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Genetic and molecular pathogenesis of primary open angle glaucoma and corticosteroid-induced glaucoma

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Genetic and molecular pathogenesis of primary open angle glaucoma and corticosteroid-induced glaucoma

Applications of research with omics data in ophthalmology

Ilona Liesenborghs

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Genetic and molecular pathogenesis of primary open angle glaucoma and corticosteroid-induced glaucoma

Applications of research with omics data in ophthalmology

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List of abbreviations

AC	anterior chamber
AH	aqueous humor
ALK	anterior lamellar keratoplasty
B	the total number of genes associated with the GO term
b	the number of genes associated with the GO term that pass the statistical criteria
BA	benzyl alcohol
cAMP	cyclic adenosine monophosphate
*CDK*s	cyclin-dependent kinases
CLANs	cross-linked actin networks
CM	ciliary muscle
CTM	corneoscleral trabecular meshwork
DALK	deep anterior lamellar keratoplasty
DAVID	Database for Annotation, Visualization and Integrated Discovery
DCT	dynamic contour tonometer
DLEK	deep lamellar endothelial keratoplasty
DLKP	deep lamellar keratoplasty
DMEK	descemet membrane endothelial keratoplasty
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DSAEK	descemet stripping automated endothelial keratoplasty
DSEK	descemet stripping endothelial keratoplasty
ECCE	extracapsular cataract extraction
ECM	extracellular matrix
EK	endothelial keratoplasty
eQTL	expression quantitative trait loci
ES	embryonic stem
EtOH	ethanol
FDR q-value	false discovery rate
FED	fuchs endothelial dystrophy
FLEK	femtosecond laser enabled keratoplasty
FN1	fibronectin 1
FPKM	fragments per kilobase million

GAT	goldmann applanation tonometer
GEO	Gene Expression Omnibus
GO	Gene Ontology
GPCR	G protein-coupled receptor
GR	glucocorticoid receptor
GWAS	genome wide association study
HGNC	HUGO Gene Nomenclature Committee
HRC	Haplotype Reference Consortium
HSCs	hematopoietic stem cells
IOL	intraocular lens
IOP	intraocular pressure
iPSCs	induced pluripotent stem cells
JCT	juxtacanalicular tissue
KC	keratoconus
Kpro	keratoprosthesis
L1000CDS^2	L1000 Characteristic Direction Signature Search Engine
LD	linkage disequilibrium
LogFC	^{2}log fold change
MAF	minor allele frequency
MMPs	matrixmetalloproteases
MUMC+	Maastricht University Medical Centre+
MYOC	*myo*cilin
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NGS	next-generation sequencing
NICE	National Institute for Healthy and Clinical Excellence
OHT	ocular hypertension
OR	odds ratio
PBMCs	peripheral blood mononuclear cells
PCA	principal component analysis
PKP	penetrating keratoplasty
POAG	primary open angle glaucoma
PPI	protein-protein interaction
QC	quality control
QTL	quantitative trait locus

RHOA	ras homolog family member A
*ROCK*s	Rho/Rho-associated kinases
SASP	senescence-associated secretory phenotype
SC	Schlemm's canal
SNP	single nucleotide polymorphisms
SS	scleral spur
SUMO	small ubiquitin-like modifier
TM	trabecular meshwork
U-DSAEK	ultra-thin descemet stripping automated endothelial keratoplasty
UTM	uveal trabecular meshwork
VKC	vernal keratoconjunctivitis
WP	Wikipathways

Chapter 1

GENERAL INTRODUCTION

This thesis describes the research that was conducted to investigate the pathogenesis of primary open angle glaucoma (POAG) and the increase in intraocular pressure (IOP) caused by corticosteroids (corticosteroid-induced increase in IOP). The trabecular meshwork (TM), situated in the anterior chamber of the eye, plays a central role in the pathogeneses of both diseases. Within this thesis two main approaches were performed. The first approach included re-using, integrating and re-analyzing previously published and publicly available gene expression data. The insight in the molecular pathways obtained through this method were also used to identify candidate drugs to treat glaucoma. The second approach entailed a genome wide association study in patients with a corticosteroid-induced increase in IOP. In this introduction we describe the key concepts and methods that are relevant for this thesis.

Trabecular meshwork

The IOP is determined by the rate of production and outflow of aqueous humor (AH). The outflow of AH is through the trabecular meshwork (TM) which is located in the iridocorneal angle of the eye. It consists of series of fenestrated beams and sheets of extracellular matrix (ECM), covered with TM-cells.[1, 2] Three distinct cell layers of the TM can be distinguished: 1) the uveal, 2) corneoscleral and 3) juxtacanalicular meshwork (figure 1). The uveal network is the innermost portion of the TM. It consists of cord-like strands which are covered with endothelial cells. The intertrabecular spaces are relatively large and therefore offer little resistance to the passage of AH. The corneoscleral meshwork lies external to the uveal meshwork and is the thickest portion of the TM. It is composed of layers of connective tissue strands with overlying endothelial-like cells. The intertrabecular spaces are smaller than those of the uveal meshwork. The juxtacanalicular meshwork is the outer part of the TM. It consists of cells embedded in a dense ECM with narrow intercellular spaces which causes a high resistance to aqueous outflow. The juxtacanalicular meshwork links the corneoscleral meshwork with the endothelium of the inner wall of the canal of Schlemm. The canal of Schlemm is the final part of the outflow pathway and enters the venous circulation.[3, 4]

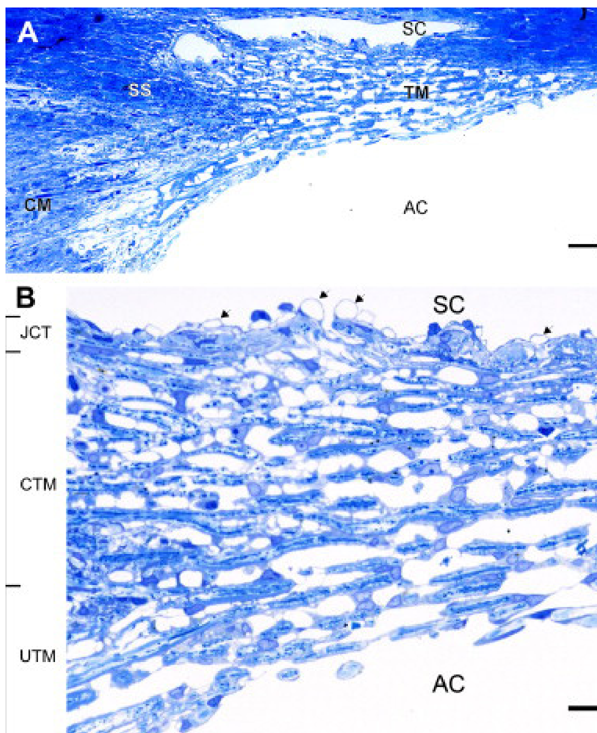

Figure 1. *Histology of TM.*
(A) shows the meridional section through the trabecular meshwork; (B) is a magnification of A.
Figure adopted from Tan et al, 2009.[5]

AC= Anterior Chamber, CM= Ciliary muscle, CTM= Corneoscleral trabecular meshwork, JCT= Juxtacanalicular tissue, SC= Schlemm's canal, SS= Scleral spur, TM= Trabecular meshwork, UTM= Uveal trabecular meshwork

Extracellular matrix in the trabecular meshwork

The ECM, an important part of the TM, is thought to be important in regulating the IOP. Many ECM proteins such as collagen, elastic fiber components, proteoglycans, glycosaminoglycans, fibronectin, matricellular proteins, and many others are present throughout the TM.[1, 2, 6-12] The ECM also appears to undergo a constant turnover and remodeling. Studies show that the TM cells can adjust the outflow resistance and restore the IOP level due to a homeostatic response. When the flow rate in perfused anterior segment organ culture is doubled, the pressure doubles as well and the TM cells respond by increasing the enzyme activity of matrixmetalloproteases (MMPs). As MMPs break down the ECM, this causes the outflow to increase and the IOP to decrease.[13-15]

Changes in the ECM have been identified in patients with primary open angle glaucoma (POAG). Studies showed that the amount of sheath-derived plaques is significantly greater in these patients.[16] Within these sheets fibronectin, vitronectin, laminin and tenascin as glycoproteins, decorin and versican as proteoglycans, as well as hyaluronan, collagen I, III, IV,V and VI, *MYOC* and fibrillin were found.[17] These plaques were both found in patients with POAG that had been treated with glaucoma medications as patients with POAG that did not use glaucoma medications, indicating that they are not caused by the glaucoma treatment.[18] Additionally, in patients with long-term treatment for corticosteroid-induced glaucoma these plaques were found as well, however their localization of depositing is different from POAG.[19] In addition, the uveal meshwork of corticosteroid responders showed no plaques but does show an accumulation of fingerprint-like depositions of material.[20] Furthermore, an accumulation of type IV collagen and fibronectin can be found in the ECM of these patients as well.[21]

Actin and myosin in the trabecular meshwork

In addition to changes in the ECM, changes in actin cytoskeleton and myosin of TM cells also plays an important role in the pathogenesis of both POAG and a corticosteroid-induced increase in IOP. The contraction of the TM is regulated by myosin light chain and myosin light chain kinase. Alterations in actin and myosin may subsequently lead to TM rigidity and increased outflow resistance.[22, 23] As both actin and myosin play an important role in the TM, the TM is also said to have smooth muscle-like contractile properties.[24] Actin has been shown to be present in the three distinct layers of the TM.[25] Now, it is clear that actin forms cross-linked actin (CLAN) networks in the TM. Studies have shown that the formation of these networks is more common in glaucomatous TM tissue compared to non-glaucomatous TM tissue.[26, 27]

In addition, TM cells treated with dexamethasone, a corticosteroid, show changes in the actin configuration. These changes are likely associated with an increased outflow resistance of the TM and could be an explanation for the increased eye pressure in patients with a corticosteroid-induced increase in IOP.[28, 29] Interestingly, the CLAN formation caused by treatment with dexamethasone was shown to be dose and time dependent and reversible when exposure to dexamethasone is withdrawn.[28]

Intraocular pressure, ocular hypertension and glaucoma

As mentioned above, the IOP is determined by the rate of production and outflow of AH. The AH is produced by the ciliary body which is located at the root of the iris, in the posterior chamber. Following, the AH flows through the pupil into the anterior chamber where it is drained. This drainage resides for 90% in the trabecular meshwork. In addition, 10% of the AH passes across the ciliary body into the subchoroidal space and is then drained by the venous system (uveoscleral outflow). A negligible part of the AH drains via the iris surface and capillaries (figure 2).

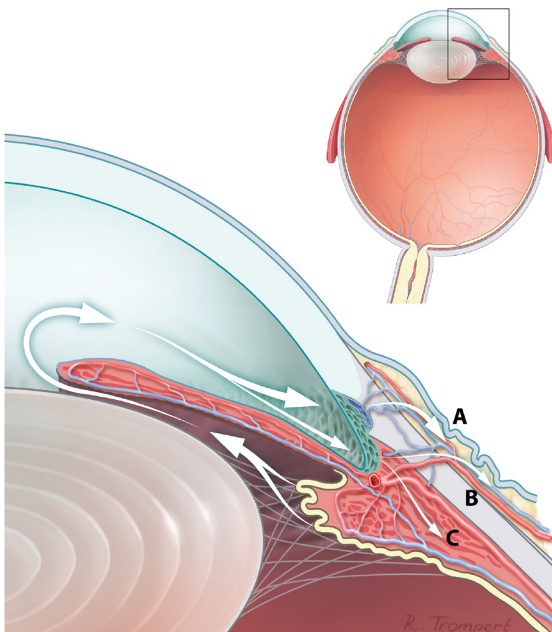

Figure 2. *Production and outflow of the AH in the anterior chamber of the eye.* *(A) shows the outflow through the TM; (B) shows the outflow via the iris surface and capillaries (figure 2); (C) shows the uveoscleral outflow.*
Figure adapted from R. Trompert.

The average IOP is around 16 mmHg and a range of 11–21 mmHg has conventionally been accepted as normal. An imbalance between the production and outflow of the AH may lead to the development of an elevated IOP, also known as intraocular hypertension. Most commonly this is defined as an IOP > 21 mmHg. Patients with an IOP above this threshold are at a higher risk to develop damage to the optic nerve and consequently visual field loss, also called glaucoma. The optic neuropathy within these patients is thought to be caused by direct mechanical damage to the retinal nerve fiber layer or by secondary ischemic damage due to the compression of blood vessels supplying the optic nerve.[30]

Traditionally, glaucoma can be classified in open and closed angle glaucoma. In open angle glaucoma, there is no identifiable underlying anatomical cause that leads to the obstruction of outflow of the AH and subsequent elevation of the IOP. In angle closure glaucoma, there is an anatomical obstruction of the outflow, located before the TM. This is most commonly caused by the iris which impedes the access of AH to the TM (figure 3).[30] Ocular hypertension can also be caused by the use of corticosteroids, also called a corticosteroid-induced increase in IOP or corticosteroid response. Corticosteroids are a specific type of drugs which are widely used for the treatment of both ophthalmological and non-ophthalmological diseases.

This thesis focuses on both POAG and a corticosteroid-induced increase in IOP and the role of the trabecular meshwork (TM) in their pathogenesis as both types are characterized by an elevated IOP without a visible obstruction of the AH outflow in the TM.

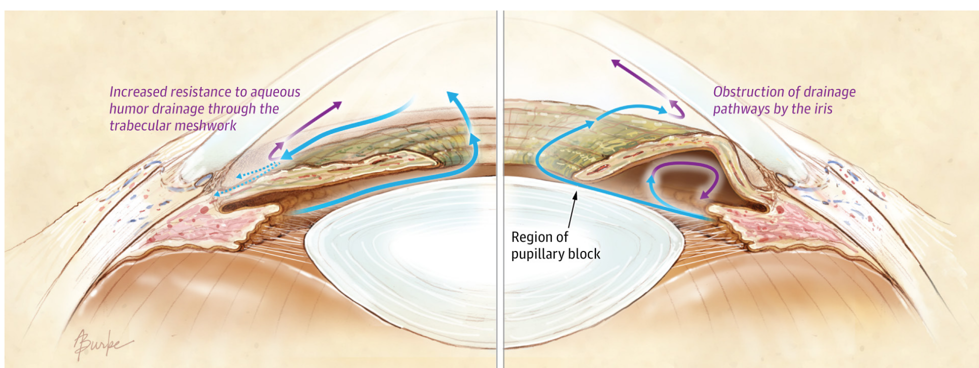

Figure 3. *Open and closed angle of the anterior chamber.*
Figure adapted from Weinreb et al., 2014[31]

Primary open angle glaucoma

POAG is the most common form of glaucoma in Europe, accounting for 70% of the cases. It is defined as an IOP > 21 mmHg, glaucomatous damage of the optic nerve and a specific type of visual field loss in eyes with an open anterior chamber angle and in the absence of signs of secondary glaucoma or a non-glaucomatous cause for the optic neuropathy. POAG is often bilateral but can be asymmetric. It is also typically insidious in onset and progresses slowly and painless. Therefore, patients are often symptomless until late stages of the disease in which a great loss of the visual fields has occurred or central vision is affected. Multiple factors have been associated with POAG such as older age, race, thin central corneal thickness and a positive family history. In addition, multiple disorders have been associated as well: myopia, obstructive sleep apnea syndrome, diabetes mellitus, hyper- or hypotension, migraine and vasospasm.[32-36] However, as mentioned before, an increased IOP is the most important risk factor.

Corticosteroid-induced increase of intraocular pressure

Glucocorticoids belong to the class of corticosteroid hormones which are produced in the adrenal cortex and play a role in multiple physiological processes. They are known to have anti-inflammatory and immunosuppressive effects for which they have been pharmaceutically derived and are now widely used within multiple medical disciplines. They are also commonly used within the field of ophthalmology, for example to treat patients with uveitis, to reduce the risk of inflammation after eye surgery or to lower the risk of rejection after corneal transplantation surgery. However, as glucocorticoids initiate multiple signaling cascades which influence the expression of hundreds to thousands of genes, they also cause multiple side effects. Within ophthalmology, one of the most common side effects is an increase in IOP, also known as a corticosteroid response, which occurs in approximately 18%-36% of the patients.[37] If the elevated IOP is sustained for a longer period of time, glaucomatous damage may occur which is often referred to as corticosteroid-induced glaucoma. This most commonly occurs after the use of topical corticosteroids, however, it is also often encountered after using corticosteroids within nasal spray or ointments applied to the skin around the eyes and even after systemic use. The definition of a corticosteroid response differs between studies. However, the most commonly used definition was formulated by Armaly and Becker as a positive change in IOP of 6 mmHg or more above normal baseline IOP (12-21 mmHg) after the use of corticosteroids.[38] In addition, Armaly and Becker defined three distinct subgroups in patients with a corticosteroid response: patients exhibiting a high response (ΔIOP > 15 mmHg), patients exhibiting a moderate response (ΔIOP 6-15 mmHg), and patients exhibiting a low response (ΔIOP < 6 mmHg).[38] As suggested by these three categories, the degree of IOP elevation after exposure to corticosteroids differ greatly.[39, 40]

In most patients with a corticosteroid-induced increase in IOP, the IOP usually returns to normal within a few weeks after discontinuation of the corticosteroids. A few patients however may experience a continued elevation of IOP and develop glaucomatous damage to the optic nerve. Furthermore, there is a well-known interplay between a corticosteroid-induced increase in IOP and POAG. Patients with POAG are not only more susceptible to develop a corticosteroid-induced increase in IOP but patients that had a corticosteroid response in the past have a higher risk of developing POAG. In addition, relatives of patients with POAG also have a higher change for developing a corticosteroid-induced increase in IOP.[37, 38, 41-44]

Current treatment modalities

Despite the fact that multiple factors seem to be involved in the pathogenesis of POAG and corticosteroid-induced glaucoma, the main therapeutic strategy is based on reducing the IOP. Currently, this often can be achieved by using IOP lowering drugs, laser therapy or surgery, or by a combination of these therapies. As the scope of this thesis focusses on the molecular pathogenesis and the identification of new drugs to treat glaucoma, we will only give more detail about IOP lowering drugs as treatment modality. As mentioned before, the IOP is determined by a balance between production and outflow of the AH, the current glaucoma medications target these processes and can be classified in four main groups: β-blockers, α2-agonists, carbonic anhydrase inhibitors and prostaglandin analogues. The first three mentioned classes of drugs decrease the production of the AH by the ciliary body. β-blockers do this by inhibiting the production of cyclic adenosine monophosphate (cAMP) in the ciliairy epithelium which reduces the production of AH. α2-agonists lower the activity of cAMP and adenylate cyclase by binding to α2-receptors on the ciliairy epithelium. Carbonic anhydrase inhibitors inhibit the activity of ciliairy epithelial carbonic anhydrase and therefore the production of AH. Prostaglandin analogues and α2-agonists both lower the IOP by increasing the uveoscleral outflow. Additionally, prostaglandin analogues are thought to not only increase the uveoscleral outflow but also to increase the outflow through the TM. The precise mechanism of action is not completely understood. However, it is hypothesized that the prostaglandins bind with prostaglandin receptors which trigger a cascade of events leading to the activation of MMP's which break down the ECM leading to a lowering of the IOP.[45] Prostaglandins are the only class of drugs that possibly target a part of the molecular pathogenesis of glaucoma, i.e. changes in the ECM.[4]

To increase the efficiency of these treatments, multiple combined preparations of the above drugs are available. Despite this, the current medical therapies can only lower the IOP by an average of 35% (ranging between 20%-35%) which may not be enough to decrease progression of the optic neuropathy.[46] In that case, laser, minimally invasive surgery e.g. Innfocus® or XEN® implants or more invasive surgical techniques such as tube shunts or trabeculectomy are available in an attempt to lower the IOP. However, there are currently no other non-invasive treatment options. As the prevalence of glaucoma continues to rise worldwide and life expectancy increases, the need for new, non-invasive therapies grows in order to reduce the number of patients with glaucoma blindness due to lack of efficient treatment. Recently some new drug treatment options have been described of which Rho kinase inhibitors are of special interest.

Rho kinase inhibitor

Rho kinase is known to regulate actin cytoskeletal dynamics, actomyosin contraction, cell adhesion, cell stiffness, cell morphology and ECM reorganization. Studies on the effect of Rho kinase inhibitors (or its upstream targets such as *RHOA*) on TM cells showed dose- and time-dependent reversible changes such as a decreased number of actin stress fibers, focal adhesions and cell-cell interactions. These changes reduce the mechanical tension and stiffness of the ECM and decrease its synthesis and rigidity.[47-53] Experiments with different types of Rho kinase inhibitors showed a dose-dependent increase in the outflow. The increase in AH outflow through the TM is associated with TM tissue relaxation, washout of extracellular material, an increase in giant vacuoles in the inner wall of the Schlemm canal and widening of this canal.[47-49, 54-57] Additionally, studies in animal models showed that these changes lead to a decrease in IOP, proving that Rho kinase inhibitors can be effective for the treatment of glaucoma.[49, 50, 56] Lastly, Rho kinase inhibitors are also believed to have neuroprotective effects on the optic nerve and retina which might inhibit the progression to glaucomatous neuropathy.[58-60]

During the past decades, biochemical and histological in vitro studies have been performed in order to identify the pathogenesis of POAG and a corticosteroid-induced increase in IOP. However, the number of molecules that can be investigated within these studies is limited. The arrival of omics technologies, such as microarray chips and next-generation sequencing (NGS), enabled the exposure of the complete human genome of individual patients and to measure the expression of several thousands of molecules from one sample of affected tissue. These data contain an abundant amount of information on the molecular mechanism of physiological and pathophysiological processes, creating new opportunities for scientific progress within ophthalmology. Within this thesis, bioinformatics analyses were used to (re-)analyze omics data.

Omics data

Transcriptomics

In transcriptomics studies, the RNA-levels for all expressed genes in a certain tissue can be measured. These studies allow to investigate the differences in gene expression between two traits, for example POAG TM versus healthy TM tissue or TM tissue exposed to corticosteroids versus TM tissue exposed to a neutral control medium. This type of studies has identified multiple genes in the TM that were differentially expressed in patients with POAG and after exposure to corticosteroids.[41, 61-70] We re-used and investigated the publicly available transcriptomic data in order to obtain new insights into the pathogenesis of POAG and corticosteroid-induced glaucoma.

Genome-wide association study

It is likely that there is a genetic or heritable basis for both POAG and corticosteroid-induced OHT as a positive family history is a risk factor.[71, 72] However, the genetic basis of both diseases are complex. Small DNA sequence variations might increase the insight in corticosteroid-induced OHT. This information can be obtained by performing a GWAS in which the genomic DNA of individuals with and without a specific trait or phenotype is genotyped and compared. These genetic variations investigated in a GWAS study are single base-pair changes in the DNA sequence, also called single nucleotide polymorphisms (SNPs). In contrast to candidate target studies, hundreds of thousands of SNPs are measured within one single GWAS. A GWAS has been proven to be a valuable method and already increased the insights in complex molecular diseases such as POAG[73], macular degeneration[74], type II diabetes[75] and many others. For POAG, various genetic studies such as linkage analyses and genome-wide association studies (GWAS) identified multiple genes such as *MYOC*, *OPTN*, *WDR36*, *SIX6* and *FOXC1* that play a role in the pathogenesis of POAG.[76] For corticosteroid-induced OHT, candidate target genes with a possible association such as *N363S*, *NR3C1*, *SFRS3*, *SFRS5*, *SFRS9*, *FKBP4*, *FKBP5* and *MYOC* have been investigated, however a significant association was only found for two SNPs: rs6559662 and rs1879370.[40, 77-81] Also, a GWAS on a small cohort of 64 individuals was performed and identified wo variants (rs2523864 and rs2251830) that could affect the expression of *HCG22*.[82]

We performed a GWAS in 339 patients with a corticosteroid-induced increase in IOP. In this case, the trait or phenotype (increase in IOP) is only triggered after exposure to corticosteroids. The identification of SNPs associated with a drug metabolism or adverse effects is called pharmocogenetics and has been proven to be one of the most successful applications of GWAS.[83] The large effect-phenotypes that are found in pharmacogenomics traits allows to find significant associations, even in studies with smaller sample sizes.

Bioinformatics

As mentioned before, omics studies generate enormous amounts of data which confronts us with the challenge of handling, analyzing and interpreting these data. Therefore, we need a way to integrate and present the results in a (clinically) meaningful way. Modern integrative bioinformatics analyses are the key to do this as they creates new possibilities for researchers to build on already obtained data and knowledge and therefore, the World Wide Web becomes the lab.

The most commonly used applications of integrative bioinformatics in this thesis are pathway and network analyses. Multiple genes have been identified through the existing gene expression studies. However, a gene is only a small fraction of the molecular processes it is involved in. Pathway diagrams represent such molecular processes and show the genes that are involved within these processes and how they are related to other molecules. By identifying the pathways in which the differentially expressed genes are involved, we can identify disease processes that are likely to play a role in the pathogenesis. A subsequent network analysis of the identified genes or pathways allows visualization of how functional categories are related to each other, how many and which mutual genes are shared between the multiple categories and investigation of the genes of interest. In addition, the obtained results can be used for performing drug repurposing or new drug development.

Drug repurposing

The identification of effective treatment modalities for a disease remains challenging. To find the appropriate drugs, two main methods are possible: developing de novo drugs or drug repurposing. The latter, also called drug repositioning, redirection or reprofiling, is defined as "the process of finding new uses outside the scope of the original medical indication for existing drugs".[84] Drug repurposing has great advantages above de novo drug discovery as it is cheaper, saves time and has a lower risk for serious adverse effects as several test-related phases, that would be part of de novo drug discovery, already took place. This approach has for example been followed to address the urgently needed treatment of COVID-19 during the SARS-CoV-2 pandemic.[85]

Most diseases are not driven by one single molecular abnormality but by multiple. POAG is also thought to be a multifactorial disease. Therefore, the drug discovery has shifted from target-centered to system-driven in which multiple targets are investigated at once. There are multiple ways to perform drug repurposing and different input data can be used. One possibility is the use of microarray gene expression data which we performed for this thesis. In addition, by using the identified pathways and networks for performing drug repurposing, neighboring (interacting, regulating) proteins can be explored for

targeting as well. Also, the downstream effects of the potential drug on other involved proteins or processes can be followed. Moreover, the identified molecular pathways can also be used to identify targets for ***de novo*** drug development.

Aim and outline of this thesis

It is well known that the trabecular meshwork plays an important role in the pathogenesis of both POAG and a corticosteroid-induced increase in IOP.[19] Therefore, the diseased TM has not only been investigated in biochemical and histological in vitro studies but also in genome-wide gene expression studies.[41, 61-70, 86] The latter has led to the identification of multiple genes and processes which might be involved in the TM of patients with POAG or in TM tissue exposed to corticosteroids. In contrast to the extensive research in diseased TM tissue, the molecular genes and processes which are active in the TM of healthy individuals are largely unknown. In **chapter two**, this is further explored by performing integrative bioinformatics analysis on the publicly available gene expression data of healthy TM tissue.

In **chapter three** the molecular pathogenesis of POAG is explored. In order to do this, we used the publicly available gene expression data of patients with POAG compared to healthy controls. Genes and molecular processes known to be involved in the pathogenesis of POAG were identified and stresses the potential role of this approach in the identification of new treatment options.

In **chapter four** the molecular pathogenesis of a corticosteroid-induced increase in IOP is investigated. First of all, we identified which processes are activated after exposing the TM to dexamethasone. In addition, we compared the gene expression data of bovine responder (those with an increase in IOP after exposure) vs. non-responder TM tissue. This allowed a more detailed identification of processes involved in a corticosteroid-induced increase in IOP.

Chapter five shows the possibilities that drug repurposing has to offer. Advantages, disadvantages and common encountered problems are discussed. Also, we identified drugs that could possibly be used in glaucoma treatment or prevention of a corticosteroid-induced increase in IOP.

Chapter six reports the results of a systematic review. This study was designed to systematically collect and summarize the current evidence on risk factors for the development of an increase in IOP after keratoplasty and followed a semi-quantitative method which allowed the identification of highly likely associated risk factors. These risk factors could be possible confounders which could play a role in the GWAS analyses in chapter seven.

A corticosteroid-induced increase in IOP occurs in about one out of three patients. As its treatment remains very challenging, it causes a clinically relevant problem. The pathogenesis of a corticosteroid-induced increase in IOP is largely unknown. In addition to changes in the trabecular meshwork, investigated in chapter four, small DNA sequence variations may also play a role in the pathogenesis. Therefore, **chapter seven**, describes the results of the first large analysis of a GWAS cohorts in which patients with and without a corticosteroid response were compared in order to identify genetic variants, single base-pair changes, also called single nucleotide polymorphisms (SNPs).

In **chapter eight**, the findings of this thesis are discussed. In addition, the future perspectives of this research are highlighted as we look forward to the use of peripheral blood mononuclear cells (PBMCs) to obtain a better insight into the individualized pathogenesis of a corticosteroid-induced increase in IOP. In addition, we make a basic step in the personalized treatment of a corticosteroid-induced increase in IOP.

References

1. Hogan MJ AJ, Weddell JE. Histology of the human eye: an atlas and textbook: Saunders: The University of Michigan, 1971.
2. Vranka JA, Kelley MJ, Acott TS, Keller KE. Extracellular matrix in the trabecular meshwork: intraocular pressure regulation and dysregulation in glaucoma. Exp Eye Res 2015;133:112-25.
3. Goel M, Picciani RG, Lee RK, Bhattacharya SK. Aqueous humor dynamics: a review. Open Ophthalmol J 2010;4:52-9.
4. Ophthalmology AAo. Glaucoma. The United States of America 2018-2019; 261.
5. Tamm ER. The trabecular meshwork outflow pathways: structural and functional aspects. Exp Eye Res 2009;88(4):648-55.
6. Francois J. The importance of the mucopolysaccharides in intraocular pressure regulation. Invest Ophthalmol 1975;14(3):173-6.
7. Gong H, Tripathi RC, Tripathi BJ. Morphology of the aqueous outflow pathway. Microsc Res Tech 1996;33(4):336-67.
8. Lutjen-Drecoll E. Functional morphology of the trabecular meshwork in primate eyes. Prog Retin Eye Res 1999;18(1):91-119.
9. Lutjen-Drecoll E, Futa R, Rohen JW. Ultrahistochemical studies on tangential sections of the trabecular meshwork in normal and glaucomatous eyes. Invest Ophthalmol Vis Sci 1981;21(4):563-73.
10. Lutjen-Drecoll E, Shimizu T, Rohrbach M, Rohen JW. Quantitative analysis of 'plaque material' in the inner- and outer wall of Schlemm's canal in normal- and glaucomatous eyes. Exp Eye Res 1986;42(5):443-55.
11. Lutjen-Drecoll E RJ. Morphology of Aqueous Outflow Pathways in Normal and Glaucomatous Eyes. 1996:89–123.
12. Richardson TM. Distribution of glycosaminoglycans in the aqueous outflow system of the cat. Invest Ophthalmol Vis Sci 1982;22(3):319-29.
13. Borras T, Rowlette LL, Tamm ER, et al. Effects of elevated intraocular pressure on outflow facility and TIGR/*MYOC* expression in perfused human anterior segments. Invest Ophthalmol Vis Sci 2002;43(1):33-40.
14. Bradley JM, Vranka J, Colvis CM, et al. Effect of matrix metalloproteinases activity on outflow in perfused human organ culture. Invest Ophthalmol Vis Sci 1998;39(13):2649-58.
15. Acott TS, Kelley MJ. Extracellular matrix in the trabecular meshwork. Exp Eye Res 2008;86(4):543-61.
16. Gottanka J, Johnson DH, Martus P, Lutjen-Drecoll E. Severity of optic nerve damage in eyes with POAG is correlated with changes in the trabecular meshwork. J Glaucoma 1997;6(2):123-32.
17. Ueda J, Wentz-Hunter K, Yue BY. Distribution of *myoc*ilin and extracellular matrix components in the juxtacanalicular tissue of human eyes. Invest Ophthalmol Vis Sci 2002;43(4):1068-76.
18. Rohen JW, Lutjen-Drecoll E, Flugel C, et al. Ultrastructure of the trabecular meshwork in untreated cases of primary open-angle glaucoma (POAG). Exp Eye Res 1993;56(6):683-92.

19. Tektas OY, Lutjen-Drecoll E. Structural changes of the trabecular meshwork in different kinds of glaucoma. Exp Eye Res 2009;88(4):769-75.
20. Rohen JW, Linner E, Witmer R. Electron microscopic studies on the trabecular meshwork in two cases of corticosteroid-glaucoma. Exp Eye Res 1973;17(1):19-31.
21. Tawara A, Tou N, Kubota T, et al. Immunohistochemical evaluation of the extracellular matrix in trabecular meshwork in steroid-induced glaucoma. Graefes Arch Clin Exp Ophthalmol 2008;246(7):1021-8.
22. Wiederholt M, Thieme H, Stumpff F. The regulation of trabecular meshwork and ciliary muscle contractility. Prog Retin Eye Res 2000;19(3):271-95.
23. Nakajima E, Nakajima T, Minagawa Y, et al. Contribution of *ROCK* in contraction of trabecular meshwork: proposed mechanism for regulating aqueous outflow in monkey and human eyes. J Pharm Sci 2005;94(4):701-8.
24. Lepple-Wienhues A, Stahl F, Wiederholt M. Differential smooth muscle-like contractile properties of trabecular meshwork and ciliary muscle. Exp Eye Res 1991;53(1):33-8.
25. Gipson IK, Anderson RA. Actin filaments in cells of human trabecular meshwork and Schlemm's canal. Invest Ophthalmol Vis Sci 1979;18(6):547-61.
26. Read AT, Chan DW, Ethier CR. Actin structure in the outflow tract of normal and glaucomatous eyes. Exp Eye Res 2007;84(1):214-26.
27. Hoare MJ, Grierson I, Brotchie D, et al. Cross-linked actin networks (CLANs) in the trabecular meshwork of the normal and glaucomatous human eye in situ. Invest Ophthalmol Vis Sci 2009;50(3):1255-63.
28. Clark AF, Wilson K, McCartney MD, et al. Glucocorticoid-induced formation of cross-linked actin networks in cultured human trabecular meshwork cells. Invest Ophthalmol Vis Sci 1994;35(1):281-94.
29. Filla MS, Schwinn MK, Nosie AK, et al. Dexamethasone-associated cross-linked actin network formation in human trabecular meshwork cells involves beta3 integrin signaling. Invest Ophthalmol Vis Sci 2011;52(6):2952-9.
30. Ophtalmology TAAo. The American Academy of Ophtalmology: Glaucoma. 2013-2014;chapter 1 (Introduction to glaucoma: Terminology, Epidemiology and Heredity) and chapter 4 (Open-Angle glaucoma).
31. Weinreb RN, Aung T, Medeiros FA. The pathophysiology and treatment of glaucoma: a review. JAMA 2014;311(18):1901-11.
32. Blumberg D, Skaat A, Liebmann JM. Emerging risk factors for glaucoma onset and progression. Prog Brain Res 2015;221:81-101.
33. McMonnies CW. Glaucoma history and risk factors. J Optom 2017;10(2):71-8.
34. Grzybowski A, Och M, Kanclerz P, et al. Primary Open Angle Glaucoma and Vascular Risk Factors: A Review of Population Based Studies from 1990 to 2019. J Clin Med 2020;9(3).
35. Coleman AL, Miglior S. Risk factors for glaucoma onset and progression. Surv Ophthalmol 2008;53 Suppl1:S3-10.
36. Yanagi M, Kawasaki R, Wang JJ, et al. Vascular risk factors in glaucoma: a review. Clin Exp Ophthalmol 2011;39(3):252-8.
37. Tripathi RC, Parapuram SK, Tripathi BJ, et al. Corticosteroids and glaucoma risk. Drugs Aging 1999;15(6):439-50.

38. Armaly MF, Becker B. Intraocular pressure response to topical corticosteroids. Fed Proc 1965;24(6):1274-8.
39. Fini ME, Schwartz SG, Gao X, et al. Steroid-induced ocular hypertension/glaucoma: Focus on pharmacogenomics and implications for precision medicine. Prog Retin Eye Res 2017;56:58-83.
40. Gerzenstein SM, Pletcher MT, Cervino AC, et al. Glucocorticoid receptor polymorphisms and intraocular pressure response to intravitreal triamcinolone acetonide. Ophthalmic Genet 2008;29(4):166-70.
41. Fan BJ, Wang DY, Tham CC, et al. Gene expression profiles of human trabecular meshwork cells induced by triamcinolone and dexamethasone. Invest Ophthalmol Vis Sci 2008;49(5):1886-97.
42. Becker B. Intraocular Pressure Response to Topical Corticosteroids. Invest Ophthalmol 1965;4:198-205.
43. Kitazawa Y, Horie T. The prognosis of corticosteroid-responsive individuals. Arch Ophthalmol 1981;99(5):819-23.
44. Lewis JM, Priddy T, Judd J, et al. Intraocular pressure response to topical dexamethasone as a predictor for the development of primary open-angle glaucoma. Am J Ophthalmol 1988;106(5):607-12.
45. Oh DJ, Martin JL, Williams AJ, et al. Effect of latanoprost on the expression of matrix metalloproteinases and their tissue inhibitors in human trabecular meshwork cells. Invest Ophthalmol Vis Sci 2006;47(9):3887-95.
46. European Glaucoma Society Terminology and Guidelines for Glaucoma, 4th Edition - Chapter 3: Treatment principles and options Supported by the EGS Foundation: Part 1: Foreword; Introduction; Glossary; Chapter 3 Treatment principles and options. Br J Ophthalmol 2017;101(6):130-95.
47. Rao PV, Deng P, Sasaki Y, Epstein DL. Regulation of myosin light chain phosphorylation in the trabecular meshwork: role in aqueous humour outflow facility. Exp Eye Res 2005;80(2):197-206.
48. Rao PV, Deng P, Maddala R, et al. Expression of dominant negative Rho-binding domain of Rho-kinase in organ cultured human eye anterior segments increases aqueous humor outflow. Mol Vis 2005;11:288-97.
49. Inoue T, Tanihara H. Rho-associated kinase inhibitors: a novel glaucoma therapy. Prog Retin Eye Res 2013;37:1-12.
50. Honjo M, Tanihara H, Inatani M, et al. Effects of rho-associated protein kinase inhibitor Y-27632 on intraocular pressure and outflow facility. Invest Ophthalmol Vis Sci 2001;42(1):137-44.
51. Zhou EH, Krishnan R, Stamer WD, et al. Mechanical responsiveness of the endothelial cell of Schlemm's canal: scope, variability and its potential role in controlling aqueous humour outflow. J R Soc Interface 2012;9(71):1144-55.
52. Zhang M, Maddala R, Rao PV. Novel molecular insights into *RhoA* GTPase-induced resistance to aqueous humor outflow through the trabecular meshwork. Am J Physiol Cell Physiol 2008;295(5):C1057-70.
53. Pattabiraman PP, Maddala R, Rao PV. Regulation of plasticity and fibrogenic activity of trabecular meshwork cells by Rho GTPase signaling. J Cell Physiol 2014;229(7):927-42.

54. Tian B, Kaufman PL. Effects of the Rho kinase inhibitor Y-27632 and the phosphatase inhibitor calyculin A on outflow facility in monkeys. Exp Eye Res 2005;80(2):215-25.
55. Rao PV, Deng PF, Kumar J, Epstein DL. Modulation of aqueous humor outflow facility by the Rho kinase-specific inhibitor Y-27632. Invest Ophthalmol Vis Sci 2001;42(5):1029-37.
56. Li G, Mukherjee D, Navarro I, et al. Visualization of conventional outflow tissue responses to netarsudil in living mouse eyes. Eur J Pharmacol 2016;787:20-31.
57. Gong H, Yang CY. Morphological and hydrodynamic correlations with increasing outflow facility by rho-kinase inhibitor Y-27632. J Ocul Pharmacol Ther 2014;30(2-3):143-53.
58. Van de Velde S, De Groef L, Stalmans I, et al. Towards axonal regeneration and neuroprotection in glaucoma: Rho kinase inhibitors as promising therapeutics. Prog Neurobiol 2015;131:105-19.
59. Bermel C, Tonges L, Planchamp V, et al. Combined inhibition of *Cdk*5 and *ROCK* additively increase cell survival, but not the regenerative response in regenerating retinal ganglion cells. Mol Cell Neurosci 2009;42(4):427-37.
60. Sugiyama T, Shibata M, Kajiura S, et al. Effects of fasudil, a Rho-associated protein kinase inhibitor, on optic nerve head blood flow in rabbits. Invest Ophthalmol Vis Sci 2011;52(1):64-9.
61. Ishibashi T, Takagi Y, Mori K, et al. cDNA microarray analysis of gene expression changes induced by dexamethasone in cultured human trabecular meshwork cells. Invest Ophthalmol Vis Sci 2002;43(12):3691-7.
62. Markus H. Kuehn CYK, Young H. Kwon. Dexamethasone induced gene expression changes in human perfusion organ cultured trabecular meshwork. 2012.
63. Matsuda A, Asada Y, Takakuwa K, et al. DNA Methylation Analysis of Human Trabecular Meshwork Cells During Dexamethasone Stimulation. Invest Ophthalmol Vis Sci 2015;56(6):3801-9.
64. Nehme A, Lobenhofer EK, Stamer WD, Edelman JL. Glucocorticoids with different chemical structures but similar glucocorticoid receptor potency regulate subsets of common and unique genes in human trabecular meshwork cells. BMC Med Genomics 2009;2:58.
65. Rozsa FW, Reed DM, Scott KM, et al. Gene expression profile of human trabecular meshwork cells in response to long-term dexamethasone exposure. Mol Vis 2006;12:125-41.
66. Lo WR, Rowlette LL, Caballero M, et al. Tissue differential microarray analysis of dexamethasone induction reveals potential mechanisms of steroid glaucoma. Invest Ophthalmol Vis Sci 2003;44(2):473-85.
67. Danias J, Gerometta R, Ge Y, et al. Gene expression changes in steroid-induced IOP elevation in bovine trabecular meshwork. Invest Ophthalmol Vis Sci 2011;52(12):8636-45.
68. Diskin S, Kumar J, Cao Z, et al. Detection of differentially expressed glycogenes in trabecular meshwork of eyes with primary open-angle glaucoma. Invest Ophthalmol Vis Sci 2006;47(4):1491-9.
69. Liton PB, Luna C, Challa P, et al. Genome-wide expression profile of human trabecular meshwork cultured cells, nonglaucomatous and primary open angle glaucoma tissue. Mol Vis 2006;12:774-90.
70. Liu Y, Allingham RR, Qin X, et al. Gene expression profile in human trabecular meshwork from patients with primary open-angle glaucoma. Invest Ophthalmol Vis Sci 2013;54(9):6382-9.

71. Chan W, Wiggs JL, Sobrin L. The genetic influence on corticosteroid-induced ocular hypertension: A field positioned for discovery. Am J Ophthalmol 2019.
72. Armaly MF. The heritable nature of dexamethasone-induced ocular hypertension. Arch Ophthalmol 1966;75(1):32-5.
73. Wiggs JL, Pasquale LR. Genetics of glaucoma. Hum Mol Genet 2017;26(R1):R21-R7.
74. Haines JL, Hauser MA, Schmidt S, et al. Complement factor H variant increases the risk of age-related macular degeneration. Science 2005;308(5720):419-21.
75. Altshuler D, Hirschhorn JN, Klannemark M, et al. The common PPARgamma Pro12Ala polymorphism is associated with decreased risk of type 2 diabetes. Nat Genet 2000;26(1):76-80.
76. Youngblood H, Hauser MA, Liu Y. Update on the genetics of primary open-angle glaucoma. Exp Eye Res 2019;188:107795.
77. Hogewind BF, Micheal S, Schoenmaker-Koller FE, et al. Analyses of Sequence Variants in the *MYOC* Gene and of Single Nucleotide Polymorphisms in the *NR3C1* and *FKBP5* Genes in Corticosteroid-Induced Ocular Hypertension. Ophthalmic Genet 2015;36(4):299-302.
78. Hogewind BF, Micheal S, Bakker B, et al. Analysis of single nucleotide polymorphisms in the *SFRS3* and *FKBP4* genes in corticosteroid-induced ocular hypertension. Ophthalmic Genet 2012;33(4):221-4.
79. Fingert JH, Alward WL, Wang K, et al. Assessment of SNPs associated with the human glucocorticoid receptor in primary open-angle glaucoma and steroid responders. Mol Vis 2010;16:596-601.
80. Szabo V, Borgulya G, Filkorn T, et al. The variant *N363S* of glucocorticoid receptor in steroid-induced ocular hypertension in Hungarian patients treated with photorefractive keratectomy. Mol Vis 2007;13:659-66.
81. Badrinarayanan L, Chitipothu S, Ramasubramanyan S, et al. Assessment of single nucleotide polymorphisms associated with steroid-induced ocular hypertension. Int J Ophthalmol 2020;13(8):1294-305.
82. Jeong S, Patel N, Edlund CK, et al. Identification of a Novel Mucin Gene *HCG22* Associated With Steroid-Induced Ocular Hypertension. Invest Ophthalmol Vis Sci 2015;56(4):2737-48.
83. Bush WS, Moore JH. Chapter 11: Genome-wide association studies. PLoS Comput Biol 2012;8(12):e1002822.
84. Ashburn TT, Thor KB. Drug repositioning: identifying and developing new uses for existing drugs. Nat Rev Drug Discov 2004;3(8):673-83.
85. Gordon DE, Jang GM, Bouhaddou M, et al. A SARS-CoV-2-Human Protein-Protein Interaction Map Reveals Drug Targets and Potential Drug-Repurposing. bioRxiv 2020.
86. Wang WH, McNatt LG, Pang IH, et al. Increased expression of serum amyloid A in glaucoma and its effect on intraocular pressure. Invest Ophthalmol Vis Sci 2008;49(5):1916-23.

Chapter 2

A SYSTEMATICALLY DERIVED OVERVIEW OF THE NON-UBIQUITOUS PATHWAYS AND GENES THAT DEFINE THE MOLECULAR AND GENETIC SIGNATURE OF THE HEALTHY TRABECULAR MESHWORK

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Abstract

Purpose: The trabecular meshwork (TM) is situated in the most frontal part of the eye and is thought to play an important role in the regulation of the eye pressure. However, this tissue is rather difficult to harvest for research. The purpose of this study is therefore to integrate the existing gene expression data of the healthy TM to increase sample size and identify its signature genes and pathways. This provides a robust reference for the study of molecular disease processes and supports the selection of candidate target genes for new treatments.

Methods: A systematic search identified microarray data of healthy TM tissue. After quality control, datasets of low quality and deviating samples were excluded. Remaining individuals were jointly normalized and integrated into one database. The average gene expression of each tested gene over all individuals was calculated. The 25% genes with the highest average expression were identified as the most active genes in the healthy TM and used as input for pathway and network analysis. Additionally, ubiquitous pathways and genes were identified and excluded from the results. Lastly, we identified genes which are likely to be TM-specific.

Results: The gene expression data of 44 individuals, obtained from 18 datasets, were jointly normalized. Ubiquitous genes (n=688) and ubiquitous pathways (n=73) were identified and excluded. Following, 1882 genes and 211 pathways were identified as the signature genes and pathways of the healthy TM. Pathway analysis revealed multiple molecular processes of which some were already known to be active in the TM, for example extracellular matrix and elastic fiber formation. Forty-six candidate TM-specific genes were identified. These consist mainly of pseudogenes or novel transcripts of which the function is unknown.

Conclusions: In this comprehensive meta-analysis we identified non-ubiquitous genes and pathways that form the signature of the functioning of the healthy TM. Additionally, 46 candidate TM-specific genes were identified. This method can also be used for other tissues that are difficult to obtain for study.

Introduction

The trabecular meshwork (TM), located in the iridocorneal angle of the eye, plays an important role in regulating the intraocular pressure (IOP). The IOP is established by a continuous balance in the production and outflow of aqueous humor.[1] The greater part of the outflow pathway resides in the TM and in case of more resistance to outflow, the IOP increases.[2] An elevated IOP is the most important risk factor for glaucoma.[3] In many types of glaucoma, such as primary open angle glaucoma (POAG) or corticosteroid-induced glaucoma, there is an increase in IOP despite any known physical obstructions of the outflow pathway. Therefore, molecular changes in the TM itself are bound to decrease the outflow of the aqueous humor.

In order to obtain a better understanding in the pathogenesis of the impaired outflow mechanism, glaucoma diseased TM tissue has been investigated extensively. Among other types of research, multiple microarray studies have been performed. These suggested that changes in the extracellular matrix (ECM), adhesion, inflammation, and collagen are involved in the pathogenesis.[4-12] In contrast, the genes and molecular processes which are required for the functioning of the TM in healthy individuals are largely unknown. As the TM is situated in the anterior segment of the eye, it is very difficult to obtain biopsies in healthy patients as this would disrupt the integrity of the eye which entails multiple risks. Therefore, samples of the healthy trabecular meshwork are relatively scarce. However, previously performed studies on diseased TM tissue also included healthy control samples. Re-using and integrating these healthy gene expression data permits to increase the sample size and leads to the establishment of the transcriptome of the healthy TM.

In this study, we performed a systematic meta-analysis using a standardized step-by-step approach which enabled us to identify, quality-check, integrate and investigate all the publicly available gene expression data of the healthy human TM tissue. This led to the establishment of the non-housekeeping genes and processes that play a role in the functioning of the TM of healthy individuals. In addition, genes specifically expressed in the TM were identified as well. A better knowledge of the identified genes and processes will help to increase the understanding of the pathogenesis of glaucoma and may support the identification of candidate target genes for new treatment options.

Material and methods

Systematic search

We conducted a systematic search in Gene Expression Omnibus (GEO) and ArrayExpress in order to identify microarray datasets in which the gene expression of TM tissue derived from healthy individuals was measured. Only 'trabecular meshwork' without any additional restrictions was used as search term in order not to miss any relevant data. The search was lastly updated on 2020-03-19. To verify that we did not miss any eligible datasets, we examined the reference lists of all included studies as well. TM samples were included if they were derived from individuals without a history of any type of glaucoma and were exposed to a neutral medium. Therefore, samples were excluded if they had been exposed to corticosteroids or any other type of drugs, or any conditions mimicking stress or stretch.

Pre-processing and quality control

Each of the identified microarray datasets were subjected to a thorough quality control and pre-processed for further analysis. If additional information was desired during any of the below described steps, the authors of the datasets were approached. We designed a step-by-step workflow in R to make sure that each dataset was subjected to a standardized pre-processing workflow (see supplemental material 1). In general, we used the raw (non-processed) data as provided by the authors. This data was quantile normalized to correct for differences in signal strength between samples. Following, the probe annotations, provided by the authors, were linked to Ensembl identifiers using Ensembl Biomart.[13] In addition, quality assessments on both the raw and normalized data per dataset were independently performed by two researchers (L.M.T.E and I.L.) as described previously.[12, 14] In short, the results of the performed quality control for each dataset were visualized in different plots. These plots were assessed for homogeneity of the data, the signal strength of the different samples in the study, the correlation of expression, and the way in which the samples cluster. In case of disagreement, consensus was achieved by discussion. In case samples behaved divergent based on multiple plots or if datasets had an overall low quality, they were excluded for further analysis.

After the quality control, the control samples were selected. If donors were already pooled (averaged) within the initial dataset, the pooled sample was counted as one individual in our study. In addition, if donors were replicated, the replicates were checked for similar behavior and intensities in the quality control and their gene expression data was pooled for each donor. Therefore, every donor was counted as one individual. Furthermore, if the number of donors in the initial study was uncertain or if samples were highly likely to be derived from the same donor after assessment of the quality control, they were also pooled and counted as one individual.

After performing the above mentioned steps, the obtained results per dataset comprised the measured genes and their mean expression per unique individual. As our main goal was to combine all data to determine which genes are expressed in healthy TM or not, genes with a low expression value were not removed from the datasets.

Integration individuals

In order to level-out study differences, we integrated the data for each of the individuals independently, i.e. separate from the original studies they were in. To obtain this, the data of the genes tested in the different individuals were merged based on their Ensembl identifiers. If a gene was tested multiple times within one individual, the measurement with the highest expression value was maintained. Therefore, only one value was assigned per tested gene and individual. Following, we performed quantile normalization over all the samples at once to make them comparable with each other. Then, the average expression value over each gene was calculated. Only genes that had been tested in at least 17 different individuals were used for further analysis. This ensured that even for genes available in the largest included study (comprising 13 individuals) at least measurements of another four individuals from other studies were required. This resulted in a set of genes that fulfilled the above described criterion and the corresponding averaged expression values. This set was used as input for pathway analysis and allowed the identification of the genes with the highest average expression.

Pathway analysis

Pathway analysis was performed on the integrated dataset as described previously.[12] In short, PathVisio was used to conduct the analysis using the human identifier mapping database from BridgeDb (version: Hs_Derby_Ensembl_91.bridge).[15, 16] Furthermore, the content of three widely adopted pathway databases, WikiPathways[17, 18], KEGG[19-21] and Reactome[22, 23] were downloaded in December 2018 and combined into one collection, which allowed integration of their content into one large pathway collection. The third quartile of the average gene expression of the integrated dataset was calculated and used as cut-off for gene activity during the pathway analysis. An overrepresentation analysis was performed in order to identify pathways with a large number of genes with an average expression above the defined cut-off in relation to the total number of measured genes in the pathway. Pathways with a Z-score ≥ 1.96, a permuted p-value < 0.05, and > 3 active genes in the pathway were considered significantly changed.

Identifying housekeeping pathways and genes

Some pathways and genes are ubiquitously expressed in virtually all tissues as they contribute to basal cellular processes and are therefore called housekeeping pathways and genes. We identified and removed them from our results as we were interested in pathways and genes that contribute more specifically to the functioning of the healthy TM. In order to do this, we downloaded the RNA-sequencing data of 32 different tissues of 122 human individuals through the Expression Atlas website (*https://www.ebi.ac.uk/gxa/home*).[24] The data were downloaded on 2019-06-19 and are available under accession number E-MTAB-2836.[25] The data consisted of the Fragments Per Kilobase Million (FPKM) values for all the samples which we put on a ^{2}log scale. We calculated the average gene expression over each tissue and evaluated the distribution (see supplemental material 2) to allow the determination of sensible cut-off values. We considered an expression value of five or higher in order to only select clearly active genes. Only pathways with a median gene expression above five over all measured genes within the pathway were defined to be active. A pathway was only considered to be housekeeping if it was significantly expressed in at least 80% of the tissues, i.e. in 26 out of 32 tissues. Additionally, housekeeping genes were defined as genes reaching a more stringent cut-off expression value of six or more in at least 80% of the tissues. The identified housekeeping pathways and genes were indicated in our results and not used for further analyses.

Network analysis

Cytoscape (*https://www.cytoscape.org*), an open access tool, was used to perform network analysis in which the genes of the significant non-housekeeping pathways were combined into a network of connected genes.[26] This allows visualization of how the identified pathways are related to each other and how many and which mutual genes are shared between them. To improve the visualization, we excluded disease pathways and only showed pathways expressed in less than 10 out of the 32 investigated tissues from E-MTAB-2836. In addition, only genes that were considered active (average expression above the third quartile) were shown.

TM active and TM specific genes

After removal of the housekeeping genes, the top 25% genes with the highest average expression within our integrated dataset were identified as the most active genes in the healthy TM. These genes were used as input for Gene Ontology (GO) analysis to determine the functions of these genes by using their structured individual annotations.[27] For this analysis, Gorilla (*http://cbl-gorilla.cs.technion.ac.il/*), a freely available online tool, was used.[28] We entered the top 25% highest expressed genes as a single-ranked list and identified the biological process GO terms that were associated with these genes.

Although the identified TM active genes are not housekeeping and therefore not expressed in almost all tissues, they may still be expressed in several other tissues beyond the TM. Consequently, we identified which genes are solely expressed in the TM and not in other tissues, from here on referred to as TM specific genes. A gene was defined as a candidate TM specific gene if it belonged to the top 25% highest expressed genes in the TM in our study and was not expressed in any of the tissues included in the latest version of the Human Protein Atlas, which was used as reference dataset (*https:www.proteinatlas.org*; RNA consensus tissue gene data as downloaded on 2019-07-01). This dataset contains the RNA gene expression data of 62 different tissues. We excluded 14 tissues with missing values for genes that had been tested in the other included tissues. Therefore, for all the 48 included tissues, the gene expression for each of the tested genes was known. The excluded tissues consisted mainly of brain regions and sexual organs. In addition, genes found on alternative haplotype versions for variable genomic regions, and as such carrying alternative identifiers, were excluded from our dataset. These genes may be expressed in other tissues as well, however, different haplotype identifiers were tested in the dataset and reference databases, resulting in false-positive results.

A flowchart overviewing the methods and results can be found in figure 1.

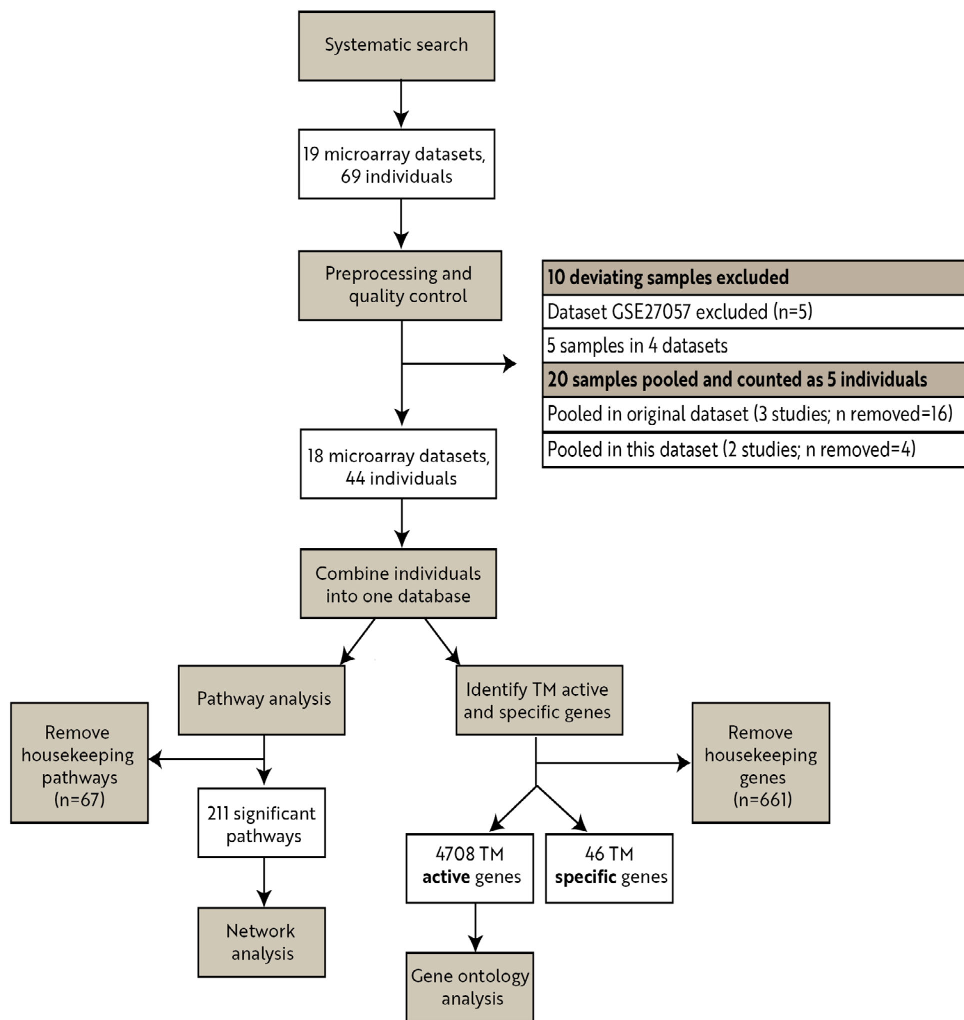


Figure 1. *Flowchart overviewing the methods and results.*

Results

Identified datasets

The search in GEO and ArrayExpress resulted in 36 and 28 datasets, respectively. After removal of duplicates, 42 unique datasets were identified of which 23 datasets were excluded. A flowchart visualizing the selection procedure can be found in figure 2. No additional datasets could be identified after examining the reference lists of all included studies. In total, 19 datasets were included in our study.[29-45]

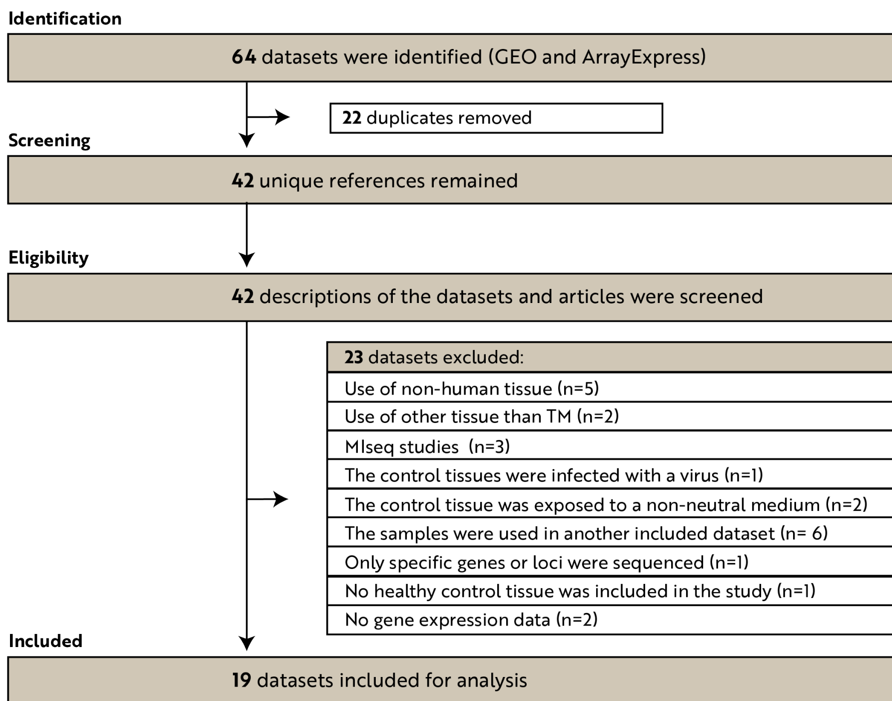


Figure 2. *Flow chart of the systematic search and inclusion of the datasets.*

All of the included datasets used post-mortem tissue. Cultured cells were used in twelve studies (63.2%), six studies used native TM tissue (31.6%), one study used both cultured and native TM tissue (5.2%), and one study used a perfusion organ culture system (5.2%). The initial datasets comprised a total of 69 healthy individuals. The largest study contained 13 healthy individuals and the smallest study only one. An overview of the identified datasets and their characteristics can be found in table 1.

Table 1. *The basic characteristics of the 19 included datasets.*

	Study	Study design	Post-mortem time	Control medium	Passage cells	# of control samples	# of control samples excluded	# of replicates excluded	Pooled in primary analysis	Pooled in this study	Total samples included	Age	Sex	Microarray	Published
1	Russel et al. GSE492 (2003)	Cell cultures	30-36h	0.1% ethanol	< 5	5	0	0 out of 2 replicates	Yes	Yes	1	30,66,73, 76,86	Not specified	Affymetrix Human Genome U95 Version 2	PMID: 12714628
2	Liton et al. GSE4316 (2006)	TM tissue + cell cultures	< 24h	Optisol	3	3 TM tissue; 3 cultured	3 cultured TM cells, Cont_A	Not applicable	No	No	2	Tissue: 70, 85, 78 Cultured: 45, 25, 58	Tissue: F,F,M Cultured: F, F, M	Affymetrix Human Genome U133 Plus 2.0	PMID: 16862071
3	Russel et al. GSE7144 (2007)	Cell cultures	± 30h	EtOH*	3-5	5 pairs of eyes	0	0 out of 2 replicates	Yes	Yes	1	16,66,67,73, 76 years	Not specified	Affymetrix Human Genome U133A	PMID: 15505052
4	Fan et al. GSE6298 (2008)	Cell cultures	Not specified	BA* 0,0025%, 0,025%	8	1	0	0 out of 3 replicates	No	No	1	52 years	Male	Stanford Human cDNA SHEW	PMID: 18436822
5	Luna et al. GSE14768 (2009)	Cell cultures	< 48h	DMEM*	3	3	1	Not applicable	No	No	2	14, 16, 25	Not specified	Duke Operon Human Oligo Arrays 35K version 3.0	PMID: 19279691
6	Luna et al. GSE18713 (2009)	Cell cultures	< 48h	DMEM*	Not specified	1	0	0 out of 3 replicates excluded	No	No	1	Not specified	Not specified	Affymetrix Human Genome U133A 2.0	PMID: 19956414
7	Nehmé et al. GSE16643 (2010)	Cell cultures	Not specified	DMSO*	3-6	2	1	Not applicable	No	No	1	3 months 35 years	Not specified	Agilent-014850 Whole Human Genome Microarray 4x44K G4112F	PMID: 19744340
8	Elahi et al. GSE27275 (2011)	Cell cultures	Not specified	DMEM*	4–6	2	0	0 out of four replicates	No	No	2	25, 30	Male	Illumina Humanref-8 v3.0 expression beadchip	PMID: 21617755
9	Diskin et al. GSE27057 (2011)	TM tissue	< 24h	Not specified	Not specified	5	5	-	-	-	0	66-87 (all samples)	Not defined	Custom Affymetrix Glyco v2 GeneChips	PMID: 16565384
10	Tay et al. GSE28679 (2012)	Cell cultures	Not specified	DMEM*	4-7	3 pairs of eyes	0	0 out of 6 replicates	No	No	3	Not specified	Not specified	Illumina humanRef-8 v2.0 expression beadchip	PMID: 22364584
11	Kwon et al. GSE37474 (2012)	Perfusion organ culture system	5-8h	Perfusion with DMEM* (2 μl/min)	Not applicable	5 pairs of eyes	0	Not applicable	No	No	5	77, 69, 51, 73, 90	4 male, 1 female (= 90 years old)	Affymetrix Human Genome U133 Plus 2.0	Full text received after contact

12	Borras et al. E-MEXP-3434 (2012)	Cell cultures	Not specified	IMEM	4	2	0	3 replicates out of 7	No	Yes	1	29, 39	Not specified	A-AFFY-44 - Affymetrix GeneChip Human Genome U133 Plus 2.0	PMID: 22615763
13	Liton et al. GSE32169 (2012)	Cell cultures	< 48h	DMEM	3	Unclear (max 4)	2 samples exposed to 40% oxygen	Unclear	No	Yes	1	30-60	Not specified	Affymetrix Human Genome U133 Plus 2.0	PMID: 22529935
14	Janssen et al. GSE50784 (2013)	TM tissue	< 24h	Not applicable	Not applicable	2	0	Not applicable	No	No	2	Not specified	Not specified	Agilent-014850 Whole Human Genome Microarray 4x44K G4112F	Method published in PMID: 23028713
15	Braun et al. GSE41102 (2013)	TM tissue	Not specified	RNALater	Not applicable	6	0	Not applicable	Yes	Yes	1	Not specified	Not specified	Affymetrix Human Exon 1.0 ST Array [transcript (gene) version]	PMID: 23500522
16	Liu et al. GSE27276 (2013)	TM tissue	< 72h	RNALater	Not applicable	13	0	1 out of 6 replicates	No	No	13	48-94	Male and female	Sentrix Human-6 Expression BeadChip	PMID: 24003086
17	Matsuda et al. GSE65240 (2015)	Cell cultures	Not specified	DMEM	Not specified	3	0	Not applicable	No	No	3	Not specified	Not specified	- Agilent-028004 SurePrint G3 Human GE 8x60K Microarray - Agilent-039494 SurePrint G3 Human GE v2 8x60K Microarray 039381	PMID: 26066748
18	Sathiyanathan et al. GSE87526 (2017)	TM tissue	< 24h	Trizol	Not applicable	3	0	Not applicable	No	No	3	50-70	Not specified	Illumina HumanRef-8 v3.0 expression beadchip	PMID: 28514956
19	Peters et al. GSE124114 (2018)	Cell cultures	Not specified	0.1% EtOH	Not specified	1	0	2 out of 9 replicates	No	No	1	27	Not specified	Affymetrix Human Gene 1.0 ST Array	Article in press

** Abbreviations: BA = benzyl alcohol ; EtOH = ethanol ; DMEM = Dulbecco's Modified Eagle's medium ; DMSO = Dimethyl sulfoxide*

Quality control

The complete results of the quality control are shown in supplemental material 3. The quality control led to the exclusion of one dataset (containing five samples), and additionally five individual samples and six replicates from other datasets. In addition, based on the provided data and the results of the quality control, samples or replicates within some studies were pooled together. This resulted in a remainder of 18 datasets, containing the gene expression data of 44 individuals. The most relevant observations of the quality control are described below. Dataset GSE27057 generated their data with a custom Affymetrix Glyco v2 GeneChip which contained both human and mouse probes for a specific chosen subset of genes. We were able to link the provided identifiers to the more generic Ensembl identifiers. This resulted in the identification of 752 unique human genes, which is a much lower number of genes compared to the other studies. Based on these findings, this dataset was excluded from further analysis. In addition, five samples from four different datasets were excluded as well. Two samples in dataset GSE32169 were defined as controls in the initial study, however, we excluded them as they were exposed to 40% oxygen which we did not consider as a neutral medium. In dataset GSE16643, a three-month-old donor was excluded as the development of the TM continues in the postnatal period.[1, 46, 47] In dataset GSE4316 and GSE14768, one sample behaved as an outlier in multiple plots of the quality control and was therefore excluded. Furthermore, from datasets E-MEXP-3434, GSE27276 and GSE124114 respectively, three, one and two replicates (in total six replicates) were excluded for further analysis as they behaved as outliers in multiple plots of the quality control.

Three datasets (GSE492, GSE7144 and GSE41102) initially pooled the gene expression data of their healthy donors. Since the data of the individuals was lacking, we used the pooled data but counted the total number of included donors as one individual per study. Furthermore, dataset E-MEXP-3434 included two different donors of which multiple replicates were made. However, as it was not possible to identify which replicates belonged to which donor, all the replicates were pooled together and the two donors were counted as one individual within our study. Lastly, in dataset GSE32169, the total number of donors was not clear. Therefore, the two provided samples were pooled and counted as one individual.

Integration of the data

The 44 individuals that endured the quality control were integrated into one dataset. As explained in the method section, the included genes were limited to those tested in at least 17 individuals to ensure that for genes available in the largest included study at least measurements of another four individuals from other studies were required. The dataset eventually contained 19,489 genes and their average gene expression levels.

Housekeeping pathways and genes

Out of the 1,200 investigated pathways, we identified 73 housekeeping pathways expressed in more than 80% of the tissues investigated in the E-MTAB-2836 database. The complete results of the pathway activity analysis in each of the 32 investigated tissues in E-MTAB-2836 can be found in supplemental material 4. This file also indicates the average gene expression of the TM-tissue over all measured genes per pathway. This allows to compare the results of the housekeeping pathways to the pathway analysis performed on the healthy TM-tissue. In addition, we identified 688 housekeeping genes which are presented in supplemental material 5.

TM active pathways

The third percentile of average gene expression (> 7.516) was used as cut-off for the pathway activity analysis and 278 pathways showed a higher median expression indicating that the pathway is active in the TM tissue. Out of the 278 active pathways, 67 were considered housekeeping (the complete results of the TM pathway activity analysis are shown in supplemental material 6). Most of the housekeeping pathways also showed high median expression in the TM-tissue. Interestingly, some pathways had a very high median expression in the healthy TM but were not identified as housekeeping pathways. Five pathways were not identified as expressed in any of the 32 tissues from dataset E-MTAB-2836 but are highly expressed in the TM (see table 2). If we look at pathways which are expressed in a maximum of ten tissues, multiple other pathways such as miRNA targets in ECM and membrane receptors (WikiPathways, WP2911), elastic fibre formation (Reactome, R-HSA-1566948) and TP53 regulates transcription of DNA repair genes (Reactome, R-HSA-6796648) were found (see table 2). On the other hand, some of the housekeeping pathways did not reach the threshold of a median expression of > 7.516 in the healthy TM. This was for example the case for RHO GTPases activate IQGAPs (Reactome, R-HSA-5626467), inhibition of exosome biogenesis and secretion by manumycin A in CRPC cells (Wikipathways, WP4301), and signaling of hepatocyte growth factor receptor (WikiPathways, WP313) (see supplemental material 4). However, these pathways had a median expression in the TM which was only just below the threshold of being considered as active. Therefore, we should take into account that these were likely overall involved, but power was lacking to retrieve them in the combined dataset of the TM-tissue.

Table 2. *TM active pathways.*

Pathways	Significant in x out of the 32 tissues	Average gene expression TM
KEGG: 2-Oxocarboxylic acid metabolism	10	8
Reactome: Glycogen metabolism	10	7.7
Reactome: Cholesterol biosynthesis	10	7.7
Reactome: HIV Life Cycle	10	7.7
KEGG: Other glycan degradation	10	7.5
KEGG: RNA transport	9	7.7
Reactome: Mitotic G1-G1/S phases	9	7.5
WP: Senescence and Autophagy in Cancer	9	7.5
Reactome: SUMOylation of RNA binding proteins	9	7.5
Reactome: Mitochondrial protein import	8	8
Reactome: Mitotic G2-G2/M phases	8	7.7
Reactome: Deadenylation-dependent mRNA decay	8	7.7
Reactome: alpha-linolenic (omega3) and linoleic (omega6) acid metabolism	8	7.7
Reactome: Host Interactions with Influenza Factors	8	7.7
Reactome: Biosynthesis of electrophilic ω-3 PUFA oxo-derivatives	7	7.9
KEGG: Propanoate metabolism	7	7.7
Reactome: Metabolism of non-coding RNA	7	7.7
Reactome: Elastic fibre formation	7	7.6
Reactome: Mitotic Metaphase and Anaphase	7	7.6
Reactome: SUMOylation of SUMOylation proteins	7	7.6
WP: ncRNAs involved in STAT3 signaling in hepatocellular carcinoma	7	7.6
Reactome: SUMOylation of ubiquitinylation proteins	7	7.6
Reactome: SUMOylation of DNA replication proteins	7	7.5
Reactome: Metallothioneins bind metals	6	9
WP: miRNA targets in ECM and membrane receptors	6	8.6
KEGG: Glyoxylate and dicarboxylate metabolism	6	7.8
Reactome: ATF4 activates genes	6	7.7
KEGG: RNA degradation	6	7.7
Reactome: Transcriptional regulation of pluripotent stem cells	6	7.7
Reactome: TP53 Regulates Transcription of DNA Repair Genes	6	7.6

WP: Sulindac Metabolic Pathway	5	9.3
Reactome: Dissolution of Fibrin Clot	5	7.9
WP: Eukaryotic Transcription Initiation	5	7.8
Reactome: Metabolism of vitamin K	5	7.7
WP: Folate-Alcohol and Cancer Pathway Hypotheses	5	7.6
WP: NLR Proteins	5	7.5
WP: Trans-sulfuration pathway	4	7.9
Reactome: Macroautophagy	4	7.7
KEGG: Biosynthesis of amino acids	4	7.6
KEGG: Aminoacyl-tRNA biosynthesis	3	7.9
Reactome: Mitochondrial iron-sulfur cluster biogenesis	3	7.8
WP: Copper homeostasis	3	7.7
KEGG: Vitamin B6 metabolism	3	7.6
KEGG: Ribosome biogenesis in eukaryotes	3	7.5
Reactome: Asparagine N-linked glycosylation	3	7.5
Reactome: Nucleobase biosynthesis	3	7.5
Reactome: tRNA processing in the nucleus	3	7.5
KEGG: RNA polymerase	2	7.9
Reactome: Mitochondrial Fatty Acid Beta-Oxidation	2	7.7
Reactome: RNA Polymerase III Transcription	2	7.6
KEGG: Autophagy	2	7.5
WP: Evolocumab Mechanism	1	7.7
WP: Proprotein convertase subtilisin/kexin type 9 (PCSK9) mediated LDL receptor degradation	1	7.7
KEGG: Nucleotide excision repair	1	7.6
WP: Transcription factor regulation in adipogenesis	1	7.6
Reactome: Sulfur amino acid metabolism	1	7.6
Reactome: DNA Damage Reversal	0	8.3
Reactome: Protein methylation	0	8.1
Reactome: Interconversion of nucleotide di- and triphosphates	0	7.9
Reactome: Synthesis of wybutosine at G37 of tRNA(Phe)	0	7.8
KEGG: Glycosaminoglycan biosynthesis	0	7.5

Abbreviations: WP = WikiPathways

2

Network analysis

A pathway-gene network (figure 3) was created with the 52 non-disease pathways that met the criteria for being active in less than 10 out of the 32 investigated tissues in E-MTAB-2836 and their highly expressed genes (median expression value > 7.516). Multiple of the identified pathways were connected by genes which cluster process-related pathways together. Clusters consisted of pathways involved in ECM and elastic fiber formation, cell cycle, RNA and DNA regulating processes, metabolic processes and SUMOylation (SUMO: small ubiquitin-like modifier) of proteins, among others. In addition, some of the pathways were not connected to any of the other pathways, for example: glycosaminoglycan biosynthesis (KEGG, 00532), interconversion of nucleotide di- and triphosphates (Reactome, R-HSA-499943), DNA damage reversal (Reactome, R-HSA-73942) and nucleobase biosynthesis (Reactome, R-HSA-8956320). The network with all pathway and gene labels can be investigated in detail on NDEx (*http://www.ndexbio.org/#/network/64573592-384f-11ea-bfdc-0ac135e8bacf?accesskey=0d128d8882c5feba867d4ba63f8a3fd38e006b5bf54c2fbe076bf18e98bfbfc1)*

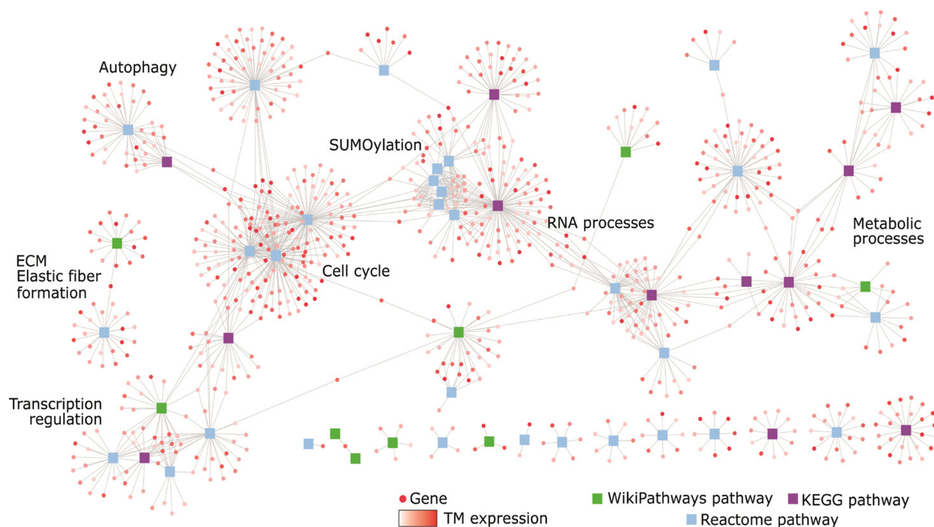


Figure 3. *Gene-pathway network including all pathways which are highly expressed in TM and expressed in less than 10 out of the 32 investigated tissues in E-MTAB-2836.*
Genes are represented as circles and their average TM expression is shown as a gradient (white to red). The pathway nodes are shown as green, purple and blue rectangles for WikiPathways, KEGG and Reactome, respectively. The network with all pathway and gene labels can be investigated in detail on NDEx (http://www.ndexbio.org/#/network/64573592-384f-11ea-bfdc-0ac135e8bacf?accesskey=0d128d8882c5feba867d4ba63f8a3fd38e006b5bf54c2fbe076bf18e98bfbfc1).

TM active and specific genes

Of the 688 housekeeping genes, 661 of these genes were presented in our integrated dataset and removed for further analysis. Thereafter, the top 25% genes with the highest average expression value (>7.404) were identified (see supplemental material 7). This comprised 4708 genes which were used as input for GO analysis to identify the process GO terms that were associated with these genes (see supplemental material 8). This revealed multiple processes such as structural developmental processes, glucose metabolism, regulation of cell communication and extracellular matrix organization. In addition, we identified candidate TM specific genes. We found 127 genes to be expressed in the healthy TM but not in any of the investigated 48 tissues of the Human Protein Atlas. Of these 127 genes, 30 genes were excluded as they were found on alternative haplotype versions for variable genomic regions. In addition, four genes referred to small nucleolar RNA and 47 to long noncoding RNA (of which one pseudogene) and were also excluded as these were not included in the reference database. Therefore, 46 genes remained. The used Human Protein Atlas RNA-sequencing dataset was annotated based on Ensembl release 92 as genome reference, a version in which all 46 genes are present. This suggests that these genes had been tested in the Human Protein Atlas dataset, but were not reported as their mRNAs were not detected above threshold in any sample. The above steps led to a list of highly likely candidates for TM specific genes which contained 46 genes (see table 3). Of these, 14 were ribosomal pseudogenes, shown in a separate part of the table. Additionally, multiple novel transcripts and (transcribed) processed pseudogenes were identified.

