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Photo-damage, Photo-protection and Age-related Macular Degeneration

Melisa Marquioni Ramella and Angela M. Suburo

Medicina Celular y Molecular, Facultad de Ciencias Biomédicas,

Universidad Austral, Pilar B1629AHJ, Buenos Aires, Argentina.

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Abstract

Age-related macular degeneration (AMD) is a degenerative retinal disease that causes

blindness in people 60-65 years and older, with the highest prevalence appearing in

people 90 years-old or more. Epidemiological estimates indicate that the number of cases

is increasing, and will almost double in the next 20 years. Preventive measures require

precise etiological knowledge. This is quite difficult, since AMD is a multifactorial condition

with intricate relationships between causes and risk factors. In this review, we describe

the impact of light on the structure and physiology of the retina and the pigment

epithelium, taking into account the continuous exposure to natural and artificial light

sources along the life of an individual. A large body of experimental evidence

demonstrates the toxic effects of some lighting conditions on the retina and the pigment

epithelium, and consensus exists about the importance of photo-oxidation phenomena in

the causality chain between light and retinal damage. Here, we analyzed the transmission

of light to the retina, and compared the aging human macula in healthy and diseased

retinas, as shown by histology and non-invasive imaging systems. Finally, we have

compared the putative retinal photo-sensitive molecular structures that might be involved

in the genesis of AMD. The relationship between these compounds and retinal damage

supports the hypothesis of light as an important initiating cause of AMD.

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1. Introduction

The clinical and social importance of Age-related Macular Degeneration (AMD)

The retina is a photo-sensitive tissue that captures light and transforms it into electrical signals. The specialized cells that capture light include rods, specialized for nightlight vision, and cones, which are in charge of daylight and color vision. Rods contain the visual pigment rhodopsin that is sensitive to blue-green light (500 nm). Cones, instead, respond to short wavelengths (S-cones, 420 nm), medium wavelengths (M-cones, 530nm) and long wavelengths (L-cones, 560 nm).

Near the center of the retina, the macula lutea appears as a yellowish spot, including four anatomical regions: the perifovea, the parafovea, the fovea and the foveola. The perifovea shows a high density of retinal vessels and a high rod:cone ratio, although the density of cones and ganglion cells is higher than in the periphery. Almost 50% of the total ganglion cell population resides in the macula[1]. Thus, the ganglion cell layer (GCL) shows more than one row, up to six cells deep, except at the foveola[2, 3]. The parafovea has a low density of retinal vessels and a rod:cone ratio close to 4:1. Cones become dominant in the slopes of the foveal pit, where vessels are restricted to a perifoveal capillary plexus. Narrowing and elongation of cones at the fovea are essential for visual acuity. Inner retinal layers are absent in the foveola, where photoreceptor cell bodies lie close to the vitreal surface[2].

AMD is a degenerative retinal disease that causes blindness in people 60-65 years and older, with the highest prevalence appearing in people 80 years-old or more[4]. Vision loss is preceded by early asymptomatic stages characterized by the presence of medium-sized

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drusen (63-125 μm). The disease progresses to intermediate AMD with larger drusen

and/or retinal pigment epithelium (RPE) alterations near the macula. The latter include

hypo- or hyperpigmentation and accumulation of autofluorescent material (lipofuscin). In

addition, multifocal electroretinography, optical coherence tomography (OCT), and

spectral domain OCT (SD-OCT) have shown the presence of various photoreceptor

changes[5, 6].

Early AMD lesions further develop into one of the two forms of late disease: geographic

atrophy (GA) or dry AMD, characterized by loss of RPE cells and photoreceptors; and

neovascular (or wet) AMD[7], where abnormally growing choroidal vessels invade the

subretinal space between the RPE and the neural retina[8].

With a global prevalence been estimated in 8.69 %[9], AMD has replaced cataracts and

refractive errors as the leading cause of blindness and severe vision impairment in higher-

income regions such as Western Europe, Australia, USA, Japan[10] and Southern Latin

America[11]. Current estimates suggest that the 2.07 million cases recorded in 2010 will

become more than 5.00 million in 2050[12].

Etiopathogenesis of AMD

Chronic oxidant RPE injury, together with a low-level inflammatory response are

important factors for development of early RPE lesions[8, 13-15]. Thus, a high risk for AMD is

associated to cigarette smoking[16], which is a well-known oxidant[17, 18]. Age is the main risk

factor for developing AMD, but in most cases, genetic factors explain the overall severity

of the disease[19]. That the most frequent factors associated to AMD are genetic variants

facilitating inflammation[20], points at the existence of sustained stress in the retina and the

RPE. Two would be the most likely causes: exposure to environmental light and the visual transduction processes. Since both circumstances are unavoidable in ordinary life, the epidemiology of AMD arguments in favor of robust endogenous mechanisms quenching photo-oxidative stress. Light radiation reaching the retina and the RPE provoke oxidative stress, which is normally restrained by endogenous antioxidant systems and by mechanisms extinguishing the associated inflammation stress[15]. The main risk factors for AMD probably reflect the failure of these systems and mechanisms but still, photo-oxidation would be the initial pathogenic factor. As previously expressed by others, "prevention or attenuation of the initial oxidative injury will reduce the risk of developing AMD, regardless of genetic background"[21].

The association between this disease and environmental light is mainly based on epidemiological grounds, and on the physical evidence explaining the interaction of light with ocular tissues. Therefore, we will first analyze transmission of light to the retina, and then we will describe the diseased macula, as shown by modern imaging procedures. Finally, we will evaluate the putative retinal photo-sensitive molecular structures that might be involved in the genesis of AMD, which have been mainly identified by experimental studies in animal species or *in vitro*.

2. Environmental light and AMD

Effects of environmental light on the course of AMD must depend on the light wavelengths and intensities reaching the retina. Ocular structures can interact with a broad portion of the spectrum, ranging between 100 and 10,000 nm and including visible

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(750-400 nm), ultraviolet (UV-A, 400-320 nm; UV-B, 320-280 nm; UV-C, <280 nm) and infrared wavelengths[22, 23]. However, almost no UV-C reaches earth's surface[24], and various natural ocular filters restrict radiation transmission to photoreceptors.

Sunlight

Retinal irradiance in daylight is in the 0.01 mW/cm^2 to 0.1 mW/cm^2 range, depending on time of day, season, presence of snow or water surfaces, wearing a hat, etc. Additionally, squinting, which prevents the formation of a sun image on the inferior retina, and photophobia, serve as biological protections against sun exposure[25, 26].

