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In vitro and _in vivo_ models of eye disease in response to neurotrophic stimulus: identification of new biomarkers and therapeutic targets

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FOOTNOTES

AH	Aqueous humor
AMD	Age-related macular degeneration
ARPE-19	Human retinal pigment epithelial
BDNF	Brain-derived neurotropic factor
CAT	Catalase
CCND1	Cyclin D1
CDH2	N-cadherin
CDH3	P-cadherin Paired Box 6
CDK1	Cyclin-dependent kinase 1
CRALBP	Retinaldehyde Binding Protein 1
DAG	Phosphatidyl inositol 4,5-bisphosphate in diacylglycerol
DAPL1	Death associated protein-like 1
DGCR8	DiGeorge syndrome critical region gene 8
DJ-1	Parkinson disease protein 7
DR	Diabetic retinopathy
ECM	Extracellular matrix
EMA	European Medicines Agency
EMT	Mesenchymal transition
Exp5	RanGTP-binding nuclear transporter exportin-5
Gadd45β	Growth Arrest and DNA Damage-inducible, beta
GEF	Guanine nucleotide exchange factor
Grb-2	Growth factor receptor-binding protein-2
HCEC	Human corneal epithelial cells
HCEK	Human corneal epithelial keratinocytes
HO-1	Heme Oxygenase-1
HP	Pressure increase
IKK	Inhibitor of kB kinase
IL-1beta	Interleukin-1 beta
IL-6	Interleukin-6

INL	Inner nuclear layer
IOP	Intraocular pressure
IP3	Inositol (1,4,5) triphosphate (IP3)
ITGAV	Integrin subunit alpha V
JNK	Jun amino-terminal kinase
MAPK	Mitogen-activated protein kinase
MERTK	MER proto-oncogene, tyrosine kinase
MiRNAs	MicroRNAs
MITF	Melanocyte Inducing Transcription Factor
ncRNAs	Non-coding RNAs
NF-kB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NGF	Nerve growth factor
NK	Neurotrophic keratitis
NRF2	Nuclear Factor Erythroid 2-Related Factor 2
NRIF	Neurotrophin receptor interacting factor
ONP	Outer nuclear layer
PAX6	Paired Box 6
PC-12	Pheochromocytoma cell line
PLC γ	Phospholipase Cγ
RB	Retinoblastoma
RDH10	Retinol Dehydrogenase 10
RECs	Retinal endothelial cells
RGCs	Retinal ganglion cells
RISC	RNA-induced silencing complex
rMC-1	Rat Müller cells
RPE	Retinal pigment epithelium
RPE6	Retinoid Isomerohydrolase
RSK	Ribosomal s6 kinase
Shc	**A**daptor protein Src
siRNAs	Small interfering RNAs
SIRT-1	Sirtuin 1

snRNA	Small nuclear RNA
SOD-1	Superoxide dismutase 1
SOX9	SRY-box transcription factor 9
TGF- β	Transformation growth factor
TYR	Tyrosinase
TRAF6	Tumor necrosis factor receptor member
tRNA	Transfer RNA
ZO-1	Zonula Occludens-1

ABSTRACT

The retinal pigment epithelium (RPE), a key cell layer in the eye, plays an important role in retinal health. Dysfunction of the RPE is linked not only to age-related macular degeneration (AMD) but also to glaucoma, a major cause of blindness. RPE dysfunction contributes to the degeneration of retinal ganglion cells (RGCs), which is central to glaucoma-related vision loss. Oxidative stress and inflammation in RPE and Müller cells exacerbate glaucoma, and dysfunction of RPE can also disrupt the blood-retinal barrier, leading to the accumulation of toxic substances. Currently, neither biomarkers nor effective therapy are available for the disease. Nerve growth factor (NGF) has shown potential as a neuroprotective agent, but mechanisms of action remain unclear. In this study, after generating a differentiated RPE cell model from ARPE-19, which confirmed the expression of specific RPE markers (e.g. RPE65, CRALBP, MITF, RDH10), an in vitro model of glaucoma was set by using an original device able to induce pressure increase (HP) on cultured cells. The above-mentioned human RPE and rat Müller cells were used for experimental procedures. Several of the most important proteins involved in oxidative stress (e.g. DJ-1, NRF2, HO-1, CAT, SOD-1) and inflammatory (IL-6, IL-1beta) responses were analyzed in HP condition, in the presence or absence of NGF. At the same way, expression levels of three microRNAs (miR-34a-5p, miR-29c-3p, miR-146a-5p) were evaluated, and a direct target of miR-34a-5p (SIRT-1) was considered as well. Interestingly, results highlighted the suitability of the model for the study of glaucoma, evidencing, on one hand, expected and coherent levels of expression of the considered markers, and, on the other, the putative role of NGF as neuroprotective factor able to revert conditions caused by HP. Such effects were evidenced in terms of both oxidative stress/inflammation-related proteins' and microRNAs' expression levels in response to specific experimental conditions.

In conclusion, the study provided interesting results and opens further analyses on preclinical models to assess the potential use of NGF for glaucoma treatment and validate candidate miRNAs here described as novel biomarkers/therapeutic targets.

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INTRODUCTION

1. Anatomy and physiology of the human eye

The eye is the main sensory organ of the visual system, which has the task of capture external stimuli and convert them into information that stimulates the nervous system's responses. The eyeball, characterized by spheroid shape, is found in the front of the orbital cavity and contains a nervous peduncle and optic nerve that connect it to the formation of the diencephalon (chiasma and optical traits) required for visual perception. The eyeball itself consists of three main layers: the outer layer, comprising cornea and sclera, the middle layer, responsible for holding the blood supply for the eye as well as the iris and the pupil, and the inner layer, or the retina (Figure 1).

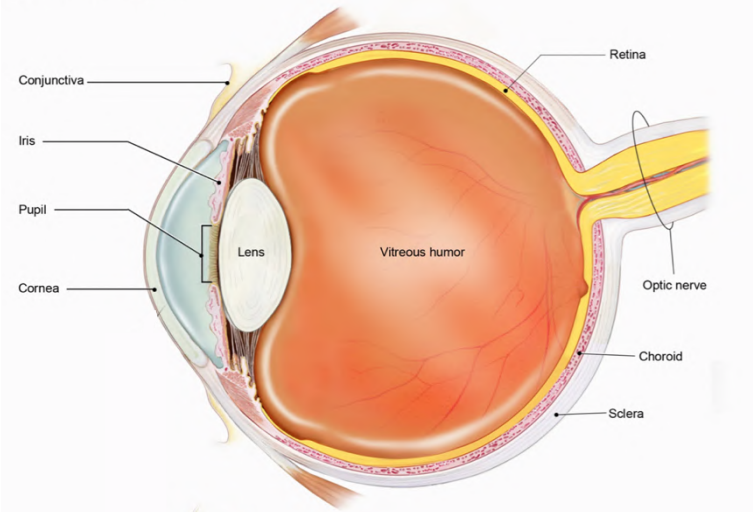


***Figure 1*. Structures of the eye.** The eyeball is divided into anterior segment (conjunctiva, iris, cornea, lens, ciliary body) and posterior segment (vitreous, optic nerve, choroid, retina) (https://www.timeofcare.com/the-anatomy-of-the-eye/).

The wall of the eye consists of three main layers that overlap each other:

- The outer layer is made of dense, fibrous, viscoelastic connective tissue known as the sclera. The sclera is a sturdy structure that supports the inner eye's visual apparatus, resists the excessive force produced by intraocular pressure, and safeguards the eye contents from external trauma (Atta et al., 2020).

  Cornea belongs to the anterior segment of the fibrous tunic of the eye. It is formed by several lamellar layers of collagen fibers immersed in a matrix of proteoglycans. Light entering the eye is refracted or deflected by the transparent, avascular cornea towards the lens, which focuses on the retina. The posterior surface of the cornea delimits the anterior chamber of the eye and is nourished by the aqueous humor (Sridhar, 2018).

- The middle layer or uvea, also known as the vascular layer, located between the sclera and the retina, is abundant in blood vessels and strongly pigmented. It is responsible for providing the nutrients necessary for proper functioning of the eye and of absorbing the light rays preventing their reflection. The iris, anterior part of the choroid, is a pigmented membrane that determines the color of our eyes. It acts as a muscular diaphragm to measure the amount of light that penetrates inside the eye. The choroid membrane is a vascular and pigmented layer rich in blood vessels that provides retina with oxygen and nutrients. The ciliary body is a muscle that sits behind the iris and before the choroid. It is used both to produce aqueous humor (saline liquid that helps to give volume to the eyeball) and for the control of the focus of images, through the ciliary muscle, which acts by adjusting the curvature of the lens.
- The inner layer is the retina which lines the back two-thirds of the eyeball. The retina is a multi-layered sensory organ responsible for receiving light stimuli, transforming them into nerve signals. The neural retina consists of six major classes of neurons: photoreceptors, bipolar cells, horizontal cells, amacrine and ganglion cells, which capture and process light signals, and the Müller glia, which acts as the organizing backbone of the neural retina. The retinal pigmented epithelium (RPE) is a monolayer of cells extending from the edge of the optic papilla to the tight hour. Externally, the cells are in contact with the Bruch membrane, and in this portion the nucleus and mitochondria are located. The innermost part, on the other hand, is linked with retinal photoreceptors, cones and rods, and contains numerous spherical or elliptical pigment granules. At this level there is a mucopolysaccharide substance, which promotes the adhesion of the epithelium to the retina and photoreceptors. The cells of the pigmented epithelium are also joined by special junctions that prevent the passage of the molecules, ions and water to the sensory retina, which define this part as "external blood-retinal barrier". The role of retinal pigment epithelium in the visual process is the renewal of the external segments of the photoreceptors. This process, that changes over the years, causes an accumulation of unmetabolized material below the retina, called lipofuscin (Purves D, 2001).

- The outer nuclear layer (ONP) contains rods and cones. When the light photons arrive in the retina, they are first captured by these two photoreceptors, which have the function of detecting the intensity and frequency of incoming light. Cone cells are responsible for perception, color recognition and distinct vision. The rods are numerous and work especially in night vision. The inner nuclear layer (INL) contains horizontal cells which are responsible for modulating the output signal from the photoreceptors. These cells communicate with bipolar and amacrine cells which contribute to the signal processing process. Through synaptic contacts, the bipolar cells communicate with retinal ganglion cells (RGC) in the inner plexiform layer. The optic nerve is formed by the layer of nerve fibers. Those are formed by the axons of the ganglion cells. Moreover, the RGC is responsible for transmitting information about retinal stimulation to the central nervous system (Purves D, 2001) (Figure 2).

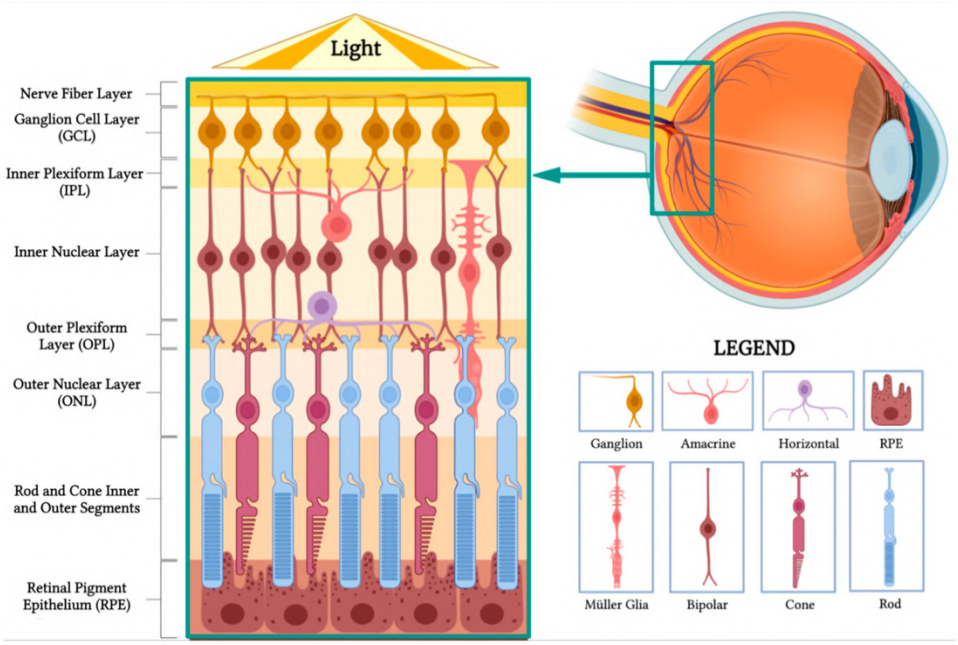


Figure 2. **The cellular layers of the retina.** Visual information is transmitted directly to the brain through photoreceptors, bipolar cells, and ganglion cells. In the outer and inner plexiform layer, the lateral interactions are mediated by horizontal cells and amacrine cells (Wu et al., 2023).

2. Glaucoma

Glaucoma disease defines a group of optic neuropathies characterized by progressive degeneration of RGC, their optic nerve axons, and synaptic loss at dendrites and axon terminals. *Cupping,* a characteristic appearance of the optic disc, and visual loss are caused by the degeneration of RGC (Allison et al., 2020).

2.1. Pathophysiology

RGC, known as neurons in the central nervous system, receive signals from photoreceptors, processing them, and transmitting them through axons into additional centers in the brain through the optic nerve. The axons travel through the lamina cribrosa (characterized by collagen) from the ganglion cell nuclei in the retina to the optic disc through the retinal vessels (Weinreb et al., 2014). The axons are surrounded by a myelin sheath and continue as the optic nerve behind the lamina cribrosa. The gradient in the lamina cribrosa is increased by elevated intraocular pressure, low perfusion pressure, and/or low cerebrospinal fluid pressure, which leads to structural changes and re-modeling of the lamina cribrosa and causes alterations in axonal transport in the optic nerve fibers (H. Tian et al., 2017). The RGC loss results in progressive impairment of the visual field, which typically starts in the peripheral area, and progresses until only a central or peripheral area of intact vision remains. Other functional issues include poor contrast and color perception, and difficulty in reading (Erb, 2015). Glaucoma shows up in two forms, according to structural changes in the anterior segment of the eye: open-angle glaucoma and closed-angle glaucoma. The primary route for draining the aqueous humor is through the trabecular meshwork and the canal of Schlemm, but some of it also passes through the uveoscleral outflow (root of the iris and ciliary body). Between the iris and the peripheral posterior surface of the cornea, lies the chamber angle, which ends with the canal of Schlemm situated under the trabecular meshwork. Open-angle glaucoma results in the chamber angle being macroscopically open, while acute angle closure results in the iris obstruction (Figure 3). A significant increase of intraocular pressure is caused by a sudden blockage of the outflow of aqueous humor through the trabecular meshwork and the canal of Schlemm. Changes in the chamber angle in secondary open-angle glaucoma, such as pigment deposition in pigmentary glaucoma and protein deposition in pseudo-exfoliation glaucoma, can be observed under the microscope (gonioscope) and cause an increase in intraocular pressure (Okafor, 2017) (Miglior S, 2007).

Although the average intraocular pressure is 15.7 mm Hg, it exhibits significant chronobiological and inter-individual variation, even in healthy individuals (Chan et al.,

2016). The regulation of the intraocular pressure is based on the balance between the ciliary body's secretion of aqueous humor and its outflow. An increase of outflow resistance may be the cause of elevated intraocular pressure. Secondary open-angle glaucoma can involve gonioscopically visible changes in the chamber angle but can occur even without such changes. Even with normal intraocular pressure, glaucomatous changes in the optic nerve may occur (normal-pressure glaucoma) (Schuster et al., 2020).

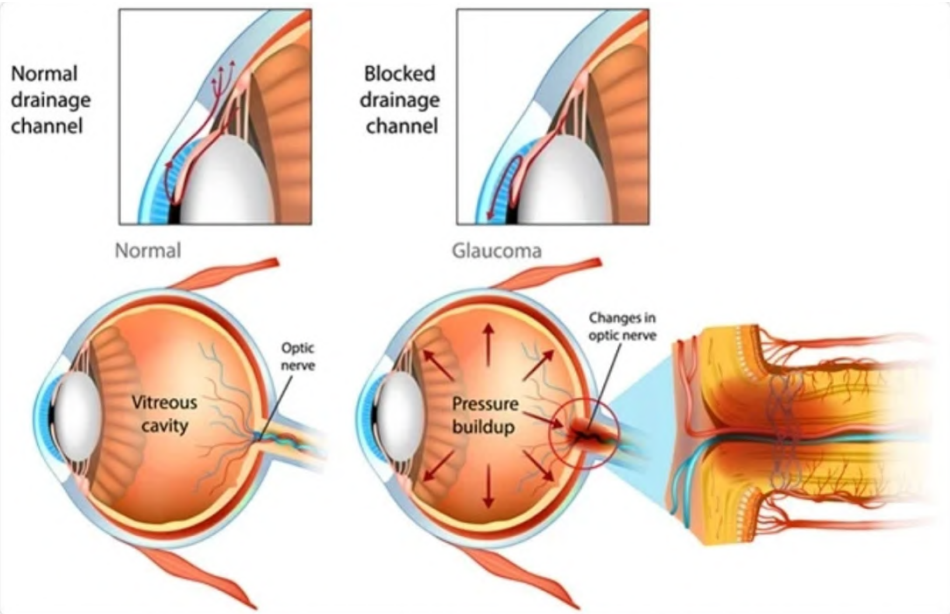


Figure 3. Glaucoma. The illustration showing open-angle glaucoma and the increase of intraocular pressure in the back of the eye (https://www.health.harvard.edu/staying-healthy/the-facts-about-glaucoma).

2.2. Epidemiology

In 2020, 57.5 million people around the world went blind because of glaucoma (Allison et al., 2020). Glaucoma is the second leading cause of irreversible blindness in western Europe, behind age-related macular degeneration (Bourne et al., 2018). In Europe, it is diagnosed in 2.93% of people between 40 and 80 years old (Figure 4). Open-angle glaucoma is mainly prevalent among individuals between the ages of 40 and 80, with a prevalence of 2.51% (Tham et al., 2014), and is becoming more common as you get older, with a prevalence of 0.4% in 40–44 years and 2.7% in 70–74 years, as well as 10.0% in people with European ancestry over 90 years (Kapetanakis et al., 2016). The prevalence of glaucoma among African ancestry is 2.8 times higher than that of Europeans, while angle-closure glaucoma and normal-pressure glaucoma are more common in Asians, according to a systematic

review (Tham et al., 2014). On the other hand, congenital and juvenile glaucoma are rare in general (Marx-Gross et al., 2017).

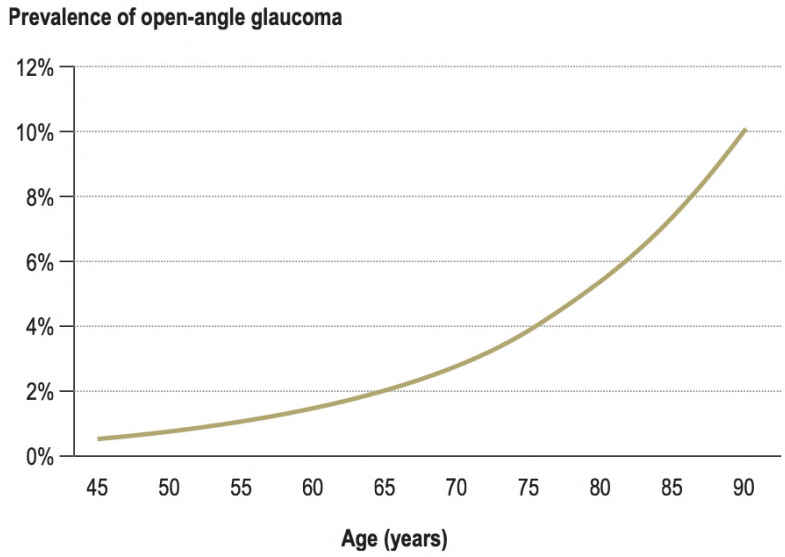


Figure 4. The prevalence of open-angle glaucoma increases with age in individuals of European descent (Kapetanakis et al., 2016).

2.3. Risk Factors

Age, positive family history, sex, increased intraocular pressure, myopic refractive error are considered risk factors for glaucoma(Schuster et al., 2021).

Family history and sex: Sweden's study by Ekström et. al., (2012) indicates that having a positive family history of glaucoma is a risk factor. Individuals with a positive family history are twice as prone to developing glaucoma. According to glaucoma patients, their mothers (50%) and sisters (26%) are more susceptible to developing glaucoma than their fathers (1.5%) and brothers (1.2%) (Mitchell P, 2002).

Intraocular pressure: increased intraocular pressure is related to higher chance of developing open-angle glaucoma. The Ocular Hypertension Treatment Study showed that, if intraocular pressure increases from 24 to 32 mm Hg without prior glaucomatous disease, the risk of developing glaucoma within 5 years is 9.5%. In fact, due high intraocular pressure reduction to 22.5%, the risk of developing glaucoma decreases from 9.5% to 4.4% (Kass et al., 2002). The European Glaucoma Prevention Study has demonstrated that ocular hypertension and thinner central corneal thickness are risk factors for open-angle glaucoma (Miglior S, 2007).

Myopic refractive error: Highly myopic eyes make it particularly hard to examine the optic disc. Myopia-related enlargement of the optic disc, with subsequent thinning of the lamina cribrosa, is thought to be a risk factor for glaucoma (Nagaoka et al., 2015). Another possible pathogenetic factor is the higher levels of shear forces in the lamina cribrosa caused by eye movements in people with highly myopic (long) eyes (X. Wang et al., 2016).

Other suspected risk factors: Migraines, hyperopia, pseudo-exfoliation and outflow facility are among the other suspected risk factors for progressive disease which have been mentioned in the literature. In current studies, outflow facility is rarely measured, but future researchers can reevaluate whether decreased outflow facility could be considered a risk factor independent of IOP (Schuster et al., 2021). The limited number of studies that evaluated these additional suspected risk factors does not support confident conclusions about their relative importance.

