

Focus on molecular events in the anterior chamber leading to glaucoma

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Abstract Primary open-angle glaucoma is a multifactorial disease that affects the retinal ganglion cells, but currently its therapy is to lower the eye pressure. This indicates a definite involvement of the trabecular meshwork, key region in the pathogenesis of glaucoma. This is the first target of glaucoma, and its functional complexity is a real challenge to search. Its functions are those to allow the outflow of aqueous humor and not the reflux. This article describes the morphological and functional changes that happen in anterior chamber. The “primus movens” is oxidative stress that affects trabecular meshwork, particularly its endothelial cells. In these develops a real mitochondriopathy. This leads to functional impotence, the trabecular meshwork altering both motility and cytoarchitecture. Its cells die by apoptosis, losing barrier functions and altering the aqueous humor outflow. All the morphological alterations occur that can be observed under a microscope. Intraocular pressure rises and the malfunctioning trabecular meshwork endothelial cells express proteins that completely alter the aqueous humor. This is a liquid whose functional proteomics complies with the conditions of the trabecular meshwork. Indeed, in glaucoma, it is possible detect the

presence of proteins which testify to what occurs in the anterior chamber. There are six classes of proteins which confirm the vascular endothelium nature of the anterior chamber and are the result of the morphofunctional trabecular meshwork decay. It is possible that, all or in part, these proteins can be used as a signal to the posterior pole.

Keywords Open angle glaucoma · Trabecular meshwork · Intraocular pressure · Endothelium · Oxidative damage · Aqueous humor · Molecular endpoints

Abbreviations

8-OHdg	8-Hydroxydeoxyguanosine
AC	Anterior chamber
AF	Actin microfilaments
AH	Aqueous humor
ECM	Extracellular matrix
ELAM-1	Endothelial leukocyte adhesion molecule-1
GSH	Reduced glutathione
IF	Intermediate filaments
IL	Interleukin
JCT	Juxtacanalicular connective tissue
MT	Microtubules
mtDNA	Mitochondrial DNA
NO	Nitric oxide
ONH	Optic nerve head
POAG	Primary open-angle glaucoma
RGCs	Retinal ganglion cells
RNFL	Retinal nerve fibre layer
ROS	Reactive oxygen species
SCS	Suprachoroidal space
SOD	Superoxide dismutase
TM	Trabecular meshwork
UV	Ultra violet radiation

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Introduction

Glaucoma refers to several disorders having the same clinical features. They are characterized by an optic neuropathy in which retinal ganglion cell degeneration leads to a characteristic cupping of the optic nerve head (ONH) associated with typical visual field defects [1]. Glaucomas have been traditionally classified as open-angle, angle-closure (referring to the configuration of the iridocorneal angle), and congenital. Each subtype has been subdivided into two categories: primary, when no cause for the glaucoma can be identified; and secondary, when the glaucoma is caused by an underlying pathological ocular or systemic condition [1]. The majority (60–70 %) of primary glaucomas are open-angle. Primary open-angle glaucoma (POAG) is the leading cause of irreversible blindness in the world [2]. Although the precise molecular mechanisms leading to glaucoma are far from being understood, three tissues are known to be involved in POAG pathogenesis: the trabecular meshwork (TM) in the anterior chamber (AC) of the eye [3]; the ONH, specifically the retinal ganglion cells (RGCs), in the posterior segment of the eye [4]; and the lateral geniculate nuclei and the visual cortex in the central nervous system [5]. Thus, we can describe POAG as an ascending disease that begins in the anterior segment with increased intraocular pressure (IOP) [6], spreads to the posterior segment, and finally travels along the neuronal chain to the visual cortex. Growing evidence obtained from clinical and experimental studies over the past decade strongly suggests the involvement of the reactive oxygen species (ROS) in glaucoma [7] and in RGCs death [8]. Free radicals can directly induce neuronal death by a protease and phosphatase-gated mechanism [9]. In glaucoma, free radicals may damage the TM [10] while, in the posterior segment of the eye, exposure of glial cells to elevated concentrations of free radicals starts the process of apoptotic retinal ganglion cell death [11]. The final neurological damage results in progressive RGCs death, axon atrophy, and degeneration, also extending to the brain cortex (visual areas), and finally leading to the characteristic optic-cup neuropathy and irreversible visual loss [12, 13]. Cell death in the various involved tissues is due to apoptosis. Different mechanisms could trigger oxidative damage. Retinal ganglion cell death has been related to high nitric oxide (NO) levels [14], glutamate excitotoxicity [15], mitochondrial damage [16], defective axonal transport [17], and glial cell pathology [18]. Visual field defects due to RGC degeneration are directly proportional to oxidative damage in the TM [19] and are therefore linked to its endothelium cellularity [10]. TM tissue alteration has been shown to be crucial in glaucoma pathogenesis. TM malfunction, which occurs in most open-angle glaucomas, causes IOP increases [20]. The relationship between IOP and glaucomatous ONH damage is still undisclosed; however, glaucoma therapy

remains focussed on decreasing IOP. Two theories were formulated to explain the glaucoma origin: the vascular one and the mechanics one. The first is based on the hypothesis that ocular blood flow is reduced [21], while according to the second hypothesis, the pressure on the axons develops a compression and loss of the neurotrophic support of the ganglion cells [22]. Neither theory explains precisely the pathogenesis of the glaucoma neuropathy, because the first does not justify the role of increased IOP, while the other one does not explain the alterations to the circulation within the ONH [23]. Indeed, the deformation of the vitreoretinal interface does not necessarily correspond to the deformation of the anterior surface of the lamina cribrosa, and the central retinal vasculature has surprisingly little effect on ONH biomechanics [24]. Thus, scleral thickness, especially near the ONH, seems a potential factor of interest in the development of glaucomatous optic neuropathy [25]. A recent paper suggested that IOP is not only transmitted via the vitreous but also via the suprachoroidal space (SCS). Increases in IOP could be efficiently applied via the SCS to the most external axons of the ONH as they leave the eye. If there were no flow in the SCS, pressure in the AC would be transmitted unmitigated to the ONH potentially causing axonal loss. Thus, the rate of flow in the SCS determines the fraction of IOP applied to the ONH [26].

Increased intraocular pressure increases create mechanical stress transmitted in an undefined manner to the back of the eye damaging the RGCs and their axons (i.e., the optic nerves). IOP may be controlled, in part, by genes that are expressed in the eye, particularly in the ciliary body and/or the TM (the ocular sites involved in aqueous production and outflow) [27] (Fig. 1). An identified genetic risk variant

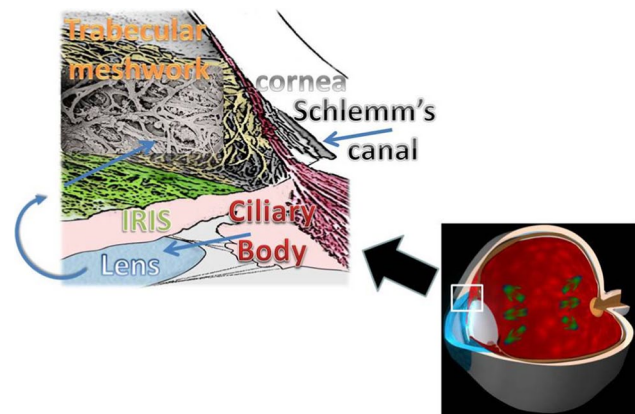


Fig. 1 Aqueous humor (AH), the biological fluid filling both the anterior and the posterior chambers of the eye. AH is secreted in the posterior chamber by the ciliary body arrives into anterior chamber (AC) through the pupil, goes above the anterior face of the iris and leaves the AC via two routes: the conventional (anterior) route is the through the TM, and the unconventional (posterior) route is through the uveoscleral pathway along the ciliary muscle fibers

for POAG is found in the caveolin gene family, members of which are expressed in the TM and RGCs [28]. This discovery suggests that the molecular pathogenesis is similar in the two regions, even if the triggering insult is different. Lütjen-Drecoll [29] and other authors [30–32] have stated that “common factors are involved in the pathogenesis of both the TM and the optic nerve changes”. Although these two structures are distinct, they share a common embryogenic origin and are linked by the pathogenetic events that determine the beginning of the glaucoma cascade. In glaucoma pathogenesis, there are two common pathways that are involved in the cellular damage to both the TM and optic nerve: oxidative stress [33] and vascular damage [21]. The purpose of this review is to examine the molecular alterations occurring in the anterior segment which activate the entire glaucoma pathogenic cascade.

Oxidative stress: endogenous and exogenous sources in the eye

Free radicals are chemical species with a single unpaired electron. This configuration is highly reactive, as it seeks to pair with another free electron. This process results in the production of another free radical. The newly produced free radical is unstable and may trigger a chain reaction that causes damage to macromolecules including DNA, protein, and lipids.

Reactive oxygen species and nitric oxide are important reactive species in living organisms. They can both initiate events resulting in cellular damage or important physiological signal transducers. Free radicals can have endogenous or exogenous sources [34]. The most common ocular diseases are mediated by ROS [35]. Oxidative stress is the result of an impaired balance between formation and removal of ROS.