The average human retina absorbs each day approximately 10^{12} to 10^{15} photons[27], which can be increased by workplace exposure or activities in high light environments. The association between sun exposure and AMD is controversial, since it has been found in some studies[28-31] but not in others[32-34]. However, a meta-analysis based on 14 epidemiological studies strongly supports the notion that more sunlight exposure increases risk for AMD[35]. Research has also emphasized the protective role of hats and eyeglasses and has suggested the possible relevance of individual differences in the reaction to sunlight exposure[36, 37]. Thus, the Age-related Maculopathy and Macular Degeneration in the elderly European populations (EUREYE) study only reported significant associations between blue-light exposure and neovascular AMD for individuals in the quartile of lowest dietary antioxidant level—vitamin C, zeaxanthin, vitamin E, and zinc[38].

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Other light sources

Welding arcs emit a wide spectrum, ranging from infrared (IR) to ultraviolet (UV). The cornea and the lens absorb UV radiation, whereas water absorbs far-IR. Visible light and near-IR may reach the retina in the unprotected eye, producing an acute macular lesion that often results in a bilateral central scotoma accompanied by pigmentation alterations[39, 40]. With only partial protection, UV light may generate corneal epithelial injury, whereas blue light destroys the center of the macula[40]. In a more recent study, macular lesions were demonstrated using OCT in 38% of welders (n = 80, age mean = 36.9 years) that did not refer visual symptoms and showed no visual acuity problems[41]. Thus, it would be of great interest to study the evolution of these lesions with age.

Concern has been raised about domestic and vehicular lighting, increasingly dependent on light-emitting diodes (LEDs). Dissemination of these devices poses a potential problem for the retina since billboards, and emergency lights extensively use blue LEDs. As it will be explained in the last part of this review, the retina is particularly vulnerable to blue-light. Current regulations establishes that for an exposure greater than 10,000 s, the exposure limit value (ELV) for blue-light radiance is about 100 W/m²/sr (or 1.0×10^6 J m^{-2} sr^{-1})[42]. Published spectral power distributions show that LEDs emit an intense blue-light component which is absent in the daylight spectra[43]. Cold-white LEDs are particularly questionable, since they emit about 3-4 times as much energy in the blue-light risk portion of the spectrum as warm-white LEDs[43]. Most important, due to their small size, it is relatively easy to produce LED sources of very high luminance that may generate visual discomfort[44]. A publication from the Department of Energy, U.S. reported that "the

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proportion of blue-light in the spectrum is not significantly higher for LEDs than it is for any other light source at the same correlated color temperature (CCT)"[45]. However, this report emphasized that safety could not be guaranteed for blue LEDs, nor for infants in close proximity to bright light sources.

Even though data from other species or *in vitro* cultures cannot be directly extrapolated to humans, we cannot disregard the experimental studies suggesting that LED blue irradiation might produce greater damage than other wavelengths. After exposure to 750 lux, retinal damage in rats occurred earlier in those exposed to blue and white (CCT 6,500 K) LEDs than in those exposed to white (CCT 6,500 K) or yellow compact fluorescent lamps (CFLs)[46]. After 9 days under blue or white LEDs, the outer nuclear layer (ONL), containing photoreceptor nuclei, was reduced to about 1/3, whereas no significant changes appeared in rats exposed to CFLs [46]. With a 3-day exposure, levels of superoxide anion in the retina were higher in those exposed to blue LEDs than in those exposed to white LEDs and CFLs. Using different commercially available blue LEDs, severe retinal damage was produced by radiances below the currently accepted ELV for blue-light[44]. Experiments *in vitro* also support the damaging potential of blue and white LED. Under the same illuminance (2,500 lux) blue LED light damaged 661W cells (a line derived from mouse cones) more severely than white and green LED lights[47]. Only blue and white LED light significantly reduced cell viability when 661W cultures were exposed under the same energy conditions (0.38 mW/cm^2)[47]. The question of artificial light sources in AMD etiopathogeny still requires more evidence; however, we cannot presently exclude their potential role as a significant hazard. Regulations are required to control glaring from billboards and emergency lights

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because, in addition to their potential role in retinal photo-toxicity, they might also conspire against security[48].

The normal eye filters UV and blue light

Cornea and lens

The cornea and lens absorb all UV-C light and most UV-B[24]. However, some UV-A radiation is transmitted, since it is about 10 times more abundant than UV-B in the solar spectrum[24, 49]. Filtering in the human lens reflects the presence of tryptophan derivatives, the kynurenines, which block most of the incident light between 295 and 400 nm[50-52]. Although kynurenins decrease with age, UV filtering properties of the human lens increase because these compounds form covalent bonds with crystallins[53]. Advanced glycation end products (AGE) also contribute to lens UV-filters[51]. UV and blue-light transmission decrease linearly as a function of age[52]. In contrast, a higher fraction of this region of the spectrum reaches the young retina(< 8-10 years)[43], determining the rapid formation of lipofuscin in children[54].

A review, including a large number of studies (2003-2014), has shown clear-cut associations between cataract surgery and AMD. However, both increased transmission of short-wavelength light to the retina[50, 55], and/or an inflammatory response[56, 57] could explain the greater risk for AMD. Since the aphakic eye loses most UV- and blue-light filtering properties [50, 55], it seems reasonable to replace cataractous lenses with intraocular lenses (IOLs) with those filtering properties. UV-filtering IOLs can be untinted or yellow-tinted. Compared with aphakic eyes, untinted IOLs allow a 60% reduction in

blue-light irradiance, whereas yellow-tinted IOLs confer an additional reduction (17 to 56%)[58].

The directly photo-sensitive retinal ganglion cells involved in the circadian rhythm are maximally sensitive to light at ~480 nm[59]; therefore, concerns about tBoth the sleep-wake cycle following implantation of filtering IOLSs have been raised[60]. Decrease in the transmission of 480 nm light may occur, but it is very small compared to that of the aged lens. By the age of 80 years, transmission of 480 nm light is only 28% of the transmission in 10 year-old children[61]. A prospective study, including 961 participants has shown that sleep quality improves after removal of cataract, notwithstanding the type of IOL used[62]. Most important, the beneficial effect of blue-light filtering IOLs is strongly supported by a very recent study measuring the enlargement of the atrophic area in patients with dry AMD. After implantation of a non-blue filter IOL, this enlargement was almost twice as after implantation of a blue-blocking IOL[63].

