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Review

# Neutrophils and neutrophil extracellular trap components: Emerging biomarkers and therapeutic targets for age-related eye diseases

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ABSTRACT

Age-related eye diseases, including dry eye, glaucoma, age-related macular degeneration, and diabetic retinopathy, represent a major global health issue based on their increasing prevalence and disabling action. Unraveling the molecular mechanisms underlying these diseases will provide novel opportunities to reduce the burden of age-related eye diseases and improve eye health, contributing to sustainable development goals achievement. The impairment of neutrophil extracellular traps formation/degradation processes seems to be one of these mechanisms. These traps formed by a meshwork of DNA and neutrophil cytosolic granule proteins may exacerbate the inflammatory response promoting chronic inflammation, a pivotal cause of age-related diseases. In this review, we describe current findings that suggest the role of neutrophils and their traps in the pathogenesis of the above-mentioned age-related eye diseases. Furthermore, we discuss why these cells and their constituents could be biomarkers and therapeutic targets for dry eye, glaucoma, age-related macular degeneration, and diabetic retinopathy. We also examine the therapeutic potential of some neutrophil function modulators and provide several recommendations for future research in age-related eye diseases.

## 1. Introduction

Consistent with the population aging and life expectancy prospects (United Nations, 2019), gerontologists expect a high prevalence of age-related conditions, including eye diseases (Akpek and Smith, 2013; Burch et al., 2014). Additionally, chronic eye conditions are the leading cause of visual impairment and blindness worldwide (GBD, 2019 Blindness and Vision Impairment Collaborators and VLEG, 2021). Given that visual loss is associated with other physical/mental disorders, and with an age-related disability, this fact is a critical issue (Court et al., 2014; National Academies of Sciences et al., 2016). Therefore, researchers are dedicating full efforts to understand the mechanisms that lead to chronic ocular diseases onsets.

### 1.1. Aging and age-related ocular changes

The Aging process involves a progressive decline of each organ-specific functions, including the eye. Like in the rest of the body, age-related ocular changes are caused by oxidative and inflammatory cellular damage (Crooke et al., 2017; De la Fuente and Miquel, 2009).

*Abbreviations:* ACPAs, Anti-citrullinated protein antibodies; ACPA-IC, Immune complexes formed by ACPAs and citrullinated proteins; AMD, Age-related macular degeneration; APCs, Antigen-presenting cells; AREDs, Age-related eye diseases; DED, Dry eye disease; DR, Diabetic retinopathy; eDNA, Extracellular DNA; FPR, Formyl peptide receptor; LCN2, Lipocalin 2; MGD, Meibomian gland dysfunction; MMP-9, Matrix metalloproteinase-9; MPO, Myeloperoxidase; NE, Neutrophil elastase; NET, Neutrophil extracellular trap; NLR, Neutrophil-to-lymphocyte ratio; oBRB, Outer blood-retinal barrier; oGVHD, Ocular graft-vs-host disease; PACG, Primary angle-closure glaucoma; POAG, Primary open-angle glaucoma; RPE, Retinal pigment epithelium; SII, Systemic immune-inflammation index; TM, Trabecular meshwork.

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**Table 1**
Summary of the main changes that ocular cells, tissues, systems, and fluids involved in AREDs suffer during aging.

| Associate disease | Cells/ Tissues/ Systems | Age-related change | Result | References |
|---|---|---|---|---|
| DED | Lacrimal gland | Inflammation. Increased amount of lipofuscin granules and oxidative lipid and DNA damage in acinar cells. Lymphocytic infiltration, acinar atrophy, periacinar and periductal fibrosis, ductal dilatation. Decreased peroxidase secretion, lacrimal gland nerves damaged, and neurotransmitter release impaired | Dysfunction of the lacrimal gland. Reduction of the volume and modification of tear film composition (ocular surface damage can subsequently occur) | (De Souza et al., 2019; Kojima et al., 2012; Ríos et al., 2005) |
| | Meibomian glands | Decreased density and diameter of acinar units. Reduced meibocyte differentiation and cell cycling. Acinar atrophy. Decreased lipid synthesis | Dysfunction of the meibomian glands. Alteration of tear film composition, tear film evaporation (ocular surface damage can subsequently occur) | (Nien et al., 2011; Wei et al., 2011) |
| | Ocular surface immune system | Increased number and response of Th17 cells. Increased number of dendritic cells able to prime Th1 cells. Increased number of Th1 cells. Decreased amount of Treg cells | Dysregulation of the ocular immune system. Amplification of the ocular surface inflammation. Promotion of corneal epithelial barrier disruption | (Bian et al., 2019; Farid et al., 2016; Foulsham et al., 2020) |
| | Ocular surface cells | Decreased number of corneal stromal and endothelial cells. Enhanced expression of corneal epithelial TGF-β gene and senescence-associated genes. Decreased corneal stromal nerve density. Reduced number and function of Goblet cells. Expression of conjunctival apoptosis-related genes and inflammation-associated genes | Corneal thinning and reduction of the corneal sensitivity. Ocular surface inflammation and cell death | (Di Zazzo et al., 2019; Giebel et al., 2005; Li et al., 2016; Taurone et al., 2020; Wei et al., 2011; Zhu et al., 2010) |
| | Tear fluid | Altered protein profile (e.g., increased expression of the inflammatory transcription factor NF-κB) | Amplification of the inflammatory damage | (Nättinen et al., 2019) |
| Glaucoma | Trabecular meshwork | Decreased number of trabecular meshwork cells and trabecular meshwork stem cells. Reduced trabecular meshwork height. Declined superoxide dismutase activity in the trabecular meshwork. Increased number of oxidized proteins and decreased proteosome activity. Increased expression of cellular senescence markers | Reduction of aqueous humor outflow. Elevation of the intraocular pressure. Reactive oxygen species accumulation and subsequent oxidative damage amplification. Amplification of the inflammatory damage | (Caballero et al., 2004; Choi et al., 2020; De La Paz and Epstein, 1996; Grierson and Howes, 1987; Sundaresan et al., 2019) |
| | Schlemm's canal | Reduced number and size of giant vacuoles. Decreased number of intracellular pores | Reduction of aqueous humor outflow. Elevation of the intraocular pressure | (Boldea et al., 2001) |
| | Ciliary muscle | Decreased mobility of the ciliary muscle | Reduction of aqueous humor drainage through trabecular meshwork and generation of optic nerve tension | (Croft et al., 2017) |
| | Extracellular matrices | Increased stiffening in the trabecular meshwork, lamina cribrosa, sclera, cornea, retina, and Bruch membrane/choroid by an aberrant accumulation of extracellular matrix proteins | Elevation of the intraocular pressure | (Liu et al., 2018) |
| | Aqueous humor fluid | Altered composition of the aqueous humor (increased expression of inflammatory-related genes) and dynamics (aqueous humor drainage reduced) | Amplification of the inflammatory damage. Intraocular pressure elevation | (Gabelt and Kaufman, 2005; Klein et al., 1992; Toris et al., 1999; Zheng et al., 2018) |
| | Inner retina and optic nerve head | Increased vulnerability and lowered recovery from injured retinal ganglion cells. Elevated axonal mitochondrial dysfunction. Altered brain-derived neurotrophic factor downstream pro-survival cell signaling, elevated amount of amyloid-β peptide | Amplification of the oxidative damage. Cell death/inner retina thinning. Structural and function deterioration of the inner retina | (Carelli et al., 2004; Gupta et al., 2014; Navarro and Boveris, 2008; Wang et al., 2007) |
| Glaucoma, DR, AMD | Retina | Induced retinal cells senescence (e.g., retinal ganglion cells, photoreceptor cells, RPE cells, endothelial cells, glial cells, etc.). Dysfunction of Müller cells by lipid peroxidation. Altered expression of calcitonin receptor gene, aquaporins genes and growth factors genes/proteins (e.g., VEGF and PEDF) | Amplification of the inflammatory, oxidative, and vascular damage | (Chen et al., 2010; Curtis et al., 2011; Lee et al., 2021; Nag et al., 2011; Ortak et al., 2013; Smith and Steinle, 2007) |
| DR | Inner retina | Partial or complete degenerated capillary endothelial and pericytes cells, and arteries/arterioles vascular smooth muscle cells (increased lipofuscin deposits). Changes possibly mediated by lipid peroxidation | Cell death. Cellular senescence. Amplification of the vascular damage | (Nag et al., 2019) |
| AMD | Outer retina and choroid | Increased deposits of amyloid-β peptide and lipofuscin. Decreased melanin granules and increased lipofuscin deposits in RPE cells. Altered expression at protein and transcript level in RPE cells (e.g., decreased expression of antioxidant enzymes, and increased gene expression of P53 and its targets genes that mediated apoptosis, senescence, and innate immune response). Increased thickening and stiffening of the Bruch membrane. Decreased thickening of the choroid and upregulated proinflammatory choroid signaling. Periphery hard drusen formation. Elevated vulnerability of photoreceptor cells. Altered microglial genes expression, compromising the supportive function of these cells, and altering their activation | Cell death. Amplification of the oxidative, inflammatory, and vascular damage | (Chaum et al., 2015; Gu et al., 2012; Gupta et al., 2017; Liu et al., 2018; Ma et al., 2013; Nag, 2021; Ratnayaka et al., 2015; Robbie et al., 2016) |

