FACULTY OF PHARMACEUTICAL SCIENCES

Laboratory for General Biochemistry and Physical Pharmacy
nanomedicines & biophotonics

NON-VIRAL/VIRAL STRATEGY AT THE LEVEL OF THE ANTERIOR SEGMENT FOR THE TREATMENT OF GLAUCOMA

Louis Bouckaert

A Master dissertation for the study programme Master in Pharmaceutical Care

Academic year: 2020 – 2021

GHENT UNIVERSITY

Master dissertation submitted to the faculty of Pharmaceutical Sciences, performed in collaboration with the Laboratory for General Biochemistry and Physical Pharmacy.

Promotor: Prof. dr. S. De Smedt
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PREAMBLE COVID-19

“This master's thesis was executed in a period where corona measures have influenced research and education activities in various ways. These unusual circumstances may have had an impact on this thesis to a greater or lesser extent, despite all the efforts of the student, daily supervisor(s) and promoters. This generic preamble aims to frame this and was approved by the faculty.”

ABSTRACT

The human eye is one of the most complex structures of the body. Inside this organ several tissues and humors perform an essential function. One of these functionalities is regulating the aqueous hydrodynamics. Located at the anterior segment of the eye, the ciliary body provides a continuous aqueous humor production, while the trabecular meshwork and Schlemm's canal are the major tissues responsible for its outflow. To maintain a normal intraocular pressure (IOP), the amount of production has to be the same as the amount that leaves the eye. Due to many reasons a disturbed modulation can occur, with an elevated eye pressure as consequence. An increased IOP is the major risk factor of developing most types of glaucoma. In this disease, the risen eye pressure causes apoptotic cell death of retinal ganglion cells and may lead to irreversible blindness. Based on different pathological mechanisms, glaucoma can be divided into four types: primary open angle glaucoma, primary angle closure glaucoma, primary congenital glaucoma and secondary glaucoma.

Currently as IOP-lowering therapy, commercially available eyedrops are commonly used. In severe cases of the disease, non-pharmacological treatments including laser therapies, surgeries and ultrasound cyclodestruction can be considered. However, both IOP-lowering strategies have various limitations. Therefore, research efforts into more efficient therapies and newer delivery methods started running.

Every type of glaucoma has causative genes or risk alleles which can also be at the basis of disease development. Although, research is still on-going to improve the knowledge about these genes and their functions, some effective nucleic acid delivery strategies have been developed. Oligonucleotide-based agents can be topically administered, but practically the same shortcomings as with commercial eye drops are experienced. They face the complexity of the extracellular matrix, gene instability by enzymatic nuclease degradation and may cause inflammatory and immune responses.

Obviously, researchers started the quest to novel gene delivery routes. In this master dissertation we made a distinction between the biochemical and physical methods. Biochemical methods included the viral and non-viral vectors. Viral carriers are non-pathogenic viruses loaded with plasmid DNA or RNA in their genome. Despite a good transducing efficiency and selectivity, commonly use is limited by a low carrying capacity and a potential risk of immunogenicity or regained pathogenicity. Non-viral vectors are nanoparticles encapsulating nucleic acids. Despite promising advantages over viruses, lower efficiency and cell specificity clearly necessitate further research. In physical methods, physical external forces are used to permeabilize the plasma membrane permeability by transient pore formation or membrane disruption. Finally, a higher transfection efficiency should be acquired by the combination of the biochemical and physical methods.

SAMENVATTING

Het menselijk oog is één van de meest complexe organen van het lichaam. Verschillende weefsels en vloeistoffen in dit orgaan vertolken verschillende functies, waaronder het regelen van kamer-vloeistofdynamiek. In het voorste oogsegment staat het ciliare lichaam in voor de continue productie van kamerwater terwijl vooral het trabeculaire netwerk en het Schlemm's kanaal verantwoordelijk zijn voor de uitstroom. Om een constante oogboldruk te behouden, is het belangrijk dat de productie en uitvloei van het kamerwater vrij simultaan gebeurt. Wegens verschillende redenen, kan een verstoorde modulatie optreden met een verhoogde intra-oculaire oogboldruk (IOP) als gevolg. Een verhoogde IOP is de grootste risicofactor om bepaalde types van glaucoom te ontwikkelen. Het zorgt voor apoptose van de ganglioncellen in de retina en leidt op die manier tot mogelijks irreversibele blindheid. Op basis van een verschillend pathologisch werkingsmechanisme is glaucoom onder te verdelen in vier types: primair open hoek glaucoom, primaire gesloten hoek glaucoom, primair congenitaal glaucoom en secundair glaucoom. Vandaag worden vooral de topicaal toegediende commercieel beschikbare oogdruppels gebruikt als IOP verlagende therapie. Bij bepaalde ernstige vormen van de ziekte kunnen niet-farmacologische behandelingen, omvattend laser therapieën, operaties of behandelingen met behulp van ultrasone geluidsgolven worden overwogen. Toch hebben beide oogdrukverlagende behandelingen hun beperkingen. Daardoor startte onderzoek naar nieuwere en efficiëntere benaderingen en afleveringsmethoden.

Voor iedere type van glaucoom zijn er ook veroorzakende genen of risico-allelen verantwoordelijk voor ziekte ontwikkeling. Hoewel niet alle genen en hun functies op dit moment gekend zijn, werden wel al enkele methoden ontwikkeld die hun genexpressie regelen. Ook oligonucleotide-gebaseerde geneesmiddelen werden oorspronkelijk topicaal toegediend. Echter traden hier dezelfde beperkingen op als bij de topicale commercieel beschikbare preparaten. Bovendien zijn deze nucleïne zuren onderhevig aan de complexiteit van de extracellulaire matrix, een versnelde afbraak door het nuclease enzym of zijn ze verantwoordelijk voor ontstekingsreacties en immuun responses.

Door deze tekortkomingen ontstond een zoektocht naar nog idealere gen-afleveringsmethoden. In deze master thesis maakten we een onderscheid tussen de biochemische en fysische methoden. De eerste omvat de virale en niet-virale vectoren. Virale dragers zijn niet-pathogene virussen die geladen worden met plasmide DNA of RNA in hun genoom. Ondanks een efficiënt en selectieve aflevering limiteren een lage draag capaciteit en de kans om virulentie te herwinnen hun veelvuldig gebruik. Niet-virale vectoren zijn nanopartikels die de nucleïnezuren omhullen. Ondanks de meest veelbelovende voordelen zorgt een verminderde efficiëntie en een lagere cel specificiteit voor de vraag naar verder onderzoek. Naast de biochemische methoden maken de fysische methoden gebruik van fysische externe krachten. Deze creëren een verhoogde permeabiliteit door de formatie van tijdelijke microporiën en membraanaantasting. Het combineren van biochemische en fysische methoden zou dan ook de transfectie-efficiëntie enkel ten goede komen.

ACKNOWLEDGEMENTS

In every master education writing a master dissertation is a must to obtain this degree. After four years of hard work in pharmaceutical sciences, it was my turn to fulfill this quest. However succeeding this mission was not possible without the help and encouragement of several people. For this reason, I would like to pronounce some words of appreciation to these important people.

First of all, I would like to thank my promotor Prof. dr. S. De Smedt for giving me the opportunity to work on this interesting subject. In addition, his critical evaluation and support were very helpful.

Moreover, I want to express my gratitude to my second promotor Dr. F. Sauvage for guiding me through this master dissertation. With weekly meetings he introduced me in the world of nanomedicines for the treatment of glaucoma. His knowledge, essential advice and assistance improved this work significantly. Your presence meant a lot to me, thank you!

A sincere word of appreciation the PhD students of the laboratory for General Biochemistry and Physical Pharmacy. They gave us the ability to join their weekly discussion group and to explain us their research field.

Finally, I want to thank my family and friends. While writing this manuscript, they supported and motivated me at the tough moments. Especially my parents acted as a stimulation by teaching me the fact that there is no time to lose or in other words, like we say in West-Flanders: 'osan deure doen' is the key to success.

Thanks for everything,

Louis Bouckaert
May 2021

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LIST OF ABBREVIATIONS

AAV:	adeno-associated virus
ALT:	argon laser trabeculoplasty
ATP:	adenosine triphosphate
cAMP:	cyclic adenosine 3′,5′-monophosphate
CAV_1:	caveolins 1
CAV_2:	caveolins 2
cGMP:	cyclic guanosine monophosphate
CPP:	cell-penetrating peptide
DM:	Descemet's membrane
ECM:	extra cellular matrix
ECP:	endoscopic cyclophotocoagulation
FAZ:	fovea avascular zone
GAG:	glycosaminoglycan
GCL:	ganglion cell layer
GON:	glaucomatous optic neuropathy
HA:	hyaluronic acid
HDR:	homology-directed repair
HFL:	Henle fiber layer
HSV:	herpes simplex virus
IL-20:	interleukine-20
IL-24:	interleukine-24
ILM:	internal limiting membrane
INL:	inner nuclear layer
IOP:	intraocular pressure
IPL:	inner plexiform layer
JAK:	janus kinase
JCT:	juxtacanalicular tissue
LCPC:	laser cyclophotocoagulation
LPI:	laser peripheral iridotomy
MMP:	matrix metalloproteinase
MYOC:	myocilin
Nd: YAG LPI:	neodymium: yttrium-aluminum-garnet laser peripheral iridotomy

NHEJ:	nonhomologous end-joining
NPE:	non-pigmented epithelial cells
NTG:	normal tension glaucoma
OLM:	outer limiting membrane
ONL:	outer nuclear layer
OPL:	outer plexiform layer
OPTN:	optineurin
PACG:	primary angle closure glaucoma
PCG:	primary congenital glaucoma
PEI:	polyethyleneimine
PGA:	prostaglandins analogs
POAG:	primary open angle glaucoma
PRS:	photoreceptor cells
RGC:	retinal ganglion cell
ROS:	reactive oxygen species
RPE:	retinal pigment epithelium
SLT:	selective laser trabeculoplasty
STAT3:	signal transducer and activator of transcription 3
TBK1:	TANK-binding kinase 1
TIMP:	tissue inhibitor for matrix metalloproteinase
TM:	trabecular meshwork
TSCPC:	transscleral cyclophotocoagulation
UTMD:	Ultrasound targeted microbubble destruction
VH:	vitreous humor
WDR36:	WD repeat domain 36

1 INTRODUCTION

1.1 THE ANATOMY AND PHYSIOLOGY OF THE EYE

The eye can be reported as one of the most complex organs of the human body. For that reason, we decided to first discuss the most relevant ocular structures to understand the treatment of glaucoma.

1.1.1 The globe of the eye

Structurally, the globe of the eye can be divided into three chambers: the anterior, vitreous and posterior chamber. Moreover, the content of the eyeball is covered by three layers. The external layer contains the opaque sclera and the transparent cornea which are fusing together at the limbus region. A more intermediate coat (uveal tract) can be subdivided into the iris, the ciliary body and the choroid. The third and innermost layer is the retina which is connected to the optic nerve. This is visible in figure 1.1.(1) Tissues inside the eyeball (i.e. lens, vitreous humor and chamber fluid) act together to induce an optimal refractive index of the entered light and to maintain an internal pressure.(1, 2)

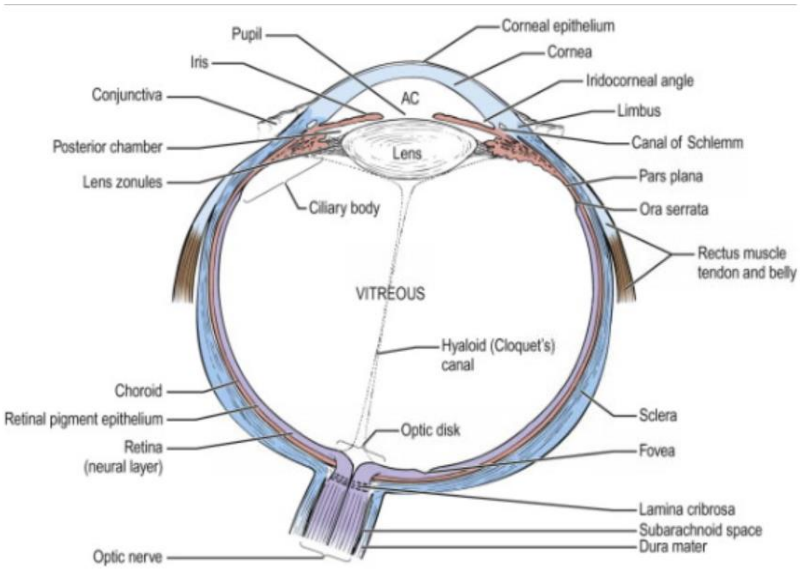

Figure 1.1: cross-section of the human eye (1)

1.1.2 Tear film

Theoretically, the tear film is not included in the anatomy of the eye. However, we did decide to discuss the tear film structure due the fact that many topically administered drugs, are subjected to this precorneal barrier. The tear film covers the cornea and has a specific composition including a lipid film, an aqueous layer and a mucus coating (figure 1.2).(3) The lipid film is produced by the meibomian glands and prevents direct contact among the outer air and the intermediate aqueous layer of the tear film. In this way, the lipid film ensures minimal evaporation of the aqueous layer. The middle aqueous layer presents multiple anti-microbial elements such as anti-bacterial lysozyme, lactoferrin and immunoglobulins. In addition, it functions as a hydration element for the cornea and the conjunctiva. Production of this layer owes to the main lacrimal gland and accessory lacrimal gland of Wolfring and Krause.(2) The tear film itself has many other differential functionalities. Firstly, it has a metabolic function by supplying oxygen or nutrients and it drains waste materials from the avascular cornea. The tear film has also an influence on the refractive index of the incoming light.(4, 5)

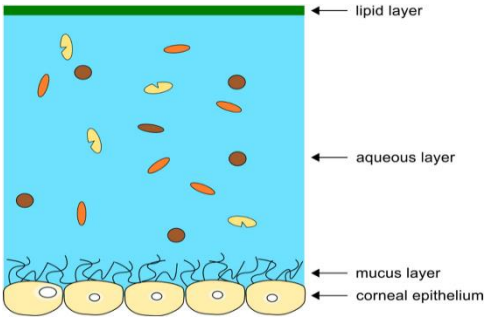

Figure 1.2: schematic cross-section of a tear film (3)

1.1.3 Cornea

The cornea is the transparent part of the outermost coat of the eye. As the most anterior part of the eye, it is a dense, innervated and avascular tissue. The cornea plays an essential role in the refractive index of the incoming light. It consists of several layers: from anterior to posterior: the epithelium, Bowman's layer, stroma, Descemet's membrane and the endothelium. The epithelium is composed of three types of cells: superficial, wing and basal cells (figure 1.3)(6). At the apical site, microvilli and microplicae present a glycocalyx coat which interacts with the precorneal tear film. Mitotic activity in the basal layer ensures a continuous regeneration of the epithelium. Stability and impermeability of the cornea is obtained by the tight junctions between the superficial cells. These junctions prevent the uptake of pathogens but also constitute a barrier to medicines.(7, 8)

The bowman's layer is a thin, collagen coat separating the epithelium from the stroma.(1) Underneath the Bowman's layer the stroma can be found. It is a hydrophilic layer, composed of extracellular matrix (ECM) and structured collagen bundles (also called fibrils). These fibrils provide cornea transparency and are wrapped together into lamellae. The innermost part of the cornea is the endothelium which is separated from the stroma by the Descemet's membrane (DM). It is a single layer of flattened cells, guaranteeing corneal clarity.(6, 9)

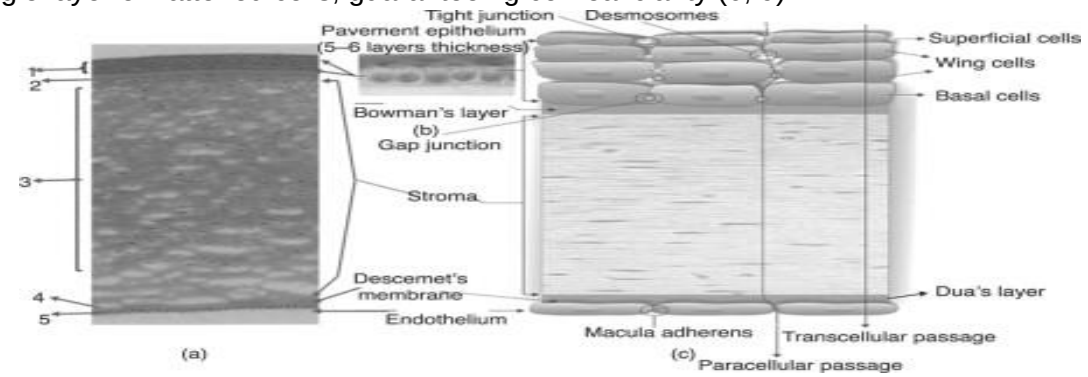

Figure 1.3: (left): Histological overview of different layers of the cornea. **(Right):** columnar basal cells, wing cells, and squamous surface cells of the corneal epithelial are shown; Bowman's layer, anterior stroma, Descemet's membrane (DM) and endothelium are also visible.(6)

1.1.4 Sclera

In comparison with the cornea, the surface of the sclera forms the principal part of the external layer of the eyeball. (figure 1.1) At the limbus the sclera flows anterior together with the cornea. While posterior, it continues the dura mater and may be seen as an outer layer of the brain. The sclera is penetrated by blood vessels and several nerves. At figure 1.1 the perforation of the optic nerve through the sclera, named by the lamina cribrosa can be noticed.(1)

The white of the eye which is a synonym for sclera is a dense connective tissue composed of irregular collagen and elastic fibers. It is comprised of three distinguishable layers. As a most external layer the episclera, subsequently the sclera proper or stroma and most internally the lamina fusca. Technically, Tenon's capsule (also called fascia bulbi) is the outermost membrane covering the eye. However in most literature, it is excluded from the anatomy of the sclera. The sclera has several functionalities. Ocular muscles are attached to the sclera, making eye movements possible. Moreover, it maintains also the shape of the globe by resisting intrinsic forces like intraocular pressure and protects the intraocular structures.(1)

The anterior end of the sclera and the inside of the eyelid are covered by the conjunctiva (fornix). As a mucus membrane on the inside of the eyelid, it is strongly vesiculated and can be recognized by its red color. This part of the fornix is called the conjunctiva palpebralis. For local effect, some eye medications (ophthalmica) are brought into the lower conjunctival sac. In case of long-term medication, the strong vascularity of the conjunctiva palpebralis can create sink conditions. Which finally will results into a lower bioavailability.(10) There is also a clear white mucosa on the sclera, the bulbar conjunctiva, functioning as first pathway to prevent local ocular drug administration along the non-corneal pathway. This is the most important entrance pathway of macromolecules and hydrophilic drugs into the eye. Cells of the conjunctiva are easier to penetrate than those from the cornea and they show a larger surface area. The limbus is the transition zone from sclera to the cornea and bulbar conjunctiva.(11)

1.1.5 Lens

As an avascular tissue, the lens contains organized lens fibers. This organization, the avascularity and shape of the lens contribute to its transparency. An elastic lens capsule envelops the lens bulk and has an important role in the accommodation process. By changing its shape, the lens assures that the light beam ends up at the photoreceptor cell of the retina. Anatomically, the lens separates the globe of the eye into an anterior segment and posterior segment. Within the anterior segment there are two spaces: the anterior chamber (between the cornea and the lens) and the posterior chamber (between the lens and the vitreous) both filled with aqueous fluid. The posterior segment of the eye consists of the vitreous humor, the retina, the choroid and the optic nerve.(1)

1.1.6 Uveal tract

As the intermediate layer of the eyeball, the uveal tract or uvea is characterized by the iris, ciliary body (anterior part) and choroid (posterior part of the uvea).