Excessive ROS levels induce apoptosis in a variety of cell types by inducing DNA damage [36]. ROS, as demonstrated for H_2O_2 , are messengers for NF- κ B activation [37]. ROS activates the transcription factor NF- κ B, which induces expression of a great variety of agents, including pro-inflammatory cytokines such as IL-1/6 and TNF- α [38]. Therefore, adequate levels of the antioxidant defenses responsible for scavenging free radicals are essential for redox homeostasis. At moderate concentrations, NO and ROS play important roles as regulatory mediators of signaling processes. Normally, the amounts of ROS in the tissues are relatively low. The increase of superoxide or NO leads to a temporary imbalance constituting the basis of redox adjustment. The persistent increase in the production of large quantities of ROS or reactive nitrosative species (RNS) can lead to persistent changes in signal transduction and gene expression that can lead to pathological conditions.

Many of ROS-mediated responses actually protect cells against oxidative stress and reestablish “redox homeostasis.”, but when ROS generation overwhelms the antioxidant defences, free radicals can interact with endogenous macromolecules and alter cellular functions [39]. There is an age-dependent increase in the fraction of ROS and free radicals that may escape these cellular defense mechanisms and exert damage to cellular constituents, including DNA, RNA, lipid, and proteins. Signals triggering ROS overproduction may induce the opening of the membrane permeability transition pore in mitochondria and the release of cytochrome c and other apoptogenic factors, which ultimately lead the cell into apoptosis [40]. Exogenous ROS sources include UV light, visible light, ionizing radiation, chemotherapeutics, and environmental toxins. Endogenous sources include the activity of peroxisomes, lipoxygenases, NADH oxidase, cytochrome P450, and mitochondrial respiration [39].

Nitric oxide has physiological functions playing an important role in controlling ocular vascular tone and blood flow in the human eye [41]. Actually, NO is a potent signaling molecule in blood vessels, where its continuous formation from endothelial cells acts on the underlying smooth muscle to maintain vasodilatation and blood flow. NO is synthesized from L-arginine by a family of nitric oxide synthase (NOS) isozymes that includes neuronal (n)NOS, endothelial (e)NOS, and inducible (i)NOS. nNOS and eNOS are constitutive Ca^{2+} (calcium)/calmodulin-dependent enzymes and are tightly controlled by mechanisms regulating physiological intracellular Ca^{2+} levels, whereas iNOS is Ca^{2+} -independent [42]. The human endothelial TM expresses mainly the eNOS isoform, with a significantly lower amount of nNOS [43]. eNOS physiologically regulates aqueous outflow in the eye by maintaining vascular endothelial cell function.

The interaction between NO and oxygen radicals that typically occurs in endothelial tissues produces peroxynitrite ($ONOO^-$) [44], which is a potent oxidant that initiates lipid peroxidation, DNA breakage and base modification, protein tyrosine nitration and nitrosylation, alterations in cell signaling, and cell necrosis and apoptosis in cases of severe damage [45]. Peroxynitrite can affect mitochondrial respiration, causing cellular energy failure, contractile dysfunction, and cell death [46, 47]. iNOS expression is regulated by signaling pathways that involve molecules such as the redox-responsive transcription factor NF- κ B and MAPK. NF- κ B is the nuclear factor kappa-light-chain-enhancer of activated B cells, which is involved in cellular responses to stimuli such as cellular stress and free radical damages. MAPK are mitogen-activated protein kinases, specific protein kinases responding to extracellular stimuli and regulating a variety of cellular activities [48]. As shown in Fig. 2, an increase in ROS generation results in sustained

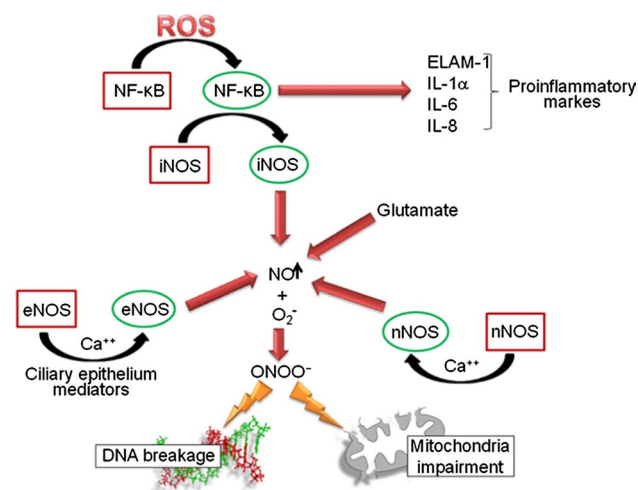


Fig. 2 Reactive oxygen species (ROS) and nitric oxide (NO) are signal transducers able to trigger a dramatic cascade of events resulting in cellular damage. Physiological NO levels play an important role in controlling ocular vascular tone and blood flow in the human eye. An enhanced level of ROS could induce an overproduction of NO which in turn, reacting with ROS, produces reactive peroxynitrite ($ONOO^-$) causing DNA breakage and mitochondrial damage

NF- κ B activation, which in turn induces the expression of proinflammatory markers, including endothelial leukocyte adhesion molecule-1 (ELAM-1), interleukin (IL)-1 α , IL-6, and IL-8 [49]. Although short-term activation of these mechanisms in the TM can contribute to IOP decrease [50], their chronic activation may exert pathological effects on the TM and contribute to glaucoma progression [51].

Nitric oxide synthase alterations are another major source of molecular damage playing a role in glaucoma pathogenesis. A NOS deficit in the TM and Schlemm's canal endothelia occurs in glaucomatous patients and is linked to altered contractile cell tone [43]. NO and peroxynitrite can affect mitochondrial respiration, causing cellular energy failure, contractile dysfunction, and cell death. ROS produced by mitochondria modulate cell signaling [52] inhibiting tyrosine phosphatase [53] leading to cell proliferation and translocation and activation of serine/threonine kinases such as protein kinase C [54].

Glutamate has also been shown to modulate NO production [55] and mediators released by the ciliary epithelium may influence eNOS activity in the cells of the inflow and outflow pathways [56].

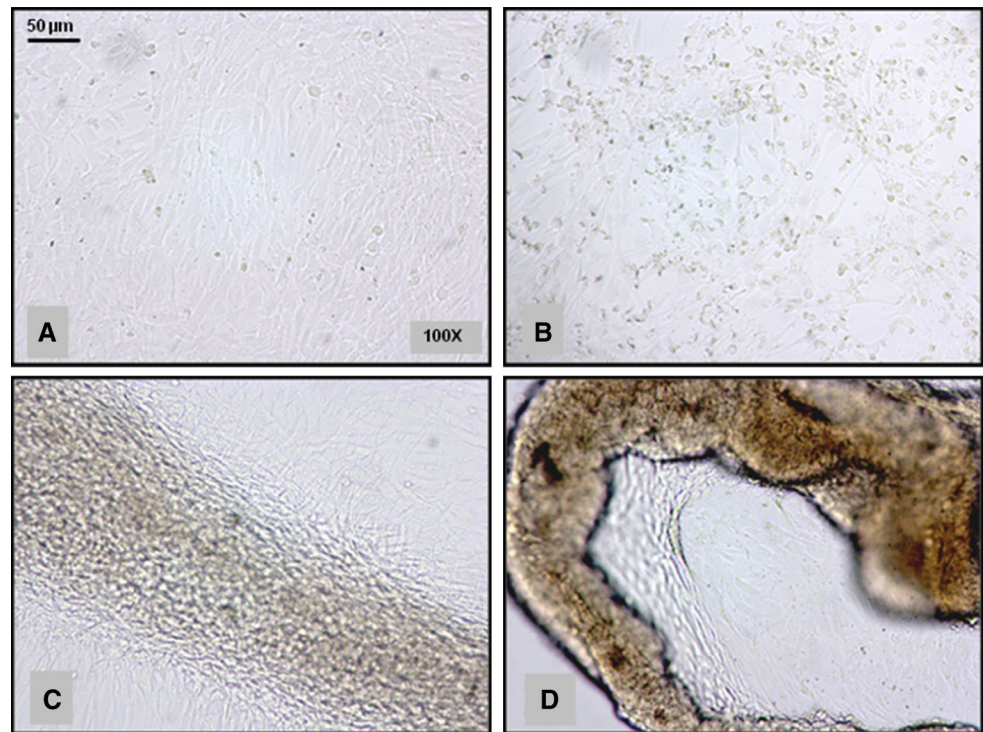
Oxidative/nitrosative stress is recognized to be a prominent feature of many acute and chronic diseases and of the normal aging process [57]. There is much evidence of the relationship between aging, increased oxidative damage, and mitochondrial dysfunction. Mitochondrial integrity declines with age, aged organelles being morphologically altered and producing more oxidants and less ATP than younger organelles [58]. Generation of ROS is a side effect

of oxidative phosphorylation which take place in mitochondria. ROS are converted to H_2O_2 by Mn-dependent superoxide dismutase (SOD) in the mitochondrial matrix [59]. Thus, mitochondria are the main endogenous source of ROS [60]. 8-hydroxy-2'-deoxyguanosine (8-OH-dG) is an established biomarker of oxidative DNA damage. The amount of 8-OH-dG in nuclear and mitochondrial DNA (mtDNA) progressively increases with normal aging. However, the rate of increase is greater in mtDNA [61–63] because of mtDNA proximity to the oxidant source, the lack of any protective histone covering, and limited mtDNA repair mechanism [64]. Such a high level of mtDNA oxidative damage results in guanosine loss and apurinic site formation, which are very fragile and whose disruption results in the formation of mtDNA deletions. The most typical and frequent mtDNA deletion related to oxidative damage is the mtDNA 4977 common deletion, causing the loss of one-third of the whole mtDNA and of mitochondrial genes encoding for enzymes involved in the oxidative phosphorylation. Mitochondria bearing this deletion are less efficient in ATP production and release more ROS than intact mitochondria [64]. Mitochondria bearing the mtDNA 4977 deletion have a shorter genome than normal mitochondria, thus replicating more quickly and prevailing in the cytoplasm throughout time. These mechanisms result in the long-term accumulation of high levels of mitochondrial damage in aged tissues targeted by endogenous oxidative stress, such as the TM [65], and causes an energy deficit and tissue atrophy [66].