This damage triggers changes in the different structures, and thus, it alters eye physiological function. For example, the formation of protein aggregates (induced by age-related oxidative stress) in the lacrimal glands causes premature senescence (increases the synthesis and secretion of inflammatory mediators) that impair their function, reducing tear secretion (Alves et al., 2005; Ríos et al., 2005; Yoon et al., 2020). This reduction evokes damage to the corneal epithelial cells, which can result in an irregular corneal surface, affecting visual quality (De Paiva, 2017). Likewise, the formation of those aggregates in the extracellular matrix of trabecular meshwork cells, retinal capillary endothelial cells, or retinal pigmented epithelial cells, increases intraocular pressure and breaks blood-retinal barriers that can also lead to a vision loss (Anand-Babu et al., 2019; Murali et al., 2020; Park and Kim, 2012; Sharif et al., 2019; Tektas and Lütjen-Drecoll, 2009; Xu et al., 2018).

Moreover, the continued exposure to solar UV radiation, and the postmitotic character of ocular cells, make the eye extremely vulnerable to age-related oxidative damage (Crooke et al., 2017). Ocular cells also become senescent during the aging process secreting pro-inflammatory mediators that damage the surrounding cells and attract immune cells (Park and Kim, 2012; Prata et al., 2018; Rocha et al., 2020; Shosha et al., 2018; Sun et al., 2018). Similarly, immune cells undergo age-related functional changes that lead to a high inflammatory environment (Fulop et al., 2017; Mashaghi et al., 2017). So, ocular non-immune senescent cells and aged immune cells generate and prolong the inflammatory cellular/tissular damage. Some authors have suggested that age-related disease onsets are due to accelerated/intensive aging (Franceschi et al., 2018). Indeed, cells and tissues involved in age-related eye diseases (AREDs) suffer more accelerated/intensive changes during illness than aging (Feher et al., 2006; Foulsham et al., 2020; Gabelt and Kaufman, 2005; Kojima et al., 2012; Lamoke et al., 2015; Lin et al., 2011; Tezel et al., 2007; Fasanella et al., 2016). The knowledge of these changes can provide valuable information to slow aging and thus prevent AREDs. Table 1 summarizes the main age-related changes in cells, tissues, systems, and fluids involved in AREDs.

### 1.2. Neutrophils and neutrophil extracellular traps

Neutrophils are one of the immunological cells that undergo functional changes during aging (Alonso-Fernández et al., 2008; Drew et al., 2018). Indeed, some authors have suggested that the host environment (e.g., age-associated inflammatory milieu) could induce expression changes in neutrophils, which might affect their cellular functions (Drew et al., 2018). In this sense, *in vitro* studies have demonstrated that neutrophils from aged individuals present lower chemotactic and phagocytic activity and produce a higher amount of intracellular reactive oxygen species than cells from young individuals (Alonso-Fernández et al., 2008; Wenisch et al., 2000). Moreover, *in vivo* neutrophil trafficking of older animals is also altered (Barkaway et al., 2021). Neutrophils from these animals reverse transendothelial migration (which allows their biological clearance) and return to circulation, causing distant tissue damage (Barkaway et al., 2021). This abnormal trafficking plays a pivotal role in both innate and adaptive immune responses. Thus. neutrophils mediate innate immunity −the first line of defense against pathogens− but are also involved in adaptive immunity as modulators of T cells, and B cells functions (Li et al., 2019; Mayadas et al., 2014). The interface between these immune functions is the termed NETosis process (Brinkmann et al., 2004; Delgado-Rizo et al., 2017; Mayadas et al., 2014). In this process, neutrophils extrude a meshwork of nuclear/mitochondrial DNA (extracellular DNA or eDNA) and cytosolic granule peptides/proteins (Brinkmann et al., 2004; Yousefi et al., 2009) (Fig. 1). Given that this meshwork serves to immobilize and kill pathogens, it is known as Neutrophil Extracellular Trap (NET). Nevertheless, the presence of these neutrophils constituents in the extracellular medium is also critical to activate adaptive immune cells and thus the inflammatory response (Delgado-Rizo et al., 2017; Li et al., 2019; Mayadas et al., 2014). For example, the neutrophil peptide LL-37 (cathelicidin) forms a complex with eDNA that promotes the synthesis of interferon-alpha (IFN-α), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNFα) from dendritic cells (Delgado-Rizo et al., 2017; Li et al., 2019; Minns et al., 2019). These cytokines activate dendritic cells and so indirectly stimulate the proliferation of T cells (Delgado-Rizo et al., 2017; Li et al., 2019). Other neutrophil proteins − myeloperoxidase (MPO), neutrophil elastase (NE), lipocalin 2 (LCN2), citrullinated histones, and peptidyl-arginine deiminases (PADs) − also have an immunomodulatory role (Cassatella et al., 2019; Floderer et al., 2014; Minns et al., 2019; Mowen and David, 2014; Zhou et al., 2017). Interestingly, PADs are crucial for NETs formation and are pathologic mediators of chronic inflammation (Mowen and David, 2014; Zhou et al., 2017). These enzymes catalyze the citrullination of proteins that results essential for the NET formation (via histones citrullination) and leads to the formation of autoantibodies (anti-citrullinated protein antibodies, ACPAs) (Mowen and David, 2014). Additionally, citrullinated histones induce cell death, supporting inflammatory damage (Fig. 1) (Saffarzadeh et al., 2012; Silvestre-Roig et al., 2019). The extracellular concentration of these proteins and the presence/absence of other local inflammatory mediators determine the pro-inflammatory or anti-inflammatory outcome (Cassatella et al., 2019; Floderer et al., 2014; Minns et al., 2019). Furthermore, some inflammatory mediators (e.g., IL-6, IL-1β, IL-8, TNF-α, and ACPAs) stimulate the NETosis process, and thus, they increase the extracellular concentration of NET proteins (Delgado-Rizo et al., 2017; Sur Chowdhury et al., 2014). Consequently, in a high inflammatory situation like aging or age-related diseases, neutrophils and their nuclear/cytosolic components could exacerbate the inflammatory damage.

Few studies (and mostly animal studies) have evaluated the impact of age on the NETosis process (Hazeldine et al., 2014; Lu et al., 2021; Ortmann and Kolaczkowska, 2018). In this context, Lu et al. have recently demonstrated that unprimed neutrophils from aged mice present a higher NETosis inducibility than young ones, particularly in female animals (Lu et al., 2021). Furthermore, these authors have observed that a priming process for NET formation, autophagy, is upregulated in aged neutrophils (Lu et al., 2021). Impairment of autophagy is also associated with inflammatory and age-related diseases, including ocular ones (Cheon et al., 2019; Crooke et al., 2017). Consequently, NETosis alteration could also play a role in age-related eye diseases (AREDs) onsets.

