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# Oxidative Stress in Anterior Segment of Primary Open Angle Glaucoma

Sergio C. Saccà<sup>1</sup> and Alberto Izzotti<sup>2</sup>

<sup>1</sup>*Department of Head/Neck Pathologies, St Martino Hospital, Ophthalmology Unit, Genoa,*

<sup>2</sup>*Department of Health Sciences, Faculty of Medicine, University of Genoa, Italy*

## 1. Introduction

The glaucomas is a group of complex and heterogeneous ocular diseases representing the second leading cause of blindness, and almost 75 million people are affected worldwide (Quigley 1999) being a major issue for public health. The prevalence of glaucoma increases with age (Friedman et al. 2006). The reported prevalence among whites in their 80s varies widely across these studies, with estimates as low as 1.9% to as high as 8.8%. Glaucoma is a syndrome characterized by a progressive optical atrophy resulting from the apoptosis of the retinal ganglion cells (RGCs). Growing evidence obtained from clinical and experimental studies over the past decade strongly suggests the involvement of the reactive oxygen species (ROS) in glaucoma. Free radicals can directly induce neuronal death by a protease and phosphatase-gated mechanism distinct from apoptosis (Sée and Loeffler 2001). In glaucoma free radicals may damage the trabecular meshwork (TM) (Saccà et al. 2005) while in the posterior segment of the eye the process of apoptotic retinal ganglion cell death starts with exposure of glial cells to elevated concentrations of free radicals (Nakazawa et al. 2006). The final neurological damage results in progressive RGCs death, axon atrophy and degeneration also extending to the brain cortex (visual areas) finally leading to the characteristic optical-cup neuropathy and irreversible visual loss (Weber and Harman 2005) (Yucel et al. 2000). In addition to the loss of the ganglion cells, the most of glaucoma types is characterized by having a high intraocular pressure (IOP). This is the most important risk factor for this disease, even if it is not yet clear what are the pathogenic events connecting IOP to glaucoma phenotype. In any case the TM damage has a key role in the increasing of IOP.

### 1.1 Trabecular meshwork: Functional anatomy

The chambers of the eye are filled with aqueous humour, a fluid with an ionic composition very similar to the blood plasma and with two main functions: to provide nutrients to the structures of the eye: cornea, iris and lens and to maintain intraocular pressure. Therefore the anterior chamber of the eye can be regarded as a highly specialized vascular compartment whose inner walls are composed of the endothelia of iris, cornea, and trabecular meshwork (Brandt and O'Donnell 1999). Aqueous humor 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 anterior chamber of the eye. At the iris-

corneal angle, the main part of this flow enters a pathway composed of the trabecular meshwork (TM), the juxtacanalicular 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 pass through the iris root and the anterior face of ciliary muscle, passing in the connective tissue interposed between the bundles of ciliary muscle to supracoroidal space. This pathway carries less than 10% of the total flow in the older adult human eye (Gabelt and Kaufman 1989). 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. The space between the beam is filled with extracellular matrix where the AH filters through (Chen and Kadlubar 2003). The beams are encapsulated by a single layer of endothelial-like cells (Polansky and Alvarado 1994) (**Figure 1**). The outermost juxtacanalicular or cribriform region has no collagenous beams, but rather several cell layers which some authors claim to be immersed in loose extracellular material/matrix (Tian et al., 2000). Histologic studies of POAG do not find a specific "plug" of the outflow pathways, suggesting instead that derangement of a cellular physiologic function may be involved (Johnson 2005). The functional aspects and morphology of the aqueous outflow pathways is still not clearly understood (Epstein and Rohen 1991). Some authors think that aqueous humor (AH) flow through TM structures in a passive way (Freddo and Johnson 2008; Tamm 2009) relegating the role of TM to a passive filter. Still others believe the TM is a tissue that is actively crossed from an active flow (Saccà et al. 2005; Alvarado et al. 2005a and b). Anyway, the locus of aqueous humor outflow resistance in the normal eye has not yet been unequivocally determined. Nevertheless experimental evidence supports the conclusion that the source of normal outflow resistance as well as the source of increased outflow resistance in glaucoma is attributable to the inner wall endothelium, its basement membrane, JCT, or some combination of all three of these tissues.

### 1.2 The juxtacanalicular tissue

The JCT, is the region of the meshwork positioned between the beams of the corneoscleral meshwork and the basal lamina of the inner wall of Schlemm's canal. Its small flow pathways would suggest a significant outflow resistance, but it is not supported by hydrodynamic studies (Ethier et al. 1986; Seiler and Wollensak 1985). Rather it manifests a decrease in extracellular matrix (ECM) components in hyaluronan (Knepper et al. 1996a) or an increase in outflow resistance to excess accumulation of glycosaminoglycans (Knepper et al. 1996b). It is possible that other extracellular matrix 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 (Vittal et al., 2005) and lower concentrations of oxidized low-density lipids stimulate ECM remodeling (Bachem et al., 1999). Interestingly, an increased fibronectin synthesis could result in concomitant increase of IOP (Fleenor et al., 2006). Transforming growth factors (TGFs) are a family of cytokines that control the production of a wide variety of ECM genes, including elastin, collagens, fibrillin, laminin, and fibulin. One of its isoform the TGF- $\beta$ 2 levels are elevated in glaucomatous human AH (Tripathi et al., 1994) and alter ECM