Table 3. *TM specific genes.*

Ensembl ID	Gene symbol	Gene description	Chromosomal location	Average gene expression in the TM	Gene type (Ensembl 99)
ENSG00000187653	TMSB4XP8	TMSB4X pseudogene 8	4	11.39	processed_ pseudogene
ENSG00000132967	HMGB1P5	High mobility group box 1 pseudogene 5	3	11.14	transcribed_ processed_ pseudogene
ENSG00000197582	GPX1P1	Glutathione peroxidase pseudogene 1	X	11.01	processed_ pseudogene
ENSG00000198134	PTMAP9	Prothymosin alpha pseudogene 9	12	10.88	processed_ pseudogene
ENSG00000270576	AC005520.4	TAF15 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 68kDa (TAF15) pseudogene	14	10.48	processed_ pseudogene
ENSG00000233369	GTF2IP4	General transcription factor IIi pseudogene 4	7	10.31	transcribed_ processed_ pseudogene
ENSG00000187534	PRR13P5	Proline rich 13 pseudogene 5	19	9.45	processed_ pseudogene

ENSG00000108958	AC130689.1	succinate dehydrogenase complex, subunit C, integral membrane protein, 15kDa (SDHC) pseudogene	17	9.38	processed_pseudogene
ENSG00000204622	HLA-J	Major histocompatibility complex, class I, J (pseudogene)	6	9.32	transcribed_unprocessed_pseudogene
ENSG00000124593	AL365205.1	Novel protein	6	9.28	protein_coding
ENSG00000213029	SPHAR	Protein SPHAR	1	9.12	protein_coding
ENSG00000167774	AC010323.1	novel transcript	19	9.02	protein_coding
ENSG00000228224	NACAP1	NACA family member 4, pseudogene	8	8.86	transcribed_processed_pseudogene
ENSG00000146556	WASH2P	WASP family homolog 2, pseudogene	2	8.74	transcribed_unprocessed_pseudogene
ENSG00000185596	WASH3P	WASP family homolog 3, pseudogene	15	8.67	transcribed_unprocessed_pseudogene
ENSG00000274791	F8A2	Coagulation factor VIII associated 2	X	8.59	protein_coding
ENSG00000173867	AC013489.1	Novel transcript	15	8.51	protein_coding
ENSG00000143429	LSP1P4	LSP1 pseudogene 4	2	8.41	transcribed_unprocessed_pseudogene
ENSG00000258427	RBM8B	RNA binding motif protein 8B pseudogene	14	8.38	processed_pseudogene
ENSG00000073905	VDAC1P1	Voltage dependent anion channel 1 pseudogene 1	X	8.26	processed_pseudogene
ENSG00000204434	POTEKP	POTE ankyrin domain family member K, pseudogene	2	8.10	unprocessed_pseudogene
ENSG00000183055	FAM133CP	Family with sequence similarity 133 member C, pseudogene	10	8.09	processed_pseudogene
ENSG00000078319	PMS2P1	PMS1 homolog 2, mismatch repair system component pseudogene 1	7	8.06	unprocessed_pseudogene
ENSG00000167807	AC011511.1	Novel transcript	19	8.01	protein_coding
ENSG00000261040	WFDC21P	WAP four-disulfide core domain 21, pseudogene	17	7.93	transcribed_unitary_pseudogene
ENSG00000174028	FAM3C2	Family with sequence similarity 3 member C2 (pseudogene)	X	7.92	processed_pseudogene
ENSG00000125695	AC046185.1	Novel transcript	17	7.74	protein_coding
ENSG00000235254	TMEM185AP1	Transmembrane protein 185A pseudogene 1	X	7.74	unprocessed_pseudogene

ENSG00000218574	HNRNPA1P37	Heterogeneous nuclear ribonucleoprotein A1 pseudogene 37	6	7.73	processed_pseudogene
ENSG00000228409	CCT6P1	Chaperonin containing TCP1 subunit 6 pseudogene 1	7	7.72	transcribed_unprocessed_pseudogene
ENSG00000171570	RAB4B-EGLN2	RAB4B-EGLN2 readthrough (NMD candidate)	19	7.68	protein_coding
ENSG00000231584	FAHD2CP	Fumarylacetoacetate hydrolase domain containing 2C, pseudogene	2	7.52	transcribed_unprocessed_pseudogene
		Ribosomal pseudogenes			
ENSG00000227063	RPL41P1	Ribosomal protein L41 pseudogene 1	20	14.22	processed_pseudogene
ENSG00000136149	RPL13AP25	Ribosomal protein L13a pseudogene 25	13	13.36	processed_pseudogene
ENSG00000227077	AC107983.1	Ribosomal protein S28 (RPS28) pseudogene	17	13.35	processed_pseudogene
ENSG00000212664	AC064799.1	Ribosomal protein L17 (RPL17) pseudogene	15	12.91	processed_pseudogene
ENSG00000139239	RPL14P1	Ribosomal protein L14 pseudogene 1	12	12.41	processed_pseudogene
ENSG00000226948	RPS4XP2	Ribosomal protein S4X pseudogene 2	20	11.94	transcribed_processed_pseudogene
ENSG00000216368	AL136226.1	Ribosomal protein L7A (RPL7A) pseudogene	6	11.92	processed_pseudogene
ENSG00000101278	RPS10P5	Ribosomal protein S10 pseudogene 5	20	10.91	processed_pseudogene
ENSG00000235581	AC005011.1	Ribosomal protein S28 (RPS28) pseudogene	7	9.87	processed_pseudogene
ENSG00000060303	RPS17P5	Ribosomal protein S17 pseudogene 5	6	9.52	processed_pseudogene
ENSG00000213063	RPL29P7	Ribosomal protein L29 pseudogene 7	1	8.28	processed_pseudogene
ENSG00000178429	RPS3AP5	Ribosomal protein S3A pseudogene 5	10	8.18	processed_pseudogene
ENSG00000224892	RPS4XP16	Ribosomal protein S4X pseudogene 16	13	7.90	transcribed_processed_pseudogene
ENSG00000229585	RPL21P44	Ribosomal protein L21 pseudogene 44	4	7.72	processed_pseudogene

Discussion

Within this study, we are the first to perform a systematic step-by-step approach on the publicly available gene expression data of TM-tissue derived from healthy individuals. This resulted in an overview of the non-housekeeping genes and processes that are involved in the functioning of the healthy TM. In addition, we identified 46 genes that are highly likely to be TM specific.

Despite the fact that the TM is thought to play a key role in the pathogenesis of multiple types of open angle glaucoma, little is known about the processes and genes involved in the functioning of the healthy TM. To our knowledge, only five studies specifically investigated the gene expression in the healthy TM.[48-52] Three of these studies used either single-pass sequencing[48], serial analysis of gene expression (SAGE)[49] or sequence tag[51]. These techniques were very innovative at that time but since the development of transcriptomics studies, even more genes can be investigated at once. The study of Carnes et al. performed RNA sequencing on the trabecular meshwork of healthy patients, however they investigated only four adult patients.[52] The study of Paylakhi et al. was the first to integrate gene expression data of the healthy TM.[50] However, the data was derived from only four studies published between 2004-2011. Since then the number of genes tested on the arrays have increased and many more relevant microarray studies have been performed. Although the five above mentioned studies used older techniques and data or included smaller samples sizes, the processes they found after performing gene ontology on their identified genes were comparable to the processes we found. However, integrating the data of the studies that are now available resulted in a larger dataset, containing more individuals and additional genes. It also allowed to increase the samples size and to filter out study-based differences related to, for example, used methods or differences between included individuals, thereby generating more robust results. In addition, we performed pathway and network analysis on the integrated data which allowed the identification of TM active processes.

Within this study, we identified 1882 non-ubiquitous TM active genes and 211 non-ubiquitous pathways. Both the results of the pathway analysis as well as the results of the GO analysis on the top 25% TM active genes revealed that processes with the highest significance are involved in developmental processes, cell communication, cell signaling, cell cycle, cell metabolism, apoptosis and cell maintenance. The identified processes are in line with the expectancies as we are looking at the functioning of a healthy tissue and are a validation of our analyses. The GO analysis reported similar results, also validating the results of the pathway analysis. Additionally, some of the identified pathways and processes are of special interest as they have been suggested to be involved in the pathogenesis of glaucoma, i.e. ECM, elastic fiber formation, elastin

crosslinking and focal adhesion.[5, 7, 32, 53] It is interesting that pathways describing some of these processes were highly expressed in the TM and to a lesser extent in other tissues (in less than 10 out of the 32 investigated tissues from E-MTAB-2836), indicating that these processes are rather characteristic for the TM tissue. We cannot designate these pathways specific for the TM tissue due to the fact that pathways generally describe relatively common, generic processes which have often been investigated in other tissues than the eye. The pathway elastic fiber formation for example was also highly expressed in lung tissue, prostate, and urinary bladder, all tissues in which elastic fibers are known to be involved. The fact that the above-mentioned processes are found to be involved in the functioning of the healthy TM, strengthens the hypothesis that they might be altered in patients with glaucoma and therefore might be causative for the development of the disease. In an earlier study, we already identified clues for the involvement of some of the above mentioned processes in the pathogenesis of POAG.[12]

Lastly, we identified 46 genes that are highly likely to be TM specific. It is of interest to note that multiple of the identified genes are pseudogenes. Pseudogenes are defined as segments of DNA that are related to a functional gene but are likely not able to code for the functioning of the related gene due to some decay in their DNA. This decay can be a point mutation, insertion, deletion, misplaced stop codon, or frame shift of a gene. The function of pseudogenes is unknown but they are thought to have important roles in gene regulation.[54, 55] Some pseudogenes are known to be able to produce peptides or proteins.[56, 57] Pseudogenes can also be involved in multiple processes and have been shown to be specifically expressed in cancers or diseases.[58] For example, *PTEN1P1*, the pseudogene of *PTEN*, represses cell growth by increasing the expression of *PTEN*. Research showed that the *PTENP1* locus was lost in some human cancer cells which caused a decreased expression of *PTEN* and an abnormal proliferation of cancer cells.[59] In addition, some siRNAs appear to be derived from pseudogenes which suggests that some pseudogenes are involved in regulating protein-coding transcripts.[60] Another possibility is that these pseudogenes are in fact protein coding genes, but have not been identified as such, because they are not expressed in most other tissues. Therefore, pseudogenes suggest a functional impact and as these pseudogenes may fulfill important and unknown functions, they are of interest for further investigation for their role in the functioning of the TM. In addition, multiple of the retrieved genes code for novel proteins or transcripts of which the function is still unknown.

Although these genes are suggested to be TM specific, we have to keep in mind that this is based on only two tissue databases. Despite the fact that these two are the most extensive databases, they do not contain the data of all human tissues. Therefore, it is possible that these genes are expressed in other tissues not covered by these two databases. However, the fact that these 46 genes were highly expressed in the TM and not at all expressed in any of the tissues in the used reference database, makes them of interest for further investigation. This is strengthened by the fact that most of these genes are annotated as pseudogenes or novel transcripts of which the function is largely unknown.

One of the strengths of this study is that we identified the available gene expression data of the TM of healthy individuals through a systematic search and integrated the data of all retrieved studies. As the TM is difficult to obtain and therefore rather sparse, integrating all available data allowed to increase the sample size significantly. This also allowed to level-out study differences and permitted the identification of the genes with an overall high average expression throughout all the studies. In addition, this method can also be used for other tissues that are difficult to obtain for study. However, similar to a regular meta-analysis, the data were derived from other researchers and therefore, a critical appraisal of the quality is necessary. We performed an extensive quality control on each of the datasets in order to obtain a better insight in the quality of the experiments and the data deducted therefrom. Samples deviating within a dataset were removed and datasets with an overall low quality were excluded as well. The importance of performing a quality control is stressed by the fact that we had to exclude one dataset, five samples and six replicates due to a low number of studied genes, insufficient quality or inconsistencies in the data. Furthermore, the included studies used different study methods. In order to minimalize the consequences of individual variability and experimental techniques, we integrated the data on the level of the individuals and not on study level. In addition, both cultured and native TM tissue samples were used in the included datasets. As Liton et al. compared the gene expression profiles of cultured and native TM cells and found more than 90% similarity between the expressed genes, we did not make a distinction between these cell types.[32] The included studies also used different methods to obtain the tissue. It is known that the isolation of pure TM-cells is difficult as the TM-tissue is closely surrounded with multiple other tissue.[61] Therefore, it is a possibility that some parts of the surrounding tissues were extracted as well. However, we performed a quality control on each of the datasets, checking the relations of the samples within one study which minimalizes the chance of including a single sample that contained little or no TM-cells. In addition, we performed pathway analysis on each of the datasets separately and the results were not only comparable between these separate datasets but also with the results of the pathway analysis of the combined dataset (data not shown).

The results that are published in this study do not only provide an overview of the processes and genes that are involved in the functioning of the healthy TM. The used methods and obtained results can be used by other researchers as well. As mentioned before, the methods presented to integrate and re-analyze publicly available gene expression data of the healthy TM can also be used to investigate the gene expression data of other tissues. In addition, we identified the housekeeping genes and pathways. The latter are presented in supplemental material 4 and 5 and can be used as a reference for those interested in identifying tissue 'specific' genes and pathways. Furthermore, in supplemental material 9, we present the complete list of genes that have been investigated in the TM and their average gene expression. This list can be used to directly check the average expression value in the TM of a gene or multiple genes of interest. In addition, the list can be used as a reference database to which the gene expression data of diseased TM-tissue (for example from patients with glaucoma) can be compared. Within supplemental material 9, we also indicated the housekeeping, TM active and TM specific genes, allowing this supplemental material to be a comprehensive reference database for investigating molecular disease mechanisms.

In conclusion, we present an overview of the processes and genes that are involved in the functioning of the healthy TM which can be used for future reference. We showed that processes which are thought to play a role in the pathogenesis of glaucoma such as those related to ECM, elastic fiber formation and actin crosslinking are important for the functioning of the healthy TM which strengthens the hypothesis of them being altered in patients with glaucoma. In addition, multiple highly likely TM specific genes have been suggested which can be used for further functional investigation and might be of special interest for drug targeting of their encoded proteins. Furthermore, the complete lists with housekeeping genes and pathways and the investigated genes in the TM with their average gene expression are provided, allowing other researchers to use this data as a reference for investigating molecular disease mechanisms.

References

1. Abu-Hassan DW, Acott TS, Kelley MJ. The Trabecular Meshwork: A Basic Review of Form and Function. J Ocul Biol 2014;2(1).
2. Goel M, Picciani RG, Lee RK, Bhattacharya SK. Aqueous humor dynamics: a review. Open Ophthalmol J 2010;4:52-9.
3. Sommer A, Tielsch JM, Katz J, et al. Relationship between intraocular pressure and primary open angle glaucoma among white and black Americans. The Baltimore Eye Survey. Arch Ophthalmol 1991;109(8):1090-5.
4. Jones R, 3rd, Rhee DJ. Corticosteroid-induced ocular hypertension and glaucoma: a brief review and update of the literature. Curr Opin Ophthalmol 2006;17(2):163-7.
5. Last JA, Pan T, Ding Y, et al. Elastic modulus determination of normal and glaucomatous human trabecular meshwork. Invest Ophthalmol Vis Sci 2011;52(5):2147-52.
6. Lutjen-Drecoll E, Futa R, Rohen JW. Ultrahistochemical studies on tangential sections of the trabecular meshwork in normal and glaucomatous eyes. Invest Ophthalmol Vis Sci 1981;21(4):563-73.
7. Rohen JW, Futa R, Lutjen-Drecoll E. The fine structure of the cribriform meshwork in normal and glaucomatous eyes as seen in tangential sections. Invest Ophthalmol Vis Sci 1981;21(4):574-85.
8. Tektas OY, Lutjen-Drecoll E. Structural changes of the trabecular meshwork in different kinds of glaucoma. Exp Eye Res 2009;88(4):769-75.
9. Yuan Y, Call MK, Yuan Y, et al. Dexamethasone induces cross-linked actin networks in trabecular meshwork cells through noncanonical wnt signaling. Invest Ophthalmol Vis Sci 2013;54(10):6502-9.
10. Kersey JP, Broadway DC. Corticosteroid-induced glaucoma: a review of the literature. Eye (Lond) 2006;20(4):407-16.
11. Raghunathan VK, Morgan JT, Park SA, et al. Dexamethasone Stiffens Trabecular Meshwork, Trabecular Meshwork Cells, and Matrix. Invest Ophthalmol Vis Sci 2015;56(8):4447-59.
12. Liesenborghs I, Eijssen LMT, Kutmon M, et al. Comprehensive bioinformatics analysis of trabecular meshwork gene expression data to unravel the molecular pathogenesis of primary open-angle glaucoma. Acta Ophthalmol 2019.
13. Smedley D, Haider S, Durinck S, et al. The BioMart community portal: an innovative alternative to large, centralized data repositories. Nucleic Acids Res 2015;43(W1):W589-98.
14. Liesenborghs I, Eijssen LMT, Kutmon M, et al. The molecular processes in the trabecular meshwork after exposure to corticosteroids and in corticosteroid-induced ocular hypertension. Invest Ophthalmol Vis Sci 2020.
15. Kutmon M, van Iersel MP, Bohler A, et al. PathVisio 3: an extendable pathway analysis toolbox. PLoS Comput Biol 2015;11(2):e1004085.
16. van Iersel MP, Kelder T, Pico AR, et al. Presenting and exploring biological pathways with PathVisio. BMC Bioinformatics 2008;9:399.
17. Kelder T, van Iersel MP, Hanspers K, et al. WikiPathways: building research communities on biological pathways. Nucleic Acids Res 2012;40(Database issue):D1301-7.

18. Kutmon M, Riutta A, Nunes N, et al. WikiPathways: capturing the full diversity of pathway knowledge. Nucleic Acids Res 2016;44(D1):D488-94.
19. Kanehisa M, Furumichi M, Tanabe M, et al. KEGG: new perspectives on genomes, pathways, diseases and drugs. Nucleic Acids Res 2017;45(D1):D353-D61.
20. Kanehisa M, Sato Y, Kawashima M, et al. KEGG as a reference resource for gene and protein annotation. Nucleic Acids Res 2016;44(D1):D457-62.
21. Kanehisa M, Goto S. KEGG: kyoto encyclopedia of genes and genomes. Nucleic Acids Res 2000;28(1):27-30.
22. Fabregat A, Sidiropoulos K, Garapati P, et al. The Reactome pathway Knowledgebase. Nucleic Acids Res 2016;44(D1):D481-7.
23. Milacic M, Haw R, Rothfels K, et al. Annotating cancer variants and anti-cancer therapeutics in reactome. Cancers (Basel) 2012;4(4):1180-211.
24. Petryszak R, Keays M, Tang YA, et al. Expression Atlas update--an integrated database of gene and protein expression in humans, animals and plants. Nucleic Acids Res 2016;44(D1):D746-52.
25. [dataset] Björn M Hallström (2015). RNA-seq of coding RNA from tissue samples of 122 human individuals representing 32 different tissues a-r, V1. *https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-2836*.
26. Shannon P, Markiel A, Ozier O, et al. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res 2003;13(11):2498-504.
27. Ashburner M, Ball CA, Blake JA, et al. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat Genet 2000;25(1):25-9.
28. Eden E, Navon R, Steinfeld I, et al. GOrilla: a tool for discovery and visualization of enriched GO terms in ranked gene lists. BMC Bioinformatics 2009;10:48.
29. Fan BJ, Wang DY, Tham CC, et al. Gene expression profiles of human trabecular meshwork cells induced by triamcinolone and dexamethasone. Invest Ophthalmol Vis Sci 2008;49(5):1886-97.
30. Janssen SF, Gorgels TG, Bossers K, et al. Gene expression and functional annotation of the human ciliary body epithelia. PLoS One 2012;7(9):e44973.
31. Kennedy KD, AnithaChristy SA, Buie LK, Borras T. Cystatin a, a potential common link for mutant *myoc*ilin causative glaucoma. PLoS One 2012;7(5):e36301.
32. Liton PB, Luna C, Challa P, et al. Genome-wide expression profile of human trabecular meshwork cultured cells, nonglaucomatous and primary open angle glaucoma tissue. Mol Vis 2006;12:774-90.
33. Liu Y, Allingham RR, Qin X, et al. Gene expression profile in human trabecular meshwork from patients with primary open-angle glaucoma. Invest Ophthalmol Vis Sci 2013;54(9):6382-9.
34. Luna C, Li G, Liton PB, et al. Alterations in gene expression induced by cyclic mechanical stress in trabecular meshwork cells. Mol Vis 2009;15:534-44.
35. Luna C, Li G, Qiu J, et al. Role of miR-29b on the regulation of the extracellular matrix in human trabecular meshwork cells under chronic oxidative stress. Mol Vis 2009;15:2488-97.
36. Markus H. Kuehn CYK, Young H. Kwon. Dexamethasone induced gene expression changes in human perfusion organ cultured trabecular meshwork. 2012.

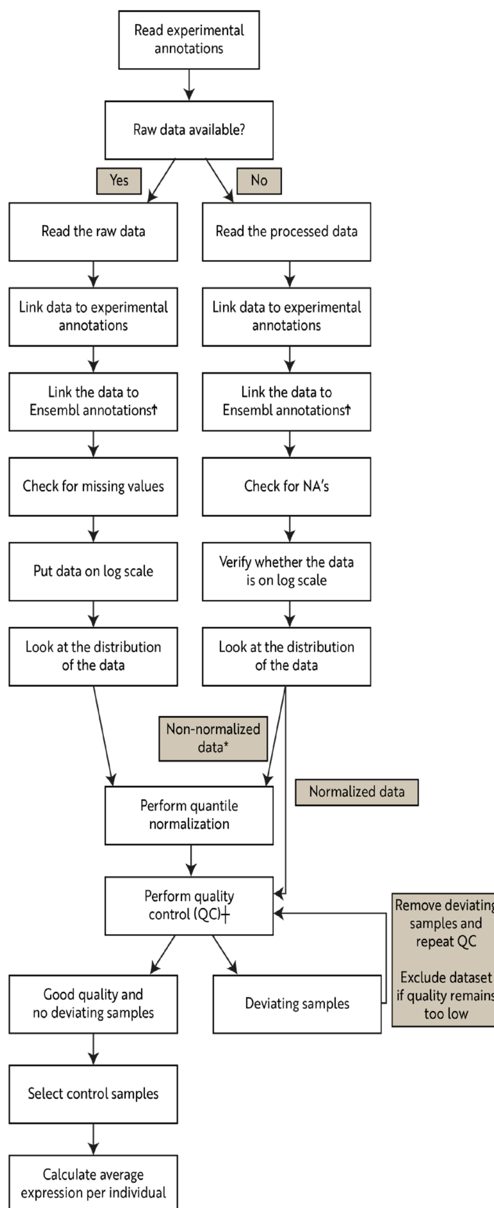
37. Matsuda A, Asada Y, Takakuwa K, et al. DNA Methylation Analysis of Human Trabecular Meshwork Cells During Dexamethasone Stimulation. Invest Ophthalmol Vis Sci 2015;56(6):3801-9.
38. Nehme A, Lobenhofer EK, Stamer WD, Edelman JL. Glucocorticoids with different chemical structures but similar glucocorticoid receptor potency regulate subsets of common and unique genes in human trabecular meshwork cells. BMC Med Genomics 2009;2:58.
39. Paylakhi SH, Fan JB, Mehrabian M, et al. Effect of PITX2 knockdown on transcriptome of primary human trabecular meshwork cell cultures. Mol Vis 2011;17:1209-21.
40. Porter KM, Epstein DL, Liton PB. Up-regulated expression of extracellular matrix remodeling genes in phagocytically challenged trabecular meshwork cells. PLoS One 2012;7(4):e34792.
41. Sathiyanathan P, Tay CY, Stanton LW. Transcriptome analysis for the identification of cellular markers related to trabecular meshwork differentiation. BMC Genomics 2017;18(1):383.
42. Tay CY, Sathiyanathan P, Chu SW, et al. Identification and characterization of mesenchymal stem cells derived from the trabecular meshwork of the human eye. Stem Cells Dev 2012;21(9):1381-90.
43. Wagner AH, Anand VN, Wang WH, et al. Exon-level expression profiling of ocular tissues. Exp Eye Res 2013;111:105-11.
44. Zhao X, Pearson KE, Stephan DA, Russell P. Effects of prostaglandin analogues on human ciliary muscle and trabecular meshwork cells. Invest Ophthalmol Vis Sci 2003;44(5):1945-52.
45. Zhao X, Ramsey KE, Stephan DA, Russell P. Gene and protein expression changes in human trabecular meshwork cells treated with transforming growth factor-beta. Invest Ophthalmol Vis Sci 2004;45(11):4023-34.
46. Gould DB, Smith RS, John SW. Anterior segment development relevant to glaucoma. Int J Dev Biol 2004;48(8-9):1015-29.
47. Johnston MC, Noden DM, Hazelton RD, et al. Origins of avian ocular and periocular tissues. Exp Eye Res 1979;29(1):27-43.
48. Gonzalez P, Epstein DL, Borras T. Characterization of gene expression in human trabecular meshwork using single-pass sequencing of 1060 clones. Invest Ophthalmol Vis Sci 2000;41(12):3678-93.
49. Liu Y, Munro D, Layfield D, et al. Serial analysis of gene expression (SAGE) in normal human trabecular meshwork. Mol Vis 2011;17:885-93.
50. Paylakhi SH, Yazdani S, April C, et al. Non-housekeeping genes expressed in human trabecular meshwork cell cultures. Mol Vis 2012;18:241-54.
51. Tomarev SI, Wistow G, Raymond V, et al. Gene expression profile of the human trabecular meshwork: NEIBank sequence tag analysis. Invest Ophthalmol Vis Sci 2003;44(6):2588-96.
52. Carnes MU, Allingham RR, Ashley-Koch A, Hauser MA. Transcriptome analysis of adult and fetal trabecular meshwork, cornea, and ciliary body tissues by RNA sequencing. Exp Eye Res 2018;167:91-9.
53. Fuchshofer R, Tamm ER. Modulation of extracellular matrix turnover in the trabecular meshwork. Exp Eye Res 2009;88(4):683-8.
54. Tutar Y. Pseudogenes. Comp Funct Genomics 2012;2012:424526.
55. Vanin EF. Processed pseudogenes: characteristics and evolution. Annu Rev Genet 1985;19:253-72.

56. Betran E, Wang W, Jin L, Long M. Evolution of the phosphoglycerate mutase processed gene in human and chimpanzee revealing the origin of a new primate gene. Mol Biol Evol 2002;19(5):654-63.
57. Pink RC, Wicks K, Caley DP, et al. Pseudogenes: pseudo-functional or key regulators in health and disease? RNA 2011;17(5):792-8.
58. Li W, Yang W, Wang XJ. Pseudogenes: pseudo or real functional elements? J Genet Genomics 2013;40(4):171-7.
59. Poliseno L, Salmena L, Zhang J, et al. A coding-independent function of gene and pseudogene mRNAs regulates tumour biology. Nature 2010;465(7301):1033-8.
60. Chan WL, Chang JG. Pseudogene-derived endogenous siRNAs and their function. Methods Mol Biol 2014;1167:227-39.
61. Keller KE, Bhattacharya SK, Borras T, et al. Consensus recommendations for trabecular meshwork cell isolation, characterization and culture. Exp Eye Res 2018;171:164-73.
62. Gautier L, Cope L, Bolstad BM, Irizarry RA. affy--analysis of Affymetrix GeneChip data at the probe level. Bioinformatics 2004;20(3):307-15.

Supplemental material

All supplemental material can be found on https://pubmed.ncbi.nlm.nih.gov/35124177/.

Supplemental material 1. *Step-by-step workflow to perform pre-processing and quality control of the datasets.*

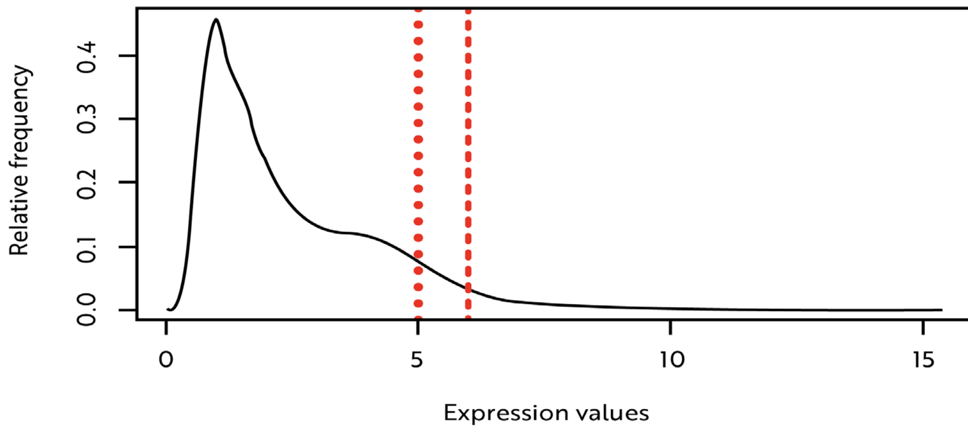


** For some datasets, the data was provided as normalized, however, the distribution of the data and or the QC revealed that it was not normalized yet. Therefore, it was handled as raw data and quantile normalized.*

† This refers to linking the used probe identifiers to a recognizable gene name. We used Ensembl ID's which were obtained through BioMart.

┼The QC was also performed on the raw data, however, to maintain the overview of the flow chart, this was not displayed.

Supplemental material 2. *Figure of the distribution of the average expression values of all measured genes over de 32 included tissues of dataset E-MTAB-2836.*



The red, dotted lines represent the cut-off values of five and six which were chosen based on the distribution of the average expression.

Chapter 3

COMPREHENSIVE BIOINFORMATICS ANALYSIS OF TRABECULAR MESHWORK GENE EXPRESSION DATA TO UNRAVEL THE MOLECULAR PATHOGENESIS OF PRIMARY OPEN ANGLE GLAUCOMA

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Abstract

Purpose: Performing bioinformatics analyses using trabecular meshwork (TM) gene expression data in order to further elucidate the molecular pathogenesis of primary open angle glaucoma (POAG), and to identify candidate target genes.

Methods: A systematic search in Gene Expression Omnibus and ArrayExpress was conducted and quality control and pre-processing of the data was performed with *ArrayAnalysis.org*. Molecular pathway overrepresentation analysis was performed with PathVisio using pathway content from three pathway databases: WikiPathways, KEGG, and Reactome. In addition, Gene Ontology (GO) analysis was performed on the gene expression data. The significantly changed pathways were clustered into functional categories which were combined into a network of connected genes.

Results: Ninety-two significantly changed pathways were clustered into five functional categories: extracellular matrix (ECM), inflammation, complement activation, senescence, and Rho GTPase signaling. ECM included pathways involved in collagen, actin, and cell-matrix interactions. Inflammation included pathways entailing *NF-κB* and arachidonic acid. The network analysis showed that several genes overlap between the inflammation cluster on the one hand, and the ECM, complement activation and senescence clusters on the other hand. GO analysis, identified additional clusters, related to development and corticosteroids.

Conclusion: This study provides an overview of the processes involved in the molecular pathogenesis of POAG in the TM. The results show good face validity and confirm findings from histological, biochemical, genome-wide association, and transcriptomics studies. The identification of known points of action for drugs, such as Rho GTPase, arachidonic acid, *NF-κB*, prostaglandins, and corticosteroid clusters support the value of this approach to identify potential drug targets.

Key words: primary open-angle glaucoma, glaucoma, trabecular meshwork, molecular pathogenesis, bioinformatics, pathway analysis, network analysis, gene ontology.

Introduction

Primary open angle glaucoma (POAG) is a potentially sight threatening and complex disease in which the trabecular meshwork (TM) has been proven to play a crucial role.[1] It is well known that an increase in intraocular pressure is the greatest and most common risk factor for POAG.[2] Despite the numerous data that has been collected in research, the molecular pathogenesis of POAG remains largely obscure. Consequently, an effective treatment option addressing these molecular changes is also still missing. Re-using, re-evaluating and integrating the existing data using state-of-the-art bioinformatics approaches, may provide an effective and efficient way to improve our understanding of the molecular pathogenesis of primary open angle glaucoma.

During the past decades, biochemical and histological in vitro studies have been performed in order to identify the pathogenesis of POAG. However, the number of molecules that can be investigated within these studies is limited. Using omics technologies, we are currently able to measure the expression of several thousands of molecules from one sample of affected tissue, leading to an exponential increase in data.

One example of omics studies are transcriptomics studies which investigate the differences in gene expression by comparing the levels of RNA for each gene in the healthy and POAG-diseased TM tissue.[3-6] Genes that are found to be differentially expressed are likely to be associated with POAG. However, a gene is only a small fraction of the molecular processes it is involved in. Pathways represent such molecular processes and shows the genes that are involved within these processes and how they are related to other molecules. By identifying the pathways in which the differentially expressed genes are involved, we can identify disease processes that are likely to play a role in the pathogenesis of POAG.

The aim of this study was to expand the understanding of the molecular pathogenesis of POAG by applying integrative bioinformatics analysis on the available human gene expression data of the TM in patients with POAG and controls. The step-by-step approach applied in this study allows a systematic quality check, investigation and interpretation of the available gene expression data from TM tissue. The obtained results enable us to identify possible drug targets to modulate the disease outcome.

Methods

Systematic search

We conducted a systematic search in two commonly used public repositories, Gene Expression Omnibus (*http://www.ncbi.nlm.nih.gov/geo/*)[7, 8] and ArrayExpress (*http://www.ebi.ac.uk/arrayexpress*)[9], to select and download publicly available data of microarray studies. In each of these studies, the gene expression profiles between human trabecular meshwork cells of participants with and without POAG were compared. The keywords used in this search were primary open angle glaucoma, POAG, glaucoma, gene expression profiling, microarray and trabecular meshwork (cells). The first search was performed on 2016-09-20 and last updated on 2018-11-19.

Quality control and pre-processing of the data

After the systematic search, the data of each identified study was subjected to a thorough quality control and pre-processed to use as input for further analysis. If necessary, the authors of the datasets were approached to obtain additional information. *ArrayAnalysis.org*[10], an online platform with standardized scripts for quality control and pre-processing of gene expression data was used to do this. Where possible, we used the raw data (non-processed data) and evaluated data quality before and after normalization. The results of the quality control of the original data were visualized in multiple plots to allow assessment of the homogeneity of the dataset, the signal strength of the different samples in the dataset, the correlation of expression, and the way in which the samples cluster or contain outliers. Studies of low quality were excluded for further analysis. Within the remaining studies, samples that appeared divergent throughout the quality control were also excluded for further analysis. The quality assessment of the studies and samples was performed by two researchers, independently from each other. Thereafter, the results were compared and in case of disagreement, consensus was achieved by discussion. After data pre-processing, statistical analysis to compare patient and control groups was performed using the limma package for R (linear regression models) as available from Bioconductor.[11, 12] Systematic application of the abovementioned steps led to high-quality data on differential gene expression, which was used as input for pathway and Gene Ontology analysis.

Pathway analysis

After the quality control and pre-processing of the data, we aimed to identify in which molecular pathways the up- and downregulated (differentially expressed) genes are present. To do this, the differential gene expression data that resulted from the statistical analysis was mapped onto known (pre-existing) pathways. As mentioned before, pathways represent molecular processes and the genes that are involved in

these processes. Therefore, if all the genes within a pathway are differentially expressed (that means up- or downregulated) between patients and controls, we can assume that this pathway, and the process it represents, is relevant in the disease process. Similarly, if no genes within a pathway are changed, we can assume that this pathway is not relevant.

However, the reality is more complicated, since mostly not all genes within a pathway are changed and the degree of change may vary as well. Consequently, we performed a pathway overrepresentation analysis using a common cut-off criterion for the degree of change in gene expression based on common practice and agreement. We chose relatively strict cut-off points and considered genes with an absolute ^{2}log fold change (logFC) in gene expression higher than 0.58 (representing a 50% change in expression) and a p-value smaller than 0.05, as significantly changed.

3

By performing pathway analysis, we can determine which pathways have the highest overrepresentation of significantly changed genes and are therefore likely to be involved in the pathogenesis of POAG. The overrepresentation analysis was performed in PathVisio 3.2.4[13, 14], an open access analysis tool. In order to recognize all the elements within the pathways, an identifier mapping database was downloaded from *www.pathvisio.org* (last updated on the 14th of April 2016, version Hs_Derby_Ensembl_85. bridge). In the results of the overrepresentation analysis, pathways with a significance score (Z-score) ≥ 1.96, a permuted p-value < 0.05, and ≥ 3 changed genes in the pathway were considered significantly changed.

As mentioned before, the pathways we studied are available from free access pathway databases. However, as none of the current pathway databases contain all the existing pathways, we combined the three most commonly used pathway databases into one large collection in order to obtain a larger pathway coverage: WikiPathways[15, 16], KEGG[17-19] and Reactome[20,21]. The pathways from each database were downloaded on August 10th 2017. The number of pathways and unique genes in each of these pathway databases are shown in Table 1. For WikiPathways only the curated collection was included. For KEGG, the disease pathways were excluded and only the general processes were added to the collection. Of the nearly 12,000 unique genes in the combined pathway collection, 42.2% are covered in one of the three databases only (Figure 1). This confirms the importance of integrating the available collections. Using PathVisio, we calculated the overrepresentation score (Z-score) in one run for all included pathways of all three databases.

Table 1. *The number of pathways and unique human genes per used pathway database.*

	Number of pathways	Number of unique genes
WikiPathways	393	5,492
Reactome	473	10,088
KEGG	234	6,595

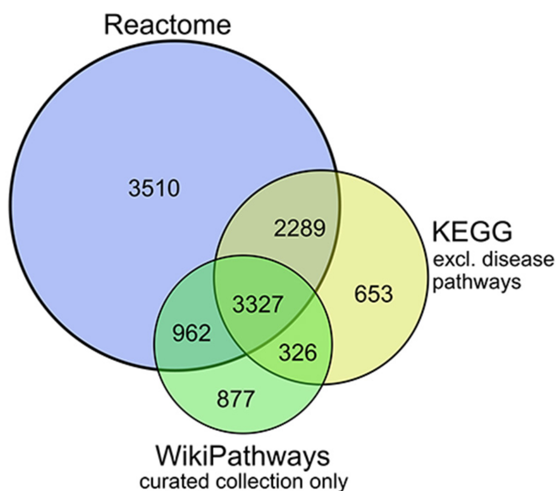

Figure 1. *Venn diagram showing the overlap in unique genes between pathway databases.*

Clustering of the pathway results

After the aforementioned pathway analysis, the significantly changed pathways were clustered into functional categories. These categories were based on the molecular mechanisms that were captured in the pathways. The clustering was performed by human curation after careful investigation of the results.

Network analysis

Cytoscape (*www.cytoscape.org*), an open access tool, was used to combine the genes involved in each of the identified functional categories into a network of connected genes.[22] It allows to vizualise how the functional categories are related to each other, to visualize how many and which mutual genes are shared between the multiple categories, to investigate these genes, and to retrieve the most relevant target genes for new drugs.

Gene ontology analysis

In addition to the pathway analysis, in which we looked at the process level, we also performed Gene Ontology (GO) analysis on the gene expression data. GO analysis allows investigating the functions of the genes by using their structured individual annotations.[23] We used Gorilla (*http://cbl-gorilla.cs.technion.ac.il/*), a freely available online tool.[24] As for pathway analysis, genes with an absolute ^{2}log fold change (logFC) in gene expression higher than 0.58 and a p-value smaller than 0.05 were considered to be significantly changed in the GO analysis.

Clustering of the GO results

After performing GO analysis, the significant functional terms were clustered into functional categories. This was also performed by human curation after careful investigation of the results. The results obtained after performing the pathway and GO analysis were compared with each other for agreement in functional categories.

Results

Systematic search

After the systematic search, the datasets of four different studies were selected for further analyses: GSE27057 and GSE27058[3], GSE4316[4], and GSE27276[5]. For three out of four datasets, we contacted the authors to obtain additional information. The characteristics of the datasets are shown in Table 2. The most important features for each of the included studies are described below.

Datasets GSE27057 and GSE27058 of Diskin et al. investigate the differences in gene expression profiles between five glaucoma cases and five and four control cases, respectively. All samples were derived from post-mortem human donor eyes. The diagnosis and medical information were supplied by the treating ophthalmologist of the donor, using a detailed questionnaire. Dataset GSE4316 shows the differences between the gene expression profiles of three cultured TM cells and the TM tissues of three control and two POAG pairs of eyes. All samples were derived from post-mortem human donor eyes. The POAG eyes were from donors with a documented history of POAG and use of glaucoma medication. Dataset GSE27276 shows the differences between the gene expression profiling of fifteen POAG and thirteen control cases. One of the POAG cases had a *MYOC*-mutation. The samples of the POAG cases were obtained from routine trabeculectomy surgery. The control TM samples were obtained from post-mortem human donor eyes. To collect these control samples, the same method was used as during the trabeculectomy. In one of the POAG cases and six of the control cases, samples of both eyes were used. A technical replicate was performed for one of the cases.

Table 2. *Characteristics of the identified gene expression datasets.*

	Diskin et al. GSE27057 and GSE27058 (2006)	**Liton et al. GSE4316 (2006)**	**Liu et al. GSE27276 (2013)**
Study design	HTM specimens of post-mortem donors with and without POAG	HTM specimens of post-mortem donors with and without POAG Cultured HTM cells of post-mortem donors without POAG	HTM specimens of POAG cases obtained from trabeculectomy and from post-mortem donor eyes using the same approach as a standard trabeculectomy
Passage cells	Not applicable	3th (cultured cells)	Not applicable
Number of included patients with POAG	5 (GSE27057 and GSE27058)	2 pairs eyes	15 (from one patient both eyes were included)
Number of included control donors	5 (GSE27057) 4 (GSE27058)	5 pairs eyes	13 (from six patients both eyes were included)
Age (years)	POAG and control: 66-87	POAG: 59 and 77 Control: 70, 85 and 87	POAG: 40-86 Control: 48-94
Microarray	Custom Affymetrix Glyco v2 GeneChips	Affymetrix Human Genome U133 Plus 2.0	Sentrix Human-6 Expression BeadChip

Quality control and pre-processing of the data

The detailed reports of the quality control of the datasets are added in supplemental material 1, containing Fig.1A-D, and supplemental material 2, containing Fig.2A-G. The most important observations concerning the quality control for each of the datasets are described below.

Excluded datasets

Diskin et al. (GSE27057 and GSE27058): The provided data were generated using custom Affymetrix Glyco v2 GeneChips. This chip contained both human and mouse probes for a specific chosen subset of genes. We were able to link the provided identifiers to the more generic Ensembl identifiers, resulting in the identification of 752 unique human genes. This is a relatively low number of genes to use as input for pathway analysis. In addition, the associated article stated that the provided samples comprised not only of POAG cases, but also normal tension glaucoma and glaucoma suspect donors. The provided metadata did not allow us to trace this back to the samples in the dataset, thereby not allowing us to identify the glaucoma samples that were diagnosed with POAG. This also could not be deduced from the results of the quality control (not shown). Based on above findings, both datasets were excluded from further analysis.

Liton et al. (GSE4316): For this study, only processed data was available. We first run quality control on the raw data, not observing any deviations. However, based on the results of the quality control, we concluded that the processed data, provided by the authors of this study, was not normalized yet. Therefore, we performed quantile normalization and made additional plots for the normalized data. The plots resulting from the quality control (supplemental material 1) demonstrated that the cultured cells behave differently from the other samples. Therefore, we removed them from the dataset, resulting in the remainder of two POAG and three control cases. When investigating these tissue samples, the quality control shows a mixed clustering of POAG and control cases (supplemental material 1). Due to the remaining small sample size and their divergent behavior, the quality of the dataset was found to be too low, despite normalization techniques. Therefore, this dataset was also excluded for further analysis.

Included dataset

Liu et al. (GSE27276): We performed pre-processing and quality control on the provided raw and normalized data. Here we only present the results of the quality control on the normalized data. The quality control (supplemental material 2) showed that one control sample (c9.2) behaved as an outlier. This sample was therefore removed from the data and the quality control was repeated with the remaining samples. No other divergent samples were detected and they were therefore included for further analyses. However, to ensure homogeneity of the dataset, we removed the samples of the *MYOC* mutation carrier (p15.1 and p15.1r). Therefore, fourteen POAG-cases and thirteen control-cases remained in the dataset. Based on the results of the quality control, one dataset containing high quality gene expression data of fourteen POAG-cases and thirteen control-cases was used for pathway and GO analysis.

Pathway analysis

Pathway analysis, performed with the combined pathway collection from WikiPathways, KEGG, and Reactome showed 92 significantly altered pathways (Z-score ≥ 1.96, a permuted p-value < 0.05, and ≥ 3 changed genes in the pathway). The complete results of the pathway analysis are shown in supplemental Table 1.

Clustering of the pathway results

The significant pathways could be clustered into the following functional categories: extracellular matrix (ECM), inflammation, complement activation, senescence, and Rho GTPase. The inflammation category could be further subcategorized in interleukin signaling, *NF-κB*, arachidonic acid, and general inflammation pathways, such as the TNF signaling pathway. Subcategories for the ECM category are collagen, actin and cell-matrix, cell-cell interactions, and general ECM pathways, such as the ECM organization pathway. The results of the clustering are presented in Table 3.

Table 3. *Clusters resulted from the pathway analysis.*

	Pathway name	Pathway database	Z-score	Permuted p-value	Positive*	Measured*
	Extracellular matrix					
ECM	Extracellular matrix organization	Reactome	5.58	<0.0001	14	41
	Degradation of the extracellular matrix	Reactome	4.94	<0.0001	12	37
	ECM-receptor interaction	KEGG	4.29	<0.0001	12	43
	miRNA targets in ECM and membrane receptors	WikiPathways	4.21	0.002	7	19
	miR-509-3p alteration of YAP1/ECM axis	WikiPathways	2.72	0.026	4	13
	Activation of Matrix Metalloproteinases	Reactome	2.53	0.014	4	14
Collagen	Collagen biosynthesis and modifying enzymes	Reactome	4.72	<0.0001	11	34
	Collagen chain trimerization	Reactome	3.86	0.001	7	21
	Assembly of collagen fibrils and other multimeric structures	Reactome	3.84	0.002	8	26
Actin	Regulation of Actin Cytoskeleton	WikiPathways	2.63	0.012	12	65
	Regulation of actin cytoskeleton	KEGG	2.36	0.018	16	101
Cell-matrix and cell-cell interactions	Cell adhesion molecules (CAMs)	KEGG	5.45	<0.0001	17	57
	Tight Junction	KEGG	3.4	0.001	17	87
	Focal Adhesion-PI3K-Akt-mTOR-signaling pathway	WikiPathways	3.29	0.003	24	141
	Focal Adhesion	WikiPathways	3.06	0.002	18	101
	Inflammation					
Interleukin signaling	Interleukin-4 and 13 signaling	Reactome	9.28	<0.0001	37	106
	IL-17 signaling pathway	KEGG	4.53	<0.0001	13	46
	Interleukin-10 signaling	Reactome	3.23	0.005	10	43
	IL17 signaling pathway	WikiPathways	2.07	0.038	4	17
NF-κB	Quercetin and Nf-kB/ AP-1 Induced Cell Apoptosis	WikiPathways	2.92	0.006	4	12
	TAK1 activates NFkB by phosphorylation and activation of IKKs complex	Reactome	2.76	0.014	5	18
	Photodynamic therapy-induced NF-kB survival signaling	WikiPathways	3.95	0.004	6	16
Arachidonic acid	Arachidonic acid metabolism	KEGG	2.48	0.014	6	26
	Arachidonic acid metabolism	Reactome	2.27	0.022	6	28

General	Inflammatory Response Pathway	WikiPathways	5.06	<0.0001	7	15
	Allograft Rejection	WikiPathways	4.05	0.001	11	40
	Intestinal immune network for IgA production	KEGG	3.95	0.0001	6	16
	Th17 cell differentiation	KEGG	2.88	0.006	11	54
	Antigen processing and presentation	KEGG	2.78	0.015	9	42
	Th1 and Th2 cell differentiation	KEGG	2.47	0.023	9	46
	Neutrophil degranulation	Reactome	2.31	0.017	38	294
	TNF signaling pathway	KEGG	2.13	0.027	9	51
Complement activation						
Complement activation	Complement Activation	WikiPathways	4.93	<0.0001	6	12
	Complement and Coagulation Cascades	WikiPathways	4.76	<0.0001	10	29
	Complement and coagulation cascades	KEGG	4.48	<0.0001	11	36
	Complement cascade	Reactome	3.86	0.001	7	21
Senescence						
Senescence	Senescence-Associated Secretory Phenotype (*SASP*)	Reactome	3.51	0.003	12	52
	DNA Damage/Telomere Stress Induced Senescence	Reactome	2.83	0.004	6	23
	Senescence and Autophagy in Cancer	WikiPathways	1.98	0.033	10	61
RHO GTPase						
RHO GTPase	RHO GTPases Activate *ROCK*s	Reactome	2.72	0.021	4	13
	RHO GTPases activate PKNs	Reactome	2.34	0.024	5	21
	RHO GTPases activate CIT	Reactome	2.37	0.019	4	15
	RHO GTPases activate PAKs	Reactome	2.21	0.023	4	16

**Positive: the number of genes on the pathway that pass the statistical criteria (absolute logFC > 0.58 and p-value < 0.05); Measured: the number of genes on the pathway that were measured in the dataset.*

Network analysis

We created an integrated network showing the genes involved in each of the five main categories to study the interplay between the processes. The network analysis shows that the clusters of ECM, complement activation, and senescence have multiple mutual genes with the inflammation cluster, showing the central role of inflammation in the connection between clusters. In addition, 12 genes are present in three or more clusters, as displayed in Figure 2.

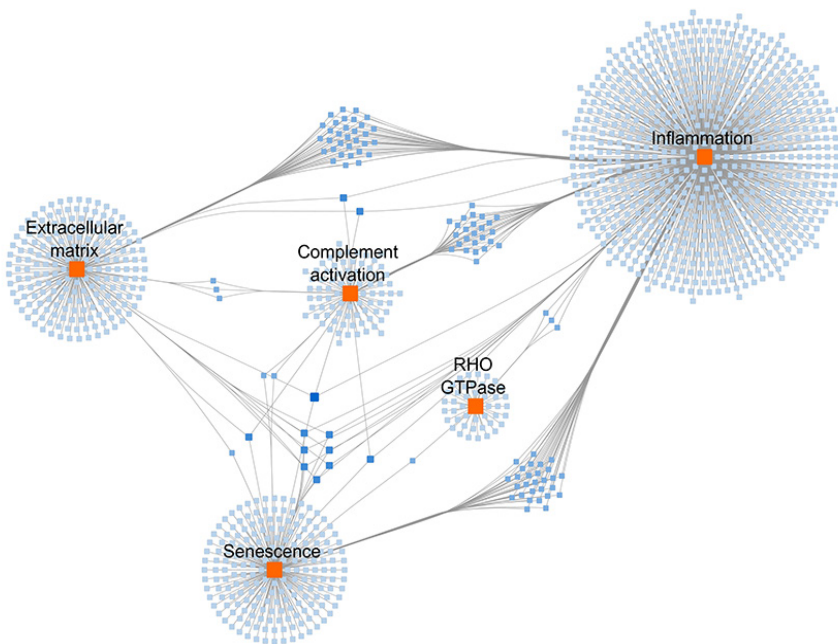

Figure 2. *Network of the five identified pathway categories.*
Orange rectangles indicate the categories; blue nodes indicate the genes present in at least one pathway of the category; darker shades of blue indicate participation of the gene in more than one category; gene labels are shown for genes present in three or more categories.

Gene ontology analysis

GO overrepresentation analysis of the differentially expressed genes resulted in fifty one significant GO functional terms (P-value < 10-6). The complete tabular results of the GO analysis are shown in supplemental Table 2.

Clustering of the GO results

The GO terms were clustered into five categories: ECM, inflammation, cell adhesion, corticosteroids and development (Table 4). Figure 3. shows an integrated flowchart of the methods and results.

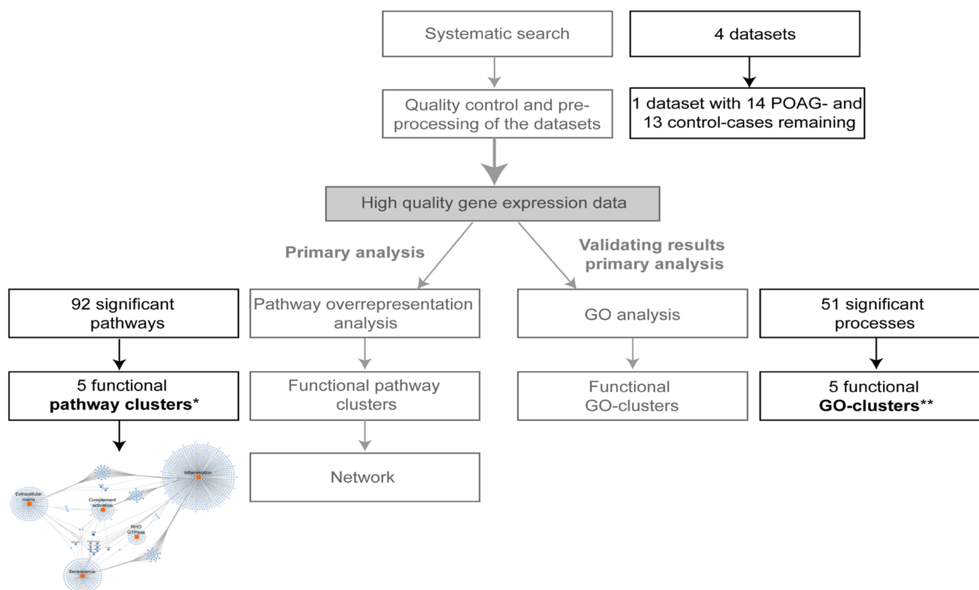

Figure 3. *Flowchart of the methods and results of this paper.*
The grey text represents the methods, black text represents the results.

Table 4. *Clusters resulting from the GO analysis.*

GO term	P-value	FDR q-value*	Enrichment (B, b)*
Extracellular matrix			
Extracellular matrix organization	1.75E-10	1.1E-6	3.08 (155,38)
Extracellular structure organization	1.75E-10	7.31E-7	3.08 (155,38)
Extracellular matrix disassembly	3.75E-6	1.27E-3	4.30 (38,13)
Inflammation			
Defense response	3.11E-8	3.24E-5	1.97 (427, 67)
Response to wounding	4.76E-8	4.25E-5	3.43 (88, 24)
Response to inorganic substance	7.67E-8	6.39E-5	2.30 (246, 45)
Response to stimulus	9.65E-8	7.54E-5	1.33 (2236, 236)
Immune response	7.25E-7	3.94E-4	1.99 (335, 53)
Regulation of immune system process	7.59E-7	3.95E-4	1.66 (667, 88)
Response to oxygen-containing compound	1.38E-6	6.41E-4	1.69 (597, 80)
Regulation of response to stimulus	1.65E-6	7.35E-4	1.35 (1767, 189)
Humoral immune response	1.83E-6	7.89E-4	3.23 (78, 20)
Regulation of immune response	2.54E-6	9.91E-4	1.81 (432, 62)
Acute inflammatory response	2.94E-6	1.05E-3	5.13 (27, 11)

Immune system process	3.42E-6	1.19E-3	1.49 (981, 116)
Regulation of cytokine production	4.13E-6	1.36E-3	1.99 (291, 46)
Response to molecule of bacterial origin	5.84E-6	1.62E-3	2.52 (135, 27)
Positive regulation of response to stimulus	6.42E-6	1.74E-3	1.47 (983, 115)
Inflammatory response	8.81E-6	2.25E-3	2.26 (178, 32)
Cell adhesion			
Cell adhesion	6.53E-9	9.07E-6	2.14 (359, 61)
Biological adhesion	1.01E-8	1.26E-5	2.11 (363, 61)
Cell-cell adhesion	2.98E-7	1.69E-4	2.58 (161, 33)
Regulation of cell adhesion	8.63E-6	2.25E-3	1.92 (308, 47)
Corticosteroids			
Response to steroid hormone	4.29E-6	1.34E-3	2.88 (96, 22)
Response to glucocorticoid	5.13E-6	1.46E-3	3.39 (63, 17)
Response to corticosteroid	7.13E-6	1.9E-3	3.19 (71, 18)
Development			
Developmental process	4.24E-11	5.3E-7	1.43 (2081, 237)
Epidermis development	1.91E-10	5.96E-7	6.11 (35, 17)
Anatomical structure development	2.98E-8	3.38E-5	1.48 (1380, 162)
Cellular developmental process	1.04E-7	7.66E-5	1.53 (1105, 134)
Cell differentiation	1.31E-6	6.29E-4	1.60 (756, 96)
Tissue development	2.24E-6	9.02E-4	2.11 (250, 42)
Regulation of developmental process	9.62E-6	2.4E-3	1.43 (1088, 124)

** FDR q-value: p-value after correction for multiple testing; B: the total number of genes associated with the GO term; b: the number of genes associated with the GO term that pass the statistical criteria (absolute logFC > 0.58 and p-value < 0.05). In total, there are 7901 number of genes in the dataset (N=7901); 628 genes are differentially expressed (n=628); Enrichment is defined as (b/n)/(B/N). Results with a p-value < 10-6 were considered to be significant.*

Discussion

Faced with the enormous amount of data obtained by omics studies, research has been directed to determine how this data can be interpreted and used in a meaningful way. In this study we have shown how developments in bioinformatics enable the needed integration of omics data to improve interpretation and understanding of biological processes and their contribution to human disease. Specifically, we demonstrated how pathway and gene ontology analysis generate new insights into the molecular pathways involved in the pathogenesis of POAG.

We applied a systematic bioinformatics approach to perform quality control and pre-processing steps on gene expression profiling datasets in which differences in gene expression of TM cells in patients with and without POAG were compared. Although only one dataset passed our quality control, pathway overrepresentation analysis identified ninety-two pathways that were significantly changed in patients with POAG, as compared to patients without POAG. These pathways were clustered into five functional categories: ECM, inflammation, complement activation, senescence, and Rho GTPase. These categories were combined into a network of connecting genes, showing overlap between the categories and the central position of the inflammation cluster. Also, the genes present in at least three categories were visualized within the network. The additional GO analysis on our pre-processed and quality controlled dataset showed that the significantly changed genes were involved in ECM, inflammation and cell adhesion, which we already found in the pathway analysis. Additionally, development and corticosteroid related clusters were found. As far as we know, this study is the first to perform a systematic, state-of-the-art step-by-step approach on the available gene expression datasets of TM-tissue from patients with POAG and controls.

For each of the five identified molecular process categories, other studies provide supportive evidence of their role in the TM and their involvement in the pathogenesis of POAG.

Multiple changes have been described in the ECM of TM derived from patients with POAG. For example, the stiffness of glaucomatous TM, especially the juxtacanalicular tissue, is higher and an increase in plaque-like depositions that consist, among others, of a core of elastic fibers is seen.[25, 26] In addition, gene expression studies have shown differences in the expression of genes encoding ECM proteins between patients with POAG and healthy controls. These differences correlate with morphological changes that occur in the ECM of the TM, for example genes that are involved in the formation of collagen, fibronectin, and integrins.[4] Furthermore, *TGF-**β*** signaling regulates the ECM turnover.[27]

TGF-β2 was identified in our study as part of the Extracellular Matrix Organization pathway and is known to regulate the ECM metabolism in TM cells and tissues. It induces elastin and collagen cross-linking enzymes (*LOX*-genes), which are associated with the pathological ECM changes observed in glaucomatous TM.[27, 28] Elastin, collagen and *LOX*-genes are also present in the Extracellular Matrix Organization pathway we identified. Moreover, *TGF-β1* is present in thirteen of our significantly changed pathways. It is known to induce the expression of smooth muscle actin in cultured TM cells and therefore influences the TM actin cytoskeleton.[29] Interestingly, corticosteroids, which we identified as a functional category in our GO analysis, also induce changes in the cytoskeleton, better known as the formation of cross-linked actin networks (CLANs).[30]

Concerning the inflammation category, inflammatory mediators such as *TGF-β1*, *TNF-α* and interleukins such as *IL-6*, can induce changes in the ECM and the cytoskeleton of the TM. This implies that an inflammatory response is also involved in the pathogeneses of POAG, which was also confirmed by other studies.[31] Moreover, interleukins, for example *IL-10*, *IL-8*, and *TGF-β1*, are upregulated in the aqueous humour of patients with POAG.[32] In addition, arachidonic acid, which we also found in our pathway analysis, is a precursor of prostaglandins. Prostaglandin analogues are currently used for the treatment of POAG and affect the outflow in the TM.[33] Furthermore, *NF-κB*, another inflammatory component, is known to be activated in the TM cells in glaucoma where it protects against oxidative stress.[34]

The category Rho GTPase is of special interest. Downstream targets of Rho GTPase are Rho/Rho-associated kinases (*ROCK*s), including *RHOA*. *ROCK*s plays an important role in focal adhesions, actin stress fibers, and cell adhesion.[35] Therefore, they are likely to play an important role in the function of the TM and in the pathogenesis of glaucoma.[35, 36] Moreover, *ROCK*-inhibitors are currently being tested to lower the intra-ocular pressure in glaucoma.[36, 37]

Finally, the category senescence is shown to be involved in POAG. Ageing is a known risk factor.[38] With ageing multiple changes in the TM have been described, for example an increase in TM-stiffness.[39, 40] Additionally, Senescence-Associated Secretory Phenotype (*SASP*) genes play a key role as their expression activates multiple inflammation factors such as *TNF-α*, and, among others, *IL-1/NF-κB* and *TGF-β/SMAD* signaling pathways.[41] There is evidence that ageing tissue is chronically inflamed, which suggests there is an interplay between senescence and inflammation.[42]

We created a network of the five molecular process categories that we have identified to be involved in the pathogenesis of POAG. This network does not only show an interplay between the senescence and inflammation cluster, as suggested above, but also shows that ECM and complement activation clusters share multiple genes with the inflammation cluster. This points out the central role of inflammation in the connection between clusters (Figure 2). Furthermore, within the network, the gene symbols of the genes that are present in at least three of the categories are shown. More than 50% of these genes have been associated with POAG. *TGF-β1* is highly expressed in glaucomatous TM cells and can be induced by mechanical stress.[43, 44] Furthermore, the exposure of the TM to *TGF-β1* or *TGF-β2* increases the expression of fibronectin (*FN1*).[43] *CD44* is increased in the aqueous humour of patients with POAG.[45] *COL3A1* and *COL1A1* are differentially expressed after exposure to oxidative stress, which is known to affect the TM in patients with POAG.[46, 47] Further, the allele frequencies of *SERPINE1* are significantly different in patients with POAG compared to a control group.[48] Lastly, *PLAU* is differentially expressed after exposure to dexamethasone or triamcinolone and it is known that patients with POAG are more susceptible to develop corticosteroid-induced ocular hypertension.[49, 50] The association of the above mentioned genes with POAG suggests a role for the other identified genes as well.

In addition to the pathway analysis, we performed GO analysis. GO annotations are based on the function of the differentially expressed genes. They do not have the level of detail of a known pathway diagram, but may add information where such detailed information is still lacking. ECM, inflammation, and cell adhesion were found in this analysis as well. Complementary, we identified development and corticosteroid related clusters in our analysis. Studies have also shown that corticosteroids cause molecular changes in TM that increase the outflow resistance.[51, 52]

The findings described in the publication of Liu et al. are of special interest as we used the dataset they made publically available. They investigated the functional distribution of the DEGs, using the Database for Annotation, Visualization, and integrated Discovery (DAVID) and showed that among others, cell adhesion, ECM, extracellular region, signal peptide, glycoprotein, and secretion are involved in the pathogenesis of POAG.[5] The GO analysis we performed in our study showed similar results. However there are some discrepancies which might partially be explained by differences in defining the cut-off for DEGs and the significance level of the GO terms.

The recently published study of Qui et al.[53] used the same publicly available dataset as we used for pathway and GO analysis. However, they did not describe quality checks and did not remove the patient with a *MYOC* mutation or the deviating control sample, from the database. In addition, they only used the KEGG database to perform pathway analysis. Our results add additional processes that have been confirmed by previous studies: ECM, inflammation, Rho GTPase, and senescence, including each of the subcategories for the ECM and inflammation categories. Information on these processes might not be completely present in the KEGG database or they may be embedded in larger less specific pathways. The result of our study emphasizes the importance of performing a systematic step-by-step approach using multiple bioinformatics techniques and the benefits of combining knowledge from multiple pathway databases, in order to include the greatest possible coverage of knowledge and to obtain the most comprehensive results.

The final study to discuss is the study of Danford et al. which identified all genes that have a confirmed link with POAG through transcriptomics, genetic association, functional and genome-wide association studies.[54] After identification, the genes were classified according to their associated ocular tissue, including the TM. The study of Fan et al. investigated the differences in gene expression between TM cells treated with and without steroid and was excluded in the analysis in order to avoid study bias. The pathway analysis Danford et al. performed on their 64 identified TM tissue-associated genes revealed the *TGF-β* signalling pathway, ECM organization, and senescence and autophagy in cancer as the most overrepresented pathways in the TM. As discussed before, the ECM organization and senescence pathways were also identified in our study and *TGF-β* is involved in thirteen of our significantly changed pathways.

One of the strengths of our study is that we integrated the pathway databases of WikiPathways, KEGG and Reactome into one collection. None of these databases captures the complete gene and pathway knowledge: 42.2% of the genes in this combined pathway collection are only covered in one of the three databases. This shows the importance of integrating the available databases. We chose WikiPathways, Reactome, and KEGG as their pathways are of high quality, they all belong to the most extensive and commonly used pathway databases, and they provide their data in a machine readable format.

Furthermore, we performed an extensive quality check on the retrieved publicly available datasets to avoid that divergent samples or an overall insufficient quality would affect the results of our following analyses. The fact that only one dataset could be maintained, stresses the importance of and the need for extensive and systematic quality control, which is often missing when performing analysis on publicly available data.

In short, the purpose of this study was to systematically analyse available gene expression data of the TM in patients with POAG and controls. The analyses performed in this study provide a comprehensive overview of the processes involved in the molecular pathogenesis of POAG. These results show good face validity and confirm findings from histological, biochemical, genome-wide association and transcriptomics studies. The appearance of already known points of action for drugs such as *ROCK* inhibitors, prostaglandins, and corticosteroids in our results supports the validity of this approach and stresses its potential in the identification of new treatment options.

This study shows how findings from decades of biochemical and histological studies of POAG can be efficiently retrieved by applying state-of-the-art bioinformatics to genome-wide data, clearly indicating the power and promise of this approach.

References

1. Tektas OY, Lutjen-Drecoll E. Structural changes of the trabecular meshwork in different kinds of glaucoma. Exp Eye Res 2009;88(4):769-75.
2. Sommer A, Tielsch JM, Katz J, et al. Relationship between intraocular pressure and primary open angle glaucoma among white and black Americans. The Baltimore Eye Survey. Arch Ophthalmol 1991;109(8):1090-5.
3. Diskin S, Kumar J, Cao Z, et al. Detection of differentially expressed glycogenes in trabecular meshwork of eyes with primary open-angle glaucoma. Invest Ophthalmol Vis Sci 2006;47(4):1491-9.
4. Liton PB, Luna C, Challa P, et al. Genome-wide expression profile of human trabecular meshwork cultured cells, nonglaucomatous and primary open angle glaucoma tissue. Mol Vis 2006;12:774-90.
5. Liu Y, Allingham RR, Qin X, et al. Gene expression profile in human trabecular meshwork from patients with primary open-angle glaucoma. Invest Ophthalmol Vis Sci 2013;54(9):6382-9.
6. Wang WH, McNatt LG, Pang IH, et al. Increased expression of serum amyloid A in glaucoma and its effect on intraocular pressure. Invest Ophthalmol Vis Sci 2008;49(5):1916-23.
7. Edgar R, Domrachev M, Lash AE. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Res 2002;30(1):207-10.
8. Barrett T, Wilhite SE, Ledoux P, et al. NCBI GEO: archive for functional genomics data sets--update. Nucleic Acids Res 2013;41(Database issue):D991-5.
9. Kolesnikov N, Hastings E, Keays M, et al. ArrayExpress update--simplifying data submissions. Nucleic Acids Res 2015;43(Database issue):D1113-6.
10. Eijssen LM, Jaillard M, Adriaens ME, et al. User-friendly solutions for microarray quality control and pre-processing on ArrayAnalysis.org. Nucleic Acids Res 2013;41(Web Server issue):W71-6.
11. Ritchie ME, Phipson B, Wu D, et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res 2015;43(7):e47.
12. RCoreTeam. R: A language and environment for statistical computing. In: Computing RFfS, ed. Vienna, Austria2013.
13. Kutmon M, van Iersel MP, Bohler A, et al. PathVisio 3: an extendable pathway analysis toolbox. PLoS Comput Biol 2015;11(2):e1004085.
14. van Iersel MP, Kelder T, Pico AR, et al. Presenting and exploring biological pathways with PathVisio. BMC Bioinformatics 2008;9:399.
15. Kelder T, van Iersel MP, Hanspers K, et al. WikiPathways: building research communities on biological pathways. Nucleic Acids Res 2012;40(Database issue):D1301-7.
16. Kutmon M, Riutta A, Nunes N, et al. WikiPathways: capturing the full diversity of pathway knowledge. Nucleic Acids Res 2016;44(D1):D488-94.
17. Kanehisa M, Furumichi M, Tanabe M, et al. KEGG: new perspectives on genomes, pathways, diseases and drugs. Nucleic Acids Res 2017;45(D1):D353-D61.
18. Kanehisa M, Sato Y, Kawashima M, et al. KEGG as a reference resource for gene and protein annotation. Nucleic Acids Res 2016;44(D1):D457-62.

19. Kanehisa M, Goto S. KEGG: kyoto encyclopedia of genes and genomes. Nucleic Acids Res 2000;28(1):27-30.
20. Fabregat A, Sidiropoulos K, Garapati P, et al. The Reactome pathway Knowledgebase. Nucleic Acids Res 2016;44(D1):D481-7.
21. Milacic M, Haw R, Rothfels K, et al. Annotating cancer variants and anti-cancer therapeutics in reactome. Cancers (Basel) 2012;4(4):1180-211.
22. Shannon P, Markiel A, Ozier O, et al. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res 2003;13(11):2498-504.
23. Ashburner M, Ball CA, Blake JA, et al. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat Genet 2000;25(1):25-9.
24. Eden E, Navon R, Steinfeld I, et al. GOrilla: a tool for discovery and visualization of enriched GO terms in ranked gene lists. BMC Bioinformatics 2009;10:48.
25. Rohen JW, Futa R, Lutjen-Drecoll E. The fine structure of the cribriform meshwork in normal and glaucomatous eyes as seen in tangential sections. Invest Ophthalmol Vis Sci 1981;21(4):574-85.
26. Last JA, Pan T, Ding Y, et al. Elastic modulus determination of normal and glaucomatous human trabecular meshwork. Invest Ophthalmol Vis Sci 2011;52(5):2147-52.
27. Fuchshofer R, Tamm ER. Modulation of extracellular matrix turnover in the trabecular meshwork. Exp Eye Res 2009;88(4):683-8.
28. Sethi A, Mao W, Wordinger RJ, Clark AF. Transforming growth factor-beta induces extracellular matrix protein cross-linking lysyl oxidase (*LOX*) genes in human trabecular meshwork cells. Invest Ophthalmol Vis Sci 2011;52(8):5240-50.
29. Tamm ER, Siegner A, Baur A, Lutjen-Drecoll E. Transforming growth factor-beta 1 induces alpha-smooth muscle-actin expression in cultured human and monkey trabecular meshwork. Exp Eye Res 1996;62(4):389-97.
30. Clark AF, Wilson K, McCartney MD, et al. Glucocorticoid-induced formation of cross-linked actin networks in cultured human trabecular meshwork cells. Invest Ophthalmol Vis Sci 1994;35(1):281-94.
31. Taurone S, Ripandelli G, Pacella E, et al. Potential regulatory molecules in the human trabecular meshwork of patients with glaucoma: immunohistochemical profile of a number of inflammatory cytokines. Mol Med Rep 2015;11(2):1384-90.
32. Micera A, Quaranta L, Esposito G, et al. Differential Protein Expression Profiles in Glaucomatous Trabecular Meshwork: An Evaluation Study on a Small Primary Open Angle Glaucoma Population. Adv Ther 2016;33(2):252-67.
33. Toris CB, Gabelt BT, Kaufman PL. Update on the mechanism of action of topical prostaglandins for intraocular pressure reduction. Surv Ophthalmol 2008;53 Suppl1:S107-20.
34. Wang N, Chintala SK, Fini ME, Schuman JS. Activation of a tissue-specific stress response in the aqueous outflow pathway of the eye defines the glaucoma disease phenotype. Nat Med 2001;7(3):304-9.
35. Wang J, Liu X, Zhong Y. Rho/Rho-associated kinase pathway in glaucoma (Review). Int J Oncol 2013;43(5):1357-67.

36. Rao PV, Pattabiraman PP, Kopczynski C. Role of the Rho GTPase/Rho kinase signaling pathway in pathogenesis and treatment of glaucoma: Bench to bedside research. Exp Eye Res 2017;158:23-32.
37. Inoue T, Tanihara H. Rho-associated kinase inhibitors: a novel glaucoma therapy. Prog Retin Eye Res 2013;37:1-12.
38. de Voogd S, Ikram MK, Wolfs RC, et al. Incidence of open-angle glaucoma in a general elderly population: the Rotterdam Study. Ophthalmology 2005;112(9):1487-93.
39. Morgan JT, Raghunathan VK, Chang YR, et al. The intrinsic stiffness of human trabecular meshwork cells increases with senescence. Oncotarget 2015;6(17):15362-74.
40. Liu B, McNally S, Kilpatrick JI, et al. Aging and ocular tissue stiffness in glaucoma. Surv Ophthalmol 2017.
41. Hubackova S, Krejcikova K, Bartek J, Hodny Z. IL1- and TGFbeta-Nox4 signaling, oxidative stress and DNA damage response are shared features of replicative, oncogene-induced, and drug-induced paracrine 'bystander senescence'. Aging (Albany NY) 2012;4(12):932-51.
42. Young AR, Narita M. *SASP* reflects senescence. EMBO Rep 2009;10(3):228-30.
43. Fatma N, Kubo E, Toris CB, et al. PRDX6 attenuates oxidative stress- and TGFbeta-induced abnormalities of human trabecular meshwork cells. Free Radic Res 2009;43(9):783-95.
44. Liton PB, Liu X, Challa P, et al. Induction of TGF-beta1 in the trabecular meshwork under cyclic mechanical stress. J Cell Physiol 2005;205(3):364-71.
45. Mokbel TH, Ghanem AA, Kishk H, et al. Erythropoietin and soluble *CD44* levels in patients with primary open-angle glaucoma. Clin Exp Ophthalmol 2010;38(6):560-5.
46. Luna C, Li G, Qiu J, et al. Role of miR-29b on the regulation of the extracellular matrix in human trabecular meshwork cells under chronic oxidative stress. Mol Vis 2009;15:2488-97.
47. Izzotti A, Bagnis A, Sacca SC. The role of oxidative stress in glaucoma. Mutat Res 2006;612(2):105-14.
48. Zhou G, Liu B. Single nucleotide polymorphisms of metabolic syndrome-related genes in primary open angle glaucoma. Int J Ophthalmol 2010;3(1):36-42.
49. Fan BJ, Wang DY, Tham CC, et al. Gene expression profiles of human trabecular meshwork cells induced by triamcinolone and dexamethasone. Invest Ophthalmol Vis Sci 2008;49(5):1886-97.
50. Tripathi RC, Parapuram SK, Tripathi BJ, et al. Corticosteroids and glaucoma risk. Drugs Aging 1999;15(6):439-50.
51. Kersey JP, Broadway DC. Corticosteroid-induced glaucoma: a review of the literature. Eye (Lond) 2006;20(4):407-16.
52. Jones R, 3rd, Rhee DJ. Corticosteroid-induced ocular hypertension and glaucoma: a brief review and update of the literature. Curr Opin Ophthalmol 2006;17(2):163-7.
53. Qiu H, Zhu B, Ni S. Identification of genes associated with primary open-angle glaucoma by bioinformatics approach. Int Ophthalmol 2017.
54. Danford ID, Verkuil LD, Choi DJ, et al. Characterizing the "POAGome": A bioinformatics-driven approach to primary open-angle glaucoma. Prog Retin Eye Res 2017;58:89-114.

Supplemental material

All supplemental material can be found on *https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7004120/*

3

Chapter 4

THE MOLECULAR PROCESSES IN THE TRABECULAR MESHWORK AFTER EXPOSURE TO CORTICOSTEROIDS AND IN CORTICOSTEROID-INDUCED OCULAR HYPERTENSION

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Abstract

Purpose: To identify processes that contribute to corticosteroid-induced ocular hypertension and candidate target genes for treatment.

Methods: A systematic search identified five human microarray datasets investigating the effect of dexamethasone versus a control medium on trabecular meshwork tissue. After thorough quality control, samples of low quality were removed and the datasets were integrated. Additionally, a bovine RNA-sequencing dataset allowed to investigate differences in gene expression profiling between cows with and without corticosteroid-induced ocular hypertension (responders vs. non-responders). The obtained datasets were used as input for parallel pathway analyses. Significantly changed pathways were clustered into functional categories and the results were further investigated. A network visualizing the differences between the responders and non-responders was created.

Results: Seven functional pathway clusters were found to be significantly changed in trabecular meshwork cells exposed to dexamethasone vs. a control medium and in trabecular meshwork cells of responders vs. non-responders: collagen, extracellular matrix, adhesion, WNT-signaling, inflammation, adipogenesis and glucose metabolism. In addition, cell cycle and senescence were only significantly changed in responders vs. non-responders. The network of the differential gene expression between responders and non-responders shows many connections between the identified processes via shared genes.

Conclusions: Nine functional pathway clusters synthesize the molecular response to dexamethasone exposure in trabecular meshwork cells and are likely to be involved in the pathogenesis of corticosteroid-induced ocular hypertension.

Introduction

Corticosteroids are widely used within the field of ophthalmology. However, they induce ocular hypertension (OHT), also known as a corticosteroid response, in approximately 18%-36% of patients. This percentage can be as high as 92% in patients with primary open angle glaucoma (POAG).[1] A sustained increase in intraocular pressure (IOP) may cause damage to the optic nerve, leading to visual field loss and eventually blindness. Corticosteroid-induced OHT is likely caused by molecular changes in the trabecular meshwork (TM) which increase the outflow resistance; however, the pathogenesis is not yet fully understood.[2-5] The current treatment attempts to lower the IOP by using traditional anti-glaucoma drugs or laser treatment. However, these don't target the pathogenic mechanisms of a corticosteroid response. Therefore, corticosteroids often have to be reduced or even ceased in order to lower the IOP which impedes the use of these clinically valuable drugs.

Multiple omics studies investigated the differential gene expression profiles in the TM after exposure to corticosteroids.[6-13] These individual studies revealed that genes involved in processes such as cell adhesion, cell cycle, extracellular matrix, inflammation, and immune response might be involved in the pathogenesis of corticosteroid-induced OHT. However, these studies comprised relatively small sample sizes, used various study methods with different cell types and a diverse duration of exposure and dosage to the used corticosteroid. In addition, most studies did not differentiate between patients with and without a corticosteroid response. It is therefore not known whether the observed processes directly relate to corticosteroid-induced OHT or only reflect the effects of corticosteroids on the TM.

Within this study, we integrated the publicly available gene expression data investigating the effect of dexamethasone on the TM. In addition, Bermudez et al. kindly provided a bovine RNA-sequencing dataset in which a distinction between eyes with and without a corticosteroid response was made.[14] Bioinformatics analyses on these datasets identified which processes are significantly changed in the TM after exposure to corticosteroids and in the TM of bovine responders. The obtained results were visualized and compared which allowed the identification of specific molecular processes that are likely to be involved in corticosteroid-induced OHT. These processes can be further explored for targeted drug therapies which specifically influence the molecular pathogenesis of corticosteroid-induced OHT.

Methods

Systematic search

A systematic search was conducted in Gene Expression Omnibus (*http://www.ncbi.nlm.nih.gov/geo/)*[15, 16] and ArrayExpress (*http://www.ebi.ac.uk/arrayexpress*)[17] to identify publicly available data of genome-wide expression studies in which the effects of corticosteroids on the gene expression profiles of TM cells had been investigated. The used search term was: "trabecular meshwork AND corticosteroids". In order to not miss any eligible studies, the search was repeated with "trabecular meshwork" as the only search term. The search was not restricted on publication status or date of publication and was lastly updated on 2019-06-04.

Five human microarray studies investigating the differences in gene expression profiles between TM cells exposed to dexamethasone and a control medium were identified. None of these studies specified whether the included individuals were responders or non-responders. However, the RNA-sequencing study of Bermudez et al. investigated the differences in gene expression profiles of TM cells of identified responders and non-responders.[14] This study allowed us to investigate the differences between responders and non-responders. In addition, the general effect of dexamethasone on the TM cells, analogous to the human microarray data, could also be investigated with the data from this study.

Pre-processing and quality control of the human data

The identified datasets were submitted to a quality control and pre-processing workflow. If necessary, the authors of the datasets were approached to obtain additional information. When available, both the raw and normalized data for each identified human microarray study was downloaded from GEO or ArrayExpress. Based on the availability of the provided data, we used the normalized data of the researchers or performed quantile normalization on the raw data ourselves. Quality control was performed on both the raw and normalized data as described previously.[18] The quality assessment of the samples and studies was independently performed by two researchers (L.M.T.E and I.L.). Thereafter, the results were compared and in case of disagreement, consensus was achieved by discussion. The results of the performed quality control for each dataset were visualized in different plots. These plots were assessed for homogeneity of the data, the signal strength of the different samples in the study, the correlation of expression, and the way in which the samples cluster (control vs. exposure). Following, in case samples appeared divergent based on combined interpretation of the plots of the quality control or in case a study showed an overall low quality, they were excluded for further analysis. After data pre-processing, statistical analysis to compare TM cells treated with and without dexamethasone was performed using the limma package for

R (linear regression models) as available from Bioconductor.[19, 20] The obtained results per dataset comprised the measured genes and their mean expression, log fold change (LogFC), T-statistic and P-value of the adapted T-test. Because our main goal was to combine all studies, we did not remove genes with a low expression from the datasets in order to obtain a dataset that was as complete as possible. As dexamethasone was investigated in all included studies and we wanted to compare studies with a similar study-design, only the samples exposed to dexamethasone were used for further analysis.

Systematic application of the abovementioned steps led to high-quality data on differential gene expression for each of the five included studies which was used for further integrated analysis.