1.1.6.1 Iris

As a circular muscle, the iris is the most anterior part of the uvea and consists of stroma and pigmented epithelial cells. These cells or melanocytes produce eumelanin and pheomelanin, variable concentrations of this pigments give us a specific eye color.(12) At the middle of the iris, the pupil can be spotted. The iris participates in regulating the incidence of light by using the sphincter and dilator muscles.

1.1.6.2 Ciliary body

The ciliary body contribute to the aqueous humor production. Since an excessive production may lead to an elevated eye pressure, the major risk factor of the disease, the ciliary body is an essential tissue to explain the mechanism behind glaucoma. It includes the ciliary muscle and ciliary epithelial cells. Its location is anteriorly from the choroid and it crosses the retina at the ora serrata. On a cross-sectional view, the ciliary body is comparable to a triangle. The shortest leg of the triangle is the border between the ciliary body and the anterior chamber, also known as the iridochoroidal angle. The outer side of the triangle is the attachment between ciliary muscle and the scleral spur, a protrusion of the sclera which runs into the anterior chamber. This leg is continuous with the trabecular meshwork. (more info 1.2.2 Aqueous humor outflow)(13)

The ciliary body can be distinguished into an anterior part or pars plicata and an posterior part or pars plana. The latter touches the anterior site of the vitreous body and is covered by zonular fibers. Zonular fibers anchor the lens to the ciliary body and the ora serrata. The pars plicata is attached to the lens surface and contains folds of inner epithelium, known as the ciliary processes. These processes consist of an epithelium bilayer within stroma and blood vessels.(13)

The ciliary muscle is a smooth muscle that regulates the outflow route of aqueous humor and the accommodation of the lens. When the ciliary muscle constricts, a lower tension on the zonular fibers gets induced. Due to this lower tension, the lens gets the ability to become more bulbous and near items can be seen clearly. Unlike when the ciliary muscle relaxes, the tension on fibers rises which leads to a flattened lens and items further away can be observed. (figure 1.5)(14)

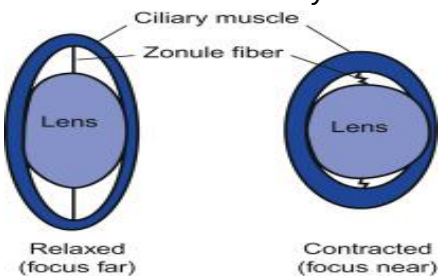

Figure 1.5: (Left plate): relaxing ciliary muscle creates higher tension on the zonule fibers, as result a flattening lens. **(Right plate)**: constricting ciliary muscle ensure lower fiber tension, a thicker lens origins. (14)

1.1.6.3 Choroid

The choroid is the most posterior membrane of the uvea which is located between the retina and the sclera. It runs from the ora serrata to the top of the optic nerve. The choroid can be subdivided into five layers: the innermost layer the Buch's membrane, the choriocapillaris, Sattler's layer, Haller's layer and adjacent to the sclera ,the suprachoroidea.(15, 16)

1.1.7 Vitreous humor

Vitreous humor (VH) is an inert, colorless, viscoelastic gel-like fluid. It consists of 99% water, chondroitin sulfate, heparan sulfates and glycosaminoglycans (GAG's) with as most important hyaluronic acid. Collagen and non-collagen vessels can also be found in the composition. The collagen bundles run parallel and constitute a network. The VH is located at the vitreous chamber and fills the space between the lens and retina. Functionally, the vitreous body is used as a shock absorber and creates an internal pressure which keeps the lens and retina at their location and maintains the shape of the eyeball.(17)

1.1.8 Fundus, retina and optic nerve

The fundus is the posterior part of the eye. The optic disc or papil is the place where the optic and ciliary nerve, blood vessels and many other nerves which regulate the sphincter or dilator muscle of the iris enter or leave the globe. Sometimes it is also called the 'blind spot' due to the fact there are no photoreceptor cells (rods and cones) located. Centrally at the optic disc, there is the optic cup. An ophthalmologist uses the cup to disk ratio as an indication for glaucoma. In healthy eyes, this ratio is usually lower than 0.5. More at the middle of the fundus, the macula lutea or 'yellow spot' is present. At the center of this region there is the fovea, the thinnest place of the retina which only contains cones.(14) Whose bottom is the foveola, surrounded by a foveal avascular zone (FAZ). The center of foveola is called the 'umbo'.(18) (figure 1.6)

Between the choroid and vitreous body, the retina can be found. It is a transparent tissue which runs from the ora serrata to the optic disc. The retina transfers light energy in neuronal signals by using photoreceptor cells, a specific type of pigment cells existing of rods and cones. Rods are responsible for scotopic vision and vision in dim light. Because they contain more visual pigments than cones, they are more sensitive to light. Cones on the other hand create a high resolutions and ensure a phototopic and in bright light vision. The retina can be divided into two regions: a central cone-rich region (macula lutea) and a more outlying, surrounding region which presents rods.

Histologically, the retina is constructed by an outer lying pigment layer and the neurosensorial retina. By following a typical direction, an impulse passes three cell types of the neural retina: a ganglion cell, a bipolar cell and a photoreceptor cell. However this inner neural layer is subdivided in ten differential layers, described from vitreous body to choroid. Firstly as innermost layer, the (i) internal limiting membrane (ILM) makes contact with the incoming light beam and will have a very important barrier function for gene therapeutics. (later more detailed information) Note that it is not visible at figure 1.6. Next, the (ii) nerve fiber layer (NFL) contains the axons of the underlying ganglion cells while the (iii) ganglion cell layer (GCL) presents its nucleus and the (iv) inner plexiform layer (IPL) its dendritic spurs. Remarkable is the absence of the GCL at the fovea. (figure 1.6) Thereafter an (v) inner nuclear layer (INL) and an (vi) outer plexiform layer (OPL) which includes the synapses of Müller, bipolar and photoreceptor cells are spotted. Even less deeper, is the (vii) Henle fiber layer (HFL) and the (viii) outer nuclear layer (ONL) containing the nucleus of the photoreceptor cells. Next, as outermost, the (ix) outer (external) limiting membrane (OLM) and (x) photoreceptor segments (PRS) consisting of a cilium connected inner segment and outer segment can be found.(18, 19). The outer segment can be a cone or rod and is encapsulated by a plasmalemma.(figure 1.7)

Due to passive forces, like intraocular pressure (IOP) and osmotic pressure, the neural layer is adjacent to the underlying pigment layer. While adhesive forces create a subretinal space between these layers. At the other side, the pigment layer touches the choroid and exists of retinal pigmented epithelium (RPE) cells which contain melanin. Melanin ensures absorption and no reflection of the light in the eyeball. In addition, these RPE cells maintain a blood-retina barrier which protects the retina from external circulating molecules in the blood circulation.(19) Once the photoreceptor cells transduce the light signal into a neural one, an action potential arises at the ganglion cells. Subsequently an impulse runs across the unmyelinated axons (nerve fibers) of the ganglion cells which make up the NFL. The optic nerve exists of approximately one million nerve

fibers whose cell body attends in the GCL. It has the characteristic of leaving at the optic disk and becoming myelinated at the lamina cribrosa.(14, 20, 21)

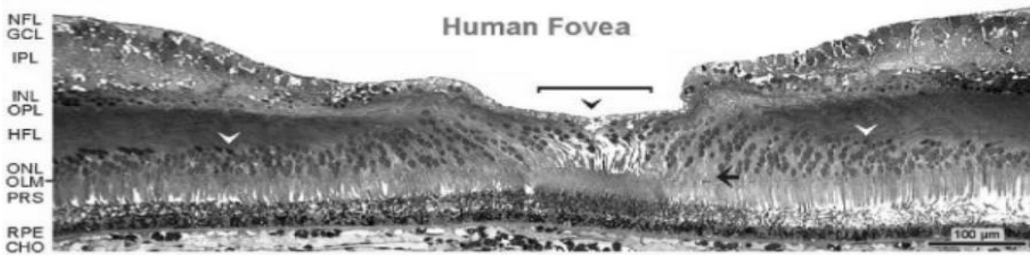

Figure 1.6: A toluidine blue-stained semithin cross-section of the human retina with the fovea, the thinnest part of the retina. At the left site, the ten layers are mentioned. Remarkable is the absence of the GCL at the fovea.(18)

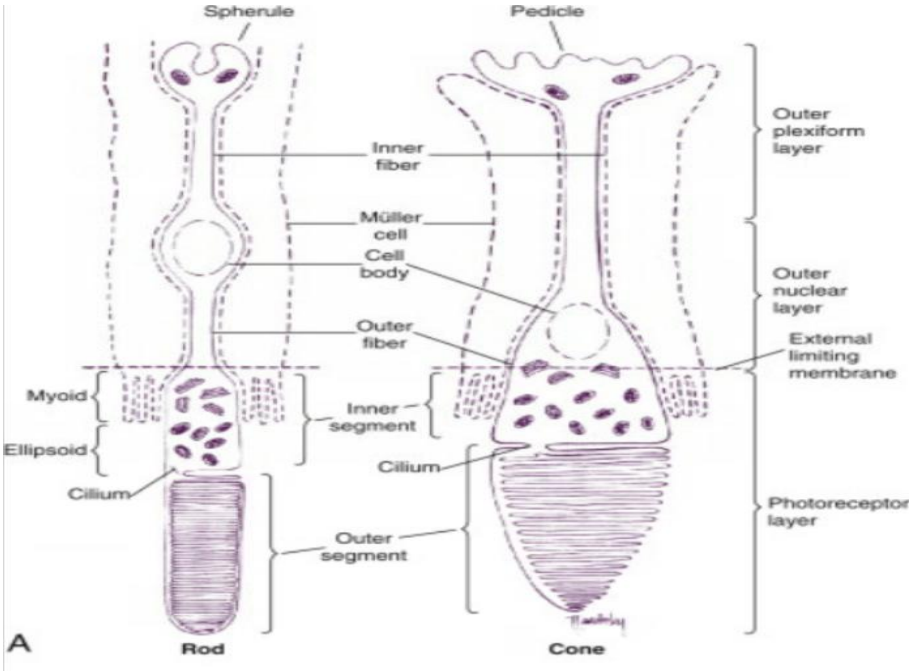

Figure 1.7: Schematic figure of photoreceptor cells. The dotted lines are portions of the Müller cells. At the right site, the retinal layers are shown in which layer the part of the photoreceptor is located. (19)

1.1.9 Aqueous humor

The aqueous humor is a transparent fluid of electrolytes which occupies the anterior and posterior chamber. It is composed of proteins, immunoglobulins, amino acids, urea and carbohydrates. The concentration of these components is significantly lower than found in plasma.(6) Functionally the circulating humor nourishes and deletes waste products of the avascular cornea, lens and the trabecular meshwork (TM). Due to a good regulated production and outflow, an IOP is maintained. An increased IOP is the primary risk factor for glaucoma. Later in this manuscript we will discuss how an elevated IOP can arise and how it is treatable.

1.2 PHYSIOLOGICAL ROLE OF AQUEOUS HUMOR

1.2.1 Aqueous hydrodynamics: humor production at homeostasis pressure

As explained in 1.1.6.2. the ciliary body is in contact with the vitreous body via its inner surface. This entire surface is surrounded by a bilayer of pigmented and non-pigmented epithelial cells (NPE). Tight junctions couple the NPE cells and create a blood-aqueous barrier. The aqueous humor is permanently formed by NPE cells. Every ciliary process has fenestrated capillaries (vascular plexus) where plasma enters the stroma of the process. The blood-aqueous barrier filters the entered plasma. This is the reason why aqueous humor is compared regularly with an ultrafiltration of the plasma.(22) Although, this is not always correct, we saw some small deviations of the concentration of many elements than expected in just an ultrafiltration. This fact demonstrates that ultrafiltration and diffusion are not the only processes, but active transport and osmotic forces have their part as well.(23)

The humor formation can be described into three steps: it starts with the uptake of ions into the pigmented cells of the processes. Sodium and chloride are taken up by Na^+/H^+ and Cl^-/HCO_3^- antiports and $Na^+/K^+/2Cl^-$ symport. The second step is the transport from these ions into the NPE cells using the gap junctions between the layers.

Besides representing a blood-aqueous barrier, the NPE cells use the enzyme carbonic anhydrase to catalyze the formation of carbonic acid by carbon dioxide and water ($CO_2 + H_2O \rightarrow H_2CO_3$). Once carbonic acid has been formed, it will dissociate in bicarbonate and a proton ($H_2CO_3 \leftrightarrow HCO_3^- + H^+$). The single negatively charged bicarbonate and as compensation for this negative charge a sodium-ion secrete actively into the intracellular channel between the NPE cells. The active secretion of Na^+ is made up of a sodium-potassium-adenosine triphosphatase (Na^+/K^+-ATPase) enzyme complex which consumes adenosine triphosphate (ATP). Due to both active secretions, particularly the sodium-ion, an osmotic pressure will be induced. This pressure stimulates the ultrafiltration of plasma and passive diffusion from ciliary stroma into the posterior chamber, the third and last step of the humor formation. (22, 24) (figure 1.8)

The production rate varies approximately from 3 µl/min in a healthy awake person to half of that in a sleeping individual. After production the humor processes into the posterior chamber of the eye, goes through the pupil into the anterior chamber and leaves at the anterior chamber angle. (25, 26)

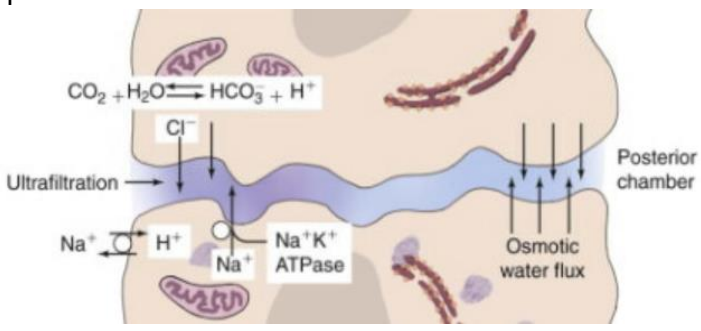

Figure 1.8: Schematic figure of an intracellular channel and two non-pigmented epithelial (NPE) cells. Due to active secretion of bicarbonate, sodium-ion and water into the channel, an osmotic pressure origins. This pressure will stimulate the passive ultrafiltration and diffusion of plasma.(22)

1.2.2 Aqueous hydrodynamics: humor outflow at homeostasis pressure

As compensation for a continuous humor production and to maintain an IOP, a good working outflow is required. After entering the anterior chamber through the pupil, the aqueous humor leaves at the anterior chamber angle (also called the iridochoroidal angle) by re-entering the venous system. Basically this return can be divided in two main outflow routes:

1) Conventional or trabecular meshwork outflow
2) Unconventional or uveoscleral outflow

1.2.2.1 Conventional or trabecular meshwork outflow

The conventional outflow is defined as the primary outflow pathway. It is a sensitive to eye pressure route where the humor follows the canalicular pathway which means the aqueous humor passes the trabecular meshwork (TM) into the Schlemm's canal. This canal is connected to an underlying connective tissue of the conjunctiva, the episcleral veins, which re-imports the humor into the venous circulation.

TM tissue

The inner segment of the limbus, the scleral sulcus, surrounds the TM. This sulcus is attached anteriorly to the Schwalbe's line which marks the edge of DM. In cross section, the TM tissue can be compared with a triangle. Its apex presents at the Schwalbe's line and the base at the scleral spur. TM is positioned between the anterior chamber and Schlemm's canal and can be divided into a pigmented filtering and a less pigmented non-filtering region. The cells of the filtering portion can be subdivided into three segments: the uveal meshwork, corneal meshwork and juxtacanalicular tissue (JCT). The anterior chamber is adjacent to the innermost layers of the TM, the uveal meshwork. Next to the uveal meshwork lies the corneoscleral meshwork. At both regions the tissue has connective trabecular beams, the lamellae, coated by a basal lamina and flat cells. Beams are formed by mostly type I and III collagen fibers and more central at the core elastic fibers.(25, 27) However not all lamellae have the same thickness. The thickness declines as the lamellae get closer to the Schlemm's canal site.(28) As final and third segment, the JCT cells border the endothelial cells of the Schlemm's canal. It is the smallest region of the TM where JCT cells are embedded in the fibrillar elements of ECM. This embedded structure forms a loose, connective tissue where some secreted matricellular proteins such as SPARC (secreted protein acidic and rich in cysteine) and thrombospondin-1 develop cell-matrix interactions. Between the JCT cells, there is a space filled with proteoglycans and hyaluronan which may function as outflow filter for the aqueous humor. Due to JCT processes, JCT cells stick not only to each other, but also to the surrounding ECM fibrils and connect to the inner wall of the Schlemm's canal.

The non-filtering region of the TM functions as a niche for adult stem cells or progenitor cells which have the capacity of rebuilding the filtering portion after an injury.(25) When the aqueous humor returns to the blood-cycle, a flow of blood into the anterior chamber is avoided by a barrier between blood and aqueous compartments, created by the filtering part of the TM tissue. In addition to performing this barrier function, TM-tissue cells perform a self-cleaning process by phagocytosis of foreign or waste particles. Otherwise they modify ECM components in the JCT under regulation of cytokines, glucocorticoids, prostaglandins, matrix metalloproteinase (MMP) and tissue inhibitor for matrix metalloproteinase (TIMP). In the literature, the TM is often described as the most IOP regulating tissue.

Schlemm's canal

The JCT lies adjacent to the epithelium of the Schlemm's canal. It is a modified endothelium-lined capillary blood vessel and has the main function of returning aqueous humor into the venous circulation. Anatomically the canal consists of an inner and outer wall of endothelial cells where tight junctions made a continuous line. Nevertheless, the endothelia at both walls have some anatomical differences and functions.

A discontinuous basal lamina rests at the cells of the inner wall. This discontinuity is important to give these cells the ability to make contact with the spaces filled with aqueous humor between the JCT cells. The membrane may realize a strong cell-ECM adhesion among the inner cells which may be essential to support a continuous barrier to fluid flow.(29) Thanks to the specialized structures of the inner endothelial cells, these cells have the capacity to transmit the humor flow in a basal-to-apical direction.