## 2. Neutrophils, neutrophil extracellular trap components and age-related eye diseases

AREDs including dry eye, glaucoma, age-related macular degeneration, and diabetic retinopathy, share underlying oxidative and inflammatory molecular processes common to aging and other age-related diseases (Crooke et al., 2017; Dogru et al., 2018; Ganesalingam et al., 2019; Johnson et al., 2015). One of these shared mechanisms seems to be the impairment of NETs formation/degradation processes. AREDs patients present local recruitment of neutrophil cells and overexpression of neutrophil extracellular trap components at both, local and systemic levels (An et al., 2019; Arafat et al., 2017; Aragona et al., 2006; Chau et al., 2007; Chen et al., 2020; Chung et al., 2016; Ghosh et al., 2019, 2017; Guo et al., 2011; Kaeslin et al., 2016; Park et al., 2016; Reyes et al., 2018; Sonawane et al., 2012; Tibrewal et al., 2013; Ueno et al., 2018; Wang et al., 2018). In this context, the neutrophil-to-lymphocyte ratio (NLR), a blood indicator of systemic inflammation and poor diseases prognosis (Azab et al., 2012; Bian et al., 2010; Jin et al., 2013), has also been studied and validated as AREDs biomarker (Ilhan et al., 2015; Kurtul and Ozer, 2016; Kurtul et al., 2016; Niazi et al., 2019; Ozgonul et al., 2016; Sekeryapan et al., 2016; Ulu et al., 2013; Yue et al., 2015).

These data suggest that neutrophils, NETs, and their constituents could be potential diagnostic biomarkers and therapeutic targets of AREDs.

### 2.1. Dry eye disease

The outer ocular surface comprises a continuous layer of epithelium (corneal and conjunctival epithelium) that provides physical/immunologic protection to the eye and contributes to the proper visual performance (Bron et al., 2017). The tear film covers this epithelium and thus, protects, nourishes, and lubricates it. This film is composed of an inner mucous/aqueous phase produced by lacrimal glands and conjunctival cells (epithelial and Goblet cells), and an external lipid phase generated by Meibomian glands (Bron et al., 2017). Due to different underlying causes, the disruption of this tear film can cause a loss of homeostasis and might result in ocular surface diseases.

According to the Dry Eye Workshop (DEWS II) report published by the Tear Film and Ocular Surface Society (TFOS) in 2017, Dry Eye Disease (DED) is defined as a "multifactorial disease of the ocular surface characterized by a loss of homeostasis of the tear film, and accompanied by ocular symptoms, in which tear film instability and hyperosmolarity, ocular surface inflammation and damage, and neurosensory abnormalities play etiological roles." (Craig et al., 2017).

The prevalence of DED established in 2017 varies from approximately 5–50% worldwide, with age considered as one of the most relevant risk factors (Stapleton et al., 2017). Indeed, aging predisposes to DED, and its critical underlying inflammatory and oxidative damage mechanisms are common to aging and age-related diseases (Craig et al., 2017; De la Fuente and Miquel, 2009; Dogru et al., 2018; Franceschi et al., 2007; Seen and Tong, 2018; Sharma and Hindman, 2014). For these reasons, some authors (including us) consider that DED is an age-related disorder (De Paiva, 2017; Farid et al., 2016; Gipson, 2013; Malet et al., 2014; Rusciano et al., 2018; Seen and Tong, 2018). This fact explains the emerging interest in these underlying signaling pathways shared by aging and age-related diseases to find more effective DED biomarkers and therapeutic agents.

Among the different causes for ocular surface inflammation, tear hyperosmolarity is considered a crucial stressor (Bron et al., 2017). Hyperosmolarity results from either aqueous tear deficiency (condition called aqueous-deficient dry eye) or increased evaporation of the aqueous tear phase (disease called evaporative dry eye), or a combination of both (Bron et al., 2009). As a result of hyperosmolarity, a cascade of inflammatory events take place in the ocular surface, which leads to epithelial cell death and alters the stability of the tear film, perpetuating the disease (Bron et al., 2017; Tibrewal et al., 2014). Immunological cells like antigen-presenting cells (APCs) and T lymphocytes sustain this inflammatory damage (Tibrewal et al., 2013). Neutrophils, which can act as APCs and modulate T cell functions (Li et al., 2019), are massively recruited to the ocular surface under non-basal conditions as stress or tissue injury (Gronert, 2010). Besides, *in vitro* hyperosmolar condition promotes the activation of the NETosis process (Tibrewal et al., 2014). Given these facts, Tibrewal et al. have suggested that DED-associated hyperosmolar stress could stimulate the NETosis process from neutrophils recruited in the ocular surface of DED patients (Tibrewal et al., 2014). In this context, various authors demonstrated the high presence of neutrophils (both at the systemic and local level) and NETs components in the tears of DED patients (Fig. 2) (An et al., 2019; Sonawane et al., 2012; Tibrewal et al., 2013).

According to Sekeryapan et al., the NLR values were significantly higher in patients with a non-autoimmune DED than in controls (Fig. 2) (Sekeryapan et al., 2016). Recently, Ozarslan Ozcan et al. have proved that the systemic immune-inflammation index (SII) -which considers neutrophil, platelet, and lymphocyte counts- is also high in patients of this DED type (Ozarslan Ozcan et al., 2020). Besides, these authors claim the superior ability of SII to measure the inflammatory status of DED patients than other inflammatory markers like NLR (Ozarslan Ozcan et al., 2020). Further studies are needed to confirm these results and to evaluate the correlation between NLR/SII and the severity of the disease.

About the local increment of neutrophils in DED patients, Reyes and colleagues have demonstrated that neutrophils promote meibomian gland obstruction in an animal model of chronic inflammation (Reyes et al., 2018). Moreover, these authors found that the tear of patients with meibomian gland dysfunction (MGD) presented high neutrophil levels correlated with MGD severity (Fig. 2) (Reyes et al., 2018). MGD, the most common cause of evaporative dry eye, is a chronic abnormality of the meibomian glands that results in a loss of tear lipids, which boosts tear evaporation leading to tearing film instability and hyperosmolarity (Bron and Tiffany, 2004; Nelson et al., 2011).

Patients with a type of aqueous-deficient dry eye named ocular graft-vs-host disease (oGVHD) also present in their tears high levels of neutrophils correlated with a more severe form of this disease (Fig. 2) (An et al., 2019). This ocular manifestation of the GVHD occurs in 40–60% of patients after allogeneic hematopoietic stem cell transplantation (Shikari et al., 2013). The tear of oGVHD patients also presents high levels of eDNA and NET-specific proteins like NE, MPO, and extracellular matrix metalloproteinases (MMP-9 and MMP-8) (Fig. 2) (An et al., 2019; Arafat et al., 2017; Sonawane et al., 2012). Likewise, the tears and ocular surface of patients with other types of aqueous-deficient DED like Sjögrenś syndrome dry eye, present high levels of NETs and their components (e.g., eDNA and LCN2) (Aragona et al., 2006; Kwon et al., 2020; Tibrewal et al., 2013). More recently, Kwon et al., have demonstrated that the ocular surface wash of both, aqueous-deficient (e.g., oGVHD and Sjögrenś syndrome) and evaporative dry eye (e.g., MGD) patients, contains an abnormal amount of citrullinated proteins, PAD4 enzyme, and ACPAs (Kwon et al., 2020). These ACPAs form complexes with citrullinated proteins that could interact with immune cells (e.g., dendritic cells or neutrophils) and the complement system, leading to the secretion of pro-inflammatory mediators (Kwon et al., 2020). Additionally, these authors have demonstrated that ocular surface ACPAs also stimulate the NETosis process *in vitro*, and in this form, they might prolong the inflammatory cellular/tissular damage (Kwon et al., 2020). In this context, Kwon and coworkers have proved that ACPAs can cause ocular surface disease in mice (Kwon et al., 2020). The ocular surface wash from the closed eye of DED patients also presents an increased number of neutrophils, which are in an enhanced state of degranulation (Postnikoff et al., 2020). Some authors have suggested that this degranulation could promote DED-related inflammatory damage (Postnikoff et al., 2020). Given that the NETosis process seems to play a critical role in DED onset and NETs are also present in the awakened eye (Mahajan et al., 2019), the connection between both processes (degranulation and NETosis) and the DED-intrinsic inflammatory damage could be a fascinating topic for future work.

