Investigating the genetic complexity of glaucoma
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CHAPTER 1

Introduction
Glaucoma

Vision is a complex sensory process. It involves the eye, the optical pathways, and the cerebral cortex. The eye is the sensory organ of sight: as light enters the eye, it travels through the cornea and the lens to the retina. Here, the sensing photoreceptors transform the light into electrical impulses through a cascade of electrical and neurochemical events. Finally, the retinal ganglion cells (RGCs) transmit the signals via the optic nerve fibres to the visual cortex and beyond, giving the sensation of seeing (Figure 1).

Many conditions affect the eye and cause visual impairment. Glaucoma is the second leading cause of blindness worldwide. It comprehends a group of clinically and genotypically heterogeneous eye conditions that ultimately damage the optic nerve. Glaucoma has traditionally been diagnosed with a triad of clinical symptoms: increased intraocular pressure (IOP), changes in the appearance of the optic disc, i.e., the optic nerve head, and visual field defects.

Histological studies have shown that there is a significant loss of RGCs and optic nerve fibres before a functional deficit become evident with current visual field examination techniques. Indeed, as the disease progresses, the retinal nerve fibre layer thins and creates a depression, called cup, in the optic nerve head. This depression can be quantified through measurement and analysis of the cup-to-disc ratio, which is the size ratio of the cup and the optic nerve head. A ratio of approximately 0.7 is the upper limit of normality and the closer the cup-to-disc ratio is to the value of 1, the more advanced is the stage of glaucoma. The primary site of glaucomatous axonal injury involves the RGCs in the lamina cribrosa, a mesh-like structure of collagen fibres located at the optic nerve head that allows the nerve fibres and the retinal blood vessels to pass through the sclera.1–3

Prevalence of glaucoma

The number of people affected by glaucoma worldwide is expected to increase from the current 64 million (3.5% of the population aged 40 to 80) to 112 million in 2040. Over the past 25 years, glaucoma patients suffering by moderate to severe vision loss grew from 3 million (1990) to 4 million (2015). Simultaneously, blindness due to glaucoma has grown from 2.5 to 3 million affected.1

Primary open-angle glaucoma (POAG) is the most common form of glaucoma, and population-based studies have reported that the prevalence rates range from 0.5 to 7.0% in adults aged 40 and older, depending on the ethnic group.1 POAG is a worldwide public health burden that requires improvement in diagnostic and monitoring approaches, particularly in those populations with high prevalence (e.g., elderly and African individuals).1
Figure 1. Anatomy of the eye seen in a cross section. From left to right: cornea allows light to enter the eye and the sclera (a continuation of the cornea). Both constitute the outer part of the eye. The iris controls the amount of light entering the eye by varying pupil size. The ciliary body produces aqueous humour, which is drained into the systemic circulation by the canal of Schlemm. The ciliary body also controls the shape of the lens. The lens focuses the light onto the retina, which converts the light images into electrical and neurochemical signals. The retina is surrounded by a vascular layer called the choroid. Subsequently, axons of retinal ganglion cells, organized as fibres of the optic nerve and surrounded by lamina cribrosa, carry the electrical signals to the cerebral cortex in the brain (not shown).

Glaucoma classification

Clinical classifications of the various forms glaucomatous disease are based on different criteria: time of onset (congenital or adult), pathogenesis (primary or secondary), shape of the anterior chamber (open-angle or closed-angle), levels of the intraocular pressure and, where applicable, DNA diagnosis.¹

Time of onset: In congenital glaucoma, the disease onset is by definition in the infantile period (<3 years old). This form of glaucoma is rare, with a prevalence of 1 in 12-18,000 births among Caucasians. It is more frequent in males (65%) and bilateral in 70% of cases. When glaucoma arises later in life, between the ages of 4 and 35, it is annotated as juvenile glaucoma, while above the age of 35, it is considered adult glaucoma. The most common type of adult-onset glaucoma is open-angle glaucoma.
The condition is asymptomatic in its early stages, a peculiarity that explains why in the western world about 50% of cases are left undiagnosed and are initially unaware of having this condition.\(^7\)