When aqueous humor presses on the basal lamina, the inner cells from giant vacuoles (also called cellular outpouchings) to make an area between them and the ECM of the JCT. This space may be important for the uptake of the humor into the Schlemm's canal which can be completed by two mechanisms. Firstly using transcellular pores and secondly pores at the tight junctions between the inner epithelium (paracellular). Via transcellular pores, particles of approximately 200-500 nanometer in diameter may pass. The motive of transport through the pores is due to hydraulic conductivity.(30) This physical process can be defined as how easily humor flows through the pores or how the humor outflow experiences a resistance.

The outer wall or corneoscleral wall consists of bundles of collagen. Parallelly orientated to the Schlemm's canal, the collector channel entrance and the intrascleral collector channels are attached to the collagen fibers of the corneoscleral wall. At the transition of the outer wall and some collector channels a flap-like, lip-like structure might regulate the outflow of fluid into the intrascleral collector channels. Thereafter smooth muscles at the collector channels contract to deduct aqueous humor into intrascleral or episcleral veins which lead to a re-import into venous circulation. (27)

1.2.2.2 Unconventional or uveoscleral outflow

Besides the primary conventional outflow where the humor exits through the TM and Schlemm's canal, a smaller amount of the aqueous humor leaves the eye by the uveoscleral route. Unlike the trabecular pathway, the uveoscleral flow is independent from the IOP. A higher eye pressure will not increase the alternative outflow. Depending on the ciliary muscle, the humor will follow the conventional or uveoscleral outflow. When this muscle constricts, the interstitial space (between the smooth muscle bundles) decreases. This suggests a decreased use of the uveoscleral outflow. Otherwise a relaxation of the ciliary muscle causes an increase of interstitial space and use of the uveoscleral flow. Some eye medication (respectively muscarine agonists or antagonists and prostaglandins) can influence this.(31)

At the second alternative route, the unconventional outflow, the fluid seeps through the ciliary muscle, iris, sclera and other tissues from the anterior segment. Firstly the humor can leave the eye by the capillaries of the ciliary body. Because there is no epithelial separation between the anterior chamber and ciliary body, the humor diffuses through the interstitial spaces of the muscle

and can reach these capillaries directly. Another possible reachable route is via the uveal meshwork. Fluid which passes this segment of TM may enter the space between the ciliary muscle bundles immediately.

Once the aqueous humor reaches the interstitial spaces, the fluid can follow different pathways to enter the venous circulation: at the (i) uveoscleral pathway, aqueous humor leaks through the sclera and episcleral straight into the orbital vessels. Secondly the choroid may absorb another portion of the humor by osmosis. The ocular choroid is drained with vortex veins in which the humor will enter.(uveovortex pathway)(ii) As a third route the lymphatic system is used. This system clears the body from excess fluids in several tissues. Recent studies have shown that lymphatic channels are present at the ciliary body. (uveolymphatic pathway)(iii) (25, 31)

1.3 GLAUCOMA

Glaucoma or glaucomatous optic neuropathy (GON) is an optic nerve disease which in most cases leads to irreversible blindness if it is left untreated. For 10% of patients with glaucoma, a loss of vision occurs despite a right treatment. According to the World Health Organization, glaucoma is, after cataract, the second leading cause of general blindness in the world. In 2013 at least 64.3 million people aged 40-80 years were diagnosed with glaucoma worldwide. By 2040, recent studies predict a value of 111.8 million diagnosed people.(32) The chance of developing glaucoma increases with multiple risk factors, for example: age, ethnicity, gender, family history, eye medication and numerous others.

Ocular tonometry, ophthalmoscopy or fundoscopy and gonioscopy are the regular diagnostic procedures to follow up a glaucoma patient. The term glaucoma includes four different types with different pathologic mechanisms: primary open angle glaucoma (POAG), primary angle closure glaucoma (PACG), primary congenital glaucoma (PCG) and secondary glaucoma. For most types of glaucoma, an increased IOP is the major risk factor, measured by ocular tonometry. The higher pressure results into death of retinal ganglion cells (RGCs) by a decrease of essential neurotrophic factors or local vascular insufficiency. This causes damage to the optic nerve and loss of visual field. Normally, at a homeostasis eye pressure, the aqueous production and outflow is strictly modulated to maintain an IOP. A regular eye pressure in a healthy eye varies approximately between 10 and 20 millimeters of mercury (mmHg) depending on the moment of the day. It is high early in the morning and decreases the first half of the day. Among both eyes, a maximum difference of 3 mmHg can be measured. When one of these standards deviates, it could be seen as a first sign of GON.(33)

The link between a measured IOP and glaucoma can be compared with the association of an elevated blood pressure and cardiovascular diseases, both are intermediary outcomes. Some patients have the possibility to develop ocular hypertension, a disease where an elevated IOP is measured without optic nerve damage or visual field loss. Furthermore an increased IOP is not the only risk factor for glaucoma. Another causative factor is due to gene mutations. This can be observed with patients where the pressure has not risen yet.(34)

Using linkage analyses and genome-wide association studies researchers identified the chromosome regions which may cause GON. In this manner, the etiology of specific genes was found. Because many sources describe a wide range of genes among different populations, only

the most significant and already discovered genes are discussed in this manuscript (Table 1). We distinguished causative genes and alleles which do not cause, but rather increase the risk of GON development.

Dependent of the type of glaucoma, the disease can be chronic or acute. Most patients with a suspicion of acute glaucoma attack exhibit some clinical symptoms: ocular pain, nausea and vomiting, red eye, headache, tenderness around the eyes, blurred vision and seeing hallows around light mostly at night.(35)

In this master thesis we made a distinction between primary and secondary glaucoma. The term 'primary' refers to the fact that there is no other eye disorder which causes glaucoma. We define the types congenital, open angle and angle closed glaucoma as primary glaucoma's, the latter two types being the most important. Secondary means that the glaucoma arises from an underlying cause, for example uveitis, cataract, a tumor, an accident and following medication. Secondary glaucoma will not be described in this manuscript.(34)

1.3.1 Primary open angle glaucoma

POAG or chronic open angle glaucoma is the most common form of GON worldwide. It is a very slowly progressive form where a lot of patients are asymptomatic for years. Once the symptoms manifest, it is often too late already. Initially the disease is unilateral, but evolves bilaterally at different rates in both eyes.(36) Studies in the USA from the National Eye Institute demonstrated clearly that aging increases the origin of POAG. Otherwise they illustrated that Afro-American populations have more risk of being diagnosed with glaucoma, but among all the glaucoma patients the Caucasians represent the majority relative to the others.(figure 1.9)(37) An anatomical clarification might be that the some people have a thinner cornea than others, a risk factor for transformation from ocular hypertension into POAG.(38) Abnormal corneal thickness may mask an accurate IOP measurement. In people with a thinner cornea, an IOP can be underestimated and be measured lower than it actually is. At this manner, an elevated IOP can left untreated and cause glaucoma and vision loss.

In some ocular structures such as the ciliary body, the retina and TM, the MYOC-gene gets expressed to myocilin, a calcium-binding protein consisting of 504 amino acids. Myocilin is a cytoskeletal protein with a unknown function found in the aqueous humor of a normal individual. People with a mutation in this gen are linked to an elevated IOP. After expression of the mutated gene, the mutated form of myocilin is assembled intracellularly inside the TM cells instead of secretion in the aqueous humor. The accumulated myocilin causes chronic endoplasmic reticulum stress and may in this way be injurious for the TM cells and their functions. The loss of these humor outflow cells indirectly causes an elevated IOP. (39) ASB10-gene is also expressed in the TM. Its function is to maintain the outflow pathway by clearing the passage from accumulated proteins. EFEMP1-gene gets expressed among others in the TM, ciliary body, cornea and retina. When a mutation emerges, the malfunctioned protein may form aggregates of the presented proteins in these tissues. The protein aggregates will influence the aqueous humor and its outflow.(40)

In normal healthy eyes, IL20RB or interleukin 20 receptor subunit β is located at the cell membrane of the TM cell and is the connecting place for the cytokines interleukine-20 (IL-20) and interleukine-24 (IL-24). After binding, Janus Kinase (JAK) gets phosphorylated which induces a phosphorylation

of STAT3 (signal transducer and activator of transcription 3). The activated STAT3 translocates to the nucleus where it stimulates the expression of inflammation-related target genes. Due to these genes a risen MMP activity and modulation of ECM are induced. This may ensure a reduction of the IOP. At the TM cells of POAG eyes, sometimes a T104M mutation at the IL20RB occurs, which means that IL-20 and IL-24 cannot bind to the receptor. As a result, the previous pathway will not perform and an elevated IOP is sustained. (41)

As a causative gene for POAG, the WDR36-gene expresses the protein WD repeat domain 36 (WDR36) in ocular and non-ocular tissues and is localized to the nuclei or cytoplasm.(42) It is involved in the synthesis of 18S rRNA. A malfunction in this gene ensures a loss of protein function which may lead to a lower concentration of 18S rRNA. However a decreased level induces the p53 stress-response pathway, a mechanism that responds to stress-signals and generates a cell cycle stop and cell apoptosis. Due to the mutation, the signal for apoptosis of RGCs may be stimulated which should be the reason of vision loss in POAG. (43) NTF4-gene or the expressed neurotrophin 4 is an example of the neurotrophic factors that may have, together with their receptors, a protective role in RGCs. The expression of NTF4-gene gets induced by an elevated IOP, ischemia or by exposing to the cytokines: glutamate or nitric oxide. As already mentioned, these factors play an essential role in POAG. NTF4 binds to the tyrosine kinase receptor B at the RGCs which is reduced by mutation of its gene. A reduction of this binding may give less protection to the possibly damaged RGCs in POAG. (44)

Via large scale genetic studies multiple genes were identified rather as risk factors than causes of POAG. Probably the most significant risk allele lies among two genes caveolins1 and 2 or respectively CAV_1 and CAV_2, but the exact location is not identified yet. CAV_1 and CAV_2-genes are detected in RGCs, TM cells and the scleral spur cells where they fulfill several functions. They regulate signal pathways and modify the cell membrane structure by forming caveolae, invaginated structures which influence the transport of molecules through the membrane. However the mutated form of the genes may affect the regulation of the humor outflow which clarifies its relation to the disease. A knocked down CAV_1 facilitates the outflow while silencing CAV_2 makes it more difficult. Dexamethasone, a corticosteroid, induces the expression of CAV_1 in TM cells in contrast to TGF-β_2.(40, 45)

Other risk alleles that might contribute to the POAG pathophysiology are CDKN2B-antisense RNA1, ABCA1 (ATP-binding cassette transporter 1),TGFBR3 (transforming growth factor beta receptor3), FOXC1 (forkhead box C1), ELOVL5 (elongation of long chain fatty acids family member 5), ATOH7 (atonal homolog7), SIX6(six homeobox 6), LOXL1 (Lysyl oxidase-like 1), CYP1B(a cytochrome P450 enzyme) and ATXN2 (ataxin isoform 2) proteins. Remarkable is the fact that the proteins of these genes are not harmful by themselves but only increase the risk of disease development or aggravate the badness of a pressure-induced injury.(46)

POAG can be subdivided into normal pressure glaucoma (also called normal tension glaucoma) or as in most cases high tension glaucoma, a type of POAG with an elevated pressure. The term ‘open angle’ refers to a normal open iridocorneal angle.(figure 1.11) Among both subtypes, pathophysiologically only slight differences can be seen.

1.3.1.1 High pressure glaucoma

Within high tension glaucoma, a disturbed modulation of the aqueous dynamics may be provoked by a decreased humor outflow and not directly by the production. Although some reports claim that for this type of GON, a hyperproduction can be a possible reason for the elevated IOP, their arguments could not be established by direct measurements. The drainage canal can be partially or totally blocked which leads to a lower outflow. Because of this, the fluid will accumulate into the eye chambers and gradually causes a higher pressure than usual. Next, the pressure pushes the lens backwards and presses on the vitreous body against the blood vessels and nerves from the retina. (figure 1.10) The consequence of an elevated eye pressure is a mechanical stress on the lamina cribrosa which leads indirectly to a degeneration of RGCs and their neurons. Loss of optic nerve tissue causes reduced visual fields and cupping of the optic disc, an increasing of the cup to disc ratio. Damage by the elevated IOP may be due to ischemia of the optic disc or NFL, neurotoxic agents and mechanical factors.(36)

In the conventional outflow, an obstruction of the drainage canal may be a result of an increased amount of ECM in some tissues. Usually ECM which is constantly modified by MMP and TIMP producing cells of the TM, has an important function in maintaining a normal outflow. At the TM and the anterior extremity of the ciliary muscle, an enhanced level of ECM is probably due to a reduction of the number of TM cells, an impairment of the phagocytic function or other functionalities of the TM cells.(47) Possibly, oxidative stress is responsible for the necrosis of TM cells and their functions. In the oxidative phosphorylation, cell mitochondria uses oxygen and transforms it to reactive oxygen species (ROS). In addition to ROS production, together with other tissues, the mitochondria is also responsible for its detoxification. The mitochondrial respiratory function or detoxification capacity decreases with age, which means that older people may own an increased amount of ROS. As a consequence, ROS causes DNA oxidative damage to the TM, the most sensitive tissue for ROS in the anterior chamber.(48) Another obstruction way is a smaller cross-section or a shorter length of the inner wall of the Schlemm's canal.

1.3.1.2 Normal pressure glaucoma

A high IOP is the most significant risk factor for glaucoma, but in 30% of the POAG cases, it is not present. When the IOP is lower than or equal to 21 mmHg, cupping of the optic disc can be spotted and the patient complains about vision loss without any other eye disorder, normal tension glaucoma (NTG) or low tension glaucoma can be diagnosed. An complete cause of this type of POAG is still unknown, although studies figured out that oxidative stress, autoimmunity and neurotoxicity may participate. However linkage and genome wide association studies identified that mutations in the genes optineurin (OPTN) and TANK-binding kinase 1 (TBK1) may contribute to the pathogenesis as well. The OPTN-gene has the usual function as regulator for apoptosis, autophagy, defense against pathogens, cell divisions and protein translocation. A mutation in this gene, an E50K mutation may result in loss of retinal ganglion cells (RGCs) and motor neurons. The expression of the gene is stimulated by exposure of tumor necrosis-α (TNF-α). This is a pro-inflammatory cytokine which augments the severity of damage to the optic nerve of normal pressure glaucoma patients. (49) In common, TBK1 has the same functionalities as OPTN and regulates inflammation and autophagy including mitophagy, a degeneration of mitochondria. Its mutation may be a duplication of the gene and may stimulate, just like the OPTN mutation, a faulty autophagy of RGCs, especially in NTG patients.(50)

A higher prevalence of NTG is noticed for patients with some risk factors: myopia, cardiovascular disorders, migraine, ocular blood flow reduction and vascular dysregulation. The treatment of NTG will be based on neuroprotection of the RGC's.(51)

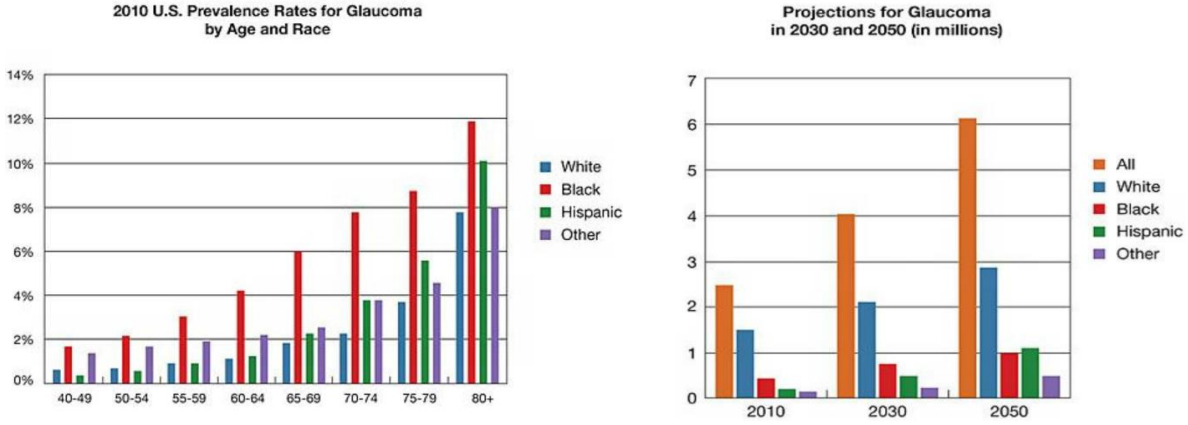

Figure 1.9: (left plate): prevalence rates of open angle glaucoma by age and race in the USA in 2010. The prevalence rate was highest for older black Americans. **(right plate):** y-as: prediction of the number of diagnosed glaucoma patients in millions in the USA. X-as: due to dividing by ethnicity and time, an increasing from 2.7 million in 2010 to 6.3 million in 2050 for all American races may be predicted.(37)

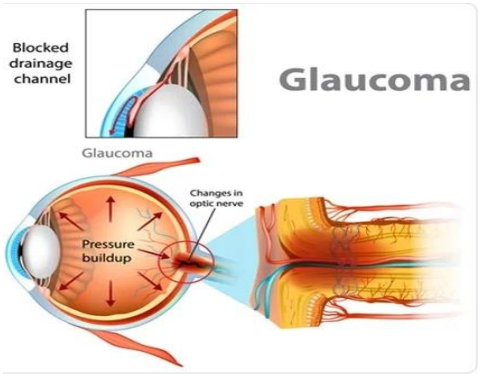

Figure 1.10: Illustration of high pressure glaucoma, a form of primary open angle glaucoma (POAG). Due to a continuous partially or totally blocked drainage channel, the humor cannot leave the anterior chamber. The pressure will rise at the eye chambers and presses the lens against the vitreous body. An elevated pressure in the eye can be measured which may damage the optic nerve.(52)

1.3.2 Primary angle closure glaucoma

After POAG, primary angle closure glaucoma (PACG) or narrow-angle glaucoma is the second common form of glaucoma worldwide. The prevalence of PACG is more often identified in the Inuits and Asian population than for Caucasian or African people.(53) Risk factors for this type of primary glaucoma are linked to the possible anatomical changes of the anterior chamber tissues. The risk increases by age due to the fact the lens may be thickened over time. The humor usually flows from posterior to anterior chamber through the pupil. Due to the raised volume of the lens, it touches the pupil and impedes the translocation. This mechanism is named the pupillary block. Next, the increased pressure in the posterior chamber pushes the iris forward and occludes the iridochoroidal angle. At figure 1.11 the pathophysiology of PACG can be noticed. The drainage outflow of humor is blocked by the iris who is now positioned against the TM. It can be a total or partial obstruction which leads to an elevated IOP.(53, 54)

Besides pupillary block, there are other causes of PACG. An example of a non-pupillary block mechanism is the plateau iris. An abnormally position of the ciliary body flattens the iris and pushes it into the angle. It is most reported in 30-50 year old women, especially with a hyperopic refractive error. Other cases of non-pupillary block mechanisms are lens-induced glaucoma or ciliary block glaucoma, but they are less commonly identified mechanisms of PACG. (53, 55)

Unlike open angle glaucoma, PACG may be an acute or chronic disease. We refer to the acute form when an abrupt onset of elevation of IOP occurs between 40-80mmHg due to a total closure of the anterior chamber angle. It is recognized by some clinical symptoms we already described above. At the chronic form of PACG the angle narrows slowly. From that moment, on gonioscopy, attachments among the iris and angle structures or peripheral anterior synechiae may be seen. These adhesions may cause an obstruction of the drainage canal as well.