As we commented above, components like eDNA can trigger the synthesis and release of pro-inflammatory cytokines that amplify the inflammatory damage. In this sense, Sonawane et al. have demonstrated that the conjunctival cells of severe DED patients (e.g., oGVHD ones) overexpress mRNAs encoding for cytosolic DNA sensors and pro-inflammatory cytokines (Sonawane et al., 2012). These results suggest that both neutrophils and the NETosis process play a critical role in severe DED onset. Therefore, the tear eDNA and the NETosis process have been evaluated as potential therapeutic targets of DED (Tibrewal et al., 2014, 2013).

Regarding the eDNA, Sandeep Jainś group tested the efficacy of DNase I eyedrops in two patients with severe DED (Tibrewal et al., 2013). This treatment reduced the tear eDNA abundance and improved the patients´ eye comfort. eDNA degradation decreases the inflammation and viscosity of the tears, thus generating comfort. This group have also performed a phase I/II clinical trial with 47 DED patients of which 25 were treated with DNase eyedrops (ClinicalTrials.gov Identifier: NCT02193490). Patients treated with DNase presented a significant lower corneal damage and presence of surface mucoid debris/strands – a mix rich in NETs and their components – than vehicle-treated patients (Fig. 2) (Mun et al., 2019). Moreover, DNase treatment was safe and well-tolerated (Mun et al., 2019). Sandeep Jainś group is now testing the efficacy of pooled ocular surface immunoglobulin (IgG)-eye drops (OSIG-eye drops) in patients with DED (e.g., Sjögrenś syndrome dry eye,

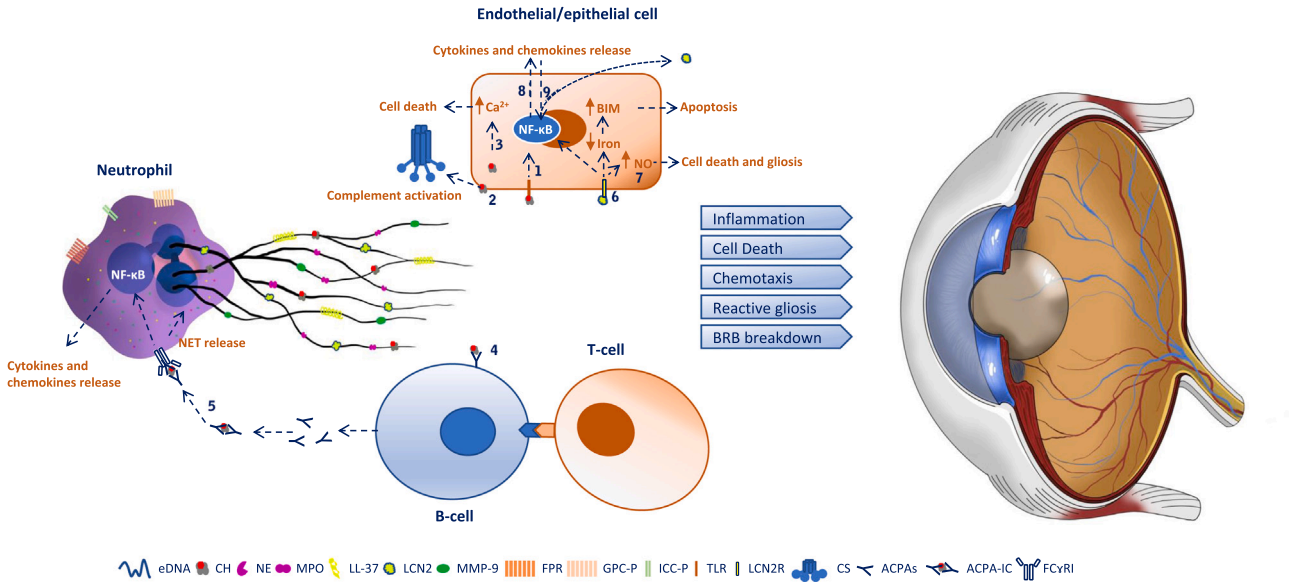


**Fig. 1.** Schematic representation of the Neutrophil Extracellular Trap (NET) and **possible harmful effects of NET constituents on ocular cells involved in AREDs**. The figure shows a neutrophil with some surface receptors [formyl peptide receptors (FPR), Fcγ receptors I (FcγRI), G protein-coupled purinergic receptors (GPC-P, e.g., P2Y2, P2Y6, A3, and A2A), and ion channel-coupled purinergic receptors (ICC-P, e.g., P2X7)] and NET components [extracellular DNA (eDNA) and components released from neutrophil primary granules (MPO, myeloperoxidase; NE, neutrophil elastase), secondary granules (LL-37, cathelicidin; LCN2, lipocalin 2), and tertiary granules (MMP-9, matrix metalloproteinase-9)]. NET components can cause inflammation, cell death, chemotaxis, reactive gliosis, and BRB breakdown on eye tissues of AREDs patients. The picture illustrates possible cell damage mechanisms evoked by NETs components in the eye: extracellular citrullinated histones (CH) activate (1) toll-like receptors (TLR), driving the NF-κB-mediated transcription of pro-inflammatory cytokines, and (2) the complement system (CS). CH can also penetrate the plasma membrane, resulting in an influx of $Ca^{2+}$ that leads to cell death (3). B-cells also recognize CH and, after T-cells stimulation, make anti-citrullinated protein antibodies (ACPAs). ACPAs can form immune complexes with citrullinated proteins (ACPA-IC) (5) that activate FcγRI of neutrophils and dendritic cells. This activation finally leads to the release of pro-inflammatory cytokines and NETs. Extracellular LCN2 binds to LCN2 receptors (LCN2R) that mediates its uptake via receptor-mediated endocytosis (6). Intracellular LCN2R can expel iron outside the cell, which induces the expression of the pro-apoptotic protein Bim. Intracellular LCN2 induces NO formation, promoting cell death and gliosis (7), and triggers the synthesis and release of cytokines and chemokines (8). Conversely, pro-inflammatory mediators evoke the synthesis and release of more LCN2 from local cells (9). For a more detailed review of CH and LCN2 harmful cell effects, see (Silk et al., 2017) and (Jha et al., 2015), respectively.

oGVHD, and MGD patients) (ClinicalTrials.gov Identifier: NCT04510428) (Kwon et al., 2020). These immunoglobulins are present in an unusual amount in the ocular surface wash of these patients, and Kwon et al. have shown their ability to reverse *in vitro* the ACPAs-induced NETosis (Kwon et al., 2020). A preliminary study with 27 DED patients has confirmed the efficacy and safety of this treatment (Kwon et al., 2020). OSIG-eye drops diminish the level of neutrophilic and NET biomarkers, and clinically, they reduce the signs and symptoms of DED (Kwon et al., 2020). These encouraging study provides the scientific justification for advancing to phase III clinical trials.

Tibrewal et al. also investigated the usefulness of direct/indirect NETosis inhibitors under a hyperosmotic condition like the one found in DED (Tibrewal et al., 2014). Among them, annexin-1 peptide and lipoxin A4 were the most effective inhibitors causing a significant decrease of the NETosis process in *ex vivo* neutrophils exposed to hyperosmolar stress (Fig. 2) (Tibrewal et al., 2014). These anti-inflammatory/pro-resolution mediators are agonists of the neutrophil surface formyl peptide receptor 2 (FPR2) (Dorward et al., 2015). The interaction of these agonists with FPR2 limits the neutrophil recruitment to the inflammation site and induces their apoptosis in this site (Dorward et al., 2015; Sugimoto et al., 2016). Consequently, annexin 1 peptide and lipoxin A4 could also have a therapeutic value in DED patients, acting as indirect anti-inflammatory agents that limit the NETosis process and promote the release of anti-inflammatory molecules on the ocular surface.

Annexin 1 peptide can also regulate neutrophil migration acting on its formyl peptide receptor 1 (FPR1) (Dorward et al., 2015). This peptide activates FRP1 that evokes autocrine release of adenosine triphosphate (ATP). Subsequently, neutrophil purinergic receptors like $P2Y_2$ and adenosine A3 receptors are activated by ATP and adenosine (nucleoside derived from the breakdown of extracellular ATP), modulating their migratory activity (Dorward et al., 2015). Interestingly, clinical studies supported the use of purinergic receptor agonists for DED therapy (Avni et al., 2010; Ohashi et al., 2020), although their role in the ocular surface recruitment of neutrophils is unknown (Fig. 2).