Integration of data

In order to obtain results that are less dependent on study differences of individual studies, we combined the high-quality and pre-processed gene expression datasets of the included studies. To make the data annotation uniform, we converted the used probe-identifiers within each dataset into Ensembl gene identifiers. If an individual was replicated multiple times within one study, the average value across these samples was computed for each gene in order to give each individual the same weight in the analysis. If a gene was tested multiple times within one dataset, we used the gene with the highest absolute value for LogFC*-$\log_{10}$(p-value) as this represents the highest change based on the LogFC and P-value. This was used in later analysis as well. Therefore, only one value was assigned per tested gene and per individual. After preparing the individual sets, we merged them based on matching Ensembl gene identifiers. Then a joint estimate of each gene's LogFC and P-value was calculated by computing their weighted averages over all datasets. In order to take differences in study size into account, weights were assigned to each separate dataset based on the total number of individuals included within that dataset. The average weighted LogFC and P-value were calculated as shown in formula 1 and 2. To ensure robust estimates, only genes that had been tested in at least four out of five studies were kept in the final combined dataset. Also, by only using the genes that had been tested in multiple studies, the potential dominance of larger studies which tested more genes was avoided. The obtained integrated human gene expression dataset containing an average weighted LogFC and P-value for each gene, was used as input for pathway analysis.

$$\text{Average weighted LogFC} = \frac{\sum_{i=1}^{n} (logFC_i * - \log_{10} p_i)}{\sum_{i=1}^{n} (- \log_{10} p_i)}$$

Formula 1. Calculation of the average weighted LogFC,
n is the number of studies for which the gene was measured.

$$\text{Average weighted P-value} = \frac{\sum_{i=1}^{n} (- \log_{10} p_i)}{n}$$

Formula 2. Calculation of the average weighted P-value,
n is the number of studies for which the gene was measured.

Pre-processing of the bovine data

Bermudez et al. kindly provided the complete statistical results of their RNA-sequencing experiment: the averaged Fragments Per Kilobase Million (FPKM) for each tested gene after exposure to dexamethasone and a control medium in both responders and non-responders. We processed the data in order to make the following comparisons: (1) the differences in gene expression between responders and non-responders and (2) the differences in gene expression after exposing the TM to dexamethasone and ethanol (control medium). In order to make the first comparison, we calculated the LogFC of the responders ($LogFC_R$) and non-responder ($LogFC_{NR}$) as shown in formula 3. The plus one was added in order to avoid minus infinity-values. Thereafter, we deducted the LogFC of the responders with the LogFC of the non-responders ($LogFC_R$ – $LogFC_{NR}$).

$$LogFC_R = log_2(A + 1/B + 1)$$
$$LogFC_{NR} = log_2(C + 1/D + 1)$$

Formula 3. A= FPKM of responders treated with dexamethasone; B= FPKM of responders treated with ethanol; C= FPKM of non-responders treated with dexamethasone; D= FPKM of non-responders treated with ethanol.

In order to make the second comparison, the mean change in LogFC between TM cells treated with and without dexamethasone was obtained by calculating the average of the $LogFC_R$ and the $LogFC_{NR}$ for each gene. As no individual sample measurements were available, we could not calculate a significance value.

Pathway overrepresentation analysis

After performing the before-mentioned steps, we performed pathway analysis on the integrated human dataset and the two datasets generated from the bovine RNA-sequencing experiment. A pathway overrepresentation analysis allows the identification of the molecular pathways in which the differentially expressed genes are significantly more present than expected by chance based on the entire dataset. In order to do so, criteria for a gene to be differentially expressed need to be defined first. In our case, a gene with an absolute LogFC > 0.58 (representing at least a 50% change on original scale of absolute numbers) and a P-value < 0.05 was defined as differentially expressed for the integrated human dataset. For the bovine set only the LogFC cut-off was used since no P-values were available. In the results of the pathway analysis, pathways with a Z-score ≥ 1.96, a permuted P-value < 0.05, and > 3 changed genes in the pathway were considered significantly changed.

The pathway overrepresentation analysis was performed in PathVisio which is an open access tool.[21, 22] To connect measured genes to the corresponding pathway elements, a human identifier mapping database is needed which was downloaded from *www.pathvisio.org* (version: Hs_Derby_Ensembl_91.bridge). For the bovine datasets, the gene identifiers provided by the authors were already converted to human HGNC symbols. Therefore, we also used the human identifier mapping database and the human pathways to perform pathway analysis on this dataset. As previously described[18], we used the pathways of three widely adopted pathway databases WikiPathways[23, 24], KEGG[25-27] and Reactome[28, 29]. Their content was downloaded on December 6, 2018 and combined into one collection in order to obtain larger pathway coverage. The overrepresentation scores (Z-scores) were calculated in one run for all included pathways of all three databases.

Clustering of the pathway results

The significantly changed pathways of each dataset were clustered into functional categories. This was based on the molecular mechanisms that are captured in the pathways and was performed by human curation after careful investigation of the results.

Network analysis

Cytoscape (*www.cytoscape.org*), an open access tool, was used to perform network analysis in which the genes of the identified functional categories were combined into one network of connected genes.[30] We created a network for the bovine data comparing responders and non-responders. To improve visualization, we only showed the differentially expressed genes (absolute LogFC > 0.58). A flowchart overviewing the methods and results is shown in figure 1.

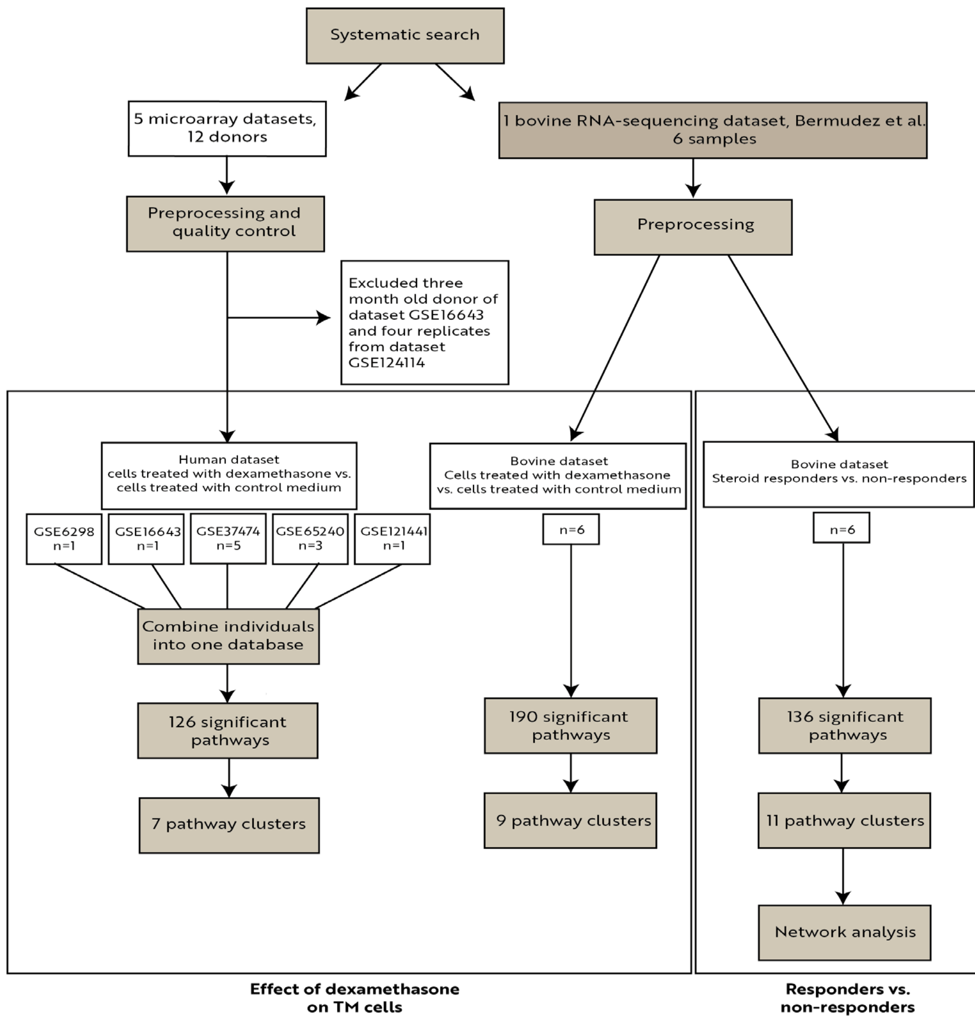


Figure 1. *Flowchart of the methods and results.*

Results

Systematic search

The systematic search in GEO identified five studies investigating the effect of corticosteroids on the TM which were all selected for further analysis. A search with the broader search term "trabecular meshwork" showed 30 datasets, however, none of them were additionally relevant. Both searches were also performed in ArrayExpress revealing no other studies.

Included datasets

After the systematic search the datasets of five different human microarray studies were selected for further analyses: GSE6298 (Fan et al., Invest Ophthalmol Vis Sci, 2008)[6], GSE16643 (Nehmé et al., BMC Med Genomics, 2009)[10], GSE37474 (Kwon et al., concept article received after contacting the authors, 2012)[8], GSE65240 (Matsuda et al., Invest Ophthalmol Vis Sci, 2015)[9], and GSE124114 (Faralli et al., Mol Vis, 2019)[31]. In addition, the statistical results of the bovine RNA-sequencing experiment by Bermudez et al. (Plos one, 2017) were used in the analysis.[14]

The key characteristics of the included studies are listed in table 1. The most important features are described here. All studies used cells derived from TM tissue which was exposed to dexamethasone and a control medium. Hereafter, total RNA was extracted and used for microarray analysis except for the study of Bermudez et al. in which RNA-sequencing was performed. Most of the included studies used cultured TM cells, however, dataset GSE37474 and Bermudez et al., used a perfusion organ culture system.

Quality control of the human data

The complete reports of the quality control of the five human microarray datasets are available in supplemental material 1. The quality control showed that all datasets were of good quality. At sample level, one donor of dataset GSE16643 and four replicates of dataset GSE124114 were identified as outliers and removed for further analysis (see supplemental material 1). The remaining high-quality data of the five datasets was combined into one dataset. This resulted in an integrated dataset consisting of 17,705 unique genes.

Table 1. *Characteristics of the included studies.*

	Fan et al. GSE6298 (2008)	Nehmé et al. GSE16643 (2009)	Kwon et al. GSE37474 (2012)	Matsuda et al. GSE65240 (2015)	Peters et al. GSE124114 (2019)	Bermudez et al. Bovine RNA-seq (2017)
Study design	Human cultured TM cells	Human cultured TM cells	Perfusion organ culture system with human donor eyes. Dissection of TM cells after exposure	Human cultured TM cells	Two human TM cell cultures from the same donor	Perfusion organ culture system with bovine donor eyes to define responders and non-responders. Contralateral eye was used for cell isolation and culture
Use of postmortem tissue	Yes	Yes	Yes	Yes	Yes	Yes
Passage cells	8th	3th-6th	Not specified	Not specified	2nd	> 3
Control medium	0,0025% and 0,025% BA*	0,1% (v/v) DMSO*	2 μl/min DMEM*	DMEM* with 10% fetal calf serum	0.1% Ethanol	0.1% Ethanol
Number of included donors	1 donor	2 donors	5 donors (pair eyes)	3 donors	1 donor	6 donors (pair eyes)
Replicates	3 replicates	3 replicates	No replicates	No replicates	18 replicates	No replicates
Age	52 years	3 months and 35 years	72 year (mean)	Not specified	27 years	Adult cows
History of glaucoma	No	Not specified	No	Not specified	Not specified	Not specified
Steroid response	Not specified	Not specified	Unclear	Not specified	Not specified	Yes
Dexamethasone	100 nM Medium changed every other day	1 μM = 1000 nM Medium not changed	100 nM Prefusion system	100 nM Medium changed every two days	500 nM Medium change not defined	100 nM Medium changed every other day
Duration of exposure (days)	7	1 (24h)	10	14	6	7
Micro-array/ RNA-seq	Stanford Human cDNA SHEW	Agilent-014850 Whole Human Genome Microarray 4x44K G4112F	Affymetrix Human Genome U133 Plus 2.0 Array	- Agilent-028004 SurePrint G3 Human GE 8x60K Microarray - Agilent-039494 SurePrint G3 Human GE v2 8x60K Microarray 039381	Affymetrix Human Gene 1.0 ST Array	Illumina human methylation 450K chips

**BA= benzyl alcohol; DMSO= dimethylsulfoxide; DMEM= Dulbecco's modified Eagle's medium*

Pre-processing of the bovine data

Within the bovine data, we firstly investigated the differential gene expression in the TM after exposure to dexamethasone compared to a control medium. Secondly, we investigated the differential gene expression in the TM between responders and non-responders. Both obtained datasets comprised 25,794 unique genes.

Pathway overrepresentation analysis

Pathway analysis was performed on the three datasets which were obtained after performing the quality control and pre-processing steps. The integrated human dataset revealed 133 significantly changed pathways. The number of genes that fulfilled the criteria for significance (i.e. an absolute LogFC > 0.58 and a P-value < 0.05) was 829. The bovine dataset comparing TM cells treated with and without dexamethasone showed 190 significantly changed pathways and the bovine dataset comparing responders and non-responders TM-cells showed 136 significantly changed pathways. The complete results of the pathway analyses are shown in respectively supplemental materials 2, 3, and 4.

Clustering of the pathway results

The significantly changed pathways of each of the three performed pathway analyses were clustered into multiple functional categories. The clusters collagen, extracellular matrix (ECM), adhesion, WNT signaling, inflammation, adipogenesis and glocuse metabolism were found in the three datasets. For nuclear factor kappa-light-chain-enhancer of activated B cells (*NF-κB*), apoptosis, G protein-coupled receptor (GPCR), and oxidative stress (see table 2), it should be noted that multiple pathways involved in these clusters were just below the significance threshold in the other datasets. However, we should take into account that the chosen threshold is an arbitrary cut-off value and should not be seen as a hard cut-off. Pathways just below the threshold are not per definition not involved and may still contain relevant changes or may have not been retrieved in each dataset due to a lack of power (see table 2). For example, some of the pathways aggregate information of multiple cellular processes. If only part of these processes are not involved in the pathogenesis of the investigated tissue, the complete pathway might be just below the chosen threshold level. On the other hand, specific pathways that contain very low numbers of measured genes, may be just below the cut-off for significance due to a lower power. Cell cycle and senescence were only found in the responder vs. non-responder datasets and not in the human or bovine dataset in which the effect of dexamethasone on the TM cells was investigated. The pathways involved in the categories for each of the analyses are presented in supplemental material 5 (sheet 1-3).

Table 2. *Overview of the clusters between the different datasets, with additional visualization for clusters that were just below the cut-off.*

Effect of dexamethasone on TM cells		**Responders vs. non-responders**
Human combined	**Bovine**	**Bovine**
Collagen	Collagen	Collagen
ECM	ECM	ECM
Adhesion	Adhesion	Adhesion
WNT signaling	WNT signaling	WNT signaling
Inflammation	Inflammation	Inflammation
Adipogenesis	Adipogenesis	Adipogenesis
Glucose metabolism	Glucose metabolism	Glucose metabolism
NF-κB	*NF-κB*	*NF-κB* *
Apoptosis*	Apoptosis	Apoptosis
GPCR	GPCR*	
		Cell cycle
		Senescence
Oxidative stress*	Oxidative stress*	Oxidative stress

Pathway clusters present in the three datasets (white)
Pathway clusters present in two out of three datasets (light)
Pathway clusters present in one out of three datasets (grey)

** Clusters that were just below the cut-off for significance in the respective dataset to form a cluster but could be overall involved.*

Abbreviations: ECM= extracellular matrix; NF-κB = nuclear factor kappa-light-chain-enhancer of activated B cells; GPCR= G protein-coupled receptor GPCR

Network analysis

In order to visualize the results after comparing the bovine responders and non-responders, we created a network based on the genes within each of the pathways per functional cluster (see figure 2). Only genes with an absolute logFC > 0.58 are shown. The network illustrates that all the identified functional clusters are connected with each other. Multiple genes are shared between multiple clusters. An overview of the genes that are present in at least two clusters is shown in table 3.

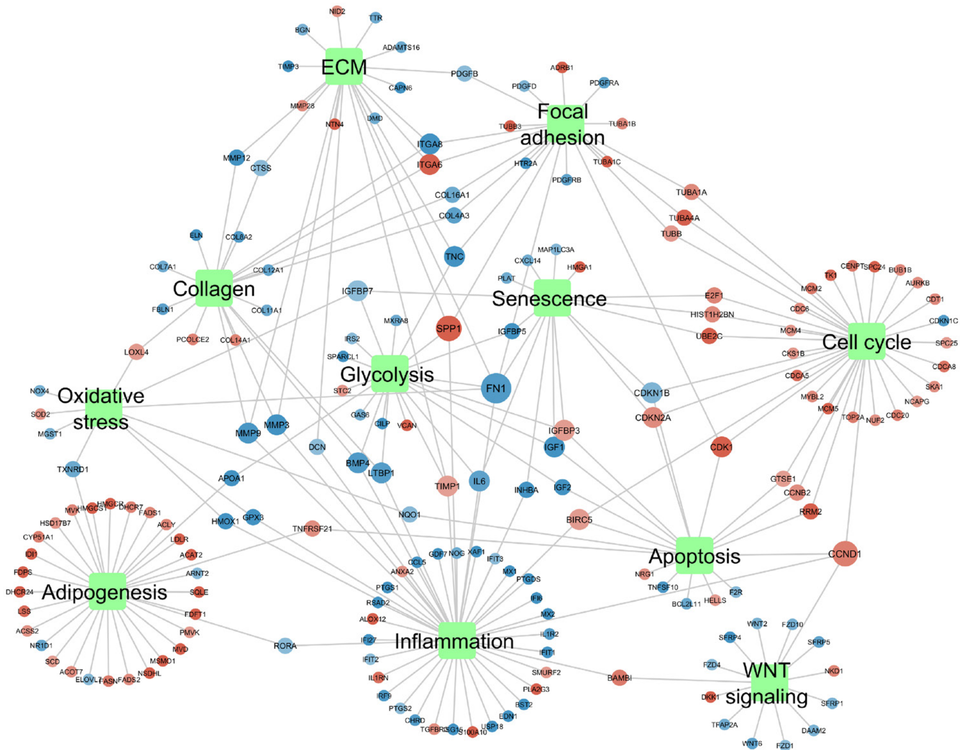


Figure 2. *Network of the bovine data comparing TM cells of responders and non-responders. Green quadrangles indicate the functional categories; Each node represents a gene. Red color indicates upregulation, blue color indicates downregulation. The larger the node, the more clusters the gene is represented in.*

Table 3. *Overview of the functional clusters between the different datasets.*

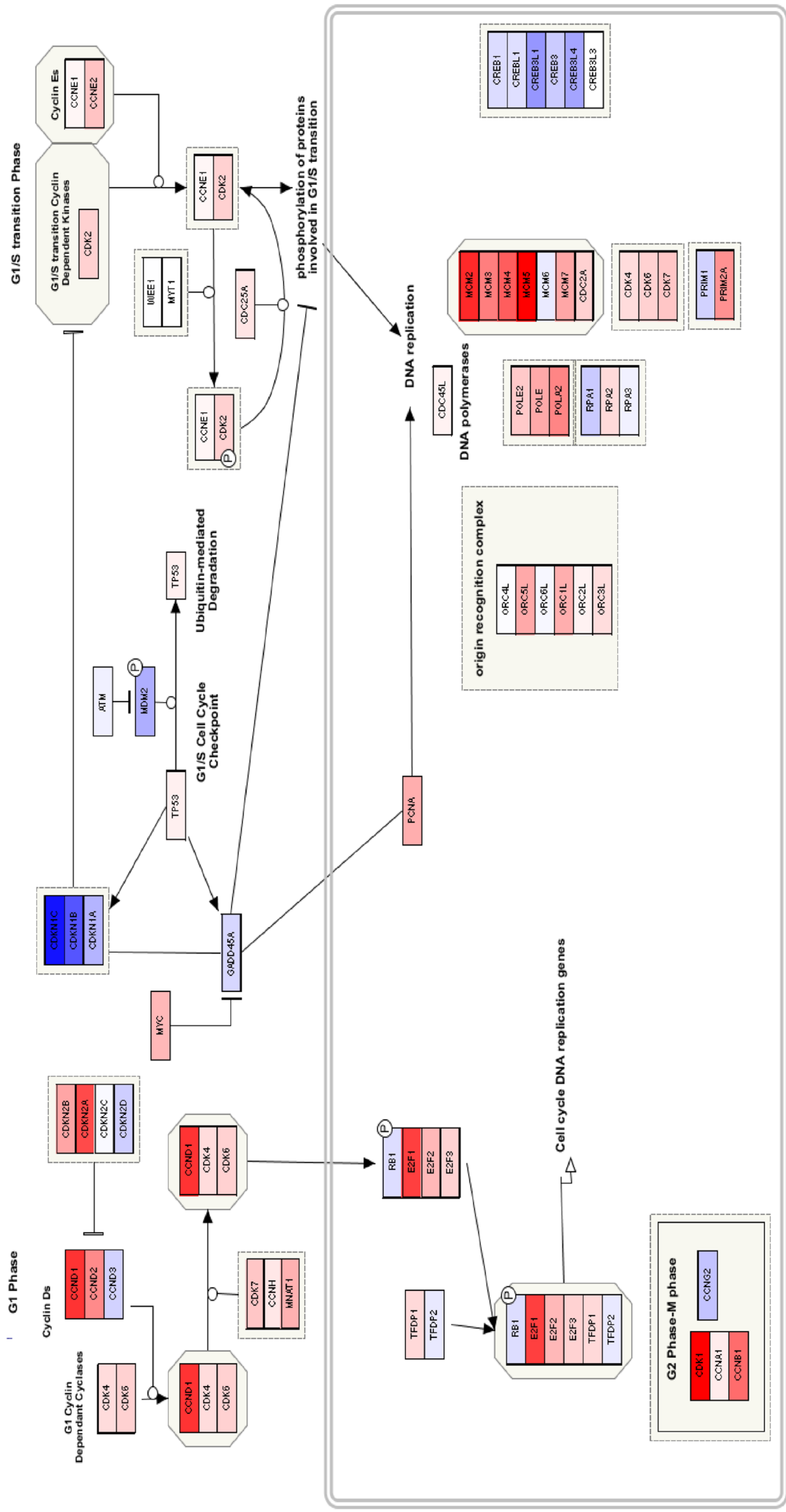
Gene symbol	Number of clusters the gene is represented in	Involved clusters
FN1	6	Inflammation, ECM, senescence, focal adhesion, oxidative stress, glucose metabolism
SPP1	4	Inflammation, ECM, focal adhesion, glucose metabolism
CCND1	4	Inflammation, cell cycle, apoptosis, WNT-signaling
IGFBP3	3	Senescence, apoptosis, glucose metabolism
IGF1	3	Senescence, apoptosis, glucose metabolism
MMP3	3	Inflammation, collagen, ECM
MMP9	3	Inflammation, collagen, ECM
LTBP1	3	Inflammation, collagen, glucose metabolism
BMP4	3	Inflammation, collagen, glucose metabolism

ITGA8	3	Collagen, ECM, focal adhesion
ITGA6	3	Collagen, ECM, focal adhesion
CDKN2A	3	Cell cycle, senescence, apoptosis
CDKN1B	3	Cell cycle, senescence, apoptosis
TIMP1	3	Inflammation, ECM, glucose metabolism
TNC	3	ECM, focal adhesion, glucose metabolism
CDK1	3	Cell cycle, apoptosis, focal adhesion
BIRC5	3	Inflammation, cell cycle, apoptosis
IGFBP7	3	Senescence, oxidative stress, glucose metabolism
IL6	3	Inflammation, senescence, glucose metabolism
E2F1	2	Cell cycle, senescence
HIST1H2BN	2	Cell cycle, senescence
UBE2C	2	Cell cycle, senescence
TUBA1A	2	Cell cycle, focal adhesion
TUBB	2	Cell cycle, focal adhesion
TUBA4A	2	Cell cycle, focal adhesion
RRM2	2	Cell cycle, apoptosis
GTSE1	2	Cell cycle, apoptosis
CCNB2	2	Cell cycle, apoptosis
COL4A3	2	Collagen, focal adhesion
COL16A1	2	Collagen, focal adhesion
CTSS	2	ECM, collagen
MMP12	2	ECM, collagen
GPX3	2	Inflammation, oxidative stress
HMOX1	2	Inflammation, oxidative stress
IGF2	2	Apoptosis, glucose metabolism
IGFBP5	2	Senescence, glucose metabolism
TNFRSF21	2	Apoptosis, adipogenesis
PDGFB	2	ECM, focal adhesion
LOXL4	2	Collagen, oxidative stress
DCN	2	Inflammation, ECM
NQO1	2	Apoptosis, oxidative stress
BAMBI	2	Inflammation, WNT-signaling
TXNRD1	2	Adipogenesis, oxidative stress
RORA	2	Adipogenesis, inflammation
INHBA	2	Inflammation, senescence
APOA1	2	Adipogenesis, glucose metabolism

Discussion

Within this study we identified seven functional pathway clusters that were significantly changed in both TM cells exposed to dexamethasone vs. a control medium as well as in TM cells of responders vs. non-responders: collagen, ECM, adhesion, WNT-signaling, inflammation, adipogenesis and glucose metabolism. In addition the functional clusters of *NF-κB*, apoptosis, GPCR and oxidative stress were just below the cut-off for significance in some of the datasets but this does not rule out their potential relevant involvement. In contrast, it is remarkable that the pathways within the functional categories cell cycle and senescence were highly significant in the bovine responder vs. non-responder data and non-significant in the other datasets. Therefore, these functional clusters are discussed in more detail below.

The network shows that most genes involved in the category cell cycle are upregulated after comparing responders and non-responders. To obtain a better understanding, we investigated the pathways involved in this cluster separately (see for example figure 3A). Within this pathway *CCND1* and *CCND2*, known to drive the G1/S phase transition by binding with multiple cyclin-dependant kinases (*CDK*s), are upregulated. *CDK*s are necessary to regulate the progression through the cell cycle and are also upregulated. In addition, *CDKN1A*, *CDKN1B* and *CDKN1C* normally inhibit *CDK*s but are here downregulated. Therefore, the gene expression of the mentioned genes, suggest an increased activity of the cell cycle. As cell cycle was only significantly changed after comparing responders and non-responders and not after comparing exposure of dexamethasone vs. a control medium, this might suggest that the pathways or multiple genes within this functional cluster behave in opposite ways in responders and non-responders. To check this hypothesis, we visualized the cell cycle pathway with the gene expression values of responders and non-responders, as calculated in formula 1 (see figure 3B). As expected, the genes within these pathways are expressed in the opposite direction or have relatively large differences in their level of expression.


A
G1/S transition Phase
G1/S transition Cyclin Dependent Kinases
CDK2
Cyclin Es
CCNE1
CCNE2
CCNE1
CDK2
WEE1
MYT1
CDC25A
CCNE1
CDK2
P
phosphorylation of proteins involved in G1/S transition
DNA replication
G1 Phase
Cyclin Ds
CCND1
CCND2
CCND3
G1 Cyclin Dependant Cyclases
CDK4
CDK6
CCND1
CDK4
CDK6
CDKN2B
CDKN2A
CDKN2C
CDKN2D
CDK7
CCNH
MNAT1
CCND1
CDK4
CDK6
MYC
GADD45A
CDKN1C
CDKN1B
CDKN1A
TP53
ATM
MDM2
P
G1/S Cell Cycle Checkpoint
TP53
Ubiquitin-mediated Degradation
PCNA
RB1
E2F1
E2F2
E2F3
P
TFDP1
TFDP2
RB1
E2F1
E2F2
E2F3
TFDP1
TFDP2
P
Cell cycle DNA replication genes
G2 Phase-M phase
CDK1
CCNA1
CCNB1
CCNG2
origin recognition complex
ORC4L
ORC5L
ORC6L
ORC1L
ORC2L
ORC3L
DNA polymerases
CDC45L
POLE2
POLE
POLA2
RPA1
RPA2
RPA3
MCM2
MCM3
MCM4
MCM5
MCM6
MCM7
CDK4
CDK6
CDK7
PRIM1
PRIM2A
CREB1
CREBL1
CREB3L1
CREB3
CREB3L4
CREB3L3

B

Figure 3. *Visualization of the cell cycle pathway from WikiPathways.*
(A) Visualizing the results after comparing the bovine responders vs. non-responders. Every colored rectangle represents a gene that had been measured in the bovine dataset. Red color indicates upregulation, blue color indicates downregulation. The higher the intensity of the color, the higher the up-or downregulation. (B) Visualizing the LogFC of the responders and the non-responders as calculated in formula 1. Every colored rectangle represents a gene that had been measured in the bovine dataset. The first part of the rectangle represents the responders and the second part of the rectangle represents the non-responders. Red color indicates upregulation, blue color indicates downregulation. The higher the intensity of the color, the higher the up-or downregulation.

It is known that corticosteroids influence the cell cycle. However, the effects have been suggested to be cell specific and differ between the dosage and types of corticosteroids. For example a study investigating the effect of different types of corticosteroids on hyperproliferant keratinocytes found that all types of corticosteroids reduced the cell proliferation.[32] On the other hand, cultured corneal epithelial cells showed an increased cell proliferation when exposed to dexamethasone less than 10^{-6} M and inhibition of the proliferation at concentrations of more than 10^{-4}.[33] In addition, we now found that the activation of the cell cycle in the TM is different between responders and non-responders.

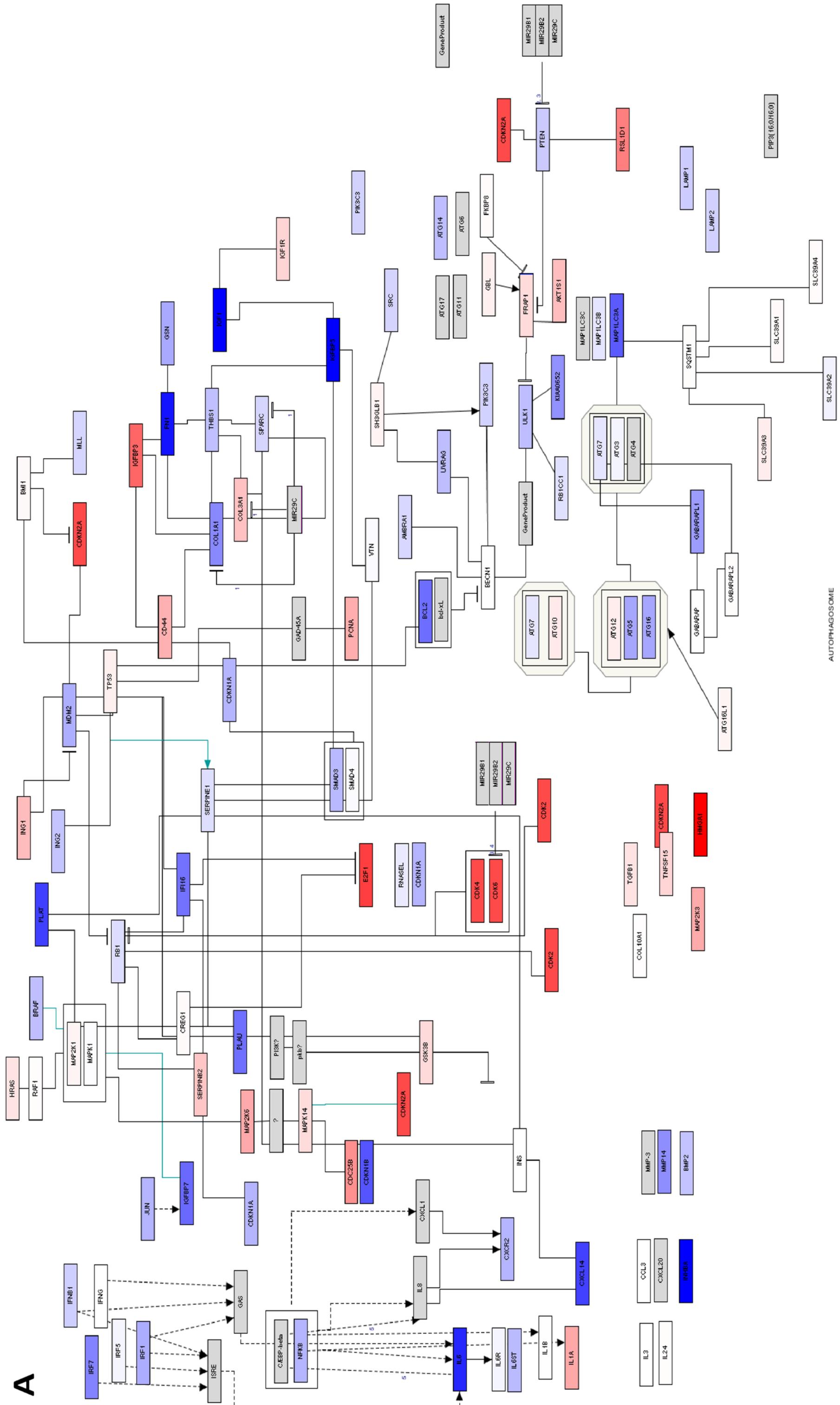
Previous studies showed that cell proliferation is inhibited in TM cells after exposure to corticosteroids. In this study, we found this process not to be significantly changed after exposure to corticosteroids. However, as stated above, we now investigated the difference between responders and non-responders and found a clear upregulation of cell proliferation in responders compared to non-responders. This suggests that there is a different reaction to corticosteroids in responders compared to non-responders and shows the need of further investigation with human TM tissue of identified responders and non-responders instead of solely TM cells exposed to corticosteroids.

Senescence was also found to be involved in corticosteroid-induced OHT. Corticosteroids have been shown to have enhancing or inhibiting effects on senescence.[34-36] In contrast to the cell cycle, it is difficult to define a certain overall up- or downregulation of the identified pathways that are involved in this functional category (see figure 4A). However, when visualizing the LogFC of the responders and non-responders on these pathways, multiple genes are expressed in the opposite direction (see figure 4B) or show large differences in gene expression. A gene of particular interest might be *UBE2C* as it is not only significantly upregulated in responders and downregulated in non-responders, it is also shared by the cell cycle cluster, as shown in the network.

Furthermore, we found the functional category senescence to be involved in the molecular pathogenesis of POAG as well.[18] It is known that patients with POAG are more susceptible to develop corticosteroid-induced OHT. The other way around, patients that had a corticosteroid response in the past are at risk to develop POAG.[1, 6, 37-40]

Multiple processes such as ECM, focal adhesion, collagen and WNT-signaling have been described to be involved in the pathogenesis of corticosteroid-induced glaucoma.[2, 6-13, 41] We found these clusters to be significantly changed in all three datasets. This indicates that the differences in gene expression profiles, for these clusters, between responders and non-responders are not as extensive as seen in cell cycle and senescence. Indeed, most genes involved in these pathways do not show opposites in gene expression (up- or downregulation) but differed in the intensity of the gene expression (see figure 5). Nevertheless, despite the fact that these clusters show differences in gene expression rather than opposites, they are also likely to be involved in the pathogenesis of corticosteroid-induced glaucoma and need further investigation.

We created a network comparing bovine eyes with and without a corticosteroid response. Notably, the visualized genes involved in the separate clusters are largely homogeneous up or down regulated. Multiple genes are shared between multiple clusters and might be of special interest. However, fibronectin (*FN1*) is shared by the largest number of clusters. Previous research showed that the gene expression of *FN1* is increased in human and bovine TM cells after exposure to dexamethasone. Interestingly, in the responders *FN1* is almost not expressed and in the non-responders it is significantly upregulated. Consequently, after comparing responders and non-responders, as shown in the network, *FN1* gene is downregulated. Bermudez et al. already addressed this finding and performed a Western Blot on *FN1*.[14] They found a significant higher expression in responders compared to non-responders after exposure to dexamethasone.


A

B

Figure 4. *Visualization of the senescence pathway from WikiPathways.*
(A) Visualizing the results after comparing the bovine responders vs. non-responders; Every colored rectangle represents a gene that had been measured in the bovine dataset. Red color indicates upregulation, blue color indicates downregulation. The higher the intensity of the color, the higher the up-or downregulation. (B) Visualizing the LogFC of the responders and the non-responders as calculated in formula 1; Every colored rectangle represents a gene that had been measured in the bovine dataset. The first part of the rectangle represents the responders and the second part of the rectangle represents the non-responders. Red color indicates upregulation, blue color indicates downregulation. The higher the intensity of the color, the higher the up-or downregulation.

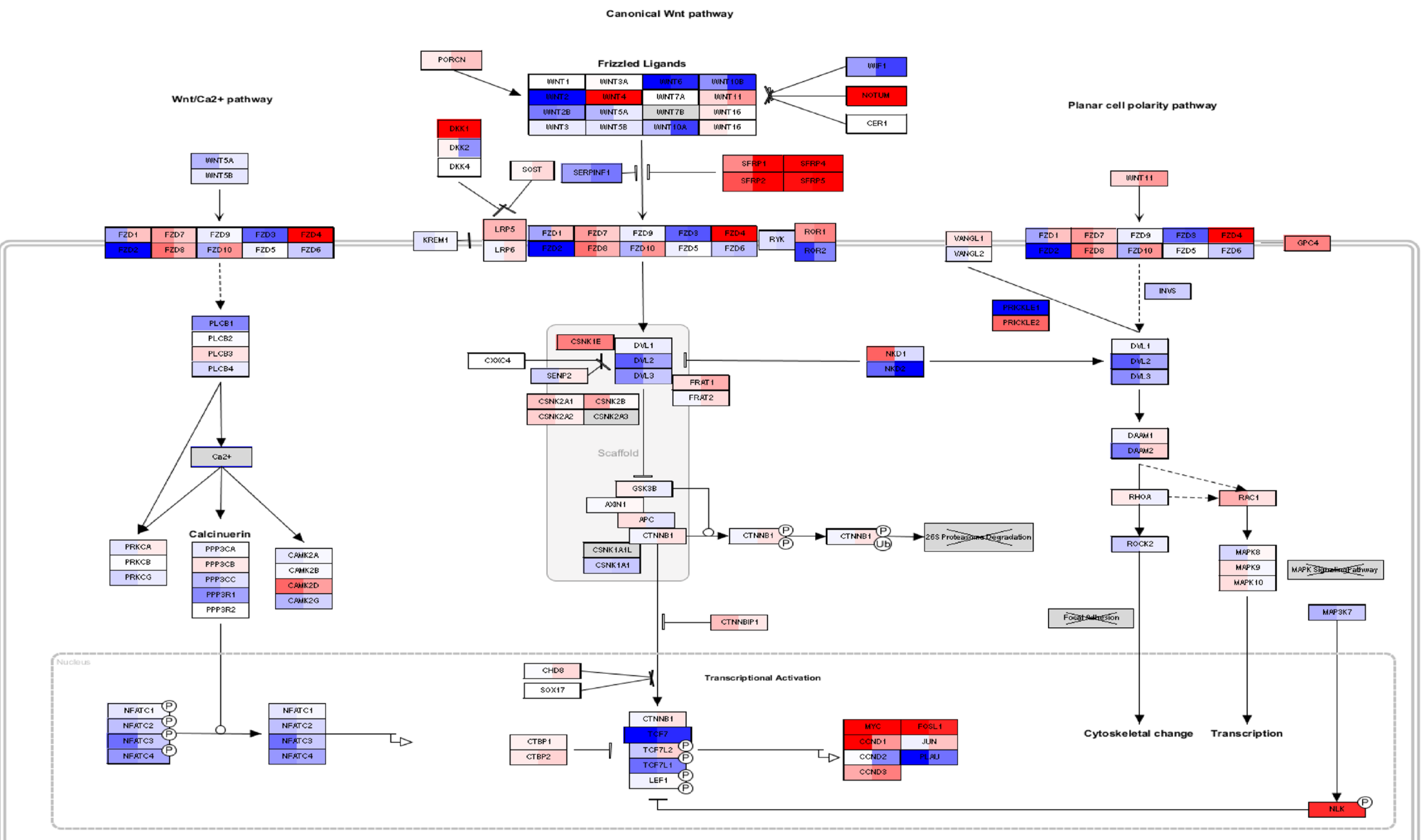


Figure 5. *WNT signaling pathway from WikiPathways after visualizing the LogFC of the responders and the non-responders as calculated in formula 1.*

Every colored rectangle represents a gene that had been measured in the bovine dataset. The first part of the rectangle represents the responders and the second part of the rectangle represents the non-responders. Red color indicates upregulation, blue color indicates downregulation. The higher the intensity of the color, the higher the up-or downregulation.

Raghunatan et al.[3] performed immunocytochemistry on human TM cells treated with dexamethasone and found fibronectin to be deposited as an organized fibrillar sheet. This was not observed in human TM cells exposed to a control medium. In another study of the group of Raghunatan et al. the same results were reported.[42] Li et al.[43] found similar results after exposing the TM of mice to dexamethasone. In contrast to this finding, Raghunatan et al.[3] reported no significant difference in the protein expression of fibronectin between human TM cells exposed to dexamethasone or those exposed to a control medium. In a similar experiment by Shan et al.[44], the protein expression of fibronectin was also not significantly changed in human TM cells exposed to dexamethasone when compared to TM cells exposed to a control medium. However, they reported that the protein expression of fibronectin was decreased in human TM cells exposed to prednisolone when compared to controls. Bollinger et al.[45] investigated both glaucomatous and non-glaucomatous human TM samples. They also found that the average relative protein abundance over all dexamethasone-treated TM cell samples (glaucomatous and non-glaucomatous together) did not show a significant change in fibronectin protein expression. In contrast, Honjo et al.[46], Peng et al.[47], Fila et al.[48] and Zhou et al.[49] reported that the protein expression of fibronectin was significantly increased after exposing human HTM cells to dexamethasone. Additionally, Li et al.[50] reported the fibronectin protein secretion to be significantly increased in four human TM cell strains but to be decreased in one human TM cell strain after exposure to dexamethasone (both after one and four weeks of exposure). Steely et al.[51] investigated the fibronectin gene expression in one glaucomatous and three non-glaucomatous human TM cell strains. They found a significant elevation of fibronectin gene expression in the glaucomatous TM cells exposed to dexamethasone and in two of the non-glaucomatous cell strains but no increase in one of the non-glaucomatous strains. Additionally to human protein expression studies, Patel et al.[52] performed an immunohistochemical analysis on the TM of wild-type mice after treatment with periocular dexamethasone-acetate injections and found an increased protein expression of fibronectin when compared to wild-type mice treated with a control medium. Wang et al.[53] exposed TM cells of rats to different concentrations of dexamethasone and found the protein expression of fibronectin to be increased as well when compared to controls. Based on the results of the above-mentioned studies, the effect of dexamethasone-treatment of TM cells on the protein expression of fibronectin is incongruent.

In addition, multiple human transcriptomics studies also investigated the gene expression of *FN1* after exposing TM cells to corticosteroids: Fan et al.[6], Nehmé et al.[10], Kwon et al.[8], Matsuda et al.[9], and Faralli et al.[31]. They all found fibronectin to be upregulated. As these studies were included in our study; the average logFC of fibronectin after exposing TM cells to corticosteroids was significantly upregulated (average LogFC: 0.68; average weight: 4.33; average P-value: 0.00005).

4

Raghunatan et al.[3] stated that an increased activation of contractility machinery and perhaps altered integrin binding in dexamethasone-treated cells may contribute to the reorganization of deposited fibronectin. Therefore, in accordance with the suggestion by Bermudez et al., the differences might be caused by post-translational processes.

Additionally, it is remarkable that in both the study of Li et al.[50] and Steely et al.[51], some of the included strains showed an increased fibronectin protein expression and others a decreased expression or even no change in expression. As a steroid response is very common (one out of three patients), it is possible that the steroid response status of a patient might explain these differences. Currently, to our best knowledge, only two studies investigated the protein expression of fibronectin in steroid responders versus non-responders. Both studies used bovine TM tissue.[14, 54] The study of Bermudez et al. reported an increased expression of fibronectin protein levels in responders but not in non-responders.[14] The study of Mao et al. used an anterior segment perfusion system and found the fibronectin protein expression to be induced in the TM of three out of eight responders and in one out of six non-responders, this difference however was not statistically significant ($p>0.5$).[54] Therefore, the role of responders and non-responders in the protein expression of fibronectin needs further investigation.

One of the strengths of this study is that we performed a systematic method in which all relevant publicly available gene expression data was used. This enabled us to integrate and build upon existing knowledge. However, as in a meta-analysis, a critical appraisal of the included studies and their quality is necessary. A recent review of Keller et al. defined the induction of Myocilin after exposure to dexamethasone to be a reliable marker for TM tissue.[55, 56] We found *MYOC* to be highly expressed in each of the five included human studies which indicates that the investigated tissue indeed is TM-tissue. In the bovine study, *MYOC* was not highly expressed. However, multiple studies found contradictive results regarding the expression of *MYOC* after exposing bovine TM tissue to dexamethasone.[13, 57] Differences in breed have been suggested to play a role.[55]

Furthermore, the age of the donors in the study ranged between three months and 72 years (see characteristics in table 1). The consensus of Keller et al. recommends using donors younger than 60 years old, however donors older than 60 years may also provide adequate primary TM cell cultures.[55] Within this study, only one donor was older than 60 years and included for further analysis. We did, however, exclude a three month old donor (dataset GSE16643) as the development of the TM continues in the postnatal period.[58-60]

Some issues need to be addressed. Ideally, gene expression data of TM tissue specifically derived from human corticosteroid responders would be the tissue of primary choice. However, to our best knowledge, this data is not available. Therefore, we used the gene expression data of bovine TM cells of responders and non-responders. The bovine TM outflow tissue is known to be morphologically different from human TM tissue.[61] However, also bovine eyes are known to develop a corticosteroid response after treatment with corticosteroids and similar to human subjects, this response declines after discontinuation of the corticosteroids.[62] The development of OHT after exposure to dexamethasone was also observed in perfusion-cultured bovine anterior segments which was used by Bermudez et al.[54] Furthermore, the physiology of the aqueous humour formation resembles that of human subjects as both have higher concentrations of chloride compared to plasma and the chloride transport is in both species inhibited by carbonic anhydrase inhibitors.[63, 64] The above described findings imply that bovine eyes and the perfusion-cultured model used by Bermudez et al. are suitable for investigating corticosteroid-induced glaucoma. In addition, the functional clusters we identified for the human and bovine data after investigation of the effect of corticosteroids on the TM, are the same which validates the used model.

We used the human pathway collections of WikiPathways, KEGG and Reactome for pathway analysis of the bovine data instead of the bovine (Bos Taurus) pathways. This was done as available bovine pathways are commonly converted from human pathways and are therefore not likely to add any new information. The human pathway collection is also more extensive than the bovine collection. Additionally, consistently using the human pathway collection allowed comparing the results of the human and bovine data with each other.

We could not alter the cut-off values for a corticosteroid response. Bermudez et al. defined a corticosteroid response as the average IOP minus the baseline IOP to be equal or higher than 2.82 mmHg. IOP was recorded every minute and the average was calculated every twenty-four hours. Multiple definitions for corticosteroid response have been defined but one of the most frequently used definitions is a one-time increase of six mmHg over baseline.[37] It is however known that diurnal IOP fluctuations can cause this difference in IOP which might result in the unjustified diagnosis of a corticosteroid response.[65-67] Bermudez et al. used a less stringent cut-off, however, they averaged the IOP over 24 hours which might make the elevation more robust. Also, since the exposure period was short, a lower cut-off is more valuable since the six mmHg or higher also reflects long term exposure to corticosteroids. A low but early increase in IOP could identify these subjects more correctly. In addition, the results show clearly differences in responders and non-responders based on gene expression and molecular processes

which strengthens the fact that responders and non-responders were identified correctly. Nevertheless, it is of value to study the molecular processes that cause early or late corticosteroid response.

Additionally, there are some differences on study level between the included datasets. Different control mediums were used and the time of exposure to corticosteroids ranged between one to fourteen days across the included datasets (see table 1). It is likely that both the use of different control mediums and a different time of exposure to corticosteroids cause differences in the transcriptome response. However, the other way around, consistently using the same control medium, without the exact knowledge on how this could affect gene expression, or the same time of exposure within every study could also mask some of the results as the transcriptome response might consistently under- or overexpress some genes. Nonetheless, as we were concerned that the differences between datasets could influence the results, we performed pathway analyses on the gene expression data of the separate datasets (not shown). This showed no major differences between the identified significantly changed pathways and processes between the different datasets. This indicates that the differences between the studies did not influence the results on pathway and process level. Nonetheless, in order to obtain results that are less dependent on study differences of individual studies and concise, we combined the high-quality and pre-processed gene expression datasets of the included studies for the reported analyses.

In conclusion, the systematically performed approach allowed the identification of the functional processes cell cycle and senescence to be highly likely involved in the pathogenesis of corticosteroid-induced OHT. Other processes such as collagen, ECM, adhesion and WNT-signaling behave differently between responders and non-responders as well. However, as these differences are mainly based on differences in intensities of gene expression rather than opposites, further investigation of these processes is needed. These pathways and their involved genes, and maybe especially the genes shared between the identified processes after comparing responders and non-responders, are of interest for drug targeting.

References

1. Tripathi RC, Parapuram SK, Tripathi BJ, et al. Corticosteroids and glaucoma risk. Drugs Aging 1999;15(6):439-50.
2. Yuan Y, Call MK, Yuan Y, et al. Dexamethasone induces cross-linked actin networks in trabecular meshwork cells through noncanonical wnt signaling. Invest Ophthalmol Vis Sci 2013;54(10):6502-9.
3. Raghunathan VK, Morgan JT, Park SA, et al. Dexamethasone Stiffens Trabecular Meshwork, Trabecular Meshwork Cells, and Matrix. Invest Ophthalmol Vis Sci 2015;56(8):4447-59.
4. Kersey JP, Broadway DC. Corticosteroid-induced glaucoma: a review of the literature. Eye (Lond) 2006;20(4):407-16.
5. Jones R, 3rd, Rhee DJ. Corticosteroid-induced ocular hypertension and glaucoma: a brief review and update of the literature. Curr Opin Ophthalmol 2006;17(2):163-7.
6. Fan BJ, Wang DY, Tham CC, et al. Gene expression profiles of human trabecular meshwork cells induced by triamcinolone and dexamethasone. Invest Ophthalmol Vis Sci 2008;49(5):1886-97.
7. Ishibashi T, Takagi Y, Mori K, et al. cDNA microarray analysis of gene expression changes induced by dexamethasone in cultured human trabecular meshwork cells. Invest Ophthalmol Vis Sci 2002;43(12):3691-7.
8. Markus H. Kuehn CYK, Young H. Kwon. Dexamethasone induced gene expression changes in human perfusion organ cultured trabecular meshwork. 2012.
9. Matsuda A, Asada Y, Takakuwa K, et al. DNA Methylation Analysis of Human Trabecular Meshwork Cells During Dexamethasone Stimulation. Invest Ophthalmol Vis Sci 2015;56(6):3801-9.
10. Nehme A, Lobenhofer EK, Stamer WD, Edelman JL. Glucocorticoids with different chemical structures but similar glucocorticoid receptor potency regulate subsets of common and unique genes in human trabecular meshwork cells. BMC Med Genomics 2009;2:58.
11. Rozsa FW, Reed DM, Scott KM, et al. Gene expression profile of human trabecular meshwork cells in response to long-term dexamethasone exposure. Mol Vis 2006;12:125-41.
12. Lo WR, Rowlette LL, Caballero M, et al. Tissue differential microarray analysis of dexamethasone induction reveals potential mechanisms of steroid glaucoma. Invest Ophthalmol Vis Sci 2003;44(2):473-85.
13. Danias J, Gerometta R, Ge Y, et al. Gene expression changes in steroid-induced IOP elevation in bovine trabecular meshwork. Invest Ophthalmol Vis Sci 2011;52(12):8636-45.
14. Bermudez JY, Webber HC, Brown B, et al. A Comparison of Gene Expression Profiles between Glucocorticoid Responder and Non-Responder Bovine Trabecular Meshwork Cells Using RNA Sequencing. PLoS One 2017;12(1):e0169671.
15. Edgar R, Domrachev M, Lash AE. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Res 2002;30(1):207-10.
16. Barrett T, Wilhite SE, Ledoux P, et al. NCBI GEO: archive for functional genomics data sets--update. Nucleic Acids Res 2013;41(Database issue):D991-5.

17. Kolesnikov N, Hastings E, Keays M, et al. ArrayExpress update--simplifying data submissions. Nucleic Acids Res 2015;43(Database issue):D1113-6.
18. Liesenborghs I, Eijssen LMT, Kutmon M, et al. Comprehensive bioinformatics analysis of trabecular meshwork gene expression data to unravel the molecular pathogenesis of primary open-angle glaucoma. Acta Ophthalmol 2019.
19. Ritchie ME, Phipson B, Wu D, et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res 2015;43(7):e47.
20. RCoreTeam. R: A language and environment for statistical computing. In: Computing RFfS, ed. Vienna, Austria2013.
21. Kutmon M, van Iersel MP, Bohler A, et al. PathVisio 3: an extendable pathway analysis toolbox. PLoS Comput Biol 2015;11(2):e1004085.
22. van Iersel MP, Kelder T, Pico AR, et al. Presenting and exploring biological pathways with PathVisio. BMC Bioinformatics 2008;9:399.
23. Kelder T, van Iersel MP, Hanspers K, et al. WikiPathways: building research communities on biological pathways. Nucleic Acids Res 2012;40(Database issue):D1301-7.
24. Kutmon M, Riutta A, Nunes N, et al. WikiPathways: capturing the full diversity of pathway knowledge. Nucleic Acids Res 2016;44(D1):D488-94.
25. Kanehisa M, Furumichi M, Tanabe M, et al. KEGG: new perspectives on genomes, pathways, diseases and drugs. Nucleic Acids Res 2017;45(D1):D353-D61.
26. Kanehisa M, Sato Y, Kawashima M, et al. KEGG as a reference resource for gene and protein annotation. Nucleic Acids Res 2016;44(D1):D457-62.
27. Kanehisa M, Goto S. KEGG: kyoto encyclopedia of genes and genomes. Nucleic Acids Res 2000;28(1):27-30.
28. Fabregat A, Sidiropoulos K, Garapati P, et al. The Reactome pathway Knowledgebase. Nucleic Acids Res 2016;44(D1):D481-7.
29. Milacic M, Haw R, Rothfels K, et al. Annotating cancer variants and anti-cancer therapeutics in reactome. Cancers (Basel) 2012;4(4):1180-211.
30. Shannon P, Markiel A, Ozier O, et al. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res 2003;13(11):2498-504.
31. Faralli JA, Desikan H, Peotter J, et al. Genomic/proteomic analyses of dexamethasone-treated human trabecular meshwork cells reveal a role for GULP1 and ABR in phagocytosis. Mol Vis 2019;25:237-54.
32. Guichard A, Humbert P, Tissot M, et al. Effects of topical corticosteroids on cell proliferation, cell cycle progression and apoptosis: in vitro comparison on HaCaT. Int J Pharm 2015;479(2):422-9.
33. Bourcier T, Forgez P, Borderie V, et al. Regulation of human corneal epithelial cell proliferation and apoptosis by dexamethasone. Invest Ophthalmol Vis Sci 2000;41(13):4133-41.
34. Poulsen RC, Watts AC, Murphy RJ, et al. Glucocorticoids induce senescence in primary human tenocytes by inhibition of sirtuin 1 and activation of the p53/p21 pathway: in vivo and in vitro evidence. Ann Rheum Dis 2014;73(7):1405-13.
35. Bose R, Moors M, Tofighi R, et al. Glucocorticoids induce long-lasting effects in neural stem cells resulting in senescence-related alterations. Cell Death Dis 2010;1:e92.

36. Laberge RM, Zhou L, Sarantos MR, et al. Glucocorticoids suppress selected components of the senescence-associated secretory phenotype. Aging Cell 2012;11(4):569-78.
37. Armaly MF, Becker B. Intraocular pressure response to topical corticosteroids. Fed Proc 1965;24(6):1274-8.
38. Becker B. Intraocular Pressure Response to Topical Corticosteroids. Invest Ophthalmol 1965;4:198-205.
39. Kitazawa Y, Horie T. The prognosis of corticosteroid-responsive individuals. Arch Ophthalmol 1981;99(5):819-23.
40. Lewis JM, Priddy T, Judd J, et al. Intraocular pressure response to topical dexamethasone as a predictor for the development of primary open-angle glaucoma. Am J Ophthalmol 1988;106(5):607-12.
41. Fini ME, Schwartz SG, Gao X, et al. Steroid-induced ocular hypertension/glaucoma: Focus on pharmacogenomics and implications for precision medicine. Prog Retin Eye Res 2017;56:58-83.
42. Raghunathan VK, Benoit J, Kasetti R, et al. Glaucomatous cell derived matrices differentially modulate non-glaucomatous trabecular meshwork cellular behavior. Acta Biomater 2018;71:444-59.
43. Li G, Lee C, Agrahari V, et al. In vivo measurement of trabecular meshwork stiffness in a corticosteroid-induced ocular hypertensive mouse model. Proc Natl Acad Sci U S A 2019;116(5):1714-22.
44. Shan SW, Do CW, Lam TC, et al. New Insight of Common Regulatory Pathways in Human Trabecular Meshwork Cells in Response to Dexamethasone and Prednisolone Using an Integrated Quantitative Proteomics: SWATH and MRM-HR Mass Spectrometry. J Proteome Res 2017;16(10):3753-65.
45. Bollinger KE, Crabb JS, Yuan X, et al. Proteomic similarities in steroid responsiveness in normal and glaucomatous trabecular meshwork cells. Mol Vis 2012;18:2001-11.
46. Honjo M, Igarashi N, Nishida J, et al. Role of the Autotaxin-LPA Pathway in Dexamethasone-Induced Fibrotic Responses and Extracellular Matrix Production in Human Trabecular Meshwork Cells. Invest Ophthalmol Vis Sci 2018;59(1):21-30.
47. Peng J, Wang H, Wang X, et al. YAP and TAZ mediate steroid-induced alterations in the trabecular meshwork cytoskeleton in human trabecular meshwork cells. Int J Mol Med 2018;41(1):164-72.
48. Filla MS, Dimeo KD, Tong T, Peters DM. Disruption of fibronectin matrix affects type IV collagen, fibrillin and laminin deposition into extracellular matrix of human trabecular meshwork (HTM) cells. Exp Eye Res 2017;165:7-19.
49. Zhou L, Li Y, Yue BY. Glucocorticoid effects on extracellular matrix proteins and integrins in bovine trabecular meshwork cells in relation to glaucoma. Int J Mol Med 1998;1(2):339-46.
50. Li G, Cui G, Dismuke WM, et al. Differential response and withdrawal profile of glucocorticoid-treated human trabecular meshwork cells. Exp Eye Res 2017;155:38-46.
51. Steely HT, Browder SL, Julian MB, et al. The effects of dexamethasone on fibronectin expression in cultured human trabecular meshwork cells. Invest Ophthalmol Vis Sci 1992;33(7):2242-50.

52. Patel GC, Millar JC, Clark AF. Glucocorticoid Receptor Transactivation Is Required for Glucocorticoid-Induced Ocular Hypertension and Glaucoma. Invest Ophthalmol Vis Sci 2019;60(6):1967-78.
53. Wang C, Li L, Liu Z. Experimental research on the relationship between the stiffness and the expressions of fibronectin proteins and adaptor proteins of rat trabecular meshwork cells. BMC Ophthalmol 2017;17(1):268.
54. Mao W, Tovar-Vidales T, Yorio T, et al. Perfusion-cultured bovine anterior segments as an ex vivo model for studying glucocorticoid-induced ocular hypertension and glaucoma. Invest Ophthalmol Vis Sci 2011;52(11):8068-75.
55. Keller KE, Bhattacharya SK, Borras T, et al. Consensus recommendations for trabecular meshwork cell isolation, characterization and culture. Exp Eye Res 2018;171:164-73.
56. Polansky JR, Fauss DJ, Zimmerman CC. Regulation of TIGR/*MYOC* gene expression in human trabecular meshwork cells. Eye (Lond) 2000;14 (Pt 3B):503-14.
57. Taniguchi F, Suzuki Y, Kurihara H, et al. Molecular cloning of the bovine *MYOC* and induction of its expression in trabecular meshwork cells. Invest Ophthalmol Vis Sci 2000;41(8):2070-5.
58. Abu-Hassan DW, Acott TS, Kelley MJ. The Trabecular Meshwork: A Basic Review of Form and Function. J Ocul Biol 2014;2(1).
59. Gould DB, Smith RS, John SW. Anterior segment development relevant to glaucoma. Int J Dev Biol 2004;48(8-9):1015-29.
60. Johnston MC, Noden DM, Hazelton RD, et al. Origins of avian ocular and periocular tissues. Exp Eye Res 1979;29(1):27-43.
61. Flugel C, Tamm E, Lutjen-Drecoll E. Different cell populations in bovine trabecular meshwork: an ultrastructural and immunocytochemical study. Exp Eye Res 1991;52(6):681-90.
62. Gerometta R, Podos SM, Candia OA, et al. Steroid-induced ocular hypertension in normal cattle. Arch Ophthalmol 2004;122(10):1492-7.
63. Gerometta RM, Malgor LA, Vilalta E, et al. Cl- concentrations of bovine, porcine and ovine aqueous humor are higher than in plasma. Exp Eye Res 2005;80(3):307-12.
64. To CH, Do CW, Zamudio AC, Candia OA. Model of ionic transport for bovine ciliary epithelium: effects of acetazolamide and HCO. Am J Physiol Cell Physiol 2001;280(6):C1521-30.
65. David R, Zangwill L, Briscoe D, et al. Diurnal intraocular pressure variations: an analysis of 690 diurnal curves. Br J Ophthalmol 1992;76(5):280-3.
66. Jonas JB, Budde W, Stroux A, et al. Single intraocular pressure measurements and diurnal intraocular pressure profiles. Am J Ophthalmol 2005;139(6):1136-7.
67. Wilensky JT. Diurnal variations in intraocular pressure. Trans Am Ophthalmol Soc 1991;89:757-90.

Supplemental material

Supplemental material 5 is provided below, all other supplemental material can be found on https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7401422/

Supplemental material 5: *Subtab 1: Overview of the pathways per cluster - Human dataset combined.*

Collagen
Reactome: Collagen degradation
Reactome: Assembly of collagen fibrils and other multimeric structures
Reactome: Elastic fibre formation
Reactome: Collagen chain trimerization
Reactome: Activation of Matrix Metalloproteinases
Reactome: Collagen biosynthesis and modifying enzymes

ECM
Reactome: Extracellular matrix organization
KEGG: ECM-receptor interaction
WP: miRNA targets in ECM and membrane receptors
WP: Matrix Metalloproteinases

Adhesion
Reactome: Integrin cell surface interactions
KEGG: Cell adhesion molecules (CAMs)
WP: Focal Adhesion

WNT- signalling
WP: ncRNAs involved in Wnt signaling in hepatocellular carcinoma
WP: LncRNA involvement in canonical Wnt signaling and colorectal cancer

Inflammation
Reactome: Interleukin-4 and Interleukin-13 signaling
WP: Prostaglandin Synthesis and Regulation
Reactome: Interleukin-10 signaling
KEGG: Cytokine-cytokine receptor interaction
Reactome: Interleukin-7 signaling
Reactome: Interferon alpha/beta signaling
WP: IL-7 Signaling Pathway
WP: TGF-B Signaling in Thyroid Cells for Epithelial-Mesenchymal Transition
KEGG: Arachidonic acid metabolism

Reactome: Interleukin-2 family signaling
KEGG: TNF signaling pathway
KEGG: Complement and coagulation cascades
WP: Complement and Coagulation Cascades
WP: Complement Activation
Reactome: Complement cascade
WP: Human Complement System

Adipogenesis
WP: Adipogenesis
Reactome: Regulation of lipid metabolism by Peroxisome proliferator-activated receptor alpha (PPARalpha)
WP: Genes targeted by miRNAs in adipocytes
WP: Fatty Acid Omega Oxidation
WP: Statin Pathway
WP: SREBF and miR33 in cholesterol and lipid homeostasis
WP: Transcription factor regulation in adipogenesis

Glucose metabolism
Reactome: Regulation of Insulin-like Growth Factor (IGF) transport and uptake by Insulin-like Growth Factor Binding Proteins (IGFBPs)
KEGG: Glycolysis / Gluconeogenesis
Reactome: Signaling by Type 1 Insulin-like Growth Factor 1 Receptor (IGF1R)

NFKB
WP: Photodynamic therapy-induced NF-kB survival signaling
WP: RANKL/RANK (Receptor activator of NFKB (ligand)) Signaling Pathway
Reactome: TAK1 activates NFkB by phosphorylation and activation of IKKs complex

GPCR
WP: GPCRs, Class A Rhodopsin-like
WP: GPCRs, Other
WP: Monoamine GPCRs
WP: Small Ligand GPCRs
WP: GPCRs, Class B Secretin-like
WP: Nucleotide GPCRs

Supplemental material 5. *Subtab 2: Overview of the pathways per cluster - Bovine dataset - Effect of dexamethasone.*

Collagen
Reactome: Collagen chain trimerization
Reactome: Collagen biosynthesis and modifying enzymes
Reactome: Assembly of collagen fibrils and other multimeric structures
Reactome: Elastic fibre formation
Reactome: Collagen degradation

ECM
WP: miRNA targets in ECM and membrane receptors
Reactome: Extracellular matrix organization
WP: Matrix Metalloproteinases
KEGG: ECM-receptor interaction
Reactome: Activation of Matrix Metalloproteinases
WP: miR-509-3p alteration of YAP1/ECM axis

Adhesion
KEGG: Focal adhesion
WP: Focal Adhesion
WP: Focal Adhesion-PI3K-Akt-mTOR-signaling pathway
Reactome: Cell surface interactions at the vascular wall

WNT signaling
WP: LncRNA involvement in canonical Wnt signaling and colorectal cancer
WP: ncRNAs involved in Wnt signaling in hepatocellular carcinoma
WP: Wnt Signaling Pathway
KEGG: Wnt signaling pathway
WP: Wnt Signaling in Kidney Disease

Inflammation
Reactome: Interleukin-4 and Interleukin-13 signaling
Reactome: Interferon alpha/beta signaling
KEGG: TNF signaling pathway
Reactome: Interleukin-10 signaling
WP: Prostaglandin Synthesis and Regulation
KEGG: TGF-beta signaling pathway
KEGG: Cytokine-cytokine receptor interaction
KEGG: *IL-17* signaling pathway
KEGG: Inflammatory mediator regulation of TRP channels

KEGG: Arachidonic acid metabolism
Reactome: Arachidonic acid metabolism
WP: TGF-beta Receptor Signaling
WP: Cytokines and Inflammatory Response
WP: TGF-B Signaling in Thyroid Cells for Epithelial-Mesenchymal Transition
KEGG: Complement and coagulation cascades
WP: Complement and Coagulation Cascades
WP: Complement Activation

Adipogenesis
WP: Adipogenesis
WP: Transcription factor regulation in adipogenesis
KEGG: Cholesterol metabolism

Glucose metabolism
WP: Glycolysis and Gluconeogenesis
Reactome: Regulation of Insulin-like Growth Factor (IGF) transport and uptake by Insulin-like Grtowth Factor Binding Proteins (IGFBPs)
KEGG: Glycolysis / Gluconeogenesis
Reactome: Glucose metabolism

NFKB
WP: Photodynamic therapy-induced NF-kB survival signaling
KEGG: NF-kappa B signaling pathway

Apoptosis
WP: Apoptosis Modulation and Signaling
KEGG: p53 signaling pathway
WP: Apoptosis
WP: TP53 Network
WP: Regulation of Apoptosis by Parathyroid Hormone-related Protein
KEGG: Apoptosis
KEGG: Apoptosis
WP: Apoptosis-related network due to altered Notch3 in ovarian cancer

Supplemental material 5. *Subtab 3: Overview of the pathways per cluster - Bovine dataset - Responder vs. Non-responder.*

Collagen
Reactome: Assembly of collagen fibrils and other multimeric structures
Reactome: Collagen chain trimerization
Reactome: Collagen degradation
Reactome: Collagen biosynthesis and modifying enzymes
Reactome: Elastic fibre formation

ECM
WP: Matrix Metalloproteinases
Reactome: Extracellular matrix organization
Reactome: Degradation of the extracellular matrix

Adhesion
KEGG: Gap junction
Reactome: Integrin cell surface interactions

WNT-signaling
WP: LncRNA involvement in canonical Wnt signaling and colorectal cancer
WP: ncRNAs involved in Wnt signaling in hepatocellular carcinoma
WP: Wnt Signaling Pathway
KEGG: Wnt signaling pathway

Inflammation
Reactome: Interferon alpha/beta signaling
Reactome: Interleukin-10 signaling
Reactome: Interleukin-4 and Interleukin-13 signaling
WP: TGF-beta Receptor Signaling
WP: Prostaglandin Synthesis and Regulation
KEGG: TGF-beta signaling pathway
KEGG: Arachidonic acid metabolism

Adipogenesis
WP: Cholesterol Biosynthesis
Reactome: Cholesterol biosynthesis
WP: SREBF and miR33 in cholesterol and lipid homeostasis
KEGG: Biosynthesis of unsaturated fatty acids

WP: Fatty Acid Biosynthesis
Reactome: Regulation of lipid metabolism by Peroxisome proliferator-activated receptor alpha (PPARalpha)
WP: Statin Pathway
KEGG: Fatty acid metabolism
Reactome: Fatty acyl-CoA biosynthesis

Glucose metabolism
Reactome: Regulation of Insulin-like Growth Factor (IGF) transport and uptake by Insulin-like Growth Factor Binding Proteins (IGFBPs)
Reactome: Signaling by Type 1 Insulin-like Growth Factor 1 Receptor (IGF1R)

Apoptosis
WP: Apoptosis-related network due to altered Notch3 in ovarian cancer
KEGG: p53 signaling pathway
WP: Apoptosis

Cell/cell cycle
Reactome: Mitotic G1-G1/S phases
WP: G1 to S cell cycle control
Reactome: Mitotic Prometaphase
KEGG: Cell cycle
WP: Cell Cycle
Reactome: Cell Cycle Checkpoints
WP: DNA Replication

Senescence
WP: Senescence and Autophagy in Cancer
Reactome: Senescence-Associated Secretory Phenotype (*SASP*)

Oxidative stress
WP: Oxidative Stress
WP: Simplified Interaction Map Between *LOX*L4 and Oxidative Stress Pathway

Chapter 5

THE PROMISING FEATURES OF DRUG REPURPOSING FOR THE IDENTIFICATION OF NEW TREATMENT OPTIONS FOR PRIMARY OPEN ANGLE GLAUCOMA

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In this chapter, we show how the research presented in this thesis can be used to identify candidate drugs for drug repurposing as treatment of primary open angle glaucoma. We present three different methods, highlight some of the results and discuss the future directions.

Introduction

The identification of effective treatment modalities for a disease remains challenging. To find the appropriate drugs, two main methods are possible: developing de novo drugs or drug repurposing. The latter, also called drug repositioning, redirection or reprofiling, is defined as "the process of finding new uses outside the scope of the original medical indication for existing drugs".[1] Drug repurposing has great advantages above de novo drug discovery.[1] The duration of de novo drug discovery is approximately 10-17 years and is very expensive.[2] Drug repurposing on the other hand is cheaper, saves time and has a lower risk for serious adverse effects as several test-related phases, that would be part of de novo drug discovery, already took place. This approach has for example been followed to address the urgently needed treatment of COVID-19 during the SARS-CoV-2 pandemic.[3]

Most diseases, like primary open angle glaucoma (POAG), are not driven by one single molecular abnormality but by multiple. POAG is a potentially blinding and very complex disease in which an increase in intraocular pressure is the greatest risk factor.[4] It has been proven that the trabecular meshwork (TM) plays a crucial role in the pathogenesis of POAG and to be involved in the increase of the intraocular pressure.[5] POAG is also thought to be a multifactorial disease. Therefore, the drug discovery has shifted from target-centered to system-driven in which multiple targets are investigated at once. There are multiple ways to perform drug repurposing and different input data can be used. One possibility is the use of gene expression data and their derived molecular pathways.

In a previous study, we obtained a high-quality dataset in which the gene expression data of fourteen patients with POAG and thirteen non-glaucomatous controls were compared.[6] Within this dataset, genes with an absolute $\log_2$FC > 0.58 and a P-value < 0.05 after comparing patients with and without POAG, were defined to be differentially expressed (n=640). These genes were used as input for pathway and network analysis. In this chapter, the results of this study were used to identify new drugs for the treatment of POAG. Three different methods to identify candidates for drug repurposing are shown. In addition, challenges of drug repurposing and possible future directions of research are discussed as well.

This chapter shows the value of our previous research since it presents some promising methods to identify candidate drugs for drug repurposing as a treatment for POAG.

Methods and results

In a previous study, we identified 640 significantly differentially expressed genes after comparing the trabecular meshwork tissue of fourteen patients with POAG and thirteen non-glaucomatous controls. Following, molecular pathway overrepresentation analysis was performed to identify significantly changed pathways. These pathways could be categorized into five functional clusters (*i.e.* extracellular matrix (ECM), inflammation, complement activation, senescence, and Rho GTPase). All the genes involved in at least one of these clusters were visualized into a network of connecting genes.[6] The obtained results were now used to perform analysis to find candidates for drug repurposing. We investigated three different methods.

Method one: Protein-protein interaction networks

The 640 identified genes expressed differentially between patients with POAG and healthy controls were imported in Cytoscape (www.cytoscape.org)[7], a widely adopted network analysis and visualization tool. We used the stringApp Cytoscape app to query the STRING database to connect the differentially expressed genes into a protein-protein interaction network.[8] The STRING database is a knowledge database storing protein-protein interaction data based on different evidence sources including primary databases, experimental evidence, and text mining. For each individual protein-protein interaction a confidence score is provided, ranging from 0 (low) to 1 (high). To lower the chance of including false positive interactions, we used a high confidence cut-off of 0.7. After that, the $\log_2$FC, of differential gene expression, as obtained previously[6], was mapped onto the corresponding protein nodes in the network. Then, by means of network community clustering to identify strongly connected submodules in the network, the largest cluster was identified and further analyzed. The results are shown in Figure 1A. The genes of the largest cluster were then used as input for Gene Ontology (GO) analysis with the Cytoscape app ClueGO.[8] This app identifies the enriched biological processes of the genes in the cluster. The results of this analysis are summarized in Figure 1B. Finally, the CyTargetLinker app[9], another Cytoscape app, was used to extend the network with known drug-target interactions from DrugBank version 5. DrugBank is an online database containing information on drugs and their targets. Version 5 of DrugBank contains 13,548 drug entries including 2,628 approved small molecule drugs, 1,372 approved biologicals (proteins, peptides, vaccines, and allergenics), 131 nutraceuticals and over 6,363 experimental (discovery-phase) drugs.[10, 11] In order to obtain as much information as possible, the complete database was used for this analysis. A total of 267 drug were identified targeting the differentially expressed genes in patients with POAG. Among the identified drugs were corticosteroids and acetazolamide, a carbonic anhydrase inhibitor, often used to treat POAG. Using network topology analysis, we identified the hub proteins in the network, which are nodes with a high number of connections (high degree).

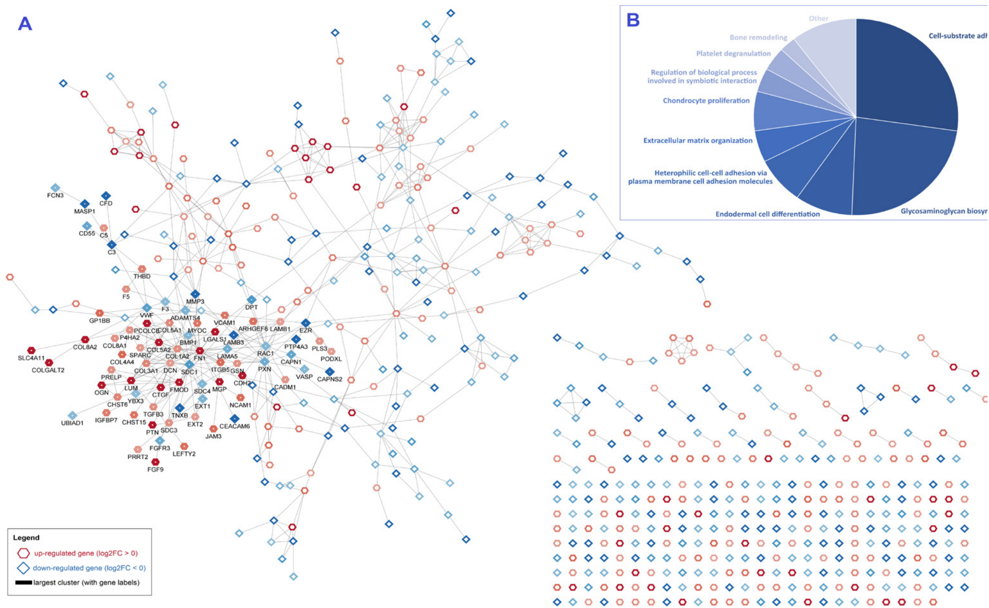

Figure 1. *Protein-protein interaction network of the differentially expressed genes in patients with POAG compared to healthy controls.*

A. Each node represents a protein, and a link between two proteins indicates a functional relationship between the two proteins. Downregulated and upregulated genes are shown as blue diamonds and red hexagons, respectively. The labeled genes in the network with a fill color are part of the largest cluster (as identified by community clustering with the clusterMaker2 app).

B. The filled, labeled nodes in the network were used for enrichment analysis using the ClueGO app to identify relevant processes for the identified protein cluster. This analysis can be repeated for other clusters if required.

Method two: Gene expression of genes within molecular pathways

As mentioned before, in our previous study, we performed overrepresentation analysis on the 640 identified significantly differentially expressed genes in order to identify significantly changed pathways. These pathways could be categorized into five functional clusters (i.e. extracellular matrix (ECM), inflammation, complement activation, senescence, and Rho GTPase). All measured genes involved in at least one of these clusters were integrated and visualized into a pathway-gene network.[6] In this method, we used this network with information about detailed molecular mechanisms affected in patients with POAG as a starting point for drug repurposing. By doing this, we not only identify drugs that target one of the 640 identified significantly differentially expressed genes but also drugs that target the other not significantly affected genes in the pathways.

Within Cytoscape, the previously obtained differential gene expression values (Log_2FC) between patients with POAG and healthy controls was mapped onto the genes in the network.[6] Genes that were not measured in the dataset were removed from the network. Thereafter, the CyTargetLinker app, a Cytoscape app, was used to extend the network with drug-target interactions from DrugBank version 5 (as described above).[9-11] NetworkAnalyzer was used to perform a topological network analysis of the drug-gene-pathway network. Calculating the degree allows the identification of the genes that have the largest number of connections to drugs within the network.

The drug-gene-pathway network is displayed in Figure 2. A gradient from blue (downregulated) to white (not changed) to red (upregulated) is used to visualize the log_2FC of the genes (circular nodes). Yellow triangles represent the 325 known drugs targeting genes in the affected pathways. Most of the drugs (218) target just one gene. Seven drugs target six or more genes. The identified drugs can be subdivided into 132 classes based on their function. These classes are defined by DrugBank and include corticosteroids, statins, rho-kinase inhibitors, non-steroidal anti-inflammatory drugs, immune modulating agents and supplements. The rho-kinase inhibitors we identified, Netarsudil and Ripasudil, target both *ROCK1* and *ROCK2* genes which are part of the Rho GTPase clusters. *ROCK1* is also part of the general inflammation cluster. Both drugs are already used to treat glaucoma patients and are known to target the trabecular meshwork.

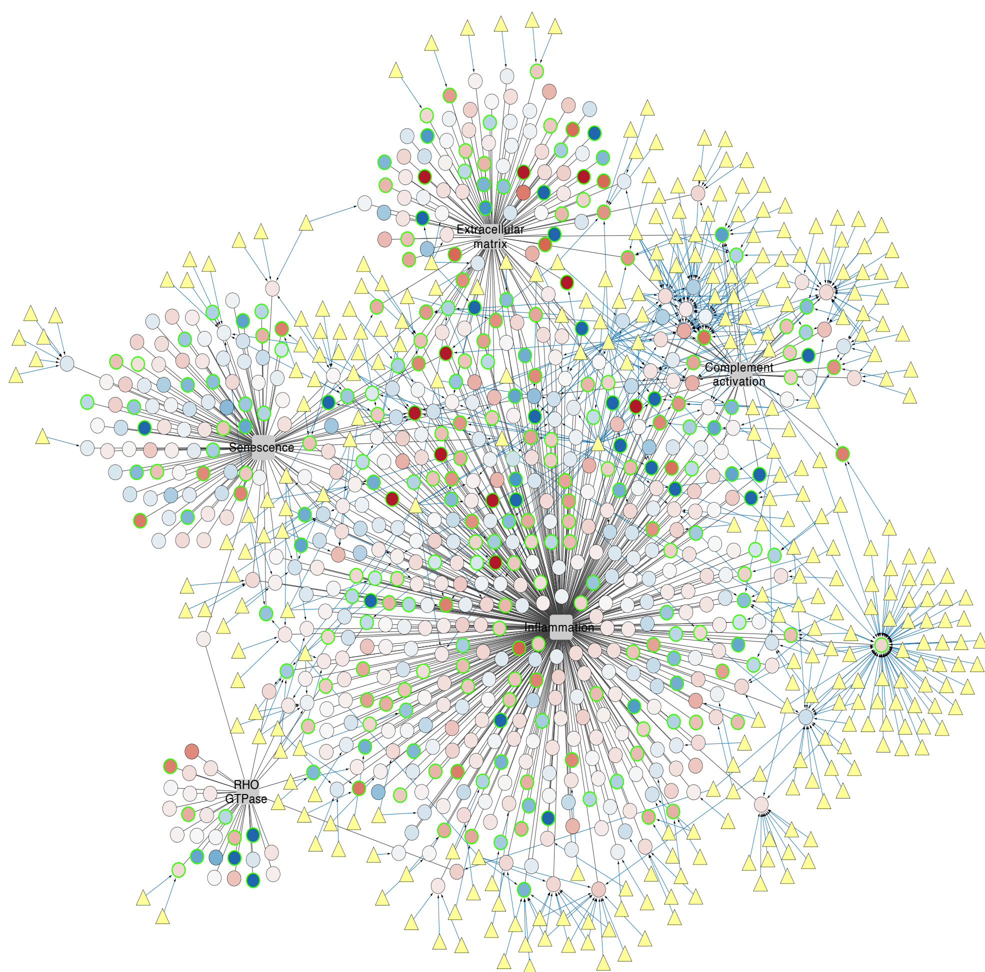

Figure 2. *Network integrating the five functional clusters involved in the pathogenesis of POAG and their drug target interactions.*

Each circular node represents a gene. A gradient from blue (downregulated) to white (not changed) to red (upregulated) is used to visualize the log_2FC of the genes. Circular nodes with a green border are genes that were significantly changed in the dataset. Yellow triangles represent the 325 known drugs targeting genes in the affected pathways. The five initial functional clusters are shown in the figure as well. The names of the individual genes and drugs are available, but have been hidden in this figure.