Pathogenesis: Primary glaucoma is an isolated glaucoma, where abnormalities of the iridocorneal angle led to decreased aqueous outflow and as consequence to an increased IOP.\(^8\) In primary glaucoma, the most frequent cause of disease is the damage to the trabecular meshwork, the area between the sclera and cornea where aqueous humour is drained out of the eye. The reduced transport of the aqueous humour out of the eye results in an increase in intraocular pressure, which subsequently causes compression of the axons of the RGCs at the level of the lamina cribrosa.\(^9\) Differentiation based on whether the angle between the iris and the cornea is open or closed determines the classification in primary open-angle glaucoma (POAG) and primary closed-angle glaucoma. In secondary glaucoma, the pathology is a consequence of other ocular and systemic disorders and is associated with anomalies in the eye, such as pseudo-exfoliation, pigment dispersion syndrome, exposure to steroid medication, and anterior segment dysgenesis (ASD).\(^10\) Nowadays, genetic tests can help clinicians to confirm a clinical diagnosis of primary glaucoma for monogenic forms of the disease such as caused by mutations in the \textit{MYOC}, \textit{OPTN} and \textit{TBK1} genes.\(^11\)

IOP levels: Normal IOP ranges from 12 to 21 mmHg. When it is greater than 21 mmHg, and is accompanied with optic nerve damage, it is referred to as high-tension glaucoma, which accounts for approximately 75% of patients diagnosed with glaucoma. In contrast, glaucoma patients with an IOP in the normal range are defined as having normal tension glaucoma.\(^12\) The phenomenon of normal tension glaucoma is yet unexplained, although this may be caused by a net translamellar pressure difference over the optic disk, between the aqueous humour (normal pressure) and the cerebrospinal fluid (low pressure).\(^13\)

**Risk factors for POAG**

Many risk factors for POAG have been recognized. The most widely acknowledged include: advanced age, elevated IOP, African descent, positive family history for glaucoma, and thin central corneal thickness (CCT).\(^14\)-\(^17\) Each risk factor is described one by one below.

Advanced age is one of the most well-documented risk factors for POAG.\(^18\)-\(^22\) Being older predispose individuals to POAG, because older eyes are themselves more susceptible to damage due to a decrease in the number of RGCs, or because the increase in risk is the result of cumulative exposure to other risk factors (e.g. various systemic diseases as diabetes, hypertension, ischemic vascular diseases, and unhealthy life-style, as smoking and alcohol consumption), which makes the optic nerve head more vulnerable to damage.\(^23\)-\(^26\)

Elevated IOP is another risk factor for glaucoma. Concerning this, without any exception, all epidemiology studies have confirmed that IOP is a highly significant risk factor.\(^27\)-\(^28\) In addition, in
a clinical prospective study, Bergea and colleagues found that IOP range, peak, and mean can predict the visual field worsening that occurs in POAG.\textsuperscript{29,30}

Ethnicity is another major risk factor for POAG, specifically for individuals of African ancestry. In the Baltimore Eye Survey, African Americans had a three to six-fold increase in risk of POAG compared to Caucasians.\textsuperscript{31,32} A similar increase in risk and a more severe presentation of symptoms of POAG has also been demonstrated in other populations of African descent.\textsuperscript{17,33–35} Analyses on ethnic differences in ocular characteristics, such as corneal thickness and optic disc structures, but also poor access to the healthcare system might explain the increased risk of glaucoma in individuals from African descent.\textsuperscript{36} From the genetic view, it has been observed that having a higher proportion of African ancestry in the genome increases the severity of POAG manifestation.\textsuperscript{37} However, fewer genetic studies of African ancestry have been performed and interestingly, the loci discovered in Caucasians have a limited role in those of African ancestries.