Given the intricate molecular interactions underlying DED pathogenesis (Bron et al., 2017; Ganesalingam et al., 2019), the combination therapy approach appears more suitable than the single-agent treatment to achieve long-term benefit in DED patients. This approach is a common practice in ophthalmology to treat diseases with complex pathogenesis (Holló et al., 2014; Peyman and Hosseini, 2011). In this context, Tibrewal and colleagues have proposed using a drug cocktail constituted by pro-resolutive, osmoprotective, and DNase 1 agents for DED treatment (Tibrewal et al., 2014).

Further studies are needed to validate the therapeutic potential of all these strategies for treating dry eye disease.

### 2.2. Glaucoma

Glaucoma is an optic neuropathy characterized by the progressive neurodegeneration and death of retinal ganglion cells and the subsequent damage to the optic nerve (Quigley, 2011). This damage causes a gradual loss of vision starting in the peripheral parts of the visual field, that leads to partial or total blindness if untreated. This disease is the second leading cause of visual impairment and blindness worldwide (GBD, 2019 Blindness and Vision Impairment Collaborators and VLEG, 2021). Despite the neurodegeneration that occurs in the retina, glaucoma is caused by impairment of aqueous humor outflow through the drainage structures found in the iridocorneal angle, the trabecular meshwork (TM) and the canal of Schlemm. Anatomically normal

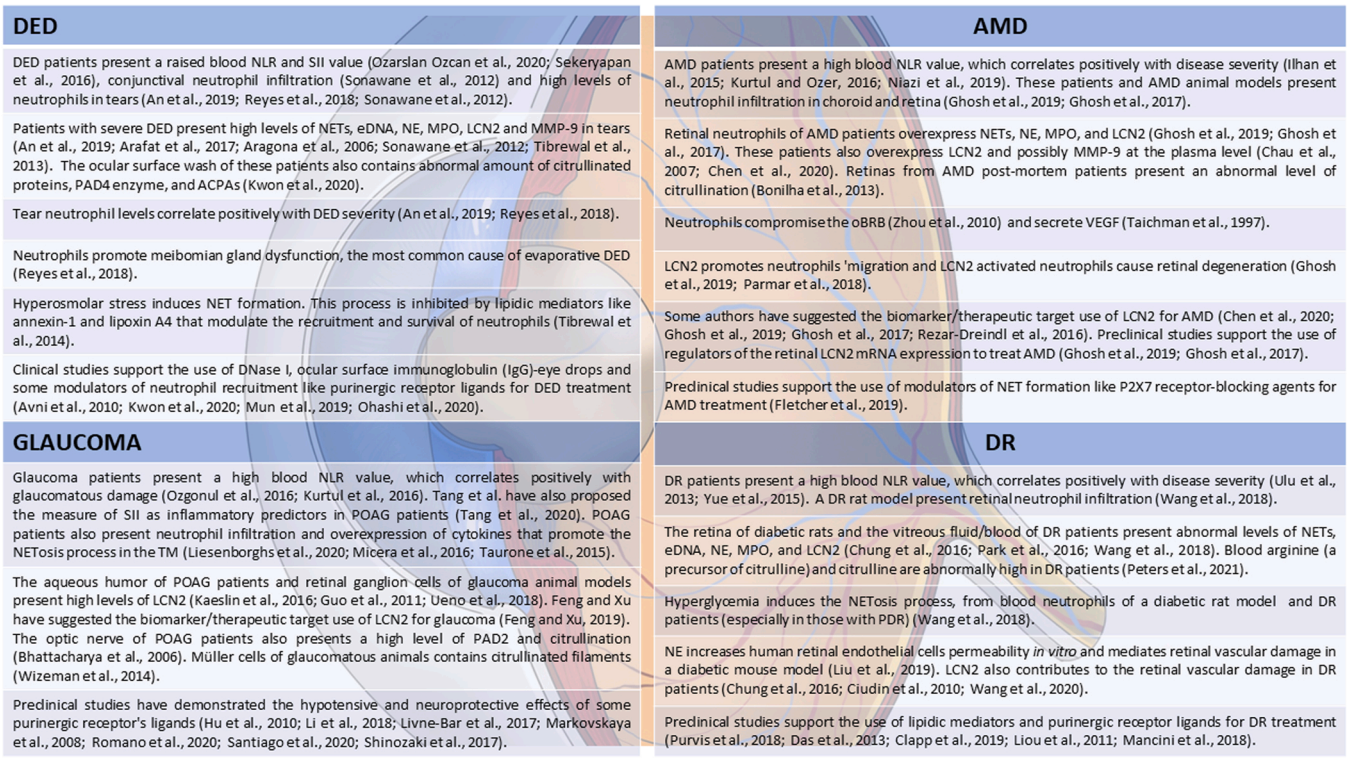


**Fig. 2.** Overview of the link between neutrophils/NETs components and age-related eye diseases. AMD, Age-related macular degeneration; eDNA, extracellular DNA; DED, dry eye disease; DR, Diabetic retinopathy; LCN2, lipocalin 2; MMP-9, matrix metalloproteinase-9; MPO, myeloperoxidase; NE, neutrophil elastase; NETs, neutrophil extracellular traps; NLR, neutrophil-to-lymphocyte ratio; oBRB, blood-retinal barrier; PAD4, peptidyl-arginine deiminase 4; POAG, primary open-angle glaucoma; SII, systemic immune-inflammation index; TM, trabecular meshwork; VEGF, vascular endothelial growth factor.

structures are found in the most frequent cause of glaucoma, primary open-angle glaucoma (POAG), that has a global prevalence of 3.1% (Tham et al., 2014). In this form of glaucoma, functional alterations in the outflow tissues are thought to reduce the outflow of aqueous humor towards the aqueous veins, thus reducing outflow and increasing intraocular pressure, the main risk factor of developing the retinal degeneration associated to this disease. A second common form of glaucoma is primary angle-closure glaucoma (PACG), with a prevalence of 0.5% in the general population (Tham et al., 2014). In this case, a partial or total impairment of aqueous humor outflow is found, together with the associated rise in intraocular pressure. This is due to an anatomically narrow iridocorneal angle or the partial or total blockade of this structure due to different problems, such as a forward displacement of the iris towards the cornea, narrowing the anterior chamber of the eye.

Given that the glaucoma prevalence increases exponentially in people older than 40 years, age is considered a key risk factor for POAG and PACG onset (Caprioli, 2013; Cheng et al., 2014; Quigley, 2011; Tham et al., 2014). In this context, various authors have demonstrated the age-related loss of trabecular meshwork cells and retinal ganglion cells that are particularly important in glaucoma (Balazsi et al., 1984; Grierson and Howes, 1987; Harman et al., 2000). Likewise, some studies have reported that these cells become susceptible to oxidative damage and senescent with age (De La Paz and Epstein, 1996; Park and Kim, 2012; Wang et al., 2007). These age-related changes also occur in glaucoma patients who seem to experiment an accelerated aging (Alvarado et al., 1984; Caprioli, 2013; Rocha et al., 2020; Tezel et al., 2007). As in all age-related diseases, oxidative stress (mainly at mitochondrial level) plays an essential role in glaucoma onset and progression (reviewed in (Crooke et al., 2017)). In addition to age, other factors such as high intraocular pressure (IOP) (the only widely accepted modifiable glaucoma risk factor) (Blumberg et al., 2015) contribute to oxidative stress-induced mitochondrial damage (Kong et al., 2009). As mentioned earlier, this elevated IOP is due to an imbalance between the aqueous humor production across the ciliary body and its drainage through the TM that decreases with age and disease (De Groef et al., 2016; Gabelt and Kaufman, 2005). Additionally, high IOP results in retinal glial cell activation (Inman and Horner, 2007; Ju et al., 2006; Woldemussie et al., 2004). Therefore, activated glial cells that release immunomodulatory mediators (e.g., TNF- α and NO) and trabecular/retinal ganglion senescent cells that secrete pro-inflammatory mediators, contribute to the chronic inflammatory process characteristic of glaucoma (Crooke et al., 2017; Neufeld, 1999; Park and Kim, 2012; Prata et al., 2018; Rocha et al., 2020; Tezel, 2011; Tezel and Wax, 2000). In this sense, some authors have demonstrated that the TM of POAG patients recruits neutrophils and overexpresses pro-inflammatory cytokines (such as TNF-α, IL-1β, and IL-6) that promote the NETosis process (Fig. 2) (Liesenborghs et al., 2020; Micera et al., 2016; Taurone et al., 2015). Similarly, the aqueous humor of POAG patients presents a high level of the NET component LCN2 (Fig. 2) (Kaeslin et al., 2016). Kaeslin et al. have claimed the role of innate immunity and particularly neutrophils in glaucoma onset as the source of this LCN2 (Kaeslin et al., 2016). However, other cells can also release this protein, contributing to the total pool of POAG patients' aqueous humor LCN2. Interestingly, Liu et al. have found that the TM of POAG patients down-express LCN2 gene (Liu et al., 2013). Therefore, more research is needed to find other cellular sources of this aqueous humor LCN2. POAG patients could also overexpress LCN2 in their retinal ganglion cells as occurs in glaucomatous rats and mice models (Guo et al., 2011; Ueno et al., 2018). Neutrophil cells release LCN2, which in turn regulates their migration to the inflammation site (Jha et al., 2015). Retinal cells such as microglial, astrocyte, and ganglion cells can also release LCN2, which can act over them, regulating the neuroinflammation process (Jha et al., 2015). Indeed, Feng and Xu have suggested the involvement of LCN2 in glaucoma pathogenesis and its potential role as a biomarker/therapeutic target against this disease (Fig. 2) (Feng and Xu, 2019). The optic nerve

