Effect of prostaglandin F2 analog treatment on proliferation and MAPK/ERK signaling in conjunctival tissues of glaucoma patients

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EFFECT OF PROSTAGLANDIN F2 ANALOG TREATMENT ON PROLIFERATION AND MAPK/ERK SIGNALING IN CONJUNCTIVAL TISSUES OF GLAUCOMA PATIENTS

Diploma thesis

Academic year:

2023/2024

Mentor:

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Split, September 2024

TABLE OF CONTENTS

ACKNOWLEDGEMENT

First and foremost, I would like to thank my parents, Ljiljana and Krešimir, for making it possible for me to fulfill my dream of studying medicine. I am deeply thankful for all the love, advice, and support you have offered me throughout my life and the last six years of my medical studies. Thank you for always having my back and believing in me.

I want to express deep gratitude to my mentor, Marin Ogorevc, MD, PhD. You started teaching us in the first year of our medical studies and continued offering us help and guidance whenever needed. Your dedication and patience helped me on my way to becoming a medical doctor, and I appreciate having you not only as my mentor but also as a good friend.

I would also like to thank the friends I have made along the way; you made the past years an incredible journey I will never forget.

LIST OF ABBREVIATIONS

CAI – carbonic anhydrase inhibitor

CO2 – carbon monoxide

ECM – extracellular matrix

EGF – epidermal growth factor

FP-receptor – prostaglandin F-receptor

IOP – intraocular pressure

MAPK/ERK – mitogen-activated protein kinase/extracellular signal-regulated kinase

MSC – mesenchymal stem cells

MYOC – myocilin

NRR – neuroretinal rim

NTG – normal tension glaucoma

OAG – open-angle glaucoma

OCT – optical coherence tomography

OHT – ocular hypertension

OPTN – optineurin

P-ERK1/2 – phosphorylated extracellular signal-regulating kinase 1 and 2

PACG – primary angle-closure glaucoma

PBS – phosphate-buffered saline

PG – prostaglandin

PI3K/AKT – phosphoinositide 3-kinase/protein kinase B

POAG – primary open angle glaucoma

RGCs – retinal ganglion cells

RNFL – retinal nerve fiber layer

ROKi – Rho kinase inhibitor

ROS – reactive oxygen species

SC – Schlemm's canal

TM – trabecular meshwork

TMC – trabecular meshwork cells

TNF – tumor necrosis factor

VEGF – vascular endothelial growth factor

1. INTRODUCTION

1.1. Structure of the eye

Vision is one of the most essential human senses, highlighted by the fact that over 50% of the body's sensory receptors are located in the eye, and a substantial portion of the cerebral cortex is responsible for the processing of visual information. The embryological development of the eye lasts from the third to the tenth week of gestation, and tissues contributing to its development are of ectodermal and mesodermal origin. More specifically, the neuroepithelium, the surface ectoderm, and mesenchyme, which consists of the neural crest and mesoderm, are involved in the developmental process. The eyes can detect visible light with a wavelength between 400 and 700 nanometers. The globe-shaped eyeball has an average diameter of 2.5 centimeters. It is situated in the anterior portion of the orbit, and only one-sixth of its volume is exposed outside the orbital cavity. Its round shape is disrupted anteriorly by the transient cornea, which projects outward and makes up about one-sixth of the total area of the eyeball. Behind the cornea, in an anteroposterior sequence, are the anterior chamber, the iris with the pupil, the posterior chamber, the biconvex lens, the vitreous chamber, occupied by the thick, clear gel-like vitreous body, and the retina. The anterior chamber is limited anteriorly by the cornea and posteriorly by the iris. The pupil is a central opening in the iris that enables the passage of light rays to the retina. The smaller posterior chamber is limited anteriorly by the iris and posteriorly by the lens. Both chambers are continuous with each other through the pupillary opening. Aqueous humor, produced by the ciliary body, is secreted into the posterior chamber and flows into the anterior chamber through the pupil. It gets absorbed by the circular scleral venous sinus, also called Schlemm's canal (SC), located at the iridocorneal angle. The aqueous humor is vital in maintaining intraocular pressure (IOP) and supplementing nutrients to avascular tissues like the cornea and lens. Any abnormality in the production or reabsorption of aqueous humor can lead to increased IOP and, eventually, the development of glaucoma. The lens is an elastic, transparent, and biconvex structure that separates the anterior one-fifth of the eye from the posterior four-fifths. Circumferentially, it is connected to the ciliary muscle through zonular fibers. Contraction or relaxation of the ciliary muscle enables the lens to change its curvature and thus its refractive ability to maintain visual acuity, a process called accommodation (1, 2).

Three main layers surround the internal structures of the eyeball. Those layers include an outer fibrous layer made up of the sclera posteriorly and the cornea anteriorly, a middle vascular layer consisting of the choroid posteriorly and continuing as the ciliary body and iris anteriorly, and an inner layer consisting of the retinal pigmented epithelium and the neuronal layer, where the photoreceptors, mainly comprised of rods for scotopic vision and cones for photopic vision, can be found. In this part, visual input is picked up, converted into electrical

impulses, and conducted via the optic nerve to the brain. The macula lutea is the central region of the retina. It contains the fovea centralis, which contains only cones and is crucial for central vision (1, 2).

Branches of the internal carotid arteries supply all ocular structures except the eyelids and the conjunctiva, which receive blood from branches of the external carotid arteries. The ophthalmic artery, originating from the internal carotid artery, further branches into the posterior ciliary, retinal, and muscular arteries and is essential for ocular blood supply. Six extrinsic eye muscles are inserted into the sclera and are responsible for eyeball movement. The superior rectus muscle, inferior rectus muscle, medial rectus muscle, and inferior oblique muscle are innervated by the oculomotor nerve. The abducens nerve innervates the lateral rectus muscle, while the trochlear nerve is responsible for innervation of the superior oblique muscle (**Figure 1**). The human eye is a highly specialized structure whose complex anatomy and embryological development are essential for understanding various ocular diseases (1, 2).

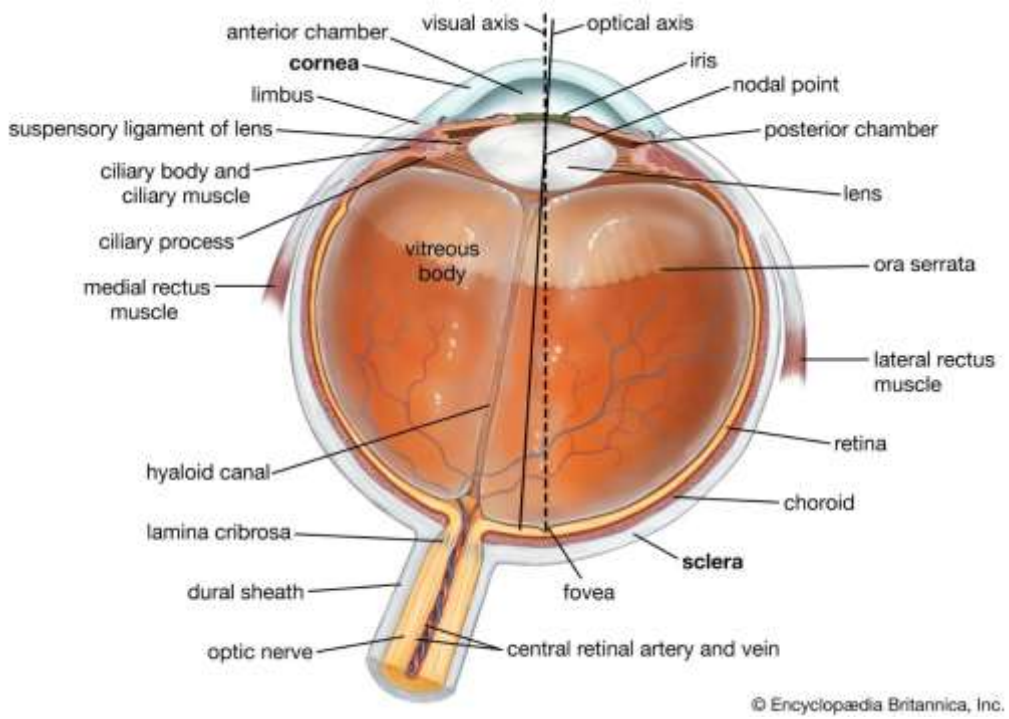

Figure 1. A horizontal cross-section of the human eye, showing the major parts of the eye. Encyclopædia Britannica, Inc. human eye: Media [Internet]. Chicago: Encyclopædia Britannica; 2019 [cited 2024 Jul 24]. Available from: https://www.britannica.com/science/human-eye/images-videos#/media/1/1688997/100415

1.1.1. The conjunctiva

The conjunctiva is a thin, transparent mucous membrane that covers the inner part of the eyelid and the sclera up to the corneoscleral limbus. Embryologically, it is derived from the ocular surface ectoderm (3). Anatomically, the conjunctiva is divided into the palpebral, forniceal, and bulbar conjunctiva. While the palpebral conjunctiva lines the eyelids, the bulbar conjunctiva can be found on the anterior surface of the globe, where it is connected to the underlying sclera by Tenon's capsule. The flexible forniceal conjunctiva connects its bulbar and palpebral counterparts and allows for movement of the globe against the eyelid. The conjunctival epithelium has a thickness of three to five cell layers and consists of a non-keratinized, stratified, squamous epithelium and a stratified columnar epithelium. Columnar cells predominantly can be found in the basal epithelial layers and become increasingly flattened as they approach more superficial layers (**Figure 2**). Interspersed are goblet cells that produce the mucinous part of the tear film and thus prevent its evaporation and drying of the eye. The conjunctival stroma, or the substantia propria, consists of two sub-layers: the superficial adenoid layer and the deep fibrous layer. It contains fibroblasts, blood vessels originating from the anterior ciliary and palpebral arteries, immune cells like lymphocytes, mast cells, plasma cells, neutrophils, and a distinct lymphatic network draining into the preauricular and submandibular lymph nodes. Accumulations of T and B lymphocytes that form patches in the fornix constitute the conjunctiva-associated lymphoid tissue (4, 5). The supraorbital nerve, the supratrochlear nerve, the infratrochlear nerve, the infraorbital nerve, the lacrimal nerve, and the long ciliary nerves are responsible for the sensory innervation of the conjunctiva (6). The conjunctiva protects and moisturizes the ocular surface by participating in tear film production; this facilitates frictionless movement between the globe and eyelids. It acts as a barrier against the entry of microorganisms and engages in the immunological surveillance of the eye. Its rich vascular network participates in nutrient supply and waste product clearance (7).

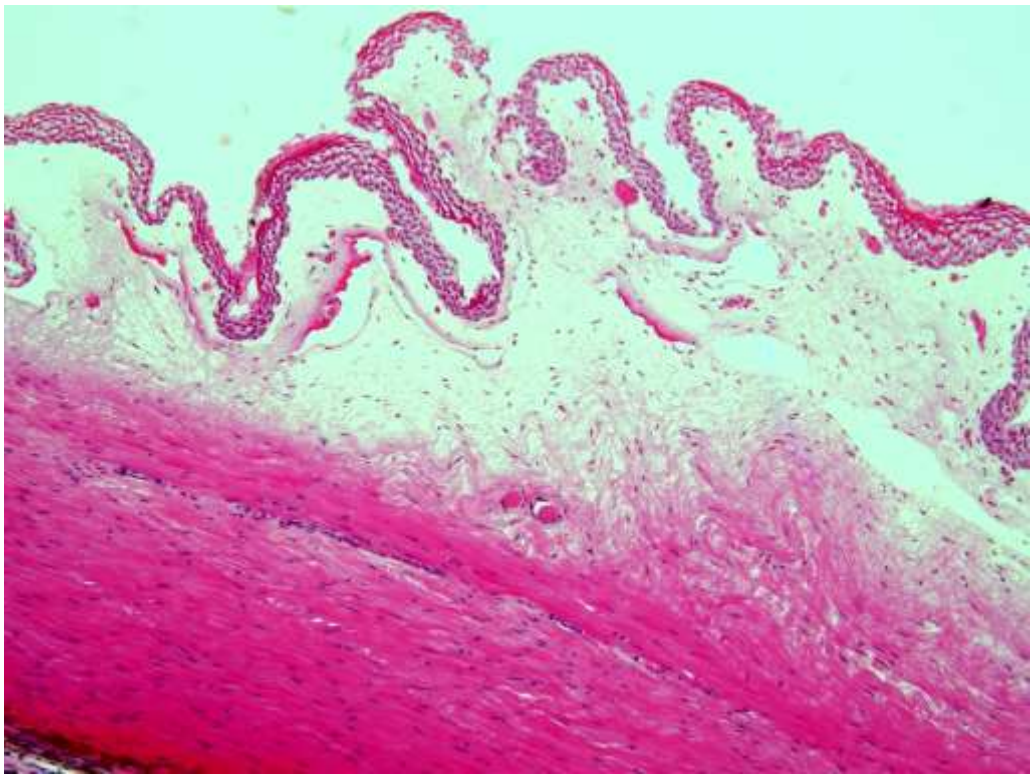

Figure 2. Hematoxylin and eosin staining of the human eye. The stratified squamous and, in parts, columnar epithelium and the highly vascularized stroma of the conjunctiva are visible above the dense connective tissue of the sclera. Source: mentor's personal archive.