Method three: Gene expression – drug effect

Another method to find candidates for drug repurposing is to identify drugs that specifically reverse the gene expression of genes that are differentially expressed between patients and controls. As these differentially expressed genes are thought to play an important role in the pathogenesis of the disease, reversing their gene expression could in theory influence the disease progression. For such an analysis it needs to be known in which direction the gene is differently expressed but also the impact of the drug on this gene. The latter knowledge is based on the mRNA expressions of cells exposed to the drug and reveals the complete impact of the drug on many possible genes, not only a limited number.

The publicly available L1000 Characteristic Direction Signature Search Engine (L1000CDS^2, *http://amp.pharm.mssm.edu/L1000CDS2/*) allows to identify drugs that reverse the gene expression profiles. This search engine uses the LINCS L1000 small molecules expression profile database which was developed in 2006 by the Connectivity Map team and allows the use of gene expression data to connect not only drugs but also other small molecules. Initially, the database contained the gene expression data of four human cancer cell lines 6 hours after exposure to one of approximately 1,300 drugs and other small molecules.[12, 13] Over the years, the database has been further expanded by using a new, low-cost, high-throughput reduced representation expression profiling method, named L1000. Now data are available on the responses of approximately 50 human cell lines to approximately 20.000 compounds across a range of concentrations for a total of over one million experiments.[14, 15] The L1000CDS^2 search engine provides prioritization of thousands of small-molecule signatures, and their pairwise combinations, predicted to either mimic or reverse an input gene expression signature. The search engine calculates a cosine distance between the input signature and every characteristic direction signature in the underlying dataset, and the top 50 signatures of either the largest (reverse mode) or the smallest (mimic mode) cosine distances are provided.[14] The drugs and small molecules that are investigated are derived from LIFE (*http://life.ccs.miami.edu*), PubChem[16] and Drugbank[10, 11].

The 640 differentially expressed genes and their logFC between patients with POAG when compared to controls, were used as input in the L1000CDS^2.[6] The L1000CDS^2 search engine calculated the top 50 signatures of the largest cosine distances in order to select small molecules and drugs that reverse the differential gene expression in patients with POAG. Most of the identified possible treatment options are small molecules. One of the identified drugs is Medrysone, a corticosteroid.

Discussion

The above performed methods show how the data obtained in this manuscript can be used to identify known candidate drugs to treat primary open angle glaucoma. Within methods one and two, the initial gene expression data is expanded with additional information. In method one, we extended the network based on known protein-protein interactions. In method two, we performed pathway analysis in which significantly changed pathways were identified. These pathways were identified based on the differentially expressed genes. For both methods drugs were added to their known targets from DrugBank. Within methods one and two, it is not known whether the identified drugs reverse the gene expression of the genes they target. Within method three, no additional data was added to the original data. However, we identified drugs that specifically reverse the gene expression of genes that are differentially expressed between patients and controls. As mentioned above, our findings included rho-kinase inhibitors, statins and carbonic anhydrase inhibitors, which are already known to be beneficial in the treatment of POAG. This can be seen as a validation of the used methods.

With the second method, we identified the rho-kinase inhibitors Netarsudil and Ripasudil. Rho-kinase inhibitors are in some countries already used as treatment for POAG.[17] They are known to regulate actin cytoskeletal dynamics, actomyosin contraction, cell adhesion, cell stiffness, cell morphology and ECM reorganization. They initiate changes such as a decreased number of actin stress fibers, focal adhesions and cell-cell interactions which reduce the mechanical tension and stiffness of the ECM and decrease its synthesis and rigidity, thereby enhancing the outflow of aqueous humor.[18-24] We found Netarsudil and Ripasudil to target both *ROCK1* and *ROCK2* which are part of the Rho GTPase clusters. *ROCK1* is also part of the general inflammation cluster. Therefore, rho-kinase inhibitors seem to affect two of the clusters we previously identified to be involved in the pathogenesis of POAG.

Furthermore, we identified statins which have previously been hypothesized to be a treatment possibility for POAG. A recent study of Cong et al. demonstrated that atorvastatin efficaciously elevated aqueous humor outflow. This effect was possibly due to changes in the cell morphology, cytoskeleton and cell junctions within the ECM.[25, 26] It has been proposed that the effects of the statins are mediated by inhibition of isoprenylation of the small GTP-binding proteins such as Rho GTPase.[27] As mentioned before, rho-kinase inhibitors, as part of the Rho GTPase cluster, were also identified within this study and are known to lower the intraocular pressure by initiating changes within the TM.

In addition, we identified acetazolamide, a carbonic anhydrase inhibitor which is used to lower the intraocular pressure. As far as we know, any effect of acetazolamide on the TM has not been described before. However, as shown in method one, it interacts with one of the proteins derived from one of the differentially expressed genes in the network. Perhaps it also influences the TM in order to lower the IOP.

The first two methods show an elegant and efficient first step to identify possible new treatments for POAG as they make use of molecular biological networks which visualize how molecular processes are related to each other and which mutual genes are shared between the multiple processes. Biological networks can help with the evaluation of the effectiveness of the proposed drugs as it also shows which targets are affected by each of them. In addition, upstream and downstream effects or targets, as well as possible collateral paths can be investigated. The networks obtained in the first two methods visualize these connections and allow us to do this. In addition, since we know in which pathways the identified genes are represented, we can go back to these pathways and predict how the drugs will influence the other genes and parts of the pathway. Perhaps the gene we want to target might play an important role in other molecular processes that are not desirable to be affected. This should be checked for each of the identified drugs. Unfortunately, automatized prediction models that take into account the complete context of the results (disease, effect and side effect) do not exist. Therefore, this has to be performed manually which is intensive and time-consuming.

Furthermore, in the first two methods we used DrugBank to identify new treatment options for POAG. Although these methods resulted in the identification of a large number of possible drugs, it also causes some difficulties. DrugBank does not contain any data on the gene expression or on how the drugs influence the gene expression, i.e. does it cause an up- or downregulation of the gene expression or protein activity? Therefore, drugs that possibly cause an elevation of the IOP due to their induced changes in the trabecular meshwork might be identified as well. This is illustrated by the fact that we found multiple corticosteroids as a possible 'treatment option' while they are known to cause corticosteroid-induced ocular hypertension. Nevertheless, as we know that these two methods don't take the direction of effect into account, this finding can be seen as a validation of our results: corticosteroids, known to influence the trabecular meshwork and raise the intra ocular pressure, were identified.

Method three has a strong potential since data on all the genes expressed after exposure to a drug in several cell lines are included. Moreover, it is known whether these drugs increase or decrease the gene expression within these cell lines. Therefore, the drugs identified with this method should in theory counteract the differentially expressed

genes in POAG. Surprisingly, medrysone, a corticosteroid was identified as a drug counteracting the differentially expressed genes in patients with POAG, despite the fact that corticosteroids are known to increase the intra-ocular pressure. However, for hydrocortisol, another type of a corticosteroid, studies have shown that its topical use lowered the eye pressure in rabbits with steroid-induced ocular hypertension after the use of dexamethasone.[28] Moreover, a study of Clarck et al., showed that tetrahydrocortisol can reverse changes in the formation of cross-linked actin networks in the TM as induced by dexamethasone.[29] These cross-linked actin networks are thought to play an important role in the pathogenesis of corticosteroid-induced ocular hypertension.[30, 31] The study of Mindel et al. showed that the IOP increased with only 1.17 mmHg after the administration of medrysone four times a day for 6 weeks. This increase was 4.82 mmHg and 9.08 mmHg after the use of respectively fluorometholone and dexamethasone.[32] As the use of medrysone does not increase the IOP significantly, it may have a comparable effect as hydrocortisol.

Drug repurposing of candidate drugs suggested by this method has already been proven to be successful. L1000CDS[2] has been able to reveal kenpaullone, a *GSK3B/ CDK2* inhibitor that has a dose-dependent efficacy in inhibiting Ebola infection.[14] The key in this success was the dose-dependent efficacy. This emphasizes that the results need additional investigations, in vitro and in vivo. In addition, although this method shows great potential, a large amount of computer capacity and time is needed to run one analysis. For example, for this study it took three days to run on a specialized server.

For methods one and two, the initial gene expression data is expanded with additional information. In method two, we performed pathway analysis in which significantly changed pathways were identified. These pathways were identified based on the differentially expressed genes. An advantage of this method is that complete processes, as found to be involved in the pathogeneses of POAG, and all the genes that are related with these processes, are taken into account and not only the genes that had been measured in the gene expression study. This is beneficial as this method has led to the identification of rho-kinase inhibitors. A possible disadvantage however is, as the network consisted of genes involved in the identified pathway clusters, that we do not necessarily know the gene expression for each gene in a significantly changed pathway. A similar situation occurred in method one for which we used a protein-protein interaction. The level of gene expression is not always correlated to its protein expression, i.e. a gene that is upregulated does not necessarily indicate an increased production or effect of its protein. Although we have shown that it can be beneficial to expand the initial results with additional information as it has more sensitivity to identify potential drugs, it could make the results more difficult to interpret.

In conclusion, this chapter shows that network analysis of disease gene expression data has the potential to find candidates for drug repurposing. Some of the identified treatment options, such as rho-kinase inhibitors, statins and corticosteroids, are known to influence the TM. In addition, we identified multiple drugs that might be effective for the treatment of POAG. However, in order to further downsize the number of drugs and select the drugs that have truly potential, additional research is needed. This research should entail, among others, a detailed investigation on the applied methods, combining the results with insights in biochemical processes and pharmacodynamics and eventually validation in animal experiments.

Please note that the drugs we identified, nor the genes they address, have not been shown in this chapter as this would hamper a possible request for a patent in the future.

References

1. Ashburn TT, Thor KB. Drug repositioning: identifying and developing new uses for existing drugs. Nat Rev Drug Discov 2004;3(8):673-83.
2. Reichert JM. Trends in development and approval times for new therapeutics in the United States. Nat Rev Drug Discov 2003;2(9):695-702.
3. Gordon DE, Jang GM, Bouhaddou M, et al. A SARS-CoV-2-Human Protein-Protein Interaction Map Reveals Drug Targets and Potential Drug-Repurposing. bioRxiv 2020.
4. Sommer A, Tielsch JM, Katz J, et al. Relationship between intraocular pressure and primary open angle glaucoma among white and black Americans. The Baltimore Eye Survey. Arch Ophthalmol 1991;109(8):1090-5.
5. Tektas OY, Lutjen-Drecoll E. Structural changes of the trabecular meshwork in different kinds of glaucoma. Exp Eye Res 2009;88(4):769-75.
6. Liesenborghs I, Eijssen LMT, Kutmon M, et al. Comprehensive bioinformatics analysis of trabecular meshwork gene expression data to unravel the molecular pathogenesis of primary open-angle glaucoma. Acta Ophthalmol 2019.
7. Shannon P, Markiel A, Ozier O, et al. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res 2003;13(11):2498-504.
8. Bindea G, Mlecnik B, Hackl H, et al. ClueGO: a Cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. Bioinformatics 2009;25(8):1091-3.
9. Kutmon M, Ehrhart F, Willighagen EL, et al. CyTargetLinker app update: A flexible solution for network extension in Cytoscape. F1000Res 2018;7.
10. Wishart DS, Feunang YD, Guo AC, et al. DrugBank 5.0: a major update to the DrugBank database for 2018. Nucleic Acids Res 2018;46(D1):D1074-D82.
11. Wishart DS, Knox C, Guo AC, et al. DrugBank: a comprehensive resource for in silico drug discovery and exploration. Nucleic Acids Res 2006;34(Database issue):D668-72.
12. Lamb J. The Connectivity Map: a new tool for biomedical research. Nat Rev Cancer 2007;7(1):54-60.
13. Lamb J, Crawford ED, Peck D, et al. The Connectivity Map: using gene-expression signatures to connect small molecules, genes, and disease. Science 2006;313(5795):1929-35.
14. Duan Q, Reid SP, Clark NR, et al. L1000CDS(2): LINCS L1000 characteristic direction signatures search engine. NPJ Syst Biol Appl 2016;2.
15. Subramanian A, Narayan R, Corsello SM, et al. A Next Generation Connectivity Map: L1000 Platform and the First 1,000,000 Profiles. Cell 2017;171(6):1437-52 e17.
16. Kim S, Chen J, Cheng T, et al. PubChem 2019 update: improved access to chemical data. Nucleic Acids Res 2019;47(D1):D1102-D9.
17. Moshirfar M, Parker L, Birdsong OC, et al. Use of Rho kinase Inhibitors in Ophthalmology: A Review of the Literature. Med Hypothesis Discov Innov Ophthalmol 2018;7(3):101-11.
18. Rao PV, Deng P, Sasaki Y, Epstein DL. Regulation of myosin light chain phosphorylation in the trabecular meshwork: role in aqueous humour outflow facility. Exp Eye Res 2005;80(2):197-206.

19. Rao PV, Deng P, Maddala R, et al. Expression of dominant negative Rho-binding domain of Rho-kinase in organ cultured human eye anterior segments increases aqueous humor outflow. Mol Vis 2005;11:288-97.
20. Inoue T, Tanihara H. Rho-associated kinase inhibitors: a novel glaucoma therapy. Prog Retin Eye Res 2013;37:1-12.
21. Honjo M, Tanihara H, Inatani M, et al. Effects of rho-associated protein kinase inhibitor Y-27632 on intraocular pressure and outflow facility. Invest Ophthalmol Vis Sci 2001;42(1):137-44.
22. Zhou EH, Krishnan R, Stamer WD, et al. Mechanical responsiveness of the endothelial cell of Schlemm's canal: scope, variability and its potential role in controlling aqueous humour outflow. J R Soc Interface 2012;9(71):1144-55.
23. Zhang M, Maddala R, Rao PV. Novel molecular insights into *RhoA* GTPase-induced resistance to aqueous humor outflow through the trabecular meshwork. Am J Physiol Cell Physiol 2008;295(5):C1057-70.
24. Pattabiraman PP, Maddala R, Rao PV. Regulation of plasticity and fibrogenic activity of trabecular meshwork cells by Rho GTPase signaling. J Cell Physiol 2014;229(7):927-42.
25. Cong L, Fu S, Zhang J, et al. Effects of atorvastatin on porcine aqueous humour outflow and trabecular meshwork cells. Exp Ther Med 2018;15(1):210-6.
26. Song J, Deng PF, Stinnett SS, et al. Effects of cholesterol-lowering statins on the aqueous humor outflow pathway. Invest Ophthalmol Vis Sci 2005;46(7):2424-32.
27. Von Zee CL, Richards MP, Bu P, et al. Increased *RhoA* and RhoB protein accumulation in cultured human trabecular meshwork cells by lovastatin. Invest Ophthalmol Vis Sci 2009;50(6):2816-23.
28. Southren AL, l'Hommedieu D, Gordon GG, Weinstein BI. Intraocular hypotensive effect of a topically applied cortisol metabolite: 3 alpha, 5 beta-tetrahydrocortisol. Invest Ophthalmol Vis Sci 1987;28(5):901-3.
29. Clark AF, Lane D, Wilson K, et al. Inhibition of dexamethasone-induced cytoskeletal changes in cultured human trabecular meshwork cells by tetrahydrocortisol. Invest Ophthalmol Vis Sci 1996;37(5):805-13.
30. Clark AF, Wilson K, McCartney MD, et al. Glucocorticoid-induced formation of cross-linked actin networks in cultured human trabecular meshwork cells. Invest Ophthalmol Vis Sci 1994;35(1):281-94.
31. Filla MS, Schwinn MK, Nosie AK, et al. Dexamethasone-associated cross-linked actin network formation in human trabecular meshwork cells involves beta3 integrin signaling. Invest Ophthalmol Vis Sci 2011;52(6):2952-9.
32. Mindel JS, Tavitian HO, Smith H, Jr., Walker EC. Comparative ocular pressure elevation by medrysone, fluorometholone, and dexamethasone phosphate. Arch Ophthalmol 1980;98(9):1577-8.

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Chapter 6

RISK FACTORS FOR THE DEVELOPMENT OF OCULAR HYPERTENSION AFTER KERATOPLASTY: A SYSTEMATIC REVIEW

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Abstract

Purpose: To identify risk factors for the development of ocular hypertension after keratoplasty.

Methods: A systematic search in Pubmed and Embase identified 67 relevant articles published between January 1990-2019. We preferentially searched for data on an intraocular pressure (IOP) increase above 21 mmHg at six months or a threshold or time-point close to that and reported whether the pre- or intraoperative status of risk factors was defined. The results were presented in evidence tables, visualizing the direction of the association, whether univariate and/or multivariate analysis were performed and the significance level ($P<0.05$). Four researchers, blinded for the risk factors, independently assigned a level of evidence (definitely, probably, possibly, not associated). Consensus was met during group meetings.

Results: From the 110 studied risk factors, pre-existing glaucoma, high preoperative IOP, combined keratoplasty with removal or exchange of an intraocular lens (IOL), and if the pre-or postoperative lens status was undefined, aphakia and pseudophakia with the IOL in the anterior or posterior chamber were definitely associated with an increased risk compared to phakia. Glaucoma in the contralateral eye, indication of bullous keratopathy, African-American descent, preoperative treatment with cyclosporine or olopatadine 0.1%, postoperative treatment with prednisolone acetate 1%, and combined surgery in general (i.e. type of surgeries undefined in primary studies) were probably associated. Multiple other identified risk factors lack sufficient evidence and need additional investigation.

Conclusion: Risk factors with a definite association can help clinicians to select patients at risk and to adjust their follow-up and treatment. The other factors need further investigation.

Introduction

Many corneal conditions like corneal dystrophies, corneal infections, trauma and iatrogenic corneal diseases may cause sight-threatening corneal opacities or scarring of the cornea for which a keratoplasty is often the only cure. A corneal transplantation is therefore one of the most frequently performed transplantations and the number of keratoplasties performed each year rises worldwide.[1, 2] Ocular hypertension (OHT) is one of the most common complications. It may cause irreversible vision loss after keratoplasty due to graft failure resulting from the impact of OHT on endothelial cell loss.[3-5] A sustained elevation of intraocular pressure (IOP) may also cause glaucomatous damage to the optic nerve, leading to progressive visual field loss and eventually blindness. The reported prevalence of the development of OHT after keratoplasty varies widely, ranging from 5.5%[6] up to 68%[7]. This is mainly due to lack of a standardized definition for OHT. Throughout the literature, OHT has been defined as a post-surgical IOP > 21 mmHg after surgery or an increase of > 10mmHg over the baseline IOP with or without the need for anti-glaucoma medication or surgery.[8]

A corticosteroid-induced elevation of the IOP is known to be the most common cause of OHT after corneal surgery.[9-11] However, various other preoperative, intraoperative and postoperative risk factors that increase the risk of developing OHT after keratoplasty have been studied. To our knowledge, only one meta-analysis, investigating eight risk factors for OHT after penetrating keratoplasty (PKP), has been performed.[8] Other reviews discussed multiple risk factors[3, 12-14]; however, they did not evaluate them systematically and a meta-analysis reporting and evaluating the available evidence of all suspected risk factors in penetrating and lamellar keratoplasty is currently missing.

We used a systematic approach to identify to what extent various risk factors have been investigated and a semi-quantitative approach to investigate which factors are associated with the development of OHT after keratoplasty. The identification of these risk factors is of clinical importance as they can help to determine the prognosis of each individual patient and facilitate preventative measures to reduce the risk of developing glaucomatous damage and/or graft failure.

Methods

Eligibility criteria for considering studies for this review

In order to provide a complete overview of the available evidence, all studies investigating at least one risk factor for the development of OHT in any type of keratoplasty were included in this review. Case series, comparative case series, cohort studies, case-control studies and randomized controlled trials were all included in this review as all of these designs were suitable to investigate risk factors. The search was restricted to articles published after January 1990. There were no restrictions on language or publication status. Only articles reporting a P-value, odds ratio (OR), or hazard ratio with or without confidence intervals were included. Articles were excluded if the full text could not be retrieved, the article was not available in Dutch, English, French or German, did not investigate risk factors, was performed ex vivo, the data was not interpretable, included patients < 18 years old, included patients before 1990, sample size < 25 eyes and follow-up ≤ 1 month or starting > 1 year after the keratoplasty. Articles were also excluded if they investigated a specific subpopulation with an a priori higher risk of developing OHT or glaucoma.

Search methods for identifying studies

A systematic search in PubMed and Embase was performed in June 2018 and an update was conducted at the beginning of February 2019. The following key words were used: corneal transplantation surgery, penetrating keratoplasty, lamellar keratoplasty, descemet membrane endothelial keratoplasty, descemet stripping (automated) endothelial keratoplasty, deep anterior lamellar keratoplasty, glaucoma, intraocular pressure, ocular hypertension and steroid-induced ocular hypertension. The complete search for both databases and all used synonyms for each of the above mentioned terms, can be found in supplemental material 1. To verify whether we did not miss any eligible articles, we searched the reference lists of all included studies and previously published reviews.

Study selection

All the references resulting from the search in Pubmed and Embase were imported in EndNote X8. After the importation of 4424 references, 1140 duplicates were removed automatically and by hand. All titles and abstracts of the 3284 unique references were studied by one author (I.L.) in order to detect all references that investigated any risk factor for the development of post-keratoplasty OHT. The titles and abstracts of references that were not selected during this first selection round were double-checked by the same author in order to not miss any relevant articles; however, no additional references were selected. Each of the selection processes entailed three

days. References not investigating any risk factors were excluded (n excluded = 2765). Following, the full-text articles of all relevant abstracts (n=519) were searched. If the full-text could not be obtained through the university portal, we contacted the authors. 443 articles out of the 519 retrieved full text did not fulfill the above mentioned selection criteria and were excluded. The remaining 76 articles were used for analysis. A flowchart visualizing the selection procedure can be found in figure 1. No additional articles could be identified after searching the reference lists of all included studies and previously published reviews.

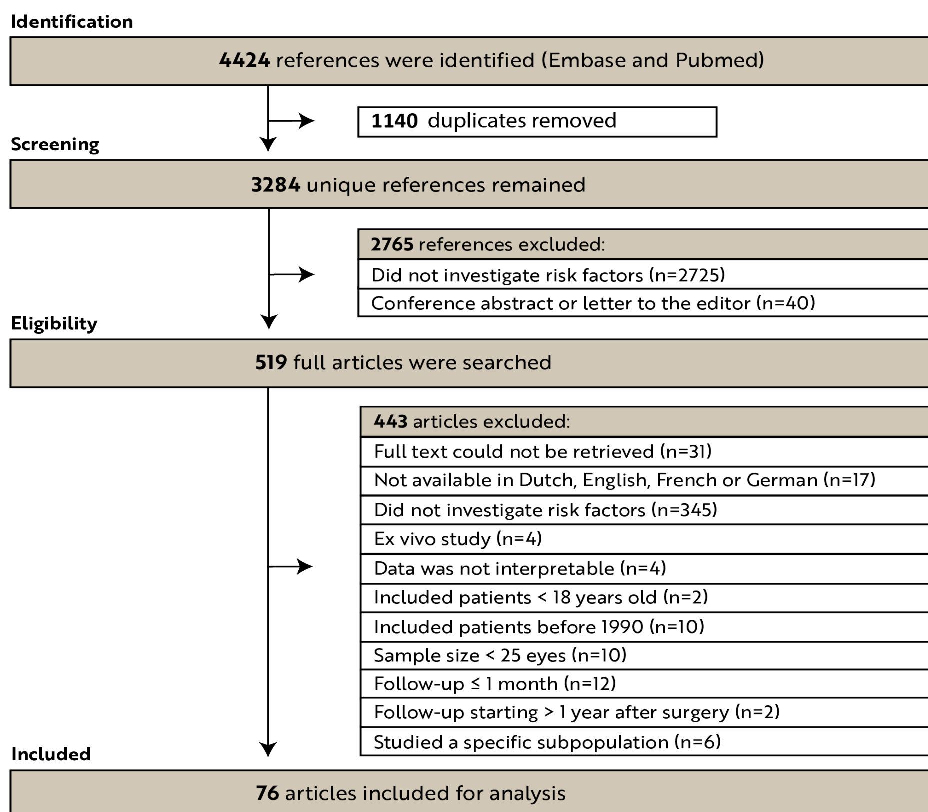

Figure 1. *Flowchart of the selection procedure of the included articles.*

Data collection and risk of bias assessment

As indicated above, case series, comparative case series, cohort studies, case-control studies and randomized controlled trials were all included in this review. We followed the Ophthalmology study design scheme to allocate the included studies properly.[15] We clustered studies with study populations that were likely to overlap into functionally related clusters. The likelihood of overlap was determined on author names, site of recruitment, trial names, period of recruitment and references to other studies.

To assess the quality of the 76 included studies, we used the checklist developed for the quality assessment of prognosis studies which is recommended by the National Institute for Health and Clinical Excellence.[16] This checklist contains six criteria which are related to the representation of the study population, risk of bias, loss to follow-up, measurement of the risk factors, outcome measurement, correction for confounders and the reproducibility of the statistical analysis.

Data synthesis and analysis

Since the risk factors have been investigated in studies using various study methods, maintaining different definitions of OHT and investigating both penetrating and lamellar keratoplasty within different study populations, we could not perform a formal meta-analysis. Therefore, as performed by Ernest et al., we used a semi-quantitative method to investigate to what extent various risk factors are most likely associated with the development of OHT after keratoplasty.[17]

We summarized the evidence for each studied risk factor in separate tables. An example of an evidence table is shown in table 1. Each outcome regarding the risk factor was classified according to the direction of the association of the risk factor with OHT (higher risk, lower risk) and the reported statistical significance (P-value <0.05). We also differentiated between univariate and multivariate results. We tried to extract one conclusion per studied risk factor within one study. If a study did not describe the direction of the risk factor, we reported the study in the middle column (relation unknown). Furthermore, we indicated which studies belonged to the same study cluster (see table 1).

Throughout the included studies, various definitions and outcome measures have been used to define the development of OHT after keratoplasty. In order to report the results as uniformly as possible, we tried to use similar outcome measures. Preferably, we reported the results investigating an increase in absolute IOP. If multiple cut-offs were mentioned, we reported the results of an IOP increase above 21 mmHg or the provided cut-off which was most adjacent to 21 mmHg.

Table 1. *Example of an evidence table summarizing all study results of a risk factor.*

Factor x	Increased risk for OHT		Direction of the relation is unknown		Decreased risk for OHT	
	Significant	**Non-significant**	**Significant**	**Non-significant**	**Non-significant**	**Significant**
Univariate analysis (n=)	1, 2†, 8*	2††, 4		7		
No. Of clusters	3	2	0	1	0	0
No. Of studies	3	2	0	1	0	0
No. Of patients	236	324	0	48	0	0
Multivariate analysis (n=)	3, 5	6				
No. Of clusters	1	1	0	0	0	0
No. Of studies	2	1	0	0	0	0
No. Of patients	496	116	0	0	0	0

Tables marked as "general" contain multiple risk factors that have been investigated together (lacking detailed information).

** Indicates a study population of solely steroid responders*

†, †† *Indicates and specifies subgroups that have been made within one study*

Study 3 and 5 are underlined as they belong to the same study cluster (as defined in table 1)

All the risk factors are presented in evidence tables. These tables were used to determine to which extent a risk factor had been investigated and whether it is not, possibly, probably or definitely associated with the development of OHT after keratoplasty.

The above mentioned table summarizes all the evidence of risk factor "X". Each number in the table represents a study. The studies are numbered according to the reference list of table 1. The studies are classified based on the direction (increased or decreased glaucoma risk or unknown direction), the significance of the association (P<0.05), and whether univariate or multivariate analysis have been performed. The number of study clusters, studies, and patients is summarized below the study numbers. Numbers between parentheses represent the total number of univariate and multivariate conclusions in the analysis.

If the absolute IOP increase was not reported, we had to use other outcome measures for which we maintained the following consecution: mean IOP (preferably compared to baseline), the need to start or change glaucoma medication and lastly the need for surgical interventions. Furthermore, if the outcome measure was mentioned for multiple time points, we reported the results after six months or the time point which was the closest to six months.

In order to make some of the results of the investigated risk factors throughout the included studies comparable to each other, we changed the reference to which a risk factor was compared and recalculated the OR using chi-square or Fisher exact test as appropriate. A P-value < 0.05 was defined as statistically significant. We did this for the indication of the surgery, the lens status and regrafting. Within multiple studies different indications had been investigated and compared to each other. In order to obtain meaningful results, we used keratoconus as reference. For the lens status, we studied the following comparisons: aphakic vs. phakic, pseudophakic vs. phakic and aphakic vs. pseudophakic. Furthermore, most studies compared graft failure as indication for the keratoplasty to a single primary indication such as for example keratoconus, not taking into account that graft failure indicates regrafting instead of performing a primary keratoplasty. Therefore, we recalculated the OR comparing graft-failure with the sum of all other studied primary indications within a respective study.

While creating the evidence tables for each risk factor, we tried to maintain the detailed information that had been provided for each risk factor as much as possible, for example keratoplasty combined with cataract extraction, vitrectomy, or the removal or exchange of an intraocular lens (IOL) implant. However, if a study did not provide this detailed information and only compared combined surgery with no combined surgery in general, we reported those results as well and marked the evidence table as 'general'. These tables might provide an overview of the available evidence of a risk factor but lack further details. Additionally, if studies specifically defined the lens status to be preoperatively or postoperatively, we maintained this differentiation as a postoperative lens status might indicate that the patients underwent a combined surgery, for example keratoplasty and cataract extraction with or without implantation of an IOL. In case initial studies did not define whether the investigated lens status was pre- or intraoperative, it was studied as a separate category.

Interpretation of the evidence tables

The obtained evidence tables were used to determine if and to what extent a risk factor was associated with the development of OHT after keratoplasty. Four different investigators (J.S.S., C.A.W., H.J.B. and I.L.) independently investigated the evidence table and judged whether the risk factor was 'not associated', 'possibly associated', 'probably associated', or 'definitely associated' with OHT. Each of the investigators were blinded for the risk factor and the articles that studied the risk factor. The following elements were taken into account during the assessment: the number of study results concerning the risk factor, the total number of participants within these studies, whether studies belonged to the same study cluster and whether univariate or multivariate results were reported. The same guidelines as applied by Ernest et al. were used for the classification. Shortly, risk factors with a consistent pattern of no relation were assigned to 'no association'. This category also comprised risk factors showing a great heterogeneity in their evidence table and risk factors that had been studied to a limited extent and for which an association was not (yet) found. Risk factors with a weak tendency towards a higher or lower risk were assigned to 'possible association'. Risk factors with an obvious tendency towards more or less progression in a moderate number of studies, with several studies having significant results were assigned to 'probable association'. 'Definite association' was assigned to risk factors with a very consistent pattern of numerous studies with multiple statistically significant results. If an identified risk factor had only been investigated in one study, we used the following criteria to assess the level of evidence: a risk factor was graded as 'possible association' if it was significantly associated in a univariate analysis and it was graded as 'probable association' if it was significantly associated in a multivariate analysis. A large cohort study showing a significant association in both the univariate and multivariate analysis was graded as 'definite association'. In all other cases, the risk factor was classified as 'no association'. During group meetings, differences in ratings between the investigators were discussed and consensus was reached. During the judgment procedure of the evidence tables, we checked the reported results of each risk factor for heterogeneity. Risk factors for which evidence was found in both directions of the association (increased and decreased risk), were marked to be heterogeneous.

Results

We identified 76 relevant articles published between 1993 and 2019: 31 case series, 32 comparative case series, 6 cohort studies, 1 case-control study and 6 randomized controlled trials. The studies are shown in supplemental material 2 and were clustered in 49 clusters. The characteristics of the included studies are shown in supplemental material 3. Most of these studies consisted of case series (76%) and were performed retrospectively (83%). A univariate analysis was executed in 87% of the studies and a multivariate analysis in 4%. In 9% of the studies, both univariate and multivariate analysis were performed. The type of surgery which was most often investigated within the included articles was penetrating keratoplasty (PKP) (57%) followed by descemet stripping automated endothelial keratoplasty (DSAEK) (27%), deep anterior lamellar keratoplasty (DALK) (15%), descemet membrane endothelial keratoplasty (DMEK) (10%), deep lamellar endothelial keratoplasty (DLEK) (4%) and endothelial keratoplasty (EK), anterior lamellar keratoplasty (ALK) and femtosecond laser enabled keratoplasty (FLEK) (together 5%). We identified 110 risk factors for the development of OHT after keratoplasty. The judgment procedure of these risk factors identified 6 definite associations, 8 probable associations, 24 possible associations and 72 no associations. The judgments were overall consistent. In case of different judgment between the investigators, consensus could easily be reached. All the evidence tables are presented in supplemental material 4.

Quality checklist

Supplemental material 5 provides an overall overview of the scores of the 76 included studies on the six quality items. In total, we found one study with 'yes' to all six criteria (1.3%), 11 studies with 'yes' to five criteria (14.5%), 34 studies with 'yes' to four criteria (44.7%) and 30 studies with 'yes' to three criteria (42%). Three studies scored 'no' to three criteria (3.9%), 49 studies 'no' to two criteria (64.5%) and 23 studies 'no' to one criterion (30.3%). A total of 39 studies (51.3%) scored 'unclear' on the same criterion of the quality checklist (outcome measurement), as these studies did not specify the IOP-measurement method. Although, it was not specifically mentioned, it is highly likely that the used method is the same before and after keratoplasty and/or between the subgroups. Therefore, we scored the quality item for these studies as 'unclear' instead of 'not'.

Baseline characteristics (criterion one) were clearly described for 74 out of 76 studies, the two other studies provided a very concise description. Although, it must be noted that 23 studies did not describe IOP values or the presence of glaucoma in the baseline characteristics. Criterion two (loss to follow-up) contained per definition 64 times 'no' as we included 63 case series and one case-control study. The other studies provided

sufficient information concerning the follow-up. Criteria three and six, describing the measurement of the risk factors and the reproducibility of the statistical analysis, were scored as 'yes' for all included studies.

Preoperative, intraoperative and postoperative risk factors

Supplemental material 6 shows a clinically relevant overview of all the risk factors. We classified the risk factors in 50 preoperative, 41 intraoperative and 13 postoperative risk factors. For five risk factors, all describing the lens status, it was unclear whether the investigated lens status was already present before surgery or was obtained during the surgery. Therefore, we classified these risk factors as 'pre- or intraoperative status not defined'. One risk factor was described as 'preoperatively and/or postoperatively' and therefore clustered separately from the above mentioned categories.

Definite risk factors

Six risk factors have been determined to be definitely associated with OHT after keratoplasty: pre-existing glaucoma, a higher preoperative IOP, combining the keratoplasty with the removal or exchange of an intraocular lens (IOL) implant, and in case the operative lens status was unknown, aphakic lens status and pseudophakic lens status with the IOL in the anterior or posterior chamber in comparison to phakic lens status. Pre-existing glaucoma had been investigated in 19 different studies leading to 24 study results of both univariate and multivariate analyses, showing a clear tendency towards an increased risk for OHT. The classification of a higher preoperative IOP and keratoplasty combined with lens removal or exchange were both based on seven conclusions. Both univariate and multivariate results of these risk factors show a clear tendency towards an increased risk for OHT and therefore, both risk factors were judged to be definitely associated. The risk factors aphakic compared to phakic lens status and pseudophakic lens status with the IOL in the anterior or posterior chamber compared to phakic eyes (pre- or intraoperative lens status unknown) were judged to be definitely associated as one large cohort study comprising 1657 participants reported a significantly higher risk in both the univariate and multivariate analysis.

Probable risk factors and possible factors

Eight risk factors have been determined to be probably associated with OHT after keratoplasty. Bullous keratopathy compared to keratoconus and combined surgery in general have been investigated in respectively 11 and 10 conclusions. Five other risk factors were probably associated with the development of OHT as well, although, their judgment was based on a lower number of studies and conclusions. These risk factors were: indication of bullous keratoplasty (yes vs. no), African-American descent, cyclosporine and olopatadine 0.1% use before keratoplasty, postoperative use of prednisolone acetate 1% vs. dexamethasone 0.1% and glaucoma in the contralateral eye (without glaucoma in the investigated eye).

Possible associated risk factors

Twenty-four risk factors were judged to have a possible association. For most of these risk factors, this was based on a limited number of studies, therefore lacking sufficient evidence to make a more certain conclusion. Five risk factors were judged to be possibly associated based on at least eight studies: a younger age of the patient, regrafting, male gender, a PKP when compared to DS(A)EK and a larger graft diameter in PKP. Despite the fact that these risk factors had been investigated in multiple studies, we were not able to find a more robust association with IOP elevation due to the heterogeneity within the evidence. Additional investigation of the possibly associated risk factors is therefore necessary. An overview of the definite, probable and possible risk factors can be found in supplemental material 7.

Non-associated risk factors

Seventy-two risk factors were judged to be not associated. Note that 56.6% (43) of these risk factors have only been based on one conclusion, 14.5% (11) on two conclusions and 14.5% (11) on three to five conclusions. In addition, these conclusions were mostly derived from studies with a low sample size. These risk factors therefore lack sufficient evidence in order to know whether they are associated with the development of OHT or not and need further investigation. Seven risk factors however were judged not to be associated based on at least six conclusions. Remarkably, for five of these risk factors, there was a considerable amount of heterogeneity, indicating contradictive study results. Therefore, we could not conclude any association for the following risk factors: corneal dystrophy as indication for the surgery, preoperative pseudophakic or phakic lens status, PKP compared with DALK and performing a triple procedure. These risk factors need further investigation as well. The evidence tables of the other two risk factors, infectious keratitis or scar compared to keratoconus, showed no heterogeneity. Their evidence is sufficient and robust which allows us to conclude that they are not associated with the development of ocular hypertension. An overview of all not associated risk factors can be found in supplemental material 8.

Heterogeneity

For 20 risk factors, we noted a considerable amount of heterogeneity during the judgment procedure (marked with 'H' in table 4 and the supplemental material 3 and 4. For four risk factors the heterogeneity consisted of significant results in the opposite direction as the majority of the results. For the other 16 risk factors, the heterogeneity only comprised non-significant results. Despites its heterogeneity, the risk factor history of glaucoma was judged to be definitively associated. It was

found to significantly increase the risk for OHT in 17 studies in both univariate and multivariate analyses (12 univariate and four multivariate significant; four univariate and two multivariate non-significant). One study with a small sample size, found a non-significant result but did not specify the direction of the association. The heterogeneity consisted therefore of only two studies reporting a non-significant decreased risk which is rather trivial compared to the 17 studies showing an increased risk. We also noticed heterogeneity for five and 15 other risk factors who were respectively judged to be possibly and not associated. Multiple of these risk factors had been investigated in a relatively large number of studies. For the possibly associated risk factors we could, despite the heterogeneity, distinguish a small association towards an increased or decreased risk. A stronger association however could not be concluded. For the not associated risk factors, the heterogeneity was too large to identify a possible direction of the association with OHT.

Discussion

This systematic review provides an overview of all investigated risk factors for the development of OHT after keratoplasty and the level of evidence that is available for each risk factor. By performing a semi-quantitative approach, we were able to identify 110 risk factors and classify them into six definite, eight probable, 24 possible and 72 no associations as shown in supplemental material 6 (Supplemental Digital Content 6).

Other reviews provide a non-systematic literature overview of the most extensively studied risk factors for the development of OHT after keratoplasty[3, 12-14]; however, a systematic overview of the available evidence is currently missing. As there is a large diversity in methods and approaches used by the different studies, it was not possible to perform a traditional meta-analysis. Therefore, we used a semi-quantitative approach as performed earlier by Ernest et al.[17] allowing us to summarize the evidence of the risk factors systematically and, as the assessors of the risk factors were blinded for both the risk factors and the included studies, objectively. This method allowed the inclusion of more studies and to summarize the evidence on more risk factors compared to a traditional meta-analysis as we reported every risk factor that had been investigated in the included studies.

For this review, articles were selected through a systematic search and strict selection criteria were applied. One criterion was the exclusion of studies investigating specific subpopulations who are known to have a higher risk for the development of OHT. We excluded studies investigating keratoplasty in patients with iridocorneal endothelial syndrome as approximately 50% to 73% develop glaucoma[18,19] and patients with endotheliitis which are prone to developing an elevated IOP during an active inflammation[20, 21]. Furthermore, patients with prior toxic anterior segment syndrome, congenital glaucoma and eyes with anterior segment alterations or disruptions were excluded from the review. We also did not include studies investigating patients who underwent keratoplasty before 1990 as the operation techniques of keratoplasty and the postoperative corticosteroid treatment, which are both likely to influence the development of OHT, has changed throughout the years. Patients used to be treated with highly potent corticosteroids for a prolonged period of time which led to a high incidence of corticosteroid-induced OHT. However, with the development of lamellar operating techniques the duration of exposure and the potency of the used corticosteroids decreased, lowering the incidence of corticosteroid-induced OHT.[22, 23]

Within the included studies, the indication of the surgery was investigated extensively as risk factor for the development of OHT. However, various indications were used as a reference which led to the identification of a great amount of indications that had only been investigated in one study. Due to the lack of evidence, most indications were judged to be not associated. In order to obtain meaningful results, we compared all indications to one reference category, i.e. keratoconus. This had been investigated in most studies and in contrast to other indications such as Fuchs endothelial dystrophy (FED), it has not been associated with the development of glaucoma.[24] Transplants performed for the treatment of keratoconus are also known to have a high graft survival.[25] As we did not have the complete databases of the studies, it was only possible to recalculate the univariate results.

To our knowledge, Wu et al. is the only meta-analysis investigating risk factors for the development of OHT after PKP.[8] They also defined OHT as an IOP > 21mmHg; however, they did not specify a time-point of IOP measurement. Similar to the study of Wu et al., we found pre-existing glaucoma to have one of the highest correlations with a rise in IOP . In addition, we found that solely a higher preoperative IOP was also definitely correlated with the development of OHT and glaucoma in the contralateral eye (in cases without glaucoma in the investigated eye) was probably associated. Wu et al. did not investigated these risk factors. In the study of Wu et al. and in this study, aphakic and pseudophakic bullous keratopathy were analyzed as one category. Wu et al. found this to be significantly associated with the development of OHT. In our study, bullous keratopathy is probably associated when compared to keratoconus and when compared to other indications (risk factor bullous keratoplasty yes vs. no). The more robust result found by Wu et al. might be due to the fact that they also included studies published before 1990 (starting from 1972). Most older studies did not differentiate between FED and bullous keratoplasty. It has been suggested that FED is associated with the development of glaucoma, which might explain why Wu et al. found a higher correlation between developing OHT and bullous keratoplasty.[24] Within our study, we could not find a clear association between FED and the development of OHT after keratoplasty. However, only two studies investigated FED separately from other corneal dystrophies. Therefore, further investigation for this risk factor is indicated. Within this review, a preoperative lens status of both aphakia and pseudophakia was possibly associated with an increased risk which was mainly due to the heterogeneity in the evidence tables. In cases where the pre- or postoperative lens status was not defined, aphakia was found to be definitely associated. For pseudophakia, there was no difference in the association of the risk and the operative status. Despite the fact that Wu et al. did not make a distinction between pre- and postoperative lens status, they also found aphakia to be significantly associated and pseudophakia to be not significantly associated. As within this study pre- and postoperative aphakic lens status

seem to differ in the risk for developing OHT, this might suggest that a keratoplasty combined with a cataract extraction is associated with the development of OHT when compared to a keratoplasty only. However, we found that the risk was not increased when comparing pseudophakic lens status pre- and postoperatively. A triple procedure, in our study defined as keratoplasty combined with cataract extraction and IOL implantation, was not associated. This finding was confirmed by the study of Wu et al. as well. The only combined surgery that we found to be definitely associated was keratoplasty combined with IOL removal or exchange. The risk factor combined surgery in general was judged to be probably associated as well; however, as its name already indicates, it contains a wide range of types of surgery and detailed information is lacking. Wu et al. also described a moderate association for regrafting. This risk factor was judged to be possibly associated in our study as well, however; its evidence table showed a considerable amount of heterogeneity. Furthermore, Wu et al. found trauma to be related with the development of OHT, although they indicated that this result should be interpreted conservatively as the robustness of the analysis was suggested. We could not find an association of trauma with the development of OHT when compared to keratoconus. Herpes simplex keratitis was not found to be significantly associated in neither the study of Wu et al., nor our study.

The use of olopatadine 0.1% or cyclosporine (any dosage) before transplantation was found to be probably associated. This might be caused by a secondary effect as patients treated with these drugs often have an allergic eye disease for which they possibly use or have used corticosteroids as well. Corticosteroids are known to cause an IOP elevation by inducing molecular alterations in the trabecular meshwork (TM) which increase the outflow resistance.[26, 27] This effect is known for all types of corticosteroids; however, we found that using prednisolone acetate 1% is probably associated with a higher risk for developing OHT compared to dexamethasone 0.1%. This is mainly due to the fact that the penetration of topical concentration through the cornea is higher for prednisolone, causing higher concentrations in the aqueous humour.[28] Unfortunately, due to a lack of evidence, we could only find a possible association for prednisolone acetate 1% vs. loteprednol etabonate 0.5% and prednisolone acetate 1% vs. fluorometholone 0.1% of which previous research showed that loteprednol etabonate and fluorometholone are weaker topical corticosteroids and therefore associated with a lower risk for the of development of OHT.[29-31] In addition, based on the data of Vajaranant, a longer duration of exposure has also been shown to increase the risk for the development of OHT.[32, 33] This article was included in our study, however, as they did not present a P-value, OR, or hazard ratio regarding this risk factor, we could not include these data in our manuscript. The two studies that investigated duration of exposure as risk factor and of which we could use the data defined the duration of exposure not homogenously and reported different results. Therefore, we could not find an

association with duration based on the current evidence. It is well known that corticosteroid-induced elevation of the IOP is the most common cause of OHT after keratoplasty.[9-11] Within this review, studies specifically reporting the results for steroid-induced OHT (marked with an asterisk in the evidence tables) do not seem to deviate from studies who reported OHT in general. Nevertheless, further research to identify the risk factors for corticosteroid-induced OHT after keratoplasty is needed.

We tried to find associations between type of surgery and risk for developing OHT, however, due to the diversity of the studies regarding methodology, presentation of results, definition of OHT, the results are not uniform and very difficult to interpret. Therefore, addition research concerning the relation between the type of keratoplasty and the development of OHT is necessary.

Twenty risk factors showed a considerable heterogeneity during the judgment procedure. We checked whether this could be explained by different types of surgery but we could not find any correlation between the heterogeneity in the results and the type of surgery. The heterogeneity might be caused by the different study populations or the use of different definitions for OHT or glaucoma within the studies. Additional investigation is needed to determine the association of these risk factors.

Some issues at study level need to be addressed. The performed semi-quantitative approach does not provide the possibility to differentiate between study differences. Throughout the included studies multiple IOP measuring devices were used. Published studies report contradictive results on the use of different IOP-measurement devices and the IOP-values after keratoplasty.[34-36] However, in most of the included studies, the same IOP-measurement devices were likely to be used before and/or after surgery and within the subgroups, minimalizing possible measurement-differences within a study.

The definition of glaucoma or OHT also differed throughout the studies. As described in the method section, we tried to use similar cut-offs and outcome measures. However, if this was not possible, we accepted the criteria set by the investigators. It would be too difficult or even impossible to recalculate the value of OHT according to other criteria. Despite this, our results are confirmed by the meta-analysis of Wu et al. and are in line with the clinical expectations. Additionally, we have to note that we defined OHT as an increase > 21 mmHg which is a commonly used cut-off in the included papers. Although, if a more strict cut-off for OHT was used, some of the risk factors might not have been found highly associated anymore. This might also apply to the six-months outcomes on which we focused. Although we could not find a specific example within the study, the use of another time-frame might influence the results as well.

In conclusion, this review provides an overview of all investigated risk factors for the development of OHT after keratoplasty and the level of evidence that is available for each risk factor. Based on the evidence tables, factors with a definitive and probable association with an increased risk for OHT have been established. This can help to identify patients at risk and to individualize patient care concerning the choice of therapy, postoperative treatment and follow-up. In addition, we have shown that many risk factors still lack sufficient evidence to determine its association and need further investigation.

References

1. Vohra R, Tsai JC, Kolko M. The role of inflammation in the pathogenesis of glaucoma. Surv Ophthalmol 2013;58(4):311-20.
2. Taurone S, Ripandelli G, Pacella E, et al. Potential regulatory molecules in the human trabecular meshwork of patients with glaucoma: immunohistochemical profile of a number of inflammatory cytokines. Mol Med Rep 2015;11(2):1384-90.
3. Micera A, Quaranta L, Esposito G, et al. Differential Protein Expression Profiles in Glaucomatous Trabecular Meshwork: An Evaluation Study on a Small Primary Open Angle Glaucoma Population. Adv Ther 2016;33(2):252-67.
4. Wang N, Chintala SK, Fini ME, Schuman JS. Activation of a tissue-specific stress response in the aqueous outflow pathway of the eye defines the glaucoma disease phenotype. Nat Med 2001;7(3):304-9.
5. Toris CB, Gabelt BT, Kaufman PL. Update on the mechanism of action of topical prostaglandins for intraocular pressure reduction. Surv Ophthalmol 2008;53 Suppl1:S107-20.
6. Hubackova S, Krejcikova K, Bartek J, Hodny Z. IL1- and TGFbeta-Nox4 signaling, oxidative stress and DNA damage response are shared features of replicative, oncogene-induced, and drug-induced paracrine 'bystander senescence'. Aging (Albany NY) 2012;4(12):932-51.
7. Young AR, Narita M. *SASP* reflects senescence. EMBO Rep 2009;10(3):228-30.
8. Guichard A, Humbert P, Tissot M, et al. Effects of topical corticosteroids on cell proliferation, cell cycle progression and apoptosis: in vitro comparison on HaCaT. Int J Pharm 2015;479(2):422-9.
9. Bourcier T, Forgez P, Borderie V, et al. Regulation of human corneal epithelial cell proliferation and apoptosis by dexamethasone. Invest Ophthalmol Vis Sci 2000;41(13):4133-41.
10. Sansregret L, Nepveu A. The multiple roles of *CUX1*: insights from mouse models and cell-based assays. Gene 2008;412(1-2):84-94.
11. Alcalay NI, Vanden Heuvel GB. Regulation of cell proliferation and differentiation in the kidney. Front Biosci (Landmark Ed) 2009;14:4978-91.
12. Hu F, Gou L, Liu Q, et al. G-patch domain containing 2, a gene highly expressed in testes, inhibits nuclear factor-kappaB and cell proliferation. Mol Med Rep 2015;11(2):1252-7.
13. Reid T, Kenney C, Waring GO. Isolation and characterization of fibronectin from bovine aqueous humor. Invest Ophthalmol Vis Sci 1982;22(1):57-61.
14. Hann CR, Springett MJ, Wang X, Johnson DH. Ultrastructural localization of collagen IV, fibronectin, and laminin in the trabecular meshwork of normal and glaucomatous eyes. Ophthalmic Res 2001;33(6):314-24.
15. Filla MS, Dimeo KD, Tong T, Peters DM. Disruption of fibronectin matrix affects type IV collagen, fibrillin and laminin deposition into extracellular matrix of human trabecular meshwork (HTM) cells. Exp Eye Res 2017;165:7-19.
16. Moshirfar M, Parker L, Birdsong OC, et al. Use of Rho kinase Inhibitors in Ophthalmology: A Review of the Literature. Med Hypothesis Discov Innov Ophthalmol 2018;7(3):101-11.
17. Tripathi RC, Li J, Chan WF, Tripathi BJ. Aqueous humor in glaucomatous eyes contains an increased level of TGF-beta 2. Exp Eye Res 1994;59(6):723-7.

18. Agarwal P, Daher AM, Agarwal R. Aqueous humor TGF-beta2 levels in patients with open-angle glaucoma: A meta-analysis. Mol Vis 2015;21:612-20.
19. Prendes MA, Harris A, Wirostko BM, et al. The role of transforming growth factor beta in glaucoma and the therapeutic implications. Br J Ophthalmol 2013;97(6):680-6.
20. Bermudez JY, Webber HC, Brown B, et al. A Comparison of Gene Expression Profiles between Glucocorticoid Responder and Non-Responder Bovine Trabecular Meshwork Cells Using RNA Sequencing. PLoS One 2017;12(1):e0169671.
21. Fan BJ, Wang DY, Tham CC, et al. Gene expression profiles of human trabecular meshwork cells induced by triamcinolone and dexamethasone. Invest Ophthalmol Vis Sci 2008;49(5):1886-97.
22. Nehme A, Lobenhofer EK, Stamer WD, Edelman JL. Glucocorticoids with different chemical structures but similar glucocorticoid receptor potency regulate subsets of common and unique genes in human trabecular meshwork cells. BMC Med Genomics 2009;2:58.
23. Markus H. Kuehn CYK, Young H. Kwon. Dexamethasone induced gene expression changes in human perfusion organ cultured trabecular meshwork. 2012.
24. Matsuda A, Asada Y, Takakuwa K, et al. DNA Methylation Analysis of Human Trabecular Meshwork Cells During Dexamethasone Stimulation. Invest Ophthalmol Vis Sci 2015;56(6):3801-9.
25. Faralli JA, Desikan H, Peotter J, et al. Genomic/proteomic analyses of dexamethasone-treated human trabecular meshwork cells reveal a role for GULP1 and ABR in phagocytosis. Mol Vis 2019;25:237-54.
26. Raghunathan VK, Morgan JT, Park SA, et al. Dexamethasone Stiffens Trabecular Meshwork, Trabecular Meshwork Cells, and Matrix. Invest Ophthalmol Vis Sci 2015;56(8):4447-59.
27. Li G, Cui G, Dismuke WM, et al. Differential response and withdrawal profile of glucocorticoid-treated human trabecular meshwork cells. Exp Eye Res 2017;155:38-46.
28. Steely HT, Browder SL, Julian MB, et al. The effects of dexamethasone on fibronectin expression in cultured human trabecular meshwork cells. Invest Ophthalmol Vis Sci 1992;33(7):2242-50.
29. Mao W, Tovar-Vidales T, Yorio T, et al. Perfusion-cultured bovine anterior segments as an ex vivo model for studying glucocorticoid-induced ocular hypertension and glaucoma. Invest Ophthalmol Vis Sci 2011;52(11):8068-75.
30. Tripathi RC, Parapuram SK, Tripathi BJ, et al. Corticosteroids and glaucoma risk. Drugs Aging 1999;15(6):439-50.
31. Armaly MF, Becker B. Intraocular pressure response to topical corticosteroids. Fed Proc 1965;24(6):1274-8.
32. Becker B. Intraocular Pressure Response to Topical Corticosteroids. Invest Ophthalmol 1965;4:198-205.
33. Kitazawa Y, Horie T. The prognosis of corticosteroid-responsive individuals. Arch Ophthalmol 1981;99(5):819-23.
34. Lewis JM, Priddy T, Judd J, et al. Intraocular pressure response to topical dexamethasone as a predictor for the development of primary open-angle glaucoma. Am J Ophthalmol 1988;106(5):607-12.

35. Gottanka J, Johnson DH, Martus P, Lutjen-Drecoll E. Severity of optic nerve damage in eyes with POAG is correlated with changes in the trabecular meshwork. J Glaucoma 1997;6(2):123-32.
36. Ueda J, Wentz-Hunter K, Yue BY. Distribution of *myoc*ilin and extracellular matrix components in the juxtacanalicular tissue of human eyes. Invest Ophthalmol Vis Sci 2002;43(4):1068-76.
37. Rohen JW, Lutjen-Drecoll E, Flugel C, et al. Ultrastructure of the trabecular meshwork in untreated cases of primary open-angle glaucoma (POAG). Exp Eye Res 1993;56(6):683-92.
38. Tawara A, Tou N, Kubota T, et al. Immunohistochemical evaluation of the extracellular matrix in trabecular meshwork in steroid-induced glaucoma. Graefes Arch Clin Exp Ophthalmol 2008;246(7):1021-8.
39. Tektas OY, Lutjen-Drecoll E. Structural changes of the trabecular meshwork in different kinds of glaucoma. Exp Eye Res 2009;88(4):769-75.
40. Clark AF, Wilson K, McCartney MD, et al. Glucocorticoid-induced formation of cross-linked actin networks in cultured human trabecular meshwork cells. Invest Ophthalmol Vis Sci 1994;35(1):281-94.
41. Hoare MJ, Grierson I, Brotchie D, et al. Cross-linked actin networks (CLANs) in the trabecular meshwork of the normal and glaucomatous human eye in situ. Invest Ophthalmol Vis Sci 2009;50(3):1255-63.
42. Bermudez JY, Montecchi-Palmer M, Mao W, Clark AF. Cross-linked actin networks (CLANs) in glaucoma. Exp Eye Res 2017;159:16-22.
43. Clark AF, Brotchie D, Read AT, et al. Dexamethasone alters F-actin architecture and promotes cross-linked actin network formation in human trabecular meshwork tissue. Cell Motil Cytoskeleton 2005;60(2):83-95.
44. de Voogd S, Ikram MK, Wolfs RC, et al. Incidence of open-angle glaucoma in a general elderly population: the Rotterdam Study. Ophthalmology 2005;112(9):1487-93.
45. Morgan JT, Raghunathan VK, Chang YR, et al. The intrinsic stiffness of human trabecular meshwork cells increases with senescence. Oncotarget 2015;6(17):15362-74.
46. Liu B, McNally S, Kilpatrick JI, et al. Aging and ocular tissue stiffness in glaucoma. Surv Ophthalmol 2017.
47. Armaly MF. Effect of Corticosteroids on Intraocular Pressure and Fluid Dynamics. I. The Effect of Dexamethasone in the Normal Eye. Arch Ophthalmol 1963;70:482-91.
48. Lam DS, Fan DS, Ng JS, et al. Ocular hypertensive and anti-inflammatory responses to different dosages of topical dexamethasone in children: a randomized trial. Clin Exp Ophthalmol 2005;33(3):252-8.
49. Hosseini M, Rose AY, Song K, et al. *IL-1* and TNF induction of matrix metalloproteinase-3 by c-Jun N-terminal kinase in trabecular meshwork. Invest Ophthalmol Vis Sci 2006;47(4):1469-76.
50. Giguere V, Yang N, Segui P, Evans RM. Identification of a new class of steroid hormone receptors. Nature 1988;331(6151):91-4.
51. Hong H, Yang L, Stallcup MR. Hormone-independent transcriptional activation and coactivator binding by novel orphan nuclear receptor ERR3. J Biol Chem 1999;274(32):22618-26.

52. Wang SC, Myers S, Dooms C, et al. An ERRbeta/gamma agonist modulates GRalpha expression, and glucocorticoid responsive gene expression in skeletal muscle cells. Mol Cell Endocrinol 2010;315(1-2):146-52.
53. Lee SW, Hwang HH, Hsu PW, et al. Whole-genome methylation profiling from PBMCs in acute-exacerbation COPD patients with good and poor responses to corticosteroid treatment. Genomics 2019;111(6):1381-6.
54. Zhang M, Maddala R, Rao PV. Novel molecular insights into *RhoA* GTPase-induced resistance to aqueous humor outflow through the trabecular meshwork. Am J Physiol Cell Physiol 2008;295(5):C1057-70.
55. Kumar J, Epstein DL. Rho GTPase-mediated cytoskeletal organization in Schlemm's canal cells play a critical role in the regulation of aqueous humor outflow facility. J Cell Biochem 2011;112(2):600-6.
56. Rao PV, Deng PF, Kumar J, Epstein DL. Modulation of aqueous humor outflow facility by the Rho kinase-specific inhibitor Y-27632. Invest Ophthalmol Vis Sci 2001;42(5):1029-37.
57. Van de Velde S, De Groef L, Stalmans I, et al. Towards axonal regeneration and neuroprotection in glaucoma: Rho kinase inhibitors as promising therapeutics. Prog Neurobiol 2015;131:105-19.
58. Bermel C, Tonges L, Planchamp V, et al. Combined inhibition of *Cdk*5 and *ROCK* additively increase cell survival, but not the regenerative response in regenerating retinal ganglion cells. Mol Cell Neurosci 2009;42(4):427-37.
59. Sugiyama T, Shibata M, Kajiura S, et al. Effects of fasudil, a Rho-associated protein kinase inhibitor, on optic nerve head blood flow in rabbits. Invest Ophthalmol Vis Sci 2011;52(1):64-9.
60. Matsuo N, Terao M, Nabeshima Y, Hoshino M. Roles of STEF/*Tiam1*, guanine nucleotide exchange factors for *Rac1*, in regulation of growth cone morphology. Mol Cell Neurosci 2003;24(1):69-81.
61. Tang AT, Campbell WB, Nithipatikom K. *ROCK1* feedback regulation of the upstream small GTPase *RhoA*. Cell Signal 2012;24(7):1375-80.
62. Maeda K, Otomo K, Yoshida N, et al. *CaMK4* compromises podocyte function in autoimmune and nonautoimmune kidney disease. J Clin Invest 2018;128(8):3445-59.
63. Kakinuma N, Roy BC, Zhu Y, et al. *Kank* regulates *RhoA*-dependent formation of actin stress fibers and cell migration via 14-3-3 in PI3K-Akt signaling. J Cell Biol 2008;181(3):537-49.
64. Price MO, Feng MT, Price FW, Jr. Randomized, Double-Masked Trial of Netarsudil 0.02% Ophthalmic Solution for Prevention of Corticosteroid-Induced Ocular Hypertension. Am J Ophthalmol 2020;222:382-7.
65. Honjo M, Tanihara H, Inatani M, et al. Effects of rho-associated protein kinase inhibitor Y-27632 on intraocular pressure and outflow facility. Invest Ophthalmol Vis Sci 2001;42(1):137-44.
66. Tian B, Kaufman PL. Effects of the Rho kinase inhibitor Y-27632 and the phosphatase inhibitor calyculin A on outflow facility in monkeys. Exp Eye Res 2005;80(2):215-25.
67. Futakuchi A, Morimoto T, Ikeda Y, et al. Intraocular pressure-lowering effects of ripasudil in uveitic glaucoma, exfoliation glaucoma, and steroid-induced glaucoma patients: *ROCK*-S, a multicentre historical cohort study. Sci Rep 2020;10(1):10308.

68. Fini ME, Schwartz SG, Gao X, et al. Steroid-induced ocular hypertension/glaucoma: Focus on pharmacogenomics and implications for precision medicine. Prog Retin Eye Res 2017;56:58-83.
69. Vajaranant TS, Price MO, Price FW, et al. Visual acuity and intraocular pressure after Descemet's stripping endothelial keratoplasty in eyes with and without preexisting glaucoma. Ophthalmology 2009;116(9):1644-50.
70. Wilkinson MD, Dumontier M, Aalbersberg IJ, et al. The FAIR Guiding Principles for scientific data management and stewardship. Sci Data 2016;3:160018.
71. Slenter DN, Kutmon M, Hanspers K, et al. WikiPathways: a multifaceted pathway database bridging metabolomics to other omics research. Nucleic Acids Res 2018;46(D1):D661-D7.
72. Kutmon M, van Iersel MP, Bohler A, et al. PathVisio 3: an extendable pathway analysis toolbox. PLoS Comput Biol 2015;11(2):e1004085.
73. van Iersel MP, Kelder T, Pico AR, et al. Presenting and exploring biological pathways with PathVisio. BMC Bioinformatics 2008;9:399.
74. Flugel C, Tamm E, Lutjen-Drecoll E. Different cell populations in bovine trabecular meshwork: an ultrastructural and immunocytochemical study. Exp Eye Res 1991;52(6):681-90.
75. Gerometta R, Podos SM, Candia OA, et al. Steroid-induced ocular hypertension in normal cattle. Arch Ophthalmol 2004;122(10):1492-7.
76. Gerometta RM, Malgor LA, Vilalta E, et al. Cl- concentrations of bovine, porcine and ovine aqueous humor are higher than in plasma. Exp Eye Res 2005;80(3):307-12.
77. To CH, Do CW, Zamudio AC, Candia OA. Model of ionic transport for bovine ciliary epithelium: effects of acetazolamide and HCO. Am J Physiol Cell Physiol 2001;280(6):C1521-30.
78. Kunisato A, Wakatsuki M, Shinba H, et al. Direct generation of induced pluripotent stem cells from human nonmobilized blood. Stem Cells Dev 2011;20(1):159-68.
79. Ding QJ, Zhu W, Cook AC, et al. Induction of trabecular meshwork cells from induced pluripotent stem cells. Invest Ophthalmol Vis Sci 2014;55(11):7065-72.
80. Keller KE, Bhattacharya SK, Borras T, et al. Consensus recommendations for trabecular meshwork cell isolation, characterization and culture. Exp Eye Res 2018;171:164-73.
81. Polansky JR, Fauss DJ, Zimmerman CC. Regulation of TIGR/*MYOC* gene expression in human trabecular meshwork cells. Eye (Lond) 2000;14 (Pt 3B):503-14.
82. Abu-Hassan DW, Li X, Ryan EI, et al. Induced pluripotent stem cells restore function in a human cell loss model of open-angle glaucoma. Stem Cells 2015;33(3):751-61.
83. Zhu W, Gramlich OW, Laboissonniere L, et al. Transplantation of iPSC-derived TM cells rescues glaucoma phenotypes in vivo. Proc Natl Acad Sci U S A 2016;113(25):E3492-500.
84. Zhu W, Jain A, Gramlich OW, et al. Restoration of Aqueous Humor Outflow Following Transplantation of iPSC-Derived Trabecular Meshwork Cells in a Transgenic Mouse Model of Glaucoma. Invest Ophthalmol Vis Sci 2017;58(4):2054-62.
85. Honda M, Orii F, Ayabe T, et al. Expression of glucocorticoid receptor beta in lymphocytes of patients with glucocorticoid-resistant ulcerative colitis. Gastroenterology 2000;118(5):859-66.
86. Goleva E, Jackson LP, Gleason M, Leung DY. Usefulness of PBMCs to predict clinical response to corticosteroids in asthmatic patients. J Allergy Clin Immunol 2012;129(3):687-93 e1.

87. Hew M, Bhavsar P, Torrego A, et al. Relative corticosteroid insensitivity of peripheral blood mononuclear cells in severe asthma. Am J Respir Crit Care Med 2006;174(2):134-41.
88. Sliwinska-Stanczyk P, Pazdur J, Ziolkowska M, et al. The effect of methylprednisolone on proliferation of PBMCs obtained from steroid-sensitive and steroid-resistant rheumatoid arthritis patients. Scand J Rheumatol 2007;36(3):167-71.
89. Jeoung JW, Ko JH, Kim YJ, et al. Microarray-based analysis of gene expression profiles in peripheral blood of patients with acute primary angle closure. Ophthalmic Genet 2017;38(6):520-6.
90. Flugel-Koch C, Ohlmann A, Fuchshofer R, et al. Thrombospondin-1 in the trabecular meshwork: localization in normal and glaucomatous eyes, and induction by TGF-beta1 and dexamethasone in vitro. Exp Eye Res 2004;79(5):649-63.

Supplemental material

All supplemental material are provided below and can also be found on *https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7015193/*

Supplemental material 1. *Search strategy used for the Pubmed and Embase database.*

Supplemental table 1. Search strategy used for the Pubmed database	
	(((((((((((((((((((((((((((((((((((((corneal transplantation surgery) OR corneal transplant surgery) OR corneal graft surgery) OR corneal grafting surgery) OR keratoplasty) OR keratoplasties) OR lamellar keratoplasty) OR lamellar keratoplasties) OR Endothelial keratoplasty) OR Endothelial keratoplasties) OR DMEK) OR Descemet Membrane Endothelial Keratoplasty) OR Descemet Membrane Endothelial Keratoplasties) OR DSAEK) OR descemet stripping automated endothelial keratoplasty) OR descemet stripping automated endothelial keratoplasties) OR ultra thin DSAEK) OR ultra-thin DSAEK) OR ultrathin DSAEK) OR ultra thin descemet stripping automated endothelial keratoplasty) OR ultra thin descemet stripping automated endothelial keratoplasties) OR ultrathin descemet stripping automated endothelial keratoplasty) OR ultrathin descemet stripping automated endothelial keratoplasties) OR ultra-thin descemet stripping automated endothelial keratoplasty) OR ultra-thin descemet stripping automated endothelial keratoplasties) OR DSEK) OR descemet stripping endothelial keratoplasty) OR descemet stripping endothelial keratoplasties) OR DALK) OR deep anterior lamellar keratoplasty) OR deep anterior lamellar keratoplasty) OR deep anterior lamellar keratoplasties) OR PKP) OR penetrating keratoplasty) OR penetrating keratoplasties) OR “Corneal Transplantation”[Mesh]) OR “Keratoplasty, Penetrating”[Mesh]) OR “Descemet Stripping Endothelial Keratoplasty”[Mesh])
AND	
	((((((((((((((((((((((((((intra-ocular pressure) OR intraocular pressure) OR IOP) OR eye pressure) OR increased IOP) OR increased intra-ocular pressure) OR increased intraocular pressure) OR ocular hypertension) OR glaucoma) OR steroid-induced ocular hypertension) OR steroid-induced glaucoma) OR steroid-induced increased intraocular pressure) OR steroid-induced increased intra-ocular pressure) OR steroid-induced increase intra-ocular pressure) OR steroid-induced increase intraocular pressure) OR steroid-induced increased IOP) OR steroid-induced increase IOP) OR steroid-response) OR steroid-responder) OR steroid-responders) OR steroid responder) OR steroid responders) OR steroid response) OR “Intraocular Pressure”[Mesh]) OR “Ocular Hypertension”[Mesh]) OR “Glaucoma”[Mesh])
Filters	Publication date from 1990/01/01 to 2019/01/31

Supplemental table 2. Search strategy used for the Embase database	
	exp cornea transplantation/
OR	
	exp penetrating keratoplasty/ or exp Descemet membrane endothelial keratoplasty/ or deep lamellar endothelial keratoplasty/ or exp endothelial keratoplasty/ or exp refractive keratoplasty/ or exp Descemet stripping endothelial keratoplasty/ or exp keratoplasty
AND	
	exp intraocular pressure/
OR	
	Glaucoma/ or intraocular pressure
OR	
	exp drug induced disease/
Filters	Publication date from 1990/01/01 to 2019/02/01

Supplemental material 2. *Clustering of the 76 included studies based on author names, site of recruitment, trial names, period of recruitment and references to other studies.**

Cluster number	Publication year	Source of study population	Case series	Comparative case series	Cohort study	Case-control studies	Randomized controlled trials
1	1993	L.V. Prasad Eye Institute, Hyderabad, India	1				
2	1998-2018	Dr Rajendra Prasad Centre for Ophthalmic Sciences, New Delhi, India	2 , 3, 4				
3	1999	Hôpital Saint Antoine, Paris, France	5				
4	2000	Augenklinik der RWTH Aachen, Aachen, Germany	6				
5	2002	Federal University of Uberlândia, Uberlândia, Brazil	7				
6	2002	University of Erlangen-Nürnberg, Erlangen, Germany					8
7	2002-2004	Tokyo Dental College, Tokyo, Japan				9	10
8	2004	Ege University Hospital, Izmir, Turkey		11			
9	2007-2018	King Khaled Eye Specialist Hospital, Riyadh, Saudi Arabia	12, 13, 14	15			
10	2008-2014	Toronto Western Hospital, Toronto, Canada ; Yonge Eglinton Laser Eye Center, Toronto, Canada	16	17, 18, 19, 20, 21, 22, 23			
11	2009	Wills Eye Hospital, Philadelphia, PA, USA		24			
12	2009	Auckland City Hospital and Eye Institute, Auckland, New Zealand	25				
13	2009	Manchester Royal Eye Hospital, Manchester, UK (through the UK Transplant database)	26				
14	2009-2015	Price Vision Group, Indianapolis, IN, USA; Gorovoy Eye Specialists, Fort Myers, FL, USA	27	28, 29			30, 31
15	2010	The University of Texas Southwestern Medical Center, Dallas, TX, USA	32				
16	2010	Dr. Lütfi Kırdar Kartal Training and Research Hospital, Istanbul, Turkey	33				
17	2010	University of Iowa Hospitals and Clinics, Iowa City, IA, USA	34				
18	2011	Eskisehir Osmangazi University Medical Faculty, Eskisehir, Turkey	35				
19	2012	Cornea Eye Institute, Beverly Hills, California, CA, USA		36			
20	2012	Affiliated Hospital of Medical College Qingdao University, Qingdao Shi, China	37				
21	2012	St. James's University Hospital, Leeds, UK	38				
22	2012	Keio University School of Medicine, Tokyo, Japan		39			
23	2012-2019	Singapore National Eye Center, Singapore, Asia; through the Singapore Corneal Transplant Study	40, 41	42, 43	44, 45, 46, 47		

24	2013	University of Düsseldorf, Düsseldorf, Germany	48			
25	2013	Tel Aviv Sourasky Medical Center; and Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel		49		
26	2013	Doheny Eye Institute, Los Angeles, Californië, Verenigde Staten		50		
27	2013	Sir Run Run Shaw Institute of Clinical Medicine of Zhejiang University, Hangzhou, China		51		
28	2013-2015	Charité – Universitätsmedizin Berlin, Berlin, Germany	52, 53	54		
29	2014	Haydarpasa Numune Education and Research Hospital, Istanbul, Turkey; Istanbul Medipol University, School of Medicine, Istanbul, Turkey		55		
30	2014	Hadassah-Hebrew University Medical Center, Jerusalem, Israel	56			
31	2014	Dr. Ashok Sharma's Cornea Centre, Chandigarh, India	57			
32	2015	General Hospital of Shenyang Military Area Command, Shenyang, China				58
33	2015	Cantonal Hospital of Lucerne, Lucerne, Switzerland		59		
34	2015	The Jules Stein Eye Institute, David Geffen School of Medicine, Los Angeles, CA, USA		60		
35	2016	Quinze-Vingts National Eye Hospital, Paris, France			61	
36	2016	Juntendo University hospital, Tokyo, Japan		62		
37	2016	Sydney Eye Hospital, Sydney, Australia		63		
38	2016	Ivey Eye Institute in London, Canada	64			
39	2016-2017	University of Cologne, Cologne, Germany		65, 66		
40	2017	Cleveland Clinic, Cleveland, Ohio, OH, USA	67			
41	2017	Dhahran Eye Specialist Hospital, Dhahran, Saudi Arabia		68		
42	2017	Adana Numune Training and Research Hospital, Adana, Turkey; Ankara Training and Research Hospital, Ankara, Turkey		69		
43	2017	Sightline Ophthalmic Associates, Pittsburgh, PA, USA		70		
44	2017	University of Muenster Medical Center, Muenster, Germany	71			
45	2017	Izmir Bozyaka Training and Research Hospital, Izmir Turkey; Kartal Training and Research Hospital, Istanbul, Turkey				72
46	2018	Ankara Training and Research Hospital, Ankara, Turkey; Ağrı State Hospital, Ağrı, Turkey		73		
47	2018	The Jules Stein Eye Institute, David Geffen School of Medicine, Los Angeles, CA, US; American Academy of Ophthalmology, San Francisco, California, CA, USA; through Centers for Medicare & Medicaid Services (CMS).			74	
48	2018	Swami Rama Himalayan University, Jolly Grant, Dehradun, Uttarakhand, India	75			
49	2018	Complejo Hospitalario La Mancha Centro, Ciudad Real, Spain		76		

**Each number between brackets represents a study corresponding with the reference list*

References of supplemental material 2

1. Sekhar, G.C., et al., Post-penetrating keratoplasty glaucoma. Indian J Ophthalmol, 1993. 41(4): p. 181-4.
2. Sihota, R., et al., Post-penetrating keratoplasty glaucoma: risk factors, management and visual outcome. Aust N Z J Ophthalmol, 1998. 26(4): p. 305-9.
3. Sharma, N., et al., Outcomes of therapeutic penetrating keratoplasty from a tertiary eye care centre in northern India. Cornea, 2014. 33(2): p. 114-8.
4. Srujana, D., et al., Long-term Functional and Anatomic Outcomes of Repeat Graft After Optically Failed Therapeutic Keratoplasty. Am J Ophthalmol, 2018. 189: p. 166-175.
5. Borderie, V.M., et al., The triple procedure: in the bag placement versus ciliary sulcus placement of the intraocular lens. Br J Ophthalmol, 1999. 83(4): p. 458-62.
6. Redbrake, C. and O. Arend, Glaucoma following keratoplasty. [German]. Ophthalmologe, 2000. 97(8): p. 552-556.
7. Franca, E.T., et al., A study of glaucoma after penetrating keratoplasty. Cornea, 2002. 21(3): p. 284-8.
8. Seitz, B., et al., Long-term follow-up of intraocular pressure after penetrating keratoplasty for keratoconus and Fuchs' dystrophy: Comparison of mechanical and excimer laser trephination. Cornea, 2002. 21(4): p. 368-373.
9. Shimazaki, J., et al., Efficacy and safety of international donor sharing: a single-center, case-controlled study on corneal transplantation. Transplantation, 2004. 78(2): p. 216-20.
10. Shimazaki, J., et al., Randomized clinical trial of deep lamellar keratoplasty vs penetrating keratoplasty. Am J Ophthalmol, 2002. 134(2): p. 159-65.
11. Egrilmez, S., S. Sahin, and A. Yagci, The effect of vernal keratoconjunctivitis on clinical outcomes of penetrating keratoplasty for keratoconus. Can J Ophthalmol, 2004. 39(7): p. 772-7.
12. Al-Mohaimeed, M., et al., Escalation of glaucoma therapy after penetrating keratoplasty. Ophthalmology, 2007. 114(12): p. 2281-6.
13. Wagoner, M.D. and R. Ba-Abbad, Penetrating keratoplasty for keratoconus with or without vernal keratoconjunctivitis. Cornea, 2009. 28(1): p. 14-8.
14. Wagoner, M.D., et al., Postoperative complications after primary adult optical penetrating keratoplasty: prevalence and impact on graft survival. Cornea, 2009. 28(4): p. 385-94.
15. Khairallah, A., Descemet stripping automated endothelialkeratoplasty (DSAEK) versus repeat penetrating keratoplasty (PKP) to manage eyes with failed corneal graft. Ann Saudi Med, 2018. 38(1): p. 36-41.
16. Kaiserman, I., I. Bahar, and D.S. Rootman, Corneal wound malapposition after penetrating keratoplasty: an optical coherence tomography study. Br J Ophthalmol, 2008. 92(8): p. 1103-7.
17. Bahar, I., et al., Comparison of posterior lamellar keratoplasty techniques to penetrating keratoplasty. Ophthalmology, 2008. 115(9): p. 1525-33.
18. Bahar, I., et al., Manual top hat wound configuration for penetrating keratoplasty. Cornea, 2008. 27(5): p. 521-526.

19. Kaiserman, I., et al., Suture-assisted vs forceps-assisted insertion of the donor lenticula during Descemet stripping automated endothelial keratoplasty. Am J Ophthalmol, 2008. 145(6): p. 986-990.
20. Bahar, I., et al., Busin Guide vs Forceps for the Insertion of the Donor Lenticule in Descemet Stripping Automated Endothelial Keratoplasty. Am J Ophthalmol, 2009. 147(2): p. 220-226 e1.
21. Kaiserman, I., et al., Half top hat wound configuration for penetrating keratoplasty: 1-year results. Br J Ophthalmol, 2009. 93(12): p. 1629-33.
22. Mashor, R.S., et al., Outcomes of deep anterior lamellar keratoplasty versus intralase enabled penetrating keratoplasty in keratoconus. Canadian Journal of Ophthalmology, 2011. 46(5): p. 403-407.
23. Shehadeh Mashor, R., et al., Zig Zag versus Top Hat configuration in IntraLase-enabled penetrating keratoplasty. Br J Ophthalmol, 2014. 98(6): p. 756-9.
24. Cohen, E.J., et al., Steroid-induced intraocular pressure elevation or glaucoma after penetrating keratoplasty in patients with keratoconus or Fuchs dystrophy. Cornea, 2009. 28(7): p. 759-764.
25. Fan, J.C., et al., Corticosteroid-induced intraocular pressure elevation in keratoconus is common following uncomplicated penetrating keratoplasty. Eye (Lond), 2009. 23(11): p. 2056-62.
26. Rahman, I., et al., Penetrating keratoplasty: indications, outcomes, and complications. Eye (Lond), 2009. 23(6): p. 1288-94.
27. Vajaranant, T.S., et al., Visual acuity and intraocular pressure after Descemet's stripping endothelial keratoplasty in eyes with and without preexisting glaucoma. Ophthalmology, 2009. 116(9): p. 1644-50.
28. Price, M.O., et al., Effect of incision width on graft survival and endothelial cell loss after Descemet stripping automated endothelial keratoplasty. Cornea, 2010. 29(5): p. 523-7.
29. Chaurasia, S., et al., Descemet's membrane endothelial keratoplasty: Clinical results of single versus triple procedures (combined with cataract surgery). Ophthalmology, 2014. 121(2): p. 454-458.
30. Price, M.O., et al., Randomized comparison of topical prednisolone acetate 1% versus fluorometholone 0.1% in the first year after descemet membrane endothelial keratoplasty. Cornea, 2014. 33(9): p. 880-6.
31. Price, M.O., et al., Loteprednol Etabonate 0.5% Gel Vs. Prednisolone Acetate 1% Solution After Descemet Membrane Endothelial Keratoplasty: Prospective Randomized Trial. Cornea, 2015. 34(8): p. 853-8.
32. Allen, M.B., et al., Risk factors for intraocular pressure elevation after descemet stripping automated endothelial keratoplasty. Eye Contact Lens, 2010. 36(4): p. 223-7.
33. Karadag, O., et al., Incidence of and risk factors for increased intraocular pressure after penetrating keratoplasty. Cornea, 2010. 29(3): p. 278-82.
34. Wandling, G.R., Jr., et al., Escalation of glaucoma therapy after deep lamellar endothelial keratoplasty. Cornea, 2010. 29(9): p. 991-5.
35. Yildirim, N., et al., Glaucoma after penetrating keratoplasty: incidence, risk factors, and management. J Ophthalmol, 2011. 2011: p. 951294.

