Genetic testing for glaucoma: lessons learned from translational genetic research and implications for genetic counselling

by

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SUMMARY

My original contribution to knowledge includes the delineation of the phenotypes associated with known glaucoma genes, the identification of novel glaucoma genes and the study of the ethical and psychological implications of genetic testing and counselling for glaucoma. There is currently a gap in knowledge regarding the role of genetic counselling in glaucoma. Genetic counselling can have a significant impact on patients and their families through discussing genetic testing and results, modes of inheritance, risk for family members and future children, and helping patients and their families adapt to the psychological implications of the genetic contribution to the disease. Glaucoma is a leading cause of irreversible blindness worldwide, with devastating implications for individuals if not diagnosed and treated appropriately. The aetiology of glaucoma is genetically heterogenous: both Mendelian and multifactorial inheritance occur, and the condition can affect all ages, from newborns to adults, each with their own implications for families. Through the publications included in this thesis, I investigated the monogenic causes of glaucoma and the benefits of the provision of genetic counselling. I demonstrated that *MYOC* and *CYP1B1* variants associated with primary open-angle glaucoma were more prevalent in individuals with severe disease, I reported on the characterisation of novel variants and I contributed to the delineation of phenotypes associates with *TBK1* duplications in primary open-angle glaucoma. I contributed to the identification of novel genes for primary congenital glaucoma (*TEK*) and nanophthalmos (*TMEM98*), I reported on the glaucoma prevalence and phenotype associated with *FOXC1* & *PITX2* variants and the contribution of *FOXC1* towards primary congenital glaucoma. Finally, I investigated participants' experience of predictive genetic testing for *MYOC* glaucoma, the benefits of predictive genetic testing in minors for childhood-onset *MYOC* glaucoma and the ethical implications of incidental findings from genetic testing. The genetic heterogeneity and the phenotypic overlap in glaucoma justifies a targeted gene panel approach using high throughput sequencing technologies to confirm or refine the clinical diagnosis based on medical and familial information. Precision medicine to tailor prevention and treatment plans based on an individual's genetic results is an appealing strategy, especially considering that preventative and restorative therapies are under development for glaucoma. Identifying at-risk individuals based on molecular diagnosis is becoming critically important in the context of clinical trials for targeted therapies, and disease registries such as the Australian and New Zealand Registry of Advanced Glaucoma can greatly facilitate the recruitment process. Current research directions in glaucoma highlight a role for genetic counsellors in translational genetic research. My work contributed towards a framework for genetic counselling in glaucoma through the incorporation of translational research outcomes to provide the best genetic counselling and genetic testing options to patients and their families.

DECLARATION

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Emmanuelle Souzeau

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Although this thesis is based on the publications that resulted from eight years of research at Flinders University, the foundations of this achievement have been laid way before I came to Australia. This journey would not have been possible without a number of individuals who believed in me or guided me over the years, and who motivated me to challenge myself and explore new trajectories.

I first started a Masters in physiology in 2002 under the supervision of Dr Christian Deschepper at the University of Montreal. I am very grateful to Christian for his great mentoring and for encouraging me to pursue in research.

Although I enjoyed research very much, at the end of my Masters I realised that I was drawn back toward a career in health. A special thank you to Isabelle Boutin-Ganache who introduced me to the field of genetic counselling. I discovered a profession that combined my desire of helping others with ongoing learning in genetics and ethics.

In 2004, I entered the first Canadian genetic counselling program in French at the University of Montreal. I shared this journey with three amazing persons who became close friends. Jacynthe, Daniela & Annie, thank you for sharing the laughter, the tears, the doubts and the fun during these two years.

In 2006, I obtained a position as a genetic counsellor at Sainte Justine's Hospital in Montreal. A number of individuals have helped me become a better genetic counsellor. A special thank you to Dr Valerie Desilets, Dr Sonia Nizard, and Dr Marie Lambert, whose dedication to patients has been inspirational and who have always believed in me, even when I had doubts.

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ABBREVIATIONS

ANZRAG	Australian and New Zealand registry of advanced glaucoma
ASD	Anterior segment dysgenesis
CDR	Cup to disc ratio
CNV	Copy number variation
GWAS	Genome wide association study
HTG	High tension glaucoma
IOP	Intraocular pressure
iPSC	Induced pluripotent stem cell
JOAG	Juvenile open-angle glaucoma
MLPA	Multiplex ligation-dependent probe amplification
NTG	Normal tension glaucoma
PACG	Primary angle-closure glaucoma
POAG	Primary open-angle glaucoma
PCG	Primary congenital glaucoma
SNP	Single nucleotide polymorphism
WES	Whole exome sequencing
WGS	Whole genome sequencing

ASSOCIATED PUBLICATIONS BY CHAPTER

This thesis forms a PhD by Published Work and includes 16 publications accumulated prior or during the enrolment in the PhD that constitute a significant original contribution to knowledge.

Chapter 2: Genetics of primary open angle glaucoma

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Chapter 4: Ethical and psychological implications of genetic counselling for glaucoma

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CHAPTER 1: BACKGROUND

1.1. Genetic counselling and genetic testing

1.1.1. Genetic counselling: Background & Definition

Genetic counselling is a fairly young discipline that emerged only 50 years ago. It followed a century of new knowledge related to human molecular genetics. From the rediscovery of Mendel's laws that paved our understanding of the classic inheritance patterns, to the discovery of DNA, the identification of genes and the sequencing of the human genome, our understanding of human genetic diseases has considerably evolved and is growing exponentially. The term genetic counselling was first introduced in 1947 by Dr Sheldon Reed, a non clinician with a doctorate in genetics, who described it as a "kind of genetic social work" [1]. In the early 70's, there was a recognition that genetic counselling should not only be about conveying genetic knowledge to patients and their families in an understandable way and a sensible manner but should also include a psychosocial perspective [2]. As a result, the first genetic counselling program, which started in 1969 at Sarah Lawrence College in the United States, incorporated the work of Carl Roger's on person-centered counselling to address the psychosocial issues of the practice [3]. Subsequently, the first definition of genetic counselling was adopted by the American Society of Human Genetics in 1975 [4]:

> "Genetic counselling is a communication process which deals with the human problems associated with the occurrence, or the risk of occurrence, of a genetic disorder in a family. This process involves an attempt by one or more appropriately trained persons to help the individual or family to (1) comprehend the medical facts, including the diagnosis, probable course of the disorder, and the available management; (2) appreciate the way heredity contributes to the disorder, and the risk of recurrence in specified relatives; (3) understand the alternatives for dealing with the risk of recurrence; (4) choose the course of action which seems to them appropriate in view of their risk, their family goals, and their ethical and religious standards, and to act in accordance with that decision; and (5) to make the best possible adjustment to the disorder in an affected family member and/or to the risk of recurrence of that disorder."

This definition was primarily centered on reproductive risks assessment and counselling. With the advances in genomics medicine, genetic counselling quickly expanded to include adult-onset conditions and complex diseases. In 2006, the National Society of Genetic Counsellors developed a broader definition that applied to the various settings in which genetic counselling takes place [5]:

> "Genetic counselling is the process of helping people understand and adapt to the medical, psychological, and familial implications of the genetic contributions to disease. This process

integrates: (1) Interpretation of family and medical histories to assess the chance of disease occurrence or recurrence, (2) Education about inheritance, testing, management, prevention, resources and research, (3) Counselling to promote informed choices and adaptation to the risk or condition".

Other countries have since developed similar definitions, including Australia[6]. Although the definition of the scope of genetic counselling might differ between countries based on the medical practice, the social background and the ethical values of each country, Rantanen et al analysed 56 documents from different countries and reported a uniform view of genetic counselling [7]. However, definitions of genetic counselling only reflect the practice at a particular time [8] and are meant to evolve as the practice, the advancements in technology and knowledge, and the ethical guidelines evolve.

Although the consultation with the patient is often considered the main component of genetic counselling, the management of a case is a lengthy process involving pre-counselling preparation and post-counselling follow up [6]. Because genetic counselling is dependent on the information gathered, it is essential to verify any clinical diagnosis for the patient or its family members, review any genetic results and their interpretation, and establish appropriate risk assessment incorporating all available data. Once information has been gathered, it needs to be communicated to the patient in an understandable and non-judgemental manner. The consultation involves educating the patient about the natural history of the condition and its inheritance and discussing testing, management and prevention options. The provision of genetic information can have a profound impact on patients and their families. Genetic counselling involves identifying the patients' needs, expectations and emotional responses, providing supportive counselling and helping patients adapt to the medical, psychological and familial implications of the genetic information with sensitivity to ethnic and cultural contexts.

1.1.2. The role of genetic testing

Genetic counselling often involves a discussion on the possibility of performing genetic tests to identify the underlying cause of a particular disease or phenotype. Genetic testing is a complex process that requires the patient to fully understand the implications of being tested and to provide informed consent and autonomy in decision-making [9-11]. Genetic counsellors have the necessary expertise to discuss available options and testing with patients, including the potentials benefits, risks and implications to enable patients to make informed decisions. They also have the ability to correctly interpret genetic findings and results, communicate them to patients in lay language, counsel them about the potential implications of results and help them cope with psychological impact. The counselling relies on the accuracy of clinical diagnosis and a molecular diagnosis can

strongly assist in providing optimised risk estimates and genetic information to patient and their families:

- A molecular diagnosis can provide information regarding the prognosis, the most appropriate treatment options and the need to monitor potential complications or associated symptoms.

- Patients and their relatives can be more accurately counselled about the mode of inheritance. A molecular diagnosis is especially valuable in the case of an affected individual with no family history where different inheritance patterns are possible.

- Recurrence risk for future children and potential reproductive options become available. Carrier testing can be offered in the case of autosomal recessive or X-linked transmission for reproductive risk assessment. Prenatal and preimplantation diagnosis can be discussed with couples in the case of severe conditions.

- Other family members might be at risk of developing the condition and a molecular diagnosis is the only way to precisely determine the risk for relatives. Predictive and presymptomatic testing assess an unaffected individual's likelihood of developing a condition present in their family. This type of testing can have significant benefits for conditions that are treatable and where early detection can change the course of the condition.

- Finally, a molecular diagnosis is important to identify individuals who could be eligible for future clinical trials and personalised treatments.

However, the absence of a molecular diagnosis does not prevent the provision of genetic counselling and risk information. The analysis of a pedigree often provides insightful information for determining the (most likely) modes of inheritance in a family. Although the pedigree is an essential tool in assessing rare Mendelian diseases, it can also provide valuable information to determine risk assessment for more common complex diseases in combination with empiric risk figures.

1.1.3. Genetic counselling in the context of glaucoma

The provision of genetic counselling is experiencing an emerging shift towards common diseases. This is the result of a fast-growing pace of medical genomics and technologies that led to an increasing understanding of the genetics of human disease and genetic testing applicability for patient care. Genetic counselling is continuously adapting and integrating new emerging genetic

knowledge. Many common diseases have a genetic basis but do not always follow Mendelian inheritance. They can be multifactorial with a contribution from both genetic and environmental factors. Because of the familial nature of such conditions, patients and their families may seek genetic counselling to obtain answers about the risk of occurrence or recurrence of the disease in the family, the availability of genetic testing and the management in the context of genetic findings. As a result, different specialties such as cardiogenetics, neurogenetics, oncogenetics and ophthalmogenetics have emerged with a strong demand for genetic counselling [12-15].

Genetic counsellors have the essential skills to address the complex issues of counselling for common disorders [16]. Risk assessment is a major component of genetic counselling and can be estimated based on familial and medical history even for common diseases. In the context of non-Mendelian diseases, risk assessment is often associated with uncertainty because of the contribution of multiple risks to the disease. However, the concept of uncertainty is not new to genetic counselling. When communicating risk, patients conceptualise the information in the context of their own perception of risk, probabilities and disease severity, all of which are routinely addressed in counselling [17]. The psychological dimension of genetic counselling facilitates adaptation to genetic information, irrespective of the genetic condition and risk estimates. Additionally, genetic counsellors are well positioned to counsel patients about the complexity of genetic risk factors and the limitation of our knowledge in the context of common diseases.

The growing demand for genetic expertise has seen the scope of genetic counsellors expand to non-clinical roles and allow them to use their training in new settings. Recent professional status surveys reflect the diverse roles held by genetic counsellors including working in laboratory, research, public and professional education, public health, public policy and consulting [18,19]. This is not surprising as the core competencies of genetic counsellors include the domains of counselling and communication, ethical practice and genetic expertise applied to the fields of clinical case management, research and education [20,21]. Genetic counsellors are experts in facilitating informed consent, critically reviewing genetic results, assessing genetic testing strategies and educating not only patients but also professionals and the general public. These skills will certainly become invaluable in the era of genomic medicine that is emerging.

A recent nationwide study in the States reported that almost half of people rated losing vision as the worst possible health outcome, greater than losing a limb, hearing, memory, or speech [22]. Glaucoma is a chronic progressive condition that is mainly pain free but requires lifelong management and treatment and can lead to blindness. The loss of vision in people with glaucoma affects their reading, driving, mobility and independence, as well as a range of social and physical daily life activities [23-25]. Several studies have demonstrated that patients with glaucoma

experienced lower quality of life than patients without the disease [26,27] and that the severity of disease correlated with the perceived quality of life [24,28-31]. Depression and anxiety have also be shown to be more common in patients with glaucoma [28,32,33].

In contrast, childhood-onset glaucoma is rare but is a devastating condition. Vision loss in children can have an impact on their education, development, social interactions, independence and career prospects and has been associated with lower perceived quality of life [34]. However, the literature on the impact of glaucoma in children is scarce. Three studies found that children with glaucoma had lower perceived quality of life compared with children without vision impairment [35-37]. A worse perceived quality of life correlated with lower visual acuity [35-37] and severity of disease [36], and the impact was greater on psychosocial than physical well-being [35]. Older children reported a better perceived quality of life, suggesting the implementation of coping strategies with time [35]. Interestingly, parents have reported a greater impact than children, which could be explained by different expectations than their children [35,38].

Glaucoma is a good example of a common disease with a high heritability and where genetic testing and analysis of medical and familial history can lead to better management for patients and their family members. The fact that glaucoma displays both Mendelian and multifactorial inheritances, can be either isolated or syndromic and affects all age groups from newborns to adults each with their own psychological and ethical implications makes it an interesting model for genetic testing. Additionally, genetic counsellors are ideally positioned to address the personal, familial and psychosocial impact of the disease.

1.2. Definition of glaucoma

The eye is a complex organ that is responsible for our vision. The anterior segment of the eye consists of an anterior chamber (between the cornea and the iris) and a posterior chamber (between the iris and the suspensory ligaments of the lens) (Figure 1-1). The anterior segment is filled with a fluid called the aqueous humor whereas the vitreous humor fills the posterior segment of the eye. In the eye, the light travels through the cornea and the lens to the retina at the back of the eye. The light sensing photoreceptors in the retina transform the light into images through a cascade of chemical and electrical events. The retinal ganglion cells then transmit the images via the retinal nerve fiber layer and the optic nerve fibers to the brain.

Figure 1-1: Anatomy of the human eye (Source: Glaucoma Research Foundation, Retrieved from: http://www.glaucoma.org/glaucoma/eye%20anatomy%20with%20descr.jpg). Figure 1-1 has been removed due to copyright restrictions.

Glaucoma is a term describing a group of heterogeneous ocular disorders affecting the optic nerve and causing irreversible vision loss. Casson et al. defined the condition as [39]:

> "Glaucoma describes a group of ocular disorders of multifactorial aetiology united by a clinically characteristic optic neuropathy with potentially progressive, clinically visible changes at the optic nerve head, comprising focal or generalized thinning of the neuroretinal rim with excavation and enlargement of the optic cup, representing neurodegeneration of retinal ganglion cell axons and deformation of the lamina cribrosa; corresponding diffuse and localized nerve-fibre-bundle pattern visual field loss may not be detectable in early stages; while visual acuity is initially spared, progression can lead to complete loss of vision; the constellation of clinical features is diagnostic."

The characteristics of glaucoma include the thinning of the retinal nerve fibre layer and the loss of retinal ganglion cells resulting in changes to the optic nerve head (structural changes) with characteristic associated visual field loss (functional changes). As the disease progresses, the retinal nerve fibre layer thins and creates a depression in the optic nerve called the cup. This depression can be measured directly or through the cup to disc ratio (CDR) which measures the ratio of the cup to the optic nerve size (Figure 1-2A). The closer the CDR is to 1.0, the more advanced the glaucoma is. The lamina cribrosa is a mesh-like structure of collagen fibers located at the optic nerve head that allow the nerve fibers of the optic nerve and the retinal blood vessels to pass through the sclera. It is considered the primary site of glaucomatous retinal ganglion cells axonal injury [40]. Substantial loss of retinal ganglion cells [41,42] and retinal nerve fibre layer [43] happen before visual field loss can be detected by automated visual field tests assessing visual function. The most common visual field test is a 24-2 test that evaluates vision at 54 locations in the central 24° of the visual field. The displayed results usually include a pattern deviation plot that shows the deviation of patient's results from an age-matched control for each test location and adjusted for decreases in sensitivity (Figure 1-2B). The performance of the visual field test can be assessed by the mean deviation which is the average of the total deviation values. A mean deviation of 0 means that there is no deviation from normal whereas negative values increase as vision loss is more advanced. Because the peripheral areas of the visual field are affected first and central vision is affected last in glaucoma [44-46], the visual acuity is usually spared until the advanced stages. Glaucoma is a progressive disease and can ultimately lead to irreversible blindness.

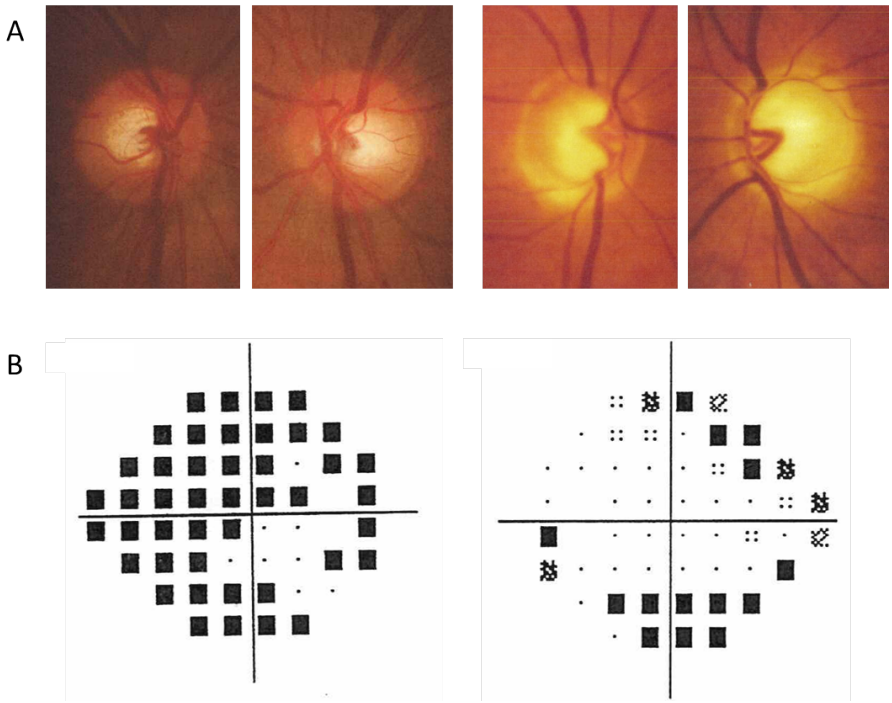

Figure 1-2: Visual fields and optic disc appearance in glaucoma. A. Healthy optic discs (left panel) compared to glaucomatous optic discs (right panel); B. Visual field defects showing severe field loss in the right eye (left panel) and peripheral mild field loss in the left eye (right panel) (Source: Flinders Ophthalmology).

1.3. Glaucoma classification

Glaucoma is a group of disorders and can be classified based on the aetiology (primary vs secondary), the anatomy of the anterior chamber (open vs closed), or the time of onset (congenital, juvenile or adult). Primary glaucoma refers to glaucoma without any underlying disease and includes primary open-angle glaucoma, primary angle-closure glaucoma and primary congenital glaucoma. Secondary glaucoma refers to glaucoma resulting from an identifiable cause such as pseudoexfoliation, pigment dispersion syndrome, exposure to steroid medication, anterior segment dysgenesis or nanophthalmos. Open-angle and closed-angle glaucomas are differentiated based on whether the angle between the iris and the cornea is open or closed (Figure 1-3). Early age of onset for POAG is termed juvenile open-angle glaucoma (JOAG) and is arbitrarily defined by an age at diagnosis before 30 to 40 years old. Childhood glaucoma refers to glaucoma diagnosed in the neonatal or infantile period. Finally, glaucoma can be subdivided based on the intraocular pressure (IOP). Approximately 75% of patients diagnosed with primary open-angle glaucoma have increased IOP (> 21 mmHg), referred to as high tension glaucoma (HTG). Normal tension glaucoma (NTG) refers to glaucoma in the absence of elevated IOP [47].

Figure 1-3: Open and closed angles between the iris and the cornea in glaucoma (Source: Glaucoma Research Foundation, Retrieved from: http://www.glaucoma.org/glaucoma/types-of-glaucoma.php). Figure 1-3 has been removed due to copyright restrictions.

1.3.1. Adult-onset glaucoma

The most common type of adult-onset glaucoma is primary open-angle glaucoma (POAG, MIM 137760). POAG is defined as an optic neuropathy with associated visual field loss, open angle, no ocular enlargement or congenital ocular anomalies and no other underlying disease. The condition is asymptomatic in its early stages which explains why as many as 50% of cases are undiagnosed [48-50]. Well recognised risk factors for open angle glaucoma include advancing age [51-54], positive family history [52,53,55], African descent [47,52,54], elevated IOP [51-53,56], thin central corneal thickness [51], and myopia [57,58]. The involvement of systemic diseases such as high blood pressure and diabetes as risk factors for glaucoma is still debated [51-54].

Risk factors for secondary open angle glaucoma include pseudoexfoliation syndrome [59], pigment dispersion syndrome [60] and exposure to steroid medication [61]. Pseudoexfoliation syndrome is an age-related disorder of the extracellular matrix characterised by abnormal fibrillar extracellular deposits on the anterior surface of the eye. It has long been recognised as a risk factor for glaucoma [59]. Pigment dispersion syndrome is characterised by pigment cells from the iris that accumulate in the anterior chamber of the eye and lead to an increased risk of glaucoma [60,62]. Finally, an elevated IOP has long been recognised as an adverse effect of corticosteroid medication [63,64] which then increases the risk of developing glaucoma (steroid-induced glaucoma).

Primary angle-closure glaucoma (PACG) is characterised by evidence of glaucomatous optic neuropathy, raised IOP and obstruction of the trabecular meshwork by the peripheral iris [65]. The sudden rise of IOP is often associated with blurry vision, ocular pain, headaches, nausea and vomiting. The risk factors for angle closure glaucoma include advancing age, female sex, Asian or Inuit descent, shallow anterior chamber depth, shorter axial length, small corneal diameter and thick lens [66,67].

Nanophthalmos, as part of the microphthalmia spectrum, can result in secondary angle closure glaucoma. Congenital microphthalmia is a developmental disorder characterised by small eyes and an axial length two standard deviations below that of the population age-adjusted mean (corresponding to an axial length <21 mm in adult eye) causing extreme hyperopia [68] (Figure 1-4). Isolated clinical variants of congenital microphthalmia include nanophthalmos (overall reduction in the size of the globe), anterior microphthalmia (shortening of the anterior segment) and posterior microphthalmia (shortening of the posterior segment) [69]. However recent genetic evidence suggests that these conditions might represent a spectrum of short axial length and high hyperopia. An increased risk of developing angle-closure glaucoma is present in nanophthalmos due to a shallow anterior chamber depth resulting in narrow irido-corneal angles [68,69].

Figure 1-4: Diagram of the eye, sagittal section, with light path shown. A: normal eye. B: nanophthalmic eye. Axial lengths are only provided as an example (Source: Sundin et al. 2005 [70]). Figure 1-4 has been removed due to copyright restrictions.

1.3.2. Childhood onset glaucoma

Childhood glaucoma comprises primary and secondary subtypes. Primary congenital glaucoma (PCG, MIM 231300) results from a development defect of the aqueous outflow system and is characterised by isolated angle anomalies leading to optic neuropathy, enlarged ocular diameter (buphthalmos) resulting from increased IOP, corneal clouding and/or Haab's striae (breaks in the Descemet membrane, the basement membrane of the cornea) (Figure 1-5) [71]. The onset is usually in the newborn or infantile period (< 3 years old) but can also be later in life (for example in individuals with spontaneous arrested glaucoma and normal IOP but typical signs of PCG) [72].

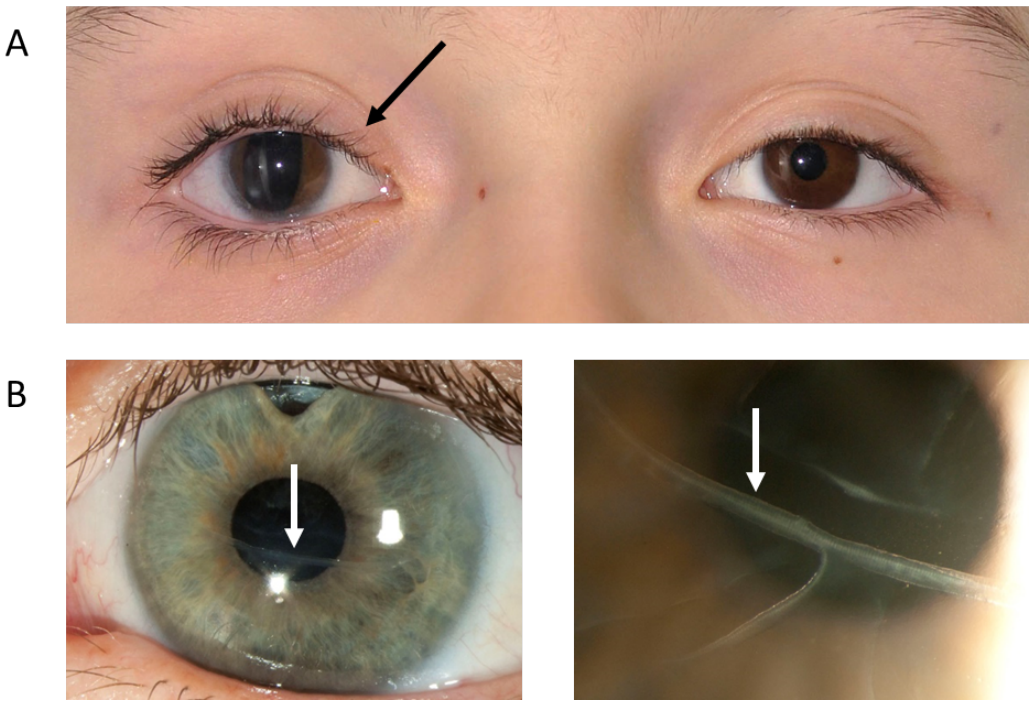

Figure 1-5: Clinical photos showing the ocular features in PCG. A. Buphthalmos (black arrow); B. Haab's striae (white arrows) (Source: Flinders Ophthalmology, Angela Chappell & Carly Emerson).

Secondary childhood glaucoma refers to glaucoma associated with nonacquired ocular anomalies and includes conditions with congenital ocular defects that increase an individual's risk of developing glaucoma [72]. Such conditions include Axenfeld-Rieger anomaly, Peters' anomaly and Aniridia as part of anterior segment dysgenesis (ASD). 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 (Figure 1-6) [73-75]. Systemic features including dental, umbilical, cardiac anomalies and/or hearing loss are among the

most frequent and can be present as part of the Axenfeld-Rieger syndrome (ARS, MIM 180500) [76,77]. Individuals with ARS face a 50% risk of developing glaucoma due to the developmental defects in the anterior chamber angle [78]. The onset is often in childhood or early adulthood but can also manifest later in life.

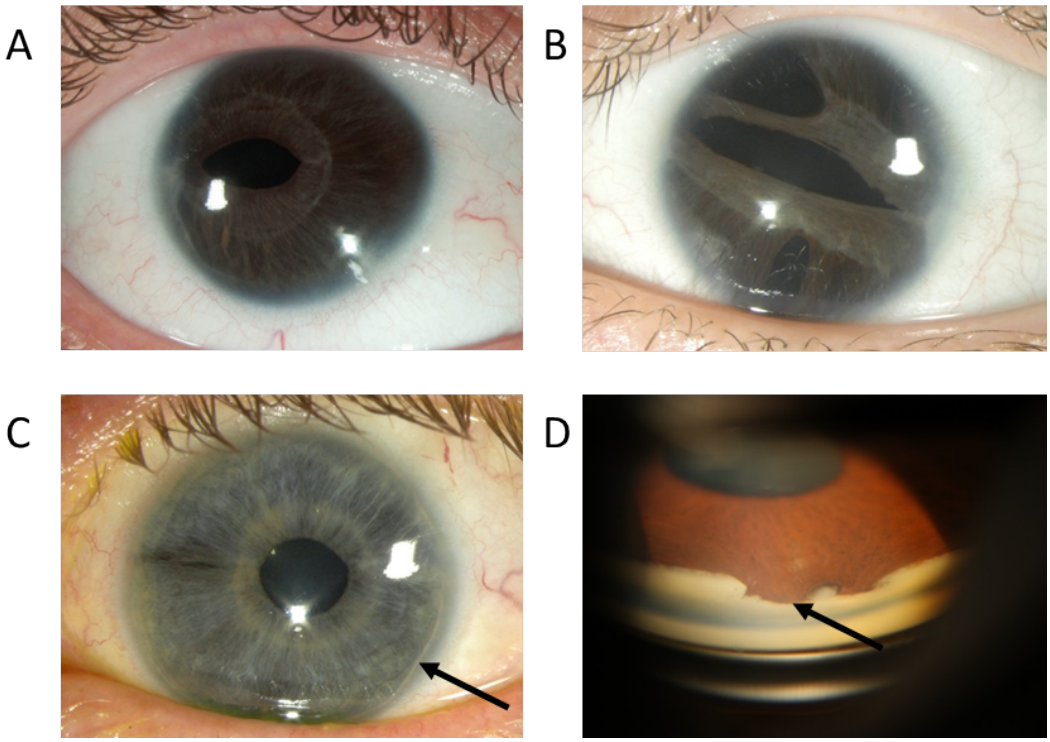

Figure 1-6: Clinical photographs showing the ocular features in Axenfeld-Rieger anomaly. A. Corectopia; B. Polycoria; C. Iris hypoplasia and posterior embryotoxon (black arrow); D. Irido-corneal adhesions (black arrow) (Source: Flinders Ophthalmology, Angela Chappell & Carly Emerson).

Peters' anomaly (MIM 604229) consists of central corneal opacity, with or without irido-corneal adhesions, corneal-lenticular adhesions and defects in the posterior layers of the cornea [67,79]. Congenital glaucoma occurs in a significant number of cases [80,81]. Aniridia (MIM 106210) is characterised by a complete or partial absence of the iris often with foveal hypoplasia resulting in nystagmus and reduced visual acuity [82,83]. Frequently associated ocular abnormalities include glaucoma, cataract, corneal changes, lens subluxation, strabismus, optic nerve hypoplasia and microphthalmia [84,85]. Half to two-third of individuals with aniridia develop glaucoma, usually with an onset in late childhood or adulthood [84,85].

1.4. Prevalence

Glaucoma is the second leading cause of blindness worldwide after cataracts, but the first cause of irreversible blindness [86]. In 2010, 60.5 million people were affected by glaucoma, including 2.65% of individuals over the age of 40 years [47]. By 2020, the total number of individuals with glaucoma is

estimated to reach 79.6 million, representing 2.86% of the population over the age of 40 years. In Australia, 3% of the population over the age of 50 years is affected [48]. POAG is the most common type, accounting for 75% of those affected by glaucoma [47]. The prevalence of POAG is higher in African, Chinese and Latin American than in European, Indian and Japanese populations [47]. In contrast, the prevalence of PACG is highest in Chinese and Japanese, with 86.5% of those affected by PACG in Asia [47], and the prevalence of pseudoexfoliation syndrome is highest in Nordic populations [87].

In comparison, developmental glaucomas are quite rare. PCG is the most common cause of paediatric glaucoma [88]. The incidence differs among populations: it has a higher incidence in populations with founder effect or consanguinity such as Slovakian Gypsies (1/1,250) [89] or Saudi Arabian (1/2,500) [90] respectively, and a lower incidence in Western populations (1/22,000-1/23,000) [89,91], with an incidence in Australia of 1/30,000 births [92]. ASD is much rarer with a prevalence of 1/200,000 for ARS often cited. However, no study has truly evaluated the prevalence of ARS and the rate is most likely underestimated due to the variable expressivity of the disease and the presence of milder cases not accounted for. Aniridia has an incidence of 1/64,000-1/100,000 worldwide [93-95] and microphthalmia has an incidence of 1/5,000-1/20,000 worldwide [81].

1.5. Pathophysiology

The pathophysiology of glaucoma leading to progressive loss of retinal ganglion cells and characteristic visual field loss patterns is still not completely understood. Although not all patients develop high IOP, it is believed that IOP plays a major role in disease onset and progression [96-98]. The IOP is determined by the production of inflow of the aqueous humour against its outflow. The aqueous humour provides nutrition to the cornea and the lens, and is responsible for inflating the eye and maintaining the shape and optical properties of the globe [99]. It is produced and secreted by the ciliary body, flows through the anterior chamber and drains from the eye by passive flow through two different routes (Figure 1-7). The regulation of the aqueous humour outflow is an important mechanism for the function of the eye. The major drainage pathway of the aqueous humour is through the trabecular meshwork into the Schlemm's canal, the collector channels, the aqueous veins and the episceral veins connected to the circulatory system (conventional pathway). A small amount of aqueous humour is drained through the uveoscleral pathway or unconventional pathway and is independent of IOP levels. In this route, the aqueous humour exits through the iris roots, the ciliary muscle bundles, the suprachoroidal space, and into the sclera where it joins the circulatory system through some lymphatic vessels.

Figure 1-7: The trabecular meshwork outflow pathway (left) and the uveoscleral outflow pathway (right) (Source: Goel et al. 2010 [99]). Figure 1-7 has been removed due to copyright restrictions.

In response to elevated IOP, the trabecular meshwork and the Schlemm's canal adjust the outflow of aqueous humour to normalise the IOP [100]. In glaucoma patients, oxidative DNA damage is increased in the trabecular meshwork [101] which then leads to cell death. Decreased cellularity of the trabecular meshwork is observed in glaucoma patients [102] and the reduction of outflow pathway cells compromises their ability to modify the outflow resistance in response to increased IOP [103].

The increase in cupping, or CDR, seen in glaucoma patients is the result of a progressive loss of retinal ganglion cell axons by apoptosis [104,105] and damage of the laminar connective tissues [106,107]. The link between trabecular meshwork death and retinal ganglion cell death is not fully understood yet. There is evidence suggesting that elevated IOP lead to remodelling of the lamina cribrosa in glaucomatous eyes [108]. Human eyes from donors show substantial stretch and compression of the lamina cribrosa when subjected to elevated IOP [109]. Changes in the micro-architecture of the lamina cribrosa have been demonstrated in glaucomatous human eyes [110]. In an animal experimental model of glaucoma with increased IOP, matrix metalloproteinases were elevated at the optic nerve head, suggesting extracellular matrix remodelling at the lamina cribrosa [111]. It is hypothesised that remodelling and damage at the lamina cribrosa may result in a loss of structural and functional support of the retinal ganglion cells and ultimately in retinal ganglion cell death. Additionally, other biological processes that trigger apoptosis could be implicated in glaucoma including impaired axonal transport, neuroinflammation, vascular dysfunction, oxidative stress and mitochondrial dysfunction [112-115].

1.6. Treatment

Early diagnostic and intervention is the key in delaying onset and slowing down the progression of the disease. Glaucoma is mainly painless, so patients are usually unaware of their loss of vision until it is significant. It is estimated that 50% of individuals with glaucoma are undiagnosed [48,49] and that 5-15% of patients with POAG progress to blindness [50,116-119]. Moreover, late diagnosis increases the risk of progression and worse visual outcome [117,119-121].

There is no curative treatment for glaucoma and the current interventions are symptomatic. The approach is similar regardless of the type of glaucoma and the only evidence-based treatment consists of lowering the IOP. This can be achieved by different interventions including topical therapy (eye drops), laser (laser trabeculoplasty) and surgery (trabeculectomy, goniotomy, tube implant). The choice of intervention depends on a number of factors including the glaucoma presentation, the progression of the disease, the response to treatment, the practicability of treatment for the patient and the patient's compliance. Different treatments can be used conjointly (for example eye drops may still be needed after laser or surgery), and subsequent interventions may be needed to control the IOP.

Several studies evaluated the effectiveness of IOP lowering therapy to prevent glaucoma onset and progression. The Ocular Hypertension Treatment Study established that IOP lowering medication was effective at preventing or delaying the onset of POAG in patients with high IOP [97]. The study showed that a 22.5% IOP reduction in ocular hypertensive patients resulted in a decreased risk of developing glaucoma over 5 years with a cumulative probability of 4.4% in the treated group compared with 9.5% in the control group. The Advanced Glaucoma Intervention Study demonstrated that a lower IOP was associated with reduced progression of visual field deterioration [98]. Patients with an IOP > 17.5 mmHg during subsequent follow-up had an estimated worsening of 1 unit of visual field defect score greater than patients whose IOP was < 14 mmHg. Similarly, the Early Manifest Glaucoma Trial showed that IOP lowering treatment significantly delayed the progression of glaucoma in both HTG and NTG [122]. Over a follow-up period of 6 years, the study showed that with a 25% IOP reduction, the progression of glaucoma in early glaucoma patients was 45% in the treatment group compared with 62% in controls. The Early Manifest Glaucoma Trial also demonstrated that the magnitude of IOP reduction is a major factor influencing the progression; with each mmHg of IOP reduction, the glaucoma progression risk decreased by 10%[120].