Family history has also been reported as a major risk factor. Results from the Rotterdam Study suggest that relatives of patients with POAG have a ten-fold increase in risk to develop the disease, suggesting a strong genetic component in the aetiology of POAG.\textsuperscript{18,22,38,39}

Finally, CCT has been identified as a risk factor and predictor of glaucoma progression. Applanation tonometry used to measure IOP is known to be influenced by CCT.\textsuperscript{40,41} In fact, CCT has a positive and linear correlation with IOP: thus, having a thick cornea increases the measured IOP compared to the actual (i.e. true) IOP.\textsuperscript{42} Therefore, a thinner CCT lead to underestimation, while a thicker one to an overestimation of IOP.\textsuperscript{43} Failure to adjust IOP for the CCT measurement leads to a wrong evaluation of the IOP and as consequence administering potentially inappropriate therapy.

## Genetics of POAG

Identifying the genetic factors influencing the susceptibility to POAG is an important step toward understanding the cause of a disease and to provide directions for the development of new treatments. Genetic studies in twins, families and populations revealed that POAG is a highly heritable and heterogeneous disease.\textsuperscript{44} Heritability is defined as the proportion of the observed phenotypic variation among individuals that is attributable to genetic variability. A recent systematic review of the literature reported heritability estimates of POAG to range between 0.17 and 0.81.\textsuperscript{45,46} Mutations in monogenic forms of glaucoma were identified in familial linkage studies with a clear segregation pattern. However, in most of the cases, POAG shows a complex inheritance where many genetic risk factors with small effect size play a role in the disease. Hundreds of genetic association studies, mostly conducted in European and Asian populations, were performed, and many POAG associated genomic locations were found. Taken together, genetic variation in these loci explain approximately 10% of POAG risk. To complicate matters further, in individuals of African ancestry most of the previously identified genetic variants do not play a role in the disease. This suggests that the genetic architecture of POAG, is different among populations, and possibly also dependent on allele frequency.\textsuperscript{37,47,48} In other words, a large
proportion of POAG heritability is still missing, and its actual estimates clearly depend on the studied population. The aims of recent genetic POAG investigations, including the ones studied in this thesis, are to elucidate the reasons behind this missing heritability. Many approaches are required for the identification of these genetic factors, and a description of them is provided in the next section.

**Gene discovery methods in POAG**

Initially, family studies, and in particular twin studies, played an important role in identifying the genetic risk of diseases. Most familial POAG cases appear to segregate as an autosomal dominant trait with reduced penetrance, although in-depth investigation of these patterns does not reflect a simple mode of inheritance or a single underlying genetic cause. Clear familial forms of POAG are more severe and have an earlier average age of onset. Congenital and juvenile forms of POAG (JOAG) are often inherited in either an autosomal recessive or autosomal dominant manner. While multi-genetic susceptibility is clearly involved in the pathophysiology of POAG, the late age of onset and its generally unnoticed progression make it impractical to discern the exact mode of inheritance.

So far, gene-findings in glaucoma have been conducted by “linkage” and “association” approaches, and by making use of the full POAG disease spectrum or of glaucoma endophenotypes. Following the rationale that close loci on chromosome have higher probability of being inherited together, the linkage analysis approach aims to identify the genome location of a disease gene (causative mutation), through its co-segregation in pedigrees. Linkage analysis and subsequent identification of the disease gene by positional cloning, has been performed to study monogenic forms of glaucoma, such as the juvenile form of POAG in large pedigrees and with multiple affected individuals. Association analysis aims to identify if a disease is significantly associated with a gene or genetic (risk) variant in a population. The association can be due to a direct role of gene or genetic variant in the disease or because they are in linkage disequilibrium with the causal variant.