1.1.2. The trabecular meshwork

The trabecular meshwork (TM) at the iridocorneal angle is crucial for regulating aqueous humor flow out of the eye. It is a three-dimensional and fenestrated structure consisting of trabecular meshwork cells (TMC) embedded into a multi-layered extracellular matrix (ECM). TMC have properties of endothelial cells, myofibroblasts, and macrophages. Those cells regulate the resistance of aqueous humor outflow, and their properties like maintenance of permeability, neutralization of reactive oxygen species (ROS), phagocytosis, immune mediation, tissue repair, contractility, and mechanotransduction depend on their location inside the TM. The TM plays a critical role in maintaining adequate IOP by modulating the outflow of aqueous humor from the anterior chamber of the eye into the SC and, ultimately, into the venous system through aqueous vein collector channels. The impaired function of the TM can disrupt the outflow of aqueous humor out of the eye, causing IOP elevation (8). Under physiological conditions, around 90% of the aqueous humor leaves the anterior chamber

through the TM, 10% via the uveoscleral tract, and a small reminder drains directly via the iris (9).

Embryologically, the TM has a mixed origin. It consists of mesodermal tissue and mesenchymal cells derived from the neural crest and migrating into the eye between the 15th and 20th week of gestation (10). The TM is located between the anterior chamber and the SC, a ring-shaped structure that collects aqueous humor and drains it into the extraocular circulatory system. It is a sieve-like structure, from Schwalbe's line anteriorly up to the connection between the ciliary body, iris, and scleral spur, where the trabecular lamellae connect posteriorly (8, 11).

Functionally, the TM is divided into two main parts. The anterior section is located close to Schwalbe's line and has no filtering properties compared to the posterior filtering section, which directly connects to the SC (**Figure 3**). The posterior section is further divided into three distinctive parts. The uveal meshwork comprises delicate, interwoven pillars consisting of collagen and elastin fibers extending from the root of the iris and ciliary body up to Schwalbe's line. They are covered with TMC lying on a basement membrane and forming openings that enable the passage of aqueous humor. The corneoscleral meshwork constitutes the largest part of the TM and consists of stacked lamellae of collagen and elastin fibers. Those lamellae have orifices that increase in number from the anterior wall of the scleral sulcus towards the scleral spur. A single layer of TMC covers the structure and facilitates the outflow of aqueous humor. The juxtacanalicular meshwork, the outermost part of the filtering TM section, exhibits histologic differences from the uveal and corneoscleral meshwork. It consists of loose connective tissue and scattered layers of TMC embedded in an ECM. This portion of the posterior filtering segment is connected to the endothelial layer of the SC and facilitates the outflow of aqueous humor into it. The number of porous orifices of the posterior filtering TM slowly decreases from the inside toward the outside (**Figure 4**). Under normal physiologic conditions, all the mentioned layers enable unobstructed drainage of aqueous humor into the extraocular circulation (8).

The aqueous humor can exit into the extraocular circulation using either the primary pathway, also called the conventional pathway, entering the SC through the TM and exiting via the episcleral and conjunctival veins, or the secondary pathway, known as the unconventional or uveoscleral pathway, draining through the ciliary muscle and other downstream tissues and exiting through canals surrounding the vortex veins or choroidal vessels (12, 13). The primary pathway is responsible for 70-95% of the outflow, while the secondary pathway accounts for only 5-30% (13). Around 75% of outflow resistance in humans originates in the TM, especially in the juxtacanalicular portion of the posterior filtering TM, while 25% originates in the SC

(**Figure 5**). Outflow resistance is determined by the anterior portion of the ciliary muscle, whose tendinous extensions transverse the TM and insert into the wall of the SC and TMC, exhibiting properties of myofibroblasts (8). Contraction of the ciliary muscle or relaxation of the mentioned cells reduces outflow resistance. Stimulation of cellular stretch-receptors or deformation of the juxtacanalicular portion are two mechanisms that regulate outflow resistance (14). In this way, the resistance exhibited can be adjusted and modified by the turnover of the ECM, the action of proteinases, and the digestion or biosynthesis of new components (8).

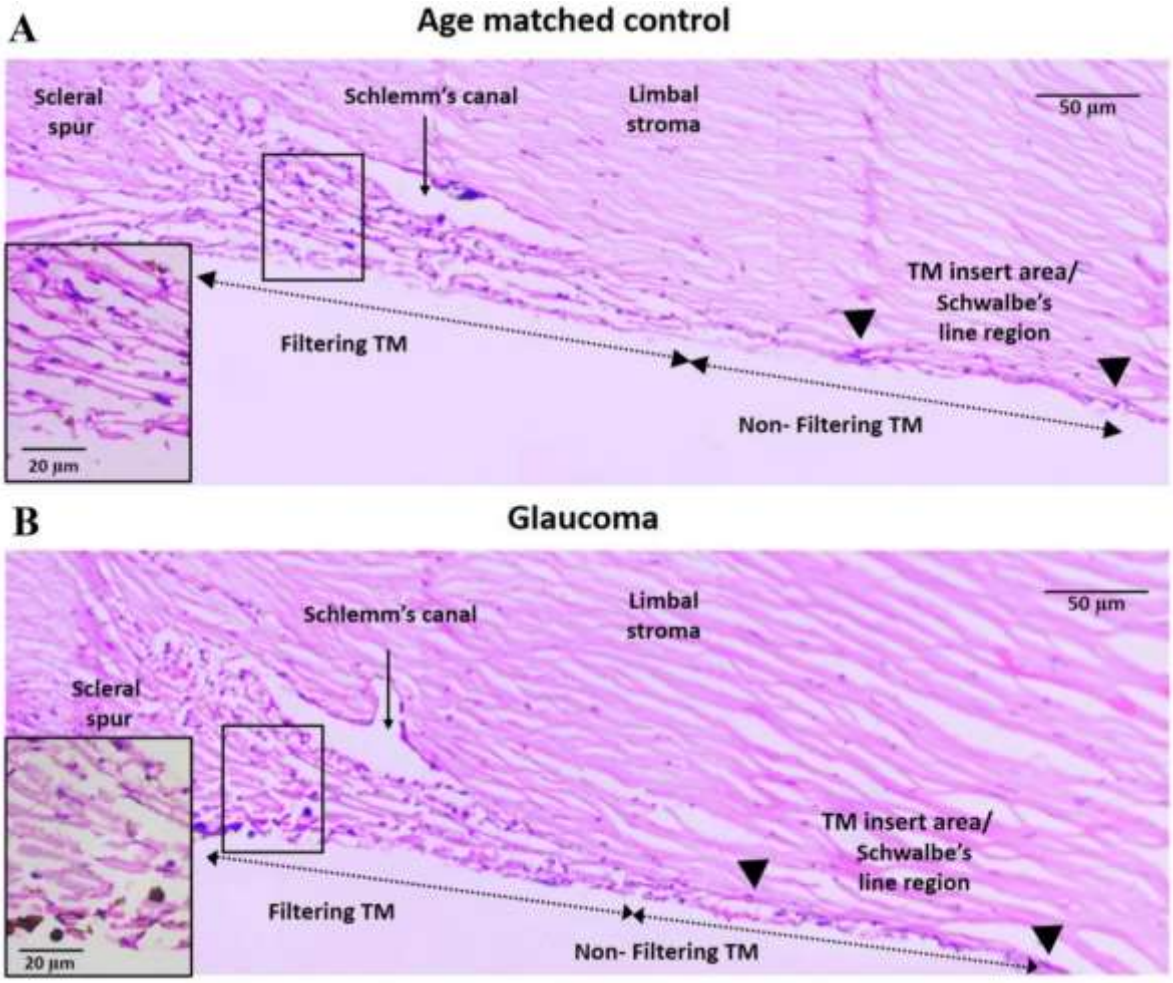

Figure 3. Histology of the trabecular meshwork in glaucoma. Source: Sundaresan Y, Manivannan LP, Radhakrishnan S, Ramasamy KS, Veerappan M, Chidambaranthan GP. Reduction in trabecular meshwork stem cell content in donor eyes with primary open angle glaucoma. Sci Rep. 2021;11:24518.

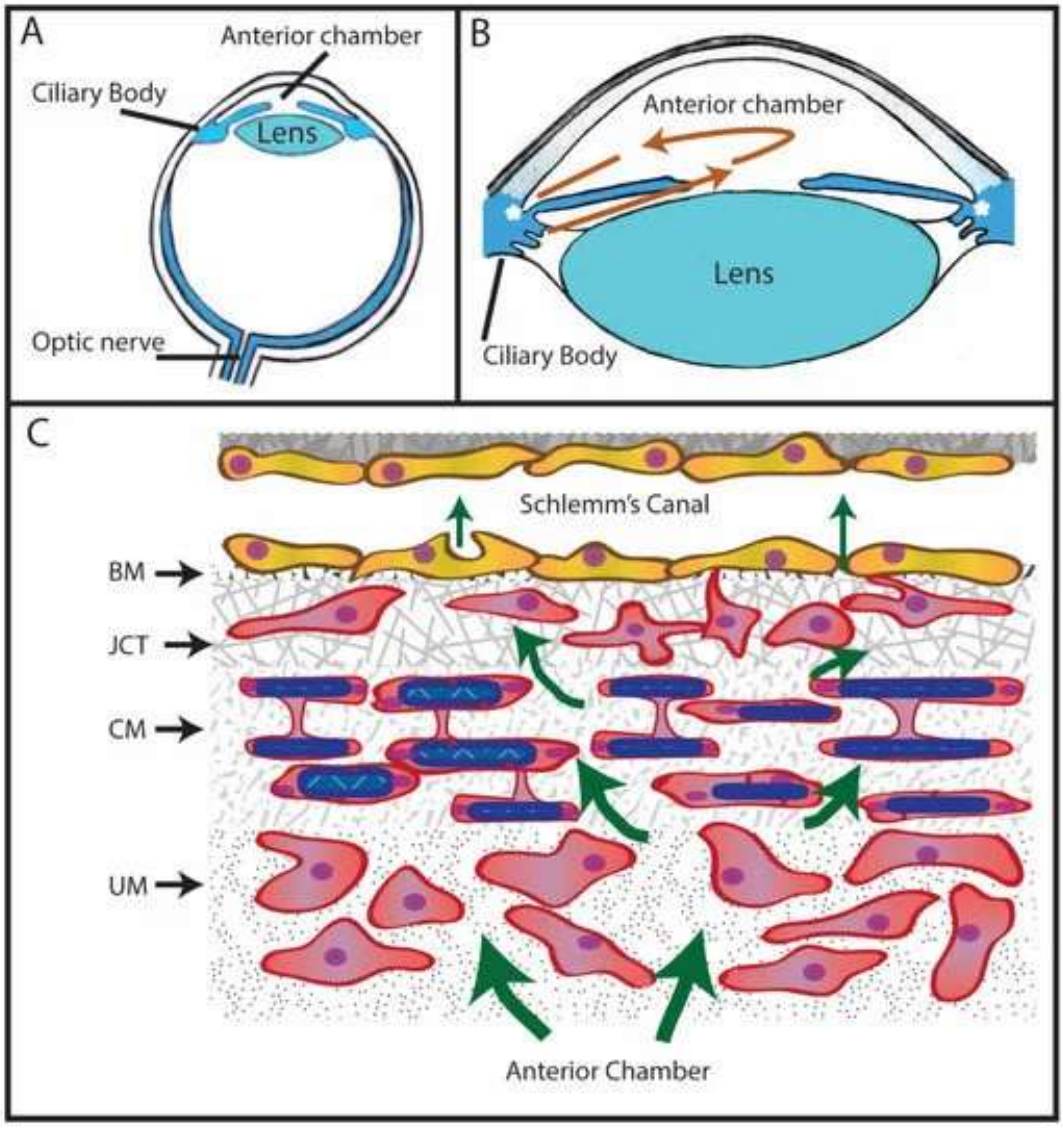

Figure 4. Anatomy of the eye and trabecular meshwork. Source: Faralli JA, Filla MS, Peters DM. Role of Fibronectin in Primary Open Angle Glaucoma. Cells. 2019;8:1518.