The treatment for angle closure is to control the sudden rise of IOP to prevent damage of the optic nerve. Medical therapy can be used but the pupil block is usually treated with laser iridotomy (opening through the iris) or iridectomy (removal of a portion of the iris tissue). Both procedures are effective in relieving the difference of pressure between the anterior and posterior chambers and widening the drainage angle [123,124]. Glaucoma control in nanophthalmos can be difficult and associated with complications following surgery [125,126].

The control of IOP in PCG is generally achieved surgically although topical therapy is sufficient in some cases. Goniotomy (incision in the trabecular meshwork from inside), trabeculotomy (incision in the trabecular meshwork from outside) and trabeculectomy (removal of part of the trabecular meshwork) are different surgical procedures used, with goniotomy being the commonly performed procedure [92]. Mackinnon et al, showed surgical success in controlling IOP with one or two goniotomies in 74% of treated eyes [92]. The management of glaucoma in ASD is similar to the management of other paediatric glaucoma but the visual outcome is usually poorer [88].

1.7. Genetics of adult-onset glaucoma

The heritable nature of glaucoma has been well recognised for a long time. From early on, studies have reported familial cases of glaucoma, suggesting that genetic factors may play a role in the condition [127-129]. A family history of glaucoma has been reported in 15-45% of glaucoma patients [130-134]. However, family self-report of glaucoma is often biased and underestimated: patients may

have poor knowledge of family history or the disease itself and patients are often undiagnosed [132,135]. The Glaucoma Inheritance Study in Tasmania examined all available relatives of POAG patients and found that 60% of POAG patients had a family member affected (Figure 1-8) [136]. In the Targeting At-Risk Relatives of Glaucoma patients for Early diagnosis and Treatment study, we examined first-degree relatives of patients with advanced glaucoma and found that 55% were glaucoma suspect or had glaucoma (manuscript under preparation).

Figure 1-8: Distribution of the proportion and relationship of affected relatives in POAG patients compared with unaffected individuals (Source: Green et al. 2007 [136]). Figure 1-8 has been removed due to copyright restrictions.

The population-based Rotterdam study examined all available family members of POAG patients and reported that first-degree relatives of affected individuals had a 22% lifetime risk of developing glaucoma compared to 2.3% in controls, corresponding to a risk ratio of 9.4 [137]. Additionally, the Glaucoma Inheritance Study in Tasmania found that familial cases had a younger age at diagnosis and a more severe disease compared to sporadic cases [138].

A recent study estimated that glaucoma is one of the most heritable multifactorial disease with an overall heritability at 70% [139]. Glaucoma displays features of Mendelian diseases and of multifactorial disorders. Both rare variants with high penetrance and common variants with smaller effects have been associated with glaucoma (Figure 1-9). On one hand, several genes displaying Mendelian inheritance (autosomal dominant and autosomal recessive) with strong odds ratio but incomplete or age-related penetrance and variable expressivity have been associated in different subtypes of glaucoma, with some overlapping between the different phenotypes. On the other hand, multiple common genetic risk factors of small size effect have been identified with little overlap between the different clinical classifications of glaucoma. In the next sections, I will discuss the known genes and the genetics risk factors associated with the different types of glaucoma.

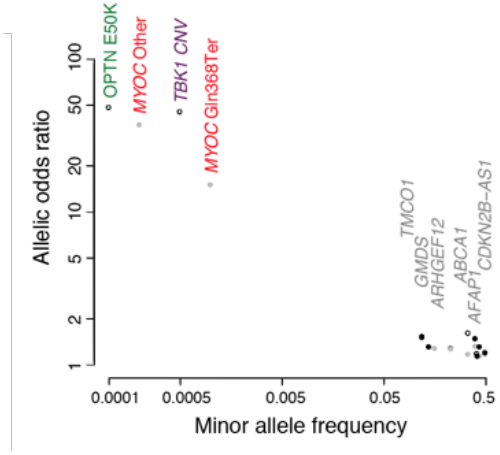

Figure 1-9: Genetic architecture of established POAG loci

1.7.1. POAG

There are currently 16 POAG chromosomal loci with a Mendelian inheritance. Most chromosomal loci were identified through family-based studies using genetic linkage studies (Table 1-1). This method relies on families with multiple related affected individuals and uses markers across the genome that co-segregate with the gene of interest to identify a specific chromosomal locus related to the studied phenotype [140]. Linkage studies are usually followed by the analysis of candidate genes within that chromosomal region to pinpoint the causative gene. Although 7 genes have been identified within these loci in association with POAG, only 3 (*MYOC*, *OPTN* and *TBK1*) have demonstrated strong evidence for a causative role in POAG (in bold in Table 1-1). Replications studies have failed to confirm a role in POAG for *ASB10*, *WDR36* and *NTF4* so far and are still awaiting for *EFEMP1* and *IL20RB*.

Table 1-1: Loci and genes associated with POAG

Locus	OMIM	Mapped	Gene	Phenotype	Ethnicity	Ref
GLC1A	137750	1q23-q25	***MYOC***	JOAG (HTG)	Caucasian	141,142
GLC1B	606689	2cen-q13		POAG (NTG/HTG)	English	143
GLC1C	601682	3q21-q24	*IL20RB*	POAG (HTG)	Caucasian/Greek	144,145
GLC1D	602429	8q23		POAG (NTG/HTG)	Caucasian	146
GLC1E	137760	10p15-p14	***OPTN***	POAG (NTG)	Caucasian	147,148
GLC1F	603383	7q35-q36	*ASB10*	POAG (HTG)	Caucasian	149,150
GLC1G	609887	5q22.1	*WDR36*	POAG (NTG/HTG)	Caucasian	151
GLC1H	611276	2p16-p15	*EFEMP1*	POAG (HTG)	Caucasian/Jamaican	152
GLC1I	609745	15q11-q13		POAG (HTG)	Caucasian/African American	153
GLC1J	608695	9q22		JOAG (HTG)	Caucasian	154
GLC1K	608696	20p12		JOAG (HTG)	Caucasian	154
GLC1L		3p21-p22		POAG	English	155
GLC1M	610535	5q22-q32		JOAG (HTG)	Filipino	156
GLC1N	611274	15q22-q24		JOAG (HTG)	Chinese	157
GLC1O	613100	19q13.33	*NTF4*	POAG (NTG/HTG)	German	158
GLC1P	177700	12q14	***TBK1***	POAG (NTG)	African American	159

1.7.1.1. Myocilin (MYOC)

The *MYOC* gene (MIM 601652) was identified by Stone et al. on the GLC1A locus and was the first gene linked to JOAG [142]. It was previously known as the Trabecular meshwork inducible-glucocorticoid response protein (TIGR). Deleterious heterozygous variants in *MYOC* have been consistently reported in 2-4% of individuals with POAG [160-163] and 8-36% of individuals with JOAG [164-168] among different populations. The *MYOC* database includes 277 variants (www.myocilin.com) [169], with 104 classified as deleterious. *MYOC* comprises 3 exons and almost all the deleterious variants are located in the highly conserved olfactomedin domain in exon 3. The p.Gln368Ter

variant is the most common variant accounting for the majority of cases. The Genome Aggregation Database (gnomAD) estimates the frequency of p.Gln368Ter at 1/330 in the general population (http://gnomad.broadinstitute.org/variant/1-171605478-G-A). A previous study of 15 Australian and 2 French Canadian POAG families identified a common disease haplotype supporting a common European founder for that variant [170,171]. *MYOC* variants are transmitted in an autosomal dominant manner and are associated with a younger age of onset than the general population, high IOP and a strong family history [161,172]. The penetrance is age-related, and although inter- and intra-familial variability is common, genotype-phenotype correlations exist: While the p.Gln368Ter variant is associated with a rather late age at diagnosis (average 52.4 ± 12.9 years) [173], other variants such as p.Pro370Leu or p.Gly367Arg cause glaucoma at a much younger age, as early as the teens or childhood [164,174].

Although the *MYOC* gene was identified almost 20 years ago, the mechanism by which the variants cause glaucoma is still not fully understood. Haploinsufficiency as a disease mechanism has been excluded since increasing or decreasing wild-type MYOC expression does not induce glaucoma [175,176] and homozygosity of some variants does not always cause a more severe phenotype [177]. Previous studies have shown that MYOC mutant proteins form dysfunctional heterodimers with wild-type MYOC, suggesting a dominant-negative mechanism [178]. Evidence supports an aggregation of misfolded protein in the endoplasmic reticulum of the trabecular meshwork [179,180], inducing abnormal accumulation of extracellular matrix proteins [181], endoplasmic reticulum stress-induced apoptosis, leading to trabecular meshwork cell death and subsequent elevation of IOP [182]. Recently, Zhu et al. demonstrated that, in a *MYOC* transgenic mouse model, very few trabecular meshwork cells were lost at 4 months but the loss was apparent at 6 months [183]. This suggests that the impairment of the aqueous humour outflow facility related to *MYOC* variants is a degenerative process, as opposed to a developmental defect.

1.7.1.2. Optineurin (OPTN)

OPTN (MIM 602432) at locus GLC1E was the second POAG gene identified. Heterozygous variants in *OPTN* were initially reported in 16.7% of familial POAG cases [148]. Some subsequent studies found an association of *OPTN* variants with POAG in some populations [184,185], while other did not [186,187]. The evidence for a role for these variants in POAG is not strong, but some variants may act as gene modifiers increasing the susceptibility to NTG. One specific variant (p.Glu50Lys) was associated with autosomal dominant NTG with high penetrance in several studies [186,188,189]: Although rare, it is associated with a younger age at diagnosis (average 41 years) and a more severe disease that requires filtration surgery more often than NTG controls [190]. Further evidence for a role of p.Glu50Lys in NTG was demonstrated by loss of retinal ganglion cells and progressive diminution of retinal nerve fibre layer at the optic nerve without IOP elevation in transgenic mice

overexpressing *OPTN* p.Glu50Lys variant [191].

1.7.1.3. Tank-binding kinase 1 (TBK1)

Fingert et al. reported a copy number variation (CNV) following linkage to chromosome 12q14 within locus GLC1P that co-segregated with NTG in a family of African American background [159]. The duplication was further identified in 1.3% of patients with NTG. All affected individuals had normal IOP and a young age at diagnosis (average 33 years old). The smallest duplication shared between individuals was 300kb and encompassed two genes (*TBK1* and *XPOT*). Increased gene expression of *TBK1* (but not *XPOT*) in affected individuals with 12q14 duplications suggested that an extra copy of *TBK1* (MIM 604834) is associated with familial autosomal dominant NTG at locus GLC1P [159]. Additionally, TBK1 is highly expressed at the nerve fibre layer and the ganglion cell layer of the retina [159] and transgenic mice that have one copy of the human *TBK1* gene in addition to their two copies develop NTG with progressive retinal ganglion cell loss despite normal IOP [192].

The phenotype caused by *OPTN* p.Glu50Lys and *TBK1* duplications is indistinguishable. Interestingly the OPTN and TBK1 proteins interact with each other [193]. OPTN is an adaptor protein that interacts with several proteins through its well defined binding domains and is involved in various biological functions including vesicle trafficking, signal transduction, autophagy and apoptosis [194]. *TBK1* encodes a serine/threonine kinase that is involved in apoptosis by regulating the activation of genes in the anti-apoptotic factor NF-κB pathway [195]. TBK1 phosphorylates OPTN, enhancing its binding to LC3 which mediates autophagy [196]. Recent studies showed that *OPTN* p.Glu50Lys enhanced binding to *TBK1* [193], which triggers the formation of insoluble aggregates of the OPTN mutant in the retina [197]. Moreover, transgenic *OPTN* mice overexpressing p.Glu50Lys showed mitochondrial dysfunction (autophagy involving mitochondria) in retinal ganglion cells [198] and *TBK1* duplications stimulated autophagy in induced pluripotent stem cells (iPSC)-derived retinal cells [199]. These results suggest that impaired autophagy and subsequent apoptosis of retinal ganglion cells might be involved in NTG caused by *TBK1* duplication and *OPTN* p.Glu50Lys.

In contrast, heterozygous *TBK1* and *OPTN* loss-of-function variants have been associated with amyotrophic lateral sclerosis and frontotemporal dementia in 1-3% of cases [200-203] and heterozygous *TBK1* loss-of-function variants in the kinase domain have been implicated in herpes simplex encephalitis [204]. However, the genetic etiologies leading to POAG are different from those causing amyotrophic lateral sclerosis, frontotemporal dementia or herpes simplex encephalitis. *TBK1* variants associated with amyotrophic lateral sclerosis and frontotemporal dementia lead to reduced levels of mRNA and protein [202,205], suggesting *TBK1* haploinsufficiency. Similarly, both point mutations and partial deletion of *OPTN* have been shown to decrease protein levels [202,203], supporting a loss-of-function mechanism in these two diseases. In herpes simplex encephalitis,

TBK1 variants has been shown to result in haploinsufficiency or dominant negative effect [204]. In summary, the *OPTN* p.Glu50Lys and the *TBK1* duplication leading to POAG act by gain-of-function whereas *OPTN* and *TBK1* variants leading to amyotrophic lateral sclerosis and frontotemporal dementia act by loss-of-function.

OPTN p.Glu50Lys and *TBK1* duplications associated with glaucoma have not been reported in patients with amyotrophic lateral sclerosis or frontotemporal dementia [206,207]. Although no study specifically evaluated the incidence of both POAG and amyotrophic lateral sclerosis/frontotemporal dementia in patients carrying *OPTN* or *TBK1* variants or CNVs, a higher incidence of one disease in the other disease cohort has not been reported. The distinct molecular mechanisms leading to either POAG or amyotrophic lateral sclerosis/frontotemporal dementia have important implications in the context of genetic counselling as patients with *OPTN* p.Glu50Lys or *TBK1* duplications are unlikely to be at increased risk of developing amyotrophic lateral sclerosis or frontotemporal dementia based on their genetic results.

1.7.1.4. Non-confirmed POAG-associated genes

In the family with POAG reported by Wirtz et al. to map to chromosome 7q35-q36 (GLC1F) [149], Pasutto et al. identified a heterozygous variant in the *ASB10* gene (*Ankyrin repeat- and socs box-containing protein 10*, MIM 615054) segregating with the phenotype [150]. They replicated their results in two cohorts and found a prevalence of variants significantly higher in POAG cases (6.0%) compared to controls (2.8%). However, another study did not support an association with POAG, identifying a similar prevalence of *ASB10* variants between cases and controls [208]. Furthermore, they found that variants reported by Pasutto et al. were also present in the general population from the National Heart, Lung and Blood Institute's Exome Sequencing Project at a similar frequency. These results suggest a selection bias due to cohort size difference between cases and controls in the initial study that identified *ASB10* as the glaucoma gene for GLC1F. Another recent study reported a significant association of non-synonymous *ASB10* variants with POAG despite that lack of segregation in their familial cases [209]. However, the most significant variant (p.Arg453Cys) found in 2.8% of cases and absent from controls had an allelic frequency of 2.2% in the Exome Aggregation Consortium (ExAC) population database, suggesting a selection bias of the cohort. Knockdown of expression of *ABS10* mRNA in human anterior segment perfusion culture showed a 50% reduction in aqueous humor outflow [150]. ASB10 may play a role in IOP regulation via the ubiquitin-mediated degradation pathway in the trabecular meshwork [210]. However, there is currently no *ASB10* animal model to study the effect of the gene on glaucoma. Overall, at present there is no convincing evidence to support a role for *ASB10* as a causative glaucoma gene.

GLC1G was initially defined as a 2Mb region on chromosome 5q22.1 in 7 families with POAG [151]. The critical genetic interval contained 7 genes and a heterozygous missense variant was identified in the *WDR36* gene (*WD repeat containing protein 36*, MIM 609669) in 5 of the families. Among additional families screened, 4 carried other variants, providing an overall prevalence of *WDR36* variants associated with POAG of 6.9%. However, subsequent studies could not replicate the results and found a prevalence of *WDR36* variants that was similar between cases and controls [211-214], or families with *WDR36* variants that did not segregate with the occurrence of the phenotype [215,216]. Additional studies reported families linked to the 5q interval but lacking *WDR36* variants [156,217]. Moreover, heterozygous *WDR36*-deficient mice do not develop glaucoma [218]. These evidences do not support a role for *WDR36* as a causative gene for POAG and the co-segregation of the variants demonstrated by Monemi et al. could be due to linkage disequilibrium with another gene in close proximity being causative. In some studies, individuals with *WDR36* variants presented a more severe phenotype than those without [215,219], which could support a role for *WDR36* as a genetic risk factor for POAG. However, a recent meta-analysis assessed the association of all reported variants in *WDR36* with POAG, HTG and NTG and their results did not support a role of *WDR36* in the genetic susceptibility of POAG and its subtypes [220]. In conclusion, the current evidence supporting a role for *WDR36* variants in the pathogenesis of POAG is limited.

Screening a large dataset of patients with POAG, Pasutto et al. identified heterozygous variants in the *NTF4* gene (*Neurotrophin factor 4*, MIM 162662) in 1.7% of cases compared to 0.1% of controls [158]. However, two subsequent studies found a higher prevalence of *NTF4* variants in controls compared to cases, including one nonsense variant in a control [221,222], and *NTF4* knockout mice do not display retinal ganglion cell death [223] questioning a causative role for *NTF4* in POAG.

In a three-generation African-American family, Mackay et al. identified a heterozygous variant predicted deleterious in the *EFEMP1* gene (*EGF-containing fibulin-like extracellular matrix protein 1*, MIM 601548) that mapped to loci GLC1H [224]. *EFEMP1* was highly expressed in the mouse ciliary body and the cornea [224]. An intronic variant in *EFEMP1* has previously been associated with optic disc area [225]. Additional studies are needed to confirm a role for *EFEMP1* in POAG pathogenesis.

A heterozygous variant in the *IL20RB* gene (*Interleukin 20 receptor, beta*, MIM 605621) was found to segregate in the family that initially mapped to GLC1C [226]. The variant was predicted to disrupt the binding site of IL2-R2 to other cytokines. Previous studies have implicated altered cytokine pathways in glaucoma [227]. A role for *IL20RB* in POAG will require replication studies.

Studies on POAG-associated genes displaying Mendelian pattern of inheritance highlight the importance of validating newly identified genes in different populations, using both family-based

studies and case-control studies, with convincing functional studies and animal model. In summary, only three genes (*MYOC*, *TBK1* and *OPTN*) have shown convincing evidence for a role in POAG pathogenesis. Together, they explain approximately 5-10% of POAG cases.

1.7.1.5. Common genetic risk factors associated with POAG

Although genes displaying a Mendelian pattern of inheritance are highly penetrant with strong odds ratios, they only account for a minority of individuals diagnosed with POAG. This means that the majority of the genetic contribution to the disease still remains to be determined. In order to unravel genetic factors involved in POAG, other methods have been used. The most common is genome wide association studies (GWAS) which is designed to test how common variants are associated with a disease. It uses large numbers of unrelated cases and controls, high-density single nucleotide polymorphism (SNP) arrays, and statistical analysis to determine the genetic contribution of common variants.

A number of GWAS have identified and validated several loci associated with POAG. The following genes were the closest identified to the association signals: *ABCA1* [228,229], *ARHGEF12* [230], *ATOH7* [231], *ATXN2* [232], *CAV1/CAV2* [229,233-235], *CDKN2B-AS* [231,235-237], FNDC3B [238], *FOXC1* [232], *GAS7* [229,239], *PMM2* [228], *SIX1/SIX6* [231,235,237], *TGFBR3* [238], *TMCO1* [229,239], *TXNRD2* [232] and an evolutionary conserved region on chromosome 8q22 [237]. In addition, some of these studies have measured the impact of SNPs on different endophenotypes of POAG: SNPs at *ARHGEF12* [230], *CAV1/CAV2* [229,233,234], *GAS7* [229,239] and *TMCO1* [229,239] have been associated with IOP, whereas SNPs at *ATOH7* [231], *CDKN2B-AS* [231,237,240], *SIX1/SIX6* [240] and the 8q22 region [237] have been associated with CDR.

No common variants have proven to be of large effect as reflected by the low magnitude of odds ratio of these loci (1.2-1.5). They do not follow Mendelian inheritance of the disease, but instead act as additive risk alleles. The majority of these variants are located in non-coding regions and the mechanisms by which they contribute to POAG are still largely unknown. Some are even located far from the named gene and might influence the expression of other genes. Additional studies are needed to understand how they contribute to the disease.

In summary, variants in POAG genes displaying a Mendelian inheritance are highly penetrant but are quite rare, whereas common variants identified through GWAS have a much higher prevalence but do not contribute largely to the disease. Additionally, most of the heritability of POAG remains unexplained and is the main focus of current research. As new genes are identified and the contribution of genetic risk factors to POAG is deciphered, they can be integrated in the current understanding of the genetics of POAG with direct applicability in genetic testing and counselling.

1.7.2. Pseudoexfoliation syndrome

Although the heritability of pseudoexfoliation syndrome has not been calculated, familial aggregation has been reported [241,242] and the disease prevalence varies between different ethnicities, suggesting the involvement of genetic factors. Thorleifsson et al. identified some SNP in the *lysyl oxidase-like 1* gene (*LOXL1*, MIM 153456) that were strongly associated with pseudoexfoliation syndrome in patients from Iceland and Sweden [243]. This finding has since been replicated in different populations including non-Nordic Europeans [244], Australia [245], United States [246], India [247] and Japan [248]. A role for *LOXL1* variants in pseudoexfoliation syndrome is further supported by the fact that LOXL1 is involved in the formation of elastin fibers [249], which are a major component of intraocular exfoliation material [250]. Interestingly, although the population attributable risk is quite high in all studied populations, the prevalence of the risk alleles is also high in controls [243-248]. This suggests limited usefulness for genetic testing for *LOXL1* due to low penetrance of the identified variants and a potential involvement of other genetic and environmental factors in the pathophysiology of the disease.

Another recent GWAS identified a SNP in the *CACNA1A* gene increasing susceptibility for pseudoexfoliation syndrome in patients from Japan, and replicated the findings in a multiethnic cohort of patients from 17 countries across 6 continents [251]. The risk increase (1.16-fold) is much less than that of *LOXL1* variants (10-fold) but it is the second most strongly associated gene for pseudoexfoliation syndrome. he SNP risk allele leads to reduce mRNA levels in blood [251]. *CACNA1A* encodes the alpha-1 subunit of the P/Q type voltage-dependent calcium channel and the protein is present in various ocular tissues [251]. Electron microscopy studies showed elevated concentration of calcium in aggregating fibrils of lens specimen with pseudoexfoliation syndrome [252]. Aung et al. hypothesised that because fibrillin uses calcium to form stable aggregates, alteration of calcium channel function could lead to a modification in calcium concentration that could play a role in lens material deposits [251]. Additionally, a number of GWAS or candidate gene approaches have identified other genes associated with pseudoexfoliation syndrome, however these associations have not been replicated [253].

1.7.3. PACG

The genetics of PACG has been the focus of several studies. The ethnicity difference [47] and familial clustering [254,255] suggest a genetic basis for PACG. Additionally, major risk factors for PACG such as anterior chamber depth and axial length have a high heritability at around 70% [256]. GWAS and candidate gene studies have identified several genetic loci associated with PACG at *ABCC5* [257], *CHAT* [258], *COL11A1* [258-262], *DPM2-FAM102A* [258], *EPDR1* [258], *FERMT2* [258], *GLIS3* [258], *HGF* [263,264], *HSP70* [265,266], *MFRP* [266], *MMP9* [267-270], *NOS3* [271,272], *PCMTD1-ST18* [258-260], *PLEKHA7*

[258-262]. However, the effect of each of these loci is relatively small. The eight loci identified through GWAS by Khor et al. had odds ratio of 1.2-1.4 and explained 1.8% of the overall disease variance in PACG [258].

1.7.4. Nanophthalmos

Both autosomal dominant and autosomal recessive transmission have been reported in patients with nanophthalmos. Four loci have been described, of which one gene has been identified (*MFRP*) (Table 1-2), with an additional gene associated with nanophthalmos (*PRSS56*).

Table 1-2: Loci and genes associated with nanophthalmos

Locus	OMIM	Mapped	Gene	Ethnicity	Ref
NNO1	600165	11p		-	[273]
NNO2	609549	11q23.3	*MFRP*	Amish	[70]
NNO3	611897	2q11-q14		Chinese	[274]
NNO4	615972	17p12-q12		Chinese	[275]
	613517	2q37.1	*PRSS56*	Tunisian	[276]

In an Amish-Mennonite family displaying autosomal recessive inheritance and linkage to chromosome 11q23.3, Sundin et al. identified a homozygous variant in the *membrane-type frizzled-related protein* (*MFRP,* MIM 606227) [70]. Other studies identified homozygous or compound heterozygous variants in individuals with nanophthalmos and extended the phenotype consisting of nanophthalmos, retinitis pigmentosa, foveoschisis and/or optic nerve drusen [277,278]. *MFRP* encodes the membrane-type frizzled-related protein which function remains unknown. However, it is expressed predominantly in the retinal pigment epithelium of the eye and the mouse model for *MFRP* variant shows retinal dystrophy supporting a role for MFRP in photoreceptor function maintenance [279].

Gal et al. identified variants in the *protease serine 56* gene (*PRSS56,* MIM 613517) transmitted in an autosomal recessive manner in a Tunisian family with nanophthalmos and microcornea as well as two families and 24 unrelated patients from the Faroe Islands with nanophthalmos and normal corneal diameter [276]. Variants were subsequently identified in additional families from Tunisia and Saudi Arabia with posterior microphthalmia [280,281] and families from Canada and Mexico with nanophthalmos [282]. The implication of *PRSS56* variants in both posterior microphthalmia and nanophthalmos suggests that they represent a continuum of phenotype rather than two separate conditions. Interestingly, the mouse model for *PRSS56* variant displays elevated IOP but variably reduced axial length [280]. The function of PRSS56 remains unknown.

1.8. Genetics of childhood glaucoma

Most cases of PCG are sporadic, with some familial cases reported, predominantly transmitted in an autosomal recessive manner [283]. Four loci (Table 1-3), of which two genes have been identified (*CYP1B1* and *LTBP2*), and one additional gene (*PXDN*), have been associated with PCG.

Table 1-3: Loci and genes associated with PCG

Locus	OMIM	Mapped	Gene	Ethnicity	Ref
GLC3A	231300	2p22.2	*CYP1B1*	Turkish	[284]
GLC3B	600975	1p36.2-p36.1		Turkish	[285]
GLC3C	613085	14q24.3		Turkish	[286]
GLC3D	613086	14q24.3	*LTBP2*	Pakistani	[287]
	605158	2p25.3	*PXDN*	Pakistani	[288]

In comparison, patients with ARS display predominantly autosomal dominant transmission with sporadic cases often reflecting a *de novo* inheritance. Four loci, including 2 genes, have been isolated so far (Table 1-4). Sequence variants and CNVs in the *FOXC1* and *PITX2* genes account for around 40% of patients with Axenfeld-Rieger Syndrome [289,290]. Peters' anomaly is mainly sporadic but familial cases usually transmitted as an autosomal recessive trait have been reported [291]. Variants in *CYP1B1*, *PAX6*, *FOXC1* and *PITX2* have been identified in patients with Peters' anomaly. Aniridia is transmitted in an autosomal dominant manner and the majority of cases are explained by variants or deletions implicating the *PAX6* gene. Finally, variants in *PXDN* have been associated with ASD [292].

Table 1-4: Loci and genes associated with ARS

Locus	OMIM	Mapped	Gene	Ethnicity	Ref
RIEG1	180500	4p25	*PITX2*		[293]
RIEG2	601499	13q14			[294]
RIEG3	602482	6p25.3	*FOXC1*		[295]
		16q24			[296]

Although genes associated with childhood glaucoma cause predominantly one phenotype, phenotypic heterogeneity and overlap exists between the implicated genes. Consequently, I will discuss the genetics of childhood glaucoma on a gene by gene basis.

1.8.1. Cytochrome P450, subfamily I, polypeptide 1 (CYP1B1)

CYP1B1 (MIM 601771) was the first gene linked to PCG on the GLC3A locus [297]. The proportion of the disease explained by deleterious variants varies between populations: The prevalence is very high in populations with high consanguinity such as Saudi Arabia or Iran (85-90%) or strong

founder effects like in Slovakian Gypsies (almost 100%), but much lower in European countries (25-42%) or in Asia (8-15%) [298]. In Australia, *CYP1B1* variants account for 22% of PCG cases [299]. The gene displays high allelic heterogeneity with over 150 deleterious variants described [300]. Deleterious variants are transmitted in an autosomal recessive manner and are spread across both coding exons of the gene with missense, nonsense, frameshift, small indels and gene deletions reported.

CYP1B1 variants can display an incomplete penetrance: some families have been reported with asymptomatic individuals carrying deleterious variants [301,302]. Families with siblings carrying the same variants but displaying either PCG or JOAG have been described [302,303]. *CYP1B1* variants (either monoallelic or biallelic) have been associated with JOAG and POAG among different populations with variable frequencies (4-19%) [304-306]. These findings support a phenotypic continuum of *CYP1B1* variants from PCG to POAG. Homozygous or compound heterozygous variants in the *CYP1B1* gene have also been identified in some patients with Peters' anomaly [307-309], corneal enlargement without optic disc cupping [310], Axenfeld-Rieger anomaly [311,312], and total or partial aniridia [313-315]. *CYP1B1* variants do not display clear genotype/phenotype correlations: The same variants, whether homozygous or in combination with another one, have been associated with different phenotypes and the associated phenotypes are caused by different variants.

CYP1B1-null mice exhibit ocular drainage structure malformations similar to those found in patients with PCG [316]. More recently, studies demonstrated irregular and decreased collagen distribution in the trabecular meshwork of *CYP1B1*-null mice, increased oxidative stress and elevated IOP [317,318]. These studies strengthen the role of *CYP1B1* in the development of the eye, including the integrity of the trabecular meshwork and the modulation of cellular oxidative state.

CYP1B1 is a member of the cytochrome P450 superfamily and the protein is responsible for the metabolism of a range of endogenous and exogenous substrates, including hormones, xenobiotics and drugs. Functional studies have demonstrated that deleterious variants act by reducing the enzymatic activity or the stability of the enzyme [319,320]. The mechanism by which they cause glaucoma is not well understood but it is hypothesised that they may alter the expression of genes or the level of regulatory molecules important during the development of the eye [321]. For example, CYP1B1 is involved in the metabolism of vitamin A by oxidising retinol to retinal and retinal to retinoic acid [322]. Early studies demonstrated severe eye malformations in an animal model deficient in vitamin A [323] and retinoic acid is involved in the morphogenesis of the anterior segment of the eye and closure of the optic fissure [324]. Vitamin A deficiency results in severe malformations of the eye. Alternatively, CYP1B1 is involved in the metabolism of melatonin [325] which has been shown to reduce IOP in animal models and in humans [326-328]. Additional functional studies are needed to

elucidate which CYP1B1 metabolic pathway might be involved in the pathogenesis of glaucoma.

In addition to its role in glaucoma, *CYP1B1* has been involved in cancers and has been suggested as a potential tumour marker [329]. An early study showed that CYP1B1 expression is enhanced in a wide range of cancers [330] and *CYP1B1*-null mice displayed reduced carcinogenesis [331]. CYP1B1 is involved in the activation of environmental procarcinogens [332], and procarcinogen-induced tumours are reduced in *CYP1B1*-null mice [333]. A number of *CYP1B1* polymorphisms affecting the catalytic activity of the protein have been implicated in different cancers such as breast, ovarian, endometrial, lung and prostate cancers [334-336]. Importantly though, the *CYP1B1* polymorphisms associated with cancers are different from the deleterious variants associated with PCG, which is relevant in the context of genetic counselling for glaucoma.

Although the pathophysiology of *CYP1B1* in glaucoma is not fully elucidated, current knowledge demonstrates a complex role for *CYP1B1* in the disease. *CYP1B1* biallelic deleterious variants are associated with PCG, JOAG and ASD, with the same variants also contributing to POAG in the heterozygous state. The incomplete penetrance and variable inter- and intra-familial expressivity add to the complexity of providing genetic counselling for patients carrying variants in the gene and their family members.

1.8.2. Forkhead box C1 (FOXC1) & Paired-like homeodomain transcription factor 2 (PITX2)

Sequence variants and deletions of the *FOXC1* gene (MIM 601090) and the *PITX2* gene (MIM 601542) [289,293,337-340], as well as duplications of the *FOXC1* gene [289,341], are associated with Axenfeld-Rieger syndrome. The ocular features associated with *PITX2* variants cannot be differentiated from those caused by *FOXC1* variants. However, the associated systemic features differ between both genes. *FOXC1* variants result in either isolated ocular defects or they can be associated with a range of systemic features including facial dysmorphism, heart anomalies, hearing defects, developmental delay, growth delay, hydrocephalus and cerebral small-vessel disease [289,290,342,343]. In comparison, individuals with *PITX2* variants usually display facial dysmorphism, dental anomalies (hypodontia and microdontia) and umbilical anomalies (periumbilical redundant skin, umbilical hernia) [289,290,342]. The prevalence of glaucoma has not been well studied but it is estimated that *FOXC1* and *PITX2* variants are associated with a 35-75% lifetime risk of developing glaucoma [289,290,342] . *FOXC1* and *PITX2* variants are transmitted in an autosomal dominant manner with strong intra- and inter-familial variable expressivity. Other ocular phenotypes have been described with *FOXC1* variants including Peters' anomaly [344,345], aniridia [346], and PCG [347]. *PITX2* variants have been identified in individuals with Peters' anomaly [348] and aniridia-like phenotype [349].

FOXC1 contains one coding exon and a forkhead DNA-binding domain. *PITX2* comprises seven exons and produces four isoforms with different spliced exons. The first three isoforms (a, b, c) represent different combinations of the first four exons but they all contains the same DNA-binding homeodomain and share the same C-terminal region that contains a protein-protein interaction domain. *PITX2d* has a truncated homeodomain and is negatively regulated by the other three isoforms. Deleterious variants are reported in *PITX2a* whose product is expressed in the developing eye. In both genes, nonsense and frameshift variants are spread across the exon but all missense deleterious variants are present in the DNA-binding domain [289]. The *FOXC1* and *PITX2* CNVs reported in affected patients all have different chromosomal breakpoints suggesting that these rearrangements are non-recurrent [289,341]. The presence of repetitive sequences and paralogous forkhead domain genes in the case of the 6p25 region can make these chromosomal regions more prone to rearrangements [341]. Additionally, conserved regulatory elements upstream of *PITX2* and in the 4q25 region have been associated with ARS [290,350]. The lack of well-defined genotype/phenotype correlations with *FOXC1* and *PITX2* CNVs suggests that no other dosage-sensitive genes are present in the deleted regions.

Interestingly, FOXC1 and PITX2 are co-expressed in the periocular mesenchyme and they both contain a DNA binding domain to bind to each other [351]. More precisely, PITX2 downregulates the functional activity of FOXC1 [351]. *FOXC1* encodes a forkhead transcription factor which regulates genes involved in cell differentiation and migration during development. *PITX2* is a member of a paired class of homeodomain transcription factors also important for the regulation of genes involved during the development.

1.8.3. Paired box gene 6 (PAX6)

Variants in *PAX6* (MIM 607108) on chromosome 11q13 have first been reported in 1992 [352]. They are identified in 90% of cases with aniridia [353] and are transmitted in an autosomal dominant manner with complete penetrance but variable expressivity [354]. A family history is present in two thirds of the cases whereas one third are sporadic [354]. Intragenic variants account for two thirds and chromosomal rearrangement account for one third of cases [353]. The *PAX6* locus-specific database (http://pax6.hgu.mrc.ac.uk) lists over 400 unique sequence variants [355]. Aniridia is typically caused by *PAX6* haploinsufficiency with three-quarters of the variants (nonsense, frameshift, splice-site, in-frame deletions and duplications) leading to premature termination codons and degradation by nonsense-mediated decay [356].

Aniridia is associated with a number of ocular complications including glaucoma, cataracts, lens dislocation, foveal hypoplasia and keratopathy. One study reported that 67% of patients with aniridia develop glaucoma [84]. The onset of glaucoma varies between birth and adulthood but there

is age-related increase of glaucoma incidence and 30% of patients with aniridia are diagnosed with glaucoma before the age of 20 years [84].

Non-aniridia phenotypes have been reported with *PAX6* variants, including Peters' anomaly [308,357], keratitis [358], congenital cataracts [359], optic nerve malformations [360], corneal dystrophy and isolated foveal hypoplasia. These phenotypes are predominantly caused by *PAX6* missense variants [356]. The majority of them are located in the paired domain, suggesting that these variants result in a PAX6 protein with impaired DNA-binding, affecting the regulation of downstream target genes, rather than haploinsufficiency.

Aniridia is predominantly an isolated ocular feature resulting from sequence variants or deletions of *PAX6*. However, it can also occur in combination with systemic features, WAGR syndrome (Wilms tumour, aniridia, genitourinary and mental retardation; MIM 194072) being the most common syndromic association with aniridia [82]. WAGR syndrome is a contiguous gene syndrome caused by a hemizygous deletion at chromosome 11p13, comprising *PAX6* resulting in aniridia and *WT1* associated with an increased risk for Wilms tumour, genitourinary and renal abnormalities [353,361]. WAGR syndrome associated with obesity is known as WAGRO syndrome, with obesity resulting from a larger deletion involving the *BDNF* gene [362].