2.4. Diagnostic evaluation

2.4.1. Symptoms

The symptoms of acute angle closure include pain radiating from the eye, visual impairment, conjunctival hyperemia, and occasionally nausea and vomiting with a tight, hard globe. To prevent severe ocular damage and blindness, immediate treatment is necessary for this ophthalmological emergency. Open-angle glaucoma is typically not symptomatic until it has reached an advanced stage, unlike other forms of glaucoma. Binocular vision usually compensates for visual field defects that occur in different parts of both eyes. Generally, individuals with open-angle glaucoma do not exhibit any symptoms, and many are unaware of their condition (Crabb DP, 2013) (Kim KE, 2016). At the time of diagnosis, one-tenth of patients already has the disease in an advanced or late stage in one eye (Heijl et al., 2013). According to Gramer et. al., (2018), 10% to 20% of patients were already unable to drive a vehicle when they were presented at the clinical examination, due to binocular visual field defects.

2.4.2. Early detection

The German ophthalmological associations suggest regular screening examinations for early detection from the age of 40 onwards since symptoms only appear when the condition has reached an advanced stage. The disorder's low prevalence and poor test sensitivity and specificity lead to high rate of false positives (>65%, and even more in younger patients)

(Vessani RM, 2009) (Abrams et al., 1994). Therefore, any positive result needs to be confirmed with additional testing. It is crucial to regularly examine risk groups with a high incidence and prevalence of the disorder, so that it can be diagnosed and treated early in its course. To perform the recommended screening examination, it is necessary to gather a clinical history, examine the papilla and peripapillary nerve layer stereoscopically, measure tonometry, and have a slit-lamp exam of the eye (Schuster et al., 2020).

2.4.3. Clinical Investigation

To diagnose glaucoma, it is important to conduct funduscopic examinations on the optic disc and retinal nerve fiber layer. Glaucomatous changes are manifested by tissue loss at the neuroretinal rim and enlargement of the optic nerve excavation, a non-physiological discrepancy between the optic nerve excavations in the two eyes, hemorrhages at the edge of the optic disc, thinning of the retinal nerve fiber layer, and parapapillary tissue atrophy (the beta zone) (Aass et al., 2022) (Jonas et al., 1999). The optical coherence tomography (OCT) (Figure 5) (Enders et al., 2019) is a morphometric technique that can be utilized to quantitatively examine the optic disc and measure the retinal nerve fiber layer and neuroretinal rim. The first visit should include objective testing (photography of the optic disc, morphometric measurement of the optic nerve head, and thickness of the retinal nerve fiber layer, e.g., with OCT) that is crucial for assessing the progression of the disease (Jonas & Budde, 1999).

Initial diagnosis requires the use of tonometry to measure intraocular pressure. To determine if the intraocular pressure measurements are artificially high or low, it is necessary to measure the thickness and curvature of the cornea simultaneously (Goldmann et al., 1957). Throughout the day, the intraocular pressure changes. For this reason, measuring intraocular pressure at different times of the day is important to create a daily pressure profile that can be used to improve treatment titration (Schuster et al., 2020). Information about the pathogenesis of the disease can be obtained through gonioscopic examination of the chamber angle during the initial diagnosis. During the first year, after the diagnosis, it is advisable to examine the visual fields at least three times, to evaluate the degree of functional impairment resulting from the loss of optic nerve fibers (The "Terminology and Guidelines, 2017).

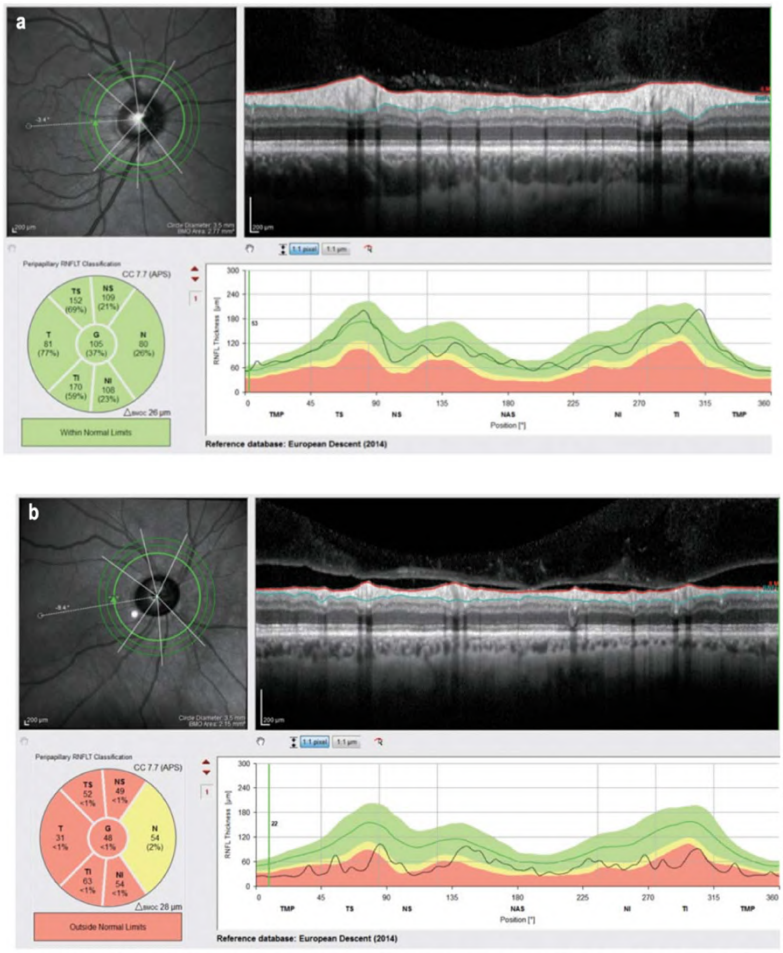


Figure 5. **Examining the thickness of the nerve fiber layer in the peripapillary area** a) in a normal eye and b) in a glaucomatous eye. The retinal nerve fiber layer is separated by the red line of the internal limiting membrane and the turquoise line of the retinal nerve fiber layer and the ganglion cell layer (Enders et al., 2019).

2.5. Treatment

To prevent glaucoma progression there is no gold standard treatment at present. The only effective and widely accepted treatment is reducing intraocular pressure (Heijl et al., 2002) (Garway-Heath et al., 2015). The intraocular pressure can be lowered in patients with open-angle glaucoma by using eyedrops (Heijl et al., 2002) (Garway-Heath et al., 2015)(T. Li et al., 2016), laser therapy (Gulati et al., 2017), and/or surgery (Table 1) (Lavia et al., 2017) (Gedde et al., 2018). The objective is to attain a target pressure that is patient-specific and will not result in progression of glaucoma, and that can be observed and documented. To determine the target pressure, the glaucoma damage, current intraocular pressure, rates of structural and functional progression, other risk factors, and potential side effects are considered. The initial treatment measures depend on these considerations (Vass et al., 2007).

Treatment category	Treatment type		Mechanism of action
Drugs	Local application	Prostaglandin analogs Beta-blockers Alpha$_2$-adrenergic agonists Carbonic anhydrase inhibitors Miotic agents	Improved uveoscleral and trabecular outflow Reduced aqueous humor production Reduced aqueous humor production, increased uveoscleral outflow Reduced aqueous humor production Widening of the chamber angle
	Systemic intake	Carbonic anhydrase inhibitors Osmotically active substances (mannitol IV)	Reduced aqueous humor production Osmotic removal of water from the globe
Operative interventions	Laser therapy	Laser trabeculoplasty	Increased outflow of aqueous humor via the canal of Schlemm
		Cyclophotocoagulation	Reduced aqueous humor production
	Surgery	Cyclocryocoagulation	Reduced aqueous humor production
		Minimally invasive procedure	For example, implantation of a stent in the canal of Schlemm to lessen the outflow resistance of the trabecular meshwork
		Non-filtering procedure	For example, deep sclerotomy: widening of the outflow pathways without incising the eye
		Filtering procedure	For example, trabeculectomy: creation of an accessory pathway for the aqueous humor to flow out of the eye under the conjunctiva

Table 1. **A summary of methods for lowering intraocular pressure in glaucoma** (Schuster et al., 2020)**.**

2.5.1. Topical Treatment

The current method to reduce intraocular pressure consists of various substance for topical use. These drugs differ based on the mechanisms of action, the reduction degree of intraocular pressure, their dosing, side effects, and cost. In a network analysis of topical first-line drugs, it was found that prostaglandin analogs, beta-blockers, alpha2-adrenergic agonists, and carbonic anhydrase inhibitors are the most effective in reducing intraocular pressure (T. Li et al., 2016). The initial treatment involves the use of prostaglandin analogs applied once daily in the evening. The drug enhances the uveoscleral and trabecular outflow,

resulting in a reduction in the intraocular pressure. Their side effects include conjunctival hyperemia, increased growth of the eyelashes, reduction of periorbital fat, and increased pigmentation of the iris and periocular skin (Alm et al., 2008). Bronchitis, severe cardiovascular conditions, and liver or kidney diseases are systemic conditions that limit the use of prostaglandin analogs. An alternative is the use of beta-blockers that are applied topically, as they reduce the intraocular pressure by diminishing the production of the aqueous humor when usually applied twice a day. The primary local side effect is dry eye disease. Systemic contraindications include bronchial asthma, sinus bradycardia, decompensated congestive heart failure, severe allergic rhinitis, cerebral hypoperfusion, and muscle weakness. The secretion of aqueous humor is decreased by alpha2-adrenergic agonists while the uveoscleral outflow is increased. The drops can cause white discoloration of conjunctiva in the local area after application, and more than one-third of patients experiences topical intolerance over time. The incidence of lid retraction, dry mouth, bradycardia, and fatigue is less common. Although miotic drugs are an alternative, they are not commonly used as the first-line treatment (Fudemberg SJ, 2008).

2.5.2. Laser therapy

Laser therapy can be used as a supplement to local treatment when it's not enough to lower the intraocular pressure or fails to achieve the target pressure. Laser therapy typically causes a moderate decrease of intraocular pressure, through increase of aqueous humor outflow after laser trabeculoplasty (Gulati et al., 2017) or decrease of aqueous humor production after cyclophotocoagulation (Aquino et al., 2015). The potential complications could include pressure reduction that is inadequate or excessive, inflammation, and pupillary deformity, which could cause a very bothersome glare. The use of micropulse laser techniques can be utilized in both applications, but their effectiveness has not been fully documented yet (Michelessi et al., 2018).

2.5.3. Glaucoma surgery

Nonsurgical treatment options do not reduce efficiently intraocular pressure and, in addition, can cause intolerable side effects: for this reason, surgery is recommended. There are different types of surgery types, including minimally invasive, filtering, and non-filtering. To reduce the outflow resistance through the trabecular meshwork, a stent can be inserted into the Schlemm canal in a minimally invasive procedure (Lavia et al., 2017), but, unfortunately, it fails unless the glaucoma is only mild (Malvankar-Mehta et al., 2015).

An accessory pathway is established during filtering operation to allow the aqueous humor to exit eye through the conjunctiva. Trabeculectomy is now the standard for this procedure. Deep sclerectomy and canaloplasty are also available as methods, with lower risk of complications like cataracts and endophthalmitis (Cabourne et al., 2015). Acute angle closure can be treated through surgical procedures, like lentectomy, with intraocular lens implantation or mechanical opening of the occluded angle (iridotomy or iridectomy) (Azuara-Blanco et al., 2016). This can be done as an emergency procedure for persistent angle closure. After the treatment of one eye, the other one should undergo surgery afterwards since the chance of acute angle closure in the other eye is 51% but can be reduced to 2% with this procedure (Lowe, 1972).

NERVE GROWTH FACTOR

Nerve growth factor (NGF), discovered by Rita Levi-Montalcini and Stanley Cohen at the Washington University in the 1950s, is the founding and best characterized member of the neurotrophin family, which also includes brain-derived neurotropic factor (BDNF), neurotropins 3 (NT-3), 4/5 (NT-4/5) and 6 (NT-6). Neurotrophins share a pair-wise sequence identity of roughly 50% and are involved in a range of signaling events on distinct but overlapping neuronal populations. The discovery occurred after the transplantation of a malignant mouse sarcoma into the body wall of a 3-day old chick embryo, where the tumor could release a chemical factor that would induce ganglion growth, production, and atypical ramification of nerve fibers, acting through humoral. Subsequent research revealed that mouse submandibular salivary glands are a superior source of NGF purification, surpassing that found in snake venom (Levi-Montalcini, 1987) (Cohen et al., 1954) (Cohen et al., 1959). NGF plays a key role in the maturation and development of the central and peripheral nervous system. The action is directed towards peripheral sensory and sympathetic neurons, promoting survival, differentiation, functional activity, and maintaining their phenotype. In tissues, NGF is synthesized as a precursor, proNGF in the reticulum of Golgi, and can be cleaved by furin at the intracellular level into mature NGF, by plasmin or matrix metalloproteinases at the extracellular level. In 1991, NGF's crystal structure was discovered with X-ray crystallography, which revealed significant information about its three-dimensional arrangement. NGF's structure is made up of two pairs of amino acids threads that are twisted and antiparallel. To support the fold on one end there are three hairpin loops, and the other end carries a cysteine-knot motif, formed by three di-sulphide bonds. Two

monomers are organized in parallel to form a close-packed homodimer when NGF becomes mature (Figure 6) (Wiesmann & De Vos, 2000).

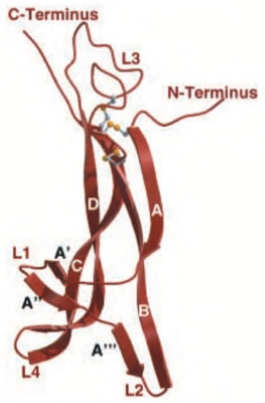


Figure 6. **The structure of the NGF monomer is depicted in a ribbon diagram.** The red is used for labeling the termini and loop regions, L1-L4, and gray and yellow represent the cysteine residues that make up the cysteine-knot motif at the top of the molecule (Wiesmann & De Vos, 2000).

NGF acts through two different classes of trans-membrane receptors, the high-affinity receptor TrkA and the low-affinity receptor $p75^{NTR}$.

Trk receptors can promote neuronal survival, differentiation, neuritic growth, myelination, and synaptic plasticity. However, the expression of $p75^{NTR}$ can determine whether a cell will survive or die depending on the type of cell, functional state, presence or absence, and the type of ligand. The TrkA receptor, which has a molecular weight of 140 kDa, is a transmembrane glycoprotein, encoded by a gene on chromosome 1, belonging to the tyrosine kinase receptors family. TrkA is present in both central/peripheral nervous systems and non-neuronal cells. Its extracellular domain contains a cysteine-rich cluster (domain 1), three leucine-rich repeats (domain 2), a second cysteine-rich cluster (domain 3), and two immunoglobulin (Ig)-like domains (domains 4 and 5) (Pattarawarapan & Burgess, 2003).

The receptor's interaction with its ligand triggers, like other tyrosine-kinase receptors, dimerization which in turn causes intracellular signals through kinase phosphorylation. Furthermore, it activates transcription factors within the nucleus that lead to the expression of specific genes. The interaction between NGF and TrkA results in the phosphorylation of two tyrosine sites: Y758 and Y490, which interact with the phospholipase Cγ (PLC γ) and the adaptor protein Src homology 2-containing protein (Shc). PLCγ catalyses the hydrolysis of phosphatidyl inositol 4,5-bisphosphate in diacylglycerol (DAG) and inositol (1,4,5) triphosphate (IP3). The release of neurotransmitters is enhanced by the release of calcium from intracellular reserves by IP3 while DAG activates a series of kinases. Furthermore, Shc

binds to growth factor receptor-binding protein-2 (Grb-2) when it is phosphorylated by TrkA. Subsequently, these proteins activate the PI3K/AKT: PI3K stimulates Akt, a protein with serine-threonine kinase activity, which induces cell survival. Akt's ability to suppress apoptotic signals is mediated by inactivating pro-apoptotic proteins, such as Bad, which prevents Bad's association with Bcl2. In contrast, phosphorylation of TrkA leads to the recruitment of a guanine nucleotide exchange factor (GEF), which facilitates GTP phosphorylation and activation of the membrane-associated G protein Ras. The Ras activation promotes the Raf phosphorylation, which then activates mitogen-activated protein kinase (MAPK) and subsequently the ribosomal s6 kinase (RSK). When Akt or RSK are activated, they phosphorylate the cyclic AMP response element binding protein (CREB) transcription factor, which then moves into the nucleus and carries out the trophic and anti-apoptotic effects of NGF. As a final effect, the Ras/MAPK cascade induces the activation of transcription factors involved in survival and cell proliferation (Figure 7) (Freund-Michel & Frossard, 2008) (Chao MV, 2003).

The $p75^{NTR}$ receptor is a transmembrane glycoprotein, with a 75 kDa molecular weight, that is part of a family of death receptors that is distinguished by a domain that is related to death and a distinct intracellular sequence. Activation of this receptor leads to the death or survival of cells in neurons and structural cells, and it depends on the signaling pathways and the recruitment of adaptor proteins or co-receptors in the $p75^{NTR}$ receptor. It belongs to the tumor necrosis factor receptor family and binds to all neurotrophins and pro-neurotrophins with nanomolar affinities (Chao MV, 2003). The $p75^{NTR}$ receptor is composed of four extracellular repeats that are negatively charged and need to be present for full-affinity NGF binding, and a specific cytoplasmic domain that is highly conserved among species. The $p75^{NTR}$ receptor is bound to neurotrophins by the negatively charged 4 cysteine-rich domains (CRD-1/-4). NGF has two bond's sites to the $p75^{NTR}$ receptor, the site I composed of the CRD1-CRD2 junction and the CRD2 domain while the site II composed of the CRD3-CRD4 junction. According to crystallographic studies, an asymmetrical complex composed of a dimer of NGF bound to a single monomer of the $p75^{NTR}$ receptor, can also bind a TrkA monomer and create a heterodimer (He and Garcia, 2004). The cellular context in which $p75^{NTR}$ is expressed determines its biological effect. In absence of the Trk, it oversees activating the Jun amino-terminal kinase (JNK) during apoptosis processes. Through the activation of proteins p53, Bad, Bim, and Bax is translocated to the mitochondria, and cytochrome c is released, leading to the phosphorylation of c-jun. This leads to caspases 3, 6 and 9 activations. By activating $p75^{NTR}$, it is possible to increase ceramide levels and

trigger the cell death pathway, which includes JNK-p53-Bax proteins and small G proteins of the Rho family, leading to the activation of MKKK and MKK4 and 7. When NGF bind to p75NTR, cytoplasmic adapter proteins recruited TRAF6 (tumor necrosis factor receptor member), resulting in activation of the inhibitor of kB kinase (IKK) and canonical NF-kB signaling. The transcription of peptides with antiapoptotic activity (AIP or BCL-2 family) or molecules that block or apoptotic pathways (Gadd45β) is induced by the activation of NF-κB. Moreover, JNK, a kinase that phosphorylates transcription factor c-Jun, can be activated by TRAF6 and neurotrophin receptor interacting factor (NRIF). Subsequently, the c-jun activation modulates the upregulation of apoptosis by the expression of pro-apoptotic genes. In the presence of TrkA, $p75^{NTR}$ boosts the affinity between TrkA and NGF, generating high-affinity binding sites, and promoting survival (Nykjaer et al., 2005) (Papa et al., 2004).

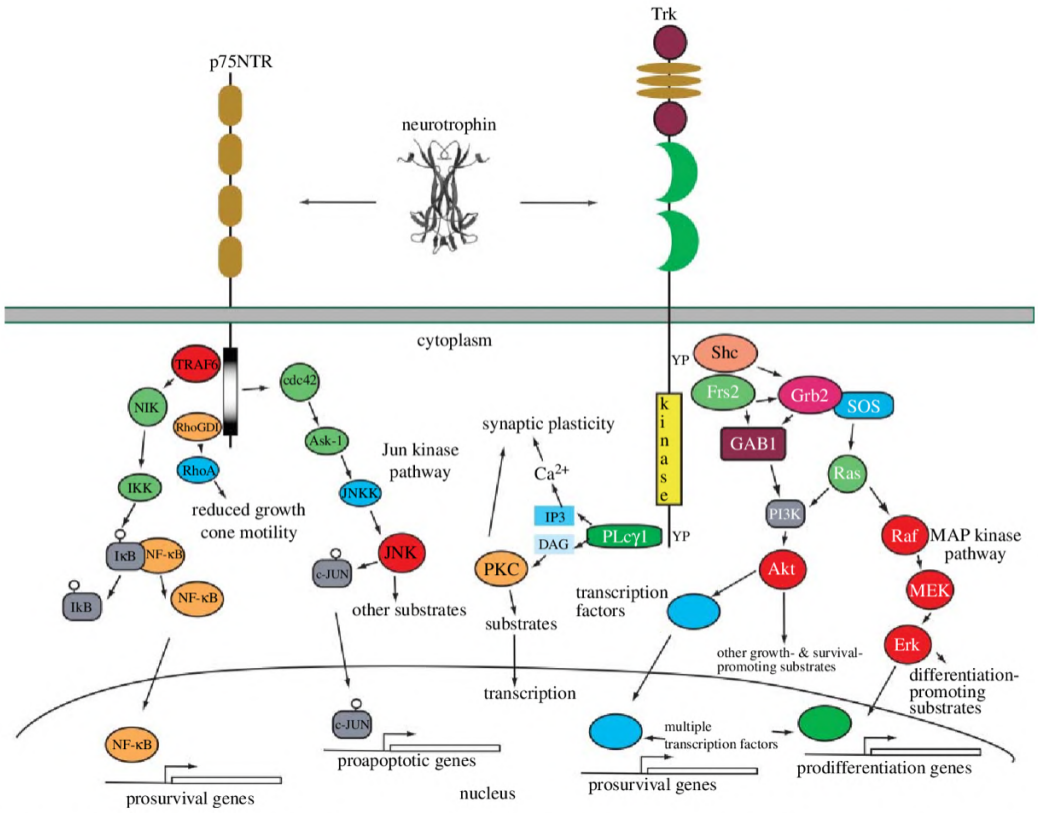


Figure 7. **Neurotrophin signaling.** NGF's interaction with the Trk receptor results in dimerization, which leads to autophosphorylation and activation of multiple signaling pathways. Trk signaling regulates differentiation, survival, plasticity, and synaptic function. The regulation of apoptosis or survival in neuronal and structural cells is regulated by p75NTR by interacting proteins (Louis F Reichardt, 2006).

