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# Inflammation in Glaucoma: From the back to the front of the eye, and beyond

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## ABSTRACT

The pathophysiology of glaucoma is complex, multifactorial and not completely understood. Elevated intraocular pressure (IOP) and/or impaired retinal blood flow may cause initial optic nerve damage. In addition, age-related oxidative stress in the retina concurrently with chronic mechanical and vascular stress is crucial for the initiation of retinal neurodegeneration. Oxidative stress is closely related to cell senescence, mitochondrial dysfunction, excitotoxicity, and neuroinflammation, which are involved in glaucoma progression. Accumulating evidence from animal glaucoma models and from human ocular samples suggests a dysfunction of the para-inflammation in the retinal ganglion cell layer and the optic nerve head. Moreover, quite similar mechanisms in the anterior chamber could explain the trabecular meshwork dysfunction and the elevated IOP in primary open-angle glaucoma. On the other hand, ocular surface disease due to topical interventions is the most prominent and visible consequence of inflammation in glaucoma, with a negative impact on filtering surgery failure, topical treatment efficacy, and possibly on inflammation in the anterior segment. Consequently, glaucoma appears as an outstanding eye disease where inflammatory changes may be present to various extents and consequences along the eye structure, from the ocular surface to the posterior segment, and the visual pathway. Here we reviewed the inflammatory processes in all ocular structures in glaucoma from the back to the front of the eye and beyond. Our approach was to explain how para-inflammation is necessary to maintain homeostasis, and to describe abnormal inflammatory findings observed in glaucomatous patients or in animal glaucoma models, supporting the hypothesis of a dysregulation of the inflammatory balance toward a pro-inflammatory phenotype. Possible anti-inflammatory therapeutic approaches in glaucoma are also discussed.

## 1. Introduction

Glaucoma is a multifactorial progressive neurodegenerative disease that constitutes the most frequent cause of irreversible blindness. It was recently estimated that the number of people with glaucoma worldwide will increase from 76.5 million in 2020 to 111.8 million by 2040, mainly due to the aging population (Tham et al., 2014; Kolko et al., 2015a; Harasymowycz et al., 2016). Glaucoma is characterised by the loss of retinal ganglion cells (RGCs), thinning of the retinal nerve fibre layer,

and cupping of the optic disc (Weinreb et al., 2014). Glaucoma represents a group of heterogeneous diseases with varying clinical features including risk factors and interrelated mechanisms. Age, increased intraocular pressure (IOP), and a genetic background are the leading risk factors for glaucoma (Weinreb et al., 2014). Primary open-angle glaucoma (POAG) is the most prevalent form in Western countries. However, 30–40% of Caucasian patients with POAG, and an even greater proportion of the Asian population present with normal tension glaucoma (NTG), typically IOP < 21 mmHg (Esporcatté and Tavares, 2016). Thus,

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the etiology of POAG is generally described as mechanical and/or vascular. The mechanical process involves compression of the axons due to elevated IOP, while the vascular process includes events in which blood flow and ocular perfusion pressure are reduced to the posterior pole leading to damage (Flammer et al., 2002; Mozaffarieh et al., 2008; Yanagi et al., 2011; Grzybowski et al., 2020). Vascular or perfusion abnormalities in NTG include the increased frequency of migraine headaches, Raynaud's phenomenon and sleep apnoea (Shields, 2008). In high IOP glaucoma, it is clear that both the anterior and posterior segments are affected, as extensive damage is detectable in the trabecular meshwork (TM) and along the inner retina-central visual pathway (Saccà et al., 2016).

Pathogenic mechanisms of the neurodegenerative process induced by the mechanical and vascular stress involve ischemia/hypoxia (Harris et al., 2005), mitochondrial dysfunction, chronic oxidative stress (Chrysostomou et al., 2013; Osborne et al., 2017), excitotoxicity (Lebrun-Julien et al., 2009), metabolic stress (Vohra et al., 2019), decreased levels of nicotinamide (Kouassi Nzoughet et al., 2019; Williams et al., 2017a), neurotrophin deprivation (Pease et al., 2000), and neuroinflammation (Russo et al., 2016; Williams et al., 2017b; Jiang et al., 2020). Oxidative stress, mitochondrial dysfunction, and cell senescence are increased in the aging retina (Pinazo-Durán et al., 2014; Zhang et al., 2015; Sreekumar et al., 2020; Eells, 2019) and are considered among the main glaucoma risk factors. In the aging retina, oxidative stress and lipid peroxidation are considered the main causes of tissue stress leading to the activation of a local para-inflammation of various magnitudes (Xu et al., 2009).

Para-inflammation is defined as a tissue adaptive response to noxious stress or malfunction, and has characteristics that are considered as intermediate between normal/basal and inflammatory states (Xu et al., 2009). A physiological level of para-inflammation is necessary to maintain tissue homeostasis and to restore its functionality, but when tissue is exposed to stress and/or malfunction for a prolonged period, inflammation may play a deleterious role and be implicated in both initiation and progression of disease (Medzhitov, 2008). One concept is the dysregulation of para-inflammation, in the retina and other structures of the eye, in response to stress stimuli especially chronic oxidative stress. Para-inflammation is an important protective homeostatic mechanism in the RGC layer and optic nerve head (ONH) which is dysregulated in glaucoma. Excessive uncontrolled para-inflammation may produce inflammatory responses with a marked release of cytokines/chemokines causing irreparable damage to the neuroretina (Chen et al., 2010). Similar para-inflammatory mechanisms exist in the anterior chamber and their dysfunction may be related to TM dysfunction and increased resistance to aqueous outflow, the main cause of increased IOP in POAG (Saccà et al., 2016).

Moreover, a chronic subclinical inflammation has been widely documented on the ocular surface as a result of long-term topical treatments, and there is a clear correlation between the duration of treatment and the number of anti-glaucoma drugs, with age and severity of glaucoma (Broadway et al., 1994a, 1994b; Baudouin et al., 2010, Baudouin et al., 2013). This inflammation may also have a detrimental effect on conjunctival and corneal barrier functions and cause activation of highly immunologically active tissues. It has also been suggested that if the surface inflammation penetrates deeper into the anterior segment it can influence TM dysfunction (Baudouin et al., 2012; Janson et al., 2018). Consequently, glaucoma appears as a disease where inflammatory changes may be present to various extents and with various consequences all along the eye structure.

In addition to the pathophysiological interest of understanding the role of inflammation in glaucoma and how inflammatory mediators may interact together and possibly with extraocular components (e.g. the central nervous system, the immune or vascular components), modulating the local immune response mediated by the glial cells and the complement system to restore a normal immune homeostasis may be an attractive therapeutic target in glaucoma (Adornetto et al., 2019).

Therefore, a comprehensive understanding of ocular inflammatory mechanisms involved in POAG is necessary before considering potential anti-inflammatory therapeutic targets (Benhar et al., 2012). Based on the scientific literature and our own research in this field, we wanted to review the current understanding of inflammatory processes which may be involved in POAG from the back to the front of the eye and beyond.

## 2. Inflammation in retinal and optic nerve head degeneration

The glaucomatous degenerative disease is characterised by a progressive loss of RGCs, thinning of the retinal nerve fibre layer, and cupping of the ONH (Jonas et al., 2017). Various stimuli including chronic mechanical stress induced by elevated IOP, hypoxia/ischemia, and oxidative stress, coupled with deprivation of neurotrophic factors may cause RGC dysfunction and death, especially in aging retina (Vohra et al., 2013). It has been suggested that increasing stress over a prolonged and cumulative period may lead to a failure in the regulation of the local protective immune response mediated by glial cells and the complement system, leading to a neuroinflammatory degenerative process, contributing to disease progression (Tezel, 2011). There is also growing evidence that a loss of immune surveillance and neuroprotection by resident glial cells in the RGC layer and/or the retinal inner plexiform layer leads to neurodegeneration (Wang et al., 2016).

### 2.1. Immune surveillance in retinal tissues

As an immune privilege tissue, the defence system in the retina consists of retinal immune cells (including microglia, astrocytes and Müller cells) and the complement system (Jiang et al., 2020). Under normal aging conditions, retinal innate immune cells and the complement system undergo a low-grade activation. Para-inflammation is induced to rapidly eliminate damaged cells, cell debris and other products of the oxidative stress. This is performed through recognition and internalisation via scavenger or toll-like receptors (TLRs) expressed on glial cells, followed by glial cells activation and the release of complement factors, cytokines/chemokines and apoptotic factors to restore the retinal tissue homeostasis (Xu et al., 2009; Xu and Chen, 2016). Sustained oxidative insults for decades is believed to induce a higher degree of chronic para-inflammation (Xu et al., 2009; Chen et al., 2019). In disease state such as in glaucoma, the para-inflammation response is dysregulated and develops into detrimental inflammation.

#### 2.1.1. Complement system

The complement cascade is activated as part of the innate immune response against pathogens or injury, and plays a major homeostatic role by clearing damaged cells and cellular debris. The complement cascade is believed to play multiple roles during different stages of glaucoma including modulation of the immune response such as cell adhesion, chemotaxis, phagocytosis or cell lysis (Xu and Chen, 2016). A low-level of complement activation is maintained under physiological conditions through the alternative pathway. A gene expression study showed that a few genes involved in the classic complement pathway (C1qa, C1qg, C3, and C4) and the alternative complement pathway (Complement factor B and C3) were upregulated in the mouse aging retina, whereas two genes involved in the lectin complement pathway (Mannan-binding lectin-associated serine protease [Masp] 1 and Masp 2) were down-regulated (Chen et al., 2010). A discrete increased deposition of complement (C3d) was evidenced by confocal examination in the ganglion cell layer to the inner plexiform layer of the neuroretina, suggesting that increased complement activation also exists in the neuroretina of aged mice (Chen et al., 2010). Such activation of the complement system may be a protective adaptive response to chronic aged-related changes.

#### 2.1.2. Retinal immune cells

Glial cells, which include microglia, astrocytes and Müller cells help maintain retinal homeostasis (Vecino et al., 2016). They are activated to

restore tissue homeostasis and facilitate tissue cleaning and healing (Jiang et al., 2020). Microglial cells are resident retinal macrophage-like cells whose main functions are phagocytosis and elimination of cellular debris from damaged cells in the ganglion cell layer, the inner and outer plexiform, and the inner nuclear layer (Kaur et al., 2015). When activated, ramified quiescent microglial cells become amoeboid with the ability to proliferate, present antigens, and produce numerous neuro-destructive molecules, inflammatory mediators, including reactive oxygen species (ROS), cytokines, chemokines, prostaglandins, nitric oxide (NO) and glutamate (Nakamura, 2002). Microglial cells are also the main sources of retinal complement gene expression in aging retina (Luo et al., 2011). They can release numerous neurotrophic factors such as brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), glial cell line-derived neurotrophic factor (GDNF), nerve growth factor (NGF), neurotrophin-3 (NT3), and basic fibroblast growth factor (bFGF) and thus play an important role in neuronal physiology and survival (Carwile et al., 1998). Microglial cells also secrete soluble angiogenic factors suggesting a contributing role in the retinal vasculature system (Alves et al., 2020). Microglial functions are controlled by communication between neurons, astrocytes, and Müller cells. When well balanced, microglial activation is reversible and does not cause secondary neuronal degeneration (Nakamura, 2002; Harada et al., 2002).

Müller cells are the most abundant glial cells in the retina and span the entire thickness of the retina (Bringmann et al., 2006). These cells are responsible for the homeostatic and metabolic support of the surrounding RGCs, including production of adenosine triphosphate (ATP) and anti-oxidants, glucose metabolism, and ion/substrate exchange (Vohra and Kolko, 2018). Müller cells protect neurons via a release of neurotrophic factors, the uptake and degradation of glutamate and the secretion of the anti-oxidant glutathione (Bringmann et al., 2009; Toft-Kehler et al., 2016, 2017). These cells play a crucial protective role by preventing accumulation of glycine, gamma amino butyric acid (GABA) and glutamate in the retina (Lebrun-Julien et al., 2009).

Astrocyte cells line the pores of the lamina cribrosa and blood vessels and are thought to play a crucial structural role in secreting extracellular matrix (ECM) molecules (Schneider and Fuchshofer, 2016). Resting astrocytes function to maintain homeostasis, regulate blood flow, recycle neurotransmitters, maintain synapses, and participate in neurogenesis. In the ONH, astrocytes facilitate myelination and myelin maintenance by clearing extracellular ions and neurotransmitters and by secreting pro-myelinating factors. They are thus critical for retinal signals transmission to visual structure of the brain (Lundgaard et al., 2014). Astrocytes produce anti-inflammatory cytokines, heat shock proteins (HSPs) and neuroprotective factors (BDNF) aiding in neural regeneration. They are also the main producers of vascular endothelial growth factor (VEGF) in response to hypoxia, which plays an important role in retinal vascularisation (Russo et al., 2016; Nutma et al., 2020).

Reactive glial responses and para-inflammation represent the initial adaptive reaction of the retina to primary stress stimuli, which can become detrimental if stress persists or repeats (Pekny and Pekna, 2014). At extreme levels of activation in response to detrimental tissue damage and inflammation, Müller cells and astrocytes can cause scarring, contribute to neurodegeneration, and hinder regenerative processes in retinal tissue (Bringmann et al., 2009; Sofroniew, 2009).

## 2.2. Early pathogenic events in glaucoma

### 2.2.1. Early astrocytes remodeling in glaucoma

As reviewed by Calkins (2012), in POAG, stressors related to age and IOP lead to progressive degeneration of the retinal projection to the brain. This implies that events distal to the retinal ganglion cell bodies in the retina are fundamental for understanding both the progression of neurodegeneration and ultimate vision loss. Glaucoma progression has been characterised by early axon expansion and astrocytes migration to the optic nerve edge, followed by astrogliosis, axon loss through

caspace-dependent apoptosis of RGC (Calkins, 2012; Cooper et al., 2016, 2018). Using the DBA2/J mice model, Cooper et al. (2018) demonstrated that astrocytes remodeling without gliosis preceded optic nerve axonopathy. They found that astrocytes in the ONH organize to form a very well connected network as an early adaptive response to the IOP stress. When disease progresses, the well parallel organisation of astrocytes is lost, concurrently with the axonal degeneration.

Previously, they showed that the pre-degenerative expansion of axon size was accompanied by a retraction of the astrocytes process to the nerves edge, and the frank loss of mitochondria (Cooper et al., 2016). However, before axons are lost, they also expand with loss of cytoskeletal integrity as also demonstrated by Tehrani et al. (2014, 2016). Diminished bioenergetics resources could be a primary inducer of progression in glaucoma (Li et al., 2015) and the number of mitochondria could decrease when axon size increases (Cooper et al., 2016). Consistent results suggesting an early rearrangement of ONH astrocyte processes orientation and early loss of gap junctions, and thus loss of homeostasis in response to chronically elevated IOP was also reported using a rat glaucoma model (Johnson et al., 2000; Tehrani et al., 2014).

### 2.2.2. Early glial cells activation in glaucoma

Several animal glaucoma models have provided evidence for the activation and recruitment of microglial cells and astrocytes during early stage glaucoma prior to RGC loss and morphologically detectable glaucomatous damage to the optic nerve and retina (Naskar et al., 2002; Bosco et al., 2011; Wang et al., 2014; Sapienza et al., 2016; Johnson et al., 2007; Lozano et al., 2019; Oikawa et al., 2020).