PAX6 is highly conserved and encodes a transcriptional regulatory protein with two DNA-binding domains (a paired domain and a paired-type homeodomain) and one transcriptional trans-activation domain. *PAX6* is expressed in the differentiating cells of the lens, cornea, retina and ciliary body [363]. Heterozygous *PAX6* variants in mice result in a small eye phenotype whereas *PAX6*-null mice display anophthalmia [364]. PAX6 plays a crucial role in key processes during the eye development such as cell proliferation, migration, adhesion and signalling by regulating downstream target genes [365,366].

1.8.4. Latent transforming growth factor-β-binding protein 2 (LTBP2)

LTBP2 (MIM 602091) was initially identified as a PCG causing gene with autosomal recessive transmission in families from Pakistan and Iran, and patients from Gypsy ethnicity [367,368]. However, more recent papers have demonstrated that variants in *LTBP2* cause congenital microspherophakia and/or megalocornea with ectopia lentis and secondary glaucoma [369,370]. The pathophysiology of *LTBP2* remains incompletely understood but the protein is a member of the latent TGF-β binding protein family and interacts with fibrillin-1, associated with Marfan syndrome which includes ectopia lentis and sometimes megalocornea and glaucoma.

1.8.5. Peroxidasin (PXDN)

Variants in the *PXDN* gene (MIM 605158) were identified in 2 families from Pakistan with congenital cataract, microcornea and corneal opacification without vascularisation, and in one family from Cambodia displaying severe developmental glaucoma with extensive corneal opacification and vascularisation in an autosomal recessive manner [288]. Systemic or neurological abnormalities were not present. Two additional Caucasian families with ASD, sclerocornea, microphthalmia, cataracts and glaucoma were identified with *PXDN* variants [292]. Hypotonia and developmental delay were present in one patient. The precise mechanism by which *PXDN* would lead to this phenotype is currently unknown but a mouse model suggested that *PXDN* is involved in cell proliferation, differentiation and adhesion and basement membrane integrity during eye development [371].

1.8.6. Summary

In summary, several genes and genetic risk factors have been identified for the different types of glaucoma. The mechanisms by which they cause glaucoma and their natural history are still not fully understood. These gaps in knowledge affect the provision of adequate genetic counselling. Gene characterisation is essential in order to provide accurate information to the patients and their families. Additionally, the phenotypic overlap between the different genes make molecular diagnosis and risk predictions challenging. The majority of glaucoma cases remain unexplained by the current genetic knowledge and a significant proportion of families with multiple individuals test negative for known glaucoma genes, limiting risk prediction for family members or reproductive options. The identification of additional rare variants with high penetrance has been proven difficult due to the lack of large families available for linkage and the lack of distinct phenotype that could be caused by single variants or genes. The development of recent technologies such as high throughput sequencing, which allows the sequencing of the entire human genome and the identification of rare variants, might be able to assist in identifying the remaining contribution of genetics to glaucoma.

1.9. This thesis

Glaucoma is a common disease and it is highly heritable. However genetic testing for glaucoma is complicated by its genetic heterogeneity. The distinction between the glaucoma subset caused by Mendelian genes and the subset caused by multiple genetic factors is important from a genetic counselling point of view because the risk estimates are different. Although only a small subset of glaucoma is caused by Mendelian genes, the identification of a monogenic cause of glaucoma in affected individuals has important implications for patients and their family members [266,267]. The mode of inheritance is not always obvious in a condition like glaucoma where variable expressivity

and incomplete penetrance are common and especially when the disease is sporadic. Genetic testing for glaucoma is complicated by the fact that the glaucoma phenotype associated with each gene is often indistinguishable and that multiple genes can account for the same glaucoma subtype.

Over the past eight years, I have worked on the Australian and New Zealand Registry of Advanced Glaucoma (ANZRAG). The genetic causes of glaucoma had only been partially unravelled and most identified genes needed to be further characterised. The aim of the ANZRAG has been to identify genes or genetic factors associated with the different types of glaucoma. Translational research has always been a strong focus of the project. As a genetic counsellor I have been in charge of all the feedback of genetic results to research participants. I have designed and conceived projects that led to the delineation of known glaucoma genes and genotype/phenotypes correlations that are used in genetic testing and genetic counselling for individuals and their families dealing with glaucoma. I have worked toward translating research outcomes to improve access to early genetic screening for glaucoma. I have participated in the development of accredited genetic testing for a number of glaucoma genes. I have established and coordinated a genetic testing program for at-risk family members of individuals with identified deleterious glaucoma variants. The publications that led to this thesis are the work of eight years of research as a genetic counsellor on the genetics of glaucoma and the translation of these outcomes into better healthcare for patients and their families.

This thesis addresses the gaps in knowledge of the different Mendelian forms of glaucoma and the issues in genetic testing that affect the effectiveness of genetic counselling for glaucoma. My original contribution to knowledge includes the delineation of the genotype/phenotypes correlations and the natural history of known glaucoma genes, the identification and characterisation of novel glaucoma genes, the investigations of patients' experience of glaucoma genetic testing and the discussion of the ethical issues related to genetic testing for glaucoma. This thesis summarises how this knowledge applies in the genetic testing setting to improve patients' care and assist health professionals in providing adequate patients' support, and provides a framework for genetic counselling in glaucoma.

CHAPTER 2: GENETICS OF PRIMARY OPEN ANGLE GLAUCOMA

This chapter includes publications focusing on the characterisation of the main glaucoma genes known to be associated with POAG, including the *MYOC*, *CYP1B1* and *TBK1* genes. A molecular diagnosis in POAG has significant benefits for the patients and their families and the gaps in knowledge in the delineation of their associated phenotypes limits the provision of adequate genetic counselling. Firstly, POAG-associated genes have not been characterised in individuals with severe glaucoma. The publications below have used the advanced POAG cohort of the ANZRAG (chapter 2-1) to assess the contribution of these genes to glaucoma blindness (chapters 2.2 and 2.4). Secondly, the interpretation of genetic variants is essential in providing feedback and counselling to patients as well as risk assessment and genetic testing for other family members. The three publications in chapter 2-3 illustrated how the characterisation of novel variants in the *MYOC* gene improved genetic counselling in the families involved. Thirdly, the identification of deleterious variants in glaucoma genes provides information about the mode of inheritance in families with POAG and access to genetic testing and counselling to those family members at risk. The evaluation of the *CYP1B1* gene reported in chapter 2-5 provided novel information in regard to the modes of transmission involved in POAG families. Finally, although predictive genetic testing is available for family members of individuals carrying *MYOC* variants, the clinical utility of the test has not been previously assessed. The publication included in chapter 2-6 evaluated the ability of the test for early identification of at-risk individuals. Collectively, my original contribution to knowledge based on the publications included in this chapter contributed to defining genotype/phenotype correlations and the natural history of known glaucoma genes for POAG, leading to improved testing options and genetic counselling in families with Mendelian forms of POAG.

2.1. The Australian and New Zealand Registry of Advanced Glaucoma (ANZRAG)

The ANZRAG (www.anzrag.com) was the first collection of advanced glaucoma patients with well-defined phenotypic information and DNA samples that aimed to investigate the genes and genetic factors leading to the worst glaucoma outcome for POAG. Because of the high heritability of POAG and the small proportion of individuals who progress to glaucoma blindness, I hypothesised that advanced POAG cases would be enriched in genetic variants associated with the disease, allowing the phenotypes associated with known glaucoma genes to be refined and identify novel genes. Additionally, patients with developmental and secondary glaucoma have also been recruited in the ANZRAG to elucidate and characterise their genetics. This publication aimed at describing the methodology and the recruitment process of the ANZRAG.

The ANZRAG has been expanding since its beginning in 2007 and as of February 2017 comprised 5000 participants, including 2200 with end stage visual loss and over 400 families, with both affected and unaffected relatives. Participants have been recruited from Australia and New Zealand and referred to the registry by their treating specialist. A main component of the ANZRAG has been the translation of genetic research outcomes into clinical practice. In this respect, I have led the development of accredited genetic testing for glaucoma, the dissemination of validated results to the participants and the development of protocols for cascade genetic testing of at-risk family members, coupled with the provision of genetic counselling.

Contribution statement

Ms Souzeau was primarily responsible for the design and coordination of the project, the continuous development of protocols, the recruitment of participants, the data analysis, and the drafting, revision, and submission of the manuscript. Prof Goldberg, Prof Healey, Prof Mills, A/Prof Landers, Prof Graham, A/Prof Grigg, Prof Casson, Prof Morgan, Dr Ruddle, A/Prof Coote, Dr White and Dr Stewart contributed to the characterisation of participants and revised the manuscript. Ms Usher, Dr Crawford and Mrs Straga contributed to the recruitment of participants, the coordination of the study and revised the manuscript. A/Prof Hewitt, Prof Mackey and A/Prof Burdon contributed to the design and conception of the study, and revised the manuscript. Prof Craig contributed to the design, conception, and supervision of the study, and revised the manuscript.

Australian and New Zealand Registry of Advanced Glaucoma: methodology and recruitment

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ABSTRACT

Background: Glaucoma is a sight-threatening disease affecting 3% of the population over the age of 50. Glaucoma is treatable, and severe vision loss can usually be prevented if diagnosis is made at an early stage. Genetic factors play a major role in the pathogenesis of the condition, and therefore, genetic testing to identify asymptomatic at-risk individuals is a promising strategy to reduce the prevalence of glaucoma blindness. Furthermore, unravelling genetic risk factors for glaucoma would also allow a better understanding of the pathogenesis of the condition and the development of new treatments.

Design: The Australian and New Zealand Registry of Advanced Glaucoma is a prospective study that aims to develop a large cohort of glaucoma cases with severe visual field loss to identify novel genetic risk factors for glaucoma blindness.

Methods: Clinical information and blood are collected from participants after referral by eye practitioners. Samples are collected across Australia and New Zealand using postage kits.

Participants: Our registry has recruited just over 2000 participants with advanced glaucoma, as well as secondary and developmental glaucomas.

Results: A positive family history of glaucoma is present in more than half of the advanced glaucoma cases and the age at diagnosis is significantly younger for participants with affected relatives, which reinforces the involvement of genetic factors in glaucoma.

Conclusions: With the collection of glaucoma cases recruited so far, our registry aims to identify novel glaucoma genetic risk factors to establish risk profiling of the population and protocols for genetic testing.

Keywords: genetics, glaucoma, open angle glaucoma

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INTRODUCTION

Glaucoma is one of the leading causes of visual impairment and blindness in the world, affecting 3% of the Australian population over the age of 50 years.[1,2] Glaucoma refers to a heterogeneous group of eye disorders that have in common characteristic cupping of the optic nerve and typical visual field defects.[3] Glaucoma may result in irreversible blindness if untreated. However, therapeutic interventions can prevent or minimize loss of visual function.[4-6] Nevertheless, half of those affected in the general population remain undiagnosed,[1,2] and the early stages are usually asymptomatic. Strategies to identify at-risk individuals before they exhibit visual loss need to be implemented to minimize the ultimate risk of blindness.

A positive family history is known to be one of the most important risk factors for developing glaucoma.[7-10] First-degree relatives of affected patients display a risk of developing glaucoma nine times higher than the general population.[11] Glaucoma can display a Mendelian mode of inheritance (autosomal dominant or recessive), but in the majority of cases, it is recognised that glaucoma is a complex disorder with the involvement of both genetic and potentially environmental factors, although these are currently less well characterized.

Several loci and some genes have been identified in association with different types of glaucoma. The type of glaucoma most studied is primary open angle glaucoma (POAG). To date, several genetic loci have been linked to POAG,[12] and four POAG genes have been identified from these loci (*Myocilin,*[13] *Optineurin,*[14] *WDR36*[15] and *NTF4*[16]). However, together, those genes account for no more than 10% of POAG cases, and therefore, the majority of genetic factors remain to be elucidated. Recent genome-wide association study approaches using large cohorts have now begun to identify common risk alleles of modest effect size in or near genes, such as *Caveolin 1* and *2,*[17] as well as *TMCO1* and *CDKN2B-AS1* for POAG.[18] It remains to be seen what proportion of the residual risk will be uncovered by these approaches.

Genetic components have been associated with other types of glaucoma (primary or secondary), including primary congenital glaucoma,[19,20] pseudoexfoliation syndrome[21] and anterior segment dysgenesis syndromes;[22-24] but once again, they do not account for the majority of cases, and in the Australian population, these forms of glaucoma are much less prevalent than POAG.

In summary, because glaucoma is underdiagnosed, is asymptomatic in the early stages, can be treatable if managed early enough and has a strong heritability, genetic testing is a promising way of identifying at-risk individuals and preventing irreversible blindness in those individuals. The numerous loci and genes involved underlie the complexity of understanding the contribution of the different genes implicated in glaucoma, and the identification of genetic factors involved in glaucoma is complicated by an age-related penetrance, genetic heterogeneity and familial heterogeneity. Monogenic forms are relatively rare, and glaucoma appears at this stage to represent a complex disorder, with most of the genetic risk factors still to be unravelled.

The Australian and New Zealand Registry of Advanced Glaucoma (the ANZRAG, http://www.anzrag.com) was established in 2007 with the aim of assembling a large cohort of participants with advanced glaucoma in order to identify new clinical and genetic risk factors for developing severe glaucoma. The registry also recruits participants with other secondary types of glaucoma for identification of genetic risk factors specific to those conditions. In addition, the registry has also developed a protocol for *Myocilin* genetic testing for individuals with advanced glaucoma, coupled with cascade screening for their at-risk relatives. In this study, we aimed to present methodology of the ANZRAG and the demographic information of participants recruited to date.

METHODS

Recruitment of participants

Ethics approval was obtained from the Southern Adelaide and Flinders University Clinical Research Ethics Committee. The study is conducted in accordance with the revised Declaration of Helsinki and following the National Health and Medical Research Council statement of ethical conduct in research involving humans.

To ensure maximal participant recruitment, brochures and referral forms were sent to all ophthalmologists in Australia and New Zealand. Community awareness was increased through a mail-out strategy to members of a glaucoma support group (Glaucoma Australia, http://www.glaucoma.org.au). The ANZRAG also collaborates closely with other studies across Australia, like the Glaucoma Inheritance Study in Tasmania (GIST)[25,26] and the Blue Mountains Eye Study,[1] to increase the number of participants for research purposes.

For open angle glaucoma (OAG) cases, patients with advanced glaucoma are recruited in

order to identify genetic risk factors associated with the worst outcomes in glaucoma. Participants with advanced glaucoma (pathway A – advanced glaucoma) need to fulfil the following criteria in the worst eye: visual field loss related to glaucoma with at least two out of the four central squares having a pattern standard deviation (PSD) <0.5% on a Humphrey 24-2 field or a mean deviation of -22dB, or in the absence of field testing, loss of central acuity related to glaucoma (Fig. 1). They also need to have evidence of glaucomatous optic disc changes (even if mild) for the better seeing eye. Participants with the following subtypes of glaucoma or glaucoma risk factors are also recruited irrespective of the presence of field loss (pathway B – developmental and secondary glaucomas): pseudoexfoliation syndrome, pigment dispersion syndrome, primary congenital glaucoma, angle closure glaucoma, anterior segment dysgenesis syndromes and steroid responders. Patients with any of these conditions do not need to have developed ocular hypertension or glaucoma to be part of the study. Some participants may be recruited via both pathways A and B (e.g. advanced visual field loss because of pseudoexfoliative glaucoma). Exclusion criteria at the current time include traumatic, rubeotic and surgically induced glaucoma as the sole diagnosis. However, the presence of the latter conditions in one eye would not preclude recruitment if advanced glaucoma was present in the other eye.

Participants are recruited across Australia and New Zealand mainly through eye practitioners' referral, but some self referrals are also received (with clinical information being verified and provided by the treating ophthalmologist). Practitioners can mail, email or fax referrals, or they can contact the registry directly to refer a participant. They can also refer their patients by completing an online interactive PDF form on our website (http://www.anzrag.com). Participants are contacted once their referring specialist has informed them about the project and they have given their consent to be contacted. The project coordinator then contacts the participants to confirm their willingness to be involved and to answer any questions before proceeding. Written informed consent and a blood sample for the purpose of DNA extraction are obtained from each participant. All assessment forms are stored in a securely locked office in the Flinders Medical Centre, and all data is stored in a password protected access database to preserve participants' confidentiality. The collection of blood samples is executed by sending kits to participants comprising the necessary equipment to have blood taken by their general practitioner or through a local pathology service (two ethylenediaminetetraacetic acid [EDTA] blood tubes [VACUETTE K3 EDTA, Greiner Bio-One, West Heidelberg, Victoria, Australia], 1x band-aid, 1x cotton ball, 1x skin-cleansing swab). As the recruitment is performed all over Australia and New Zealand, a reply paid satchel for return through Australia Post is provided. The

shipping of blood samples complies with Australia Post requirements (International Air Transport Association Packing Instruction 650) using LabPak-1 packaging (Thermo Fisher Scientific, Scoresby, Victoria, Australia). In addition, international samples (e.g. New Zealand) comply with New Zealand Post requirements, and Australian Quarantine and Inspection Service quarantine requirements.

Detailed clinical assessment is performed and documented by the patient's usual clinical ophthalmologist on our referral form. Required clinical details include diagnosis, ethnicity, family history, age at diagnosis, best-corrected visual acuity, maximum recorded intraocular pressure, refraction, central corneal thickness, vertical cup:disc ratio (CDR), glaucoma surgery/laser history and results from visual field tests (mean deviation and presence of central field loss) (supplementary information). Participants are specifically asked if anyone in their family is affected with glaucoma. For the purpose of the study, a positive family history is defined as the presence of a fourth degree or closer relative affected by glaucoma. Details are sought as to the number and relationship of the affected relatives.

Statistical analysis

For all analyses, data was combined from the ANZRAG, and cases meeting the ANZRAG criteria enrolled in the GIST, prior to establishment of the ANZRAG. In the case of analysis of family history, only participants recruited directly through the ANZRAG were considered, as the collection of this data was made differently by the ANZRAG and the GIST. Family history in the ANZRAG is self-reported by participants, whereas glaucoma status was determined by examining consenting family members in the GIST.

PAWS Statistics, Rel. 18.0.1.2009, Chicago, IL, USA: SPSS, Inc., was used for statistical analyses. Data are presented as mean ± standard deviation. Mann-Whitney *U*-test was used for the assessment of differences in nonparametric data. A *P*-value < 0.05 was considered statistically significant.

RESULTS

A total of 2072 subjects with various types of glaucoma have been recruited to date in this ongoing study. Participants from every state and territory of Australia as well as New Zealand have been recruited (Fig. 2): 9 from the Australian Capital Territory, 408 from New South Wales, 17 from the Northern Territory, 22 from Queensland, 1004 from South Australia, 357 from Tasmania, 186 from Victoria, 49 from Western Australia and 20 from

New Zealand. We have sent more than 600 kits to participants to date and have obtained a return rate of 96.5% with the majority of the kits being sent back within a month.

The total number of participants with developmental or secondary glaucomas is presented in Figure 3. The most prevalent secondary glaucoma in our cohort is pseudoexfoliation syndrome (42%), followed by angle closure glaucoma (25%), steroid responders (12%) and pigment dispersion syndrome (9%). Developmental glaucomas (primary congenital glaucoma and anterior segment dysgenesis syndromes) account for only 12%. We have also recruited 1214 participants with advanced glaucoma. Of those, some will have advanced glaucoma because of secondary or developmental glaucoma. Figure 4 shows that 7.2% of our advanced glaucoma participants also have pseudoexfoliation syndrome, 4.4% have angle closure glaucoma, 2.0% have pigment dispersion syndrome, 1.3% are steroid responders, and 0.6% have primary congenital glaucoma.

Table 1 presents the demographic details of the advanced glaucoma cohort. Of those, 51.4% are women, and 48.6% are men. Most of the participants are Caucasians (92.9%). Family history of glaucoma is present in 55.5% of the 866 participants with advanced glaucoma recruited through the ANZRAG, and 88% of these have a first-degree relative affected. The mean age at diagnosis in the group with a family history of glaucoma is 54.7 ± 14.8 years old *versus* 63.0 ± 14.0 years old in the group with no family history of glaucoma ($P < 0.001$). The age distribution at the time of recruitment for participants with advanced glaucoma recruited through the ANZRAG with no secondary or developmental glaucoma is shown in Table 1. Their mean age at the time of recruitment is 74.4 ± 12.2 years old, and 70% of participants were ≥70 years old.

DISCUSSION

Glaucoma is one of the leading causes of blindness in the world but only a proportion of those affected will progress to blindness, and rates of progression vary among patients. Moreover, little is known about who might develop glaucoma even though a positive family history of the condition is a major risk factor. The condition is treatable and glaucoma blindness might be prevented if managed early enough. Therefore, identifying genetic risk factors for severe glaucoma could benefit affected individuals in identifying those at risk of progressing to blindness. It would also benefit asymptomatic at-risk individuals, as it may facilitate earlier and more aggressive treatment for individuals at high risk and save treatment costs for individuals at low risk. To our knowledge, the ANZRAG is the largest cohort of individuals assembled with advanced glaucoma.

We now have participants from every state and territory of Australia as well as from New Zealand. The recruitment is progressing steadily, and we are still in the process of recruiting participants with any of the following diagnoses: advanced OAG, pseudoexfoliation syndrome, pigment dispersion syndrome, angle closure glaucoma, primary congenital glaucoma, steroid responders and anterior segment dysgenesis syndromes. The majority of participants have been referred by their eye practitioner (96.5%). Any practitioner interested in referring a patient or obtaining more information about the project can do so via the ANZRAG website: http://www.anzrag.com. To the best of our knowledge, our use of kits to collect blood samples from participants across the country is unique. We demonstrated the feasibility of using such kits with an excellent return rate of 96.5% in a reasonable timeframe. Most participants provide a positive feedback regarding the kits, as this system allows them to have their blood sample taken at their own convenience without having to attend a hospital facility. We have also developed some strategies to overcome difficulties encountered in regard to the referral process. The amount of information on the referral form needs to be balanced, as requesting too many details leads to a lower response rate. However, some phenotypic and demographic information is quite critical when doing genetic studies. We overcame these conflicting demands with two different strategies. First, we made sure the referral process was as simple and rapid as possible for eye practitioners by allowing them to fax, email, post or complete the referral form online. Second, we created a follow-up questionnaire sent to all our participants, which allowed us to obtain any missing information. The questionnaire focused specially on the detailed family history and ethnicity, which are very important in genetic studies and were not always detailed enough on the initial referral form. Subsequent verbal follow-up with subjects by telephone also allowed any unresolved questions to be answered in regard to the genetic testing and consent process.

As of July 2011, 2072 participants have been recruited in the ANZRAG, the majority of which have advanced glaucoma (*n* = 1214). In our advanced glaucoma cohort, 55.5% report a positive family history of glaucoma, which is higher than previous studies assessing a family history of glaucoma as self-reported by participants with POAG among various populations (16-46%[8,9,27-34]). Those studies usually considered a family history positive if first- or second-degree relatives were affected with glaucoma, but half of them do not provide any specific details on their criteria. It has been demonstrated that self-reported family histories are potentially inaccurate and underestimated, as patients are often unaware of their positive family history and glaucoma remains frequently undiagnosed.[27,35] The GIST assessed directly the presence of glaucoma in relatives up to the fourth degree of patients with POAG and found a positive family history in 60% of cases.[7] The higher prevalence of affected

relatives in our cohort can be explained by the fact that a positive family history is associated with more advanced glaucoma.[33,36] Also, the age at diagnosis in the positive family history group is significantly younger than in the negative family history group which highlights the fact that heritable factors are responsible for the development of glaucoma at an earlier age. Alternatively, the increased knowledge and awareness of glaucoma in those with a positive family history can lead to earlier diagnosis in those at risk but in the asymptomatic stage.[37,38] We speculate that both mechanisms apply; there is a propensity for those with a strong family history to develop more severe disease, but that awareness of the situation can mitigate against the worst outcomes.

In conclusion, The ANZRAG aims to provide the world's largest collection of advanced glaucoma cases, along with other subtypes of secondary glaucoma, to ascertain new genetic glaucoma profiles. Glaucoma risk variants have already been identified near the *Caveolin 1* and *2* genes as well as the *TMCO1* and *CDKN2B-AS1* genes recently in genome-wide association studies using the ANZRAG participants.[17,18] The main objective of the study is to identify novel genetic and clinical risk factors for poor outcome in glaucoma. This will aid in identification of at-risk individuals, cascade screening of family members, risk profiling of the population, implementation of genetic tests and the development of novel glaucoma treatments.

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Figure 1. Participant with advanced glaucoma: (a) visual field loss related to glaucoma with two out of the four central squares having a pattern standard deviation (PSD) <0.5% on a Humphrey 24-2 field, (b) evidence of glaucomatous optic disc changes.

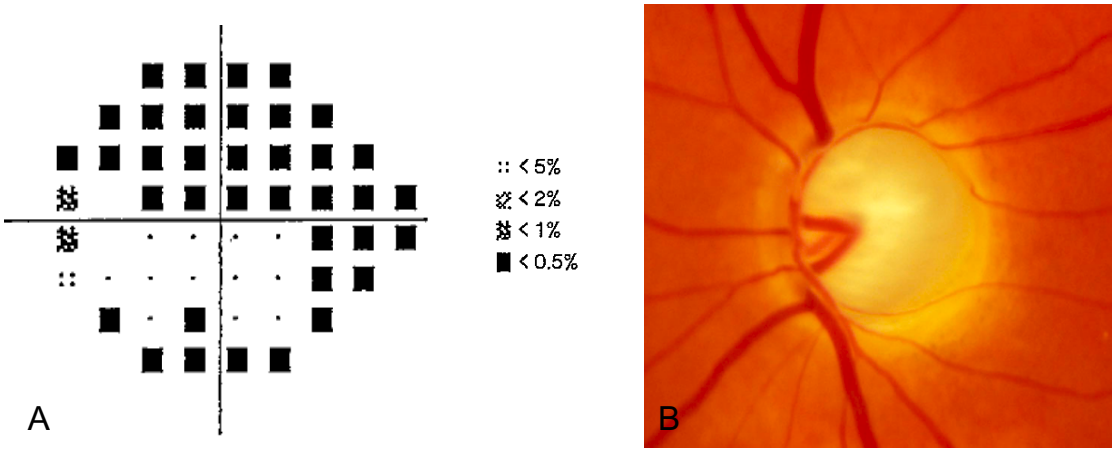

Figure 2. Distribution of all participants according to state. ACT: Australian Capital Territory, NSW: New South Wales, NT: Northern Territory, NZ: New Zealand, QLD: Queensland, SA: South Australia, TAS: Tasmania, VIC: Victoria, WA: Western Australia.

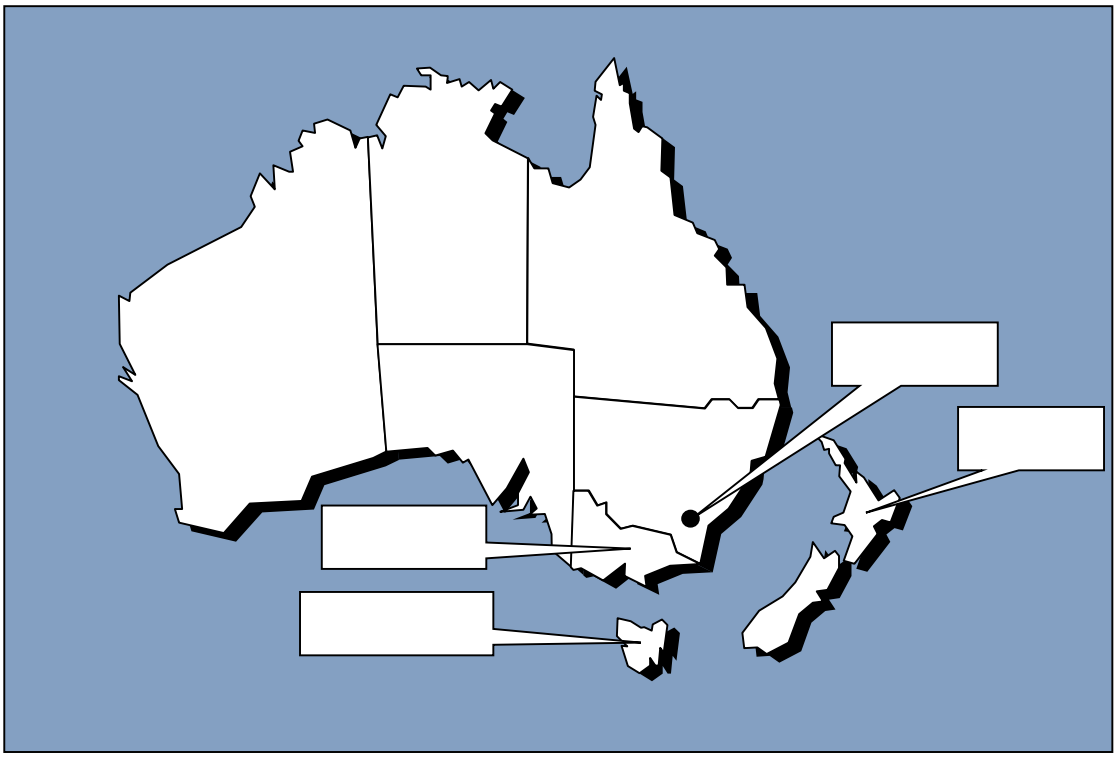

Figure 3. Distribution and number of participants recruited with developmental and secondary glaucoma.

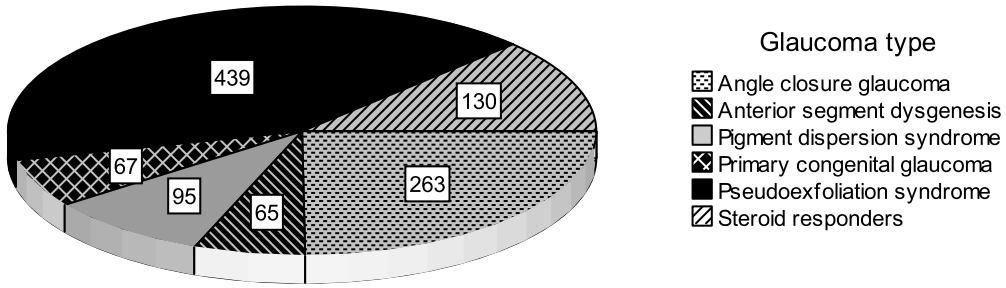

Figure 4. Distribution of developmental and secondary glaucomas in the advanced glaucoma cohort.

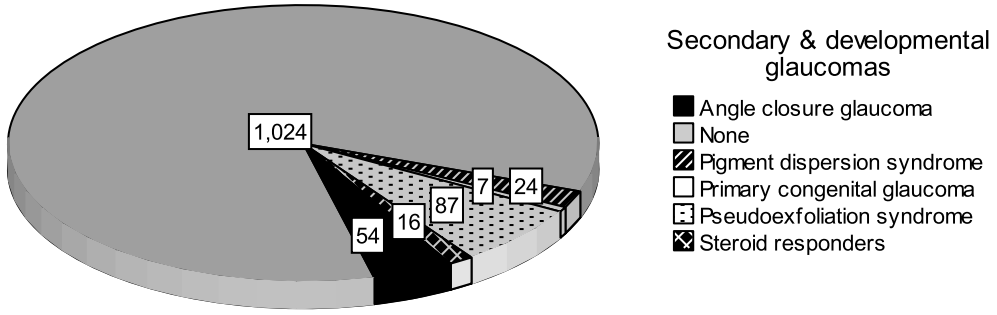

Table 1. Demographic information of advanced glaucoma patients (age distribution at recruitment is only including participants recruited through the Australian and New Zealand Registry of Advanced Glaucoma with no secondary or developmental glaucoma).

	Prevalence (%)
Age at recruitment (years)	
<40	1.5
40-49	2.6
50-59	7.3
60-69	18.3
70-79	28.9
80-89	35.3
>89	6.1
Gender	
Female	51.4
Male	48.6
Ethnicity	
Caucasian	92.9
African	1.0
Asian	4.1
Australian Aboriginal	0.4
Hispanic	0.4
Mixed Ethnicity	1.2

2.2. High prevalence of *MYOC* variants in individuals with advanced visual field loss

The study in this chapter was the first to assess whether *MYOC* deleterious variants were more prevalent among individuals with severe visual loss. A total of 1060 individuals with advanced POAG (including 103 with JOAG) and 320 individuals with non-advanced POAG were sequenced for the *MYOC* gene. In this study, I showed a significant enrichment of *MYOC* deleterious variants among advanced POAG cases compared with non-advanced (4.2% vs 1.6%, $p = 0.02$), suggesting a more severe glaucoma phenotype in *MYOC* carriers.

These results led to improve the delineation of the phenotype associated with *MYOC* variants. Among individuals with advanced POAG, those with *MYOC* deleterious variants were significantly younger at diagnosis (46.2 ± 17.8 vs 60.9 ± 14.3 years old, $p < 0.001$) and had higher IOP (33.1 ± 10.1 vs 26.5 ± 9.9 mmHg, $p < 0.001$) compared with those without *MYOC* deleterious variants. The majority of the carriers (80%) reported a positive family history of glaucoma. A high detection rate (up to 40%) was achieved among cases selected for young age at diagnosis, high IOP, and positive family history, which is useful when prioritising cases for genetic testing. Although these findings were generated in a Caucasian Australian population, the majority of Australians have a European background with no prominent founder effect. Therefore, these results could be extrapolated to other European or Caucasian populations. The outcomes of my research have important translational implications for the patients: The phenotypic characteristics of *MYOC* variants can greatly assist clinicians and genetic counsellors in prioritising cases for genetic testing, and counselling patients about the expected course of the disease and the most appropriate management options.

The ANZRAG oversees a cascade genetic testing program for family members of individuals with a confirmed *MYOC* deleterious variant. Asymptomatic individuals who carry the familial variant are at a very high risk of developing glaucoma and can benefit from close monitoring and early interventions. Similarly, individuals who do not carry the familial variant have a similar risk of developing glaucoma than the general population and are advised to follow the standard recommendations. The fact that *MYOC* variants are associated with a young age of glaucoma onset is important for family members at risk of having inherited these variants and of developing glaucoma. Although the identification of a *MYOC* variant in an asymptomatic individual does not predict the age of glaucoma onset, the range of age of onset in the family and in other families with the same variant can be used to counsel individuals about glaucoma surveillance and management.

Contribution statement

Ms Souzeau was primarily responsible for the design and conception of the study, the collection, analysis and interpretation of data, the feedback of results and the genetic counselling to the participants, as well as the drafting and submission of the manuscript. A/Prof Burdon contributed to the design, conception, and data analysis of the study, and revised the manuscript. Dr Dubowsky and Dr Grist contributed to genetic and data analysis, and revised the manuscript. Ms Usher, Dr Fitzgerald and Dr Crawford contributed to the coordination of the study and revised the manuscript. A/Prof Hewitt, Prof Goldberg, Prof Mills, Dr Ruddle, A/Prof Landers and Prof Mackey contributed to the characterisation of participants and revised the manuscript. Prof Craig contributed to the design, conception, data analysis and supervision of the study, and revised the manuscript.

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Higher prevalence of *Myocilin* mutations in advanced glaucoma in comparison with less advanced disease in an Australasian disease registry

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This article contains additional online-only material: Tables 1 and 5.

Running Head: *Myocilin* prevalence in advanced POAG

ABSTRACT

Objectives: To determine the proportion of all *Myocilin* coding mutations responsible for advanced primary open-angle glaucoma (POAG) in early-age-at-onset individuals and to investigate the prevalence of exon 3 *Myocilin* mutations in advanced POAG at any age at onset in a large Australasian cohort.

Design: Cross-sectional study using national disease registry.

Participants: One thousand sixty individuals with advanced POAG (103 with age oat onset of 40 years or younger) and 320 with nonadvanced POAG all recruited by the Australian and New Zealand Registry of Advanced Glaucoma.

Methods: Participants were examined and referred by their eye practitioner, and *Myocilin* genetic testing was performed by direct sequencing. Cascade genetic testing was made available for relatives of participants found to carry a *Myocilin* mutation.

Main outcome measures: Advanced glaucoma diagnosis based on strict visual field entry criteria. Prevalence and spectrum of *Myocilin* mutations in individuals with advanced and nonadvanced POAG.

Results: This is the first study to report *Myocilin* mutations in an advanced POAG cohort. No pathogenic *Myocilin* mutations were identified in exons 1 and 2 in early-age-at-onset advanced POAG cases. Exon 3 *Myocilin* mutations were identified in 45 advanced POAG patients (4.2%), which is significantly higher (P = 0.02) compared with nonadvanced POAG patients (1.6%). A novel mutation (Trp373X) and a new variant of uncertain pathogenicity (Ala447Thr) also were reported. The prevalence of *Myocilin* mutations rose from 16% to 40%, in selected advanced POAG subgroups based on different thresholds of maximum recorded intraocular pressure, age at diagnosis, and the presence and strength of positive family history. Twenty-six individuals with *Myocilin* mutations were identified through cascade genetic testing of first-degree relatives of affected mutation carriers.

Conclusions: The prevalence of *Myocilin* mutations in glaucoma cases with severe visual field loss is significantly greater than in nonadvanced glaucoma patients. *Myocilin* screening in phenotypically selected cases can have a much higher yield than in previous unselected series. Identifying individuals who have *Myocilin* mutations provides an opportunity to screen at-risk clinically unaffected relatives and to reduce glaucoma blindness through early management and intervention.