1.2. Glaucoma

The term glaucoma refers to a group of eye conditions sharing features of irreversible and progressive optic nerve damage with cupping of the optic disc, reduction in the number of retinal ganglion cells (RGCs), and a decrease of peripapillary retinal nerve fiber layer (RNFL) thickness. These degenerative processes cause permanent visual field loss and make glaucoma one of the leading causes of blindness worldwide and the second most common cause of irreversible blindness in Western Europe. Despite the standard features, there are essential differences in the pathophysiology, causative factors, treatment options, and prognosis (15). The condition can be either congenital or acquired, and further distinction is made between two major forms of glaucoma: open-angle glaucoma, where impaired aqueous outflow leads to a

gradual increase in IOP, despite a normal appearing anterior chamber angle, and angle-closure glaucoma, in which increased IOP is caused by impaired aqueous humor outflow secondary to appositional or synechial blockage of the iridocorneal angle. Further differentiation between primary and secondary forms depends on whether an underlying ocular or systemic condition, medications, or trauma can be identified as the causative factor. Increased IOP is a significant risk factor and a critical modifiable factor (7). In people over the age of 40, approximately 2-3% are affected by glaucoma, and up to 50% remain undiagnosed; this number dramatically varies depending on the country, ethnicity, and socioeconomic status (7, 16).

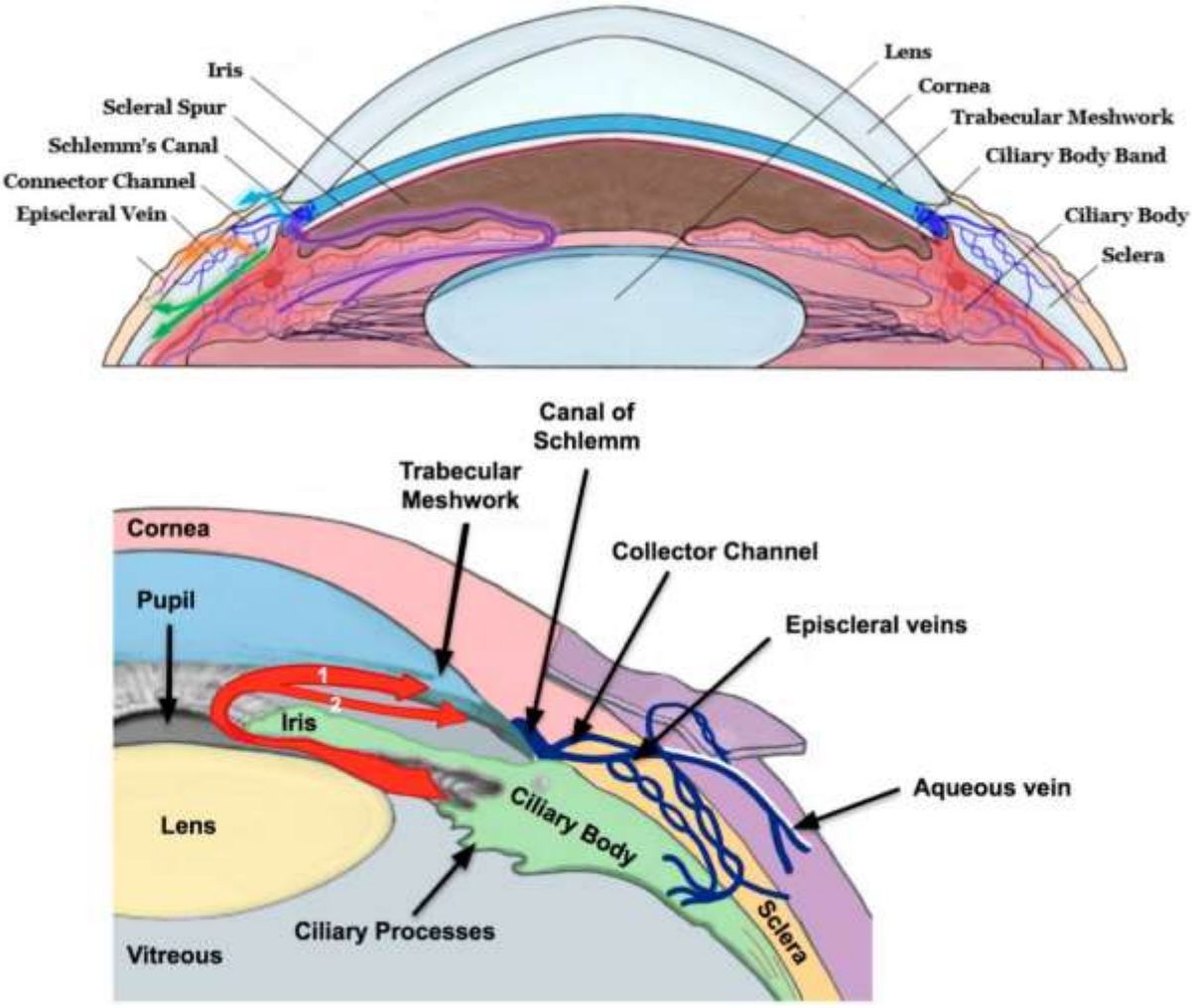

Figure 5. Illustration of aqueous humor flow dynamics. Source: Dave B, Patel M, Suresh S, Ginjupalli M, Surya A, Albdour M, Kooner KS. Wound modulations in glaucoma surgery: a systematic review. Bioengineering. 2024;11:446.

1.2.1. Primary open-angle glaucoma

Primary open-angle glaucoma (POAG) is the most prevalent form of glaucoma in people of European and African descent and affects both genders equally. It is a chronic disease with a progressive course, typically presenting with an adult-onset (7). Characteristically, the TM in the iridocorneal angle appears open, but the outflow of aqueous humor is impaired (17). The impaired flow is caused by trabecular stiffening, aging, TMC apoptosis, and changes in ECM structure. Although those changes resemble aging processes, they are accelerated in glaucoma cases (8). In a study by Hamard et al., the outer trabecular membrane was extracted during non-penetrating deep sclerotomy, and subsequent microscopic examination revealed a substantial decrease in cellular density of juxtacanalicular and corneoscleral meshwork tissues compared to healthy controls (18). An increased IOP or a difference in IOP of more than four mmHg between the right and left eye is a significant risk factor and sole modifiable factor (7, 9, 19). Despite the consistent connection between increased IOP and primary open-angle glaucoma, 25-50% of affected individuals present with normal pressures within the range of 10-21 mmHg (7, 17, 19). Further essential risk factors include advanced age, ethnicity, high-grade myopia, and a positive family history. Mutations in two genes are broadly accepted to increase the likelihood of POAG. Those include the MYOC gene, coding for the TM protein myocilin. Mutations in the MYOC gene are typically connected to juvenile or early-onset adult POAG and affected individuals usually present with an increased IOP. In contrast, individuals with a mutation in the OPTN gene, coding for the protein optineurin, typically present with a normal IOP (7, 20). In about one-third of patients, POAG is diagnosed in an advanced stage since affected individuals often remain asymptomatic for many years, and the disease progresses silently, causing substantial damage to the optic nerve (20). Persistent IOP elevation leads to excavation or cupping of the optic nerve head, which can be visualized as a pathologic increase in cup-to-disc ratio, posterior displacement and thinning of the lamina cribrosa, and narrowing of the neuroretinal rim (NRR). An ongoing loss of RGCs and axons causes those changes. The characteristic appearance of the optic nerve head and RNFL are key diagnostic features of glaucomatous neuropathy and can be visualized during an ophthalmologic examination (20).

1.2.2. Primary angle-closure glaucoma

Primary angle-closure glaucoma (PACG) is mainly caused by pupillary block. Despite its prevalence among glaucoma patients being only 26%, it accounts for around 50% of glaucoma-related blindness cases worldwide (21). In PACG, the flow of aqueous humor from

the posterior into the anterior chamber through the iris-lens channel is impaired; this causes an increase in the pressure differential between those two compartments, which is around 0,23 mmHg under physiologic conditions, and leads to bowing of the iris anteriorly. The abnormally displaced, convex iris ultimately leads to appositional closure of the iridocorneal angle and impaired aqueous humor outflow from the eye's anterior chamber through the TM. The resulting increase in IOP leads to optic nerve damage and visual field loss. Anterior non-pupillary block is another possible cause of PACG (22). This can be observed in the case of plateau iris, where outflow impairment persists or progresses despite a patent iridotomy. Plateau-iris configurations can be mainly observed in women aged 30-50 who have a short axial eye length due to hyperopia. The iris root gets compressed against the TM by a large ciliary body positioned anteriorly; this facilitates angle-closure development even though a normal anterior chamber depth and flat iris profile can be observed (23). The most significant risk factors for the development of PACG include advanced age, female gender, Asian ethnicity, hyperopia, larger iris volume and thickness, a shallow anterior chamber, decreased corneal diameter, a steep corneal curvature, a shallow limbal chamber, and a thick lens that is positioned anteriorly (22). Certain drugs with mydriatic effects, like antihistamines, adrenergics, and anticholinergics, may also increase the probability of an acute PACG attack in patient groups with the mentioned risk factors (24). Similar to POAG, the majority of patients with PACG are asymptomatic for long periods of time, commonly diagnosed with other chronic diseases, and are not always aware of visual field losses. Gonioscopy is an essential diagnostic technique for evaluation and direct visualization of the anterior chamber angle, and iridotrabecular contact of at least 180° in conjunction with elevated IOP or peripheral anterior synechiae strongly indicates the presence of PACG (22, 25). In the case of acute angle closure, patients can experience symptoms like sudden onset of pain and usually unilateral vision loss due to an acute increase in IOP. Additionally, they may present with halos around lights due to corneal edema, intense sensation of pain around the eye, as well as nausea and vomiting. These symptoms increase the likelihood of potentially misdiagnosing the condition as a migraine episode. Examination findings like a mid-dilated, unreactive pupil, a cloudy cornea, and an injected conjunctiva can typically be found in acute PACG and can help differentiate the condition from migraines (24).

1.2.3. Classification of secondary glaucoma

Secondary glaucoma can be divided into two main categories: open-angle and closed-angle. Secondary open-angle glaucoma can be further subdivided by the position of aqueous

humor outflow obstruction, which can be pre-trabecular, trabecular, or post-trabecular. Pre-trabecular outflow obstruction is usually caused by a membrane that covers the trabeculum. This membrane may consist of fibrovascular tissue or endothelial and epithelial cellular membranous proliferation. Trabecular obstruction is caused by congestion of the TM and secondary degenerative changes. Factors leading to the described changes include pigment particles, red blood cells, degenerate red cells, macrophages, and lens proteins in phacolytic glaucoma, proteins as an element of uveitis, pseudoexfoliative materials and edematous alterations in trabecular fiber structure and post-traumatic, -infectious, or -surgical scarring. In post-trabecular causes of secondary open-angle glaucoma, the TM structure is normal, and elevated episcleral venous pressure due to a carotid-cavernous fistula, Sturge-Weber syndrome, or obstruction of the superior vena cava impairs proper outflow (7).