3.1. NGF role in the eye

In physiological and pathological conditions, the nerve growth factor and its receptors play an important role in the eye. In anterior segment of the eye, NGF, in basal condition, plays a role in trophism and maintaining the integrity of the corneal epithelium, and when it is co-expressed with the TrkA receptor, it suggests that there is an autocrine and/or paracrine mechanism of action. This mechanism supports the survival and function of epithelial cells. The corneal tissue reacts to the mechanical insult with an increase of the NGF synthesis levels when it gets damaged (Lambiase et al., 2000). NGF has been shown to have a potential role in conjunctival cells, where it is produced, stored, released, and used. The homeostasis of the tear film is maintained by NGF which is basally released from the lacrimal gland in both aqueous humor and tissue. After eye injury, NGF is overexpressed and released by the iris and ciliary body (Lambiase et al., 2002). In the posterior segment of the eye, vitreous, choroid, optic nerve, and retina (retinal pigment epithelium, Müller cells, and RGC) express NGF (Micera et al., 2004). The development and differentiation of the retina and optic nerve can be controlled by NGF, which also promotes the survival and recovery of RGC. In retinal detachment experiments, it has been demonstrated, that NGF plays a protective role, when injected intravenously, diminishing the degeneration of retinal cells (Bai et al., 2010). In different cell types in the retina, NGF promotes several effects including proliferation, plasticity, transmitter synthesis, reorganization, cytoskeleton changes, and synaptic transmission (Figure 8). Studies have demonstrated NGF is able to prevent the deterioration of ganglion cells and nerve fibers in the optic nerve (Carmignoto et al., 1989).

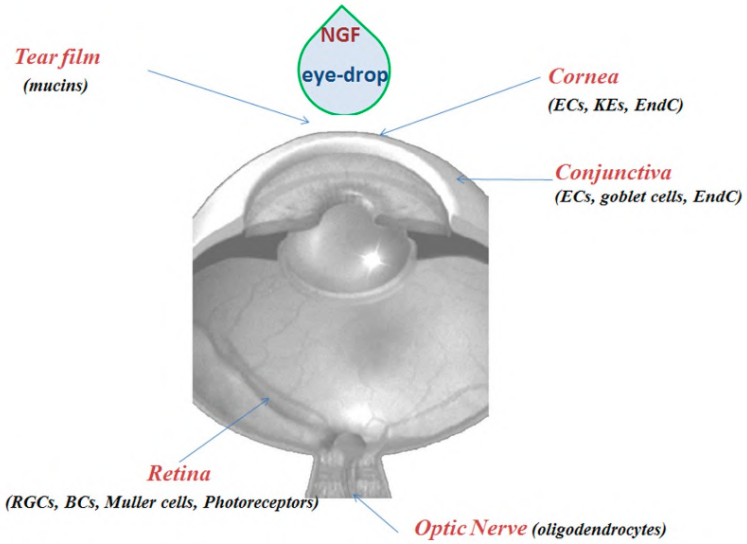


Figure 8. **NGF role in the eye.** The types of eye cells that respond to NGF. ECs: epithelial cells, KEs: keratocytes, EndC: endothelial cells, RGCs: RGC, BCs: bipolar cells (Aloe et al., 2015).

3.2. NGF and eye diseases

As previously mentioned, the anterior and posterior segments of the eye express NGF and its receptors, which perform various tissue functions in both physiological and pathological conditions. The onset or severity of ocular pathologies can be attributed to changes in NGF expression and its receptors in these ocular segments in both animal models and patients. Moreover, the use of NGF in wound healing processes has been evaluated based on its biological activities in neuronal and non-neuronal cells. Several studies have demonstrated the potential therapeutic efficacy of NGF, which can be injected directly into the eye, thus acting on the ocular surface and the optic nerve, retina, and brain. Based on the results, its efficacy in preventing tissue damage and loss of visual functions it has been suggested, thus considering NGF a candidate pharmacological molecule for healing the visual system.

3.2.1. Dry eye

NGF may play a crucial role in dry eye pathology and could be used as a therapeutic treatment due to its ability to regulate lacrimal production and homeostasis. In vivo research has demonstrated that the topical use of NGF in a dry-eye dog model can enhance tear production and conjunctival goblet cell density (Coassin et al., 2005). A phase II clinical trial also demonstrated significant improvement in dry eye patients after 4 weeks of rhNGF therapy. Current treatments mainly focus on lubricating the eye or reducing inflammation, as there is no specific therapy available (Lambiase et al., 2011).

3.2.2. Neurotrophic Keratitis

Neurotrophic keratitis (NK) is a rare degenerative corneal condition caused by impaired trigeminal innervation due to surgery, diabetes, or multiple sclerosis, leading to reduced corneal sensitivity and epithelial damage. Classified as an orphan disease, NK has no standard treatment. However, the European Medicines Agency (EMA) has approved a molecule derived from *Escherichia coli* that is structurally identical to human NGF as a first-class treatment for NK. Administered as eye drops, this drug promotes corneal epithelium survival, stimulates tear production, provides lubrication, and helps maintain corneal innervation (Bonini et al., 2018) (Versura et al., 2018).

3.2.3. Retinitis Pigmentosa

Faktorovich et al. (1992) discovered that neurotrophic factors, such as NGF, could have a protective effect against retinal cell degeneration in rodents with retinitis pigmentosa (RP), a genetic disorder that results in progressive photoreceptor degeneration and vision loss. In 1996, NGF was shown to delay photoreceptor degeneration in a mouse model of RP (Lambiase Luigi Aloe et al., 1996). Further studies in mice and rats demonstrated that topical NGF could also delay degeneration, while in humans, patients treated with NGF eye drops reported temporary improvements in visual performance and field (Falsini et al., 2016).

3.2.4. Glaucoma

Turner et al. (1979) found that NGF could protect retinal cells and promote optic nerve regeneration in goldfish. Later studies in cats and rodents confirmed that NGF influences neuroplasticity in the visual cortex and geniculate nucleus (Maffei et al., 1992). It may also promote functional recovery of RGCs after ischemia, while intracerebral anti-NGF administration in rodents caused a delayed consolidation of the visual cortex synapses (Siliprandi et al., 1993) (Carmignoto et al., 1989) (Domenici et al., 1991). Various research has demonstrated that the application of NGF promoted the recovery of axotomized optic nerves, ocular ischemia, and ocular hypertension (Lambiase et al., 1997). Furthermore, it was observed, using a rodent model of glaucoma induced through injection of hypertonic saline into the episcleral vein, that the levels of NGF and NGF-receptors in the retina were significantly down regulated. However, the administration of NGF in the eye reduced loss and promoted recovery of damaged RGCs (Crimmins & Maeder, 2009) (Sharon Kingman, 2004).

TrkA is expressed by RGCs, and NGF interacts with it, leading to upregulation of Bcl-2, which protects cells from apoptosis by preventing caspase activation.

Subsequent experiments on patients with glaucoma, who presented progressive visual field defects, showed that individuals treated with NGF presented a lower reduction of RGC, associated with inhibition of cell death by apoptosis, in comparison with the untreated ones (Sposato et al., 2009). In addition, patients treated with NGF presented improvements in optic nerve function, contrast sensitivity and visual acuity (Lambiase, A et al., 2009). NGF also had neuroprotective effects in glaucomatous rabbits by decreasing RGC death and improving visual function (Morrison et al., 1997).

3.2.5. Macular Degeneration

The macula, a highly pigmented area in the center of the retina, is affected in maculopathy, a condition commonly linked to age-related macular degeneration (Yuan D, 2013) (Jager et al., 2008). Retinal cells express NGF receptors and respond to NGF. A 94-year-old patient with macular degeneration, unresponsive to existing treatments, was given topical NGF eye drops for 6 months. After 3 months, the patient showed significant improvement in visual acuity and electrofunctional parameters, with no adverse effects observed. This suggests that topical NGF may be a promising therapy for maculopathy (Lambiase A, 2009).

miRNAs

MicroRNAs, also known as miRNAs, are a group of small non-coding RNAs (~22 nucleotides) that play a role in regulating gene expression at the post-transcriptional level, in both plants and animals (Bartel, 2004a). MiRNAs can specifically modulate the expression of target genes by pairing with sequence motifs in the 3' UTR of mRNAs that have perfect or near-perfect complementarities, leading to degradation of the target mRNAs or, alternatively, inhibition of translation. MiRNAs play a role in maintaining cell homeostasis and regulating fundamental biological processes like growth, proliferation, apoptosis, and cell metabolism. Dysregulation of miRNAs expression levels has been described associated with relevant human diseases, such as viral, immune, neurodegenerative, and cancer-related diseases (Li & Kowdley, 2012). MiRNAs can be also easily detected in all body fluids, including blood, saliva, urine, tears, where they are highly stable and can be released either by active (i.e. secretion by exosomes or vesicles) or passive mechanisms (i.e. cell death). For these reasons, miRNAs are considered highly promising candidate biomarkers. In addition, due to the availability of synthetic molecules that can restore their normal expression (e.g., mimic miRNAs and antagomir), they are also considered as putative therapeutic targets: this is considered very intriguing for ocular diseases, as the eye is well demarcated and easily accessible.

4.1. The Discovery of miRNAs

MicroRNAs (miRNAs) were first discovered in *Caenorhabditis elegans* in 1981 by Gary Ruvkun's group, when they identified the lin-4 gene, which regulates post-embryonic development. They found that mutations in this gene led to continuous larval cuticle synthesis, suggesting its role in developmental control. Lee’s study in 1993 further clarified

that lin-4 produces small RNA molecules (22 and 61 nucleotides) that don't code for proteins but regulate gene expression post-transcriptionally by interacting with target mRNAs, like lin-14 (Olsen & Ambros, 1999). The discovery of let-7, the second miRNA, extended this concept, showing that miRNAs play a role in regulating developmental processes. Let-7 is highly conserved across species, indicating a broader regulatory role in gene networks (Schulman & Ball, 2014). MiRNAs such as let-7 and lin-41 are found in many multicellular organisms, highlighting their importance in regulating genes across different species (Hammond et al., 2001). MiRNAs are found exclusively in multicellular organisms, and their sequences are conserved across animal species. About 55% of *C. elegans* miRNAs are similar to human miRNAs, suggesting their evolutionary importance (Ibáñez-Ventoso et al., 2008). Unlike plant miRNAs, which have different biogenetic mechanisms, animal miRNAs have evolved independently. Computational analysis shows that miRNA genes make up about 1% of various species' genomes, and it's estimated that 30% of genes are regulated by miRNAs (Bartel, 2004b). In humans, over 2000 miRNAs have been identified (Kozomara & Griffiths-Jones, 2011). MiRNAs are critical for human development, physiology, and disease. They regulate key processes like cell differentiation, proliferation, and apoptosis. As miRNAs are implicated in numerous diseases, their potential as biomarkers for diagnosis and prognosis is being explored (Saeidimehr et al., 2015). MiRNAs belong to a broader category of non-coding RNAs (ncRNAs), including siRNAs, tRNA, and snRNA, which do not code for proteins but are crucial in gene regulation. MiRNAs can be located in various genomic regions: 40% in introns of protein-coding genes, 10% in intronic sequences of RNA transcripts, and 50% as independent transcription units (Kim & Nam, 2006).

4.2. MiRNAs biogenesis

MiRNAs must undergo a maturation process that starts within the nucleus and ends in the cytoplasm. Introns of protein-coding genes often harbor first-messenger miRNA genes, which are transcribed by either RNA polymerase II (Pol II) or RNA polymerase III (Pol III). The microRNAs are transcribed as long primary transcript, called pri-miRNA, with the length of several hundred nucleotides, that in the nucleus folds to form a double filament hairpin structure, often containing the sequence for different mature miRNAs. The stem-loop structure of pri-miRNAs includes a 5'-7-methylguanosine cap and a poly(A) tail, and contains sequences of uridine residues, which would be expected to prematurely terminate pol III transcription. In the nucleus, Drosha RNase III endonuclease, which is roughly

160kDa, performs the nuclear cleavage of the pri-miRNA. Drosha in cooperation with protein DiGeorge syndrome critical region gene 8 (DGCR8), known also Pasha in flies and nematodes, cleaves both strands of the stem at sites near the base of primary stem loop, releasing 60-70 nucleotides stem loop intermediate known as pre-miRNAs (miRNA precursor), with 5' phosphate and 2-nucleotide 3′-overhang. Cropping is the term used for this phase (Lee et al., 2002). The RanGTP-binding nuclear transporter exportin-5 (Exp5) exports pre-miRNAs from the nucleus to the cytoplasm (Yi et al., 2003). Once inside the cytoplasm, these hairpin precursors are cleaved by RNaseIII endonuclease Dicer into a small, imperfect dsRNA duplex (miRNA: miRNA*), about 25 bases in length. MiRNA is a mature miRNA whereas miRNA* is the opposing arm of the miRNA. The Dicer is composed of helicase domains, DUF283 domain, PAZ (Piwi–Argonaute-Zwille) domain, two tandem RNase-III domains, and a dsRNA-binding domain (dsRBD). The Paz domain identifies the 3' overhang. Both strands are cleaved by the two RNase-III domains after the helicase domain identifies the loop region. Once the miRNA complex is loaded into the Dicer, the process takes place (He et al., 2004).

The mature miRNA is incorporated in the RNA-induced silencing complex (RISC), assembled by the interaction between argonaute family proteins (Ago1-Ago4) and Dicer in mammals. In the presence of RNA-dependent RNA polymerase, the other strand is either degraded to Ago2 or can serve as a template to synthesize new dsRNA, leading to the creation of more miRNAs. Within the RISC protein complex, mature miRNAs are usually capable of matching by 2-8 nucleotides present at the 5'end, known as "seed sequence", to complementary sequences in the 3'UTR region of messenger RNA (mRNA) and to regulate gene expression at the post-transcriptional level (Diederichs & Haber, 2007). The stability of the 5′ ends of the two arms of the miRNA: miRNA* duplex can vary depending on how Dicer processes the pre-miRNA into the miRNA: miRNA* duplex. The active miRNA is incorporated into the RISC to the relative instability (lower thermodynamic energy) at the 5' end of the mature miRNA (Schwarz et al., 2003). Mature miRNAs control target genes' expression by base pairing to the sequence motif in the 3'UTR of mRNA: perfect or near perfect complementarity between miRNA and target produces the cleavage of the mRNA by Ago2, whereas imperfect base pairing lets the mRNA's destabilization by miRNA/RISC complex through deadenylation and subsequent decapping (Figure 9). Translation is terminated prematurely in this way (Xie et al., 2005). Multiple target sites can exist within the mRNA transcript of a downstream gene for one miRNA, and multiple miRNAs can target one mRNA simultaneously. Thus, microRNAs play a crucial role in the development and

normal functioning of almost all tissues and organ systems through their newly recognized level of gene expression regulation.

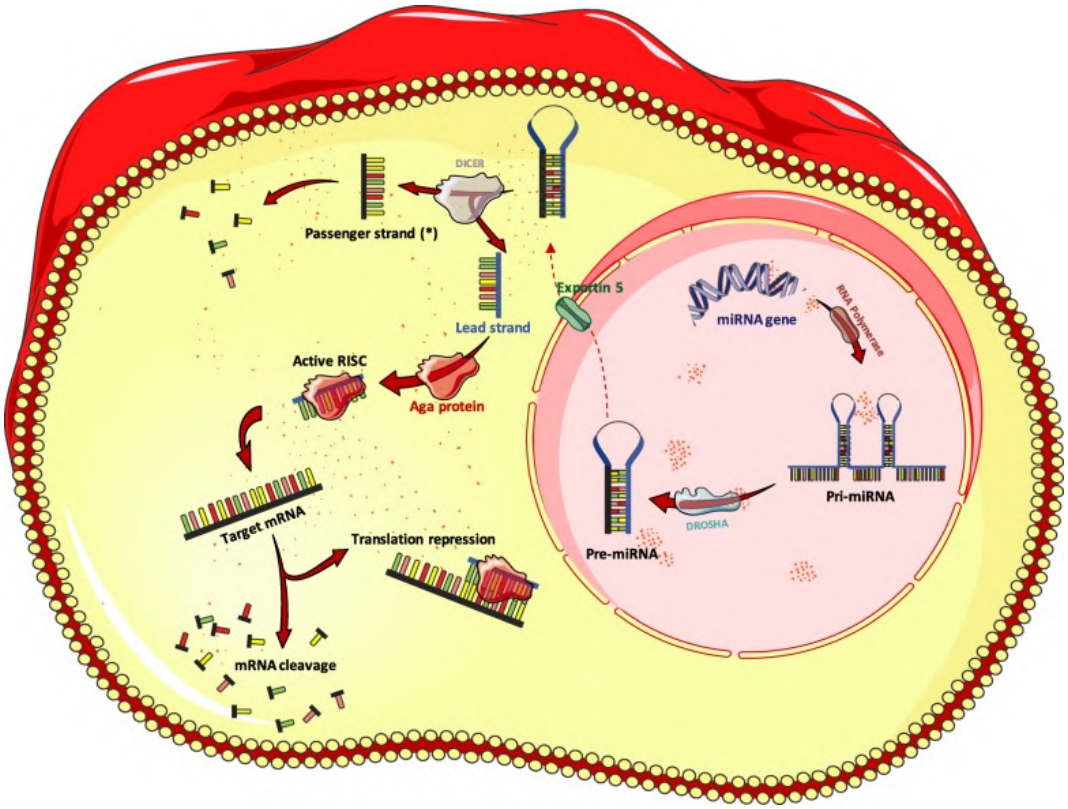


Figure 9. **Biogenesis of miRNAs.** pri-miRNAs are transformed into pre-miRNA by Drosha and DGCR8 within the nucleus. Then, they are exported from the cytoplasm nucleus by Exp 5 and Ran-GTP. Later, the pre-miRNAs are processed by Dicer in mature double-stranded microRNA (dsRNA, miRNA: miRNA*). Dicer also processes long dsRNA molecules in small interferent RNA (siRNA) duplex. These dsRNAs are subsequently bound by AGO2 and incorporated by RISC that allows the degradation of the previously unused complementary filament, while the filament remained associated with AGO2 constitutes the mature miRNA. Once incorporated by RISC, the miRNA induces the mRNA cleavage if there is sufficient complementarity between the two, while it represses the translation, if the mRNA has a partial complementarity. The degradation of the mRNA occurs after recognition of the ORF (open reading frame) by the miRNA (Daoud et al., 2019).

4.3. Role of miRNAs in the eye

Numerous miRNAs show expression patterns that are specific to tissue and developmental stages, suggesting potential unique functions in the ocular tissues. The abundance of miRNAs in the eye suggests that they may be responsible for maintaining visual health, and even in pathological conditions, through gene regulation.

MiRNAs have been identified to have crucial functions in the eye development. One of the first studies on the specific expression of miRNA in the eye was conducted by Lagos-Quintana et al. (2003), who identified 21 new miRNAs in various mouse tissues. Among these, it was observed that specific tissues had higher expression of some miRNAs, like miR-124, which was found both in the eyes and the spinal cord, probably due to the presence of neuronal cells. Through subsequent studies, has been shown that miR-124a persists in the neural retina from development until adulthood. All eye layers, including photoreceptors, neurons within the inner nuclear layer, and the ganglion cell layer, show strong expression of miR-124a (Loscher et al., 2008) which targets different developmental genes (Rdh10), confirming its involvement in retinal development (Arora et al., 2007). MITF, a transcription factor that is highly expressed in RPE and crucial for maintaining neuroretinal identity, is another target (Adijanto et al., 2012). Subsequently, through a profiling of 78 miRNAs in the mouse retina, heart and brain, Xu et al. (2009) confirmed that 19 of the 21 miRNAs emerged in the Lagos-Quintana's study, were expressed in the adult mouse retina: in particular, six of them were expressed exclusively in the retina (mir-96, mir-182, mir-183, mir-184, mir-210, mir-140AS). Thus, it appears that the normal retinal functions may be regulated by these miRs. Analysis of miRNA expression both during embryogenesis and after birth has demonstrated that specific miRNAs can be expressed during various stages of retinal development to control both proliferation and sequential differentiation of retinal progenitor cells (RPCs) (Xu, 2009).

MiR-204 is expressed at a high level in RPE, the ganglion cell layer, lens epithelial cells, the ciliary body, and the retina of the mouse embryos. This indicates that miR-204 can be involved in the differentiation of RPE and neuronal cells from their shared precursors as well as in the development of the retina (Loscher et al., 2008). Conte et al. (2010) demonstrated that the dysregulation of Meis transcription factor led to the loss of mir-204 function in the development of the eye, which resulted in macroscopic abnormalities. Through targeting Meis, the miRNA indirectly fed back Pax6 (Shaham et al., 2013).