INTRODUCTION

Glaucoma is one of the leading causes of visual impairment and blindness worldwide, affecting approximately 60 million people.[1] It is characterized by cupping of the optic nerve and typical visual field defects that can lead to irreversible blindness if untreated. Its molecular basis is not understood completely, but it presents as a complex and heterogeneous disorder resulting from the interaction of multiple genes and environmental factors.[2] Family history is one of the most important risk factors for glaucoma.[3-5] First-degree relatives of affected patients are almost 10 times more likely to demonstrate glaucoma than the general population.[6]

Primary open angle glaucoma (POAG) is the most common type of glaucoma in which optic nerve damage occurs without evidence of angle closure, and elevated intraocular pressure (IOP) is a major risk factor.[7] POAG with early-age-at-onset tends to be more severe and is more likely to segregate in an autosomal dominant fashion.[8-9] POAG is genetically heterogeneous, and at least 16 loci contributing to susceptibility have been identified. Of these, only 4 genes have been isolated: *Myocilin,*[10] *Optineurin,*[11] *WDR36*[12] and *NTF4,*[13] with a fifth gene for normal-tension glaucoma, *TBK1*, still requiring independent replication.[14] More recently, genome-wide association studies have begun to shed light on the common variants of smaller effect size associated with POAG, with several loci reaching genome-wide significance.[15-16]

The *Myocilin* gene, within the GLC1A locus on chromosome 1q21-q31, was the first gene discovered to be associated in a causal manner with POAG.[10] Mutations in *Myocilin* are inherited in an autosomal dominant fashion and have been identified consistently in 2% to 4% of POAG patients,[10, 17-20] and in 8% to 36% of early-age-at-onset POAG patients[21-23] (diagnosed before 40 years of age) among different populations. More than 70 disease-causing mutations have been reported, and 97% of these occur within exon 3.[24] *Myocilin* mutations typically are associated with a younger age at diagnosis of glaucoma, elevated IOP, and a strong family history of glaucoma,[17-18, 21, 25] but also can show incomplete penetrance and variable interfamilial and intrafamilial expressivity in severity and age at onset.[25] The mechanism by which *Myocilin* mutations cause glaucoma has not yet been elucidated fully. However, it seems that most mutations act through a dominant negative mechanism, potentially forming dysfunctional heterodimers with wild-type *Myocilin*, leading to inappropriate intracellular accumulation of the protein in the trabecular meshwork, with consequent decrease in outflow facility leading to marked elevation of IOP.[26]

Glaucoma is a treatable condition, and appropriate treatment can delay or prevent glaucoma blindness.[27-29] Population studies in Australia have demonstrated that half of those affected in the general population remain undiagnosed[30-31] because the early stages usually are asymptomatic. Genetic testing is a promising way of identifying at-risk individuals and providing them the opportunity of early treatment, which could prevent irreversible glaucoma blindness. The Australian and New Zealand Registry of Advanced Glaucoma (ANZRAG; www.anzrag.com, accessed March 9, 2012) was established in 2007 with the aim of assembling a large cohort of patients with advanced glaucoma to identify new clinical and genetic risk factors for developing the condition.[32] In addition, the ANZRAG offers *Myocilin* full coding sequence analysis to individuals with early-age-at-onset advanced glaucoma and sequencing of *Myocilin* exon 3 to individuals with adult-age-at-onset advanced glaucoma. Cascade genetic testing for family members of participants with a *Myocilin* mutation is also offered to identify presymptomatic mutation carriers. This article presents the results of screening for *Myocilin* mutations in a large cohort of individuals with advanced glaucoma and their relatives.

PATIENTS AND METHODS

Participant recruitment

Ethics approval was obtained from the Southern Adelaide and Flinders University Clinical Research Ethics Committee. The study was conducted in accordance with the revised Declaration of Helsinki and following the National Health and Medical Research Council statement of ethical conduct in research involving humans.

To ensure this was the first population-based genetic study involving advanced glaucoma cases, the Pubmed database was searched using the keywords *advanced glaucoma* or *severe glaucoma* in combination with *Myocilin*, *MYOC*, *genetic testing* or *genetic study*. No other similar study was found.

Participant recruitment has been described previously.[32] In brief, participants with advanced glaucoma had to meet the following criteria in the worst affected eye to be included in the registry: visual field loss related to glaucoma with at least 2of the 4 central fixation squares having a pattern standard deviation of less than 0.5% on a reliable Humphrey 24-2 field (Carl Zeiss, Dublin, CA), or a mean deviation of less than -22 dB, or in the absence of field testing, best-corrected visual acuity worse than 20/200 because of glaucoma. They also needed to have evidence of glaucoma in the less severely affected eye characterized by glaucomatous

visual field defects on a reliable Humphrey 24-2 field, with corresponding optic disc rim thinning. The ANZRAG collaborates closely with other studies across Australia, such as the Glaucoma Inheritance Study in Tasmania (GIST),[33] to increase the number of participants for research purposes. Participants with evidence of POAG, but not meeting the threshold for advanced POAG in either eye, also were recruited concurrently to provide a comparison group for the results on the advanced glaucoma cohort. Nonadvanced POAG was defined by glaucomatous visual field defects on a reliable Humphrey 24-2 field, with corresponding optic disc rim thinning, including an enlarged cup-to-disc ratio (≥0.7) or cup-to-disc ratio asymmetry (≥0.2) between both eyes.

Participants were recruited across Australia and New Zealand mainly through referrals from their eye practitioner. Written informed consent and a blood sample for the purpose of DNA extraction were obtained from each participant. Clinical information was collected by the patient's usual clinical ophthalmologist. Following the same protocol, affected relatives with advanced POAG also were recruited if they met the above criteria. Family history of glaucoma was self-reported. For the purpose of the study, a positive family history was defined as the presence of a fourth-degree or closer relative affected by glaucoma, and early-age-at-onset POAG was defined by diagnosis before 40 years of age.

Genetic testing

All early-age-at-onset advanced (n = 103) POAG patients were screened for mutations in all 3 exons of *Myocilin*, but because no mutations were identified in exons 1 and 2, the rest of the cohort was screened for mutations in exon 3 only. Testing was performed through the National Association of Testing Authorities (NATA)-accredited laboratories of the Institute of Medical and Veterinary Science Pathology at the Flinders Medical Centre (Bedford Par, Australia).

Genomic DNA was prepared from a 200 μL sample of venous blood collected into ethylenediaminetetraacetic acid tubes and extracted with an Illustra Blood Genomic Prep MiniSpin kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK) according to the manufacturer's protocols. Alternatively, patients who were either needle phobic or remote from a pathology collection center provided a saliva specimen collected into an Oragene DNA Self-Collection Kit (DNA Genotek, Inc, Kanata, ON, Canada), and the DNA was isolated from 500 μL of sample and extracted as described in the manufacturer's instructions.

Each polymerase chain reaction (PCR) was performed with 100 ng purified genomic DNA as a template in a reaction mix containing 1.5 mM $MgCl_2$, 200 µM of each deoxyribonucleotide triphosphate (dNTP), 0.5 µM of each primer (Table 1, available at http://aaojournal.org), 1 U of Platinum *Taq* DNA polymerase (Invitrogen, Mulgrave, VIC, Australia), and 1x Platinum *Taq* PCR reaction buffer, in a final volume of 25 µL. Samples were denatured for 5 min at 94˚ C, then incubated for 15 cycles under the following conditions: 94˚ C for 30 seconds, 61˚ C for 50 seconds (reduced 1˚ C every 5 cycles), 72˚ C for 60 seconds. Then the samples were incubated for 35 cycles under the following conditions: 94˚ C for 30 seconds, 58˚ C for 50 seconds, 72˚ C for 60 seconds, and the last elongation step at 72˚ C for 5 minutes on a Veriti (Life Technologies, Mulgrave, VIC, Australia) thermal cycler.

Polymerase chain reaction amplicons were prepared for DNA sequencing with a 10 µL sample of each PCR reaction treated with 5 U Exonuclease I (Biolabs, Ipswich, MA) and 1 U shrimp alkaline phosphatase (USB Corporation, Cleveland, OH) to remove residual primers and dNTPs. Bi-directional BigDye Terminator Cycle Sequencing (Life Technologies) reactions of the appropriate template and *Myocilin* PCR primer were resolved and base called on an Applied Biosystems 3130XL Genetic Analyzer (Life Technologies).

Detection of sequence variants was performed with the aid of the Mutation Surveyor version 3.10 (SoftGenetics LLC, State College, PA) software; all forward and reverse sequence trace files for both overlapping upstream and downstream PCR fragments were assembled by the software into a single contiguous sequence following alignment against the *Myocilin* gene GenBank reference (build 37.3, mRNA NM_000261.1). Significant differences in the relative peak heights of the sequence traces observed between that of the patient sample and a normal control were called automatically as a sequence variant by Mutation Surveyor; all such calls were inspected visually for confirmation. The Human Genome Variation Society (HGVS) nomenclature was used to describe the variants, and all reference to the *Myocilin* gene sequence was consistent with RefSeq mRNA NM_000261.1 derived from contig NT_004487.19 of Build GRCh37.3. The *Myocilin* database (www.myocilin.com; accessed March 9, 2012),[24] established in 2007, was used to call the pathogenicity of sequence variants.

The results were then provided in writing directly to the participants and their referring practitioner by a trained genetic counselor (ES).[34] When a mutation was identified in the *Myocilin* gene, genetic counseling was provided to the participant to help him/her understand the information and cope with the medical, psychological and familial implications.

Cascade genetic testing

As *Myocilin* mutations are inherited in an autosomal dominant fashion, first-degree relatives of individuals with a *Myocilin* mutation have a 50% risk of carrying the same mutation. This puts them at a very high risk of developing glaucoma in their lifetime due to the high age-dependent penetrance of *Myocilin* mutations.[24] Through the ANZRAG, genetic testing is offered to first-degree relatives over the age of 18 years. Exceptions are made when the age at onset within a family is known to be less than 18, in which case testing can be offered at a younger age. No contact to first-degree relatives is made by the ANZRAG; individuals interested in genetic testing are asked by the index case in their family to contact us, in order to promote a voluntary decision and avoid any pressure from third parties.

We had previously established the acceptability of *Myocilin* predictive genetic testing.[35] Information regarding the process of genetic testing and its implications was discussed at the first contact with the genetic counselor (ES), including, but not limited to, description of the condition, transmission and initial glaucoma risk, familial and personal implications of being tested, insurance issues, and the interpretation of results.[36] A consent form and a blood sample were required from the individual (postage kits were used for interstate and overseas individuals),[32] and genetic testing was carried out through the Institute of Medical and Veterinary Science Pathology. The ANZRAG does not provide treatment or glaucoma monitoring for people who are found to carry a mutation, but in all cases, facilitates referral to appropriately trained local ophthalmologists with all supporting documentation of the *Myocilin* mutation.

Statistical analysis

For all analyses, data were combined from the ANZRAG, and cases meeting the ANZRAG criteria enrolled in the GIST, except for analysis of family history. In this case, only participants recruited directly through the ANZRAG were considered, because the collection of these data was not uniform between the ANZRAG and the GIST. In the ANZRAG, family history was self-reported by participants, whereas in the GIST, glaucoma status was determined in many cases by examining consenting family members.

PASW Statistics release 18.0.1.2009(SPSS, Inc, Chicago, IL) was used for statistical analyses. Data are presented as mean ± standard deviation. Mann-Whitney *U* test was used for the assessment of differences in nonparametric data. A *P* value less than 0.05 was considered statistically significant.

RESULTS

A total of 1311 subjects with advanced glaucoma were recruited in this ongoing study. Advanced glaucoma resulting from secondary or developmental glaucoma accounted for 203 participants (15.5%). These participants were excluded from the subsequent analysis to ensure the findings reflect only POAG, leaving 1108 patients with advanced POAG. The demographic details of this cohort are presented in Table 2. Every state and territory of Australia as well as New Zealand was represented.[32] Most (95%) of the participants were white persons of European descent. The most prevalent ancestries reported by participants were English (60%), Scottish (15%), and Irish (13%), with these 3 accounting for 74% of white participants (some participants reported more than 1 ethnic background). The mean age of individuals at the time of recruitment was 74.1 ± 12.2 years old, with the mean age of diagnosis 60.0 ± 14.7 years old. Early-age-at-onset was present in 103 subjects (9%) with advanced POAG.

Three hundred twenty subjects with nonadvanced POAG were recruited for comparison purposes. Fifty-eight percent of these cases were women and 42% were men, with most being white (98%). The mean age of the nonadvanced POAG cohort at the time of recruitment was 68.9 ± 12.9 years old, and the mean age at diagnosis was 60.1 ± 13.5 years old.

For the advanced POAG cohort, the mean cup-to-disc ratio (CDR) in the most affected eye was 0.92 ± 0.09, the mean highest recorded IOP in the most affected eye was 26.8 ± 10.0 mmHg and the mean central corneal thickness was 514.1 ± 42.6 μm. High-tension glaucoma (defined as a maximum recorded IOP >21 mmHg in at least 1 eye) was present in 68%, and filtration surgery had been performed in at least 1 eye in 56%. Family history of glaucoma was present in 58% of the participants with advanced POAG recruited through the ANZRAG excluding the GIST, and 88% of these had a first-degree affected relative. The mean age at diagnosis in the group with a family history of glaucoma was 55.1 ± 14.3 years old versus 63.3 ± 13.9 years old in the group with no family history of glaucoma (P<0.001).

A total of 1060 participants with advanced POAG completed exon 3 *Myocilin* gene screening, including 103 participants with early-age-at-onset screened for all 3 exons. No pathogenic mutations were identified in exon 1 or 2 in the 103 early-age-at-onset cases. Forty-five of the advanced POAG patients were found to carry a pathogenic *Myocilin* mutation in exon 3, representing 4.2% of our cohort, compared with 5/320 of the nonadvanced POAG patients (1.6%, P = 0.02). When combining the advanced and

nonadvanced glaucoma cases, *Myocilin* mutations were present in 3.6%. Of the 103 participants with early-age-at-onset advanced POAG (diagnosed at younger than 40 years) screened for the *Myocilin* gene, 16 had a mutation in exon 3 (17%).

The mean age at diagnosis for advanced *Myocilin* POAG patients was 46.2 ± 17.8 years old compared with 60.9 ± 14.3 years old for advanced non-*Myocilin* POAG patients (P<0.001; Fig 1). The prevalence of *Myocilin* mutations was correlated inversely with the age at diagnosis (Table 3). The peak IOP in the worst eye for advanced *Myocilin* POAG patients was 33.1 ± 10.1 mmHg, which was significantly higher than the peak IOP in the worst eye for advanced non-*Myocilin* POAG patients (26.5 ± 9.9 mmHg, P<0.001), and 89% of advanced *Myocilin* POAG patients had high-tension glaucoma. The prevalence of *Myocilin* mutations increased with increasing peak IOP (Table 3). Among advanced *Myocilin* POAG participants recruited directly through the ANZRAG, 80% reported having at least 1 relative affected with glaucoma. The prevalence of *Myocilin* mutations in the group with a positive family history was 4.6% compared with 1.6% in the group with no family history of glaucoma (P = 0.02). The prevalence of *Myocilin* mutations also increased with the number of affected relatives (Table 3). Figure 2 shows the prevalence of mutations according to age at diagnosis, highest IOP, family history, or any combination of these 3 features. The prevalence of *Myocilin* mutations in advanced POAG patients with high-tension glaucoma (>21 mmHg), onset before 50 years of age, and at least 2 affected family members was 16%, compared with 4.2% in unselected advanced patients (P = 0.03) or with 1.9% in patients meeting none of these 3 criteria (P = 0.01). Applying even more stringent filters in the advanced POAG group, the group with at least 2 affected relatives, a maximum recorded IOP of 35mmHg or more, and an age a onset of 35 years or younger had a 40% chance of having a *Myocilin* mutation. This combination of clinical features is relatively uncommon, occurring in 1.3% of the ANZRAG participants with advanced POAG.

Twenty-eight of the 45 pathogenic mutations (62%) identified in the advanced POAG cohort were the previously well-studied Gln368X (rs74315329) variant,[25] the most common mutation reported so far.[24] Other mutations identified included Gly252Arg (rs74315341),[37] Trp286Arg, Gly367Arg (rs74315334), Pro370Leu (rs74315330), Trp373X, Thr377Met, Asp380Gly, Ala445Val (rs140967767), and Pro481Ser. All mutations had been reported (www.myocilin.com; accessed March 9, 2012), except Trp373X, which was novel. The clinical features associated with mutations identified are presented in Table 4. Two variants of uncertain pathogenicity also were identified: Thr353Ile (1058C>T, rs137853277), already reported, and a novel variant, Ala447Thr (1339G>A), both of which were identified in 1

patient each. All the polymorphisms identified are shown in Table 5 (available at http://aaojournal.org).

So far, 50 family members have contacted the authors requesting testing for the familial *Myocilin* mutation. In line with expectations, 26 were found to carry the familial mutation. The mean age for those individuals found to carry the familial mutation was 41.3 ± 14.2 years old (range, 20-76 years old). Seventeen had never seen an eye practitioner and 19 were unaware of having glaucoma. One patient had been treated by an ophthalmologist with laser, but subsequently was lost to follow-up. This individual subsequently required trabeculectomy surgery for uncontrolled IOP with progressive field loss. The remainder had seen an eye practitioner because of their family history of glaucoma or their own previously made diagnosis of glaucoma.

DISCUSSION

The ANZRAG is the largest genetic repository of selected individuals with severe glaucoma. This is the first study to report the prevalence of *Myocilin* mutations in a population of only advanced POAG patients. The combined results of subjects with advanced and nonadvanced POAG show a similar prevalence of *Myocilin* mutations to what has been reported previously in this and other white populations (3.6%).[10, 17-20] However, this study showed for the first time that the prevalence of *Myocilin* mutations in advanced POAG patients (4.2%) is significantly higher (P = 0.02) than in nonadvanced POAG patients (1.5%) in the Australian population. The data demonstrate that *Myocilin* mutations are associated with severe, potentially blinding glaucoma with a prevalence 3 times what is found in nonsevere glaucoma cases.[21] One might have expected the prevalence to be even higher in a cohort of advanced glaucoma cases. However, *Myocilin* patients respond well to treatment and are less likely to develop severe glaucoma if diagnosed and appropriately managed early. Furthermore, there is a heightened awareness in families with multiple relatives affected with glaucoma (who are more likely to carry *Myocilin* mutations), and a stronger family history can reduce the likelihood of late diagnosis, which in turn is associated with advanced disease.[38] The finding that 95.8% of advanced POAG is not explained by *Myocilin* mutations highlights the role of other genetic factors, some of which we have shown to be common susceptibility variants such as *CDKN2B-AS1* and *TMCO1*,[15] and others as yet unidentified, in the most severe cases of POAG. The genetic makeup of white persons in Australia results from more than 2 centuries of continuous migration from Europe, with a predominance of English, Scottish, and Irish backgrounds. Therefore, these findings could

be extrapolated to other white populations because there are not strong founder effects in the Australian population as a whole. It would be interesting to test cohorts with advanced POAG from different ethnic backgrounds to see if *Myocilin* may account for a similar prevalence of severe cases.

Myocilin mutations have been reported to be associated with high IOP, early-age-at-onset, and a strong family history.[17-18, 21, 25] In the present cohort, the peak IOP in the worst eye of *Myocilin* patients was significantly higher than in non-*Myocilin* patients, and 89% demonstrated high-tension glaucoma. Some of the ANZRAG participants had been diagnosed many years previously, and as such, records of the initial presenting IOP may no longer exist. This could account for the cases not meeting the definition of high-tension glaucoma. The present results show that *Myocilin* patients are diagnosed at a significantly younger age than those with no *Myocilin* mutation. The prevalence of *Myocilin* mutations is substantially higher in patients with a family history of glaucoma. Eighty percent of the present *Myocilin* patients have a positive family history, which is similar to that reported previously.[18, 39] Conversely, one-fifth of *Myocilin* patients do not know of any affected member with glaucoma at their time of diagnosis. This can be explained by the following factors: (1) some families may be too small to have any affected relatives, (2) affected family members may be deceased or not in contact, (3) participants may not be aware of the diagnosis in their family, and (4) because *Myocilin* mutations show age-dependent penetrance, some relatives may be too young to demonstrate glaucoma yet. In conclusion, these results show a positive correlation between the prevalence of *Myocilin* mutations and the peak IOP in the worst eye, a positive correlation with the number of glaucoma-affected relatives, as well as a negative correlation with the age at diagnosis in patients with advanced POAG.

Interestingly, screening patients with advanced glaucoma, with more than 2 affected relatives, with a maximum recorded IOP of 35mmHgor more, and wit an age at onset of 35 years or younger yields a 40% chance of having a *Myocilin* mutation. Even with less stringent criteria like high-tension glaucoma (>21 mmHg), onset before 50 years of age and at least 2 affected family members, the prevalence of *Myocilin* mutations was substantially higher (16%) than in unselected advanced POAG patients (4.2%). These factors can be taken into account when one considers *Myocilin* screening for a particular patient. According to the present data, the strongest predictor for having a *Myocilin* mutation is the age at onset, which is in accordance with the fact that *Myocilin* mutations are much more frequent among early-age-at-onset POAG cases.[21-23] Family history alone is not a sufficiently strong

predictor because of the many familial cases explained by other genes. However, one should not exclude the possibility of a *Myocilin* mutation in a glaucoma patient based on the absence of a positive family history because, as discussed above, *Myocilin* patients are not always aware of having glaucoma-affected relatives. The implications for blindness prevention are likely to be higher for families identified with *Myocilin* mutations who previously were unaware of the familial nature of glaucoma. In contrast, the age at diagnosis and the highest recorded pressure are more reliable predictive factors and should be available for any glaucoma patient.

According to the *Myocilin* gene database (www.myocilin.com; accessed March 9, 2012), 97% of the mutations lie in exon 3. One hundred three samples were screened for exons 1 and 2, and no pathogenic variant was identified in these exons. It seems that pathogenic mutations in these 2 exons are not common in the Australian population. The samples were not screened for copy number variants in the *Myocilin* gene because this mechanism has not yet been implicated for *Myocilin* glaucoma, and POAG is known not to result from *Myocilin* haploinsufficiency[40-41]. In addition, a recent study published results on 400 POAG patients and found no copy number variants in *Myocilin*[42].

All the mutations identified in our cohort lie in exon 3 of the *Myocilin* gene, most of which have been previously reported. Trp373X is a novel pathogenic mutation and was identified in a patient with a positive family history. Another new variant was identified in 1 patient (Ala447Thr). At the moment, there is insufficient evidence to confirm its pathogenicity, and its interpretation therefore must remain equivocal. The most commonly identified mutation in the cohort was Gln368X, and this mutation has been reported consistently in individuals from white (European, North American and Australian), Hispanic, and black descent.[17-19, 21, 39, 43] In these populations, Gln368X accounts for 28% to 100% of the identified mutations. This study showed that the Gln368X mutation is the most common *Myocilin* mutation among the Australian population (62%), which is in accordance with a previous study reporting a prevalence of 72% in a smaller Australian cohort of unselected severity.[19] Previous studies from Australia and Canada showed that the Gln368X mutation is derived from a common founder,[19, 44] which is likely to explain its high prevalence among white persons.

Considering that half of the individuals affected with glaucoma are not aware of it, the main challenge with glaucoma is to identify the at-risk individuals before they demonstrate visual loss. Previous work from the GIST suggested that individuals with a family history of glaucoma have more severe glaucoma.[45] Thus, cascade genetic testing in *Myocilin* families

is a good approach to prevent glaucoma blindness in at-risk individuals. In a previous study, Healey et al. showed that predictive testing for *Myocilin* was acceptable when appropriate guidelines are followed and genetic counseling is provided.[35] The present study again demonstrated that the identification of presymptomatic *Myocilin* individuals by cascade genetic testing is feasible and acceptable. Participants are counseled that a positive result does not in themselves determine the age at onset, the severity, or the progression of the condition, but puts them at a very high risk of having glaucoma in their lifetime. As a consequence, increased surveillance and early intervention are beneficial regardless of their age. Furthermore, the authors implemented a recall system for presymptomatic carriers to minimize the risk of future loss to follow-up. Negative results indicate that their risk of having glaucoma is approximately similar to the population risk, and it is recommended that they follow routine population screening. Pedigrees need to be analyzed carefully, because multiple genes or genetic factors may segregate in some large families, and the risk of individuals not carrying the familial *Myocilin* mutation may be higher than in the general population in these specific families. Of the 26 individuals found to be *Myocilin* mutation carriers who were identified as first-degree relatives of individuals in this study, 73% had not been diagnosed with POAG, and of these, 79% had never seen an eye professional, despite their markedly increased risk of developing POAG. These young at-risk individuals (usually those younger than 40 years) now can benefit from early management and intervention to reduce glaucoma blindness. Recent animal studies showed that topical ocular 4-phenylbutiric acid can reduce glaucomatous phenotype in mice carrying a *Myocilin* glaucoma-associated mutation[46]. The breakthrough of treatments for POAG patients with a *Myocilin* mutation emphasize ever more the need to identify at-risk individuals in glaucoma families with *Myocilin* mutations.

This was a population-based, which means that some participants may be from the same family. The authors previously reported that the *Myocilin* Gln368X mutation has a common genetic origin, even when ascertained in unrelated Australian and Canadian families[44, 47]. As a result, all patients carrying Gln368X mutation in fact are distantly related. However, families with phenocopies have been reported, which means that glaucoma in affected relatives can be caused by different genetic factors[25, 37]. The recruitment of relatives affected by POAG was made regardless of genetic results, and *Myocilin* mutations account for only 4.6% of advanced POAG patients reporting a positive family history of glaucoma. The authors do not believe that the recruitment of affected relatives created a significant bias, but acknowledge the limitation of this design. The authors believe that this limitation is preferable to an artificial exclusion of affected relatives who do indeed contribute to the disease burden

of advanced glaucoma in our region.

In summary, data from the ANZRAG have established that *Myocilin* mutations are 3 times more prevalent among advanced POAG cases than no-advanced POAG cases in the Australian population. This study confirms the association between *Myocilin* mutations and phenotype, including young age at onset, high IOP, and a strong family history. In selected groups based on these 3 variables, *Myocilin* mutations can be identified in up to 40% of advanced glaucoma cases. Cascade genetic testing for *Myocilin* mutations is offered as part of the ANZRAG and has proven to be effective at identifying presymptomatic individuals, most of whom had never seen an eye professional. This should reduce glaucoma blindness in families with *Myocilin* glaucoma and reduce the overall population disease burden.

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Figure 1: Mean age at diagnosis in individuals with advanced primary open-angle glaucoma according to their *Myocilin* mutation status. *** P<0.001

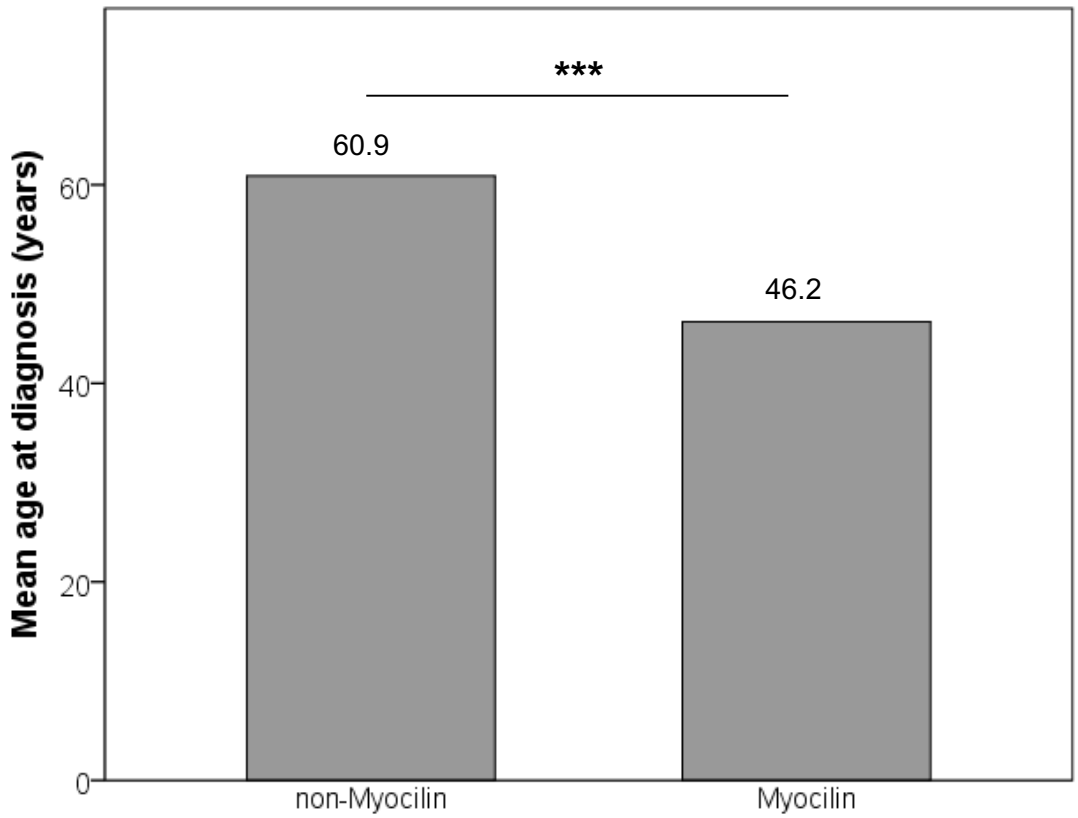
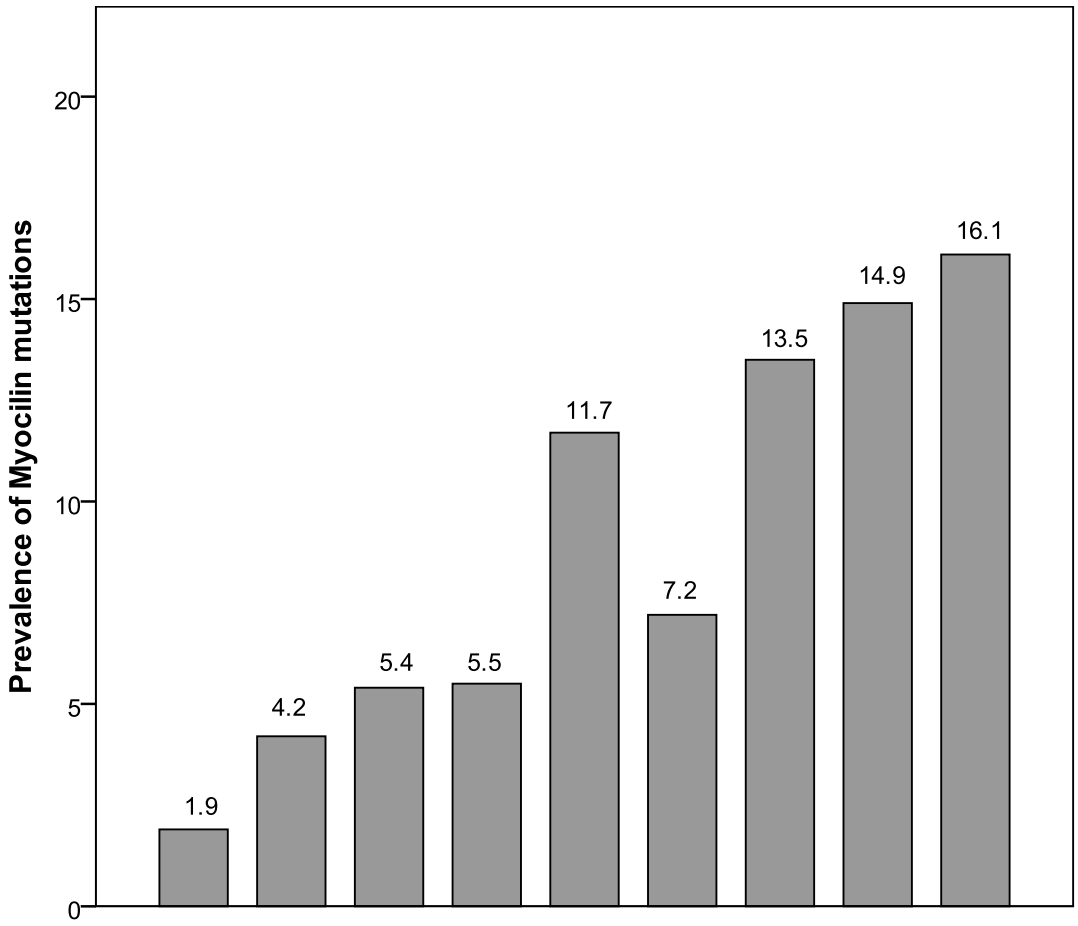


Figure 2: Prevalence of *Myocilin* mutations in individuals with advanced primary open-angle glaucoma in relation to age at diagnosis, peak intraocular pressure and family history of glaucoma.



>2 affected relatives	**-**	Unselected	**-**	**+**	**-**	**+**	**+**	**-**	**+**
Age of diagnosis <50 years old	**-**		**-**	**-**	**+**	**-**	**+**	**+**	**+**
Peak intraocular pressure >21mmHg	**-**		**+**	**-**	**-**	**+**	**-**	**+**	**+**

Table 1: Primers used for amplification of the *Myocilin* gene.

Exons	Primer sequence (forward/reverse)	Size (bp)
1.1	5'-CACCTCTCAGCACAGCAGAG-3'	478
	5'-GTAGGCAGTCTCCAACTCTCTG-3'	
1.2	5'-CCATGTCAGTCATCCATAACTTAC-3'	505
	5'-TAGGAGAAAGGGCAGGCAG-3'	
2	5'-CAACATAGTCAATCCTTGGGC-3'	269
	5'-ATACTGATTCTCTGAACACAGCAC-3'	
3.1	5'-GGGCTGTCACATCTACTGGC-3'	555
	5'-GCTGTAAATGACCCAGAGGC-3'	
3.2	5'-GCTGAATACCGAGACAGTGAAG-3'	590
	5'-AACTTGGAAAGCAGTCAAAGC-3'	

Table 2: Demographic details of advanced primary open-angle glaucoma patients. The number of patients reflects those for which this information is available.

	Number (%)
Age at time of recruitment (years)	
<40	18 (1.6)
40-49	29 (2.6)
50-59	74 (6.7)
60-69	204 (18.4)
70-79	353 (31.9)
80-89	376 (33.9)
>89	54 (4.9)
Gender	
Female	571 (51.5)
Male	537 (48.5)
Ethnicity	
Caucasian	889 (95.4)
African	5 (0.5)
Asian	22 (2.4)
Australian Aboriginal	2 (0.2)
Hispanic	3 (0.3)
Mixed Ethnicity	11 (1.2)
Country and State of residence	
Australia	1090 (98.4)
Australian Capital Territory	2 (0.2)
New South Wales	158 (14.5)
Northern Territory	11 (1.0)
Queensland	11 (1.0)
South Australia	525 (48.2)
Tasmania	281 (25.8)
Victoria	60 (5.5)
Western Australia	42 (3.8)
New Zealand	18 (1.6)

Table 3: Prevalence of *Myocilin* mutations in individuals with advanced primary open-angle glaucoma in relation to age of onset, maximum recorded intraocular pressure and family history of glaucoma.

	Prevalence of *Myocilin* mutations (%)
Age at diagnosis (years)	
<41	16.8
41-50	6.0
51-60	4.5
>60	1.4
Maximum recorded intraocular pressure (mmHg)	
<22	1.8
22-30	4.1
>30	8.7
Number of affected relatives	
0	1.6
1-2	4.1
>2	5.8

Table 4: Clinical features of advanced primary open-angle glaucoma patients with *Myocilin* mutations. IOP: intraocular pressure, RE: right eye, LE: left eye, NA: not available

Identifier	Mutation	Ethnicity	Age at diagnosis (years)	Family history of glaucoma	Highest IOP (mmHg) RE/LE
AG0021	Gln368X	Caucasian	16	Yes	45/26
AG0093	Gln368X	Caucasian	76	No	21/21
AG0107	Gln368X	Caucasian	47	Yes	NA/23
AG0136	Gln368X	Caucasian	32	Yes	37/19
AG0242	Gln368X	Caucasian	57	No	35/35
AG0301	Gln368X	Caucasian	71	Yes	40/46
AG0315	Gln368X	Caucasian	40	Yes	42/21
AG0542	Gln368X	Caucasian	NA	No	20/20
AG0633	Gln368X	Caucasian	53	Yes	18/18
AG0697	Gln368X	Caucasian	41	Yes	48/38
AG0720	Gln368X	Caucasian	55	No	22/22
AG0792	Gln368X	Caucasian	40	Yes	28/25
AG0857	Gln368X	Caucasian	60	Yes	27/27
AG0982	Gln368X	Caucasian	58	Yes	31/26
AG0984	Gln368X	Caucasian	33	Yes	28/34
AG0985	Gln368X	Caucasian	70	Yes	33/25
AG1110	Gln368X	Caucasian	62	NA	50/50
AG1111	Gln368X	Caucasian	49	NA	40/21
AG1139	Gln368X	Caucasian	46	NA	40/40
AG1141	Gln368X	Caucasian	49	NA	50/27
AG1176	Gln368X	Caucasian	77	Yes	27/24
AG1315	Gln368X	Caucasian	NA	Yes	30/42
AG1335	Gln368X	Caucasian	54	Yes	23/25
AG1408	Gln368X	Caucasian	55	Yes	20/20
AG1432	Gln368X	Caucasian	55	No	28/29
AG1485	Gln368X	Caucasian	NA	Yes	44/NA
AG1486	Gln368X	Caucasian	87	Yes	21/30
AG1487	Gln368X	Caucasian	67	Yes	40/40

AG1123	Gly252Ala	Caucasian	36	Yes	30/30
AG1205	Gly252Ala	Caucasian	32	Yes	12/10
AG1491	Gly252Ala	Caucasian	57	Yes	25/27
AG0229	Trp286Arg	Caucasian	57	No	24/19
AG0629	Gly367Arg	Caucasian	13	Yes	40/40
AG1458	Gly367Arg	Caucasian	14	Yes	NA/NA
AG1191	Pro370Leu	Caucasian	21	Yes	50/48
AG1192	Pro370Leu	Caucasian	27	Yes	27/27
AG0868	Trp373X	Caucasian	70	Yes	20/33
AG0603	Thr377Met	Caucasian	45	Yes	39/38
AG1144	Thr377Met	Caucasian	41	Yes	25/25
AG1145	Thr377Met	Caucasian	36	Yes	48/30
AG1146	Thr377Met	Caucasian	42	Yes	24/24
AG1148	Thr377Met	Caucasian	38	Yes	24/24
AG1156	Asp380Gly	Caucasian	28	NA	44/10
AG0063	Ala445Val	Caucasian	48	Yes	22/17
AG1356	Pro481Ser	Caucasian	17	Yes	40/40

Table 5: Polymorphic sequence changes identified in the *Myocilin* gene for primary open-angle glaucoma patients recruited directly from the Australian and New Zealand Registry of Advanced Glaucoma.