Secondary closed-angle glaucoma can be present with or without pupillary block. If pupillary block is present, possible causes are seclusion of the pupil after recurrent iridocyclitis, a subluxated lens, phacomorphic glaucoma, capsular block syndrome, aphakic pupillary block, and a lens implanted into the anterior chamber without patent iridotomy. In the absence of a pupillary block, factors causing secondary peripheral anterior synechiae, like chronic anterior uveitis, are potential triggers. It is estimated that up to 20% of all glaucoma cases can be attributed to some secondary cause like other ocular and systemic diseases or trauma (7, 26).

1.2.4. Diagnosis of glaucoma

IOP, which ranges between 10 and 21 mmHg under physiologic conditions, is a critical factor in the development and progression of glaucoma. Lowering the IOP is the main goal of glaucoma treatment, so its determination is crucial for screening, diagnosis, and monitoring. Tonometry involves measuring the IOP and is important for diagnosing glaucoma, but it can also be used as a screening method or in ophthalmologic follow-up examinations. Goldmann applanation tonometry is the gold standard for determining IOP due to its high accuracy and reliability. This method is based on the Imbert-Fick law, according to which the pressure inside a sphere is equal to the force required to flatten its surface divided by the area of the flattening. In practice, the patient's eye is anesthetized with eye drops, and a fluorescein dye is applied to the tear film. In this way, the indented area can be visualized with a slit-lamp biomicroscope. The tonometer consists of a biprism with a flat, applanating surface that is 3.06 mm in diameter. The tonometer is gently brought into contact with the cornea by the ophthalmologist and compressed until the inner edges of two fluorescein-stained semicircles come into contact, and

the indented surface area amounts to 7.35 mm2. Corneal thickness and properties can influence the results of the measured IOP, and the technique is highly user-dependent (27).

Visual field testing is mandatory for diagnosing glaucoma, and automated perimetry currently constitutes the gold-standard method. It evaluated the patients' visual field, specifically evaluating the sensitivity and extent of the peripheral vision in a quantitative manner. The method is user-friendly and provides standardized data, meaning that obtained results are consistent over time and can be compared to monitor disease progression or treatment success. Most commonly, a static perimetry approach is used, where the intensity of the light stimulus varies at fixed locations in the visual field. The Humphrey Field Analyzer is routinely used for static perimetry. The Octopus perimeter is another commonly used device that allows static and kinetic perimetry, where the light stimulus is moving, and the patient must point out when seeing the light for the first time. In automated static perimetry, the patients sit straight while watching a fixation point. Subsequently, light stimuli of different intensities and locations are presented to the patient, who must press a button each time they see one. In this way, a detailed map of the individual visual field and its defects is created. The analysis involves threshold testing, where the dimmest detectable light stimulus is determined. The values are compared to healthy controls in the corresponding age group and visual field location. In suprathreshold testing, a very bright stimulus is used to detect existing defects quickly. Pattern and total deviations provide a comprehensive visual field analysis, making distinguishing between localized and generalized defects possible. Significant limitations of automated perimetry include patient compliance and fatigue. It is important to state that a substantial learning effect can be observed commonly, and results may improve with practice (28).

Indirect fundoscopy is a diagnostic procedure used to evaluate the back of the eye. It is particularly useful for visualizing the retina, the optic nerve head, and retinal vasculature. The examiner uses a slit-lamp biomicroscope in combination with hand-held lenses to obtain an enlarged and clear image of background structures through a mydriatic pupil. Indirect fundoscopy is an excellent method for detecting glaucomatous changes like optic neuropathy. Death of RGCs and a reduction of optic nerve fibers lead to a characteristic appearance of the optic nerve head and RNFL (20). The mentioned changes can be visualized during an ophthalmoscopic examination of the optic nerve head, showing irreversible and pathologic cupping with an increase of cup-to-disc ratio, pointing to a diminished number of nerve fibers, glial cells, and blood vessels; thinning and notching of the NRR, asymmetry in appearance of the optic nerve heads between right and left eye, disc hemorrhage, baring of circumlinear blood vessels, bayoneting of blood vessels, laminar dot sign, indicating an advanced stage of

glaucoma and presenting as gray dot like fenestrations in the lamina cribrosa, and sharpening of the NRR edge (7, 20).

The main feature differentiating POAG from PCAG is the patency of the iridocorneal angle, where aqueous humor leaves the anterior chamber of the eye. Per definition, an anatomically closed angle is present when at least 270° of the angle is occluded. Gonioscopy can visualize this distinctive feature directly. During this examination, the ophthalmologist uses a slit-lamp biomicroscope in combination with a gonioscope, a hand-held tool consisting of high-quality lenses or prisms, multiple mirrors angled in different directions, and a contact lens that is directly placed onto the patient's eye, with the help of coupling gel or fluid. During gonioscopy, the examiner can determine whether angle closure or peripheral anterior synechiae are present. The downsides of this method are a high degree of subjectivity and poor reproducibility. The amount of light and pressure used on the eye during the examination may also influence the results obtained (20).

Optical coherence tomography (OCT) is a non-invasive, in vivo, high-resolution, cross-sectional 2D imaging technique that allows visualization of the retinal layers and other ocular structures using low-coherence interferometry. The structural assessment of the retina and the optic nerve is crucial for diagnosing and evaluating disease progression in glaucoma patients. OCT enables objective quantification of retinal tissue layers like the macula, area surrounding the papilla, and optic nerve head. The obtained results are then compared with normative databases. OCT is crucial because it allows for the detection of glaucomatous changes before visual field loss has occurred. Damage of RGCs and their axons leads to thinning of the RNFL, one of the earliest detectable signs suggesting the presence of glaucoma. Glaucoma primarily affects the three innermost retinal layers: the RNFL, the ganglion cell layer, and the inner plexiform layer. Those layers are also known as the ganglion cell complex, usually measured in the macular region, and essential for transmitting visual information from the retina to the brain. Visualization of the optic nerve head, with a particular focus on cup-to-disc ratio, rim area, and volume, is also important for diagnosis and monitoring of disease. An OCT of the anterior segment provides valuable information about anterior segment structures like the iridocorneal angle, anterior synechiae, angle recession, and corneal properties. OCT is a valuable tool for screening, detecting, and monitoring glaucoma. It is a cost-effective and quick technique that helps to establish a baseline data assessment against which future results can be compared (29).

Corneal properties like central corneal thickness and corneal hysteresis have been shown to play an essential role in determining IOP correctly. The average corneal thickness measures

approximately 542 μm, and it is proven that excessively thick corneas can lead to an overestimation of IOP, while excessively thin corneas result in the contrary (30). Thin corneas have additionally been associated with an increased risk for glaucoma development, particularly normal tension glaucoma. Corneal thickness can be determined using ultrasound pachymetry, OCT, specular or confocal microscopy, and Scheimpflug imaging (31).

1.2.5. Treatment of glaucoma

1.2.5.1. Prostaglandins

Prostaglandins (PGs) belong to the group of eicosanoids. This group also includes related compounds like prostacyclins, thromboxanes, leukotrienes, and lipoxins (32). PGs are pro-inflammatory molecules and a byproduct of arachidonic acid metabolism by cyclooxygenase 1 and 2 enzymes. There are five different classes of PGs: PGE2, PGF2, PGI2, PGD2, and thromboxane A2. PGs exhibit their actions by coupling to matching G-protein coupled receptors, of which nine specific ones are known and expressed on surfaces of different cells (33).

The prostaglandin F-receptor (FP-receptor) is found in the human eye, specifically in the corneal and ciliary epithelia, the circular portion of the ciliary muscle, as well as stromal and smooth muscle cells of the iris (34, 35). It activates the phosphatidylinositol metabolism, which leads to an increase in intracellular free calcium concentration and the initiation of different signaling pathways. PGF2 and its agonists cause a decrease in IOP by potentiating the uveoscleral outflow of aqueous humor. Those effects are based on the relaxation of the ciliary vasculature and smooth musculature, changes in cytoskeletal structure, and ECM modification but are still not fully understood (33). By activating matrix metalloproteinases, PGF2 analogs cause ECM disruption in the ciliary body and decrease the outflow resistance of the uveoscleral tract (36). Latanoprost was the first PG agonist approved for glaucoma treatment in 1996. Since then, similar agonists like bimatoprost, travoprost, and tafluprost have been developed and brought to the market (33).

PG analogs are the first-line agents for glaucoma treatment since they most effectively target the IOP, which is currently the sole modifiable risk factor (36). They replaced beta-blockers as first-line agents due to their superior efficacy, once-a-day usage, better daytime control of IOP, and fewer systemic side effects. They have an IOP-lowering potential of 28% – 31%, while the drug-elimination period ranges from four to six weeks. Additional drug classes used for the treatment of elevated IOP include carbonic anhydrase inhibitors (CAI) and α-2-

adrenergics. Indications for using PG analogs include POAG, PACG, ocular hypertension (OHT), NTG, secondary glaucoma (e.g., pseudoexfoliative glaucoma, pigmentary glaucoma), and any form of glaucoma without inflammation, macular edema or keratitis. PGs are well-known for their pro-inflammatory effects, which make uveitis and macular edema absolute contraindications for their usage. Relative contraindications for the treatment with PG analogs include patients with an increased risk for the development of cystoid macular edema, like aphakic or pseudophakic eyes with posterior capsular rupture, patients with a history of macular edema or herpetic keratitis, recent intra-ocular surgery, and women who are pregnant or nursing (32). The responsiveness of patients to the effects of PG analogs can be highly variable. Those patients commonly need the coadministration of other drugs like beta-blockers or CAIs. Rarely there are patients who only respond to specific PG analogs while being unresponsive to others (37).

Latanoprost is a prodrug-analog of PGF2 that is selective for the FP-subtype of prostanoid receptors and becomes biologically active through the action of hydrolases in the cornea. It is a compound with highly lipophilic properties, enabling it to penetrate the cornea and reach its maximal concentration in the aqueous humor after one to two hours. After administration of one dose, it exhibits actions on the IOP for 20 – 24 hours. Studies have shown that latanoprost leads to an average reduction of the mean IOP by 7.9 mmHg in patients with PACG and mainly induces side effects limited to the eye, including conjunctival hyperemia and darkening of the iris. Latanoprost additionally improves perfusion of the optic nerve head (32, 38).

Travoprost is a PGF2-agonist with strong agonistic effects on the FP-receptor. It is used once daily and is more effective in reducing IOP than timolol in patients with OAG and OH. IOP is mainly reduced by potentiated uveoscleral outflow, and a meta-analysis showed a pressure-reducing potential of 29% – 31%. Travoprost is available in a formulation with benzalkonium chloride, but studies have shown no significant advantages over the benzalkonium chloride free formulation (32, 39).

Tafluprost was the first PG analog for treating OAG and OH without added preservatives. It acts similarly to the previously mentioned PG analogs and is administered once daily before bedtime. Studies have shown that preservative-free tafluprost in combination with timolol effectively reduces IOP in patients who responded poorly to other treatment regimens. According to a meta-analysis, tafluprost and latanoprost have similar efficacy in reducing IOP, but conjunctival hyperemia can be observed more frequently as a side effect after tafluprost administration (32).

Bimatoprost is a prostamide, a fatty acid with an ethyl amide at the C-1 carbon of the alpha chain. Bimatoprost improves the uveoscleral outflow, while activation of prostamide receptors in the TM increases the conventional outflow of aqueous humor. Compared to other PG analogs, conjunctival hyperemia can be observed more frequently in patients using bimatoprost. It is also used to treat hypotrichosis and chemotherapy-induced madarosis (32, 40).