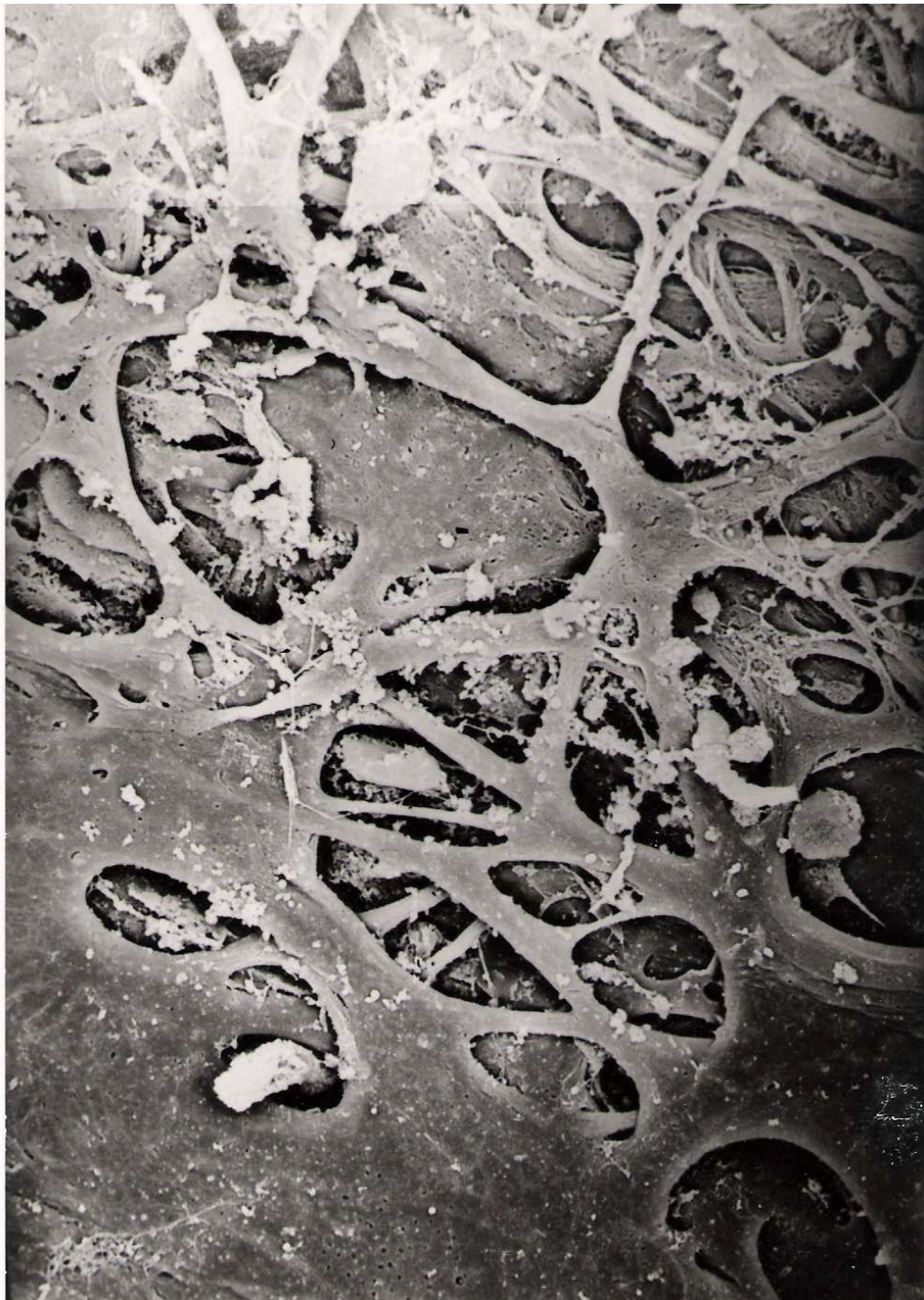


Fig. 1. Scanning electron microscope photograph of the human sclerocorneal trabecular meshwork (magnification 2000 X). The conventional outflow pathway consists of trabecular lamellae covered with human trabecular meshwork (HTM) cells, in front of a resistor consisting of juxtacanalicular HTM cells and the inner wall of Schlemm's canal. This tissue has unique morphologic and functional properties involved in the regulation of AH outflow. Endothelial cells of TM seem to have a leading role in outflow: probably, their tridimensional architecture and allocation on the trabecular beams considerably increases the filtration surface whose degeneration, resulting in the decay of HTM cellularity, causes IOP increase and triggers glaucoma (Saccà and Izzotti 2008).