Location	rs number	Nucleotide change	Coding effect	Frequency (%)
Exon 1	rs12082573	39T>G	Pro13Pro	0.14
Exon 1	rs2234926	227G>A	Arg76Lys	17.71
Exon 1	rs61730977	477A>G	Leu159Leu	0.14
Intron 1	rs113416006	604+50G>A		0.14
Intron 2	rs2032555	730+35G>A		57.29
Exon 3	rs79255460	731-73C>T		3.58
Exon 3	rs146606638	855G>T	Thr285Thr	0.57
Exon 3	rs61730976	975G>A	Thr325Thr	0.43
Exon 3	rs61730974	1041T>C	Tyr347Tyr	3.73
Exon 3	rs56314834	1193A>G	Lys398Arg	1.15
Exon 3	-	*52G>T		0.14
3'UTR	rs74403899	*73G>C		0.14

2.3. Characterisation of *MYOC* deleterious variants

Characterisation of genetic variants and genotype/phenotype correlations assist in the accurate interpretation of genetic results and improve prognosis for counselling purposes. Here are three studies describing the phenotype associated with novel *MYOC* variants, or novel combination of *MYOC* variants. These findings allowed the families to be counselled adequately regarding the mode of inheritance and risk prediction (recurrence or occurrence) in the family.

In the first study, I described the first co-occurrence of the two most common *MYOC* deleterious variants in a family: p.Gln368Ter and p.Thr377Met *MYOC* variants were identified in four siblings, three of which were diagnosed with JOAG or ocular hypertension before the age of 35 years. The results indicated that the two variants were on different alleles. The mother carried the p.Gln368Ter variant while the father could not be tested. However, he and eight members of his family had POAG suggesting an autosomal dominant inheritance, and his family was from a region in Croatia where the p.Thr377Met variant is prevalent [372], suggesting the variant might have been inherited from the father. These two variants are well characterised *MYOC* deleterious variants: p.Gln368Ter and p.Thr377Met cause adult-onset glaucoma with a mean age at diagnosis of 53 and 42 years respectively [169,173]. In this family, individuals carrying both variants were diagnosed before 35 years old, indicating a more severe phenotype associated with the combination of both variants than each variant alone. The characterisation of the phenotype associated with these two *MYOC* variants allowed for accurate genetic counselling of the different members of the family and would benefit future families carrying the two most common *MYOC* variants.

Contribution statement

Ms Souzeau was primarily responsible for the conception of the study, the data analysis and interpretation, the provision of genetic counselling, drafting and revising the manuscript. Dr Young was responsible for characterising the family, drafting and submitting the manuscript as corresponding author. Dr Liu and Mrs Kearns contributed to the characterisation of the family and revised the manuscript. A/Prof Burdon contributed to the data analysis and revised the manuscript. Prof Craig contributed to the conception of the study, data analysis and revised the manuscript. Dr Ruddle contributed to recruiting and characterising the family, data analysis and revised the manuscript.

In the second study, I reported a novel *MYOC* nonsense variant likely to be deleterious. The p.(Trp373Ter) was identified in two affected siblings from an Australian family. The phenotype was similar to the p.Gln368Ter variant with an age at diagnosis in the fifties, and high IOPs requiring surgical intervention for control. The interpretation of the variant and its classification as deleterious had clear benefits for the family members through the provision of genetic counselling and the availability of predictive genetic testing. Three asymptomatic family members subsequently benefited from cascade genetic testing.

Contribution statement

Ms Souzeau was responsible for the conception of the study, collected and interpreted the data, provided genetic counselling, drafted, revised and submitted the manuscript as corresponding author. Dr Crawford contributed to characterising the family, analysing the data and drafting the manuscript. Dr Agar characterised the participants and revised the manuscript. Ms Ridge recruited the family in the study and revised the manuscript. Dr Dubowsky was responsible for the molecular genetic studies, the interpretation of the results and revised the manuscript. A/Prof Burdon participated in the design of the study, data analysis and revised the manuscript. Prof Craig participated in the design of the study and data analysis, characterised the participants and revised the manuscript.

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In the third study, I reported a novel and *de novo MYOC* variant. The variant was considered deleterious based on the evidence and was present in the individual with sporadic JOAG but neither of his unaffected parents. This is the second report of a *de novo* deleterious variant in *MYOC* and it is still unknown if this mechanism occurs more often. The identification of the *MYOC* variant allowed for genetic counselling regarding the mode of transmission and the risk of recurrence in the family. The siblings of the affected individual were tested for the *MYOC* variant to exclude a recurrence risk due to the possibility of germline mosaicism, but none carried the variant.

Contribution statement

Ms Souzeau was responsible for designing the study, collecting and interpreting the data, providing genetic counselling, drafting and submitting the manuscript as corresponding author. A/Prof Burdon participated in the data analysis and revised the manuscript. Ms Ridge recruited the family in the study and revised the manuscript. Dr Dubowsky was responsible for the molecular genetic studies, the interpretation of the results and revised the manuscript. Dr Ruddle characterised the family and revised the manuscript. Prof Craig participated in the design of the study, data analysis and revised the manuscript.

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Compound heterozygote myocilin mutations in a pedigree with high prevalence of primary open-angle glaucoma

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Purpose: To describe the phenotype of ocular hypertension and primary open-angle glaucoma in a family with individuals compound heterozygote for Gln368STOP and Thr377Met *myocilin (MYOC)* mutations.
Methods: Family members of the proband underwent comprehensive ocular clinical examination and DNA sequencing for *MYOC* mutations.
Results: A 34-year-old woman with marked ocular hypertension was found to carry Gln368STOP and Thr377Met *MYOC* mutations. Three other siblings carried both mutations, while one carried Gln368STOP alone. Three of five siblings had received treatment for ocular hypertension or early glaucoma, with the average age of diagnosis 28 years; one required trabeculectomy at age 27. The mother of the proband was found to be a carrier for Gln368STOP alone, which indicates that her offspring with both Gln368STOP and Thr377Met carry variants on opposing alleles.
Conclusions: This pedigree is the first report with individuals compound heterozygote for the two most common glaucoma-causing *MYOC* variants. The combination of mutations manifests a more severe phenotype than either alone. Identification of gene changes associated with glaucoma within the family has enabled unaffected members to stratify their risk of future disease and institute closer monitoring and early treatment.

Primary open-angle glaucoma (POAG) is a complex genetic disease and one of the most common causes of visual loss worldwide. Mutations in the myocilin gene (*MYOC*, formerly known as the trabecular meshwork-induced glucocorticoid response gene) associated with POAG were discovered in 1997 and mapped to the long arm of chromosome 1 [1]. *MYOC* variants account for almost 4% of adult POAG cases, and 10% of juvenile open-angle glaucoma [2].

POAG attributable to *MYOC* gene changes is inherited in an autosomal dominant manner. Carriers tend to display elevated intraocular pressure (IOP) or open-angle glaucoma from an early age, although there may be variability in the phenotype depending on the underlying mutation [2]. Two of the most common glaucoma-causing variants of *MYOC* worldwide are Gln368STOP and Thr377Met [3]. As with most disease-causing mutations of this gene, both occur in exon 3 [4]. We describe a pedigree that to our knowledge is the first identified with individuals compound heterozygous for these *MYOC* mutations.

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METHODS

Members of a family with high prevalence of glaucoma were recruited into the study. Six individuals living in Australia, comprising 4 males and 2 females were available for direct clinical examination and genotyping. Five of the patients were siblings aged 26 to 35 years. The proband's mother age 63 was also assessed. This study was approved by the Human Research and Ethics Committee of the Royal Victorian Eye and Ear Hospital, Melbourne, and was conducted in accordance with the revised Declaration of Helsinki. Informed patient consent was obtained before enrolment.

The proband (patient V:3) was first diagnosed with ocular hypertension by her optometrist. She was referred to the Australian and New Zealand Registry of Advanced Glaucoma (ANZRAG) [5] for *MYOC* genetic testing due to her young age and strong family history of POAG. Following identification of the mutations, additional family members were ascertained and offered genetic testing through ANZRAG after providing signed consent and a blood sample.

First-degree relatives of the index case (patient V:3) lived in Australia and were available for direct assessment, except the father (patient IV:8), who was deceased. Relatives on the father's side lived in Croatia and were not available for examination. Comprehensive medical and family history was taken by a glaucoma subspecialist ophthalmologist (JBR). Clinical

details for disease-affected family members were obtained from their medical records, while unaffected individuals were invited to present for ocular examination. Data recorded included demographic details, general medical history, past ocular history, best-corrected visual acuity (BCVA), Goldmann IOP, gonioscopy, dilated fundus examination, central corneal thickness (Pachmate DGH55, DGH Technology Inc., Exton, PA), and Humphrey visual fields (HFA II, Carl Zeiss, North Ryde, Australia). For each patient, venous blood was collected by peripheral venepuncture in 2×10 ml EDTA tubes. The blood samples were stored at 2–8 °C before processing.

The criterion for ocular hypertension was IOP on repeated measurement ≥24 mmHg. A diagnosis of POAG was made in patients with glaucomatous visual field defects on a reliable Humphrey 24–2 field, including an enlarged cup-disc ratio (≥0.7) or cup-disc ratio asymmetry (≥0.2) between both eyes.

Genotyping: The testing was performed through the National Association of Testing Authorities (NATA) accredited laboratories of the Institute of Medical and Veterinary Science (IMVS) Pathology at the Flinders Medical Centre (Bedford Park, Australia). Genomic DNA was prepared from a 200 µl sample of venous blood and extracted using an Illustra Blood Genomic Prep Mini Spin kit (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's protocols.

Each PCR was performed using 100 ng of purified genomic DNA as the template in a reaction mix containing 1.5 mM $MgCl_2$, 200 µM of each deoxynucleoside triphosphate (dNTP), 1 U of Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA), 1x Platinum Taq PCR reaction buffer, and 0.5 µM of each primer (for exon 3: 3.1F: 5′-GGG CTG TCA CAT CTA CTG GC-3′, 3.1R: 5′-GCT GTA AAT GAC CCA GAG GC-3′; 3.2F: 5′-GCT GAA TAC CGA GAC AGT GAA G-3′, and 3.2R: 5′-AAC TTG GAA AGC AGT CAA AGC-3′), in a final volume of 25 µl. Samples were denatured for 5 min at 94 °C and then incubated for 15 cycles under the following conditions: 94 °C for 30 s, 61 °C for 50 s (reduced 1 °C every five cycles), and 72 °C for 60 s. The samples were then incubated for 35 cycles under the following conditions: 94 °C for 30 s, 58 °C for 50 s, and 72 °C for 60 s. The last elongation step was at 72 °C for 5 min on a Veriti thermal cycler (Life Technologies, Carlsbad, CA).

PCR amplicons were prepared for DNA sequencing with the ExoSAP method using a 10 µl sample of each PCR reaction treated with 5 U of Exonuclease I (New England Biolabs, Ipswich, MA) and 1 U of Shrimp Alkaline Phosphatase (USB) to remove residual primers and deoxynucleoside triphosphate (dNTPs). Bidirectional BigDye Terminator Cycle Sequencing (Life Technologies) reactions of the appropriate template and exon 3 *MYOC* PCR primer were resolved and base called on an Applied Biosystems 3130XL Genetic Analyzer (Life Technologies).

Detection of sequence variants was performed with the Mutation Surveyor v3.10 (SoftGenetics LLC, State College, PA) software; all forward and reverse sequence trace files for overlapping upstream and downstream PCR fragments of exon 3 were assembled by the software into a single contiguous sequence following alignment against the *MYOC* gene GenBank reference NM_000261.1. Significant differences in the relative peak heights of the sequence traces observed between that of the patient sample and a normal control were automatically called a sequence variant by Mutation Surveyor; all such calls were visually inspected for confirmation.

RESULTS

The entire pedigree of this *MYOC* glaucoma family is shown in Figure 1. There are six generations with 46 known members. The family of the proband's mother (patient IV:9) were Dutch, while the father (patient IV:8) was of Croatian ancestry.

Clinical data of examined family members are summarized in Table 1. All patients had open anterior chamber angles bilaterally with gonioscopy. The average age at diagnosis for individuals with POAG or ocular hypertension was 28 years.

The most severe phenotype is exhibited in patient V:6. This patient presented with a right ischemic central retinal vein occlusion and elevated IOP in both eyes at age 25. He subsequently developed anterior chamber angle neovascularization requiring treatment with indirect scatter laser photocoagulation and intravitreal bevacizumab. The patient's blood pressure was normal, and physician evaluation for a hypercoagulable state unremarkable. The only identified risk factor for the central retinal vein occlusion was ocular hypertension.

Fundus examination showed a cup-disc ratio of 0.5 in the right eye and 0.4 in the left with normal neuroretinal rims in both (Figure 2A). Visual field testing was normal in the left eye, but showed an arcuate loss in the right, likely due to the panretinal photocoagulation (Figure 2B).

Two years after his initial treatment, his right IOP became uncontrolled on medical treatment alone, and he proceeded to trabeculectomy with mitomycin C. His IOP is also poorly controlled in the left eye despite the use of three antiglaucoma medications, and glaucoma surgery has been recommended.

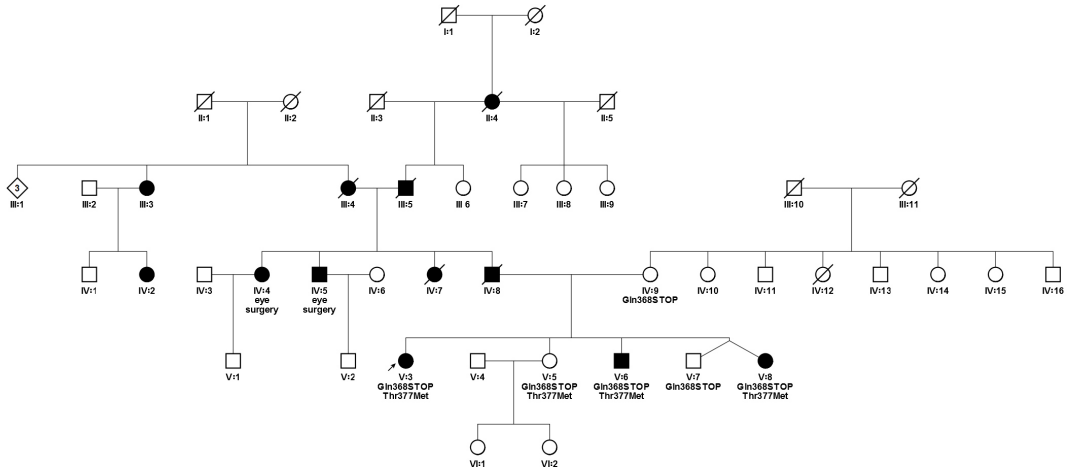

Figure 1. Pedigree chart with Gln368STOP and Thr377Met *MYOC* mutations. Round symbols indicate women; square symbols, men; diamond, gender unspecified; fully filled symbols, primary open-angle glaucoma; unfilled symbols, unaffected; diagonal line, deceased; arrow, proband.

The proband's mother had no evidence of ocular hypertension or glaucoma at age 63. Her seven siblings (ranging from 59 to 73 years old) have refused examination, but are all believed to be unaffected.

The proband's father was diagnosed with glaucoma at age 45 years (patient IV:8). On this side of the family, nine of 28 individuals were known to have POAG or thought to have glaucoma based on reports of previous eye surgery.

Analysis of the *MYOC* gene in the proband (patient V:3) showed a heterozygous C>T substitution at nucleotide 1102 in exon 3, predicted to generate a nonsense codon in place of the normal glutamine codon at position 368 (Gln368STOP). In addition, a second DNA sequence variant was discovered: a heterozygous C>T substitution at nucleotide 1130 in exon 3, predicted to generate a missense substitution of methionine for threonine normally present at codon 377 (Thr377Met).

Four of five children were carriers of both Gln368STOP and Thr377Met (Figure 1). Of these, three had already been diagnosed with glaucoma or ocular hypertension (ages 25, 26, and 34).

Genotyping could not be performed on the proband's father as the patient was deceased. The unaffected mother (patient IV:9) was found to carry the Gln368STOP mutation. One of her brothers was screened for the Gln368STOP mutation and does not carry it. The rest of her siblings have not requested genetic testing.

DISCUSSION

To date, more than 80 disease-causing *MYOC* variants have been identified [3]. Gln368STOP and Thr377Met are the two most common worldwide [4]. We present here the first report of glaucoma-affected individuals carrying both variants. In this pedigree, the proband's mother (patient IV:9, Figure 1) carries the Gln368STOP mutation alone; therefore, we concluded that the Thr377Met variant was passed down from the proband's father (patient IV:8) and that children with both would carry mutations on opposing alleles.

The clinical features of *MYOC* glaucoma reflect the underlying mutation. The proband's father (patient IV:8) was diagnosed with POAG at the age of 45. Glaucoma patients with Thr377Met usually have a disease of intermediate severity with an age at diagnosis of 41.6±13.2 years and a mean maximum IOP of 32.5±10 mmHg [6]. The same mutation has been described in the isolated Croatian village of Veli Brgud, whose population has an unusually high prevalence of early-onset glaucoma [7]. The family of patient IV:8 was of Croatian background and came from a village 10 km from Veli Brgud. It is likely that they share a common genetic basis. Hewitt et al. previously reported that families from Greece, the USA, and Australia, all known to be of Greek or Macedonian ethnicity, shared a common haplotype [6].

The severity of phenotype in Gln368STOP carriers is variable, ranging from ocular hypertension to advanced glaucomatous neuropathy with severe visual field loss [8].

Table 1. Summary of known clinical features of the affected family members

Pedigree number	Age	Sex	Age at diagnosis	BCVA		Highest recorded IOP (mmHg)		Centralcorneal thickness (µm)		Visual field defect		Cup/Disc Ratio		IOP treatment	MYOC mutations
				OD	OS	OD	OS	OD	OS	OD	OS	OD	OS		
V:3	35	F	34	6/6	6/6	37	36	569	566	N	N	0.4	0.3	Latanoprost OU	Gln368STOP/ Thr377Met
V:5	34	F	-	6/6	6/6	20	18	567	566	N	N	0.4	0.4	-	Gln368STOP/ Thr377Met
V:6	29	M	26	6/18	6/5	54	29	582	584	Y	Y	0.5	0.4	3 anti-glaucoma agents OS Trabeculectomy OD age 27	Gln368STOP/ Thr377Met
V:7	26	M	-	6/5	6/6	19	19	552	556	N	N	0.2	0.2	-	Gln368STOP
V:8	26	F	25	6/5	6/5	41	37	547	547	N	N	0.4	0.7	Bimatoprost OU	Gln368STOP/ Thr377Met
IV:9	63	F	-	6/9	6/9	17	16	566	561	N	N	0.5	0.5	-	Gln368STOP

BCVA: Best Corrected Visual Acuity, IOP: Intraocular Pressure, OD: Right eye, OS: Left eye, OU: Both eyes.

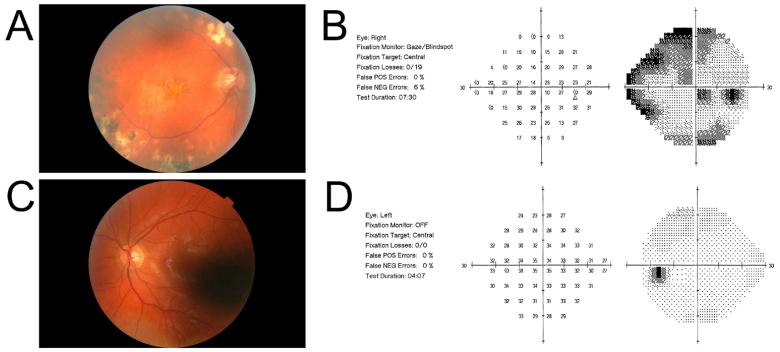

Figure 2. Fundus photographs for Patient V:6. The cup/disc ratio is 0.5 in the right eye (**A**) and 0.4 in the left eye (**B**). Humphrey visual fields for the same patient show a right eye visual field defect due to previous panretinal photocoagulation (**C**).

The mean maximum IOP for affected patients carrying Gln368STOP is 29.5±4.5 mmHg, and the mean age at diagnosis is 53±10.5 years [4]. The age at diagnosis may vary considerably; newly diagnosed patients ranging from age 32 to 80 years have been described [4,8]. The proband's mother carried Gln368STOP alone, and was unaffected at 63 years. As the penetrance at this age lies somewhere between 49% and 98%, this is not surprising [4].

Individuals with both Gln368STOP and Thr377Met appear more likely to develop ocular hypertension or glaucoma from an earlier age than carrying either mutation alone would predict. The three affected individuals carrying both mutations were diagnosed between 25 and 35 years old. The penetrance at 25 years is low for both mutations (3% for Thr377Met and 1% for Gln368STOP) [4]. Juvenile affected carriers of Thr377Met have been described [9]. However, the father carrying Thr377Met in our pedigree was diagnosed with POAG at age 45, so we feel it is less likely Thr377Met al.one is responsible for the young age at diagnosis in the children. Detailed clinical and genotype data for affected members on the father's side would be of considerable interest, but were unobtainable.

The only patient with both mutations without ocular hypertension was 34 years old at the time of review. It is highly probable she will develop glaucoma in the future, and therefore early treatment was offered and regular reviews scheduled.

To the best of our knowledge, there is only one other report of individuals carrying Thr377Met combined with another *MYOC* variant. In Greece, Thr377Met has been described in three individuals also carrying the Arg76Lys variant [10]. However, Arg76Lys is considered a neutral polymorphism [4], and in the Greek study, several control subjects carried this mutation [10]. Two patients were homozygous for Thr377Met, and demonstrated a more severe glaucoma phenotype than heterozygous cases for this variant [10]. This however was not the case for a patient homozygous for Gln368STOP, who showed no signs of glaucoma at the age of 49 [11].

Currently, the mechanism through which mutant MYOC protein contributes to the pathogenesis of glaucoma is unknown. Gln368STOP and Thr377Met are predicted to change the secondary structure of the MYOC protein [12]. Gln368STOP results in premature termination of protein synthesis [12]. Thr377Met is thought to cause the loss of phosphorylation of the Thr377 site by casein kinase II [10]. Both produce a Triton assay–insoluble protein [12]. The presence of these variants on opposing alleles would predict the assembly of aberrant heterodimeric protein, though further investigation is required to determine why this leads to a more severe phenotype.

Other rare compound heterozygote *MYOC* variants have been associated with glaucoma of earlier onset than predicted by a single mutation. An 11-year-old from Quebec with aggressive juvenile-onset glaucoma was found to be a compound Arg126Trp/Lys423Glu carrier [13]. In addition, a patient from eastern India compound heterozygote for Asn480Lys/Thr353Ile was diagnosed with glaucoma aged 14 years [14], though the Thr353Ile mutation is of uncertain pathogenicity [4].

In summary, we have described the first known pedigree containing compound heterozygotes for the two most common glaucoma-causing *MYOC* mutations, Gln368STOP and Thr377Met. Within this family, both mutations in combination predict a more severe phenotype than either in isolation. Early identification of genetic risk in unaffected family members has strengthened the case for early treatment and monitoring. Our findings will assist clinicians in providing

more suitable treatments for affected individuals and appropriate preventive management for unaffected individuals who are compound heterozygotes for these two mutations. Our findings also provide resources for genetic counselors and clinicians to educate patients about their genetic risk profiles.

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Identification of a novel *MYOC* mutation, p.(Trp373*), in a family with open angle glaucoma

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Conflict of interest

The authors of this manuscript declare that they have no conflict of interest.

ABSTRACT

MYOC gene variants are associated with autosomal dominant primary open angle glaucoma (POAG). In this study, we describe a previously unreported *MYOC* variant segregating with a POAG phenotype in an Australian family. Two individuals affected with POAG and three unaffected individuals from the same family were recruited through the Australian and New Zealand Registry of Advanced Glaucoma (ANZRAG). Direct sequencing of all *MYOC* coding exons identified the novel heterozygous single nucleotide transition MYOC:c.1119G>A, p.(Trp373*), predicted to encode an aberrant truncated MYOC protein in two affected siblings. Two unaffected siblings and an unaffected niece were negative for the *MYOC* sequence variant.

Keywords: Myocilin; POAG; glaucoma; genetic testing

INTRODUCTION

Glaucoma is the most common cause of irreversible blindness worldwide [1]. It is an optic neuropathy characterized by changes in the optic nerve head with corresponding visual field loss. Primary open angle glaucoma (POAG) is the most common subtype of glaucoma [2]. Family history of POAG is a significant risk factor, with relatives of affected patients having a 9 fold increased risk of developing the disease [2]. In Australia, 55-60% of affected individuals have an affected family member [3,4].

Glaucoma is a genetically heterogeneous disorder. Several loci have been identified in association with POAG, of which 5 genes have been isolated so far: *MYOC* [5], *OPTN* [6], *WDR36* [7], *NTF4* [8] and *TBK1* [9]. These genes only account for 10% of POAG cases. Common risk alleles of smaller effect size have also been identified through genome-wide association studies [10,11]. The *MYOC* gene, at the GLC1A locus on chromosome 1q24.3, was first discovered by linkage analysis of a large pedigree with juvenile open angle glaucoma [5,12]. Pathogenic sequence variants in the *MYOC* gene consistently cause 2-4% of adult-onset POAG among different populations [13,14]. To date more than 70 pathogenic *MYOC* sequence variants have been identified [14,15] (http://www.myocilin.com, last accessed February 2014). *MYOC* has three exons encoding a protein comprised of two major domains, the N-terminal myosin-like domain and the C-terminal olfactomedin-like domain [4]. Most disease causing variants are clustered within exon 3 in the olfactomedin domain [16]. Pathogenic variants in *MYOC* are usually associated with high intraocular pressure (IOP), younger age at diagnosis and strong family history consistent with an autosomal dominant transmission [13-15]. Although *MYOC* variants display some genotype/phenotype correlations with some variants associated with a more severe phenotype and/or younger age of onset than others, intra- and inter-family variability is usually observed. For example, the age at diagnosis for the most common variant, p.Gln368*, varies between 30 and 80 years old with a variable proportion requiring surgery to control their IOP [13,17-19]. In contrast, another common variant, p.Pro370Leu, is usually associated with a much younger age at diagnosis (5-30 years old) and a higher proportion requiring surgery [20-22]. *MYOC* pathogenic variants also display an incomplete and age-related penetrance [13,17,19,20]. The MYOC protein is expressed in most ocular tissues and is found in the aqueous humor [23]. Although the exact mechanism is still unclear, it is thought that *MYOC* pathogenic sequence variants lead to the expression and accumulation of misfolded MYOC protein in the trabecular meshwork thereby reducing outflow facility and elevating IOP [24,25].

In this report we describe the clinical findings from the study of an Australian family affected with POAG; the novel MYOC:p.(Trp373*) variant was identified in the affected individuals.

METHODS

Patient recruitment

Ethics approval was obtained from the Southern Adelaide Clinical Human Research Ethics Committee. The study followed the National Health and Medical Research Council statement of ethical conduct in research involving humans.

The index case was referred through his treating ophthalmologist to the Australian and New Zealand Registry of Advanced Glaucoma (ANZRAG) [3]. After genetic testing results were reported to the index case, the patient was asked to invite family members for genetic testing. A further four family members agreed to participate in this process and received *MYOC* genetic testing.

Genetic testing

Genetic testing was performed through the National Association of Testing Authorities (NATA) accredited laboratories of SA Pathology at the Flinders Medical Centre in Adelaide, Australia. The proband and family members were sequenced for the 3 coding exons as previously described [13]. Genomic DNA was extracted with an Illustra™ Blood Genomic Prep MiniSpin kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK) from venous blood.

Sequence variants of all 3 exons of *MYOC* and flanking intron-exon junctions were detected using Mutation Surveyor™ v3.10 (SoftGenetics LLC, State College, PA, USA) and by comparison to the *MYOC* gene GenBank reference sequence (NM_000261.1). Sequence variants were compared with known variants in the *MYOC* database which was established in 2007 (http://www.myocilin.com, last accessed February 2014) [15].

Results were provided in writing to the patient and his referring ophthalmologist. Genetic counseling was provided by a trained genetic counselor (ES). Predictive genetic testing was offered through the affected patient inviting family members to undergo genetic testing as *MYOC* glaucoma is a highly penetrant autosomal dominant disorder.

RESULTS

The pedigree of the family is shown in Fig. 1. A 53 year old Caucasian male (II-9) from rural

Australia was initially seen in 2005 with a history of elevated IOP. His ophthalmic details are described in Table 1. Past ophthalmic history included trauma to the left eye resulting in a corneal scar. He had no evidence of pseudoexfoliation syndrome or pigment dispersion. Optic disc photos and retinal nerve fiber layer analysis with optical coherence tomography were consistent with inferior retinal nerve fiber layer loss (Fig. 2). Visual field testing on the Humphrey Automated 24-2 Field Analyzer (HFA II, Carl Zeiss, North Ryde, Australia) showed a right superior nasal step and a left superior arcuate scotoma (Fig. 3). His IOPs were initially controlled with topical therapy (latanoprost and brinzolamide). The latter was ceased as the patient did not tolerate it. As he showed progressive field loss, he underwent selective laser trabeculoplasty (SLT) to both eyes. No visual field progression was observed over the following year, however his IOP continued to remain borderline so brimonidine was added to his treatment regimen but was later ceased as the patient developed follicular conjunctivitis judged to be consistent with brimonidine allergy. He was treated with a second SLT to the right eye, as he declined glaucoma surgery.

His sister (II-4) presented to her ophthalmologist at the age of 70 years with subjective blurring of vision in the left eye. Her clinical details are shown in Table 1. She had high IOP and significant visual field defects in the left eye (Figure 3). She was also found to have pseudoexfoliation of the left eye. She had previously undergone left trabeculectomy and currently is on topical therapy with latanoprost, brinzolamide and brimonidine to stabilize her glaucoma.

Two other family members (I-1 and II-2) were reported to be affected. According to the family, the proband's father (I-1) developed glaucoma in his sixties. The other affected relative (II-2) never expressed interest in being tested and no clinical details were available. The other two unaffected siblings who have not expressed interest in being tested (II-3 and II-8) were 81 and 62 years old respectively.

MYOC screening revealed a heterozygous substitution of Guanine to Adenine at nucleotide 1119 in the *MYOC* coding sequence of exon 3, (c.1119G>A), in individuals II-4 and II-9 (Fig. 4). This encodes a nonsense stop codon in place of Tryptophan at position 373, p.(Trp373*), which is predicted to result in premature termination of protein synthesis. This variant was not present in unaffected individuals II-5, II-6 and III-2. The rest of the family declined participation, including another affected sibling (II-2). No other variants were identified in the *MYOC* gene.

DISCUSSION

In this study we report the novel *MYOC* nonsense variant, MYOC:c.1119G>A, p.(Trp373*), that co-segregates with the glaucoma phenotype in a Caucasian Australian family. *MYOC* was the first gene found to be associated with POAG and is responsible for 2-4% of adult-onset cases [13,14]. There are 16 recorded DNA sequence variants predicted to result in a truncated MYOC protein in the Myocilin database, accessed February 2014 (http://www.myocilin.com). Twelve are classified as disease causing. A further 3 PTC variants in exon 3 remain of unknown clinical importance and a single nonsense change in exon 1 is listed as non-pathogenic; all 4 are located greater than 150 nucleotides from p.(Trp373*). Significantly, the most common *MYOC* pathogenic variant identified to date is a nonsense variant, p.Gln368*[15], and is located only 5 amino acids from p.(Trp373*). We can therefore expect that the deleterious nature observed for p.Gln368* will also be exhibited by a MYOC protein similarly truncated and of approximately the same size, such as p.(Trp373*), which appears to exhibit a similar POAG phenotype. The sequence variant is not reported in publicly available variation databases accessed February 2014, including dbSNP (http://www.ncbi.nlm.nih.gov/snp/) or the Exome Variant Server (http://evs.gs.washington.edu/EVS/), further suggesting that it is a pathogenic variation.

A dominant negative model for *MYOC* POAG has been proposed [24,25] whereby the mutant protein interferes with the function of the protein translated from the wild type allele. However, simple association of POAG with the presence of a heterozygous premature terminating codon (PTC) within the *MYOC* gene is not universally observed. An alternative model may be haploinsufficiency brought about by nonsense mediated decay (NMD) of the mutant allele. This may explain the pathogenicity of 4 PTC variants within exon 1 as listed in the Myocilin database although this has not been tested. There are no reports of whole *MYOC* gene deletions associated with this disease in humans suggesting that haploinsufficiency is not a common mechanism. The presence of two pathogenic PTC variants (p.Tyr453Metfs*11 and p.Glu483*) [14] in the terminal end of the final exon of the *MYOC* gene coupled with the absence of evidence for whole *MYOC* gene deletions responsible for the glaucoma phenotype suggest that mRNA transcribed from the allele containing MYOC:c.1119G>A evades the surveillance and or efficient removal by NMD likely as there are no downstream introns from these mutations. Thus the resulting predicted translation product punctuated by p.(Trp373*) would be consistent with the recently postulated dominant negative etiology for glaucoma [24].

MYOC glaucoma is associated with high IOP which is consistent with the 2 cases screened positive for p.(Trp373*) who had recorded peak IOP of 39 mmHg in the right eye and 35

mmHg in the left eye for the index case (II-9), and 20 mmHg in the right eye and 33 mmHg in the left eye for his sibling (II-4). The index case presented at the age of 53 years which is comparable with the average age of POAG diagnosis in those with p.Gln368* [17]. His sibling presented later at the age of 70 years by which time she had very advanced glaucoma indicating the disease onset was likely to have been some years earlier. If she was screened at a younger age she would certainly have received earlier intervention. The proband's father was also reported to be diagnosed in his sixties. It is likely that this novel variant produces a similar phenotype to p.Gln368* given the high IOP at presentation, however further clinical and genetic screening of family members is required to define a better genotype/phenotype correlation.

It is evident in the glaucoma history of the two *MYOC* positive individuals that control of their disease required more than pharmacological management. The IOP of the proband gradually elevated despite the combination of multiple eye drops and laser treatment. The older affected sibling required surgical management to control her IOP. Interestingly, she also had pseudoexfoliation in the left eye which was the eye that had poorer visual field and higher IOP. It is possible that the combination of the *MYOC* variant with pseudoexfoliation leads to a more severe phenotype supporting a model of glaucoma pathogenesis in which multiple genetic variants interact in an additive fashion. Patients with *MYOC* pathogenic variants usually have substantially elevated IOP requiring multiple treatment strategies to obtain adequate IOP control. Glaucoma surgery has been reported in 28% of patients with p.Gln368* [17]. As POAG associated with *MYOC* pathogenic variants often requires surgical treatment to control the disease, early identification of carriers is essential for their glaucoma management. Predictive genetic testing has been offered to the unaffected at-risk individuals through the family members already tested, and they can now make their own decision as to whether or not they wish to learn their glaucoma risk.

In conclusion, we identified a novel *MYOC* sequence variant, segregating with the POAG phenotype in an Australian family. Further evaluation of family members and independent case reports are required to correlate the severity of the phenotype for this variant. The identification and characterization of novel *MYOC* sequence variants allows for better treatment strategies in affected individuals and early monitoring and detection of disease in unaffected at-risk family members.

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Table 1. Ophthalmic clinical details of the two recruited individuals with glaucoma.

Clinical details (RE, LE)	Individual II-9	Individual II-4
Highest recorded IOP (mmHg)	39, 35	20, 33
BCVA	20/20, 20/25	20/30, 20/40
Central corneal thickness (μm)	504, 529	533, 533
Cup to disc ratio	0.5, 0.75	0.7, 0.9
Glaucoma surgery	Nil	RE Trabeculectomy

RE, right eye; LE, left eye; IOP, intraocular pressure; BCVA, best corrected visual acuity.

Fig. 1. Pedigree of the family. Round symbols indicate female; square symbols, male; diamond symbols, unspecified gender; fully filled symbols, open angle glaucoma; unfilled symbols, unaffected; diagonal lines, deceased; brackets, adopted in; arrow, proband; stars, sample; plus/minus, presence/absence of the *MYOC* p.(Trp373*) variant.