In common with POAG, linkage studies and genome wide association studies discovered genes that may contribute to the disease. So far the mutation of this gene MMP-9 gene brings the MMP protein to expression that remodels the ECM. Change in composition of ECM influences the humor outflow and raises the IOP in PACG. Otherwise eight other important risk alleles were associated with PACG: PLEKHA7, COL11A1, PCMTD1-ST18, EPDR1, GLIS3, DPM2-FAM102A, CHAT and FERMT2. (56)

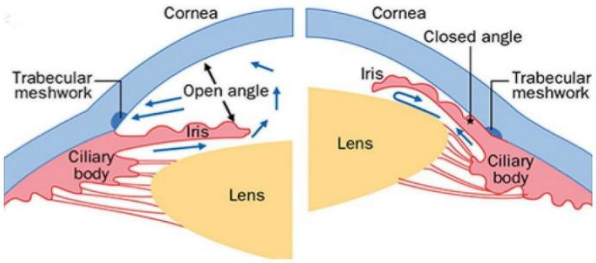

Figure 1.11: Schematic view of the anterior segment. (Left plate): Open angle glaucoma. A clear space between iris and cornea, a normal iridochoroidal angle, is be shown. A total of partial obstruction of the drainage canal causes an elevated IOP. **(Right figure):** Angle closure glaucoma. The peripheral iris pushes against the cornea which creates an obstruction for the aqueous humor with a risen IOP as consequence. (46)

1.3.3 Primary congenital glaucoma

In western countries, PCG is an autosomal recessive disease and is normally diagnosed at birth in 1 of 10000-20000 newborn babies. It is the most common type of childhood glaucoma in children to an age of 3 and is their major cause of blindness. Typical features or symptoms of the disease are mostly bilateral and characterize: tearing, eyelid squeezing, corneal edema, a decreased thickness of the cornea and photophobia, a phenomenon where the patient is intolerant to light.

The enzyme CYP1B1, a family member of the cytochrome P450 enzymes is associated with PCG. It is expressed in multiple tissues including the cornea, ciliary body, iris and retina. Functionally the protein should help develop the TM. Malfunctions in this gene determine a loss of protein function. According to several clinical studies, problems at the development of the TM and Schlemm's canal caused by a CYP1B1-knockout, can be compared between mice and humans. (57, 58) Mutations might lead to dysplasia of the anterior chamber angle or TM and eventually cause a small or absent Schlemm's canal, with a decreased outflow and elevated IOP as a result. (59) Besides optic nerve

damage and partial or total vision loss, the risk of an enhanced IOP in infancy is also an augmentation of the eyeball, named bull's eye or buphthalmos. (33)
Today, research is linking the genes MYOC, LTBP2 (latent transforming growth factor-b-binding protein 2), PITX2 (paired-like homeodomain 2), PITX3 (paired-like homeodomain 3), FOXC1, FOXE3 (forkhead box protein E3), PAX6 (paired box protein 6) and LMX1B (LIM homeobox transcription factor 1-β) to PCG. Mutations in these genes may also cause abnormalities of the iridochoroidal angle.(60)

Table 1.1: candidate genes whose mutations may contribute to the pathogenesis of GON

Causative genes	Type of GON	Possible mechanism after mutation
MYOC	POAG, PCG	Accumulation of proteins intracellularly and humor outflow reduction
ASB10	POAG	Reduced humor outflow and incorrect protein degradation
EFEMP1	POAG	Abnormal formation of protein aggregates
IL20RB	POAG	Lowered MMP expression and ECM modulation
WDR36	POAG	Stimulating p53-stress response pathway and apoptosis of RGC's
NTF4	POAG	Decreased tyrosine kinase B receptor activity which leads to a reduced protection of RGC's
OPTN	POAG, NTG	Malfunctioned autophagy of RGC's
TBK1	POAG, NTG	Malfunctioned autophagy of RGC's
MMP-9	PACG	Higher IOP by changed ECM-composition
CYP1B1	POAG,PACG, PCG	Inducing an abnormal structure of iridochoroidal angle
Risk alleles		
CAV_1/CAV_2	POAG	Lower elimination of the aqueous humor outflow
CDKN2B-AS1 ABCA1, FOXC1, ELOVL5, ATOH7, SIX6, TXNRD2, LOXL1, CYP1B, ATXN2	POAG	CDKN2B-AS1: increasing IOP and damage RGC's after 1 year. SIX6: causing reduced thickness of the NFL and aging RGC's ABCA1: inflammation of the retina and loss of RGC's FOXC1: may lead to problems at the development the anterior segment TXNDR2: elevated damage of RGC's due to a decreased protection against ROS For the other genes, a working mechanism is insufficiently known.
PLEKHA7,COL11A1, PCMTD1-ST18, EPDR1, GLIS3, DPM2-FAM102A, CHAT , FERMT2	PACG	A mechanism is as good as unknown for all these genes. However they may associate to the pathogenesis of the disease.
LTBP2,PITX2,PITX3, FOXC1,FOXE3,PAX6, LMX1B	PCG	The association among their mutation and PCG is recently discovered. There are suspicions about their contribution of the pathogenesis, but nothing is certainly proved yet.

1.4 PHARMACOLOGICAL TREATMENT OF GLAUCOMA

1.4.1 Commercially available anti-glaucoma medication

For most types of the disease a risen eye pressure is a major modifiable risk factor. For that reason most commercially available treatments are developed to decrease the IOP by lowering the humor production or increasing the outflow. These medications are often for topical use and has a lot of advantages and disadvantages. Firstly, the patient need to apply the eye drops in general once or twice daily into the lower conjunctival sac. It goes without saying that this requires a sufficient patient adherence and compliance to the medication. The therapeutic dose has to be similar to the absorbed portion into the ocular tissues which may consist of only five percent of the applied dose. Ocular absorption comprises a primary, corneal route for small lipophilic drugs and a secondary, conjunctival and sclera route for large hydrophilic drugs. However at least 95% is subjected to systemic absorption or gets lost for example into the drainage duct. (61) As already written in 1.1.4. the sclera and the conjunctiva palpebralis are strongly vascularized which leads to sink conditions and indirectly to a lower bioavailability.

In addition, side effects are not excluded. Long term use of eye drops may cause symptoms of allergies like redness and itchiness. Due to the presence of preservatives, a normal bottle filled with eyedrops can only be used for approximately 30 days after opening. Use of these preservatives for a longer period can induce instability of the tear film. A possible solution for this problem is packaging the eye drops in for example specialized continuous monodose (comod)-system bottles where preservatives are not needed. From investigation, at least ten percent of blindness of glaucoma is directly caused by a bad compliance to the daily prescribed eye medication. Reasons for this impairment are often forgetfulness, a too complex medication scheme, an insufficient knowledge of the disease or the physical disability of an older person.

An obvious example of a typical case is in elderly patients diagnosed with GON and who have arthrosis in their hands. Specifically for these patients, it becomes more difficult to apply the eyedrops correctly by themselves. Despite these obstacles, the effectiveness of topical eye medication acting in the anterior segment is accurate when used properly. However in future, these problems could be avoided by new sustained-release drug therapies like scleral plugs, punctal plugs, cul-de-sac rings or nanomedicines.

1.4.1.1 Sympatholytics (β-blockers)

Topical β-blockers are one of the most prescribed treatments of glaucoma due to among others, their low cost. β-blocking agents can be deployed for all types of GON, particularly POAG and PACG. Applying the drug once or twice a day in monotherapy or in combination with other anti-GON medication, should suffice. There are two types of β-blockers, the non-selective and selective or cardioselective which can both be used as a treatment. Their working mechanism is based on an inhibition of aqueous humor production and differs slightly among both types. A decreased IOP effect is lower for the selective in comparison with the non-selective β-blockers.

Non-selective β-blockers includes carteolol, levobunolol, metipranolol and the most effective lowering IOP agent, timolol. According to the BCFI, levobunolol and metipranolol are absent on the Belgian market. They are competitive antagonists of the β_1 and β_2-receptors located at several tissues such as the ciliary epithelium. By blocking the binding between the catecholamines (epinephrine or norepinephrine) and the β-adrenergic receptor, an activation of those receptors is

prevented. Generally, some studies claim that mostly inhibiting the β_2-receptor activation and a decrease of cyclic adenosine 3′,5′-monophosphate (cAMP) levels may be the cause of decreased humor production. It would induce vasoconstriction of the ciliary arteries and thus indirectly lower the production. Betaxolol, a selective β-blocker exhibits a higher affinity for the β_1-receptor. Despite this fact, a small portion may still bind to the β_2-receptor and have a weak antagonistic effect. However, it is suggested that a higher concentration of betaxolol will arrive at the ciliary arteries and compensate for the lower β_2-adrenoceptor binding capacity. (62, 63)

Local side effects of the non-selective beta-blocking agents are conjunctival hyperemia, ocular discomfort, decreased tear flow and deteriorating of dry eye. Besides the ciliary epithelium, β_1 and β_2-receptors are also located at other tissues including the heart and lungs. Therefore, systemic side effects such as bradycardia, heart block, arrhythmia, bronchospasm and worsening of asthma or chronic obstructive pulmonary disease can be explained. An advantage of the cardioselective β-blockers is that they may develop fewer side effects than the non-selective. Because these drugs show as good as single affinity for the β_1-receptors, they bind logically only to these receptors in unwanted tissues which can cause side effects and not to different β_2-receptors. (64)

Since the production of the endogenous adrenergic neurotransmitters is slightly when a person is sleeping, the effect of β-blockers is minimalized at night. A newer type of anti-glaucoma medication, the prostaglandins might be a solution for this problem.

1.4.1.2 Carbonic anhydrase inhibitors

In the section 1.2.1, we already mentioned the importance of the enzyme carbonic anhydrase for the humor production. Brinzolamide and dorzalamide are the two available carbonic anhydrase inhibitors that lower the production. This leads indirectly to a IOP reduction around 20% which is lower than other topical anti-glaucoma medication. In addition, these inhibitors may rise the ocular blood flow as well which may attribute to the risen CO_2 concentration. It has multiple side effects such as: a bitter mouth taste, stinging, itching and a blurred vision. In terms of side effects, brinzolamide has practically the same side effects but to a lesser extent than dorzalamide.(63)

1.4.1.3 Sympathomimetics

Another type of IOP lowering medication are the sympathomimetics. They consist of non-selective and selective adrenergic agonists. Non-selective agonists including phenylephrine stimulate the α1, α2 and β2 adrenoreceptors. Clinical use of them is rather limited due to the many adverse side effects they produce. On the other hand apraclonidine and brimonidine, the available selective sympathomimetics or α_2-adrenergic agonists can both be used for PACG or POAG. They reduce the IOP within a range of 20-30% with a working mechanism based on activation of the α_2-adrenergic receptors located at the ciliary epithelium. As a consequence of this stimulation, the cAMP levels may be suppressed which leads to a lower humor production. In addition, apraclonidine should elevate the trabecular outflow route while brimonidine increases the uveoscleral outflow. A strict theory about how they influence the outflow routes is still unknown, but there are suspicions that alterations in contractility of the TM and ciliary muscle may be involved.(47)

Furthermore, brimonidine shows a higher affinity for the α_2-adrenergic receptors than apraclonidine and should be more lipophilic. Due to this last determination, brimonidine is allowed to be applied

at lower concentrations and may have less local side effects. Other rarely reported systemic side effects for both drugs are dry mouth, sedation and general malaise. The disadvantage of a higher lipophilicity, is facilitated transport through the blood-brain barrier which may increase the chance of a central nervous system depression development. For that reason, brimonidine is absolutely contraindicated for children. (63, 64)

1.4.1.4 Prostaglandins analogs

Typically prostaglandins analogs (PGA) are known as lipophilic derivates of arachidonic acid. Often they are associated with a diversity of physiological functions including inflammation, vascular permeability, relaxation of smooth muscles and blood vessels. At the end of last century, the therapeutic use for different clinical applications was noted. Especially in the USA, prostaglandins are mostly the first line pharmacological treatment for GON. They consist of several types highly expressed in ocular tissues. Especially $PGF_{2\alpha}$-analogs, a PGA-type has an IOP-lowering effect of approximately 30%. Bimatoprost, latanoprost, tafluprost and travoprost are the most popular $PGF_{2\alpha}$-analogs which facilitates the uveoscleral outflow route. The trabecular outflow and humor production should not be influenced by the $PGF_{2\alpha}$-analogs. A potential mechanism behind the PGF-receptor agonists may be stimulating the expression of MMP- genes present in the ciliary muscle cells. MMP modifies ECM which lowers the outflow resistance by enlarging the extracellular empty spaces among the muscle bundles. Furthermore, $PGF_{2\alpha}$-analogs should help to relax the TM and ciliary muscle which decreases the pressure as well. (47, 64)

PGA are still mediators of ocular inflammation where ocular side effects such as strengthening of iris or skin pigmentation, hyperemia of the conjunctiva, hypertrichosis of the eyelashes and reactivation of uveitis are reported. Patients with a history of aphakia, a condition where the lens has been removed after eye surgery should have a higher risk to develop cystoid macular edema in combination with PGA.(64)

Different with the β-blockers, the therapeutic efficacy is independent of the sleep or awake status of the patient. Sometimes PGA is combined with other ocular hypotensive medications including β-blockers, adrenergic and cholinergic agonists or carbonic anhydrase inhibitors to obtain an additional reduced pressure. (65)

1.4.1.5 Cholinomimetics

Cholinomimetics or parasympathomimetic agents activate muscarinic receptors by elevating acetylcholine levels via two pathways. These receptors are located in several tissues including the ciliary muscle and iris sphincter. A first type of cholinomimetics are the cholinesterase inhibitors which create a downsize of the acetylcholine degradation. But due to serious long-term side effects, this type is not designated anymore for the treatment of GON. Secondly, drugs such as pilocarpine are direct-stimulating muscarine agonists used for POAG and an acute attack of PACG. Activation of the muscarine receptors causes a contraction of the ciliary muscle and iris sphincter. The contracted ciliary muscle enlarges the extracellular spaces in TM by pulling this tissue backward. This should facilitate the trabecular outflow route. Its fast performance of iris sphincter contraction induces miosis which ensures a loss of touch among lens and iris at the moment of an acute PACG attack. Thus, ophthalmologists apply pilocarpine as an emergency treatment.(47, 63) Just like the other commercially sold anti-GON medication, pilocarpine involves adverse side effects. Some patients might complain about a blurred vision, local irritation and headache.

1.4.2 Novel or investigational anti-glaucoma agents

1.4.2.1 Cytoskeleton acting agents

The cytoskeleton is a dynamic network of cytoplasmic proteins and functions for cell shape, motility, contractility and cell adhesion to other cells or to the ECM. Cells of humor outflow regulating tissues such as TM and Schlemm's canal have an extensive cytoskeleton. Each in their own way, some drugs will influence this cytoskeleton and their functions which facilitate the humor outflow. Firstly the cytochalasins should dilate the TM and generate ruptures in the inner wall of the Schlemm's canal.(47) A second example of a cytoskeleton acting agent, is a type of the macrolides, the latrunculins. They disrupt the cytoskeleton which induces a change in cell shape, adhesion with other cells or ECM. In addition, latrunculins ensure relaxation of the ciliary muscle and other morphological alterations including a space expansion among the inner wall cells of Schlemm's canal and the TM.(47, 66) The biggest issue for cytoskeleton acting agents may be their non-selectivity, since practically every cell has a cytoskeleton which can be influenced. More and more clinicians start questioning the long-term safety of this kind of medication.