metabolism (Wordinger et al., 2007). TGF in the AH is also responsible for anterior chamber-associated immune deviation, a mechanism that protects the eye from inflammation and immune-related tissue damage (Wilbanks et al., 1992). Indeed, TGF- $\beta$ 2 is one of the most important immunosuppressive cytokines in the anterior chamber of the eye and has a fibrogenic effect in trabecular cells (Alexander et al., 1998). Finally ECM production in the TM may be mediated by vitamin C (Epstein et al., 1990; Sawaguchi et al., 1992). Ascorbic acid is reported to stimulate increased hyaluronic acid synthesis in glaucomatous TM cells compared with normal human TM cells (Schachtschabel and Binninger, 1993). Also, ascorbate reduces the viscosity of hyaluronic acid, thus increasing outflow through the trabeculum (McCarty, 1998). Indeed, Virno already in 1966 discovered that high doses of vitamin C decreases IOP (Virno 1966). Other molecules that seem to play a very important role on collagen remodeling are the metalloproteinases (MMPs). MMPs are a family of calcium- and zinc-dependent extracellular endoproteases that degrade ECM proteins (Nagase and Woessner, 1999). Matrix metalloproteinases (MMPs) comprise a family of at least 25 secreted zinc proteases, which are of eminent importance not only for the ECM turnover, but also for interactions between cells and their surrounding structures (Sternlicht and Werb 2001). Indeed, increased MMP activity decreases collagen deposition, and AH outflow facility is increased by stimulating MMP activity (Saccà and Izzotti 2008). Anyway, it remains unclear what fraction of total resistance is attributable to the JCT and how ECM or specific ECM molecules might be involved in generation of this resistance (Overby et al. 2009). Anyway, ECM turnover is required to maintain the appropriate outflow resistance. (Bradley et al. 1998).

By analogy to other basement membranes in the body, the inner wall basement membrane has the potential to generate a significant portion of outflow resistance. This is discontinuous (Gong et al. 1996) and this characteristic may be related to the flow of aqueous humor into Schlemm's canal (Buller and Johnson, 1994). The resistance by this tissue seems to be substantially limited (Overby et al. 2009).

On the basis of electron microscopy studies, it has been proposed that aqueous humor 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 (Epstein and Rohen 1991) and a transcellular pathway through intracellular pores of the same cells (Johnson and Erickson 2000).

Nevertheless trabecular meshwork pores contribute only 10% of the aqueous outflow resistance (Sit et al. 1997). Furthermore characteristics of inner wall pores depend on fixation conditions. In particular, the density of inner wall pores increases with the volume of fixative perfused through the outflow pathway (Johnson et al. 2002). Scott et al. (2009) provided by confirmation that the inner wall and underlying juxtacanalicular connective tissue work together to regulate outflow resistance.

### 1.3 Meshwork endothelial cells

According to Alvarado, we know that in conventional aqueous outflow pathway there are two endothelial cell barriers separating the venous circulation from the aqueous humor, which are specialized and positioned in series: the trabecular meshwork endothelial (TME) cells and then, subsequently, the endothelial cells that line the lumen of Schlemm's canal (SCE) cells. Between these two barriers, there is the juxtacanalicular tissue, which contains a loose extracellular matrix through which the AH flows (Alvarado et al. 2004). The TME cells release factors into the AH, and these ligands 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 (Alvarado et al. 2005a) inducing a 400% enhance in SCE conductivity by means of the activation of specific TME genes (Alvarado et al. 2005b). In particular interleukin-1 $\alpha$  and 1 $\beta$  and tumor necrosis factor- $\alpha$  released by TME cells induce cell division and migration (Bradley et al. 2000) in those cells near Schwalbe's line, while inducing the release of matrix metalloproteinases (Kelley et al. 2007) and an increase of fluid flow across extracellular matrix tissues near JCT (Alvarado et al. 2005b). In a recent research (Izzotti et al. 2010a) for the first time we have provided evidence that aqueous humour molecular alterations reflect glaucoma pathogenesis. The expression of 1,264 proteins was analysed detecting remarkable changing in the aqueous humour proteins of glaucomatous patients as compared to matched controls. Among the others AH proteins we have observed that in patients with glaucoma those cytokines referred by Alvarado are expressed in significantly greater amount compared with controls. This finding is likely related to the fact that these cytokines are produced to improve the TM working but in the case of glaucoma TM does not respond properly because malfunctioning and therefore we are seeing an over-expression of these cytokines. Therefore, the cytokines released by TME cells regulate the permeability of the SCE barrier in active way (Alvarado et al. 2005b). Regulatory volume responses of TM cells influence the tissue permeability too; indeed, hyperosmotic solutions increased and hyposmotic solutions decreased outflow facility, respectively (Al-Aswad et al. 1999; Gual et al. 1997). The molecular mechanisms for regulating water balance in many tissues are unknown, but TM cells express aquaporin-1, a multiple water channel protein transporting water through membranes that can modulate cell volume (Stamer et al. 2001). Aquaporins also facilitate cell migration, (Verkman 2005), cell proliferation, neuroexcitation, fat metabolism, hydration, and others cell functions (Tradtrantip et al. 2009). Aquaporin may be implicated in the pathogenesis of glaucomatous optical neuropathy, indeed in animal model elevated IOP reduce its expression (Naka et al. 2010). Anyway, chronic sublethal injury due to cellular stress is a common theme in the pathogenesis of diverse diseases including atherosclerosis, glomerulonephritis and pulmonary fibrosis (Dunn 1991; Ross 1995). During glaucoma course, sublethal damage to the outflow pathways is developing, being the result of accumulated oxidative stress arising from the environment, vascular dysregulation, aging and/or the pathogenic processes (Flammer et al. 1999). Molecular changes in the surviving cells determine the expression of new genes (Dunn 1991; Ross 1995) dependent on the nature of the damaging stimulus and on tissue type (Mercurio and Manning 1999; Itoh and Nakao 1999). Glaucomatous eyes exhibit a high level of TM cell loss, above and beyond that of age-matched controls (Alvarado et al. 1984). The decline of human TM cellularity is linearly related to age (Alvarado et al., 1984). Grierson has calculated that at 20 years of age the estimated TM cell number for the whole meshwork is 763,000 (Grierson and Howes, 1987) and the cells number decreases to 403,000 by the age of 80 years, with a loss rate of 6000 per year (Grierson et al., 1982).