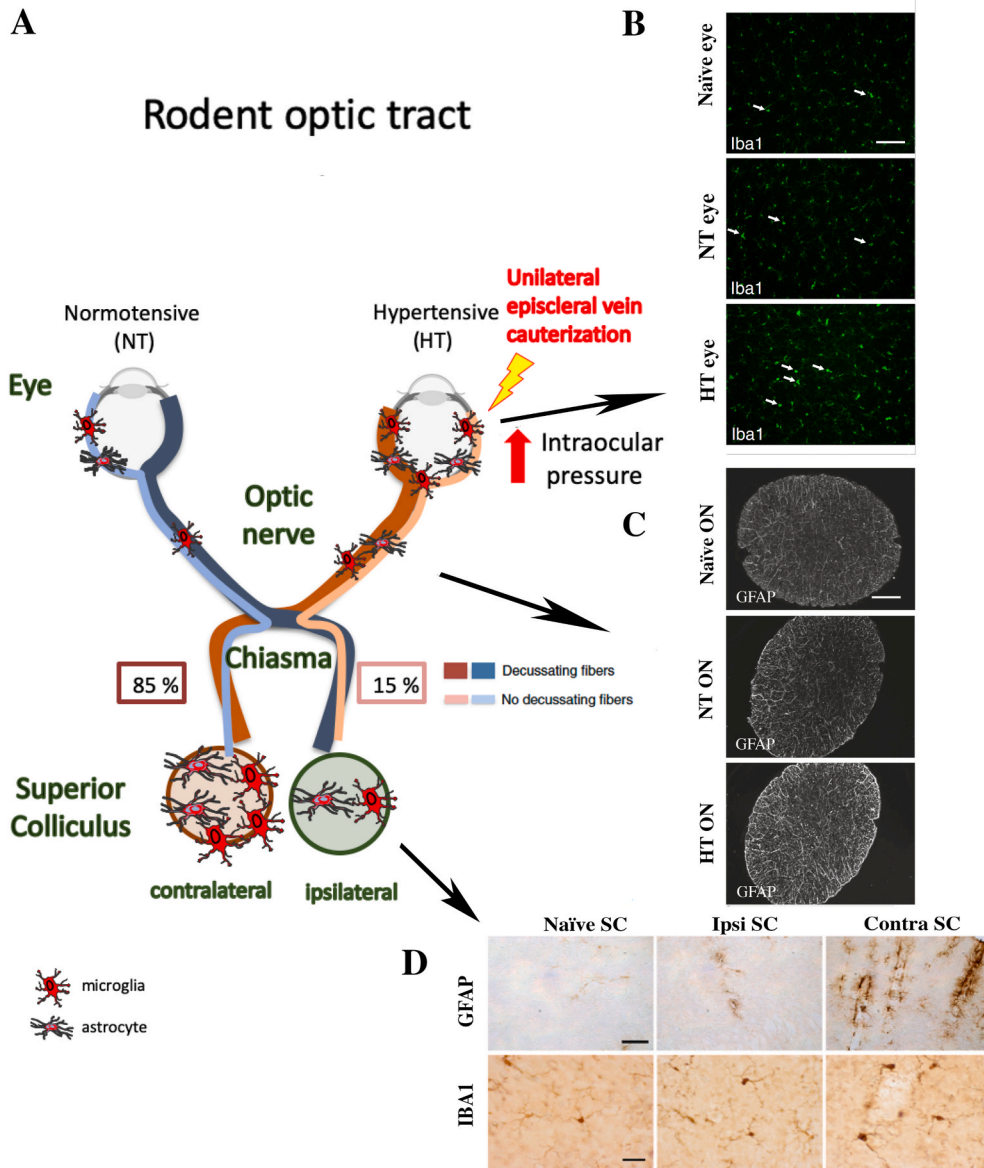
In DBA/2 J mice, which is an established model of inherited pigmentary glaucoma, the clustering, activation and migration of microglial cells was detected in the retinal periphery and in the unmyelinated portion of the ONH several months prior to detectable neuronal pathology (Bosco et al., 2011). Early microglial activation was also strongly correlated with the severity of ONH degeneration, suggesting that neurodegenerative severity could be predicted from early changes in microglia (Bosco et al., 2015). The alterations in microglia were initially detected in the ONH and further progress along the retinal paretchima overtime (Bosco et al., 2015). Additionally, inhibition of microglial activation in irradiated mice or minocycline-treated mice was shown to protect against neurodegenerative changes in DBA/2 J mice demonstrating that these cells play a major role in glaucoma progression (Bosco et al., 2008, 2012; Wang et al., 2014).

Recently, Tribble et al. (2020) suggested that mitochondrial/metabolic changes rather than inflammatory changes of microglial cells in the ONH may be an early pre-degenerative disease feature in DBA/2 J mouse glaucoma model. The transcriptomic profile of ONH microglial cells suggests an upregulation of mitochondrial phosphorylation and of glycolysis/gluconeogenesis in microglial cells suggesting an increased capacity to metabolise energy from various sources. Genes that regulate the immune surveillance and phagocytosis mediated by the TREM2-TYROBP signaling pathway were downregulated. By contrast, there was no change in gene expression of pro-inflammatory mediators, unless decreased expression of chemokine C-C ligand (CCL)-5 and increased expression of transforming growth factor (TGF)- $\beta$ 2, known to display an anti-inflammatory and survival signal for microglial cells.

In the hypertensive rat glaucoma model (unilateral episcleral vein injection of hypertonic saline), proliferation of various cell types including astrocytes, microglial cells and oligodendrocytes were shown to be activated by the elevation of IOP and the optic nerve injury (Johnson et al., 2007). Similarly, Sapienza et al. showed that ocular hypertension (OHT) induced retinal inflammation, tissue macrophage activation, retinal astrogliosis, macrogliosis, and mRNA upregulation of pro-inflammatory cytokines (monocyte chemoattractant protein [MCP]-1/CCL-2, interleukin (IL)-1 $\beta$ , and tumor necrosis factor [TNF]- $\alpha$ ) (Sapienza et al., 2016) (Fig. 1).

Complementary deoxyribonucleic acid (DNA) microarray analysis in the hypertensive rat glaucoma model showed a dramatic increase in





**Fig. 1. Elevated IOP induces astrogliosis and microgliosis in the optic nerve and superior colliculus.** Elevated intraocular pressure (IOP) over 40 days after episcleral vein cauterization (EVC) in rat induced astrogliosis and microgliosis in the retina, optic nerve and superior colliculus. (A) Diagram showing the rodent neuronal visual pathway. Immunofluorescent labeling of glial fibrillary acidic protein (GFAP) and ionized calcium binding adaptor molecule 1 (Iba1) in the retina (B), optic nerves (C) superior colliculus (D) after episcleral vein cauterization in rat. Scale bar, 100  $\mu$ m. From Sapienza, A. et al., 2016. Bilateral neuroinflammatory processes in visual pathways induced by unilateral ocular hypertension in the rat. *J. Neuroinflammation* 13, 44.

gene expression related to cell proliferation and immune response in the ONH (including myelinated and unmyelinated segments) (Johnson et al., 2007). In this model, a minimal exposure to elevated IOP results in an upregulation of a number of genes in the ONH including genes associated with cell proliferation, inflammation (IL-6), and vascular function, and ECM remodeling, consistent with early immune cell responses and microglial activation but apparently in the absence of a significant astrocyte activation in the ONH (Johnson et al., 2011). Recently, it was shown that early astrocytes proliferation predominated in the anterior (unmyelinated) and transition (partially myelinated) region of the ONH (Lozano et al., 2019). The early glial proliferative response may be initiated by the activation of the Jak-Stat signaling pathways which up-regulates the generation of IL-6 type molecules (Johnson et al., 2011; Lozano et al., 2019).

Recently, using a feline congenital glaucoma model (ortholog of a human primary congenital glaucoma), Oikawa et al. (2020) showed ONH remodeling and functional deficits following the onset of IOP elevation and prior to axon loss compared with age-matched control animals. Upregulation of cell proliferation and expression of inflammatory-related genes was shown in the ONH in early stage. According to the sub-region of glaucomatous ONH, glial cell proliferation

was more pronounced in the retro-laminar region, with almost all glial/macrophage cell expression in the pre-laminar and lamina cribrosa, and oligodendrocyte precursor cells (OPCs) in the retro-laminar space. In this model, microglia and OPCs specific genes were upregulated while neurons and myelinated oligodendrocytes genes were down-regulated. In addition, there was no significant cell proliferation in late stage chronic glaucoma, suggesting that microglia proliferation may be restricted to early-stage glaucoma. On the other hand, enrichment and activation of ONH astrocytes in early stage were less prominent, in contrast with the rat hypertensive glaucoma model (Lozano et al., 2019; Sapienza et al., 2016). The transcriptome analysis of the ONH in this feline glaucoma model showed up-regulation of various pathways in early glaucoma including ribosome, cytokine-cytokine receptor interaction, complement coagulation cascades and ECM receptor interaction pathways. Two signaling pathways were up-regulated the nuclear factor (NF)- $\kappa$ B and MyD88-dependent TLR pathway including damage-associated molecular patterns (DAMPs) which are endogenous ligands of TLRs (Oikawa et al., 2020).

Using an in vitro model of retinal cells exposed to elevated hydrostatic pressure (EHP), Aires et al. (2020) showed that the content of exosomes of retinal microglial cells exposed to EHP increased the

reactivity of naïve microglial cells including increased production of proinflammatory cytokines (TNF- $\alpha$  and IL-1 $\beta$ ), expression of major histocompatibility complex class II (MHC-II) molecules, enhanced microglia motility, phagocytic efficiency, and proliferation (Aires et al., 2020). In addition, the exosomes of microglial cells exposed to EHP increased neural cell death and increased the ROS production. Intravitreal injection of exosomes derived from EHP exposed cells were able to induce a sustained activation of retinal microglia, mediated cell death, and impacted RGC number in C57BL/6 J mice. Exosomes are nanometer-sized vesicles that are released by cells in a controlled fashion and mediate a plethora of extra- and intercellular activities including cell-cell communication, immune modulation, extracellular matrix turnover, stem cell division/differentiation, neovascularization and cellular waste removal (Klingeborn et al., 2017). Thus, this model suggests that exosomes derived from retinal microglia may have an autocrine function in the propagation of the inflammatory signal in conditions of elevated pressure, contributing to retinal degeneration in glaucomatous conditions (Aires et al., 2020).

### 2.3. Glial cells phenotypes

A growing field of glaucoma research has focused on understanding whether glial cells exert a protective function via the production of neurotrophic factors or contribute to neuronal death by producing toxic inflammatory factors. Retinal glia cell response in glaucoma may also change as the disease progresses from early to advanced injury (Nickells, 2007; Johnson and Morrison, 2009).

As an intermediate between basal/normal and inflammatory states, para-inflammation can induce different degrees of glial cells activation (Xu et al., 2009). As shown in the mouse central nervous system, retinal microglial cells and astrocytes are very heterogeneous cell populations characterised by various metabolic states and degrees of activation (Hammond et al., 2019). Microglial cells are also characterized by transcriptionally distinct states as demonstrated in brain mice (Hammond et al., 2019). It is still not known if these states are transient or represent terminal differentiation subsets of microglia. It was also suggested that some microglial cell populations may be primed by age-related changes and neurodegenerative disease leading to a more vigorous uncontrolled maladaptive response to inflammatory challenges contributing to disease progression (Perry and Teeling, 2013).

The mechanisms that cause the progression from a protective inflammatory response to chronic neurotoxic inflammation in glaucoma are largely unknown (Zeng and Shi, 2018). It seems that the microglial responses to injury become altered with aging and aged microglia may be less capable of maintaining homeostasis of the immune environment resulting in microglial inflammation and neurotoxic phenotype (Ma and Wong, 2016; Chen et al., 2019). Under physiological conditions, endogenous factors downregulate inflammation and provide a negative feedback that switches off the inflammatory response and prevents inflammatory-mediated damage. Sun et al. demonstrated that mild IOP elevation led to a reversible activation of astrocytes in mice (Sun et al., 2013). In contrast, astrocytes exposed to proinflammatory injury in the central nervous system (CNS) exhibited a phenotype that led to direct damage of the axons with ROS release, pro-inflammatory cytokines, and modulation of the ECM, forming a scar opposed to axon regeneration (Zamanian et al., 2012). As suggested, prolonged exposure to glaucomatous stress may shift the balance from a physiological process toward a pro-inflammatory neurodegenerative process (Tezel, 2013). The glial cell phenotype may depend on the disease stage and severity as observed in other neurodegenerative diseases (Tang and Le, 2016; Seitz et al., 2013; Zeng and Shi, 2018; Wie et al., 2019).

The cytotoxic phenotype (M1 phenotype) in microglia may promote the activation of NF- $\kappa$ B, a transcriptional factor which generates high levels of pro-inflammatory cytokines promoting tissue inflammation, oxidative metabolites and RGC death (Dvorianchikova et al., 2009; Yang et al., 2011).

In contrast, the protective phenotype (M2 phenotype) may be maintained by anti-inflammatory cytokines (e.g. IL-5, IL-10) which are thought to inhibit inflammation, enhance tissue repair and wound healing, and also to promote RGC survival (Huang et al., 2009). Another possible dual phenotype could be the differential expression of TNF receptor (TNFR)1 and TNFR2 on glial cells leading to different TNF- $\alpha$  signaling pathways followed by the formation of pro- or anti-inflammatory mediators, respectively. In response to stress stimuli, astrocytes may express the TNFR1 phenotype and release a number of pro-inflammatory and immune mediators linked to NF- $\kappa$ B activation (Tezel et al., 2012). By contrast, activation of TNFR2 in microglia may promote induction of anti-inflammatory pathways like those driven by granulocyte colony-stimulating factor (G-CSF), adrenomedullin and IL-10 (Veroni et al., 2010). Yet, the oligodendrocyte and RGC death induced by TNF- $\alpha$  was also shown to be mediated by the TNFR2 receptors in a model of angle closure IOP glaucoma thus inconsistent with the TNFR2 signaling of cell survival (Nakazawa et al., 2006).

In addition, the expression of membrane CX3C chemokine (fractalkine) receptor 1 (CX3CR1) seems to play a role, as its deficiency has been shown to increase microglial neurotoxicity in an experimental mouse glaucoma model with transient IOP elevation (Wang et al., 2014). Similarly, another experimental study in DBA/2 J mice has suggested a protective role of fractalkine against infiltration of CC chemokine receptor (CCR) 2+ macrophages in the retina and in the protection against axonal transport dysfunction, through enhanced nitric oxide synthase (NOS)-2 expression in myeloid cells (Breen et al., 2016).

Similarly, as demonstrated in mouse brain, a dual phenotype, i.e. neurotoxic (A1) upregulating inflammatory mediators or neuroprotective (A2) promoting healing, has been characterized for astrocytes (Liddelov and Barres, 2015; Liddelov et al., 2017). A1 reactive astrocytes were shown to lose many normal astrocyte functions including synapses formation in RGCs and phagocytic capacity (Liddelov et al., 2017).

### 2.4. Complement system activation

As part of the innate response, complement pathways are also intrinsically linked with the process of glial activation in the pathogenesis of glaucomatous damage within the retina (Soto and Howell, 2014). Increased protein expression of C1q, the initiating protein of the classical complement cascade, in the retina has been reported in animal glaucoma models, including the monkey, and in some human glaucomatous retinal specimens obtained after enucleation (Kuehn et al., 2006; Stasi et al., 2006; Stevens et al., 2007; Tezel et al., 2010; Williams et al., 2016). Uncontrolled complement activation in glaucoma is believed to contribute to the progression of degenerative injury to RGCs, their synapses, and axons. The increased levels of expression of C1q may be initially induced by the elevated IOP before the loss of RGCs, and then parallel the RGC loss (Kuehn et al., 2006; Stasi et al., 2006; Williams et al., 2016).

Components of the C1 complex may be upregulated in multiple cell types in the retina and ONH during early stages of glaucoma, particularly myeloid-derived cells and RGCs (Howell et al., 2011, 2014; Williams et al., 2016). It was also suggested that C1q might be rapidly upregulated within microglia and neurons concurrently with the appearance of reactive astrocytes and/or Müller cells (Stasi et al., 2006; Stevens et al., 2007).

The role of complement system in neurodegenerative disease is not clear since these molecules may have beneficial or damaging effects (Xu and Chen, 2016). Activation of C1q was shown to promote synapse opsonisation and phagocytosis by microglial cells in animal models, which could explain early synapse loss and dendritic atrophy before axon and soma loss in animal models (Williams et al., 2016). One postulated mechanism may be the aberrant reactivation of synapses elimination mediated by the classical complement cascade leading to early synapse loss associated with glaucoma (Stevens et al., 2007; Rosen

and Stevens, 2010).

In addition, membrane attack complex has been shown to be formed in the RGC layer in both human and rat glaucoma (Kuehn et al., 2006). In animal models of glaucoma, inhibition of C1q has been shown to reduce RGCs loss, and induction of C5 to aggravate the ONH degeneration confirming the link between classical complement activation and the development of glaucoma. By contrast, upregulation of C3 by astrocytes in the ONH may be protective from glaucomatous damages (Howell et al., 2013; Williams et al., 2016; Harder et al., 2017). The apparent paradoxical role of C1q and C3 complement components may be explained by their generation through different independent pathways, as suggested for autoimmune disease (Scott and Botto, 2016).

In addition, a trend toward downregulation of the complement factor H expression was observed in human retina of glaucomatous donors (Tezel et al., 2010). Such an alteration suggests increased vulnerability of RGCs to complement-mediated lysis in glaucoma.