36. Gaster, R.N., O. Dumitrascu, and Y.S. Rabinowitz, Penetrating keratoplasty using femtosecond laser-enabled keratoplasty with zig-zag incisions versus a mechanical trephine in patients with keratoconus. British Journal of Ophthalmology, 2012. 96(9): p. 1195-1199.
37. Li, C., et al., Effect of corneal graft diameter on therapeutic penetrating keratoplasty for fungal keratitis. Int J Ophthalmol, 2012. 5(6): p. 698-703.
38. Musa, F.U., et al., Long-term risk of intraocular pressure elevation and glaucoma escalation after deep anterior lamellar keratoplasty. Clin Exp Ophthalmol, 2012. 40(8): p. 780-5.
39. Ozeki, N., et al., Intraocular pressure elevation after Descemet's stripping endothelial keratoplasty. Jpn J Ophthalmol, 2012. 56(4): p. 307-11.
40. Huang, O.S., et al., Incidence and Risk Factors of Elevated Intraocular Pressure Following Deep Anterior Lamellar Keratoplasty. Am J Ophthalmol, 2016. 170: p. 153-160.
41. Huang, O.S., et al., Incidence and Outcomes of Intraoperative Descemet Membrane Perforations During Deep Anterior Lamellar Keratoplasty. American Journal of Ophthalmology, 2019. 199: p. 9-18.
42. Chan, E.W., et al., De novo ocular hypertension after Descemet stripping endothelial keratoplasty: comparative 3-year incidence, risk factors, and outcomes. Clin Ophthalmol, 2013. 7: p. 1829-41.
43. Ang, M., et al., Endothelial keratoplasty after failed penetrating keratoplasty: an alternative to repeat penetrating keratoplasty. Am J Ophthalmol, 2014. 158(6): p. 1221-1227.e1.
44. Ang, M., et al., Endothelial cell counts after Descemet's stripping automated endothelial keratoplasty versus penetrating keratoplasty in Asian eyes. Clinical Ophthalmology, 2012. 6(1): p. 537-544.
45. Ang, M., et al., Indications, outcomes, and risk factors for failure in tectonic keratoplasty. Ophthalmology, 2012. 119(7): p. 1311-1319.
46. Ang, M., et al., Descemet's stripping automated endothelial keratoplasty with anterior chamber intraocular lenses: Complications and 3-year outcomes. British Journal of Ophthalmology, 2014. 98(8): p. 1028-1032.
47. Soh, Y., et al., Five-Year Graft Survival Comparing Descemet Stripping Automated Endothelial Keratoplasty and Penetrating Keratoplasty. Ophthalmology, 2016. 123(8): p. 1646-1652.
48. Huber, K.K., et al., Glaucoma in penetrating keratoplasty: risk factors, management and outcome. Graefes Arch Clin Exp Ophthalmol, 2013. 251(1): p. 105-16.
49. Moisseiev, E., et al., Intraocular pressure after penetrating keratoplasty and Descemet's stripping automated endothelial keratoplasty. Can J Ophthalmol, 2013. 48(3): p. 179-85.
50. Nguyen, P., et al., Descemet stripping with automated endothelial keratoplasty: A comparative study of outcome in patients with preexisting glaucoma. Saudi J Ophthalmol, 2013. 27(2): p. 73-8.
51. Zhang, Y.M., S.Q. Wu, and Y.F. Yao, Long-term comparison of full-bed deep anterior lamellar keratoplasty and penetrating keratoplasty in treating keratoconus. J Zhejiang Univ Sci B, 2013. 14(5): p. 438-50.
52. Maier, A.K., et al., Intraocular pressure elevation and post-DSEK glaucoma after Descemet`s stripping endothelial keratoplasty. Graefes Arch Clin Exp Ophthalmol, 2013. 251(4): p. 1191-8.

53. Maier, A.K., et al., Intraocular pressure elevation and post-DMEK glaucoma following Descemet membrane endothelial keratoplasty. Graefes Arch Clin Exp Ophthalmol, 2014. 252(12): p. 1947-54.
54. Maier, A.K., et al., Anterior segment analysis and intraocular pressure elevation after penetrating keratoplasty and posterior lamellar endothelial keratoplasty. Ophthalmic Res, 2015. 53(1): p. 36-47.
55. Acar, B.T., O. Muftuoglu, and S. Acar, Comparison of sulfur hexafluoride and air for donor attachment in Descemet stripping endothelial keratoplasty in patients with pseudophakic bullous keratopathy. Cornea, 2014. 33(3): p. 219-22.
56. Blumenthal, E.Z., J. Frucht-Pery, and A. Solomon, Risk factors and incidence of ocular hypertension after penetrating keratoplasty. Journal of Glaucoma, 2014. 23(9): p. 599-605.
57. Sharma, A., et al., Post penetrating keratoplasty glaucoma: cumulative effect of quantifiable risk factors. Indian J Ophthalmol, 2014. 62(5): p. 590-5.
58. Chen, Y., et al., Efficacy and Safety of Corneal Transplantation Using Corneas from Foreign Donors versus Domestic Donors: A Prospective, Randomized, Controlled Trial. J Ophthalmol, 2015. 2015: p. 178289.
59. Muller, L., et al., Changes in intraocular pressure after descemet stripping automated endothelial keratoplasty: a retrospective analysis. Cornea, 2015. 34(3): p. 271-4.
60. Tannan, A., et al., Comparison of ACIOL Retention With IOL Exchange in Patients Undergoing Descemet Stripping Automated Endothelial Keratoplasty. Cornea, 2015. 34(9): p. 1030-4.
61. Borderie, V.M., et al., Incidence of Intraocular Pressure Elevation and Glaucoma after Lamellar versus Full-Thickness Penetrating Keratoplasty. Ophthalmology, 2016. 123(7): p. 1428-34.
62. Nakatani, S. and A. Murakami, Descemet stripping automated endothelial keratoplasty using corneas from elderly donors. Graefe's Archive for Clinical and Experimental Ophthalmology, 2016. 254(6): p. 1135-1140.
63. Sandhu, S., et al., Elevated Intraocular Pressure in Patients Undergoing Penetrating Keratoplasty and Descemet Stripping Endothelial Keratoplasty. J Glaucoma, 2016. 25(4): p. 390-6.
64. Sharma, R.A., et al., Comparison of intraocular pressure post penetrating keratoplasty vs Descemet's stripping endothelial keratoplasty. Can J Ophthalmol, 2016. 51(1): p. 19-24.
65. Schaub, F., et al., Impact of Donor Age on Descemet Membrane Endothelial Keratoplasty Outcome: Evaluation of Donors Aged 17-55 Years. American Journal of Ophthalmology, 2016. 170: p. 119-127.
66. Schaub, F., et al., One-year outcome after Descemet membrane endothelial keratoplasty (DMEK) comparing sulfur hexafluoride (SF6) 20% versus 100% air for anterior chamber tamponade. Br J Ophthalmol, 2017. 101(7): p. 902-908.
67. Kaleem, M., et al., Rates of Intraocular Pressure Elevation and Use of Topical Antihypertensive Medication After Descemet Stripping Automated Endothelial Keratoplasty. Cornea, 2017. 36(6): p. 669-674.
68. Khattak, A., et al., Comparison of outcomes and complications of deep anterior lamellar keratoplasty and penetrating keratoplasty performed in a large group of patients with keratoconus. Int Ophthalmol, 2017.

69. Kocluk, Y., E.A. Sukgen, and A. Burcu, Comparison of outcomes in patients who underwent deep anterior lamellar keratoplasty and those converted to penetrating keratoplasty. Turkish Journal of Ophthalmology, 2017. 47(2): p. 63-67.
70. Phillips, P.M., et al., Experienced DSAEK Surgeon's Transition to DMEK: Outcomes Comparing the Last 100 DSAEK Surgeries With the First 100 DMEK Surgeries Exclusively Using Previously Published Techniques. Cornea, 2017. 36(3): p. 275-279.
71. Treder, M., M. Alnawaiseh, and N. Eter, Descemet membrane endothelial keratoplasty (DMEK) early stage graft failure in eyes with preexisting glaucoma. Graefes Arch Clin Exp Ophthalmol, 2017. 255(7): p. 1417-1421.
72. Yuksel, B., et al., Comparison of visual and topographic outcomes of deep-anterior lamellar keratoplasty and penetrating keratoplasty in keratoconus. Int J Ophthalmol, 2017. 10(3): p. 385-390.
73. Singar-Ozdemir, E., et al., Effect of Donor Cornea on the Surgical Outcomes of Penetrating Keratoplasty: Imported Cornea Versus Domestic Cornea. Exp Clin Transplant, 2018.
74. Zheng, C., et al., Risk of Glaucoma Surgery after Corneal Transplant Surgery in Medicare Patients. Am J Ophthalmol, 2018.
75. Raj, A., R. Dhasmana, and H. Bahadur, Incidence and risk factors for postkeratoplasty glaucoma in tertiary care center, India. Oman J Ophthalmol, 2018. 11(3): p. 220-226.
76. Infantes Molina, E.J., et al., Deep anterior lamellar keratoplasty versus penetrating keratoplasty in corneas showing a high or low graft rejection risk. European Journal of Ophthalmology., 2018.

Supplemental material 3. *Overview of the characteristics of the 76 included studies.*

Publication	Prospective/ Retrospective	Number of patients	Time of follow-up (months)	Method of IOP measurement	Univariate/ multivariate	Type of surgery
1	Retrospective	185	14.5 months*	GAT, digital tonometer	Univariate	PKP
2	Retrospective	747	23 months*	GAT, Shiotz tonometer	Univariate	PKP
5	Prospective	40	12 months	Not defined	Univariate	PKP
6	Retrospective	75	5 years	GAT	Univariate	PKP
7	Retrospective	228	17.14 months*	Not defined	Univariate	PKP
8	Prospective	170	3.4 years*	GAT	Univariate	PKP
10	Prospective	24	6 months†	Not defined	Univariate	PKP or DLEK
11	Retrospective	76	KC + VKC 34.0; KC alone 41.0 months*	Not defined	Univariate	PKP
9	Prospective	236	31.3 months*	Not defined	Univariate	PKP
12	Retrospective	678	32.5 months*	Not defined	Univariate	PKP
17	Prospective	161	13.6 months*	Not defined	Univariate	PKP, DLEK, DSEK or DSAEK
18	Retrospective	71	12 months†	Not defined	Univariate	PKP
19	Prospective	28	Stitch-assisted: 7.13; Forceps-assisted 12.8 months*	Not defined	Univariate	DSAEK
16	Retrospective	25	5 years*	Not defined	Multivariate	PKP
20	Prospective	63	6 months	Not defined	Univariate	DSAEK
24	Retrospective	158	4-7 months	GAT, Tono-Pen	Univariate	PKP
25	Retrospective	48	12 months	GAT	Univariate	PKP
21	Retrospective	87	At 12 months	Not defined	Univariate	PKP
26	Retrospective	186	61 months*	Not defined	Univariate	PKP
27	Retrospective	400	6 months	GAT, Tono-Pen, Pneumatonometer	Univariate	DSEK
13	Retrospective	464	With VKC 58.6; without VKC 57.7 months*	Not defined	Univariate	PKP
14	Retrospective	910	At least 3 months	Not defined	Univariate	PKP
32	Retrospective	57	11.38 months	GAT, Pneumatonometer	Both	DSAEK
33	Retrospective	729	Early and late (not further defined)	Not defined	Univariate	PKP
28	Retrospective	167	1 year	Not defined	Univariate	DSAEK

34	Retrospective	80	27.1 months*	Tono-Pen	Univariate	DLEK
22	Retrospective	36	DALK 17.83; IEK 11.3 months*	Not defined	Univariate	DALK, FLEK
35	Retrospective	115	38.9 months*	Tono-Pen	Univariate	PKP
44	Retrospective	241	1 year	Not defined	Univariate	PKP, DSAEK
45	Retrospective	362	3 months	Not defined	Univariate	PKP, ALK
36	Retrospective	116	6 months	Not defined	Univariate	PKP
37	Retrospective	116	2 years	Not defined	Univariate	PKP
38	Retrospective	59	55.6 months*	GAT, Tono-Pen	Univariate	DALK
39	Retrospective	46	6 months*	GAT, noncontact tonometer	Univariate	DSEK
42	Retrospective	324	3 years	GAT, Tono-Pen	Both	DSAEK
48	Retrospective	160	6 months	GAT, digital palpation	Univariate	PKLP
52	Retrospective	59	437 days*	GAT	Univariate	DSEK
49	Retrospective	53	12 months	GAT	Univariate	PKP, DSAEK
50	Retrospective	298	1.85 years*	Tono-Pen	Univariate	DSAEK
51	Retrospective	115	DALK 46.9; PKP 60.2 months*	Not defined	Univariate	DALK, PKP
55	Prospective	44	6 months†	GAT	Univariate	DSAEK
43	Retrospective	113	3 years	Not defined	Univariate	PKP, EK
46	Retrospective	132	1 year	Not defined	Univariate	DSAEK
56	Retrospective	146	24.4 months*	GAT	Both	PKP
29	Retrospective	451	6 months	Not defined	Univariate	DMEK
53	Retrospective	117	12 months	GAT, Pneumatonometer	Multivariate	DMEK
30	Prospective	264	12 months	GAT	Univariate	DMEK
57	Retrospective	445	32 months*	GAT	Both	PKP
3	Retrospective	506	26.7 months*	Not defined	Univariate	PKP
23	Retrospective	33	Zig Zag 13.24; Top Hat: 14.14 months*	Not defined	Univariate	PKP
58	Prospective	108	Foreign 301; domestic: 326 days*	Not defined	Univariate	PKP
54	Retrospective	112	658 days*	GAT	Univariate	PKP, DSAEK
59	Retrospective	176	1 year	GAT, DCT	Univariate	DSAEK
31	Prospective	167	36 months	GAT	Univariate	DMEK
60	Retrospective	41	18.3 months*	Not defined	Univariate	DSAEK

61	Retrospective	1657	10 years	GAT	Both	PKP, ALK, DSAEK
40	Retrospective	116	5 years	Pneumatonometer, GAT, Tono-Pen	Multivariate	DALK
62	Retrospective	90	2 years	Not defined	Univariate	DSAEK
63	Retrospective	61	12 months	GAT, Tono-Pen	Univariate	DSEK
65	Retrospective	529	12 months	Not defined	Univariate	DMEK
64	Retrospective	71	24 weeks	GAT, Pneumatonometer	Univariate	DSEK
47	Retrospective	828	5 years	Not defined	Univariate	DSAEK, PKP
67	Retrospective	379	1 year	GAT, Tono-Pen	Both	DSAEK
68	Retrospective	183	DALK 28.06; PKP 29.29 months*	Not defined	Univariate	PKP, DALK
69	Retrospective	54	PKP 14; DALK 14.8 months*	GAT	Univariate	PKP converted from DALK, PKP
70	Retrospective	132	6 months†	Not defined	Univariate	DMEK
66	Retrospective	854	6 months†	Icare	Univariate	DMEK
71	Retrospective	59	3 months†	GAT	Univariate	DALK
72	Prospective	71	1 year	GAT, Tono-Pen	Univariate	DALK
76	Retrospective	98	2 years†	GAT	Univariate	PKP, DALK
15	Retrospective	45	6 months†	Tono-Pen	Univariate	RE-PKP, DSEAK
75	Retrospective	155	6 months	Applanation tonometer or Tono-Pen	Both	PKP
73	Retrospective	200	6 months	Not defined	Univariate	PKP
4	Prospective	32	18,4 months*	Not defined	Univariate	RE-PKP
74	Retrospective	3098	1 year	Not defined	Univariate	PKP, EK, ALK
41	Retrospective	540	1 year	Not defined	Univariate	DALK

*† IOP or glaucoma was measured at the indicated point of time, * IOP or glaucoma was measured at any point during the indicated mean time of follow-up, in all other cases IOP or glaucoma was measured at any point within the indicated time of follow-up.*

Abbreviations: (D)ALK = (deep) anterior lamellar keratoplasty; DCT = dynamic contour tonometer; DLEK = deep lamellar endothelial keratoplasty; DLKP = deep lamellar keratoplasty; DMEK = descemet membrane endothelial keratoplasty; DS(A)EK = descemet stripping (automated) endothelial keratoplasty; EK = endothelial keratoplasty; GAT = goldmann applanation tonometer; FLEK = femtosecond laser enabled keratoplasty; KC = keratoconus; PKP = penetrating keratoplasty; VKC = vernal keratoconjunctivitis

Supplemental material 4. *Evidence tables of all identified risk factors for the development of OHT after.*

Legend

These evidence tables summarize the univariate and multivariate results of all identified risk factors for the development of OHT after keratoplasty. Conform table 3, all evidence tables are ranked according to the strength of association (high to low) and the preoperative (p2), intraoperative(p19), postoperative (p33), and pre- and postoperative (p38) status. The last category exists of risk factors of which the pre- or intraoperative (p39) status was not defined. Each number in the table represents a study corresponding with the reference list used in table 1. The number of study clusters, studies, and patients is summarized below the study numbers. Numbers between parentheses represent the total number of multivariate and univariate conclusions in the analysis. Significant refers to statistically significance (P<0.05)

Underlined studies belong to the same study cluster.
* Indicates specifically steroid responders.
†, †† Indicates and specifies subgroups that have been made within one study and are further specified under the table.
Abbreviations: (D)ALK = (deep) anterior lamellar keratoplasty, DLEK = deep lamellar endothelial keratoplasty, DMEK = descemet membrane endothelial keratoplasty, DS(A) EK = descemet stripping (automated) endothelial keratoplasty, ECCE = extracapsular cataract extraction, EK = endothelial keratoplasty, FED = Fuchs endothelial dystrophy, IOL = intraocular lens, IOP = intraocular pressure, Kpro = keratoprosthesis, PKP = penetrating keratoplasty

Preoperative risk factors

Pre-existing glaucoma	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=17)	61, 59, 56,32, 26, 33†, 12, 52 and 52*, 54†, 6, 57, 74†	67, 27, 74††		49	71	
No. Of clusters	11	3	0	1	1	0
No. Of studies	12	3	0	1	1	0
No. Of patients	7418	3877	0	53	59	0
Multivariate analysis (n=7)	61, 56, 32, 57	40, 53			67	
No. Of clusters	4	2	0	0	1	0
No. Of studies	4	2	0	0	1	0
No. Of patients	2305	233	0	0	379	0

33† early and late post-op period;

54† In eyes with Fuchs and bullous keratopathy

74† PK, EK ALK, Kpro, PK + EK and in PK separately ; †† EK; ALK

Preoperative IOP	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=4)	35, 39	42		25		
No. Of clusters	2	1	0	1	0	0
No. Of studies	2	1	0	1	0	0
No. Of patients	161	324	0	48	0	0
Multivariate analysis (n=3)	67, 53	40				
No. Of clusters	2	1	0	0	0	0
No. Of studies	2	1	0	0	0	0
No. Of patients	496	116	0	0	0	0

Glaucoma in contralateral eye (no glaucoma in investigated eye)	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)	42					
No. Of clusters	1	0	0	0	0	0
No. Of studies	1	0	0	0	0	0
No. Of patients	324	0	0	0	0	0
Multivariate analysis (n=1)	42					
No. Of clusters	1	0	0	0	0	0
No. Of studies	1	0	0	0	0	0
No. Of patients	324	0	0	0	0	0

History of pseudo-exfoliation syndrome	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)	59					
No. Of clusters	1	0	0	0	0	0
No. Of studies	1	0	0	0	0	0
No. Of patients	176	0	0	0	0	0
Multivariate analysis (n=7)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Preoperative treatment of glaucoma with medication	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)	50					
No. Of clusters	1	0	0	0	0	0
No. Of studies	1	0	0	0	0	0
No. Of patients	298	0	0	0	0	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Preoperative treatment of glaucoma with medication and/or surgical	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)	34					
No. Of clusters	1	0	0	0	0	0
No. Of studies	1	0	0	0	0	0
No. Of patients	80	0	0	0	0	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Preoperative treatment of glaucoma surgical	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=3)	61	50			27	
No. Of clusters	1	1	0	0	1	0
No. Of studies	1	1	0	0	1	0
No. Of patients	1657	298	0	0	400	0
Multivariate analysis (n=1)		61				
No. Of clusters	0	1	0	0	0	0
No. Of studies	0	1	0	0	0	0
No. Of patients	0	1657	0	0	0	0

Preoperative treatment with medication vs. surgical	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)		12				
No. Of clusters	0	1	0	0	0	0
No. Of studies	0	1	0	0	0	0
No. Of patients	0	678	0	0	0	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Preoperative treatment with one vs. two or more medications	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)		12				
No. Of clusters	0	1	0	0	0	0
No. Of studies	0	1	0	0	0	0
No. Of patients	0	678	0	0	0	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Cyclosporine use before transplantation	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0
Multivariate analysis (n=1)	40					
No. Of clusters	1	0	0	0	0	0
No. Of studies	1	0	0	0	0	0
No. Of patients	116	0	0	0	0	0

Olopatadine 0.1% use before transplantation	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0
Multivariate analysis (n=1)	40					
No. Of clusters	1	0	0	0	0	0
No. Of studies	1	0	0	0	0	0
No. Of patients	116	0	0	0	0	0

Age patient (old vs. young age)	Increased risk for OHT		Direction of the relation is unknown		Decreased risk for OHT	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=12)	61, 12, 57	39		56, 25, 49, 34, 74	75, 42††	42†
No. Of clusters	3	1	0	5	2	1
No. Of studies	3	1	0	5	2	1
No. Of patients	2780	46	0	3425	479	324
Multivariate analysis (n=9)	57	61	16	53	42††,40, 67	75,42†
No. Of clusters	1	1	1	1	2	2
No. Of studies	1	1	1	1	3	2
No. Of patients	445	1657	25	117	819	479

42† age < 60 years vs. ≥70 years; †† age 60-69 years vs. ≥70 years

Gender (male vs. female)	Increased risk for OHT		Direction of the relation is unknown		Decreased risk for OHT	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=9)	74, 57	42, 75		56, 49, 25	39, 34	
No. Of clusters	2	2	0	3	2	0
No. Of studies	2	2	0	3	2	0
No. Of patients	3543	479	0	247	126	0
Multivariate analysis (n=4)	57	75, 40	16			
No. Of clusters	1	2	1	0	0	0
No. Of studies	1	2	1	0	0	0
No. Of patients	455	271	25	0	0	0

History of ocular surgery	Increased risk for OHT		Direction of the relation is unknown		Decreased risk for OHT	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)						56
No. Of clusters	0	0	0	0	0	1
No. Of studies	0	0	0	0	0	1
No. Of patients	0	0	0	0	0	146
Multivariate analysis (n=1)					56	
No. Of clusters	0	0	0	0	1	0
No. Of studies	0	0	0	0	1	0
No. Of patients	0	0	0	0	146	0

Preoperative presence of peripheral anterior synechiae	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)	33					
No. Of clusters	1	0	0	0	0	0
No. Of studies	1	0	0	0	0	0
No. Of patients	729	0	0	0	0	0
Multivariate analysis (n=1)				53		
No. Of clusters	0	0	0	1	0	0
No. Of studies	0	0	0	1	0	0
No. Of patients	0	0	0	117	0	0

Age donor (older age)	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=2)		62			65	
No. Of clusters	0	1	0	0	1	0
No. Of studies	0	1	0	0	1	0
No. Of patients	0	90	0	0	529	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Diabetes mellitus	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)				49		
No. Of clusters	0	0	0	1	0	0
No. Of studies	0	0	0	1	0	0
No. Of patients	0	0	0	53	0	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Hypertension	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)				49		
No. Of clusters	0	0	0	1	0	0
No. Of studies	0	0	0	1	0	0
No. Of patients	0	0	0	53	0	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Family history of keratoconus	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)				25		
No. Of clusters	0	0	0	1	0	0
No. Of studies	0	0	0	1	0	0
No. Of patients	0	0	0	48	0	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

History of steroid use (systemic + topical)	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)				25		
No. Of clusters	0	0	0	1	0	0
No. Of studies	0	0	0	1	0	0
No. Of patients	0	0	0	48	0	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Indication bullous keratoplasty (yes vs. no)	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0
Multivariate analysis (n=0)	67†	67††				
No. Of clusters	1	1	0	0	0	0
No. Of studies	1	1	0	0	0	0
No. Of patients	379	379	0	0	0	0

[67]† *pseudophakic bullous keratoplasty;* †† *aphakic bullous keratoplasty*

Microbial keratitis with vs. without corneal perforation	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)	3					
No. Of clusters	1	0	0	0	0	0
No. Of studies	1	0	0	0	0	0
No. Of patients	506	0	0	0	0	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Inflammatory vs. non-inflammatory indication	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)	33					
No. Of clusters	1	0	0	0	0	0
No. Of studies	1	0	0	0	0	0
No. Of patients	729	0	0	0	0	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Indication (general)	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)		35		56, 49		
No. Of clusters	0	1	0	2	0	0
No. Of studies	0	1	0	2	0	0
No. Of patients	0	115	0	199	0	0
Multivariate analysis (n=0)				53		
No. Of clusters	0	0	0	1	0	0
No. Of studies	0	0	0	1	0	0
No. Of patients	0	0	0	117	0	0

History of vernal keratoconjunctivitis	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)					13	
No. Of clusters	0	0	0	0	1	0
No. Of studies	0	0	0	0	1	0
No. Of patients	0	0	0	0	464	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Non-optical vs. optical indication	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0
Multivariate analysis (n=0)		40				
No. Of clusters	0	1	0	0	0	0
No. Of studies	0	1	0	0	0	0
No. Of patients	0	116	0	0	0	0

Keratoconus + vernal keratoconjunctivitis vs. keratoconus only	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)		11*				
No. Of clusters	0	1	0	0	0	0
No. Of studies	0	1	0	0	0	0
No. Of patients	0	76	0	0	0	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Bullous keratoplasty vs. keratoconus	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=11)	33, 12, 7, 61, 26††, 1†	2, 1††, 26††, 48†		48††		
No. Of clusters	6	4	0	1	0	0
No. Of studies	6	4	0	1	0	0
No. Of patients	3663	1278	0	160	0	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

[1]† *aphakic bullous keratoplasty;* †† *pseudophakic bullous keratoplasty*

[26]† *pseudophakic bullous keratoplasty;* †† *aphakic bullous keratoplasty*

[48]† *aphakic bullous keratoplasty;* †† *pseudophakic bullous keratoplasty*

Corneal dystrophy (general) vs. keratoconus	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=8)	61	33, 61, 1		26, 12, 7, 14		
No. Of clusters	1	3	0	3	0	0
No. Of studies	1	3	0	4	0	0
No. Of patients	1657	2571	0	2002	0	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

FED vs. keratoconus	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=3)		8		48	24	
No. Of clusters	0	1	0	1	1	0
No. Of studies	0	1	0	1	1	0
No. Of patients	0	170	0	160	158	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Scar vs. keratoconus	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=6)	33, 12, 14	48, 61, 1				
No. Of clusters	2	3	0	0	0	0
No. Of studies	3	3	0	0	0	0
No. Of patients	2317	2002	0	0	0	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Trauma vs. keratoconus	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=3)	61	48, 7				
No. Of clusters	1	2	0	0	0	0
No. Of studies	1	2	0	0	0	0
No. Of patients	1657	388	0	0	0	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Herpetic keratitis vs. keratoconus	Increased risk for OHT		Direction of the relation is unknown		Decreased risk for OHT	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=3)		48, 7, 33				
No. Of clusters	0	3	0	0	0	0
No. Of studies	0	3	0	0	0	0
No. Of patients	0	1117	0	0	0	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Infectious keratitis vs. keratoconus	Increased risk for OHT		Direction of the relation is unknown		Decreased risk for OHT	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=8)	26†, 33, 12	26††, 2, 7†		48, 7††		
No. Of clusters	3	3	0	2	0	0
No. Of studies	3	3	0	2	0	0
No. Of patients	1593	1161	0	388	0	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

[7]† *infectious keratitis;* †† *interstitial keratitis*

[26]† *fungal infection;* †† *ulcerative keratitis, viral, bacterial*

Others (general) vs. keratoconus	Increased risk for OHT		Direction of the relation is unknown		Decreased risk for OHT	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=5)	12	48, 1			26, 7	
No. Of clusters	1	2	0	0	2	0
No. Of studies	1	2	0	0	2	0
No. Of patients	678	345	0	0	414	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Adherent leucoma vs. keratoconus	Increased risk for OHT		Direction of the relation is unknown		Decreased risk for OHT	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)		2				
No. Of clusters	0	1	0	0	0	0
No. Of studies	0	1	0	0	0	0
No. Of patients	0	747	0	0	0	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Descemetocele vs. keratoconus	Increased risk for OHT		Direction of the relation is unknown		Decreased risk for OHT	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)		33				
No. Of clusters	0	1	0	0	0	0
No. Of studies	0	1	0	0	0	0
No. Of patients	0	729	0	0	0	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Band keratopathy vs. keratoconus	Increased risk for OHT		Direction of the relation is unknown		Decreased risk for OHT	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)		33				
No. Of clusters	0	1	0	0	0	0
No. Of studies	0	1	0	0	0	0
No. Of patients	0	729	0	0	0	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Dysgenesis vs. keratoconus	Increased risk for OHT		Direction of the relation is unknown		Decreased risk for OHT	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)				48		
No. Of clusters	0	0	0	1	0	0
No. Of studies	0	0	0	1	0	0
No. Of patients	0	0	0	160	0	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Corneal edema vs. keratoconus	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)	14					
No. Of clusters	1	0	0	0	0	0
No. Of studies	1	0	0	0	0	0
No. Of patients	910	0	0	0	0	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Preoperative: Aphakic vs. phakic	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=6)	2, 33	35, 75				[3] 56 [56]
No. Of clusters	2	2	0	0	0	2
No. Of studies	2	2	0	0	0	2
No. Of patients	1476	270	0	0	0	652
Multivariate analysis (n=1)					56	
No. Of clusters	0	0	0	0	1	0
No. Of studies	0	0	0	0	1	0
No. Of patients	0	0	0	0	146	0

Preoperative: Pseudophakic vs. Phakic	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=6)	33	2, 35, 75			42	56
No. Of clusters	1	3	0	0	1	1
No. Of studies	1	3	0	0	1	1
No. Of patients	729	1017	0	0	324	146
Multivariate analysis (n=1)					56	
No. Of clusters	0	0	0	0	1	0
No. Of studies	0	0	0	0	1	0
No. Of patients	0	0	0	0	146	0

Preoperative: Aphakic or pseudophakic vs. Phakic	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)		34				
No. Of clusters	0	1	0	0	0	0
No. Of studies	0	1	0	0	0	0
No. Of patients	0	80	0	0	0	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Preoperative: Lens status in general	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=6)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0
Multivariate analysis (n=1)				53		
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	117	0	0

Preoperative: placement of IOL: Sulcus vs. bag	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)					33	
No. Of clusters	0	0	0	0	1	0
No. Of studies	0	0	0	0	1	0
No. Of patients	0	0	0	0	729	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Preoperative: placement of IOL: Scleral fixated vs. Bag	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)					33	
No. Of clusters	0	0	0	0	1	0
No. Of studies	0	0	0	0	1	0
No. Of patients	0	0	0	0	729	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Ethnicity: Caucasian vs. African-American descent	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)						74
No. Of clusters	0	0	0	0	0	1
No. Of studies	0	0	0	0	0	1
No. Of patients	0	0	0	0	0	3098
Multivariate analysis (n=1)						67
No. Of clusters	0	0	0	0	0	1
No. Of studies	0	0	0	0	0	1
No. Of patients	0	0	0	0	0	379

Ethnicity: Maori or Pacefic (new Zealand Europeans, Samoan, other Pacefic people) ethnicity vs. others (Indian, other European en Middel Eastern)	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)						25
No. Of clusters	0	0	0	0	0	1
No. Of studies	0	0	0	0	0	1
No. Of patients	0	0	0	0	0	48
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Ethnicity: Non-Chinese vs. Chinese	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)		42				
No. Of clusters	0	1	0	0	0	0
No. Of studies	0	1	0	0	0	0
No. Of patients	0	324	0	0	0	0
Multivariate analysis (n=1)					40	
No. Of clusters	0	0	0	0	1	0
No. Of studies	0	0	0	0	1	0
No. Of patients	0	0	0	0	116	0

Ethnicity: Region of the United States (East, West, Midwest, South)	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)				74		
No. Of clusters	0	0	0	1	0	0
No. Of studies	0	0	0	1	0	0
No. Of patients	0	0	0	3098	0	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Intraoperative risk factors

Type of surgery: PKP vs. DS(A)EK	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=9)	49, 61, 54†	63, 44		64	[42] 47, [47], 54††, 54*†††	
No. Of clusters	3	2	0	1	2	0
No. Of studies	3	2	0	1	3	0
No. Of patients	1822	302	0	71	1264	0
Multivariate analysis (n=1)	61					
No. Of clusters	1	0	0	0	0	0
No. Of studies	1	0	0	0	0	0
No. Of patients	1657	0	0	0	0	0

[54]† in eyes with bullous keratoplasty; †† in eyes with Fuchs; [54]††† in eyes with a steroid response (for both Fuchs and bullous keratoplasty)

Type of surgery: DSEK	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)	27					
No. Of clusters	1	0	0	0	0	0
No. Of studies	1	0	0	0	0	0
No. Of patients	400	0	0	0	0	0
Multivariate analysis (n=1)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Type of surgery: Regraft (due to failed or rejected graft)	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=11)	61, 1, 33, 12, 75, 57	32, 26, 48			7	2
No. Of clusters	6	3	0	0	1	1
No. Of studies	6	3	0	0	1	1
No. Of patients	3849	403	0	0	228	747
Multivariate analysis (n=3)		32, 67†			67††	
No. Of clusters	0	2	0	0	1	0
No. Of studies	0	2	0	0	1	0
No. Of patients	0	436	0	0	379	0

[67]† in re-DSAEK vs. no re-DSAEK; †† PK failure vs. no PK failure as indication

Type of surgery: Re-PKP vs. DSAEK after failed PKP	Increased risk for OHT		Direction of the relation is unknown		Decreased risk for OHT	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)						15
No. Of clusters	0	0	0	0	0	1
No. Of studies	0	0	0	0	0	1
No. Of patients	0	0	0	0	0	45
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Type of surgery: PKP vs. DALK	Increased risk for OHT		Direction of the relation is unknown		Decreased risk for OHT	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=7)	51, 72, 61	10, 68*, 76			22	
No. Of clusters	3	3	0	0	1	0
No. Of studies	3	3	0	0	1	0
No. Of patients	1843	305	0	0	36	0
Multivariate analysis (n=1)		61				
No. Of clusters	0	1	0	0	0	0
No. Of studies	0	1	0	0	0	0
No. Of patients	0	1657	0	0	0	0

Type of surgery: Re-PKP vs. EK after failed PKP	Increased risk for OHT		Direction of the relation is unknown		Decreased risk for OHT	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)		43				
No. Of clusters	0	1	0	0	0	0
No. Of studies	0	1	0	0	0	0
No. Of patients	0	113	0	0	0	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Type of surgery: ALK vs. DSAEK	Increased risk for OHT		Direction of the relation is unknown		Decreased risk for OHT	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)		61				
No. Of clusters	0	1	0	0	0	0
No. Of studies	0	1	0	0	0	0
No. Of patients	0	1657	0	0	0	0
Multivariate analysis (n=1)		61				
No. Of clusters	0	1	0	0	0	0
No. Of studies	0	1	0	0	0	0
No. Of patients	0	1657	0	0	0	0

Type of surgery: DALK converted to PKP vs. DALK	Increased risk for OHT		Direction of the relation is unknown		Decreased risk for OHT	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)		69, 69*				
No. Of clusters	0	1	0	0	0	0
No. Of studies	0	1	0	0	0	0
No. Of patients	0	54	0	0	0	0
Multivariate analysis (n=1)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Type of surgery (general)	Increased risk for OHT		Direction of the relation is unknown		Decreased risk for OHT	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=2)				74, 17*		
No. Of clusters	0	0	0	2	0	0
No. Of studies	0	0	0	2	0	0
No. Of patients	0	0	0	3259	0	0
Multivariate analysis (n=1)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Type of surgery: DMEK	Increased risk for OHT		Direction of the relation is unknown		Decreased risk for OHT	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)					71	
No. Of clusters	0	0	0	0	1	0
No. Of studies	0	0	0	0	1	0
No. Of patients	0	0	0	0	59	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Type of surgery: DALK	Increased risk for OHT		Direction of the relation is unknown		Decreased risk for OHT	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)		38				
No. Of clusters	0	1	0	0	0	0
No. Of studies	0	1	0	0	0	0
No. Of patients	0	59	0	0	0	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Combined surgery: Keratoplasty + IOL removal or exchange	Increased risk for OHT		Direction of the relation is unknown		Decreased risk for OHT	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=5)	61, 1, 60	32, 2				
No. Of clusters	3	2	0	0	0	0
No. Of studies	3	2	0	0	0	0
No. Of patients	1883	804	0	0	0	0
Multivariate analysis (n=2)	61, 32					
No. Of clusters	2	0	0	0	0	0
No. Of studies	2	0	0	0	0	0
No. Of patients	1714	0	0	0	0	0

Combined surgery (general)	Increased risk for OHT		Direction of the relation is unknown		Decreased risk for OHT	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=7)	56, 42, 33	26, 7		54, 75		
No. Of clusters	3	2	0	2	0	0
No. Of studies	3	2	0	2	0	0
No. Of patients	1199	414	0	267	0	0
Multivariate analysis (n=3)	56, 42	40				
No. Of clusters	2	1	0	0	0	0
No. Of studies	2	1	0	0	0	0
No. Of patients	470	116	0	0	0	0

Combined surgery: Keratoplasty + vitrectomy	Increased risk for OHT		Direction of the relation is unknown		Decreased risk for OHT	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=3)	61, 2, 1					
No. Of clusters	3	0	0	0	0	0
No. Of studies	3	0	0	0	0	0
No. Of patients	2589	0	0	0	0	0
Multivariate analysis (n=1)		61				
No. Of clusters	0	1	0	0	0	0
No. Of studies	0	1	0	0	0	0
No. Of patients	0	1657	0	0	0	0

Combined surgery: Keratoplasty + retaining anterior IOL vs. Keratoplasty posterior chamber lens left in place	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)	46					
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	132	0	0	0	0	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Combined surgery: Triple procedure (yes vs. no)	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=5)		42, 8, 35		49	29, 39	
No. Of clusters	0	3	0	1	2	0
No. Of studies	0	3	0	1	2	0
No. Of patients	0	609	0	53	497	0
Multivariate analysis (n=2)		42		53		
No. Of clusters	0	1	0	1	0	0
No. Of studies	0	1	0	1	0	0
No. Of patients	0	324	0	117	0	0

Combined surgery: Keratoplasty + ECCE	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=5)	1				57	
No. Of clusters	1	0	0	0	1	0
No. Of studies	1	0	0	0	1	0
No. Of patients	185	0	0	0	445	0
Multivariate analysis (n=2)					57	
No. Of clusters	0	0	0	0	1	0
No. Of studies	0	0	0	0	1	0
No. Of patients	0	0	0	0	445	0

Combined surgery: Keratoplasty + anterior segment reconstruction	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=2)	2	57				
No. Of clusters	1	1	0	0	0	0
No. Of studies	1	1	0	0	0	0
No. Of patients	747	445	0	0	0	0
Multivariate analysis (n=1)		57				
No. Of clusters	0	1	0	0	0	0
No. Of studies	0	1	0	0	0	0
No. Of patients	0	445	0	0	0	0

Combined surgery: Keratoplasty + secondary IOL	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)		1				
No. Of clusters	0	1	0	0	0	0
No. Of studies	0	1	0	0	0	0
No. Of patients	0	185	0	0	0	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Combined surgery: Keratoplasty + cataract extraction with IOL in ciliary sulcus vs. IOL in bag	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)		5				
No. Of clusters	0	1	0	0	0	0
No. Of studies	0	1	0	0	0	0
No. Of patients	0	36	0	0	0	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Half top-hat vs. regular PKP	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)	21					
No. Of clusters	1	0	0	0	0	0
No. Of studies	1	0	0	0	0	0
No. Of patients	87	0	0	0	0	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Zig Zag vs. top-hat in PKP	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)		23				
No. Of clusters	0	1	0	0	0	0
No. Of studies	0	1	0	0	0	0
No. Of patients	0	33	0	0	0	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Zig Zag with femtosecond vs. mechanical trephine in PKP	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)		36				
No. Of clusters	0	1	0	0	0	0
No. Of studies	0	1	0	0	0	0
No. Of patients	0	116	0	0	0	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Manual top-hat vs. regular PKP	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)		18				
No. Of clusters	0	1	0	0	0	0
No. Of studies	0	1	0	0	0	0
No. Of patients	0	71	0	0	0	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Manual half top-hat vs. top-hat PKP	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)		21				
No. Of clusters	0	1	0	0	0	0
No. Of studies	0	1	0	0	0	0
No. Of patients	0	87	0	0	0	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Width of the incision (large vs. small) in DSAEK or DLEK	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)		28		34		
No. Of clusters	0	1	0	1	0	0
No. Of studies	0	1	0	1	0	0
No. Of patients	0	167	0	80	0	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Trephination with excimer vs. motor	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)					8	
No. Of clusters	0	0	0	0	1	0
No. Of studies	0	0	0	0	1	0
No. Of patients	0	0	0	0	170	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Busin Guide-assisted vs. forceps-assisted DSAEK	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)		20				
No. Of clusters	0	1	0	0	0	0
No. Of studies	0	1	0	0	0	0
No. Of patients	0	63	0	0	0	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Forceps-assisted DSAEK vs. stitch-assisted	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)		19				
No. Of clusters	0	1	0	0	0	0
No. Of studies	0	1	0	0	0	0
No. Of patients	0	28	0	0	0	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Interrupted vs. interrupted + single continuous	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=2)				56, 25		
No. Of clusters	0	0	0	2	0	0
No. Of studies	0	0	0	2	0	0
No. Of patients	0	0	0	194	0	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Foreign vs. domestic donor grafts	Increased risk for OHT		Direction of the relation is unknown		Decreased risk for OHT	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=2)		9, 73			58	
No. Of clusters	0	2	0	0	1	0
No. Of studies	0	2	0	0	1	0
No. Of patients	0	436	0	0	108	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Laterality: Left vs. right	Increased risk for OHT		Direction of the relation is unknown		Decreased risk for OHT	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)		75				
No. Of clusters	0	1	0	0	0	0
No. Of studies	0	1	0	0	0	0
No. Of patients	0	155	0	0	0	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Graft diameter in PKP (large vs. small)	Increased risk for OHT		Direction of the relation is unknown		Decreased risk for OHT	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=6)	3, 37, 75			56	4	12
No. Of clusters	3	0	0	1	1	1
No. Of studies	3	0	0	1	1	1
No. Of patients	777	0	0	146	32	678
Multivariate analysis (n=2)	75		16			
No. Of clusters	1	0	1	0	0	0
No. Of studies	1	0	1	0	0	0
No. Of patients	155	0	25	0	0	0

Graft diameter in DSAEK (per mm increase)	Increased risk for OHT		Direction of the relation is unknown		Decreased risk for OHT	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)		42				
No. Of clusters	0	1	0	0	0	0
No. Of studies	0	1	0	0	0	0
No. Of patients	0	324	0	0	0	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Graft diameter in DALK (per mm increase)	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0
Multivariate analysis (n=1)					40	
No. Of clusters	0	0	0	0	1	0
No. Of studies	0	0	0	0	1	0
No. Of patients	0	0	0	0	116	0

Graft oversize in PKP (large vs. small)	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=3)	56			26	12	
No. Of clusters	1	0	0	1	1	0
No. Of studies	1	0	0	1	1	0
No. Of patients	146	0	0	186	678	0
Multivariate analysis (n=2)		56	16			
No. Of clusters	0	1	1	0	0	0
No. Of studies	0	1	1	0	0	0
No. Of patients	0	146	25	0	0	0

Size of malapposition	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0
Multivariate analysis (n=1)			16			
No. Of clusters	0	0	1	0	0	0
No. Of studies	0	0	1	0	0	0
No. Of patients	0	0	25	0	0	0

ANWAR big bubble technique	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0
Multivariate analysis (n=1)		40				
No. Of clusters	0	1	0	0	0	0
No. Of studies	0	1	0	0	0	0
No. Of patients	0	116	0	0	0	0

Sulfar hexafluoride SF6 20% vs. 100% air (bubble technique)	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=2)		66			55	
No. Of clusters	0	1	0	0	1	0
No. Of studies	0	1	0	0	1	0
No. Of patients	0	854	0	0	44	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Rebubbling	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)					32	
No. Of clusters	0	0	0	0	1	0
No. Of studies	0	0	0	0	1	0
No. Of patients	0	0	0	0	57	0
Multivariate analysis (n=1)					32	
No. Of clusters	0	0	0	0	1	0
No. Of studies	0	0	0	0	1	0
No. Of patients	0	0	0	0	57	0

Intraoperative perforation of the Descemet membrane during DALK	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)		41				
No. Of clusters	0	1	0	0	0	0
No. Of studies	0	1	0	0	0	0
No. Of patients	0	540	0	0	0	0
Multivariate analysis (n=1)		40				
No. Of clusters	0	1	0	0	0	0
No. Of studies	0	1	0	0	0	0
No. Of patients	0	116	0	0	0	0

Postoperative risk factors

Type of steroid use: Prednisolone acetate 1% vs. dexamethasone 0.1%	Increased risk for OHT		Direction of the relation is unknown		Decreased risk for OHT	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0
Multivariate analysis (n=1)	40					
No. Of clusters	1	0	0	0	0	0
No. Of studies	1	0	0	0	0	0
No. Of patients	116	0	0	0	0	0

Type of steroid use: Prednisolone acetate 1% vs. loteprednol etabonate 0.5%	Increased risk for OHT		Direction of the relation is unknown		Decreased risk for OHT	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)	31					
No. Of clusters	1	0	0	0	0	0
No. Of studies	1	0	0	0	0	0
No. Of patients	167	0	0	0	0	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Type of steroid use: Prednisolone acetate 1% vs. Fluorometholone 0.1%	Increased risk for OHT		Direction of the relation is unknown		Decreased risk for OHT	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)	30					
No. Of clusters	1	0	0	0	0	0
No. Of studies	1	0	0	0	0	0
No. Of patients	264	0	0	0	0	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Type of steroid use: Prednisolone acetate 0.12% vs. dexamethasone 0.1%	Increased risk for OHT		Direction of the relation is unknown		Decreased risk for OHT	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0
Multivariate analysis (n=1)		40				
No. Of clusters	0	1	0	0	0	0
No. Of studies	0	1	0	0	0	0
No. Of patients	0	116	0	0	0	0

Duration of steroid use (longer use)	Increased risk for OHT		Direction of the relation is unknown		Decreased risk for OHT	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)		32				
No. Of clusters	0	1	0	0	0	0
No. Of studies	0	1	0	0	0	0
No. Of patients	0	57	0	0	0	0
Multivariate analysis (n=2)		32		40		
No. Of clusters	0	1	0	1	0	0
No. Of studies	0	1	0	1	0	0
No. Of patients	0	57	0	116	0	0

Ocular surgery after keratoplasty	Increased risk for OHT		Direction of the relation is unknown		Decreased risk for OHT	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0
Multivariate analysis (n=1)		40				
No. Of clusters	0	1	0	0	0	0
No. Of studies	0	1	0	0	0	0
No. Of patients	0	116	0	0	0	0

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Cataract surgery after keratoplasty	Increased risk for OHT		Direction of the relation is unknown		Decreased risk for OHT	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1		35				
No. Of clusters	0	1	0	0	0	0
No. Of studies	0	1	0	0	0	0
No. Of patients	0	115	0	0	0	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Postoperative procedures or complications	Increased risk for OHT		Direction of the relation is unknown		Decreased risk for OHT	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)	42					
No. Of clusters	1	0	0	0	0	0
No. Of studies	1	0	0	0	0	0
No. Of patients	324	0	0	0	0	0
Multivariate analysis (n=0)	42	40				
No. Of clusters	1	1	0	0	0	0
No. Of studies	1	1	0	0	0	0
No. Of patients	324	116	0	0	0	0

Postoperative corneal oedema vs. corneal scar	Increased risk for OHT		Direction of the relation is unknown		Decreased risk for OHT	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=2)	33†	33††				
No. Of clusters	1	1	0	0	0	0
No. Of studies	1	1	0	0	0	0
No. Of patients	729	729	0	0	0	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

[33]† *late post-op (not further specified);* †† *early post-op (not further specified)*

Postoperative graft failure/rejection	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)					45	
No. Of clusters	0	0	0	0	1	0
No. Of studies	0	0	0	0	1	0
No. Of patients	0	0	0	0	362	0
Multivariate analysis (n=1)		40				
No. Of clusters	0	1	0	0	0	0
No. Of studies	0	1	0	0	0	0
No. Of patients	0	116	0	0	0	0

Postoperative graft status: Clear graft vs. graft with bullous keratoplasty	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)		39				
No. Of clusters	0	1	0	0	0	0
No. Of studies	0	1	0	0	0	0
No. Of patients	0	46	0	0	0	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Postoperative presence of peripheral anterior synechiae	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)					70	
No. Of clusters	0	0	0	0	1	0
No. Of studies	0	0	0	0	1	0
No. Of patients	0	0	0	0	132	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Graft clarity (high to low clarity)	**Increased risk for OHT**		**Direction of the relation is unknown**		**Decreased risk for OHT**	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)				75		
No. Of clusters	0	0	0	1	0	0
No. Of studies	0	0	0	1	0	0
No. Of patients	0	0	0	155	0	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Pre- and postoperative risk factor

pre- and postoperative: Presence of peripheral anterior synechiae	Increased risk for OHT		Direction of the relation is unknown		Decreased risk for OHT	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)		48				
No. Of clusters	0	1	0	0	0	0
No. Of studies	0	1	0	0	0	0
No. Of patients	0	160	0	0	0	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Pre-or intraoperative risk factor not specified

Aphakic vs. phakic: Status pre- or intraoperative not defined	Increased risk for OHT		Direction of the relation is unknown		Decreased risk for OHT	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=2)	61, 1					
No. Of clusters	2	0	0	0	0	0
No. Of studies	2	0	0	0	0	0
No. Of patients	1842	0	0	0	0	0
Multivariate analysis (n=1)	61					
No. Of clusters	1	0	0	0	0	0
No. Of studies	1	0	0	0	0	0
No. Of patients	1657	0	0	0	0	0

IOL in anterior chamber vs. Phakic: Status pre- or intraoperative not defined	Increased risk for OHT		Direction of the relation is unknown		Decreased risk for OHT	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)	61					
No. Of clusters	1	0	0	0	0	0
No. Of studies	1	0	0	0	0	0
No. Of patients	1657	0	0	0	0	0
Multivariate analysis (n=1)	61					
No. Of clusters	1	0	0	0	0	0
No. Of studies	1	0	0	0	0	0
No. Of patients	1657	0	0	0	0	0

IOL in posterior chamber vs. Phakic: Status pre- or intraoperative not defined	Increased risk for OHT		Direction of the relation is unknown		Decreased risk for OHT	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)	61					
No. Of clusters	1	0	0	0	0	0
No. Of studies	1	0	0	0	0	0
No. Of patients	1657	0	0	0	0	0
Multivariate analysis (n=1)	61					
No. Of clusters	1	0	0	0	0	0
No. Of studies	1	0	0	0	0	0
No. Of patients	1657	0	0	0	0	0

Pseudophakic vs. phakic: Status pre- or intraoperative not defined	Increased risk for OHT		Direction of the relation is unknown		Decreased risk for OHT	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=1)	1					
No. Of clusters	1	0	0	0	0	0
No. Of studies	1	0	0	0	0	0
No. Of patients	185	0	0	0	0	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

Aphakic or pseudophakic vs. phakic: Status pre- or intraoperative not defined	Increased risk for OHT		Direction of the relation is unknown		Decreased risk for OHT	
	Significant	Non-significant	Significant	Non-significant	Non-significant	Significant
Univariate analysis (n=2)	12	34				
No. Of clusters	1	1	0	0	0	0
No. Of studies	1	1	0	0	0	0
No. Of patients	678	80	0	0	0	0
Multivariate analysis (n=0)						
No. Of clusters	0	0	0	0	0	0
No. Of studies	0	0	0	0	0	0
No. Of patients	0	0	0	0	0	0

References of supplemental material 4

1. Sekhar GC, Vyas P, Nagarajan R, et al. Post-penetrating keratoplasty glaucoma. Indian J Ophthalmol 1993;41(4):181-4.
2. Sihota R, Sharma N, Panda A, et al. Post-penetrating keratoplasty glaucoma: risk factors, management and visual outcome. Aust N Z J Ophthalmol 1998;26(4):305-9.
3. Sharma N, Jain M, Sehra SV, et al. Outcomes of therapeutic penetrating keratoplasty from a tertiary eye care centre in northern India. Cornea 2014;33(2):114-8.
4. Srujana D, Kaur M, Urkude J, et al. Long-term Functional and Anatomic Outcomes of Repeat Graft After Optically Failed Therapeutic Keratoplasty. Am J Ophthalmol 2018;189:166-75.
5. Borderie VM, Touzeau O, Bourcier T, et al. The triple procedure: in the bag placement versus ciliary sulcus placement of the intraocular lens. Br J Ophthalmol 1999;83(4):458-62.
6. Redbrake C, Arend O. Glaucoma following keratoplasty. [German]. Ophthalmologe 2000;97(8):552-6.
7. Franca ET, Arcieri ES, Arcieri RS, Rocha FJ. A study of glaucoma after penetrating keratoplasty. Cornea 2002;21(3):284-8.
8. Seitz B, Langenbucher A, Nguyen NX, et al. Long-term follow-up of intraocular pressure after penetrating keratoplasty for keratoconus and Fuchs' dystrophy: Comparison of mechanical and excimer laser trephination. Cornea 2002;21(4):368-73.
9. Shimazaki J, Shinozaki N, Shimmura S, et al. Efficacy and safety of international donor sharing: a single-center, case-controlled study on corneal transplantation. Transplantation 2004;78(2):216-20.
10. Shimazaki J, Shimmura S, Ishioka M, Tsubota K. Randomized clinical trial of deep lamellar keratoplasty vs penetrating keratoplasty. Am J Ophthalmol 2002;134(2):159-65.
11. Egrilmez S, Sahin S, Yagci A. The effect of vernal keratoconjunctivitis on clinical outcomes of penetrating keratoplasty for keratoconus. Can J Ophthalmol 2004;39(7):772-7.
12. Al-Mohaimeed M, Al-Shahwan S, Al-Torbak A, Wagoner MD. Escalation of glaucoma therapy after penetrating keratoplasty. Ophthalmology 2007;114(12):2281-6.
13. Wagoner MD, Ba-Abbad R. Penetrating keratoplasty for keratoconus with or without vernal keratoconjunctivitis. Cornea 2009;28(1):14-8.
14. Wagoner MD, Ba-Abbad R, Al-Mohaimeed M, et al. Postoperative complications after primary adult optical penetrating keratoplasty: prevalence and impact on graft survival. Cornea 2009;28(4):385-94.
15. Khairallah A. Descemet stripping automated endothelialkeratoplasty (DSAEK) versus repeat penetrating keratoplasty (PKP) to manage eyes with failed corneal graft. Ann Saudi Med 2018;38(1):36-41.
16. Kaiserman I, Bahar I, Rootman DS. Corneal wound malapposition after penetrating keratoplasty: an optical coherence tomography study. Br J Ophthalmol 2008;92(8):1103-7.
17. Bahar I, Kaiserman I, McAllum P, et al. Comparison of posterior lamellar keratoplasty techniques to penetrating keratoplasty. Ophthalmology 2008;115(9):1525-33.
18. Bahar I, Kaiserman I, Srinivasan S, et al. Manual top hat wound configuration for penetrating keratoplasty. Cornea 2008;27(5):521-6.

19. Kaiserman I, Bahar I, McAllum P, et al. Suture-assisted vs forceps-assisted insertion of the donor lenticula during Descemet stripping automated endothelial keratoplasty. Am J Ophthalmol 2008;145(6):986-90.
20. Bahar I, Kaiserman I, Sansanayudh W, et al. Busin Guide vs Forceps for the Insertion of the Donor Lenticule in Descemet Stripping Automated Endothelial Keratoplasty. Am J Ophthalmol 2009;147(2):220-6 e1.
21. Kaiserman I, Bahar I, Slomovic AR, Rootman DS. Half top hat wound configuration for penetrating keratoplasty: 1-year results. Br J Ophthalmol 2009;93(12):1629-33.
22. Mashor RS, Rootman DB, Bahar I, et al. Outcomes of deep anterior lamellar keratoplasty versus intralase enabled penetrating keratoplasty in keratoconus. Canadian Journal of Ophthalmology 2011;46(5):403-7.
23. Shehadeh Mashor R, Bahar I, Rootman DB, et al. Zig Zag versus Top Hat configuration in IntraLase-enabled penetrating keratoplasty. Br J Ophthalmol 2014;98(6):756-9.
24. Erdurmus M, Cohen EJ, Yildiz EH, et al. Steroid-induced intraocular pressure elevation or glaucoma after penetrating keratoplasty in patients with keratoconus or Fuchs dystrophy. Cornea 2009;28(7):759-64.
25. Fan JC, Chow K, Patel DV, McGhee CN. Corticosteroid-induced intraocular pressure elevation in keratoconus is common following uncomplicated penetrating keratoplasty. Eye (Lond) 2009;23(11):2056-62.
26. Rahman I, Carley F, Hillarby C, et al. Penetrating keratoplasty: indications, outcomes, and complications. Eye (Lond) 2009;23(6):1288-94.
27. Vajaranant TS, Price MO, Price FW, et al. Visual acuity and intraocular pressure after Descemet's stripping endothelial keratoplasty in eyes with and without preexisting glaucoma. Ophthalmology 2009;116(9):1644-50.
28. Price MO, Bidros M, Gorovoy M, et al. Effect of incision width on graft survival and endothelial cell loss after Descemet stripping automated endothelial keratoplasty. Cornea 2010;29(5):523-7.
29. Chaurasia S, Price Jr FW, Gunderson L, Price MO. Descemet's membrane endothelial keratoplasty: Clinical results of single versus triple procedures (combined with cataract surgery). Ophthalmology 2014;121(2):454-8.
30. Price MO, Price FW, Jr., Kruse FE, et al. Randomized comparison of topical prednisolone acetate 1% versus fluorometholone 0.1% in the first year after descemet membrane endothelial keratoplasty. Cornea 2014;33(9):880-6.
31. Price MO, Feng MT, Scanameo A, Price FW, Jr. Loteprednol Etabonate 0.5% Gel Vs. Prednisolone Acetate 1% Solution After Descemet Membrane Endothelial Keratoplasty: Prospective Randomized Trial. Cornea 2015;34(8):853-8.
32. Allen MB, Lieu P, Mootha VV, et al. Risk factors for intraocular pressure elevation after descemet stripping automated endothelial keratoplasty. Eye Contact Lens 2010;36(4):223-7.
33. Karadag O, Kugu S, Erdogan G, et al. Incidence of and risk factors for increased intraocular pressure after penetrating keratoplasty. Cornea 2010;29(3):278-82.
34. Wandling GR, Jr., Parikh M, Robinson C, et al. Escalation of glaucoma therapy after deep lamellar endothelial keratoplasty. Cornea 2010;29(9):991-5.

35. Yildirim N, Gursoy H, Sahin A, et al. Glaucoma after penetrating keratoplasty: incidence, risk factors, and management. J Ophthalmol 2011;2011:951294.
36. Gaster RN, Dumitrascu O, Rabinowitz YS. Penetrating keratoplasty using femtosecond laser-enabled keratoplasty with zig-zag incisions versus a mechanical trephine in patients with keratoconus. British Journal of Ophthalmology 2012;96(9):1195-9.
37. Li C, Zhao GQ, Che CY, et al. Effect of corneal graft diameter on therapeutic penetrating keratoplasty for fungal keratitis. Int J Ophthalmol 2012;5(6):698-703.
38. Musa FU, Patil S, Rafiq O, et al. Long-term risk of intraocular pressure elevation and glaucoma escalation after deep anterior lamellar keratoplasty. Clin Exp Ophthalmol 2012;40(8):780-5.
39. Ozeki N, Yuki K, Shiba D, et al. Intraocular pressure elevation after Descemet's stripping endothelial keratoplasty. Jpn J Ophthalmol 2012;56(4):307-11.
40. Huang OS, Mehta JS, Htoon HM, et al. Incidence and Risk Factors of Elevated Intraocular Pressure Following Deep Anterior Lamellar Keratoplasty. Am J Ophthalmol 2016;170:153-60.
41. Huang OS, Htoon HM, Chan AM, et al. Incidence and Outcomes of Intraoperative Descemet Membrane Perforations During Deep Anterior Lamellar Keratoplasty. American Journal of Ophthalmology 2019;199:9-18.
42. Chan EW, Wong TT, Htoon HM, et al. De novo ocular hypertension after Descemet stripping endothelial keratoplasty: comparative 3-year incidence, risk factors, and outcomes. Clin Ophthalmol 2013;7:1829-41.
43. Ang M, Ho H, Wong C, et al. Endothelial keratoplasty after failed penetrating keratoplasty: an alternative to repeat penetrating keratoplasty. Am J Ophthalmol 2014;158(6):1221-7.e1.
44. Ang M, Mehta JS, Anshu A, et al. Endothelial cell counts after Descemet's stripping automated endothelial keratoplasty versus penetrating keratoplasty in Asian eyes. Clinical Ophthalmology 2012;6(1):537-44.
45. Ang M, Mehta JS, Sng CCA, et al. Indications, outcomes, and risk factors for failure in tectonic keratoplasty. Ophthalmology 2012;119(7):1311-9.
46. Ang M, Li L, Chua D, et al. Descemet's stripping automated endothelial keratoplasty with anterior chamber intraocular lenses: Complications and 3-year outcomes. British Journal of Ophthalmology 2014;98(8):1028-32.
47. Soh Y, Htoon HM, Mehta JS, Tan D. Five-Year Graft Survival Comparing Descemet Stripping Automated Endothelial Keratoplasty and Penetrating Keratoplasty. Ophthalmology 2016;123(8):1646-52.
48. Huber KK, Maier AK, Klamann MK, et al. Glaucoma in penetrating keratoplasty: risk factors, management and outcome. Graefes Arch Clin Exp Ophthalmol 2013;251(1):105-16.
49. Moisseiev E, Varssano D, Rosenfeld E, Rachmiel R. Intraocular pressure after penetrating keratoplasty and Descemet's stripping automated endothelial keratoplasty. Can J Ophthalmol 2013;48(3):179-85.
50. Nguyen P, Khashabi S, Chopra V, et al. Descemet stripping with automated endothelial keratoplasty: A comparative study of outcome in patients with preexisting glaucoma. Saudi J Ophthalmol 2013;27(2):73-8.
51. Zhang YM, Wu SQ, Yao YF. Long-term comparison of full-bed deep anterior lamellar keratoplasty and penetrating keratoplasty in treating keratoconus. J Zhejiang Univ Sci B 2013;14(5):438-50.

52. Maier AK, Klamann MK, Torun N, et al. Intraocular pressure elevation and post-DSEK glaucoma after Descemet`s stripping endothelial keratoplasty. Graefes Arch Clin Exp Ophthalmol 2013;251(4):1191-8.
53. Maier AK, Wolf T, Gundlach E, et al. Intraocular pressure elevation and post-DMEK glaucoma following Descemet membrane endothelial keratoplasty. Graefes Arch Clin Exp Ophthalmol 2014;252(12):1947-54.
54. Maier AK, Gundlach E, Gonnermann J, et al. Anterior segment analysis and intraocular pressure elevation after penetrating keratoplasty and posterior lamellar endothelial keratoplasty. Ophthalmic Res 2015;53(1):36-47.
55. Acar BT, Muftuoglu O, Acar S. Comparison of sulfur hexafluoride and air for donor attachment in Descemet stripping endothelial keratoplasty in patients with pseudophakic bullous keratopathy. Cornea 2014;33(3):219-22.
56. Blumenthal EZ, Frucht-Pery J, Solomon A. Risk factors and incidence of ocular hypertension after penetrating keratoplasty. Journal of Glaucoma 2014;23(9):599-605.
57. Sharma A, Sharma S, Pandav SS, Mohan K. Post penetrating keratoplasty glaucoma: cumulative effect of quantifiable risk factors. Indian J Ophthalmol 2014;62(5):590-5.
58. Chen Y, Liao C, Gao M, et al. Efficacy and Safety of Corneal Transplantation Using Corneas from Foreign Donors versus Domestic Donors: A Prospective, Randomized, Controlled Trial. J Ophthalmol 2015;2015:178289.
59. Muller L, Kaufmann C, Bachmann LM, et al. Changes in intraocular pressure after descemet stripping automated endothelial keratoplasty: a retrospective analysis. Cornea 2015;34(3):271-4.
60. Tannan A, Vo RC, Chen JL, et al. Comparison of ACIOL Retention With IOL Exchange in Patients Undergoing Descemet Stripping Automated Endothelial Keratoplasty. Cornea 2015;34(9):1030-4.
61. Borderie VM, Loriaut P, Bouheraoua N, Nordmann JP. Incidence of Intraocular Pressure Elevation and Glaucoma after Lamellar versus Full-Thickness Penetrating Keratoplasty. Ophthalmology 2016;123(7):1428-34.
62. Nakatani S, Murakami A. Descemet stripping automated endothelial keratoplasty using corneas from elderly donors. Graefe's Archive for Clinical and Experimental Ophthalmology 2016;254(6):1135-40.
63. Sandhu S, Petsoglou C, Grigg J, Veillard AS. Elevated Intraocular Pressure in Patients Undergoing Penetrating Keratoplasty and Descemet Stripping Endothelial Keratoplasty. J Glaucoma 2016;25(4):390-6.
64. Sharma RA, Bursztyn LL, Golesic E, et al. Comparison of intraocular pressure post penetrating keratoplasty vs Descemet's stripping endothelial keratoplasty. Can J Ophthalmol 2016;51(1):19-24.
65. Schaub F, Enders P, Zachewicz J, et al. Impact of Donor Age on Descemet Membrane Endothelial Keratoplasty Outcome: Evaluation of Donors Aged 17-55 Years. American Journal of Ophthalmology 2016;170:119-27.
66. Schaub F, Enders P, Snijders K, et al. One-year outcome after Descemet membrane endothelial keratoplasty (DMEK) comparing sulfur hexafluoride (SF6) 20% versus 100% air for anterior chamber tamponade. Br J Ophthalmol 2017;101(7):902-8.

67. Kaleem M, Ridha F, Shwani Z, et al. Rates of Intraocular Pressure Elevation and Use of Topical Antihypertensive Medication After Descemet Stripping Automated Endothelial Keratoplasty. Cornea 2017;36(6):669-74.
68. Khattak A, Nakhli FR, Al-Arfaj KM, Cheema AA. Comparison of outcomes and complications of deep anterior lamellar keratoplasty and penetrating keratoplasty performed in a large group of patients with keratoconus. Int Ophthalmol 2017.
69. Kocluk Y, Sukgen EA, Burcu A. Comparison of outcomes in patients who underwent deep anterior lamellar keratoplasty and those converted to penetrating keratoplasty. Turkish Journal of Ophthalmology 2017;47(2):63-7.
70. Phillips PM, Phillips LJ, Muthappan V, et al. Experienced DSAEK Surgeon's Transition to DMEK: Outcomes Comparing the Last 100 DSAEK Surgeries With the First 100 DMEK Surgeries Exclusively Using Previously Published Techniques. Cornea 2017;36(3):275-9.
71. Treder M, Alnawaiseh M, Eter N. Descemet membrane endothelial keratoplasty (DMEK) early stage graft failure in eyes with preexisting glaucoma. Graefes Arch Clin Exp Ophthalmol 2017;255(7):1417-21.
72. Yuksel B, Kandemir B, Uzunel UD, et al. Comparison of visual and topographic outcomes of deep-anterior lamellar keratoplasty and penetrating keratoplasty in keratoconus. Int J Ophthalmol 2017;10(3):385-90.
73. Singar-Ozdemir E, Burcu A, Oral B, et al. Effect of Donor Cornea on the Surgical Outcomes of Penetrating Keratoplasty: Imported Cornea Versus Domestic Cornea. Exp Clin Transplant 2018.
74. Zheng C, Yu F, Tseng VL, et al. Risk of Glaucoma Surgery after Corneal Transplant Surgery in Medicare Patients. Am J Ophthalmol 2018.
75. Raj A, Dhasmana R, Bahadur H. Incidence and risk factors for postkeratoplasty glaucoma in tertiary care center, India. Oman J Ophthalmol 2018;11(3):220-6.
76. Infantes Molina EJ, Celis Sanchez J, Tenias Burilllo JM, et al. Deep anterior lamellar keratoplasty versus penetrating keratoplasty in corneas showing a high or low graft rejection risk. European Journal of Ophthalmology 2018.

Supplemental material 5. *Quality items of the 76 included studies following the NICE checklist.*

Quality items from the NICE checklist	Yes	No	Unclear	Explanation for scoring studies
The study sample represents the population of interest with regard to key characteristics, sufficient to limit bias to the results.	74 (97.4%)	2 (2.6%)	0 (0%)	We rated studies that investigated patients that underwent or still had to undergo corneal transplantation surgery and of whom the baseline IOP or presence of glaucoma was established and or the mean IOP or the presence of glaucoma was compared between two or more potential risk factors. Baseline characteristics (i.e., age, sex and IOP/glaucoma) should be adequately described.
Loss to follow-up is unrelated to key characteristics (i.e., the study data adequately represent the sample), sufficient to limit potential bias.	12 (15.8%)	64 (84.2%)	0 (0%)	We rated cohort studies that either reported similar baseline characteristics in the group lost to follow-up and the analysed group, or had no loss to follow-up. By definition, case series and case-control studies were rated as "No".
The prognostic factor of interest is adequately measured in study participants, sufficient to limit bias.	76 (100%)	0 (0%)	0 (0%)	We rated studies that measured risk factors in patients in which an increase in IOP/high IOP after corneal surgery could occur or studies that investigated whether the presence of a studied risk factor influenced the IOP/presence of glaucoma.
The outcome of interest is adequately measured in study participants, sufficient to limit bias.	36(47.4%)	1 (1.3%)	39 (51.3%)	We rated the outcome as adequately measured if the same method of measurement for IOP was used in all groups/before and after surgery.
Important potential confounders are appropriately accounted for, limiting potential bias with respect to the prognostic factor of interest.	10 (13.2%)	66 (86.8%)	0 (0%)	We rated studies that performed a multivariable analysis.
The statistical analysis is appropriate for the design of the study, limiting potential for the presentation of invalid results	76 (100%)	0 (0%)	0 (0%)	We rated studies that adequately described the statistical analysis in a way that it is reproducible for others. All candidate variables considered for the analysis should be listed.

**NICE: National Institute for Healthy and Clinical Excellence*

Values are shown as n (%)

Supplemental material 6. *Overview of all the investigated risk factors for the development of ocular hypertension after keratoplasty, ranged in a clinically relevant order.*

Risk factor	Level of association*	Increased risk with	Number of study results		
			Univariate	Multivariate	Total
Preoperative					
Pre-existing glaucoma	4 (H)	Presence	17	7	24
Preoperative IOP	4	Higher preoperative IOP	4	3	7
Glaucoma in contralateral eye (no glaucoma in investigated eye)	3	Presence	1	1	2
History of pseudo-exfoliation syndrome	2	Presence	1	0	1
Preoperative treatment of glaucoma with medication	2	Presence	1	0	1
Preoperative treatment of glaucoma with medication and/or surgical	2	Presence	1	0	1
Preoperative treatment of glaucoma surgical	1 (H)	-	3	1	4
Preoperative treatment with medication vs. surgical	1	-	1	0	1
Preoperative treatment with one vs. two or more medications	1	-	1	0	1
Cyclosporine use before transplantation	3	Presence	0	1	1
Olopatadine 0.1% use before transplantation	3	Presence	0	1	1
Age patient (old vs. young age)	2 (H)	Younger age	12	9	21
Gender (male vs. female)	2 (H)	Male gender	9	4	13
History of ocular surgery	2	Absence	1	1	2
Preoperative presence of peripheral anterior synechiae	2	Presence	1	1	2
Age donor (older age)	1 (H)	-	2	0	2
Diabetes mellitus	1	-	1	0	1
Hypertension	1	-	1	0	1
Family history of keratoconus	1	-	1	0	1
History of steroid use (systemic + topical)	1	-	1	0	1
Indication bullous keratoplasty (yes vs. no)	3	Presence	0	2	2
Microbial keratitis with vs. without corneal perforation	2	Presence	1	0	1
Inflammatory vs. non-inflammatory indication	2	Inflammation	1	0	1
Indication (general)	1	-	3	1	4
History of vernal keratoconjunctivitis	1	-	1	0	1
Non-optical vs. optical indication	1	-	0	1	1
Keratoconus + vernal keratoconjunctivitis vs. keratoconus only	1	-	1	0	1
Indication keratoconus					

Bullous keratoplasty vs. keratoconus	3	Bullous keratoplasty	11	0	11
Corneal dystrophy (general) vs. keratoconus	1	-	8	0	8
FED vs. keratoconus	1 (H)	-	3	0	3
Scar vs. keratoconus	1	-	6	0	6
Trauma vs. keratoconus	1	-	3	0	3
Herpetic keratitis vs. keratoconus	1	-	3	0	3
Infectious keratitis vs. keratoconus	1	-	8	0	8
Others (general) vs. keratoconus	1 (H)	-	5	0	5
Adherent leucoma vs. keratoconus	1	-	1	0	1
Descemetocele vs. keratoconus	1	-	1	0	1
Band keratopathy vs. keratoconus	1	-	1	0	1
Dysgenesis vs. keratoconus	1	-	1	0	1
Corneal edema vs. keratoconus	1	-	1	0	1
Preoperative: Aphakic vs. phakic	1 (H)	-	6	1	7
Preoperative: Pseudophakic vs. Phakic	1 (H)	-	6	1	7
Preoperative: Aphakic or pseudophakic vs. Phakic	1	-	1	0	1
Preoperative: Lens status in general	1	-	0	1	1
Preoperative: Placement of IOL: sulcus vs. bag	1	-	1	0	1
Preoperative: Placement of IOL: scleral fixated vs. Bag	1	-	1	0	1
Ethnicity: Caucasian vs. African-American descent	3	African-American descent	1	1	2
Ethnicity: Maori or Pacefic (new Zealand Europeans, Samoan, other Pacefic people) ethnicity vs. others (Indian, other European en Middle Eastern)	2	Others	1	0	1
Ethnicity: Non-Chinese vs. Chinese	1 (H)	-	1	1	2
Ethnicity: Region of the United States (East, West, Midwest, South)	1	-	1	0	1
Intraoperative					
Type of surgery: PKP vs. DS(A)EK	2 (H)	Higher in PKP	9	1	10
Type of surgery: DSEK	2	Presence	1	0	1
Type of surgery: Regraft (due to failed or rejected graft)	2 (H)	Presence	11	3	14
Type of surgery: Re-PKP vs. DSAEK after failed PKP	2	DSAEK	1	0	1
Type of surgery: PKP vs. DALK	1 (H)	-	7	1	8
Type of surgery: Re-PKP vs. EK after failed PKP	1	-	1	0	1
Type of surgery: ALK vs. DSAEK	1	-	1	1	2
Type of surgery: DALK converted to PKP vs. DALK	1	-	2	0	2

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Type of surgery (general)	1	-	2	0	2
Type of surgery: DMEK	1	-	1	0	1
Type of surgery: DALK	1	-	1	0	1
Combined surgery: Keratoplasty + IOL removal or exchange	4	Presence	5	2	7
Combined surgery (general)	3	Presence	7	3	10
Combined surgery: Keratoplasty + vitrectomy	2	Presence	3	1	4
Combined surgery: Keratoplasty + retaining anterior chamber IOL vs. Keratoplasty posterior chamber lens left in place	2	Presence	1	0	1
Combined surgery: Triple procedure (yes vs. no)	1 (H)	-	6	2	8
Combined surgery: Keratoplasty + ECCE	1 (H)	-	2	1	3
Combined surgery: Keratoplasty + anterior segment reconstruction	1	-	2	1	3
Combined surgery: Keratoplasty + secondary IOL	1	-	1	0	1
Combined surgery: Keratoplasty + cataract extraction with IOL in ciliary sulcus vs. IOL in bag	1	-	1	0	1
Half top-hat vs. regular PKP	2	Half top hat	1	0	1
Zig Zag vs. top-hat in PKP	1	-	1	0	1
Zig Zag with femtosecond vs. mechanical trephine in PKP	1	-	1	0	1
Manual top-hat vs. regular PKP	1	-	1	0	1
Manual half top-hat vs. top-hat PKP	1	-	1	0	1
Width of the incision (large vs. small) in DSAEK or DLEK	1	-	2	0	2
Trephination with excimer vs. motor	1	-	1	0	1
Busin Guide-assisted vs. forceps-assisted DSAEK	1	-	1	0	1
Stitch-assisted vs. forceps-assisted DSAEK	1	-	1	0	1
Interrupted vs. interrupted + single continuous	1	-	2	0	2
Foreign vs. domestic donor grafts	1 (H)	-	3	0	3
Laterality: Left vs. right	1	-	1	0	1
Graft diameter in PKP (large vs. small)	2 (H)	Larger diameter	6	2	8
Graft diameter in DSAEK (per mm increase)	1	-	1	0	1
Graft diameter in DALK (per mm increase)	1	-	0	1	1
Graft oversize in PKP (large vs. small)	1 (H)	-	3	2	5
Size of malapposition	1	-	0	1	1
ANWAR big bubble technique	1	-	0	1	1
Sulfar hexafluoride SF6 20% vs. 100% air (bubble technique)	1 (H)	-	2	0	2
Rebubbling	1	-	1	1	2

Intraoperative perforation of the Descemet membrane during DALK	1	-	1	1	2
Postoperative					
Type of steroid use: Prednisolone acetate 1% vs. dexamethasone 0.1%	3	Prednisolone	0	1	1
Type of steroid use: Prednisolone acetate 1% vs. loteprednol etabonate 0.5%	2	Prednisolone	1	0	1
Type of steroid use: Prednisolone acetate 1% vs. fluorometholone 0.1%	2	Prednisolone	1	0	1
Type of steroid use: Prednisolone acetate 0.12% vs. dexamethasone 0.1%	1	-	0	1	1
Duration of steroid use (longer use)	1	-	1	2	3
Ocular surgery after keratoplasty	1	-	0	1	1
Cataract surgery after keratoplasty	1	-	1	0	1
Postoperative procedures or complications	2	Presence	1	2	3
Postoperative corneal scar vs. corneal oedema	2	Corneal scar	2	0	2
Postoperative graft failure/rejection	1 (H)	-	1	1	2
Postoperative graft status: Clear graft vs. graft with BK	1	-	1	0	1
Postoperative presence of peripheral anterior synechiae	1	-	1	0	1
Graft clarity (high to low clarity)	1	-	1	0	1
Pre- and postoperative					
Pre- and postoperative: Presence of peripheral anterior synechiae	1	-	1	0	1
Pre- or intraoperative not defined					
Aphakic vs. phakic: Status pre- or intraoperative not defined	4	Aphakic	2	1	3
Pseudophakic with IOL in anterior chamber vs. Phakic: Status pre- or intraoperative not defined	4	Anterior chamber	1	1	2
Pseudophakic with IOL in posterior chamber vs. Phakic: Status pre- or intraoperative not defined	4	Posterior chamber	1	1	2
Pseudophakic vs. phakic: Status pre- or intraoperative not defined	2	Pseudophakic	1	0	1
Aphakic or pseudophakic vs. phakic: Status pre- or intraoperative not defined	2	Aphakic or pseudophakic	2	0	2

** Level of association: 4 = definitely associated, 3 = probably associated, 2 = possibly associated, 1 = not associated. (H) = heterogenity*

Abbreviations: (D)ALK = (deep) anterior lamellar keratoplasty, DLEK = deep lamellar endothelial keratoplasty, DMEK = descemet membrane endothelial keratoplasty, DS(A)EK = descemet stripping (automated) endothelial keratoplasty, ECCE = extracapsular cataract extraction, EK = endothelial keratoplasty, FED = Fuchs endothelial dystrophy, IOL = intraocular lens, IOP = intraocular pressure, PKP = penetrating keratoplasty

Supplemental material 7. *Overview of all the definitely, probably and possibly associated risk factors, ranked according to the level of association.*

Risk factor	Level of association*	Direction Increased risk with	Number of study results		
			Univariate	Multivariate	Total
Pre-existing glaucoma	4 (H)	Presence	17	7	24
Preoperative IOP	4	Higher preoperative IOP	4	3	7
Combined surgery: Keratoplasty + IOL removal or exchange	4	Presence	5	2	7
Aphakic vs. phakic: Status pre- or intraoperative not defined	4	Aphakic	2	1	3
Pseudophakic with IOL in anterior chamber vs. Phakic: Status pre- or intraoperative not defined	4	Anterior chamber	1	1	2
Pseudophakic with IOL in posterior chamber vs. Phakic: Status pre- or intraoperative not defined	4	Posterior chamber	1	1	2
bullous keratoplasty vs. keratoconus	3	bullous keratoplasty	11	0	11
Combined surgery (general)	3	Presence	7	3	10
Glaucoma in contralateral eye (no glaucoma in contralateral eye)	3	Presence	1	1	2
Indication bullous keratoplasty (yes vs. no)	3	Presence	0	2	2
Ethnicity: Caucasian vs. African-American descent	3	African-American descent	1	1	2
Cyclosporine use before transplantation	3	Presence	0	1	1
Olopatadine 0.1% use before transplantation	3	Presence	0	1	1
Type of steroid use: Prednisolone acetate 1% vs. dexamethasone 0.1%	3	Prednisolone	0	1	1
Age patient (old vs. young age)	2 (H)	Younger age	12	9	21
Type of surgery: Regraft (due to failed or rejected graft)	2 (H)	Presence	11	3	14
Gender (male vs. female)	2 (H)	Male gender	9	4	13
Type of surgery: PKP vs. DS(A)EK	2 (H)	Higher in PKP	9	1	10
Graft diameter in PKP (large vs. small)	2 (H)	Larger diameter	6	2	8
Combined surgery: Keratoplasty + vitrectomy	2	Presence	3	1	4
Postoperative procedures or complications	2	Presence	1	2	3
History of ocular surgery	2	Absence	1	1	2
Preoperative presence of peripheral anterior synechiae	2	Presence	1	1	2
Postoperative corneal scar vs. corneal oedema	2	Corneal scar	2	0	2
Aphakic or pseudophakic vs. phakic: Status pre- or intraoperative not defined	2	Aphakic or pseudophakic	2	0	2

History of pseudo-exfoliation syndrome	2	Presence	1	0	1
Preoperative treatment of glaucoma with medication	2	Presence	1	0	1
Preoperative treatment of glaucoma with medication and/or surgical	2	Presence	1	0	1
Microbial keratitis with vs. without corneal perforation	2	Presence	1	0	1
Inflammatory vs. non-inflammatory indication	2	Inflammation	1	0	1
Ethnicity: Maori or Pacefic (new Zealand Europeans, Samoan, other Pacefic people) ethnicity vs. others (Indian, other European en Middle Eastern)	2	Others	1	0	1
Type of surgery: DSEK	2	Presence	1	0	1
Type of surgery: Re-PKP vs. DSAEK after failed PKP	2	DSAEK	1	0	1
Combined surgery: Keratoplasty + retaining anterior chamber IOL vs. Keratoplasty posterior chamber lens left in place	2	Presence	1	0	1
Half top-hat vs. regular PKP	2	Half top hat	1	0	1
Type of steroid use: Prednisolone acetate 1% vs. loteprednol etabonate 0.5%	2	Prednisolone	1	0	1
Type of steroid use: Prednisolone acetate 1% vs. fluorometholone 0.1%	2	Prednisolone	1	0	1
Pseudophakic vs. phakic: Status pre- or intraoperative not defined	2	Pseudophakic	1	0	1

** level of association: 4 = definitely associated; 3 = probably associated; 2 = possibly associated; H= heterogeneity*

Abbreviations: DMEK = descemet membrane endothelial keratoplasty, DS(A)EK = descemet stripping (automated) endothelial keratoplasty, IOL = intraocular lens, IOP = intraocular pressure, PKP = penetrating keratoplasty

Supplemental material 8. *Overview of the risk factors not associated with ocular hypertension after keratoplasty.*

Risk factor	Level of association*	Number of study results		
		Univariate	Multivariate	Total
Infectious keratitis vs. keratoconus	1	8	0	8
Corneal dystrophy (general) vs. keratoconus	1	8	0	8
Type of surgery: PKP vs. DALK	1 (H)	7	1	8
Combined surgery: Triple procedure (yes vs. no)	1 (H)	6	2	8
Preoperative: Aphakic vs. phakic	1 (H)	6	1	7
Preoperative: Pseudophakic vs. Phakic	1 (H)	6	1	7
Scar vs. keratoconus	1	6	0	6
Others (general) vs. keratoconus	1 (H)	5	0	5
Graft oversize in PKP (large vs. small)	1 (H)	3	2	5
Indication (general)	1	3	1	4
Preoperative treatment of glaucoma surgical	1 (H)	3	1	4
Trauma vs. keratoconus	1	3	0	3
Herpetic keratitis vs. keratoconus	1	3	0	3
Combined surgery: Keratoplasty + anterior segment reconstruction	1	2	1	3
Duration of steroid use (longer use)	1	1	2	3
FED vs. keratoconus	1 (H)	3	0	3
Combined surgery: Keratoplasty + ECCE	1 (H)	2	1	3
Foreign vs. domestic donor grafts	1 (H)	3	0	3
Type of surgery: ALK vs. DSAEK	1	1	1	2
Type of surgery: DALK converted to PKP	1	2	0	2
Type of surgery (general)	1	2	0	2
Width of the incision (large vs. small) in DSAEK or DLEK	1	2	0	2
Interrupted vs. interrupted + single continuous	1	2	0	2
Rebubbling	1	1	1	2
Intraoperative perforation of the Descemet membrane during DALK	1	1	1	2
Age donor (older age)	1 (H)	2	0	2
Ethnicity: Non-Chinese vs. Chinese	1 (H)	1	1	2
Sulfar hexafluoride SF6 20% vs. 100% air (bubble technique)	1 (H)	2	0	2
Postoperative graft failure/rejection	1 (H)	1	1	2
Preoperative treatment with medication vs. surgical	1	1	0	1
Preoperative treatment with one vs. two or more medications	1	1	0	1
Diabetes mellitus	1	1	0	1
Hypertension	1	1	0	1

Family history of keratoconus	1	1	0	1
History of steroid use (systemic + topical)	1	1	0	1
History of vernal keratoconjunctivitis	1	1	0	1
Non-optical vs. optical indication	1	0	1	1
Keratoconus + vernal keratoconjunctivitis vs. keratoconus only	1	1	0	1
Adherent leucoma vs. keratoconus	1	1	0	1
Descemetocele vs. keratoconus	1	1	0	1
Band keratopathy vs. keratoconus	1	1	0	1
Dysgenesis vs. keratoconus	1	1	0	1
Corneal edema vs. keratoconus	1	1	0	1
Preoperative: Aphakic or pseudophakic vs. Phakic	1	1	0	1
Preoperative: Lens status in general	1	0	1	1
Preoperative: Placement of IOL: sulcus vs. bag	1	1	0	1
Preoperative: Placement of IOL: scleral fixated vs. Bag	1	1	0	1
Ethnicity: Region of the United States (East, West, Midwest, South)	1	1	0	1
Type of surgery: Re-PKP vs. EK after failed PKP	1	1	0	1
Type of surgery: DMEK	1	1	0	1
Type of surgery: DALK	1	1	0	1
Combined surgery: Keratoplasty + secondary IOL	1	1	0	1
Combined surgery: Keratoplasty + cataract extraction with IOL in ciliary sulcus vs. IOL in bag	1	1	0	1
Zig Zag vs. top-hat in PKP	1	1	0	1
Zig Zag with femtosecond vs. mechanical trephine in PKP	1	1	0	1
Manual top-hat vs. regular PKP	1	1	0	1
Manual half top-hat vs. top-hat PKP	1	1	0	1
Trephination with excimer vs. motor	1	1	0	1
Busin Guide-assisted vs. forceps-assisted DSAEK	1	1	0	1
Stitch-assisted vs. forceps-assisted DSAEK	1	1	0	1
Laterality: Left vs. right	1	1	0	1
Graft diameter in DSAEK (per mm increase)	1	1	0	1
Graft diameter in DALK (per mm increase)	1	0	1	1
Size of malapposition	1	0	1	1
ANWAR big bubble technique	1	0	1	1
Type of steroid use: Prednisolone acetate 0.12% vs. dexamethasone 0.1%	1	0	1	1
Ocular surgery after keratoplasty	1	0	1	1
Cataract surgery after keratoplasty	1	1	0	1
Postoperative graft status: Clear graft vs. graft with bullous keratoplasty	1	1	0	1

Postoperative presence of peripheral anterior synechiae	1	1	0	1
Graft clarity (high to low clarity)	1	1	0	1
Pre- and postoperative: Presence of peripheral anterior synechiae	1	1	0	1

** Level of association: 1 = not associated; H= heterogeneity*

Abbreviations: (D)ALK = (deep) anterior lamellar keratoplasty, DLEK = deep lamellar endothelial keratoplasty, DMEK = descemet membrane endothelial keratoplasty, DS(A)EK = descemet stripping (automated) endothelial keratoplasty, ECCE = extracapsular cataract extraction, EK = endothelial keratoplasty, FED = Fuchs endothelial dystrophy, IOL = intraocular lens, PKP = penetrating keratoplasty

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Chapter 7

A GENOME-WIDE ASSOCIATION STUDY FOR A CORTICOSTEROID-INDUCED INCREASE IN INTRAOCULAR PRESSURE AFTER USING TOPICAL CORTICOSTEROIDS

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Abstract

Purpose: An increased intraocular pressure is a common side effect of topical and systemic corticosteroids, also called a corticosteroid response, and may cause visual field loss and blindness. The treatment can't be based on the molecular pathogenesis since it is still largely unknown. To investigate genes potentially involved in the molecular pathogenesis, we performed a genome-wide association study (GWAS).