1.2.5.2. Other pharmacological treatment options

Beta-blockers, mainly known for their antiarrhythmic and antihypertensive properties, can also be used as eye drops as a topical treatment for glaucoma. Cardioselective beta-blockers target the beta-1 receptor, while non-selective beta-blockers target beta-1 and beta-2 receptors and have greater antihypertensive effects. Due to the increased beta-2 receptor affinity, nonselective beta-blockers are more effective in lowering IOP (41). It is important to note that patients who already take systemic beta-blockers may not profit from the concomitant use of topical preparations or even suffer from adverse effects. Topical beta-blocker preparations are usually used twice daily, and IOP is decreased by reducing aqueous humor production. Ocular side effects include dryness of the eye or worsening of existing conditions like keratoconjunctivitis sicca. Contraindications include the presence of sinus bradycardia, atrioventricular block, bronchial asthma, decompensated congestive heart failure, allergic rhinitis, cerebral hypoperfusion, and muscle weakness (15). The most used beta-blocker for glaucoma treatment is the nonselective agent timolol; others include betaxolol, levobunolol, carteolol, and metipranolol.

CAIs belong to the oldest treatment options for OAG. CAIs reduce bicarbonate formation by inhibiting the enzyme carbonic anhydrase, which is present in the ciliary body and typically catalyzes the interconversion of carbon dioxide (CO2) and water to bicarbonate and protons. This mechanism is crucial for the production and release of aqueous humor. Furthermore, they improve circulation by reducing vascular resistance and improving blood flow velocity. The increased CO2 concentration caused by CAIs leads to a locally decreased pH and, in turn, vasodilatory effects, particularly affecting circulatory systems in the brain, retina, and choroid. Collectively, those mechanisms reduce the production and secretion of aqueous humor, thus leading to a decrease in IOP. They are mainly used as topical preparations due to their potentially severe side effects. These side effects include paresthesia, diuresis, metabolic acidosis, electrolyte imbalance, nausea, and weakness (42). The sulfonamide structure of CIAs can lead to allergic reactions that rarely may result in Stevens-Johnsons

syndrome or toxic epidermal necrolysis (43). Frequently used agents include dorzolamide and brinzolamide; both are topical agents used locally, significantly reducing their adverse effect profile. They are commonly combined with other IOP-lowering drugs, such as dorzolamide-timolol, brinzolamide-timolol, or brinzolamide-brimonidine (42).

Alpha2-adrenergic agonists are a possible treatment option for patients with glaucoma, and they reduce IOP by reducing the production of aqueous humor and increasing the rate of uveoscleral outflow. Through the stimulation of alpha2-adrenergic receptors located in the ciliary body of the eye, they inhibit the enzyme adenylate cyclase, thus causing a decrease in cyclic adenosine monophosphate levels (44). Low cyclic adenosine monophosphate levels reduce ciliary process activity and aqueous humor production. Locally observable adverse effects include white discoloration of the conjunctiva and the development of topical intolerance in up to one-third of patients. Systemic contraindications include the concomitant use of monoamine oxidase inhibitors, sympathomimetics, and tricyclic antidepressants. Topical use is also absolutely contraindicated in children under the age of twelve years, hence the possibility of severe side effects that may go as far as causing coma in toddlers (15). Routinely used drugs from this group are brimonidine and apraclonidine. Brimonidine is a selective alpha2-receptor agonist with a superior side effect profile compared to non-selective ones and has the potential to lower IOP by 20% – 25% from baseline. Studies indicate that brimonidine exhibits neuroprotective properties independently from the IOP-lowering effect and protects RGCs from damage (45). Apraclonidine targets the same receptor and is commonly used when a short-term IOP reduction is needed. Due to its rapid onset of action and significant IOP lowering potential, it is preferentially used to reduce IOP spikes post-ocular surgery or laser therapy (46).

Parasympathomimetic drugs stimulate muscarinic receptors in the eye. Stimulation of M3 receptors leads to contraction of the ciliary muscle, which pulls on the TM and widens its spaces. This mechanism facilitates the flow of aqueous humor out of the anterior chamber into SC (47, 48). Additionally, they cause miosis by contraction of the sphincter pupillae muscle. The most prominent representative of this drug group is pilocarpine, with an IOP-lowering potential of 20% – 25%. Acetylcholine, physostigmine, or carbachol can be used alternatively. Parasympathomimetic drugs present with a large span of systemic and ocular side effects like gastrointestinal upset, increased salivation and sweating, bronchoconstriction, blurred vision and difficulty seeing in low-light conditions due to miosis, ocular hyperemia, ciliary cramps, retinal detachment, and pupillary block with potential exacerbation of angle-closure glaucoma. The adverse effects make them a less tolerable drug for many patients and are even

contraindicated in patients younger than 40 years of age. According to studies, pilocarpine has a neuroprotective effect, reduces the incidence of glutamate-induced cell death, and maintains calcium homeostasis. The neuroprotective effects may be mediated by stimulation of M1 receptors and subsequent release of NF-E2-related factor-2, which protects against oxidative stress (45).

Rho kinase inhibitors (ROKis) are administered as eye drops once or twice daily. Rho kinase is a serine/threonine protein kinase, and its activation leads to ECM reorganization, modulation of cytoskeletal structures, and cellular properties, including stiffness and adhesions. Netarsudil is a commonly used agent from this drug group that increases aqueous humor outflow via the conventional pathway by relaxation of endothelial cells in the TM and SC. The inhibition of norepinephrine transporters causes a reduction in episcleral venous pressure and aqueous humor production. The mentioned mechanisms act synergistically and cause a decrease in IOP. Studies suggest that netarsudil also exhibits neuroprotective and anti-fibrotic actions. Patients treated with ROKis show an increased tendency for adverse effects compared to other treatment options. Possible adverse effects include conjunctival hyperemia due to vasodilatory effects, cornea verticillata causing bilateral whorl-like, golden brown opacification of the corneal epithelium, and ocular pain or blurred vision (49).

1.2.5.3. Surgical management

Indications for surgical management of glaucoma include a failure to reach target IOP values with drug therapy, progressing optic nerve damage despite maximal medical therapy and target IOP achievement, intolerability of drug therapy due to side effects or contraindications, and patient non-compliance to the prescribed treatment. The first invasive procedures indicated in those scenarios are usually laser trabeculoplasty or surgical trabeculectomy (50). Laser trabeculoplasty is relatively safe and a potential treatment approach for OAG or closed-angle glaucoma with a patent iridotomy. Commonly used approaches include argon laser trabeculoplasty, which causes coagulative necrosis in the TM, and selective laser trabeculoplasty with a Q-switched Neodymium: Yttrium-Aluminum-Garnet laser, disrupting the TM endothelial cells. Laser treatment promotes the recruitment of macrophages and release of interleukin-1 and tumor necrosis factor (TNF) alpha in the TM, which causes remodeling of the ECM and leads to increased patency of the TM and SC, thus increasing aqueous humor outflow and resulting in an IOP reduction (9). Trabeculectomy is still the gold standard incisional procedure for the management of glaucoma. During the procedure, the conjunctiva is lifted to create a partial thickness scleral flap, and parts of the TM lying underneath the flap

are removed. Additionally, an entrance is created by perforating the anterior chamber; in this way, a drainage path for the aqueous humor is created. After repositioning the scleral flap and closure of the conjunctiva, the aqueous humor accumulates in the subconjunctival space and forms a fluid-filled bleb. The fluid-filled bleb acts as a reservoir and alternative pathway for aqueous humor outflow and contributes to the decrease in IOP (**Figure 6**). Over time, the aqueous humor inside the bleb diffuses into surrounding conjunctival and episcleral tissues and eventually gets absorbed into the bloodstream (51). Trabeculoplasty and trabeculectomy can be conducted in the outpatient setting, and laser trabeculoplasty can even be considered a first-line treatment if some of the factors mentioned above are present. Surgical trabeculectomy is usually considered if medical or laser treatment does not bring the desired outcomes due to possible visual risks following the procedure (50). Deep sclerotomy is another surgical technique used for glaucoma treatment, which has a lower rate of side effects and inferior IOP lowering potential compared to trabeculectomy (52). The first step involves creating a superficial scleral flap involving up to one-third to one-half of the scleral thickness. Subsequently, a smaller and deeper rectangular scleral flap is created under the superficial one. The SC is partially removed to improve drainage further, often involving the juxtacanalicular TM. A space-maintaining absorbable or non-absorbable collagen or hyaluronic acid implant is usually placed to maintain aqueous drainage long-term. Finally, the scleral flap and conjunctiva are closed tightly by sutures (53, 54). Compared to trabeculectomy, deep sclerotomy has a superior safety profile because of its non-penetrating technique; there is no iridectomy, and the deeper parts of the TM are not surgically manipulated. This way, complications connected to hypotony are minimized (52, 55). Applying 5-fluorouracil and mitomycin-C positively influences postoperative scar formation while increasing the probability of complications like wound leakage and postoperative ocular hypotony (56-58).

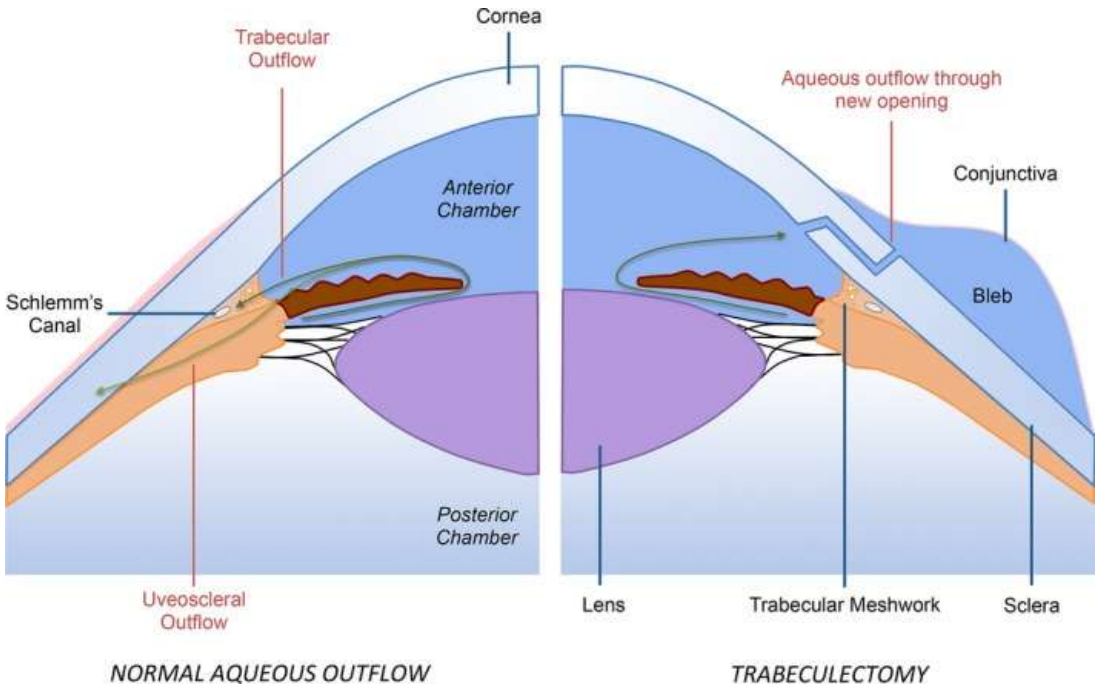

Figure 6. Aqueous outflow in a normal eye (left) vs. following trabeculectomy glaucoma filtration surgery (right). Source: Lee RMH, Bouremel Y, Eames I, Brocchini S, Khaw PT. Translating Minimally Invasive Glaucoma Surgery Devices. Clin Transl Sci. 2020;13:14-25.

1.3. The MAPK/ERK signaling pathway

Current studies suggest that the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) signaling pathway is crucial in organ regeneration. Injury leads to rapid activation of the MAPK/ERK pathway, which regulates crucial cell functions via regenerative events, including cell cycle progression, migration, proliferation, differentiation, adhesion, growth, and transcriptional and translational modification (59).