1.4.2.2 Rho-associated Protein Kinase Inhibitors

In December 2019, the European Medicine Agency approved a new IOP-lowering eye medication, the Rho-associated coiled coil-forming kinase or ROCK-inhibitors such as ripasudil and netarsudil. An increased outflow of the major TM pathway gets done by blocking the activity of the enzyme named Rho kinase, a serine-threonine kinase expressed in the TM, Schlemm's canal, ciliary muscle and optic nerve. Rho is a member of the Rho family existing of three isomer types (Rho A, Rho B and Rho C) and is described as a small G-protein. When this kind of proteins is activated, they are typically bound to a guanosine triphosphate (GTP), while binding to guanosine diphosphate (GDP) indicates its inactivation. An activation mechanism is due to binding to the ECM or to the secreted bioactive molecules receptors including TGF-β2, other cytokines, thrombin or endothelin-1. GTP bounded Rho activates its effector molecules ROCK-1 and ROCK-2. Once activated, ROCKs phosphorylate and influence the functions of multiple proteins expressed at the level of the TM which polymerize actin stress fibers and at this turn creating focal adhesion. Moreover, they play an important role in cell contractility, shape and motility. In other words, ROCK-inhibitors decrease actomyosin-driven cellular contraction which leads to a relaxation of the TM and inner wall of the Schlemm's canal, more empty space in the JCT area, less cell binding to the ECM and finally a lower IOP. This new therapy works directly on the TM and cells of the Schlemm's canal, it can be thus used for all types of POAG.(67)

Not all patients respond well to anti-GON medication, making non-pharmacological treatments indispensable. Regrettably these treatments often fail whereby excessive postoperative wound healing is unavailable. In addition ROCK-inhibitors act as anti-scarring agents and may reduce postoperative scarring after glaucoma filtration surgery. They do this by lowering cytokinesis such as TGF-β_2, inhibiting cell migration and invasion which play an essential role in scar formation. Besides GON, ROCK-inhibitors have been examined for several other indications, like myocardial ischemia, hypertension, kidney problems and neurological diseases including spinal cord injury, Alzheimer's disease and multiple sclerosis. The reason for research on those last neurological diseases is that ROCK-inhibitors have, in addition to the IOP-lowering effect, a neuroprotective activity. Since GON is a progressive optic neuropathy, this neuroprotective activity may be an

interesting treating element. By increasing the optic nerve blood flow they may be helpful for recovering injured optic neurons and axon regeneration. (68, 69)

1.4.2.3 Statins

In literature statins are mostly known as first line therapy for hypercholesterolemia. Usually they function as inhibitors of the 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoA reductase). An inhibition of this enzyme creates a lower formation of farnesyl pyrophosphate and geranylgeranyl pyrophosphate, the next products in the biosynthesis of cholesterol. These isoprenoids are also used in the posttranslational modification of small G-proteins like Rho. As already described above, reduced stimulation of Rho leads indirectly to an inhibition of ROCK activation. Hence, the effect of the statins including lovastatin, mevastatin and simvastatin is largely comparable with the ROCK-inhibitors. The humor outflow facility due the use of lovastatin would increase to 110% relative to healthy control eyes.(70) Another investigation claims a different theory around the IOP-lowering effect of statins. According to this research, they should downregulate the expression of SPARC. We already mentioned in chapter 1.2.2.1, the presence of matricellular protein SPARC at the ECM of the TM. Matricellular proteins are glycoproteins which control and communicate with their surrounding ECM. Furthermore SPARC is linked with tissue fibrosis and divergent tissue remodeling, processes that may disturb ECM homeostasis. An overexpression of SPARC is dependent on TGF-β_2, which is elevated in GON-patients and would at this manner contribute to POAG pathogenesis. (71, 72)

1.4.2.4 Nitrous Oxide Agents

In the USA, early 2018 the first commercial use of nitrous oxide donating agent, latanoprostene bunod was prescribed as novel IOP-lowering treatment for all types of GON. This medicine is a hybrid molecule existing of a combination of latanoprost (more information 1.4.1.4 Prostaglandins analogs) and butanediol mononitrate, a nitric oxide (NO) donating molecule. NO is a soluble, short-lived gas which can arise endogenous through the conversion of L-arginine to L-citrulline located at the TM, Schlemm's canal and ciliary body. It activates soluble guanylyl cyclases which increases the intracellular cyclic guanosine monophosphate (cGMP) levels. Elevated cGMP levels create a relaxation of smooth muscles including the ciliary muscle, TM and Schlemm's canal. Investigations discovered that POAG patients have a suppressed level of NO and cGMP present in their aqueous humor. Based on this theory, exogenous NO, like in latanoprostene bunod strengthen the possibility of smooth muscle relaxation into the eye which leads to less outflow resistance.(63, 64) The IOP-lowering effect of this combination therapy should be higher than when latanoprost is given as a single solution. (73) Ocular side are practically equal to those from prostaglandins analogues.(74)

1.4.2.5 Increasing neurotrophic factors

Neurotrophic factor delivery should be a preventing strategy for the treatment of GON. Neurotrophin-4 (NT-4), ciliary neuronotrophic factor, sciatic nerve derived medium and brain-derived neurotrophic factor (BDNF) are neurotrophins (NTF). Eventually carried by viral vectors, they can be injected intravitreal and may avoid apoptotic death of RGC after axonal injury. Having an essential role in neuroprotection and RGC survival, BDNF is the most active NTF produced at several tissues including RGC and braincells. It is transported by the terminal axon of the RGC to its receptor at the retina. In GON-patients disturbed levels of endogenous NTF can be found. A

view of the NTF-biology in glaucoma helps to understand the difficult pathogenesis of the disease and could be the basis of drug development in novel medical therapies.

1.4.2.6 Adenosine Receptor Agonists

This relatively recent eye medication lowers the IOP by increasing the conventional outflow route and should have a neuroprotective effect. Adenosine is a ribonuclease released from practically all cells and is extracellularly known as an important part of ATP. It binds to G-protein-coupled adenosine receptors located for example in the ciliary epithelium and can be divided into four subtypes: A1, A2A, A2B, and A3. With the aim of lowering the IOP, not all receptors may be stimulated. The mode of action is focused on selectively activating the A1-receptor. Hereby A1-agonists may lower IOP by expressing and secreting more of the protein MMP-2, a member of the MMP-family. These proteins should decrease cell volume, adjust ion transport and remodel the ECM at the level of JCT, TM and Schlemm's canal cells. The clinical importance of selective activation is proven by the fact that stimulating the A3-receptor should lead to opening of the chloride channels in the NPE cells and in this manner raise the aqueous humor secretion. This induces the opposite of the intended purpose.(75)

Trabodenoson or INO-8875 is an example of a A1-agonist that has been developed as IOP lowering medicine for open angle glaucoma and ocular hypertension. Despite the hopeful results of the phase II clinical trials, the drug failed at the first pivotal phase III trial. It couldn't achieve the primary endpoint by a probably stronger placebo-effect than expected. Currently further investigations are running to improve this result and continuing the targeted clinical secondary endpoints.(76)

1.4.2.7 Oligonucleotide-based Agents

Oligonucleotides are developed to target specific genes or RNA's and can regulate the expression of these genes. Currently, small interfering RNA (siRNA), antisense oligonucleotides (ASOs) and microRNAs (miRNAs) are the classes of oligonucleotides where research is still being done with the aim of using them as potential therapeutic agents.(77) The main advantage of these new therapies is a longer-lasting effect in comparison with the commercial pharmaceutical products.(78)

<u>Small interfering RNA (siRNA)</u>

As a natural biological cellular process, RNA-interfering (RNAi) regulates the expression of specific genes by neutralizing the corresponding mRNA-molecules. Based on this principle and as a possible treatment for GON, scientists in biotechnology tried to engineer siRNA which suppresses the translation of several genes to proteins. Characteristically, siRNA is a double-strand RNA (dsRNA) and has a length from 21 to 23 base pairs. An optimal anatomical eye structure, immune privilege, the fact that it is highly compartmentalized and the possibility of local topical delivery are explaining why the eye is a good target for siRNA drug delivery. Nevertheless, a high sensitivity to enzyme hydrolysis, fast clearance of the circular system, insufficient cellular absorption and biological barriers for topical ocular gene therapy clarify why the clinical outcome is still in research. As bypass for the barriers of topical gene administration, several other delivery routes can be used e.g. intravitreal injections, periocular routes, intracameral injection, suprachoroidal injections and subretinal injection.(figure 3.1). For RNAi-delivery systems multiple viral and non-viral carriers as routes of delivery are designed, which we will discuss more in detail later this master thesis.(79)

In TM and retina, specific siRNA targeting genes, should be neuroprotective and decreasing secretion or elevate outflow of the aqueous humor. Via topical gene administration, siRNA can inhibit humor production by silencing the carbonic anhydrase genes and alfa and beta receptor genes. Finally the effect of IOP-lowering should be comparable to those from the commercially available agents. (78)

Gene silencing by RNAi happens by a specific working mechanism which is shown in figure 1.12. Naturally a RNase III-like enzyme, the Dicer, assimilates via an ATP-dependent mode dsRNA into 21-25 nucleotides siRNA. Otherwise synthetically engineered siRNA can affect RNAi as well. The formed or added siRNA infiltrates into the RISC (RNA-induced silencing) complex and cleaves the region of mRNA which is complementary to the siRNA. This infiltration can happen directly into RISC while other siRNA needs an initial prior process by Dicer before entering RISC. RISC is an endonuclease complex possessing guide RNA and Argonaute proteins. Guide RNA is single-stranded and directs the RISC-complex to the target mRNA. Endonuclease Argonaute destroys target mRNA by hydrolyzing the mRNA region fully complementary to the original guide strand of siRNA.(80, 81) Since the cleaved mRNA strands lost the 5'-cap structure or poly(A) tail, which is essential for the stability and survival of the mRNA strand, they will degrade. This means that the translation or protein syntheses is inhibited. (82)

Currently, two candidate siRNA drugs for treatment of GON are still in research phase. On the one hand SYL040012 discovered by the Spanish pharmaceutical company Sylentis and on the other hand QPI-1007 developed by Quark Pharmaceuticals, Inc.

SYL040012 or bamosiran is an example of topically applied naked siRNA which suppresses the gene that gets translated to the beta-2 adrenergic receptor. Indirectly, this should lead to the same effects as the commercially used β-blockers and lowering the IOP by decreasing the humor production.(77) In October 2014, 'SYL040012, Treatment for Open Angle Glaucoma (SYLTAG)' a phase IIb clinical trial started. According to clinical trials.gov, it was a multicenter, international, randomized, double blinded, Timolol- controlled study that tried to select an optimal effective dose. They analyzed the eyedrops of bamosiran administered once a day against twice daily timolol administrations in each eye for 28 consecutive days. Earlier this year, January 2021 the results of the study were published. In the intervention arm of healthy volunteers, a lowered IOP was measured, just like they resulted in animal models in previous studies.(83, 84)

Another siRNA compound is QPI-1007 or cosdosiran, a double-stranded neuroprotective drug candidate for GON which silences the apoptotic stimulating caspase-2 gene. It already passed three clinical studies and is approved by the U.S. Food and Drug Administration (FDA) for the indication of non-arteritic ischemic optic neuropathy. A disease similar to glaucoma where suddenly loss of vision and RGCs occurs. Right now, cosdosiran is under phase II clinical trials. Despite the minor side effects and the promising results, further clinical research and development as an anti-GON medication is still required. (85, 86)

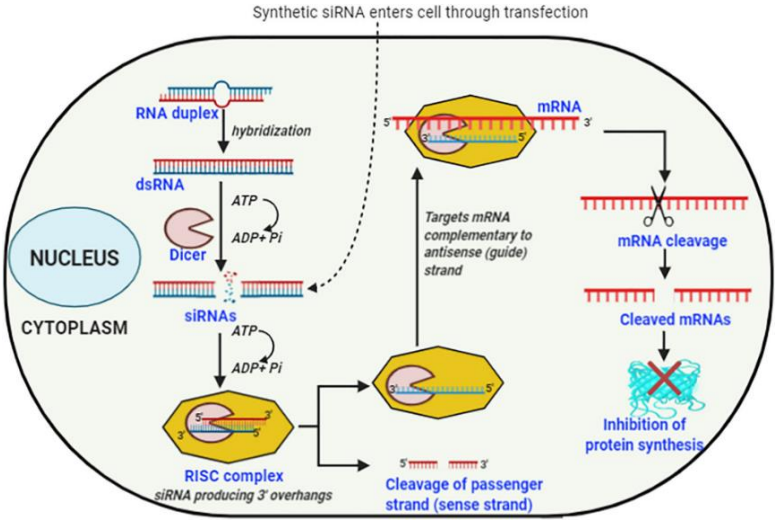

Figure 1.12: mechanism of RNA interference (RNAi) (79)

Antisense Oligonucleotides (ASOs)

ASOs are small sized single-stranded RNA or DNA with a length of circa 15-25 base pairs and modulate mRNA using Ribonuclease H (RNase-H)-mediating degradation. As endonuclease enzyme, RNase-H recognizes a RNA-DNA duplex and catalyzes the cleavage of the RNA of this duplex via hydrolyses. Its mode of action is based on removing RNA primers from the Okazaki-fragments at the moment of DNA replication. Using this manner, the mRNA and protein expression should be reduced by at least 80%.(87) Aganirsen and ISTH0036 are the current potential therapeutic anti-glaucoma medication. Aganirsen is a 25 base pairs ASO which inhibits the transcription of the insulin receptor substrate-1. This protein is expressed in the cornea and functions as a factor in the blood vessel development. It should stimulate vascular endothelial growth factor-1 (VEGF-1), a protein acting as promotor of neovascularization. Moreover, this medicine has as advantage that it should not inhibit normal vessel growth. In Europe, topically administered Aganirsen can be used as treatment for various ocular eye-indications including neovascular glaucoma, an example of secondary GON.(88) As latter explained ASO, ISTH0036 targets the mRNA of TGF-β_2. As we already mentioned, TGF-β_2 is elevated in the anterior chamber, vitreous body and optic nerve of glaucoma eyes. It plays a critical role in cell differentiation, epithelial-to-mesenchymal transition, fibrosis and immune responses. ISTH0036, a product of Isarna Therapeutics is under investigation and has very encouraging results after a phase I clinical trial.(89)

microRNAs (miRNAs)

Besides siRNA, another type of noncoding RNA or small RNA is endogenous miRNA. They both function at a posttranscriptional manner to silence genes and have the same stability issues in vivo, delivery problems and off-target effects. Though they use some enzymes in common such as RISC and Dicer, their mechanism of action may be different. Distinct between them, is that siRNA decreases expression of one specific target mRNA while miRNA inhibits the expression of multiple mRNAs. In the nucleus, transcription of the miRNA-gene starts by the enzyme RNA polymerase II which forms into a hairpin folded primary miRNA. Next, some other enzymes transform the primary miRNA to precursor miRNA (pre-miRNA). This pre-miRNA will be transported from the nucleus to the cytoplasm where its gets changed by Dicer to a mature double-stranded miRNA existing of 18-

25 nucleotides. As mode of action miRNA should inhibit protein expression by two pathways, blocking the mRNA translation and cleaving of the mRNA just like siRNA.(87) In GON, miRNA levels change, which suggests their contribution to these disorders.(90) The fact that one miRNA can regulate multiple genes such as MYOC, CAV_1/CAV_2, OPTN, WDR36 etc. makes this type of RNA interesting for the treatment of GON. (91)

1.4.2.8 CRISPR-CAS-9 mechanism

As a treatment for ocular diseases including GON, gene therapy has been explored for many years. Comparable with RNA-interference is the mechanism behind CRISPR-CAS-9. Clustered regularly interspaced short palindromic repeats or CRISPR is a DNA sequence originating of mostly bacteria or archaea which were infected earlier by bacteriophages. It functions as a guide strand for the enzyme CAS-9 (CRISPR associated protein 9) and delivers it to its complementary target DNA in for example humans. Once the CAS-9 endonuclease has arrived, it cleaves the dsDNA in two parts. Because of this, the human body has two endogenous cellular repair mechanisms: nonhomologous end-joining (NHEJ) and homology-directed repair (HDR). These processes can recover the now broken dsDNA by DNA ligation. However HDR will repair the induced break sites practically flawlessly. It uses other very homologue intact sequence DNA, located in the nucleus, as a repairing tool. Unlike to HDR, when the homologue donor DNA is not present in the nucleus the other process, NHEJ is employed. NHEJ is an error-sensitive repair method which may induce inserts and deletions which can lead to a frameshift in the coding regions. As consequence, this frameshift creates a forward replacement of the stop codon causing an earlier stop of the protein translation. It is obvious that there is a higher efficiency of NHEJ-repair tool than HDR.(92, 93)
Last year, Prof. dr. Charpentier and Dr. Doudna received the Nobel prize in Chemistry for their research on the CRISPR-CAS-9 mechanism.(94)

For POAG, this technique should be used to silence the causative gene MYOC, since myocilin would not be necessary for ocular health. An efficient gene delivery route of the CRISPR components to ocular tissues can be achieved by especially the use of viral vectors, but non-viral vectors as well. (more information see later) By lowering the expression of the mutant myocilin, they could prevent an elevated IOP indirectly.(93)

1.5 NON-PHARMACOLOGICAL TREATMENT OF GLAUCOMA

Currently today the non-pharmacological treatment of GON including surgery and laser are still often used, especially when the topical administration fails or in severe cases of GON. Similar as the pharmacological drugs, they can enhance the outflow of the aqueous humor or reduce the production of it. It depends on the choice of the ophthalmologist and after agreement with the patient which type of surgery or laser treatment will be used. In this section, only the most commonly used procedures will be described. Since this is not the main theme we will not discuss this more in detail.

1.5.1 Types of anti-GON surgeries

1.5.1.1 Trabeculectomy

Trabeculectomy or filtration surgery is a treatment for most forms of open-angle glaucoma and chronic angle glaucoma. It is an IOP-lowering method where the blockage of the drainage canal is undone. Hereby an incision at the bulbar conjunctiva is made, followed by a disconnection of a flap

of the sclera. Next, underlying tissue including a part of the TM and eventually a portion of the iris is removed to create an opening into the eye and to create a new outflow route for the aqueous humor. Finally the flap of the sclera can be replaced and be attached temporarily with stitches which helps avoid too much humor draining at once. After a successful operation, the humor can drain into a filtering bleb, an area located underneath the conjunctiva that is hidden under the eyelid. There it gets absorbed into the circulatory system or is filtered into tears. Scar tissue formation can be a significant reason why a blockage unfortunately reoccurs. Other complications of traditional filtration surgery are among others endophthalmitis and postoperative hypotony (95, 96)

1.5.1.2 Drainage implant surgery

Glaucoma drainage implant surgery is a process where an artificial IOP-lowering device is placed at the sclera of the patient. Structurally the device consits of a scleral attached silicone rubber tube that drains the aqueous humor into an end plate. This plate can be seen as a space to catch the arrived humor. As complications, too low eye pressure, loss of IOP-control, injury to the cornea or sclera, abnormal accumulation of humor, blocked implanted tube or position-loss of the tube and several others can occur after this type of surgery.(97)

1.5.1.3 Minimally-Invasive Glaucoma Surgery

Minimally-Invasive Glaucoma Surgery (MIGS) is a new surgery method with less complications than the typical anti-GON surgeries. Other advantages of this novel procedure are a higher safety profile and shorter operation time and high efficacy. It includes multiple IOP-lowering devices which are distinguishable into trabecular, suprachoroidal and subconjunctival based procedures and are mostly placed via corneal incision ab interno. This means that they create an opening through the TM, with the result of making direct contact between the drainage angle and the Schlemm's canal or collector channels.(98, 99)

1.5.2 Laser therapies as anti-GON treatment

The introduction of laser therapies made the treatment of GON a lot simpler and more efficient. Ophthalmologists opt for these procedures routinely due to their similar effectiveness to eye medication but without the potential side effects.