In the 1990s, using linkage approaches, the first studies conducted in families affected by POAG led to the identification of autosomal inherited mutations in the myocilin (MYOC), optineurin (OPTN), and WD Repeat Domain 36 (WDR36). These mutations have high penetrance and account for 3-6% of POAG cases. The MYOC was the first gene discovered to be associated with POAG. MYOC mutations cause 2 to 4% of POAG in the population, and around 40% of mutations in this gene are disease-causing mutations. MYOC is a secreted protein that is involved in extracellular matrix turnover and cytoskeleton function. The OPTN gene stands for “optineurin” and rare mutations in this gene can cause normal-tension glaucoma. Most of these mutations cause oxidative stress, apoptosis in RGCs, and influence aqueous humour regulation. The disease gene WDR36 is involved in cell cycle progression, signal transduction, apoptosis, and gene regulation. A study conducted in 2005 reported that mutations in this gene could cause POAG. However, the association between WDR36 mutations and POAG has not been replicated in other studies, suggesting that mutations in the WDR36 gene
are not sufficient to cause the disease. By using linkage analysis more than 20 loci have been associated with POAG and JOAG.

Later on, new methods, based on association approach, started to search for genes (candidate gene association study) and genetic variants (genome-wide association studies) associated with the disease. The gene association study selects candidate disease genes based on biologically driven hypotheses, or for their role in the aetiology. Nowadays, more than hundreds of candidate genes have been associated to POAG. These candidate gene studies represent an efficient and economic approach for the discovery of genes involved in a disease, however they are limited by current knowledge of the pathobiology. The genome-wide association studies (GWAS) investigate genetic variants spanning the entire genome to calculate the heritability, accounting for the variance explained by common genetic variants. GWAS is an hypothesis-free investigation method that scans the entire genome to identify the potential associations between genetic variation in loci and traits. GWAS has been increasingly used for the identification of genetic risk factors in POAG due to a number of advantages: it does not rely on a priori hypothesis, it is applicable to case-control datasets, and it does not depend on familial aggregates. However, commonly recognized disadvantages of GWAS approach are the reliance on genetic variants present in genome reference panels and the fact that it cannot detect rare variants contributing to traits. Since its first implementation in 2007 in a POAG study, many successful GWASs have been conducted. These studies contributed to finding more than 120 loci associated with POAG, spanning an equal number of candidate disease genes. The workflow of a GWAS involves several steps, which are depicted in Figure 2.

The endophenotype approach of GWAS has been conducted also in studying quantitative traits of POAG, such as central corneal thickness (CCT), IOP, and vertical cup-disc ratio (VCDR). This approach requires that the endophenotype must be heritable and correlated to POAG, but that it is not disease causative by itself. Furthermore, since quantitative GWAS does not require categorizing individuals as cases or controls, it avoids the inconvenience of misclassification. The presence of genetic overlap between the endophenotype, IOP for example, and POAG indicates that genes found associated with the endophenotype are more likely to be involved in POAG risk.
Chapter 1

**Figure 2.** Workflow for conducting GWAS. 

**a.** Data from cases and controls is collected from study cohorts.  
**b.** Genotypic data is collected using arrays.  
**c.** Quality control is performed on genotype calling.  
**d.** Genotypic data is imputed using information from matched reference populations.  
**e.** Genetic association tests run for each genetic variant.  
**f.** Results from multiple cohorts are combined.  
**g.** Results are replicated in an independent cohort.  
**h.** Post-GWAS analyses can be performed to identify the genetic causes of disease. Source: https://www.nature.com/articles/s43586-021-00056-9

### Missing heritability in POAG

Early studies on heritability were performed by comparing the phenotypic resemblance of monozygotic and dizygotic twins. With the advent of GWAS, thousands of genetic variants have been identified associated with many different traits. Still, in general, GWAS can explain only a small fraction of the total heritability. The gap between heritability estimates from twins and genotype data, the so-called missing heritability, has drawn the attention of researchers.72