Methods: A GWAS was performed in 339 corneal transplant patients who had used long-term topical corticosteroids according to protocol. Corticosteroid responders were carefully defined to avoid misclassification (39.5%).

Results: Comparing responders vs. non-responders revealed 172 SNPs in 18 genes. These genes are involved in the expression of the glucocorticoid receptor, development or functioning of the trabecular meshwork and extracellular matrix, or refer to molecular processes like the cell cycle. We also found that small genetic variances in one of the identified genes (*UBL5*) might determine whether a patient develops a corticosteroid response. Multiple of the identified genes are targeted by rho-kinase inhibitors.

Conclusions: Genes that play a role in the extracellular matrix and the cell cycle of the trabecular meshwork, the expression of the glucocorticoid receptor, the development and functioning of the trabecular meshwork are involved in the pathogenesis of a corticosteroid response. Rho-kinase inhibitors address several of the identified genes and are therefore a rational choice as a treatment. In addition, the identified genes help to find biomarkers to predict patients at risk for a corticosteroid response and new candidate genes as targets for new treatments.

Introduction

Glucocorticoids are a class of corticosteroid hormones which are produced in the adrenal cortex and play a role in multiple physiological processes. They are known to have anti-inflammatory effects for which they have been pharmaceutically derived and are now widely used within multiple medical disciplines. However, as glucocorticoids initiate multiple signaling cascades which influence the expression of hundreds to thousands of genes, they also cause multiple side effects. Within ophthalmology, one of the most common side effects after the use of topical corticosteroids is the development of an increase in intraocular pressure (IOP), also known as a corticosteroid response. This occurs in approximately 18%-36% of the patients.[1] However, in patients with primary open angle glaucoma (POAG), this percentage can be as high as 92%.[2] A prolonged increase in IOP may cause damage to the optic nerve, leading to visual field loss and eventually blindness. The identification of patients at risk for a corticosteroid-induced increase in IOP can therefore be of clinical importance.

The pathogenesis of a corticosteroid-induced increase in IOP is largely unknown. Biochemical and in vitro studies have shown that molecular changes and differences in gene expression occur in the trabecular meshwork (TM).[3-11] In addition, a genetic component has also been suggested as first degree relatives of high corticosteroid responders had a greater likelihood of being high corticosteroid responders as well.[12, 13] Therefore, candidate target genes with a possible association such as *N363S*, *NR3C1*, *SFRS3*, *SFRS5*, *SFRS9*, *FKBP4* and *FKBP5* have been investigated, however a significant association was only found for three single nucleotide polymorphisms (SNPs): *N363S* (glucocorticoid receptor SNP), rs6559662 and rs1879370.[14-19] In contrast to candidate target studies, genome-wide association studies (GWAS) allow the investigation of millions of genetic variants. Jeong et al. performed the first GWAS for a corticosteroid-induced increase in IOP in patients treated with an intravitreal triamcinolone injection and identified two intergenic quantitative trait loci (QTL) that could affect the expression of *HCG22*.[20] However, a relatively small cohort of 64 subjects was investigated. One of their identified SNPs was replicated, using a cohort of 49 subjects.

In this study, we performed a GWAS in 339 participants of which 39.5% developed a corticosteroid response. As far as we know, this is the largest cohort investigating a corticosteroid-induced increase in IOP until now. In addition, the response was not only defined by a cut-off in IOP raise but this elevation also had to be clearly associated with the use of corticosteroids and could not be explained by other possible causes of IOP increase. All participants in our study were exposed to corticosteroids for a longer period of time in comparison with the study of Jeong et al. as they all underwent a keratoplasty after which corticosteroids were used for at least one year and in a protocolled fashion. Therefore, the chance of incorrectly classifying a responder as non-responder and vice versa is very low. In addition, we investigated whether there are differences in high responders (increase in IOP of 15 mmHg or more within 6 months) compared to low responders. Determination of highly likely associated genes that are involved in the pathogenesis of a corticosteroid-induced increase in IOP might help to find biomarkers for the identification of patients at risk and new candidate genes as targets for more efficient prevention or treatment of the IOP increase.

Methods

Study cohort

We performed a GWAS in a retrospective cohort study of patients using corticosteroid eye drops after undergoing a keratoplasty. All patients were included at the University Eye Clinic Maastricht, Maastricht University Medical Centre⁺ (MUMC⁺), the Netherlands. The DNA samples needed for GWAS-sampling were collected prospectively and the clinical data retrospectively. Before the start of the study, approval of the Institutional Review Board was obtained (Medical Ethical Committee of the Maastricht University Medical Centre, MUMC⁺ in the Netherlands). All participants had to sign an informed consent prior to participation and the study was performed in accordance with the tenets of the Declaration of Helsinki.

Patients were invited for participation between September 2017 and February 2019. They underwent a keratoplasty between January 2010 and December 2018 at the University Eye Clinic Maastricht and were eligible for inclusion if they had one of the following types of keratoplasty: penetrating keratoplasty (PKP), Descemet stripping automated endothelial keratoplasty (DSAEK), ultra-thin Descemet stripping automated endothelial keratoplasty (U-DSAEK), Descemet membrane endothelial keratoplasty (DMEK), deep anterior lamellar keratoplasty (DALK), anterior lamellar keratoplasty (ALK) or retransplantation of any of the before mentioned surgeries. Patients were not included if they were 18 years or younger, mentally incompetent, did not master the Dutch language or had a history of uveitis that could have influenced the IOP. They were approached at least three months after undergoing the keratoplasty.

Sample and data collection and handling

An anticoagulated venous blood sample was collected from each patient. The samples were immediately transferred to the BioBank Maastricht where buffy coat was extracted and saved by -20 degrees. Thereafter, DNA was isolated. Upon inclusion patients were asked the nature of their ethnicity and current medicinal use, both general as ophthalmological. The following parameters were collected by retrospective chart review for three pre-operative visits and all available post-operative visits with a minimum follow-up time of one year after the keratoplasty: IOP, measurement method of the IOP (Goldman applanation tonometry, pneumotonometry, rebound tonometry or unknown), dosage and type of corticosteroids used. If patients used IOP-lowering drugs, the type and dosage was also noted.

Surgical techniques and post-operative care

All patients were treated according to a standardized protocol. The first three months after a ***penetrating keratoplasty***, patients used dexamethasone six times a day. Starting from the fourth month postoperatively dexamethasone was used four times a day and from then on further reduced with one drop every month. Dexamethasone was continued once a day until one year postoperatively. Then, dexamethasone was stopped and fluorometholone was used once a day. Patients had to keep using it once a day in order to decrease the chance of transplant rejection. After a ***non-penetrating keratoplasty***, patients used dexamethasone six times a day during the first month, four times a day during the second month and thereafter, the frequency was reduced with one drop every month. Again, dexamethasone was continued once a day until one year postoperatively and then switched to fluorometholone once a day. Patients that underwent a DALK were allowed to quit the use of fluorometholone after removal of all corneal sutures. Patients that had a DMEK, DSAEK or U-DSAEK had to keep using fluorometholone once a day or once in two days in order to decrease the chance of transplant rejection. If patients developed (signs of) a rejection of their corneal transplant, they were treated with prednisolone eye drops eight times a day and prednisolone eye ointment for the night. In case of severe rejection, subconjunctival betamethasone was administered. In case of a corticosteroid response (see below for definition), the corticosteroids were decreased in administration frequency. If this was not possible, the used corticosteroid was changed to another agent that was less likely to cause a corticosteroid response. Most often, fluorometholone was used.[21, 22] In case this was not sufficient or if the corticosteroids could not be tapered, patients were treated with IOP lowering eye drops as well. In some cases, cyclosporines were started in order to replace the corticosteroid therapy.

Defining corticosteroid responders (phenotype definition)

A patient was defined to be a corticosteroid responder if all of the following criteria were met:

1. The IOP was at least 6 mmHg higher compared to the baseline IOP or compared to the other eye in which no corticosteroids were used. Baseline IOP was defined as the highest IOP out of three pre-operative IOP measurements.

2. There had to be a clear relation between the eye pressure and the use of corticosteroids, i.e. a decrease in IOP when the frequency of the corticosteroid administration was reduced or when it was switched to a corticosteroid which is known to be less potent to cause a corticosteroid response; and conversely a raise

in IOP after increasing the frequency of administration of the corticosteroid eye drops or the use of a corticosteroid with a higher potency to cause a corticosteroid response.

3. The change in IOP could not be explained by other obvious factors such as for example method of IOP-measurement, use of IOP-lowering drugs, corneal edema or a rebubbling.

4. Any IOP elevation within the first week after the surgery was considered to be due to surgery-related factors and was not defined as a corticosteroid response.

In case a patient had shown a corticosteroid response before undergoing a keratoplasty and this response fulfilled the three first criteria as described above, the patient was classified as a corticosteroid responder in this study as well. In order to define patients as a corticosteroid responder or as a non-responder, the above criteria were independently assessed by two ophthalmology residents (I.L., PhD candidate, and M.F.C.). In case of disagreement, consensus was reached by discussion. When no agreement could be obtained or in case of doubt, a third assessor, C.A.B.W., a glaucoma specialist, was asked to make a final decision concerning these patients. In case it was not clear whether the IOP elevation was due to a corticosteroid response or due to other factors, the participant was excluded for further analysis. Additionally, some patients underwent filtering surgery or placement of a glaucoma drainage device before the keratoplasty. As such types of glaucoma surgery might mask a corticosteroid response, these patients were excluded for further analysis. Patients who fulfilled the criteria of corticosteroid responder were subdivided in high responders and low responders. A high responder was defined as an increase in eye pressure of at least 15 mmHg within six months after keratoplasty. All other responders were defined as low responders (increase 6-14 mmHg). Statistical analyses to investigate differences between group characteristics (responders vs. non responders; high responder vs. non responder and high responder vs. low responder) were performed within SPSS Statistics 25 for Windows (SPSS, Inc.) by using χ^2 tests. A p-value < 0.05 was considered statistically significant.

Genotyping

All DNA samples were genotyped using the Illumina GSA beadchip GSA MD v2 (Illumina GSA Arrays “Infinium iSelect 24x1 HTS Custom Beadchip Kit”). This analysis was performed by the HUman GEnomics Facility (HUGE-F), Genetic Laboratory Rotterdam, The Netherlands, using protocols according to the manufacturer’s instructions. A standard quality control analysis of the individual samples and the single nucleotide

polymorphisms (SNPs) was performed. In the first step of the quality control the individuals and SNPs with a call rate < 97.5%, and SNPs with a deviation from the Hardy–Weinberg equilibrium ($P < 1 \times 10^{-4}$) were excluded. In addition, individuals were excluded in case of excess heterozygosity (defined as samples with a F-value smaller than the mean -(4*SD) = -0.0553) and homozygosity (defined as samples with a F-value bigger than the mean +(4*SD) = 0.06491), and discrepancy in recorded and genetically determined gender. During the final quality control, the exclusion was based on a low call-rate (<99%), a deviation from the Hardy–Weinberg equilibrium ($P < 1 \times 10^{-4}$) or in case of excess heterozygosity. We adopted a minor allele frequency (MAF) cutoff of ≥ 0.05 as an inclusion criterion for the SNPs. The MAF was calculated for each analysis separately using RvTests.[23]

Imputation

Imputation was performed using a two-step procedure using Minimac and MaCH for the phasing and imputation respectively.[24, 25] The Haplotype Reference Consortium (HRC) reference panel r1.1 was used as the reference panel.[26, 27] The quality of the imputation was assessed by means of MaCH r^2 in which a good quality was defined as MaCH $r^2 > 0.3$.

Statistical analysis

Statistical analysis of the imputed genetic data was performed with PLINK, an open-source and freely available whole genome association analysis toolset.[28] We used this toolset to determine a P-value of association for each SNP by performing Monte Carlo label-swapping adaptive permutation, with a minimum of 5 and a maximum of 1,000,000 permutations per SNP. This analysis was performed for four different groups: (i) all responders vs. non-responders, (ii) high responders vs. non-responders, (iii) high responders vs. low responders and (iv) low responders vs. non-responders (results only shown in supplemental material). In addition, X-chromosomal analyses for the male and female subcohorts were performed for each of the above mentioned groups.

Functional annotation of significantly associated SNPs

FUMA SNP2GENE function was used to annotate the identified lead SNPs with their biological functionality and to map them to genes based on their positional and *expression quantitative trait loci (*eQTL) information.29 The lead SNPs were defined as SNPs with a P-value ≤ 1×10^{-5}. The eQTL catalogue as provided by FUMA (GTEx v8) was used to perform the eQTL mapping. For the eQTL, a cut-off of $r^{\wedge 2}=0.6$ was maintained. Only significant SNP-gene pairs were selected (False discovery rate (FDR) ≤ 0.05). The maximal distance was defined as 10 kb. The reference population used to define the linkage disequilibrium (LD) was the 1000G Phase 3 European database. Ensembl version 85 was used to annotate the genes.

Results

Study population

In total 367 patients were enrolled in the study. Based on the quality control of the DNA samples (see further), four samples were excluded. In addition, four participants did not complete the year of follow-up after the keratoplasty, for six participants it was not possible to define whether an increase in IOP was due to a corticosteroid response or other factors and fourteen patients underwent glaucoma surgery before having a keratoplasty. Therefore, a total of 339 participants were included in the GWAS analysis. In this study cohort 39.5% were corticosteroid responders and 60.5% non-responders. Of the included patients 97.3% was Caucasian. The other 2.7% were from American (0.9%), African (0.6%) and Asian (1.2%) descent. The most common indication to perform a keratoplasty was Fuchs endothelial dystrophy (56.05%). U-DSAEK (27.4%) and DSAEK (n=24.5%) were the most commonly performed procedures. In 78.5% of the patients the keratoplasty was not combined with any other type of surgery. In 19.2% it was combined with a phacoemulsification with implantation of an IOL (triple procedure). The baseline characteristics for responders and non-responders are described in table 1. Only age and gender were significantly different between responders and non-responders.

Additionally, the mean time to develop a corticosteroid response was 166 days (median 114.5; 1st quartile 78.3 and 3th quartile 201.3; SD 151.1). The cumulative number of patients that developed a corticosteroid response over time is shown in figure 1. 50.8% developed the response within 4 months after keratoplasty, 71.5% within 6 months and 95.4% showed the response before or at their one-year postoperative control. The mean IOP at the moment of response was 26.2 (median 25, minimum 17 mmHg, maximum 45 mmHg; SD 5.6 mmHg) and the mean highest IOP reached during the response was 29.5 mmHg (median 28, minimum 18 mmHg, maximum 67 mmHg; SD 7.6 mmHg). Topical dexamethasone sodium phosphate 0 .1% was used by 93.1% of the patients at the moment the response was detected. At the moment the response was defined by us, 40.8% used the corticosteroid eye drops six times a day, 11.5% four times a day, 22.3% three times a day, 9.2% twice a day and 10% once a day.

Of the corticosteroid responders 27.6% were high responders and 72.4% were low responders. In the subgroup of the high responders, the mean time to develop a corticosteroid response was 122 days (median 111; 1st percentile 40 and 3th percentile 184; SD 82.5) compared to 183 days (median 118; 1st quartile 83 and 3th quartile 221; SD 168) in the low responder group (P = 0.037). The mean IOP at the moment of response was 30.7 (median 30; minimum 17 mmHg; maximum 45 mmHg; SD 7.1) in the group of high responders and 24.4 mmHg (median 24; minimum 17 mmHg; maximum 38 mmHg; SD

3.6) in the group of the low responders (P < 0.001). The mean highest IOP reached in the group with high responders was 36 mmHg (median 36; minimum 20 mmHg; maximum 67 mmHg; 8.4 mmHg) and 26.9 mmHg (median 26; minimum 18 mmHg; maximum 42 mmHg; SD 5.5) in the group of low responders (P < 0.001).

Baseline characteristics for high responders vs. non responders are shown in table 2. Age, gender and ethnicity were significantly different between both groups. Baseline characteristics for high responders vs. low responders are shown in table 3.

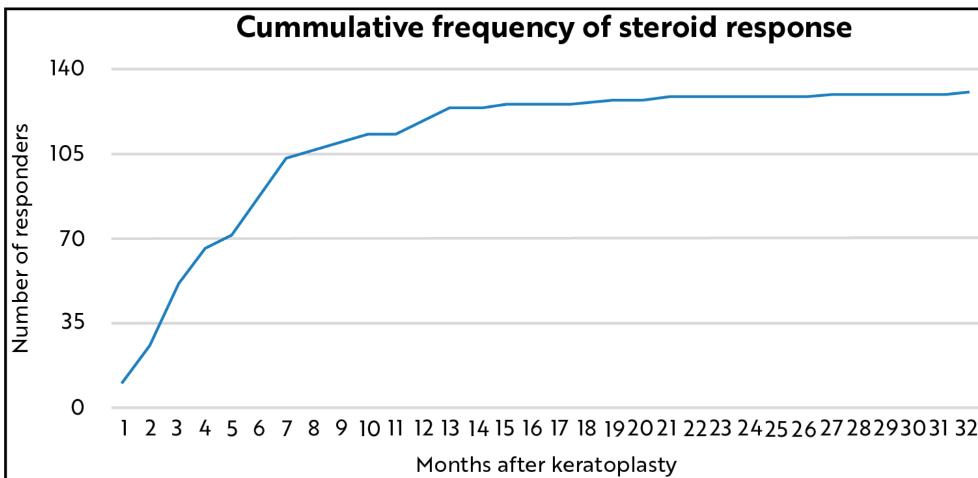

Figure 1. *Cumulative frequency of corticosteroid response over time (months after the keratoplasty; all responders included in figure).*

Table 1. *Baseline characteristics comparing responders and non-responders.*

	Responder (n=134)	Non-responder (n=205)	P-value (chi-square test)
Age at time of surgery (years), mean (SD)	62.2 (15.2)	66 (14.6)	0.02*
Male sex, n (%)	70 (52.2%)	82 (40%)	0.027*
Caucasian ethnicity, n (%)†	129 (96.3%)	201 (98%)	0.877
Operated eye OD, n (%)	76 (56.7%)	107 (52.2%)	0.414
Had rebubbling, n (%)	9 (6.7%)	26 (12.7%)	0.078
Surgery after which the steroid response occurred			0.927
First	125 (93.3%)	193 (94.1%)	
Second	8 (6.0%)	11 (5.4%)	
Third	1 (0.7%)	1 (0.5%)	
Lens status before surgery			0.155
Phakic	65 (48.5%)	79 (38.5%)	
Pseudophakic	65 (48.5%)	122 (59.5%)	
Aphakic	3 (2.2%)	4 (2.0%)	
Anterior chamber angle supported IOL	1 (0.7%)	0	
Type of surgery			
Primary graft vs regrafting			0.075
Primary graft	109 (81.3%)	181 (88.3%)	
Regraft	25 (18.7%)	24 (11.7%)	
Type of primary graft			0.082
DSAEK	36 (29.8%)	47 (24.4%)	
U-DSAEK	30 (24.8%)	63 (32.6%)	
DMEK	23 (19%)	53 (27.5%)	
PKP	19 (15.7)	17 (8.8%)	
DALK	12 (9.9%)	12 (6.2%)	
ALK	1 (0.8%)	0	
PKP à chaud	0	1 (0.5%)	
Combined surgery			0.807
No	103 (76.9%)	163 (79.5%)	
Combined with phaco	28 (20.9%)	37 (18%)	
Combined with others †	3 (2.2%)	5 (2.4%)	
Indication for surgery			0.088
Fuchs endothelial dystrophy	63 (47.0%)	127 (62.0%)	
Keratitis	15 (11.2%)	18 (8.8%)	
Graft failure	14 (10.4%)	21 (10.2%)	
Keratoconus	14 (10.4%)	8 (3.9%)	
Corneal decompensation	14 (10.4%)	18 (8.8%)	
Other dystrophies besides Fuchs	3 (2.2%)	3 (1.5%)	
Others †	11 (8.2%)	10 (4.9%)	

** P-value < 0.05*

† Included non-Caucasian ethnicities: Asian (n=4), African (n=2), Native American (n=3)

Combined surgery, others: Extracapsular cataract extraction with implantation of an intraocular lens (IOL) (n=2), explantation intra-corneal ring segments (n=1), explantation artisan and implantation of an IOL (n=1), injection (n=1), phaco combined with the removal of a corpus alienum (n=1), reconstruction corneal wound (n=1), removal of cortex rests (n=1)

Indication of surgery, others: complicated DMEK (n=1), cornea scrophulosa (n=2), corneal decompensation with Fuchs heterochrome iridocyclitis (n=1), corneal perforation after trauma (n=1), corneal scar (n=1), corneal ulcer (n=1), decompensation after multiple surgical procedures (n=8), descemetolyse after phaco (n=1), Fuchs and viral keratitis (n=1), Fuchs heterochrome iridocyclitis (n=1), iridocorneal endothelial (ICE) syndrome (n=1), keratoconus and viral keratitis (n=1), re-perforation of a PKP (n=1)

Table 2. *Baseline characteristics comparing high responders and non-responders.*

	High responder (n=37)	Non responder (n=205)	P-value (chi-square test)
Age at time of surgery (years), mean (SD)	58.2 (15.6)	66 (14.6)	0.003*
Male sex, n (%)	22 (59.5%)	82 (40.0%)	0.028*
Caucasian ethnicity, n (%)†	34 (91.9%)	201 (98.0%)	0.04*
Operated eye			0.925
OD, n (%)	19 (51.4%)	107 (52.2%)	
OS, n (%)	18 (48.6%)	98 (47.8%)	
Had rebubbling, n (%)	36 (97.3%)	179 (87.3%)	0.076
Surgery after which the steroid response occurred			0.391
First	34 (91.9%)	193 (94.1%)	
Second	2 (5.4%)	11 (5.4%)	
Third	1 (2.7%)	1 (0.5%)	
Lens status before surgery			0.645
Phakic	17 (45.9%)	79 (38.5%)	
Pseudophakic	19 (51.4%)	122 (59.5%)	
Aphakic	1 (2.7%)	4 (2.0%)	
Anterior chamber angle supported IOL	0 (0.0%)	0 (0.0%)	
Type of surgery			
Primary graft vs regrafting			0.756
Primary graft	32 (86.5%)	181 (88.3%)	
Regraft	5 (13.5%)	24 (11.7%)	
Type of primary graft			0.086
DSAEK	8 (22.9%)	47 (24.4%)	
U-DSAEK	7 (20.0%)	63 (32.6%)	
DMEK	9 (25.7%)	53 (27.5%)	
PKP	7 (20.0%)	17 (8.8%)	
DALK	3 (8.6%)	12 (6.2%)	
ALK	1 (2.9%)	0 (0.0%)	
PKP à chaud	0 (0.0%)	1 (0.5%)	
Combined surgery			0.962
No	30 (81.1%)	163 (79.5%)	
Combined with phaco	6 (16.2%)	37 (18.0%)	
Combined with others †	1 (2.7%)	5 (2.4%)	
Indication for surgery			0.370
Fuchs endothelial dystrophy	18 (48.6%)	127 (62.0%)	
Keratitis	5 (13.5%)	18 (8.8%)	
Graft failure	2 (5.4%)	21 (10.2%)	
Keratoconus	4 (10.8%)	8 (3.9%)	
Corneal decompensation	4 (10.8%)	18 (8.8%)	
Other dystrophies besides Fuchs	1 (2.7%)	3 (1.5%)	
Others †	3 (8.1%)	10 (4.9%)	

† Included non-Caucasian ethnicities: Asian (n=3), African (n=1), native American (n=3)

Combined surgery, others: Extracapsular cataract extraction with implantation of an intraocular lens (IOL) (n=2), explantation intra-corneal ring segments (n=1), injection (n=1), reconstruction corneal wound (n=1), removal of cortex rests (n=1)

Indication of surgery, others: complicated DMEK (n=1), corneal perforation after trauma (n=1), corneal scar (n=1), corneal ulcer (n=1), decompensation after multiple surgical procedures (n=6), Fuchs and viral keratitis (n=1), keratoconus and viral keratitis (n=1), re-perforation of a PKP (n=1)

Table 3. *Baseline characteristics comparing high responders and low responders.*

	High responder (n=37)	Low responder (n=97)	P-value (chi-square test)
Age at time of surgery (years), mean (SD)	58.2 (15.6)	63.7 (14.9)	0.06
Male sex, n (%)	22 (59.9%)	48 (49.5%)	0.301
Caucasian ethnicity, n (%)†	34 (91.9%)	95 (97.9%)	0.099
Operated eye			0.439
OD, n (%)	19 (51.4%)	57 (58.8%)	
OS, n (%)	18 (48.6%)	40 (41.2%)	
Had rebubbling, n (%)	36 (97.3%)	89 (91.8%)	0.252
Surgery after which the steroid response occurred			0.265
First	34 (91.9%)	91 (92.8%)	
Second	2 (5.4%)	6 (6.2%)	
Third	1 (2.7%)	0 (0%)	
Lens status before surgery			0.9
Phakic	17 (45.9%)	48 (49.5%)	
Pseudophakic	19 (51.4%)	46 (47.4%)	
Aphakic	1 (2.7%)	2 (2.1%)	
Anterior chamber angle supported IOL	0 (0%)	1 (1.0%)	
Type of surgery			
Primary graft vs regrafting			0.343
Primary graft	32 (86.5%)	77 (79.4%)	
Regraft	5 (13.5%)	20 (20.6%)	
Type of primary graft			
DSAEK	8 (22.9%)	28 (32.6%)	
U-DSAEK	7 (20%)	23 (26.7%)	
DMEK	9 (25.7%)	14 (16.3%)	
PKP	7 (20.0%)	12 (14.0%)	
DALK	3 (8.6%)	9 (10.5%)	
ALK	1 (2.9%)	0 (0%)	
PKP à chaud	0 (0%)	0 (0%)	
Combined surgery			0.704
No	30 (81.1%)	73 (75.3%)	
Combined with phaco	6 (16.2%)	22 (22.7%)	
Combined with others †	1 (2.7%)	2 (2.1%)	
Indication for surgery			0.954
Fuchs endothelial dystrophy	18 (48.6%)	45 (46.4%)	
Keratitis	5 (13.5%)	10 (10.3%)	
Graft failure	2 (5.4%)	12 (12.4%)	
Keratoconus	4 (10.8%)	10 (10.3%)	
Corneal decompensation	4 (10.8%)	10 (10.3%)	
Other dystrophies besides Fuchs	1 (2.7%)	2 (2.1)	
Others †	3 (8.1%)	8 (8.2%)	

† *Included non-Caucasian ethnicities: Asian (n=2), African (n=1), American (n=2)*

Combined surgery, others: explantation artisan and implantation of an IOL (n=1), injection (n=1), phaco combined with the removal of a corpus alienum (n=1), reconstruction corneal wound (n=1)

Indication of surgery, others: complicated DMEK (n=1), cornea scrophulosa (n=2), Fuchs heterochrome iridocyclitis (n=1), corneal scar (n=1), decompensation after multiple surgical procedures (n=3), descemetolyse after phaco (n=1), iridocorneal endothelial (ICE) syndrome (n=1), corneal decompensation with Fuchs heterochrome iridocyclitis (n=1)

Quality control of the individuals and the SNPs

Quality control was performed on the complete study population (n=367). After quality control, one sample was excluded based on a low call-rate (<97.5%) and three samples were excluded due to excess of heterozygosity. Therefore, a total of four participants were excluded for further analysis. None of the included individuals showed a mismatch in reported and genetically determined gender. A total of 14,660 SNPs were excluded due to a low call-rate (<97.5%) or because they showed a deviation from the Hardy–Weinberg equilibrium ($P < 1 \times 10^{-4}$). Therefore, a total of 726,702 SNPs have been included in the statistical GWAS analysis. The number of SNPs imputed was 39,131,556. The mean MaCH r-squared for the whole data was of 0.324728 (median: 0.421615, standard deviation: 0.01609). After imputation, the number of SNPs with $r^2 > 0.3$ and MAF > 1% is 7.772.212. After removal of variants with MAF < 1%, the MaCH r^2 is 0.900070649 (standard deviation 0.08276642).

Results of the GWAS study

Responders vs. non-responders

After comparing all responders vs. non-responders, we identified 172 unique SNPs associated with a corticosteroid-induced increase in IOP. A Manhattan plot of the results is shown in figure 2. Based on the positioning, eQTL and chromatin interaction information, these SNPs could be assigned to 18 different genes. An overview of the identified genes and their SNP with the lowest P-value can be found in table 4. On the X-chromosome, 4 additional SNPs, associated with one gene could be identified for the female subcohort and 2 additional SNPs, associated with one gene could be identified for the male subcohort (table 4). Supplemental material 1 provides the complete results.

High responders vs. non-responders

After comparing high responders vs. non-responders, we identified 253 unique SNPs which were associated to 13 unique genes based on their positioning, eQTL and chromatin interaction information. An overview of the identified genes and their SNP with the lowest P-value can be found in table 5. On the X-chromosome, 2 additional SNPs, associated with one gene could be identified for the female subcohort (table 5). Supplemental material 2 provides the complete results of this analysis. A Manhattan plot of the results is shown supplemental material 3.

High responders vs. low responders

After comparing high responders vs. low responders, we identified 433 unique SNPs which were associated to 19 unique genes based on their positioning, eQTL and chromatin interaction information. An overview of the identified genes and their SNP with the lowest P-value can be found in table 6. Supplemental material 4 provides the complete results of this analysis. A Manhattan plot of the results is shown supplemental material 5.

The complete results after comparing low responders and non-responders are provided in supplemental material 6. Supplemental material 7 provides the regional association plots for the four above mentioned analyses.

Comparing all the analyses

When comparing the results of all analyses, 8 genes and 47 SNPs overlap between the comparisons of responders vs. non-responders and low responders vs. non-responders. In addition, 3 genes and 55 SNPs were overlapping between high responders vs. low responders and high responders vs. non-responders. None of the other possible comparisons had overlapping genes or SNPs.

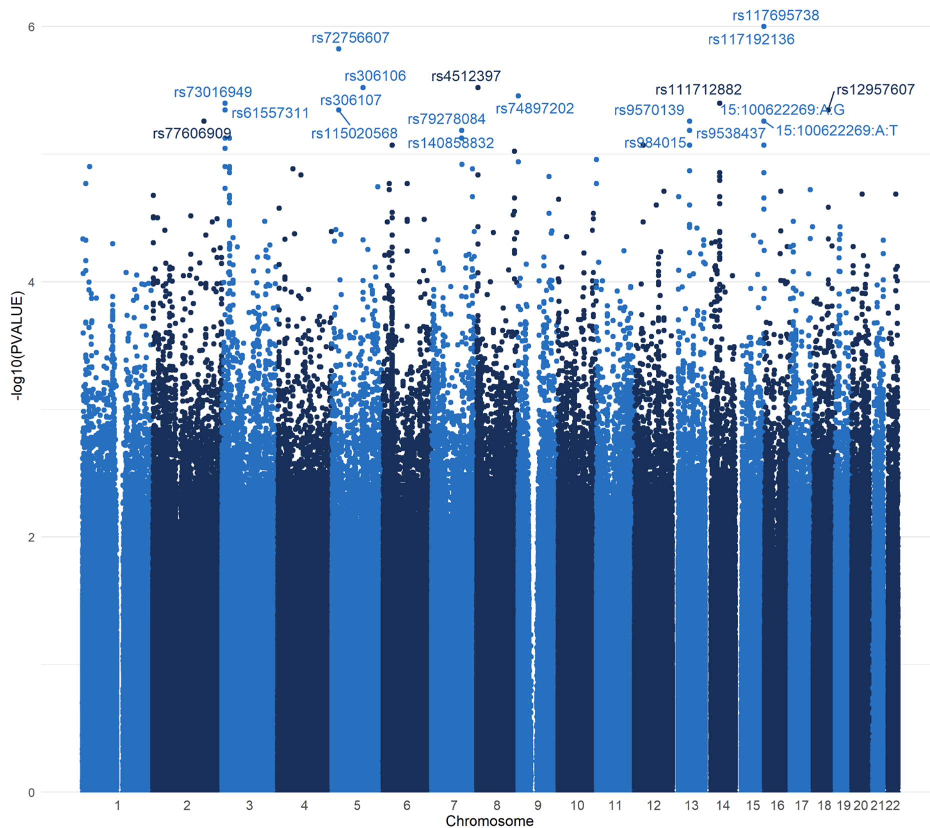

Figure 2. *Manhattan plot for the association of genome-wide SNPs with a corticosteroid-induced increase in IOP. The* $-\log_{10}$ *transformed P-value for all SNPs is shown.*

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Table 4. *Summary of the identified genes after comparing responders vs. non-responders.*

SNP	Unique ID	Gene symbol	Ensembl ID	Gene name	Gene type	MAF	P-value*
rs117192136	15:100618735:G:T	*ADAMTS17*	ENSG00000140470	ADAM metallopeptidase with thrombospondin type 1 motif 17	Protein coding	0.058997	1.00E-06
rs72756607	5:23480294:C:T	CTD-2351A8.1				0.081121	1.50E-06
rs306106	5:110620830:C:T	*CAMK4*:CTC-499J9.1				0.470501	3.00E-06
rs4512397	8:5776116:A:C	RN7SKP159	ENSG00000222589	RN7SK pseudogene 159	Misc RNA	0.20944	3.00E-06
rs73016949	3:11778555:C:G	AC090939.1				0.076696	4.00E-06
rs111712882	14:52292212:C:T	RP11-280K24.4				0.113569	4.00E-06
rs12957607	18:53621933:C:T	CTD-2008L17.1				0.467552	4.50E-06
rs115020568	5:23512325:C:T	PRDM9	ENSG00000164256	PR/SET domain 9	Protein coding	0.057522	4.50E-06
rs9570139	13:60062817:C:G	RNU7-88P	ENSG00000239003	RNA, U7 small nuclear 88 pseudogene	SnRNA	0.377581	5.50E-06
rs72902589	3:27754726:C:G	RP11-222K16.2				0.284661	7.50E-06
rs302327	12:29898518:C:T	TMTC1	ENSG00000133687	Transmembrane O-mannosyltransferase targeting cadherins 1	Protein coding	0.544248	8.50E-06
rs2597345	8:133473576:C:T	KCNQ3	ENSG00000184156	Potassium voltage-gated channel subfamily Q member 3	Protein coding	0.914454	9.50E-06
rs77768661	14:52293395:A:T	GNG2	ENSG00000186469	G protein subunit gamma 2	Protein coding	0.10177	1.60E-05
rs73061262	3:27743953:A:C	AC098614.3				0.222714	2.10E-05
rs9962249	18:53561170:A:G	RP11-214L13.1:CTD-2008L17.1				0.557522	2.60E-05
rs2581183	3:27759404:C:T	*EOMES*	ENSG00000163508	Eomesodermin	Protein coding	0.20649	5.00E-05
rs4637006	18:53516138:C:T	RP11-397A16.3				0.469027	0.000104
rs11402418	18:53533470:G:GA	RP11-214L13.1					
				X- Chromosome, female subcohort			
rs5935385	23:12707690:C:T	FRMPD4	ENSG00000169933	FERM and PDZ domain containing 4	Protein coding	0.194595	1.00E-06
				X- Chromosome, male subcohort			
rs6640597	23:10095261:C:G	WWC3	ENSG00000047644	WWC family member 3	Protein coding	0.112583	9.00E-06

** P-value of the genes assigned to the SNPs that were found to be significantly associated with a corticosteroid-induced increase in IOP. Some*

of the SNPs were selected based on eQTL and LD and therefore have no assigned P-value or a P-value > 1×10^{-5}

Table 5. *Summary of the identified genes after comparing high responder vs. non-responders.*

SNP	Unique ID	Gene symbol	Ensembl ID	Gene name	Gene type	MAF	P-value*
rs66617331	13:71220627:A:G	RNU6-54P	ENSG00000202433	RNA, U6 small nuclear 54, pseudogene	SnRNA	0.05992	2.00E-06
rs9619254	22:32627264:C:T	RP1-90G24.10:SLC5A4				0.18388	3.50E-06
rs4846403	1:217400809:C:T	*ESRRγ*	ENSG00000196482	Estrogen related receptor gamma	Protein coding	0.13636	4.00E-06
rs9372593	6:120623020:C:G	*RNU6-214P*	ENSG00000206857	RNA, U6 small nuclear 214, pseudogene	SnRNA	0.58678	6.00E-06
rs9397223	6:155430945:C:T	*TIAM2*	ENSG00000146426	*TIAM Rac1* associated GEF 2	Protein coding	0.20041	6.00E-06
rs10778335	12:104860627:A:G	CHST11	ENSG00000171310	Carbohydrate sulfotransferase 11	Protein coding	0.06818	7.00E-06
rs3813887	11:3663669:G:T	ART5	ENSG00000167311	ADP-ribosyltransferase 5	Protein coding	0.23554	7.50E-06
rs13222538	7:113606227:C:T	PPP1R3A	ENSG00000154415	Protein phosphatase 1 regulatory subunit 3A	Protein coding	0.1095	9.00E-06
rs4697551	4:25128560:A:G	SEPSECS	ENSG00000109618	Sep (O-phosphoserine) tRNA:Sec (selenocysteine) tRNA synthase	Protein coding	0.51446	9.00E-06
rs12168696	22:32611862:G:T	RP1-90G24.10				0.18595	1.15E-05
rs9374873	6:120579336:C:T	MIR3144	ENSG00000265725	MicroRNA 3144	miRNA	0.36777	4.35E-05
rs4846411	1:217461144:A:C	*GPATCH2*	ENSG00000092978	G-patch domain containing 2	Protein coding	0.13636	0.00022
rs11937742	4:25163672:A:G	PI4K2B	ENSG00000038210	Phosphatidylinositol 4-kinase type 2 beta	Protein coding	0.53512	0.00055
				X- Chromosome, female subcohort			
rs12839005	23:31251156:C:T	*DMD*	ENSG00000198947	Dystrophin	Protein coding	0.188406	0.0000095

** P-value of the genes assigned to the SNPs that were found to be significantly associated with a corticosteroid-induced increase in IOP. Some of the SNPs were selected based on eQTL and LD and therefore have no assigned P-value or a P-value > 1×10^{-5}*

Table 6. *Summary of the identified genes after comparing high responders vs. low responders.*

SNP	Unique ID	Gene symbol	Ensembl ID	Gene name	Gene type	MAF	P-Value*
rs11117811	1:217398583:A:G	*ESRRγ*	ENSG00000196482	Estrogen related receptor gamma	Protein coding	0.287313	1.00E-06
rs10953351	7:101437863:C:G	*CUX1*	ENSG00000257923	Cut like homeobox 1	Protein coding	0.391791	1.50E-06
rs4145890	6:120808245:A:C	*RNU6-214P*	ENSG00000206857	R, U6 small nuclear 214, pseudogene	SnRNA	0.470149	3.00E-06
rs35390466	19:9943017:G:T	CTD-2623N2.11				0.08209	4.00E-06
rs56281400	19:9977320:C:T	*OLFM2*	ENSG00000105088	Olfactomedin 2	Protein coding	0.08209	4.00E-06
rs11044252	12:18847238:C:T	PLCZ1	ENSG00000139151	Phospholipase C zeta 1	Protein coding	0.074627	4.00E-06
rs34575329	19:9942730:A:T	*UBL5*	ENSG00000198258	Ubiquitin like 5	Protein coding	0.08209	4.00E-06
rs12368494	12:18911845:C:G	CAPZA3	ENSG00000177938	Capping actin protein of muscle Z-line subunit alpha 3	Protein coding	0.100746	6.00E-06
rs13302651	9:29848380:C:T	RP11-460C6.1				0.089552	6.00E-06
rs58492625	21:20873271:A:G	RPL37P4	ENSG00000230198	Ribosomal protein L37 pseudogene 4	Processed pseudogene	0.11194	6.00E-06
rs35458555	3:192247942:A:G	FGF12	ENSG00000114279	Fibroblast growth factor 12	Protein coding	0.533582	7.00E-06
rs4343874	5:89731013:A:G	MBLAC2	ENSG00000176055	Metallo-beta-lactamase domain containing 2	Protein coding	0.425373	7.00E-06
rs2914674	5:89769194:G:T	MBLAC2:POLR3G				0.425373	7.00E-06
rs771135	1:34615027:A:T	CSMD2	ENSG00000121904	CUB and Sushi multiple domains 2	Protein coding	0.817164	8.00E-06
rs11117841	1:217456125:C:T	*GPATCH2*	ENSG00000092978	G-patch domain containing 2	Protein coding	0.130597	8.50E-06
rs4539131	1:115891156:A:G	RP4-663N10.1				0.794776	8.50E-06
rs1158316	6:120947919:C:G	R5SP215	ENSG00000200310	R, 5S ribosomal pseudogene 215	RNA pseudogene	0.425373	1.65E-05
rs73056413	12:18875354:G:T	PLCZ1:RP11-361I14.2				0.089552	3.50E-05
rs9387888	6:121055821:C:T	COX6A1P3	ENSG00000216710	Cytochrome c oxidase subunit 6A1 pseudogene 3	Processed pseudogene	0.41791	0.0001236

** P-value of the genes assigned to the SNPs that were found to be significantly associated with a corticosteroid-induced increase in IOP. Some of the SNPs were selected based on eQTL and LD and therefore have no assigned P-value or a P-value > 1×10^{-5}.*

Discussion

As far as we know, the current study is the largest cohort that investigated the pharmacogenomics of a corticosteroid-induced increase in IOP. Furthermore, previous studies often investigated targeted SNPs instead of a genome-wide analysis as was performed in this study.[14-19] Within the cohort 39.5% was corticosteroid responder. This is in accordance with previous studies on the occurrence of a corticosteroid response.[1, 2] After comparing all responders and non-responders, we identified 172 unique genome-wide significant SNPs, which could be assigned to 18 genes of which *EOMES*, *ADAMTS17* and *CaMK4* are of special interest and will be discussed below. In addition, Armaly and Becker already published in 1963 that the degree of IOP elevation after exposure to corticosteroids differs greatly and defined three distinct subgroups in patients with a corticosteroid response: patients exhibiting a high (>15 mmHg), moderate (6-15 mmHg) and low (<6 mmHg) response.[30, 17, 31] As in the current study, a small subgroup, defined as high responders, were phenotypically different from low responders, we investigated whether there are genetic differences between these groups as well by performing additional GWA analyses with these subgroups. After comparing high responders vs. non-responders and high responder vs. low responders, respectively *TIAM2*, *DMD* and *OLFM2*, *UBL5*, *CUX1* were identified and are of special interest for further discussion. *ESRRγ* and *GPATCH2* were identified after comparing high responders vs. non-responders and after comparing high responder vs. low responders and will be discussed as well.

To obtain a better understanding of the performed analyses, we compared the SNPs and genes that were identified in the different analyses. We found a considerable overlap in SNPs and genes between low responders vs. non-responders and responders vs. non-responders. This was to be expected as most of the responders were low responders (72.4%). On the other hand, only 3 genes (*ESRRγ*, *RNU6-214P* and *GPATCH2*) and 55 SNPs were overlapping between high responders vs. non-responders and high responders vs. low responders. In addition, none of the remaining comparisons had overlapping genes. As these findings supported our hypothesis that high responders and low responders are not only phenotypically but also genetically different, we also compared the P-values of all the significant SNPs throughout the four comparisons. To visualize these results, a heat map was created (see supplemental material 8). This showed that the SNPs in the comparisons responder vs. non-responder and low responder vs non-responder are overall similar. Again, this was to be expected as these groups are similar (most responders were low responder). In addition, the P-values of the SNPs comparing high responders vs. non-responders and high responders vs. low responders are overall different, i.e. SNPs with a low P-value in one analysis have high P-values in the other and vice versa. This supports our hypothesis that high responders and low responders are not only phenotypically but also genetically different.

TIAM2 and *CaMK4*, who were both identified in this study, suppress *RhoA* activity.[32-34] There is a close interaction between *Rac1*, *RhoA* and *ROCK*. Both *TIAM2* and *CaMK4* are known to target *Rac1*. *CaMK4*, also known as Calcium/Calmodulin Dependent Protein Kinase IV, has been shown to play an important role in the podocyte motility in the kidney. *CaMK4* achieves this by regulating the ratio of activated *Rac1* and *RhoA* GTPases.[34] Tang et al. previously published an insightful pathway of this feedback loop.[33]

TIAM2 or *TIAM Rac1* Associated *GEF2*, as identified in our study, and *TIAM1* activate *Rac1*. There is a close interaction between *Rac1* and *RhoA* as *Rac1* inhibits *RhoA* and *RhoA* in turn can inhibit *Rac1* by activating *ROCK*. *ROCK* has been shown to inhibit *TIAM* which leads to a decreased activity of *Rac1* and an increase in the activity of *RhoA*. Therefore, there seems to be a feedback loop for the regulation of *Rac1* and *RhoA*, through *TIAM1* and *TIAM2*. Fusadil, a *ROCK*-inhibitor has been shown to increase *TIAM1* which in turn activates *Rac1* which leads to a diminished activity of *RhoA*.[33] Interestingly, rho-kinase inhibitors such as netarsudil and ripasudil are currently already used in some countries to treat glaucoma. Not only do these inhibitors influence the outflow of aqueous humor through the TM (see below), they are also believed to have neuroprotective effects on the optic nerve and retina which might inhibit the progression to glaucomatous neuropathy.[35-38] A recently published study found that ripasudil significantly lowered the IOP in patients with a corticosteroid-induced increase in IOP.[39] In addition, a randomized, double-masked trial, investigated whether the use of netarsudil compared to a placebo after corneal transplantation lowered the risk to develop a corticosteroid-induced increase in IOP. They showed that 14% of patients using netarsudil had an IOP-increase defined as an increase of ≥ 10 mmHg from baseline or an achieved IOP of ≥ 24 mmHg compared to 21% after treatment with placebo.[40] Although this difference was not statistically significant, the trend showed a lower risk for the group using netarsudil. Previous studies have shown that rho-kinase inhibitors have a dose- and time-dependent increase in the outflow of the aqueous humor through the TM.[41-43] Therefore, the use of a different dosage or the use of one of the other rho-kinase inhibitors might be more effective.

As rho-kinase inhibitors show promising results to treat glaucoma and/or a corticosteroid-induced increase in intraocular pressure, we also performed an additional targeted analysis and investigated whether the results of our GWA analysis contained other genes that are involved in the rho-kinase pathway and are targeted by rho-kinase inhibitors. Being a targeted analysis, we were mainly interested whether any genes in the rho-kinase pathway had just not met the criteria for the global analysis, but were strong enough to be considered in a contextual and knowledge driven approach. This analysis retrieved *KANK1* and *SPATA18* which are targeted by (directly or indirectly) rho-kinase inhibitors (the GWAS P-values and MAF for these genes are provided in supplemental material 9).

It is of interest that *KANK1* (KN motif and ankyrin repeat domains 1) is involved in the formation of actin, an important component of the extracellular matrix (ECM) in the TM. As the aqueous humor exits the eye predominantly through the TM, changes in this tissue are thought to play an important role in the pathogenesis of POAG and a corticosteroid-induced increase in IOP. *KANK1*, regulates the polymerization of actin. Actin is known to be an important component of the TM and is believed to be altered in patients with a corticosteroid-induced increase in IOP.[44] Cross-linked actin networks (CLANs) are formed in the TM. These formations are more common in patients with glaucoma and after treating the TM with corticosteroids.[44, 45] The formation of these CLANs are thought to be responsible for a reduced outflow of aqueous humor and thereby for the increase in eye pressure.[46, 47] The study of Kakunima et al. showed that *KANK* regulates the actin polymerization through the inhibition of *RhoA*. They found that when *KANK* was expressed, the amount of active *RhoA* was significantly reduced. Furthermore, knockdown of *KANK* significantly increased the amount of active *RhoA*.[48] The activation of *RhoA* in the aqueous humor outflow pathway has been shown to decrease the outflow. This was caused by alterations in the actin stress fibers.[49] *ROCK1*, rho-associated protein kinase 1, is a downstream effector of *RhoA*. Rho-kinase inhibitors have been shown to disrupt the actin cytoskeleton and cause a decrease in TM stiffness and enhance the outflow of aqueous humor.[42, 50] In a previous study, we found that *KANK1* was significantly downregulated after comparing bovine responders and non-responders.[51] *SPATA18* (spermatogenesis associated 18), was previously found to be upregulated in cultured TM cells of monkeys after exposure to Y-27632, a rho-kinase inhibitor.[52] Interestingly, *TIAM2* was identified after comparing high responders vs. non-responders and *CaMK* after comparing responder vs. non-responder and low responders vs. non-responders. *KANK1* and *SPATA18*, identified through a targeted analysis did just not meet the criteria of the global analysis after comparing responders vs. non-responders and high responders vs. non-responders. In addition, *KANK1* did just not meet the criteria of the global analysis after comparing low responders vs. non-responders. As the above mentioned genes do not seem to be specifically bound to a high or a low response, this suggests that rho-kinase inhibitors are efficient to treat both high and low responders.

EOMES and *OLFM2*, as identified in the current study, are involved in the development of the TM or retinal ganglion cell layers. *EOMES* (eomesodermin) is crucial for embryonic development of mesoderm and the central nervous system in vertebrates. The TM is found to be not only derived from neural crest cells but it also has a mesoderm origin.[53, 54] In addition, a previous study showed that *EOMES* is required for the development of retinal ganglion cells and the optic nerve.[55, 56] Loss of the retinal ganglion cells in patients with glaucoma leads to visual field loss.

The study of Sulatana et al. investigated the expression of *OLFM2* (olfactomedin 2) in the developing mouse eye. They showed that this gene was preferentially expressed in the developing retinal ganglion cell layer, suggesting that *OLFM2* may play an important role in the course of retinal and eye development. In addition, in a study with Japanese patients with POAG, twelve sequence variances were identified within *OLFM2*.[57] It is known that patients with POAG are more susceptible to develop a corticosteroid-induced increase in IOP. The other way around, patients that had a corticosteroid response in the past are at risk to develop POAG.[1, 3, 30, 58-60] *DMD*, identified after comparing high responders vs. non-responders on the X-chromosome for the female subcohort, was recently found to be significantly expressed in the optic nerve head of patients with POAG compared to healthy controls.[61]

ADAMTS17 (ADAM metallopeptidase with thrombospondin type 1 motif 17) is a member of the *ADAMTS* family of extracellular proteases and is known to be expressed in the trabecular meshwork of the eyes of mice and neonates.[62, 63, 64] In one of our other studies (not published yet), we found *ADAMTS17* to be expressed as well but it was not identified as a TM-specific gene. However, we found multiple of the *ADAMTS* genes to be TM-specific. Mutations in *ADAMTS17* are involved in multiple anterior segment diseases such as glaucoma, ectopia lentis and Weill-Marchesani-like syndrome (short stature and brachydactyly in combination with lens dislocation, thickened skin, and joint contractures).[62, 65] Phenotypic similarities and functional evidence between *ADAMTS* associated disease and Fibrillin1 (*FBN1*) associated disease have been shown which supports a role for *ADAMTS* family members in microfibril assembly and function.[66, 67] In addition, a novel pathogenic variant in *ADAMTS17* in a patient with Weill-Marchesani type 4, was linked to abnormal fibrillin, fibronectin, and collagen type I deposition in the ECM of the skin and in skin-derived fibroblasts. An abnormal ultrastructure of elastic fibers a collagen type I vessels was also observed.[68] Changes in collagen, fibrillin and fibronectin as import structures within the ECM of the TM, have been thought to cause a decrease in outflow of the aqueous humor through the TM.[69, 70]

Furthermore, we identified two genes, *ESRRγ* and *UBL5*, that appear to play a role in how the body reacts to corticosteroids. *ESRRγ* (estrogen related receptor gamma) is one of the subgroups of the estrogen related receptors. Despite what the name suggests, these receptors are not modulated by estrogen. However, the molecules that target these receptors are not known yet.[71, 72] A study of Wang et al. showed that there is a crosstalk between the *ESRRγ* and glucocorticoid receptor (GR) signaling in skeletal muscle cells. Treatment of skeletal muscle cells with an *ESRRγ* receptor agonist led to a significant increase in the expression of GR*α* protein. In addition, genes that are targeted by this receptor were induced as well. After using an *ESRRγ*

receptor antagonist, the opposite effect was observed.[73] Furthermore, the formation of CLANs after exposing the TM to corticosteroids, have been found to be mediated through the GR.[44] As mentioned previously, the study of Wang et al. showed that there is a crosstalk between the *ESRRγ* and GR signaling in skeletal muscle cells. In addition we identified *UBL5* (ubiquitin like 5 gene). In one of our other studies (not published yet), we found this gene to be highly expressed in the healthy TM. This shows that *UBL5* plays an important role in the functioning of this tissue. The study of Lee et al. performed a GWA analysis to compare the methylation levels of peripheral blood mononuclear cells (PBMCs) of patients with an acute exacerbation of chronic obstructed pulmonary disease (COPD). Some patients with an exacerbation have a good response to the use of corticosteroids while others will respond not or insufficiently. Their study showed that in patients with a good response to corticosteroids during an exacerbation, genes in the ubiquitin conjugation pathway are involved.[74] This suggests that *UBL5* and genes closely related to this gene might be involved in how patients with COPD react to their corticosteroid treatment. Analogue to the above, small genetic variances in *ESRRγ* or *UBL5* and their related genes might determine whether a patient develops a corticosteroid-induced increase in IOP or not. *ESRRγ* was identified after comparing high responders vs. non-responders and high responder vs. low responders and *UBL5* was identified after comparing high responder vs. low responders. Neither *ESRRγ* nor *UBL5* was identified after comparing responders vs. non-responders or after comparing low responders vs. non-responders. This suggests that patients with a high response, might react differently to corticosteroids compared to low responders.

In a previous study, we integrated the publicly available gene expression data investigating the effect of dexamethasone on the TM from human and bovine subjects. In addition, we investigated the differences in gene expression data of bovine corticosteroid responders and non-responders. Within this previously performed study, we found that the activation of the cell cycle in the TM seemed to be different between responders and non-responders.[51] *CUX1*, as identified in the current study, has been shown to play a role in the cell cycle progression.[75, 76] In addition, *GPATCH2*, also identified in this study, has been found to inhibit the G1-S phase within the cell cycle. *GPATCH2* has also been shown to inhibit NF-κB.[77] We previously identified multiple pathways related to *NF-κB* (functional pathway cluster) after exposing the TM of human and bovine subjects to dexamethasone.[51] In addition, multiple pathways related to *NF-κB* were just below the threshold after comparing the gene expression in TM of bovine corticosteroid-responders and non-responders, suggesting that the functioning of *NF-κB* might be different between responders and non-responders. Furthermore, *NF-κB* is also known to be activated in the TM cells of glaucoma patients where it protects against oxidative stress.[78]

Interestingly, *CUX1* and *CPATCH2* were identified after comparing high responder vs. low responders. *GPATCH2* was also identified after comparing high responders vs. non-responders. The fact that both genes were only found in the high responders, might suggest that the role of the cell cycle is larger in the pathogenesis of a high response compared to a low response. Jeong et al. performed the first GWAS for a corticosteroid-induced increase in IOP in patients treated with an intravitreal triamcinolone injection and identified two intergenic quantitative trait locus (rs2523864 and rs2251830) that could affect the expression of *HCG22*.[20] These SNPs did not reach the significance threshold, neither based on P-value, nor based on eQTL or LD in any of our four analysis. However, these SNPs are linked to the region of the major histocompatibility complex (MHC). This region has high levels of variation and LD which causes the interpretation of SNPs and associated genes found in this region more difficult. In addition, in the study of Jeong et al., only a small cohort of 64 subjects was investigated. In addition, some other studies investigated candidate target genes. As mentioned before, SNPs associated with the glucocorticoid receptor or following genes have been investigated previously: *SFRS3*, *FKBP4*, *SFRS5*, *SFRS9*.[14-19] Only in the study of Badrinarayanan, 2 SNPs were found to be significant after comparing responders and non-responders: rs6559662 and rs1879370.[19] However, in our GWAS study, these SNPs were not significant in neither of our four analysis.

One of the strengths of this study is the careful selection of our study population as the long exposure to corticosteroids and the criteria used to identify a corticosteroid responder both lowered the risk to misclassify non-responder as responders. Corticosteroids are widely used within ophthalmology, mostly to control postoperative inflammation or to treat auto-immune disorders such as uveitis. As the latter are known to affect the IOP, it can be difficult to determine whether an IOP increase in these patients is due to the use of corticosteroids or due to other factors related to the disorder.[79] Therefore, participants using corticosteroids to control postoperative inflammation are more suitable compared to uveitis patients receiving corticosteroids to investigate the genetics of a corticosteroid response. In addition, most patients have to use corticosteroids for a prolonged period of time before developing a corticosteroid response. As reported in the study of Vajaranant et al, the cumulative incidence of experiencing an increase in IOP in patients with no history of glaucoma was 2.5% by 1 month, 10% by 3 months, 23% by 6 months, and 35% 1 year after keratoplasty. The incidence increased each month by 3% to 4% up to 8 months and with 1% between 8 and 12 months after the keratoplasty.[2] As our study population consists of corneal transplantation patients without underlying auto-immune disorders and had to use corticosteroids for a prolonged period of time (at least one year), this group of patients offer a unique opportunity to evaluate the genetics of a corticosteroid response.

The definition of a corticosteroid response differs throughout studies. The most commonly used definition, however, was formulated by Armaly and Becker as a positive change in IOP of more than 6 mmHg above normal baseline IOP after the use of corticosteroids.[30] We used the same cut-off in our study, however, the above definition does not take into account any other possible post-operative causes of an increase in IOP. Therefore, we carefully assessed whether any elevation in IOP was due to a corticosteroid response or could be explained by other factors. In addition, the IOP increase also had to be related to changes in corticosteroid used as described in the method section. As the last two criteria are more or less susceptible to personal interpretation, all participants were independently assessed by two ophthalmology residents (M.F.C. and I.L.). After the first screening, they already reached consensus for 80% of the participants. The other cases were discussed with a glaucoma specialist to make a final decision. In case it was not clear whether the IOP elevation was due to a corticosteroid response or due to other factors, the participant was excluded for further analysis, achieving that classified responders are true responders and avoiding that the classified non-responders include true responders. Within our study cohort, 39.5% of the patients had a corticosteroid response which lies in the range of expectations as reported by previously performed studies.[1, 2]

For most complex diseases, large cohorts are required to detect significant differences in GWAS. However, within this study, we are investigating a drug response which are known to be strongly related to genetic differences.[80] For example, a GWAS in 85 subjects and 90 controls revealed genome-wide statistically significant variants in *SLC01B1* that are strongly associated with an increased risk of statin-induced myopathy.[81] We were not able to find a suitable validation cohort. An ideal validation cohort would need to have similar features to ours. However, most patients that have to use topical corticosteroids have an auto-immune or hyper-reactivity disorders such as uveitis, which are, as indicated before, associated with an increase in IOP, complicating the identification of true corticosteroid responders. In addition, most patient groups don't have to use topical corticosteroids for a prolonged period of time, increasing the chance of incorrectly classified non-responders. As we are performing a study to obtain more information on the genetic components of a corticosteroid-induced increase in IOP, the classification of responders and non-responders has to be as absolute as possible. In order to prevent false positive results, we identified the significant SNPs by performing an adaptive permutation analysis. Within this method, SNPs that are clearly not going to be significant are excluded earlier in the analysis than SNPs that might reach the genome-wide significant threshold. Moreover, the analysis was based on a permutation test, i.e. the samples are continuously randomized and tested against each other which increased the likelihood of the identified significant SNPs to be truly associated.

In conclusion, for as far as we know, the current study is the largest cohort that investigated the pharmacogenomics of a corticosteroid-induced increase in IOP. Changes in the extracellular matrix and the cell cycle of the trabecular meshwork seem to be involved in the pathogenesis of a corticosteroid response. In addition, we identified genes that are involved in the expression of the glucocorticoid receptor and the development or functioning of the trabecular meshwork. We also found that small genetic variances in one of the identified genes (*UBL5*) might determine whether a patient develops a corticosteroid-induced increase in IOP or not. Furthermore, rho-kinase inhibitors warrant further investigation as treatment for a corticosteroid-induced increase in IOP, with potential target genes identified in both high and low responders.

References

1. Tripathi RC, Parapuram SK, Tripathi BJ, et al. Corticosteroids and glaucoma risk. Drugs Aging 1999;15(6):439-50.
2. Vajaranant TS, Price MO, Price FW, et al. Visual acuity and intraocular pressure after Descemet's stripping endothelial keratoplasty in eyes with and without preexisting glaucoma. Ophthalmology 2009;116(9):1644-50.
3. Fan BJ, Wang DY, Tham CC, et al. Gene expression profiles of human trabecular meshwork cells induced by triamcinolone and dexamethasone. Invest Ophthalmol Vis Sci 2008;49(5):1886-97.
4. Ishibashi T, Takagi Y, Mori K, et al. cDNA microarray analysis of gene expression changes induced by dexamethasone in cultured human trabecular meshwork cells. Invest Ophthalmol Vis Sci 2002;43(12):3691-7.
5. Markus H. Kuehn CYK, Young H. Kwon. Dexamethasone induced gene expression changes in human perfusion organ cultured trabecular meshwork. 2012.
6. Matsuda A, Asada Y, Takakuwa K, et al. DNA Methylation Analysis of Human Trabecular Meshwork Cells During Dexamethasone Stimulation. Invest Ophthalmol Vis Sci 2015;56(6):3801-9.
7. Nehme A, Lobenhofer EK, Stamer WD, Edelman JL. Glucocorticoids with different chemical structures but similar glucocorticoid receptor potency regulate subsets of common and unique genes in human trabecular meshwork cells. BMC Med Genomics 2009;2:58.
8. Rozsa FW, Reed DM, Scott KM, et al. Gene expression profile of human trabecular meshwork cells in response to long-term dexamethasone exposure. Mol Vis 2006;12:125-41.
9. Lo WR, Rowlette LL, Caballero M, et al. Tissue differential microarray analysis of dexamethasone induction reveals potential mechanisms of steroid glaucoma. Invest Ophthalmol Vis Sci 2003;44(2):473-85.
10. Danias J, Gerometta R, Ge Y, et al. Gene expression changes in steroid-induced IOP elevation in bovine trabecular meshwork. Invest Ophthalmol Vis Sci 2011;52(12):8636-45.
11. Liesenborghs I, Eijssen LMT, Kutmon M, et al. The molecular processes in the trabecular meshwork after exposure to corticosteroids and in corticosteroid-induced ocular hypertension. Invest Ophthalmol Vis Sci 2020.
12. Chan W, Wiggs JL, Sobrin L. The genetic influence on corticosteroid-induced ocular hypertension: A field positioned for discovery. Am J Ophthalmol 2019.
13. Armaly MF. The heritable nature of dexamethasone-induced ocular hypertension. Arch Ophthalmol 1966;75(1):32-5.
14. Hogewind BF, Micheal S, Schoenmaker-Koller FE, et al. Analyses of Sequence Variants in the *MYOC* Gene and of Single Nucleotide Polymorphisms in the *NR3C1* and *FKBP5* Genes in Corticosteroid-Induced Ocular Hypertension. Ophthalmic Genet 2015;36(4):299-302.
15. Hogewind BF, Micheal S, Bakker B, et al. Analysis of single nucleotide polymorphisms in the *SFRS3* and *FKBP4* genes in corticosteroid-induced ocular hypertension. Ophthalmic Genet 2012;33(4):221-4.

16. Fingert JH, Alward WL, Wang K, et al. Assessment of SNPs associated with the human glucocorticoid receptor in primary open-angle glaucoma and steroid responders. Mol Vis 2010;16:596-601.
17. Gerzenstein SM, Pletcher MT, Cervino AC, et al. Glucocorticoid receptor polymorphisms and intraocular pressure response to intravitreal triamcinolone acetonide. Ophthalmic Genet 2008;29(4):166-70.
18. Szabo V, Borgulya G, Filkorn T, et al. The variant *N363S* of glucocorticoid receptor in steroid-induced ocular hypertension in Hungarian patients treated with photorefractive keratectomy. Mol Vis 2007;13:659-66.
19. Badrinarayanan L, Chitipothu S, Ramasubramanyan S, et al. Assessment of single nucleotide polymorphisms associated with steroid-induced ocular hypertension. Int J Ophthalmol 2020;13(8):1294-305.
20. Jeong S, Patel N, Edlund CK, et al. Identification of a Novel Mucin Gene *HCG22* Associated With Steroid-Induced Ocular Hypertension. Invest Ophthalmol Vis Sci 2015;56(4):2737-48.
21. Mindel JS, Tavitian HO, Smith H, Jr., Walker EC. Comparative ocular pressure elevation by medrysone, fluorometholone, and dexamethasone phosphate. Arch Ophthalmol 1980;98(9):1577-8.
22. Price MO, Price FW, Jr., Kruse FE, et al. Randomized comparison of topical prednisolone acetate 1% versus fluorometholone 0.1% in the first year after descemet membrane endothelial keratoplasty. Cornea 2014;33(9):880-6.
23. Zhan X, Hu Y, Li B, et al. RVTESTS: an efficient and comprehensive tool for rare variant association analysis using sequence data. Bioinformatics 2016;32(9):1423-6.
24. Howie B, Fuchsberger C, Stephens M, et al. Fast and accurate genotype imputation in genome-wide association studies through pre-phasing. Nat Genet 2012;44(8):955-9.
25. Li Y, Willer CJ, Ding J, et al. MaCH: using sequence and genotype data to estimate haplotypes and unobserved genotypes. Genet Epidemiol 2010;34(8):816-34.
26. McCarthy S, Das S, Kretzschmar W, et al. A reference panel of 64,976 haplotypes for genotype imputation. Nat Genet 2016;48(10):1279-83.
27. Loh PR, Danecek P, Palamara PF, et al. Reference-based phasing using the Haplotype Reference Consortium panel. Nature Genetics 2016;48(11):1443-8.
28. Purcell S, Neale B, Todd-Brown K, et al. PLINK: a tool set for whole-genome association and population-based linkage analyses. Am J Hum Genet 2007;81(3):559-75.
29. Watanabe K, Taskesen E, van Bochoven A, Posthuma D. Functional mapping and annotation of genetic associations with FUMA. Nat Commun 2017;8(1):1826.
30. Armaly MF, Becker B. Intraocular pressure response to topical corticosteroids. Fed Proc 1965;24(6):1274-8.
31. Fini ME, Schwartz SG, Gao X, et al. Steroid-induced ocular hypertension/glaucoma: Focus on pharmacogenomics and implications for precision medicine. Prog Retin Eye Res 2017;56:58-83.
32. Matsuo N, Terao M, Nabeshima Y, Hoshino M. Roles of STEF/*Tiam1*, guanine nucleotide exchange factors for *Rac1*, in regulation of growth cone morphology. Mol Cell Neurosci 2003;24(1):69-81.

33. Tang AT, Campbell WB, Nithipatikom K. *ROCK1* feedback regulation of the upstream small GTPase *RhoA*. Cell Signal 2012;24(7):1375-80.
34. Maeda K, Otomo K, Yoshida N, et al. *CaMK4* compromises podocyte function in autoimmune and nonautoimmune kidney disease. J Clin Invest 2018;128(8):3445-59.
35. Van de Velde S, De Groef L, Stalmans I, et al. Towards axonal regeneration and neuroprotection in glaucoma: Rho kinase inhibitors as promising therapeutics. Prog Neurobiol 2015;131:105-19.
36. Bermel C, Tonges L, Planchamp V, et al. Combined inhibition of *Cdk*5 and *ROCK* additively increase cell survival, but not the regenerative response in regenerating retinal ganglion cells. Mol Cell Neurosci 2009;42(4):427-37.
37. Sugiyama T, Shibata M, Kajiura S, et al. Effects of fasudil, a Rho-associated protein kinase inhibitor, on optic nerve head blood flow in rabbits. Invest Ophthalmol Vis Sci 2011;52(1):64-9.
38. Moshirfar M, Parker L, Birdsong OC, et al. Use of Rho kinase Inhibitors in Ophthalmology: A Review of the Literature. Med Hypothesis Discov Innov Ophthalmol 2018;7(3):101-11.
39. Futakuchi A, Morimoto T, Ikeda Y, et al. Intraocular pressure-lowering effects of ripasudil in uveitic glaucoma, exfoliation glaucoma, and steroid-induced glaucoma patients: *ROCK*-S, a multicentre historical cohort study. Sci Rep 2020;10(1):10308.
40. Price MO, Feng MT, Price FW, Jr. Randomized, Double-Masked Trial of Netarsudil 0.02% Ophthalmic Solution for Prevention of Corticosteroid-Induced Ocular Hypertension. Am J Ophthalmol 2020;222:382-7.
41. Honjo M, Tanihara H, Inatani M, et al. Effects of rho-associated protein kinase inhibitor Y-27632 on intraocular pressure and outflow facility. Invest Ophthalmol Vis Sci 2001;42(1):137-44.
42. Rao PV, Deng PF, Kumar J, Epstein DL. Modulation of aqueous humor outflow facility by the Rho kinase-specific inhibitor Y-27632. Invest Ophthalmol Vis Sci 2001;42(5):1029-37.
43. Tian B, Kaufman PL. Effects of the Rho kinase inhibitor Y-27632 and the phosphatase inhibitor calyculin A on outflow facility in monkeys. Exp Eye Res 2005;80(2):215-25.
44. Clark AF, Wilson K, McCartney MD, et al. Glucocorticoid-induced formation of cross-linked actin networks in cultured human trabecular meshwork cells. Invest Ophthalmol Vis Sci 1994;35(1):281-94.
45. Hoare MJ, Grierson I, Brotchie D, et al. Cross-linked actin networks (CLANs) in the trabecular meshwork of the normal and glaucomatous human eye in situ. Invest Ophthalmol Vis Sci 2009;50(3):1255-63.
46. Bermudez JY, Montecchi-Palmer M, Mao W, Clark AF. Cross-linked actin networks (CLANs) in glaucoma. Exp Eye Res 2017;159:16-22.
47. Clark AF, Brotchie D, Read AT, et al. Dexamethasone alters F-actin architecture and promotes cross-linked actin network formation in human trabecular meshwork tissue. Cell Motil Cytoskeleton 2005;60(2):83-95.
48. Kakinuma N, Roy BC, Zhu Y, et al. *Kank* regulates *RhoA*-dependent formation of actin stress fibers and cell migration via 14-3-3 in PI3K-Akt signaling. J Cell Biol 2008;181(3):537-49.
49. Zhang M, Maddala R, Rao PV. Novel molecular insights into *RhoA* GTPase-induced resistance to aqueous humor outflow through the trabecular meshwork. Am J Physiol Cell Physiol 2008;295(5):C1057-70.

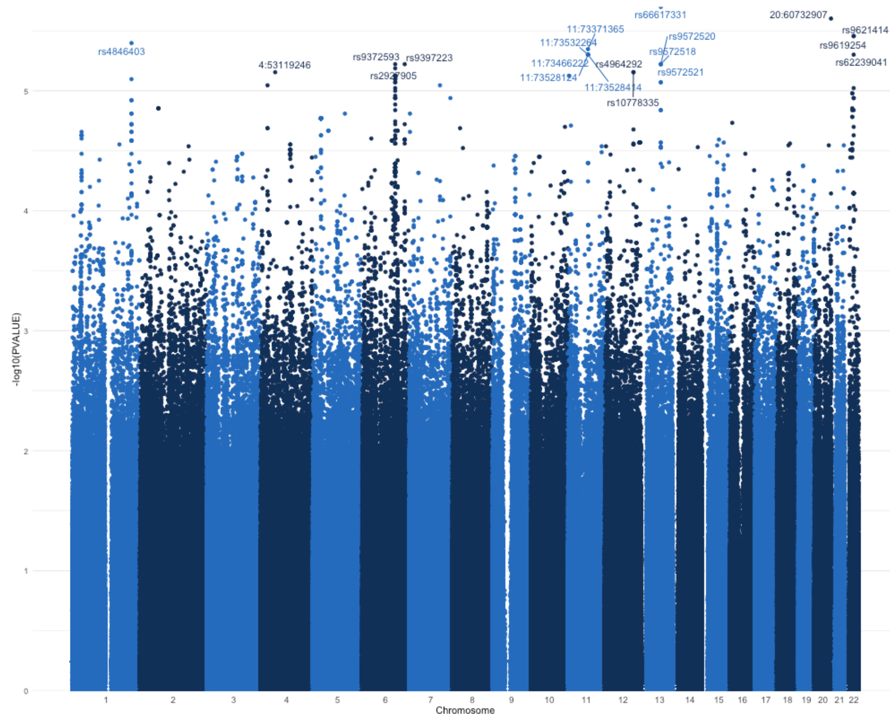
50. Kumar J, Epstein DL. Rho GTPase-mediated cytoskeletal organization in Schlemm's canal cells play a critical role in the regulation of aqueous humor outflow facility. J Cell Biochem 2011;112(2):600-6.
51. Liesenborghs I, Eijssen LMT, Kutmon M, et al. The Molecular Processes in the Trabecular Meshwork After Exposure to Corticosteroids and in Corticosteroid-Induced Ocular Hypertension. Invest Ophthalmol Vis Sci 2020;61(4):24.
52. Fujimoto T, Inoue T, Ohira S, et al. Inhibition of Rho Kinase Induces Antioxidative Molecules and Suppresses Reactive Oxidative Species in Trabecular Meshwork Cells. J Ophthalmol 2017;2017:7598140.
53. Gould DB, Smith RS, John SW. Anterior segment development relevant to glaucoma. Int J Dev Biol 2004;48(8-9):1015-29.
54. Sowden JC. Molecular and developmental mechanisms of anterior segment dysgenesis. Eye (Lond) 2007;21(10):1310-8.
55. Mao CA, Kiyama T, Pan P, et al. Eomesodermin, a target gene of Pou4f2, is required for retinal ganglion cell and optic nerve development in the mouse. Development 2008;135(2):271-80.
56. Sultana A, Nakaya N, Senatorov VV, Tomarev SI. Olfactomedin 2: expression in the eye and interaction with other olfactomedin domain-containing proteins. Invest Ophthalmol Vis Sci 2011;52(5):2584-92.
57. Funayama T, Mashima Y, Ohtake Y, et al. SNPs and interaction analyses of noelin 2, *myoc*ilin, and optineurin genes in Japanese patients with open-angle glaucoma. Invest Ophthalmol Vis Sci 2006;47(12):5368-75.
58. Becker B. Intraocular Pressure Response to Topical Corticosteroids. Invest Ophthalmol 1965;4:198-205.
59. Kitazawa Y, Horie T. The prognosis of corticosteroid-responsive individuals. Arch Ophthalmol 1981;99(5):819-23.
60. Lewis JM, Priddy T, Judd J, et al. Intraocular pressure response to topical dexamethasone as a predictor for the development of primary open-angle glaucoma. Am J Ophthalmol 1988;106(5):607-12.
61. Zhang DW, Zhang S, Wu J. Expression profile analysis to predict potential biomarkers for glaucoma: BMP1, *DMD* and GEM. PeerJ 2020;8:e9462.
62. Hubmacher D, Schneider M, Berardinelli SJ, et al. Unusual life cycle and impact on microfibril assembly of *ADAMTS17*, a secreted metalloprotease mutated in genetic eye disease. Sci Rep 2017;7:41871.
63. Cal S, Obaya AJ, Llamazares M, et al. Cloning, expression analysis, and structural characterization of seven novel human *ADAMTS*s, a family of metalloproteinases with disintegrin and thrombospondin-1 domains. Gene 2002;283(1-2):49-62.
64. Porter S, Clark IM, Kevorkian L, Edwards DR. The *ADAMTS* metalloproteinases. Biochem J 2005;386(Pt 1):15-27.
65. Morales J, Al-Sharif L, Khalil DS, et al. Homozygous mutations in *ADAMTS*10 and *ADAMTS17* cause lenticular myopia, ectopia lentis, glaucoma, spherophakia, and short stature. Am J Hum Genet 2009;85(5):558-68.