The relative amount of mtDNA deletions correlates with the levels of 8-OH-dG [67]. The level of mtDNA damage detected in glaucomatous TMs is remarkably high [16]. The bioenergetics consequences of mtDNA and nuclear DNA derangements, with the associated mitochondrial dysfunction, contribute to the development of POAG [68]. MtDNA lesions tend to accumulate with age, possibly influencing mitochondrial function [69, 70] and providing a link between ageing and glaucoma.

Izzotti et al. [65] demonstrated that mitochondrial deletions specifically accumulate more in POAG and pseudoexfoliative glaucoma (PEXG) but not in other glaucoma types compared with unaffected control TM samples. Similar findings have also been obtained for oxidative damage to nuclear DNA, which was significantly increased in POAG and PEXG but not in other glaucoma types. By contrast, the total number of cells in the TM decreased in all glaucoma types. These findings imply that the mechanism causing glaucomatous damage in the TM is not identical in all types of glaucoma, even if the target tissues are the same. Furthermore, analysing molecular proteomic endpoints in the AH, oxidative and mitochondrial damage as occurring in glaucomatous TM has also been demonstrated [71].

Fig. 3 Oxidant effect of H_2O_2 (hydrogen peroxide) on the adhesion of HTM cells to ECM proteins results in a rearrangement of cytoskeletal structures that may lead to TM disruption. **a** Untreated HTM cells, **b** HTM cells 0 min after treatment with H_2O_2 (400 μ M for 15 min), **c** HTM cells after 1 h since H_2O_2 treatment, **d** HTM cells after 3 h since H_2O_2 treatment. Magnification $\times 100$



The high sensitivity of mtDNA to oxidative damage led to the concept of a “vicious cycle” in which an initial ROS-induced mitochondrial impairment leads to increased oxidant production that, in turn, leads to further mitochondrial damage [60]. Oxidative damage to mitochondria influences different structural and functional components of mtDNA, proteins and membrane lipids. TM cells of POAG patients have a decreased activity in the mitochondrial respiratory chain complex I [72]. Human trabecular meshwork (HTM) cells exposed to 1 mmol of hydrogen peroxide show reduced adhesiveness to the extracellular matrix (ECM) proteins fibronectin, laminin, and collagen types I and IV, with the consequence that the effect of H_2O_2 on the adhesion of HTM cells to ECM proteins results in a rearrangement of cytoskeletal structures that may lead to HTM disruption [73]. Figure 3 reports the oxidant effect of H_2O_2 on the adhesion of TM cells to extra-cellular matrix proteins resulting in a rearrangement of cytoskeletal structures leading to TM disruption. Under physiological conditions, this effect is reversed by reduced GSH, catalase and ascorbic acid [20]. The GSH redox system is thought to protect ocular tissues from the damage induced at low H_2O_2 concentrations, whereas catalase protects tissues at higher H_2O_2 concentrations [74]. Patients with glaucoma have decreased levels of circulating GSH as compared to controls [75]. The TM is the most sensitive tissue of the anterior chamber to oxidative damage. Indeed, after H_2O_2 in vitro exposure of fresh human biopsies, oxidative damage dramatically increased in the TM but not in the cornea

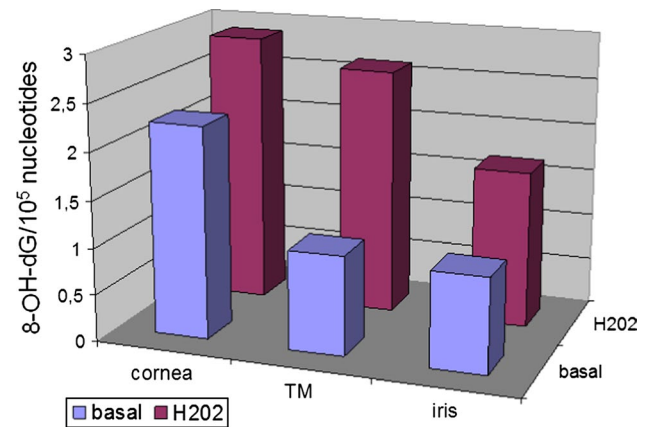
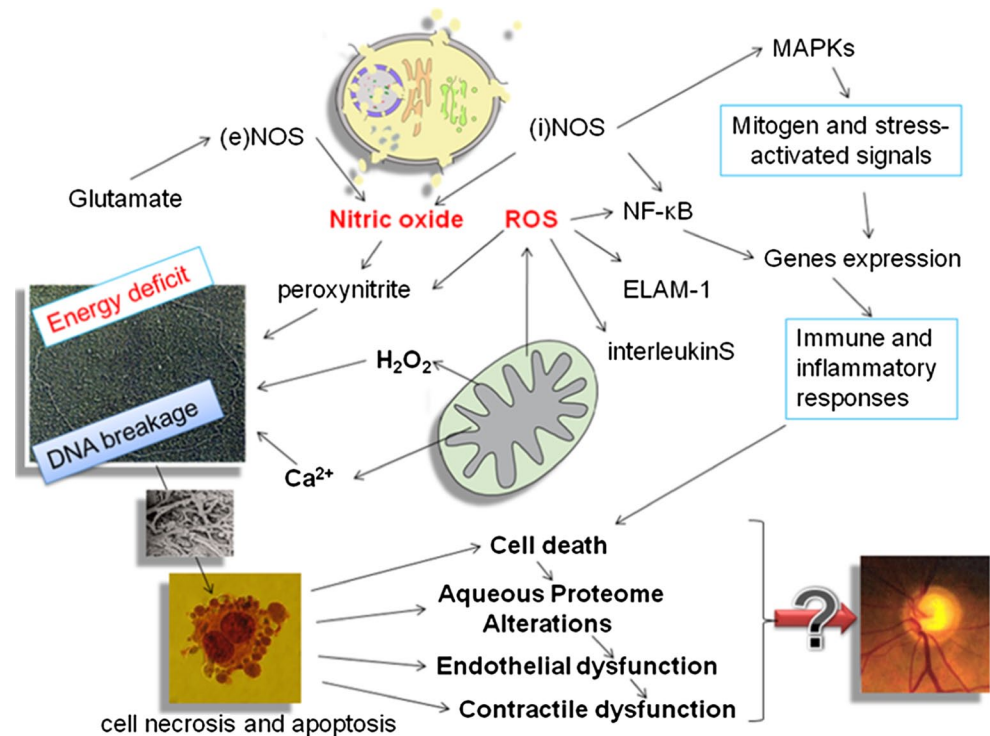


Fig. 4 Different sensibility to oxidative damage of the anterior chamber tissues. Basal damage (*blue columns*) is greater in cornea than in trabecular meshwork and iris. Cornea is the most exposed tissue to exogenous ROS sources. After treatment with H_2O_2 (*red columns*) the most relevant increase in oxidative DNA damage is detected in trabecular meshwork (TM), whose natural defences against reactive oxygen species are minimal due to its position repaired from the direct action of ROS exogenous sources (data from Izzotti et al. [76])

and iris [76] (Fig. 4). TM sensitivity to oxidative stress has been explained by the fact that ocular tissues directly exposed to light, i.e. cornea and iris, possess effective antioxidant defence mechanisms that are not activated in the TM, which being in the sclero-cornal angle is not directly exposed to light [76]. This bears relevance for glaucoma therapy, because a direct exposure of TM to light has been

Fig. 5 The complex network of mechanisms interlacing oxidative stress, mitochondria impairment and pathogenic events leading to glaucoma