## 2.5. Fas ligand signaling

The constitutive expression of FasL, a transmembrane protein of the TNF family with the capacity to induce apoptosis in Fas receptor positive (Fas+) cells, including RGCs in the retina, is believed to maintain immune privilege in part by inducing apoptosis of infiltration T cells. Studies conducted by Ksander and coworkers showed that FasL when expressed as a membrane-bound protein (mFasL) in microglial cells was pro-apoptotic and pro-inflammatory, and when elevated and released as a soluble isoform (sFasL), it was non-apoptotic, and non-inflammatory (Krishnan et al., 2019). Increased expression of membrane FasL has been detected in microglial cells in the chronic hypertensive rat glaucoma model (Ju et al., 2006), and cleavage of the membrane FasL was considered as an important mechanism protecting retinal tissue from extensive degeneration (Gregory et al., 2011). Interestingly, they found that induction of soluble FasL correlated with inhibition of glial activation, reduced production of TNF- $\alpha$ , and decreased apoptosis of RGCs and loss of axons, even in the presence of elevated IOP (Krishnan et al., 2016).

## 2.6. Immune cells infiltration

Glaucoma appears to be a neuroinflammatory disease with damages coordinated through dysfunctional RGCs, reactive glial cells and infiltration of circulating immune cells.

There is a growing consensus that sustained and excessive glial reactions lead to extended immune responses, including adaptive immunity, characterized by T-cell infiltration, contributing to progressive nerve damage in glaucoma (Jiang et al., 2020).

Studies have suggested that the immune adaptive response may be associated with RGC death and glaucomatous retinal degeneration (Tezel and Wax, 2000; Wax et al., 2008). In this context, there is evidence to suggest that the retinal blood barrier is dysfunctional in patients with glaucoma, and that infiltration of immune cells, including monocytes and lymphocytes, occurs in the retina of patients with glaucoma (Gramlich et al., 2013; Tezel, 2009). Monocyte infiltration has been shown to be coincident with the onset of glaucoma in the DBA/2 J mouse, while irradiated DBA/2 J mice lacking infiltrating monocytes, did not develop retinal neurodegeneration (Howell et al., 2012). Inhibition of monocyte-like cell extravasation clearly protected from neurodegeneration in DBA/2 J glaucoma (Williams et al., 2019). Consistently, macrophages have been detected in axon bundles of post-mortem ONH of patients with mild or severe glaucoma (Margeta et al., 2018). Increased retinal deposition of immunoglobulin G (IgG) autoantibodies has been detected in eyes of glaucoma patients, and infiltration of T and B lymphocytes into the retina has also been detected (Gramlich et al., 2013). It is possible that activated T cells may be directly cytotoxic to RGCs and induce RGC apoptosis mainly through death receptor-mediated signaling.

Previous animal studies have found that HSP27 and HSP60 immunisation in rats induces a rapid and transient retinal T cell infiltration associated with microglial activation and RGC apoptosis via the release of the proapoptotic cytokine FasL, a member of the TNF- $\alpha$  family (Wax et al., 2008).

Increased expression of MHC class II antigens by activated glial cells in glaucomatous retina and ONH may therefore promote T-cell activation, suggesting an innate/adaptive interplay in glaucoma (Luo et al., 2010).

On the other hand, elimination of T cells by apoptosis is considered as an essential protective mechanism to prevent inflammation in immune-privileged tissue (Wax et al., 2008). Such protective role of inflammation signaling was also evidenced in animal studies. Experimental intraocular inflammation in rat was shown to stimulate macrophage infiltration, Müller cell activation, and expression of growth factors in RGC, leading to enhanced RGC survival and axon regeneration in the optic nerve (Leon et al., 2000; Yin et al., 2009).

## 2.7. Inflammatory findings from glaucomatous human tissues

Studies using animal models of glaucoma can not predict what's happen in human primary open glaucoma, which is a multifactorial disease. Nevertheless, histological studies in human suggest the activation of glial cells and the resulting intense inflammatory reaction associated with glaucomatous neural damage (Yuan and Neufeld, 2001). Compared to normal specimens, ONH specimens from advanced glaucoma of human donors have shown a proliferation of microglial cells in all regions of the ONH, including the lamina cribrosa, as indicated by the elevated expression of proliferating cell nuclear antigen (PCNA)-positive microglial cells (Yuan and Neufeld, 2001). The expression of immunomodulating (TGF- $\beta$ 2, prostaglandin E2 [PGE2]) and pro-inflammatory mediators (TNF- $\alpha$ , inducible nitric oxide synthase [iNOS]) seems to be upregulated, as well as the expression of various metalloproteinases (MMPs) (MMP-1, MMP-2, MMP-3, MMP-14), and tissue-inhibitor metalloproteinases (TIMPs) (TIMP-1, and -2), suggesting an intense inflammatory and remodeling reaction in the glaucomatous ONH. More recently, increased protein levels of pro-inflammatory cytokines (TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-8, and interferon [IFN]- $\gamma$ ) in human glaucomatous retinal tissue have been reported (Gramlich et al., 2013). ONH specimens of post-mortem glaucomatous eyes were also characterised by abundant glial fibrillary acidic protein (GFAP)-positive astrocytes, which co-localised with high levels of human leukocyte antigen class II antigens (HLA-DR) (Yang et al., 2001; Tezel et al., 2003). Cell adhesion proteins (e.g. integrins) have also been found to be overexpressed in glaucomatous astrocytes, indicating that these cells could promote the adhesion and migration of immune cells to damaged regions of the ONH (Soto et al., 2014). In human glaucomatous ONHs, astrocytes also expressed high levels of TNF- $\alpha$  and TNFR, which parallels the progression of ONH retina from glaucomatous donors showed signs of chronic oxidative stress, as revealed by the increased levels of oxidative by-products immunolabeling in glaucomatous retina compared to control donors (Tezel, 2006). Proteomic analyses showed increased expression of TLRs including TLR-2, -3, and -4 which localized on retinal microglia and astrocytes (Luo et al., 2010). Various inflammasome components including NOD-like receptor pyrin (NLRP) 3, caspase 1, and caspase 8 are also increased in glaucomatous compared with normal eyes (Yang et al., 2011).

TLRs are the first line of host defence mechanisms against pathogens and non pathogens stress stimuli. They are known to induce a rapid innate response by recruiting adaptor proteins and activating the transcription factor NF- $\kappa$ B, thereby leading to the expression of pro-inflammatory cytokines. TLRs induce transcriptional activation of pro-IL-1 $\beta$ , which is proteolytically processed via inflammasome activation (Netea et al., 2008; Schroder and Tschopp, 2010). In vitro experiments showed that oxidative stress and heat shock proteins could activate glial cells via the TLRs signaling. Both TLRs and inflammasome seem to be



dysregulated in glaucoma. Animal models showed upregulation of the TLR signaling in early glaucoma (Oikawa et al., 2020).

These findings are therefore in accordance with the retinal inflammation observed in animal models of glaucoma.

### 2.8. Neuroinflammation along the visual pathways

Several studies on glaucomatous neuropathy strongly suggest that axon impairment spreads through the optic nerve to the posterior visual pathways of the CNS (Gupta and Yucel, 2007; Yucel and Gupta, 2008). Changes in the expression patterns of several synaptic plasticity markers in the lateral geniculate nucleus (LGN) have been observed in patients or primate models of glaucoma, demonstrating a lower functional connectivity (Lam et al., 2003). Post-mortem histopathologic analysis of brain sections of patients with advanced glaucoma indicated lower neuron density in the LGN (Weber et al., 2000). This lower connectivity was correlated with LGN degeneration and shrinkage of the cellular body, causing loss of neurons and reduced metabolic activity, which lead to visual alteration (Yucel et al., 2001; Sasaoka et al., 2008). The shrinkage of the cellular body and loss of neurons has also been documented in the visual cortex of glaucoma patients (Chaturvedi et al., 1993; Gupta et al., 2006). In murine models of OHT (episcleral vein perfusion with saline solution or DBA/2 J), the shrinkage and dendritic loss of neurons has also been detected in the superior colliculus, the major optic nerve fibre relay in the rodent brain (Liu et al., 2014; Dengler-Crish et al., 2014). In these ocular hypertension models, an increase in the number of GFAP-positive astrocytes throughout the superficial layers of the contralateral superior colliculus could be detected (Zhang et al., 2009; Dekeyster et al., 2015). Similarly in non-human primates, astrogliosis has been reported in the LGN and visual cortex following IOP increase induced by photocoagulation of the trabeculum (Lam et al., 2009).

By contrast, Crish et al. (2013) showed using DBA/2 J mice, that focal deficits in anterograde axonal transport from retina to the superior colliculus induced a concurrent elevation of BDNF, especially in hypertrophic astrocytes, that is spatially coincident with retinotropic location of transport loss, suggesting a local intrinsic protective mechanism to slow loss of retino-collicular synapses.

Microglial activation has been also described in relation to degenerating neurons in glaucoma. In a preclinical model of glaucoma (obtained by unilateral episcleral vein cauterisations in the rat), Sapienza et al. demonstrated the presence of inflammation along the visual pathways beyond the back of the eye (Sapienza et al., 2016) (Fig. 1). RGC death was shown in the cauterised eye and, interestingly, in the contralateral eye as well. Thus, retinal inflammation characterised by tissue macrophage activation and elevated pro-inflammatory cytokines was present bilaterally, although at a lower level in the normotensive eye. It was suggested that retinal neuroinflammation was transmitted along the optic nerve via the superior colliculus. Inflammatory mediators in one eye could be activated and circulate along RGC fibres and induce inflammation in the two superior colliculus, which may activate inflammation in the other eye by retrograde transport via RGC fibres (Sapienza et al., 2016). In this model, retinal inflammation, tissue macrophage activation and elevated levels of pro-inflammatory cytokines were present bilaterally (normotensive and hypertensive eyes). These findings raise important hypotheses on the possible risk of diffusion of IOP-related RGC degeneration in unilateral or asymmetrical glaucoma to the visual pathways, both in ipsilateral and contralateral modes. If these hypotheses are confirmed in the future using more sensitive neuroimaging technologies, clinicians should be warned about possible bilateralisation of the disease and the need for careful monitoring of the contralateral eye, with the potential for more aggressive interventions.

## 3. Neuro inflammatory mechanisms in glaucoma

A number of molecular and signaling pathways that are key regulators of neuroinflammation in both human and animal models have been proposed in the etiology of glaucoma (Danford et al., 2017). These mechanisms are complex and involve a close interaction between glial cells, immune cells, endothelial cells and RGC through autocrine/paracrine pathways. One hypothesis is the chronic mechanical and/or vascular stress at the ONH and retina, leading to a persistent maladaptive inflammatory phenotype in the context of increased oxidative stress and aging/senescence, associated with a loss of neuroprotective and remodeling functions of glial cells, release of apoptotic signals, RGC degeneration, blood retinal barrier disruption, and ONH tissue remodeling (Almasieh et al., 2012).

### 3.1. Ischemia/hypoxia

Hypoxia is believed to play an important role in initiating and driving retinal damage in glaucoma. According to the vascular hypothesis of glaucoma, RGC axons traversing the ONH, undergo oxygen and nutrient insufficiency resulting from compromised local blood flow, which ultimately leads to their degeneration (Chidlow et al., 2017). The unstable ocular blood flow produced by chronically elevated and/or oscillating IOP, together with possible vascular dysfunction (e.g. decreased perfusion pressure, autoregulation deficit or vasospasm) can lead to repeated hypoperfusion and chronic oxidative stress associated with inflammation (Flammer et al., 2002; Mozaffarieh et al., 2008). The resulting ischemic damage and hypoxia can initiate acute or prolonged inflammatory reactions characterised by the generation of pro-inflammatory mediators and infiltration of various inflammatory cells into the ischemic tissue (Vohra et al., 2013). Increased expression of hypoxia inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ) has been found in post-mortem human glaucomatous retinas. The location of increased HIF-1 $\alpha$  in the retina of glaucomatous eyes concurred with the location of visual field defects, supporting the presence of hypoxia in the retina and ONH of glaucomatous patients (Tezel and Wax, 2004). In a rat model of hypertensive glaucoma, high levels of HIF-1 $\alpha$  were present specifically in astrocytes and Müller cells (Ergorul et al., 2010). HIF-1 $\alpha$  has also been found to induce the production of VEGF and NOS, which may be responsible for blood-retinal barrier disruption in glaucoma (Kaur et al., 2008). Increased expression of HIF- $\alpha$  in isolated retina has also been associated with up-regulation of p53 and pro-apoptotic caspases (Tezel, 2006). The expression of TNF- $\alpha$  and IL-1 $\beta$  in primary cultures of microglial cells was shown to be dramatically increased in response to hypoxic exposure, and conditioned medium derived from hypoxic microglia enhanced apoptosis, which was significantly decreased when cells were pre-treated with anti-TNF- $\alpha$ /IL-1 $\beta$  antibodies (Sivakumar et al., 2011). Recruitment of microglia was also observed in the RGC layer in the rat retinas subjected to hypoxia (Kaur et al., 2015). Furthermore, hypoxia produced mitochondrial damage in retinal cells including RGCs and Müller cells, leading to abnormal accumulation of glutamate, activation of N-methyl-D-aspartate (NMDA) and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, increased intracellular calcium influx, caspase signaling and RGC death (Harris et al., 2005).

### 3.2. Endothelins (ETs)

ETs are other vascular factors closely related to glial cell activation, ischemia and blood-retinal barrier disruption. The ET receptors have been shown to mediate RGC loss and ONH degeneration (Howell et al., 2011; Minton et al., 2012). ET-1 is a potent vasoconstrictor produced by vascular endothelial cells, but also by microglia/macrophages that play a role in local vascular tone (Howell et al., 2011; Kowalczyk et al., 2015). Increased plasma levels of ET-1 have been found in POAG patients and this may be linked to vasoconstriction, decreased ocular blood



flow and ischemia/hypoxia (Li et al., 2016). Acute intraocular injection of ET-1 in rats has been shown to increase GFAP expression in Müller cells, indicating glial cell hyperactivity and RGC death (Lau et al., 2006). ET-1 has also been shown to induce proliferation of human ONH astrocytes in culture through ET (A/B) receptor activation (Prasanna et al., 2003). In DBA/2 J mice, ET-2 was shown to be produced in astrocytes as well as in microglia/macrophages in ONH at an early stage before retinal damage (Howell et al., 2014). Both ETA and ETB receptors were upregulated during the early stage of retinal neurodegeneration (ETA expression was present in endothelial cells and ETB was more specifically found in astrocytes in the ONH). Oral administration of an ETA/B receptor antagonist was shown to lead to a partial inhibition of RGC death (Howell et al., 2014). Intravitreal injection of ET-2 peptide was also shown to cause RGC axon damage (Howell et al., 2011), and reverse the inhibition of monocyte infiltration (Howell et al., 2012). In an ocular hypertensive rat model, elevated IOP resulted in overexpression of ETA and ETB in the retina and was associated with cell death (McGrady et al., 2017). Finally, Wang et al. reported upregulation of ETB expression in ONH astrocytes in an ET-1-induced chronic optic neuropathy, causing RGC loss (Wang et al., 2009).

### 3.3. Chronic oxidative stress

The retina is constantly exposed to ROS due to intense mitochondrial activity and oxidative stress is considered as an important pathogenic risk factor in glaucoma (Nita and Grzybowski, 2016). Oxidative stress reflects an imbalance between the production of ROS generated by the oxygen consumption during mitochondrial respiration and antioxidant defences, in which oxidative processes exceed antioxidant systems (Chrysostomou et al., 2013; Pinazo-Durán et al., 2015). The excess of ROS generation is normally counteracted by protective antioxidant mechanisms including antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase, vitamins and vitamin-like molecules (polyphenols). If not rapidly recycled, the unstable oxygen species can attack lipid membranes, cellular proteins and nucleic acids, leading to apoptosis and cell death. Antioxidant enzymes expression is tightly regulated through the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway (Nakagami, 2016), which was shown to be impaired in aging (Zhang et al., 2015). Overall, senescent cells are associated with high level of intracellular ROS and the oxidative stress increase with age while the ability to respond to the oxidative stress decline with age (Pinazo-Durán et al., 2014; Zhang et al., 2015). In aging animal, para-inflammation was associated with an accumulation of oxidative stress products including oxidized proteins and lipids, oxidized lipoproteins and metabolic products e.g. the advanced glycation end products (AGEs) (Xu et al., 2009).