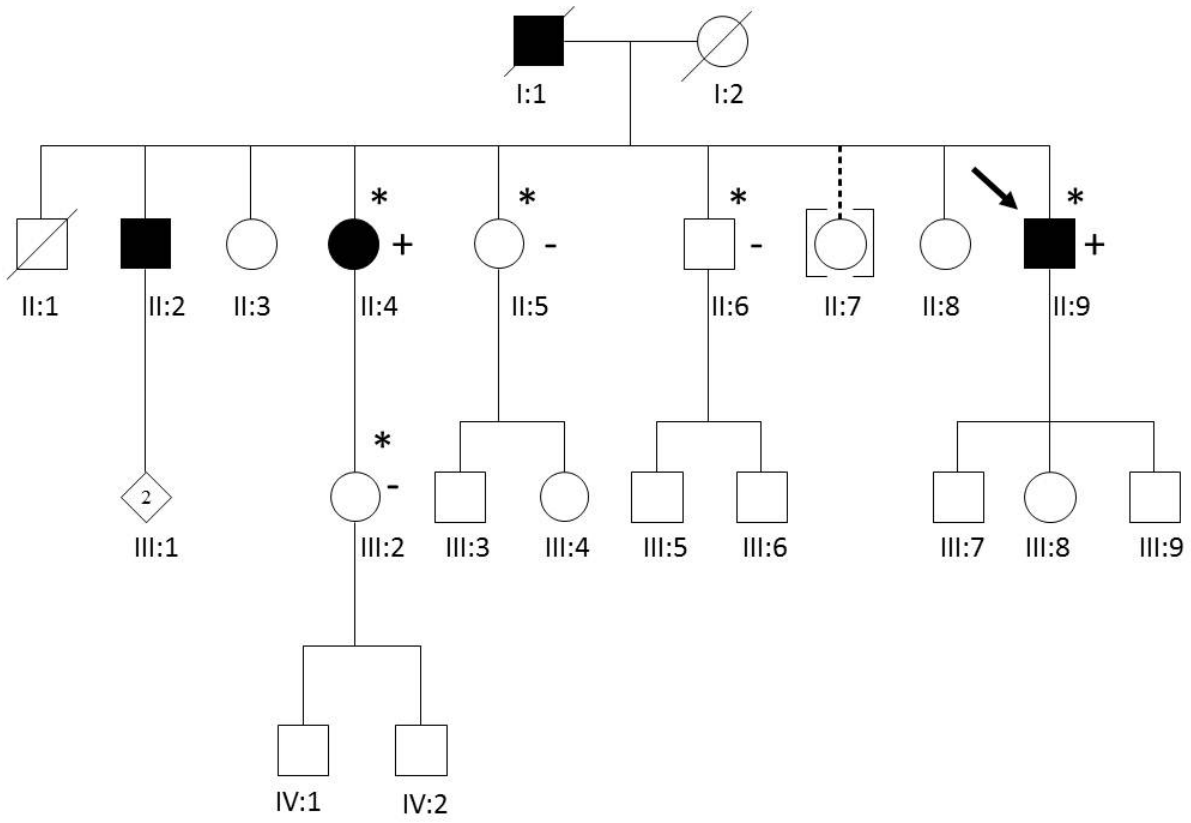


Fig. 2. Glaucomatous defects in index case II-9. A: optic discs photos of the right eye and left eye respectively. B: Retinal nerve fiber layer analysis with optical coherence tomography.

Top: right eye, bottom: left eye.

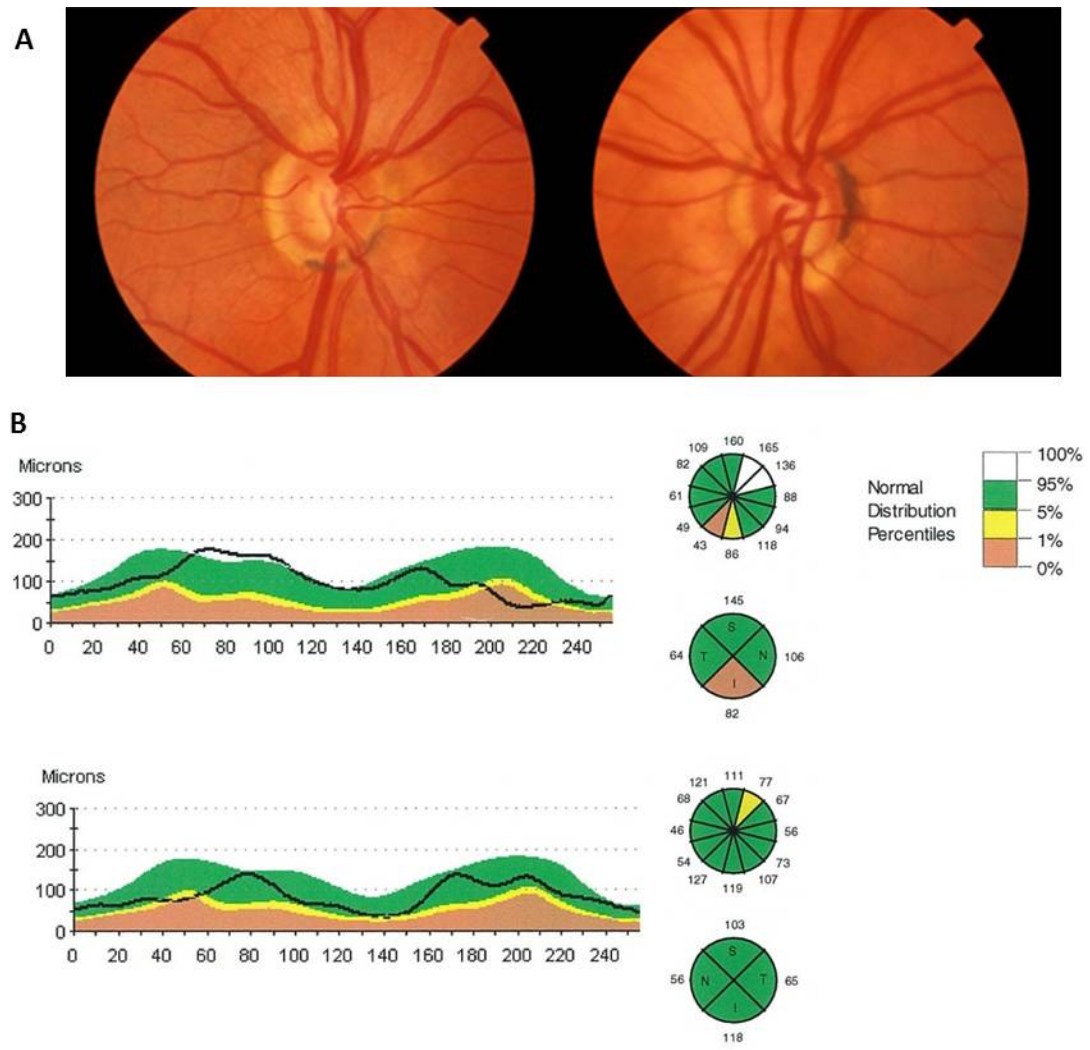


Fig. 3. Visual field tests. A: index case II-9 (mean deviation right -5.84dB, left -13.14dB). B: individual II-4 (mean deviation right -2.15dB, left -29.94dB). RE, right eye; LE, left eye.

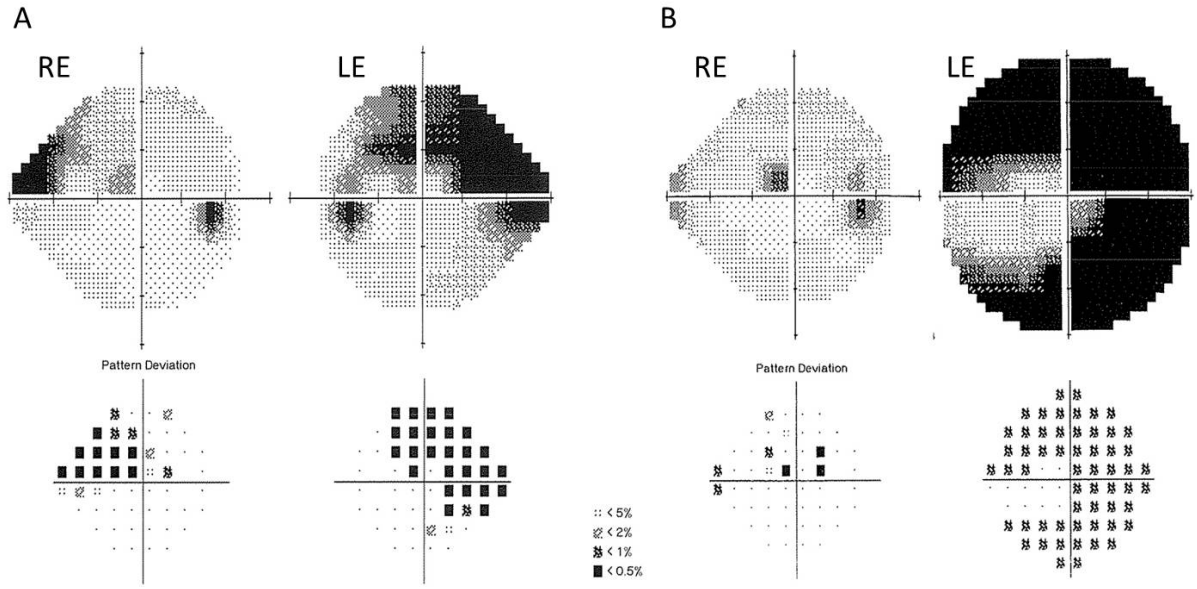
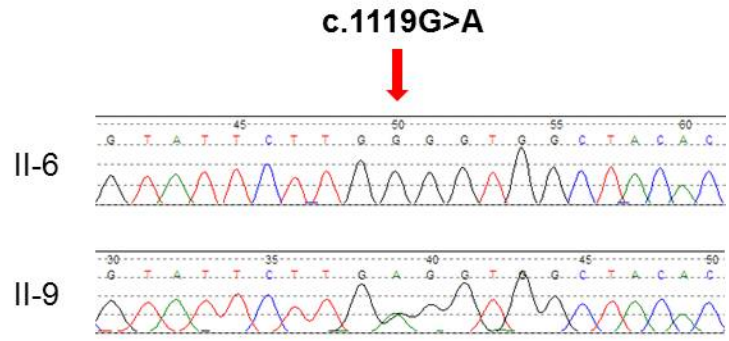


Fig. 4. MYOC:c.1119G>A, p.(Trp373*) sequence variant in individuals II-6 (normal) and II-9 (affected). The red arrow marks the variant.



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CASE REPORT Open Access

A novel de novo *Myocilin* variant in a patient with sporadic juvenile open angle glaucoma

Emmanuelle Souzeau[1*], Kathryn P. Burdon[2], Bronwyn Ridge[1], Andrew Dubowsky[3], Jonathan B. Ruddle[4] and Jamie E. Craig[1]

Abstract

Background: Glaucoma is a leading cause of irreversible blindness. Pathogenic variants in the *Myocilin* gene (*MYOC*) cause juvenile open angle glaucoma (JOAG) in 8–36 % of cases, and display an autosomal dominant inheritance with high penetrance. Molecular diagnosis is important for early identification as therapies are effective in minimizing vision loss and *MYOC* variants can be associated to severe glaucoma. *MYOC* variants are usually inherited, however a fifth of carriers do not report a family history. The occurrence of *de novo MYOC* variants is currently unknown.

Case presentation: In this study we investigated a 14 year old male Caucasian patient diagnosed with JOAG, and no family history of glaucoma. A novel probably deleterious MYOC:p.(Pro254Leu) variant was identified in the index case. This variant was not present in the parents or the siblings.

Conclusion: This is the second report of a *de novo MYOC* variant in a sporadic case of JOAG and it is currently unknown if this mechanism occurs more frequently. This finding emphasizes the importance of screening individuals with JOAG for *MYOC* mutations irrespective of a negative family history.

Keywords: De novo variant, Juvenile open angle glaucoma, Genetic testing, Glaucoma, Myocilin

Background

Glaucoma is one of the leading causes of irreversible blindness affecting over 60 million individuals worldwide [1]. Primary open angle glaucoma (POAG, MIM 137760) is the most common type and is characterized by changes in the optic nerve head with corresponding visual field loss in the presence of an open anterior chamber angle [2]. Juvenile open angle glaucoma (JOAG) refers to a younger age at diagnosis usually defined by an onset before 30–40 years old and associated with a more severe phenotype [3, 4]. Therapies for POAG aim at controlling intraocular pressure (IOP) and are usually effective in minimizing disease progression [5–7]. However, the early stages are often asymptomatic and half of the cases remain undiagnosed, making it challenging to implement treatment before irreversible vision loss occurs.

Pathogenic sequence variants in the *MYOC* gene (MIM 601652) have been first described in association with JOAG in 1997 [8]. Since then, they have been consistently identified in 2–4 % of adult-onset POAG [9, 10] and in 8–36 % of JOAG [9, 11, 12] among different ethnicities. *MYOC* comprises three exons which encode a protein consisting of two major domains, an N-terminal myosin-like domain and a C-terminal olfactomedin-like domain [13]. Most disease causing variants are clustered within exon 3 in the olfactomedin domain [14]. The pathophysiology is not fully understood but it has been postulated that the accumulation of misfolded proteins lead to endoplasmic reticulum stress, which compromises the trabecular meshwork cells regulating the IOP [15]. *MYOC* pathogenic variants are inherited in an autosomal dominant fashion and are often associated with high IOP, younger age at diagnosis and strong family history and can result in severe glaucoma and blindness if left untreated [9, 10, 16].

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The majority of *MYOC* carriers report a family history of glaucoma, however sporadic cases still account for 20 % of mutation carriers [9]. It is currently unknown whether sporadic cases could be explained by *de novo* variants. In this study, we report a JOAG sporadic case with a novel *de novo MYOC* variant, and discuss the occurrence of *de novo* variants in *MYOC* associated glaucoma and the implications for the patient and his family.

Case presentation

Clinical presentation

The pedigree of the family is shown in Fig. 1a. The index case and his family were referred to the Australian and New Zealand Registry of Advanced Glaucoma (ANZRAG) through his treating ophthalmologist [17]. The proband was a 14 year old Caucasian male patient (II-1). He was referred to an ophthalmologist following a routine optometrist review for his glasses prescription which revealed high IOP. Following examination, he was diagnosed with JOAG. His IOP at presentation were 31 mmHg in the right eye and 32 mmHg in the left. His vertical cup-to-disc ratio was 0.85 right and 0.8 left, and he had central field loss involving fixation in the right eye (Humphrey Field Analyzer, Zeiss) (Fig. 2a). His visual acuity was 20/20 in both eyes. His IOP was initially under control with latanoprost and brimonidine/timolol. However he underwent bilateral trabeculectomies following his most recent IOP which were 40 mmHg. Optic nerve appearances and retinal nerve fiber layer loss (Spectralis®, Heidelberg Engineering) are depicted in Fig. 2b and c. His parents and two siblings had normal eye examinations.

Genetic testing

Genetic testing was performed through the National Association of Testing Authorities (NATA) accredited laboratories of SA Pathology at the Flinders Medical Centre in Adelaide, Australia. The proband was sequenced for the 3 coding exons of the *MYOC* gene as previously described [9]. A heterozygous substitution of Thymine for Cytosine at nucleotide 761 of the *MYOC* exon 3 coding sequence was identified (MYOC:c.761C > T), encoding a missense substitution of Proline to Leucine at position 254 (p.(Pro254Leu)) (Fig. 1b). No other variants were identified in the *MYOC* gene of the proband. JOAG can also be associated with *CYP1B1* variants [18]. The coding region of the *CYP1B1* gene was sequenced to exclude other causative genes. No disease-causing variants were identified in *CYP1B1*.

The p.(Pro254Leu) variant is novel since it was absent from the *MYOC* Database (www.myocilin.com), NCBI dbSNP (www.ncbi.nlm.nih.gov/SNP/), and the Exome Aggregation Consortium (http://exac.broadinstitute.org/) which comprises exome sequence data spanning 60 706 unrelated individuals. A search of the scientific literature also failed to identify any reference to this variant. However, a recent study reported a *MYOC* variant at the same residue p.(Pro254Arg) in a patient with JOAG and his affected mother [19]. SIFT and Polyphen-2 both predicted this variant to be deleterious, with sequence alignment demonstrating this position to be highly conserved among vertebrates and other olfactomedin domain-containing proteins (Fig. 1c). *MYOC* is a well characterized gene and codon position 254 resides in the core hydrophobic β-sheet belt of the olfactomedin domain, which is important in protein-protein interactions and is sensitive to aggregation in the presence of substitutions [20]. The p.(Pro254Leu) variant is likely pathogenic based on bioinformatics prediction, invariant conservation of this residue, and characterization of the protein structure. *MYOC* disease-causing variants can be associated with severe glaucoma and blindness [9]. In the view of the genetic result and the patient's most recent IOP, bilateral trabeculectomies were performed to better control his IOP and minimize damage on his optic nerves.

This variant was not detected in either parent of the index case (Fig. 1b). The marker profile comparison using the AmpFLSTR® Identifiler® PCR Amplification Kit confirmed a profile consistent with the proband being the biological child of the stated parents, indicating p.(Pro254Leu) has arisen *de novo* in the proband. A *de novo MYOC* pathogenic variant, p.(Val251Ala), has been previously reported once in a JOAG case [21]. Interestingly, this variant was located three amino acids from p.(Pro254Leu) which was identified in this study.

While the occurrence of *de novo* pathogenic variants in the genome vary considerably based on genomic location, they are estimated to be common and have been linked to many sporadic diseases [22]. Conditions with dominant inheritance and modest fitness effect are more commonly inherited than caused by *de novo* variants, and this is the situation for *MYOC* associated glaucoma which is usually inherited. For example, a founder effect with an origin prior to the European settlement of Australia has been suggested for the most common *MYOC* disease-causing variant, p.Gln368Ter, in some families [23]. However, we previously reported that 20 % of *MYOC* carriers do not report a family history of the disease [9]. Although this may be explained by individuals not being aware of a diagnosis in their families, or relatives being undiagnosed, it is possible that variants occur *de novo* in some families. *MYOC* variants are often identified in older individuals with parents usually unavailable for testing, making it difficult to evaluate whether variants are inherited or sporadic. This case is the second report of a *de novo MYOC* variant, emphasizing that a sporadic variant should

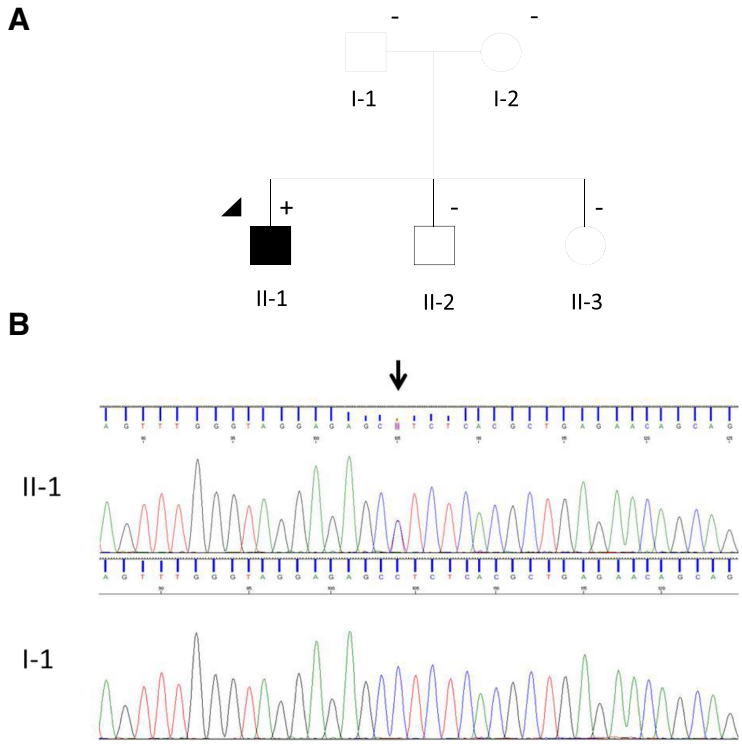

Fig. 1 (See legend on next page.)

(See figure on previous page.)
Fig. 1 Pedigree and genetic analysis. **a** Pedigree of the family. Round symbols indicate female; square symbols, male; fully filled symbols, open angle glaucoma; unfilled symbols, unaffected; arrow, proband; plus/minus, presence/absence of the MYOC:p.(Pro254Leu) variant. **b** Chromatogram showing the presence of MYOC:c.761C > T, p.(Pro254Leu) sequence variant in individual II-1 at the top (affected) and its absence in individual I-1 at the bottom (unaffected). The black arrow marks the heterozygous variant. **c**. Alignment of MYOC protein sequences corresponding to residues 248 through 262 (NP_000252.1), against different species, and of different human olfactomedin proteins. The residue of interest, p.(Pro254Leu), is highlighted in yellow. Reference sequences IDs of the genes/species aligned are shown in brackets

be considered when evaluating the likelihood of *MYOC* variants in cases with no family history of JOAG or POAG.

De novo variants arise either in the germline or during embryogenesis. If present in the germline cells of one parent, they can represent a recurrence risk in siblings of the variant carrier. We have previously shown that *MYOC* genetic testing is important for early identification of at-risk individuals and appropriate interventions to minimize irreversible vision loss [9, 24]. To exclude a recurrence risk resulting from germline mosaicism, both siblings of the proband were subsequently tested for the *MYOC* variant. Our testing revealed that neither sibling carried the *MYOC* p.(Pro254Leu) variant, eliminating an inherited risk of developing *MYOC* associated glaucoma.

Conclusion

In conclusion, we report a novel *de novo MYOC* variant considered pathogenic in a patient with sporadic JOAG. This is the second report of a *MYOC de novo* variant, and it is currently unknown if this mechanism occurs more

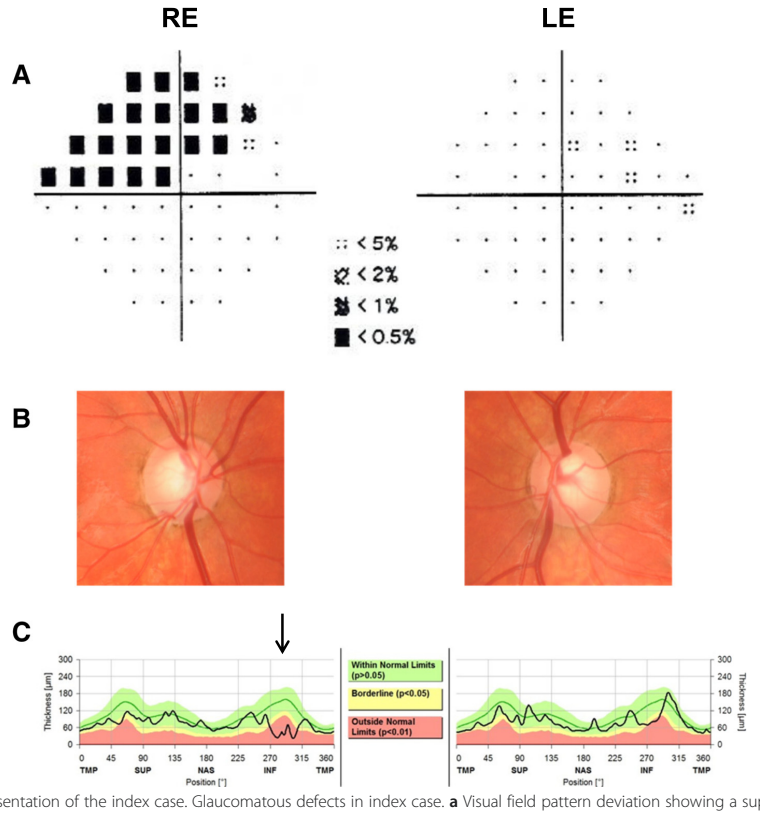

Fig. 2 Clinical presentation of the index case. Glaucomatous defects in index case. **a** Visual field pattern deviation showing a superior arcuate defect involving fixation in the right eye (Humphrey Field analyser, Zeiss). **b** Optic discs photos showing a right infratemporal notch and disc haemorrhage. **c** Optical coherence tomography showing inferior retinal nerve fibre layer loss more prominent in the right eye than the left as shown by the black arrow (Spectralis®, Heidelberg Engineering). RE: right eye, LE: left eye, TMP: temporal, SUP: superior, NAS: nasal, INF: inferior

frequently. This case also highlights that *MYOC* testing should not be restricted to individuals with a positive family history of glaucoma.

Consent

Ethics approval was obtained from the Southern Adelaide and Flinders University Clinical Research Ethics Committee. The study conformed to the tenets of the Declaration of Helsinski and follows the National Health and Medical Research Council statement of ethical conduct in research involving humans. Written informed consents were obtained from each participating family member. A copy of the written consent is available for review by the Series Editor of this journal.

Abbreviations

ANZRAG: Australian and New Zealand Registry of Advanced Glaucoma; IOP: intraocular pressure; JOAG: juvenile open angle glaucoma; MYOC: Myocilin; NATA: National Association of Testing Authorities; POAG: primary open angle glaucoma.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

ES participated in the design of the study, interpreted the data and drafted the manuscript. KPB participated in the design of the study and critically revised the manuscript. BR recruited the family in the study and critically revised the manuscript. AD carried out the molecular genetic studies, interpreted the results and critically revised the manuscript. JBR performed ophthalmological examination of the patients and critically revised the manuscript. JEC participated in the design of the study and critically revised the manuscript. All authors read and approved the final manuscript.

Acknowledgments

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2.4. Copy number variations of *TBK1* are associated with NTG

TBK1 duplications were first associated with normal tension POAG in 1.3% (2/152) individuals from the USA [159]. Replication studies confirmed the involvement of *TBK1* duplications in a minority of NTG cases from Japan (0.4%, 1/252) [373] and the USA (1.0%, 1/96) [374].

This study represented the largest cohort of NTG patients and the first to evaluate *TBK1* in a cohort with advanced visual loss from glaucoma. *TBK1* CNVs were detected in 1.2% (4/334) Australian patients with NTG and none of the 1045 HTG cases screened. The detection rate was slightly higher in cases with advanced POAG (1.4%, 3/212) compared to non-advanced glaucoma (0.8%, 1/122), suggesting a severe phenotype associated with *TBK1* CNVs similar to what has been reported with *MYOC* variants [375]. The CNVs identified in the individuals from this study differed in size from those reported previously, suggesting that they have happened independently. This was also the first study to investigate *TBK1* CNVs in HTG patients with advanced glaucoma. None of the 1045 HTG cases had *TBK1* duplications. These results suggest that *TBK1* is not a major contributor of HTG, and that NTG cases should be prioritised for genetic testing instead. In summary, our data indicates that *TBK1* can lead to severe glaucoma which has implications for genetic counselling and predictive genetic testing of at-risk family members.

Contribution statement

Ms Souzeau was responsible for the study design, feedback of results and the provision of genetic counselling, data collection and interpretation, and revision of the manuscript. Dr Awadalla was responsible for the conception and design of the study, data collection and analysis, drafting and submitting the manuscript as corresponding author. A/Prof Fingert contributed to the conception and design of the study, data analysis and revised the manuscript. Mr Roos contributed to data collection and analysis and revised the manuscript. Dr Chen, Dr Holmes, Prof Graham, Dr Chehade, Dr Galanopoulos, A/Prof Hewitt and Prof Mackey contributed to the characterisation of participants and revised the manuscript. Ms Ridge contributed to the recruitment of participants and revised the manuscript. Dr Zhou contributed to data collection and revised the manuscript. Dr Siggs contributed to data analysis and revised the manuscript. A/Prof Burdon contributed to the conception and design of the study, data analysis and revised the manuscript. Prof Craig contributed to the conception and design of the study, characterisation of participants, data analysis and revised the manuscript.

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Copy number variations of *TBK1* in Australian patients with primary open-angle glaucoma

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ABSTRACT

Purpose: To investigate the presence of TBK1 copy number variations in a large, well-characterized Australian cohort of patients with glaucoma comprising both normal-tension glaucoma and high-tension glaucoma cases.

Design: A retrospective cohort study.

Methods: DNA samples from patients with normal-tension glaucoma and high-tension glaucoma and unaffected controls were screened for *TBK1* copy number variations using real-time quantitative polymerase chain reaction. Samples with additional copies of the *TBK1* gene were further tested using custom comparative genomic hybridization arrays.

Results: Four out of 334 normal-tension glaucoma cases (1.2%) were found to carry *TBK1* copy number variations using quantitative polymerase chain reaction. One extra dose of the *TBK1* gene (duplication) was detected in 3 normal-tension glaucoma patients, while 2 extra doses of the gene (triplication) were detected in a fourth normal-tension glaucoma patient. The results were further confirmed by custom comparative genomic hybridization arrays. Further, the TBK1 copy number variation segregated with normal-tension glaucoma in the family members of the probands, showing an autosomal dominant pattern of inheritance. No *TBK1* copy number variations were detected in 1045 Australian patients with high-tension glaucoma or in 254 unaffected controls.

Conclusion: We report the presence of *TBK1* copy number variations in our Australian normal-tension glaucoma cohort, including the first example of more than 1 extra copy of this gene in glaucoma patients (gene triplication). These results confirm *TBK1* to be an important cause of normal-tension glaucoma, but do not suggest common involvement in high-tension glaucoma.

INTRODUCTION

Glaucomas are a group of eye diseases with a common feature of progressive irreversible degeneration of the optic nerve with corresponding loss of the peripheral visual field (1). Glaucomas are the leading cause of irreversible blindness worldwide, and primary open-angle glaucoma is the most prevalent subtype worldwide (2). The main risk factor for glaucoma is elevated intraocular pressure; however approximately 20-50% of all primary open-angle glaucoma cases present with normal intraocular pressure range (10-21 mmHg) and are termed normal-tension glaucoma (3).

The genetic contribution to primary open-angle glaucoma is well documented (4). Around half of all primary open-angle glaucoma patients have a positive family history (5), and first-degree relatives of primary open-angle glaucoma patients have an approximately 9-fold increased risk of developing glaucoma (6, 7). The first gene identified to be associated with familial normal-tension glaucoma was *Optineurin* (*OPTN*) in the GLCE1 region on chromosome 10p15-14 (8). Subsequent studies reported that mutations in OPTN cause 1%-2% of primary open-angle glaucoma or normal-tension glaucoma (9-12). Despite several studies on the OPTN gene, it exact role in causing primary open-angle glaucoma remains elusive (3, 13, 14).

Recently, a novel genetic locus (GLC1P) on chromosome 12q14 was reported to be linked to normal-tension glaucoma in an African-American pedigree (15). A duplication that spans the TANK binding kinase 1 (TBK1) gene was subsequently detected in this pedigree, as well as in 2 out of 153 unrelated normal-tension glaucoma subjects from Iowa (1.5%), 1 out of 252 unrelated Japanese normal-tension glaucoma patients (0.4%), and 1 out of 96 unrelated patients from New York (1.0%) (15-17). These data suggest that abnormal TBK1 dosage (duplication) causes normal-tension glaucoma in these patients. The association between copy number variations of the TBK1 gene and normal-tension glaucoma is supported by several additional observations. First, copy number variations are known to be involved in influencing gene expression and are risk factors for primary open-angle glaucoma (GALC gene) (17, 18) and Axenfeld-Rieger syndrome (FOXC1 gene) (19), as well as a number diseases such as HIV dementia complex (20), autism (21), and Alzheimer disease (22). Second, TBK1 is specifically expressed in the ganglion cells and the nerve fiber layer of the human retina, which are involved in the pathogenesis of glaucoma (15, 23). Third, OPTN binds the TBK1 protein, particularly in the presence of the recurrent severe glaucoma-causing mutation E50K in the OPTN gene (24). Interestingly, 3 known normal-tension

glaucoma genes (TBK1, OPTN, and TLR4) each encode proteins that directly interact with each other in a biological pathway that activates autophagy (25, 26), a process by which intracellular materials (eg, proteins, organelles, or pathogens) are degraded. Together these data further implicate the role of the TBK1 gene in the pathogenesis of normal-tension glaucoma.

In this study, we aimed to investigate the presence of copy number variations of the *TBK1* gene in unrelated normal-tension glaucoma cases and unaffected controls recruited from the Australian population. We also explored the presence of the gene copy number variations in patients with high-tension glaucoma, thus attempting to define an overall contribution of *TBK1* copy number variations to glaucoma blindness.

METHODS

Approval of this retrospective cohort study was obtained from the Southern Adelaide Clinical Human Research Ethics Committee. This study has been conducted in accordance with the Declaration of Helsinki and its subsequent revisions. The committee prospectively approved the recruitment of individuals and family members with primary open-angle glaucoma and its subtypes, the collection of blood or saliva samples for deoxyribonucleic acid extraction, the screening for genetic mutations, the data analysis, and the making of genotype and phenotype correlations. Written informed consent was obtained from each individual to participate in this study. Recruitment was conducted through the Australian and New Zealand Registry of Advanced Glaucoma (5). The unaffected control cohort was collected from retirement villages in Adelaide, South Australia, as previously described (27).

Each participant was examined by his or her specialist and received a complete eye examination, including slit-lamp examination of the anterior chamber, gonioscopy, measurement of central corneal thickness (CCT), visual acuity, intraocular pressure, fundus examination with special attention to optic disc health and size, and automated perimetry. The diagnosis of glaucoma followed the definition of the International Society of Geographical and Epidemiological Ophthalmology described by Foster and associates (28), with optic nerve damage and corresponding visual loss detected in at least 1 eye. Patients recruited in the study and identified as having normal-tension glaucoma followed the same criteria described by Fingert and associates (15) (intraocular pressure less than or equal to 21 mmHg in both eyes, unadjusted for CCT). High-tension glaucoma patients were diagnosed with intraocular pressure greater than 21 mmHg in at least 1 eye, along with glaucomatous optic nerve and visual field damage. Patients diagnosed with advanced

glaucoma presented with either fixation involving visual field loss (at least 2 of the 4 central fixation squares having a pattern standard deviation of less than 0.5% on a reliable Humphrey 24-2 field) or severe global field loss at baseline (mean deviation of less than -22 dB) in at least 1 eye (5). Family members of *TBK1* copy number variation carriers were recruited when available. The controls had no evidence of glaucomatous optic nerve damage, intraocular pressure of less than or equal to 21 mmHg, and no family history of glaucoma, and were slightly older than cases by design for this aging disease. The study was first conducted using a total of 334 unrelated patients with normal-tension glaucoma and 254 unaffected controls. Sixty-three percent of patients (n = 212) had advanced normal-tension glaucoma, while the remainder (n= 122) had less severe (nonadvanced) normal-tension glaucoma. A positive family history of glaucoma was present in 133 patients (40%).

Venous blood samples were obtained from the participants for the study. Genomic DNA was extracted from peripheral whole blood using the QiaAmp Blood Maxi Kit (Qiagen, Valencia, California, USA). DNA from each subject was tested for *TBK1* duplications using TaqMan Copy Number Assays (Life Technologies, Carlsbad, California, USA). The segment of the *TBK1* gene was amplified in 4 replicates for each DNA sample. The experiment was conducted using the StepOne Plus real-time polymerase chain reaction instrument, which quantitates the gene of interest, normalizes to an endogenous reference gene (RNase P) known to be present in 2 copies in a diploid genome. Evaluation of the copy number of genomic DNA targets was performed using the CopyCaller 2.0 software (Life Technologies) with default settings. For detailed mapping of duplication events, patients with detected *TBK1* duplications were analyzed using custom 8x60K SurePrint G3 Human custom comparative genomic hybridization microarrays (Agilent, Santa Clara, California, USA) that interrogated over 55000 probes in the GLC1P locus that spans 9.5 Mbp between rs12227270 and rs7488555 on chromosome 12q14, using the manufacturer's protocol (15).

To further explore the relationship between 2 apparently unrelated individuals with an identical duplication, we analyzed the haplotypes surrounding the duplication region. The 3 carriers with primary open-angle glaucoma were also part of a previously reported genome-wide association scan (GWAS) (29). Along with 590 other participants with primary open-angle glaucoma, they were genotyped on the Omni1 array (Illumina, San Diego, California, USA). The most likely haplotype pair across the duplication region (chr12:64173733-65613733, hg19) in each participant in the GWAS were estimated using Beagle3.3.2 (http://faculty.washington.edu/browning/beagle/beagle.html) (30). The haplotypes across the whole region were visually compared between patients AG624 and AG724. The haplotypes

for AG604 with a different duplication were also compared.

Mutation screening of *TBK1* was performed on 95 unrelated cases with high-tension glaucoma, 100 unrelated cases with normal-tension glaucoma, and 104 unaffected unrelated controls from Australia. Exome capture was performed using the SureSelect system (Agilent) and paired-end libraries were sequenced on an Illumina HiSeq 2000 by Macrogen Inc (Seoul, South Korea). Reads were mapped to the human reference genome (hg19) using BWA (http://bio-bwa.sourceforge.net/), and duplicates were marked and removed using picard. Variants were called using SAMtools and annotated with ANNOVAR (http://annovar.openbioinformatics.org/en/latest/). Variants were described according to the recommendations of the Human Genome Variation Society (http://www.hgvs.org/) and referenced against the NHLBI Exome Variant Server (http://evs.gs.washington.edu/EVS/ [July 2014]), 1000 Genomes (31), and dbSNP v138 databases (https://www.ncbi.nlm.nih.gov/SNP/).