66. Hubmacher D, Apte SS. Genetic and functional linkage between *ADAMTS* superfamily proteins and fibrillin-1: a novel mechanism influencing microfibril assembly and function. Cell Mol Life Sci 2011;68(19):3137-48.
67. Hubmacher D, Apte SS. *ADAMTS* proteins as modulators of microfibril formation and function. Matrix Biol 2015;47:34-43.
68. Karoulias SZ, Beyens A, Balic Z, et al. A novel *ADAMTS17* variant that causes Weill-Marchesani syndrome 4 alters fibrillin-1 and collagen type I deposition in the extracellular matrix. Matrix Biol 2020;88:1-18.
69. Gong H, Tripathi RC, Tripathi BJ. Morphology of the aqueous outflow pathway. Microsc Res Tech 1996;33(4):336-67.
70. Lutjen-Drecoll E, Shimizu T, Rohrbach M, Rohen JW. Quantitative analysis of 'plaque material' in the inner- and outer wall of Schlemm's canal in normal- and glaucomatous eyes. Exp Eye Res 1986;42(5):443-55.
71. Giguere V, Yang N, Segui P, Evans RM. Identification of a new class of steroid hormone receptors. Nature 1988;331(6151):91-4.
72. Hong H, Yang L, Stallcup MR. Hormone-independent transcriptional activation and coactivator binding by novel orphan nuclear receptor ERR3. J Biol Chem 1999;274(32):22618-26.
73. Wang SC, Myers S, Dooms C, et al. An ERRbeta/gamma agonist modulates GRalpha expression, and glucocorticoid responsive gene expression in skeletal muscle cells. Mol Cell Endocrinol 2010;315(1-2):146-52.
74. Lee SW, Hwang HH, Hsu PW, et al. Whole-genome methylation profiling from PBMCs in acute-exacerbation COPD patients with good and poor responses to corticosteroid treatment. Genomics 2019;111(6):1381-6.
75. Alcalay NI, Vanden Heuvel GB. Regulation of cell proliferation and differentiation in the kidney. Front Biosci (Landmark Ed) 2009;14:4978-91.
76. Sansregret L, Nepveu A. The multiple roles of *CUX1*: insights from mouse models and cell-based assays. Gene 2008;412(1-2):84-94.
77. Hu F, Gou L, Liu Q, et al. G-patch domain containing 2, a gene highly expressed in testes, inhibits nuclear factor-kappaB and cell proliferation. Mol Med Rep 2015;11(2):1252-7.
78. Wang N, Chintala SK, Fini ME, Schuman JS. Activation of a tissue-specific stress response in the aqueous outflow pathway of the eye defines the glaucoma disease phenotype. Nat Med 2001;7(3):304-9.
79. Daniel E, Pistilli M, Kothari S, et al. Risk of Ocular Hypertension in Adults with Noninfectious Uveitis. Ophthalmology 2017;124(8):1196-208.
80. Harper AR, Topol EJ. Pharmacogenomics in clinical practice and drug development. Nat Biotechnol 2012;30(11):1117-24.
81. Group SC, Link E, Parish S, et al. SLCO1B1 variants and statin-induced myopathy--a genomewide study. N Engl J Med 2008;359(8):789-99.

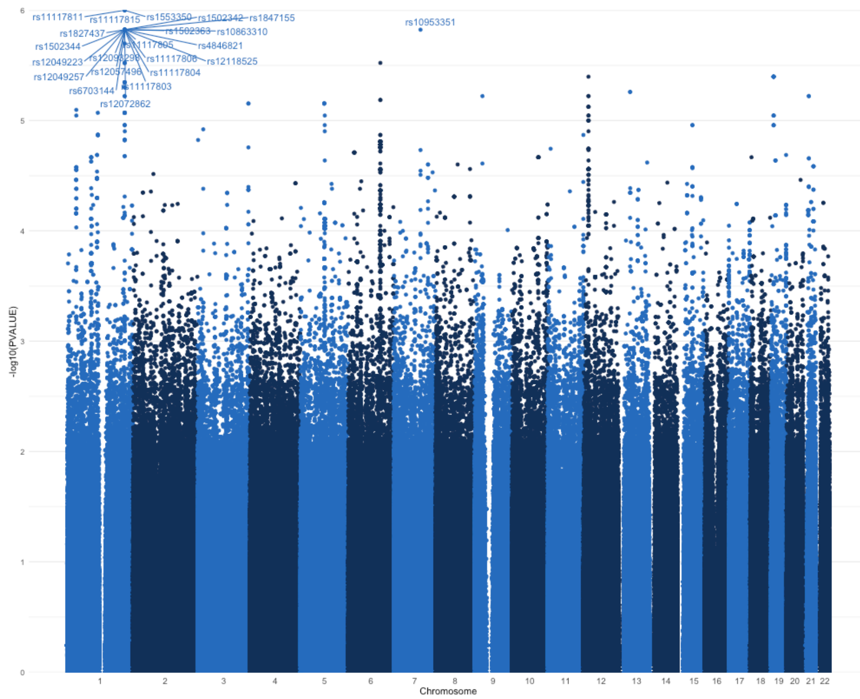
Supplemental material

Supplemental material 3, 5, 7, 8 and 9 are provided below, all other supplemental material can be obtained by contacting the author (*ilona.liesenborghs@mumc.nl*)

Supplemental material 3. *Manhatton plot of high responders vs. non-responders.*



Supplemental material 5. *Manhatton plot of high responders vs. low responders.*



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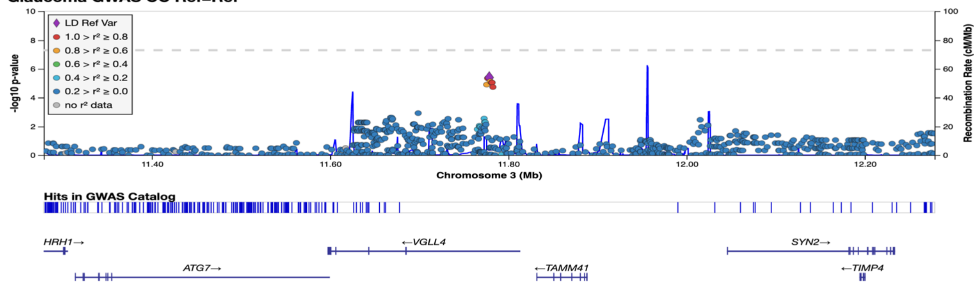
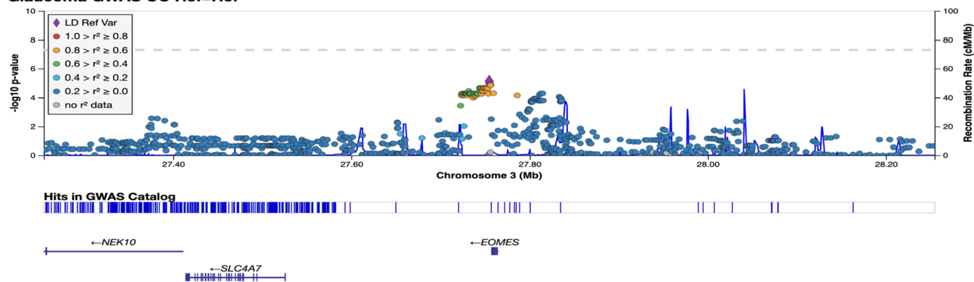
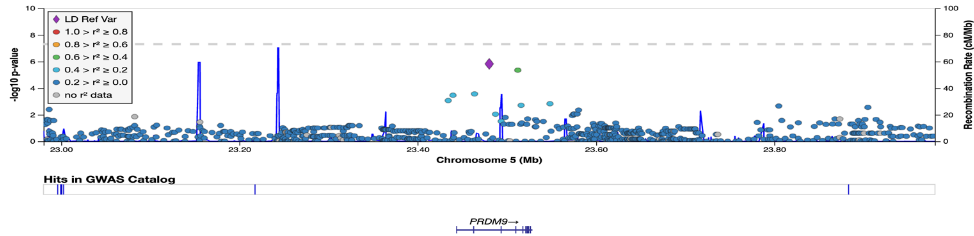
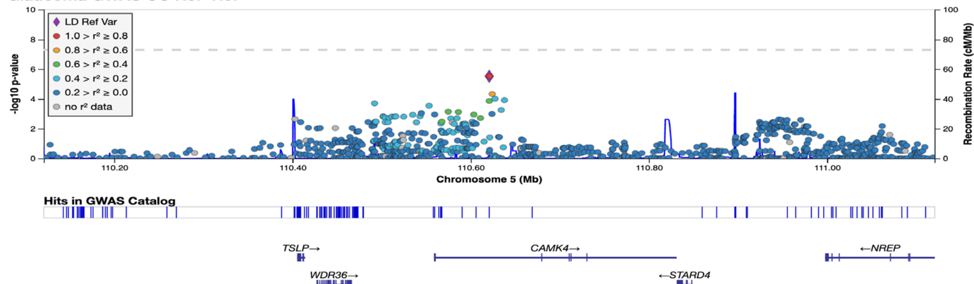
Supplemental material 7. *Regional association plots.*

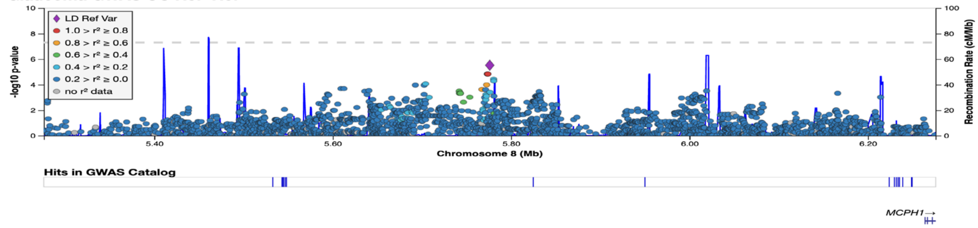
Regional association plots for responder vs. non-responder







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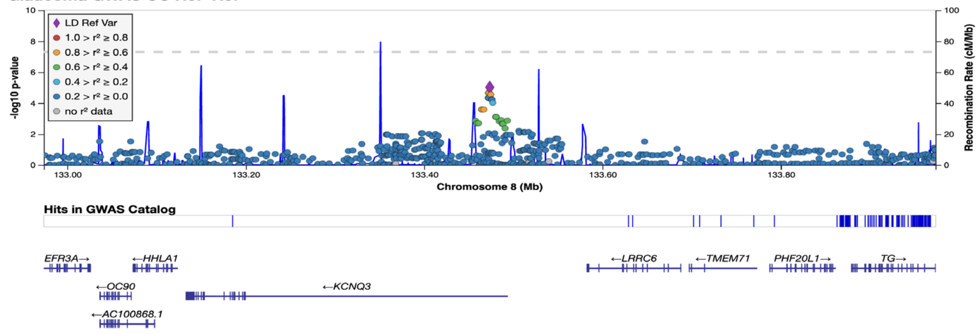
0.8 > r² ≥ 0.6
0.6 > r² ≥ 0.4
0.4 > r² ≥ 0.2
0.2 > r² ≥ 0.0
no r² data
-log10 p-value
Recombination Rate (cM/Mb)
Chromosome 8 (Mb)
5.40
5.60
5.80
6.00
6.20
Hits in GWAS Catalog
MCPH1→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no r² data
-log10 p-value
Recombination Rate (cM/Mb)
Chromosome 8 (Mb)
133.00
133.20
133.40
133.60
133.80
Hits in GWAS Catalog
EFR3A→
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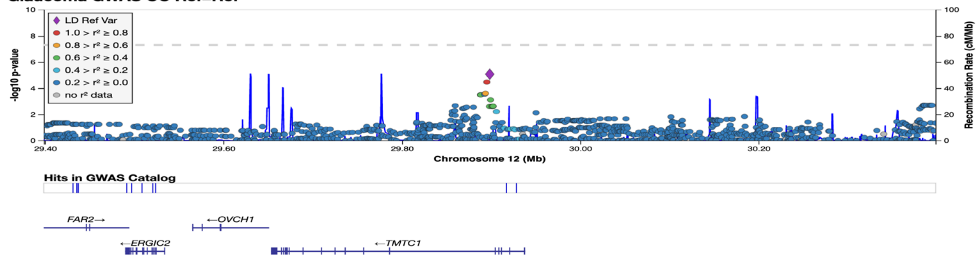
←OC90
←AC100868.1
←KCNQ3
←LRRC6
←TMEM71
PHF20L1→
TG→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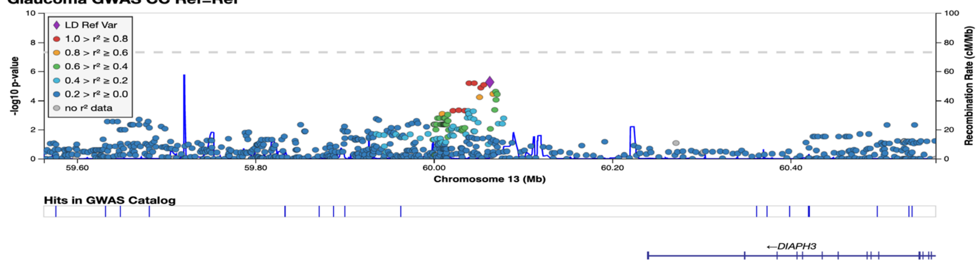
0.2 > r² ≥ 0.0
no r² data
-log10 p-value
Recombination Rate (cM/Mb)
Chromosome 12 (Mb)
29.40
29.60
29.80
30.00
30.20
Hits in GWAS Catalog
FAR2→
←OVCH1
←ERGIC2
←TMTC1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0.4 > r² ≥ 0.2
0.2 > r² ≥ 0.0
no r² data
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Recombination Rate (cM/Mb)
Chromosome 13 (Mb)
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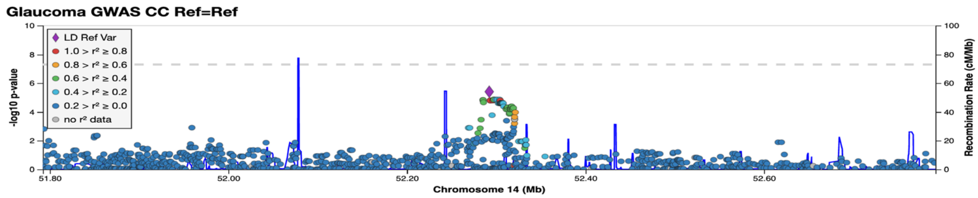
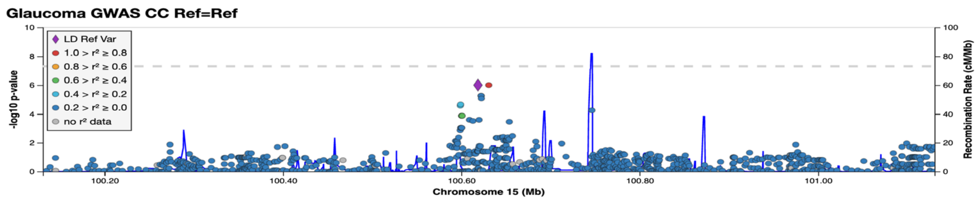
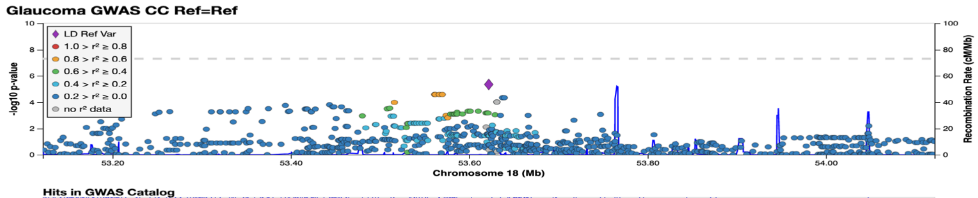
59.80
60.00
60.20
60.40
Hits in GWAS Catalog
←DIAPH3

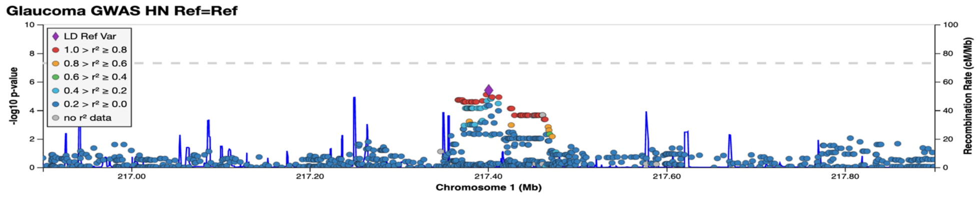
7







Regional association plots for high responder vs. non-responder



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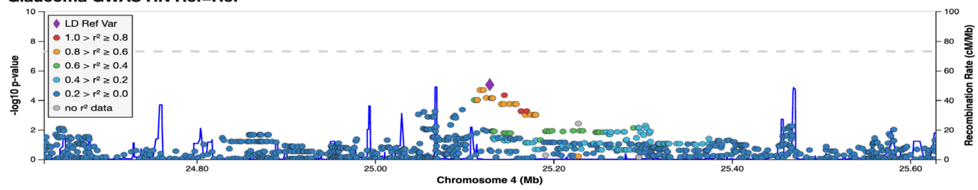
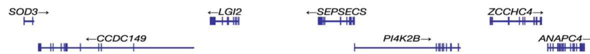


Hits in GWAS Catalog



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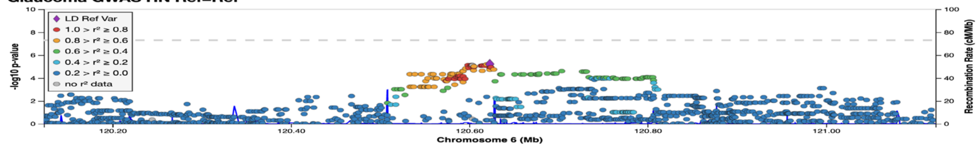

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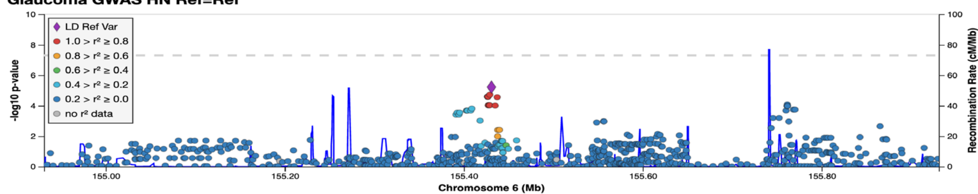

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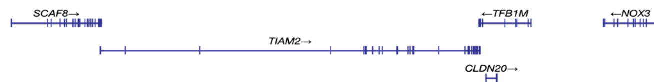

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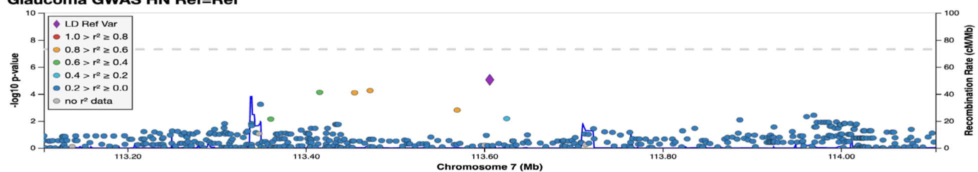

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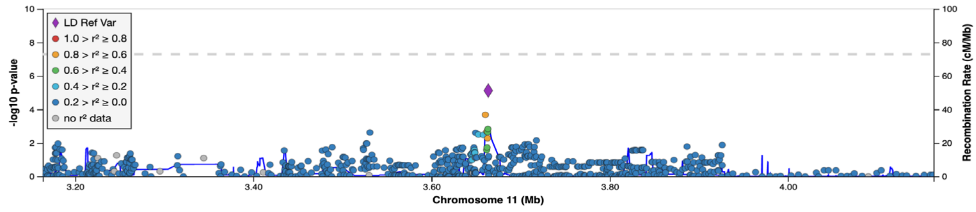

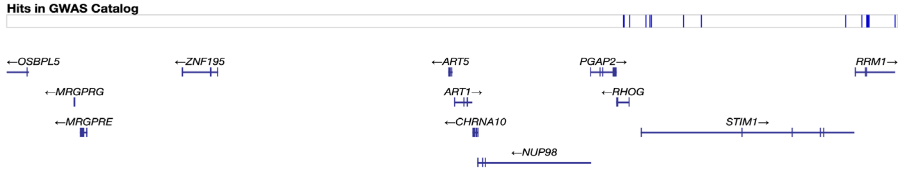

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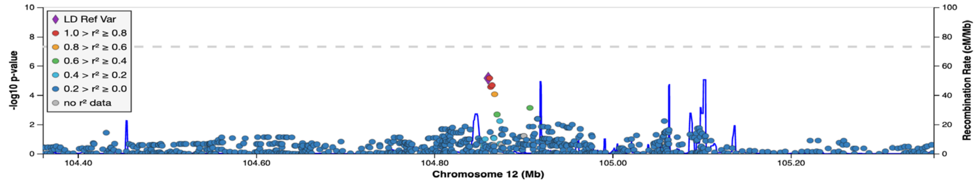

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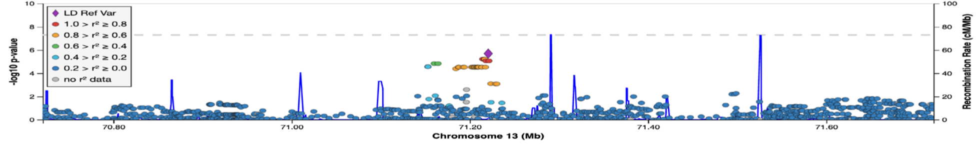

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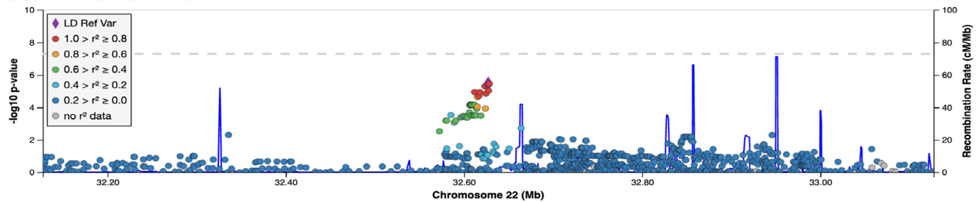
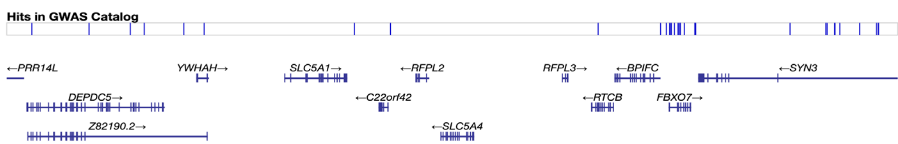
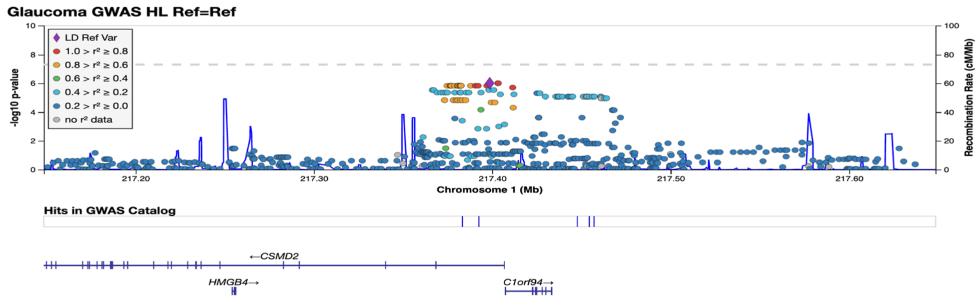
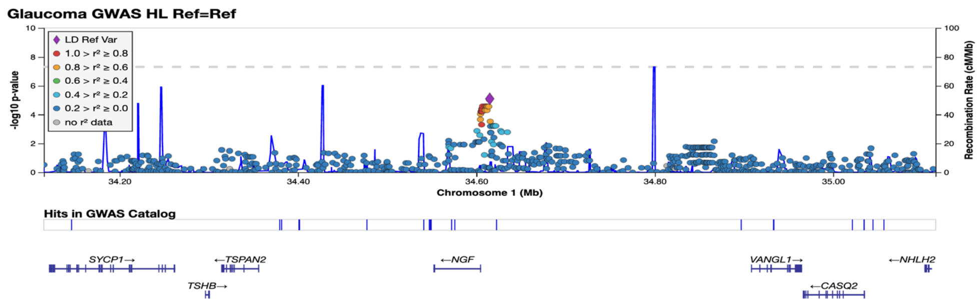
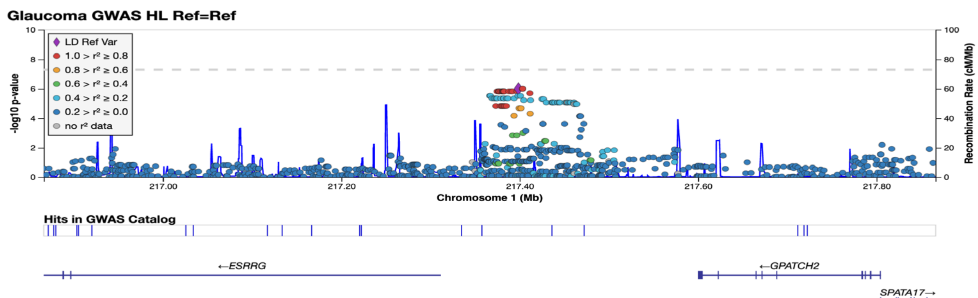


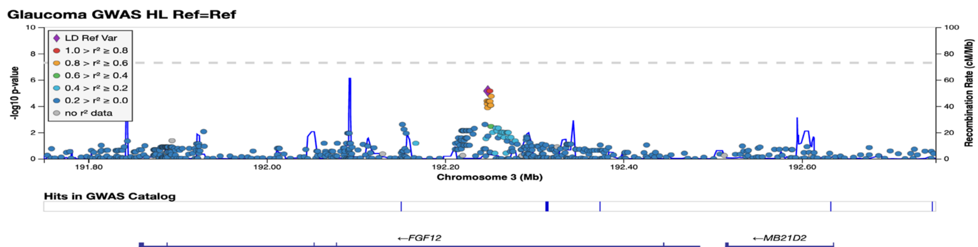


Regional association plots for high responder vs. low responder









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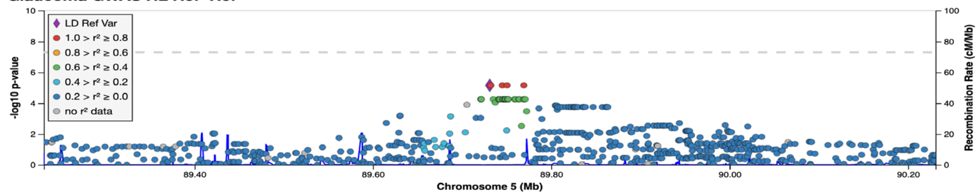

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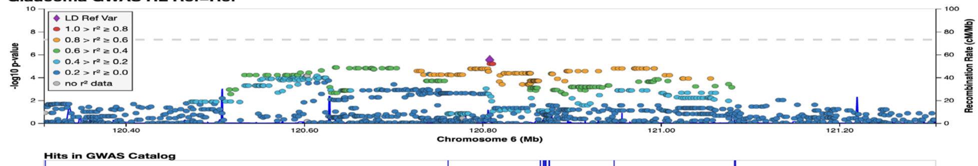

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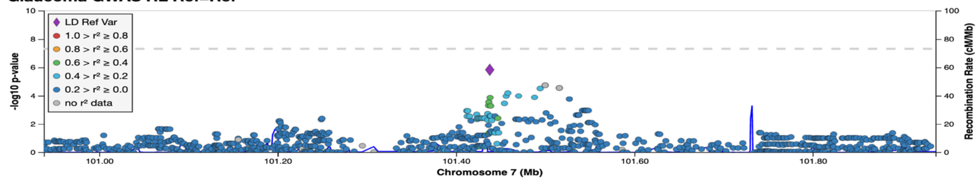

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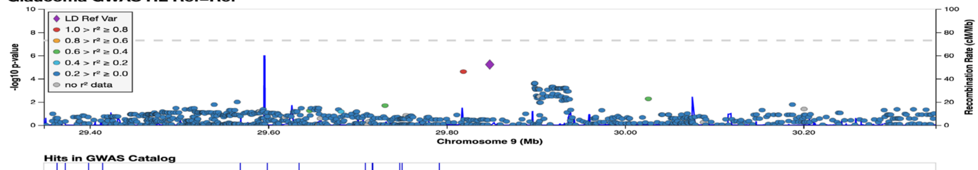

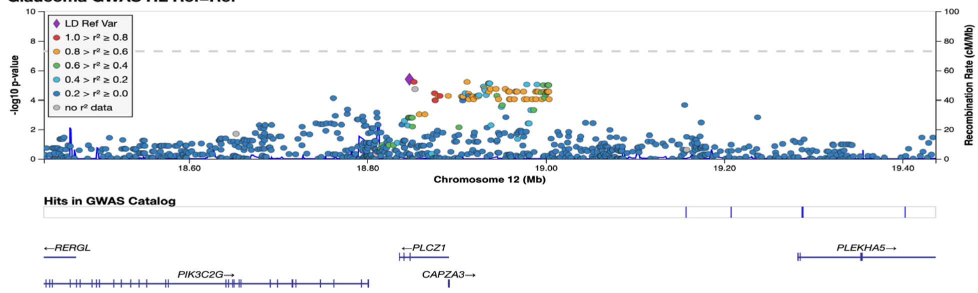
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0.6 > r² ≥ 0.4
0.4 > r² ≥ 0.2
0.2 > r² ≥ 0.0
no r² data
-log10 p-value
Recombination Rate (cM/Mb)
18.60
18.80
19.00
19.20
19.40
Chromosome 12 (Mb)
Hits in GWAS Catalog
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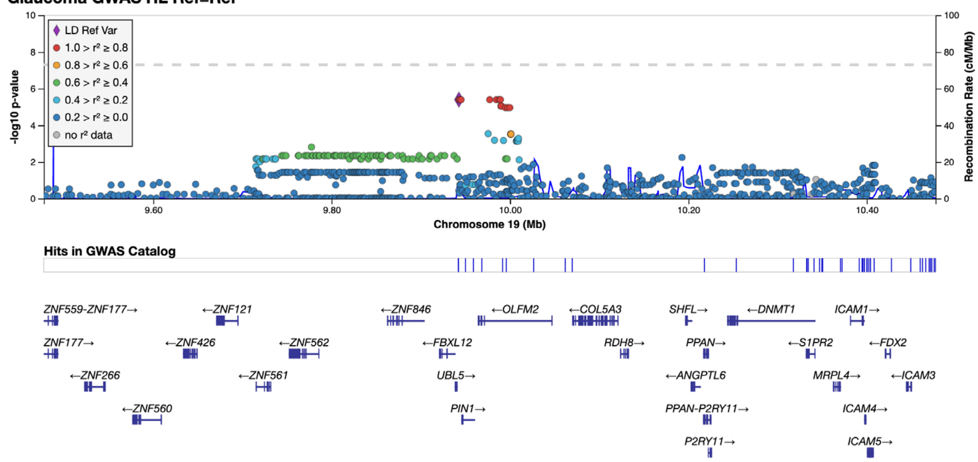
←PLCZ1
CAPZA3→
PLEKHA5→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LD Ref Var
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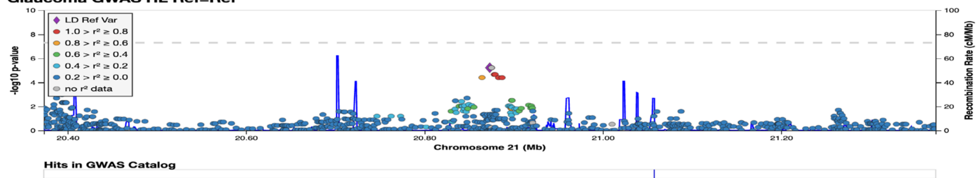
0.8 > r² ≥ 0.6
0.6 > r² ≥ 0.4
0.4 > r² ≥ 0.2
0.2 > r² ≥ 0.0
no r² data
-log10 p-value
Recombination Rate (cM/Mb)
9.60
9.80
10.00
10.20
10.40
Chromosome 19 (Mb)
Hits in GWAS Catalog
ZNF559-ZNF177→
←ZNF121
←ZNF846
←OLFM2
←COL5A3
SHFL→
←DNMT1
ICAM1→
ZNF177→
←ZNF426
←ZNF562
←FBXL12
RDH8→
PPAN→
←S1PR2
←FDX2
←ZNF266
←ZNF561
UBL5→
←ANGPTL6
MRPL4→
←ICAM3
←ZNF560
PIN1→
PPAN-P2RY11→
ICAM4→
P2RY11→
ICAM5→


Glaucoma GWAS HL Ref=Ref
LD Ref Var
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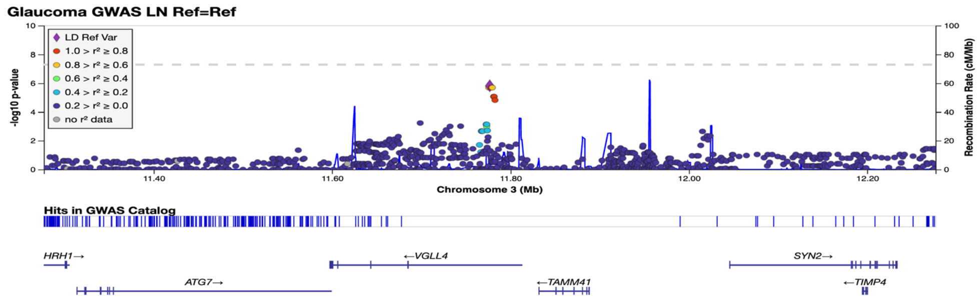
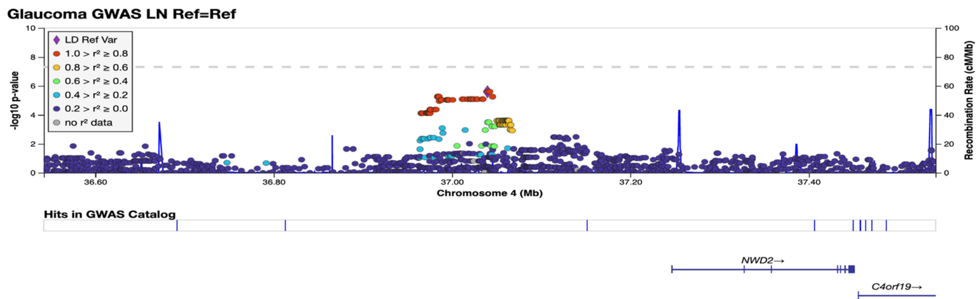
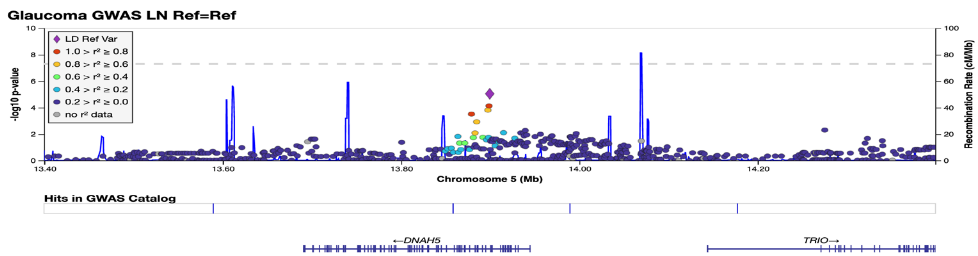
0.8 > r² ≥ 0.6
0.6 > r² ≥ 0.4
0.4 > r² ≥ 0.2
0.2 > r² ≥ 0.0
no r² data
-log10 p-value
Recombination Rate (cM/Mb)
20.40
20.60
20.80
21.00
21.20
Chromosome 21 (Mb)
Hits in GWAS Catalog

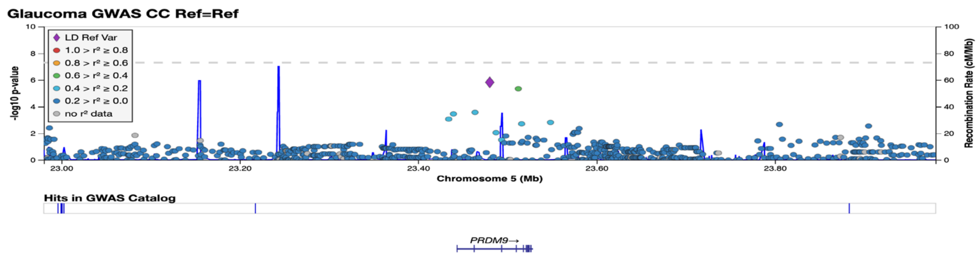
7

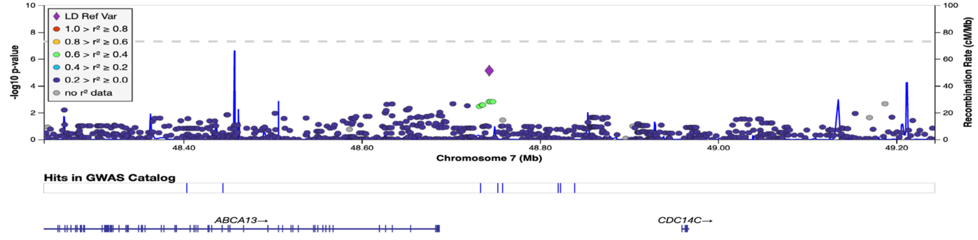
Regional association plots for low responder vs. non-responder

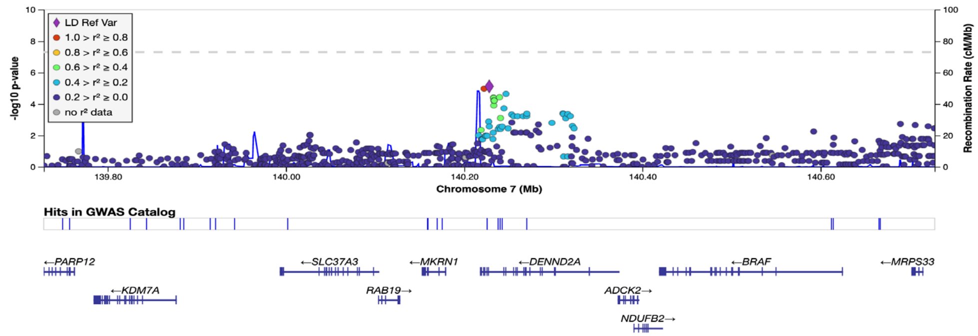


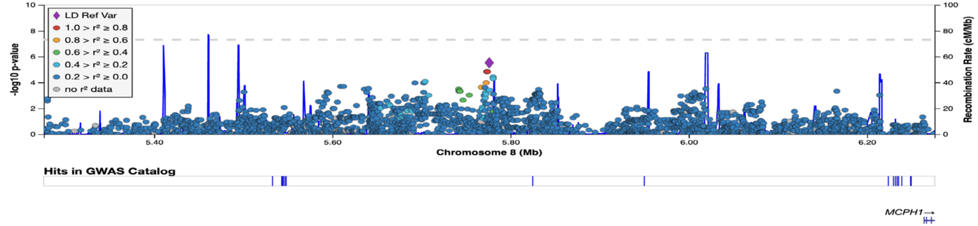


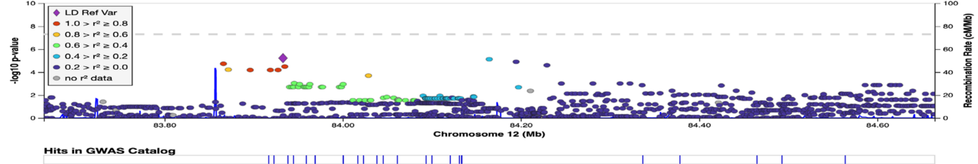




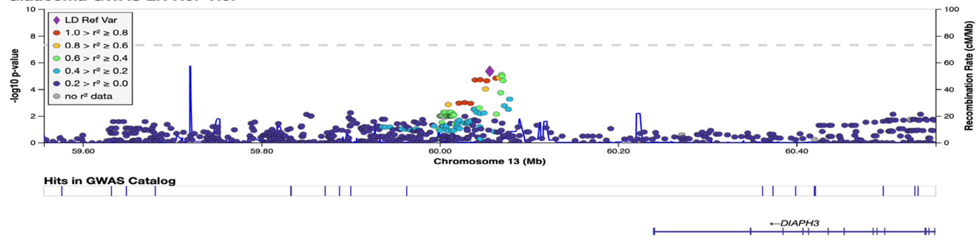

Glaucoma GWAS LN Ref=Ref
-log10 p-value
Recombination Rate (cM/Mb)
LD Ref Var
1.0 > r² ≥ 0.8
0.8 > r² ≥ 0.6
0.6 > r² ≥ 0.4
0.4 > r² ≥ 0.2
0.2 > r² ≥ 0.0
no r² data
48.40
48.60
48.80
49.00
49.20
Chromosome 7 (Mb)
Hits in GWAS Catalog
ABCA13→
CDC14C→


Glaucoma GWAS LN Ref=Ref
-log10 p-value
Recombination Rate (cM/Mb)
LD Ref Var
1.0 > r² ≥ 0.8
0.8 > r² ≥ 0.6
0.6 > r² ≥ 0.4
0.4 > r² ≥ 0.2
0.2 > r² ≥ 0.0
no r² data
139.80
140.00
140.20
140.40
140.60
Chromosome 7 (Mb)
Hits in GWAS Catalog
←PARP12
←SLC37A3
←MKRN1
←DENND2A
←BRAF
←MRPS33
←KDM7A
RAB19→
ADCK2→
NDUFB2→


Glaucoma GWAS CC Ref=Ref
-log10 p-value
Recombination Rate (cM/Mb)
LD Ref Var
1.0 > r² ≥ 0.8
0.8 > r² ≥ 0.6
0.6 > r² ≥ 0.4
0.4 > r² ≥ 0.2
0.2 > r² ≥ 0.0
no r² data
5.40
5.60
5.80
6.00
6.20
Chromosome 8 (Mb)
Hits in GWAS Catalog
MCPH1→


Glaucoma GWAS LN Ref=Ref
-log10 p-value
Recombination Rate (cM/Mb)
LD Ref Var
1.0 > r² ≥ 0.8
0.8 > r² ≥ 0.6
0.6 > r² ≥ 0.4
0.4 > r² ≥ 0.2
0.2 > r² ≥ 0.0
no r² data
83.80
84.00
84.20
84.40
84.60
Chromosome 12 (Mb)
Hits in GWAS Catalog

7


Glaucoma GWAS LN Ref=Ref
LD Ref Var
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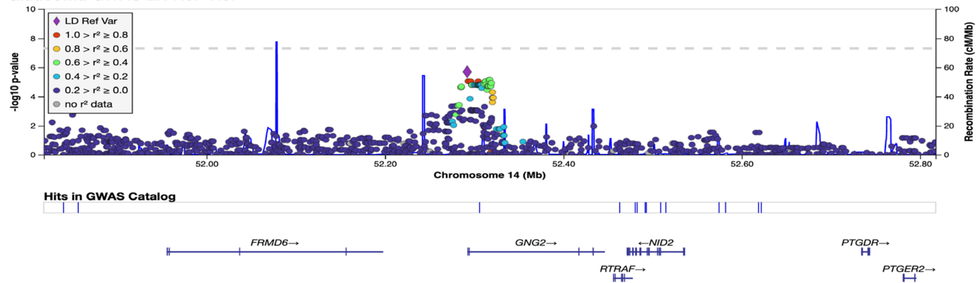
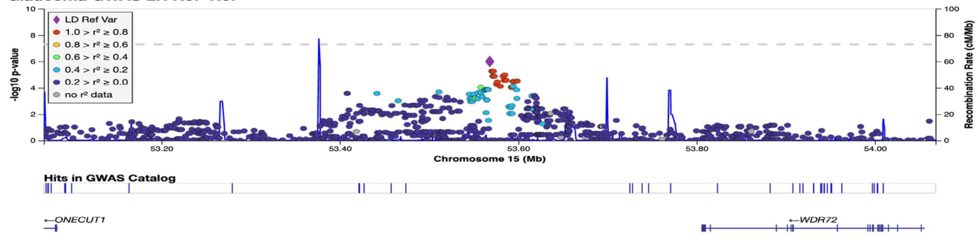
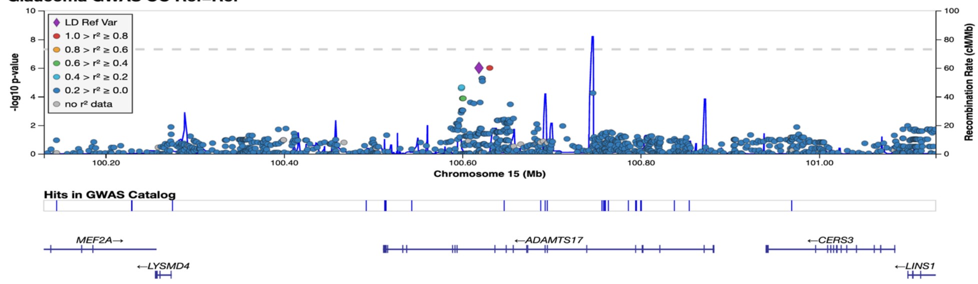
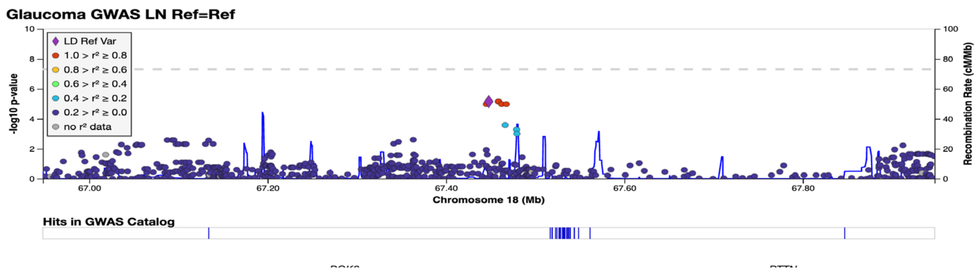
0.8 > r² ≥ 0.6
0.6 > r² ≥ 0.4
0.4 > r² ≥ 0.2
0.2 > r² ≥ 0.0
no r² data
-log10 p-value
Recombination Rate (cM/Mb)
Chromosome 13 (Mb)
Hits in GWAS Catalog
←DIAPH3


Glaucoma GWAS LN Ref=Ref
LD Ref Var
1.0 > r² ≥ 0.8
0.8 > r² ≥ 0.6
0.6 > r² ≥ 0.4
0.4 > r² ≥ 0.2
0.2 > r² ≥ 0.0
no r² data
-log10 p-value
Recombination Rate (cM/Mb)
Chromosome 14 (Mb)
Hits in GWAS Catalog
FRMD6→
GNG2→
←NID2
RTRAF→
PTGDR→
PTGER2→


Glaucoma GWAS LN Ref=Ref
LD Ref Var
1.0 > r² ≥ 0.8
0.8 > r² ≥ 0.6
0.6 > r² ≥ 0.4
0.4 > r² ≥ 0.2
0.2 > r² ≥ 0.0
no r² data
-log10 p-value
Recombination Rate (cM/Mb)
Chromosome 15 (Mb)
Hits in GWAS Catalog
←ONECUT1
←WDR72


Glaucoma GWAS CC Ref=Ref
LD Ref Var
1.0 > r² ≥ 0.8
0.8 > r² ≥ 0.6
0.6 > r² ≥ 0.4
0.4 > r² ≥ 0.2
0.2 > r² ≥ 0.0
no r² data
-log10 p-value
Recombination Rate (cM/Mb)
Chromosome 15 (Mb)
Hits in GWAS Catalog
MEF2A→
←LYSMD4
←ADAMTS17
←CERS3
←LINS1



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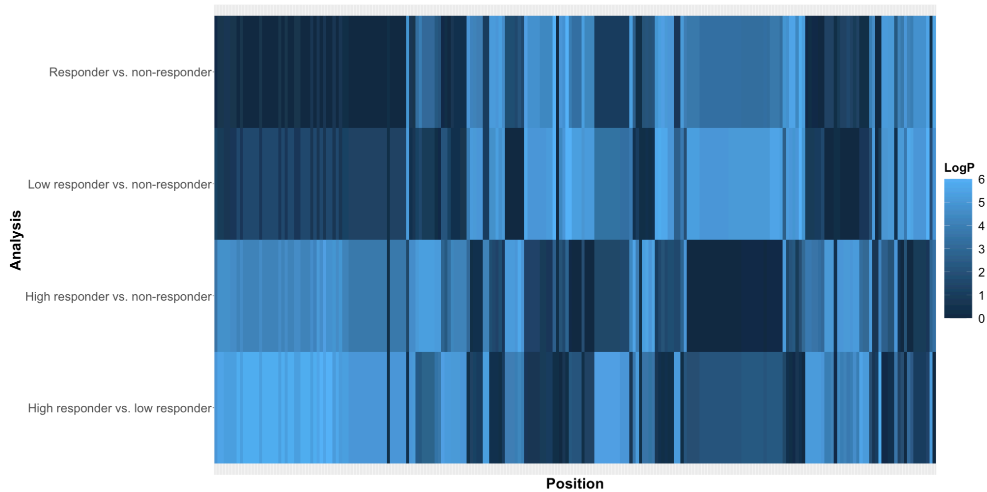
Supplemental material 8. *Heatmap comparing the P-values of all the significant SNPs throughout the four comparisons.*



Supplemental material 9. *GWAS P-value and MAF for genes identified after targeted analysis.*

Responder vs. non-responder	**SNP**	**GWAS P-value**	**MAF**
KANK1	rs142110636	1.15E-05	0.044248
	rs74897202	3.50E-06	0.044248
SPATA18	rs150319632	1.30E-05	0.020649

High responder vs. low responder	**SNP**	**GWAS P-value**	**MAF**
KANK1	rs142110636	8.75E-01	0.085821
	rs74897202	9.29E-01	0.089552
SPATA18	rs150319632	3.88E-02	0.048508

High responder vs. non responder	**SNP**	**GWAS P-value**	**MAF**
KANK1	rs142110636	9.15E-04	0.028926
	rs74897202	4.34E-04	0.02686
SPATA18	rs150319632	7.00E-06	0.016529

Low responder vs. non responder	**SNP**	**GWAS P-value**	**MAF**
KANK1	rs142110636	8.66E-05	0.03808
	rs74897202	1.40E-05	0.03808
SPATA18	rs150319632	2.99E-03	0.011589

7

Chapter 8

GENERAL DISCUSSION

The research presented in this thesis shows how re-using, integrating and re-evaluating publicly available data and knowledge serves towards a better understanding of the functioning of the healthy TM tissue and the pathogenesis of POAG and a corticosteroid-induced increase in IOP. This was done by using multiple bioinformatics approaches and integrative analyses. We also performed a GWAS to obtain a better insight into the genetic components of a corticosteroid-induced increase in IOP. In addition, this thesis also shows how the obtained gene expression data can be used for drug repurposing in order to identify new treatment options for POAG and a corticosteroid-induced increase in IOP. In this chapter we discuss the most important highlights of the thesis. Following, we look ahead and propose two perspectives we created for further research.

Key role for inflammation in POAG?

In chapter three, we identified 92 significantly changed pathways in the TM of patients with POAG compared to individuals without glaucoma. These pathways could be subcategorized into five functional subcategories: extracellular matrix (ECM), inflammation, complement activation, senescence, and Rho GTPase signaling. A network analysis based on these clusters showed that the inflammation cluster shared multiple genes with three of the other clusters (ECM, complement activation, and senescence), suggesting a central role for inflammation in the connection between the involved clusters and in the pathogenesis of POAG. The inflammation cluster could be further subcategorized into interleukin signaling, *NF-κB*, arachidonic acid, and general inflammatory pathways, such as the TNF signaling pathway. Inflammation has already been hypothesized to play a key role in POAG. Multiple studies described that the main focus of influence is the retinal ganglion cell layer, however, there is proof that inflammation also influences the TM.[1] Taurone et al. showed that inflammatory mediators such as *TGF-β1*, *TNF-α* and interleukins such as *IL-6*, induce changes in the ECM and the cytoskeleton of the TM.[2] Moreover, *TGF-β1*, *TGF- β2* and interleukins such as *IL-10* and *IL-8* are upregulated in the aqueous humor of patients with POAG.[3] *NF-κB*, another inflammatory component, is known to be activated in the TM cells in glaucoma where it protects against oxidative stress.[4] Another interesting finding in chapter three are the significantly changed pathways in the inflammation cluster involving arachidonic acid. The latter is a precursor of prostaglandins, and prostaglandin analogues are currently used for the treatment of POAG. They are the only glaucoma drugs that are thought to affect the outflow in the TM.[5] The other treatment possibilities for glaucoma focus on lowering the intraocular pressure (IOP) by decreasing the production of aqueous humor in the ciliary body or by increasing the uveoscleral outflow. The fact that we found prostaglandins as a treatment option in our analyses, is a sound validation of our approach and results. Furthermore, Senescence-Associated Secretory Phenotype (*SASP*) genes, which we found as part of the senescence cluster, might play a key role in POAG as their expression activates multiple inflammation factors such as *TNF-α*, and, among others, *IL-1/NF-κB* and *TGF-β/SMAD* signaling pathways.[6] There is evidence that ageing tissue is chronically inflamed, suggesting a close interplay between senescence and inflammation.[7] This was also confirmed in our network analysis and it could explain the increase in IOP with age.

The role of the cell cycle in a corticosteroid-induced increase in intraocular pressure

In chapter four, we found multiple molecular pathway clusters to be significantly changed in bovine responders compared to bovine non-responders. Most of these clusters were also identified after comparing TM tissue exposed to corticosteroids with TM tissue exposed to a neutral medium (in both human and bovine subjects). Interestingly, the cell cycle cluster, was only significantly changed after comparing responders and non-responders. This suggested that the pathways or multiple genes within this functional cluster had to behave in opposite ways in responders compared to non-responders. Upon visualization of the cell cycle pathway, we demonstrated that the gene expression changes of multiple genes involved in this pathway were indeed opposite or showed relatively large differences between bovine eyes with and without a corticosteroid-induced increase in IOP. It is known that corticosteroids influence the cell cycle. However, their effect on the cell cycle (activation or inhibition) is found to be different between cell types and depends on the dosage and types of corticosteroids.[8, 4, 9] Our new finding that the activation of the cell cycle in the TM is different between responders and non-responders adds to this knowledge. Upon further investigation of the genes involved in the cell cycle, *CCND1* and *CCND2*, known to drive the G1/S phase transition by binding with multiple cyclin-dependent kinases (*CDK*s), were upregulated between responders and non-responders. *CDK*s are necessary to regulate the progression through the cell cycle and were also upregulated. In addition, *CDKN1A*, *CDKN1B* and *CDKN1C* normally inhibit *CDK*s but were downregulated in our results. Therefore, the gene expression of the mentioned genes, suggest an increased activity of the cell cycle in the TM of eyes with a corticosteroid response. Furthermore, in chapter seven, in which we performed a GWAS study comparing patients with and without a corticosteroid-induced increase in IOP, we also found clues for the involvement of the cell cycle in the pathogenesis of a corticosteroid response. Some of the identified SNPs were associated with *CUX1* and *GPATCH2*. *CUX1* has been shown to play a role in the cell cycle progression and *GPATCH2* has been found to inhibit the G1-S phase within the cell cycle.[10-12] In addition, within chapter seven, we also made a distinction between high and low responders and found that these groups are likely to be not only phenotypically but also genetically different. Interestingly, *CUX1* and CPATCH2 were only found in the high responders, which might suggest that the role of the cell cycle is larger in the pathogenesis of a high response compared to a low response. The role of the cell cycle in a corticosteroid-induced increase in IOP has not been described before and is of interest for further investigation.

The role of fibronectin in POAG and a corticosteroid-induced increase in IOP

In chapter three, we found fibronectin to be present in three out of the five functional categories (i.e. ECM, general inflammation and senescence) we identified to be involved in the pathogenesis of POAG. In addition, in chapter four, fibronectin was found to be shared by six different clusters related to corticosteroid response, which was the highest number of clusters a gene in this study was represented in. Therefore, fibronectin seems to play a central role in both POAG and a corticosteroid-induced increase in IOP. Fibronectin has been shown to be one of the ECM proteins and it can be found in the aqueous humor as well.[13,14] In chapter two, we found fibronectin to be expressed in the TM of healthy patients. Even though it belonged to the top 50% highly expressed genes and not the top 25% highly expressed genes, its expression indicates a role in the functioning of the healthy TM. As identified in chapter three, the gene expression of fibronectin is significantly upregulated in patients with POAG. Currently, the role of fibronectin in the pathogenesis of POAG is not yet completely understood. However, it has been shown that it regulates the deposition of collagen IV, laminin and fibrillin in the ECM of the TM. An increase in fibronectin is therefore hypothesized to increase the outflow resistance through the ECM of the TM.[15] In addition, the assemblage of fibronectin into fibrillin is thought to be *RhoA*-related. Studies have shown that an increase in *RhoA* causes an increase in fibril formation. Currently Rho-kinase inhibitors, which target *RhoA*, are in some countries already used to treat an increased IOP (see further).[16] Although not represented as a direct connection in our network, the above finding connects fibronectin also to the identified RHO GTPase cluster, making our analysis and the possible key role of fibronectin even more robust. We did not find a connection between fibronectin and RHO GTPase in the network. This may be due to the fact that, although a connection has been described in literature, this has, for as far as we know, not been incorporated yet in one of the pathways in the used pathway databases (i.e. WikiPathways, KEGG and Reactome). In addition to a role of fibronectin in the ECM and a link to RHO GTPase, there is also evidence that senescence and inflammation, clusters in which fibronectin was represented, are involved in an increased expression of fibronectin in POAG. An increase in age has shown to increase the fibronectin expression values in the TM. In addition, *TGF-β2*, an inflammatory component, is thought to increase the expression of fibronectin in the aqueous humor.[17-19]

In contrast to the increased expression of fibronectin in patients with POAG, we found fibronectin to be downregulated in bovine responders as compared to non-responders to corticosteroids. As discussed in chapter four, the bovine dataset we used (Bermudez et al.[20]) described a discrepancy between the gene expression of fibronectin and its protein expression in bovine eyes with a corticosteroid response. Multiple studies investigated the fibronectin gene and protein expression in TM after

exposure to dexamethasone. Human transcriptomics studies which investigated the gene expression of fibronectin after exposing TM cells to corticosteroids (Fan et al.[21], Nehmé et al.[22], Kwon et al.[23], Matsuda et al.[24], and Faralli et al.[25]) all found fibronectin to be upregulated. As these studies were included in our study, we found the average gene expression of fibronectin after exposing TM cells to corticosteroids to be significantly upregulated. For the fibronectin protein expression however, the results are not uniform (see also chapter four). Nevertheless, immunocytochemistry on human TM cells treated with dexamethasone showed fibronectin to be deposited as an organized fibrillary sheet. This was not observed in human TM cells exposed to a control medium and might, similar to the mechanism as described above for POAG, increase the outflow resistance.[26] Interestingly, this was reported by a study that found no significant difference in the protein expression of fibronectin between human TM cells exposed to dexamethasone or those exposed to a control medium.[26] In addition, the study of Li et al.[27] and Steely et al.[28] reported the fibronectin protein expression to be different between samples. As a corticosteroid response occurs in approximately 30% of the population, we hypothesized that these discrepancies might be explained by the corticosteroid response status of a patient. Currently, to our best knowledge, only two studies investigated the protein expression of fibronectin in corticosteroid responders versus non-responders. Both studies used bovine TM tissue.[20, 29] The study of Bermudez et al. reported an increased expression of fibronectin protein levels in responders but not in non-responders.[20] The study of Mao et al. used an anterior segment perfusion system and found the fibronectin protein expression to be induced in the TM of three out of eight responders and in one out of six non-responders, this difference however was not statistically significant ($p>0.05$).[29] Therefore, the role of responders and non-responders in the protein expression of fibronectin needs further investigation.

In conclusion, we found fibronectin to play a central role in multiple of the identified functional clusters in both POAG and a corticosteroid-induced increase in IOP. Although the precise role of fibronectin protein and gene expression in corticosteroid responders is currently unknown, our finding adds to existing clues such as the changes seen in the fibrillary deposition after exposing TM to corticosteroids and in POAG. Further research is needed to completely unravel the role of fibronectin in both POAG and a corticosteroid-induced increase in IOP.

The overlap in molecular processes between POAG and a corticosteroid-induced increase in IOP

As indicated in the introduction of this thesis, there is a well-known interplay between a corticosteroid-induced increase in IOP and POAG. Patients with POAG are not only more susceptible to develop a corticosteroid-induced increase in IOP but patients

that had a corticosteroid response in the past have a higher risk of developing POAG. In addition, relatives of patients with POAG also have a higher chance to develop a corticosteroid-induced increase in IOP.[21, 30-34] The pathway analysis for POAG and a corticosteroid-induced increase in IOP revealed four shared functional clusters: ECM, collagen, senescence and inflammation. The evidence for these clusters in both POAG and a corticosteroid-induced increase in IOP is discussed below.

Changes in the ECM and collagen have been identified in patients with POAG and after exposing the TM to corticosteroids. Studies showed that the amount of sheath-derived plaques is significantly greater in patients with POAG when compared to non-glaucomatous controls.[35] Multiple components have been identified within these sheets: collagen I, III, IV,V and VI, fibrillin and fibronectin.[36] It has also been shown that these plaques are not induced by the use of glaucoma medication.[37] TM exposed to corticosteroids show an accumulation of type IV collagen and fibronectin in the ECM as well.[38] In addition, patients with long-term treatment for corticosteroid-induced glaucoma showed similar plaques as found in POAG, however their localization within the TM was different.[39] Lastly, above, we already described that we identified fibronectin in chapters three and four. In addition, in chapter three, actin was identified as a subcluster of ECM. Although, actin was not identified as a functional cluster after comparing bovine responders and non-responders, or after comparing human TM tissue exposed to corticosteroids, *KANK1* was significantly downregulated after comparing bovine responders and non-responders (chapter four). This gene regulates the polymerization of actin and was also identified in additional targeted analysis in our GWAS study (chapter seven). Actin is known to be an important component of the TM and is believed to be altered in patients with POAG and a corticosteroid-induced increase in IOP.[40] Cross-linked actin networks (CLANs) are formed in the TM. These formations are more common in patients with glaucoma and after treating the TM with corticosteroids.[40, 41] The formation of these CLANs are thought to be responsible for a reduced outflow of aqueous humor and thereby for the increase in IOP. [42, 43]

Furthermore, we found senescence to be involved in the pathogenesis of POAG and to be highly likely to play a role in corticosteroid-induced increase in IOP as well. Ageing is a known risk factor for POAG.[44] The older an individual gets, the higher the chance of developing POAG. This has directly been correlated with changes in the TM. For example, aging causes an increase in TM-stiffness.[45, 46] In contrast, the frequency of corticosteroid-induced glaucoma is hypothesized to occur in a bimodal distribution as both children and older patients seem to be at risk to develop a corticosteroid response. Armaly et al. described a significant higher increase in eye pressure in older patients after the use of topical corticosteroids (age groups compared were 18-32 and 40-62).[47] The study of Lam et al. showed that the frequency of corticosteroid response

in children ranges around 59.2-71.2%, depending on age and frequency of topical corticosteroid use.[48] As the frequency of a corticosteroid response is around 18-36% in adults[30], children seem to be more likely to develop a corticosteroid response. In chapter four, we integrated the publicly available gene expression data investigating the effect of dexamethasone on the TM from human and bovine subjects. In addition, we investigated the differences in gene expression data of bovine corticosteroid responders and non-responders. As indicated in that chapter, we saw opposites in gene expression between responders and non-responders for cell cycle related processes. For the senescence cluster, we saw a similar pattern. Therefore, we stated in chapter four that it is highly likely that both cell cycle and senescence are involved in the pathogenesis of a corticosteroid-induced increase in IOP.

We already described an association between senescence and inflammation earlier in this chapter. Additionally, as discussed above, previous research has implicated a large role of inflammation in the TM of patients with POAG.[2, 3, 4, 5, 49] We also found that the inflammation cluster (chapter three), plays a central role in the pathogenesis of POAG. NF-κB was in chapter three defined as a subcluster of inflammation and might be of special interest as it was also identified after exposing the TM of human and bovine subjects to dexamethasone. In addition, multiple pathways related to *NF-κB* were just below the threshold after comparing the gene expression in TM of bovine corticosteroid-responders and non-responders, suggesting that the functioning of *NF-κB* might be different between responders and non-responders. In addition, in chapter seven, we identified multiple SNPs that are associated to *GPATCH2*. This gene has been shown to inhibit NF-κB.[12] Therefore, multiple findings within this thesis that indicate that NF-κB might be involved in the pathogenesis of POAG and a corticosteroid-induced increase in eye pressure.

Differences in molecular processes between POAG and a corticosteroid-induced increase in IOP

The comparison between POAG and a corticosteroid-induced increase in IOP is not as straight forward as it seems. For the four clusters that we found to be involved in both POAG and a corticosteroid-induced increase in IOP, we cannot assume that their gene expression is similar. Or in other words, have these processes the same direction and pattern of activation in patients with POAG and a corticosteroid-induced increase in IOP? Therefore, we first identified which genes are significantly changed in patients with both POAG and a corticosteroid-induced increase in IOP and found only 58 shared genes. In addition, the gene expression of 42 of these genes were opposites (up in POAG and down in a corticosteroid-induced increase in IOP or vice versa). Following, we visualized the gene expression for all genes measured in at least one of both diseases

on the pathways involved in the shared clusters. This showed that the gene expression of multiple genes within the pathways are different between patients with POAG and a corticosteroid-induced increase in IOP. An example of a pathway with mapped data is presented in figure 1.

In conclusion, POAG and a corticosteroid-induced increase in IOP share several clusters of functional processes. These may partially represent shared mechanisms, but there are also clear differences in the changes observed within the identified functional clusters between these patient groups. In addition, some clusters we identified were not shared between the two diseases. Therefore, the pathogenesis of POAG and a corticosteroid-induced increase in IOP might show some similarities such as changes in the ECM, fibronectin and formation of plaques, however there are clear differences between both diseases as well.

Figure 1. *Visualization of the differentially expressed genes in POAG and corticosteroid-induced glaucoma for the focal adhesion pathway (WP306). Every rectangle represents a gene. The first part of the rectangle represents the differential gene expression in the POAG dataset, and the second part of the rectangle represents the differential gene expression in the corticosteroid-induced increase in IOP dataset. Red color indicates upregulation, blue color indicates downregulation. The brighter the intensity of the color, the higher the up- or downregulation. A grey color indicates that the gene was not measured in the respective dataset.*

Susceptibility to corticosteroids important in developing a corticosteroid response?

In chapter seven, we identified multiple SNPs associated to a corticosteroid-induced increase in IOP in *ESRRγ* and *UBL5*, both genes that might play a role in how patients react to corticosteroids. *ESRRγ* (estrogen related receptor gamma) is one of the subgroups of the estrogen related receptors. Despite what the name suggests, these receptors are not modulated by estrogen. However, the molecules that target these receptors are not known yet.[50, 51] A study of Wang et al. showed that there is a crosstalk between the *ESRRγ* and glucocorticoid receptor (GR) signaling in skeletal muscle cells. Treatment of skeletal muscle cells with an *ESRRγ* receptor agonist led to a significant increase in the expression of GR*α* protein. In addition, genes that are targeted by the latter receptor were induced as well. After using an *ESRRγ* receptor antagonist, the opposite effect was observed.[52] Furthermore, the formation of CLANs after exposing the TM to corticosteroids, have been found to be mediated through the GR.[40] In addition we identified *UBL5* (ubiquitin like 5 gene). In one of our other studies (chapter two), we found this gene to be highly expressed in the healthy TM. This shows that *UBL5* plays an important role in the functioning of this tissue. The study of Lee et al. performed a epigenome-wide association analysis to investigate the methylation levels of peripheral blood mononuclear cells (PBMCs) of patients with an acute exacerbation of chronic obstructed pulmonary disease (COPD). Some patients with an exacerbation have a good response to the use of corticosteroids while others will respond not or insufficiently. Their study showed that in patients with a good response to corticosteroids during an exacerbation, genes in the ubiquitin conjugation pathway are involved.[53] This suggests that *UBL5* and genes closely related to this gene might be involved in how patients with COPD react to their corticosteroid treatment.

To explore this further, in chapter seven, as indicated before, we made a distinction between high and low responders. Interestingly, both *ESRRγ* and *UBL5* were only identified in high responders. Therefore, these patients might react differently to corticosteroids compared to low responders.

Rho-kinase inhibitors are likely to be an effective treatment for POAG and a corticosteroid-induced increase in IOP.

Throughout the thesis, multiple of the identified functional processes and genes are involved in the rho-kinase pathway. Some rho-kinase inhibitors such as netarsudil and ripasudil are currently already used to treat glaucoma. Rho-kinase inhibitors influence the outflow of aqueous humor through the TM and do this mainly by causing alterations in the actin stress fibers.[54] They have been shown to disrupt the actin cytoskeleton and cause a decrease in TM stiffness and enhance the outflow of aqueous humor.[55, 56] In addition, they are also believed to have neuroprotective effects on the optic nerve and retina which might inhibit the progression to glaucomatous neuropathy.[16, 57-59]

As already mentioned above, it has been shown that fibronectin regulates the deposition of, among others, fibrillin in the ECM of the TM. The assemblage of fibronectin into fibrillin is thought to be *RhoA*-related. An increase in fibronectin is hypothesized to increase the outflow resistance through the ECM of the TM.[15] In chapter two, we found that fibronectin is likely to play a role in the functioning of the TM of healthy patients. In addition, in chapter three, the gene expression of fibronectin was significantly upregulated in patients with POAG. In chapter three, we also found the functional category of Rho GTPase to be significantly different between patients with POAG and healthy controls. Downstream targets of Rho GTPase are among others Rho/Rho-associated kinases (*ROCK*s), *RhoA* and myosin light chain genes. In chapter five, the chapter on drug repurposing, we showed that netarsudil and ripasudil, both known rho-kinase inhibitors, target *ROCK1* and *ROCK2* which are part of the Rho GTPase cluster.

In addition to clues for rho-kinase inhibitors to treat POAG, we also found multiple indications that rho-kinase inhibitors might be particularly effective to treat a corticosteroid-induced increase in IOP. In addition to a role for fibronectin in corticosteroid-induced increase in IOP as well (chapter four), some of the identified genes in chapter seven are known to be targeted (directly or indirectly) by rho-kinase inhibitors. We identified *TIAM2* and *CaMK4*, who both suppress *RhoA* activity through *Rac1*.[60-62] *TIAM2*, as identified in our study, and *TIAM1* both activate *Rac1*. There is a close interaction between *Rac1*, *RhoA* and *ROCK*. *Rac1* inhibits *RhoA* and *RhoA* in turn can inhibit *Rac1* by activating *ROCK*. *ROCK* has been shown to inhibit *TIAM1* and *TIAM2* which leads to a decreased activity of *Rac1* and an increased activity of *RhoA*. Therefore, there seems to be a feedback loop for the regulation of *Rac1* and *RhoA* through *TIAM1* and *TIAM2*. Furthermore, Fusadil, a *ROCK*-inhibitor has been shown to increase *TIAM1* which in turn activates *Rac1* which leads to a diminished activity of *RhoA*.[61] *CaMK4* is also thought to regulate the ratio of activated *Rac1* and *RhoA* GTPases.[62] Interestingly, *CaMK4* and *TIAM2* were not found to be significantly changed between bovine responders compared to non-responders, nor after exposing the human or bovine TM to dexamethasone (chapter four).

In chapter seven, we also performed an additional targeted analysis and investigated whether the results of our GWAS analysis contained other genes that are involved in the rho-kinase pathway and are targeted by rho-kinase inhibitors. We were mainly interested whether any genes in the rho-kinase pathway had just not met the criteria for the global analysis, but were strong enough to be considered in a contextual and knowledge driven approach. This analysis retrieved *KANK1* and *SPATA18* which are targeted (directly or indirectly) by rho-kinase inhibitors. *KANK1* (KN motif and ankyrin repeat domains 1) regulates the polymerization of actin.

As mentioned above, actin, and more specific cross-linked actin networks (CLANs), are known to be an important component of the TM and are known to be increased in patients with a corticosteroid-induced increase in IOP.[40, 41, 42, 43] *KANK* has been shown to regulate the actin polymerization through the inhibition of *RhoA*, which, as mentioned before, is a downstream target of rho-kinase inhibitors. When *KANK* was expressed, the amount of active *RhoA* was significantly reduced. Furthermore, knockdown of *KANK* significantly increased the amount of active *RhoA*.[63] The activation of *RhoA* in the aqueous humor outflow pathway has been shown to decrease the outflow. This was caused by alterations in the actin stress fibers.[54, 55, 56] Furthermore, in chapter four, we found *KANK1* to be significantly downregulated after comparing bovine responders and non-responders. As knockdown of this gene causes an increase in *RhoA*, this finding supports our hypothesis that rho-kinase inhibitors might not only be effective to treat POAG but also shows high potential to be effective to treat a corticosteroid-induced increase in IOP as well.

Based on the results above, rho-kinase inhibitors might be particularly effective to treat a corticosteroid-induced increase in IOP. Furthermore, as most of the identified genes in the GWAS study were found in both high and low responders, rho-kinase inhibitors are likely to be effective to treat both patients groups.

A recently published randomized, double-masked trial, investigated whether the use of netarsudil compared to a placebo after corneal transplantation lowered the risk to develop a corticosteroid-induced increase in IOP. They showed that 14% of patients using netarsudil had an IOP-increase defined as an increase of ≥ 10 mmHg from baseline or ≥ 24 mmHg compared to 21% after treatment with placebo.[64] Although this difference was not statistically significant, the trend shows a lower risk for the group using netarsudil. Previous studies have shown that rho-kinase inhibitors induce a dose- and time-dependent increase in the outflow of the aqueous humor through the TM.[56, 65, 66] Therefore, the use of a different dosage or the use of one of the other rho-kinase inhibitors may be more effective. In addition, a recently published study found that ripasudil significantly lowered the IOP in patients with a corticosteroid-induced increase in IOP.[67]

Strengths of the research presented in this thesis

During the past decades, biochemical and histological in vitro studies have been performed in order to identify the pathogenesis of POAG and a corticosteroid-induced increase in IOP. The number of molecules that can be investigated within these studies, however, is limited. Using omics technologies, we are currently able to measure the expression values of several thousands of molecules from one sample of affected tissue, leading to an exponential increase in data. One example of omics studies are transcriptomics studies which investigate the differences in gene expression by comparing the levels of RNA for each gene in the healthy and diseased TM tissue. Genes that are found to be differentially expressed are likely to be associated with the disease. However, a gene is only a small fraction of the molecular processes it is involved in. Pathways represent such molecular processes and shows the genes (or, mostly, their encoded products) that are involved within these processes and how they are related to other genes. The presented methods in chapters two to four are examples of the system biological approaches which can be applied to re-use, integrate and re-analyze publicly available (transcriptomic) data. As these methods allow to build on the shoulders of previously performed research, this will eventually spare the scientific world a lot of expenses as basic experiments do not have to be repeated to the same extend. In chapters two to four, we performed multiple pre-processing steps on all publicly available studies in order to be able to use the data for further overarching investigation. After performing a thorough quality control, the high-quality data were integrated in high-quality databases containing all currently available data of the healthy TM, POAG, and a corticosteroid-induced increase in IOP which can be used as future reference or for further research. As already discussed above, using these data we were able to identify some known aspects of the functioning of the healthy TM and the pathogenesis of POAG and a corticosteroid-induced increase in IOP which validates our research. In additionour analytical approaches revealed multiple additional clues which can be used to not only obtain a better understanding in the pathogenesis of POAG and a corticosteroid-induced increase in IOP but also to identify new treatment options. Furthermore, the tools we used in these chapters are all freely available and easy accessible. Therefore, the methods we described can be applied by other researchers and for other diseases as well. This might especially be beneficial for the investigation of rare diseases or tissues that are difficult to obtain. Lastly, the methods can also be applied for individualized treatment as will be discussed later in this chapter.

One of the other strengths in this thesis is the careful selection of our study population and the criteria used to identify a corticosteroid responder in chapter seven. The definition of a corticosteroid response differs throughout studies. The most commonly used definition, however, was formulated by Armaly and Becker as a positive change in IOP of more than 6 mmHg above normal baseline IOP after the use of corticosteroids.[31]

However, this definition does not take into account whether the IOP elevation is caused by the use of corticosteroids or by other factors. Therefore, we carefully assessed whether an elevation of 6 mmHg in IOP was truly due to a corticosteroid response or not. Furthermore, the IOP increase also had to be related to changes in corticosteroid use. As the last two criteria are more or less subject to personal interpretation, all participants were independently assessed by two ophthalmology residents. They also took into account the results of chapter six in which we identified which risk factors cause an increase in IOP after keratoplasty. After the first screening, they already reached consensus about 80% of the participants. The other cases were discussed with a glaucoma specialist to make a final decision. In case it was not clear whether the IOP elevation was due to a corticosteroid response or due to other factors, the participant was excluded for further analysis. This lowered the risk to incorrectly classify a non-responder as responder and vice versa. Furthermore, the study of Fini et al. showed that most patients develop a corticosteroid response within the first 8 months of corticosteroid use.[68] As our study population consists of corneal transplantation patients which had to use corticosteroids for at least one year, this risk to incorrectly classify a responder as non-responder is very low. Within our study cohort, 39.5% of the patients had a corticosteroid response which lies in the range of expectations as reported by previously performed studies.[30, 69]

Methodological challenges

In chapters two to four we re-used, integrated and re-analyzed publicly available data. Although this enabled us to obtain a better insight into the molecular processes of the healthy TM, POAG and a corticosteroid-induced increase in IOP, we also faced multiple challenges. Similar to a regular meta-analysis, the data were derived from other researchers and therefore, a critical appraisal of the quality was necessary. Consequently, we performed an extensive quality control on each of the datasets in order to obtain a better insight in the quality of the experiments and the data deducted therefrom. Samples deviating within a dataset were removed and datasets with an overall low quality were excluded as well. The importance of performing a quality control was stressed by the fact that we had to exclude multiple datasets and samples in all three chapters in which we re-used data. As the data were derived from different research groups and their original research questions might have been different from ours, there were variabilities between the studies, the study methods and their included individuals. In order to obtain results that are less dependent on differences between individual studies, we combined and similarly pre-processed the selected high-quality gene expression datasets of the included studies for the reported analyses. In addition, for multiple of the included datasets, we had to contact the researchers to obtain additional information. Although most researchers responded to our request, we noticed that often several

details could not be retrieved anymore or that additional information we required, had not been noted at the time of the research. The fact that more and more often researchers re-use data of previous research and bioinformatics methods that allow these analyses are improving at a rapid pace, stresses the need for FAIR data, i.e. data that is Findable, Accessible, Interoperable and Reusable. Since the outline of the FAIR guiding principles for scientific data and stewardship was published, data is already getting more FAIR than it used to be, although there is still room for improvement.[70] In addition, researchers should aim to target the collection of a large number of biological parameters of the biological system and their included individuals (metadata) as these are crucial for a detailed in silico representation and analysis of the complex biological systems. However, this requires a change in the mindset of current research and will probably need some time. Especially the collection of metadata beyond that needed for the original study, requires awareness within the community.

In chapters two to five, we performed pathway analysis. As pathways represent and visualize molecular processes, after mapping data they can quickly indicate which processes are likely to be involved in a certain disease. However, there are also some disadvantages. Not all molecular knowledge is represented in pathways yet. As indicated earlier in this chapter for example, we found a link between fibronectin and RHO GTPase in the literature but this was not represented in a pathway yet. However, the coverage of genes and knowledge in pathway databases increases continuously and therefore the power of the type of analyses performed in our studies will further increase over time, showing its promise already.[71]

Furthermore, as is the case with the P-value of 0.05, the used cut-off for significantly changed pathways is also arbitrary. It does not discern between true and not true. Therefore, it should not be interpreted as a hard cut-off and we should keep an open mind for pathways just below this threshold as well. With pathway analyses there are some reasons that pathways might not meet the chosen cut-off value. For example, some of the pathways are very large and contain information of multiple processes. If one of these processes is not involved in the pathogenesis of the investigated tissue, the complete pathway might be just below the chosen threshold level. This is also the case with pathways that contain a small number of measured genes, which leads to a lower statistical power for those pathways. Similarly, if several of the genes were not measured within the included datasets, a part of the information is lacking which might cause the pathway to lie just below the cut-off of significance. Therefore, we should be careful with the interpretation of the pathway statistics results and interpret them in a biological context. The advantage of tools that, besides performing overrepresentation statistics, also display the data on the pathway diagrams, such as PathVisio (*www.pathvisio.org*)[72,73], is that the user can easily investigate what is happening and whether this is relevant.