Macular Pigment

Macular pigment (MP) is composed of the xanthophyll carotenoids: lutein, zeaxanthin, and meso-zeaxanthin[64]. Xanthophylls gradually increase towards the center of the macula, and in the human fovea, they reach concentrations greater than 1 mM[65]. The cause of this elevated concentration might be explained by the low activity of β,β-carotene-9′,10′-dioxygenase, the only known mammalian enzyme that cleaves xanthophylls, which is much weaker in humans and primates than in other mammals[66]. Xantophylls accumulate preferentially in the outer and inner plexiform layers (ONL and INL) where they may be inserted in the plasma membrane, or associated with specific binding proteins[65]. The

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lutein : zeaxanthin : meso-zeaxanthin ratio changes progressively from 1 : 1 : 1 at the fovea to a ratio approaching 3 : 1 : 0 in the periphery. Since their peak absorbance is at 460 nm, and because they are located in the anterior (vitreal) portion of individual photoreceptors, macular pigments attenuate the amount of blue-light incident on the photoreceptors in the most sensitive region of the retina[67].

Macular pigment optical density (MPOD), which may be measured *in vitro* or *in vivo*[68, 69], is positively related to visual performance[65, 70]. Blue-light filtering improves the visibility of distant objects, most likely because scattered light from haze aerosols suspended on the horizon is predominantly blue[71]. The implantation of blue-light filtering IOLs after cataract surgery is associated with augmentation of MPOD in the absence of raised serum concentrations of lutein and zeaxanthin[57], highlighting the efficacy of these molecules as blue filters. By contrast, in a sample of healthy volunteers (n = 828), MPOD levels were significantly and independently reduced by age, current and past smoking and AMD family history[72].

After the Age-related Eye Disease Study (AREDS) provided level 1 evidence that supplementation with vitamins C and E, β-carotene and zinc resulted in a 25% risk reduction of progression from intermediate to advanced AMD[73], numerous clinical and epidemiological studies have tried to ascertain the putative protecting role of macular xanthophylls. Addition of lutein + zeaxanthin to the AREDS formulation did not further reduce the risk of progression to advanced AMD[74], and only a mild beneficial effect on visual acuity has been observed after a one-year lutein supplementation[75]. However, functional abnormalities of the central retina in early AMD can be ameliorated by lutein

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and zeaxanthin supplementation, an effect attributed to elevations in MPOD[76]. A recent

review concluded that supplementation with macular carotenoids is probably the best

available measure to strengthen the antioxidant defenses of the macula, thus reducing

the risk of AMD and/or its progression[67]. Xanthophyll carotenoid supplementation in AMD

would not only be significantly associated with improvements in visual acuity and contrast

sensitivity, but also with a concomitant increase of MPOD[77]. Results of carotenoid

supplementation may depend on previous nutritional conditions and genetic risk status.

Thus, in the Blue Mountains Eye and the Rotterdam studies, an interaction between

lutein/zexanthin intake and early AMD incidence was only found in participants with high

genetic risk (carriers of ≥ 2 risk alleles of CFH or ARMS2)[78]. The impact of supplements

containing different combinations of lutein, zeaxanthin and meso-zeaxanthin on visual

function in normal subjects and subjects with early AMD is under investigation[79].

Independently of their filtering function in the macula, carotenoids could serve as

antioxidants in the macula and in the RPE. They protect against singlet oxygen mediated

photo-oxidation reactions and can also react with free radicals[80]. Thus, they would also

reduce photo-oxidation of retinyl derivatives (such as A2-phosphatidylethanolamine and

A2E, see below)[81, 82]. Cultured RPE cells actively uptake lutein and zeaxanthin and these

xanthophylls prevent photo-oxidative inactivation of the proteasome, and photo-

oxidation induced changes in the expression of MCP-11, IL-8, and CFH[83]. Zeaxanthin has

direct anti-oxidant actions on RPE cells, including the induction of Nrf2-mediated phase II

enzymes such as heme-oxygenase-1, NAD(P)H:quinone oxidoreductase and γ-glutamyl-

cysteine ligase[84].

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Melanin

Melanins, the heterogeneous polymers formed by tyrosinase (TYR) oxidation products of tyrosine, and L-DOPA (L-3,4-dihydroxyphenylalanine), are essential instruments for defense against UV exposure[85]. Uveal melanocytes and RPE cells contain eumelanin and trace amounts of pheomelanin[86]. Eumelanin, which has a broadband absorption spectrum smoothly decaying to the lower-energy end, can rapidly dissipate UV and blue-light energy as heat[87]. Thus, eumelanin light absorption followed by rapid thermal relaxation could quench potentially harmful photo-chemical reactions. Melanin can also scavenge free radicals and reduce the oxidative stress resulting from lipid peroxidation and reactive oxygen species (ROS) production[88].

The function of melanin in sun photo-protection seems to be undeniable[86]. Therefore, if sunlight is a stressing factor involved in the etiopathogenesis of AMD, melanin might be one of the anti-AMD defense mechanisms. In line with this hypothesis, AMD is more frequent in white persons than in persons of black African inheritance[89]. Most studies also agree that white subjects with light blue-colored irises have a higher AMD prevalence than those with dark-colored irises[89]. In the Beaver Dam Eye Study, increased risk of early AMD was found for persons with high sunlight exposure and light colored eyes (gray/blue), or light colored hair (blond/red)[90]. Remarkably, initial recovery of patients with neovascular AMD after anti-VEGF treatment shows a seasonal oscillation that is inversely correlated with global radiation intensity[91], and functional improvement is significantly higher in patients with dark-colored eyes than in those with light-colored eyes[91]. In addition, a

recent report suggests a possible relationship between early AMD and TYR single

nucleotide polymorphisms (SNPs) previously associated with skin and eye pigmentation[92].

Quantitative observations in eyes from human cadaveric donors indicate a decrease in

RPE melanin with age, most likely related to photo-oxidation[93, 94]. Aging also affects

melanosomes, and by age 90, most RPE melanin appears as melanolipofuscin[95]. The latter

can generate ROS upon excitation with blue light[96]. Studies *in vitro* have shown that

melanin may reduce the accumulation of lipofuscin in RPE cells[97], and the photo-oxidation

of its components[98].

Pupillary diameter

The pupil modulates retinal illumination; consequently, it would also regulate retinal

susceptibility to photo-toxicity. In eyes with little pigmentation, light might reach the

retina by transmission through the iris and the sclera, possibly increasing the risk of light-

induced damage[99].