of POAG patients also presents a high level of PAD2 and citrullination (Bhattacharya et al., 2006). Interestingly, Bhattacharya et al. have suggested that elevated IOP could induce PAD2 expression (Bhattacharya et al., 2006). Given that arterial hypertension evokes *ex vivo* NETosis (Hofbauer et al., 2017), high IOP might also promote NETosis releasing more PAD2. Müller cells (a type of retinal glial cells) of a mouse model of glaucoma also express citrullinated filaments, which could mediate pathological gliosis (Wizeman et al., 2014).Therefore, citrullination could play a role in POAG, leading to retinal and optic nerve damage (Bhattacharya et al., 2006; Wizeman et al., 2014).

In this context, Ozgonul et al. have proposed the NLR parameter as a diagnostic biomarker of POAG (Ozgonul et al., 2016). The authors found an abnormally high value of the NLR parameter in POAG patients, which correlated well with a high visual field defect (glaucomatous damage) (Fig. 2) (Ozgonul et al., 2016). Recently, Tang et al. have confirmed the validity of this parameter and proposed the measure of SII as inflammatory predictors in POAG patients (Tang et al., 2020). Similarly, patients with pseudoexfoliation glaucoma present a remarkably high NLR value (Kurtul et al., 2016). However, other authors found no difference in NLR between subjects with normal-tension glaucoma and the control group (Atalay et al., 2019).

Altogether, these results suggest that neutrophils and probably the NET components play a role in glaucoma onset and could be biomarkers and therapeutic targets for this disease. In this sense and like in dry eye disease, some preclinical studies have suggested the anti-inflammatory and neuroprotective role of annexin-1 and lipoxin A4 (indirect NETosis inhibitors) in experimental glaucoma (Fig. 2) (Li et al., 2018; Livne-Bar et al., 2017).

Similarly, preclinical works have proved the hypotensive and neuroprotective effects of different purinergic receptor ligands (Fig. 2) (Fonseca et al., 2017; Markovskaya et al., 2008; Romano et al., 2020; Santiago et al., 2020; Shinozaki et al., 2017; Soto et al., 2005). So, the activation of purinergic receptors like $P2Y_1$ and $P2Y_6$ reduces IOP (Fonseca et al., 2017; Markovskaya et al., 2008; Soto et al., 2005). These effects are possible because cells involved in glaucoma pathogenesis (e.g., ciliary cells, trabecular meshwork cells, retinal ganglion cells, and glial cells) express these receptors on their surface (Crooke et al., 2008; Fonseca et al., 2017; Markovskaya et al., 2008; Soto et al., 2005). Indeed, the dysregulation of these receptors in animals results in glaucomatous optic neuropathy (Hamada et al., 2021; Shinozaki et al., 2017). In this sense, various authors have demonstrated the role of glial $P2Y_1$ and $P2Y_6$ receptors in neuroinflammation (Kita et al., 2019; Yang et al., 2017). However, this neuroprotective effect could be also due to neutrophil $P2Y_1$/$P2Y_6$ receptors that are involved in neutrophil activation and recruitment, and NET formation (Grbic et al., 2012; Sil et al., 2017; Wang and Chen, 2018). Likewise, the $P2X_7$ receptor, which is also present on the surface of retinal ganglion cells, glial cells, and immune cells, seems to be involved in glaucoma-related neuroinflammation (Fletcher et al., 2019). The ATP-activated receptor mediates retinal ganglion cell death (Hu et al., 2010; Romano et al., 2020). Moreover, the activation of $P2X_7$ receptors can lead to pannexin-1 channel-dependent ATP release in all cells mentioned contributing to retinal ganglion cell death (Fletcher et al., 2019). Thus, neutrophils that express both $P2X_7$ receptor and pannexin-1 channel could mediate ganglion cell death but also the secretion of pro-inflammatory mediators and NETosis process in the glaucomatous retina (Pelegrin and Surprenant, 2009; Sofoluwe et al., 2019). Further studies are needed to confirm the therapeutic potential of all these molecules for treating glaucoma.

### 2.3. Age-related macular degeneration

Age-related macular degeneration (AMD) is a progressive degenerative disease of the outer retina and the third leading cause of vision loss worldwide (GBD, 2019 Blindness and Vision Impairment Collaborators and VLEG, 2021). Although age is the principal risk factor for AMD, other factors such as genetic predisposition, environmental and behavioral factors may also increase the risk of its onset (Mitchell et al., 2018). Aged-related retinal changes, mainly over retinal pigment epithelial and retinal glial cells, generate a favorable environment to AMD onset (Crooke et al., 2017; Telegina et al., 2018). Besides, many of these changes are exacerbated in AMD, promoting oxidative damage and the subsequent immune-inflammatory, drusen formation, and neovascularization events characteristic of this disease (Ardeljan and Chan, 2013; Nowak, 2013; Telegina et al., 2018). Drusen are extracellular deposits (rich in photooxidative products) associated with the early/intermediate AMD and localized beneath retinal pigment epithelium (RPE), impairing its metabolic connection with the choroid (Crooke et al., 2017; Mitchell et al., 2018). The choroidal neovascularization or growth of new frail vessels is associated with late AMD and is the principal cause of severe vision loss (Mitchell et al., 2018; Telegina et al., 2018).

Regarding the immune-inflammatory events (e.g., the complement system activation, the change to senescent RPE cells, and the exacerbated activation of glial cells) are also involved in drusen formation and choroidal neovascularization events (Bradley et al., 2011; Jensen et al., 2020; Marazita et al., 2016; Telegina et al., 2018; Wang et al., 2019). Like in glaucoma, AMD retinas present an abnormal level of citrullination (Bonilha et al., 2013). Nevertheless, non-AMD and AMD post-mortem patients express similar levels of retinal PAD2 (Bonilha et al., 2013). These data suggest that AMD retinas present a reduced rate of turnover of citrullinated proteins, contributing to AMD-associated adverse events.

As mentioned above, LCN2 can regulate the neuroinflammation process present in age-related retinal pathologies. Given that LCN2 receptors are present in the surface of retinal cells like glial and RPE cells, this protein can modulate their functions (e.g., glial activation and RPE survival) and thus the inflammatory response (Ghosh et al., 2020; Parmar et al., 2018; Tang et al., 2018). In this sense, the retina of AMD patients overexpresses LCN2 (Fig. 2) (Ghosh et al., 2019, 2017). Ghosh and coworkers have proved that the choroid and retina of early AMD patients have a higher infiltration of LCN2 expressing neutrophils than age-matched controls (Ghosh et al., 2019, 2017, 2020). These neutrophils also express other NET proteins such as MPO and NE (Fig. 2) (Ghosh et al., 2019, 2017). Furthermore, Ghosh et al. have insinuated that these infiltrated cells could also overexpress MMP-9, which may form a complex with LCN2 in the outer retina-choroid of AMD patients (Ghosh et al., 2020). Therefore, the LCN2/MMP-9 complex could facilitate AMD choroidal vascularization as seems to occur in corneal vascularization (Shen et al., 2016).