MiR-204 and miR-211 are both widely expressed in RPEs. The seed region sequences shared classified them as a family with the same targets. Wang FE (2010) revealed that high

expression of miRs-204/211 can preserve epithelial phenotype, while maintaining high expression levels of tight junction proteins and membrane channels, which helped to preserve the interaction between retinal photoreceptors and RPE, physiology and epithelial barrier function. In vivo studies were conducted on rats to demonstrate the protective role of miR-211 on RGC, and it was found that up-regulation of this miR down-regulated Frs2 expression, which resulted in a decrease in cell death (Yang et al., 2018). Karali et al. (2007) demonstrated that miR-182, miR-183, and miR-96 all belong to the same genomic region. The targeted of one of these three caused visible alterations in the retina and this explained the overlap in functions, as they have shared targets and sometimes one can replace the other. The presence of these miRs was apparent in all retinal layers (miR-182), ganglion cell, inner plexiform, and inner nuclear layer (miR-181), whereas miR-183 was only observed in the outer nuclear layer. The role of this miRNA in photoreceptor homeostasis and as a pro-survival factor under stressful conditions has been explained by its neurogenic effects and association with neurotransmitter transporters, Casp2, and survival genes (Rac1) (Zuzic et al., 2019). It has been shown that the cluster miR-183 targets Casp2, given that an increase of both was detected in wild-type mice in response to light stimuli. The degeneration of the retina caused by light was prevented in transgenic mice by pharmacological inhibition of Casp2. This study demonstrated that the miR-183 cluster is responsible for protecting and maintaining photoreceptors (Zhu et al., 2011). In addition, to demonstrate that miRNAs play a key role in maintaining homeostasis and retinal functions, mice Dicer CKO (conditional knockout) models were created, which allow to detect the phenotypic consequences of the loss of miRNA gene regulation in the visual system. Different levels of phenotypes were observed in retinal progenitors after Dicer was deleted, including retinal degeneration, progressive disorganization of retinal morphology, abnormalities in electroretinogram responses, abnormal differentiation of retinal cell types, and diffuse apoptosis (Damiani et al., 2008) (Sundermeier et al., 2016). Ryan et. al. (2006) found that miR-184 was expressed more frequently in the lens and corneal epithelium and did not change during wound healing after injury to the epithelium. Corneal epithelium differentiation and extracellular matrix synthesis, transparency maintenance, and avascularity are all modulated by miR-184 expression in corneal epithelium and lens. In the same study the expression of miR-205 was found to be expressed in all layers of the corneal, limbal and conjunctival epithelium. The healing process of human corneal epithelial cells (HCEC) involves this microRNA due to its ability to inhibit KCNJ10 at every stage of the healing process, from migration to proliferation (Lin et al., 2013). The migration of keratinocytes is also influenced by miR-

205 and miR-184. It was shown that miR-205 repressed SH2-containing phosphoinositide 5'-phosphatase 2 (SHIP2) that dephosphorylated phosphatidyinositol 3,4,5-triphosphate (PIP3), which was involved in many signaling pathways, including that of Akt. The latter was improved by miR-205's suppression of SHIP2, increasing cell survival. According to Yu J (2010), the use of miR-205-antagomiR caused SHIP2 level decrease, inducing lower keratinocyte migration. However, down-regulation of miR-205 resulted in an increase in Rho-ROCKI activity and decrease in actin cytoskeleton due to cytoskeleton rearrangement events. Their research revealed that the removal of mir-184 from human corneal epithelial keratinocytes (HCEK) improved Akt signaling, and wound seals were faster, allowing miR-205 to inhibit SHIP2. MiR-26a, miR-31, miR-125b, miR-181, and miR-204 have been found highly expressed in corneal and lens epithelium. It could be also considered that the transcription regulation of these miRs may be directed by eye specific transcription factors such as Pax6 (Collinson et al., 2003). This indicates that miRNAs, if specific to tissues, can have impact on preserving tissue identity. Cieślar-Pobuda et al. (2016) confirmed, through a miRNA profiling, the connection between Pax6 and the ocular diseases using pluripotent stem cells as a model of corneal epithelial embryogenesis. It was discovered that miR-450-5p plays a crucial role in Pax6's molecular switch. MiR-450-5p plays a role in the fate of corneal epithelial cells by replacing Pax6, but its deletion allows for the expression of Pax6 on the ocular surface, which facilitates the formation of the corneal epithelium. In addition, this research demonstrated that miR-184 reduction caused Pax6 and K3 level decrease, which play a role in corneal integrity, likely via indirect mechanisms since the 3'UTRs of Pax6 and K3 lack binding sites for miR-184. In the limbal and corneal tissues, miR-143 and miR-145 were discovered be expressed by Lee et al. (2011). The development and differentiation of corneal epithelium cells is influenced by miR-145, which controls the formation and integrity maintenance of the epithelium cells by regulating integrinβ8 (ITG8B). In vitro, ITG8B was found express in epithelial and neuronal cells and was responsible for regulating the activation of transforming growth factor β (TGFβ) in matrix modeling, cell growth, epithelial homeostasis, and vasculogenesis (Cambier et al., 2005). The up-regulation of IFNB1, which has anti-inflammatory activity, was observed in human corneal epithelium cells (HCE) after miR-145 transfection. This could contribute to the improvement of anti-inflammatory activity of corneal cells. The inhibition of apoptosis in corneal keratinocytes caused by MiR-122 involves the downregulation of cytoplasmic polyadenylation element-binding protein-1 (CPEB1). By preventing the expression of inflammatory cytokines caused by apoptosis, it also minimizes the risk of immune rejection

following corneal transplantation (Wang et al., 2017). MiR-206, a miRNA that is normally absent in the mouse cornea, was found to be overexpressed in alkali-burned cornea models and led to inflammation by modulating Connexina 43 (Cx43), which is associated with inflammatory responses in the injured cornea, as demonstrated by Li et al. (2015) (Table 2).

miRNAs	Layer of eye	Target genes	References
miR-124a	All eye layers	RDH10; MITF	Arora et al., 2007; Loscher et al., 2008
miR-204	RPE	Meis; PAX6	Conte et al. 2010; Shaham et al., 2013
miR-211	RPE	FRS2	Yang et al., 2018
miR-181	Ganglion cell, inner plexiform, and inner nuclear layer	Casp-2; Rac1	Zuzic et al., 2019 ; Zhu et al., 2011
miR-182	All retinal layers		
miR-183	Outer nuclear layer		
miR-184	Lens and corneal epithelium	KCNJ10; PAX6	Lin et al., 2013
miR-205	All layers of the corneal	KCNJ10; SHIP2; PIP3; Rho-ROCK1	Lin et al., 2013; Yu J. 2010
miR-26a	Lens and corneal epithelium	PAX6	Collinson et al., 2003
miR-31a			
miR-125b			
miR-181			
miR-204			
miR-450-5p	Corneal epithelial cells	PAX6	Cieślar-Pobuda et al. 2016
miR-143	Corneal tissues	ITG8B; TGFβ	Cambier et al., 2005
miR-145		ITG8B; TGFβ; IFNB1	
miR-122	Corneal tissues	CPEB1	Wang et al., 2017
miR-206	Mouse cornea	Cx43	Li et al. 2015

Table 2. **MiRNAs show expression patterns that are specific in ocular tissues.**

4.4. MiRNAs involved in retinal diseases

The regulation of pathological processes such as age-related macular degeneration, retinoblastoma, diabetic retinopathy, glaucoma, that are diseases that affect the posterior segment and pterygium, cataract, keratoconus, dry eye, uveitis, uveal melanoma that affect the anterior segment, have been linked to the dysregulation of miRNA expression by several studies (Table 3).

4.4.1. Retinoblastoma

Retinoblastoma (RB), the most common eye cancer in children, is primarily caused by alterations or loss of the RB1 gene. It is also linked to other genetic changes, including the inactivation of the p53 pathway due to MDMX amplification, which promotes tumor progression. Studies have shown that p53 activates miR-34 family members, suppressing tumors through cell cycle arrest and apoptosis (He et al., 2007). MDMX is a target of miR-34a, and its expression in RB could inhibit tumor growth. Additionally, Sirt1, a deacetylase that regulates p53 activity, plays a role in RB progression (Dalgard et al., 2009). Research has identified several miRNAs, such as miR-494, let-7e, miR-129, and miR-373*, that are upregulated in RB compared to normal retina (Zhao et al., 2009). MiR-365b-3p, a tumor suppressor, is downregulated in RB and inhibits PAX6, leading to reduced cell growth, cell cycle arrest, and apoptosis (Wang et al., 2013).

4.4.2. Diabetic Retinopathy

Diabetic retinopathy (DR) is a common complication of diabetes, characterized by microvascular changes leading to retinal permeability, ischemia, neovascularization, and macular edema. These changes are driven by growth factors like VEGF, which stimulates neovascularization, and miRNAs, which have been shown to regulate retinal and choroidal neovascularization. For example, miR-31, miR-150, and miR-184 can reduce VEGF levels, impacting neovascularization (Shen et al., 2008). In diabetic rats, alterations in miRNA expression were observed, particularly in retinal endothelial cells (RECs), which are among the first affected in DR pathogenesis (Ryan et al., 2006) (Kovacs et al., 2011). Increased levels of NF-κB-responsive miRNAs, including miR-146a and miR-21, were found in RECs, and p53-responsive miR-34 family members were elevated, suggesting their role in preventing apoptosis in neuroretinal and endothelial cells. Other studies have identified dysregulated miRNAs in the retina and serum of diabetic mice. MiR-20a, miR-20b, and

miR-106 were down-regulated, while miR-27a and miR-206 were up-regulated, with effects on VEGF and HIF expression, influencing retinal neovascularization and angiogenesis (Platania et al., 2019) (Nunes DN, 2015). MiR-206 and miR-381-3p also modulated brain-derived neurotrophic factor (BDNF), a key factor in DR pathology. Additionally, miR-195, elevated in retinal cells exposed to high glucose, down-regulated SIRT1, contributing to aging, vascular permeability, and fibronectin increase (Mortuza et al., 2014). These findings highlight the significant role of miRNAs in the regulation of retinal changes in DR.

4.4.3. Age related macular degeneration (AMD)

Age-related macular degeneration (AMD) is the leading cause of blindness in developed countries and is classified into dry and wet forms. Dry AMD is marked by abnormal RPE pigment distribution and drusen bodies, while wet AMD involves choroidal blood vessel growth, leading to retinal damage and edema (Velez-Montoya et al., 2014). Studies show that miRNA expression differs between wet AMD patients and healthy controls, with miRNAs like miR-21, miR-25-3p, and miR-146a being significantly dysregulated. These miRNAs may work as early detection biomarkers for AMD (Ertekin et al. 2014). MiR-146a is up-regulated in both forms of AMD and plays a role in inflammation, being controlled by NF-κB and elevated by oxidative stress and pro-inflammatory cytokines. Its up-regulation is linked to the down-regulation of complement factor H (CFH), contributing to AMD progression (Pogue et al., 2009) (Berber et al., 2017). MiR-146a interacts with NF-κB modulators like IRAK1 and TRAF6, inhibiting pro-inflammatory cytokine signaling (Taganov et al., 2006). Other miRNAs, including miR-9, miR-125b, and miR-155, also contribute to AMD progression through CFH-mediated inflammation (Ménard et al., 2016) (Lukiw et al., 2012). Additionally, miR-9 regulates retinal pigment epithelium (RPE) function and inflammatory cytokines like TNF-α and IL-1β (Askou AL, 2018). MiR-184, which negatively regulates Wnt signaling, is down-regulated in AMD and is essential for photoreceptor efficiency in RPE cells (Murad et al., 2014) (Shen et al., 2008). The involvement of miRNAs in inflammation, angiogenesis, and oxidative stress suggests that they could be used as biomarkers and therapeutic targets for AMD (Murad et al., 2014) (Ménard et al., 2016).

4.4.4. Glaucoma

MiRNAs play a significant role in glaucoma pathogenesis by influencing the balance of aqueous humor (AH), trabecular meshwork variability, and retinal cell apoptosis. They impact the trabecular meshwork by regulating extracellular matrix (ECM) deposition, trabecular meshwork cell contraction, and structural anomalies. For example, exposure to H2O2 up-regulates miR-183 in trabecular meshwork cells, influencing genes like ITGB1 and KIF2A, which affect cell functionality in aging and glaucoma (Li et al., 2010). The transformation growth factor (TGF-β) also regulates ECM changes in primary open-angle glaucoma, and miRNAs such as the miR-29 family modulate ECM protein synthesis, influencing intraocular pressure (Villarreal et al., 2011). Chronic oxidative stress down-regulates miR-29b, leading to increased ECM deposition and cell loss (Luna et al., 2009). MiR-24 can reduce TGF-β expression by targeting FURIN, which activates TGF-β (Luna et al., 2011). High expression of miR-200c in trabecular meshwork cells suppresses contraction-related genes, and its intraocular injection has been shown to lower intraocular pressure, offering a potential therapeutic approach for glaucoma (Luna et al., 2012). In glaucomatous retinas, several miRNAs, such as miR-204, miR-106b, and miR-25, are down-regulated, affecting TGF-β signaling, while miR-27a is up-regulated, influencing mTORC-1 signaling and axonal development (Jayaram et al., 2015). Additionally, miR-96 up-regulation decreases retinal ganglion cell (RGC) activity and promotes apoptosis through caspase-2 activation (Wang & Li, 2014).

Retinal diseases	miRNAs		Target genes	References
Retinoblastoma	miR-34a	↑	MDMX	He et al., 2007; Dalgard et al., 2009
	miR-494	↑	There is no functional study reported of these miRNAs in tumor development	Zhao et al., 2009
	Let-7e	↑		
	miR-129	↑		
	miR-373	↑		
	miR-365b-3p	↓	PAX6	Wang et al., 2013
Diabetic Retinopathy	miR-31	↑	VEGF	Shen et al., 2008
	miR-150	↑	NF-κB	
	miR-184	↑		
	miR-146a	↑		
	miR-21	↑	p53	Platania et al., 2019
	miR-34	↑		
	miR-20a	↓	VEGF; HIF	Nunes DN, 2015
	miR-20b	↓		
	miR-106	↓		
	miR-27a	↑	BDNF	Mortuza et al., 2014
	miR-206	↑		
	miR-381-3p	↑		
	miR-195	↓	SIRT-1	
Age related macular degeneration (AMD)	miR-21	↑	Biomarkers	Ertekin et al. 2014
	miR-25-3p	↑		
	miR-146a	↑	IRAK1; TRAF6	Askou AL, 2018; Ménard et al., 2016; Lukiw et al., 2012
	miR-9	↑	CFH-mediated inflammation; TNF-α; IL-1β	
	miR-125b	↑	CFH-mediated inflammation	
	miR-155	↑		
	miR-184	↓	Wnt signaling	Murad et al., 2014; Shen et al., 2008
Glaucoma	miR-183	↑	ITGB1; KIF2A	Li et al., 2010
	miR-29b	↓	TGF-β	Luna et al., 2009; Luna et al., 2011
	miR-24	↑	FURIN	
	miR-200c	↑	TGF-β; OPTN; FOXC1	Luna et al., 2012
	miR-204	↓	TGF-β	Jayaram et al., 2015
	miR-106b	↓		
	miR-25	↓		
	miR-27a	↑	mTORC-1	
	miR-96	↑	Caspase-2	Wang & Li, 2014

Table 3. **Main miRNAs implicated in retinal diseases (↑up-regulated; ↓down-regulated).**

4.5. MiRNAs and NGF relationships

Currently, there are few studies that have explored the relationship between NGF and microRNAs. The regulation of multiple protein components of signaling pathways involved in neuronal development and diseases can be attributed to NGF-modulated miRNAs, as demonstrated by different research. It has been showed that miR-21 improves NGF signaling and controls neuron differentiation induced by NGF, as revealed by studies on the pheochromocytoma cell line (PC12). This miRNA is also effective in preventing neurodegeneration, maintaining the neuronal network and the viability of neurons in an NGF-removal situation (Montalban et al., 2014). NGF was found to upregulate 8 and downregulate 12 miRNAs in another study on PC12. Among them emerged miR-221, whose expression was greatly increased. MiR-221 has a protective function against apoptosis in PC12 cells by reducing the expression of Foxo3 and Apaf-1. Moreover, in pathological conditions, like Alzheimer disease, miR-221 is believed to compensate for defective NGF signaling, as it appears to enhance neuronal differentiation infected with low doses of NGF (Hamada et al., 2012).

AIM OF THE STUDY

In this study, a differentiated human RPE was established, and innovative *in vitr*o cell models of glaucoma, specifically designed using an original device, were used to assess the neuroprotective efficacy of NGF. Oxidative stress, inflammatory factors and miRNAs' expression levels were analyzed with the purpose of providing new insights on some of the molecular mechanisms underlying glaucoma and NGF biological response. The study was aimed at identifying novel candidate biomarkers/therapeutic targets as well.

The PhD program includes Dompé S.p.A. as the industry partner.

The project also includes some analyses, in which I took part, on a mouse model of glaucoma, the results of which cannot be shown due to ongoing patent procedures.

MATERIALS AND METHODS

5.1. Cells and culture conditions

Human retinal pigment epithelial ARPE-19 cells were purchased by the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium/F12 (DMEM F12) (Euroclone, Thermo-Fisher Scientific, Monza, Italy) and supplemented with 10% Fetal Bovine Serum (FBS) (Corning, New York, NY, USA), 1% Glutamine, and 1% Penicillin/Streptomycin (Gibco, Thermo-Fisher Scientific, Monza, Italy) at 37°C and 5% CO_2. Afterwards, ARPE-19 were differentiated in RPE for at least one month in DMEM with high glucose/pyruvate and 1% FBS (Euroclone, Thermo-Fisher Scientific, Monza, Italy). Cells were maintained at 37 °C in a humidified environment of 5% CO2 and were allowed to grow for 1 months with media exchange performed twice a week. Rat Muller cell line (rMC-1) was purchased from American Type Culture Collection (ATCC) and cultured in DMEM/F12 supplemented with 10% FBS at 37 °C in an incubator. NGF was used at a 200 ng/ml concentration.

5.2. Immunocytochemistry

Undifferentiated ARPE-19 and differentiated RPE cells were blocked for one hour with 2% bovine serum albumin (BSA) and stained for 1 h at room temperature with the following antibodies: rabbit anti-zonula occludens-1 (ZO-1, 1:200; Thermo Fisher Scientific, Waltham, MA, Cat # 61–7300), rabbit anti-occludin (1:250; Thermo Fisher Cat # 40–4700). Antibodies were prepared in 0,1% BSA. After three 5 min washes, the cells were stained with Alexa Fluor secondary antibodies (Thermo Fisher Scientific) at 1:1000 in 0,1% BSA 1h. The cells were washed three times each for 5 min, stained with 4',6-diamidino-2-phenylindole (DAPI) for 5 min, rinsed, and mounted on glass slides. Images were captured using an EVOS M5000 using the 20X Plan objective.

5.3. Flow Cytometry Analysis

Undifferentiated ARPE-19 and differentiated RPE, cultured for either 4 days or 1 months, respectively, were used for flow cytometry analysis to evaluate the expression of NGF receptor. Cells were incubated with 1X trypsin in a humidified atmosphere at 37 °C with 5% CO_2 for 5 min and then centrifuged (1300 rpm for 10 min at 4 °C). For permeabilization, the cells were fixed with eBioscience™ Fixation (Invitrogen) for 30 min h in the dark. Washed twice with the permeabilization buffer and stained with a mixture solution of primary

antibodies of TrkA (Bio-Techne,1:1000, #AF175) and p75 (Merck, 1:1000, #o6-574). For the membrane analysis we used the same protocol but without the fixation/permeabilization buffer. After wash, stained with Alexa Fluor secondary antibodies (Thermo Fisher Scientific) at 1:1000 for 30 min. Data from 10,000 events per sample were collected and analyzed. The data were analyzed using FlowJo software (FlowJo).

5.4. RNA-Seq library preparation and data processing

Undifferentiated ARPE-19 and differentiated RPE cells, cultured as described for either 4 days or 1 months, respectively, were used for total RNA extraction using the Qiagen RNeasy Mini Kit following manufacturer's instructions (Qiagen, Hilden, Germany). The libraries were sequenced using a 2 × 150 bp paired end protocol with the median number of 350 million reads across all samples on the Illumina NovaSeq 6000 (Illumina). Once sequencing was completed, the base call files were converted into FASTQ files using Illumina Software (CASAVA) and Expression Analysis developed open source programs. A subset of the reads (about 1 million reads) was aligned to other spiked-in control sequences (PhiX and other Illumina controls used during library preparation), residual sequences (globin and rRNA), and poly-A/T sequences that persisted after clipping. On average, about 98% of the sort-pair-end reads was mapped using the CLC genomics workbench on the GRCh38. The Wald test was used to generate padj and log2 fold change values. Genes with a padj value < 0.01 and log2 fold change <-2 and > 2 were considered as differentially expressed genes for each comparison. The differential gene expression analysis (DESeq2) software package was used to identify differentially expressed transcripts.

5.5. Dynamic 2D culture condition

RPE and rMC-1 cells were analyzed using the LiveFlow millifluidic bioreactor, a sophisticated model of a milli-scaled multi-organ device in a single-flow configuration (LB1, IVTechsrl, Massarosa, Lucca, Italy), equipped with a peristaltic pump (LF, IVTechsrl), transparent culture chambers, and a mixing bottle (Figure 10). The system allows precise dynamic flow (CTRL) regulation to be generated in order to provide nutrients in a controlled and constant way. In addition, to mimic the increase of pressure in the culture chamber (that is the condition of high pressure), the platform uses an auxiliary device consisting of a small piston (Live Pa, IVTechsrl). The pressure exerted by the pistons on the outflow tube of a chamber causes an increase in hydrostatic pressure (HP). The experimental

design was based on four different treatments to RPE and three treatments for rMC-1, maintained in dynamic conditions for 24h, 48h and 96h:

- Group I (Flow Vehicle): RPE exposed to dynamic flow (20 µl/min) of culture medium.
- Group II (HP Vehicle): RPE subjected to dynamic flow (20 µl/min) and increased pressure of 1.000% of flow condition.
- Group III (HP+NGF): RPE subjected simultaneously to dynamic flow (20 µl/min) and increased pressure (of 1.000% of flow condition) treated with NGF the concentration of 200 ng/ml.

As control of the entire system for RPE, was used a transparent culture chamber of the bioreactor filled with the same volume of culture medium (same height), kept in static condition (without flow and high pressure). For the treatment conditions cells were maintained in DMEM High Glucose with 2,061 mg/ml of BSA. We used this culture medium to increase the stability of NGF in a depletion condition of FBS.