The mechanism of cell loss and the environmental factors contributing to it are not yet known. However, this phenomenon may be brought about by cell death caused by noxious insult, such as free radical attack (Yan et al., 1991; Padgaonkar et al., 1994).

In the anterior chamber (AC), oxidized lipoproteins and free radicals are considered to be major causes of tissue stress and serve as local triggers for tissue inflammation (Xu et al. 2009). The up-regulation of a large number of inflammatory genes, including genes involved



in complement activation and inflammatory cytokine/chemokine production, which in turn cause abnormal leukocyte-endothelial interactions and ultimately vascular damage (Xu et al. 2009). Furthermore, the innate immune system in general and monocytes in particular play an important role in aqueous outflow homeostasis: presumably under the influence of chemotactic signals, the monocytes circulate through the trabecular meshwork in the normal state and cytokines regulate the permeability of Schlemm's canal endothelial cells (Shifera et al. 2010) and monocytes increase aqueous outflow (Alvarado et al. 2010).

This last mechanism is most easily understood if we think AC as a vase and its endothelium as that of specialized vase in which flows AH; the endothelia of this vase is the TM whose complex structure represent a system to increase the area of contact between the TME cells and the AH. Indeed, the TM has been shown to be composed of contractile elements, which helps to regulate the outflow facility (Wiederholt et al. 2000). Therefore, opening or fastening its slots, TM can change the quantity of cells involved in the passage of AH from AC to SC. Its malfunction thus leads to the intraocular pressure increase. Finally, it is to remember that fluid flow is not equal within the trabecular meshwork and that preferential pathways or flow to areas of lower resistance exist in the trabecular meshwork. (De Kater et al. 1989; Tripathi 1971). One factor contributing to preferential flow may be changes in extracellular matrix interactions with Schlemm's canal cells in collector channel regions. As collector channels become altered with age or disease, other collector channels are available to assume the functional burden (Hann and Fautsch 2009).

## 2. Oxidative stress and Trabecular Meshwork

The interaction of many risk factors converge on intracellular signaling pathways, affecting the balance between protein synthesis and breakdown, inducing mitochondrial damage and apoptosis, which cause the primary glaucoma pathology through a significant loss of TM endothelial cells. It is not known the exact sequence in which this disease starts, but it is a fact that glaucoma is not a condition relating to IOP alone. Oxidative stress (Izzotti et al. 2006), vascular abnormalities (vasospasm, systemic hypotension, reduced vascular perfusion in the optic nerve head and/or retina) (Flammer et al. 2002), glial activity (Kirwan et al. 2005), immune system (Tezel 2007), inflammatory stimulus (Rönkkö et al. 2007) are involved in glaucoma injury.

The “trait de union” of all these components is the oxidative stress (Kumar and Agarwal 2007). Oxidative DNA damage is an inevitable consequence of cellular metabolism and it is secondary to free-radical formation (Cooke et al., 2003).

The oxidative stress and related molecular damages occur in a cell, tissue, and organ in response to the exposure to oxidizing agents. The mayor types of free radicals are reactive oxygen species (ROS) and reactive nitrogen species. The free radicals are molecule fragments equipped with an unpaired electron (odd number of electrons in the last orbital, when normally the electrons are coupled). Under normal physiological conditions, a small fraction of the oxygen consumed by mitochondria is constantly converted to superoxide anions, hydrogen peroxide, hydroxyl radicals, and other ROS. To cope with the ROS, human cells express an array of antioxidant enzymes, including Mn<sup>2+</sup>-dependent superoxide dismutase (SOD), copper/zinc SOD, glutathione peroxidase (GP), glutathione reductase (GR), and catalase (CAT). SOD convert superoxide anions to hydrogen peroxide, which is then transformed to water by CAT. The NO radical is produced in organisms by the oxidation of one of the terminal guanido- nitrogen atoms of L-arginine (Palmer et al. 1988).

This process is catalyzed by the enzyme NOS. Depending on the microenvironment, superoxide and NO are readily converted by enzymes or nonenzymic chemical reactions into reactive non radical species such as singlet oxygen, hydrogen peroxide, or peroxynitrite, i.e., species which can in turn give rise to new radicals. At moderate concentrations, however, nitric oxide (NO), superoxide anion, and related reactive oxygen species (ROS) play an important role as regulatory mediators in signaling processes. Many of the ROS-mediated responses actually protect the cells against oxidative stress and reestablish "redox homeostasis." (Dröge 2002). Anyway, 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. Any signal or stimulus that triggers overproduction of ROS may induce the opening of the membrane permeability transition pore in mitochondria and release of cytochrome *c* and other apoptogenic factors, which ultimately lead the cell into apoptosis (Tatton and Olanow 1999).