Neovascularization may occur after the outer blood-retinal barrier (oBRB) breakdown and the subsequent recruitment of circulating immune cells like neutrophils, which, together with senescent RPE cells, secrete vascular endothelial growth factor (VEGF) and promote the growth of new vessels (Marazita et al., 2016; Taichman et al., 1997). Therefore, neutrophil-derived MMP-9 could compromise oBRB, formed by the tight junctions between adjacent RPE cells (Zhou et al., 2010), and permit the pass of systemic immune and inflammatory components into the retina, amplifying the vascular damage and neuroimmune response (Yang et al., 2020). In this context, a current phase II clinical trial tries to evaluate the efficacy of an MMP-9 inhibitor against recalcitrant neovascular AMD (ClinicalTrials.gov Identifier: NCT04504123). Moreover, Chau et al. found a high plasma level of MMP-9 in subjects with AMD (Fig. 2) (Chau et al., 2007). Nevertheless, a recent study has proved that neovascular AMD patients only overexpress LCN2, but not MMP-9 or LCN2/MMP-9 complex, in their plasma (Fig. 2) (Chen et al., 2020). Therefore, the potential expression of MMP-9 on retinal neutrophils and their role in AMD vascular and neuroinflammatory damage is an exciting topic to explore in future research.

Regarding the potential role of LCN2 in AMD damage, this NET protein also regulates neutrophils' migration into the retina, where in conjunction with retinal cells (mostly RPE cells), synthesize and release new LCN2, potentiating outer retinal degeneration (Ghosh et al., 2019).

Parmar et al. have suggested that the compromised oBRB may contribute to leakage of LCN2 produced in the retina into the plasma, and so could activate neutrophils migration (Parmar et al., 2018). In this sense, and as we mentioned above, some authors have found abnormally elevated levels of LCN2 and neutrophils activated in the plasma of AMD patients (Fig. 2) (Chen et al., 2020; Ghosh et al., 2019; Parmar et al., 2018). In agreement with this last result, Ilhan et al. have suggested that AMD patients (with both non-vascular and vascular disease) present a high neutrophil-to-lymphocyte ratio (NLR) compared to controls (Fig. 2) (Ilhan et al., 2015). However, other authors have only found an increased NLR value in neovascular AMD patients (Kurtul and Ozer, 2016; Niazi et al., 2019). Consequently, these authors suggest that the LCN2 level and NLR value could be biomarkers of AMD. Rhezar-Dreindl and colleagues have also suggested that the level of LCN2 in the aqueous humor could be a biomarker for monitoring the treatment of persistent/recurrent neovascular AMD patients (Fig. 2) (Rezar-Dreindl et al., 2016).

Regarding the therapeutic target role of LCN2, Ghosh et al. have demonstrated that the intravitreal injection of a serine/threonine-protein kinase AKT2 inhibitor in early AMD-like phenotype mice can mitigate drusen formation, inflammation, neutrophil's infiltration, and activation of retinal glial cells (Ghosh et al., 2019). Therefore, targeting AKT2 an upstream regulator of the retinal LCN2 mRNA expression could be a feasible strategy for treating early AMD (Fig. 2) (Ghosh et al., 2019, 2017).

Other potential AMD therapeutic agents are ligands of neutrophil receptors like lipoxin A4, which has been suggested with a limited experimental basis (Bazan, 2009, 2010; Das, 2012), and purinergic blocking agents (Fig. 2) (Fletcher et al., 2019; Notomi et al., 2013).

Fletcher et al. have recently suggested targeting the $P2X_7$ receptor for treating AMD (Fletcher et al., 2019). Indeed, the blockage of the $P2X_7$ receptor delay photoreceptors' death in experimental-late AMD (Notomi et al., 2013). Under inflammatory stress, immune cells like neutrophils release ATP that activates $P2X_7$-induced cell death (Dosch et al., 2018). Besides, ATP can activate the neutrophil $P2X_7$ receptor modulating functions like the secretion of pro-inflammatory mediators, and NET formation (Dosch et al., 2018; Pelegrin and Surprenant, 2009; Sofoluwe et al., 2019; Wang and Chen, 2018). These data explain the potential neuroprotective effect of $P2X_7$ receptor-blocking agents (e.g., antagonists and silencers) in AMD. However, further preclinical, and clinical works are necessary to prove the efficacy of these agents in AMD, including the newer blood-brain barrier-permeable $P2X_7$ antagonists developed (Fletcher et al., 2019).

### 2.4. Diabetic retinopathy

Diabetic retinopathy (DR) is a common retinal microvascular complication of diabetes (Cheung et al., 2010). Chronic hyperglycemia associated with diabetes leads to oxidative and inflammatory cellular damage that alters the inner blood-retinal barrier (iBRB), allowing plasma leakage, which causes injury to retinal cells (Chiu and Taylor, 2011; Crooke et al., 2017; Eshaq et al., 2017; Yang et al., 2020). Moreover, in the disease's late stage called proliferative DR (PDR), these damages evoke the growth of vessels that invade the vitreous humor, causing blindness (Cheung et al., 2010; Eshaq et al., 2017). Indeed, and according to a 2021 Vision Loss Expert Group report, DR is one of the most common causes of blindness worldwide (GBD, 2019 Blindness and Vision Impairment Collaborators and VLEG, 2021).

The iBRB impairment can also allow neutrophils migration to the retina generating critical events in the pathogenesis of the DR, such as retinal leukostasis and inflammatory response (Wang et al., 2018). In this sense, Wang et al. have demonstrated that the retina of diabetic rats presents an abnormal proportion of neutrophils and overexpresses pro-inflammatory cytokines (e.g., IL-6, IL-8, and IL-1β) (Fig. 2) (Wang et al., 2018). These cytokines, together with hyperglycemia, promote the NETosis process (Delgado-Rizo et al., 2017; Wang et al., 2018). Consequently, various authors have found high levels of NETs and their components (e.g., eDNA, NE, MPO, and LCN2) in the retina of diabetic rats and the vitreous fluid/blood of DR patients (Fig. 2) (Chung et al., 2016; Park et al., 2016; Wang et al., 2018). Furthermore, blood arginine (a precursor of citrulline) and citrulline are abnormally high in DR patients. Nevertheless, the level of systemic/retinal citrullination in these patients remains unknown (Peters et al., 2021).

Regarding the NE enzyme, Liu and coworkers have confirmed the role of this protease in the vascular damage generated in the disease (Liu et al., 2019). Likewise, and given that there is a significant correlation between vitreous LCN2 and VEGF levels in DR patients, LCN2 could also contribute to vascular impairment (Wang et al., 2020). Indeed, some authors have reported that LCN2 can induce the production of hypoxia inducible factor-1α (HIF-1α) and the downstream gene, VEGF, to stimulate angiogenesis (Hu et al., 2018).

The MMP-9/LCN2 complex could also be involved in DR vascular damage, as some authors have suggested that this occurs in other eye vascular diseases (Ghosh et al., 2020; Shen et al., 2016). This complex avoids the inactivation of MMP-9, which might increase the retinal vascular permeability and neovascularization (Kowluru et al., 2012). Additionally, LCN2 can enhance the binding of advanced glycation end-products (AGEs) to their receptors (RAGEs), amplifying diabetic vascular and inflammatory damage (Chiu and Taylor, 2011; Chung et al., 2016; Ciudin et al., 2010). Conversely, these AGEs increase the expression of LCN2 by vascular cells (Chung et al., 2013). Therefore, LCN2 secreted under AGEs- and inflammatory stimuli by retinal cells (vascular, ganglion, glial and recruited-neutrophil cells) could also modulate, via binding to its receptors on these cells, diabetic retinal neuroinflammation as seems to occur in AMD (Chung et al., 2013; Gerhardinger et al., 2005; Ghosh et al., 2020; Parmar et al., 2018).