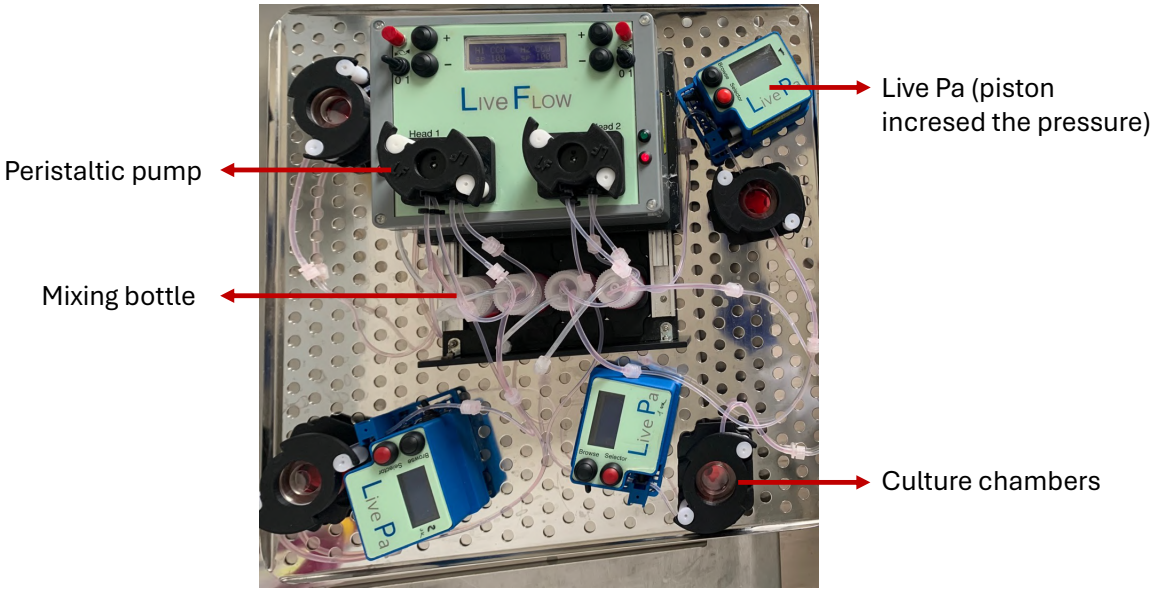


Figure 10. **Schematic representation of the LiveFlow millifluidic bioreactor.**

5.6. Protein extraction and western blot analysis

ARPE-19, RPE and rMC-1 cells were lysed in 200 µL modified RIPA Buffer (PBS 1X, NP40 1%, sodium deoxycholate 0.5%, SDS 1%, Complete-Mini protease inhibitor cocktail tablet (Roche Diagnostics), PMSF 10 mg/mL, aprotinin 10 mg/mL, and sodium orthovanadate 0.1 M (Sigma-Aldrich). Three sonication cycles at amplitude 30 with 0.8 duty cycle for a duration of 10 sec per cycle were performed. Cell lysates were incubated on ice for 30 min and then centrifuged at 14,000× g for 15 min at 4 °C. The supernatants were

recovered and stored at −80 °C until use. For immunoblotting the samples were diluted in reducing 10X Bolt™ Sample Reducing Agent (ThermoFisher) and denatured (85°C, 2 min). Following sample separation in a 4-12% Bis- Tris Plus, proteins were transferred onto a nitrocellulose membrane (Bio-Rad) using a Trans-Blot turbo transfer system. Membranes were blocked with 10% milk (1 h) and incubated with specific primary antibodies (2% milk, overnight at 4°C) (Table1). Detection was performed using peroxidase-conjugated anti-species antibodies (1 h): anti-mouse IgG-Fcγ-POD (Cell Signaling Technology), anti-rabbit IgG-Fcγ-POD (Cell Signaling Technology) and anti-goat IgG-Fcγ-POD (Santa Cruz). Actin (sc-1615) was used as endogenous control. Chemiluminescent detection system (SuperSignal™ West Pico Plus, Thermo Scientific and ECL Western BloSng Detection Reagent, GE Healthcare Life Sciences), and autoradiography films were used to detect signals. Experiments were replicated three times for RPE and twice for rMC-1.

Antibody	Company	Catalogue number	MW (kDa)	Dilution
Actin	Santa Cruz	#sc-1615	42	1:5000
Catalase	Cell Signaling	#14097S	60	1:7000
Claudin-2	Thermo Fisher	#51-6100	20	1:1000
CRALBP	Thermo Fisher	#MA1-813	36	1:1000
DJ-1	Santa Cruz	#sc-55572	24	1:7000
GAPDH	Cell Signaling	#5174	36	1:10000
HO-1	Cell Signaling	#43966S	31	1:1000
IL-6	Abcam	#Ab-9324	18	1:5000
NRF2	Cell Signaling	#20733S	97-100	1:1000
IL-1beta	Bios	#bs-6319R	30	1:1000
p-AKT (T308)	Cell Signaling	#13038S	60	1:1000
p-AKT (S473)	Cell Signaling	#4060S	60	1:3000
p-p70S6K	Cell Signaling	#9205S	70-85	1:1000
p75	Immunological Science	#94728	75	1:1000
p-STAT3	Cell Signaling	#9131S	79-86	1:1000
RPE65	Santa Cruz	#390787	65	1:5000
SIRT-1	LsBio	#LS-B1564	130-110	1:1000
SOD-1	Cell Signaling	#37385S	18	1:7000
Sortilin	Cell Signaling	#20681S	100	1:3000
TrkA	Merck	#06-574	160-140	1:1000
TNF-alpha	Abcam	#ab6671	17	1:1000

5.7. MiRNA analysis

The Quick-RNA Microprep Kit (Zymo) was used for total and miRNA extraction. Total RNA (10 ng) was reverse transcribed using the high-capacity cDNA archive kit (Thermo Fisher Scientific). A miRNA profiling was performed on an in vivo glaucoma mouse model, using microfluidic Thermo-Fisher cards (Applied Biosystems) following the manufacturer's directions, and analyzed using ViiA7 (Thermo Fisher). Several interesting miRNAs were selected and assayed in the in vitro systems. TaqMan Advanced miRNA cDNA synthesis kit (Applied Biosystem) and the TaqMan miRNA RT kit (Applied Biosystem, Thermo Fisher Scientific, Waltham, MA, USA) was used for cDNA synthesis from miRNA. MiRNA assay miR-146a-5p, miR-34a-5p and miR-29c-3p were analyzed on a ViiA7 instrument (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA), and data were processed by QuantStudio Real-Time PCR software v1.2 (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA). MicroRNA's expression levels (RQ, relative quantification) were evaluated by comparative assay ($2^{-\Delta\Delta Ct}$). snU6 RNA and ath-miR159a were used as endogenous control.

5.8. MiRNAs' target genes/pathway analysis

Comparative data analysis was carried out by Expression Suite v1.1 (Thermo Fisher). Among the differentially expressed miRNAs (Relative Quantification -RQ- fold change ≤ 0.5 or ≥ 2), those showing significant P value ($P<0.05$) were analyzed by Diana tools (Diana-miRpath v.4.0, TarBase v8.0) to identify target genes and pathways (https://www.mirnet.ca) (Tastsoglou et al., 2023). We used KEGG and GO database annotation with standard statistics and genes union settings for an exploratory functional analysis.

RESULTS

6.1. ARPE-19 cells exhibit RPE characteristics after one-month differentiation

ARPE-19 cells' ability to acquire physiological RPE characteristics is influenced by the culture conditions. During culture, these cells undergo dedifferentiation, and it appears that this particular phenomenon is a result of the cell substrate and growth media (Luo et al., 2006) (Ahmado et al., 2011) (Grisanti et al., 1995). Figure 11A illustrates the typical growth of the ARPE-19 cells cultured on standard conditions show a non-epithelial morphology while they are sub confluent and mitotically active. Upon achieving 100% confluence and appropriately changing culture medium, cells develop a morphology that resembles fibroblasts (Figure 11B) and remain in elongated fibroblastic morphology for more than 2 weeks. The cells cultured in such condition for one month show islands of hexagonally packed epithelial-like cells that are typical of the RPE (Figure 11C). As the cells in culture mature, they develop the characteristics of the RPE, such as well-defined cell borders an increase in pigmentation that indicates melanin pigment de novo synthesis, the typical cobblestone epithelial morphology and heavy pigmentation, which can be also seen by the naked eye (Figure 11D). These cells maintain a well-defined differentiation pattern and create a monolayer of pigmented cells that resembles RPE cells.

To further define the phenotypic changes that occur during differentiation, we investigated the existence of tight junctions (Figure 12A) between adjacent cells, which is a crucial structural feature of natural RPE monolayers in differentiated cells. We also analyzed the expression and localization of ZO-1 and occludin, which are related to tight junctions, through immunocytochemistry and fluorescent microscopy. Differentiated cells express the tight junction complex protein ZO-1, predominantly localized to the cell edges, suggesting that these cells form continuous cell–cell contact with tight junctions. Figure 12B displays occludin expression being uniformly distributed to the tight junction complex across the monolayer in the differentiated RPE cells. We also analyzed the expression of claudin-2, another marker of tight junction expressed only in differentiated cells (Figure 12C). RPE-specific differentiation can be characterized by the expression of key enzymes in the visual cycle.

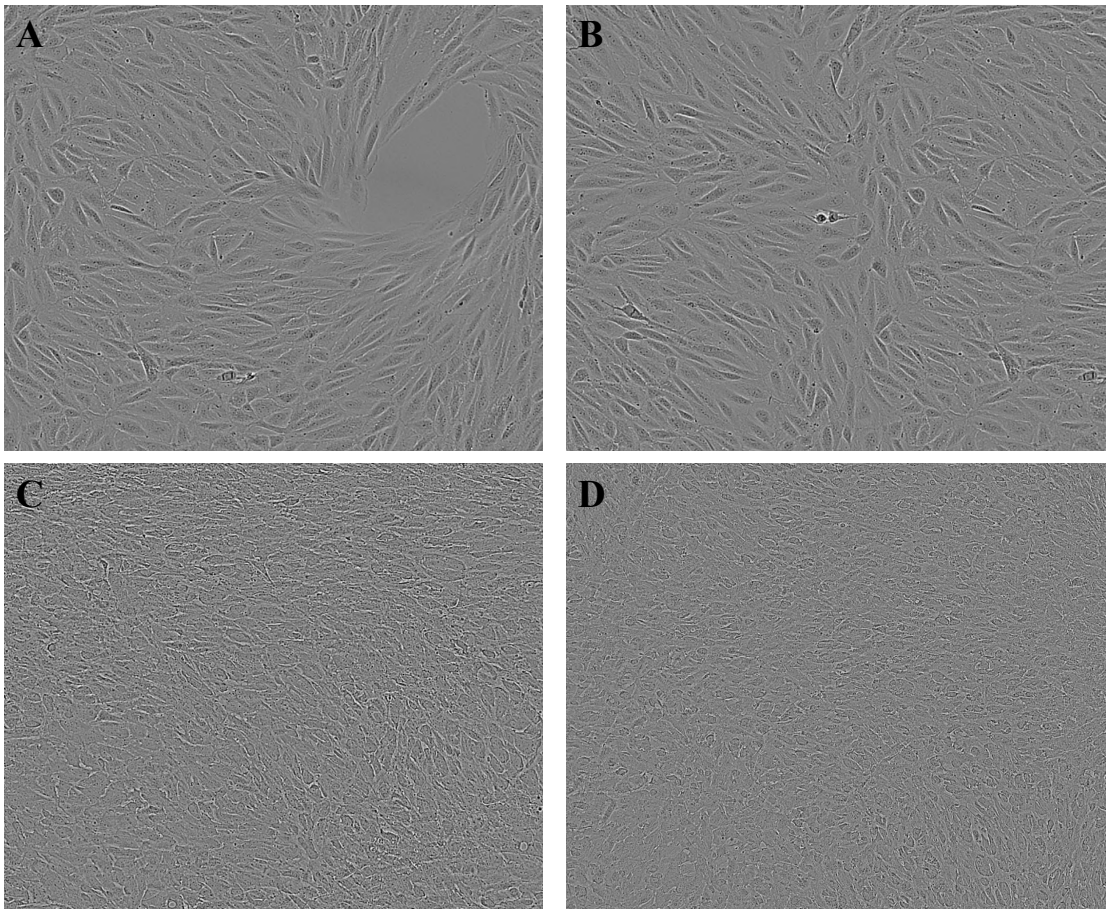


Figure 11. **During differentiation, ARPE-19 cells exhibit RPE phenotype characteristics.** ARPE-19 cells were grown for one month, as outlined in the methods section, and the morphology of the cells was examined with phase-contrast microscopy. **A:** typical growth of the ARPE-19 **B:** Cells grown for 4 days **C:** Cells grown for 2 weeks **D:** Cells grown for one month.

We utilized qRT-PCR and western blot analysis to examine the expression of visual cycle genes in ARPE-19 cells that were cultured for either 4 days or 1 month. All genes associated with the visual cycle (RPE65, CRALBP/RLBP1) showed increased expression in the differentiated ARPE-19 cells. A slight expression increase was also observed for MITF and RDH10 (Figure 12B). Protein expression increase was closely linked to increase of the visual cycle protein for CRALBP and claudin-2 markers (Figure 12C). However, immunoblot did not detect the expression of the RPE65 protein in both differentiated and undifferentiated RPE cells.

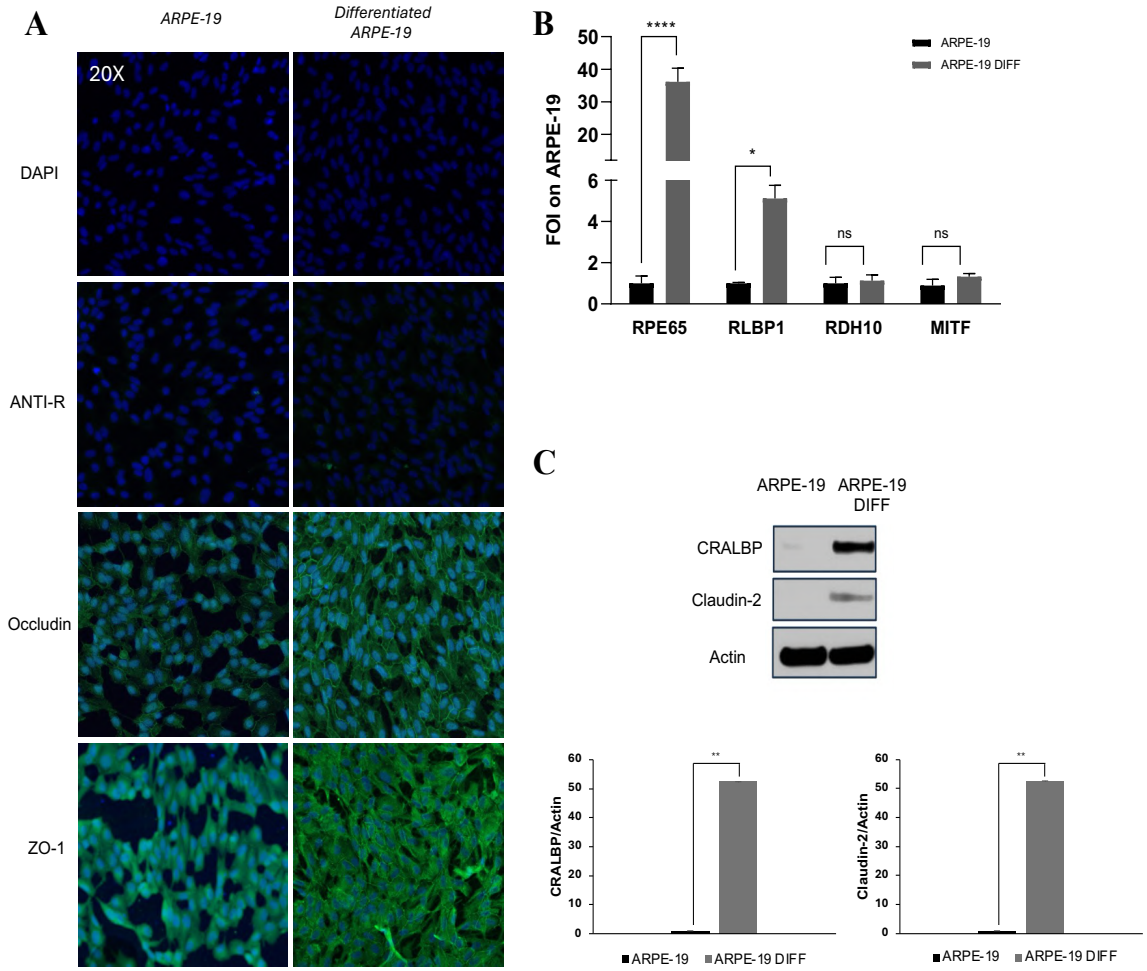


***Figure 12*. A:** Fluorescent micrographs of ARPE-19 cells cultured for 4 day or one month. ZO-1 and occludin immunostaining shows good junctional development and localization at
the cell borders containing a mixture of elongated and polygonal cells. Images were captured using an EVOS M5000 and the 20X Plan objective. **B:** Real-time PCR analysis of RPE-specific mRNA expression of visual cycle genes. The values are mean ± standard deviation (SD) and statistical analysis was performed using two-way ANOVA test followed by Bonferroni test (n = 4). *p<05, **p<01, ***p<001 compared with undifferentiated ARPE-19. **C:** Western blot and densitometric analysis of the expression of the RPE-specific proteins (normalization target/actin). Statistical analysis was performed using multiple t tests. * $p < 0.05$; **p<0.01 *** $p < 0.001$ (n =2).

6.2. Differentiation-induced changes in gene expression: RNA-seq.

In order to determine if gene expression changes could be responsible for the acquisition of the RPE phenotype, we conducted a whole transcriptomic RNA-Seq analysis of RPE cells that were cultured for 4 days and for 1 month. After seeding at a low density, ARPE-19 cells were collected either within 4 days while still sub confluent, or after 1 month once they had acquired the characteristic cobblestone epithelial morphology with heavy pigmentation. RNA-Seq produced a total of approximately 30 million reads for each of the six cDNA libraries (three from the cells cultured for 4 days and three from the cells cultured for 1 months) obtained from the pooled cultured cells. The reads were mapped to the human genome issue GRCh38-hg38.

The transcriptome was aligned with an average of 98% of the reads, overall. RNA-seq showed 5,453 significant genes with detectable signals. Among them, about 1,135 genes were upregulated, and approximately 1,182 genes were downregulated. Gene ontology (GO) enrichment analysis of the statistically significant differentially expressed genes revealed different pathways involved in apoptosis, migration, transcription, angiogenesis and adhesion (Figure 13A). Regarding up-regulated genes, we focused analysis on the pathways mainly associated with the differentiation, such as visual cycle, pigment synthesis, cell differentiation and cell adhesion, while downregulated genes were principally associated with cell cycle (Figure 13B).

Among the most highly upregulated genes, ranked by fold change, are the pigmentation-related genes, TRPM1, TYRP1, and TYR. Tyrosinase (TYR) is a copper-containing enzyme and a type I membrane protein that is involved in the generation of melanin, the main pigment in vertebrates. The RPE cells cultured for 1 month displayed high level of genes that have been reported to be associated with phagocytosis. Specifically, the expression of MERTK, which is crucial for POS internalization, and ITGAV, which facilitates POS recognition and binding, was found elevated.

The RPE cells cultured for one month showed high increase of genes correlated with cell confluence status, which are usually expressed in differentiated cells. In the retina/RPE transcriptome, the death associated protein-like 1(DAPL1), implicated in the early stages of epithelial differentiation, showed expression as well.

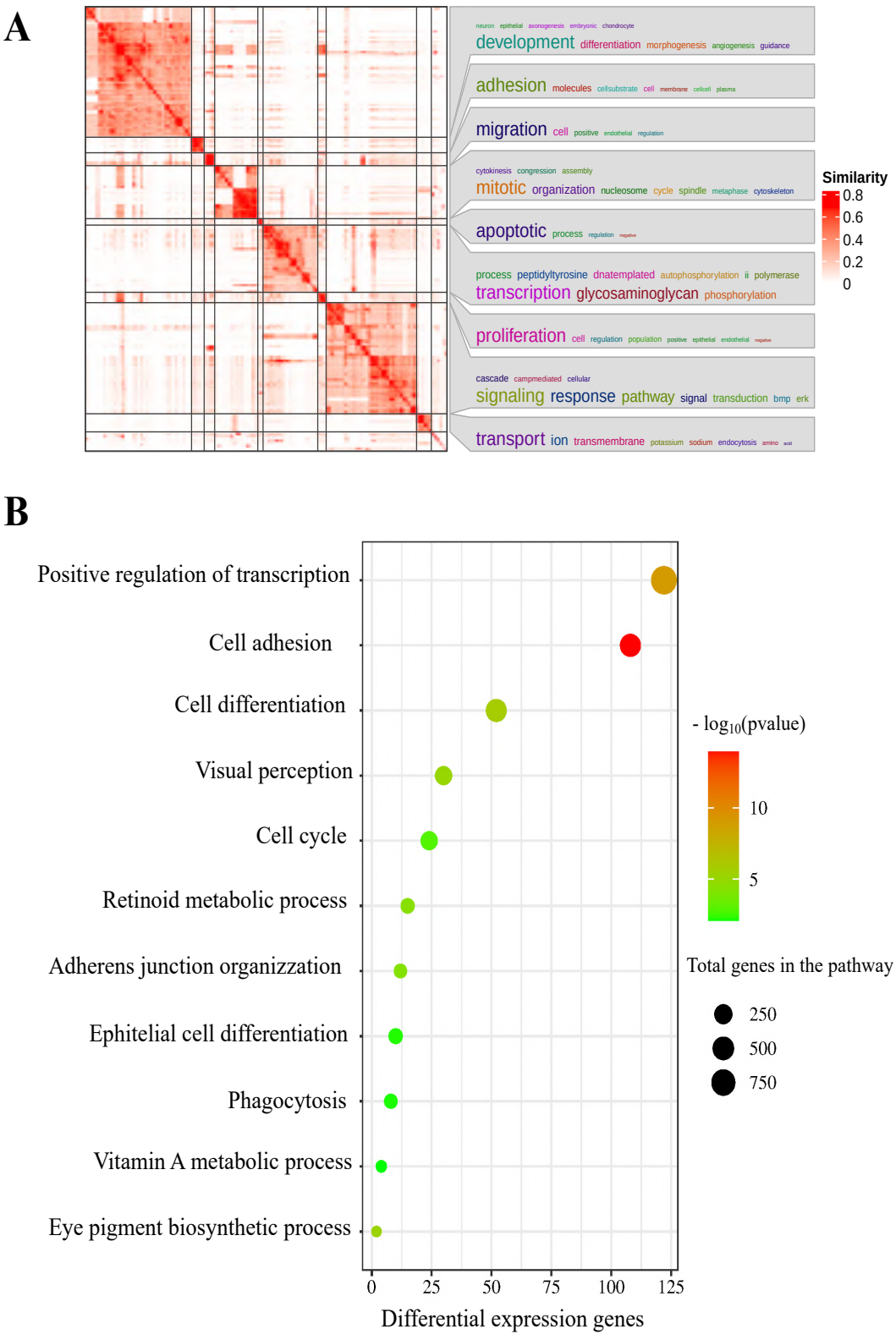


Figure 13. **A:** Similarity heatmap of the significant GO terms (adjusted p-value ≤ 0.01) clustered and annotated with word clouds by using the simplified Enrichment R package. **B:** Gene Ontology (GO) terms associated with differentially expressed genes in the RPE cells.

The epithelial marker p-cadherin (CDH3), which is expressed in the RPE monolayer, showed a significant increase when compared to cells cultured for 4 days.

The differentiation of ARPE-19 cells resulted in a significant upregulation of several RPE-associated transcription factors, including PAX6 (which combines with MITF to activate the expression of genes involved in pigment biogenesis) and SOX9 (which is a key regulator of visual cycle genes).