The anterior chamber (AC) is a highly specialized structure of the eye. It is composed of several tissues and structures, including the posterior surface of the cornea, the anterior surface of the iris, the pupil, the pupillary portion of the lens, and peripherally, the sclerocorneal angle, where the trabecular meshwork (TM), the scleral spur, the ciliary body, and the iris root are located.

Both vitamin C and glutathione operate in fluid outside the cell and within the cell (Cardoso et al., 1998); the ascorbate content is higher in diurnal than in nocturnal aqueous humor (Reiss et al. 1986). Aqueous humor could act as a liquid ultraviolet-light filter for the lens by virtue of the ascorbic acid in the anterior chamber (Ringvold 1980). Also in vitreous vitamin C has a very important role; indeed human vitreous gel consumes oxygen by an ascorbate dependent mechanism. Anyway, the concentration of ascorbate in human vitreous is remarkably high (Shui et al. 2009). Hence, the vitamin might protect against oxidative or photo-oxidative damage (Garland 1991; Rose et al. 1998) in both the central corneal epithelium and aqueous humour (Giblin et al., 1984) and reacts with  $O_2$  to form  $H_2O_2$ , which in turn is neutralized by SOD and CAT. Continuous exposure of HTM cells to oxidative stress via  $H_2O_2$  results in ROS generation in mitochondria. This, in turn, stimulated NF- $\kappa$ B activation and subsequent production of interleukins and the induction of inflammatory mediators. (Li et al., 2007). A high level of ascorbic acid is necessary to maintain oxidative balance in the AH (Izzotti et al. 2009). A synergism between vitamin E and C has been envisaged, because vitamin C reduces oxidized vitamin E, which is crucial for protecting cell membranes from lipid peroxidation; thus, this synergism may have a role in the pathogenesis of glaucoma (Varma 1991; Kang et al. 2003). Indeed, resistance to AH outflow increases in the presence of high levels of  $H_2O_2$  in eyes with a glutathione (GSH)-depleted TM (Kahn et al. 1983). GSH plays a critical role as an intracellular defense system providing detoxification of a broad spectrum of reactive species and allowing their excretion as water-soluble conjugates (Meister, 1989). Glaucomatous patients exhibit low levels of circulating glutathione (Gherghel et al. 2005) and glutathione participates directly in the neutralization of free radicals and reactive oxygen species, and maintains exogenous antioxidants such as vitamins C and E in their reduced (active) forms (Saccà et al. 2007). Insufficient glutathione combined with exogenous  $H_2O_2$  may induce collagen matrix remodeling and TM cell apoptosis independently of mitochondria (Veach 2004). Therefore in glaucoma course occurs a decline in total antioxidant defenses and in particular in AH (Ferreira et al. 2004) that have a great impact on the TM endothelium: because this is not more protected



properly and because TM is the most sensitive tissue to oxidative radicals in the AC (Izzotti et al. 2009). This leads to the reduction in the TM cellularity, to TM failure and subsequently to IOP increase. Of course, oxidative DNA damage in the TM of patients with primary open-angle glaucoma is significantly higher than in controls (Izzotti et al. 2003). Furthermore, oxidative DNA damage in patients with glaucoma correlate significantly with intraocular pressure and with visual field defects (Saccà et al. 2005, Izzotti et al. 2003; Fernández-Durango et al. 2008). Therefore it is possible that the process that occurs in AC and at the level of the optic nerve head is the same, and that in both districts the oxidative stress play an important role. Indeed, in glaucoma animal model, through the induction of oxidative damage, mechanical and vascular factors working synergistically lead to the same final pathologic consequence, i.e. glaucoma (Prasanna et al., 2005).

### 3. Mitochondrial dysfunction

The most important and frequent ocular degenerative diseases including cataract, glaucoma and age-related macular degeneration are caused by multiple interacting factors. A large number of environmental and genetic factors play a role in common eye pathologies. Many of these risk factors are genotoxic therefore being emerging evidence that DNA damage play a role in the development of the degenerative diseases of the eye (Saccà et al. 2009). ROS production has been principally implicated in the pathogenesis of eye disease. In particular, it is possible to believe that the ROS are responsible for the decline of cells that occurs in TM during aging and glaucoma that is the basis of its bad functioning and failure (Alvarado et al. 1984 and 2005a; Saccà et al. 2005).

Nowadays it is established that POAG patients bear a spectrum of mitochondrial abnormalities (Abu-Amro et al. 2006). Mitochondrial theory of ageing, a variant of free radical theory of ageing, proposes that the accumulation of damage in mitochondria, and in particular in mitochondrial DNA (mtDNA), leads to human and animal ageing. Oxidative modification and mutation of mtDNA easily and frequently occur, and the extent of such alterations of mitochondrial DNA increases exponentially with age. Oxidative modification in mtDNA is much more extensive than that in nuclear DNA (Ames et al. 1993; Yakes and Van Houten 1997). Age-related alterations in the respiratory enzymes not only decrease ATP synthesis but also enhance the production of reactive oxygen species (ROS) through increasing electron leakage in the respiratory chain. With the accumulation of genetic defects in mechanisms of mitochondrial energy production, the issue of neuronal susceptibility to damage as a function of ageing becomes important (Parihar and Brewer 2007). Damage to mtDNA induces alterations to the polypeptides encoded by mtDNA in the respiratory complexes, with a consequent decrease in electron transfer efficiency, leading to further production of ROS, thus establishing a vicious circle of oxidative stress and energy decline. This deficiency in mitochondrial energy capacity is regarded as the cause of ageing and age-related degenerative diseases (Genova et al. 2004). On the basis of the fact that mitochondria are the major intracellular source and vulnerable target of ROS (Linnane et al. 1989) accumulation of somatic mutations in mtDNA is a major contributor to human aging and degenerative diseases. Hence, a vicious cycle contributes to the progression of degenerative process. In this cycle, first a primary mitochondrial mutations induces a mitochondrial respiratory defect, which increases the leakage of ROS from the respiratory chain. Then the ROS would trigger accumulation of secondary mtDNA mutations in