RESULTS

TBK1 copy number variations were detected in 4 out of 334 Australian cases with normal-tension glaucoma (1.2%) using quantitative polymerase chain reaction assays (Figure 1). Three unrelated probands, GFMC524, AG604, and AG624, were found to have 3 copies of the gene (1 extra dose), while AG724 participant was found to carry 4 total copies of *TBK1* (2 extra doses). No copy number variations were detected in any of the unaffected controls. This rate is similar to previously published data where overlapping copy number variations were found in 1.3% of white normal-tension glaucoma subjects from Iowa and in 1% of normal-tension glaucoma patients from New York (15, 17). Affected siblings of the probands AG724 and GFMC524 (AG724.1 and GFMC524.1, respectively) were also shown to carry *TBK1* duplications using the quantitative polymerase chain reaction assay. The inheritance of *TBK1* copy number variations and normal-tension glaucoma is shown in Figure 2 for these pedigrees. Interestingly, all of the members of 1 pedigree that were diagnosed with normal-tension glaucoma (AG604, AG604.1, and AG604.2) had 2 extra copies of *TBK1* (triplication), while previously reported cases had 1 extra copy (Figure 1). All families display an autosomal dominant inheritance pattern of *TBK1* copy number variations and normal-tension glaucoma, providing further evidence that these copy number variations are pathogenic. Moreover, these data also suggest that the extra copies of the *TBK1* are tandem repeats on the same allele, that is, a gene duplication in pedigrees with 1 extra copy of *TBK1* and a gene triplication in pedigrees with 2 extra copies.

The borders of the copy number variations detected in normal-tension glaucoma probands GFMC524, AG604, AG624, and AG724 were assessed using comparative genomic hybridization (Figure 3). The copy number variation in these Australian normal-tension glaucoma patients are all novel and differ from previously reported copy number variations in the extent of chromosome 12q14 that is involved. Both probands AG624 and AG724 had a duplication, extending from approximately 64,68 Mbp to 65,09 Mbp on chromosome 12. These probands were not known to be related; however, detection of identical copy number variation borders suggested a founder effect. This hypothesis was investigated by comparing haplotypes spanning the *TBK1* locus using genotypes obtained from a prior genome-wide association study. These 2 patients were found to share a common haplotype over a greater than 1.4Mb segment of chromosome 12q14 (between rs10506464 and rs1909340), which further supports a founder effect in these 2 individuals. A 300kbp duplication was detected in normal-tension glaucoma proband AG604 that has similar borders as a previously reported copy number variation in a Japanese normal-tension glaucoma patient, GGJ-414 (Figure 3) (15). Genotype data were not available to explore a possible founder effect between these 2 patients. When the copy number variations from the current report and those from prior report were analysed (15-17), the overlap defined a critical region (~131 kbp), which harbors the *TBK1* gene and part of the *XPOT* gene (Figure 3).

Table 1 shows the clinical features of patients carrying the *TBK1* copy number variations. All of them presented with a family history of glaucoma, large cup-to-disc ratio (ranges from 0.80 to 0.95), and intraocular pressure in the normal range (the maximum recorded untreated intraocular pressures ranged from 12 mmHg to 17 mmHg). However, the central corneal thickness varied between the affected probands. GFMC524 had thin CCT (496 µm OD, 505 µm OS), and AG624 had thick CCT (622 µm OD, 621 µm OS). Most of the patients who carry the *TBK1* copy number variations were diagnosed at a relatively young age, except for Patient AG604, who was diagnosed at age 60. However, the onset of the disease in Patient AG604 is likely to have been much earlier, given the advanced visual field loss that was observed at the time of diagnosis.

To further explore the role of *TBK1* copy number variations in primary open-angle glaucoma in general, 1045 patients with high-tension glaucoma were screened by quantitative polymerase chain reaction. No *TBK1* copy number variations were identified, indicating that in our dataset *TBK1* duplications were found only in normal-tension glaucoma cases. The demographic features and clinical differences between the 2 subtypes of primary open-angle glaucoma and normal controls are illustrated in Table 2.

A cohort of 195 Australian cases with primary open-angle glaucoma (including 100 normal-tension glaucoma cases) and 104 unrelated unaffected controls were screened for disease-causing variants in the coding sequence of *TBK1*. A total of 3 single nucleotide variants were identified. Two synonymous variants were detected in 87 unaffected controls (p.N22N, p.I326I). One previously published (15), nonsynonymous variant (p.V464V) was found in 3 normal-tension and 5 high-tension glaucoma cases and 7 unaffected controls. None of these variants is likely to account for disease.

DISCUSSION

Primary open-angle glaucoma is known to be a genetically heterogeneous disease. Recently, Fingert and associates identified a large duplication within a novel locus (GLC1P) to be associated with primary open-angle glaucoma and its subtype, normal-tension glaucoma, located on chromosome 12q14 (15). Although the overlapping duplication encompassed 4 genes (*TBK1, XPOT, RASSF3,* and *GNS*), *TBK1* was considered the strongest candidate gene for normal-tension glaucoma by virtue of its biology and the critical region defined by duplications in multiple patients. *TBK1* is expressed in cells affected by glaucoma (human retina) (15), with a clearly documented direct interaction with *OPTN*, another gene known to cause normal-tension glaucoma (24, 32). *TBK1* encodes a protein kinase that participates in both autophagy and NF-κB signaling pathways (25, 26). The specific mechanism by which *TBK1* duplication causes normal-tension glaucoma is still undetermined; however, there is a plausible hypothesis that copy number variations of *TBK1* cause a dysregulation either of autophagy or of NF-*κ*B signaling pathways that ultimately leads to apoptosis of retinal ganglion cells and the development of normal-tension glaucoma (7).

In addition to confirming the association of the *TBK1* gene copy number variations with normal-tension glaucoma, we also provide the first report of a *TBK1* gene triplication in a family with normal-tension glaucoma. After making this discovery, we retested our American pedigrees that were previously reported to have *TBK1* gene duplications, and we found compelling evidence that 1 of these pedigrees (GGA-458) (15) in fact has a *TBK1* triplication (data not shown). It is tempting to hypothesize that patients with 2 extra doses of the *TBK1* gene may have a more severe phenotype than those patients with 1 extra dose (ie, earlier onset of disease). Moreover, such a genotype-phenotype relationship might be mediated by increased *TBK1* gene expression. A previous study reported a 1.60-fold increase in the expression level of *TBK1* in patients carrying the duplication than in controls (15). It would be

interesting to examine the expression level in our patients with a *TBK1* triplication.

The absence of *TBK1* duplication in the Australian high-tension glaucoma cohort provides confirmation that *TBK1* duplications appear to occur specifically associated with the normal-tension glaucoma phenotype. Nonetheless, it is interesting to speculate that the likely phenotype if a *TBK1* copy number variation carrier had elevated intraocular pressure by chance could be significantly more severe. The Australian cohort shows a similar rate of mutation in normal-tension glaucoma as other studies of white subjects. *TBK1* copy number variations are responsible for 0.4%-1.3% of normal-tension glaucoma cases in different populations (15-17). As such, it is a rare but easily detectable marker for significant disease, which appears to be highly penetrant within families. Analysis of coding variants in our Australian cohort did not show any mutations likely to cause disease. Considering these data in conjunction with the previously published data by Fingert and associates (15) indicates that coding variants in TBK1 are not a common cause of normal-tension glaucoma.

As no mutations have been reported in unaffected controls, this assay may be an important predictor of normal-tension glaucoma risk in select patient populations (ie, strongly familial normal-tension glaucoma, or in relatives of patients with known *TBK1* copy number variations), leading to regular clinical screening of carriers of *TBK1* copy number variants. Identifying the genetic risk(s) will facilitate early diagnosis and treatment of any complications arising from this condition and prevent the advanced vision loss seen in 3 of our 4 *TBK1*-associated normal-tension glaucoma cases.

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Figure 1. Assessment of *TBK1* gene dosage by quantitative polymerase chain reaction in Australian patients with primary open-angle glaucoma. The x-axis shows the number of copies of the *TBK1* gene that were detected in each subject. The normal dosage of 2 copies of *TBK1* was detected in the control (Subject 1466). Three probands from unrelated pedigrees (AG624, AG724, and GFMC524) were found to have 1 extra copy of *TBK1* (3 total copies) while the proband AG604 was found to have 2 extra copies of *TBK1* (4 total copies). AG, advanced glaucoma; GFMC, nonadvanced glaucoma; the positive control was from a normal-tension glaucoma patient previously reported to carry a *TBK1* gene duplication.

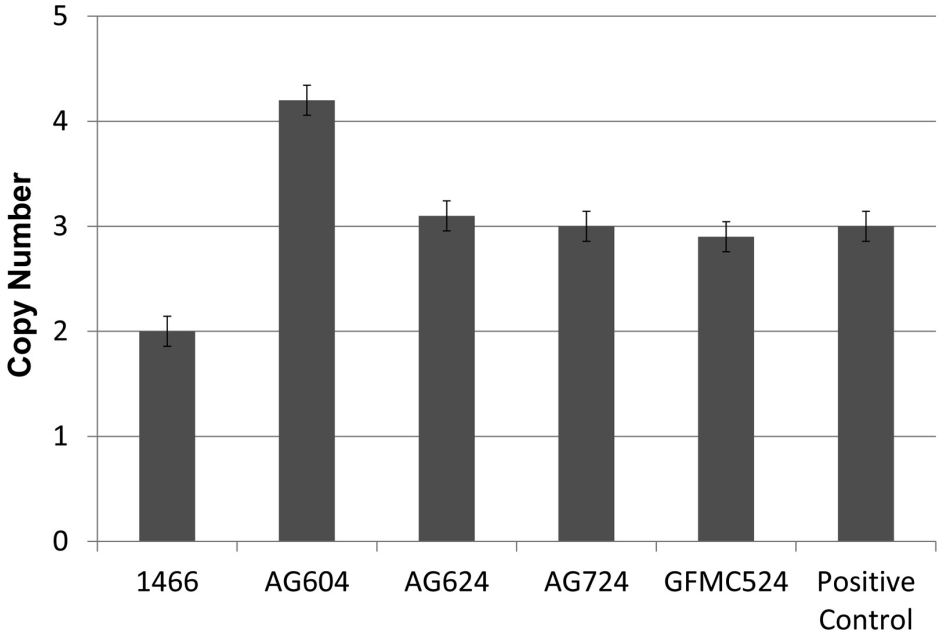

Figure 2. Pedigrees of the Australian probands with normal-tension glaucoma carrying the *TBK1* copy number variations. Black symbol indicates individuals with normal-tension glaucoma. The proband is indicated by an arrow. Participants carrying a *TBK1* duplication or triplication are indicated by a (+), and tested wild-type individuals are denoted with a (-).

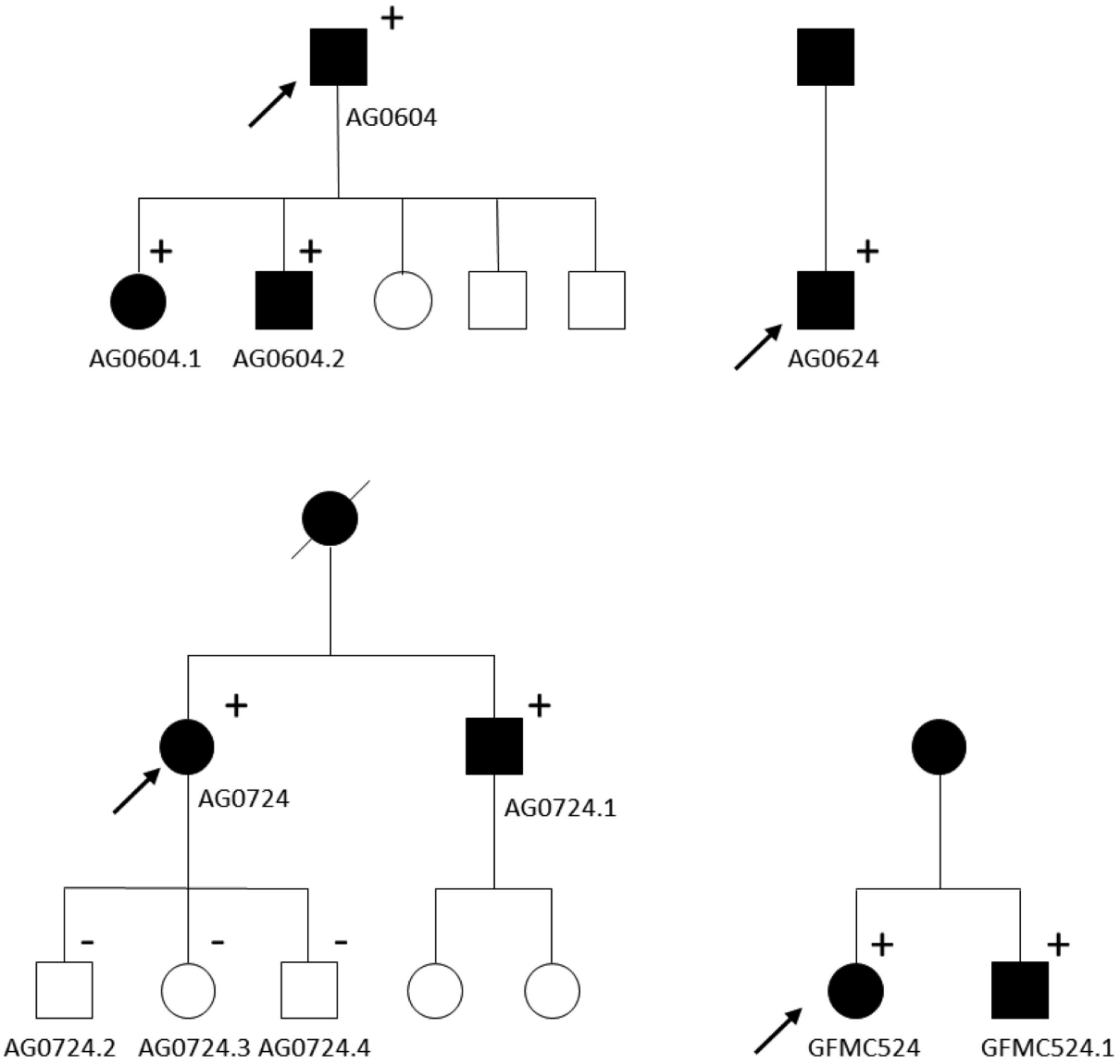

Figure 3. Relative positions of copy number variations detected in Australian cases (AG604, AG624, AG724, and GFMC524) with normal-tension glaucoma in the current report and copy number variations in pedigrees GGO-441, GGA-458, GGj-414, and GGR-590, which were previously reported. The extent of each copy number variation in base pairs is in parentheses (hg19 build) and is also depicted by black boxes; the genes encompassed by duplications are depicted as gray boxes.

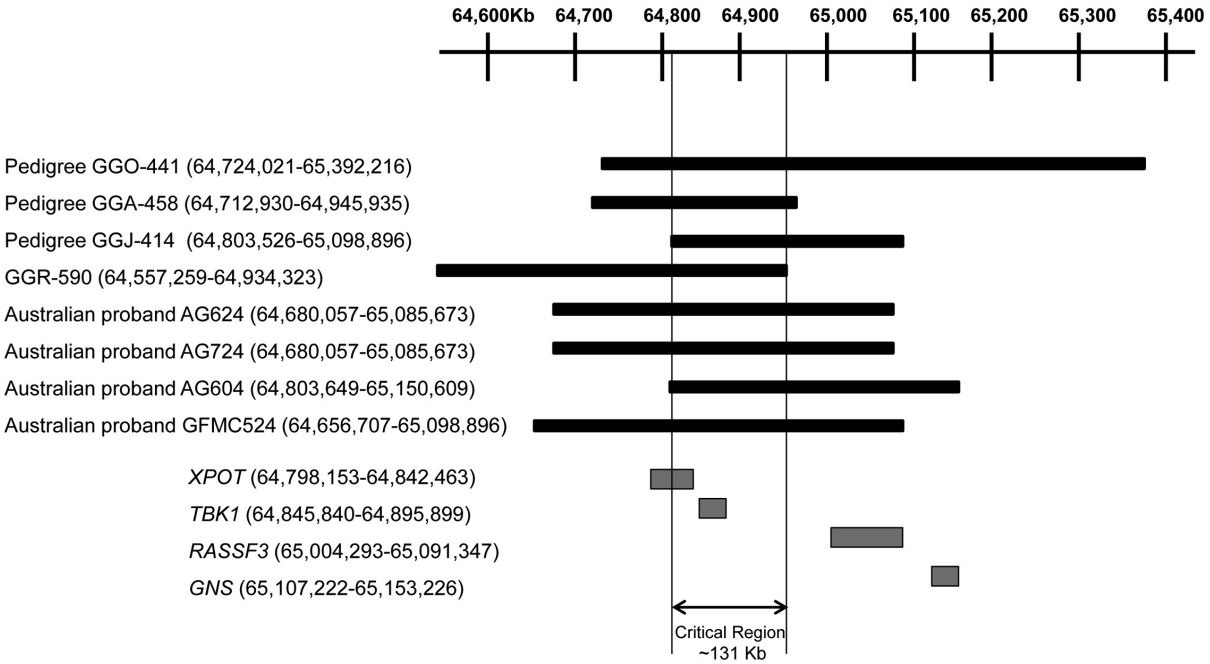

Table 1. Clinical features of normal-tension glaucoma patients carrying *TBK1* copy number variations

Patient ID	Age of Diagnosis (y)	Highest IOP OD (mmHg)	Highest IOP OS (mmHg)	CCT OD (µm)	CCT OS (µm)	CDR OD	CDR OS
GFMC524	32	13	13	496	505	0.85	0.85
AG604	60	12	12	N/A	N/A	0.95	0.95
AG624	44	17	17	622	621	0.95	0.90
AG724	43	14	14	560	550	0.80	0.90

AG = advanced glaucoma; CCT = central corneal thickness; CDR = cup to disc ratio; GFMC = nonadvanced glaucoma; IOP = intraocular pressure; N/A = not available.

Table 2. Demographic and clinical characteristics of the Australian cohort including normal-tension glaucoma patients, high-tension glaucoma patients, and normal unaffected controls.

Cohort	Mean age, years (SD)	Sex (% female)	Mean IOP, mmHg (SD)	Mean CCT, µm (SD)	Mean CDR (SD)
NTG (n = 334)	62.4 (11.4)	61%	16.9 (2.4)	510.7 (40.2)	0.8 (0.1)
HTG (n = 1045)	53.0 (14.6)	50%	25.8 (8.9)	519.8 (43.3)	0.8 (0.2)
Normal controls (n = 254)	75.9 (8.9)	58%	12.8 (2.3)	544.7 (7.2)	0.2 (0.12)

CCT = central corneal thickness; CDR = cup-to-disc ratio; HTG = high-tension glaucoma patients; IOP = intraocular pressure; NTG = normal-tension glaucoma patients.

2.5. *CYP1B1* deleterious variants are involved in JOAG with advanced visual field loss

CYP1B1 is a well-known gene for PCG and deleterious variants are transmitted in an autosomal recessive manner. More recently, a few studies reported *CYP1B1* variants in JOAG or POAG. However, most studies included small cohorts, and none investigated whether the variants could be associated with a more severe phenotype. In this study, I investigated for the first time *CYP1B1* deleterious variants in a large well-characterised cohort of JOAG cases with severe visual loss. *CYP1B1* allele frequency was significantly higher in advanced JOAG cases (4.7%, 11/236) compared to normal controls (0.6%, 1/160). Moreover, individuals with *CYP1B1* variants had a younger age at diagnosis (23.1 vs 31.5 years, $p = 0.008$) and worse mean deviation from visual field test (-24.5 vs 15.6 dB, $p = 0.02$) than individuals *CYP1B1*-negative. Additionally, biallelic *CYP1B1* deleterious variants explained 2.5% of advanced JOAG cases diagnosed before 40 years old and 11% of cases diagnosed before 25 years old. These findings suggest that genetic testing for *CYP1B1* should be considered for POAG cases with young age of onset and negative family history of glaucoma. *CYP1B1* variants can result in both PCG and JOAG, suggesting that these two diseases might be part of a phenotypic spectrum. The findings from my research have important implications for genetic testing and counselling: Family members of adult patients with *CYP1B1* variants can benefit from appropriate risk prediction. The autosomal recessive transmission of *CYP1B1* variants means that siblings have the highest risk of being affected and that the risk for other family members is low.

Contribution statement

Ms Souzeau was responsible for the study concept and design, data acquisition, analysis and interpretation, feedback of results and provision of genetic counselling, drafting and submitting the manuscript as corresponding author. Dr Hayes contributed to the genetic studies, the data analysis and revised the manuscript. Dr Zhou, Dr Siggs and Dr Awadalla contributed to data analysis and revised the manuscript. Ms Ridge contributed to the recruitment of participants, the data acquisition and revised the manuscript. Dr Smith, Dr Ruddle, Prof Elder, A/Prof Hewitt, Prof Goldberg, Prof Morgan and A/Prof Landers contributed to the characterisation of participants, the data acquisition and revised the manuscript. Prof Mackey contributed to the study concept and design, data acquisition and revised the manuscript. Dr Dubowsky contributed to the genetic studies, data analysis and interpretation and revised the manuscript. A/Prof Burdon contributed to the data analysis and interpretation and revised the manuscript. Prof Craig contributed to the study concept and

design, data acquisition, analysis and interpretation and revised the manuscript.

The manuscript is freely available at:
https://jamanetwork.com/journals/jamaophthalmology/fullarticle/2289998

Occurrence of *CYP1B1* mutations in juvenile open-angle glaucoma with advanced visual field loss

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ABSTRACT

Importance: Juvenile open-angle glaucoma (JOAG) is a severe neurodegenerative eye disorder in which most of the genetic contribution remains unexplained.

Objective: To assess the prevalence of pathogenic *CYP1B1* sequence variants in an Australian cohort of patients with JOAG and severe visual field loss.

Design, Setting and Participants: For this cohort study, we recruited 160 patients with JOAG classified as advanced (n = 118) and nonadvanced glaucoma (n = 42) through the Australian and New Zealand Registry of Advanced Glaucoma from January 1, 2007, through April 1, 2014. Eighty individuals with no evidence of glaucoma served as a control group. We defined JOAG as diagnosis before age 40 years and advanced JOAG as visual field loss in 2 of the 4 central fixation squares on a reliable visual field test. We performed direct sequencing of the entire coding region of *CYP1B1*. Data analysis was performed in October 2014.

Main outcome and measures: Identification and characterization of *CYP1B1* sequence variants.

Results: We identified 7 different pathogenic variants among 8 of 118 patients with advanced (6.8%) but none among the patients with nonadvanced JOAG. Three patients were homozygous or compound heterozygous for *CYP1B1* pathogenic variants, which provided a likely basis for their disease. Five patients were heterozygous. The allele frequency among the patients with advanced JOAG (11 in 23 [4,7%]) was higher than among our controls (1 in 160 [0.6%]; *P* = .02; odds ratio, 7.8 [95% CI, 0.02-1.0]) or among the control population from the Exome Aggregation Consortium database (2946 of 122960 [2.4%]; *P* = .02; odds ratio, 2.0 [95% CI, 0.3-0.9]). Individuals with *CYP1B1* pathogenic variants, whether heterozygous or homozygous, had worse mean (SD) deviation on visual fields (-24.5 [5.1] [95% CI, -31.8 to -17.2] vs -15.6 [10.0] [95% CI, -17.1 to -13.6] dB; $F_{1,126}$ = 5.90; *P* = .02; partial η_p^2 = 0.05) and were younger at diagnosis (mean [SD] age, 23.1 [8.4] [95% CI, 17.2-29.1] vs 31.5 [8.0] [95% CI, 30.1-33.0] years; $F_{1,122}$ = 7.18; *P* = .008; partial η_p^2 = 0.06) than patients without *CYP1B1* pathogenic variants.

Conclusions and relevance: Patients with advanced JOAG based on visual field loss had enrichment of *CYP1B1* pathogenic variants and a more severe phenotype compared with unaffected controls and patients with nonadvanced JOAG.

AT A GLANCE

- This is the first study, to our knowledge, to assess the prevalence of *CYP1B1* pathogenic variants with juvenile open-angle glaucoma (JOAG) and severe visual field loss.
- *CYP1B1* allele frequency was higher in patients with advanced compared with nonadvanced JOAG and unaffected controls.
- *CYP1B1*-positive individuals had worse mean deviation on visual field test results and were younger at diagnosis than *CYP1B1*-negative individuals.
- *CYP1B1* pathogenic variants explained approximately 2.5% of patients with advanced JOAG but 11% of those diagnosed before age 25 years.
- The study suggests that at-risk individuals should be identified early through genetic testing for adequate glaucoma intervention and to prevent irreversible blindness.

INTRODUCTION

The term glaucoma describes a heterogeneous group of neurodegenerative eye disorders characterized by cupping of the optic nerve and typical visual field defects. Glaucoma is one of the leading causes of irreversible blindness worldwide[1] and affects 3% of the Australian population older than 50 years.[2] Primary open-angle glaucoma (POAG [phenotype OMIM 137760]) is the most common type of glaucoma in which the anterior chamber angle is open and is often, but not always, associated with high intraocular pressures (IOP). We can subclassify POAG into early and late onset; early-onset disease is termed *juvenile open-angle glaucoma* (JOAG) and is defined arbitrarily by onset before age 30 to 40 years.[3,4] Primary open-angle glaucoma is a treatable condition, and therapeutic and/or surgical interventions can minimize the loss of visual function.[5-7]

Family history is one of the strongest risk factors for POAG. First-degree relatives of affected individuals have a risk of developing glaucoma that is 9 times greater than that of the general population.[8] Primary open-angle glaucoma displays a strong heritability but is genetically heterogeneous. The *MYOC* gene (OMIM 601652) was the first gene identified as causative[9] and accounts for the most cases. The Australian and New Zealand Registry of Advanced Glaucoma (ANZRAG)[10] has previously shown that mutations in *MYOC*, which are inherited in an autosomal dominant fashion, account for 4% of advanced POAG cases and 17% of advanced JOAG cases. Pathogenic variants in the *CYP1B1* gene (OMIM 601771) were first associated with primary congenital glaucoma (PCG; OMIM 231300)[11] and are inherited in an autosomal recessive fashion. Primary congenital glaucoma is a much rarer condition than POAG[12] that results from a developmental defect of the aqueous filtration system and generally manifests in the neonatal or early infantile period.[13] The *CYP1B1* gene is a member of the cytochrome P450 superfamily and is involved in the metabolism of endogenous and exogenous substrates. The mechanism by which the gene causes glaucoma is still unknown, but investigators[14-16] have hypothesized that *CYP1B1* pathogenic variants may affect the enzymatic activity or the substrate specificity of the protein, thereby influencing the concentration of metabolites that modulate the expression of targeted genes essential during development.

Pathogenic variants of *CYP1B1* have been associated with JOAG among different populations with variable frequencies.[17-28] However, most studies involving cases of JOAG included small cohorts, and none assessed severe cases as defined by their visual field loss. In this study, we investigated *CYP1B1* in a large cohort of patients with severe JOAG to

assess the prevalence of mutations in this gene in the Australian population with JOAG and to evaluate whether it was more prevalent in cases with severe visual field loss.

METHODS

Recruitment of Participants

We obtained ethics approval for this study from the Southern Adelaide Clinical Human Research Ethics Committee. The study was conducted in accordance with the revised Declaration of Helsinki. Participants were recruited from January 1, 2007, through April 1, 2014.

Individuals with advanced and nonadvanced JOAG were recruited through the ANZRAG as described previously.[10,29] In brief, the visual field at recruitment was used to classify participants as having advanced or nonadvanced disease. *Advanced JOAG* was defined as visual field loss in the worse eye related to glaucoma with at least 2 of the 4 central fixation squares having a pattern standard deviation of less than 0.5% on a reliable Humphrey 24-2 field result or a mean deviation (MD) of less than -22dB. *Nonadvanced JOAG* was defined by glaucomatous visual field defects on a reliable field test that did not meet the criteria for advanced POAG, with corresponding optic disc rim thinning. *Juvenile open-angle glaucoma* was defined as a diagnosis after age 4 years but before age 40 years.[30] Patients with buphthalmus/congenital glaucoma or secondary glaucoma were excluded from this analysis. Ethnicity was self-reported by participants and classified as white or other.

Patients were referred by their ophthalmic practitioner. Informed written consent and a blood sample for DNA extraction purposes were obtained. Clinical information was collected by the patient's usual clinical ophthalmologist. Control participants were examined by an ophthalmologist (J.E.C.) to determine that they had no evidence of glaucomatous optic nerve damage and had an IOP no greater than 22 mm Hg. Control individuals were selected to be older than cases.

Genetic testing

Sequence variant analysis of *CYP1B1* was performed in 2014 through the National Association of Testing Authorities-accredited laboratories of SA Pathology at the Flinders Medical Centre (Adelaide, Australia). The entire coding region of the *CYP1B1* gene was sequenced. Each polymerase chain reaction (PCR) analysis was performed using 100 ng of purified genomic DNA in a reaction mix containing 1.5mM magnesium chloride, 200μM each

deoxynucleotide, 0.5μM each primer (Table 1), 1 U of DNA polymerase (Platinum *Taq*; Invitrogen) and 1x PCR reaction buffer (Platinum *Taq*) in a final volume of 25 μL. The PCR steps included initial denaturation at 95˚C for 5 minutes, 40 cycles of denaturation at 94˚C for 30 seconds, annealing at 58˚C for 30 seconds, elongation at 72˚C for 30 seconds, and final elongation at 72˚C for 1 minute on a thermal cycler (Veriti; Life Technologies).

Residual primers and deoxynucleotides were removed by incubating 10 μL of PCR products with 5 U of *Escherichia coli* (Exonuclease I; Biolabs) and 1 U of shrimp alkaline phosphatase (USB Corporation). We used cleaned PCR amplicons to perform bidirectional cycle sequencing reactions (BigDye Terminator; Life Technologies) with a genetic analyser 3130XL; Applied Biosystems, Life Technologies).

We performed detection of sequence variants using commercially available software (Mutation Surveyor, version 3.10; SoftGenetics LLC). All forward and reverse chromatograms were assembled against the National Center for Biotechnology Information genomic reference sequence NT_022184.16 (GRCh38) containing *CYP1B1*. We used 2 software programs (SIFT [Ssorting intolerant from tolerant; http://sift.jcvi.org] and PolyPhen-2 [http://genetics.bwh.harvard.edu/pph2]) to predict the potential effect of amino acid substitutions on the protein. We used the Homologene system (http://www.ncbi.nlm.nih.gov/homologene) to assess the conservation among mammalian species. Allelic frequencies were compared with the population frequencies from the Exome Aggregation Consortium (ExAC) (http://exac.broadinstitute.org).

Mutation screening of *CYP1B1* for the controls used exome sequencing data. Exome capture was performed using a DNA library (SureSelect system; Agilent). Paired-end libraries underwent sequencing (Illumina HiSeq 2000; Macrogen). All exomes had a mean read depth of at least 60 times, and more than 97% of the genome was covered at 10 times or better. Reads were mapped to the human reference genome (hg19) using the BWA (Burrows-Wheeler Aligner) software package (http://bio-bwa.sourceforge.net), and duplicates were marked and removed using Picard sequencing tools (https://broadinstitute.github.io/picard/picard-metric-definitions.html). Variants were called using the Samtools suite of programs (http://www.htslib.org/) and annotated with ANNOVAR software tool (http://annovar.openbioinformatics.org/en/latest/). Variants were described according to the recommendations of the Human Genome Variation Society (http://www.hgvs.org) and referenced against the ExAC database. All called variants were inspected. They were considered pathogenic if they were predicted to be damaging by SIFT

or Polyphen-2 and if they had a minor allele frequency of less than 1%.

Statistical analysis

Data analysis was performed in October 2014. We used commercially available software (PASW Statistics, release 18.0.1.2009; SPSS, Inc) for statistical analyses. Data are presented as mean (SD) unless otherwise indicated. We used the Fisher exact and Mann-Whitney tests for the assessment of differences in nonparametric data. We performed multivariate analysis of variance to investigate differences between advanced and nonadvanced cases and between cases positive and negative for *CYP1B1*pathogenic variants. The following 2 groups of correlated dependent variables were identified: IOP, central corneal thickness, and age at diagnosis (group 1) and cup-disc ratio, MD, and trabeculectomy (group 2). When variables were not normally distributed, we applied appropriate transformation. However, age at diagnosis, cup-disc ratio, and MD were not normally distributed and could not be transformed.

RESULTS

We recruited 160 patients with JOAG who met the entry criteria. The demographic details are presented in Table 2. Advanced JOAG was documented in 118 patients (73.8%) and nonadvanced JOAG, in 42 patients (26.2%). The mean age at recruitment was 56.0 (18.1 [range, 10-86]) years. As expected, we found a statistical difference between advanced and nonadvanced JOAG in the group 2 combined dependent variables ($F_{3,124}$ = 60.4; P < .001; Pillai trace = 0.59; partial η_p^2 = 0.59). Using a Bonferroni-adjusted α level of .017, the mean (SD) cup-disc ratio (0.92 [0.08] [95% CI, 0.90-0.94] vs 0.74 [0.10] [95% CI, 0.71-0.77]; $F_{1,126}$ = 106.7; P < .001; η_p^2 = 0.46), the MD on visual field test (-20.2 [7.9] [95% CI, -22.0 to -19.2] vs -4.0 [3.6] [95% CI, -6.5 to -2.2] dB; $F_{1,126}$ = 160.0; P < .001; η_p^2 = 0.56), and prevalence of trabeculectomy (74.5% [95% CI, 0.7%-0.8%] vs 33.3% [95% CI, 0.2%-0.5%]; $F_{1,126}$ = 25.5; P < .001, η_p^2 = 0.17) were all higher in the patients with advanced compared with nonadvanced JOAG. The age at diagnosis, highest IOP, mean central corneal thickness, and family history were not different between the 2 groups. Nineteen patients carried an *MYOC* pathogenic variant.

We included 80 controls (all white). Forty-four controls (55%) were women, and their mean age at recruitment was 65.7 (18.1) years.

Sequencing of the *CYP1B1* gene identified 15 different sequence variants among the

patients with JOAG. Six variants were known polymorphisms (eTable in the Supplement). Their allelic frequency did not differ between the patients and controls. The other 9 sequence variants were identified among 10 patients with advanced JOAG (Table 3 and Table 4). No *CYP1B1* variants were identified in the patients with nonadvanced JOAG, nor were any variants identified in individuals carrying an *MYOC* pathogenic variant.

Two sequence variants were not present in ExAC database or the literature and were therefore considered novel. The variant p.A64V (c.191C>T) was predicted to be benign by SIFT and PolyPhen-2. Alanine is conserved between mammals, bird, and fish sequences but not amphibian sequences (eFigure in the Supplement). The variant p.M244I (c.732G>T) was predicted to be benign using SIFT and possibly damaging using PolyPhen-2. This position is occupied by methionine or leucine among different species (eFigure in the Supplement). Isoleucine has similar biophysical and chemical properties to methionine and leucine. Based on this evidence, these 2 sequence variants were considered unlikely to be pathogenic.

The other 7 sequence variants were all considered pathogenic based on the findings of mutation prediction software, conservation among species and previously published literature (Table 3). Therefore, 8 of 160 patients with JOAG (5.0%), including 8 of 118 patients with advanced JOAG (6.8%), had *CYP1B1* pathogenic variants. Three patients carried 2 pathogenic sequence variants. Patient AG0180 was compound heterozygous for p.A179Rfs*18 and p.E387K, patient AG1751 was homozygous for p.R290Pfs*37, and patient AG1791 was homozygous for p.A237E. Two other sequence variants were present in the heterozygous state in 1 patient each (p.Y81N and p.E229K), and p.R368H was present in 3 individuals.

Apart from the 6 known polymorphisms described above, 6 other variants were identified in controls. They consisted of 4 synonymous (p.L47L, p.G236G, p.V243V, and p.L360L), 1 non-synonymous (p.A443G), and 1 nonsense (p.W57*) sequence variant. A previous report [24,28] described p.A443G as a polymorphism. We identified p.W57* in the heterozygous state in 1 control. As a known pathogenic variant, it has been reported in association with other known mutations in several patients with PCG [24,28,31,32]. This finding equates to an allele frequency of *CYP1B1* pathogenic variants of 1 in 160 (0.6%) among controls, which is decreased compared with patients with advanced JOAG cases (11 in 236 [4.7%]) (*P* =.02; odds ratio, 7.8 [95% CI, 0.02-1.0]).

In the ExAC database, 139 *CYP1B1* variants were predicted to be pathogenic, occurring

1262 times in 39 657 white individuals (allele frequency of 1.6%). Among all ethnicities, 147 pathogenic variants occurred 2946 times in 61 480 individuals (allele frequency of 2.4%). Among the 8 pathogenic variants identified in our patients and controls, 7 were present in the ExAC database. Our findings showed a higher prevalence of *CYP1B1* pathogenic variants in patients with advanced JOAG compared with those in white individuals in the ExAC database (P < .001; odds ratio, 3.4 [95% CI, 0.2-0.6]) and when including all ethnicities (P = .02; adds ratio, 2.0 [95% CI, 0.3-0.9]).