ERK1 and ERK2 are essential serine/threonine protein kinases of the MAPK family, transmitting extracellular signals to intracellular targets. They show structural similarities and share activators and substrates (60). ERK usually can be found in the cytoplasm but transfers into the nucleus upon activation, regulating transcription factors and, thus, gene expression (61). Both protein kinases are structurally comprised of 23 amino acids, of which 22 are identical (60, 62). Furthermore, ERK1 and ERK2 have two distinct substrate binding domains, consisting of D- and F-recruitment sites (63). On an additional motif, activation is initiated by catalytic processes (62). They can be found in all vertebrates and are crucial in cellular regulatory processes. Growth factors like fibroblast growth factor 2 and insulin-like growth factor 1, platelet-derived growth factors, neurotrophins such as brain-derived neurotrophic factor and

serotonin, cytokines including TNF and transforming growth factor-alpha, ROS, and DNA damage are examples of extrinsic signals that initiate the signaling cascade (59). PGF2 has also been shown to cause time-dependent phosphorylation of ERK1/2. Furthermore, studies demonstrated that stimulating the prostanoid FP receptor by agents like bimatoprost leads to activation of the phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) and MAPK/ERK pathways in RGCs (64). ERK1/2 activation happens through the rat sarcoma/rapidly accelerated fibrosarcoma/MAPK/ERK signaling cascade, controlled by phosphorylation processes starting on the cell membrane. Once phosphorylated and activated, ERK1/2, in turn, phosphorylates various substrates located in cellular membranes, organelles, and cytoplasm. After dimerization, ERK can even transfer into the nucleus, regulating transcriptional processes crucial for gene expression (**Figure 7**).

Studies show that the ERK-signaling cascade is crucial for retinal regeneration independently of species. Studies conducted on adult newts show an immediate ERK activation in RGCs, followed by nuclear translocation of phosphorylated ERK (P-ERK) following retinal removal. Three days after injury, β-catenin is translocated into the nucleus. The retinal pigmented epithelium cells reenter the cell cycle, transdifferentiate, and proliferate through combined actions of ERK, β-catenin, and other signaling factors. ERK signaling in the cytoplasm and nucleus leads to the expression of transdifferentiation-related transcription factors. Besides the eye, studies showed the importance of ERK-signaling in the heart, liver, central nervous, and peripheral nervous systems. The ERK-signaling pathway interacts with several other signaling networks in a synergistic or parallel manner. Examples include PI3K/AKT and β-Catenin signaling (59).

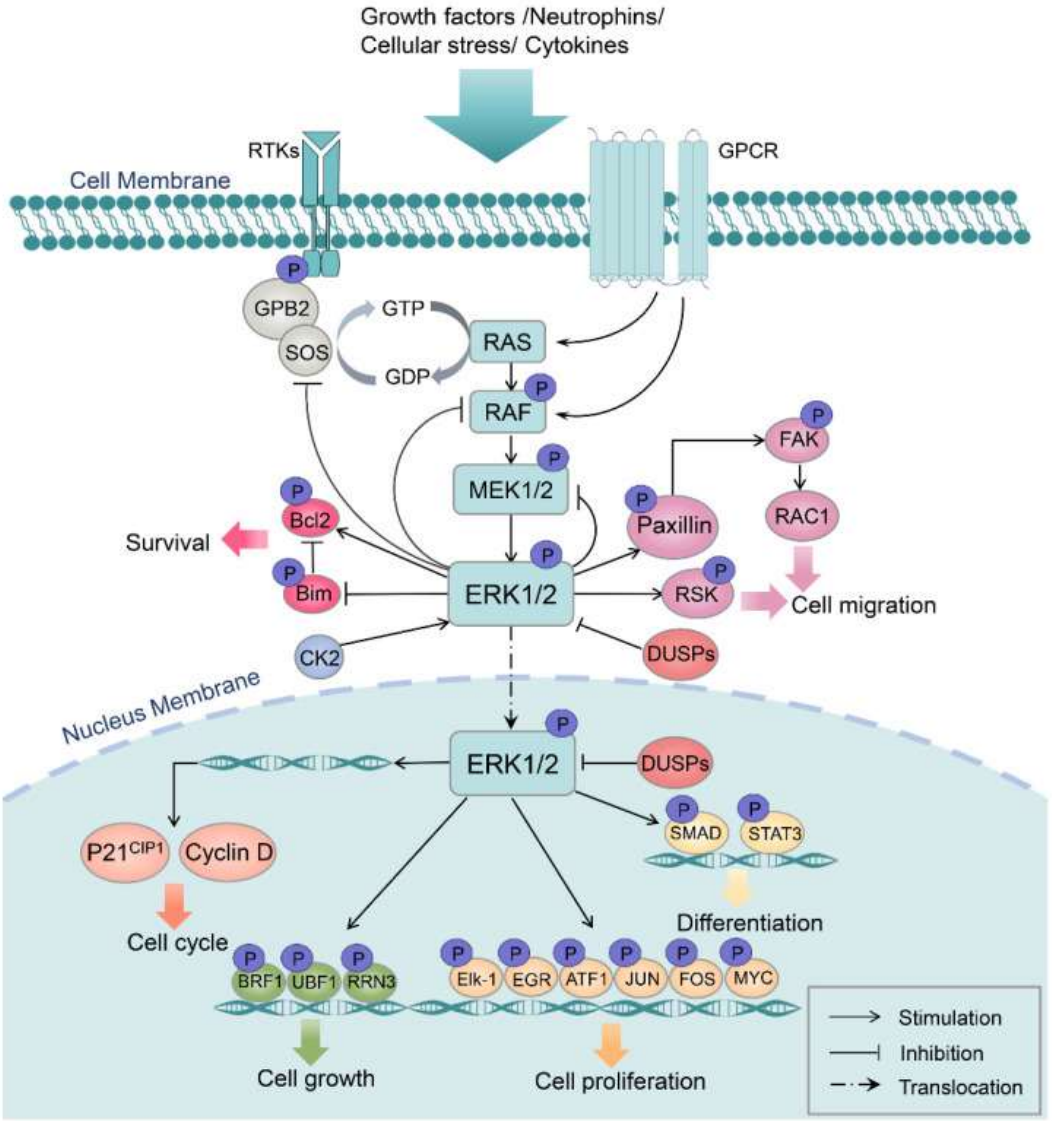

Figure 7. Simplified schematic of the regulatory mechanism and functions of the MAPK/ERK pathway. Source: Wen X, Jiao L, Tan H. MAPK/ERK Pathway as a Central Regulator in Vertebrate Organ Regeneration. Int J Mol Sci. 2022;23:1464.

2. OBJECTIVES

Aim:

The aim of this study was to analyze the proliferation and activation of MAPK/ERK signaling in the conjunctival cells of glaucoma patients treated with PGF2 analogs and those without PGF2 analog treatment to determine the effect of PGF2 analog treatment on these processes.

Hypotheses:

1. The proliferation of conjunctival cells will be increased in glaucoma patients treated with PGF2 analogs compared to those without PGF2 analog treatment.

2. The activation of MAPK/ERK signaling in conjunctival cells will be increased in glaucoma patients treated with PGF2 analogs compared to those without PGF2 analog treatment.

3. Changes in the proliferation of conjunctival cells will be positively correlated with activation of MAPK/ERK signaling in glaucoma patients.

3. MATERIALS AND METHODS

3.1. Tissue collection and sectioning

Conjunctiva samples were originally collected during deep sclerotomy procedures for the treatment of glaucoma performed at the Department of Ophthalmology of the University Hospital in Split between January 2011 and December 2022. Briefly, the procedure began by opening the conjunctiva and Tenon's capsule in the upper quadrant of the limbus. The exposed scleral surface was then abraded, and bleeding episcleral veins were cauterized. A superficial scleral flap was created, followed by a deeper scleral flap to expose the trabeculodescement membrane. The inner wall of Schlemm's canal was removed. Finally, the superficial scleral flap and conjunctiva were sutured, and a sample of the conjunctiva over the limbus was excised., submerged into a 4% paraformaldehyde solution in phosphate-buffered saline (PBS) for fixation, and sent to the Department of Pathology, Forensic Medicine and Cytology of the University Hospital in Split. Informed consent was obtained from all patients for the use of their tissues. The procedures adhered to the Declaration of Helsinki and other relevant national guidelines and regulations. All samples underwent the same initial processing steps. After fixation, the samples were dehydrated using ethanol solutions with an increasing concentration gradient (70% – 100%). Clearing was performed by submerging the samples in xylol solutions, followed by infiltration with paraffin after heating in an oven. The samples were left to cool at room temperature to finish the embedding process. The samples embedded in paraffin blocks were sectioned into 5 μm slices using a microtome and were mounted on glass slides. Each 10th slice underwent staining with hematoxylin and eosin to confirm appropriate tissue preservation and morphology. Some of the tissue slices were then used for research, while the remaining slices were archived for use in future studies at the Department of Histology and Embryology of the University of Split School of Medicine. The use of these archived samples for the purpose of this thesis was approved by the Ethics committee from the University of Split School of Medicine (class: 029-01/24-02/0001, registry number: 2181-198-03-04-24-0040). Samples were taken from 4 glaucoma patients who never used PGF2 analogs for treatment (PGF2-) and 6 patients with PGF2 analogs in their regular treatment regimen (PGF2+).

3.2. Immunofluorescence staining

After deparaffinizing and rehydrating the sample slices, antigen retrieval was performed by submerging the sections in a 0.01 M sodium citrate buffer solution (pH 6.0) and heating them in a steam cooker at 95°C for 30 minutes. The slices were then cooled to room temperature and rinsed in a 0.1 M PBS solution for 5 minutes. To prevent the nonspecific binding of antibodies, the slices were treated with a protein-blocking buffer (Protein Block, Abcam,

Cambridge, United Kingdom) for 20 minutes in a humid chamber. Next, the slices were incubated overnight with either a Ki-67 (dilution 1:200, AB9260, Sigma-Aldrich, Inc., St. Louis, United States of America) or phospho-p44/42 MAPK (P-ERK1/2) (dilution 1:200, 4370S, Cell Signaling Technology, Danvers, United States of America) rabbit monoclonal primary antibody diluted in PBS in a humid chamber, followed by two 5-minute washes in PBS solutions. An Alexa Fluor®488-conjugated anti-rabbit lgG secondary antibody (dilution 1:300, 711-545-152, Jackson Immuno Research Laboratories, Baltimore, United States of America) was then applied, and the slices were incubated in a humid chamber for one hour before undergoing three additional 5-minute washes in PBS solutions. A 4',6-diamidino-2-phenylindole solution was applied for 2 minutes to counterstain the nuclei. The stained slices were examined using an Olympus BX51 microscope (Olympus, Tokyo, Japan), and images were captured with a Nikon DS-Ri2 mounted digital camera (Nikon, Tokyo, Japan) using NIS-Elements F software (Nikon, Tokyo, Japan).

3.3. Proliferation and MAPK/ERK pathway activation analysis

In order to increase the accuracy of the analysis and to allow for better comparison between the two groups of samples, we have performed the staining in replicates. Two technical replicates were used for each sample of the PGF2+ group (N = 6) and three replicates for the PGF- group (N = 4), which resulted in twelve slices for analysis per group. Quantification of the immunofluorescence signal was performed by calculating the percentage of cells demonstrating positive staining in the captured images. Only nuclear staining was considered positive for analysis of the proliferation marker Ki-67, while the presence of nuclear and/or cytoplasmic staining was considered positive for P-ERK1/2 (Soliman, 28154782, Vicent, 14997206). For each slice, we captured five nonadjacent representative images at x400 total magnification. The images were analyzed using ImageJ software (National Institutes of Health, Bethesda, United States of America). In each image, the number of cells displaying positive staining and the total number of cells present were manually counted. The percentage of cells displaying positive staining in a replicate was calculated by dividing the sum of cells displaying positive staining by the sum of total cell counts in the individual images. These values were used for further statistical analyses. Cells of the conjunctival epithelium and the conjunctival stroma were counted and analyzed separately. Adobe Photoshop version 21.0.2 (Adobe, San Jose, United States of America) was used to create the figures.