ROS production and oxidative retinal damage and imbalance between pro-oxidative and antioxidant capacity may be crucial in early retinal injury. Glaucomatous ONH astrocytes compared to normal ONH astrocytes have lower basal levels of reduced glutathione, which may imply a compromised oxidation-reduction system or a reduced antioxidant response in the glaucomatous optic nerve (Malone and Hernandez, 2007). As reviewed by Nita and Grzybowski (2016), oxidative stress produced by elevated IOP and/or hypoxia can have detrimental effect resulting in glaucomatous optic nerve atrophy and optic disc cupping enlargement. In glaucoma, oxidative stress produces oxidative mitochondrial DNA (mtDNA) damage leading to apoptosis of RGCs (Chrysostomou et al., 2013) and was shown to impair basal cytoprotective autophagy. Oxidative stress also induced ECM remodeling through MMPs activation. Oxidative stress also alters the glutamate/glutamine cycling, leading to the accumulation of neurotoxic levels of glutamate. On the other hand, intracellular and extracellular accumulation of advanced glycation end products (AGEs) is increased as well as TNF- $\alpha$  and NO secretion by glial cells. In addition, ROS can scavenge NO radicals and thus increase the vascular tone and impair the ocular blood flow, leading to RGCs apoptosis. There is some evidence for a role of ROS

in glial dysfunction. Chronic oxidative stress is considered as the main initial trigger of retinal para-inflammation in aging retina, and dysregulated para-inflammation in glaucoma as reviewed by Xu et al. (2009).

ROS also play an essential role as signaling molecules driving gene transcriptions involved in inflammation. Oxidative stress activates the redox-sensitive transcription factor NF- $\kappa$ B, which is known to regulate the expression of inflammatory molecules (including IL-6, TNF- $\alpha$ , iNOS, intercellular adhesion molecule [ICAM] 1, MMP9) and apoptosis-inhibiting factors, such as B-cell lymphoma 2 (Bcl-2) (Kabe et al., 2005; Harari and Liao, 2010). Inactivation of NF- $\kappa$ B in transgenic mice astrocytes was found to promote survival of retinal neurons after ischemic injury (Dvorianchikova et al., 2009). On the other hand, there is some evidence for an inhibition of the anti-oxidative Nrf2 pathway in glaucoma (Ahmed et al., 2017; Wang et al., 2020). This activation of Nrf2 was shown to inhibit the NF- $\kappa$ B pro-inflammatory pathways and to promote neuronal survival in neurodegeneration and acute nerve damage (Xiong et al., 2015).

### 3.4. Mitochondrial dysfunction

The retina is one of the most energy and oxygen demanding tissues in the body (Lee, 2011). In this context, mitochondria are essential for retinal homeostasis, and substantial evidence has shown that retinal damage is triggered and perpetuated by mitochondrial dysfunction. Mitochondria produce ROS as a by-product of electron leak along the electron transport chain during cellular respiration (Murphy, 2009). This activity is essential to maintain the energy requirements for neuronal function, and RGCs have an absolute requirement for optimal mitochondrial function to maintain survival (Osborne et al., 2008). Mitochondrial activity may be impaired by ischemia/hypoxia or by oxidative stress most likely associated with glaucoma. A lack of nutrients or oxygen can not only result in bioenergetically compromised RGC axons, but can also dramatically increase ROS production, which leads to further oxidative stress and retinal damage (Chrysostomou et al., 2013; Eells, 2019; Kolko, 2017). Dysfunctional mitochondria also release multiple DAMPs, such as ATP, ROS, and mtDNA, which activate the NLRP3 inflammasome. This triggers the proteolytic maturation of proinflammatory cytokine precursors, such as IL-1 $\beta$ , and leads to the activation of the NF- $\kappa$ B pathway (Martinon, 2010).

Under conditions of increased IOP and/or unstable oxygen tension, the simultaneous production of NO in astrocytes and of superoxide in the mitochondria of neuronal cells lead to the production of peroxynitrate (ONOO-) (Neufeld et al., 1997; Mozaffarieh et al., 2008; Konieczka et al., 2014). Peroxynitrate has strong oxidising properties against a wide variety of molecules, including DNA and proteins, leading to RGC death. In addition, oxidative stress may alter cycling of glutamate/glutamine, leading to the accumulation of neurotoxic levels of glutamate (Atlante et al., 2001; Toft-Kehler et al., 2016) and to Müller cell dysfunction during starvation, potentially causing impaired glutamate uptake and excitotoxicity (Toft-Kehler et al., 2016).

Recently, it was confirmed that mitochondrial dysfunction was an early driver of neuronal dysfunction, before detectable degeneration in DBA/2 J mice (Williams et al., 2017a). Retinal levels of nicotinamide adenine dinucleotide (NAD<sup>+</sup>) were decreased in aging animals, rendering neurons vulnerable to disease-related insults. The main function of NAD as a redox cofactor consists in providing electrons from oxidized nutrients to the mitochondrial respiratory chain complex I, thus sustaining ATP production. Nicotinamide treatment and gene therapy to enhance the NAD producing enzymes were shown to be neuroprotective against glaucoma in this model. This is consistent with the nicotinamide deficiency in POAG (Kouassi Nzoughet et al., 2019).

### 3.5. Glutamate

One protective function of astrocytes and Müller cells is the regulation of glutamate homeostasis. Accumulation of glutamate is the result

of a number of insults, including inappropriate electrical activity, ischemia/reperfusion injury and neurotrophin deprivation. This may initiate the death of neurons containing ionotropic glutamate (NMDA) receptors (Harris et al., 2005). Activation of NMDA receptors on RGC induces the influx of calcium and the generation of free radicals, leading to cell death. It has been suggested that Müller cells may increase RGC susceptibility to stress signals in response to elevated IOP, thereby contributing to disease progression (Bringmann et al., 2009). In support of this, it has been shown that Müller cells have a reduced ability to regulate glutamate homeostasis upon release of TNF- $\alpha$  and other inflammatory molecules (Vohra et al., 2013; Toft-Kehler et al., 2017; De Groef et al., 2015).

### 3.6. Tumour necrosis factor alpha

TNF- $\alpha$  is found in high levels in the retina and ONH of glaucoma patients and appears to play a key role in the regulation of neuroinflammation (Yuan and Neufeld, 2001; Tezel et al., 2001; Gramlich et al., 2013). This cytokine is primarily produced by glial cells, including microglia, astrocytes, and Müller cells, and its production seems to be upregulated in glaucoma in response to vascular (ischaemic) or oxidative stress as previously described (Tezel et al., 2001; Lebrun-Julien et al., 2009; Sivakumar et al., 2011). In the rat hypertensive glaucoma model, increased IOP resulted in a dramatic increase in TNF- $\alpha$  levels produced by microglial cells around the ONH within a few days, associated with axonal degeneration and a 38% loss of RGCs after several weeks (Roh et al., 2012). Similarly, Cueva Vargas et al. showed that TNF- $\alpha$  was upregulated by Müller cells and microglia/macrophages soon after induction of ocular hypertension (Cueva Vargas et al., 2015). TNF- $\alpha$  may affect RGC survival through different pathways, including the activation of NOS expression and NO production (Yuan and Neufeld, 2000), mitochondrial dysfunction (Kaur et al., 2013), modulation of NMDA and AMPA in Müller cells (Lebrun-Julien et al., 2009; Cueva Vargas et al., 2015), induction of membrane FasL expression on retinal microglia and/or infiltrating macrophages (Krishnan et al., 2016), modulation of tissue remodeling via the synthesis and secretion of MMPs (including MMP-9) (Shubayev et al., 2006), and stimulation of ET-1 synthesis in several ocular cell types, including ONH astrocytes (Desai et al., 2004).

Some contradictory studies have also shown that TNF- $\alpha$  released by activated microglia causes astrocytes to produce neuroprotective factors in response to relatively mild hypertensive glaucomatous injury (Lee et al., 2014). TNF- $\alpha$  is involved in a complex interplay between pro-inflammatory, pro-apoptotic, and pro-survival pathways mediated by two distinct types of receptors TNFR1 and TNFR2 (Tezel, 2008; Orticañ et al., 2019). Mac Nair et al. have shown that RGC loss induced by optic nerve crush in mice was attenuated by the pre-injection of intravitreal TNF- $\alpha$ , suggesting that it may have a protective role early in the RGC death process (Mac Nair et al., 2014). This effect may be mediated by TNFR2, which is expressed by microglial cells and when activated leads to the production of anti-inflammatory cytokines such as G-CSF and IL-10 (Veroni et al., 2010). Opposite effect of TNFR2 receptor signaling was found by Nakazawa et al. using an angle-closure hypertensive model of glaucoma (Nakazawa et al., 2006).

Conversely, upon binding to TNFR1 receptors on the RGCs, soluble TNF- $\alpha$  induces apoptosis, via activation of the caspase (caspase 8) signaling cascade, loss of mitochondrial membrane potential and generation of ROS (De Groef et al., 2015). These processes are consistent with the high level of TNFR1 in inner retinal layers of glaucomatous eyes (Yan et al., 2000; Yuan and Neufeld, 2001; Tezel et al., 2001) and the significant decrease in RGC death due to neutralisation of TNF effects with anti-TNF- $\alpha$  antibodies (Tezel and Wax, 2000). In addition, inhibition of TNF- $\alpha$  activity, by a soluble TNF- $\alpha$  receptor antagonist, inhibited microglial response and prevented axonal degeneration and loss of RGCs in a rat model of hypertensive glaucoma (Roh et al., 2012). Another study showed that blocking TNF- $\alpha$  minimised the detrimental effect of

TNFR1 activation while preserving TNFR2-mediated signaling (Cueva Vargas et al., 2015). It was suggested that TNF- $\alpha$  acts as an important regulatory cytokine with differential signaling through the two distinct TNFRs that determine its contribution to degenerative or regenerative processes. Therefore, depending on the type and stage of glaucoma, environmental/external factors, cellular composition of the affected tissue and intracellular availability of TNFR signaling components, TNF- $\alpha$  may function to aggravate or ameliorate the disease (Marchetti et al., 2004).

### 3.7. Metalloproteinases

There is evidence to suggest a close association between inflammation and ECM remodeling in the ONH. Pathological conditions in glaucoma may be due to a disordered deposition of elastic and collagen fibres and a typical, pronounced thickening of the connective tissue septae that surround the nerve fibres in the lamina cribrosa (Schneider and Fuchshofer, 2016). Increased elastin expression by astrocytes in response to elevated IOP has been documented in the lamina cribrosa (Pena et al., 2001). The regulation of ECM deposition is normally based on balanced synthesis and degradation of ECM. It has been speculated that the remodeling of the ONH in glaucoma involves an impaired astrocyte response and changes in ECM composition and distribution under the influence of MMPs.

MMPs belong to a superfamily of structurally similar proteolytic enzymes that cleave ECM components including collagens (MMP-1, MMP-8, and MMP-13), gelatin (MMP-2, MMP-9) and proteoglycans (MMP-3, MMP-10, and MMP-11). Under normal conditions, astrocytes are thought to maintain low MMP activity by high constitutive expression of TIMPs (Hernandez, 2000). In contrast, there is evidence of abnormally increased production of MMPs and TIMPs by activated astrocytes in the ONH of glaucoma patients (Agapova et al., 2001). Pro-inflammatory cytokines (IL-1, IL-8, and TNF- $\alpha$ ) are known to upregulate MMPs expression, resulting in elastin and collagen deposition in the surrounding tissues. Hence, changes in MMPs and TIMPs appear to be closely related to the inflammatory responses. Treatment of human ONH astrocytes with the anti-inflammatory TGF- $\beta$ 2 has been shown to result in a shift towards the inhibitory mechanisms of MMP activity due to elevated TIMP expression (Neumann et al., 2008).

In addition to collagenase proteolysis, MMPs may cleave other substrates, such as inflammatory cytokines from their inactivated to active forms. In this context, experiments in MMP-2 deficient mice showed that this attenuated the loss of RGC induced by intravitreal administration of NMDA, which was associated with a marked reduction in soluble TNF- $\alpha$ . Some have postulated that MMP-2 could cleave a transmembrane precursor of TNF- $\alpha$  from membrane glial cells and release soluble, biologically active TNF- $\alpha$  from the cell membrane (De Groef et al., 2015).

## 4. Inflammation and trabecular meshwork in glaucoma

Upstream of ONH degeneration and RGC loss characteristic of glaucoma neuropathy, aqueous humour (AH) outflow resistance and elevated IOP are key features of POAG and constitute the most documented risk factor. Increased resistance to outflow results from a progressive dysfunction of the remodeling of TM cells and the imbalance between the rate of ECM synthesis and breakdown, leading to increased ECM deposition in aqueous outflow pathways (De Groef et al., 2013). The precise pathophysiological mechanisms involved in the outflow resistance are not fully understood. IOP is maintained due to the balance between production of AH by the ciliary processes and hydrodynamic resistance to its outflow through the conventional outflow pathway comprising the TM and Schlemm's canal (SC).

### 4.1. Structure and function of the trabecular meshwork

The TM is a porous structure located between the iris and the cornea

innermost surface in the iridocorneal angle. The TM is composed of a complex elastic network of connective tissue beams and sheets or lamellae covered by TM cells. TM cells have both mesodermal and ectodermal origins, and behave like endothelial cells. The TM is composed of three main consecutive regions which define the filtering portion of the TM: the uveal meshwork, the corneoscleral meshwork and the juxtacanalicular region (JCT). The aqueous humour flows through the intercellular spaces of the TM and crosses the SC inner wall. It first encounters the uveal and corneoscleral TM, which function as self-cleaning biological filters before reaching the resistance generating region of the JCT directly underneath the inner wall of Schlemm's canal. JCT-TM cells and SC cells are the main regulators of the outflow resistance (Abu-Hassan et al., 2014; Stamer and Clark, 2017).

TM cells have various functions including synthesis and degradation of collagen, proteoglycans and other ECM components, and phagocytosis of extracellular debris (Vranka et al., 2015; Stamer and Clark, 2017). TM cells express prominent cytoskeletal, complex cell-cell and cell-matrix attachment and water channels to facilitate rapid changes in cell volume following stretch and thus to regulate the aqueous outflow (Stamer and Clark, 2017). Thus, the outflow occurs both between the cells and through the trabecular cells, and a source of inflammation is precisely the alteration of the barrier function of the trabecular meshwork. TM cells normally sense increased mechanical stretching forces due to IOP elevation and respond by upregulating the secretion of MMPs and TIMPs (Bradley et al., 1998; De Groef et al., 2013). It seems that MMPs contribute to conventional aqueous outflow homeostasis in their capacity to remodel extracellular matrices (Acott et al., 2020).

#### 4.2. Inflammatory cytokines and aqueous outflow homeostasis

There is some evidence that local para-inflammation plays a regulatory role in outflow facility through the TM. TM cells secrete various growth factors and cytokines in response to mechanical stretching (Shifera et al., 2010; Saccà et al., 2015), including IL-1, IL-6, IL-8, and TNF- $\alpha$  (Alvarado et al., 2005a; Liton et al., 2005a). Some of these inflammatory cytokines have been shown to promote AH outflow facility in human and animal models, suggesting an autocrine feedback loop aimed at restoring normal IOP values in response to the mechanical stress produced by IOP elevations (Alvarado et al., 2015). Birke et al. (2011), demonstrated increased TM outflow in response to perfusion of IL-1 $\alpha$  and IL-1 $\beta$  in human TM. This was partly mediated by the induction of the expression of MMPs (MMP-3 and MMP-9), and by the induction of endothelial leukocyte adhesion molecule (ELAM)-1 (E-selectin), which is a well-known mediator in the first step of inflammation before diapedesis (Bradley et al., 1998). Another inflammatory cytokine IL-6 partially increased outflow facility in perfused human organ cultures, through modulation of endothelial permeability and induction of MMPs, resulting in ECM degradation (Liton et al., 2005a). Furthermore, TNF- $\alpha$  combined with IL-1 $\alpha$  or IL-1 $\beta$  was shown to increase outflow facility associated with an intense synergistic increase in MMP-3 and MMP-12 through a mechanism partially mediated by the induction of IL-1 receptors (Kelley et al., 2007).

It has also been suggested that cytokines produced by TM cells could act rapidly to increase the endothelium permeability, especially at the level of the juxtacanalicular tissue, and by inducing the expressions of TM MMPs leading to ECM degradation and remodeling. Alvarado et al. demonstrated that some cytokines, including IL-1 $\alpha$ , IL-1 $\beta$ , IL-8 and TNF- $\alpha$  increased the permeability of cultured SC endothelial cells exposed to laser irradiation (Alvarado et al., 2005a, 2005b). They also demonstrated that intracameral infusion of autologous macrophages in rabbits increased outflow facility 2-fold in a rapid and sustained manner, suggesting that the innate immune system, especially monocytes/macrophages, could play a role in aqueous outflow homeostasis (Alvarado et al., 2010). It was subsequently confirmed that cultured human TM cells from non-glaucomatous subjects were able to secrete various chemotactic cytokines (IL-8, chemokine C-X-C ligand (CXCL)-6

and MCP-1) under resting conditions, which supports other observations that monocytes, presumably under the influence of chemotactic signals, circulate through the TM in the normal state (Shifera et al., 2010). Blondin et al. have reported that human TM cells secrete CCL2/MCP-1 in a constitutive manner, whereas the secretion of IL-8 occurs only after the cells were exposed to stimuli such as TNF- $\alpha$  (Blondin et al., 2003). CCL2/MCP-1 was shown to increase the AH outflow facility when perfused in enucleated porcine eyes and this may be mediated by reduced resistance of SC endothelial cells via altered cell-cell contact (Tsuboi et al., 2012). TM cells also secrete VEGF in response to mechanical stress and this was suggested as a paracrine pathway for conventional outflow facility regulation (Reina-Torres et al., 2017; Fujimoto et al., 2016).