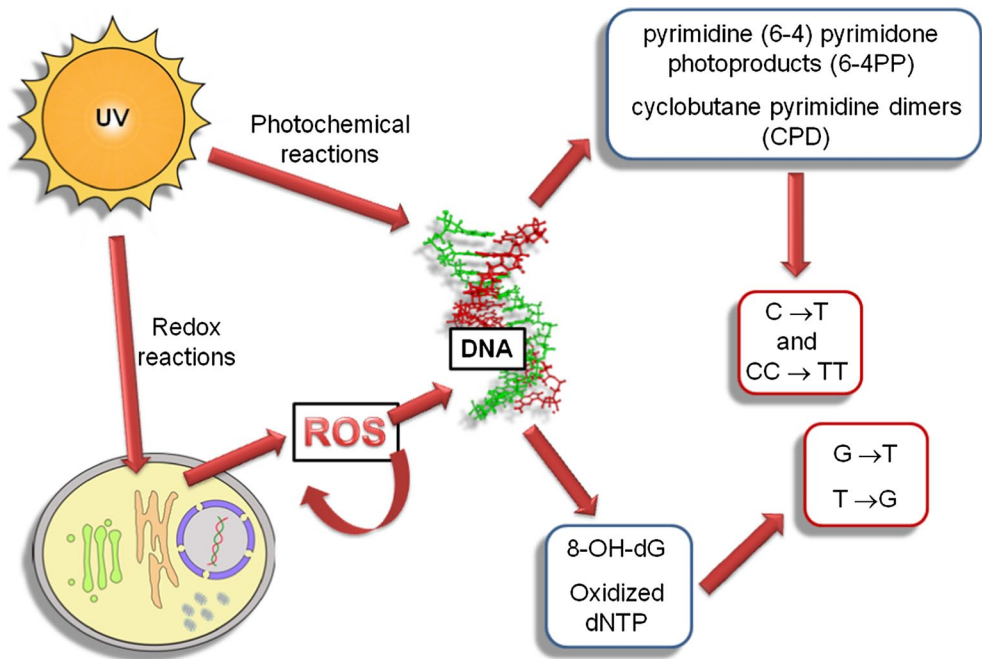
demonstrated to activate antioxidant defences in in vitro cultured HTM cells [77]. Nerve cells are also quite sensitive to oxidative damage, and the oxidative protein modifications occurring during glaucomatous neurodegeneration increase neuronal susceptibility to damage and lead to glial dysfunction [30]. In the aging eye and in several neurodegenerative diseases, there is a decline in the normal antioxidant defence mechanisms, which increase the vulnerability of the eye to the deleterious effects of oxidative damage [78].

Figure 5 resumes the complex network of mechanisms interlacing oxidative stress, mitochondria impairment and pathogenic events leading to glaucoma. These mechanisms could be at the base of the decrease in the TM cellularity and its malfunction in anging and glaucoma but also in ONH.

Both oxidative stress and AH antioxidant defences plays an important role in glaucoma pathogenesis. Antioxidant enzymes including SOD, catalase, glutathione peroxidase, and glutathione reductase display reduced activities in the eyes of patients with glaucoma [20, 79]. Microarray gene-expression analysis identified antioxidant downregulation and endothelial stress-response upregulation as hallmarks of glaucomatous TM [51]. 8-OH-dG levels were significantly higher in the TM of patients affected by glaucoma than in the control group [19]. The decline in the human TM endothelial cells cellularity is linearly related to age and plays a major role in glaucoma pathogenesis; [80–82], glaucomatous subjects having a lower

TM cellularity than non-glaucomatous subjects [82], UVB light comparable to solar irradiation levels readily causes the formation of intracellular ROS in human corneal epithelial cells [83]. In particular, UV induces oxidative stress in irradiated cells through the production of ROS by activating riboflavin, tryptophan and porphyrin, that in their turn activate cellular oxygen [84]. Iris surface and corneal endothelium are clearly visible and exposed to direct light; the TM is hidden between them in the iris corneal corner. As a consequence of that, the TME have fewer antioxidant defences [85]. Light can cause cell dysfunction through the action of ROS on DNA, [86]. Light has a direct oxidising effect that is exerted locally and at a systemic level in exposed organisms [87, 88]. At the eye level, the apoptotic death of photoreceptor cells by light-induced stress in retinal degenerative disorders is well documented [85, 89, 90]. In conjunctiva, UV radiation induces a typical caspase-dependent apoptotic cell death [91]. In the AC, light induces the formation of oxidative radicals that can indirectly target the TM by altering the oxidant–antioxidant balance in the AH, thus contributing to glaucoma pathogenesis. This mechanism generates both a decrease in the total antioxidant potential of the AC [79] and TM damage in terms of 8-oxo-dG formation and DNA fragmentation [19], contributing to endothelial dysfunction. Thus, UV represents an exogenous ROS source targeting the ocular structures. As reported in Fig. 6, UV can cause DNA lesions directly through the formation of photochemical products, such as pyrimidine-(6-4) pyrimidone and

Fig. 6 UV can cause DNA lesions directly through photochemical reaction (6-4 PP and CPD) or indirectly through redox reaction-generating ROS, which in turn produce DNA lesions (8-OH-dG and oxidized dNTPs). If repair mechanisms are ineffective, such damage results in stable DNA damage and mutations



cyclobutane T–T dimers, or indirectly generating ROS, which in turn produce DNA lesions, such as 8-OH-dG and oxidized nucleotides. If repair mechanisms are ineffective, these damages result in stable DNA damage and mutations, which, after a long-term accumulation, are in perennial cells, such as those composing TM, a stimulus towards apoptosis and cell loss [92].

Anterior Chamber: Functional anatomy and antioxidant defences

AC is a space filled by aqueous humor (AH) between the inner endothelial face of cornea and the anterior limiting layer of the iris, and surrounded peripherally for 360° by TM (Fig. 1). AH has an ionic composition very similar to the blood plasma and has two main functions: to provide nutrients to the tissues surrounding AC and to maintain IOP.

The cornea

The cornea protects inner ocular tissues, such as the lens and retina, against external environmental insults as a physical and biochemical barrier. The human cornea absorbs 92 % of ultra violet (UV)-B at 300 nm, [93]. UVB light comparable to solar irradiation levels readily causes the formation of intracellular ROS in human corneal epithelial cells [83]. The cornea contains both low molecular weight antioxidants such as ferritin [94], ascorbic acid [95], reduced glutathione (GSH) [96], α -tocopherol [97], and high molecular weight antioxidants such as catalase, SOD,

glutathione peroxidase, and reductase [98]. L-Ascorbate is present in aqueous compartments (e.g. cytosol, plasma, and other body fluids) and can reduce the tocopheroxyl radical; it also has a number of metabolically important cofactor functions in enzyme reactions, notably hydroxylations. The water-soluble antioxidant vitamin C can reduce tocopheroxyl radicals directly or indirectly thus supporting the antioxidant activity of vitamin E; such functions can be performed also by other appropriate reducing compounds such as GSH [99]. GSH is ubiquitously synthesized in all cell types [100] and directly protects cells from ROS through the direct scavenging of radicals and acting as a cofactor for glutathione peroxidases during the metabolism of H_2O_2 and lipid peroxides [101]. GSH plays a fundamental role in cellular metabolism, including differentiation, proliferation, cellular senescence, and apoptosis [102, 103]. In the cornea GSH is differentially distributed and the highest levels of GSH are found in the epithelium fivefold higher than that in the stroma [104, 105]. Its presence is related with its pivotal role in maintaining optimum hydration of the cornea [106]. The α -tocopherol is the major peroxy radical scavenger in biological lipid phases such as membranes or low-density lipoproteins. It can also regenerate other antioxidants, including GSH and ascorbate [99]. The synergistic effect between vitamin E and ascorbate significantly reduce oxidative stress by preventing lipid peroxidation and apoptosis in corneal endothelial cells [107]. Another antioxidant enzyme relatively abundant in the human cornea is SOD. SOD isoenzymes exert their actions in their respective compartments: the CuZn-SOD in cytosol and in extracellular compartments, while Mn-SOD is located in the

mitochondrial matrix [108]. The total level of SOD activity is much higher in the normal corneal epithelium than the activity of catalase and this is higher than the activity of glutathione peroxidase [109]. SOD neutralizes H_2O_2 producing superoxide anion, which is still a potent ROS and need to be neutralized by catalase. Accordingly, SOD exerts antioxidant effect only when coupled with catalase. Even if catalase is a major enzyme involved in the detoxification of H_2O_2 from cells, its expression under oxidative stress is not predictable and its activity may be decreased, increased, or unchanged [110]. Anyway, H_2O_2 induces a great amount of catalase expression, and smaller increases in glutathione peroxidase (GPx) and Mn-SOD expression [111]. The regulation of SOD isoenzymes in mammalian tissues primarily occurs in a manner coordinated by inflammatory cytokines, rather than as a response of individual cells to oxidants [112].

UVB modulates corneal epithelial cell expression of antioxidants and proinflammatory mediators by distinct mechanisms [113]. The suppression of proinflammatory cytokines strongly reduces matrix metalloproteinase and xanthine oxidase expression in the UVB-irradiated corneal epithelium [114]. Modulation of the expression of these mediators is important in regulating inflammation and protecting the cornea from UVB-induced oxidative stress [85].

The glutathione peroxidase catalyzes the reduction of lipid hydroperoxides to water or alcohol and H_2O_2 to water and molecular oxygen with concomitant oxidation of GSH [115] and is localized predominantly in the epithelium and endothelium of the cornea [116]. This reaction thereby minimizes the destructive effects of ROS in these corneal tissue layers, preventing the propagation of peroxide-dependent chain reactions that result in cell membrane degeneration [115]. Antioxidant enzyme activity decreases in aged corneas [117]; these enzymes are able to handle basal or low levels of ROS, but can be overwhelmed during acute stress [116].

The iris

The iris epithelium consists of two monocellular layers. The anterior epithelium is highly specialized; it is a myoepithelium that has an epithelial portion and a muscular portion that functions as the radially oriented dilator muscle [118]. The posterior epithelium is deeply pigmented to prohibit scattering of light.