GWAS have identified common variants typically with a minor allele frequency greater than 5%, leaving variants with lower frequencies (rare variants) undetected. The reasons why rare variants are excluded in GWAS are different: they may not be present on the SNP arrays, or not well imputed due to the low linkage disequilibrium with common variants, or because they are present only in specific populations or families. These are the reasons why GWAS fail to account for a
fraction of disease heritability. Indeed, rare variants have been proposed as a source of missing heritability. Although they are not always fully penetrant, the most powerful way to investigate them is through family studies with multiple affected, even if these families do not have an entirely clear mendelian segregation pattern. In fact, analysing families of many individuals affected by POAG has helped to identify rare variants, mainly because they are found at a higher frequency than in the general population. Furthermore, rare variants are more likely to have larger effect size and functional consequence, making an important contribution to the POAG missing heritability. Not only rare variants, but also non-coding genomic variants, structural variations as insertion, inversions, DNA deletions, as well as copy number variations (CNVs) have been proposed as a source of missing heritability. CNVs are genomic insertions or deletions that are larger than 1 kb. CNVs are now recognized as a major contributor to human genetic variation and have been associated with other genetically complex disorders. However, for POAG phenotype, CNV detection is still quite an uncharted path. The discovery of new associations between structural variations and POAG can help to identify genes and improve our understanding of the aetiology underlying POAG.

Another potential limitation of the majority of current studies that aim to explain the missing heritability of POAG is their sole focus on the nuclear genome. It has been postulated that part of the remaining POAG heritability might also be found in variants in the mitochondrial DNA (mtDNA). Mutations in the mtDNA have been previously implicated in cellular energy deficits that lead to optic nerve damage and as a consequence to mitochondrial optic neuropathy. Indeed, the mitochondria generate energy for the cell, so it is expected that mitochondrial disorders tend more frequently to affect tissues with high-energy demand, such as the retina, brain, muscles, and heart.

In conclusion, complex diseases such as POAG exhibit substantial genetic heterogeneity. Therefore, identifying POAG susceptibility factors requires the use of multiple complementary techniques, bioinformatics approaches and last but not least, a precise phenotyping. Indeed, there is a necessity to investigate further sources of genetic variations in POAG and to continue research that aim to understand the pathobiological impact of genes and pathways.

**Clinical features and gene discovery of congenital glaucoma**

Primary congenital glaucoma is an inherited form of glaucoma that arises in the first months of life or even at birth. It is caused by a development defect of the aqueous outflow system, which leads to isolated angle anomalies leading to an increase in IOP, enlarged ocular diameter (buphthalmos), corneal clouding and/or Haab's striae, and finally optic neuropathy. Primary congenital glaucoma implies a huge impact on the child's development and long-term quality of life. Early treatment of affected children consists mainly of surgery, an approach that can make a major difference in the preservation of visual function and in the prevention of lifelong disability. In this form of glaucoma, a strong genetic component is evident because its prevalence is 5 to 10 times higher in patients who have first-degree relatives affected by the same pathology.
majority of primary congenital glaucoma cases are familial cases, predominantly transmitted in an autosomal recessive manner. Mutations in three disease genes and in one genetic locus are implicated in congenital glaucoma (2p22.2 CYP1B1, 1p36.2-p36.1, 14q24.3 LTBP2, and 2p25.3 PXDN), as well as the newly discovered genes such as TEK, ANGPT1, and CPAMD8. The CYP1B1 (Cytochrome P450 Family 1 Subfamily B Member 1) gene encodes a member of the cytochrome P450 superfamily of enzymes and its protein is expressed in cornea, ciliary body, iris, and retina. The LGP2 (Latent Transforming Growth Factor Beta Binding Protein 2) gene is expressed in the trabecular meshwork and is involved in cell adhesion and the regulation of aqueous humor. The PXDN (peroxidisin) gene is involved in extracellular matrix formation. The PXDN proteins are localized in the cornea and lens, and are required for the normal development of the anterior chamber of the eye.