1.5.2.1 Laser trabeculoplasty

As an IOP-lowering therapy, laser trabeculoplasty can be used as a primary or adjunct treatment for POAG, ocular hypertension and PACG. It can be divided into two main types: argon laser trabeculoplasty (ALT) and selective laser trabeculoplasty (SLT). In literature, similar for both types, three possible theories on a mechanism of actions are suggested. First of all trabeculoplasty should realize thermal changes in the TM bundles where the laser beam is focused. This causes a contraction of these bundles followed by a shrinking of the surrounded TM bundles as well. At their turn the intertrabecular spaces may be enlarged which leads to a facilitated outflow. A second biochemical theory starts by the damage of the TM-tissue due to the laser beam. This may stimulate on the one hand the attraction of macrophages which raise the ECM secretion and on the other hand elevating some cytokines which upregulate MMP expression, both causing an increased outflow. Finally a cell division theory suggests that the laser beam elevates the division of TM cells. In addition some endothelial cells around TM bundles will be injured and as a compensation for this, the division of corneal endothelial cells should be extra activated, replacing the lost prior cells. These new corneal endothelial cells secrete different ECM, raising the outflow.(100, 101) An

alternative IOP-lowering mechanism to ALT is SLT, a procedure with less or minimal scarring or coagulative damage of the TM. At the tissue-zone where the laser beam arrives, SLT handles the principle of selective photothermolysis which means it permits only to affect the pigmented cells of the TM. Pigmented TM cells distinguish themselves from the non-pigmented cells by the presence of intracellular chromophores such as melatonin. This is one of the base principles used for SLT.(102)

1.5.2.2 Laser peripheral iridotomy

Neodymium: Yttrium-Aluminum-Garnet Laser peripheral iridotomy(Nd: YAG LPI) is designated as most common used laser surgery for the treatment of acute PACG. It can be given in monotherapy or in combination with other types of Laser peripheral iridotomy (LPI) including argon laser iridotomy as pretreatment. When a patient signs up with pupillary block (see section 1.3.2), a YAG LPI can be chosen to relieve the acute closure attack and to decrease the elevated IOP. Otherwise non-pupillary block mechanisms such as lens-induced glaucoma are contraindicated for this therapy. With Nd: YAG LPI a new outflow space of approximately 150-200 micrometer is shot at the iris. This should significantly increase the angle width.(103) The place where the laser beam will fall in, depends on the thickness of the iris, the existence of a crypt and the choice of the ophthalmologist. Normally two to four shots are sufficient. Although this method is a relative safe procedure, the potential complications are corneal decompensation, endothelial damage and visual restrictions. (104)

In laser iridoplasty or gonioplasty, a laser beam burns into the peripheral iris which causes contraction of the iris pigmented cells and pulls the iris stroma away from the angle structures and deepens the angle recess. This should open up the closed angle. Argon laser peripheral iridoplasty, a type of gonioplasty is used for PACG caused by a nonpapillary block mechanism, since normal LPI is contraindicated or when a closed angle after LPI treatment reoccurs. If the normal eye medication cannot remedy an acute attack of closed angle glaucoma iridoplasty can be an option.(105)

1.5.2.3 Cyclophotocoagulation

Cyclophotocoagulation is a surgery often employed in GON patients when filtration surgery is contraindicated or when damage is already done. A laser beam goes through the sclera, gets absorbed by the pigmented ciliary epithelium and affects the ciliary body by heating. Since this tissue is responsible for the aqueous production, this action will indirectly cause a lowering IOP-effect. Partial coagulation necrosis of the ciliary epithelium can be obtained by an heating ablation procedure. Cyclophotocoagulation includes three types: laser cyclophotocoagulation (LCPC), trans-scleral cyclophotocoagulation (TSCPC) and endoscopic cyclophotocoagulation (EPC). More recently, those last two types should create less external damage to the surrounding tissues. In a TSCPC surgery, the laser light falls in with intense eruption and rest intervals. This causes an effect with minimalized surrounding tissue absorption. In addition to the reduced humor production, TSCPC should increase the uveoscleral outflow by augmenting the permeability of the ciliary body and sclera for the humor. EPC permits direct entering of the laser energy in the ciliary epithelium resulting in lower humor production while a normal cellular structure is still maintained. (106) Together with the other surgeries it has similar disadvantages: failure to lower eye pressure, too low eye pressure, swelling of the eye, pain, sensitivity to light, bleeding in the eye, decreased or permanent loss of vision. Although, most of them are very rare.(107)

1.5.3 Ultrasound Cyclodestruction

With the aim of resulting in partially destroying the ciliary epithelium, the heating procedure can be achieved by laser energy (cyclophotocoagulation) or a novel method based on ultrasound, named ultrasound ciliary-plasty. Creating high-frequency sound waves or high-intensity focused ultrasound (HIFU), the epithelial cells of the ciliary body can be impaired. Ultrasound based transscleral cyclodestruction has advantages compared to laser energy. Because the sclera scatters the laser light, an excessive amount of laser energy is required to have a clinical effect and ablate a portion of the ciliary body. Employing such quantity of laser energy increases the risk of injuring the collateral tissues including the sclera, the lens and the retina. During and after the procedure, the patient may observe some discomfort and may develop an inflammation one month later. Besides, in some cases, no efficiency is observed with this type of therapy.(106)

2 OBJECTIVES

As the second leading cause of blindness, glaucoma requires the most optimal and efficient treatment. An elevated IOP is the major risk factor to develop the disease. With the aim of decreasing this IOP, commercially available medications are usually applied topically. Despite an approved significant clinical effect, this type of delivery contain various limitations. Poor patient compliance, biological barriers, systemic absorption, relative fast metabolizing, long-term side effects and a low bioavailability are the reasons to search for some optimized or other delivery routes. Via an invasive delivery such as ocular injection, performed by an ophthalmologist, the drug could be delivered more precisely into the target area whereby most of the limitations could be bypassed. Nevertheless, this type of medication might be expensive, increases the risk of eye damaging, pain, blurred vision and sensitivity to light. For these reasons and for the compliance of the patient, extensive research is currently performed to reduce the number of those injections or topical administrations. The interest in delivering routes of IOP-lowering medication with a longer-lasting effect, sustained-and localized release devices increased in no time. Besides an increased IOP, some causative genes or risk alleles can also be responsible of developing GON. Since the commercially available agents or non-pharmacological treatments do not have an influence on these genes, multiple significant gene therapies with a gene silencing or stimulating effect were designed.(referring to chapter 1.4.2 novel anti-GON agents) However the question which can be arise is: *'how can these oligonucleotide-based agents or CRISPR-CAS-9 elements be delivered in a safe and efficient way to the target tissues?'*

A first attempt was topical administration of the nucleic acids. But just like the commercially available drugs, they have practically the same limitations and inconveniences. Also bad patient adherence and the non-patient-related problems such as biological barriers, ectopic absorption, rapid clearance, nuclease degradation, a low bioavailability and no acting site at posterior segment were the motives to find some alternative methods and delivery routes. Surface installation can still be interesting to reach the corneal epithelium, but due to their size and charge, nucleic acids, such as DNA are unable to pass the corneal barrier.

Another trial was trying to inject 'naked' nucleic acids. Depending on the type of injection (figure 4.1), a direct injection into target tissue can experience less of the topical limitations. In common with the commercially available injected medication, they face the same inconveniences. Moreover they still have to cross the barriers of ECM or have the risk of originating inflammatory or immune responses. More specifically, intracameral injections encounter practically the same inconveniences as topical installation while the use of a sub-conjunctival injection is constrained by the blood-retina barrier. As more deeper systemic installations, the intravitreal and subretinal injections are reachable for the posterior part of the eye including optic nerve, lens and retina. Nonetheless, direct administration via posterior injection of naked oligonucleotides (e.g. siRNA) is limited by the short half-life (within a few days) of the siRNA in the vitreous or ECM due to an enzymatic nuclease degradation. Moreover the single applied oligonucleotides carry a negative charge and may experience electrostatic repulsion with the also negatively charged cell membrane of the target cell, which impedes cellular binding and uptake. It goes without saying, that after multiple injections, damage to the eye is often inevitable.

In this master dissertation we performed a literature survey to report the current-state-of-the-art on novel and more ideal gene delivery routes for the treatment of glaucoma.

3 NANOMEDICINES FOR OCULAR DELIVERY

In the introduction of the commercially available anti-GON medication and objectives, we already mentioned the limitations of applying the first-line topical eye medications. These were the reason to discover some improved or other delivery routes. A first manner was enhancing the viscosity of the eye drop with e.g. cellulose, thermo-reversible poloxamer gels, chitosan or hyaluronic acid with the intention of increasing the residence time at the cornea. However this was not very efficient. A second method was using penetration enhancers, prodrugs or colloidal systems such as nanoparticles. For example benzalkonium chloride is a penetration enhancer which can destabilize the corneal junction and in this turn increasing the corneal permeability. Also with multiple types of injection, this kind of medication could be entered straight into target area, but due to several other drawbacks including off-target effects and rapid clearance, this delivery method was not successful either.

One of the new targeted delivering system is obtained by nanotechnology-based antiglaucoma therapies or drug-loaded nanoparticles. They include nano-emulsion, liposomes, dendrimers, nanospheres, hydrogels, nanocrystals, cyclodextrins, nanodiamonds, microspheres, niosomes, nanofibers and nanocapsules. Various traditionally used drugs like PGA, β-blockers, carbonic anhydrase inhibitors, cholinomimetics and sympathomimetics have been investigated in nanomedicine formulations but aren't clinically used yet. They can be applied topically or via injection, for example a sub-conjunctival or intravitreal injection of nanomedicines can be obtained. Using the example of latanoprost-liposome we quoted the several advantages and drawbacks of drugs encapsulated nanoparticles, which are also summarized in table 2.1. Since delivering of the commercially available IOP lowering agents via nanocarriers is not the main goal of this master dissertation, we decided to not explain each type of nanoparticle in detail. For this we would like to refer to other detailed reviews in the literature.

As the hydrolyzed active form of the lipophilic latanoprost, latanoprost acid has a more hydrophilic character and exhibits more penetration resistance through the cornea which may result in a lower bioavailability. Liposomes are nano-sized vesicles existing of a phospholipid bilayer forming a hydrophobic outer membrane and an inner aqueous core or hydrophilic area. The surrounding membrane can be composed of a single or multiple layers of lipid bilayers, named unilamellar or multilamellar vesicles respectively. Liposomes have the ability to deliver hydrophilic or lipophilic drugs. They can encapsulate hydrophilic or amphiphilic drugs in the aqueous core or stock lipophilic drugs in the outer lipid bilayer. Nevertheless, the loading capacity in the bilayer is limited to maintain the spherical shape of the nanoparticle. Delivering those drugs like latanoprost and its active component in this manner improves the drug solubility and stability. The liposome protects its content of enzymatic hydrolysis in the biochemical environment. Another advantage of nanosized particles is that they can easily cross the anatomical eye structures straight to the target issue when they are applied topically.

Nanocarriers participate in a sustained release effect due to partially controlled release rather than diffusion controlled release, since the loaded drug prefers to reside in the bilayer of the particle rather than in the surrounding liquid. When you inject them in a space of limited volume of liquid a controlled release over longer periods of time is caused. In addition the drug should have polar- and pi-pi interactions with the particle which stabilizes the drug into the bilayer and helps in the controlled long-term release. (108, 109)

Besides targeting the often employed drugs, nanotechnology can also be used in the biochemical part of non-viral vectors in ocular gene delivery. As explained in the objectives (chapter 2), direct delivery of naked genes to treat GON has several constraints as well. As physical barriers, corneal epithelium and conjunctival cells show a low permeability, the tear film can wash off the nucleic acid delivery carrier and the blood-eye barriers including blood-aqueous and blood-retina barriers complicate the delivery. Furthermore siRNA for example, is sensitive to serum proteins interactions, uptake by phagocytes, possible endosomal or nuclease degradation and cellular internalization. With their own characteristics, size, biocompatibility and biodegradability nanocarriers can load a relatively high amount of RNAi therapeutics and avoid enzymatic degradation and fast elimination. Just like the frequently used IOP-lowering drugs, oligonucleotides can form a complex with the nanocarrier which controls sustained release. Later, the specific types of non-viral vector and their mode of action will be discussed more in detail. (more information chapter 4.1.2 Non-viral vectors)

Table 2.1: Summary of the common properties of nanocarriers encapsulating often used IOP-lowering drugs or siRNA (108-110)

Advantages	Disadvantages
- Elevating drug stability by reducing enzymatic hydrolysis - Drug delivery through anatomic eye structures runs more efficiently due to nanoparticle's size - Sustained release over various weeks to months by formation - Improved solubility of the drugs - Possibility of extra coating the nanocarriers which can enhance the drug solubility, prevent opsonization and attacking of macrophages - Less tissue irritation due to selectively targeted drug delivery - Engineered nanoparticle helps to find the drug's target - Less elimination by conventional, uveoscleral and uveolymphatic pathways - Smaller nanoparticles have enhanced drug loading capacity than larger ones due to a higher surface area - Improving wound healing after filtration surgery - Reduce toxicity and less side effects - Practically no discomfort after the injections - Lower immunogenicity - or higher loading capacity of the drug or siRNA - Protecting of enzymatic degradation and fast removal or elimination.	- Nanoparticle stability is dependent on: size, bilayer fluidity and surface charge and hydration - Limited amount of drug particles can be stored in the nanoparticle - After injection, a bleb can be formed. But this disappears after 24 hours - Low endosomal escape of the nanoparticle's core - Fusion between nanoparticle and lysosome leading to nanoparticle degredation

4 OCULAR GENE-DELIVERY ROUTES

In 1.4.2.6 oligonucleotide-based agents, we already mentioned the mode of action and advantages of using nucleic acids to modulate the expression of the causative or risk alleles of GON. The nucleic acids could be applied topically or via multiple types of injection.(figure 4.1). Especially intravitreal or subretinal injections were attractive routes, because the posterior segment of the eye is a reachable area. But due to numerous limitations of 'naked' nucleic acids injections or topical applications, an introduction of a novel and more ideal gene delivery route was requested. In that sense, an ideal gene delivery vector is one that shows practically no systemic absorption, do not impair eye tissues, is able to deliver successfully an intact gene into the target eye-tissue and protects its core of nuclease degradation. In this master dissertation, we will divide these newer and more ideal delivery routes into the biochemical and physical methods whereby each of them is subdivided and will be explained in detail with the current knowledge.

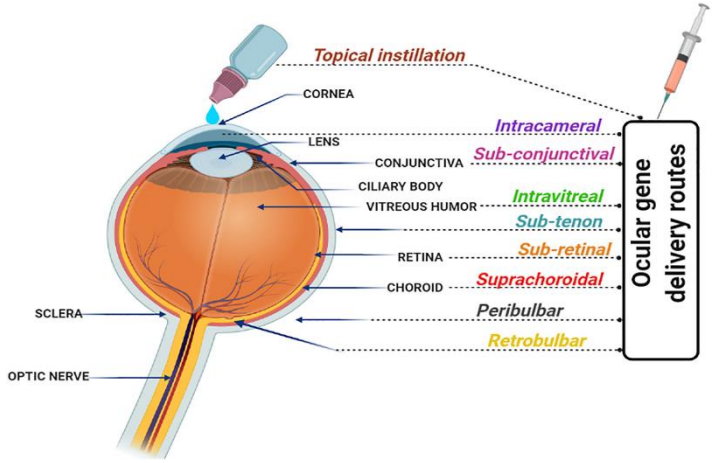

Figure 4.1: Ocular gene delivery routes including topical application and the several types of injections.(79)

4.1 BIOCHEMICAL METHODS

4.1.1 Viral vectors

Viruses are small, infectious, intracellular entities with a relatively simple structure. In comparison with other microorganisms, they do not possess their own metabolism and ability to produce proteins or enzymes which are essential for self-dividing. Viruses enter and infect host cells to fulfill their replication. Containing their own specific genetic material makes them independent of the host cell genome. Extracellular viruses, also named virions consists of viral DNA or RNA enclosed by a capsid. This is a protein shell that protects the genetic material from the enzymatic degradation, helps interacting with the host cell and may play a delivering role of the genome. Among the viruses, a classification system is made by their viral genome. A virus contains mostly linear, single or double stranded genetic material which can be DNA or RNA.

The cycle of viral replication exists of five common phases. Firstly the virion will attach with its capsid to the receptors on the surface of the host cell. This ensures that the virus binds only to its target cells with the corresponding receptors of the cells which have the capacity to replicate the virus. As a second or viral entry phase, the nucleic acids of the virion penetrates in the host cell. Subsequently the host cell will be forced to start the synthesis of newly viral nucleic acids and proteins. After the production of the viral components, the virus maturation phase initiates. The capsid is assembled and the viral genome is packaged. Finally the new formed mature virions can

be released from the host cells and are ready to affect other cells. In most cases, when a virus infect a host cell, this leads to the death of this cell or a part of the viral genome can undergo uptake by the host cell DNA which will be passed on to the daughter cells.(111, 112)

Viral vectors are genetically engineered viruses that transfer genetic material into host cells. They encode oligonucleotides and efficiently transport their core into the human cells. Making it possible for clinical use, multiple viral elements of the viral vectors were adjusted while the gene delivery efficiency and expression should remain unchanged. In this way, unneeded pathogenic features or the ability to damage the host cell were removed. Pathogenic viruses will only be recruited as vector after reversing their pathogenicity. In addition, a part of the viral genome critical for replication is deleted, preventing replication of the vector. Also their tropism is changed by genetic modifications of the viral capsid, responsible for targeting specific commissioned cells. Via their capsid, these vectors are used to target a wide range of cells or are engineered to recognize only specific target cells. However some viruses ,which should be avoided as vectors, have the ability to rearrange their genome quickly. This can cause genome-instability and complicates targeting specific cells. Moreover, there are some safety concerns and drawbacks for viral vectors reported which impede the development of clinical applications. Engineered viruses have a risk of mutagenesis, a potential immunogenicity, limited loading capacity and relatively high production cost.

Thanks to a high in vivo transfection efficiency and target cell selectivity, research in ocular gene therapy provides a variety of vector options. The most commonly employed are adenoviral vectors, adeno-associated viral vectors, lentiviral vectors, retroviral vectors and herpes simplex viral vectors.(79, 113) Besides transferring nucleic acids, some of these vectors can also be eligible for the delivery of CRISPR components. However these may have a bigger size which will require a large delivering capacity of the vectors. (92)

4.1.1.1 Adenoviral vectors

Originally, human adenoviruses were known to be responsible for some respiratory and ocular infections. Adenoviral virions are medium sized (90-100nm), existing of double stranded linear DNA and viral proteins surrounded by an icosahedral capsid. This capsid consists of protruding fibers which provide the interactions between the virion and the host cellular receptors. Once attached, the virion is taken up via endocytosis. After internalization, the virus and its DNA escape the endosome, are transported to the nucleus and will start viral replication. However, using this virus as a gene delivery vector, the viral DNA replication will be blocked by deleting some genes. They are an attractive option of gene delivery because their genome is easily manipulated and they can transfect both the dividing and non-dividing cells. Adenoviruses have the ability to transduce cells of the humor outflow pathway cells such as the TM cells. Other advantages are preventing the possibility of mutagenesis and a large loading capacity of foreign DNA. As a potential danger, the virus can trigger an immune response rapidly.