In summary, there is some evidence for local para-inflammation involved in the regulation of AH outflow, mediated by the constitutive expression of inflammatory cytokines and infiltration of immune cells.

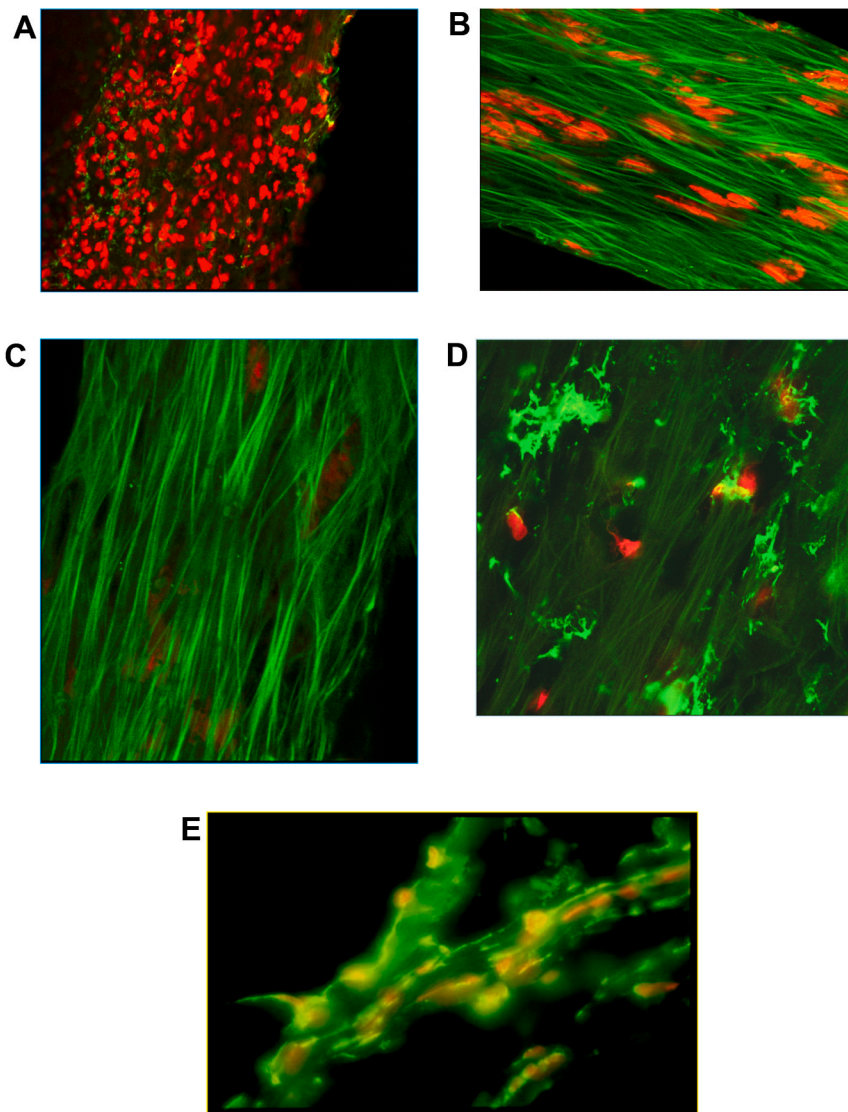
#### 4.3. Increased inflammation in the glaucomatous trabecular meshwork

Various histologic studies using specimens from glaucoma patients at the time of trabeculectomy compared with samples from normal (post-mortem) non-glaucomatous subjects showed an upregulation of inflammation-associated genes in the TM, which may be involved in the initiation or progression of glaucoma. Using immunohistochemistry and confocal microscopy, Baudouin & his colleagues clearly showed dendritiform inflammatory cells infiltrating the TM, associated with a dramatic decrease of TM cells in glaucoma patients (Baudouin et al., 1999, Baudouin et al., 2012; Hamard et al., 2002) (Fig. 2). Liton et al. showed statistically significant upregulation of several genes associated with inflammation and the acute-phase response, including ELAM-1, CXCL-6, CCL5, immune-associated nucleotide and IL-1 receptor type II in cultured TM samples of POAG patients compared to human non-glaucomatous TM samples (Liton et al., 2006). More recently, Taurone et al. (2015), confirmed the infiltration and activation of inflammatory cells, including macrophages, T cells and plasma cells, in TM sections of glaucomatous compared to normal specimens. They found significantly increased expression levels of IL-6, IL-1 $\beta$  and TNF- $\alpha$  in TM specimens of POAG patients with high IOP. They suggested that macrophages produce pro-inflammatory cytokines, which leads to an acute inflammatory response and recruitment of other immune cells, including T cells. Micera et al. (2016), showed increased expression of various cytokines (IL-10, IL-6, IL-7, IL-12, mitochondrial intermediate peptidase (MIP) 1 $\alpha/\delta$  and soluble TNFR1), Th1/Th2 (IL-2, IL-3, IL-4, IL-5), pro-fibrogenic growth factors (VEGF, TGF- $\beta$ 1) in the glaucomatous TM, suggesting an intense inflammatory reaction in patients undergoing trabeculectomy. Fractalkine (CX3CL1) is another pro-inflammatory chemokine, which induces adhesion and retention of monocytes and T cells into inflammatory tissue (Jones et al., 2010). The expression of CX3CL1 and its receptor (CX3CR1) were found to be increased in TM specimens of patients undergoing trabeculectomy (Baudouin, 2012) (Fig. 3). Interestingly, a lower inflammatory cell density and a significantly lower level of CX3CL1 expression were observed when patients were treated with an anti-inflammatory agent (topical 0.1% indomethacin, or fluorometholone) prior to surgery (Baudouin, 2012).

#### 4.4. Aqueous humour cytokines in glaucoma

The immune privilege of the aqueous humour is maintained by immune regulatory and/or suppressive soluble and membrane-bound molecules, along with an efficient blood-aqueous barrier, which prevents inflammation. The abnormal immune-inflammatory response mediated by infiltration of immune cells in POAG is supported by the altered cytokines profile in the anterior chamber (Pinazo-Durán et al., 2013). The source of these cytokines in the anterior chamber of glaucoma patients is not clear, and may be the result of secretion from





**Fig. 2.** Inflammatory damage of the glaucomatous trabecular meshwork. **A:** En-face image of a normal trabecular meshwork with many trabecular cells, stained red by propidium iodide, few vimentin-positive cells (bright green), and autofluorescent extracellular matrix fibers (mild green). Confocal microscopy in a donor bank eye after dissection of the trabecular meshwork.

**B:** Glaucomatous trabeculum in a surgically taken specimen: very few remaining cells (red) and high level of extracellular matrix.

**C:** higher magnification. The trabeculum is mainly composed of paucicellular extracellular matrix (from Baudouin C et al. *In vitro and in vivo experimental studies on trabecular meshwork degeneration induced by benzalkonium chloride* (an American Ophthalmological Society thesis). *Trans Am Ophthalmol Soc.* 2012;110:40-63, with permission from The American Ophthalmological Society).

**D:** Inflammatory dendritiform cells (green vimentin staining) in the cribriform layer of a trabecular meshwork surgically removed at time of trabeculectomy (same reference). **E:** Cryosection of trabeculectomy specimen infiltrated by many inflammatory dendritiform cells (vimentin staining). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

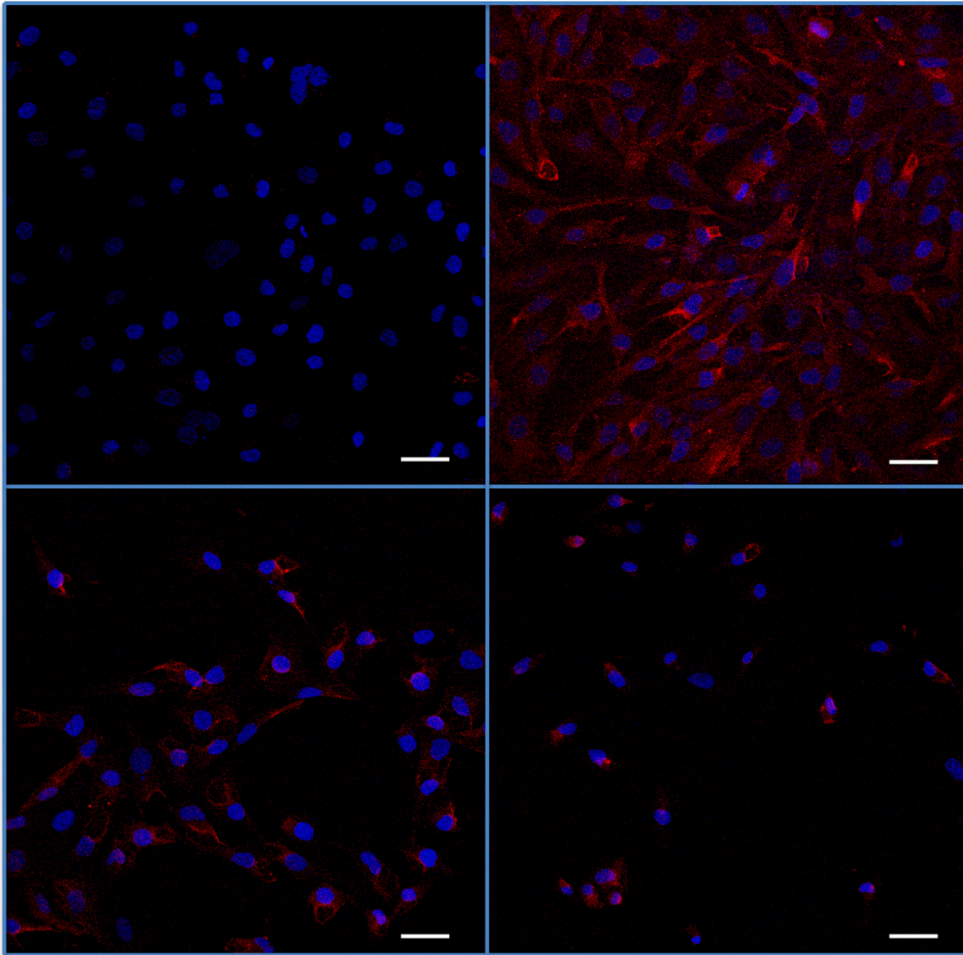
various ocular resident cells in the TM, iris and ciliary body, and/or from diffusion from the vitreous humour or the corneal surface (Wong et al., 2015; Noma et al., 2010). Alternatively, the AH proteome of glaucoma patients indicates the presence of significantly higher levels of some proteins known to play a role in mediating oxidative stress, apoptosis, inflammation and/or immunity. Most of these are plasma proteins, suggesting that some glaucoma disease stages may be associated with a breakdown in the blood-aqueous barrier (Anshu et al., 2011). Furthermore, it has been suggested that proteins expressed in the anterior chamber, in addition to reflecting TM dysfunction, could diffuse to the posterior segment, triggering activation of glial cells and participating in RGC apoptosis (Saccà et al., 2016).

Most studies have compared AH cytokines expression between glaucoma and non-glaucoma patients undergoing trabeculectomy or routine cataract surgery, respectively. As shown by Freedman et al. patients with open-angle glaucoma (OAG) have higher aqueous levels of cytokines (IL-6, IL-8) and chemokines (CCL2/MCP-1, CXCL1) associated with elevated IOP, confirming that mechanical stress produced by IOP may be responsible for cytokine production (Freedman et al., 2013). Increased aqueous IL-8 levels were consistently reported in several studies in glaucoma patients (Kuchtey et al., 2010; Takai et al., 2012; Engel et al., 2014). Kuchtey et al. found that patients with severe visual field defects had higher aqueous concentrations of IL-8 compared to

patients with mild visual field defects, supporting a correlation between elevation of IL-8 and the severity of POAG (Kuchtey et al., 2010). Takai et al. reported increased IL-8 levels in the AH of patients with POAG or exfoliation glaucoma, together with increased level of TGF- $\beta$ 1 compared with aqueous levels in non-glaucoma patients (Takai et al., 2012). IL-8 was overexpressed in conjunction with serum amyloid A (SAA), which is expressed in high levels in glaucomatous TM cells compared to non-glaucomatous TM cells, and is known to play a role in inflammation and tissue repair (Wang et al., 2008; Takai et al., 2012). Increased levels of aqueous TNF- $\alpha$  in POAG patients have also been reported in some studies (Cvenkel et al., 2010; Sawada et al., 2010; Balaiya et al., 2011), but not in others (Kuchtey et al., 2010; Takai et al., 2012).

In contrast, decreased levels of IL-2 and IL-6 were shown in the anterior chamber of glaucoma patients (Hautala et al., 2012; Takai et al., 2012; Borkenstein et al., 2013). IL-6 is known to induce a Th2-type of immune-inflammation (Diehl & Rincon, 2002) and it was suggested that this plays a role in maintaining normal outflow facility in TM (Liton et al., 2005a), which may have neuroprotective properties in glaucoma (Sappington et al., 2006). Chua et al. (2012) found increased levels of IL-12, INF- $\gamma$  and CXCL9 (IL-9) in the AH of POAG patients. Increased aqueous levels of IL-12 and INF- $\gamma$  suggest the activation of a Th1-type immune response involving T-cells (Chua et al., 2012) and increased expression of CCL2/MCP-1 suggests the activation of





**Fig. 3. Assessment of fractalkine (CXCL3) in trabecular meshwork cells submitted to benzalkonium or TNF- $\alpha$ .** Indirect immunostaining for fractalkine (in red) in trabecular cells in vitro (nuclei are counterstained in blue by DAPI). A: Low, almost negative fluorescence intensity in basal condition; B: strong expression following TNF- $\alpha$ ; C: BAK  $5 \times 10^{-5}\%$  stimulation for 24 h; D:  $5 \times 10^{-4}\%$  stimulation for 24 h. After BAK stimulations, cells lost their elongated form and cell density was reduced. Bars = 50  $\mu$ m (from Baudouin C et al. *In vitro and in vivo experimental studies on trabecular meshwork degeneration induced by benzalkonium chloride* (an American Ophthalmological Society thesis). *Trans Am Ophthalmol Soc.* 2012;110:40-63, with permission from The American Ophthalmological Society). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

monocytes/macrophages.

#### 4.5. Mechanisms of chronic inflammation in the glaucomatous TM

In glaucomatous TM specimens, the increased extracellular material and thickness of elastic fiber sheaths are associated with oxidative stress, premature senescence, altered autophagy and apoptosis which leads to cell death and excessive loss of TM (Alvarado et al., 1984; Hamard et al., 2002; Baudouin et al., 2012; Tektas et al., 2009) (Fig. 3). As described above, local para-inflammation seems to be involved in the regulation of AH homeostasis, through the constitutive expression of inflammatory cytokines and infiltration of immune cells. A disruption of the cytokine-mediated feedback loop in response to mechanical stress has been proposed as a mechanism for the progression to elevated IOP (Alvarado et al., 2015). Other stress stimuli, including oxidative, toxic or vascular stress may shift the physiological immune balance over a chronic-cumulative period leading to a chronic inflammatory degenerative process.

##### 4.5.1. Chronic oxidative stress, mitochondria, senescence and inflammation

Oxidative stress and vascular damages are considered as two major alterations in the TM related to glaucoma (Zhao et al., 2016). During a chronic disease such as glaucoma, the affected tissues and TM first suffer from a chronic low-grade oxidative insult, which lasts for decades and increases in level with age (Saccà et al., 2016). Even small concentrations of ROS can produce changes in cellular redox state and affect enzyme activity, protein-protein and DNA-protein interactions and transcription factors (Finkel, 1998; Finkel and Holbrook, 2000). Chronic

oxidative stress arises from the impaired balance between ROS and anti-oxidant mechanisms and can promote cell death via mitochondrial damage, apoptosis, and loss of tissue integrity (Saccà et al., 2015). The chronic oxidative stress in the TM may originate from ROS generated by many cellular sources, but also from light radiation (Osborne et al., 2017), endogenous mitochondrial respiration and possibly from mechanical stress (Gonzalez et al., 2000). Glaucoma-affected patients also have a significant depletion in the total anti-oxidant potential in the aqueous humour compared to non-affected subjects (Ferreira et al., 2004).