Moreover, other genes were found to be downregulated. The majority of these genes plays a role in the cell cycle and its regulation, based on the Gene Ontology analysis. In fact, transforming growth factor beta 1 (TGFβ1), which is a key factor in the epithelial–mesenchymal transition (EMT), was less abundant in RPE cells cultured for 1 month than in cells cultured for 4 days. In the differentiated RPE, there was a decrease of cyclin-dependent kinase 1 (CDK1), a key factor in determining the timing of mitosis by interfering with cyclin B. The RPE cells were unable to progress from G1 to S phase as regulated by cyclin D1 (CCND1), which is an activator of cell cycle progression, when grown for 1 month, compared to cells cultured for 4 days. The cells cultured for 1 month had reduced levels of N-cadherin (CDH2), which is typically present in non-epithelial cells. In addition, the expression of a non-epithelial marker that is commonly used, vimentin, was reduced. Vimentin mRNA levels were elevated in cells cultured for 4 days, but they decreased in differentiated cells cultured for 1 month (Figure 14).

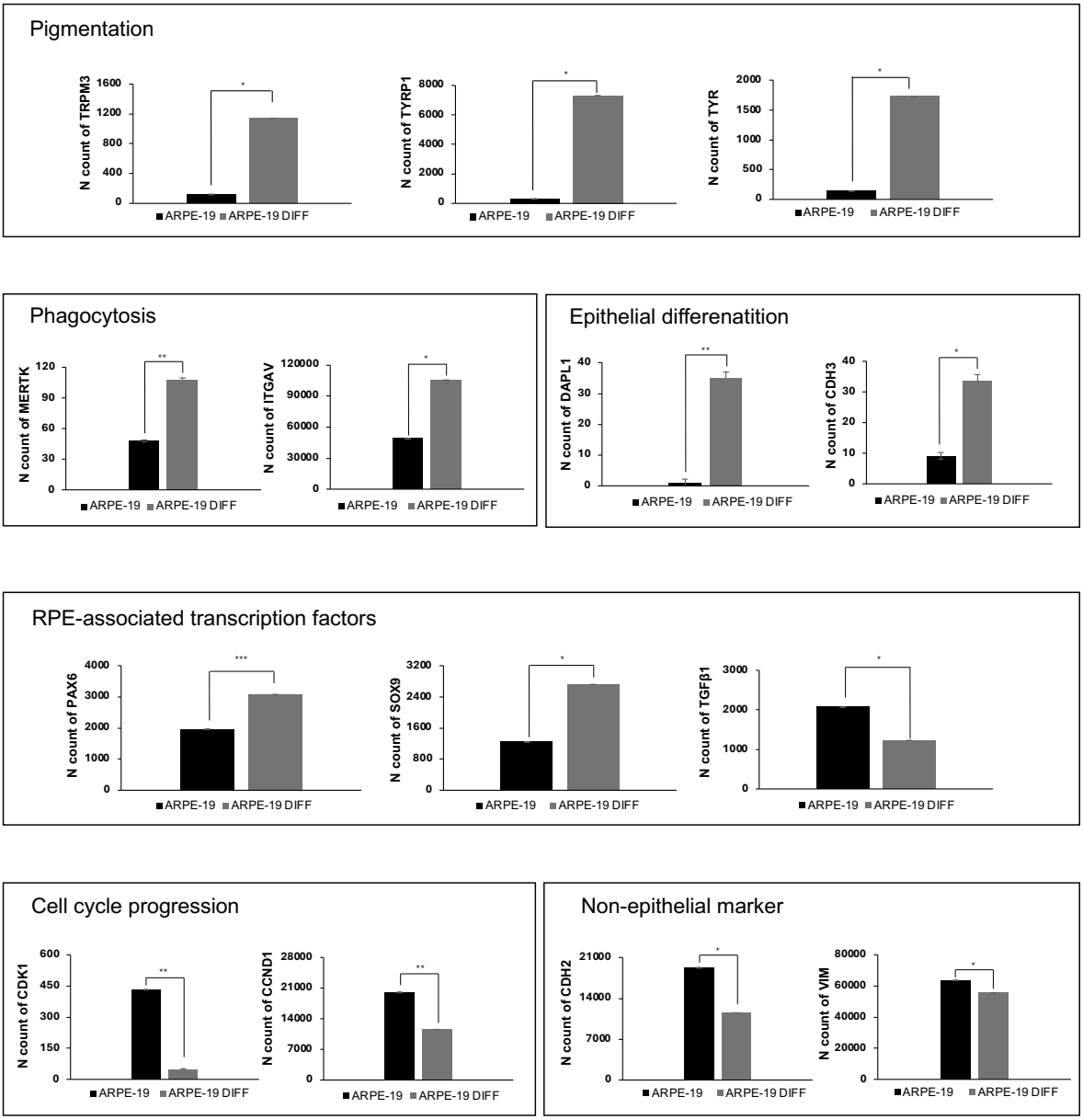


Figure 14. Expression of genes involved in the differentiation of RPE. Statistical analysis was performed using multiple t tests. * $p < 0.05$; **p<0.01 *** $p < 0.001$ (n =3).

6.3. TrkA and p75^{NTR} NGF receptors' expression was reduced in RPE

We investigated the expression and localization of the receptors mediating the biological actions of NGF, namely TrkA and p75^{NTR}, in cells cultured for 4 days and 1 month. In the first series of experiments carried out using qRT-PCR, we found that TrkA mRNA was detected just in late cycles, with expression increase in differentiated cells. In contrast, no changes in p75^{NTR} mRNA expression were observed (Figure 15A).

In order to provide complementary results to the bio-molecular experiments described above, flow cytometry and western blot assays were performed to assess the expression and localization of TrkA and p75^{NTR} proteins in both cell models. These experiments showed that the undifferentiated and the differentiated cells expressed both receptors. In particular, the flow cytometry assay showed an increase in the membrane (41%) and total expression (94.5%) of TrkA in the cells cultured for 4 days. But the p75^{NTR} expression was found increased only in total analysis (35.4%). The same increase was identified by western blot analysis. For differentiated cells we observed a reduction of membrane (20%) and total TrkA receptor (17%), and we found p75^{NTR} downregulation in terms of total expression (3%) compared to undifferentiated cells. Western blot analysis shows the same decrease (Figure 15B-C). In conclusion, differentiated RPE was demonstrated a cell model closer to the physiological one and suitable for in vitro analyses.

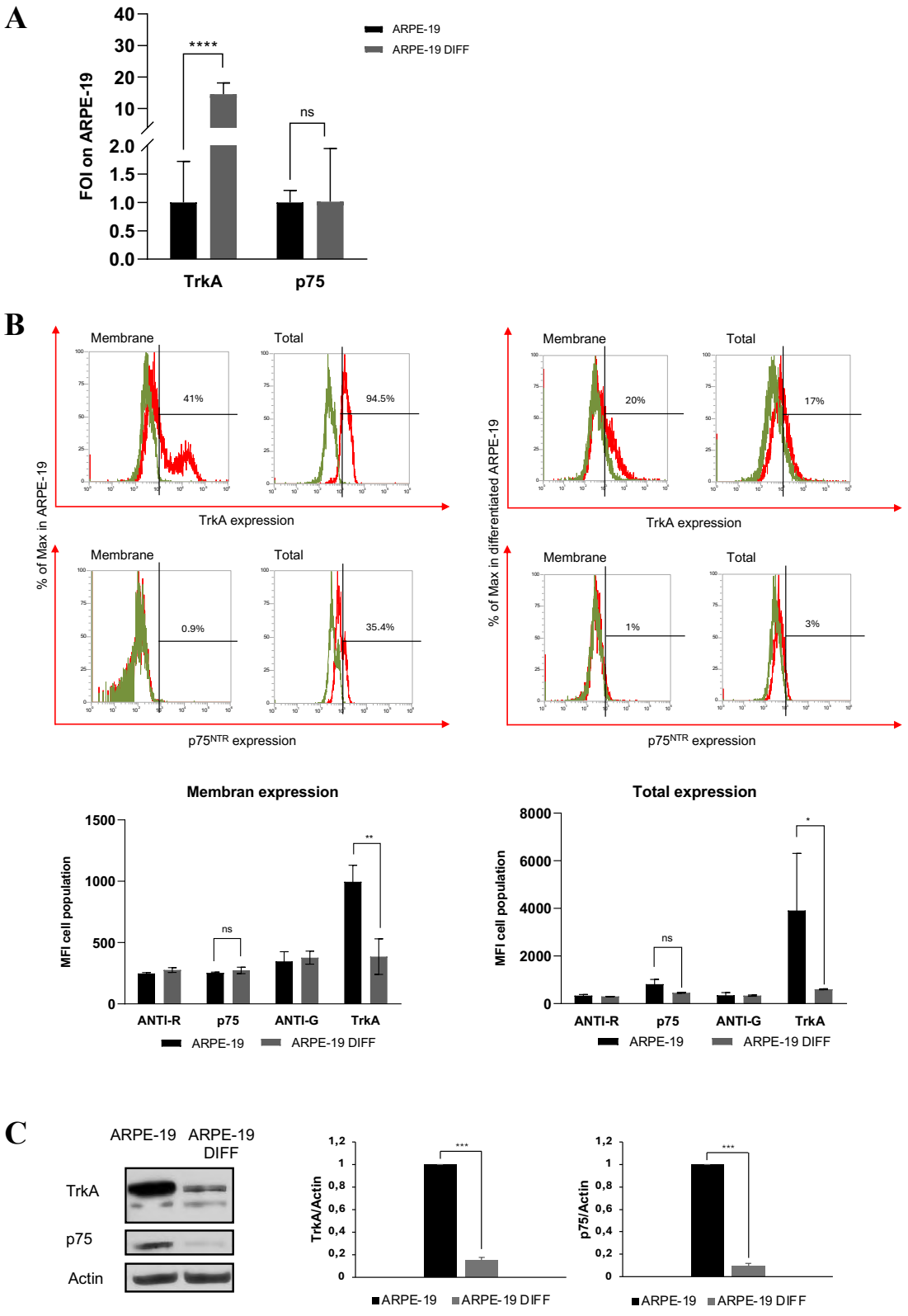


Figure 15. **A:** mRNA expression of NGF receptor TrkA and p75^{NTR}. The values are mean ± standard deviation (SD) and statistical analysis was performed using two-way ANOVA test followed by Bonferroni test (n = 4). *p<05, **p<01, ***p<001 compared with undifferentiated ARPE-19. **B:** Cytofluorimetry analysis of localization of NGF receptor, in cells cultured for 4 days and 1 month.

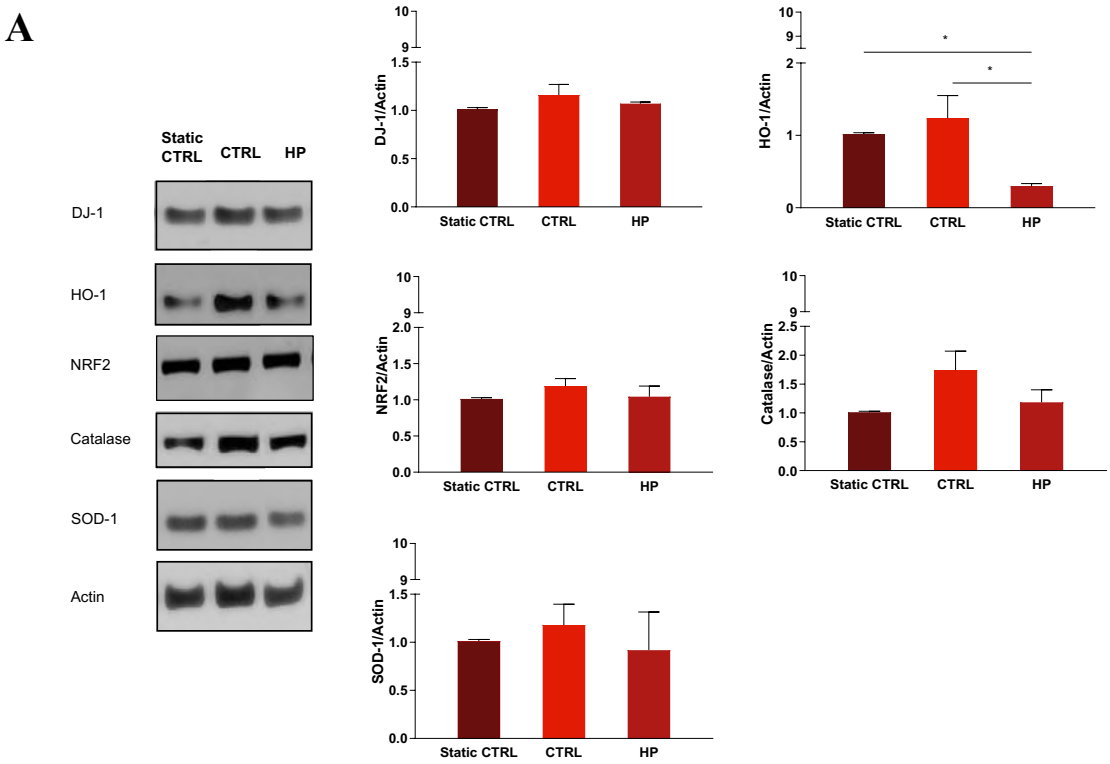
We used MFI to measure the fluorescence intensity shift of a population of cells. Statistical analysis was performed using two-way ANOVA test followed by Bonferroni test. *p<05, **p<01, ***p<001 versus ARPE-19 (n=2). **C:** Protein expression and densitometric analysis of TrkA and $p75^{NTR}$ (normalization target/actin). Statistical analysis was performed using multiple t tests. * $p < 0.05$; **p<0.01 *** $p < 0.001$ (n =2).

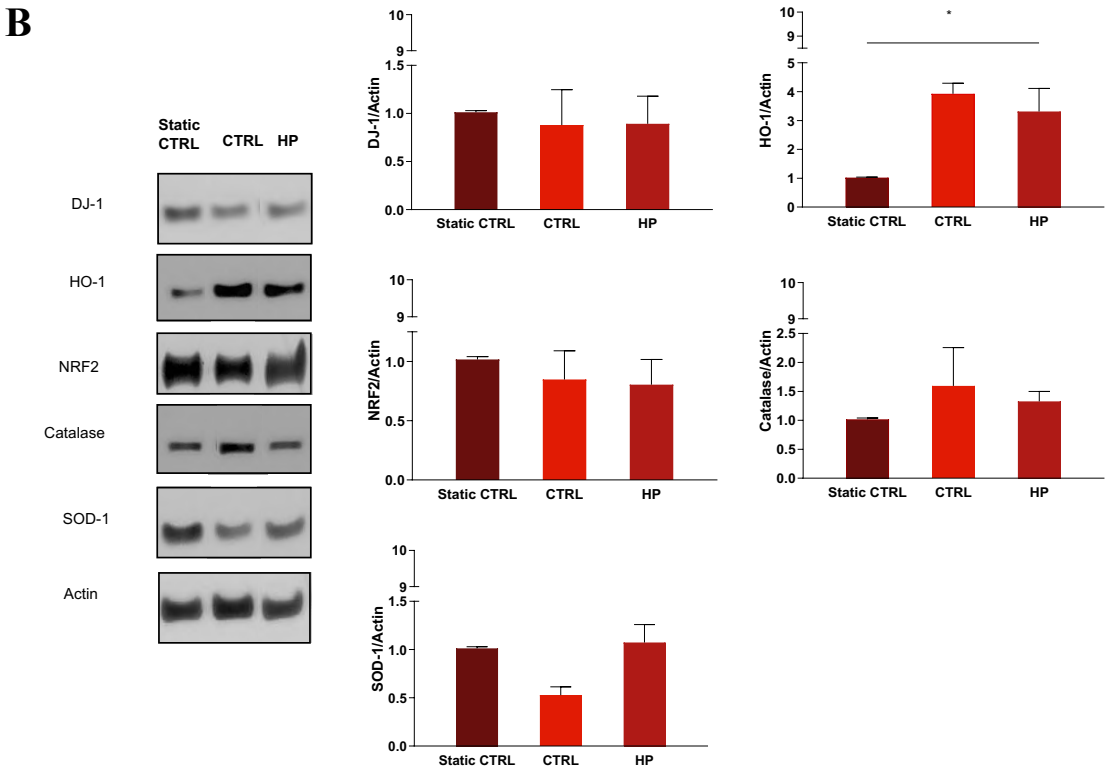
6.4. High pressure induces oxidative stress factors in 2D cell models

In order to simulate glaucomatous disease *in vitro*, a millifluidic bioreactor (LiveFlow) was used. During the setting phase, different experimental times were evaluated to optimize the experimental procedure. Preliminary analysis performed on differentiated RPE, at 24h, 48h and 96h, were necessary to assess the time needed to create damage through pressure increase (HP). At the end of the experiments, some factors involved in oxidative stress were analyzed.

After 24h HP, we observed no variations of Parkinson disease protein 7 (DJ-1), sentinel of oxidative stress. At the same way, very slight, no significant changes were observed for superoxide dismutase 1 (SOD-1) and catalase (CAT). A reduction of HO-1, one of the major sensor of oxidative stress response, in the HP condition compared to the static CTRL was detected, whereas no changes were identified for NRF2 expression (Figure 16A). Such 24h condition seems to be not optimal, due to the markers' levels not reflecting the expected one for glaucomatous conditions (e.g. HO-1 is described hyper-expressed in plasma in glaucoma patients -Salinaro et al., 2014-).

The 48h analysis also showed no significant changes in the expression of markers analyzed, except for HO-1 and CAT, the latter less marked but unsignificant (Figure 16B). We ultimately considered the timing of 96h, in which significant increase of HO-1 was detected in HP condition compared to static and flow CTRLs. On the other hand, NRF2 increase was shown in HP and flow CTRL conditions compared to static control (Figure 16C). Expression differences in flow CTRL condition (20 µl/min) might be attributed to a mechanical stress also inducing pressure increase (as indicated by technical notes from the device's manufacturer), with consequences in terms of cell responses. Very slight, not-significant, expression differences were observed for CAT and SOD-1.


A
Static CTRL
CTRL
HP
DJ-1
HO-1
NRF2
Catalase
SOD-1
Actin
DJ-1/Actin
HO-1/Actin
NRF2/Actin
Catalase/Actin
SOD-1/Actin
Static CTRL
CTRL
HP


B
Static CTRL
CTRL
HP
DJ-1
HO-1
NRF2
Catalase
SOD-1
Actin
DJ-1/Actin
HO-1/Actin
NRF2/Actin
Catalase/Actin
SOD-1/Actin
Static CTRL
CTRL
HP

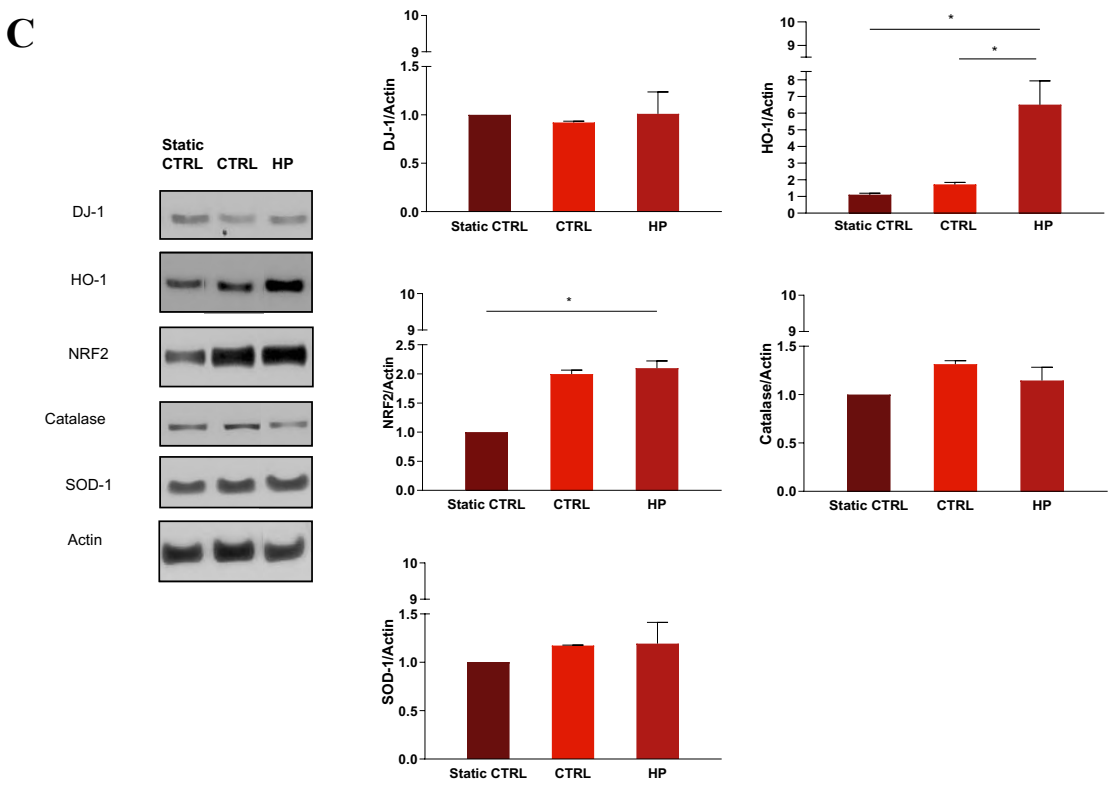


Figure 16. Western blot and densitometric analysis of the expression of oxidative stress proteins in RPE cells (normalization target/actin). **A:** 24hrs; **B:** 48hrs; **C:** 96hrs. Statistical analysis was performed using multiple t tests. * $p < 0.05$; **p<0.01 *** $p < 0.001$ (n =3) compared to static CTRL.

We also analyzed the effect of high pressure on the rMC-1 rat cell line by examining only the 96h time-point. Results showed significant increase of the oxidative stress marker DJ-1. At the same way, we detected NRF2, Cat and SOD-1 increase in HP condition (Figure 17).