Also, the iris tissue contributes to the antioxidant defenses of AC, the protective antioxidant system of the iris including low-molecular-weight antioxidants that are present in this pigmented tissue at high concentrations [119]. This tissue contains melanocytes bearing the pigment melanin, whose function is to prevent light scattering. This is a potent antioxidant likely to be a major

contributor to the poor sensitivity of the iris to hydrogen peroxide [76]. Further melanin absorbs UV radiation and blue light more efficiently than visible light of longer wavelengths [120]. Not much is known about the role of the pigment in the glaucoma pathogenesis. Melanin granules, released from the iris, are present in the circulating AH of the human AC in low but quantifiable numbers [121] many of the retained granules being phagocytized by TM cells [122]. There is little evidence suggesting that melanin is digested in situ in TM; it remains within cells and gradually leads to pigmentation in the ageing meshwork, as can be observed by gonioscopy [123]. Patients with POAG have a higher incidence of iris transillumination defects than controls [124]. Together with melanin, the spectral properties of the ECM contribute to the antioxidant activity defending the iris [125]. The opening in the iris, the pupil, expands and contracts to control the amount of incoming light. Therefore, the iris modulates the amount of the damage that the light may cause to the posterior segment.

The aqueous humor

The AH is a crystal-clear fluid filling the AC. The concept of the AH as a stagnant liquid was refuted in the early twentieth century by Leber, Lauber and Troncoso. In 1923, Seidel, working with stains, concluded that there is continuous AH flow from the AC. In 1965, it was discovered that AH passes through the TM and uveoscleral pathways [126, 127]. AH is continuously formed from plasma by the epithelial cells of the ciliary processes. It is secreted into the posterior chamber, passes from the posterior chamber through the pupil into the anterior chamber, and is drained at the AC angle. There is a small loss of liquid through the limbar sclera [128]. AH drainage is also influenced by the selective uptake of certain substances by the iris [129]. The volume of the AC AH is approximately 0.25 ml, and the posterior chamber AH volume is 0.06 ml. It is estimated that the daytime flow is approximately 2.5 $\mu\text{l}/\text{min}$ in the normal eye, and the tonography value of "C" (i.e., the coefficient of aqueous outflow facility) is $0.3 \mu\text{l} \times \text{min}^{-1} \times \text{mmHg}^{-1}$ [130]. Approximately 1 % of the AC and 3 % of the posterior chamber volume of AH is replaced each minute [131]. IOP in ocular-hypertensive patients is due to a reduced TM and uveoscleral outflow, while aqueous production remains normal [132]. Conversely, in the healthy aging eye, there are both reduced production of AH and reduced drainage through the uveoscleral outflow pathway [133]. AH production shows an age-related decline that approximately amounts to 15–35 % over the age range of 20–80 years [133–135]. Also, outflow facility decreases with age. The age-related decrease is about 30 % from 40- to 60-year-old subjects [134].

Increased intraocular pressure is associated with increased fluorophotometric outflow facility as has been demonstrated by administering apraclonidine to patients with ocular hypertension [136]. The AH provides nutrients for the avascular lens and cornea and an egress for the waste products of these structures. It contains approximately 1/100 of the serum protein concentration [137] that directly enters the AC by diffusion through the root of the iris. The aqueous fluid has 0.1–0.2 % the concentration of plasma proteins and a higher amino acid concentration than plasma. Ascorbate [138], lactate [139], and bicarbonate concentrations are also elevated in the AH [140]. Hydrogen peroxide, the main oxidant in AH, is normally present [141] as a result of reactions of ascorbic acid and trace metals [142]. Additional hydrogen peroxide and ROS are generated by light-catalyzed reactions, metabolic pathways, and phagocytic or inflammatory processes [141, 142]. Antioxidant defenses of the aqueous humor include vitamins, enzymes, and proteins such as albumins that have a protective role toward the TM [20, 143]. A high level of ascorbic acid in the AH is necessary for maintaining a filter-like function against UV radiation in the central corneal epithelium and the AH [138, 144].

The trabecular meshwork and the conventional outflow pathways

Anterior chamber inner cornea and trabecular meshwork walls are lined with endothelium, while the anterior surface of the iris does not have it. During embryologic development, the anterior surface of the iris is covered with a continuous layer of endothelium, which disappears at or soon after birth [145]. Anyway, the AC endothelium is constantly immersed in AH having both plastic and trophic functions [146]. AH is secreted by the ciliary body into the posterior chamber of the eye. Aqueous humor cannot traverse the intact iris and thus it passes through the pupil to reach the AC of the eye. At the iris–corneal angle, the main part of this flow enters a pathway composed of the TM, the juxta canalicular connective tissue (JCT), the endothelial lining of the inner wall of Schlemm’s canal, Schlemm’s canal itself, and the collecting channels that lead to the episcleral veins and episcleral vessels. This outflow pathway is called the “conventional way” to distinguish it from the non-conventional outflow called the uveoscleral way. The posterior way or uveoscleral outflow passes through the iris root and the anterior face of ciliary muscle in the connective tissue interposed between the bundles of ciliary muscle to SCS. This pathway carries less than 10 % of the total flow in the older adult human eye [147]. The TM resides in the ocular limbus between the cornea and the sclera and comprises perforated, interlacing collagenous lamellae, called the TM beams. These have a core of collagenous and elastic fibers,

and are covered by flat cells which rest on a basal lamina. Although some authors still believe that spaces between the beams are occupied only by AH, other authors propose that the space between the beams is filled in with ECM where the AH filters through [148]. The beams are encapsulated by a single layer of endothelial-like cells [149]. The outermost JCT or cribriform region has no beams, but rather several cell layers which some authors claim to be immersed in loose extracellular material/matrix [150]. Between the beams of the corneoscleral meshwork and the basal lamina of the inner wall of Schlemm’s canal there is the JCT. ECM components have a major role in contributing to outflow resistance in human eyes. Several ECM proteins may contribute to homeostatic modifications of AH outflow resistance, being up- or downregulated [151]. Low concentrations of oxidized low-density lipoproteins stimulate ECM remodeling [152]. An increased fibronectin synthesis in ECM could result in concomitant increase of IOP [153]. Thus, ECM turnover is important in the regulation of aqueous humor outflow facility [154]. Transforming growth factors (TGFs) controls the expression of a wide variety of ECM genes, including elastin, collagens, fibrillin, laminin, and fibulin. Levels of one of its isoform, the TGF- β 2, are elevated in glaucomatous human AH [155] altering ECM metabolism [156]. TGF- β 2 is also responsible for anterior chamber-associated immune deviation, a mechanism that protects the eye from inflammation and immune-related tissue damage [157]. TGF- β 2 is associated with glaucomatous neuropathy, primarily via the increased synthesis and secretion of ECM proteins and remodeling of the ONH [158]. In TM, ECM production may be modulated by vitamin C [159, 160] that stimulate hyaluronic acid synthesis in glaucomatous TM cells [161] and increase outflow through the TM reducing the viscosity of hyaluronic acid [162]. Thus, it has long been known that high doses of vitamin C decreases IOP [163].

Other molecules that seem to play a very important role in collagen remodeling are the metalloproteinases (MMPs) matrix. These calcium- and zinc-dependent extracellular endoproteinases degrade ECM proteins [164] and interact with cells and their surrounding structures [165], decreasing collagen deposition and increasing AH outflow facility [3]. Nevertheless, it remains unclear what fraction of total resistance is attributable to the JCT and how ECM or specific ECM molecules might be involved in the generation of this resistance [166]. AH mainly crosses the inner endothelium wall of Schlemm’s canal by two different mechanisms: a paracellular route through the junctions formed between the endothelial cells [167] and a transcellular pathway through intracellular pores of the same cells [168]. Nevertheless, TM pores contribute only 10 % of the aqueous outflow resistance [169] and the density of inner wall pores increases with the volume of fixative perfused

through the outflow pathway on experimental conditions [170]. Anyway, the inner wall and underlying JCT work together to regulate outflow resistance [171]. In the conventional aqueous outflow pathway, there are two endothelial cell barriers separating the venous circulation from AH, which are specialized and positioned in series: the TM endothelial cells (TME) and subsequently the endothelial cells that line the lumen of Schlemm's canal (SCE). Between these two barriers, there is the JCT, which contains a loose ECM through which the AH flows [172]. The TME cells release factors into the AH that flow downstream from TMEs to bind and actively regulate the permeability properties of the SCEs. These factors, upon binding to SCE cells, increase the permeability of the SCE barrier [173], inducing a 400 % enhance in SCE conductivity by means of the activation of specific TME genes [174]. In particular IL-1 α and 1 β and tumor necrosis factor- α released by TME cells induce cell division and migration [175] in the cells near Schwalbe's line, while inducing the release of matrix metalloproteinases [176] and an increase of fluid flow across ECM tissues near JCT [173]. Therefore, the cytokines released by TME cells regulate the permeability of the SCE barrier in an active way [173].