In clinically defined secondary congenital glaucoma, the pathological picture is more complex. Here, the pathology is a final consequence of one or more causes combined, such as ocular injuries, inflammation, or other primary congenital ocular anomalies, referred to as ASD. This includes Axenfeld-Rieger anomalies, Peters’ anomaly, and aniridia. Axenfeld-Rieger anomaly refers to congenital ocular anomalies including abnormal angle tissue, iris stromal hypoplasia, pseudopolycoria (multiple pupils), corectopia (eccentric pupil), posterior embryotoxon (thickened and anteriorly displaced Schwalbe’s line), and/or irido-corneal adhesions. Systemic features including dental, umbilical, cardiac anomalies, and/or hearing loss are among the most frequent and can be included in the Axenfeld-Rieger syndrome (ARS). Individuals with ARS face a 50% risk of developing glaucoma due to the developmental defects in the anterior chamber angle. Peters’ anomaly consists of central corneal opacity, with or without irido-corneal adhesions, corneal-lenticular adhesions, defects in the posterior layers of the cornea, and congenital glaucoma. Aniridia is characterized by a complete or partial absence of the iris often with foveal hypoplasia resulting in nystagmus and reduced visual acuity. Frequently associated ocular abnormalities include glaucoma, cataract, corneal changes, lens subluxation, strabismus, optic nerve hypoplasia, and microphthalmia. Individuals with aniridia develop glaucoma, usually with an onset in late childhood or adulthood. Although ARS, Peter anomaly and/or aniridia are clinically complex and heterogeneous entities, these disorders may be derived from unique single disease genes (in a family).

Patients with ARS display predominantly autosomal dominant transmission with sporadic cases often reflecting a de novo inheritance. Two genetic loci (16q24 and 13q14) and mutations in two disease genes (FOXC1 and PITX2), have been implicated in ARS so far. The gene, FOXC1, is a member of the forkhead box transcription factor family and has a key role in cardiac, renal, and ocular morphogenesis. Mutations in FOXC1 are mainly detected in ARS patients with ocular anomalies. The gene PITX2 is a member of the bicoid-like homeodomain transcription factor family and plays an important role in the development of eyes, heart, brain, limb, umbilicus, and teeth. Many of these tissues are derived from neural crest cells, implying that the clinical features of ARS are probably due to a failure of the migration and differentiation processes during embryonic development. Mutations in FOXC1 and PITX2 genes account for approximately 35% of patients with ARS, with the remainder of cases yet unresolved. It is undeniable that,
genetic mutation analysis combined with a precise clinical diagnosis are necessary in order to improve differential diagnosis and genetic counselling.

Unravelling the genetic causes of these monogenic disorders can lead to an improved diagnosis. Furthermore, genetic studies of these conditions can also enhance the knowledge of complex adult polygenic of glaucoma diseases, since they can share underlying biological mechanisms.

Scope of the thesis

The occurrence in families as well as the high heritability estimates of glaucoma in population studies and GWAS results strongly suggest that many genetic factors play a major role in glaucoma. Each of these individual genetic factors apparently contributes only relatively little to the pathogenesis.

Family and population studies explain the genetic POAG load only partially, leading to the so-called “missing heritability” issue. In the literature, there are several hypotheses about the source of this missing heritability, such as, variations in the mitochondrial genome, rare variants, and copy number variations. In addition, multi-ancestry studies are warranted today to explain the missing heritability and describe differences in clinical presentation of POAG across ethnic groups.

The aim of this thesis was to investigate different sources of genetic risk factors that contribute to glaucoma susceptibility and that may explain part of its missing heritability. This was achieved by (i) investigating the ethnic risk factors for POAG (Chapter 2), (ii) identifying causal mutations in patients affected by a secondary form of congenital glaucoma (Chapter 3), (iii) investigating whether copy number variations are implicated in molecular mechanisms underlying POAG (Chapter 4), (iv) analysing potential association between mitochondrial DNA variants and POAG (Chapter 5). Finally, (v) performing a GWAS meta-analysis across cross multiple ancestry in a large-scale study, in order to identify novel risk loci for POAG and explore their biological and functional contributions with post-GWAS analyses (Chapter 6).

In the discussion section (Chapter 7), I place the findings in a wider perspective and speculate on future research directions. As new genes are identified and the contribution of genetic risk factors to POAG is deciphered, these findings need to be integrated in the current understanding of the genetics of POAG, exploring also a possible direct applicability in genetic testing and counselling.
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