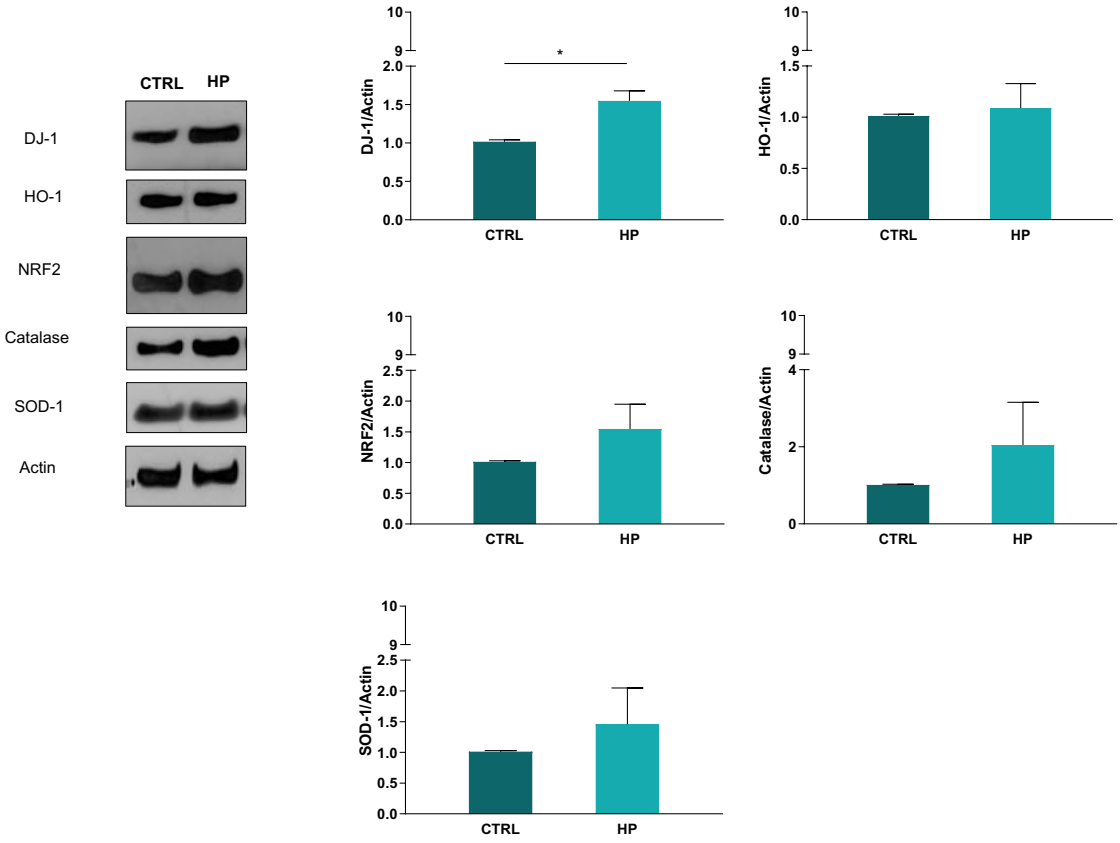


Figure 17. Western blot and densitometric analysis of the expression of oxidative stress proteins in rMC-1 cells (normalization target/actin). Statistical analysis was performed using multiple t tests. * $p < 0.05$; **p<0.01 *** $p < 0.001$ (n =2) compared to CTRL.

6.5. High pressure induces pro-inflammatory interleukins in 2D cell models

Inflammation is one of the main active processes in neuropathy. For this reason, we evaluated pro-inflammatory interleukins in both human and rat cell models under HP for 96h. We have evidenced significant increase of interleukin 6 (IL-6) under high-pressure conditions in RPE, whereas slight increase was detected in the expression of interleukin 1 beta (IL-1β) (Figure 18A). The same analysis was also performed on rMC-1, in which different degrees of expression increase were also observed for interleukins in HP samples compared to control (Figure 18B). This data shows that mechanical stress can cause not only increase of oxidative, but also inflammatory factors.

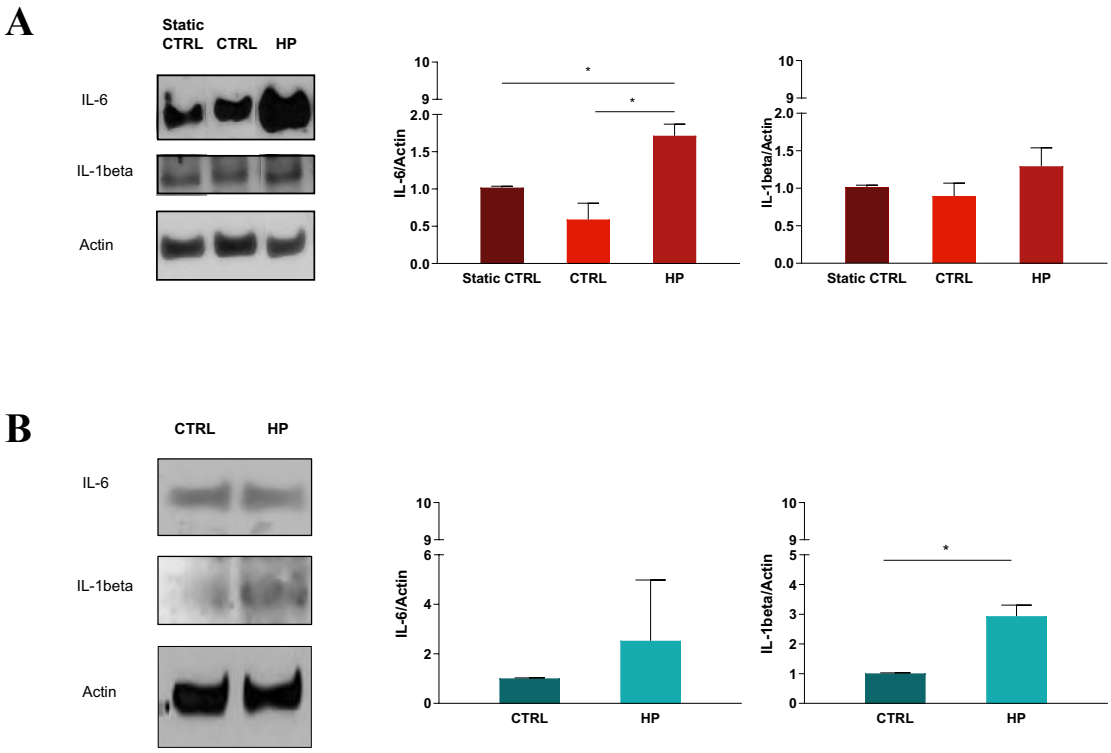
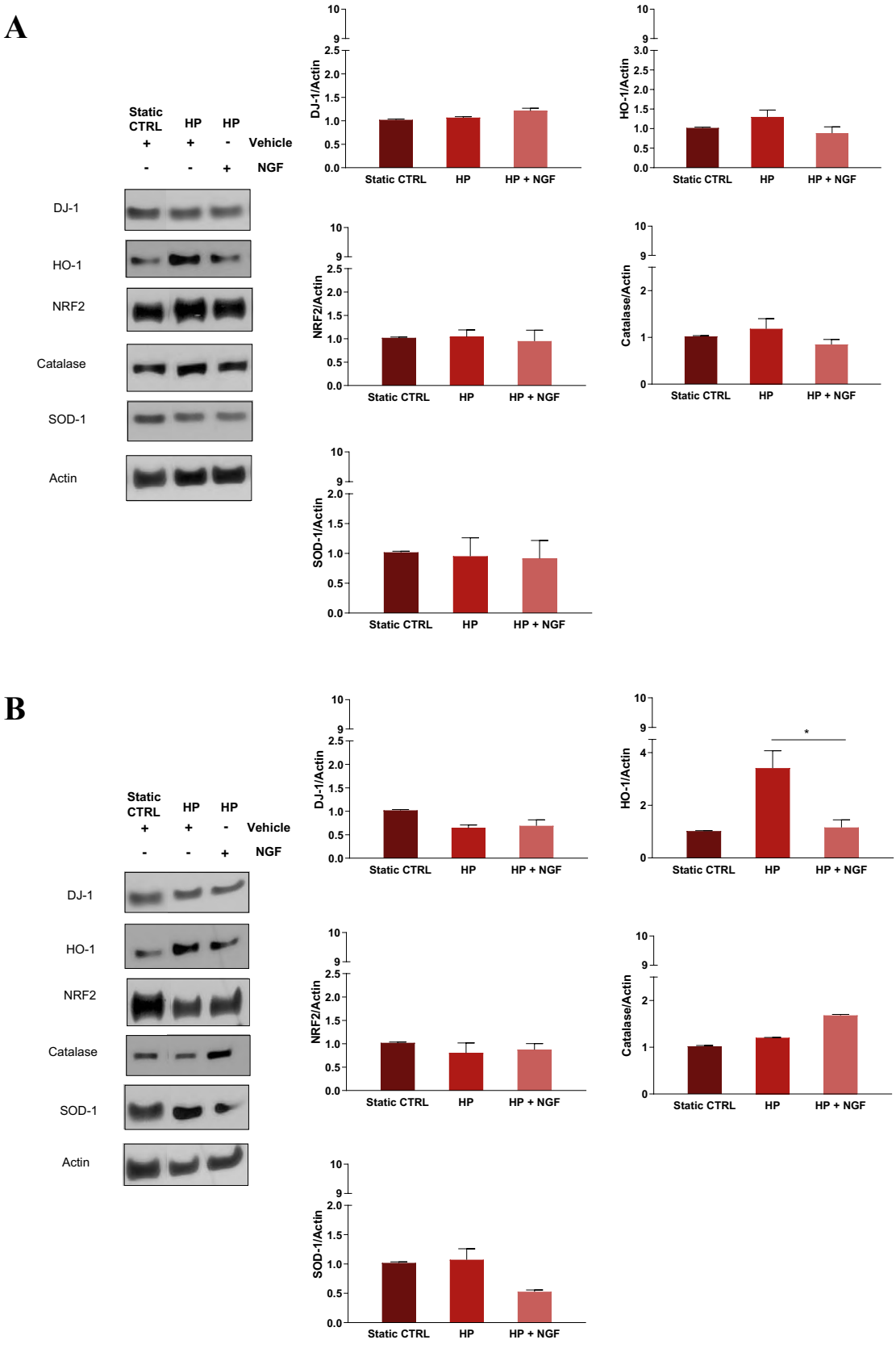


Figure 18. Western blot and densitometric analysis showing the expression of pro-inflammatory proteins (normalization target/actin) in **A:** RPE (n =3) and **B:** rMC-1 cells (n =2). Statistical analysis was performed using multiple t tests. * $p < 0.05$; **p<0.01 *** $p < 0.001$ compared to static CTRL.

6.6. NGF treatment reduced expression of oxidative stress and inflammation protein factors

To assess the possible role of NGF on modulating oxidative stress and inflammation protein expression in our models, we analyzed again samples after 24, 48 and 96h of HP treatment.
After 24h NGF under HP condition, any significant changes in oxidative stress markers were not detected, except for a decrease of HO-1 and, less marked, for CAT (Figure 19A).
After 48h, NGF treatment induced a reduction of HO-1 (significant) and SOD-1. Moreover, a slight, unsignificant increase of catalase was observed (Figure 19B).
Finally, we considered the NGF 96h time-point, in which significant reduction of HO-1 and NRF2 was shown. In addition, a slight expression decrease of DJ-1and, less marked, of CAT and SOD-1 was detected (Figure 19C).
We also analyzed the effect of NGF on the rMC-1 cell line by examining only the 96h time-point. Results showed a significant reduction of DJ-1 as well as a decrease, even though not significant, of HO-1, SOD-1 and CAT (Figure 19D).


A
Static CTRL
HP
HP
Vehicle
NGF
DJ-1
HO-1
NRF2
Catalase
SOD-1
Actin
DJ-1/Actin
HO-1/Actin
NRF2/Actin
Catalase/Actin
SOD-1/Actin
Static CTRL
HP
HP + NGF
B
*

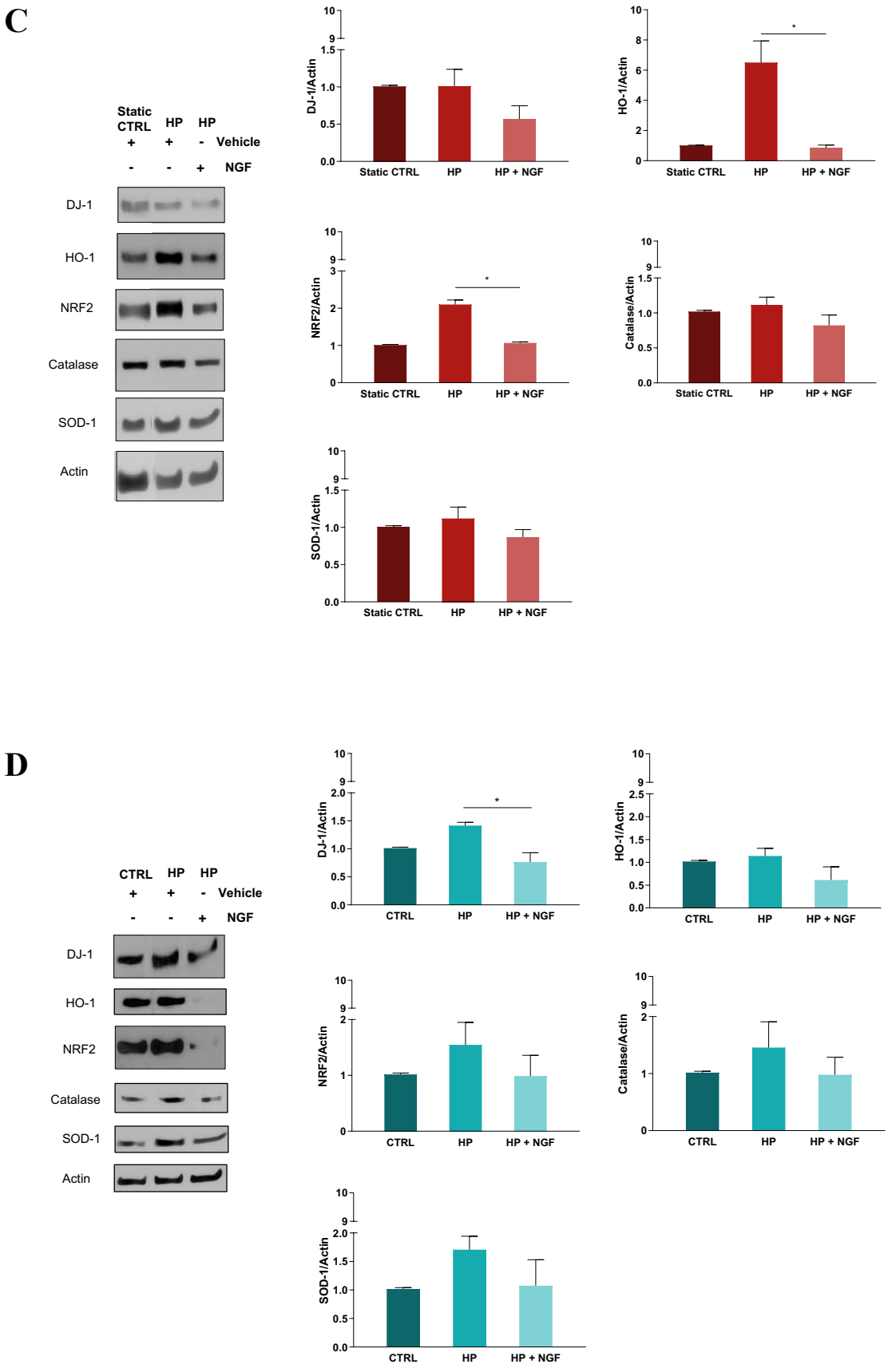


Figure 19. Western blot and densitometric analysis of oxidative stress proteins in RPE cells treated with NGF (normalization target/act) **A:** 24hrs; **B:** 48hrs; **C:** 96hrs. Statistical analysis was performed

using multiple t tests. * $p < 0.05$; **p<0.01 *** $p < 0.001$ (n =3) compared to high-pressure (HP). **D:** oxidative stress markers in rMC-1 cell line (n=2).

As shown in Figure 20, IL-6 and IL-1beta significant decrease was observed in RPE cells after 96h NGF, whereas data was consistent only for IL-1beta in rMC-1 cells.

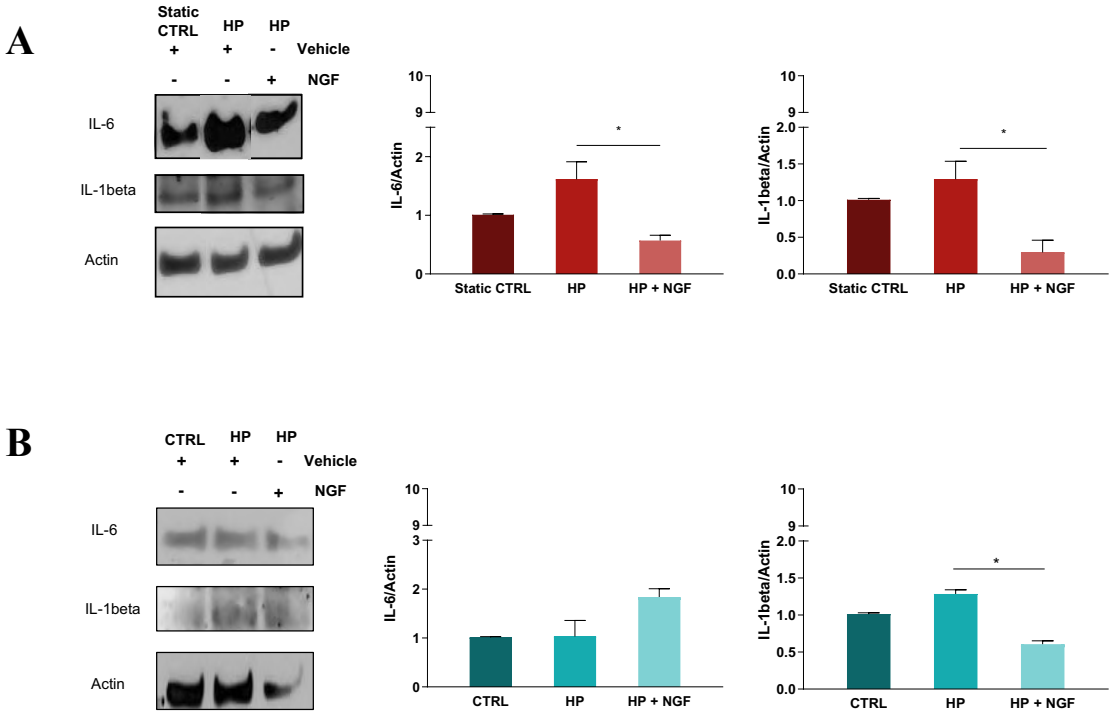


Figure 20. Western blot and densitometric analysis showing expression of pro-inflammatory proteins (normalization target/actin) in **A:** RPE (n =3) and **B:** rMC-1 cells (n =2) after NGF treatment. Statistical analysis was performed using multiple t tests. * $p < 0.05$; **p<0.01 *** $p < 0.001$ compared to high-pressure (HP).

6.7. MicroRNAs' expression levels in 2D cell models

MicroRNA profiling, performed in an *in vivo* murine model of glaucoma, revealed differences in several miRNAs (data not shown due to ongoing patent procedures). Levels of miR-29c-3p, miR-146a-5p and miR-34a-5p were evaluated in cell models in static, HP and HP+NGF conditions. Interestingly, an expression increase of miR-29c-3p and miR-34a-5p in HP condition and, conversely, decrease in response to NGF was detected in both cell lines. The same trend was shown by miR-146a-5p only in RPE cells, being this not expressed in r-MC1 (Figure 21).

