovascular unit recently described in the CNS. In line with this, in the retina the rate of autophagy within rods varies according to light cycle being diurnally regulated. This occurs according to the metabolic requests induced by vision. In this way, it is possible to define a retinal

neurovascular unit, where the activity of the outer segment of photoreceptors and RPE cells are coordinated with blood supply through the CC and such a coordinated activity largely depends on synchronized autophagy status between all these cell types. In fact, (1) light synchronizes autophagy by epigenetic regulation of 23 genes, all coding for autophagy proteins. As a consequence, more autophagy structures are generated during periods of light within "inner choroid/outer retina" neurovascular unit, according to "*momenta*" of light exposure. This is in line with the fact that autophagy in the retina is induced during the process of photo-transduction. These autophagy-related, light-induced proteins include ezrin inhibition (2), which removes lysosomal inhibition (3), thus making the autophagy pathway faster. Remarkably, also the protein RPEDF is induced (4), which is key in regulating the process of vision. This produces a physiological blood flow which inhibits abnormal angiogenesis (5) and increases blood supply (6), which occurs concomitantly with light exposure and autophagy induction.

This in turn depends on the activity of pericytes and endothelial cells. The kernel of such a coordinated activity may largely depend on a synchronized autophagy status between all these cell types. Light-induced autophagy activity may be the key to trigger such a coordination. This may explain why autophagy machinery in the retina is finely tuned at the level of gene and protein expression to allow visual acuity [77,78]. In fact, more autophagy structures are generated within "inner choroid/outer retina" neurovascular unit during periods of light. Such a cycling does not occur according to a circadian rhythm but it is directly stimulated by "*momenta*" of light exposure [76]. This is confirmed by the fact that, when measuring autophagy in the retina, this is induced during the process of photo-transduction. In fact, autophagy stimulation in the outer retina generates a protein named RPEDF, which is key in regulating the process of vision [77].

The hypothesis of a cyclic regulation of the "inner choroid/outer retina" neurovascular unit by the autophagy status is further confirmed by the studies of Datta and colleagues [79], who attributed fluctuations in autophagy and blood supply to specific variation of metabolic demands in the outer retina. This is expected when considering the need of active autophagy to phagocytose the outer segment of photoreceptors by RPE cells following photo-transduction. This also applies to the cyclic metabolism of retinal and the clearance of photo-induced oxidative stress. Thus, it is not surprising that daily light as much as artificial light depending on their specific wavelengths may sort robust epigenetic effects in the outer retina. This is needed to cope with light-induced metabolic demand. In fact, light regulates the expression of 23 autophagy-related genes [81]. In detrimental conditions, when autophagy is inhibited by systemic metabolic disorders such as diabetes even this light-induced epigenetic cycling is attenuated.

The specific role of autophagy in the visual process also relies downstream of merging between autophagosomes with lysosomes. In fact, Santo and Conte [81] indicate how critical is the final lysosomal step to provide an effective metabolic support for photoreceptors to respond to light exposure. We expect (and dedicated experiments are needed) that both timing and spacing of cyclic regulation of autophagy may occur in alternating retinal fields, likely to match the ON/OFF mosaic of retinal receptive fields. Accordingly, this may depend on activation of either excitatory or inhibitory receptors to glutamate, which release is inhibited by the effects of light. In this scenario, an ON retinal field should undergo autophagy activation when impacted by light, whereas an OFF retinal field should feature autophagy suppression following the same light exposure. As shown by Intartaglia et al. [77] timing and spacing in retinal regulation of autophagy is under the influence of a specific molecules known as "ezrin". Intartaglia et al. [77] consider the alternation of the autophagy status and light exposure as an appropriate stimulus to keep retinal integrity. It is likely that pulses of light, when owing the right wave length may produce ezrin inhibition and autophagy activation to sustain burst of trophic retinal activity. In fact, ezrin acting as an autophagy inhibitor poses the external retina into a resting state and needs to be inhibited in order to stimulate autophagy and protect from neuronal

damage. In fact, autophagy stimulation in the outer retina, within RPE, stimulates the expression of RPEDF which restores the visual cycle [49,77].

The energy loss which accompanies autophagy activation and visual processing, combined with the need of light in order to activate autophagy in the ON retinal field may explain why light exposure in order to be beneficial needs to be administered according to a pulsatile pattern, where blood flow fueling energy is coordinated. Similarly, due to the specific sensitivity of various photoreceptors to various wavelengths, it is expected that in order to sustain retinal integrity and visual functions, various wavelengths should be combined during pulsatile light exposure as recently suggested [17]. The marked autophagy demand in the outer retina is explained by the site-specificity of visual transduction and recycling of visual structures, which is matched by strong mitochondrial activity and metabolic networks dedicated to neutralize oxidative species. The coupling of these demands with appropriate functional changes in the CC is summarized by the novel concept of "inner choroid/outer retina neurovascular unit", where alternating patches of the retina in alternating time frames are either inhibited or activated.

7. Downstream of outer retina

Although most studies focus on the transition zone between the epithelial/mesenchymal part of the eye where the choroid-retinal junction occurs [40,73,82], facts and hypothesis discussed so far are limited to the CC, the RPE and the external segment of photoreceptor. Nonetheless, the relevance of autophagy downstream in the inner retina is reported [65]. This may rely on a number of biochemical issues. The role of autophagy within the whole photoreceptor cells is worth to be investigated. In fact, Intartaglia et al [77,78] recently found that autophagy prevents cell death of retinal photoreceptors in the course of retinal degeneration.

In keeping with retinal neuroanatomy, a key role may be attributed to those glial cells which connect the outer retina with its inner part. In fact, the autophagy status within Muller cells is likely to be relevant to trans-synaptically modulate retinal homeostasis. It is shown that Muller cells, depending on their autophagy status may induce neo-angiogenesis and inflammation which occurs during retinal degeneration [83]. Even Muller cells viability is promoted by autophagy activation which impedes Muller cells' apoptosis during retinal degeneration [84].

The spreading of autophagy-dependent retinal activity to the inner retina may also occur as the consequence of the natural stream of excitation and inhibition through bipolar, amacrin down to ganglionic cells. In fact, the concept of retinal fields is counting mostly downstream of glutamate receptors placed on bipolar cells suggesting a patchy pattern of retinal degeneration depending on this fact. It is not surprising indeed that retinal degeneration in the course of AMD is patchy by definition.

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