These results suggest that the neutrophils and NET components, such as LCN2, also play a critical role in DR pathogenesis and could be biomarkers and therapeutic targets for this disease. In this sense, some authors have found elevated NRL values in DR patients mainly in PDR ones compared to controls and suggested its potential as a diagnostic marker of DR severity (Fig. 2) (Ulu et al., 2013; Yue et al., 2015). Moreover, some preclinical studies have suggested the therapeutic potential of indirect NETosis-inhibitors against DR (Fig. 2). In this context, Purvis and coworkers have demonstrated the protective effects of annexin-1 against microvascular complications in an animal model of type 1 diabetes (Purvis et al., 2018). The treatment of diabetic mice with human recombinant annexin-1 inhibits mitogen-activated protein kinases (MAPKs), which are activated in hyperglycemic condition, attenuating the cardiac and renal dysfunction of these animals (Purvis et al., 2018). Given that MAPKs also mediate DR, annexin-1 could have a therapeutic effect against this microvascular complication (Purvis et al., 2018). Likewise, some authors have proposed the therapeutic potential of lipoxin A4 against diabetes-associated vascular complications (Brennan et al., 2018; Das, 2012, 2013). Indeed, Brennan et al. have demonstrated that lipoxin A4 and its analog benzo-lipoxin A4 protect against vascular inflammatory damage in aortic tissue of diabetic rats and human carotid atherosclerotic plaques (*ex-vivo*) (Brennan et al., 2018). These lipidic mediators reduce the production of pro-inflammatory cytokines and VEGF-induced angiogenesis (Das, 2012). Therefore, lipoxin A4 and its analogs could protect against PDR (Das, 2012, 2013).

Other regulators of neutrophil functions with therapeutic potential in DR are the ligands of purinergic receptors (Fig. 2) (Clapp et al., 2019; Liou et al., 2011; Mancini et al., 2018; Sugiyama, 2014; Vindeirinho et al., 2016). The continuous ATP released by retinal cells like ganglion, glial and vascular cells and infiltrated neutrophil cells during diabetes activates the purinergic receptors expressed by these cells (Costa et al., 2009; Mancini et al., 2018). So, the activation of retinal $P2X_7$ receptors can evoke vascular cell death and regulate the secretion of pro-inflammatory mediators (Clapp et al., 2019; Sugiyama et al., 2005). Indeed, the blockage of $P2X_7$ receptors reverses increased vascular

permeability, VEGF accumulation, and IL-6 expression in diabetic rat retinas (Clapp et al., 2019). Similarly, Mancini et al. have proved that the inhibition of the $P2X_2$ receptor decreases retinal cell death in diabetic rats (Mancini et al., 2018). In contrast, the effect of adenosine receptor ligands in the retina during diabetes is controversial. For example, adenosine A2 receptor (ADORA2A) agonists could prevent or promote vascular damage depending on the cell type and the experimental approach (Barletta et al., 2012; Charles, 2014; Liou et al., 2011; Vindeirinho et al., 2016). These agonists can decrease neutrophil adherence to endothelial cells (prevent vascular damage) and increase the production of retinal VEGF or glucose transport into retinal vasculature (promote vascular damage) (Barletta et al., 2012; Takagi et al., 1998, 1996).

On the other hand, inhibitors of the adenosine reuptake (adenosine transporters) are under investigation as a therapeutic alternative of adenosine and its analogs against DR (Liou et al., 2011). These inhibitors have been successfully probed in experimental diabetes, decreasing microglial activation and retinal vasculature damage (De la Cruz et al., 1997; Liou et al., 2008).

The translation of all these preclinical data into clinical practice will require a better characterization of the DR underlying molecular mechanisms mediated by all these components of the adenosinergic system.

## 3. Conclusions and future directions

Age-related eye diseases, including dry eye, glaucoma, age-related macular degeneration, and diabetic retinopathy, represent a major global health issue based on their increasing prevalence and disabling action, with an enormous personal and economic consequences (GBD, 2019 Blindness and Vision Impairment Collaborators and VLEG, 2021; Uchino and Schaumberg, 2013). In this sense, The Lancet Global Health Commission on Global Eye Health argues that to achieve eye health (and thus facilitates the UN Sustainable Development Goals) is necessary to understand the mechanisms underlying age-related eye diseases (Burton et al., 2021).

One of these mechanisms shared with other age-related diseases seems to be the impairment of neutrophil extracellular traps formation/degradation processes. Indeed, age-related eye disease patients present local recruitment of neutrophils and overexpression of neutrophil extracellular trap components at local and systemic levels (An et al., 2019; Arafat et al., 2017; Aragona et al., 2006; Chau et al., 2007; Chen et al., 2020; Chung et al., 2016; Ghosh et al., 2019, 2017; Guo et al., 2011; Kaeslin et al., 2016; Park et al., 2016; Reyes et al., 2018; Sonawane et al., 2012; Tibrewal et al., 2013; Ueno et al., 2018; Wang et al., 2018).

These data suggest the potential role of neutrophils and their constituents as pathologic mediators, biomarkers, and therapeutic targets of AREDs. In this context, future studies should explore the validity of AREDs biomarkers linked to neutrophils at local and particularly at ocular surface level. Tear film and ocular surface cells represent a power source of biomarkers of ocular and systemic diseases like neurodegenerative disorders (Hagan et al., 2016; Roda et al., 2020). Furthermore, the techniques employed to obtain tear and ocular surface samples are less invasive than blood/intraocular sampling methods. So, it will be interesting to determine the value of the blood indicator of systemic inflammation and poor disease prognosis NLH at the tear level of AREDs patients (including age-related retinal diseases ones). Likewise, and given the pivotal role of LCN2 as a neuroinflammatory and vascular damage modulator, the measure of this NET component in the ocular surface of age-related retinal diseases patients could supply quite beneficial for the diagnostic of these diseases. Indeed, several authors have already proposed the LCN2 diagnostic use (and therapeutic potential of targeting LCN2) for other neurodegenerative disorders (e.g., Alzheimer's, Parkinson's, multiple sclerosis, and Huntington's diseases) (reviewed in (Suk, 2016)).

Regarding the therapeutic potential of targeting the NET components in AREDs, the use of DNase inhibitors and pooled ocular surface immunoglobulin (IgG)-eye drops, which neutralize ACPAs effects, has been particularly successful in DED patients (Kwon et al., 2020; Mun et al., 2019). In this context, Chirivi et al. have proposed as drug candidates to treat NET-mediated inflammatory diseases the so-called therapeutic ACPAs (ACPAs that selectively bind to citrullinated histones 2A and 4) (Chirivi et al., 2021). These therapeutic ACPAs diminish NET release and promote NET clearance *in vivo* (Chirivi et al., 2021). These results could open new therapeutic avenues to combat AREDs. An upstream modulator of LCN2 mRNA has also provided an encouraging result in experimental AMD (Ghosh et al., 2019, 2017). These results should inspire future research concerning the employ of NET components-blocking agents like DNase inhibitors and LCN2 modulators against age-related eye diseases.

Other potential and emerging AREDs therapeutic agents are ligands of neutrophil receptors like pro-resolution mediators and purinergic ligands, which neutralize neutrophils recruitment avoiding local NET components accumulation (Clapp et al., 2019; Das, 2013; Li et al., 2018; Liou et al., 2011; Mancini et al., 2018; Purvis et al., 2018; Romano et al., 2020; Shinozaki et al., 2017; Tibrewal et al., 2014). Future research should confirm these initial (preclinical) findings enabling their translation into clinical practice.

Finally, we also believe that combination therapy approaches including at least one of the above-commented agents targeting neutrophils or NET components are the unique choice to achieve long-term health benefits, given the complexity of AREDs. Consequently, we consider that age-related eye diseases future research should also explore these multi-target drug cocktails.

## Author Contributions

**Irene Martínez-Alberquilla:** Investigation and Writing – original draft. **Xavier Gasull:** Writing – review & editing. **Patricia Pérez-Luna**: Investigation. **Rubén Seco-Mera:** Investigation. **Javier Ruiz-Alcocer:** Investigation, Conceptualization. **Almudena Crooke:** Conceptualization, Supervision, and Writing – original draft.

## Declaration of Competing Interest

The authors report no declarations of interest.

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