3.4. Statistical analysis

Statistical analyses were performed in GraphPad Prism version 9.0.0 software (GraphPad Software, San Diego, United States of America). The results are presented as the mean and standard deviation of the percentages of positive cells for the analyzed proteins. The Shapiro-Wilk test was used to determine the normality of the data distribution. The existence of statistically significant differences in the percentage of positive cells between the analyzed groups was assessed using a two-way analysis of variance with Tukey's post hoc test. The significance of correlations between the analyzed groups regarding the percentage of positive cells was tested using linear regression modeling. The goodness of fit was described by the coefficient of determination (R2), while linear trends were expressed as the slope of a linear regression line (β). Statistical significance was set at $P < 0.05$. All graphs were constructed in GraphPad Prism 9.0.0.

4. RESULTS

We first analyzed the proliferation rate of cells in the samples. The proliferation marker Ki-67 was mostly expressed in the nuclei of the basal and suprabasal cells in the conjunctival epithelium of patients from the PGF2- group. When analyzing the epithelium of the PGF2+ group, many Ki-67 positive cells could also be seen in the more superficial layers. The conjunctival stroma of PGF2- patients contained a few Ki-67 positive endothelial cells and fibroblasts, while the stroma of PGF2+ patients had comparatively more cells expressing Ki-67, especially endothelial cells. The proliferation rate of cells in the conjunctival epithelium was significantly higher ($P = 0.024$) in the PGF2+ group ($8.69\% \pm 5.57\%$) compared to the PGF2- group ($5.20\% \pm 1.39\%$). The same was true for the cells of the conjunctival stroma with a significantly higher proliferation rate ($P = 0.024$) in the PGF2+ group ($3.48\% \pm 2.50\%$) than the PGF2- group ($1.61\% \pm 0.64\%$). The proliferation rate of cells in the conjunctival epithelium was significantly higher than in the stroma for both groups ($P < 0.001$). There was a significant positive linear trend ($R2 = 33.12\%$, $\beta = 0.268 \pm 0.168$, $P = 0.003$) between the proliferation rate of cells in the conjunctival epithelium and stroma of the same samples (**Figure 8**).

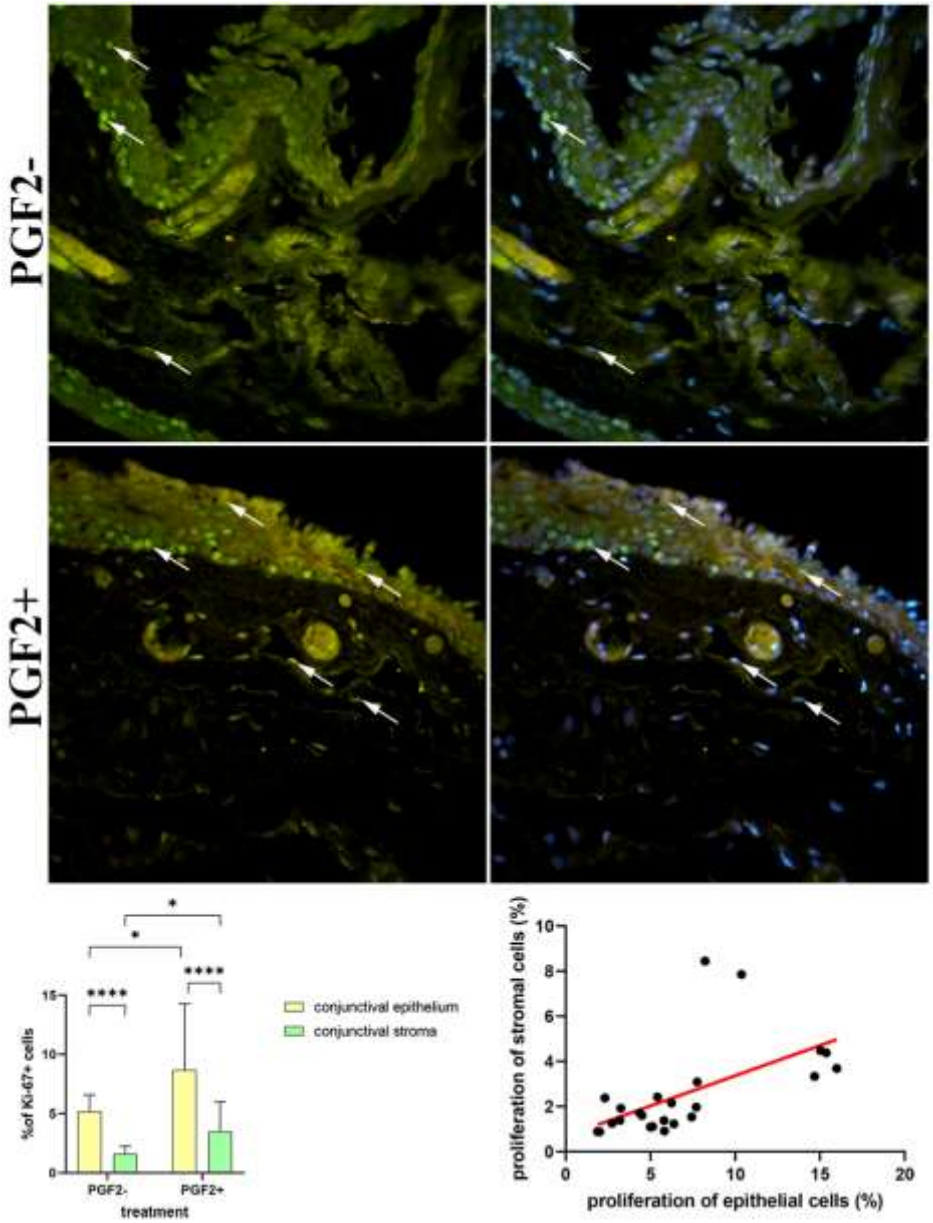

Figure 8. Ki-67 staining of conjunctival tissues of glaucoma patients. PGF2- – patients without prostaglandin F2α analog treatment, PGF+ – patients treated with prostaglandin F2α analogs. The first panels represent staining with Ki-67 (green signal). The second panels represent merged images of Ki-67 and 4',6-diamidino-2-phenylindole nuclear staining (blue signal). Arrows mark proliferating cells. The left graph represents the expression of Ki-67. The values are given as the means of the percentage of positive cells. Error bars represent the standard deviation. The statistical significance of differences was determined by a two-way analysis of variance followed by Tukey's post hoc test. * $P < 0.05$, **** $P < 0.0001$. The right graph represents the linear regression modeling of the percentage of Ki-67 positive cells between the epithelium and the stroma.

The activation of the MAPK/ERK pathway was assessed by analyzing the accumulation of P-ERK1/2 in the cells. The conjunctival epithelium of the PGF2- group demonstrated cytoplasmic P-ERK1/2 staining mainly in the basal layer, while the staining in the PGF2+ group was both nuclear and cytoplasmic and even present in the superficial layers. Strong P-ERK1/2 staining was present in the stroma of both patient groups; however, more cells demonstrated P-ERK1/2 positivity in the PGF2+ group. The percentage of cells positive for P-ERK1/2 in the conjunctival epithelium was significantly higher ($P = 0.014$) in the PGF2+ group ($18.63\% \pm 6.29\%$) compared to the PGF2- group ($12.69\% \pm 2.77\%$). The same was true for the cells of the conjunctival stroma, with a significantly higher percentage of P-ERK1/2 positive cells ($P = 0.014$) in the PGF2+ group ($13.36\% \pm 5.24\%$) than the PGF2- group ($10.08\% \pm 5.15\%$). The percentage of P-ERK1/2 positive cells in the conjunctival epithelium was significantly higher than in the stroma for both groups ($P = 0.044$). There was a significant positive linear trend ($R2 = 50.05\%$, $\beta = 0.671 \pm 0.296$, $P < 0.001$) between the percentage of P-ERK1/2 positive cells in the conjunctival epithelium and stroma of the same samples (**Figure 9**). There was no significant linear trend between the proliferation rate and percentage of P-ERK1/2 positive cells in the conjunctival epithelium ($R2 = 23.92\%$, $\beta = 0.614 \pm 0.892$, $P = 0.151$) or the conjunctival stroma ($R2 = 21.89\%$, $\beta = 1.076 \pm 1.656$, $P = 0.172$).

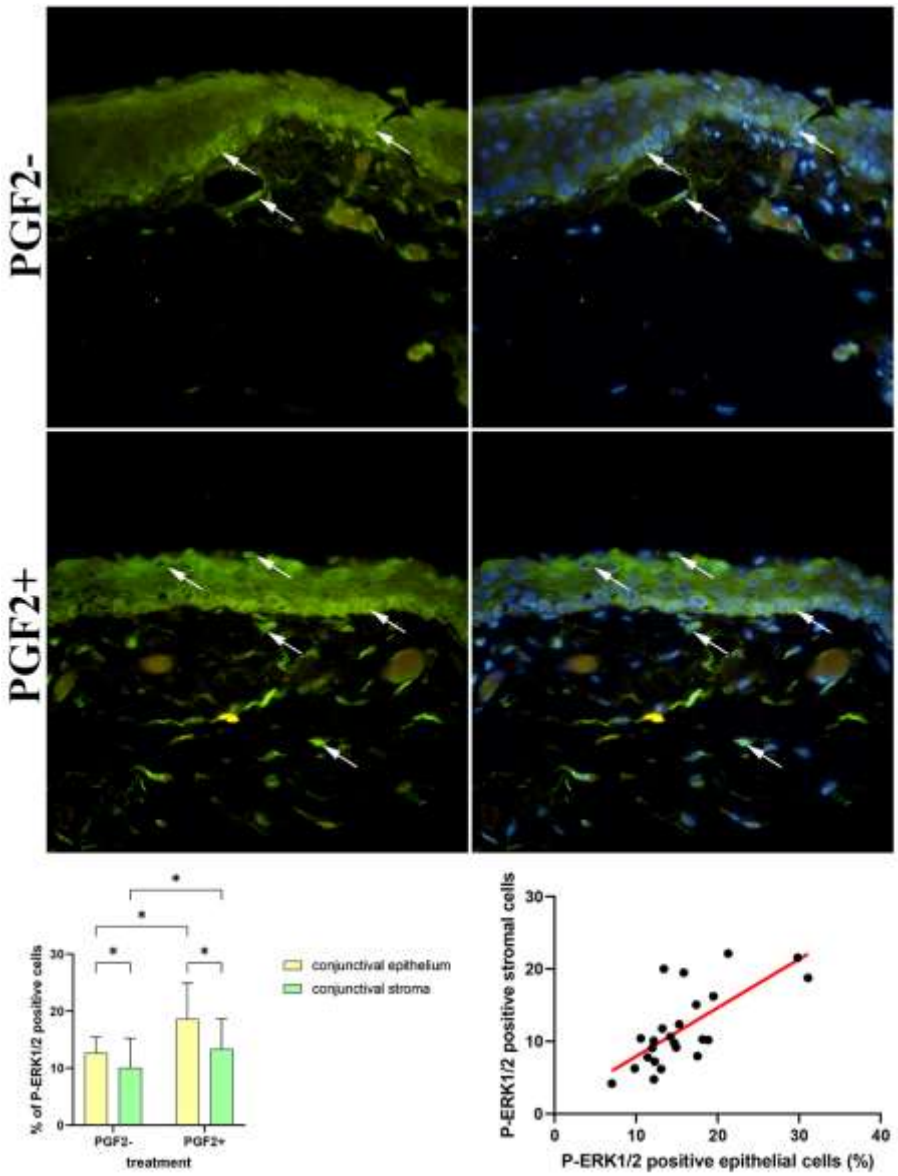

Figure 9. Phosphorylated ERK1/2 (P-ERK1/2) staining of conjunctival tissues of glaucoma patients. PGF2- – patients without prostaglandin F2α analog treatment, PGF+ – patients treated with prostaglandin F2α analogs. The first panels represent staining with P-ERK1/2 (green signal). The second panels represent merged images of P-ERK1/2 and 4',6-diamidino-2-phenylindole nuclear staining (blue signal). Arrows mark cells with MAPK/ERK pathway activation. The left graph represents the expression of P-ERK1/2. The values are given as the means of the percentage of positive cells. Error bars represent the standard deviation. The statistical significance of differences was determined by a two-way analysis of variance followed by Tukey's post hoc test. * $P < 0.05$. The right graph represents the linear regression modeling of the percentage of P-ERK1/2 positive cells between the epithelium and the stroma.