Light sensitivity of the pupil constriction reflex seems to be unaffected by age[100]; however,

AMD patients confronted by a navigation task display larger pupillary diameters than

controls of the same age and sex[101]. A larger pupillary diameter under the same luminance

conditions might increase retinal light exposure and contribute to progression of the

disease.

Light-induced damage

Electromagnetic radiation in the 100 nm^{-1} mm range is widely known as "optical

radiation"[42]. Light absorption by biological material implies energy transfer, which may be

damaging for absorbing tissues. Light-inflicted damage will depend on the specific

combination of radiation wavelength, exposure time, tissue properties and volume[22].

Photo-chemical damage arises when a cromophore, or photo-sensitive molecule,

undergoes physico-chemical changes after the absorption of a photon. In the eye,

chromophores include visual pigments in the photoreceptors, the macular pigments,

absorbing in the 400-530 nm range, and the broadband absorbers melanin and lipofuscin

in the RPE and choroid[23]. Effects of chromophore excitation may be transmitted to

neighboring molecules, dissipating extra energy in various ways, including chemical bond

splitting, hydrogen exchange and ROS production, such as singlet oxygen, superoxide,

hydrogen peroxide and hydroxyl radicals. In turn, these radicals react with nearby

molecules, inducing diverse photo-oxidative changes. Thus, photo-chemical damage is

almost synonymous with photo-oxidative damage[22, 102]. Cells may or may not repair these

lesions depending on the irradiation intensity and the exposure time[22, 23, 27, 103]. Spreading

of photo-oxidative effects is particularly damaging in tissues with high concentration of

cell membranes, such as photoreceptor outer segments[104]. Oxidative stress contributes to

photoreceptor cell death in animal models of retinal degeneration, including light-induced

retinopathy[105, 106].

3. Macular damage in aging and AMD

Photoreceptors

Eyes from 40-year or older persons without significant ocular disease show loss of

photoreceptor nuclei in the macular ONL, together with disappearance of outer segments,

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but without defects in the RPE or the choriocapillaris[107]. Quantitative microscopy studies

in donor eyes demonstrated a steady decline in central rod number with age, without

concomitant changes in cone numbers[108]. Cones, however, displayed some morphological

abnormalities, including lipofuscin deposition[109, 110]. More recent studies have detected a

significant thinning of the RPE and the choroid, together with an increase in the thickness

of the OPL[3], which in the macular region is known as the fiber layer of Henle. Greater OPL

thickness most likely reflects activation and hypertrophy of Müller cells following

photoreceptor loss[111]. Aging eyes also exhibited a reduced thickness of the retinal nerve

fiber layer (RFNL), GCL, inner plexiform layer (IPL), INL and photoreceptor inner segments,

except at the fovea[3, 112]. By contrast, width of the photoreceptor outer segment layer

correlated positively with age, presumably reflecting the age-related decrease in RPE

phagocytosis[112]. The amount of parafoveal rods significantly decreased in aging retinas[108].

Although changes in foveal cone numbers were not detected histologically[108], adaptive

optics have shown that, in old age, cone packing density decreases up to 25% within 0.45

mm of the foveal center, but not in peripheral regions[113-115].

Histological evaluation of dry AMD showed RPE irregularities and atrophy, whereas wet

AMD samples displayed both RPE defects and fibrovascular scars[108]. Foveal cone numbers

showed few changes, but rods were almost completely lost in the parafovea. In the wet

AMD samples, photoreceptors surviving in the neighborhood of disciform scars were

largely cones[108]. Since external light is focused on the cone-rich fovea, sparing of foveal

cones suggests that they may be more resistant than rods to light-induced damage.

Nevertheless, since they depend on the rod-derived cone viability factor (RdCVF)[116], they

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would disappear after demise of parafoveal rods. It has been demonstrated that RdCVF protects 661W cells from photooxidative damage[117] and, most important, that RdCVF-deficient mice are extraordinarily sensitive to light-induced damage[118].

Histological findings are supported by analysis of rod function[119], and adaptive optics scanning laser ophthalmoscopy[120]. Besides, AMD retinas also displayed reduced cone reflectivity, suggesting mild structural abnormalities[120]. Additionally, scanning laser polarimetry studies indicated that the number of central cone photoreceptors may be lower, and/or structural alterations of their axons significantly higher, than in non-AMD eyes of the same age[121]. Reduction of the RPE/photoreceptor and ONL layers overlying drusen has been consistently found[6, 122, 123], but reports about their thinning in drusen-free areas[122] need confirmation.

Loss of rod photoreceptors, with cone sparing, resembles the consequences of white light-induced damage in rodents, where cones remained after complete disappearance of rods[124]. Remarkably, in rats maintained under cyclic lighting, the retinas of older animals suffered more damage from exposure to intense light than those of younger animals[125].

Retinal Pigment Epithelium and Lipofuscin

The outer segments of rods and cones are under constant renewal, with old discs being shed from the apical tip and phagocytosed by RPE cells[126, 127]. A current development is the *in vivo* study of disc renewal in human cones through changes in their reflectance[128]. Daily shed outer segments are phagocytosed by the RPE and processed using a combination of phagocytic and autophagic mechanisms where lysosomes are fused with autophagosomes. Since some autophagy characteristic proteins (LC3 and Atg5) appear in

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the membrane of phagosomes, the process is known as non-canonical autophagy or LC3-associated phagocytosis[129, 130]. Most of the material is recycled to the photoreceptors; however, the RPE accumulates lipofuscin, a non-digested heterogeneous substance, within the residual bodies of the lysosomal compartment[131].

Lipofuscin distribution in the RPE shows a defined pattern, increasing from the equator to the posterior pole with a consistent dip at the macula. Curiously, melanin follows a contrasting distribution, decreasing from the equator to the posterior pole, but with a regular peak at the macula. This polarization fades by the age of 50, presumably because most melanin becomes incorporated into melanolipofuscin granules[132].