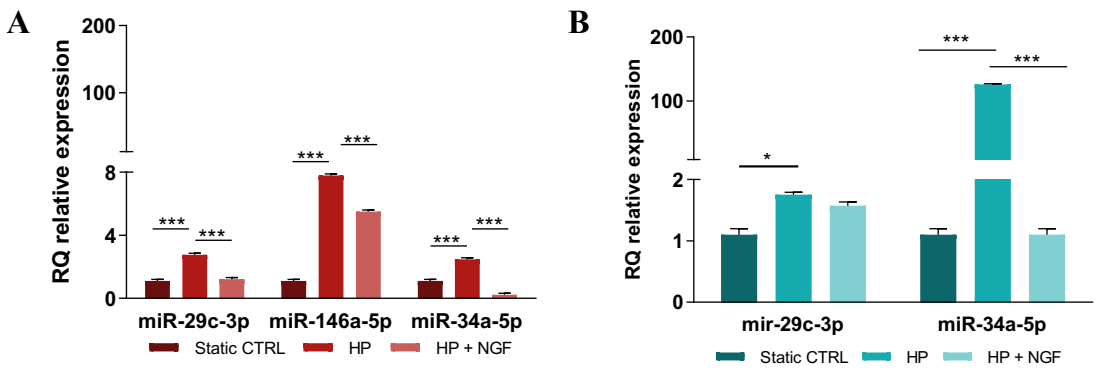
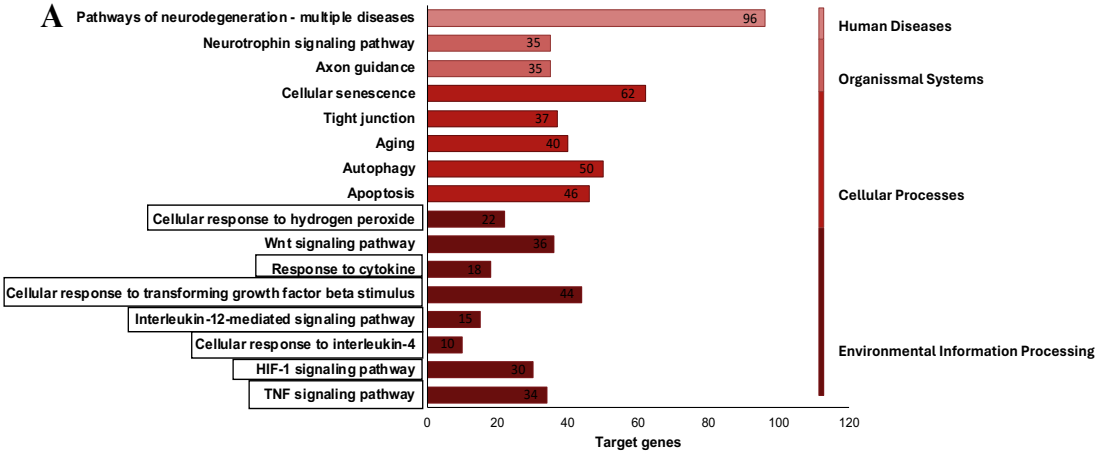


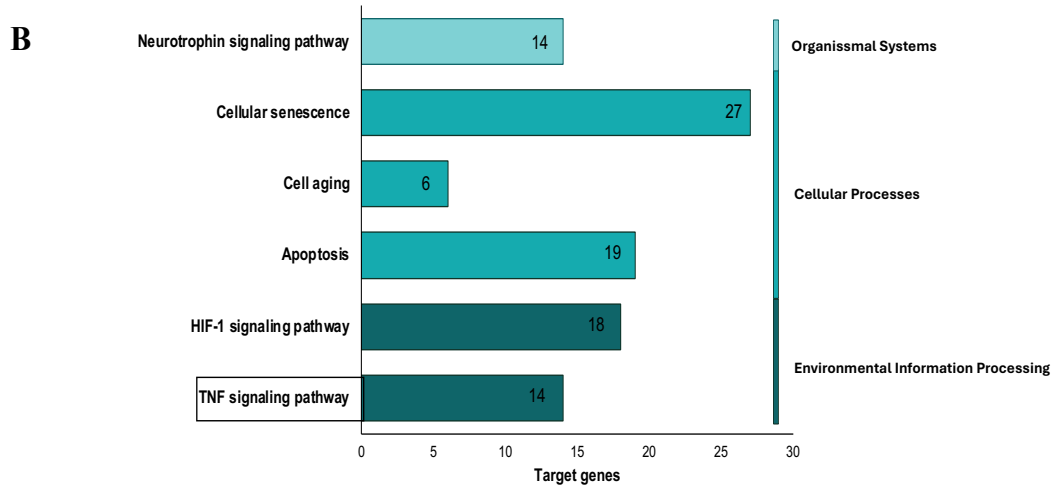
Figure 21. **A:** Histograms showing miR-29c-3p, miR-146a-5p and miR-34a-5p expression levels in RPE cell line **(A)** and rMC-1 **(B)**. Statistical analysis was performed using multiple t tests. * $p < 0.05$; **p<0.01 *** $p < 0.001$

6.8. MiRNAs' pathway and gene targets

A global enrichment analysis of miR-29c-3p, miR-146a-5p and miR-34a-5p was performed on the RPE cell line. In this case, *H. sapiens* was considered as reference. The analysis returned to 37 pathways, including some more closely associated with glaucoma (e.g. apoptosis, TNF signaling pathway, tight junction, cellular response to hydrogen peroxide, autophagy, cellular response to transforming growth factor beta stimulus) (Figure 22A). The same enrichment analysis was also performed on rMC-1, considering just miR-29c-3p and miR-34a-5p. In this case, the reference organism was *R. norvegicus*, but, unfortunately, no pathways emerged, most probably due to too few studies carried out on this species. Therefore, enrichment analysis was performed considering the very close species *M. musculus* as reference. In this case, 20 pathways (apoptosis, TNF signaling pathway, HIF-1 signaling pathway, cellular senescence) were revealed, all associated with neuropathies

(Figure 22B). In addition, target genes of the miRNAs analyzed were identified (n=1213 unique genes) (Figure 22C), among which genes associated with oxidative stress and inflammation pathways emerged as well (Figure 23). Subsequently, the analysis was focused on SIRT-1, an experimentally validated miR-34a-5p target, since it was already described as involved in oxidative stress and inflammation processes (ZHU et al., 2021).





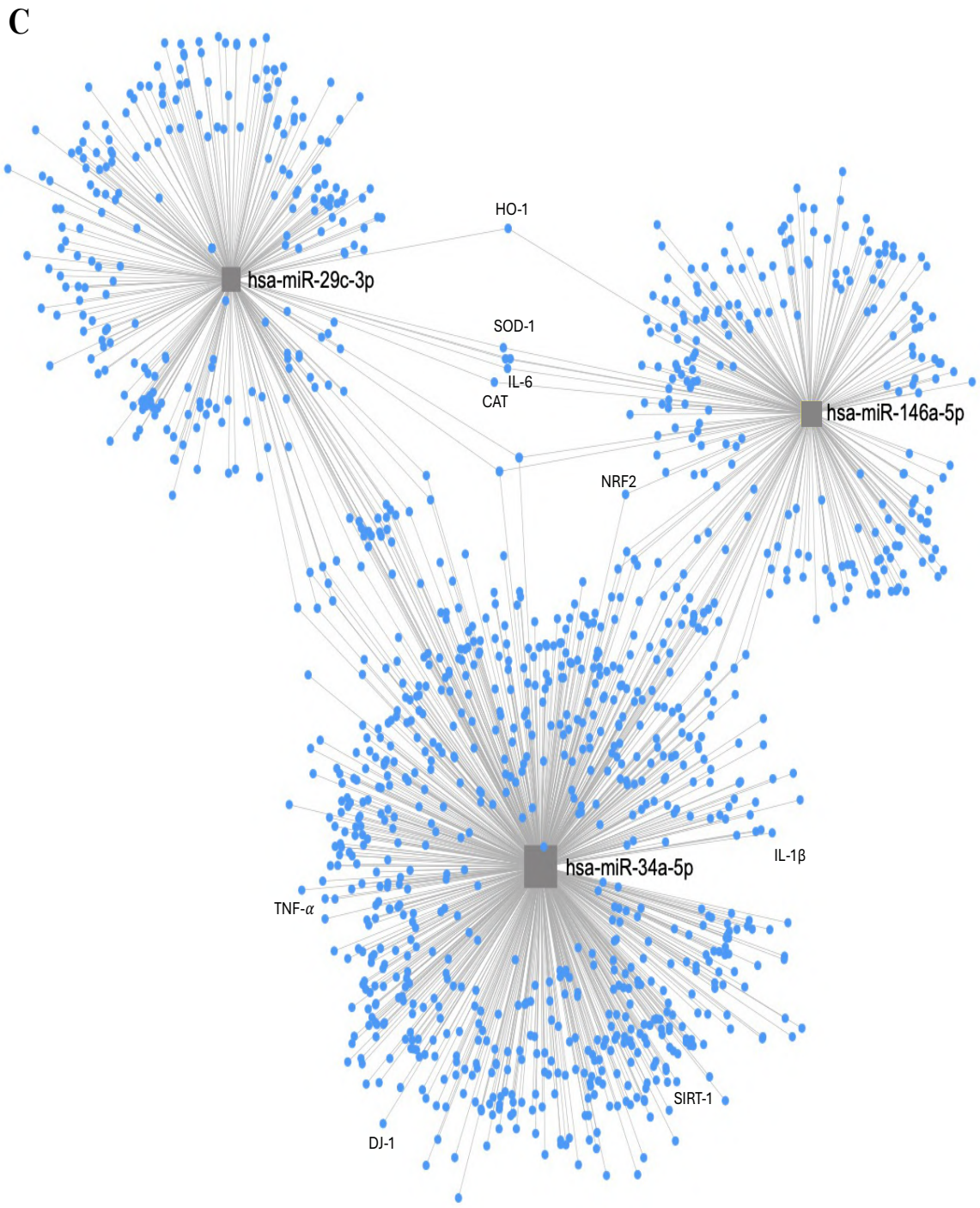


Figure 22. Enrichment pathway analysis in relation of miR-29c-3p, miR-146a-5p and miR-34a-5p in RPE (**A**) and rMC-1 (**B**) cell line**. C:** MiRNA-centric network analytics for a global representation of the target genes regulated by miR-29c-3p, miR-146a-5p and miR-34a-5p**.**

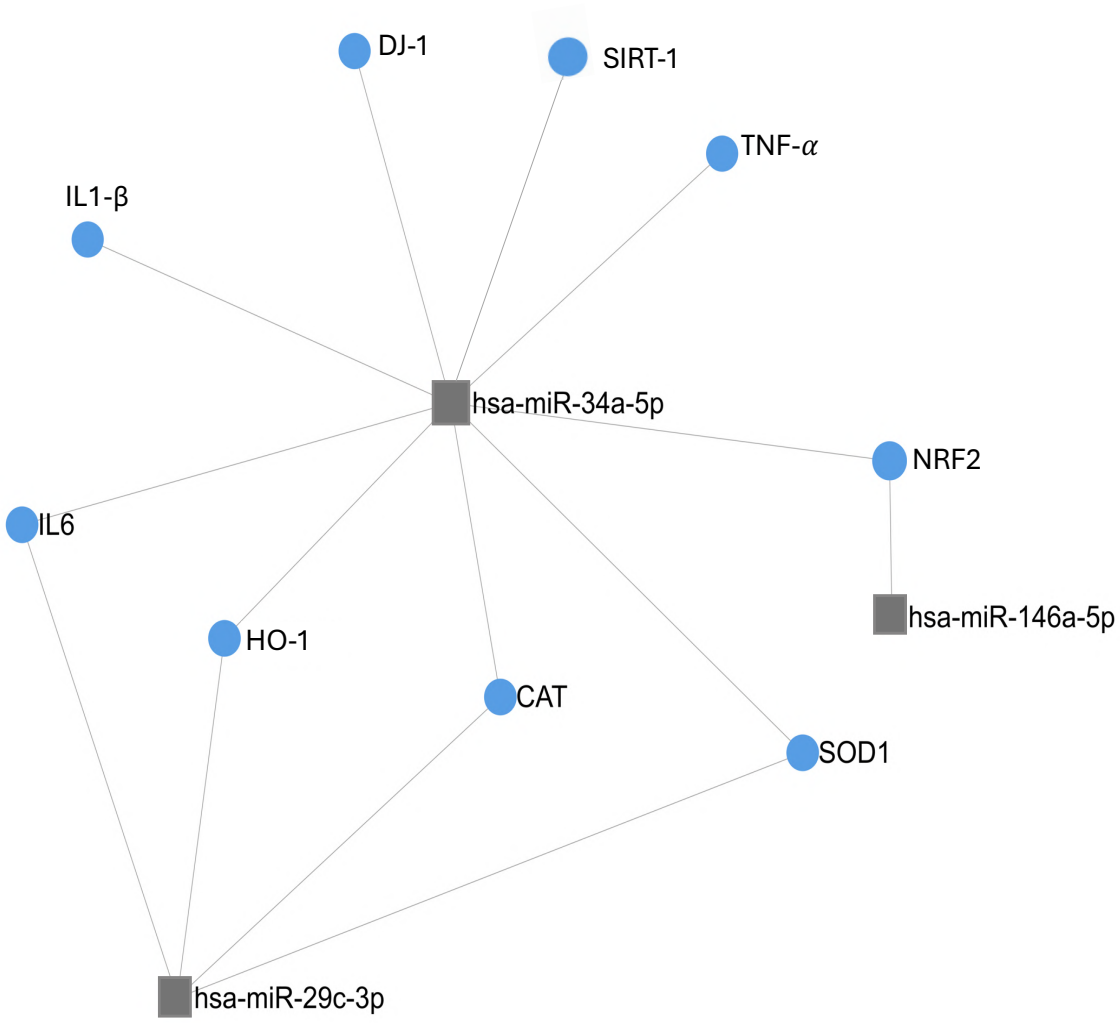


Figure 23. MiRNA-centric network analytics for interpretation of target genes of oxidative stress and inflammation regulated by miR-29c-3p, miR-146a-5p and miR-34a-5p**.**

6.9. SIRT-1 is modulated by miR-34a-5p and involved in the NGF-induced response

The relationship between miR-34a-5p and SIRT-1 was assessed in the RPE cell model. As shown in Figure 21A, miR-34a-5p is significantly increased in RPE cells in HP condition compared to static control but is decreased after NGF treatment. Accordingly, SIRT-1 expression was higher in static CTRL condition, to decrease in HP RPE cells and increase again in response to NGF (Figure 24). Data confirms the interconnection between miR-34a-5p and SIRT-1 as well as the effect on NFG in further increasing SIRT-1 expression.

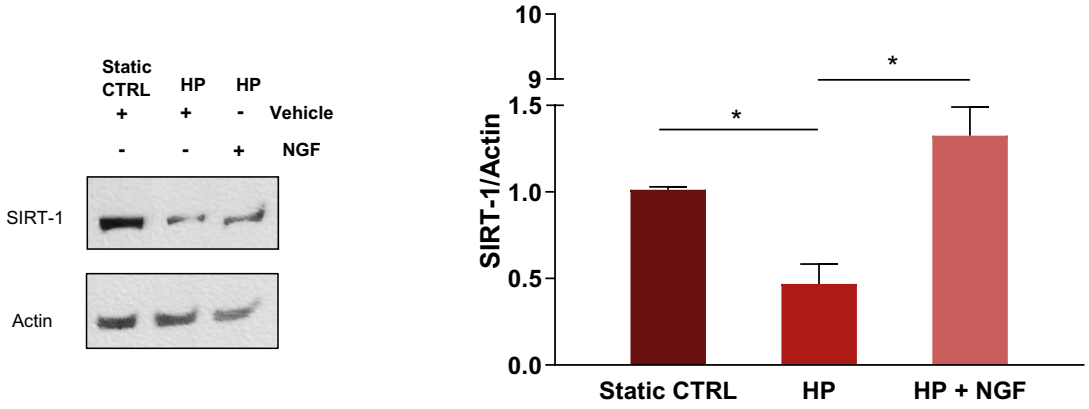


Figure 24. Western blot and densitometric analysis of SIRT-1 protein in RPE cells (normalization target/actin) (n=3). Statistical analysis was performed using multiple t tests * $p < 0.05$; **p<0.01 *** $p < 0.001$.

DISCUSSION

The retinal pigment epithelium (RPE) is a polarized monolayer of highly differentiated epithelial cells that resides between the neural retina and the vascular choriocapillaris. It is responsible for supporting photoreceptor cells by performing specific actives that are necessary to maintain the function and survival of the photoreceptors and other retinal cells (Strauss, 2005). Aotaki-Keen et al. (1996) established from a 19-year-old male the ARPE-19, a spontaneously arising retinal pigment epithelia cell line expressing the RPE-specific markers CRALBP and RPE65 (Hamel et al., 1993). The cells have been extensively utilized in studies on various aspects of cell growth and differentiation (Alizadeh et al., 2001) (Luo et al., 2006). However, reproducing more specific characteristics of differentiated RPE has become difficult using ARPE-19 cells, because they lose specialized properties after multiple (more than 30) passages (Samuel et al., 2017). In this study, we demonstrated that 1 month culturing of ARPE-19 cells leads to the development of a phenotype similar to human RPE. Another study demonstrated the suitability of RPE from differentiated ARPE-19, even though it was for a longer time (4 months instead of 1) (Samuel et al., 2017). The cells here described showed increased expression of genes related to the visual cycle, pigment synthesis, phagocytosis, and RPE differentiation, while genes linked to cell proliferation were downregulated. This resulted in an increase in both mRNA and protein levels, indicating that the cultured ARPE-19 cells can be suitable as a reliable model for studying functions of more differentiated RPE (Luo et al., 2006) (Cai et al., 2006) (J. Tian et al., 2005) (Sharma et al., 2005). Differentiated cells exhibited distinctive characteristics such as a cobblestone epithelial phenotype and high pigmentation (TYR, TYRP1 expression), typical of RPE. They also expressed key proteins involved in epithelial differentiation and tight junction formation, including ZO-1, claudin-2 and occludin (Furuse et al., 2001) (Van Itallie et al., 2003) (Lee J, 2008) (H. Li et al., 2011). In addition, ARPE-19 cells cultured for one month displayed features in terms of photoreceptor outer segments (POS) phagocytosis, demonstrated by the expression of phagocytosis-related genes, such as ITGAV and MERTK (Finnemann et al., 1997) (Feng et al., 2002) (Nandrot et al., 2004). Moreover, cells showed an increase in transcription factors like PAX6 and SOX9, which are crucial for differentiation, melanogenesis, and visual cycle gene expression (Masuda & Esumi, 2010) (Masuda et al., 2014). Overall, results highlight that ARPE-19 cells cultured for longer periods develop a more mature, functional RPE-like phenotype, making them a useful and appropriate in vitro cell model for studying RPE-related diseases. RPE dysfunction is primarily associated with age-related macular degeneration (AMD), but

several evidence show that it can be also involved in the pathophysiology of glaucoma, a major cause of irreversible blindness. In fact, RPE plays a crucial role in cellular repairing and maintaining retinal homeostasis; in this context, its dysfunction is linked to retinal ganglion cell (RGC) death, a hallmark of glaucoma (Gupta, 2009). Glaucoma, characterized by optic nerve damage leading to blindness is often caused by increased intraocular pressure (IOP). At the molecular level, oxidative stress, caused by an imbalance between reactive oxygen species (ROS) production and antioxidant defense, is described as a key factor in glaucoma (Tezel, 2006). Furthermore, when retinal cells experience stress or damage, they release pro-inflammatory cytokines and chemokines, which activate microglia and astrocytes. In turn, activated microglia releases also a variety of inflammatory mediators such as tumor necrosis factor-alpha (TNF-α), interleukins (IL-1β, IL-6) which exacerbate retinal inflammation and damage (Fodelianaki et al., 2019). In this work a novel device able to mimic glaucomatous condition was used to analyze biological responses of the above-described differentiated human RPE and the rat rMC-1 ganglion cells, both involved in glaucoma development, to high pressure (HP) stimuli. Such responses were evaluated in the presence or absence of a neurotrophic factor (NGF). As expected, an increase of proteins working in oxidative stress response mechanisms (e.g. NRF2, HO-1, SOD-1 and CAT) and inflammation (e.g. IL-6 and IL-1β) was observed in HP, indicating the suitability of our model for the study of glaucoma. Such results suggest the vulnerability of RPE cells to oxidative stress and ROS, likely impairing their role also in the photoreceptor cycle: elevated IOP exacerbates oxidative damage in both RPE and Müller cells, further compromising retinal function and contributing to glaucoma progression (Tezel, 2006). Additionally, RPE dysfunction is linked to the breakdown of the blood-retinal barrier and the accumulation of toxic substances, which may further expedite disease progression, as observed in dog with primary glaucoma (Mangan BG, 2007). Studies on animal models of glaucoma have shown that NRF2 increases in response to elevated IOP, indicating that NRF2 is a key part of the cellular response to oxidative damage in glaucoma (Liu et al., 2018). In addition, HO-1 increase has been observed in plasma from glaucoma patients (Salinaro et al., 2014). This phenomenon could be considered as a form of fine-tuning of the antioxidant response, and the initial increase of antioxidant factors induced by high pressure might be intended as an adaptive mechanism designed to maintain cellular homeostasis.

Unfortunately, glaucoma is considered as a disease with high need of cure: early interventions with medications or surgery, can help manage IOP and prevent vision loss (Rudnicka et al., 2006); however, there is no gold standard treatment to stop glaucoma

progression. NGF has been extensively studied for its neuroprotective properties in many neurodegenerative diseases, including some of the eye (diabetic retinopathy, glaucoma, age-related macular degeneration). For this purpose, the response to NGF was assessed in the in vitro glaucoma models. Results here shown are very encouraging, since, after high pressure induction, with consequent responses in terms of oxidative stress, inflammation and likely cell damage, NGF appears to be able to restore conditions similar to those observed in absence of pressure increase. In fact, reduction of NRF2, HO-1, IL-6 and IL-1beta is evidenced, suggesting that NGF could induce biological responses aimed at counteracting those generated by high pressure. Accordingly, NGF has been reported to lower the production of IL-6 in a variety of cell types, including microglial cells and other immune cells in the retina (Fodelianaki et al., 2019), and NGF's ability to reduce IL-1β production is particularly relevant in the context of microglial activation. NGF was found to inhibit the overproduction of IL-1β by microglial cells, in in vitro experimental glaucoma models, thus contributing to a reduction in the inflammatory cascade and limiting the damage to retinal neurons (Fodelianaki et al., 2019).

The identification of specific miRNAs that are dysregulated in glaucoma provides an opportunity for their use either as biomarkers for early diagnosis/disease progression or therapeutic targets. With regard to the latter, the use of synthetic molecules (miRNA mimics or inhibitors), holds promise as a candidate, novel treatment for glaucoma, being the eye a well delimited easily accessible organ. This study highlights three interesting miRNAs (miR-29c-3p, miR-146a-5p and miR-34a-5p) whose expression is very similar in both in the vitro models used: levels of all the 3 miRNAs increase under high pressure to decrease after NGF treatment. Accordingly, with regard to their possible involvement/role in glaucoma, up-regulation of miR-29c-3p was observed in exosomes of glaucomatous patients (Zhang et al., 2023), miR-146a-5p is known for its ability to suppress toll-like receptors pathway activation, a crucial mediator of inflammation and studies suggest that miR-146a-5p can reduce the expression of NF-kB, a key transcription factor in both oxidative stress and inflammation (Krishnan Kutty et al., 2013). MiR-34a-5p plays a role in the regulation of the inflammatory response, promoting the expression of inflammatory cytokines such as IL-6 and TNF-α (Hwang et al., 2013). Furthermore, miR-34a-5p has been described in NF-kB signaling pathway activation, a major mediator of inflammatory response. Therefore, the alteration of miR-34a-5p levels can contribute to immune dysfunction and inflammation, as seen in neurodegenerative diseases, necrotizing enterocolitis, cardiovascular diseases (Zhu et al., 2021). Several target genes of miR-29c-3p, miR-146a-5p and miR-34a-5p, involved

in inflammatory/oxidative responses, were also identified: among them, SIRT1, a miR-34a-5p direct target which plays a role in reducing inflammation (Zhu et al., 2021), expression of which was confirmed coherent with miR-34a-5p levels, and, interestingly, reverted by NGF treatment up to normal condition.

CONCLUSION

This study makes available differentiated human RPE, closer to the physiological one, suitable for further studies, and here used, together with a rat ganglion cell line, for generating an original glaucoma in vitro model. The latter was based on the use of a device able to mimic glaucomatous conditions through pressure increase on the same cultured cells. The model demonstrated its appropriateness, confirmed by the expression level changes, as expected, of factors known to be involved in glaucoma, with reference to oxidative stress and inflammatory responses. In parallel, novel miRNAs showing modulated expression in glaucomatous condition were identified, encouraging further studies about their possible role as biomarkers and/or therapeutic targets. Lastly, very interestingly, considering both protein factors and microRNAs, NGF treatment was revealed to be able to repristinate conditions very similar to those described in absence of high pressure, suggesting possible roles in terms of candidate therapeutic applications for glaucoma treatment.

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Asse IV “Istruzione e ricerca per il recupero – REACT-EU”

Azione IV.4 “Dottorati e Contratti di ricerca su tematiche dell'innovazione”