postmitotic cells, leading to further aggravation of mitochondrial respiratory defects and increased production of ROS and lipid peroxides from mitochondria, and thus resulting in degeneration of cellular components (Tanaka et al. 1996). This is the basis of mitochondrial dysfunction that occurs in glaucoma. In the anterior chamber of POAG patients the relationship between mitochondria and TM is still rather obscure. From a morphological point of view, we know that in the cribriform layer often contained small mitochondria (Rohen et al. 1993). Besides using an in vitro culture system of bovine trabecular meshwork cells Dexamethasone-treatment developed an increased number of mitochondria (Sibayan et al. 1998). TM cells from patients with POAG cells have high endogenous reactive oxygen species, low ATP, and that mitochondrial complex I defect is associated with the degeneration of TM cells (He et al. 2008). Therefore other information is taken not “in vivo”, but only from the study of cell cultures. Recently, it has been demonstrated in vitro that expression of mutant myocilin, a mitochondrial protein whose role in the arising of early glaucoma is established, sensitizes Cells to Oxidative Stress-Induced Apoptosis (Myung Kuk et al., 2010).

Recently we have demonstrated that mtDNA damage occurs in the target tissue of POAG: the TM, but not in other anterior chamber districts, e.g. the iris (Izzotti et al. 2010b). Furthermore genetic polymorphisms for antioxidant and apoptosis related genes affect the amount of mtDNA damage in TM (Izzotti et al. 2010b). The remarkable interindividual variability observed in this study could be due to differences in diseases status because all patients were carriers of advanced unbalanced POAG or related to mitochondrial haplotypes, which are characterized by different sensitivity to oxidative damage (Kofler et al. 2009). The lack of mtDNA damage in tissues different from TM observed in this study in iris is in agreement with the negative findings reported by other studies in blood lymphocytes of patients with POAG (Abu-Amero et al.: 2007). These findings indicate that glaucoma may be envisaged as a mitochondriopathy specifically occurring in TM.

Mitochondrial damage and loss occurring in TM trigger both degenerative and apoptotic phenomena resulting in cell loss, as specifically occurring only in primary open-angle glaucoma and in pseudoexfoliative glaucoma and not in other glaucoma types (Izzotti et al. 2011). Decreased cellularity of the TM appears to be a particular characteristic of POAG, but other authors reported that it does not seem to play a role in the pathogenesis of PEX glaucoma (Schlotzer-Schrehardt and Naumann 1995). Anyway, in all types of glaucoma in which IOP is high, TM malfunction resulting from cellularity decrease is the key to the development of the disease, in acute and chronic angle closure glaucoma too (Sihota et al. 2001).

Furthermore, glaucoma itself could also produce apoptosis of TM cells through mechanical stress (Grierson 1987) or through trabecular hypoperfusion (Rohen et al. 1993). An increase in oxidative stress may also contribute to cell loss or alterations in the functioning of TM cells (Izzotti et al. 2003; Saccà et al. 2005; Dela Paz et al. 1996). Mitochondrial damage has been detected only in primary open angle and pseudo-exfoliation glaucoma, as the outflow dysfunction in the other glaucomas studied may have a different underlying bases (Izzotti et al. 2011). A further confirmation of this type of pathogenesis is given by the study of AH.

#### **4. Aqueous Humour proteome reflects glaucoma pathogenesis**

The AC endothelium (ACE) is not only a group of cells that act 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. In physiological conditions the ACE plays an active role cellular interchange, being able to adapt functionally and structurally to changes in the environment (Verma et al. 2003). The normal endothelial function depends on both the continuity of cellular anatomical monolayer and by its functional integrity (Furchgott and Zawadzki 1980). In vessels, the endothelial dysfunction is characterized by vasoconstriction, platelet aggregation, proliferation of smooth muscle cells, and is related to a reduced bioavailability of nitric oxide (NO), to an excess oxidative burden, and to an increased action of endothelin ET-1 (Monnink et al. 2002; Landmesser et al. 2002).

NO is one of the more important substances produced by endothelium; it is a powerful vasodilator, an inhibitor of endothelial cell growth and inflammation. NO is a major intracellular and extracellular effective agent against oxidative stress, and it has a beneficial antioxidant effects against reactive oxygen species, such as  $H_2O_2$ , whose detrimental effects on aqueous humor outflow are established (Lutjen-Drecoll 2000). A prominent role in endothelial dysfunction must be assigned to NO inactivation by ROS. ROS react with NO producing peroxy-nitrites, which are cytotoxic agents, thus decreasing NO bioavailability or directly inactivating NO (Aslan et al. 2008).