Lipofuscin has a broad excitation range (300-600 nm) and a broad emission spectrum (480-800 nm), allowing histological and non-invasive studies of fundus autofluorescence[133]. Wholemount studies of human donor retinas have shown that the topography of RPE autofluorescence follows the distribution of rod photoreceptors, being highest in the vicinity of the rod-rich perifoveal annulus[134]. The highest autofluorescence levels were found in 80 years or more retinas[134]. Older retinas displayed an increase of non-hexagonal shapes, without changes in RPE cell density[134]. Degranulation of RPE cells appeared in healthy and AMD aged eyes, whereas granule aggregation was only observed in AMD eyes. In the latter, some RPE cells were greatly enlarged and displayed cytoskeletal alterations[135]. In GA patients, the atrophic patches were usually surrounded by a junctional region of abnormal autofluorescence. Distinct patterns have been described and some of them may have a genetic basis[136, 137].

4. Main targets of photo-toxicity

Understanding the role of light exposure in the course of AMD requires identification of the molecular targets that initiate photo-oxidation reactions, which we may call primary targets. Some other molecules, the secondary targets, would not be directly affected by light, but they would become the immediate targets of ensuing photo-oxidation. Some compounds could be both primary and secondary targets, for example all-*trans*-retinal or lipofuscin. Available information about light molecular targets results from a large amount of experimental work that has been mainly done in animals or *in vitro*[23, 27, 96, 138-140]. Early work in albino rats showed photoreceptor damage after light exposure through blue (360-530 nm) and green (490-580 nm) filters. Electroretinogram (ERG) alterations, however, were most efficiently induced by exposure to 500 nm[141]. The RPE was sometimes involved, depending on age of the animal, temperature, previous illumination conditions and the intensity and duration of the damaging light[142]. Results suggested that these lesions depended, directly or indirectly, on rhodopsin excitation. Indirect damage would require the activation of other photo-sensitive molecules appearing under light adaptation conditions, perhaps including products of the rhodopsin bleaching process, such as vitamin A derivatives[27, 139, 142]. By contrast, experiments using blue light (441 nm), done in monkeys, showed an initial damage of the RPE, followed by alteration of the photoreceptor outer segments and remarkable recovery 10-11 days after exposure[143]. Available data for monochromatic-induced retinal damage support the existence of at least two damage action spectra. Irradiation in the 320-440 nm range predominantly affected photoreceptors[144], whereas in the 440-550 nm range injured the RPE and/or the

photoreceptors[103, 141, 142]. Rhodopsin, and also other chromophores such as lipofuscin, intermediate products of the visual cycle, and even melanin, could be the photo-sensitive targets converting light into retinal damage[27]. Rhodopsin, however, is not only affected by 500 nm light, but can also be a target for blue light-induced photoreversal of bleaching. This phenomenon increases the photon-catch capacity of the retina and its susceptibility to light damage, thus explaining why blue light has a greater damage potential than green light[145].

Of note, most spectral data for retinal damage has been obtained in anesthetized animals, often using funduscopic visible changes as threshold damage[103]. Therefore, lesions described in these experiments do not resemble aging or AMD changes, but those found in welders[40, 41] and laser or sun-gazing accidents[146, 147]. Their relationship with AMD is conceivable, but is far from proven, particularly since these experiments provide little information about the damage spectra of very long exposures in freely moving subjects. Curiously, exposure of albino mice or rats to diffuse white light induces photoreceptor death, without overt RPE damage[124, 148].

Rhodopsin

White light did not cause photoreceptor degeneration in mice lacking functional rhodopsin, thus, rhodopsin must play an essential role in the retinal response to excessive lighting[138]. This role is further supported by the correlation between rates of visual pigments regeneration and light-induced damage thresholds[138, 149]. Moreover, white light did not induce retinal damage in mice and rats under halothane anesthesia, which blocks

rhodopsin metabolic regeneration. In these animals, however, exposure to blue light (403 nm) induced photoreceptor apoptosis and RPE swelling[148].

The absence of functional transducin, which blocks signaling from light-activated rhodopsin, did not protect from bright light-induced degeneration. However, mutant mice with persistent rhodopsin activation, which are extremely sensitive to low-intensity cyclic light, were protected[138].

Photo-transduction and oxidative stress in the outer segments

Oxidative metabolism, which is required to support the light pathway, could also induce or aggravate photoreceptor damage[150]. Since a significant fraction of the O_2 used by cells is converted to ROS, excessive activation of photo-transduction might determine a higher activity of the respiratory complexes, and a higher oxidative stress. These phenomena might occur within the outer segments, which contain their own machinery for ATP synthesis, including mitochondrial-like electron transport chains, F1-ATP synthase and the TCA cycle enzymes, as has been demonstrated in bovine retinas using proteomic procedures and immunogold transmission electron microscopy[151-153]. Remarkably, bovine and mouse outer segments are selectively stained with mitochondrial dyes[151, 154].

The nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX) are also involved in light-induced oxidative stress. The primary function of NOX enzymes is the reduction of oxygen into superoxide anion using NADPH as an electron donor and oxygen as an electron acceptor[155]. Increase of these species is observed in mouse outer segments when whole eyeball cultures are irradiated with blue light (405 nm), and can be prevented by the NOX inhibitor apocynin[154].

The visual cycle and the retinoids

Photo-excitation of rhodopsin and other visual pigments leads to isomerization of their chromophore 11-*cis*-retinal to all-*trans*-retinal, which dissociates from the opsin protein. Regeneration of the visual pigments requires the restoration of 11-*cis*-retinal. Visual pigments in rods and cones recover at very different rates, about 40 min in rods but only 2-3 min in cones[156]. These are the times required by their specific regeneration processes: the rod and the cone visual cycles[156, 157].

The rod visual cycle

Both 11-*cis*- and all-*trans*-retinal form Schiff base adducts with phosphatidylethanolamine (PE). The ATP-binding cassette subfamily member 4 (ABCA4) flips this N-retinylidene-PE to the disk cytoplasmic leaflet[158], and cytoplasmic dehydrogenases (RDHs) reduce it to all-*trans*-retinol[156]. This retinoid is released and bound by the interphotoreceptor retinoid-binding protein (IRBP). It is then captured by RPE cells, becoming bound to a retinaldehyde-binding protein (CRALBP). All-*trans*-retinol is esterified by lecithin-retinol acyltransferase (LRAT) and turned into 11-*cis*-retinol by the isomerase (RPE-specific 65 kDa protein; Rpe65). Further oxidation produces 11-*cis*-retinal, which abandons the RPE and is taken up by photoreceptors, regenerating a functional visual pigment[156].