ROS have a role in the pathogenesis of glaucoma by stimulating the apoptotic and inflammatory pathways at the level of the TM. It was suggested that oxidative stress might induce premature senescence of TM cells. *In vitro*, TM cells exposed to chronic oxidative stress showed a marked decline in proteasome activity, associated with premature senescence and decreased cell viability (Caballero et al., 2003). Elevated cellular oxidative stress was shown to activate senescence markers such as cyclin-dependent kinase inhibitors p16 and p21 (Fatma et al., 2009). This was associated with several mechanisms, including vascular dysfunction, mitochondrial dysfunction, declined autophagy, altered gene expression, oxidative stress and apoptosis (Caballero et al., 2003; Izzotti et al., 2006; Liton et al., 2005b, 2009; Baleriola et al., 2008). Senescent cells are metabolically, highly active and secrete an array of proinflammatory cytokines and chemokines, growth factors and extracellular degrading protein (Coppé et al., 2008; Sreekumar et al., 2020).

Mitochondria are not only the main endogenous source of ROS, but they are also one target of the oxidative stress leading to mtDNA damage (Izzotti et al., 2010; Saccà et al., 2015). Thus, oxidative stress can cause

mitochondrial failure and increase endogenous production of oxidative stress from the mitochondria, thus establishing a vicious cycle (Saccà et al., 2016). Mitochondrial oxidative stress is associated with a significant decrease in mitochondrial potential membrane, and a rapid activation of transcription factors involved in caspase-mediated apoptosis and excessive autophagy leading to cell death (Saccà et al., 2015; Osborne et al., 2017).

On the other hand, the Nrf2 signaling pathway, which is known to protect from the effect of oxidative stress, was shown to be down-regulated in human glaucomatous TM cells compared with normal TM cells (Cheng et al., 2017). Oxidative stress not only causes direct TM cell damage and participates in TM matrix accumulation, but also activates the redox-responsive factors NF- $\kappa$ B and mitogen-activated protein kinases (MAPKs), resulting in the transcription of inflammatory cytokines. When exposed to chronic oxidative stress, TM cells in primary culture have been shown to produce inflammatory markers, including IL-1 $\alpha$ , IL-6, IL-8 and ELAM-1, through sustained activation of the NF- $\kappa$ B pathway (Li et al., 2007; Liton et al., 2005a). These inflammatory stress markers have been associated with high-tension glaucoma of different aetiologies (Wang et al., 2001). The IL-1/NF $\kappa$ B pathway has been suggested as protective in the short term, but amplification of this pathway due to prolonged stress may lead to pathologic tissue changes. In summary, the inflammatory reaction in the glaucomatous TM is likely to be initiated by oxidative stress, leading to activation of NF- $\kappa$ B signaling and the release of pro-inflammatory mediators, including TNF- $\alpha$ , which in turn are able to modify the ECM (Saccà et al., 2016b).

#### 4.5.2. TGF- $\beta$ 2

TGF- $\beta$ 2 is believed to play a major role in TM degeneration. Elevated levels of TGF- $\beta$ 2 have been found in the AH of POAG patients compared to normal patients (Tripathi et al., 1994a; Inatani et al., 2001; Ozcan et al., 2004; Min et al., 2006; Trivedi et al., 2011; Agarwal et al., 2015). This factor is known to promote fibrosis through increased ECM synthesis and deposition (Fuchshofer and Tamm, 2009; Zhavoronkov et al., 2016). TGF- $\beta$ 2 has been shown to reduce outflow facility when perfused into cultured human anterior segments and to increase IOP in human organ culture models (Gottanka et al., 2004; Fleenor et al., 2006). TGF- $\beta$ 2 is thought to contribute to the development of glaucoma by increasing the secretion of ECM materials, facilitating ECM crosslinking, increasing size and rigidity of TM cells, reducing protease activity and the formation of cellular stress fibres in the TM (Fleenor et al., 2006; Wordinger et al., 2007; Tovar-Vidales et al., 2013). In addition, TGF- $\beta$ 2 was found to induce senescence-associated changes in human TM cells (Yu et al., 2010) and to induce synthesis and secretion of ET-1, a potent vasoconstrictor that elicits TM cell contraction through endothelin-A receptor signaling (Von Zee et al., 2012). TGF- $\beta$ 2 has been shown to increase lipid peroxidation and ROS production in TM cells (Yu et al., 2010), reinforcing the cascade of events occurring in POAG, in an order that remains to be determined, TM cell senescence, TGF- $\beta$  dysregulation and overexpression, TM oxidative stress, TM cell loss, matrix accumulation and further reduction in AH outflow (Baudouin, 2012).

The mechanisms responsible for regulating endogenous synthesis and secretion of TGF- $\beta$ 2 within anterior chamber are not well known. Nevertheless, it has been shown that human TM cells can secrete TGF- $\beta$ 2 (Tripathi et al., 1994b), that TGF- $\beta$ 2 activation is enhanced by senescence (Chhunchha et al., 2017), and that senescent cells may release more pro-inflammatory mediators (Sreekumar et al., 2020), and possibly more TGF- $\beta$ 2.

Thus, a localised constitutive expression and release of TGF- $\beta$ 2 by TM cells may promote or exacerbate elevation of IOP in POAG (Pervan et al., 2016).

However, this growth factor also has a dual effect since it is known to have immunosuppressive properties and is responsible for anterior chamber-associated immune deviation, a mechanism that protects the eye from inflammation and immune-related tissue damage (Wilbanks et al., 1992; Streilein, 1999).

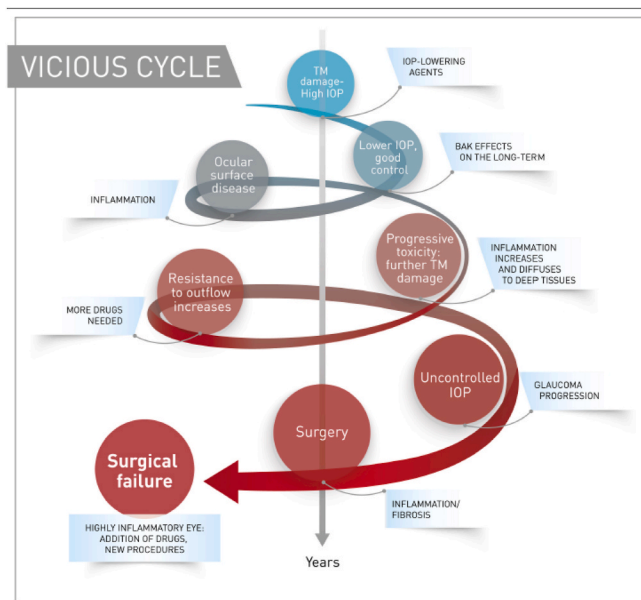
#### 4.5.3. Glaucoma medications toxicity

Most of the human studies looked at specimens taken at time of surgery, mostly in late stages of glaucoma. However, they have rarely investigated the relationship between increased aqueous levels of cytokines and preoperative treatments, as patients undergoing glaucoma surgery are likely to have received more treatments over a longer duration. In one study, aqueous levels of IL-8, TGF- $\beta$ 1, and SAA were positively associated with the number of topical glaucoma medications (Takai et al., 2012). Therefore, it is possible that the increased exposure to toxic preservatives (i.e. benzalkonium chloride [BAK]) could induce local inflammation. This could result in a vicious cycle, i.e. the more treatments administered, the greater the quantity of toxic compounds and inflammatory cytokines diffusing from the surface to deeper structures, which leads to TM inflammation and a higher resistance to AH outflow, a subsequent increase in IOP, and the requirement for more treatments (Baudouin et al., 2010; Baudouin et al., 2012). Indeed, penetration of BAK deeper into the eye, including the TM and the lens, was shown in rabbit eyes exposed to standard concentrations of BAK (Brignole-Baudouin et al., 2012; Desbenoit et al., 2013). In patients with OHT or POAG, increased flare levels were also shown in the anterior chamber of eyes receiving BAK-preserved anti-glaucomatous eye drops compared to preservative-free eye drops, suggesting that BAK exerts its effect on the blood-aqueous barrier in the anterior chamber (Stevens et al., 2012; Kestelyn et al., 2019). In a rat model, subconjunctival injections of BAK 0.01% were shown to cause TM cell death and induce toxic trabecular damage resulting in a significant reduction of the AH outflow facility and a sustained rise in IOP (Baudouin, 2012). In human TM in culture, BAK induced cell death, DNA fragmentation, and gene expression alterations (Izzotti et al., 2015).

BAK also increased the generation of inflammatory mediators (Fractalkine, also called CX3CL1) and decreased TM cell expression of stromal cell-derived factor-1 (SDF-1, CXCL12), a protective chemokine with anti-apoptotic properties (Khan et al., 2008). Consequently, long-term administration of preserved anti-glaucoma treatments containing BAK may play a role in TM cell loss through oxidative stress, chronic inflammatory changes, and apoptosis within the glaucomatous TM (Baudouin et al., 2012; Hamard et al., 2003; Izzotti et al., 2015). This vicious cycle might clinically appear as an apparent progressive loss of efficacy of topical IOP-lowering drugs, namely the more drugs, the greater the toxic reaction, leading to a higher IOP despite initial efficiency of drugs (Baudouin, 2012). Therefore, a progressive cyclic evolution may occur over the long term in glaucomatous patients, characterised by progressive inflammation caused by inflammatory infiltrates and cytokine release, which is either pre-existing and/or aggravated by topical treatments or purely iatrogenic. The subsequent diffusion of these infiltrates into the deep structures within the eye participate in an increased resistance to aqueous humour outflow, which requires additional drugs to control IOP and eventually surgery, whose prognosis will be negatively influenced by inflammatory changes within the anterior segment. Such hypothetical model is proposed in Fig. 4.

## 5. Ocular surface inflammation in glaucoma

Inflammation, free radical production, and apoptosis are closely related processes in ocular cells. The most prominent and visible consequences of inflammation in glaucoma are those occurring at the ocular surface level. Ocular surface disease (OSD) is therefore very common in these patients (Erb et al., 2008; Ghosh et al., 2012; Baudouin et al., 2013). In an observational cross-sectional study, 51% of patients with glaucoma or OHT had significant OSD, which was mild to moderate in 30% of patients and severe in 21% (Baudouin et al., 2013). Glaucoma patients commonly complain of ocular symptoms, including foreign body sensation, eye redness, pruritus, pain, photosensitivity and blurred vision. Adverse changes in tear film stability, tear osmolarity, conjunctival hyperaemia, and conjunctival or corneal damage are frequently observed (Baudouin et al., 2013; Wong et al., 2018) (Fig. 5). Ocular



**Fig. 4.** Schematic proposal of possible evolution of glaucomatous patients treated over the long term and experiencing potential aggravation of the disease through immunoinflammatory and/or toxic mechanisms.

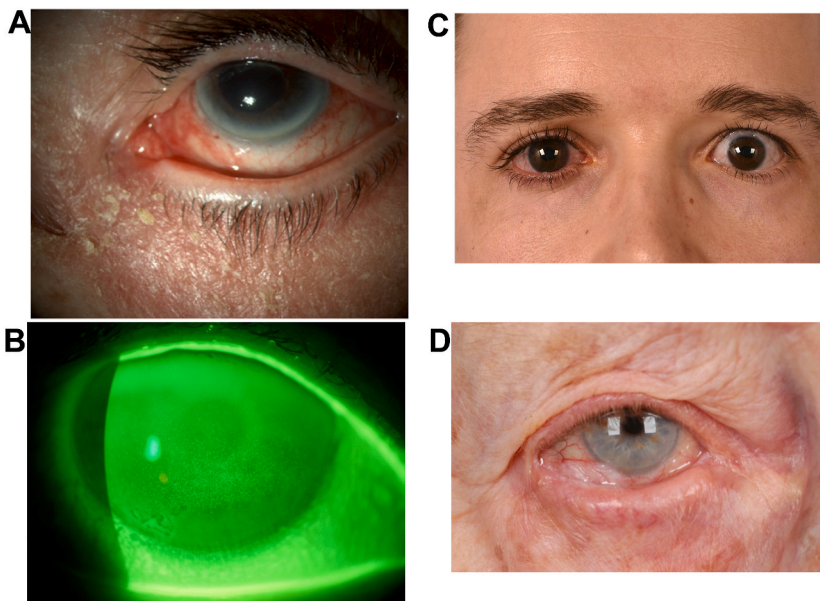
surface changes also include effects which are not clinically visible but highly relevant to ocular surface physiology, including decreased goblet cell density, infiltration by inflammatory cells, a significant decrease in the number and density of the central corneal subbasal nerve fibers, and a reduction in corneal sensitivity (Broadway et al., 1994a, 1994b; Martone et al., 2009; Baudouin et al., 2010; Saini et al., 2017; Tiedemann et al., 2019). Changes in the meibomian gland morphology and function have also been frequently reported. Indeed meibomian gland dysfunction is strongly associated with treatments over the long-term, causing both eyelid inflammation, toxic or immune-allergic blepharitis and dry eye through tear film instability (Messmer et al., 2005; Arita et al., 2012; Mocan et al., 2016; Lee et al., 2018).

### 5.1. Subclinical inflammation at the ocular surface

Patients on long-term glaucoma treatment often show significant subclinical ocular surface inflammation suggestive of overexpression of HLA-DR, ICAM-1, cytokines, chemokines, infiltration of immunoinflammatory cells (monocytes and T-cells) in the conjunctiva and cornea, and fibroblast activation in the conjunctiva and subconjunctival space (Baudouin et al., 1999) (Fig. 6). Increased secretion of cytokines (IL-6, IL-8, and IL-10) have been reported in conjunctival epithelial cells of glaucoma patients treated with preserved latanoprost or timolol eye drops compared to normal eyes (Pisella et al., 2004; Baudouin et al., 2004, 2008). In addition, increased levels of cytokines (IL-1 $\beta$ , IL-2, IL-5, IL-6, IL-8, IL-10, IL-13, IL-15, IL-17, MCP-1/CCL2, TNF- $\alpha$ ) have been reported in tears of POAG patients on medical therapy (Malvitte et al., 2007; Benitez-Del-Castillo et al., 2019). Levels of both Th1 (IFN- $\gamma$ , IL-2) and Th2 (IL-4, IL-5, IL-10) cytokines have also been found at significantly higher levels in the tears of patients on topical glaucoma therapy (Malvitte et al., 2007). Interestingly, Th1 cytokines have shown the most marked increase, suggesting a chronic pro-inflammatory reaction resembling dry eye disease (Baudouin et al., 2008), whereas Th2 cytokines are associated more with allergic reactions, demonstrating a possible combination of the two mechanisms. This is consistent with the overexpression of both CCR5 and CCR4 found in the conjunctival epithelium of glaucoma patients, two markers of the Th1 and Th2 activation pathways, respectively (Baudouin et al., 2008).