The Endothelin-1(ET-1), a potent vasoconstricting peptide of endothelial production acts on specific receptors present only on smooth muscle cells and cause vasoconstriction and cell growth. ET-1 determine stimulates the NO production, which acts as negative feedback by inhibiting further ET-1 production (Heitzer et al. 2001). In case of reduced NO bioavailability this negative feedback mechanism is compromised, and consequently ET-1 vasoconstricting effect is increased (Haynes and Webb 1998).

A balance between vasoconstrictors and vasodilators is necessary for the maintenance of the physiological structure and function of endothelia (Gibbons 1997). Whenever the balance between vasoconstriction and vasodilatation is disrupted, as in glaucoma, the outcome is endothelial dysfunction and injury that has as consequence cellular proteins loss in AH.

Anterior chamber (AC) is a lumen of a vessel constituted by the cornea and iris and joint together by means of TM. The AC contains the aqueous humor (AH). The volume of the AC is approximately 0.25 mL, whereas the volume of the posterior chamber is 0.06 mL. AH is needed to guarantee optical transparency, structural integrity, and nutrition in the absence of blood vessels (Izzotti et al. 2009). Furthermore, this liquid has the task of protecting and supplying nutrients to the cornea, lens, and TM (Izzotti et al. 2006; Fuchshofer and Tamm 2009). Other functions ascribed to AH inflow have been less clearly defined (Krupin and Civan 1996) and include the delivery of antioxidant, such as ascorbate, and participation in local immune responses. The ciliary epithelium concentrates ascorbate in AH rendering its concentrations 40-fold higher in AH than in blood plasma (Krupin and Civan 1996). It is possible that ascorbate is not only a scavenger of ROS, but may be also a regulator of ion channel activity functioning as an endogenous modulator of neuronal excitability (Nelson et al. 2007). In any case, cells in the outflow pathway are subjected to chronic oxidative stress and go towards an impaired proteasome activity in TM (Govindarajan et al. 2008). Therefore, any alteration in proteasome function due to oxidative stress or aging would be also expected to increase the rate of accumulation of misfolded mutant myocilin in the endoplasmic reticulum and contribute to the pathogenic effect of this mutant protein in the mitochondrial functions in human TM cells (He et al. 2009). AH represents a protein-containing biological fluid fundamental for eye pathophysiology (Civan 2008). However,

the relationship between TM and AH proteins as related to POAG pathogenesis has not yet been explored. Many proteins expressed at high levels in healthy patients are reduced in POAG patients, while other proteins detected at low levels in normal subjects are increased in POAG patients.

Recently we have discovered 6 classes of protein specifically present in the AH of advanced glaucomatous patients (Izzotti et al. 2010a).

## 5. Proteins in glaucomatous Aqueous Humour

The first class of proteins were **mitochondrial proteins** involved in the electron transport chain, trans-membrane transport, protein repair, and mitochondrial integrity maintenance.

The second class were proteins involved in **apoptosis induction**, through the intrinsic, i.e., mitochondrial-dependent, pathway.

The third class were **proteins connecting cells**. These include catenins, junctional plaque protein, dynein, and cadherins.

The fourth class is composed by **neuronal proteins** like optineurin or growth and differentiation factors involved in neurogenesis and neuron survival.

The fifth class includes the **protein kinase** involved in apoptosis activation and signal transduction.

Finally, the sixth class concerns **the oxidative stress** and includes: nitric oxide synthetase, superoxide dismutase and microsomal glutathione S-transferase 1.

All these proteins testify that the AH composition is affected and reflects the mechanisms of glaucoma pathogenesis. Mitochondrial proteins presence, segregated into intact mitochondria under normal conditions, reflects that TM endothelium cells is affected by mitochondria loss and dysfunction, as specifically occurring only in TM and not other AC tissues during glaucoma. The presence of mitochondrial proteins in AH indicates that TM undergoes structural alterations and that in particular its endothelial cells loss mitochondrial proteins as a consequence of mitochondrial DNA deletion that leads to mitochondrial TM malfunction and destruction leading to apoptosis. Cellular loss is determined not only by proapoptotic proteins but also by other mechanisms involved and revealed by the presence of other protein: inflammation, vascular dysregulation, and hypoxia (Choi and Benveniste 2004; Li et al. 2006). Indeed, proteins of these functional groups are expressed in the AH of glaucomatous patients. This is the situation for BIK protein, normally located inside mitochondria, which activates apoptosis process through the intrinsic pathway, and for FAS protein, that is responsible for the activation of apoptosis through the extrinsic pathway in response to inflammation and/or oxidative stress. Furthermore, FAS has been demonstrated to provoke apoptosis increasing myocilin release from mitochondria to cytosolic compartments of TM cells (Sakai et al. 2007).