The cone visual cycle

The cone visual cycle is intraretinal. Instead of trafficking to the RPE, all-*trans*-retinol diffuses from cones to the Müller cells, where it is isomerized to 11-*cis*-retinol, probably by dihydroceramide desaturase-1 (DES1)[159]. This is a type 2 isomerase that, at difference with Rpe65, acts directly on all-*trans*-retinol[159]. 11-*cis*-retinol is rapidly sterified by

multifunctional O-acyltransferase (MFAT)[160]. CRALBP plays an important role in the cone visual cycle, since its absence desensitizes cone-driven vision in humans and mice[161]. Both cone and rod dark adaptation depend on the presence of CRALBP[161].

Photoxicity of all-trans-retinal

The possible role of all-*trans*-retinal as the agent of light-induced damage, initially discussed by Noell (1966)[141], has been extensively described[132, 149]. Remarkably, the instantaneous concentration of this retinoid in the light-exposed outer segment could be as high as 5 mM[162, 163]. Bleaching less than 0.5% of all rhodopsin would still generate toxic levels of all-*trans*-retinal[164]. Thus, this molecule could either be an indirect damage target of rhodopsin activation and/or the direct target of short-wavelength light. Peak absorption of all-*trans*-retinal is at 380 nm, which is almost completely filtered by the human lens. However, this retinoid still shows substantial absorption at > 410 nm wavelengths [165]. UV-A (355 nm) and blue (422 nm) light excitation of all-*trans*-retinal in the presence of oxygen generates singlet oxygen, which can in turn oxidize all-*trans*-retinal[166]. The degradation products, including several endoperoxides, shorter-chain aldehydes and epoxides, significantly increase all-*trans*-retinal cytotoxic effects on RPE cells *in vitro*[166].

Rod photoreceptors would be the primary site of all-*trans*-retinal attack[167]. Damage is induced through different mechanisms, including photo-damage of its own transporter ABCA4[165], impairment of mitochondrial function, increase in the production of superoxide through the activation of NOX enzymes[168], and/or the activation of Toll-like-receptor 3 (TLR-3)[169], followed by microglial activation[170]. In addition, *in vitro* irradiation (400-700

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nm) of rod outer segments in the presence of all-*trans*-retinal impairs the ability of rhodopsin to regenerate[171], indicating another probable cause of photoreceptor degeneration.

All-*trans*-retinal arrives to the RPE together with phagocytosed outer segment discs, but can also be synthesized in the RPE from β,β carotene or all-*trans*-retinol[172]. All-*trans*-retinal is highly cytotoxic to human RPE cells in primary cultures, and potentiates the effect of light irradiation[172].

The pharmacological control of visual chromophore biosynthesis has been proposed as a preventive method for retinal diseases depending on light-induced damage, increase of retinoid byproducts and hyperoxia[173]. Emixustat hydrochloride, presently in clinical trial for dry AMD is an RPE65 inhibitor and retinal scavenger. This drug has significant adverse effects; however, it has shown that all-*trans*-retinal sequestration is a crucial function for photo-toxicity protection[174].

Lipofuscin and A2E

Although lipofuscin has been extensively described, its composition is still poorly understood and might differ between the diverse regions of the retina. Lipofuscin, which contains little protein, would mainly derive from all-*trans*-retinal, docosahexaenoic acid (DHA), and other components from outer segments[175-177]. Its best known constituents are the bisretinoids, a complex mixture of autofluorescent compounds[132, 176]. Retinal isomers, including all-*trans* and 11-*cis*, covalently react with the amine group of PE forming N-retinylidene-PE. The addition of a second retinal molecule produces N-retinylidene-N-retinylphosphatidylethanolamine (A2PE). A2E forms after removal of the A2PE

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phospholipid moiety[176]. Lipofuscin also contains all-*trans*-retinal dimers, which are more abundant than A2E in the retina of Abca4$^{-/-}$ mice[27]. RPE bisretinoids exhibit diverse excitation maxima, but they all emit fluorescence centered around 600 nm, which is similar to the maximum emission of the fundus autofluorescence[176].

Numerous experiments, *in vivo* and *in vitro*, support the role of lipofuscin, all-*trans*-retinal and A2E as targets for blue light. In primate eyes, visible light (488 and 568 nm) may photo-bleach RPE cells autofluorescence at levels previously considered safe. Experiments *in vitro* suggested that A2E might be involved in this response[27]. At higher intensity irradiation levels, but still not higher than the maximum permissible exposure, the RPE developed long-term structural disruption. At present, it is unclear whether these lesions represent a lipofuscin- or photopigment-dependent damage mechanism[27]. However, damage induced in RPE cell cultures fed isolated lipofuscin granules, and exposed to short-wavelength visible light (390–550 nm)[97], but in the absence of photoreceptors, cannot be attributed to a rhodopsin effect.

A2E may be less damaging than retinaldehydes[172], and it has been postulated that the formation of A2E and its precursor A2PE would reduce the photo-reactivity of all-*trans*-retinal[172]. In contrast with this hypothesis, a damage spectrum has been described for A2E-loaded porcine RPE cells, with lesions occurring between 390 and 552 nm (maximal at 420-450 nm)[178]. This apparent contradiction can be explained by the increased photo-toxicity of A2E oxidation products[179], which would contribute to RPE photo-damage in rat retinas exposed to blue light[180]. Photo-oxidation and photo-degradation of bisretinoids release small carbonyls involved in the formation of Advanced Glycation End-products,

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which may accumulate in drusen and laminar deposits[181]. It has also been suggested that

photo-activation and cleavage of bisretinoids promote complement attack on RPE cells[182, 183]. These similarities in the cytotoxicity of lipofuscin and A2E, plus the fact that both

molecules show the same distribution in mice RPE[184], were taken as an indication that A2E

might be the target triggering and maintaining the course of AMD[185].

A2E and its oxides have been studied *in situ* using high-resolution matrix-assisted laser

desorption/ionization imaging mass spectrometry (MALDI-IMS). Whereas the RPE central

area displayed the highest lipofuscin fluorescence intensities, the highest A2E densities

were found in the far periphery[186, 187]. Comparison of A2E distribution in human and

mouse retinas suggests that this bisretinoid is characteristic of rod-rich areas. Low levels in

the cone-rich area macular area suggest that the cone visual cycle does not favor the

transformation of all-*trans*-retinal into A2E. Thus, light-induced damage in the central

retina would not depend on A2E. Nevertheless, the distribution of lipofuscin in a

perifoveal ring corresponds to the localization of perifoveal rods[134], which are the first

photoreceptors to perish in aging and AMD[108, 119, 120].