Trabecular meshwork cells express aquaporin-1, a multiple water channel protein transporting water through membranes that can modulate cell volume [177] and tissue permeability. Indeed, outflow facility is increased by hyperosmotic solutions and decreased by hyposmotic solutions [178, 179]. Aquaporins also facilitate cell migration, [180], hydration, neuroexcitation, cell proliferation, fat metabolism, and other cell functions [181]. In experimental animal models, elevated IOP reduces aquaporin expression, suggesting a role for this molecule in the pathogenesis of glaucomatous optic neuropathy [182]. During glaucoma course, accumulated oxidative stress arising from the environment, vascular dysregulation, aging and/or the pathogenic processes could induce a sublethal damage to the outflow pathways [183]. These molecular changes in the surviving cells determine the expression of new genes [184] dependent on the nature of the damaging stimulus and on the other tissue type [185, 186]. In the AC, oxidized lipoproteins and free radicals are considered to be major causes of tissue stress and serve as local triggers for tissue inflammation [187]. Primary glaucoma is associated with an aqueous inflammatory response and is associated with changes in the aqueous cytokine profile [188]. A large number of inflammatory genes, including genes involved in complement activation and inflammatory cytokine/chemokine production, are upregulated, this in turn causing abnormal leukocyte-endothelial interactions and ultimately vascular damage [187]. Furthermore, the innate immune system in general and monocytes in particular play an important role in aqueous outflow homeostasis. Indeed,

under the influence of chemotactic signals, the monocytes circulate through the TM in the normal state and cytokines regulate the permeability of Schlemm's canal endothelial cells [189] and monocytes increase aqueous outflow [190].

Glaucomatous eyes exhibit a higher level of TM cell loss, than age-matched controls [82]. The decline of human TM cellularity is linearly related to age [82]. Noxious insults, such as free radical attack, trigger and enhance this mechanism [191, 192].

Contractile elements composing TM help to regulate the outflow facility [193]. Human TM cells contain smooth muscle-specific α -actin and have been identified as functional contractile cells, and in intact eye, the contractility balance between ciliary muscle and TM determines the total AH outflow [194]. Therefore, opening or fastening its slots, TM can change the quantity of cells involved in the passage of AH from AC to SC. As collector channels become altered with age or disease, other collector channels are available to assume the functional burden [195].

Similarity between anterior chamber and blood vessels

From a structural point of view, the AC may be seen as a specialized circulatory vessel in which the walls are lined with endothelial cells (i.e., corneal and trabecular) and the AH acts as the "blood". Malfunction in this endothelium may lead to outflow deterioration and result in increased IOP. The three-dimensional architecture of the human TM considerably increases the filtration surface between the TME and AH. The adjacent Schlemm's canal is a continuous endothelium-lined channel that drains AH to the general venous circulation [150]. These cells display an endothelial cell-like morphology, are avid phagocytes [196], possess a contractile and migratory apparatus [197, 198], have the capacity to produce ECM elements [199], and can transduce signals after stress-induced protein kinase C (PKC) attaches to the ECM [200]. The AC endothelium (ACE) not only acts as a barrier between AH and the surrounding tissues but, like in vessels, is a real organ with the function of modulating the tone and the flow rate in response to humoral, nervous, and mechanics stimuli. Under physiological conditions, the ACE plays an active role in cellular interchange, being able to adapt functionally and structurally to changes in the environment [201]. The normal endothelial function depends on both the anatomical continuity of cellular monolayer and its functional integrity [202].

The endothelium plays an important role in AC homeostasis. In particular, the TM endothelium plays a fundamental role in AH transit from the AC to Schlemm's canal (SC). TM endothelium releases into the media cytokines, which increase the SC endothelium barrier permeability

Table 1 Vascular protein as detected in AH of POAG patients by antibody microarray (data from Saccà et al. [206])

Protein	Role	Target
ELAM1	Inflammatory responses	Endothelium and cytoskeleton
Apo B	Inflammatory responses	Endothelium
Apo E	Oxidative stress response	Endothelium
VASP	Cell adhesion and motility	Endothelium
Hsp60	Mitochondrial chaperonin	Endothelium
Hsp70	Immunoreactivity	Cytoskeleton
Myogenin	Muscle growth and regeneration	Cytoskeleton
Myogenic factor 3	Regulator of skeletal myogenesis	Cytoskeleton
Myotrophin	Myofibrillar growth pattern	Cytoskeleton
Ankyrin	Anchors cytoskeletal components	Cytoskeleton
Phospholipase C	PKC activator	Cytoskeleton
Ubiquitin	Regulation of endothelial NOS activity	Endothelium

The presence of these proteins in AH indicates that POAG course damages the trabecular meshwork and in particular its endothelial and cytoskeletal components thus altering both TM functionality and motility

upon SC endothelium binding [174] and flow downstream with the AH to influence SCE barrier function and regulate the egress of AH [172]. When the IOP is greater than the venous pressure, the increased tension makes the trabecular beams and cords taut, which then triggers the stretch receptors to activate the TM endothelium to release vasoactive factors that will increase the flow across SC endothelium [174]. The presence of stretch receptor in TM is supported by recent literature indicating that epithelial sodium channel proteins may function as sensors of pressure-induced vascular stretch and laminar flow [203]. Indeed, IOP is regulated by the transport of AH across epithelial eye structures, which in turn is associated with ion flux. The specific upregulation of epithelial sodium channel proteins acts as a protection mechanism against IOP increase [204]. Therefore, the endothelial cells of the TM and Schlemm's canal constitute a system that governs the outflow through complex interactions. These interactions proceed in both directions and involve TM–SC mutual relationships [174].

A further element supporting the similarity between AC and blood vessel is the presence of protein marker typically detected in atherosclerotic vascular lesions. ELAM-1 has been detected in TM and its increase observed in POAG [205]. Recently, our group discovered in AH the presence of several proteins usually linked to the atherosclerotic plaque. Levels of these vascular proteins (Table 1) were significantly increased in AH of glaucomatous patients compared with expression levels in healthy controls [206].

Endothelial dysfunction

Vascular dysfunction is commonly observed in many diseases and is closely related to oxidative stress [207, 208]. Higher levels of reactive oxygen and nitrogen species occurring in vasculature have been found in hypertension, hyperlipidemia, atherosclerosis, and diabetes mellitus

being established that these reactive species induce oxidative damage in vascular tissue [209].

Chronic eye diseases are characterized by decreased biosynthesis and/or bioavailability of NO [210], excess of superoxide [211], and endothelin production [212]. TNF- α (tumor necrosis factor-alpha) regulates NOS expression and activity, which exert direct effects on NO production. TNF- α increases iNOS expression by activating NF- κ B. Increased TNF- α expression induces ROS production. TNF- α also activates NF- κ B transcription, which regulates the expression of genes involved in inflammation and oxidative stress [213–215]. Glaucoma syndromes are characterized by these bio-humoral changes. A balance between vasoconstrictors and vasodilators is necessary for maintaining the physiological structure and function of endothelia [194], modulating the permeability of the endothelial barrier, and releasing of ET and NO. This balance is impaired in glaucoma, with major consequences. Cyclic GMP and NO₂ concentrations were lower in the AH of POAG patients than in normal eyes [216]. NO donors decrease IOP by increasing the aqueous outflow facility in the TM and/or Schlemm's canal through cellular mechanisms known to regulate outflow facility, including changes in cell volume and cell contractility. In fact, the NO promotes the conversion of GTP to cGMP. This process activates protein kinase G (PKG), which then determines the phosphorylation and therefore the activation of the BKCa ionic channel [217]. These potassium (K⁺) ion channels are activated by calcium (Ca⁺⁺), are located at the cell membrane, and are activated by electric potential changes across the membrane or by an increase of intracellular Ca⁺⁺ concentration. Potassium ion channel activation determines the exit of K⁺ from cells and the consequent reduction of cell volume and osmotic pressure [218]. TM cell volume reduction allows for enlarged intertrabecular spaces and consequently greater exposure of the cell surface and enhanced

AH outflow. Furthermore, the NO-dependent soluble guanylate cyclase/cyclic guanosine monophosphate system plays an important role in regulating the AH dynamics that control AH production in the ciliary processes [219, 220], with subsequent decreases in IOP [221].

Endothelins also participate in IOP regulation; despite TM mobility, ET-1 (endothelin 1) induces TM contraction and increased outflow resistance, whereas TM relaxation increases outflow [194]. ET-1 is the most powerful constricting substance produced by endothelium, and it acts on specific ETA and ETB receptors. ETA receptors are present only in smooth muscle cells and cause vasoconstriction and cell growth. ETB receptors (which also lead to vasoconstriction) are present on smooth muscle cells, the expansion of which stimulates NO production and provides negative feedback by inhibiting the further production of ET-1. This negative feedback mechanism is compromised when the bioavailability of NO is reduced, and vasoconstriction effect is consequently increased [222]. The effects of endothelin-induced vasoconstriction in the anterior part of the eye cause a decrease in ocular blood flow, followed by pathological changes in the retina and the ONH; these processes are assumed to contribute to the degeneration of the RGCs. Nevertheless, trabecular outflow is modulated by TM contractility, which is affected by endothelin [223]. High ET-1 levels have been reported in the AH of patients with glaucoma [224, 225], and abnormal vascular responses to endothelin or its receptor antagonists have been shown in patients with normal tension glaucoma (NTG) [226]. Although direct evidence for local ocular endothelial dysfunction (EDf) is difficult to obtain, Henry et al. [227] demonstrated general EDf in a group of patients with NTG. This association could be due to attenuated ETA receptor-mediated tone, increased ETB receptor-mediated contraction, or impaired ETB receptor-mediated endothelial NO release [228]. There may be both a dysregulated vascular response to increased endothelin levels and a direct endothelin effect on target tissues, depending on the expression and distribution of their endothelin receptors [229]. NO can modulate the expression, sensitivity, and signal termination of endothelin receptors [230].