The adenoviral vector is a plasmid vector containing a portion of the viral genome including a deletion of the viral replication genes and secondly a multiple cloning site, a region where the cloned and desired genes can be placed. Over the years, two generations of the vector and a helper dependent adenoviral vector were developed. In the second generation, other and additional replication genes were removed to enlarge the carrying capacity. In addition it has an impaired safety and increased transgene expression for a longer duration but can still cause a severe

immune system reaction.(114) Theoretically, the helper dependent or gutted adenoviral vector can be seen as a third generation. They are called helper dependent because they are produced by another helper virus. In this type of vector, practically all viral-coding sequences are removed which makes it a high-capacity adenoviral vector, less immunogen or toxic and long-term transgene expression. In this way, the gutted vector should have an incorporation capacity of 36 thousand foreign base pairs.(115) In 2010, a first adenoviral vector was designed carrying the MMP-1-gene. In combination with the IOP-elevating drugs, dexamethasone, the vector overproduced MMP-1. This is a protein which benefits the outflow by modulating the ECM. The vector could constitute a treatment for steroid-induced POAG or ocular hypertension.(116)

Since the administration of siRNA depends on an efficient delivery route into the target cells, the adenoviral vector can also encode the sequence of siRNA.(117) As benefits, the recombinant adenoviral short RNA vectors ensure a regulated expression of siRNA, have a wide spectrum of cell infectivity and are not reliant of active cell division.(114) Thanks to a large gene delivery, adenoviral vectors also have the ability to carry the sequence of CRISPR components. The intention of transducing CRISPR-CAS-9 with an adenoviral vector was to silence the mutated MYOC-gene in POAG patients. With a transducing efficacy of 60-70% in the TM cells, a significant IOP-lowering effect was measured and slight inflammation in the anterior chamber was reported. (92)

4.1.1.2 Adeno-associated viral vectors

Adeno-associated virus (AAV) is a contaminant of adenovirus preparations. It is a small, naked, helper-dependent parvovirus existing of single stranded DNA encapsulated by an icosahedral capsid. Since the recombinant AAV-vector is long-lived, non-toxic and non-pathogenic with low immunogenicity, it can be seen as the most efficient and leading choice for ocular viral vector gene delivery. Moreover other advantages are that in some rare cases AAV may incorporate its genome into that of the host cell and secondly it can also infect dividing and non-dividing cells, just like the adenovirus did. In the nucleus of the host cell, AAV forms a episomal concatamer. This is a continuous DNA molecule which exists of various copies of the same DNA sequences coupled in series. In postmitotic cells, this concatamer stay intact, while in dividing cells the viral DNA gets lost through replication. At the production of the recombinant vector, genes encoding for some structural capsid and replication proteins are deleted. As a replacement for them, the AAV-vector can be carried with specific genes of interest. However a known drawback is their limited carrying capacity of maximum five thousand foreign base pairs.(112, 118)

Currently multiple serotypes of the AAV-vectors have been developed. Each of them should have a different tropism and host cell binding. The numerous vector types can be divided in subgroups which all transduce other target tissues via different delivery routes. For example a first subgroup reaches the superficial cells of the cornea after topical administration, while another subgroup affects TM cells via intracameral or intravitreal injection. Recently, a research group developed an AAV-vector which contains the genes of the exoenzyme C3 transferase and transduces the TM cells after intracameral injection. The C3 exoenzyme transferase is a toxin isolated from Clostridium botulinum and is commonly used as inhibitor of Rho kinase. With a similar mode of action as the ROCK-inhibitors (see section 1.4.2.2), the C3 exoenzyme can induce morphological changes in the human TM cells by disrupting the actin cytoskeleton. This caused an significantly enhance of the outflow facility and a decrease of the IOP in mice and monkeys.(119)

In vivo, it was discovered that AAV-mediated CRISPR/Cas9 gene therapy can also be an option to protect RGC's and optic nerves. These results demonstrate that AAV-vectors are a promising gene vector as a neuroprotective treatment for GON.(92, 118)

4.1.1.3 Retroviral vectors

Retroviruses are RNA viruses containing double stranded RNA surrounded by an icosahedral capsid. They can be divided into two classes: the simple and complex retroviruses. Simple refers to the virus which enters a host cell via endocytosis after attachment to the host cell receptor. On the other hand, complex retroviruses such as the lentiviruses or human immunodeficiency virus (HIV) will penetrate the host cell also via direct fusion or endocytosis but firstly it requires a co-receptor stimulating signal beside the usual main entry receptor. After uptake, the retrovirus transduces its dsRNA genome into the cytosol of the host cell where the enzyme reversed transcriptase transforms it to dsDNA. Subsequently the simple retrovirus can integrate the nucleus of only dividing cells while the complex viruses will infect both dividing and resting cells. Once the nucleus penetrated a second enzyme, integrase, incorporates the foreign DNA into the host genome which turns the inserted viral DNA into a 'provirus'. Since the viral DNA is now a part of the host genome, it can replicate together with the host cell and the viral genome will pass vertically to the daughter cells. The integrated provirus creates new dsRNA which is stored into a core particle. (114)

Retroviral vectors originated from simple retroviruses have some advantages and limitations. They can integrate the host cell very efficiently, have the ability to carry bulky DNA inserts and contain no viral genes which ensures a long term and stable genetic expression. Nevertheless they only enter the host cell nucleus at the moment the cell is in its dividing phase which explains the absence of its transducing capacity in resting cells. Although the most genes with sequences compatible to retroviral life cycle can be plugged into the vector, its carrying size is confined. The clinical use of the retroviral vector is limited due to a reported occurrence of T-cell leukemia in immuno-deficient patients generated by insertional mutagenesis.(79, 120)

4.1.1.4 Lentiviral vectors

Lentiviruses are complex single stranded retroviruses of which transducing capacity is independent of the living status of the host cell. Most of their features are relatively similar to those of the simple retroviruses. In vivo, lentiviruses excite low or no immunological complications and have the ability to integrate their insert into the host genome. The latter feature provides a persistent gene expression in already transduced host cells. Due to the fact that the insertion location into the host genome cannot be strictly predicted, lentiviruses may enhance the risk of insertional mutagenesis. Just as with the simple retroviruses, this impedes their clinical use as gene delivery vectors. For that reason researchers developed non-integrational lentiviral vectors which do not adjust the host genome. Biosafety caused by the pathogenicity of the virus was another concern, since the first vector generations resulted from primate lentiviruses including simian immunodeficiency virus and HIV. Newer generations were originated rather from non-primate lentivirus presented in for example cats, bovine animals or horses. Although the AAV-vectors transduce more efficiently, a long term gene expression and a high packaging capacity makes this vector beneficial in GON gene therapy. As target tissues lentiviral vectors transfect anteriorly TM or corneal epithelial cells but can also affect RPE, RGC and Müller cells posteriorly. (92, 112)

In glaucoma investigations, via intravitreal injection this vector has already been used to deliver genes with neuroprotective features. A significantly increased RGC's survival and axon extension has been determined. Furthermore since 2010, some lentiviral vectors have been designed to express cyclo-oxygenase-2, a rate-limiting enzyme in prostaglandin biosynthesis, and other components of the biosynthesis such as the $PGF_{2\alpha}$-receptor. For this application the tissues targeted were TM cells, ciliary epithelium and other tissues responsible for the production or outflow regulation of the aqueous humor.(118, 121) The working mechanism behind prostaglandins and analogues has already been discussed in more detail in section 1.4.1.4.

The same research group which recently developed an AAV-vector carrying the genes of exoenzyme C3 transferase, also investigated the use of the lentiviral vector encoding this C3 exoenzyme. With the aim of targeting the human TM cells and changing its morphological structure, a significant decrease of the IOP was also reported.(122) As the consequence of its sufficiently large packaging capacity, a single lentiviral vector can accommodate all the genes expressing the elements of CRISPR-CAS-9 either.

4.1.1.5 Herpes simplex viral vectors

As a large dsDNA virus, the virion of a Herpes simplex virus (HSV) is composed of linear dsDNA environed by an icosahedral capsid. In vitro, the virus infects more than 70% of the cells and can transfect non-dividing cells as well. Its original genome exists mostly of non-essential genes for growth in tissues and genes leading to viral toxicity and virulence. For the use as gene delivery vector, this portion of the viral genome is logically removed. This opens up a lot of packaging space for foreign DNA inserts. The helper-independent HSV-vector transduces a broad host cell range including mainly neurons, especially those of RGC's and several other tissues like the ciliary epithelia or TM of rat and monkey eyes. Another variant of the HSV-vector is the replication-defective or helper-dependent amplicon vector. It also has a large cargo packaging capacity, a wide range of cell tropism and can transduce both dividing and resting cells. After subretinal injection the amplicon vectors can transfect RPE cells efficiently but temporarily. However, both HSV and amplicon vector have the constrains of cytotoxicity, restricted duration of gene expression and a severe inflammatory response.(114, 123)

4.1.2 Non-viral carriers

In viral vectors, viruses lost their pathogenicity and replication capacity by deleting some typical DNA/RNA sequences. However in some cases, the virus can in vivo reform itself to its original form and regain for example its pathogenicity. Together with the other limitations of viral vectors, this paved the way to investigate in non-viral vectors. Due to a lower production cost, the ability of high-titer productions and most importantly a better safety profile including a minimalized immune response and toxicity, the non-viral vectors are promoted as an attractive alternative in delivering synthetic oligo-nucleotide agents such as plasmid DNA (pDNA), siRNA, miRNA and even the ASOs. In contrast to the viral vectors, the non-viral carriers are not a limited in carrying capacity. However for this gene delivery method, the most notable drawbacks are less efficiency and a rarely achieved transgene expression at therapeutic levels. The biochemical part of the non-viral vectors is powered by the use of nanocarriers including lipid-based passed particles, polymer-based vectors, dendrimers and cell-penetrating peptides.

4.1.2.1 Lipid-based vectors

Lipid-based particles or liposomes are potent delivery carriers for nucleic acids to accomplish a gene stimulating or suppression effect efficiently. These are commonly used in ocular gene therapy and can for example be injected intravitreally to silence genes in specific retinal layers or other ocular mammalian cell lines. Basically liposomes consist of a phospholipid bilayer forming a hydrophobic outer membrane and an inner hydrophilic core. In general, their bilayer is composed of cationic lipids which are amphiphilic molecules with a positively charged hydrophilic head and a hydrophobic tail, held together by a linker molecule. Cytotoxicity of the liposome will depend on the biodegradability of this linker molecule. The positive charge of the lipid is due to the presence of for example primary, secondary, tertiary amines or quaternary amine salts as hydrophilic head. Functionally the positive charge ensures a complex formation among the cationic lipids and the negatively charged phosphate group of core-encapsulated nucleic acids. This leads to a controlled release and stabilized effect of the liposome and its content. Structural characteristics of the lipid and the charge ratio play an essential role in the development of lipid-DNA electrostatic interactions or lipoplexes. In other words, lipoplexes are the fusion of cationic liposomes and negatively charged nucleic acids.(124) Determined by the number of positively charged lipids and negatively charged phosphate groups, liposomes can be defined as anionic, cationic or neutral. It should be assumed that negative and neutral liposomes are rapidly eliminated from the eye within a few hours. Moreover the ratio of slightly more positive charges enhances the escape of the oligo-nucleotide content and helps cellular uptake by facilitating the fusion among the liposome and endosomal bilayers.(125-127) In addition cationic lipids have detergent and buffering properties which ease the DNA/RNA release. Sometimes in lipid-based vectors, a neutral lipid or helper lipid such as cholesterol or a zwitterionic lipid is placed between the cationic lipids to improve the particle stability and transfection efficiency. (113)

However in classic lipoplexes, the nucleic acids are encapsulated in multiple cationic lipid layers. They can be internalized in the target cell via a specific main mechanism or other routes including micropinocytosis and clarthrin-mediated endocytosis. Which internalization pathway the lipoplex will follow depends on the dose and the charge ratio of the lipids. The main lipoplex-entering pathway makes use of endosomes. Firstly the lipid-DNA complex enters the endosome and destabilizes the endosomal membrane together with the possibly presented helper lipids. A destabilized membrane enables a spontaneous uptake of anionic cytoplasm lipids into the endosome. This neutralizes the cationic lipids of lipoplex via ion pairs formation which should cause a weakened lipid/DNA interaction and in this way indirectly induce a more facilitated DNA release out of the lipoplex. Note that only when all cationic lipids are neutralized, the DNA will start its escape of the complex. (124, 128)

Intravitreal injection was an attractive approach for lipoplex delivery to the inner retina. Nevertheless its innermost layer, the ILM and the vitreous appeared to be some critical barriers to cross. Sometimes an extra electrostatic coating of for example hyaluronic acid (later more information) was brought around the lipoplex to improve their intravitreal mobility and protect it against extracellular interactions. Moreover the coating would not influence internalization efficiency. At our faculty of Ghent University, a study in bovine eyes concluded that a hyaluronic acid-coating of lipoplexes had the ability of crossing the vitreous. Unfortunately, a significant concentration couldn't penetrate into the inner retina. Overcoming the predominant ILM barrier with this modification was not achieved and an accumulation at ILM was determined. Further research

to reach the inner retina successfully in other animals or even humans is stimulated by this study.(129)

Currently the largest part of lipid nanocarriers are biodegradable, non-toxic and non-immunogenic delivery systems. However, their instability in biological fluids is still a problem. The cationic lipids can interact with the serum and form aggregation plaques which lead to a release of the DNA and a faster degradation by nuclease enzymes. This unwanted aggregation clarifies among others the lower transducing efficiency than the viral carriers.(128)

As an approval of a successful delivery method using liposomes, an in-vitro study developed a modified lipid-based particle carrying a higher concentration of VEGF-siRNA cargo. It connects selectively with integrins presented at the surface of RPE cells and is internalized mostly via endocytosis without damaging the cell. By targeting the VEGF-genes in RPE cells, the nanocarriers could be a potential option to treat retinopathy. This causes in some cases neovascular glaucoma, an example of secondary GON which is not explained in this manuscript.(130)

4.1.2.2 Polymer-based vectors

Another type of gene delivering nanoparticles are polymer-based vectors which can encapsulate RNAi therapeutics or pDNA for the purpose of knocking down or stimulating some typical genes. With other benefits and limitations than the lipid-based vectors, these carriers were explored in recent years. Polymers consist of iterated long-chained molecules or monomers. They can be homopolymers or heteropolymers. In the first case, the monomers are all identical in contrast to the heteropolymers. Both (i)natural polymers including peptides, polysaccharides and proteins and (ii)synthetic polymers like dendrimers, polyethylene amine and phospho-esters can function as an important substitute of non-viral gene delivery to GON affected target tissues. In addition, as a successful gene carrier, polymer-based vectors provide a protection against repulsion between the negatively charged phosphate nucleic acids and anionic surface of the target cell. Here too, the negative phosphate group of DNA can interact with cationic polymers forming a nanosized complex familiar as polyplexes. The binding affinity in a polyplex is related to some intrinsic characteristics. Firstly the number of protonated functional groups at physiological pH (mostly amines, lysine or arginine groups) as a building unit of the polymer making it cationic. Other intrinsic properties are the distance among the charged molecules within the polyplex, a branching degree of the polymer and its hydrophobicity. An overly strong internal binding can cause a decreased transfection efficiency by delayed escape of the DNA into the cytoplasm. On the one hand polyplexes make enzymatic intracellular nuclease degradation of the DNA less evident and on the other hand they facilitate target cell linking, incorporation of the genetic core, endosomal release and internalization into the nucleus of the target cell. Using three different mechanisms, polyplexes can enter the cell. These are based on pinocytosis, phagocytosis and mainly endocytosis.

In treatment of GON various types of polymers were examined, whereby most of them are cationic. The most relevant include polylactic-co-glycolic acid (PLGA), polyethyleneimine (PEI), poly-L-lysine (PLL), polylactide, polyamidoamine (PAMAM) dendrimers and the polysaccharides such as chitosan and hyaluronic acid (HA). Each of them has its own benefits and limitations which are shown in table 3.1. Some of their drawbacks can be circumvented by specific modifications of the nanoparticle.

In contrast to cationic lipids, positively charged polymers form smaller complexes with DNA which increases the transfection efficiency. Furthermore they are soluble in aqueous solvents due to the absence of a hydrophobic tail in comparison with the lipids. In recent years, lipolyplexes were introduced as a novel generation of gene delivery vectors. Therefore the advantageous properties of both lipids and polymers were combined to temper the limitations of their gene delivery vectors. An example of a lipolyplex is the merge of the saturated lipid, stearic acid and the backbone of PEI as siRNA delivery system. This led in vivo to a higher transfection capacity compared to an unmodified PEI.(113)

Table 3.1: Intrinsic properties including the advantages and constraints of the most relevant polymer-based vectors delivering genes, explored for the treatment of GON.(79, 127, 128, 131)

Types of polymer-based vectors	Advantages	Constraints
polylactic-co-glycolic acid (PLGA): is a copolymer of lactic acids and glycolic acids linked by ester bonds. (132)	- non-toxicity and good safety profile - small particle size - sustained core release - siRNA can be loaded in the core or absorbed on the particle surface - protected siRNA sensitive RNase activity - good biocompatibility	- slightly electrostatic interactions among nucleic acids and the particle - poor endosomal release
polyethyleneimine (PEI): is a linear or branched polymer. It is the most used synthetic polymer carrier in gene delivery. PEI also contains several protonated amines at physiological pH.	- very efficient in pDNA delivery - possible toxicity can be undone by using linear or branched PEI - modified PEI protects its core of siRNA sensitive RNase or other nuclease enzymes - proton sponge effect [a] - ability of incorporation in another polymeric vector causing more stability in serum and a good biocompatibility	- low electrostatic interactions - possible cytotoxicity
Poly-L-lysine(PLL): is a cationic polymer due to the positive charge of the basic amino acid lysine.	- strong electrostatic interactions - easily to induce modifications - small particle size - biodegradable - proton sponge effect [a]	- low stability due to lysosome degradation - poor endosomal release - limited transfection efficiency - cytotoxicity - sufficiently high molecular weight (MW) of the vector to form stable polyplexes
Polyamidoamine (PAMAM) dendrimers: are hyperbranched, mono-dispersive,	- controlled shape, nanoparticle size - electrostatic interactions forming soluble and stable dendrites - proton sponge effect [a]	- High production cost - Earlier generations had a low

spherical and cationic polymers.(133)	- production is possible on large scale and in a wide MW range - high transfection efficiency in RPE cells - strong gene condensation and protection ability	endocytosis capacity - Strong cytotoxicity - Inefficient drug loading - Not biodegradable
Chitosan: natural, cationic copolymer of D-glucosamine and N-acetyl-D-glucosamine (134)	- decreased cytotoxicity and immuno-genicity - good biocompatibility and low cost - frequently available in nature and easy to produce - polyplex formation is possible due to hydrogen bonding, hydrophobic interactions and electrostatic interactions - efficiently cellular uptake - modification possibility to improve their limitations	- insolubility in water at physiological pH - inadequate charge - low transfection efficiency
Hyaluronic acid (HA): is an anionic glycosamino-glycans, consisting of repeated units of glucuronic acid and glucosamines con-nected by glycoside bonds.	- good biocompatibility - induces no toxicity, immunogenicity or inflammation - naturally presented in the ECM of practically all human tissues including the vitreous - can act as an extra electrostatic coating	- fast degradation

[a] This is a facilitated endosomal escape due to presence of multiple protonable amino groups. They act as a buffering system and lead to a endosomal swelling and destruction.