On the other hand, photo-oxidative damage (448 nm) of lipofuscin-loaded primary human

RPE cells and ARPE-19 cells activated the inflammasome, suggesting a link between photo-

oxidative damage and innate immune activation[188].

The ABCA4 gene and clearance of all-trans-retinal

Clearance of all-*trans*-retinal is delayed when certain variants of the ABCA4 gene are

present, as in recessive Stargardt's disease (STGD1), a juvenile form of macular

degeneration[189]. Two variants of the human gene have been associated with increased

risk for AMD[190].

STGD1 patients show a distinctive fundus autofluorescence pattern, the granular pattern

with peripheral punctate spots (GPS+), that also appears in 2-3 % of GA AMD patients[137].

About half of the GPS+ patients carried a monoallelic ABCA4 variant, whereas only 10% of

the GPS- patients carried these variant alleles[137]. Of note, light deprivation might

contribute to reduced progression of decreased autofluorescence in STDG1 patients[191].

Although the vast majority of AMD cases are not related to ABCA4 gene variants, the

aforementioned associations support a role of all-*trans*-retinal in AMD development.

Data obtained in mice carrying Abca4 mutations suggest a complex and still controversial

scenario. Abca$^{-/-}$ mice, which are more vulnerable to light-induced retinal degeneration,

accumulate RPE lipofuscin and A2E[192]. Rdh8$^{-/-}$Abca4$^{-/-}$ mice, with a delayed all-*trans*-retinal

clearance, develop retinal lesions resembling human AMD (RPE/photoreceptor dystrophy,

lipofuscin, drusen-like deposits under the RPE and choroidal neovascularization), and

show an acute retinopathy under irradiation levels harmless for Rdh8$^{+/+}$Abca4$^{+/+}$ mice[149].

Abca4$^{-/-}$ mice increased the expression of proteins activating the complement system, and

downregulated the complement regulatory proteins. Besides, they showed basal laminar

deposits along the Bruch's membrane[193]. Moreover, all-*trans*-retinal sensitized human RPE

cells *in vitro* to alternative complement pathway attack[194], suggesting another likely link

between light exposure, the visual cycle and AMD.

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Retinal lipids

DHA is the most abundant fatty acid in whole retinas (22-24%)[195]. Prolonged light exposure and high-light rearing environments reduce DHA levels in rod outer segments[196]. Interestingly, acute exposure to bright light did not damage photoreceptor outer segments in rats with dietary DHA or linolenic acid deprivation[196, 197].

Involvement of N-retinylidene-PE in the clearance of all-*trans*-retinal probably explains the extraordinarily high content of PE and its long-chain DHA in photoreceptor membranes. In the disc membranes, PE would act as a sink preventing diffusion of 11-*cis*-retinal[198].

As a precursor of neuroprotectin D1, DHA may also shield retinal cells from oxidative stress[199]. Importantly, photo-activation of rhodopsin may be regulated by the relative proportion of polyunsaturated lipids, such as DHA, and cholesterol, in the disc membranes. Thus, quantum yield of all-*trans*-retinal depends on the availability of DHA in the retina[96].

Lipid peroxidation significantly increases in the retina of rats exposed to light[200]. Moreover, it has been shown that the oxidative potential of the posterior region of the human eye, including the macula, increases with age[201]. Exposure to light induces phospholipid oxidation and immunoreactivity for oxidized phosphatidylcholine appears in photoreceptors and RPE cells at the healthy human macular area. Its levels increase with age and eyes with AMD show stronger immunoreactivity than age-matched normal eyes[202]. Increase of oxidized phospholipids multiplies the expression of monocyte chemoattractant protein-1 (MCP-1), followed by macrophage accumulation, and these

effects are prevented by antioxidants. Moreover, subretinal application of oxidized

phospholipids induces choroidal neovascularization, typical of the wet-type AMD[203].

Carboxyethylpyrrole adducts

Carboxyethylpyrrole protein (CEP)-adducts are oxidative products derived from

fragmentation of DHA-containing lipids. They are elevated in ocular tissues and plasma in

AMD patients, where they can be detected in drusen[204]. Purified lipofuscin granules also

contain CEP-adducts[175, 205]. Likewise, CEP adducts are found in the retina of rodents

exposed to intense light[206]. The photo-oxidative processes that generate CEP-adducts

could occur in photoreceptor cells, but may also take place after disc shedding in the RPE

autophagosomes and lysosomal bodies[176]. CEP-adducts may well be another pathway to

macular degeneration, since autoantibodies are present in the blood of AMD patients.

Moreover, mice immunization with CEP-seroalbumin induced, after 12-14 months,

numerous sub-RPE deposits and accumulation of complement proteins in the Bruch's

membrane[207].

Isolevuglandins

Levuglandins (LGs) and isolevuglandins are γ-keto-aldehydes derived from the oxidation of

arachydonyl phospholipids[208, 209]. These molecules are highly reactive toward free primary

amines such as the ε-amine of lysine residues in proteins and the primary amino groups of

phosphatidylethanolamines[210]. They also react with mitochondrial cytochrome P450 27A1

(CYP27A1), impairing its function in sterol elimination[211]. Isolevuloglandins are highly

abundant in the human retina, where immunoreactivity is mainly localized in

photoreceptor inner segments. They are not detected in retinas of mice reared under dim

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light, but can be found in inner segments and RPE cells after exposure to a bright light

source (10,000 lux 2 h)[212]. Iso[4]levuglandin E_2 adducts have been found in purified

lipofuscin granules[175].

5. Concluding Remarks

Evidence presented here supports the concept that light reaching the retina and the RPE

provokes oxidative stress, leading to a buildup of toxic compounds that induce

inflammation and cell death. Experimental and clinical findings indicate that light can

affect oxidative homeostasis in the outer retina, either by excessive activation of photo-

transduction processes or by the impairment of waste disposal mechanisms. All-*trans*-

retinal and its subproducts appear as the major offenders in the retinal degeneration

circuit.