Primary open-angle glaucoma is also associated with peripheral vascular EDf [231]. This dysfunction can include the TM endothelium, which exhibits mitochondrial dysfunction in POAG patients [16] and a higher susceptibility to ROS than other tissues that constitute the AC [76]. Siasos et al. [232] have reported that POAG patients have significantly impaired endothelial function, which has been linked to an increased inflammatory status that is a causative mechanism of EDf. Furthermore, ET-1 has been linked to various other glaucoma-associated effects on the optic nerve and RGC including astrogliosis, ECM remodeling, and NO-induced damage [233]. Additional evidence of

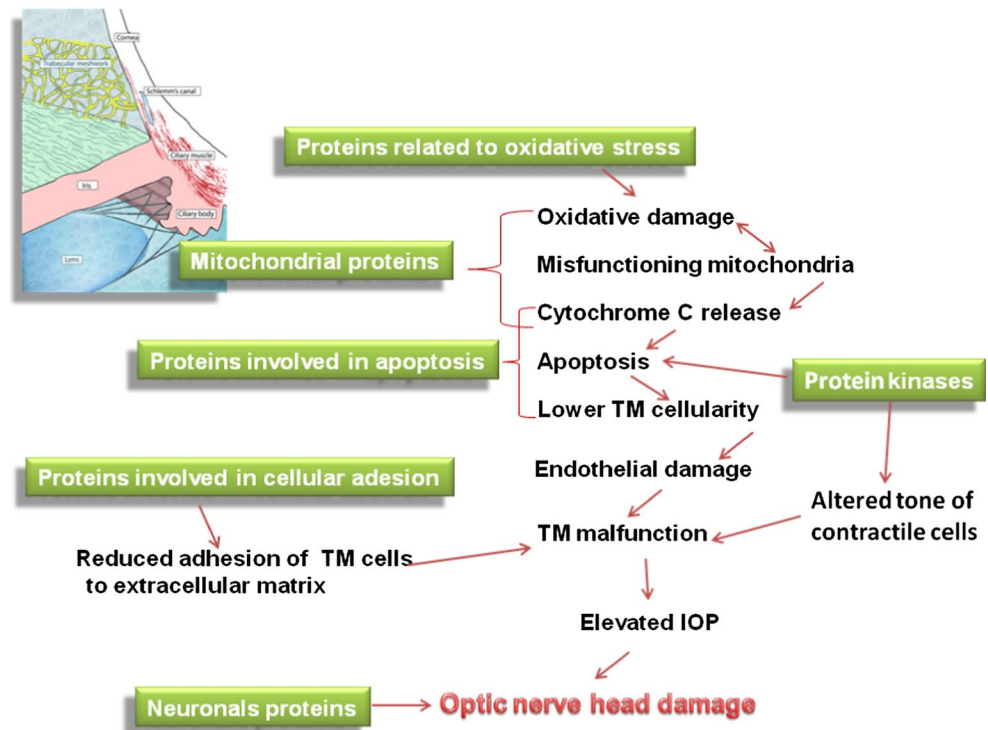
EDf arises from data showing increased levels of ELAM-1 in the AH. ELAM-1 is the earliest marker of atherosclerotic plaques in the vasculature. It is activated in human TM cells collected from patients with glaucoma [205], and it represents the sustained activation of a stress response that results in the expression of pro-inflammatory markers [234] and an index of endothelial damage.

Aqueous humor proteome during glaucoma course

An intriguing component of the glaucomatous AH is its protein fraction. Some stimuli, including elevated IOP, exert physical forces on HTM cells, causing mechanical stretch. This stretching can have a profound effect on their gene expression profile and may dramatically change the AH proteomics during glaucoma. To date, several molecules that appear to be involved in POAG pathogenesis have been identified. Many of these molecules are involved in the control of ECM turnover in TM [235]. Evidence that molecular alterations in the AH reflect glaucoma pathogenesis has been published by Izzotti et al. [71, 236]. The expression of 1,264 proteins was analyzed by detecting remarkable changes in the AH proteins of glaucomatous patients relative to matched controls. These findings shed light on the biomolecular mechanisms involved in human glaucoma pathogenesis. AH proteome profile undergoes dramatic changes in POAG patients compared to matched controls, even if total protein amount was only slightly, and not to a statistically significant extent, increased.

Proteins having a significant (more than two-fold) variation in expression between POAG patients and controls can be classified into 6 groups reflecting glaucoma pathogenesis. The first group includes mitochondrial proteins that are involved in the electron transport chain, trans-membrane transport, protein repair, and mitochondrial integrity maintenance. Under physiological conditions, these proteins are segregated inside cells in functional mitochondria. The presence of these proteins in AH provides evidence of both mitochondrial dysfunction and distinct injury occurring during glaucoma. This molecular event is linked to myocilin and its mutations impairing mitochondrial functions in HTM cells [237], affecting the actin cytoskeletal structure [238], and conferring differential sensitivity to oxidative stress [239]. Myocilin mutations are typically associated with high IOP, while optineurin (OPTN) mutations are associated with normal tension glaucoma. These associations imply that myocilin alteration has more impact on the cells of the aqueous outflow pathway, i.e. the TM, than those in the ONH and in the retina, i.e. RGCs, and vice versa in the case of OPTN [240]. OPTN expression in TM is increased by IOP increases [241, 242]. OPTN expression is regulated by various cytokines, particularly NF- κ B

Fig. 7 Histopathological and ultrastructural functional alterations occurring in glaucomatous TM as highlighted by AH proteome analysis



[243], which can be activated by increased IOP, ageing, vascular diseases, and oxidative stress. Furthermore, OPTN also plays an important role in regulating several genes, including myocilin [244].

The second group consists of proteins involved in apoptosis induction through the intrinsic (i.e., mitochondrial-dependent) pathway. The relationship between TM cell loss and the apoptosis observed in POAG patients can be triggered by many events, including oxidative stress [10, 245], intense phagocytosis, and inflammation, either alone or in combination with [246] mechanical stress [247]. Nevertheless, these pathways induce endothelial cell death, which first leads to Edf and then to complete TM malfunction with the consequent increase in IOP (Fig. 7). Cytochrome c is prominent in this group of proteins. It is released from the mitochondrial cristae following elevated hydrostatic pressure or oxidative stress and leads to apoptotic cell death. Cytochrome c release on day 3 of pressure treatment is correlated with caspase-3 activation and apoptotic cell death in differentiated RGC-5 cells [248]. The presence of cytochrome c in the AC is due to an apoptotic mechanism led towards to TM cells. It is conceivable that AH proteome alteration reflect from anterior to the posterior ocular segment thus establishing a communication mediated by proteins between AC and ONH. Many proteins enter the AH of the AC by diffusing through the root of the iris [249]. From there, they reach the supraciliary space and move as far as the suprachoroid layer of the peripapillary retina in the posterior segment [250]. This process demonstrates how

different molecules, particularly proteins, spread freely in the anterior and posterior segments. Therefore, these proteins may acquire the task of transmitting signals to both the AC and posterior segment cells. This situation bears pathogenic relevance in particular for AH proteins highly expressed in glaucoma and having neurological relevance.

Indeed, the third group is composed by neuronal proteins. It includes optineurin and growth and differentiation factors involved in neurogenesis and neuronal survival. Their presence in the AC reflects the neural embryologic origin of the TM cells, which have a neuro-ectodermal origin and, in part, a neural-like phenotype [32]. Indeed, TM cells are derived from the mesenchymal cells of the ectodermal neural crest [251]. This is unique situation in human body because all vascular endothelial are of mesodermal origin. Conversely, TM is an endothelium of ectodermal origin, thus bearing cross-reacting antigens with ONH lamina cribrosa. Indeed, TM and lamina cribrosa display remarkable similarity in protein expression [252]. Neural protein may be detected by proteome analysis in AH under physiological conditions [253] and are enhanced in vitreous humor in case of proliferative retinopathy [254]. Accordingly, it has been proposed [71] that the high amount of neural proteins detectable in POAG AH derives from ONH damage, reflecting on both vitreous and AH composition due to the protein exchange occurring through the uveo-scleral pathway [250].

The fourth group consists of proteins involved in intercellular adhesion. These proteins include catenins,

junctional plaque protein, dynein, and cadherins. The presence of these proteins confirms that Edf plays an important role in the pathogenesis of TM damage by permeabilizing the barrier between the AC and Schlemm's canal. Thus, outflow decrease in glaucoma is related to loss of mechanical integrity in the TM as a consequence of reduced cell–cell and cell–matrix adhesion. Catenins are WNT proteins, and the WNT signaling pathway controls the activity of the genes needed at specific times during development and regulates the interactions between cells as organs and tissues are forming. Interestingly, normal myocilin is a modulator of WNT signaling and provides a useful tool for reorganizing the cytoskeleton of TM cells and regulating IOP [255]. E-cadherin is one of the most important cell surface glycoproteins involved in cell morphogenesis. It is closely related to metalloproteinase and has an important but not well-understood role in the onset and progression of POAG [256]. N-cadherin, a Ca^{2+} -dependent cell–cell adhesion molecule, is an effector of the optomedin gene, which is a neural protein playing an important role in differentiating the brain and retina by modulating cytoskeleton organisation, cell–cell adhesion, and migration [257]. Proteins interconnecting TM cells exert a role in glaucoma pathogenesis because their alteration hampers TM function and integrity. Indeed, it has been recently reported that phosphorylation of Tyr14 in caveolin-1 (CAV-1) and transcriptional regulation of CAV-1 expression have a role in glaucomatous alterations in TM cells [258]. Furthermore, polymorphisms of genes encoding for caveolins have been established to act as risk factor for POAG by genome-wide association studies [28].