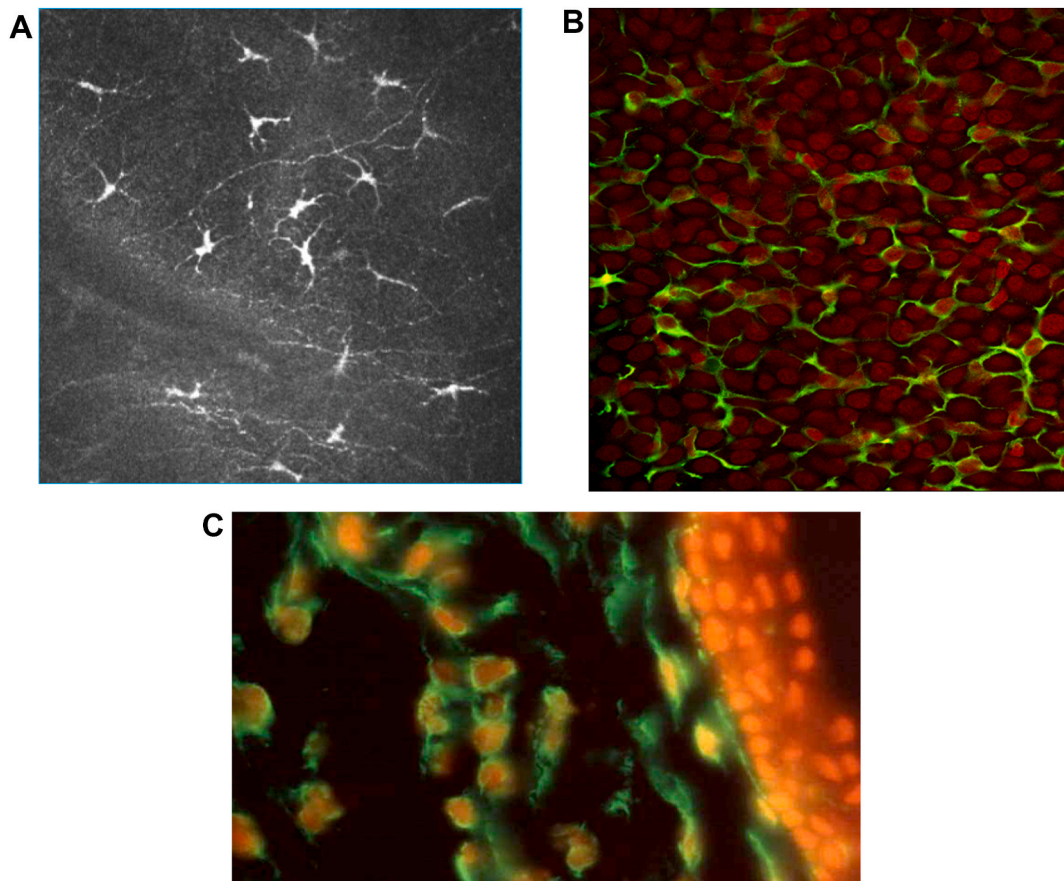
### 5.2. Glaucoma medications and ocular surface inflammation

Factors affecting the stability and osmolarity of the tear film induce ocular surface damage and initiate inflammatory cascades that generate innate and adaptive immune responses, mediated by infiltration of monocytes and lymphocytes. In this context, it was reported that untreated OAG patients had decreased basal tear turnover, which may have been caused by glaucoma itself (Kuppens et al., 1995). However, it has also been consistently shown that the number of antiglaucoma drugs and the duration of treatment correlates with the severity of glaucoma and are key predictors of significant OSD (Ghosh et al., 2012; Baudouin et al., 2013). The cumulative exposure of the most commonly used preservative (BAK), appears to be one of the major factors leading to ocular surface inflammation and damage (Ghosh et al., 2012; Iester et al., 2014; Mastropasqua et al., 2016). BAK induces time- and concentration-dependent cytotoxic effects on the cells of the ocular



**Fig. 5.** OSD in glaucomatous patients. A: Allergic reaction to topical drug: eczematous eyelid. B: Weakly symptomatic toxic keratopathy marked by extended fluorescein staining. C: Post-surgical difference between left eye, free of eyedrops after trabeculectomy and right eye severely affected by topical treatments. D: Toxic pseudomphigoid with eyelid retraction and symblepharon.





**Fig. 6.** Inflammatory cells in ocular surface tissues in glaucoma. A: Dendritic cells infiltrating the cornea in a glaucomatous patient (*in vivo* confocal microscopy, 400 $\mu$ m $\times$ 400 $\mu$ m).

B: Immunofluorescence staining of dendritiform cells in impression cytology in a multitreated patient: vimentin staining (green), nuclei stained with propidium iodide (red), confocal microscopy.

C: Conjunctival biopsy taken at the time of trabeculectomy in a multitreated patient: infiltration of the substantia propria by numerous inflammatory cells expressing vimentin (green). Nuclei are stained in red with propidium iodide. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

surface, likely mediated by chronic oxidative stress, which causes inflammation and induction of signals leading to apoptosis and cell death (Baudouin et al., 2010).

However, the contribution of the active ingredients in the medication or the interaction with pathological ocular surface tissues at the initiation of therapy cannot be ruled out (Pflugfelder and Baudouin, 2011; Villani et al., 2016; Mastropasqua et al., 2016). As an example of the direct effects of an active compound, topical application of preservative-free latanoprost was shown to decrease tear production, induce conjunctival goblet cells loss, disrupt corneal epithelial barrier, and promote cell apoptosis and inflammation of the ocular surface (Yang et al., 2018a). Beta-blockers, with or without preservatives, have also been shown to induce ocular surface damage, including corneal staining, conjunctival goblet cell loss and squamous metaplasia (Aydin Kurna et al., 2014). In mice, topical application of preservative-free latanoprost has also been shown to increase CD4<sup>+</sup> T cells infiltration in the conjunctiva. This was associated with increased production of IFN- $\gamma$ , IL-17 A, TNF- $\alpha$ , IL-1 $\beta$ , activation of MMPs in the conjunctiva, and decreased production of IL-13, which may be a result of the activation of P38-NF- $\kappa$ B signaling (Yang et al., 2018a).

In summary, OSD in glaucoma is likely to be the result of interactions between the ocular surface and the active compounds acting alone or in combination with the preservatives and excipients. In this context, OSD may manifest from a progressive toxic and/or immune-inflammatory reaction or be aggravated when eye drops are administered on an

already damaged or pathological ocular surface.

### 5.3. Corneal barrier dysfunction in glaucoma

The corneal cell layers are critical in maintaining a healthy and clear cornea. In medically controlled glaucoma patients, the cornea is one of the most altered tissues, with modifications involving all epithelial layers, subbasal nerve plexus, stroma and endothelium. Glaucoma is also associated with deleterious effects on corneal endothelium due to topical treatments, elevated IOP and glaucoma surgical procedures (Janson et al., 2018).

Ocular inflammation is also associated with OSD and has been shown to affect corneal structure and function (Stern and Pflugfelder, 2004). In patients with glaucoma, the dendritic cell density has been found to be two to three times higher at the limbus, and more than ten times higher in the central cornea compared to healthy controls (Mastropasqua et al., 2016) (Fig. 6A and B). Inflammatory cytokines (e.g. TNF- $\alpha$ , IL-1 $\beta$  and IFN- $\gamma$ ) and toxic compounds like BAK, have been shown to induce corneal epithelial barrier dysfunction through downregulation of corneal epithelial cell adhesion molecules and tight-junction proteins such as zonula occludens 1 (ZO-1) or occludin (Pauly et al., 2009; Kimura et al., 2013).

In general, the ocular surface is extensively supplied by sensory and autonomic nerve fibers that play a crucial role in maintaining ocular surface homeostasis (Belmonte et al., 2017). Chronic ocular



inflammation can therefore lead to decreased corneal sensitivity and a consequent neurosecretory block that reduces reflex tear secretion (Lambiase et al., 2011). Neurogenic inflammation is produced principally through release of neuromodulators, such as substance P and calcitonin-gene related peptide (CGRP), leading to edema and breakdown of the blood-tissue barrier (Beuerman and Stern, 2005).

Corneal barrier dysfunction may cause cytokine penetration, thereby contributing to inflammation and damage of the corneal endothelium as well as possibly causing damage to deeper structures in the anterior segment. The influence of toxic compounds and/or inflammatory cytokines that diffuse from the ocular surface to deeper structures, such as TM, is still speculative, but studies have suggested that this may contribute to degenerative anterior processes associated with POAG (Baudouin, 2012).

#### 5.4. Subconjunctival inflammation and fibrosis in glaucoma

The development of subconjunctival fibrosis has been reported in patients treated with antiglaucomatous drugs over a long duration, is potentially due to an increase in fibroblast density in the subepithelial substantia propria, related to an increase in inflammatory cells (Sherwood et al., 1989; Baudouin et al., 1999; Broadway et al., 1994a, 1994b). It has also been shown that POAG patients undergoing a deep sclerectomy, and with a long history of anti-glaucomatous eye drops have increased expression of MMPs, especially MMP-9 and TIMPs, which could impair the healing process (Helin-Toiviainen et al., 2015).

More evidence has been found that sub-clinical, abnormal pre-operative inflammation due to a cumulative exposure to BAK-preserved eye drops are likely to be responsible for an exaggerated wound healing process that blocks aqueous outflow in the subconjunctival space. This results in external bleb scarring and failure of filtration surgery (Broadway et al., 1993; Sherwood et al., 1989; Baudouin, 2012; Helin et al., 2011) (Fig. 6C).

Prolonged use of some topical anti-glaucomatous eye drops may be associated with enhanced Tenon's capsule fibroblasts proliferation and

abnormal expressions of TGF- $\beta$  and MMPs, which may promote scarring of the filtering blebs following glaucoma-filtering surgery (Leng et al., 2011). In this context, specific pro-inflammatory cytokines, including TNF- $\alpha$  and IL-6, have been suggested as responsible for bleb scarring and failed trabeculectomy when diffusing in the subconjunctival spaces (Cvenkel et al., 2010). Chong et al. (2010) found increased levels of MCP-1 in the tears of patients treated with topical anti-glaucomatous drugs, which was associated with early postoperative scarring in need of postoperative bleb needling. Recently, Hayek et al. (2019) used angio-optical coherence tomography (OCT) and demonstrated that a higher conjunctival vascular density is associated with poorer prognosis after surgery, including a higher postoperative IOP and a greater need for needling. Furthermore, it has been suggested that goblet cells may act as aqueous humour pathways after surgery (Amar et al., 2008), and that preoperative goblet cell reduction induced by BAK may have deleterious effects (Baudouin et al., 2019; Rolando et al., 1991). In line with this, Agnifili et al. (2016) reported negative outcomes in cases with reduced goblet cell density, due to chronic inflammation and/or toxic drugs. Similarly, Souchier et al. (2006) found a higher mucin 5AC (MUC5AC) expression and lower subclinical inflammation (HLA-DR) in successful glaucoma surgery compared with failed surgery.

#### 5.5. From subclinical to severe ocular surface changes in glaucoma

It has become clear that inflammatory responses induced by glaucoma drugs lead to further ocular surface damage and the development of a self-perpetuating vicious cycle of inflammation (Stevenson et al., 2012; Baudouin et al., 2012, 2018). A model of frequency of ocular surface changes from subclinical to extremely severe alterations is proposed in Fig. 7. Most likely, such changes will gradually worsen as a result of aging and the need for additional IOP-lowering eye drops, with the increasing damage to the ocular surface making it more sensitive and vulnerable to further treatments. One difficulty in assessing subclinical changes and subtle clinical abnormalities is that it remains impossible to determine whether such subclinical inflammation will progressively

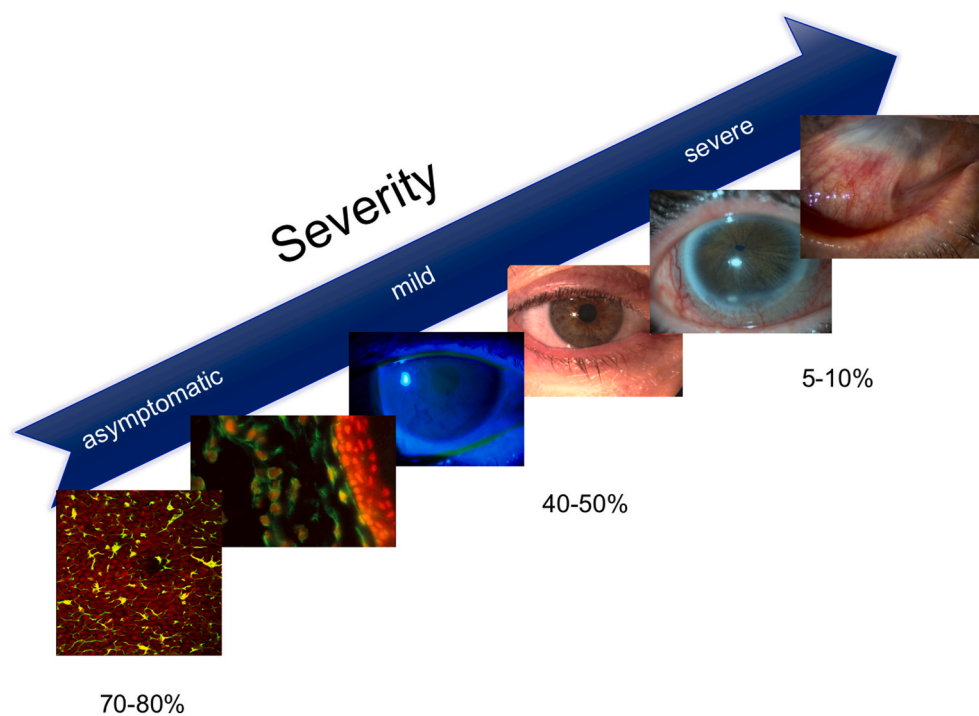


Fig. 7. Proposal for a schematic representation of increasing severity levels and estimated frequency of various levels of ocular surface disease in glaucoma patients. Cumulative frequencies are given by including the lower level of severity, namely asymptomatic (with only histological lesions), mildly symptomatic and more severe cases.

increase and become clinically relevant or remain stable.

## 6. Modulation of inflammation in glaucoma: perspectives and future directions

Innate and adaptive inflammatory mechanisms mediated by resident cells are necessary to maintain healthy eye structures. However, the para-inflammation can quickly go from being positive to becoming toxic and become a contributing factor to the degeneration of ocular tissues. There is evidence that stress stimuli, including increased IOP or decreased ocular blood flow, associated with chronic oxidative stress can tip the balance from beneficial para-inflammation to toxic levels participating in TM and retinal/ONH degeneration in glaucoma (Xu et al., 2009; Jiang et al., 2020). In addition to its potential involvement in TM and retinal/ONH degeneration, inflammation also plays a major role in initiating and perpetuating OSD in glaucomatous patients, which may decrease the effectiveness of IOP-lowering treatments and increase the risk of surgical failure (Baudouin et al., 2010, Baudouin et al., 2012). Various therapeutic approaches have been proposed to prevent or modulate the excessive inflammatory reactions in glaucomatous eyes. Some well-known products appear to have beneficial effects on neuro-inflammation in animal glaucoma models, but have not yet been tested in clinical studies.

### 6.1. Tentative targets for treating neuroinflammation

Therapies aimed at modulating the immune and inflammatory responses are attractive approaches for limiting ONH damage and the loss of RGCs associated with glaucoma (Adornetto et al., 2019; Karlstetter et al., 2015). Studies using animal models have shown that a number of drugs have the ability to modulate the reactivity of glial cells and their inflammatory response. However, even though neuroprotection has been shown in rodent models, many drugs that have demonstrated potential in experimental models have failed or are missing in a clinical setting and further investigations are needed in primates and humans.

#### 6.1.1. Suppression of glial cell activity

**6.1.1.1. Minocyclines.** Minocycline is a second-generation tetracycline with well-known anti-inflammatory activity (Abcouwer et al., 2013). The neuroprotection provided by minocycline appears to be mediated by two mechanisms: attenuation of innate and adaptive immunity, in particular the prevention of microglia activation, and blockade of the apoptotic cascade. Long-term treatment of DBA/2 J mice with minocycline was found to suppress early microglial activation and to significantly improve neuronal retrograde tracing from the superior colliculus, the main retinal synaptic target in rodents (Bosco et al., 2008). In an experimental model of glaucoma using intracameral injection of chondroitin sulfate in rats, minocycline (30 mg/kg per day for 2 weeks, i. p.) significantly reduced microglial (Iba, ED1) and astrocyte (GFAP) markers and myelin alteration, without effect on IOP (Bordone et al., 2017). Minocycline also preserved the anterograde transport of cholera toxin B (CTB), a reliable tracer, from the retina to the superior colliculus and LGN. Although these results suggest protection in the early stage of glaucoma, it was also demonstrated that minocycline (22 mg/kg, i. p.) improved RGC survival in other experimental glaucoma models with elevated IOP (Levkovitch-Verbin et al., 2006). One of the mechanisms may be the increased gene expression of Bcl-2, as indicated by the observations from a rat glaucoma model with optic nerve transection (Levkovitch-Verbin et al., 2014a, Levkovitch-Verbin et al., 2014b). In addition to showing neuroprotective qualities in rat glaucoma models, minocycline treatment was also effective in preventing vascular permeability and inflammation following retinal ischemia-reperfusion (IR) injury in a model of retinal neurodegeneration with breakdown of the blood-retinal barrier (Abcouwer et al., 2013). Here, minocycline

significantly inhibited the expression of the monocyte chemoattractant CCL2/MCP-1 and the leukocyte adhesion molecule ICAM-1 while at the same time reducing the infiltration of MHC class-II positive inflammatory leukocytes (Abcouwer et al., 2013).

**6.1.1.2. Azithromycin.** Azithromycin is a widely used semi-synthetic macrolide antibiotic with anti-inflammatory and immunomodulatory properties independent of its antibacterial effects (Zarogoulidis et al., 2012). Varano et al. recently reported the evidence of the neuro-protective effect of azithromycin in an experimental model of acute glaucoma induced by transient elevation of IOP (Varano et al., 2017). A single dose of azithromycin given systemically at the end of ischemia produced a down-regulation of gene products related to necrotic and apoptotic cell death, including reduced calpain activity and prevention of Bcl-2-associated death promoter (Bad) upregulation. The observed neuroprotection was also associated with a significant inhibition of MMP-2/MMP-9 activity and extracellular signal-regulated kinases (ERK) 1/2 phosphorylation. Azithromycin may therefore provide neuroprotection by modifying the inflammatory state of the retina following ischemia/reperfusion injury that, in turn, affects neuronal survival in central areas (Varano et al., 2017; Adornetto et al., 2019).