In experimental models, accumulations of all-*trans*-retinal in photoreceptors, and

bisretinoids and lipofuscin in the RPE, are light-dependent processes. In addition, these

compounds are both photo-reactive, and photo-toxicity inducers as well. Experimental

evidence indicates that all-*trans*-retinal accumulation in photoreceptors suffices for the

initiation of their degeneration. Therefore, early AMD might represent the direct effect of

all-*trans*-retinal on photoreceptors, perhaps reinforced by lipofuscin accumulation in

cones. In a second stage, lipofuscin, A2E, and related compounds, would increasingly

accrue in the RPE, giving rise to a new target site for photo-toxicity. The course of the

disease would then accelerate, since the light attack becomes possible at two different

fronts. Differences between early and late AMD could perhaps be explained by this

temporal pattern. In addition, all-*trans*-retinal photo-toxicity includes disruption of ABCA4, the N-retinylidene-PE transporter. Since photoreceptor PE molecules are highly enriched in DHA, all-*trans*-retinal photo-toxicity might be involved in the formation of CEP-adducts that appear in drusen and lipofuscin granules.

The availability of precise and fast analytic tools has also brought to light that rods and cones follow different death pathways. Most important, both histological and modern imaging procedures have shown that perifoveal rods die before foveal cones. This sequence could be associated to differences in the management of visual pigment regeneration, which requires an RPE step for rods, but is mainly intraretinal for cones. Moreover, cones seem to be more resistant to light-damage than rods. Since their light-resistance and survival depends on RdCVF availability, loss of perifoveal rods predicts the future demise of foveal cones. Of note, experiments suggest that a replacement therapy might extend cone survival.

Ample evidence shows that light-induced photoreceptor and/or RPE injury would trigger the inflammatory component, amplifying the initial damage. These processes explain the importance of certain gene variants for complement regulatory proteins as risk factors for AMD development.

As shown by data presented in this review, the hypothesis of light as an initiation cause of AMD is mainly supported by the existence of molecular targets in the retina and the pigment epithelium which light can transform into photoreceptor toxics.

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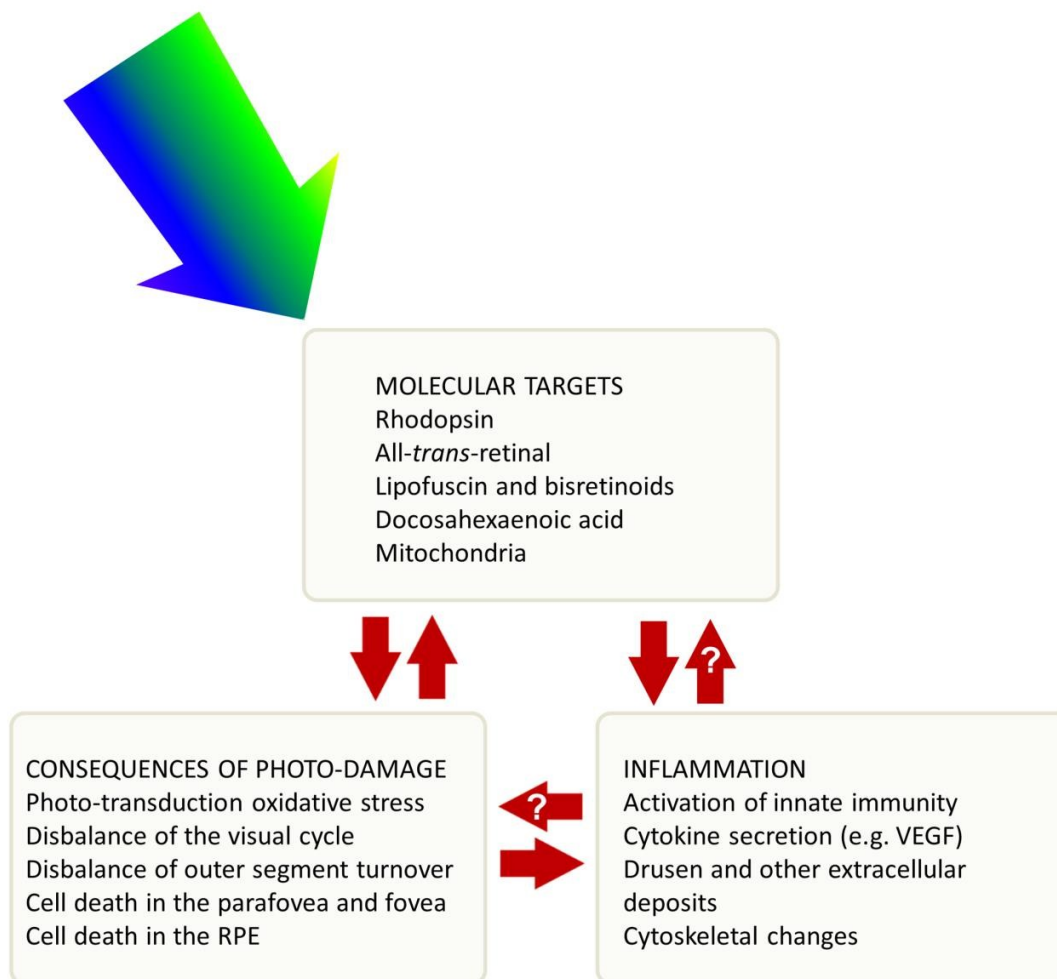
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Photochemical & Photobiological Sciences Accepted Manuscript

Graphical abstract

MOLECULAR TARGETS
Rhodopsin
All-*trans*-retinal
Lipofuscin and bisretinoids
Docosahexaenoic acid
Mitochondria

CONSEQUENCES OF PHOTO-DAMAGE
Photo-transduction oxidative stress
Disbalance of the visual cycle
Disbalance of outer segment turnover
Cell death in the parafovea and fovea
Cell death in the RPE

INFLAMMATION
Activation of innate immunity
Cytokine secretion (e.g. VEGF)
Drusen and other extracellular deposits
Cytoskeletal changes

Legend for Graphical Abstract

The course of Age-related Macular Degeneration (AMD) is described as the effect of light

(400-580 nm) on several molecular targets in photoreceptors and the retinal pigment

epithelium (RPE). Photo-oxidative changes determine damage of cellular structures, and

may determine the appearance of secondary molecular targets, such as bisretinoids and

lipofuscin. Photo-damage results in severe disturbances followed by death of macular

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photoreceptors and RPE cells. Photo-oxidation and photo-damage elicit several inflammatory processes that support development of characteristic drusen and VEGF-induced angiogenesis, typical of AMD. Question marks indicate that photo-damage- and inflammatory-derived products could perhaps contribute other photo-sensitive molecules, thus increasing the initial damage.

Short statement of Novelty

The course of Age-related Macular Degeneration (AMD) is described as the effect of light (400-580 nm) on various molecular targets in photoreceptors and the retinal pigment epithelium (RPE).