Future perspectives: Using peripheral blood mononuclear cells for further research

In chapter four, we obtained an overview of the molecular processes that are likely to be involved in the pathogenesis of a corticosteroid-induced increase in IOP. We found evidence that also at TM gene expression level there is a different reaction to corticosteroids in responders compared to non-responders. An important part of these results, however, were obtained after investigating bovine data. Although we found no differences in identified molecular processes in human and bovine tissue exposed versus non-exposed to corticosteroids and bovine tissue has been proven to be suitable for this purpose[29, 74, 75, 76, 77], it is necessary to validate these findings in human subjects. This emphasizes the need of further investigation with human TM tissue of identified responders and non-responders instead of solely exposing TM cells to corticosteroids.

However, TM cells are situated in the anterior segment of the eye and are very difficult to obtain. It is not possible to simply take a biopsy as this would disrupt the integrity of the eye which entails multiple risks such as infection, detachment of the retina and cataract formation. Currently, there are only two possibilities for collecting this tissue. The first is to collect this tissue post-mortem, however, very often it is not known whether the patient would have been a corticosteroid responder or not. The second possibility is to collect it from patients that undergo a specific glaucoma-operation (trabeculectomy). This is performed very rarely in patients with a corticosteroid-induced elevation of the IOP and is therefore not only scarce but would also comprise only a very specific subgroup of patients with a corticosteroid response.

In chapter seven we described the GWAS we performed in a retrospective cohort study. Based on retrospective chart investigation, we carefully determined which patients of this cohort were corticosteroid responders and which patients were non-responders. Upon inclusion, we collected venous blood samples from all 367 patients. From these blood samples we did not only isolate DNA, but also PBMCs. PBMCs originate from hematopoietic stem cells (HSCs) that reside in the bone marrow. PBMCs are all blood cells with round nuclei and consist of monocytes, lymphocytes, T cells, B cells, NK cells and dendritic cells. The relative frequencies of these different cell types within the PBMCs show differences between individuals but lymphocytes make up the largest part (70-90%).

In conclusion, this review provides an overview of all investigated risk factors for the development of OHT after keratoplasty and the level of evidence that is available for each risk factor. Based on the evidence tables, factors with a definitive and probable association with an increased risk for OHT have been established. This can help to

identify patients at risk and to individualize patient care concerning the choice of therapy, postoperative treatment and follow-up. In addition, we have shown that many risk factors still lack sufficient evidence to determine its association and need further investigation.

Blood sampling rather than trying to obtain in vivo TM cells is a far less invasive and more practical approach. The collected PBMCs offer multiple possibilities for the investigation of the corticosteroid response in blood cells of patients identified as responders or non-responders to a corticosteroid-induced increase in IOP. It not only allows us to further investigate the response on a population or group level, but also for each patient individually:

- How does an individual patient react to corticosteroids?
- What are the individual molecular processes?
- How can this reaction be counteracted by a drug?
- How would an individual patient react to a possible new treatment?

Therefore, forming the first steps to individualized insight in pathogenic mechanisms and treatment. Furthermore, this collected material can help us to further investigate the molecular processes that are involved in the pathogenesis of a corticosteroid-induced increase in IOP in these patients. Two possible study designs are proposed below:

1. TM-like cells generated from PBMCs

A possibility is to use the PBMCs to generate TM-like cells and use these cells for further research. For as far as we know, human PBMCs have not been transformed into TM cells yet. However, it has been shown that induced pluripotent stem cells (iPSCs) can be generated from PBMCs. These iPSCs resemble embryonic stem (ES) cells and are therefore suitable to be used as a stem cell.[78] Therefore, iPSCs can in theory be used to generate TM cells. Previous studies have shown that TM-like cells have been obtained successfully from iPSCs. A study of Ding et al. for example used mouse iPSCs generated from fibroblasts which were co-cultured with human TM cells and differentiated in TM-like cells.[79] The induced cells did not only resemble morphological features of TM cells, they also showed an increase in the production of *MYOC* after exposure to corticosteroids which has recently been defined by Keller et al. to be a reliable marker for TM tissue.[80, 81] Additionally, the expression of specific pluripotent markers decreased and the expression of TM related markers such as caveolin (*Cav1*) and collagen IV (*Col4A5*) increased.[79] Another example is the study of Abu-Hassan et al. in which glaucomatous damage was

mimicked in a perfused anterior segment organ culture by exposing it to Saponin which increased the eye pressure. Following, they successfully transplanted human TM-like iPSCs which integrated into the TM of the perfused anterior segment organ culture and restored the IOP homeostasis.[82] Zhu et al. transplanted TM-like iPSCs into the anterior chamber of a transgenic glaucoma mouse model with mild-severe TM dysfunction. This led to a significant reduction in IOP and improved the aqueous humor outflow.[83, 84]

As shown above, TM-like cells have successfully been derived from iPSCs and are of interest for further investigation. The following experiment is therefore proposed by us. As we identified which participants within our study cohort (chapter seven) are responders and non-responders, we can use their PBMCs and differentiate them into IPSCs and eventually TM-like cells. Following, the PBMC-derived TM cells of both responders and non-responders can be exposed to corticosteroids. Performing high throughput RNA-sequencing on these cell cultures allows the identification of differentially expressed genes in the TM of human responders compared to non-responders. In addition, the results of these analyses can be used as input for pathway and network analysis in order to obtain a better insight in which molecular pathways are involved in the individual pathogenesis of a corticosteroid responder. As the gene expression data of the TM in human responders is currently lacking, this experiment might be of great value towards a better understanding of a corticosteroid-induced increase in IOP. Moreover, once the TM-like cells have been derived from the PBMCs, this would also enable additional research. We could for example investigate how the gene expression changes after exposing these cells to new treatment possibilities. It would also allow an individualized approach. In addition, by selecting patients with certain SNPs from our GWAS, we can study specific pathways in which these SNPs are involved and even individualize the investigation and eventually treatment even more.

Although the use of stem cell derived TM cells would be preferable, it is still very expensive, difficult and time-consuming to perform, limiting its clinical application. Therefore, we suggest the experiment below as well.

2. RNA sequencing on PBMCs exposed to corticosteroids

PBMCs are known to express the glucocorticoid receptors (GCR*α* and GCR*β*) with which corticosteroids interact. Multiple studies have shown that there is a correlation in the expression of one or both receptors and the sensitivity to corticosteroids. It has been shown that the expression of mRNA for GCR*β* was higher in patients with corticosteroid-resistant colitis ulcerosa[85] and corticosteroid-resistant asthma[86]. However, not only the expression of these receptors was found to be different. Multiple studies have shown

that genes or gene products are affected after exposure to corticosteroids and that this can be different between patient groups. Goleva et al. for example showed that the mRNA expressions of cytokines such as *MKP-1* and *IL-8* are significantly higher in patients with corticosteroid-resistant asthma.[86] Hew et al. also showed that the nuclear histone deacetylase (HDAC) activity in PBMCs of patient with severe asthma is lower than in patients with less severe asthma. In addition, HDAC activity reduction correlated directly to the degree of corticosteroid insensitivity in the release of certain cytokines.[87] Moreover, the needed concentration of dexamethasone to inhibit the proliferation of PBMCs was higher in patients with vs. without corticosteroid-resistant asthma.[86] This effect was also seen in patients with corticosteroid-resistant rheumatoid arthritis.[88] Interestingly, in chapter four, we identified cell cycle/cell proliferation as one of the molecular processes that are activated in patients with a corticosteroid induced ocular hypertension. Not all patients develop a corticosteroid induced increase in IOP after using corticosteroids. As mentioned above, it is likely that an increased or decreased sensitivity (resistance) for corticosteroids might play an important role. The processes that are thought to be relevant in corticosteroid-induced glaucoma are rather general processes such as cell cycle and senescence which can be studied in PBMCs as well. Therefore, performing RNA-sequencing and pathway analysis on PBMCs may indicate which molecular processes could play a role in the pathogenesis of a corticosteroid induced increase in IOP. In addition, a previously performed study investigated the gene expression in PBMCs between patients that had an acute primary angle-closure glaucoma and healthy controls. They found the TSP1 gene to be highly upregulated in patients with acute primary angle-closure glaucoma. This gene plays, among others, a role in cell adhesion/migration.[89] Within this thesis, we already found these processes to play a role in both the healthy TM and POAG and likely to be involved in the pathogenesis of a corticosteroid-induced increase in IOP. In addition, *TSP-1* is also known to be highly expressed in the TM of patients with POAG.[90] Although these studies do not investigate a corticosteroid-induced increase in IOP, their findings in the PBMCs resemble findings in the TM of glaucoma-diseased TM. This suggests that PBMCs might be suitable for investigating a corticosteroid-induced increase in IOP as well. Therefore, we propose the following study: Performing RNA-sequencing on the PBMCs of responders vs. non responders with and without treatment of corticosteroids. The derived gene expression data can then be used as input for pathway and network analysis, enabling us to identify which molecular processes are involved in the pathogenesis of a corticosteroid-induced increase in IOP and to investigated how these processes are connected with each other. In addition, the PBMCs might also be used to perform a targeted mRNA expression study of the glucocorticosteroid receptors (GCR***α*** and GCR***β***) in PBMCs derived from patients with and without a response, before and after treatment. These individual profiles of response can then be used to predict a corticosteroid induced increase in IOP before treatment with corticosteroids is started. It can also specify the drugs that may prevent or affect the corticosteroid-induced increase in IOP.

Finally, one may question how long it will take to implant derived and possibly genetically modified TM-cells generated from PBMC based stem cells from the individual patient with POAG. Our biological samples can be used to set the first step into further research to individualize treatment and transplantation of TM-like iPSCs.

References

1. Vohra R, Tsai JC, Kolko M. The role of inflammation in the pathogenesis of glaucoma. Surv Ophthalmol 2013;58(4):311-20.
2. Taurone S, Ripandelli G, Pacella E, et al. Potential regulatory molecules in the human trabecular meshwork of patients with glaucoma: immunohistochemical profile of a number of inflammatory cytokines. Mol Med Rep 2015;11(2):1384-90.
3. Micera A, Quaranta L, Esposito G, et al. Differential Protein Expression Profiles in Glaucomatous Trabecular Meshwork: An Evaluation Study on a Small Primary Open Angle Glaucoma Population. Adv Ther 2016;33(2):252-67.
4. Wang N, Chintala SK, Fini ME, Schuman JS. Activation of a tissue-specific stress response in the aqueous outflow pathway of the eye defines the glaucoma disease phenotype. Nat Med 2001;7(3):304-9.
5. Toris CB, Gabelt BT, Kaufman PL. Update on the mechanism of action of topical prostaglandins for intraocular pressure reduction. Surv Ophthalmol 2008;53 Suppl1:S107-20.
6. Hubackova S, Krejcikova K, Bartek J, Hodny Z. IL1- and TGFbeta-Nox4 signaling, oxidative stress and DNA damage response are shared features of replicative, oncogene-induced, and drug-induced paracrine ‘bystander senescence’. Aging (Albany NY) 2012;4(12):932-51.
7. Young AR, Narita M. *SASP* reflects senescence. EMBO Rep 2009;10(3):228-30.
8. Guichard A, Humbert P, Tissot M, et al. Effects of topical corticosteroids on cell proliferation, cell cycle progression and apoptosis: in vitro comparison on HaCaT. Int J Pharm 2015;479(2):422-9.
9. Bourcier T, Forgez P, Borderie V, et al. Regulation of human corneal epithelial cell proliferation and apoptosis by dexamethasone. Invest Ophthalmol Vis Sci 2000;41(13):4133-41.
10. Sansregret L, Nepveu A. The multiple roles of *CUX1*: insights from mouse models and cell-based assays. Gene 2008;412(1-2):84-94.
11. Alcalay NI, Vanden Heuvel GB. Regulation of cell proliferation and differentiation in the kidney. Front Biosci (Landmark Ed) 2009;14:4978-91.
12. Hu F, Gou L, Liu Q, et al. G-patch domain containing 2, a gene highly expressed in testes, inhibits nuclear factor-kappaB and cell proliferation. Mol Med Rep 2015;11(2):1252-7.
13. Reid T, Kenney C, Waring GO. Isolation and characterization of fibronectin from bovine aqueous humor. Invest Ophthalmol Vis Sci 1982;22(1):57-61.
14. Hann CR, Springett MJ, Wang X, Johnson DH. Ultrastructural localization of collagen IV, fibronectin, and laminin in the trabecular meshwork of normal and glaucomatous eyes. Ophthalmic Res 2001;33(6):314-24.
15. Filla MS, Dimeo KD, Tong T, Peters DM. Disruption of fibronectin matrix affects type IV collagen, fibrillin and laminin deposition into extracellular matrix of human trabecular meshwork (HTM) cells. Exp Eye Res 2017;165:7-19.
16. Moshirfar M, Parker L, Birdsong OC, et al. Use of Rho kinase Inhibitors in Ophthalmology: A Review of the Literature. Med Hypothesis Discov Innov Ophthalmol 2018;7(3):101-11.
17. Tripathi RC, Li J, Chan WF, Tripathi BJ. Aqueous humor in glaucomatous eyes contains an increased level of TGF-beta 2. Exp Eye Res 1994;59(6):723-7.

18. Agarwal P, Daher AM, Agarwal R. Aqueous humor TGF-beta2 levels in patients with open-angle glaucoma: A meta-analysis. Mol Vis 2015;21:612-20.
19. Prendes MA, Harris A, Wirostko BM, et al. The role of transforming growth factor beta in glaucoma and the therapeutic implications. Br J Ophthalmol 2013;97(6):680-6.
20. Bermudez JY, Webber HC, Brown B, et al. A Comparison of Gene Expression Profiles between Glucocorticoid Responder and Non-Responder Bovine Trabecular Meshwork Cells Using RNA Sequencing. PLoS One 2017;12(1):e0169671.
21. Fan BJ, Wang DY, Tham CC, et al. Gene expression profiles of human trabecular meshwork cells induced by triamcinolone and dexamethasone. Invest Ophthalmol Vis Sci 2008;49(5):1886-97.
22. Nehme A, Lobenhofer EK, Stamer WD, Edelman JL. Glucocorticoids with different chemical structures but similar glucocorticoid receptor potency regulate subsets of common and unique genes in human trabecular meshwork cells. BMC Med Genomics 2009;2:58.
23. Markus H. Kuehn CYK, Young H. Kwon. Dexamethasone induced gene expression changes in human perfusion organ cultured trabecular meshwork. 2012.
24. Matsuda A, Asada Y, Takakuwa K, et al. DNA Methylation Analysis of Human Trabecular Meshwork Cells During Dexamethasone Stimulation. Invest Ophthalmol Vis Sci 2015;56(6):3801-9.
25. Faralli JA, Desikan H, Peotter J, et al. Genomic/proteomic analyses of dexamethasone-treated human trabecular meshwork cells reveal a role for GULP1 and ABR in phagocytosis. Mol Vis 2019;25:237-54.
26. Raghunathan VK, Morgan JT, Park SA, et al. Dexamethasone Stiffens Trabecular Meshwork, Trabecular Meshwork Cells, and Matrix. Invest Ophthalmol Vis Sci 2015;56(8):4447-59.
27. Li G, Cui G, Dismuke WM, et al. Differential response and withdrawal profile of glucocorticoid-treated human trabecular meshwork cells. Exp Eye Res 2017;155:38-46.
28. Steely HT, Browder SL, Julian MB, et al. The effects of dexamethasone on fibronectin expression in cultured human trabecular meshwork cells. Invest Ophthalmol Vis Sci 1992;33(7):2242-50.
29. Mao W, Tovar-Vidales T, Yorio T, et al. Perfusion-cultured bovine anterior segments as an ex vivo model for studying glucocorticoid-induced ocular hypertension and glaucoma. Invest Ophthalmol Vis Sci 2011;52(11):8068-75.
30. Tripathi RC, Parapuram SK, Tripathi BJ, et al. Corticosteroids and glaucoma risk. Drugs Aging 1999;15(6):439-50.
31. Armaly MF, Becker B. Intraocular pressure response to topical corticosteroids. Fed Proc 1965;24(6):1274-8.
32. Becker B. Intraocular Pressure Response to Topical Corticosteroids. Invest Ophthalmol 1965;4:198-205.
33. Kitazawa Y, Horie T. The prognosis of corticosteroid-responsive individuals. Arch Ophthalmol 1981;99(5):819-23.
34. Lewis JM, Priddy T, Judd J, et al. Intraocular pressure response to topical dexamethasone as a predictor for the development of primary open-angle glaucoma. Am J Ophthalmol 1988;106(5):607-12.

35. Gottanka J, Johnson DH, Martus P, Lutjen-Drecoll E. Severity of optic nerve damage in eyes with POAG is correlated with changes in the trabecular meshwork. J Glaucoma 1997;6(2):123-32.
36. Ueda J, Wentz-Hunter K, Yue BY. Distribution of *myoc*ilin and extracellular matrix components in the juxtacanalicular tissue of human eyes. Invest Ophthalmol Vis Sci 2002;43(4):1068-76.
37. Rohen JW, Lutjen-Drecoll E, Flugel C, et al. Ultrastructure of the trabecular meshwork in untreated cases of primary open-angle glaucoma (POAG). Exp Eye Res 1993;56(6):683-92.
38. Tawara A, Tou N, Kubota T, et al. Immunohistochemical evaluation of the extracellular matrix in trabecular meshwork in steroid-induced glaucoma. Graefes Arch Clin Exp Ophthalmol 2008;246(7):1021-8.
39. Tektas OY, Lutjen-Drecoll E. Structural changes of the trabecular meshwork in different kinds of glaucoma. Exp Eye Res 2009;88(4):769-75.
40. Clark AF, Wilson K, McCartney MD, et al. Glucocorticoid-induced formation of cross-linked actin networks in cultured human trabecular meshwork cells. Invest Ophthalmol Vis Sci 1994;35(1):281-94.
41. Hoare MJ, Grierson I, Brotchie D, et al. Cross-linked actin networks (CLANs) in the trabecular meshwork of the normal and glaucomatous human eye in situ. Invest Ophthalmol Vis Sci 2009;50(3):1255-63.
42. Bermudez JY, Montecchi-Palmer M, Mao W, Clark AF. Cross-linked actin networks (CLANs) in glaucoma. Exp Eye Res 2017;159:16-22.
43. Clark AF, Brotchie D, Read AT, et al. Dexamethasone alters F-actin architecture and promotes cross-linked actin network formation in human trabecular meshwork tissue. Cell Motil Cytoskeleton 2005;60(2):83-95.
44. de Voogd S, Ikram MK, Wolfs RC, et al. Incidence of open-angle glaucoma in a general elderly population: the Rotterdam Study. Ophthalmology 2005;112(9):1487-93.
45. Morgan JT, Raghunathan VK, Chang YR, et al. The intrinsic stiffness of human trabecular meshwork cells increases with senescence. Oncotarget 2015;6(17):15362-74.
46. Liu B, McNally S, Kilpatrick JI, et al. Aging and ocular tissue stiffness in glaucoma. Surv Ophthalmol 2017.
47. Armaly MF. Effect of Corticosteroids on Intraocular Pressure and Fluid Dynamics. I. The Effect of Dexamethasone in the Normal Eye. Arch Ophthalmol 1963;70:482-91.
48. Lam DS, Fan DS, Ng JS, et al. Ocular hypertensive and anti-inflammatory responses to different dosages of topical dexamethasone in children: a randomized trial. Clin Exp Ophthalmol 2005;33(3):252-8.
49. Hosseini M, Rose AY, Song K, et al. *IL-1* and TNF induction of matrix metalloproteinase-3 by c-Jun N-terminal kinase in trabecular meshwork. Invest Ophthalmol Vis Sci 2006;47(4):1469-76.
50. Giguere V, Yang N, Segui P, Evans RM. Identification of a new class of steroid hormone receptors. Nature 1988;331(6151):91-4.
51. Hong H, Yang L, Stallcup MR. Hormone-independent transcriptional activation and coactivator binding by novel orphan nuclear receptor ERR3. J Biol Chem 1999;274(32):22618-26.

52. Wang SC, Myers S, Dooms C, et al. An ERRbeta/gamma agonist modulates GRalpha expression, and glucocorticoid responsive gene expression in skeletal muscle cells. Mol Cell Endocrinol 2010;315(1-2):146-52.
53. Lee SW, Hwang HH, Hsu PW, et al. Whole-genome methylation profiling from PBMCs in acute-exacerbation COPD patients with good and poor responses to corticosteroid treatment. Genomics 2019;111(6):1381-6.
54. Zhang M, Maddala R, Rao PV. Novel molecular insights into *RhoA* GTPase-induced resistance to aqueous humor outflow through the trabecular meshwork. Am J Physiol Cell Physiol 2008;295(5):C1057-70.
55. Kumar J, Epstein DL. Rho GTPase-mediated cytoskeletal organization in Schlemm's canal cells play a critical role in the regulation of aqueous humor outflow facility. J Cell Biochem 2011;112(2):600-6.
56. Rao PV, Deng PF, Kumar J, Epstein DL. Modulation of aqueous humor outflow facility by the Rho kinase-specific inhibitor Y-27632. Invest Ophthalmol Vis Sci 2001;42(5):1029-37.
57. Van de Velde S, De Groef L, Stalmans I, et al. Towards axonal regeneration and neuroprotection in glaucoma: Rho kinase inhibitors as promising therapeutics. Prog Neurobiol 2015;131:105-19.
58. Bermel C, Tonges L, Planchamp V, et al. Combined inhibition of *Cdk*5 and *ROCK* additively increase cell survival, but not the regenerative response in regenerating retinal ganglion cells. Mol Cell Neurosci 2009;42(4):427-37.
59. Sugiyama T, Shibata M, Kajiura S, et al. Effects of fasudil, a Rho-associated protein kinase inhibitor, on optic nerve head blood flow in rabbits. Invest Ophthalmol Vis Sci 2011;52(1):64-9.
60. Matsuo N, Terao M, Nabeshima Y, Hoshino M. Roles of STEF/*Tiam1*, guanine nucleotide exchange factors for *Rac1*, in regulation of growth cone morphology. Mol Cell Neurosci 2003;24(1):69-81.
61. Tang AT, Campbell WB, Nithipatikom K. *ROCK1* feedback regulation of the upstream small GTPase *RhoA*. Cell Signal 2012;24(7):1375-80.
62. Maeda K, Otomo K, Yoshida N, et al. *CaMK4* compromises podocyte function in autoimmune and nonautoimmune kidney disease. J Clin Invest 2018;128(8):3445-59.
63. Kakinuma N, Roy BC, Zhu Y, et al. *Kank* regulates *RhoA*-dependent formation of actin stress fibers and cell migration via 14-3-3 in PI3K-Akt signaling. J Cell Biol 2008;181(3):537-49.
64. Price MO, Feng MT, Price FW, Jr. Randomized, Double-Masked Trial of Netarsudil 0.02% Ophthalmic Solution for Prevention of Corticosteroid-Induced Ocular Hypertension. Am J Ophthalmol 2020;222:382-7.
65. Honjo M, Tanihara H, Inatani M, et al. Effects of rho-associated protein kinase inhibitor Y-27632 on intraocular pressure and outflow facility. Invest Ophthalmol Vis Sci 2001;42(1):137-44.
66. Tian B, Kaufman PL. Effects of the Rho kinase inhibitor Y-27632 and the phosphatase inhibitor calyculin A on outflow facility in monkeys. Exp Eye Res 2005;80(2):215-25.
67. Futakuchi A, Morimoto T, Ikeda Y, et al. Intraocular pressure-lowering effects of ripasudil in uveitic glaucoma, exfoliation glaucoma, and steroid-induced glaucoma patients: *ROCK*-S, a multicentre historical cohort study. Sci Rep 2020;10(1):10308.

68. Fini ME, Schwartz SG, Gao X, et al. Steroid-induced ocular hypertension/glaucoma: Focus on pharmacogenomics and implications for precision medicine. Prog Retin Eye Res 2017;56:58-83.

69. Vajaranant TS, Price MO, Price FW, et al. Visual acuity and intraocular pressure after Descemet's stripping endothelial keratoplasty in eyes with and without preexisting glaucoma. Ophthalmology 2009;116(9):1644-50.

70. Wilkinson MD, Dumontier M, Aalbersberg IJ, et al. The FAIR Guiding Principles for scientific data management and stewardship. Sci Data 2016;3:160018.

71. Slenter DN, Kutmon M, Hanspers K, et al. WikiPathways: a multifaceted pathway database bridging metabolomics to other omics research. Nucleic Acids Res 2018;46(D1):D661-D7.

72. Kutmon M, van Iersel MP, Bohler A, et al. PathVisio 3: an extendable pathway analysis toolbox. PLoS Comput Biol 2015;11(2):e1004085.

73. van Iersel MP, Kelder T, Pico AR, et al. Presenting and exploring biological pathways with PathVisio. BMC Bioinformatics 2008;9:399.

74. Flugel C, Tamm E, Lutjen-Drecoll E. Different cell populations in bovine trabecular meshwork: an ultrastructural and immunocytochemical study. Exp Eye Res 1991;52(6):681-90.

75. Gerometta R, Podos SM, Candia OA, et al. Steroid-induced ocular hypertension in normal cattle. Arch Ophthalmol 2004;122(10):1492-7.

76. Gerometta RM, Malgor LA, Vilalta E, et al. Cl- concentrations of bovine, porcine and ovine aqueous humor are higher than in plasma. Exp Eye Res 2005;80(3):307-12.

77. To CH, Do CW, Zamudio AC, Candia OA. Model of ionic transport for bovine ciliary epithelium: effects of acetazolamide and HCO. Am J Physiol Cell Physiol 2001;280(6):C1521-30.

78. Kunisato A, Wakatsuki M, Shinba H, et al. Direct generation of induced pluripotent stem cells from human nonmobilized blood. Stem Cells Dev 2011;20(1):159-68.

79. Ding QJ, Zhu W, Cook AC, et al. Induction of trabecular meshwork cells from induced pluripotent stem cells. Invest Ophthalmol Vis Sci 2014;55(11):7065-72.

80. Keller KE, Bhattacharya SK, Borras T, et al. Consensus recommendations for trabecular meshwork cell isolation, characterization and culture. Exp Eye Res 2018;171:164-73.

81. Polansky JR, Fauss DJ, Zimmerman CC. Regulation of TIGR/*MYOC* gene expression in human trabecular meshwork cells. Eye (Lond) 2000;14 (Pt 3B):503-14.

82. Abu-Hassan DW, Li X, Ryan EI, et al. Induced pluripotent stem cells restore function in a human cell loss model of open-angle glaucoma. Stem Cells 2015;33(3):751-61.

83. Zhu W, Gramlich OW, Laboissonniere L, et al. Transplantation of iPSC-derived TM cells rescues glaucoma phenotypes in vivo. Proc Natl Acad Sci U S A 2016;113(25):E3492-500.

84. Zhu W, Jain A, Gramlich OW, et al. Restoration of Aqueous Humor Outflow Following Transplantation of iPSC-Derived Trabecular Meshwork Cells in a Transgenic Mouse Model of Glaucoma. Invest Ophthalmol Vis Sci 2017;58(4):2054-62.

85. Honda M, Orii F, Ayabe T, et al. Expression of glucocorticoid receptor beta in lymphocytes of patients with glucocorticoid-resistant ulcerative colitis. Gastroenterology 2000;118(5):859-66.

86. Goleva E, Jackson LP, Gleason M, Leung DY. Usefulness of PBMCs to predict clinical response to corticosteroids in asthmatic patients. J Allergy Clin Immunol 2012;129(3):687-93 e1.

87. Hew M, Bhavsar P, Torrego A, et al. Relative corticosteroid insensitivity of peripheral blood mononuclear cells in severe asthma. Am J Respir Crit Care Med 2006;174(2):134-41.
88. Sliwinska-Stanczyk P, Pazdur J, Ziolkowska M, et al. The effect of methylprednisolone on proliferation of PBMCs obtained from steroid-sensitive and steroid-resistant rheumatoid arthritis patients. Scand J Rheumatol 2007;36(3):167-71.
89. Jeoung JW, Ko JH, Kim YJ, et al. Microarray-based analysis of gene expression profiles in peripheral blood of patients with acute primary angle closure. Ophthalmic Genet 2017;38(6):520-6.
90. Flugel-Koch C, Ohlmann A, Fuchshofer R, et al. Thrombospondin-1 in the trabecular meshwork: localization in normal and glaucomatous eyes, and induction by TGF-beta1 and dexamethasone in vitro. Exp Eye Res 2004;79(5):64

ADDENDUM

SUMMARY

The subjects of this thesis are the pathogenesis of an increase in intraocular pressure (IOP) in primary open angle glaucoma (POAG) and a corticosteroid-induced increase in IOP. A sustained increase in eye pressure causes damage to the optic nerve, also called glaucoma. This results in visual field loss which slowly progresses over time and eventually causes blindness. Glaucoma is the second leading cause of visual impairment and blindness worldwide. The increase in eye pressure is the only manageable risk factor and since the precise pathogenesis of an increased IOP is largely unknown, current treatment is focused on lowering the eye pressure.

A small part of the eye, the trabecular meshwork (TM) tissue, is thought to play an important role in the pathogenesis of an increase in eye pressure. In the eye, a specific type of fluid (aqueous humor) is produced. The eye pressure is regulated by a balance between the production and outflow of this fluid. The outflow is regulated by the TM as this is the part of the eye through which this fluid leaves the eye. There is substantial evidence that the TM plays an important role in increasing the IOP .

An increase in eye pressure may also be caused by corticosteroids. These drugs are often used by ophthalmologists for the treatment of several eye diseases or to prevent inflammation after eye surgery. It is known that approximately 3 out of 10 patients who use corticosteroid eye drops develop an increase in eye pressure. This is called a steroid response. When this response takes place over a prolonged period of time, the sustained elevation of eye pressure will damage the optic nerve. When this occurs, this is called corticosteroid-induced glaucoma. As in other forms of an increased eye pressure, changes in the TM are thought to be the cause of a corticosteroid-induced increase in eye pressure, however, the precise pathogenesis is unknown. At this time, the only final way to lower the eye pressure is to stop the treatment with corticosteroids. However, the corticosteroids are prescribed for a reason and stopping them is not always possible due the underlying disease of the patient. In addition, conventional treatment of an increased IOP may be ineffective or invasive. If there is no alternative for corticosteroids, the development of an increased eye pressure upon the use of corticosteroids can create a difficult dilemma for the treating ophthalmologist.

In this thesis we describe how we obtained a better insight into the molecular pathogenesis of an increased IOP in primary open angle glaucoma, a corticosteroid-induced increase in IOP and the molecular mechanisms in the healthy TM tissue.

Throughout the years several studies have been performed, generating large amounts of biological data, among which, omics data. However, due to lack of analytical techniques, these data were not always explored to its full extent. The techniques are now available

and are summarized by the term 'bioinformatics'. Bioinformatics is the science that enriches the biological knowledge by applying methods from informatics to the biological data. These analyses create the opportunity to integrate the existing data and to use it to its full potential.

In chapter two to four, we build on the shoulders of previously performed research by integrating and re-analyzing publicly available datasets by means of bioinformatics analysis. The type of data that we used is gene expression data as it is known that the level of the expression of genes is different between diseased tissue and healthy control tissue. Within these studies, we did not only re-use data which allowed to save resources and costs, we also used freely accessible tools. Therefore, the methods used in this thesis can easily be applied by other researchers and for other diseases.

The first step towards a better understanding of how an increase in IOP develops and the role of the TM in it, is to obtain knowledge about the functioning of the healthy trabecular meshwork. Therefore, in **chapter two**, we performed a systematic search and collected all gene expression data (micro-array) on healthy TM tissue. After a check of the quality of the data, 18 datasets containing gene expression data of the healthy TM of 44 different deceased individuals remained. These data were integrated by means of bioinformatics analysis. In this study, we investigated which genes are overall highly expressed in the TM and therefore form the signature genes of this tissue. This study led to the identification of 1882 genes. However, a gene is only a small part of a complete molecular process in which it participates. For the human body, these molecular processes are presented as pathway diagrams of interacting molecules. Therefore, we also investigated in which pathways or molecular processes the identified signature genes of the healthy TM are involved. Pathway analysis revealed multiple molecular processes, including some that were already known to be active in the trabecular meshwork, for example extracellular matrix, elastic fiber formation, elastin crosslinking and focal adhesion. Also, 46 candidate TM-specific genes were identified. These consist mainly of pseudogenes or novel transcripts of which the function is unknown. The signature genes and processes identified in this study can be used as a reference to compare diseased TM tissue. The candidate TM-specific genes are of interest for further investigation and characterization.

In **chapter three**, we compared the gene expression of the TM tissue in patients with and without POAG. The data consisted of 14 patients with POAG and 13 control cases, all derived from one previously published and publicly available study. We identified the genes that are differently expressed between the two groups. Following, we investigated in which molecular processes these genes are involved. These processes could be

grouped in five large clusters: extracellular matrix, inflammation, complement activation, senescence, and Rho GTPase signaling. Some of these clusters were already known to play a role in POAG, for the others, a clear path of involvement could be hypothesized. Of special interest is Rho GTPase as Rho kinase inhibitors are known to lower the eye pressure. In addition, to obtain a better insight in how these genes in the retrieved pathway are working together and how they are interconnected, we made a network of connecting genes. This showed a central role for inflammation. Furthermore, some genes such as *TGF-β1*, *CD44*, *COL3A1* and *COL1A1*, *SERPINE1* and *PLAU* were shared by multiple clusters and could be linked to known biochemical processes causing POAG, confirming a possible important role in the pathogenesis of the disease.

In **chapter four**, we investigated the gene expression data of TM tissue exposed to corticosteroids. For this study, we were able to include data from five publicly available studies. The data were integrated and re-analyzed by means of bioinformatics analysis. The differentially expressed genes were found to be involved in multiple processes such as collagen, extracellular matrix, adhesion and inflammation. These processes have been researched and hypothesized by other research groups to play a role in the development of a corticosteroid-induced increase in eye pressure. However, for patients included in this part of the study, it was unknown whether they developed a steroid response or not after exposure to corticosteroids. Therefore, it is not known whether the identified processes are causative for the development of a corticosteroid response or only represent the effect of corticosteroids on the TM tissue.

In the second part of the study presented in chapter four, we included data on bovine eyes for which it was known whether they developed a corticosteroid-induced increase in IOP or not. The processes we identified after exposure of the bovine TM to corticosteroids were identical to the process identified in the first part of the study. As this validated the suitability of the bovine data, we compared the gene expression in this TM tissue with and without a corticosteroid response. The same clusters as identified in the first part of the study were identified again. In addition, two other clusters were found to be highly involved as well: cell cycle and senescence. Further investigation showed that the gene expression of the genes involved in these two clusters are in the opposite direction for those with and those without a corticosteroid response. This finding of even an opposite response instead of a different quantitative response suggests that these two clusters play an important role in the pathogenesis of corticosteroid-induced increase in IOP.

In **chapter five**, we showed how gene expression data of chapters two to four can be used for the identification of new treatment possibilities for POAG. Three different methods were described that allowed the identification of new medical treatments for POAG by focusing the search for known drugs that are related to the identified genes

and molecular processes that are known to be involved in the pathogenesis. Some drugs that are known to influence the TM and the eye pressure, such as rho kinase inhibitors, corticosteroids and statins were identified. However, to further downsize the number of potentially effective drugs, additional research, among which a detailed investigation on the applied methods, combining the results with insights in biochemical processes and pharmacodynamics and eventually validation in animal experiments, is necessary.

After obtaining a better insight into the molecular processes involved in a corticosteroid-induced increased IOP (chapter four), we also aimed to obtain a better insight into the role of relevant genes in a different way. We performed a genome-wide association analysis (GWAS) in which we investigated single base-pair genetic variances, also called single nucleotide polymorphisms (SNPs), between patients with and without a steroid response (see further). As it was very important for this study to correctly identify which patients are steroid responders and which are not and to exclude other causes of an increase in IOP, we performed an additional study in **chapter six.** In this chapter we identified which risk factors are associated with an increase in eye pressure (other than the use of corticosteroids or in interaction with these drugs) after a corneal transplantation. We did this based on the results of 76 studies. All risk factors were identified and per risk factor, all the evidence was visualized in tables. These tables allowed to judge which risk factors are highly likely to cause an increase in eye pressure after corneal transplantation. The following risk factors were identified: pre-existing glaucoma, high eye pressure before the surgery, corneal transplantation combined with the removal or exchange of an intraocular lens, and aphakia and pseudophakia with the intraocular lens placed in the anterior or posterior chamber.

In **chapter seven**, we performed a GWAS in which small variances in the genomes between patients with and without a corticosteroid response were identified. These variances help to identify associated genes. In turn, these genes enable us to increase the understanding of the pathogenesis of a corticosteroid response and can be investigated as candidate target genes for new treatment options. We included a study cohort of 339 patients and collected their blood samples for genome-wide association analysis. All the included patients had undergone a corneal transplantation and had to use steroid eye drops, in a protocolled fashion, for at least a year after this procedure. Since most patients develop a corticosteroid-induced increase in IOP when corticosteroids are used between one and eight months, this group is the ideal study population to investigate the genetic components of a corticosteroid response. Two ophthalmology residents, and when needed a glaucoma specialist, independently identified the responders and non-responders based on strict criteria. In addition, a distinction between high and low responders was made. Within our study cohort, 39.5% developed a corticosteroid

response of which 27.6% were high responders. Genome-wide association analysis on all responders vs. non-responders revealed 172 SNPs which were assigned to 18 genes. These genes are involved in the expression of the glucocorticoid receptor, the development or functioning of the trabecular meshwork, or refer to molecular processes like the extracellular matrix and cell cycle. We also found that small genetic variances in one of the identified genes (*UBL5*) determine whether a patient develops a corticosteroid-induced increase in IOP. Multiple of the identified genes are targeted by rho-kinase inhibitors, indicating that these drugs might be an effective treatment by addressing its molecular pathogenesis. In addition, a distinction was made between high and low responders. This revealed that there are genetic differences between these groups, however, this requires more research.

In **chapter eight**, the general discussion, we reflect on the findings within this thesis. In addition, the future perspectives of this research are highlighted as we look forward to the use of peripheral blood mononuclear cells (PBMCs) to obtain a better insight into the individualized pathogenesis of a corticosteroid-induced increase in IOP.

ADDENDUM

SAMENVATTING

De onderwerpen van deze thesis zijn de pathogenese van primair open kamerhoek glaucoom en een corticosteroïd-geïnduceerd oogdrukstijging. Een langdurig verhoogde oogdruk veroorzaakt schade aan de oogzenuw, ook wel glaucoom genoemd. Dit leidt tot verlies van het gezichtsveld en uiteindelijk volledige blindheid. Glaucoom is wereldwijd de tweede meest voorkomende oorzaak van visuele beperking en blindheid. Aangezien de precieze pathogenese van een verhoogde oogdruk onbekend is, is de verhoogde oogdruk de enige risicofactor die behandeld kan worden. Alle huidige therapieën, zowel medicamenteus als chirurgisch, zijn dan ook gericht op het verlagen van de oogdruk.

Het precieze mechanisme van glaucoom en met name de oorzaak van de oogdrukstijging, is nog onduidelijk. In het oog wordt kamerwater geproduceerd. De balans tussen aanmaak en afvoer van dit kamerwater bepaalt de oogdruk. De afvoer van het kamerwater gebeurt via het trabekelsysteem. Er wordt dan ook gedacht dat dit weefsel een belangrijke rol speelt in de oorzaak van glaucoom.

Een verhoogde oogdruk kan ook veroorzaakt worden door het gebruik van corticosteroïden. Dit is een geneesmiddel dat door oogartsen vaak voorgeschreven wordt om oogaandoeningen te behandelen of om een ontsteking na een oogheelkundige operatie te voorkomen. Het is bekend dat ongeveer 3 op de 10 patiënten die oogdruppels met een corticosteroïd gebruiken een stijging van de oogdruk ontwikkelen. Dit wordt ook wel een steroïd-response genoemd. Wanneer de oogdruk voor een te lange tijd te hoog blijft, zal de oogzenuw beschadigd raken. Net zoals bij andere vormen van een verhoogde oogdrukstijging, is de verwachting dat veranderingen in het trabekelsysteem de oogdrukstijging veroorzaken. De precieze pathogenese is echter nog niet duidelijk. Op dit moment is de enige oplossing bij een steroïd-response het stoppen van het gebruik van steroïden. Echter wordt dit geneesmiddel niet zonder reden voorgeschreven en simpelweg stoppen kan niet altijd door het onderliggende ziektebeeld. Als er geen alternatief mogelijk is voor corticosteroïden, kan een steroïd-response een lastig dilemma vormen in de spreekkamer van de oogarts.

In deze thesis beschrijven we hoe we een beter inzicht konden verkrijgen in de pathogenese van glaucoom, een steroïd-geïnduceerde oogdrukstijging en de moleculaire mechanismen die bijdragen aan de werking van het gezonde trabekelsysteem. Doorheen de jaren werden verschillende studies uitgevoerd door andere onderzoekers. Dit heeft ervoor gezorgd dat er een enorme hoeveelheid aan data gegenereerd werd. Hierbij gaat het dan met name om de zogenaamde ‘omics data’. Echter, vanwege het ontbreken van meer geavanceerde technologie en technieken ten tijde van deze data generatie, kon de data niet altijd even grondig of volledig onderzocht worden. Deze technieken zijn nu wel beschikbaar en worden verzameld onder de term ‘bioinformatica’.

Bioinformatica is de wetenschap die de biologische kennis verrijkt door informatica-methoden toe te passen op biologische data. Deze analyses creëren de mogelijkheid om de bestaande data met elkaar te integreren en grondig te onderzoeken.

In hoofdstuk twee tot vier bouwen we op de schouders van reeds uitgevoerde onderzoeken door de data met elkaar te integreren en opnieuw te analyseren. Hiervoor maken we gebruik van bioinformatica analyses. We weten dat de expressie van genen tussen patiënten met een aandoening en gezonde controles verschillend is. Het gebruikte type van data in deze thesis is dan met name ook genexpressie data. We hebben in deze hoofdstukken niet alleen de data hergebruikt, we hebben hierbij ook enkel gebruik gemaakt van online programma's die gratis te gebruiken zijn. De methoden die in de thesis getoond worden, kunnen dan ook makkelijk toegepast worden door andere onderzoekers en voor andere aandoeningen.

De eerste stap om een beter begrip te krijgen van hoe glaucoom veroorzaakt wordt en wat de rol van het trabekelsysteem hierin is, is een beter beeld te krijgen van hoe het gezonde trabekelsysteem werkt. Daarom hebben we in **hoofdstuk 2** op een systematische manier alle genexpressie data van het gezonde trabekelsysteem verzameld. Vervolgens hebben we de kwaliteit van de data onderzocht waarbij data van onvoldoende kwaliteit verwijderd werd. Er bleven 18 datasets, die de data van 44 verschillende overleden individuen omvatte, over. Deze data werd vervolgens geïntegreerd waarna we konden kijken welke genen over het algemeen hoog tot expressie gebracht werden in het gezonde trabekelsysteem. Deze genen vormen dan ook de handtekening van dit weefsel. Het onderzoek leidde tot de identificatie van 1882 genen. Echter, een gen is slechts een klein onderdeel van een moleculair proces waar het een rol in speelt. Voor het menselijke lichaam worden deze moleculaire processen weergegeven in pathways van moleculen (waaronder genen) die met elkaar communiceren. Daarom hebben we ook gekeken in welke pathways - of moleculaire processen - de genen die we identificeerden in de studie een rol spelen. We vonden verschillende processen waarvan voor sommigen reeds bewezen werd dat ze een rol spelen in het trabekelsysteem. Voorbeelden hiervan zijn de extracellulaire matrix en de vorming van elastine vezels. De genen en pathways die in deze studie geïdentificeerd werden, kunnen gebruikt worden als referentiekader om te vergelijken met ziek trabekelsysteem, zoals dat van patiënten met glaucoom. Daarnaast werden ook 46 genen geïdentificeerd die, voor zover we nu weten, enkel in het trabekelsysteem tot expressie gebracht worden. Deze genen bestaan met name uit pseudogenen of nieuwe genen waarvan de functie nog niet bekend is. Net daarom zijn deze pseudogenen interessant voor aanvullend onderzoek.

In **hoofdstuk drie** hebben we de genexpressie van het trabekelsysteem in patiënten met en zonder primair open kamerhoek glaucoom met elkaar vergeleken. De data bestond uit 14 patiënten met primair open kamerhoek glaucoom en 13 controles, allen afkomstig van één publiekelijk beschikbare studie. We identificeerden de genen die verschillend tot expressie komen tussen beide patiëntengroepen. Vervolgens onderzochten we in welke moleculaire processen deze genen betrokken waren. Deze processen konden onderverdeeld worden in vijf grote clusters: extracellulaire matrix, inflammatie, complement activatie, veroudering en Rho GTPase. Voor sommige van deze clusters had eerder onderzoek reeds aangetoond dat ze een rol spelen in glaucoom, voor de andere processen konden we een duidelijke hypothese formuleren. Het cluster Rho GTPase is met name interessant gezien we reeds weten dat Rho-kinase remmers de oogdruk kunnen verlagen. Om een beter inzicht te verkrijgen in hoe de genen in de pathways samenwerken en hoe ze met elkaar in connectie staan, maakten we een netwerk van connecterende genen. Deze analyse toonde een centrale rol voor ontsteking. Daarnaast, meerdere genen zoals *TGF-β1*, *CD44*, *COL3A1*, *COL1A1*, *SERPINE1* en *PLAU* werden door meerdere clusters gedeeld en konden gelinkt worden aan processen die een rol spelen in glaucoom. Dit bevestigt een mogelijk belangrijke rol voor inflammatie in de pathogenese van glaucoom.

In **hoofdstuk vier** onderzochten we de genexpressie van het trabekelsysteem nadat het blootgesteld werd aan corticosteroïden. Voor dit onderzoek konden we de data van vijf publiekelijk beschikbare studies met elkaar integreren en re-analyseren door gebruik te maken van bioinformatica technieken. De genen die verschillend tot expressie gebracht werden, spelen een rol in verschillende processen zoals collageen, extracellulaire matrix, adhesie en inflammatie. Eerder onderzoek heeft reeds aangetoond dat deze processen een rol kunnen spelen in de ontwikkeling van een corticosteroïd-geïnduceerde oogdrukstijging. Echter, van de patiënten die in deze studies werden geïncludeerd, is niet geweten of ze een steroïd-response zouden ontwikkelen. Met andere woorden, we weten niet of de gevonden processen een steroïd-response veroorzaken of dat ze enkel het effect van corticosteroïden op het trabekelsysteem representeren.

In het tweede gedeelte van hoofdstuk vier, includeerden we genexpressie data van koeienogen waarbij geweten was of de koeien een steroïd-response ontwikkelden na blootstelling aan corticosteroïden of niet. Ondanks het feit dat de gebruikte data van koeien afkomstig was, waren de processen die we identificeerden na blootstelling van het trabekelsysteem van deze koeien identiek aan de processen die gevonden werden na blootstelling van het trabekelsysteem van mensen. Dit toont aan dat de data van koeien geschikt was voor verder onderzoek. Vervolgens vergeleken we dan ook de genexpressie van het trabekelsysteem tussen koeien met en zonder een steroïd-response. Naast dezelfde clusters die we reeds eerder identificeerden, vonden we dat ook cel cyclus

en veroudering betrokken waren. Verder onderzoek toonde aan dat de genexpressie van de genen van deze twee clusters een tegenovergestelde richting laten zien tussen responders en non-responders. De bevinding dat er zelfs een tegenstelde response is en niet alleen een kwantitatieve response, suggereert dat cel cyclus en veroudering een belangrijke rol spelen in de pathogenese van corticosteroïd-geïnduceerd glaucoom.

In **hoofdstuk vijf** hebben we laten zien hoe genexpressie data gebruikt kan worden voor de identificatie van nieuwe behandelingsmogelijkheden voor glaucoom. Om dit te doen, werden de resultaten van hoofdstuk drie gebruikt als input voor drie verschillende methoden voor "drug repurposing". Dit laatste bestaat uit het onderzoeken of reeds bestaande geneesmiddelen ook voor andere indicaties dan oorspronkelijk bedoeld, gebruikt kunnen worden. De getoonde methoden in dit hoofdstuk onderzochten welke geneesmiddelen aangrijpen op de genen en moleculaire processen waarvan we reeds eerder aantoonden dat ze betrokken zijn bij de pathogenese van primair open kamerhoek glaucoom (hoofdstuk drie). Sommige geneesmiddelen waarvan bekend is dat ze het trabeculaire netwerk en de oogdruk beïnvloeden, zoals rho-kinaseremmers, corticosteroïden en statines, werden geïdentificeerd. Om het aantal potentieel effectieve geneesmiddelen verder te verkleinen is echter aanvullend onderzoek nodig. Hiervoor kunnen onder andere een gedetailleerd onderzoek naar de toegepaste methoden, het combineren van de resultaten met inzichten in biochemische processen en farmacodynamiek en uiteindelijk validatie in dierproeven meer duidelijk brengen.

Nadat we een beter inzicht verkregen in de moleculaire processen die betrokken zijn in een corticosteroïd-geïnduceerde oogdrukstijging, wilden we ook een beter inzicht krijgen in de genetische verschillen tussen responders en niet-responders. Daarom deden we een genoom-wijde associatie studie (GWAS) in patiënten na een corneatransplantatie waarbij we kleine verschillen in genetische variaties, ook wel SNPs genoemd, tussen responders en niet-responders onderzochten (zie hieronder). Aangezien het voor deze studie van groot belang was om patiënten met een steroïd-response correct te identificeren, deden we een additionele studie in **hoofdstuk zes**. In dit hoofdstuk stelden we vast welke factoren het risico op oogdrukstijging vergroten na een corneatransplantatie (naast het gebruik van corticosteroïden). Dit hebben we gedaan op basis van de resultaten van 76 studies. Alle risicofactoren werden geïdentificeerd waarna het bewijs per risicofactor in een tabel werd weergegeven. Deze tabellen lieten toe om per risicofactor te beoordelen of deze aannemelijk was een oogdrukstijging te veroorzaken. De volgende risicofactoren werden geïdentificeerd: glaucoom voorgaand aan de ingreep, hoge oogdruk voorgaand aan de ingreep, corneatransplantatie gecombineerd met het verwijderen of verwisselen van een intra-oculaire lens, en afakie en pseudofakie waarbij de intra-oculaire lens in de voorste oogkamer geplaatst werd.

In **hoofdstuk zeven** deden we een genoom-wijde associatie studie (GWAS) waarbij kleine verschillen in genetische variaties (SNPs) tussen responders en niet-responders onderzochten werden. De genen die geassocieerd zijn met deze variaties helpen om een beter begrip te verkrijgen van de pathogenese van een corticosteroïd-geïnduceerde oogdrukstijging en kunnen verder onderzocht worden als kandidaat targetgenen voor nieuwe behandelingsmogelijkheden. Voor deze genoom-wijde associatie studie includeerden we het bloed van 339 patiënten. Al deze patiënten ondergingen een bepaald type corneatransplantatie en moesten na deze ingreep gedurende minstens een jaar oogdruppels met steroïden gebruiken. Aangezien de meeste patiënten een corticosteroïd-geïnduceerde oogdrukstijging ontwikkelen wanneer de corticosteroïden tussen de één tot acht maanden gebruikt worden, is deze groep de ideale studiepopulatie om de genetische componenten van een corticosteroïd-response te onderzoeken. Twee oogartsen in opleiding en, zo nodig een professor gespecialiseerd in glaucoom, identificeerden, onafhankelijk van elkaar, de responders en niet-responders. Binnen onze studie ontwikkelde 39.5% een corticosteroïd-response waarvan 27.6% extreme responders waren (hoge oogdrukstijging). De genoom-wijde associatie studie tussen responders en niet-responders leverde 172 SNP's op die konden worden toegewezen aan 18 genen. Deze genen zijn betrokken in de expressie van de glucocorticosteroïd receptor, de ontwikkeling en functioneren van het trabekelsysteem of verwijzen naar moleculaire processen zoals de cel cyclus en de extracellulaire matrix. We vonden ook dat kleine genetische variaties in een van de geïdentificeerde genen (*UBL5*) bepalen of een patiënt een corticosteroïd-response ontwikkelt of niet. Meerdere van de geïdentificeerde genen worden aangegrepen door rho-kinase remmers. Dit kan er op wijzen dat rho-kinase remmers een effectieve behandeling zijn die ook daadwerkelijk aangrijpen op de moleculaire pathogenese van een corticosteroïd-response. Tot slot maakten we in deze studie een onderscheid tussen patiënten met een hoge en een lage oogdrukstijging na blootstelling aan corticosteroïden. Dit toonde aan dat er genetische verschillen lijken te zijn tussen deze 2 groepen, echter is hier nog aanvullend onderzoek naar nodig.

In **hoofdstuk acht**, de algemene discussie, reflecteren we op de bevindingen van deze thesis. Daarnaast bespreken we de mogelijkheid van het gebruik van perifere mononucleaire bloedcellen (PBMCs) om een beter inzicht te verkrijgen in de geïndividualiseerde pathogenese van een corticosteroïd-geïnduceerde oogdrukstijging.

ADDENDUM

REFLECTING ON IMPACT

This thesis:

- Shows a new approach in ophthalmology to synthesize current knowledge on the pathogenesis of eye diseases and to identify the involved genes and molecular processes. It provides a solid genetic foundation to further investigate the molecular processes in healthy TM, POAG and a corticosteroid-induced increase in IOP.

- Brings the identification of new interventions from all existing drugs close to new clinical applications in ophthalmology. It opens opportunities for the Maastricht University to patent these new applications.

- Contains the largest GWAS study on corticosteroid-induced increase in IOP conducted until now and shows new insights into its genetic mechanisms.

- It's data and new biomaterial give an interesting and promising foundation for future research and is available for other researchers from the Maastricht University or somewhere else.

- Opens the doors for individualized treatment. The study of individualized molecular mechanisms, especially in those with a corticosteroid-induced increase in IOP with specific SNP's, can be based on the development of TM from lymphocytes derived stem cells. The impact of exposure to steroids in specifically changed molecular processes can then be tested. Bringing this to the clinic it is then possible to target the treatment on the molecular mechanisms of individuals.

Research

What is the main objective of the research described in the thesis and what are the most important results and conclusions?

POAG is a chronic neurodegenerative disease of the optic nerve in which there is a progressive loss of retinal ganglion cells. POAG leads to a specific type of visual field loss and may eventually lead to blindness. It is the second leading cause of visual impairment and blindness worldwide.[1] The IOP is the most important risk factor. Treating an increased IOP leads to a slower progression of the loss of retinal ganglion cells and related visual field loss. An increased IOP can also be caused by topical corticosteroids, also known as a corticosteroid response. This occurs in approximately 18%-36% of patients and this percentage can be as high as 92% in patients with POAG.[2] A corticosteroid response may also occur when corticosteroids are used systemically, intranasal or after application on the skin around the eye. A sustained increase in IOP caused by the use of these drugs can also lead to visual field loss and eventually blindness.

POAG and a corticosteroid response are likely caused by molecular changes in the TM which increase the outflow resistance, thereby causing an increased IOP.[3-6] The precise molecular pathogenesis of both diseases is still largely unknown. One of the main objectives of the research described in this manuscript is therefore to further unravel their molecular pathogenesis.

In chapters two to four we described our approach and results. In more detail, we re-used, integrated and re-analyzed all publicly available gene expression data on healthy TM tissue, TM tissue exposed to corticosteroids and TM tissue of patients with POAG or a corticosteroid response. This allowed us to obtain an overview of the signature genes and molecular pathways of the functioning of the healthy TM, the molecular processes involved in the pathogenesis of POAG and a corticosteroid response.

In chapter five, we illustrated how the data retrieved in chapters two to four can be used to identify possible drug targets. The methods to identify new medical treatments allow to focus the search for drugs that are related to the identified genes and molecular processes that are known to be involved in the pathogenesis. This approach has the advantage that also already registered drugs are identified. Their adverse events are known and medical specialist have experience with these registered drugs. Three different methods to identify relevant drugs were shown, all specifically selected to modulate the disease outcome.

In chapter six, we report the results of a systematic review. This study was designed to systematically collect and summarize the current evidence on risk factors for the development of an increase in IOP after keratoplasty and followed a semi-quantitative method which allowed the identification of highly likely associated risk factors. These risk factors could be possible confounders which were taken into account in the identification of corticosteroid responders in chapter seven.

In chapter seven, we report about our GWAS study which was performed to identify which genetic variants are linked to a corticosteroid response. In order to do so, the study population was carefully selected as the long exposure to corticosteroids and the criteria used to identify a corticosteroid responder both lowered the risk to misclassify non-responder as responders. We compared responders vs. non-responders and we made a distinction between high and low responders.

Another objective of the thesis was to identify new treatment options that target the identified molecular processes that causes POAG and a corticosteroid response. In chapter seven, multiple of the identified genes are involved in the rho-kinase pathway, suggesting that rho-kinase inhibitors are highly suitable to treat a corticosteroid response. In chapter four, we also identified clues that rho-kinase inhibitors might be effective to treat a corticosteroid-induced increase in IOP. Therefore, rho-kinase inhibitors warrant further investigation as treatment for a corticosteroid-induced increase in eye pressure, with potential target genes identified in both high and low responders. A recently published study found that ripasudil (a rho-kinase inhibitor) significantly lowered the IOP in patients with a corticosteroid-induced increase in IOP.[7]

Relevance

What is the (potential) contribution of the results from this research to science, and, if applicable, to social sectors and social challenges?

We integrated all current gene expression data and developed a model for the molecular pathogenesis of POAG and corticosteroid induced ocular hypertension. The relevance of this for science is that it helps other researchers to build on this model of current evidence for new research. It is the reference to specifically investigate genes or molecular processes of interest for POAG or a corticosteroid response to further unravel the pathogenesis of both diseases. In addition, it is the reference to find diagnostic or prognostic markers, new drugs or markers for effect modification of treatments.

In chapter two, we present an overview of the processes and genes that are involved in the functioning of the healthy TM which can be used as a future reference to study physiological processes of the TM. We showed that processes related to ECM, elastic fiber formation and actin crosslinking are important for the functioning of the healthy TM. In addition, multiple highly likely TM specific genes have been suggested which can be used for further functional investigation and might be of special interest for drug targeting of their encoded proteins. Furthermore, the complete lists with housekeeping genes and pathways and the investigated genes in the TM with their average gene expression are provided. This allows other researchers to use this data as a reference for investigating molecular mechanisms. Several identified molecular processes in the healthy TM have shown concordance with molecular processes in patients with POAG.

In chapter three we provide a comprehensive overview of the processes involved in the molecular pathogenesis of POAG. We identified multiple pathways which were clustered into five functional categories: ECM, inflammation, complement activation, senescence, and Rho GTPase. These categories were combined into a network of connecting genes, showing overlap between the categories and the central position of the inflammation cluster. Also, the genes present in at least three categories were visualized within the network. The additional Gene Ontology analysis on our pre-processed and quality controlled dataset showed that the significantly changed genes were involved in ECM, inflammation and cell adhesion, which we already found in the pathway analysis. Additionally, development and corticosteroid related clusters were found. The identified clusters and the genes involved in these clusters can be used by other researchers to further unravel the pathogenesis of POAG.

In chapter four we found the functional processes cell cycle and senescence to be highly likely involved in the pathogenesis of corticosteroid-induced increase in IOP. Other processes such as collagen, ECM, adhesion and WNT-signaling behave differently between responders and non-responders as well. However, as these differences are mainly based on differences in intensities of gene expression rather than opposites, further investigation of these processes is needed. These pathways and their involved genes, and maybe especially the genes shared between the identified processes after comparing responders and non-responders, are of interest for further investigation. Fibronectin 1 was shared by the largest numbers of clusters, however, as discussed in chapter four, the precise role of this gene in het pathogenesis of a corticosteroid-induced increase in IOP needs further investigation. This fuels the research initiatives of other researchers.

In chapter five, we showed how the obtained gene expression data can be used for the identification of new treatment possibilities for primary open angle glaucoma. As an example, the results of chapter three were used as input for three different methods for drug repurposing. Three different methods were described that allowed the identification of new medical treatments for POAG by focusing the search for known drugs that are related to the identified genes and molecular processes that were previously found to be involved in the pathogenesis. Some drugs that are known to influence the trabecular meshwork and the eye pressure, such as rho kinase inhibitors, corticosteroids and statins were identified.

In chapter six we performed a review on the risk factors for the development of an increased IOP after keratoplasty and the level of evidence that is available for each risk factor. Based on the evidence tables, factors with a definitive and probable association with an increased risk for IOP elevation have been established. This can help to identify patients at risk and to individualize patient care concerning the choice of therapy, postoperative treatment and follow-up. However, we have also shown that many risk factors still lack sufficient evidence to determine its association and need further investigation.

In chapter seven we performed a GWAS study to identify which genetic variants are linked to a corticosteroid response. Comparing responders vs. non-responders, revealed 172 SNPs and 18 genes. These genes are involved in the expression of the glucocorticoid receptor, the development or functioning of the trabecular meshwork, or refer to molecular processes like the extracellular matrix and cell cycle. We also found that small genetic variances in one of the identified genes (*UBL5*) might determine whether a patient develops a corticosteroid-induced increase in IOP or not. In addition,

a distinction was made between high and low responders. This revealed that there are genetic differences between these groups, however, this requires more research. We also found that several of the identified genes are involved in the rho-kinase pathway, suggesting that rho-kinase inhibitors are highly suitable to treat a corticosteroid response. A recently published study confirmed our hypothesis that ripasudil significantly lowered the IOP in patients with a corticosteroid-induced increase in IOP, however, they only included a small study cohort.[7] This gives a clue for ophthalmologists to effectively treat patients with a steroid response.

In addition to our findings, our data and biomaterial can also be used by other researchers. We therefore described two new research proposals in chapter eight to illustrate how the results, data and biomaterial collected for this thesis may form the foundation for further research. We presented that the collected blood samples for the GWAS studies contain peripheral blood mononuclear cells (PBMCs) which can be used to generate TM-like stem cells. As TM cells are rather difficult to obtain in vivo this can be a way to study the molecular pathways in TM cells of individual patients, especially in those patients with specific genetic changes. In addition, we would be able to investigate how the gene expression changes after exposing these cells to new treatment possibilities which would also facilitate an individualized approach. Developing cells and tissue from stem cells can be seen as the biopsy of the twenty-first century.

The blood samples for the GWAS study were collected from patients who underwent a corneal transplantation mainly due to Fuchs endothelial dystrophy and keratoconus. Therefore, the data from our GWAS study can also be used by other researcher to study the genetics of these corneal diseases. The obtained samples and data also create the opportunity to join international collaborations.

The societal impact of our results is not only based on the opportunities to influence the outcome of glaucoma but also on the opportunities to influence the cost of glaucoma. Besides an impact on quality of life, visual impairment and blindness also have a significant impact on economic costs of which a substantial part is related to caretaking and home-help.[8-10] This economic impact will grow on the population level due to the high prevalence and increasing number of elderly people affected by glaucoma. It has already been shown that decreasing the IOP prevents the occurrence of blindness and reduces the costs of glaucoma for society.[10] A better understanding of the pathogenesis and the development of effective drugs, with fewer or no side effects, will not only improve quality of life but also reduce costs of blindness.

Target group

To whom are the research results interesting and/or relevant? And why?

The results are relevant for ophthalmologists and glaucoma specialists. The pathways and networks in the thesis show how multiple processes interfere with each other, increasing the understanding of the pathogenesis and emphasizing which processes eventually have to be targeted. It also shows ophthalmologists that there is a promising future for the medical treatment of glaucoma as multiple opportunities for new drugs have been proposed in this manuscript. More specifically, we have shown that Rho-kinase is involved in the pathogenesis of a corticosteroid-induced increase in IOP. Therefore, Rho-kinase inhibitors should be introduced in the Netherlands urgently. In addition, in chapter six, we identified the risk factors for developing an increased IOP after corneal transplantation. This can help clinicians to select corneal transplant patients at risk of developing an increased IOP and adjust their follow-up and treatment accordingly.

The results are also of interest for other researcher in the glaucoma field who want to compare their results to ours or can use our databases as a reference or starting point. As already mentioned above, in chapters two to four we laid the foundations for applied research to develop new treatment options for glaucoma.

As mentioned above, researchers in the field of Fuchs endothelial dystrophy or keratoconus may also benefit from our findings and data.

Lastly, omics research is booming. This type of research creates large amounts data which contain a lot of information. For example, it allows us to obtain information about the gene expression of thousands of genes within one single tissue of one single patient. Therefore, we need a way to integrate and present the results in a (clinically) meaningful way. Bioinformatics analyses are the key to do this. All the information we need is available online, even the data. This creates new possibilities for researchers to build on already obtained data and knowledge and therefore: “The World Wide Web is our lab”. The methods presented in chapters two to four and chapter eight of this manuscript are based on freely available tools. Therefore, researchers in other fields can apply our methods to explore other gene expression datasets for other diseases as well.

Activity

In what way can these target groups be involved in and informed about the research results, so that the knowledge gained can be used in the future?

Our data are the final summary and overview of the known molecular pathways based on all publicly available omics data. Other researchers can use our articles and data to develop new hypothesis and to start their investigations.

The articles have been published in, or has been submitted to, peer-reviewed journals which target researchers in the same field. The findings have also been presented at multiple national and international conferences such as the European Glaucoma Society Conference (EGS) and the Association for Research in Vision and Ophthalmology (ARVO). In addition, the methods have been presented on national bioinformatics conferences among which the Netherlands Bioinformatics and Systems Biology research school (BioSB) and the Maastricht Centre for Systems Biology (MaCSBio) Science Days.

Moreover, the opportunities for drug repurposing, and in the future possible patents, that follows form our results have been discussed with the head of the Department for Toxogenomics, professor Kleinjans, at the University of Maastricht.

References

1. Quigley HA, Broman AT. The number of people with glaucoma worldwide in 2010 and 2020. Br J Ophthalmol 2006;90(3):262-7.
2. Tripathi RC, Parapuram SK, Tripathi BJ, et al. Corticosteroids and glaucoma risk. Drugs Aging 1999;15(6):439-50.
3. Yuan Y, Call MK, Yuan Y, et al. Dexamethasone induces cross-linked actin networks in trabecular meshwork cells through noncanonical wnt signaling. Invest Ophthalmol Vis Sci 2013;54(10):6502-9.
4. Raghunathan VK, Morgan JT, Park SA, et al. Dexamethasone Stiffens Trabecular Meshwork, Trabecular Meshwork Cells, and Matrix. Invest Ophthalmol Vis Sci 2015;56(8):4447-59.
5. Kersey JP, Broadway DC. Corticosteroid-induced glaucoma: a review of the literature. Eye (Lond) 2006;20(4):407-16.
6. Jones R, 3rd, Rhee DJ. Corticosteroid-induced ocular hypertension and glaucoma: a brief review and update of the literature. Curr Opin Ophthalmol 2006;17(2):163-7.
7. Futakuchi A, Morimoto T, Ikeda Y, et al. Intraocular pressure-lowering effects of ripasudil in uveitic glaucoma, exfoliation glaucoma, and steroid-induced glaucoma patients: *ROCK*-S, a multicentre historical cohort study. Sci Rep 2020;10(1):10308.
8. Koberlein J, Beifus K, Schaffert C, Finger RP. The economic burden of visual impairment and blindness: a systematic review. BMJ Open 2013;3(11):e003471.
9. Thygesen J, Aagren M, Arnavielle S, et al. Late-stage, primary open-angle glaucoma in Europe: social and health care maintenance costs and quality of life of patients from 4 countries. Curr Med Res Opin 2008;24(6):1763-70.
10. van Gestel A, Webers CA, Severens JL, et al. The long-term outcomes of four alternative treatment strategies for primary open-angle glaucoma. Acta Ophthalmol 2012;90(1):20-31.

ADDENDUM

CURRICULUM VITAE

Ilona Liesenborghs was born on August 1, 1992 in Jette, Belgium. She graduated from secondary school in 2010 (Koninklijk Atheneum in Sint-Truiden) and started her medical studies at the University of Maastricht that same year. In October 2016 she received her medical degree at the same university. At the start of her internships in 2013 she participated in a research project under supervision of prof. C.A.B. Webers, dr. J.S.A.G. Schouten and dr. E.E.B. de Clerck. This project was part of the Maastricht Study and entailed the clinical assessment of optical coherence tomography images of a large amount of participants and epidemiological interpretation of the results. In august 2015 she started her senior year at the University Eye Clinic Maastricht, MUMC+ in which the scientific participation entailed a bioinformatics project on primary open angle glaucoma under supervision of dr. J.S.A.G. Schouten. This resulted in a PhD trajectory under supervision of prof. C.A.B. Webers, prof. C. Evelo, dr. J.S.A.G. Schouten and dr. L.M.T. Eijssen which started in August 2016. This PhD project included a collaboration between three different departments of the University of Maastricht: Maastricht Centre for Systems Biology, BigCAT and the University Eye Clinic Maastricht, MUMC+. In October 2019 she started as a trainee in Ophthalmology at the University Eye Clinic Maastricht, MUMC+.

ADDENDUM

LIST OF PUBLICATIONS

Related to this thesis

- **Liesenborghs I**, Eijssen LMT, Kutmon M, Gorgels TGMF, Evelo CT, Beckers HJM, Webers CAB, Schouten JSAG. Comprehensive bioinformatics analysis of trabecular meshwork gene expression data to unravel the molecular pathogenesis of primary open-angle glaucoma. Acta Ophthalmol. 2020 Feb;98(1):48-57.
- **Liesenborghs I**, Schouten JSAG, Berendschot TTJM, Beckers HJM, Nuijts RMMA, Visser N, Webers CAB. Risk Factors for the Development of Ocular Hypertension After Keratoplasty: A Systematic Review. Cornea. 2020 Mar;39(3):394-402.
- **Liesenborghs I**, Eijssen LMT, Kutmon M, Gorgels TGMF, Evelo CT, Beckers HJM, Webers CAB, Schouten JSAG. The molecular processes in the trabecular meshwork after exposure to corticosteroids and in corticosteroid-induced ocular hypertension. Invest Ophthalmol Vis Sci. 2020 Apr 9;61(4):24.
- **Liesenborghs I**, Schouten JSAG, Kutmon M, Gorgels TGMF, Evelo CT, Hubens WHG, Beckers HJM, Webers CAB, Eijssen LMT. A systematically derived overview of the non-ubiquitous pathways and genes that define the molecular and genetic signature of the healthy trabecular meshwork. Genomics. 2022 Mar;114(2):110280.

Submitted

- **Liesenborghs I**, van Beek D, Adriaens ME, Berendschot TTJM, Gorgels TGMF, Boesten I, Cornelissen MF, Ramdas WD, Nuijts RMMA, Beckers HJM, Webers CAB, Eijssen LMT, Schouten JSAG. A genome-wide association study for a corticosteroid-induced increase in intraocular pressure after using topical corticosteroids. Translational Vision Science & Technology.

Beyond the scope of this thesis

- **Liesenborghs I**, De Clerck EEB, Berendschot TTJM, Goezinne F, Schram MT, Henry RMA, Stehouwer CDA, Webers CAB, Schouten JSAG. Prevalence of optical coherence tomography detected vitreomacular interface disorders: the Maastricht Study. Acta Ophthalmol. 2018;96(7):729–736.
- Hubens WHG, Mohren RJC, **Liesenborghs I**, Eijssen LMT, Ramdas WD, Webers CAB, Gorgels TGMF. The aqueous humor proteome of primary open angle glaucoma: An extensive review. Exp Eye Res. 2020 Aug;197:108077.
- Hubens WHG, Mohren RJC, **Liesenborghs I**, Eijssen LMT, Ramdas WD, Webers CAB, Gorgels TGMF. Aqueous humor proteome of primary open angle glaucoma: A combined dataset of mass spectrometry studies. Data Brief. 2020 Sep 21;32:106327.
- De Clerck EEB, Schouten JSAG, Berendschot TTJM, Goezinne F, **Liesenborghs I**, Dagnelie PC, Schaper NC, Kroon AA, Henry RMA, Reesink KD, Schram MT, Stehouwer CDA, Webers CAB. Vascular risk factors for optical coherence tomography-detected macular cysts: The Maastricht Study. Acta Ophthalmol. 2020 Nov 30. doi: 10.1111/aos.14677.

ADDENDUM

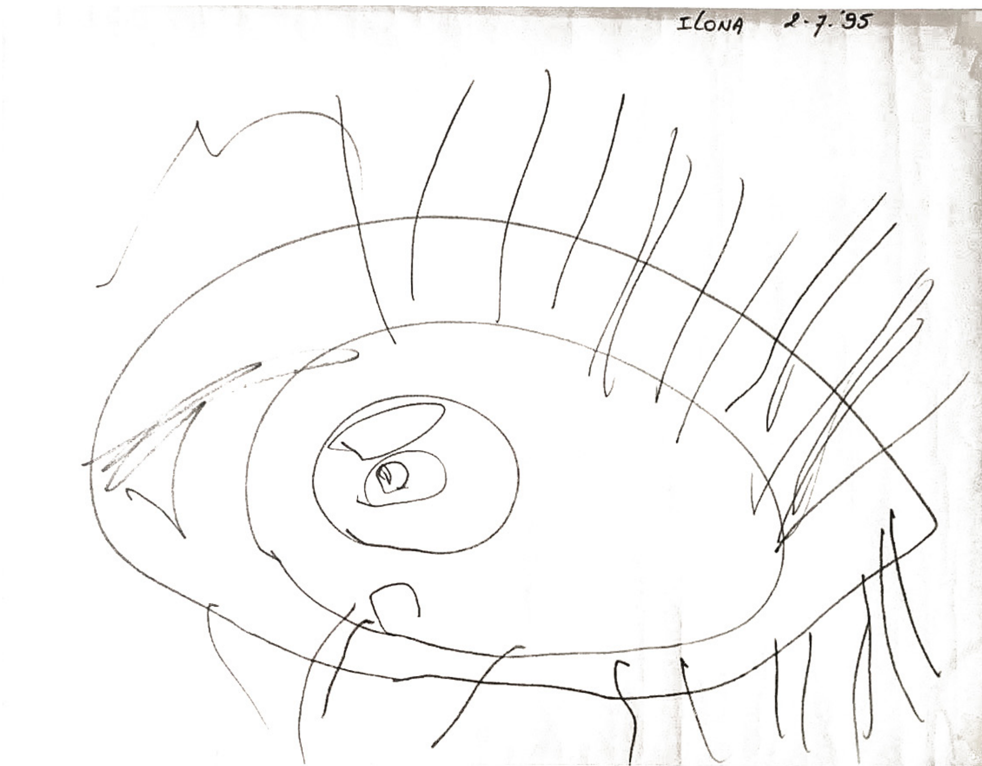
LIST OF PRESENTATIONS

- **I. Liesenborghs,** J.S.A.G. Schouten, T.T.J.M. Berendschot, H.J.M. Beckers, R.M.M.A. Nuijts, N. Visser, C.A.B. Webers; Risk factors for the development of ocular hypertension after keratoplasty: A systematic review; Jaarcongres Nederlands Oogheelkundig Gezelschap (NOG), June 2022, Groningen, The Netherlands
  Oral presentation
- **I. Liesenborghs**, D. van Beek, M.E. Adriaens, T.T.J.M. Berendschot, L.M.T. Eijssen, T.G.M.F. Gorgels, I. Boesten, M.F. Cornelissen, W.D. Ramdas, R.M.M.A. Nuijts, H.J.M. Beckers, C.A.B. Webers, J.S.A.G. Schouten; A genome-wide association study for a corticosteroid-induced increase in intraocular pressure after using topical corticosteroids; Jaarcongres Nederlands Oogheelkundig Gezelschap (NOG), Maastricht, March 2021, Virtual meeting
  Oral presentation
- **I. Liesenborghs**, L.M.T. Eijssen, M. Kutmon, T.G.M.F. Gorgels, C. T. Evelo, H.J.M. Beckers, C.A.B. Webers, J.S.A.G. Schouten; The molecular processes in the trabecular meshwork after exposure to corticosteroids and in corticosteroid response; European Glaucoma Society Congress (EGS), December 2020, Virtual meeting
  Poster presentation
- **I. Liesenborghs**, L.M.T. Eijssen, T.G.M.F. Gorgels, M. Kutmon, W.H.G. Hubens, C. T. Evelo, H.J.M. Beckers, C.A.B. Webers, J.S.A.G. Schouten; The signature of the healthy trabecular meshwork; Annual Meeting of the Association for Research in Vision and Ophthalmology (ARVO), April 2019, Vancouver, Canada
  Poster presentation
- **I. Liesenborghs**, L.M.T. Eijssen, T.G.M.F. Gorgels, M. Kutmon, W.H.G. Hubens, C. T. Evelo, H.J.M. Beckers, C.A.B. Webers, J.S.A.G. Schouten; The signature of the healthy trabecular meshwork; Jaarcongres Nederlands Oogheelkundig Gezelschap (NOG), Maastricht, March 2019, Maastricht, The Netherlands
  Oral presentation
- **I. Liesenborghs**, T.G.M.F. Gorgels, Prof. C. T. Evelo, M. Kutmon, L.M.T. Eijssen, C.A.B. Webers, J.S.A.G. Schouten; Bioinformatical pathway analyses to discover the molecular pathogenesis of primary open angle glaucoma; European Glaucoma Society Congress (EGS), May 2018, Florence, Italy
  Poster presentation
- **I. Liesenborghs**, L.M.T. Eijssen, T.G.M.F. Gorgels, M. Kutmon, C.T. Evelo, H.J.M. Beckers, C.A.B. Webers, J.S.A.G. Schouten; Comprehensive bioinformatics analysis of trabecular meshwork gene expression data to unravel the molecular pathogenesis of primary open angle glaucoma; Jaarcongres Nederlands Oogheelkundig Gezelschap (NOG), Groningen, March 2018, Maastricht, The Netherlands
  Oral presentation
- **I. Liesenborghs**, L.M.T. Eijssen, M. Kutmon, T.G.M.F. Gorgels, C.T. Evelo, W.H.G. Hubens, H.J.M. Beckers, C. A.B. Webers, J.S.A.G. Schouten; A bioinformatics approach to identify molecular pathways that characterize the trabecular meshwork; Annual Meeting of the Association for Research in Vision and Ophthalmology (ARVO), May 2017, Baltimore, Maryland, US
  Poster presentation

- **I. Liesenborghs**, L.M.T. Eijssen, M. Kutmon, T.G.M.F. Gorgels, C.T. Evelo, W.H.G. Hubens, H.J.M. Beckers, C. A.B. Webers, J.S.A.G. Schouten; Challenges to see patterns in reused data, identifying molecular pathways that characterize the trabecular meshwork; Science Café BigCat Maastricht university, April 2017, Maastricht, The Netherlands
  Oral presentation
- **I. Liesenborghs**, L.M.T. Eijssen, M. Kutmon, T.G.M.F. Gorgels, C.T. Evelo, W.H.G. Hubens, H.J.M. Beckers, C. A.B. Webers, J.S.A.G. Schouten; Challenges to see patterns in reused data, identifying molecular pathways that characterize the trabecular meshwork; Conference on Bioinformatics and Systems Biology (BioSB), April 2017, Lunteren, The Netherlands
  Poster presentation
- **I. Liesenborghs**, L.M.T. Eijssen, T.G.M.F. Gorgels, , M. Kutmon, W.H.G. Hubens, C. T. Evelo, H.J.M. Beckers, C.A.B. Webers, J.S.A.G. Schouten; Bioinformatica analyses voor de identificatie van moleculaire pathways in normaal functionerend trabeculair meshwork; Jaarcongres Nederlands Oogheelkundig Gezelschap (NOG), Maastricht, March 2017, Maastricht, The Netherlands
  Oral presentation
- **I. Liesenborghs**, L.M.T. Eijssen, M. Kutmon, Prof. C. T. Evelo, T.G.M.F. Gorgels, H.J.M. Beckers, C.A.B. Webers, J.S.A.G. Schouten; Molecular pathway analysis in trabecular meshwork cells after treatment with corticosteroids; European Conference on Computational Biology (ECCB), September 2016, The Hague, The Netherlands
  Poster presentation
- **I. Liesenborghs**, T.G.M.F. Gorgels, Prof. C. T. Evelo, M. Kutmon, L.M.T. Eijssen, C.A.B. Webers, J.S.A.G. Schouten; Molecular pathway analysis in patients with primary open angle glaucoma; European Glaucoma Society Congress (EGS), June 2016, Prague, Czech Republic
  Poster presentation
- **I. Liesenborghs**, T.G.M.F. Gorgels, Prof. C. T. Evelo, M. Kutmon, L.M.T. Eijssen, C.A.B. Webers, J.S.A.G. Schouten; Molecular pathway analysis in patients with corticosteroid induced glaucoma; Conference on Bioinformatics and Systems Biology (BioSB), April 2016, Lunteren, The Netherlands
  Poster presentation
- **I. Liesenborghs**, T.G.M.F. Gorgels, Prof. C. T. Evelo, M. Kutmon, L.M.T. Eijssen, C.A.B. Webers, J.S.A.G. Schouten; Moleculaire pathway analyse in patiënten met primair open hoek glaucoom; Jaarcongres Nederlands Oogheelkundig Gezelschap (NOG), Maastricht, April 2016, Maastricht, The Netherlands
  Oral presentation
- **I. Liesenborghs**, J.S.A.G. Schouten, T.G.M.F. Gorgels, M.Kutmon, L.M. Eijssen
  Gene networks of POAG and corticosteroid induced glaucoma; Overkoepelend overleg glaucoomspecialisten, bioinformatici, systeembiologen en studenten MUMC+, February 2016, Maastricht, The Netherlands
  Oral presentation
- **I. Liesenborghs**, J.S.A.G. Schouten, T.G.M.F. Gorgels, Prof. C.T. Evelo, M. Kutmon, L.M.T. Eijssen, C.A.B. Webers; Moleculaire pathway analyse in patiënten met primair open hoek en corticosteroïd geïnduceerd glaucoom; Refereeravond oogheelkunde MUMC+, March 2013, Elsloo, The Netherlands
  Oral presentation

ADDENDUM

DANKWOORD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