4.1.2.3 Cell-penetrating peptides

Cell-penetrating peptides (CPP) are short peptides existing of 5 to 40 amino acids. Based on the presence of typical amino acids, they can be sub-classified into polycationic, hydrophobic and amphipathic peptides. Cationic CPP contain particularly lysine or arginine amino acids as most abundant element, while amphipathic peptides show rather an alternating pattern of polar and non-polar amino acids and the hydrophobic category only non-polar residues. CPP are interesting as a new alternative topically administrable or injectable delivery vector due to a low toxicity and a high transfection efficiency via facilitating cellular uptake of nanoparticles, nucleic acids or other chemical compounds through a plasma membrane. Covalent, non-covalent and electrostatic interactions connect the peptides with their delivery content including oligonucleotides and plasmid DNA. For all types of CPP, cellular uptake can be achieved in three following steps. The first step is the attachment of the CPP to the plasma membrane. This is driven by (i) electrostatic interactions, especially between cationic peptides and the negatively charged cell membrane, and (ii) hydrophobic interactions for both hydrophobic and amphipathic peptides. Subsequently the CPP gets internalized and finally releases its cargo. In the internalization step the CPP can follow two routes: a passive, energy-independent and an active, energy-dependent process. The passive uptake can be defined as a direct or spontaneous penetration of small cationic CPP through the lipid membrane stimulated by the plasma membrane potential. It happens without a membrane disruption. However when small CPP aggregate together to larger complexes, a temporary

translocation pore or a transient membrane disruption can occur. In the latter case, a good working membrane recovery is required to avoid cytotoxicity.

Besides the passive entering process, there is also an energy-dependent mechanism like endocytosis. According to the literature, this should be the main CPP translocation pathway. In contrast with direct penetration, the cargo should be first released out of the endosome before it reaches the cytosol. Endosomal escape may occur like in some polymer-based vector, namely via an proton sponge effect. Although both entering-processes act complementary, one route preferred above the other. Which internalization routes is preferred will depend on the target tissue and the aim of the medication carried by the CPP. When the cargo needs to be delivered in the nucleus or in one of the cell organelles, endocytosis is considered. Otherwise, a high CPP-concentration or accumulation at the cell membrane can facilitate the choice of direct penetration. (127, 135, 136)

4.2 PHYSICAL METHODS

In the translocation of oligonucleotides, other methodologies besides non-viral and viral vectors have been developed. Within the physical methods, various external forces are used to enhance transfection efficiency. In general, an enhanced permeability due to micropore formation or a transient disruption of the plasma membrane is introduced. This may lead to a passage of the plasmid through the cell membrane. Physical delivery circumvents some constraints of the other gene delivery approaches including limited carrying capacity of larger genes, lower production cost, better safety profile and non-immunogenicity. For the treatment of GON, electroporation, iontophoresis, sonoporation, magnetofection, gene gun and laser poration can be used as physical methods.(128, 137) However, only the most significant method for the treatment of GON are explained in more detail below.

4.2.1.1 Electroporation

In electroporation, electropermeabilization or electrotransfer, which are synonyms, a high intensity electric field and short electric pulses are applied, inducing a reversible membrane destabilization and a temporary formation of aqueous micropores within nanoseconds. This causes an enhanced permeability for the entrance of small quantities of material, drugs or genetic material like pDNA and oligonucleotide agents. The translocation of foreign molecules into the cell is mainly driven by diffusion but also local electrophoresis and electro-osmosis complete their part. As a preliminary step, the drugs or nucleic acids are injected (e.g. intravitreally) or topically delivered. Shortly after, the electrotransfer can start in vivo. Hereby two electrodes are deposited into the target tissues or the tissue is held with plate-type electrodes. The effect of electroporation has been approved in the retina, cornea, skin, lungs, liver, kidney, solid tumors, brain, spinal cord and skeletal muscles. Besides the numerous advantages, however, electroporation can cause tissue damage and may have a limited transfection efficiency.(128) A research group explored a gene therapy using electroporation to transfect MMP-3 gene after trabeculectomy. The study concluded that an MMP-3 expression was measured 30 days after transfection. Filtration surgery followed by MMP-3 transfection prolonged the survival of the formed filtering bleb and reduced the elevated IOP.(138) Since skeletal muscle cells can also be affected with gene transfer electroporation, the ciliary muscle can be targeted optimally as well. Via an intramuscular injection followed by specifically designed electrodes, a plasmid was transfected, causing reduced levels of TNF-α (more information chapter 1.3.1.2. normal pressure glaucoma) in the aqueous humor of rats.(139)

4.2.1.2 Iontophoresis

Another electric field using gene delivery method is iontophoresis. While electroporation relies on a relatively high intensity of electric field and very short pulses, iontophoresis is based on an application of direct low voltage and continuous current. Here, the electric field increases the plasma membrane or tissue permeability for small charged oligonucleotide compounds, ionized medication or pDNA. Improved intraocular delivery of topically applied or injected oligonucleotides is reached with trans-corneal, scleral or corneoscleral iontophoresis. Many studies confirmed that trans-scleral iontophoresis, as a non-invasive treatment, efficiently ameliorates gene transfection for ocular diseases. (137, 140)

4.2.1.3 Sonoporation

For more than a decade already, the interest in ultrasound in the ophthalmology is high. It made its entrance as a diagnostic imaging tool and recently as a newer laser therapy in the treatment of GON.(referring to chapter 1.5.2.3 cyclophotocoagulation) Ultrasound targeted microbubble destruction (UTMD) uses sonophoresis, also called ultrasound waves, to enhance drugs translocation through biological membranes. In UTMD, ultrasound is applied externally and microbubbles, which can be loaded with low molecular weight drugs, genes and proteins, are injected straight into the systemic circulation. For example the genetic material, is mostly connected via electrostatic interaction to the shell of the bubble or co-administered with the microbubble. As gas-filled spheres, microbubbles are cavitated due to high-amplitude oscillations, originating from the ultrasound waves. Because of the compressible nature of gas, microbubbles have the ability of resonating. They may expand volumetrically or contract in response to the applied pressure's rarefaction and compression of the ultrasound. However at certain point, the acoustic pressure reaches a threshold and triggers a violent collapse of the cavitation bubble. This causes some shock waves, heat or shear forces which temporarily disrupt the adjacent cell membrane or perturb vascular endothelial integrity transiently. The now generated pores on the surface of the cell membrane (also known as sonoporation) increase the cellular incorporation of the microbubble content or co-administered elements (e.g. oligonucleotides, nanoparticles). Applying a longer treatment or elevated acoustic pressure can enlarge the pore size and thus cellular uptake.(141) UTMD has numerous advantages such as a good safety profile, non-invasiveness, repetitive applicability and target-specific gene delivery. (140, 142, 143)

In 2011, a research group explored sonoporation to deliver genes into the ciliary muscle of rat eyes. They injected pDNA, a green fluorescent protein and microbubbles near to the muscle cells. Subsequently, they exposed this location with ultrasound waves to enhance the translocation of this injected elements. Via the fluorescent protein the uptake into the muscle cells could be confirmed. In comparison with the control groups, a significant increase of fluorescent signal and thus, a higher cellular uptake was measured. This study approved that sonoporation can be possible an effective physical delivery method to treat glaucoma.(144)

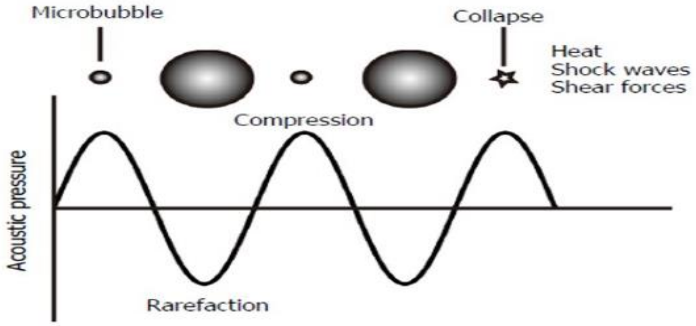

Figure 3.2: Mechanism of sonoporation. Due to fluctuations of the acoustic pressure, the microbubble can expand volumetrically or contract. After a while, a threshold is reached and the microbubble will collapse. This induces temporary tissue damaging by the formed heat, shock waves and shear forces.(141)

4.2.1.4 Magnetofection

In literature, the frequently used term magnetofection refers to magnetically guided and enhanced nucleic acid delivery. It relies on the encapsulation of oligonucleotides into magnetic nanocarriers and administering of magnetic forces. Via intravenous injection and the aid of an external appropriate magnetic field, the magnetic nanoparticle can concentrate and translocate the nucleic acids into some posterior tissues (e.g. deeper retinal layers). Obviously the magnetic capacity of the magnetic nanoparticle plays an important role. This must have a sufficient strength to let them escort by the external magnetic field to the target tissue. In addition the magnetic nanoparticle can carry for example a positive charge or contain hydrophobic structures and interact electrostatically or hydrophobically with nucleic acids alone or complexed in a viral or non-viral vector (e.g. lipoplex or polyplex). Once the gene-magnetic vector complex arrives at the target cell, it can transfect its nucleic acids into this cell in a comparable way to non-magnetic gene delivery vectors, more specifically by the uptake and release of an endosome.(145, 146) However, a more recent study claims another transfection pathway by enzymatic division, degeneration of the ECM or charge inter-action.(79) Although non-viral vectors have the characteristics of being safe and easily to produce in large amounts, their transfection efficiency is one of their major limitations. Magnetofection has the ability of enhancing this transfection significantly. (146)

4.2.1.5 Gene gun

Gene gun or also called DNA-coated particle bombardment or bio-ballistics or microprojectile is a high-voltage electric current device which delivers DNA-coated nanoparticles into the target tissue and eventually straight into target cell nucleus. The device launches the DNA-coated particle with a very high acceleration and velocity and causes a clear embedding through the target tissues. DNA can be recruited to coat some microparticles including gold, wolfram and silver particles to enhance transfection efficiency and to target cornea epithelium. More precisely this action can be accomplished without injuring the eye or cornea. Besides a DNA modification, the gold particle can also be functionalized by cationic polymer-based vectors (e.g. PEI) to transfect the cornea.(128) However studies from March this year questioned its effectiveness in cornea penetration. Hereby stromal tissue should limit microprojectile delivery into the inner layers of the cornea and penetration depth should be sensitive to particle size and density.(147) Nonetheless, the device avoids the use of dangerous chemicals and injection needles, its effectiveness is independent of specific receptor binding and a the loading capacity is less limited. This makes gene gun still an option as a potential treatment tool of GON in future.

4.2.1.6 Laser-induced photoporation

By offering more advantages over the other physical methods, laser-induced photoporation will be discussed as last intracellular delivery approach. This technique uses pulsed or continuous laser light to cause an enhanced membrane permeability and to facilitate nanoparticle or foreign DNA diffusion into the target cell. The term photoporation is dividable into two procedures: direct focused laser light photoporation and nanoparticle sensitized photoporation.

Basically in the first method, high-intensity femtosecond laser light with pulse duration is focused on a very specific and selected region of the membrane. This has the aim of attaining a local high photon density and creating some temporary pores. Pore formation in the method of direct focused laser light is possible by three theoretical mechanisms: photothermal, photomechanical and photochemical. Which mechanism contributes, will depend on laser pulse duration and the intrinsic factors of the laser light including intensity and wavelength. Photothermal pore formation refers to the use of heat, created by the laser beam. This ensures thermal denaturation of some proteins inside the cell membrane, causing an increased membrane permeability. On the other hand the principle of photomechanical pore formation is based on the formation of some cavitated nanobubbles by the laser beam. Comparable to sonoporation, the nanobubbles can resonate and expand volumetrically, until a threshold is reached. From that moment a collapse is induced which causes shock waves and forms temporary pores into the membrane. When the laser pulses are too low to induce nanobubble formation, the pore opening is up to a third photochemical mechanism. Here, pulsed ultraviolet-light can stimulate the development of ROS which damages the cell membrane. However this last process is not often used, since ultraviolet-light can be toxic.

In recent years, as a newer and more efficiency-enhanced process, a second method of photoporation has been developed. Hereby, they made use of light-sensitized nanocarriers. The procedure itself is dividable into three major steps. Firstly light-sensitive nanoparticles (e.g. plasmonic nanostructures like gold nanoparticles) are brought near the target host cell. They have the ability to attach to the cell membrane. Those which remain unattached can now be removed in a washing intermediate step. After injection of the foreign DNA or non-viral vectors, the target cells linked with gold particles are ready to be focused by the laser light. Its effect on the now irradiated cells will depend on the intensity of the incident laser light. In literature, most studies obtain lower intensity laser pulses or more continuous light to create a heating effect of the light-sensitive nanoparticles. These heated nanoparticles (10-100°C) induce the formation of temporary hydrophilic pores by thermal denaturation of integral glycoproteins inside the lipid bilayer. Advantageous to lower intensity laser light is its decreased cost price and high availability, but a duration within ten seconds to a few minutes before opening the pores can be assumed as a limitation.

Nevertheless, when shorter and higher laser intensity is employed, the even hotter nanoparticles (100-1000°C) can evaporate water of the surrounding environment and form vapor nanobubbles. Once a threshold is reached, these nanobubbles explode and cause high-pressure shock-waves which are responsible for opening some pores into the cell membrane. An interesting property of this process is the absence of disrupting the environment by heat due to an insulating effect of the vapor nanobubbles. This means only a slight heat transfer to the surrounding tissues is measured which ensures a better cell viability. In both cases, the now injected foreign DNA or nanoparticles should undergo a more facilitated diffusion into the cells.(148, 149) At the laboratory for General

Biochemistry and Physical Pharmacy at Ghent university, research is currently running which tries, among others, with this laser shooting method to overcome the ILM and reaching the inner retina with gene therapeutics.

5 DISCUSSION AND FUTURE PERSPECTIVES

In general we agree that the biochemical and physical methods are a more ideal gene delivery route in comparison with the topically applied or injected naked nucleic acids. However, multiple challenging limitations especially around the biochemical methods are open to discussion. One of the main shortcomings of gene therapy are toxicity concerns. As written above, the viral vectors are viruses of which the genome is modulated by deleting partially or totally their original viral genes with the aim of losing their pathogenicity and replication capacity. Regrettably, in some cases the virus can reform itself and regain its old features. For example in the first generation of adenoviral vectors, immune responses were certainly reported. After several attempts and progressing newer generations including the last generation helper-dependent adenovirus, the risk of immunogenicity may significantly be tempered. Nevertheless, viral vectors contain a high selective tropism, limited loading capacity, immunogenicity, long-term adequate transgenic expression and genotoxicity concerns will always be questioned. This lead to a minor clinical interest in the viral vectors and enhances the attractiveness of the non-viral vectors and the physical methods.

As drawbacks, non-viral vectors are less efficient, contain a lower cell specificity and also have the chance of causing cytotoxicity. This stimulated further investigation. A manner to address the lower target cell selectivity of the non-viral vectors is by embedding them for example with antibodies or ligands as targeting fragments. An investigation group made the use of CD44 receptors located at TM and Schlemm's canal cells. Previous research showed that the concentration of CD44 receptors may be 2 to 6 times higher in glaucomatous eyes compared to healthy ones. In addition, they combined this fact with the binding feature of HA to the CD44 receptor. By developing nanoparticles (e.g. PEI) which can be electrostatically coated by a CD44 ligand such as HA, a higher cell specificity was reached.(150) With these two examples, we concretized that optimization research for the deficiencies of non-viral vectors is still ongoing and very actual.

The physical methods can also play an essential role in upgrading the efficiency of the viral/non-viral carriers. Most of this kind of methods seemed interesting to facilitate cellular uptake of the vectors, especially sonoporation and photoporation. Because of earlier clinical use of ultrasound and laser light in the non-pharmaceutical treatments of GON, knowledge of these methods was already at a certain level. Although, further investigation of the physical methods may be interesting. For example, the method of photoporation could be a possible option to overcome the ciliary epithelial cells and to translocate genes into the ciliary body.

At present multiple gene silencing siRNA medicines including bamosiran (SYL040012), cosdosiran (QPI-1007) and bevasiranib are running clinical trials. Unfortunately only the first two drugs show sufficiently promising results to continue clinical trials. The development of bevasiranib ceased after poor phase III clinical trial results. A lack of selective interaction to the target cells and a limited stability in vivo were the reasons for diminished efficacy and some serious side effects.(127)

The Spanish company Sylentis delivers their drug SYL040012, a naked siRNA, via topical eyedrops. According to the information we collected and summarized in this manuscript, it could be interesting to use biochemical, physical methods or eventually the combination of both to enhance transfection efficiency. A temporary reason may be that this gene silencing drug and especially the newer gene delivery methods are still both in research phase and require further investigations and improvements to be clinically relevant. Despite the various limitations, the

company will currently select topical administration due to its safety and convenience. (151) With the knowledge that we acquired while writing this master thesis, we are convinced that when the concerns of the biochemical methods are alleviated and the further clinical stages of SYL040012 shows promising results, these together could be a new interesting approach in the treatment of glaucoma.

6 CONCLUSION

Untreated glaucoma is the second leading cause of general blindness with a considerable gradual rise of incidence. In order to prevent this serious consequence, an optimal treatment is absolutely required. An elevated IOP is the major risk factor for developing most types of glaucoma. In the introduction, we made a review of, among other things, the most used commercially available and invasive non-pharmacological treatments. These had the aim of reducing this IOP directly, each with their own mode of action. Despite several limitations, these therapies are still an optional choice. However increased eye pressure is not the only risk factor. Several clinical studies discovered the presence of mutated or risk alleles genes in glaucomatous eyes responsible for disease development. Due to this, the interest in gene therapy for the treatment of GON increased. Research evolved so quickly, that currently the main hindrance of gene therapy application is not the lack of an ultimate gene therapeutic strategy. Multiple effective gene targeting or neuroprotective strategies were explained in detail in the introduction as well. Although there is still a lack of a safe and ideal delivery process to selectively transduce nucleic acids into the target tissues and cells. Often in clinical research, naked gene therapeutic agents are intravitreally or subretinally injected. This method has the risk of provoking inflammation reactions and the performance of gene instability. The main goal of this manuscript was to explain and to list the current state of using biochemical methods including the multiple viral and non-viral strategies as a more ideal delivery tool. Both types of vectors have their advantages and limitations.

At present, non-viral vectors are less effective in transducing efficiency and may bind less selectively to their target cell than the viral vectors. Viral vectors, on the other hand, have a lower loading capacity and, more importantly, they introduce a risk of immunogenicity. In some cases the virus can reform itself and regain its pathogenicity or replication capacity, despite the fact that its viral genes were partially or totally deleted. As a last part of this manuscript, the most relevant physical methods were explained. With the aim of enhancing gene transduction efficiency or viral/non-viral uptake into the target cell, this method may be an innovative treatment for glaucoma as well.

In general, we can conclude that especially the biochemical non-viral vectors, possibly in combination with some physical methods, shows the most promising future. However, further research will be essential to eliminate the last inconveniences and to have a better knowledge of the causative genes and their effects for glaucoma. For a disease like glaucoma, gene therapy resets the original treatment from a general to a more personalized one. Moreover with a higher efficiency and patient compliance, we are convinced that this newer strategy is part of the future medicine including the treatment of GON, but several other genetic diseases as well.

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