5. DISCUSSION

Our study analyzed the proliferation and activation of the MAPK/ERK signaling pathway in the conjunctival epithelium and stroma of glaucoma patients with and without PGF2 analog treatment. After performing immunofluorescence staining and quantitative analysis, we found a significant increase in the proliferation rate (Ki-67 staining) and activation of the MAPK/ERK signaling pathway (P-ERK1/2 staining) in both the conjunctival epithelium and stroma of glaucoma patients treated with PGF2 analogs compared to those without PGF2 analog treatment.

A recent study has investigated the effect of prostaglandin PGF2 on the secretion of vascular endothelial growth factor (VEGF) and the cellular proliferation and migration of mesenchymal stem cells (MSC). They concluded that PGF2 significantly enhances MSC proliferation and migration. Furthermore, they discovered that specific concentrations of PGF2 potentiate VEGF secretion in MSCs (65). The effect of PGF2 on endometrial epithelial cells, demonstrating a dose-dependent increase in proliferation and a potential vasoconstrictive effect mediated by the action of PGF2 on perivascular smooth muscle cells within the endometrium, was proven by another study (66). Takada Y et al. elucidated the role of PGF2 derivatives like travoprost, which is commonly used as an IOP-lowering agent, on the expression of epidermal growth factor (EGF) on epithelial cell lines in the cornea. Treatment with PGF2 derivatives caused a significant increase in EGF mRNA, which is a crucial factor for cellular proliferation and epithelial regeneration. The observed increase in EGF levels implicates the importance of prostaglandins in causing proliferative and regenerative effects in the corneal epithelium (67). Among other ocular tissues, studies confirmed the presence of PGF2 mRNA in conjunctival tissues and PGF2 protein, especially abundant in conjunctival epithelial cells. The findings indicate that PGF2 may be crucial in modulating conjunctival inflammatory responses, regenerative processes, and tear production (68). After microscopically examining the conjunctival tissues of patients treated with PG analogs and comparing them with a control group, we found that treatment with PG derivatives causes a marked increase in cellular proliferation of the conjunctival stroma and especially the conjunctival epithelium. Furthermore, the proliferation of stromal endothelial cells was found in patient samples exposed to PG derivatives; this could be potentially explained by potentiated VEGF secretion described in a previous study. Additionally, this relation may help in explaining the development of conjunctival hyperemia, one of the most common side effects of many PG analogs used for glaucoma treatment. Our results regarding the proliferation of epithelial cell lines are consistent with the mentioned studies, which also may aid their explanation.

PGF2 is known to be positively related to the activation of the MAPK/ERK signaling pathway. A study investigating the expression and signaling mechanism of PGF2 receptors in human adenocarcinoma further evaluated this relation, showing that activation of PGF2 receptors leads to increased activation of the MAPK pathway, proven by increased phosphorylation of ERK1/2 (69). Another study conducted on human endometrial adenocarcinoma stated the involvement of the MAPK/ERK signaling pathway in tumor growth and metastasis by activation of angiogenic mechanisms after initial stimulation of PGF2 receptors (70). Moreover, Choi et al. state that FP-receptor stimulation by the PG derivative bimatoprost leads to the activation of the MAPK/ERK pathway in RGCs (64). By detecting P-ERK1/2, we proved that conjunctival tissue samples of patients treated with PGF2 analogs show a significant increase in MAPK/ERK signaling activity compared to the control group. Cells of the conjunctival epithelium showed higher MAPK/ERK activity than cells of the conjunctival stroma. The results obtained from the mentioned studies are consistent with our observations, and the higher abundance of PGF2 receptors can potentially explain superior MAPK/ ERK activity in epithelial cells.

PGs belong to potential stimuli triggering the MAPK/ERK signaling pathway, which can interact with other signaling networks like PI3K/AKT and β-Catenin synergistically or parallelly (59). Activation regulates cellular functions like proliferation, differentiation, turnover, and gene expression. Besides proving the activation of the MAPK/ERK signaling pathway following stimulation of PGF2 receptors in human endometrial adenocarcinoma cells, Sales et al. linked its increased activity to upregulated proliferation of endometrial adenocarcinoma cells (69). In contrast, a study by Milne et al. on the effects of PGF2 on the proliferation of endometrial epithelial cells emphasized the link between the phospholipase C pathway activation by exogenous PGF2 and a resulting upregulation of endometrial epithelial cells. Furthermore, the study demonstrated that this process is ERK1/2 independent and that the MAPK/ERK pathway may be significant for cell survival and inhibition of apoptosis in the endometrial epithelial cells (66). Despite the upregulated proliferation of epithelial cells and increased MAPK/ERK activity in the patient group treated with PGF2 analogs, our results indicate no significant correlation between those two factors. This conclusion is supported by Milne et al., where endometrial cell proliferation was MAPK/ERK independent. This indicates that other pathways triggered by prostaglandins may be crucial in the proliferative activity of conjunctival epithelial cells, and upregulated MAPK/ERK is potentially important for other cellular mechanisms linked to the conjunctival epithelium.

The main limitations of our study are the small sample size and the fact all procedures were done in a single center, which could significantly affect the ability to extrapolate our results to the general population. Additionally, it should be taken into account that immunofluorescence staining is primarily a qualitative analysis method and quantitative results obtained via this method are less robust than those obtained from flow cytometry or Western blotting. We could not perform these methods due to our samples being processed with formalin. Even with these limitations in mind, our results demonstrate that PGF2 analog treatment can have significant off-target effects on other ocular tissues. Although we have found increased proliferation and MAPK/ERK pathway activation associated with PGF2 analog use, they were not significantly correlated. Future studies could focus on other PGF2-mediated pathways and their effect on proliferation in the conjunctiva, which could lead to novel targets in managing the side effects of PGF2 analog treatment, like conjunctival hyperemia.

6. CONCLUSIONS

1. The proliferation of conjunctival epithelial and stromal cells was significantly increased in glaucoma patients treated with PGF2 analogs compared to those without PGF2 analog treatment.

2. Conjunctival epithelial cells had a significantly higher proliferation rate compared to stromal cells in glaucoma patients, regardless of treatment with PGF2 analogs.

3. The proliferation rate of conjunctival epithelial and stromal cells was positively correlated in glaucoma patients.

4. The activation of MAPK/ERK signaling in conjunctival epithelial and stromal cells was significantly increased in glaucoma patients treated with PGF2 analogs compared to those without PGF2 analog treatment.

5. Conjunctival epithelial cells had a significantly higher activation of MAPK/ERK signaling compared to stromal cells in glaucoma patients, regardless of treatment with PGF2 analogs.

6. The activation of MAPK/ERK signaling in conjunctival epithelial and stromal cells was positively correlated in glaucoma patients.

7. There was no significant correlation between the proliferation rate and activation of MAPK/ERK signaling in the conjunctival cells of glaucoma patients.

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8. SUMMARY

Objectives: Our study aimed to investigate the proliferation and activation of MAPK/ERK signaling in the conjunctival cells of glaucoma patients who received PGF2 analogs in their treatment and those without PGF2 analog treatment. We hypothesized that the patient group treated with PGF2 analogs would show increased conjunctival cellular proliferation, MAPK/ERK activation, and a positive correlation between those two processes.

Material and methods: Conjunctival samples were collected during deep sclerotomy procedures for the treatment of glaucoma and subsequently processed by standard preparation procedures for tissues before sectioning. Following immunofluorescence staining for Ki-67 and P-ERK1/2, the sections were examined by digital image analysis to quantify the emitted fluorescent signals. For the evaluation of statistically significant differences between the expression of the tested proteins in our sample groups, we used a two-way analysis of variance with Tukey's post hoc test.

Results: We found an increased proliferation rate, measured by Ki-67 expression, in the conjunctival cells of glaucoma patients treated with PGF2 analogs compared to patients without PGF2 analog treatment. The same trend was present when analyzing the activation of the MAPK/ERK pathway by measuring the expression of P-ERK1/2. Cellular proliferation and MAPK/ERK activation were markedly higher in the conjunctival epithelium compared to the stroma. There was no significant correlation between the proliferation rate and activation of MAPK/ERK signaling in the conjunctival cells of our samples.

Conclusions: Our study has demonstrated a significant increase in conjunctival epithelial and stromal cell proliferation and activation of the MAPK/ERK pathway in glaucoma patients treated with PFG2 analogs. However, these two processes were not significantly correlated, indicating that MAPK/ERK activation may have other effects on the conjunctiva. Future studies on other PG-associated signaling pathways could elucidate the mechanism of increased proliferation in the conjunctiva and lead to potential novel targets for treating the side effects of PGF2 analog treatment.

9. CROATIAN SUMMARY

Naslov: Učinak liječenja analozima prostaglandina F2 na proliferaciju i MAPK/ERK signaliziranje u tkivu spojnice bolesnika s glaukomom

Ciljevi: Cilj našeg istraživanja bio je odrediti stopu proliferacije i aktivacije mitogenima aktivirane protein kinaze / kinaze regulirane izvanstaničnim signalima (MAPK/ERK) signalnog puta u stanicama spojnice bolesnika s glaukomom koji su tijekom liječenja primali analoge prostaglandina F2 (PGF2), odnosni onih koji nisu liječeni analozima PGF2. Pretpostavili smo da će skupina bolesnika liječena analozima PGF2 pokazati povećanu proliferaciju i aktivaciju MAPK/ERK signalnog puta u stanicama spojnice te pozitivnu korelaciju između ta dva procesa.

Materijali i metode: Uzorci spojnica prikupljeni su tijekom provođenja zahvata duboke sklerotomije za liječenje glaukoma i naknadno obrađeni standardnim postupcima za obradu tkiva prije izrade rezova. Nakon imunofluorescencijskog bojenja na Ki-67 i fosforilirani ERK1/2 (P-ERK1/2), provedena je digitalna analiza slika u svrhu kvantifikacije fluorescentnih signala. Za procjenu statistički značajnih razlika između izražaja ispitivanih proteina u našim uzorcima koristili smo dvosmjernu analizu varijance s Tukeyjevim post hoc testom.

Rezultati: Pronašli smo povećanu stopu proliferacije, mjerenu izražajem Ki-67, u stanicama spojnice bolesnika s glaukomom liječenih analozima PGF2 u usporedbi s pacijentima bez liječenja analozima PGF2. Isti trend bio je prisutan kada se analizirala aktivacija MAPK/ERK signalnog puta mjerenjem izražaja P-ERK1/2. Stanična proliferacija i aktivacija MAPK/ERK signalnog puta bili su značajno veći u epitelu spojnice u usporedbi sa stromom. Nije bilo značajne povezanosti između stope proliferacije i aktivacije MAPK/ERK signalnog puta u stanicama spojnice naših uzoraka.

Zaključci: Naše je istraživanje pokazalo značajno povećanje proliferacije epitelnih i stromalnih stanica spojnice i aktivaciju MAPK/ERK signalnog puta u bolesnika s glaukomom liječenih analozima PFG2. Međutim, nije postojala značajna povezanost ova dva procesa što ukazuje da aktivacija MAPK/ERK signalnog puta vjerojatno ima druge učinke na spojnicu. Daljnja istraživanja o drugim signalnim putevima povezanim s prostaglandinima mogla bi razjasniti mehanizam povećane proliferacije u spojnici te dovesti do potencijalnih novih meta za liječenje nuspojava analoga PGF2.