The presence of “proteins connecting cells” in glaucomatous AH, reflects the impairment of cytoskeleton organization, cell-cell adhesion and migration (Lee and Tomarev 2007). Calnexin presence in AH can be linked with the presence of mutant myocilin. Myocilin, the first protein genetically associated with the development of glaucoma, is a constituent of human AH and is expressed in many ocular tissues, with highest expression observed in cells of the trabecular meshwork (Adam et al., 1997;). While calnexin is a calcium-binding protein playing a major role in the quality control apparatus of the endoplasmic reticulum by the retention of incorrectly folded proteins. Normally, located in melanosomes, calnexin



presence in AH is likely to be caused by the death of pigmented HTM cells induced by mutant and wild-type myocilin depending upon the presence of misfolded protein in the ER (Liu Y, Vollrath 2004).

Myocilin is a signal secretory protein (Hebert and, Molinari 2007). It has both intracellular and intercellular functions (Ueda et al. 2002) and can be found in various organelles such as the endoplasmic reticulum (Liu Y, Vollrath 2004), Golgi apparatus (O'Brien et al. 2000), and moreover mitochondria (Wentz-Hunter et al. 2002). Myocilin increases both calcium concentration in cytoplasm and in mitochondria, possibly through the dysregulation of calcium channels (He et al. 2009). Excessive cytoplasmic  $\text{Ca}^{2+}$  leads to mitochondrial  $\text{Ca}^{2+}$  overload, which triggers ROS overproduction, mitochondrial membrane depolarization, and ATP production inhibition, all hallmark events of mitochondrial dysfunction and eventual apoptosis (Jackson JG, Thayer 2006; Dahlem et al. 2006). Hence mutant myocilin impairs mitochondrial functions in human trabecular meshwork cells (He et al. 2009) and may confer different sensitivity to oxidative stress depending on the mutation of this protein (Joe and Tomarev 2010) as induced on genetic basis in juvenile glaucoma or on degenerative ROS-mediated basis in POAG.

The presence of neuronal proteins in AH is not surprising, because TM cells have a neuroectodermic origin, expressing, at least in part, a neural-like phenotype (Steely et al. 2000) TM cells deriving from mesenchymal cells of the neural crest (Cvekl and Tamm 2004). The role of neural proteins in glaucoma pathogenesis is established. Optineurin plays a neuroprotective role in the eye and optic nerve. Optineurin protects the cell from oxidative damage and blocks the release of cytochrome c from mitochondria (De Marco et al. 2006). Its presence in glaucomatous AH suggests an antiapoptotic attempt by TM cells through NF $\kappa$ -B pathway regulation (Ray and Mookherjee 2009). Conversely, the presence of ankyrin bears witness to the TM cells degenerative process (Scotland et al. 1998).

Of great interest seems to be the presence of Kinase proteins in AH. Indeed, protein kinases C (PKC) could influence AH outflow affecting cellular relaxation, contraction, and morphological changes in TM and sclerocorneal cells (Khurana et al. 2003) and leading to secretion of matrix metalloproteinase (Husain et al. 2007). Cyclic mechanical stress induces changes in a large number of genes that are known to affect the AH outflow facility altering extracellular matrix composition, cellular cytoskeleton, and cell adhesion (Luna et al. 2009). The finding that AH proteome alterations reflects glaucoma pathogenesis confirms the importance of TM motility. TM malfunction is multifactorial being related not only to mitochondrial dysfunction leading to TM endothelial cell loss but also to the alteration of extracellular cell matrix and to the altered expression of genes governing TM functions and motility.

It is necessary the action of many factors for developing glaucoma, i.e. ageing, genetic predisposition, exogenous environmental and endogenous factors. Environmental factors can interact with genetic predisposition. In some families, the disease has a clearly dominant inheritance, but it is very rare cases. In a greater number of cases there is a certain genetic predisposition, witnessed by the presence of another member of a family, even though having a far relationship, affected by the disease. In particular, a concerning aspect is the individual sensitivity to light: in fact, this could encourage the radical free production that could induce damage. Indeed, reactive oxygen species are increased in glaucomatous AH determining decrease of the total antioxidant potential (Ferreira et al. 2004). In particular, we demonstrated that in the AH of glaucomatous patients as compared to normal subjects the antioxidant enzymes superoxide dismutases 1/2 and glutathione S transferase 1 are

significantly lower in POAG patients than in controls while the pro-oxidant enzyme nitric oxide synthetase 2 and glutamate ammonia ligase are significantly higher in POAG patients than in controls (**Figure 1**). This unbalance results in a pro-oxidative status mainly affecting in the AC of the eye TM, which indeed is particularly sensitive to oxidative damage thus triggering the glaucoma's pathogenic cascade (Izzotti et al. 2009).

The importance of oxidative stress in glaucoma pathogenesis is further highlighted by the recent discovery that some active antiglaucomatous drugs like Timolol (Izzotti et al. 2008) and Dorzolamide (Saccà et al. 2011), commonly used in glaucoma treatment, have antioxidant properties and counteracts adverse consequences of oxidative damage as occurring in whole TM and in specifically in its endothelial component. Timolol has an antioxidant effect on the whole cell while Dorzolamide exerts protective activity towards oxidative stress only in presence of intact mitochondria. Therefore, drugs targeting basic mitochondrial processes such as energy production and free radical generation, or specific interactions of disease-related protein with mitochondria, hold great promise for glaucoma therapy.

## 6. References

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Unit 405, Office Block, Hotel Equatorial Shanghai  
No.65, Yan An Road (West), Shanghai, 200040, China  
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元  
Phone: +86-21-62489820  
Fax: +86-21-62489821



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