The fifth group includes protein kinases, which are key regulators of cell function constituting one of the largest and most functionally diverse gene families. PKC pathway modulation is relevant in glaucoma. PKC inhibitors relax TM and affect expression of matrix metalloproteinase and PGF2 alpha thus increasing AH outflow by regulating myosin light chain phosphorylation and the morphological and cytoskeletal characteristics of TM and SC cells [259]. PKC has been implicated in activating iROS production by NAD(P)H oxidase in endothelial and smooth muscle cells and contribute to induce iROS production [260]. PKC gamma is a unique PKC isoform that is found in neuronal cells and eye tissues. This isoform is activated by ROS, such as H_2O_2 , which activates protein kinase $\text{C}\gamma$ through the C1 domain, this activation resulting in the inhibition of gap junctions [261]. This confirms the key role of EDF in the course of glaucoma. PKCs are involved in apoptosis activation and signal transduction [262, 263].

Finally, the sixth group of AH proteins altered in glaucoma is related to oxidative stress and includes nitric oxide synthase, SOD, and microsomal glutathione S-transferase 1. In a study by our group [264], the levels of the

antioxidant enzymes SOD and glutathione transferase in the AH were significantly lower in POAG patients than in controls, whereas the levels of the pro-oxidant enzyme NOS and glutamine synthase in the AH were significantly higher in patients with POAG than in controls. This finding confirms that the AH antioxidant defenses in glaucoma patients are insufficient, and failure of these antioxidant defenses results in damage to the TM due to the remarkable susceptibility of this tissue to oxidative injury [76].

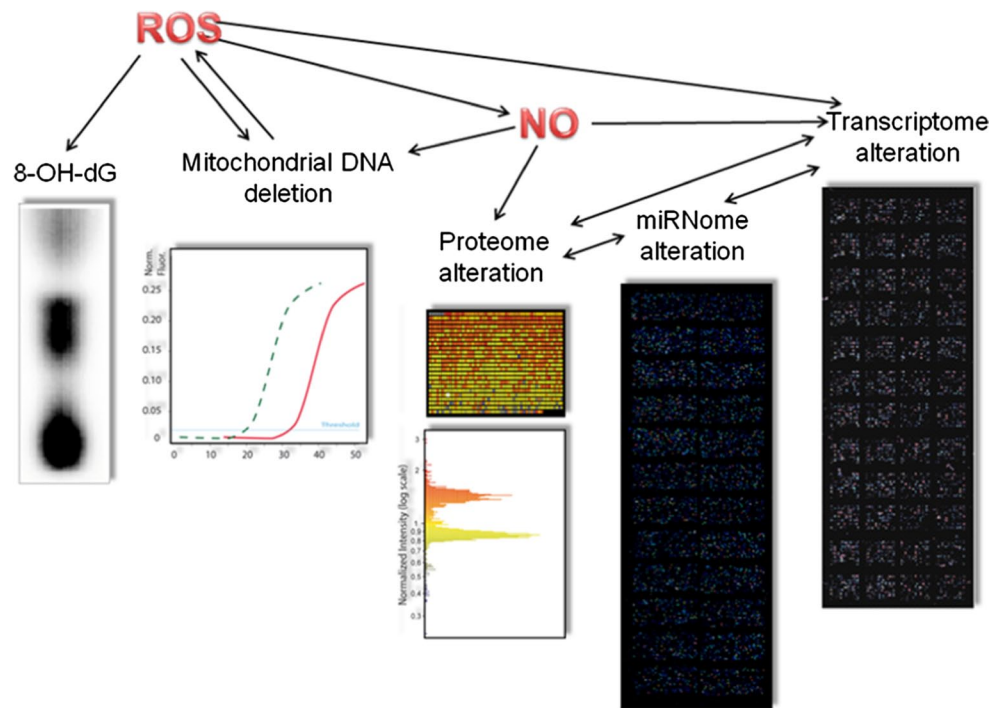
The origin of these AH proteins may leave some doubt. However, it is unlikely that their origin is in either iris or cornea. In fact, cornea endothelia do not suffer specific abnormalities in the course of glaucoma, and iris loses the endothelium after the birth. Plasma-derived proteins enter AH from the ciliary body at the iris root [265], but a reflux in the course of POAG from Schlemm's canal or from the iris root has never been disclosed. TM damages both functional and morphological are in contrast well known, validating the hypothesis of a TM origin for AH protein changes.

Furthermore, vascular endothelial growth factor (VEGF) is increased in AH of glaucomatous eyes [266]. VEGF induces endothelial cell proliferation, promotes cell migration, and inhibits apoptosis. Its presence in glaucomatous AH might be justified with the mechanism of activation of endothelial cells and then with an increase in endothelial permeability. Indeed, the permeability-inducing factor and the endothelial cell growth factor are encoded by a single VEGF gene [267]. In addition, vascular permeability factor had been characterized as a protein that promotes extravasation of proteins from tumor-associated blood vessels [268]. Therefore, a trabecular origin of AH's proteins is strongly plausible.

Conclusions

The molecular alterations described as occurring in TM are the first events triggering POAG. Shedding light on this early TM molecular damage can be useful to develop preventive strategies aimed at protecting TM before the occurrence of irreversible neural damage. Ageing, ROS, and other pathogenic events can decrease TM cellularity until levels of inadequacy for the passage of AH from AC to Schlemm's canal. This situation results in IOP increase and unleashes a vicious circle of molecular events leading to POAG development, including mitochondrial dysfunction [66], endothelial cell dysfunction [269], and finally functional TM impairment [3]. When severe damage occurs in TM endothelial cells in tight contact with AH, intracellular proteins leak from damaged cells, thereby changing the AH composition [71]. The presence of neurological proteins in glaucomatous AH [71] reflects the

Fig. 8 Omics techniques so far applied to the study of glaucoma. Genomic methods include analysis of nuclear DNA oxidative damage (8-OH-dG) by ^{32}P postlabeling and analysis of mitochondrial DNA deletion by qPCR. Microarray-based postgenomic methods include expression analysis of gene (transcriptome), micro-RNA (miRNome), and proteins (proteome)



occurrence of a similar pathological condition affecting both TM and ONH [252].

Trabecular meshwork actively modulates AH outflow from AC to Schlemm's Canal and not a mere passive way of egress. Little is known about the mechanism transmitting glaucomatous molecular signals from the AC to the retina and then to the central nervous system. It is conceivable that protein produced by damaged TM behave as a communicating signal for the posterior segment. Early molecular events happening in AC and AH can be studied by mean of newly developed biomolecular techniques thus disclosing new horizons for understanding glaucoma patho-physiology. The understanding of this molecular language of communication between AC and ONH is a new frontier for glaucoma. Thanks to the use of new omics methodologies molecular bases of POAG are beginning to be understood (Fig. 8). In the near future, this knowledge ought to be applied in the diagnostic and therapeutic field.

Microarray-based omics techniques able to detect early molecular damage in glaucoma include gene expression profiling as applied *in vitro* in endothelial cells [270] and *in vivo* in rat filtering belbs [271] and in mouse retina [272]. In human, microarray gene expression analysis in blood leukocytes has been recently reported to discriminate between glaucomatous patients and controls [273]. Micro-RNA expression profiling has been applied *in vitro* in cultured TM cells [274, 275].

The antibody microarray method seems to be very promising [71], and, at variance with other methods, is able to quantify glaucoma protein hallmarks by a moderately

invasive sample such as corneal puncture. It is conceivable that the invasiveness of this approach might be improved by the application of micropuncture techniques [236]. This method relies on the identification of different proteins differentially expressed in AH in healthy or sick subjects by means of specific antibody- and fluorescence-labeled probes. Fluorescent signals are acquired by means of a laser scanner and analyzed by means of software able to evaluate expression patterns of sets of proteins, thus being able to distinguish a normal from a POAG expression pattern.

This protein signature distinguishes different glaucoma types and could be useful for making a diagnosis at early stage. These techniques will furnish a very useful instrument of diagnosis and prevention, leading to a reduction of costs for public health. Indeed, the performances of the diagnostic test are increased by increasing the number of tested AH proteins. Performances include sensitivity (number of false negative results), specificity (number of false positive results), and accuracy (the transferability of the results from a low to a great number of subjects). AH antibody microarray analysis is able to test up to thousands of proteins in a single analysis using hallmark proteins to identify glaucoma-affected patients [71, 236].

The knowledge of molecular mechanisms leading to POAG onset will further help to develop new therapeutic approaches, counteracting TM oxidative stress and its molecular consequences, improving the energetic metabolism, correcting mitochondrial impairment, and decreasing the apoptosis rate. This molecular approach will further provide us with the tools to prevent the propagation of

damage leading to apoptosis from the anterior chamber and ONH.

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