**6.1.1.3. Adenosine receptor antagonists.** Caffeine, by blocking A<sub>2A</sub> receptors is known to prevent synaptotoxicity, excitotoxicity and neuronal loss. In addition, it has been shown to have anti-inflammatory properties in the CNS, namely by attenuating microglia-mediated neuroinflammation (Brothers et al., 2010). In this context, caffeine and adenosine receptor antagonists have been suggested as possible therapeutic options to manage RGC loss in glaucoma (Madeira et al., 2016a, 2016b). In rats with OHT induced by laser photocoagulation of the trabecular meshwork and limbal veins, caffeine (1 g/L) administered ad libitum in the drinking water was shown to inhibit the overall inflammatory response as well as prevent the retinal microglia-mediated neuroinflammatory response. The elevated retinal expression of TNF- $\alpha$  and IL-1 $\beta$  induced by the ocular hypertension also decreased. In addition, caffeine was able to prevent OHT-induced microglia reactivity, including in the contralateral eye. Overall, the study suggested that caffeine preserves RGC survival (Madeira et al., 2016b). Similar effects were recently obtained with caffeic acid phenethyl ester (CAPE) in a rat glaucoma model with optic nerve crush injury (Jia et al., 2019). RGC loss was significantly reduced in CAPE-treated animals compared with the non-treated group. This was associated with decreased expression of inflammatory cytokines, including IL-8, IL-6, iNOS, cyclo-oxygenase-2, TNF- $\alpha$  and CCL2/MCP-1, and by inhibition of the NF- $\kappa$ B signaling in caffeine-treated rats. Hypertrophy of astrocytes and Müller cells caused by optic nerve crush was also attenuated (Jia et al., 2019).

**6.1.1.4. TNF- $\alpha$  receptor antagonists.** Etanercept is a recombinant chimeric protein generated by fusing the ligand-binding portion of human TNFR2 and the Fc portion of human IgG1. It is approved for various autoimmune diseases, including rheumatoid arthritis, juvenile idiopathic arthritis, ankylosing spondylitis, and psoriatic arthritis (Nanda and Bathon, 2004; Ramiro et al., 2011). When administered intraperitoneally in mice with OHT produced by episcleral vein cauterization, etanercept inhibited TNF- $\alpha$  generation in the retina, and reduced the reactive microglia and astrogliosis around the ONH. Overall, etanercept was shown to prevent RGC loss and axon degeneration in mice with OHT (Roh et al., 2012).

**6.1.1.5. cAMP phosphodiesterase (PDE) inhibitors.** Ibudilast, a PDE type 4 (PDE4) inhibitor, has been proposed as a potential treatment of neurodegenerative diseases (Chen et al., 2020) and is currently under clinical investigations for the treatment of multiple sclerosis (Fox et al., 2018). In ocular hypertensive rats, Cueva Vargas et al. (2016) demonstrated that intravitreal administration of ibudilast dampened macroglia

and microglia reactivity in the retina and optic nerve compared to vehicle injection. Ibudilast also reduced to baseline level proinflammatory cytokines (including TNF- $\alpha$ , IL-1 $\beta$ , IL-6, migratory macrophage inhibitory factor [MIF-1]). It also promoted RGC survival, prevented axonal degeneration, and improved anteretrograde axonal transport. Thus, the PDE4 signaling may be considered as a potential target in glaucomatous neuroinflammation.

**6.1.1.6. Fas receptor antagonists.** Krishnan et al. (2019) recently report the inhibition of neuroinflammation and prevention of axon degeneration and RGC death in an inducible mouse model of glaucoma treated with an intravitreal injection of a Fas receptor antagonist (ONL1204). Neuroprotection was correlated with a significant reduction of microglial and/or infiltrating macrophages activation, and inflammatory cytokines (including TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-18) and chemokines (MIP-1 $\alpha$ , MIP-1 $\beta$ , MIP-10, MCP1, and IP10).

### 6.1.2. Suppression of oxidative stress

In view of the role of oxidative stress-related mechanisms in neuroinflammation, anti-oxidant strategies represent a possible approach as an immunomodulation strategy in glaucoma.

**6.1.2.1. Coenzyme Q10.** Coenzyme Q10 is a mitochondrial-targeted anti-oxidant with known neuroprotective activity (Davies et al., 2017; Martucci et al., 2019). This product is claimed to protect RGCs (Nucci et al., 2007) and it is currently under investigations in clinical trials. In an open randomised controlled trial, topical coenzyme Q10 (one drop twice daily for 12 months) associated with vitamin E administered in OAG patients improved the inner retinal function (pattern electroretinogram) with an enhancement of the visual cortical responses (Parisi et al., 2014). Significant levels of co-enzyme Q10 were detected in the vitreous humour after a single drop administration (Fato et al., 2010), and there was a significant reduction in the levels of superoxide dismutase in the aqueous humour after administration in patients with pseudo-exfoliative glaucoma (Ozates et al., 2019). A randomised, double-blind and controlled study is currently being performed to assess the neuroprotective effect of co-enzyme Q and vitamin E on the time to visual field progression up to 36 months in POAG patients (Quaranta et al., 2019) (Clinicaltrials.gov NCT03611530).

**6.1.2.2. Ginkgo biloba.** In a randomised, placebo-controlled study, oral administration of *Ginkgo biloba* extracts (40 mg 3 times daily for one month) improved pre-existing visual field damage in patients with normal tension glaucoma (Quaranta et al., 2003, 2014). The mechanism of action may be due to free radical scavenger effects, but also on the glutamate signaling and the preservation of the mitochondrial function (Kolko, 2015). Nevertheless, these results have not been confirmed in other clinical trials (Guo et al., 2014a, Guo et al., 2014b), and therefore further clinical studies are required.

**6.1.2.3. Tempol.** Tempol is a superoxide dismutase mimetic and free radical scavenger. When tempol was given subcutaneously by implanted osmotic mini-pumps (constant infusion for 6 weeks) in mice with unilaterally induced IOP elevation, it was shown to reduce the activation of NF- $\kappa$ B, which limits the expression of proinflammatory cytokines including IL-1, IL-2, IFN- $\gamma$ , and TNF- $\alpha$  in eyes of ocular hypertensive rats (Yang et al., 2016).

**6.1.2.4. Lipoic acid.** It was demonstrated that dietary administration of the anti-oxidant  $\alpha$ -lipoic acid, an organosulfur compound derived from octanoic acid to DBA/2 J mice, it resulted in a significant decrease in RGC death and a generally decrease in upregulation of specific genes and proteins related to oxidative stress (Inman et al., 2013).

## 6.2. Treatment of anterior segment inflammation

Based on TM degeneration pathophysiology, there is a need for anti-oxidative and anti-inflammatory strategies to treat inflammation in the anterior segment and restore TM function. Although timolol has been shown to exert a direct anti-oxidant activity that protects human endothelial cells from oxidative stress *in vitro* (Izzotti et al., 2008), no studies have provided evidence of this *in vivo* protective mechanism in glaucoma patients. Animal studies of IOP-lowering eye drops, such as latanoprost, showed that although these medications can lower IOP, they did not reverse or improve the oxidative stress or damage caused by glaucoma (Fahmy et al., 2018).

### 6.2.1. Flavonoids

Flavonoids have several bioactive properties including anti-oxidant, anti-inflammatory and neuroprotective effects (Milbury, 2012). It has also been reported that they may reduce oxidative stress and improve ocular blood flow in POAG (Khoo et al., 2010). Recently, a flavonoid, myricetin was shown to reduce the ROS-induced oxidative stress, to down-regulate senescence markers, and to lower the expression levels of inflammatory cytokines including (IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, IL-8, TNF- $\alpha$ , VEGF, TGF- $\beta$ 1 and TGF- $\beta$ 2) in TM cells from POAG patients (Yang et al., 2018b). When administered in rats with chronic OHT induced by intracameral injection of hyaluronic acid for 6 weeks, oral myricetin (25, 50 or 100 mg) effectively prevented IOP elevation. Moreover, myricetin reduced ROS levels and inflammatory cytokines (IL-6, IL-8) in the aqueous humour and in the TM cells of these hypertensive rats (Yang et al., 2018b).

### 6.2.2. Omega-3 fatty acids

Omega-3 fatty acids, like docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), have well-documented anti-inflammatory properties (San et al., 2005). These essential fatty acids may have therapeutic potential in glaucoma or may be beneficial in terms of glaucoma prevention (Saccà et al., 2019). Recently, three months supplementation of oral omega-3 supplementation was shown to significantly reduce IOP in normotensive adults and in pseudoexfoliative glaucoma (Downie et al., 2018; Villadoniga et al., 2018).

## 6.3. Treatment of ocular surface inflammation

Treatment of the ocular surface may be useful in glaucomatous patients with OSD and before filtering surgery. The most appropriate way to control and inhibit surface inflammation is to identify the components responsible for OSD, whether the active compound or one of the excipient of a treatment, and remove or replace it by a more tolerated compound. This may also be achieved by using laser therapy or surgery to reduce IOP. Indeed, such a subtractive strategy consisting of stopping the harmful compounds associated with the treatment of OSD has been shown to be effective in several case series, not only to improve the ocular surface but also to reduce IOP, probably through reduction of inflammatory reactions within the TM (Batra et al., 2014; Dubrulle et al., 2018). When preventive and subtractive strategies are not feasible, some anti-inflammatory options may improve OSD in glaucoma patients.

### 6.3.1. Cyclosporine A

Cyclosporine A is an immunomodulatory, anti-inflammatory, and anti-fibroblastic agent. Its topical formulation is currently indicated for the treatment of inflammation in dry eye disease. In a prospective comparative study in POAG patients, treatment with topical cyclosporine 0.05%, twice daily for 6 months concurrently with glaucomatous eye drops was shown to improve OSD as assessed by Schirmer's test, ocular staining, ocular surface disease index (OSDI), corneal sensations and corneal sub-basal nerve fiber bundle density (Saini et al., 2015). This confirms the beneficial effect of topical cyclosporine in the treatment of ocular inflammation as shown in dry eye patients, with a good



overall safety profile with only mild adverse events (Zhou et al., 2014).

### 6.3.2. Rebamipide

Rebamipide, a quinolone derivative, is an ophthalmic solution indicated for the treatment of dry eye disease (Kinoshita et al., 2012). This product has anti-inflammatory and anti-oxidant properties and induces mucin secretion when applied on the ocular surface (Ohguchi et al., 2013). It has been shown to inhibit the increase of TNF- $\alpha$ -induced IL-6 and IL-8 in a human corneal epithelial cell line (Tanaka et al., 2013). In glaucoma patients treated with BAK-preserved eye drops, rebamipide, administered four times daily, resulted in improved corneal barrier function and improved tear break-up time after 2 months (Tokuda et al., 2015).

### 6.3.3. Ketorolac

Although the association of nonsteroidal anti-inflammatory drugs and prostaglandins analogues is currently not approved in the treatment of glaucoma, one small open-labelled contro-lateral eye controlled clinical trial showed that topical ketorolac, a non-selective cyclooxygenase inhibitor, significantly enhanced the IOP-lowering effect of prostaglandins analogues (PGAs), including latanoprost, travoprost, and bimatoprost in POAG patients (Turan-Vural et al., 2012). Such results need to be confirmed in larger double-asked randomized clinical trials but are consistent with other studies and was attributed to inhibiting the generation of endogenous prostaglandins that may compete with PGAs to improve aqueous outflow facility (Costagliola et al., 2008; Zhu et al., 2018).

### 6.3.4. Nutraceuticals

Nutraceutical intervention could be a valuable option for OSD in glaucoma-treated patients. One open-label randomised study showed that administration of an oral supplementation of anti-oxidants and essential fatty acids for three months led to decreased levels of inflammatory cytokines (IL-6 and TNF- $\alpha$ ) in tears of POAG patients (Galbis-Estrada et al., 2013). In two large open-label studies, administration of an oral supplement containing vitamins, anti-oxidants, minerals, and omega-3 fatty acids for three or six months showed improvement of dry eye signs and symptoms associated with a small but significant improvement of IOP in glaucoma patients treated with IOP-lowering eye drops (Tellez-Vazquez, 2016; Romeo Villadoniga et al., 2018).

## 6.4. Treatment of ocular inflammation before filtering surgery

Treating the ocular surface with anti-inflammatory drugs before trabeculectomy may be advisable as initially suggested by Broadway et al. Preoperative 1% fluorometholone eye drops one month before surgery can improve the ocular surface and the success rate of filtration surgery (Broadway et al., 1996). Indomethacin (unpreserved) and fluorometholone (preserved) eye drops administered one month before filtering surgery in glaucomatous patients also reduced subclinical conjunctival inflammation as measured by ocular surface HLA-DR expression (Baudouin et al., 2002). Recently, ketorolac eye drops administered for one month in 61 patients with uncontrolled IOP before surgery were shown to be associated with improved trabeculectomy outcome in the likelihood of postoperative needling (41% in the placebo group versus 6% in the ketorolac group, and 5% in the fluorometholone group,  $p = 0.006$ ) (Breusegem et al., 2010).

## 7. Concluding remarks

In summary, there are consistent findings from animal models and from post-mortem human ONH specimen supporting the role of neuro-inflammation in glaucoma. Various mechanisms have been proposed but they cannot be currently translated into clinical science, due to the slow age-related progression of this multifactorial disease where numerous factors interact and also to the inherent difference in eye structure

between species. Thus there is clearly a need for innovative animal models such as the microbead occlusion model in non-human primate (Lambert et al., 2019), or the hyaluronic acid glycidyl methacrylate (HAMA) mouse model (Guo et al., 2018) which seem attractive models. New genetic glaucoma models may also improve the translatability of current models (Harada et al., 2019).

The pathophysiology of glaucoma involves complex inflammatory responses, at various levels with an interaction of various pathways. Para-inflammation seems necessary in response to various stimuli in order to maintain healthy ocular tissues including the retina, the anterior chamber, and the ocular surface. However, the magnitude of this para-inflammation is variable and is likely increased during aging (Xu et al., 2009). Within the eye, the inflammatory responses are mainly regulated by glial cells in the retinal/neuronal tissue and by TM cells in the trabecular meshwork. These cells are essential to maintain tissue repair and recovery, and to avoid the development of inflammation with immune cell infiltration. The feedback process to recovery is likely impaired in glaucoma where sustained stress is elicited by mechanical, vascular and oxidative stress. When this stress is maintained over time, it may result in chronic inflammation, causing TM cell and RGC loss or ONH degeneration. There is also evidence that even at subclinical levels, inflammation occurs at the ocular surface of glaucoma patients on active therapy, resulting in OSD, corneal dysfunction and subconjunctival fibrosis. The potential effects of severe chronic surface inflammation to deeper structures like the trabecular meshwork, the lens or even the retina, remain speculative. However, several arguments support the detrimental consequences that may be masked by the IOP-lowering effects of the topical medications, rendering such interactions very difficult to identify. Therefore, a careful assessment of OSD in glaucomatous patients especially when treated with preserved eyedrops is highly advisable. When suppression or reduction of the compounds responsible for such inflammation is not possible, treating ocular surface inflammation with anti-inflammatory drugs may be recommended. Control of inflammation to restore a normal immune homeostasis constitutes an attractive therapeutic approach in glaucoma. Various therapeutic targets aimed at suppressing oxidative stress or chronic neuroinflammation are considered promising based on animal glaucoma models. However, interventional studies in humans are still lacking. Animal experimental glaucoma models have generally suggested that the inflammatory processes are initiated in early stages of glaucoma. Therefore, it would be appropriate to treat inflammation early in the development of the disease before the excessive and intractable cell loss and tissue remodeling abnormalities occur. This remains particularly challenging in this slowly developing disease because of the lack of biomarkers of disease progression and chronic inflammation (Agnifili et al., 2015). A question remains to determine whether the anti-inflammatory therapy would have to be delivered at the TM level to prevent or control IOP increase, at the ONH level, which is less easily accessible to therapy, or both. Lastly, another unsolved issue would be the dual activity of the targeted inflammatory mediators and the level of inhibition to restore the inflammatory balance. It is questionable whether a complete and uncontrolled inhibition would be beneficial. Thus, further research is required and should be encouraged.

## Conflicts of interest

Christophe Baudouin: Consulting and/or research grants from Aerie, Alcon, Allergan, Dompe, Horus Pharma, Santen, SIFI, and Théa. Patent from EyePrim® OPIA.

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