Clinical variables were compared between the patients with JOAG with and without *CYP1B1* pathogenic variants (Table 5). We found a difference in the combined group 1 of dependent variables ($F_{3,120}$ = 3.15; P = .03; Pillai Trace, 0.073; η_p^2 = 0.073) and in group 2 of dependent variables ($F_{3,126}$, = 3.51; P = .02; Pillai Trace = 0.078; η_p^2 = 0.078). Using a Bonferroni-adjusted α level of .017, patients with *CYP1B1* pathogenic variants were younger at diagnosis (mean [SD] age, 23.1 [8.4] [95% CI, 17.2-29.1] vs 31.5 [8.0] [95% CI, 30.1-33.0] years; $F_{1,122}$ = 7.18; P = .008; η_p^2 = 0.06) and had worse mean [SD] deviation on visual fields tests (-24.5 [5.1] [95% CI, -31.8 to -17.2] vs -15.6 [10.0] [95% CI, -17.1 to -13.6] dB; $F_{1,126}$ = 5.90; P = .02; partial η_p^2 = 0.05) than those without *CYP1B1* pathogenic variants. Individuals with the *CYP1B1* variants also required trabeculectomy more often (100%) than those without *CYP1B1* variants (56.6%). No differences in IOP, family history, cup-disc ratio, or central corneal thickness were identified.

DISCUSSION

Several studies[17-28] have indicated that *CYP1B1* sequence variants may play a role in JOAG and POAG among different populations. However, all studies including cases of JOAG were small, with the largest reporting 61 cases.[25] To our knowledge, the present study is the largest cohort of patients with JOAG assessed for *CYP1B1* sequence variants and is the first to include a cohort of severely affected cases based on visual field loss.

Our findings showed an enrichment of *CYP1B1* pathogenic variants among patients with advanced JOAG (6.8%) compared with a control group without glaucoma and compared with the general population. No *CYP1B1* mutations were identified in the group with nonadvanced JOAG. The ExAC database reflects the general population that has not been examined for glaucoma, which means that a small percentage might have or might develop JOAG. Our findings are similar to those reported in other white populations (8-10%)[24,28] but lower than those in studies from India (12-20%),[19,22] Iran (17-24%),[20,21] or Saudi Arabia (93%)[26] and higher than those reported in Taiwan (3%)[25] or Japan (0%).[33] The prevalence is

expected to be higher in populations with high rates of consanguinity or common founder mutations, such as Iran or Saudi Arabia. However, one has to be cautious when drawing comparisons because most studies included patients with a younger cutoff at diagnosis for JOAG[19,22,25,33] or patients with high-tension glaucoma only.[22,24-26,33] In this study, all individuals diagnosed as having JOAG before age 40 years were included regardless of their IOP or family history.

Three individuals carried 2 *CYP1B1* mutations. One individual was compound heterozygous for p.A179Rfs*18 and p.E387K. This combination has been reported to segregate in 2 Spanish siblings, with one diagnosed as having PCG at birth and the other diagnosed as having JOAG at 10 years of age.[27] The other 2 individuals were homozygous. The p.A237E variant has only been reported in the compound heterozygous state in patients with PCG,[27,34] whereas p.R290Pfs*37 has been reported in the compound heterozygous and homozygous states in patients with PCG.[27,34-37] Neither variant has been found in conjunction with JOAG or POAG. These 3 individuals all received the diagnosis in their third decade of life and do not display the characteristic features of PCG. Genetic modifiers might account for the different phenotypes and ages at onset in this autosomal recessive model.

The ANZRAG study[10] previously demonstrated that *MYOC* pathogenic variants accounted for 17% of advanced JOAG in patients from the Australian population. In comparison, *CYP1B1* variants were present in 6.8% of patients with advanced JOAG and in the homozygous/compound heterozygous state in 3 patients (2.5%). However, when considering individuals diagnosed as having advanced JOAG by 25 years of age, 6 of 27 (22%) carried an *MYOC* pathogenic variant and 3 of 27 (11%) carried 2 *CYP1B1* pathogenic variants, all likely to account for their disease. Therefore, our results show that these 2 genes together can explain up to one-third of selected JOAG cases based on severity and younger age at diagnosis.

Five individuals were heterozygous for *CYP1B1* mutations. How heterozygous *CYP1B1* pathogenic variants are associated with JOAG and whether *CYP1B1* acts as a causative gene or more likely as a contributing modifier gene, remain unclear. Carrier parents of individuals with *CYP1B1*-enriched PCG have not been shown to be at a higher risk for developing glaucoma, which suggests that variants in the heterozygous state are not sufficient to cause glaucoma. However, Vincent et al[17] described a family with *MYOC* and *CYP1B1* mutations. Among these individuals, those carrying the *MYOC* variant only had a later age at diagnosis than those carrying both *MYOC* and *CYP1B1* variants, suggesting that

CYP1B1 may act as a gene modifier for *MYOC* in patients with JOAG. In our large cohort, no individual carried mutations in *CYP1B1* and *MYOC*, suggesting that this specific combination is an extremely rare cause of disease.

All *CYP1B1* pathogenic variants were identified in the group with advanced JOAG, the diagnosis of which was based on central visual field loss. Presumably, a small proportion of patients with JOAG that has not yet progressed to advanced disease will have *CYP1B1* mutations; however, a larger study will be required to detect these mutations. Our findings show that individuals with *CYP1B1* pathogenic variants had a statistically worse MD on a visual field test results and received a diagnosis at a younger age. They all required trabeculectomy to control their glaucoma compared with only 56.6% in the non *CYP1B1* group. A few studies previously reported genotype/phenotype correlations; 1 study[18] found the age at diagnosis to be younger among patients with POAG and *CYP1B1* variants, whereas 2 others[22,23] did not find differences in the age at diagnosis, IOP, disc changes, and visual field defects when comparing both groups . One possible explanation for the severity of glaucoma in *CYP1B1* carriers is that most do not have risk factors, such as family history, to prompt a diagnosis in the early stages of disease. Among the 3 patients with 2 *CYP1B1* mutations, two had no family history of glaucoma and the relatives of the third received a diagnosis at a much later age. This finding is an important contrast to *MYOC*-associated glaucoma, in which the rate of positive family history is extremely high and severity of disease typically matches that of other family members.

CONCLUSIONS

Our results showed an enrichment of *CYP1B1* mutations in patients with advanced JOAG compared with patients with nonadvanced JOAG and controls. This finding is in keeping with those of previous smaller studies among white individuals. It reinforces the hypothesis that JOAG is a complex disorder displaying genetic heterogeneity and that heterozygous *CYP1B1* variants may act as a modifier to some unknown other genetic factors. We also demonstrated that individuals with *CYP1B1* mutations tend to have more severe glaucoma than patients without the *CYP1B1* mutations. Because these individuals are at high risk for preventable blindness, early identification through genetic testing for adequate glaucoma intervention is important.

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Table 1. Primers used for amplification of the *CYP1B1* gene.

Exons	Primer sequence, Forward/Reverse	Size, Base Pair
2.1	5'-CACCCAACGGCACTCAGTC-3'	646
	5'-CAGCAGCGCCACCAGCTC-3'	
2.2	5'-CACTACTCGGAGCACTGGAAGG-3'	713
	5'-ACTCAGCATATTCTGTCTCTACTCC-3'	
3	5'-AGCCTATTTAAGAAAAAGTGGAA-3'	761
	5'-CTGAATTTTACTCCTCATCTCC-3'	

Table 2. Demographic details of patients with primary JOAG

Characteristic	Patient group		
	All (n = 160)	Advanced JOAG (n = 118)	Nonadvanced JOAG (n = 42)
Age at diagnosis, mean (SD) [range], y	31.0 (8.4) [11-40]	31.3 (8.5) [11-40]	30.3 (8.3) [11-40]
Sex			
Female	73 (45.6)	45 (38.1)	28 (66.7)
Male	87 (54.4)	73 (61.9)	14 (33.3)
Ethnicity			
White	130 (81.3)	92 (78.0)	38 (90.5)
Other	30 (18.7)	26 (22.0)	4 (9.5)
Family history			
Positive	117 (73.1)	82 (69.5)	35 (83.3)
Negative	42 (26.3)	35 (29.7)	7 (16.7)
Unknown	1 (0.6)	1 (0.8)	0 (0.0)

Table 3. *CYP1B1* sequence variants identified in this study in juvenile open-angle glaucoma patients and controls

Exon Location	Nucleotide change	Amino acid change	Reference SNP No.	SIFT score[a]	PolyPhen-2 prediction, HumDiv	Conserved[b]	Allelic Frequency in ExAC, %	Previously reported ethnicity
2	c.171G>A	p.W57*	rs72549387	NA	NA	NA	0.00041	White [24,28,31,32,34,38,39] Hispanic [40]
2	c.241T>A	p.Y81N	rs9282671	0.00	Probably damaging	Yes	0.00642	White [18,23,24,27,28,32,41] Indian [22]
2	c.535delG	p.A179Rfs*18		NA	NA	NA	0.00004	White [31,42] Hispanic [27,28,40,41,43,44] Northern African [38,45,46]
2	c.685G>A	p.E229K	rs57865060	0.00	Benign	Yes	0.01423	White [18,23,24,28,31,38] Middle Eastern [21,37,47,48] Indian [19,22,49-51]
2	c.710C>A	p.A237E		0.00	Probably damaging	Yes	NA	White [27,34]
2	c.868dupC	p.R290Pfs*37	rs67543922	NA	NA	NA	0.00005	White [34,42] Hispanic [27] Middle Eastern [11,35-37,52]
3	c.1103G>A	p.R368H	rs79204362	0.00	Probably damaging	Yes	0.00616	Asian [53] White [18,24,31,34] Hispanic [27,28,40] Indian [19,22,49-51,54] Middle Eastern [20,21,37,48,55,56]
3	c.1159G>A	p.E387K	rs55989760	0.00	Probably damaging	+	0.00034	White [18,31,32,34,35,38,39,57-59] Hispanic [27,35,40,59,60]

ExAC, Exome Aggregation Consortium database; JOAG, juvenile open-angle glaucoma; NA not applicable; SIFT, sorting intolerant from tolerant; SNP, single-nucleotide polymorphism.

[a] score of less than 0.05 is considered damaging

[b] Indicates among mammalian species.

Table 4. Clinical details of patients with JOAG and *CYP1B1* sequence variants.

Patient No.	Mutation	Sex	Country of origin	Age at diagnosis, y	Family history	Dependent variable, OD; OS				Surgery, RE/LE
						Highest IOP, mmHg	BCVA	CDR	CCT, µm	
AG0180	p.A179Rfs*18/ p.E387K	M	Australia	24	Yes	40;50	20/20;20/200	0.6;0.95	585;578	Yes/Yes
AG0521	p.Y81N	M	Ireland/ Germany	25	No	29;23[a]	20/125;20/25	0.95;0.95	434;433	Yes/Yes
AG0654	p.R368H	F	Australia	36	Yes	28;13[a]	20/20;20/20	0.4;0.3	541;536	Yes/Yes
AG1196	p.R368H	M	Australia	11	Yes	31;37	20/15;CF	NA;1.0	NA	NA
AG1427	p.E229K	M	Denmark	38	No	NA	20/30;20/40	1.0;1.0	NA	Yes/Yes
AG1751	p.R290Pfs*37/ p.R290Pfs*37	F	Italy	16	No	21;16[a]	20/40;20/80	0.8;0.9	627;624	Yes/Yes
AG1791	p.A237E/ p.A237E	M	Italy	20	No	48;38	20/60;20/25	0.95;0.8	675;674	Yes/Yes
AG1858	p.R368H	F	India	30	Yes	23;25[a]	LP;20/20	1.0;0.9	564;557	Yes/Yes
KSA008	p.W57*	M	Australia		No	12;12	20/30;20/30	0.5;0.4	684;575	No/No

BCVA, best corrected visual acuity; CCT, central corneal thickness; CDR, cup-disc ratio; IOP, highest intraocular pressure; JOAG; juvenile open-angle glaucoma; LP, light perception; NA, not available

[a] The IOP at diagnosis is not available

Table 5. Comparison of clinical details between patients with JOAG with and without *CYP1B1* pathogenic sequence variants.

	CYP1B1, variant finding (n = 118)[a]	
Variable	positive	negative
No. (%) of patients	8 (6.8)	110 (93.2)
Age at diagnosis, y	23.1 (8.4)	31.5 (8.0)
IOP, mm Hg	34.0 (11.3)	31.3 (11.2)
Family history positive, %	50.0 (5.4)	75.0 (4.4)
CDR	0.94 (0.07)	0.87 (0.12)
CCT µm	566.7 (75.5)	531.4 (39.5)
Mean deviation, dB[b]	-24.5 (5.1)	-15.6 (10.0)
Trabeculectomy, %	100.0 (0.0)	56.6 (4.9)

CCT, central corneal thickness; CDR, cup-disc ratio; IOP, intraocular pressure, JOAG, juvenile open-angle glaucoma.

[a] Unless otherwise indicated, data are expressed as mean (SD).

[b] Obtained from visual field tests.

2.6. Clinical utility of predictive genetic testing for *MYOC*

Predictive genetic testing for *MYOC*-associated glaucoma has the potential to identify asymptomatic at-risk individuals. There is evidence supporting clinical validity and patients' acceptance for the test [376]. However, no study examined the clinical benefits and utility of *MYOC* predictive genetic testing.

In this study, I examined the difference in disease severity at the time of presentation to an ophthalmology clinic of patients with *MYOC* variants between those who presented through the classic clinical pathway (Clinical cases) and those who were examined following genetic results (Genetic Cases). I collected data for 73 participants including 43 Clinical cases and 30 Genetic cases. I showed that Genetic cases were significantly younger at presentation than Clinical Cases. The majority of Genetic cases were asymptomatic at presentation (83%) or had early signs of POAG (17%) whereas half of the Clinical cases had early signs of POAG (44%) and the other half already had POAG (56%), including half with severe POAG (28%). All clinical parameters related to glaucoma were better among Genetic cases compared with Clinical cases. This study showed the ability of cascade genetic testing for glaucoma to identify gene carriers at high risk of developing glaucoma before they exhibit symptoms of the disease. The findings from my research contribute to closing the gap in knowledge regarding the clinical utility of genetic testing for glaucoma and are relevant when discussing risks and benefits of genetic testing with at-risk family members.

Contribution statement

Ms Souzeau was primarily responsible for the design, conception, data collection and analysis of the study as well as the drafting and submission of the manuscript. Dr Tram and Dr Whitney contributed to data collection and revised the manuscript. Dr Ruddle, Prof Graham, Prof Healey, Prof Goldberg, Prof Mackey and A/Prof Hewitt contributed to the characterisation of participants and revised the manuscript. A/Prof Burdon contributed to data analysis and revised the manuscript. Prof Craig contributed to the conception, design and supervision of the study, characterisation of participants, data analysis and revised the manuscript.

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Myocilin Predictive Genetic Testing for Primary Open-Angle Glaucoma Leads to Early Identification of At-Risk Individuals

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Purpose: To assess the difference in severity of disease in primary open-angle glaucoma (POAG) patients with a *Myocilin* (*MYOC*) disease-causing variant who presented through normal clinical pathways (Clinical cases) versus those who were examined following genetic testing (Genetic cases).

Design: Retrospective clinical and molecular study.

Participants: Seventy-three *MYOC* mutation carriers identified through the Australian and New Zealand Registry of Advanced Glaucoma.

Methods: Individuals were classified based on how they first presented to an ophthalmologist: Clinical cases were referred by their general practitioner or optometrist, and Genetic cases were referred following positive results from genetic testing for the previously identified familial *MYOC* variant (cascade genetic testing). All cases were then sub-classified into 4 groups (unaffected, glaucoma suspect, glaucoma, advanced glaucoma) according to the severity of disease at the time of their first examination by an ophthalmologist.

Main Outcome Measures: Glaucoma clinical parameters and age at presentation.

Results: At their first examination, 83% of Genetic cases were unaffected and 17% were glaucoma suspect, whereas among Clinical cases 44% were glaucoma suspect, 28% had glaucoma, and 28% had advanced glaucoma. Genetic cases were significantly younger at presentation than Clinical cases (40.6±12.5 vs. 47.5±16.7 years; $P = 0.018$). The mean highest intraocular pressure (32.2±9.7 vs. 17.6±3.6 mmHg; $P < 0.001$), cup-to-disc ratio (0.65±0.27 vs. 0.48±0.13; $P = 0.006$), and mean deviation on visual field testing (−10.0±10.3 vs. −1.2±1.2; $P < 0.001$) were all significantly worse in Clinical cases compared with Genetic cases. Individuals with common *MYOC* p.Gln368Ter variant were further analyzed separately to account for the phenotypic variability of different disease-causing variants. All findings remained significant after adjusting for the common *MYOC* p.Gln368Ter variant.

Conclusions: Our findings demonstrated that *MYOC* cascade genetic testing for POAG allows identification of at-risk individuals at an early stage or even before signs of glaucoma are present. To our knowledge, this is the first study to demonstrate the clinical utility of predictive genetic testing for *MYOC* glaucoma. *Ophthalmology 2016;■:1–7 © 2016 by the American Academy of Ophthalmology*

Glaucoma is the leading cause of irreversible and preventable blindness worldwide.[1] It refers to a heterogeneous set of progressive eye disorders characterized by optic disc cupping and corresponding visual field defects.[2] Primary open-angle glaucoma (POAG) is the most common subset and affects 3% of the Australian population above the age of 50 years.[3] Symptoms are usually not apparent until substantial irreversible damage has occurred. Therefore it is important to facilitate early diagnosis to prevent vision loss. Approximately half of those affected remain undiagnosed,[3,4] suggesting that current screening strategies lack efficacy.

POAG has a strong genetic component.[5] Individuals with an affected first-degree relative are 9 times more likely to develop glaucoma compared with the general population.[6] The *Myocilin* (*MYOC*) gene was the first gene associated with POAG.[7,8] *MYOC* disease-causing variants have been identified in 2% to 4% of unselected POAG patients and in 8% to 36% of POAG patients diagnosed before 40 years of age.[9–11] The variants are inherited in an autosomal dominant fashion with high penetrance, and carriers usually demonstrate elevated intraocular pressure (IOP) with a younger age at onset than POAG patients without *MYOC* variants.[10] There is an enrichment of *MYOC* variants in patients with advanced POAG, indicating a progression to a more severe disease, particularly without treatment.[10] Since the discovery of the *MYOC* gene in 1997, over 80 disease-causing variants have been described; the p.Gln368Ter variant is the most common.[12] Although clear genotype–phenotype correlations exist, inter- and intrafamilial phenotypic variability is also

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well acknowledged. The p.Gln368Ter variant has a variable age-related penetrance, with 50% of carriers diagnosed with glaucoma by 50 years of age.[13] Other disease-causing variants such as p.Pro370Leu and p.Gly367Arg are more severe and are associated with complete penetrance by 50 years of age.[9,10,14,15] The exact mechanism of *MYOC* variants leading to disease has not yet been fully elucidated. There is evidence to suggest that the abnormal gene protein products accumulate in the trabecular meshwork, contributing to outflow obstruction and ultimately increasing IOP.[16,17]

POAG is treated by lowering IOP; it is an effective strategy to slow progression or to prevent disease development, provided that patients are identified early in the disease process.[18,19] Lowering IOP is achieved with medical therapy, with laser, or with incisional surgical interventions. In the era of personalized medicine, the ability to predict disease development can allow tailored, specific treatment plans for individuals. Considering the difficulties in diagnosing glaucoma early, the younger age at onset for *MYOC* carriers compared with the general population, and the availability of effective preventive measures for treating POAG, genetic testing of relatives for the previously identified familial *MYOC* variant (cascade genetic testing) offers the potential to improve patient care and to prevent glaucoma blindness.[20,21] No previous study has examined the possible clinical benefits of *MYOC* cascade genetic testing.

Established in 2007, the Australian and New Zealand Registry of Advanced Glaucoma (ANZRAG) has gathered the largest cohort of patients with advanced glaucoma with the aim to identify genetic risk factors for glaucoma blindness.[22] The ANZRAG offers all participants with *MYOC* disease-causing variants the opportunity to have cascade genetic testing performed on all first-degree family members over the age of 18 years. Using the ANZRAG, this study aimed to assess the clinical utility of performing cascade genetic testing by comparing the disease severity of POAG patients with a *MYOC* disease-causing variant who presented through usual clinical care pathways with those who were examined following genetic testing.

Methods

Ethics committee approval was obtained through the Southern Adelaide and Flinders University Clinical Research Ethics Committee. The study adhered to the tenets of the Declaration of Helsinki and followed the National Health and Medical Research Council statement of ethical conduct in research involving humans. Informed consent was obtained from all participants.

Participant recruitment into the ANZRAG has been described previously.[22] Patients with all levels of glaucoma could be referred to the ANZRAG by clinicians. Advanced glaucoma was defined as central visual field loss related to glaucoma with at least 2 of the 4 central fixation squares having a pattern standard deviation probability less than 0.5% on a reliable Humphrey 24-2 field, or a mean deviation (MD) greater than −22 dB or, in the absence of visual field testing, best-corrected visual acuity (BCVA) worse than 20/200 owing to glaucoma. Participants also needed evidence of glaucoma in the less severely affected eye, characterized by glaucomatous visual field defects with corresponding optic disc rim thinning. Nonadvanced glaucoma was defined by glaucomatous visual field defects, with corresponding optic disc rim thinning, including an enlarged cup-to-disc ratio (CDR) (≥0.7) or CDR asymmetry (≥0.2) between both eyes. Glaucoma suspects had ocular hypertension, defined by IOP >21 mmHg, or had preperimetric glaucoma with no glaucomatous field changes.

Advanced and nonadvanced POAG cases recruited in the ANZRAG were screened for *MYOC* as previously described.[10] Glaucoma suspects who did not meet the advanced or nonadvanced criteria but had a combination of ocular hypertension, young age, and positive family history of glaucoma were also screened. Through the proband, cascade genetic testing and counseling were offered to first-degree family members older than 18 years who were either affected or unaffected.

This study retrospectively identified the manner in which patients with an underlying *MYOC* disease-causing variant first presented to an ophthalmologist and aimed to capture a clinical picture of the patients at the time of their first presentation. All participants with *MYOC* variants were categorized into 2 main groups: participants who were referred to an ophthalmologist for the first time by their general practitioner or optometrist (Clinical group) and those who were referred to an ophthalmologist for the first time following genetic testing results (Genetic group). Clinical parameters recorded at the time of participants' first presentation to an ophthalmologist were collected. The data collected included demographic information, IOP, CDR, central corneal thickness (CCT), BCVA, and reliable visual field testing parameters including MD. Once cases were classified according to their mode of presentation, they were further sub-classified into 4 groups according to the severity of disease at the time of their first presentation: normal, glaucoma suspect, nonadvanced glaucoma, and advanced glaucoma, as described previously.

Data were analyzed for all participants with *MYOC* disease-causing variants identified in the ANZRAG that satisfied inclusion criteria. BCVA was transformed into decimal fractions for analysis purposes. Owing to the phenotypic variations of underlying *MYOC* variants, additional analysis was also performed on participants carrying p.Gln368Ter only, as it is the most common disease-causing variant. Clinical data were analyzed with PASW Statistics, Rel. 18.0.1.2009 (SPSS Inc., Chicago, IL). Data are presented as mean ± standard deviation. The Mann-Whitney *U* test was used for the assessment of differences in nonparametric data and chi-square tests for categorical data.

Results

Ninety-seven participants with a *MYOC* disease-causing variant were identified in the ANZRAG. Of these, clinical details at presentation could be obtained for 73 participants (75%) included in the study. They consisted of 43 Clinical cases (59%) and 30 Genetic cases (41%). There were 39 (53%) female and 34 (47%) male patients. The mean current age was 60.9±17.7 years (range, 16−87 years) for Clinical cases and 44.7±11.9 years (range, 24−77 years) for Genetic cases. Genetic cases were significantly younger at presentation than Clinical cases (40.6±12.5 vs. 47.5±16.7 years; $P = 0.018$). At their first examination, 25 Genetic cases (83%) were unaffected and 5 (17%) were glaucoma suspect, whereas among Clinical cases 19 (44%) were glaucoma suspect, 12 (28%) had nonadvanced glaucoma, and 12 (28%) had advanced glaucoma (Fig 1). Among the Genetic cases, unaffected individuals were significantly younger compared with glaucoma suspects (42.5±10.4 vs. 55.8±13.7 years; $P = 0.037$).

The mean highest IOP (17.6±3.6 vs. 32.2±9.7 mmHg; $P < 0.001$), highest CDR (0.48±0.13 vs. 0.65±0.27; $P = 0.006$), worst MD (−1.2±1.2 vs. −10.0±10.3; $P < 0.001$), and worst BCVA (0.96±0.30 vs. 0.70±0.38; $P = 0.004$) were all significantly less severe among Genetic cases compared with Clinical

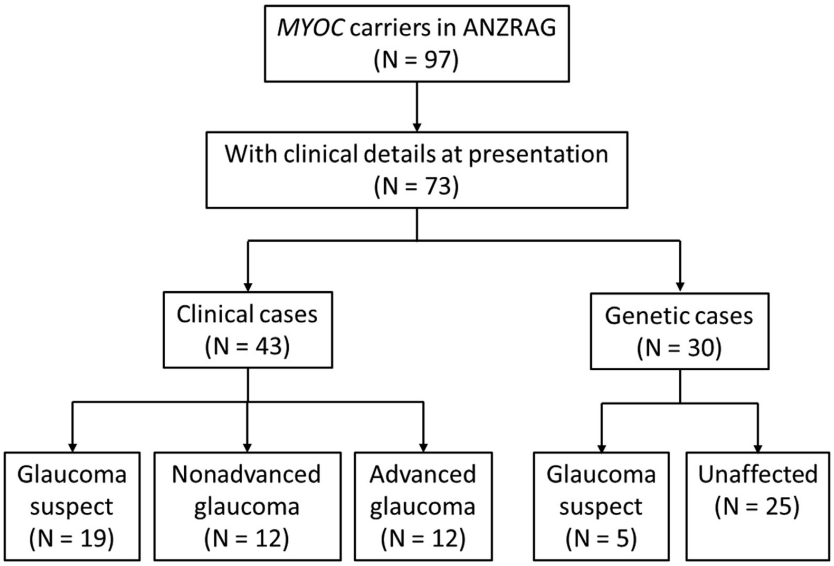

Figure 1. Diagram of the study showing the number of participants in the Clinical and Genetic groups and their glaucoma status at first presentation. Clinical cases were referred by their general practitioner or optometrist, and Genetic cases were referred following genetic test results. ANZRAG = Australian and New Zealand Registry of Advanced Glaucoma; MYOC = *Myocilin* gene.

cases (Fig 2). The mean CCT was similar between the groups (561.3±37.2 Genetic vs. 538.7±42.6 μm Clinical; $P = 0.52$). Elevated IOP at presentation was recorded for 91% (39/43) of Clinical cases vs. 10% (3/30) of Genetic cases. We conducted the same analyses including only 1 relative per family to account for the characteristics that individuals from the same family may share and obtained similar results (data not shown).

Probands and Siblings

We then analyzed separately the probands and their siblings, including 38 Clinical and 9 Genetic cases. The mean age at presentation was similar in both groups (48.29±17.0 years Clinical vs. 45.3±15.2 years Genetic; $P = 0.401$). At presentation, 16 were glaucoma suspect, 11 had nonadvanced glaucoma, and 11 had advanced glaucoma among Clinical cases, whereas 5 were unaffected and 4 were glaucoma suspect among Genetic cases.

The mean highest IOP (20.2±3.2 vs. 32.2±10.0 mmHg; $P < 0.001$), highest CDR (0.46±0.18 vs. 0.66±0.27; $P = 0.026$), and worst MD (−1.3±1.1 vs. −10.9±10.4; $P = 0.017$) were all significantly less severe among Genetic cases compared with Clinical cases. Although not significant, the worst BCVA was also less severe in Genetic cases compared with Clinical cases (0.91±0.27 vs. 0.70±0.39; $P = 0.128$). The mean CCT was significantly different between both groups (569.7±29.6 μm Genetic vs. 536.0±42.8 μm Clinical). Elevated IOP was reported for 92% (35/38) of Clinical cases versus 22% (2/9) of Genetic cases.

Probands and Offspring

Next, we analyzed probands and their offspring, comprising 35 Clinical and 21 Genetic cases. The mean age at presentation was significantly lower among Genetic (41.7±9.4 years) compared with Clinical cases (62.1±17.1; $P = 0.002$). Among the Clinical

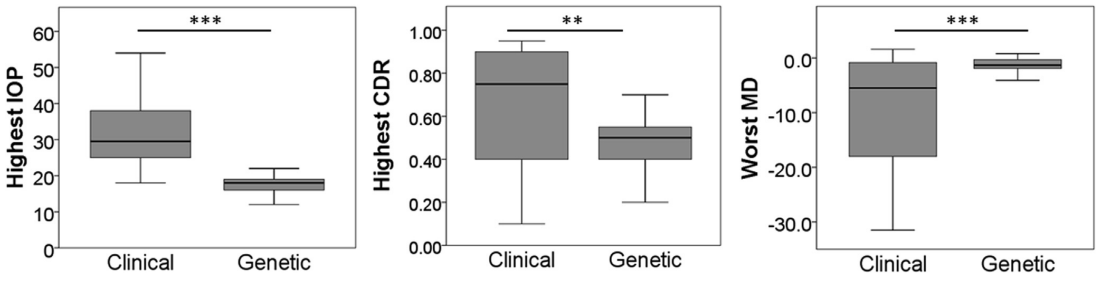

Figure 2. Comparison of the clinical characteristics between Clinical and Genetic cases with a *Myocilin* variant. CDR = cup-to-disc ratio; IOP = intraocular pressure; MD = mean deviation from a reliable visual field test. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.

cases, 14 were glaucoma suspect, 11 had glaucoma, and 11 had advanced glaucoma at presentation, whereas 20 Genetic cases were unaffected and 1 was a glaucoma suspect.

The mean highest IOP (16.5±3.1 vs. 32.2±9.8 mmHg; $P < 0.001$), highest CDR (0.49±0.11 vs. 0.67±0.26; $P = 0.004$), worst MD (−1.0±1.0 vs. −10.3±10.0; $P < 0.001$), and worst BCVA (1.01±0.31 vs. 0.71±0.38; $P = 0.003$) were all significantly less severe in Genetic cases compared with Clinical cases. The mean CCT was similar between both groups (553.5±42.0 vs. 533.5±44.4 μm; $P = 0.204$). Elevated IOP was recorded in 89% (31/35) of the Clinical cases, compared with 5% (1/21) of the Genetic cases.

Carriers of *MYOC* p.Gln368Ter

A total of 52 cases had the p.Gln368Ter variant, 71% of the total study population. Of the 52 p.Gln368Ter cases, 28 (54%) were Clinical cases and 24 (46%) were Genetic cases. The mean current age was 68.4±8.8 years (range, 53−87 years) for Clinical cases and 44.7±12.7 years (range, 24−77 years) for Genetic cases. The mean age at presentation was significantly younger among Genetic cases compared with Clinical cases (40.5±13.3 vs. 55.0±9.8 years; $P < 0.001$). Among Genetic cases, 19 were unaffected and 5 were glaucoma suspect at presentation, whereas 12 Clinical cases were glaucoma suspect, 8 had nonadvanced glaucoma, and 8 had advanced glaucoma.

The mean highest IOP (18.0±3.7 vs. 29.9±9.3 mmHg; $P < 0.001$), highest CDR (0.49±0.14 vs. 0.66±0.27; $P = 0.016$), worst MD (−1.3±1.2 vs. −9.2±10.0; $P = 0.010$), and worst BCVA (0.95±0.29 vs. 0.67±0.41; $P = 0.009$) were all significantly less severe among Genetic cases compared with Clinical cases with p.Gln368Ter (Fig 3). The mean CCT was significantly higher among Genetic cases compared with Clinical cases (569.4±32.5 vs. 530.1±40.8 μm; $P = 0.004$). Increased IOP at presentation was recorded for 86% (24/28) of Clinical cases vs. 13% (3/24) of Genetic cases. Figure 4 shows higher IOP and lower MD with a later age at presentation for Clinical cases compared with Genetic cases.

Response to Treatment

The IOP before and after treatment was available for 83% (35/42) of the glaucoma suspects and affected individuals included in the study who were receiving treatment. All individuals attained IOP within the normal range using IOP-lowering therapy. The mean highest IOP before treatment was 31.8±1.4 mmHg (range, 21−52 mmHg) versus 16.8±0.4 mmHg (range, 12−21 mmHg) after initiation of treatment ($P < 0.001$).

Discussion

Glaucoma can lead to irreversible blindness if left untreated and often remains undiagnosed until substantial damage has occurred. It is crucial to identify at-risk individuals at the earliest opportunity because there are medical and surgical treatment options that are effective for slowing down the progression of glaucoma or even preventing glaucoma from developing.[18,19] *MYOC* disease-causing variants exhibit a strong age-dependent penetrance, and affected individuals present with more advanced disease if not identified and treated early.[10] Despite evidence supporting clinical validity and patient acceptance for *MYOC* genetic testing,[20,21] there is a lack of outcome measures and evidence-based clinical utility for genetic testing for the monogenic forms of glaucoma. To our knowledge, this study is the first to investigate the clinical utility of cascade genetic testing for *MYOC* by examining the clinical parameters at time of presentation of *MYOC* carriers.

We showed that patients identified via cascade genetic testing presented 7 years younger than those identified following ophthalmic referral. The majority (83%) of carriers identified through genetic testing were asymptomatic at the time of presentation, whereas half of the patients who had an ophthalmic referral had early signs of glaucoma and the other half already had glaucoma, including 28% with advanced disease. All clinical parameters related to glaucoma (IOP, CDR, and MD on visual field test) were significantly worse at presentation among Clinical cases compared with Genetic ones.

We conducted separate analyses of probands/siblings and probands/offspring to evaluate whether the age difference affected our findings. As expected, the age at presentation was significantly younger in Genetic cases compared with Clinical cases within the probands/children group, whereas the age at presentation was similar between Clinical and Genetic cases within the probands/siblings group. There were fewer siblings than offspring in the Genetic group, which can be explained by a proportion of siblings already affected by glaucoma and not identified through genetic testing. In both analyses, the clinical parameters associated with glaucoma were significantly less severe among Genetic cases compared with Clinical cases. Of the siblings, 44% were identified as glaucoma suspect following genetic

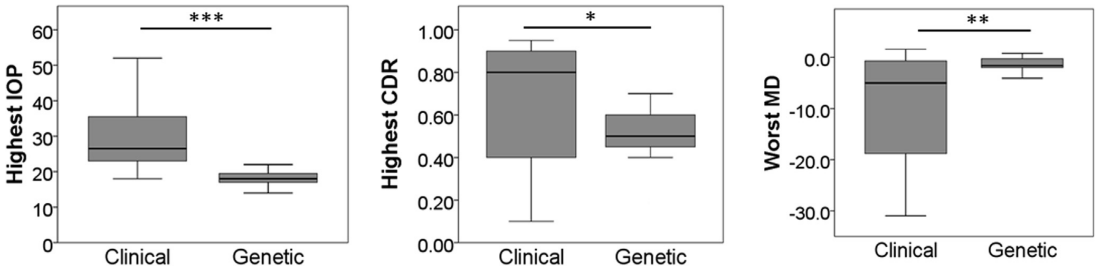

Figure 3. Comparison of the clinical characteristics between Clinical and Genetic cases with the p.Gln368Ter *Myocilin* variant. CDR = cup-to-disc ratio; IOP = intraocular pressure; MD = mean deviation from a reliable visual field test. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.

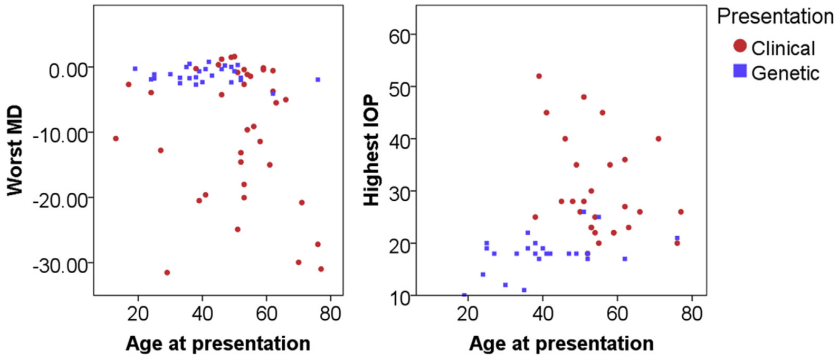

Figure 4. Clinical details in relation to the age at presentation between Clinical and Genetic cases with the p.Gln368Ter *Myocilin* variant. IOP = intraocular pressure; MD = mean deviation from a reliable visual field test.

testing results. However, the siblings in the Genetic group had better glaucoma parameters than the probands, despite the fact that they presented at a similar age as the probands and that almost half of them had early signs of glaucoma. These findings highlight the usefulness of cascade genetic testing irrespective of the age of the family members.

Genotype–phenotype correlations have been well described for *MYOC* variants.[10–12] To reduce the variability accounted for by disease-causing variants of different severity, we analyzed individuals carrying only the most common *MYOC* variant (p.Gln368Ter) separately. p.Gln368Ter is usually associated with a moderate severity and displays an age-related penetrance, with half of the carriers being diagnosed with glaucoma by 50 years of age and almost all carriers diagnosed by 75 years of age.[13] When p.Gln368Ter carriers only are considered, individuals diagnosed early because of more severe *MYOC* variants are excluded, as shown by the older age at presentation among p.Gln368Ter carriers. Our results showed that p.Gln368Ter carriers identified through genetic testing presented 15 years younger than those who presented clinically. They also show better clinical parameters at presentation, as illustrated by lower IOP, CDR, and MD on visual field test than their clinically diagnosed counterparts. Glaucoma suspects identified by ophthalmic presentation were on average in their early 50s, which is in accordance with the age-related penetrance for this variant. Unaffected individuals identified through genetic testing were on average in their late 30s (37.1±2.5 years), an age group where a minority of p.Gln368Ter carriers are affected. This shows the ability of cascade genetic testing to identify gene carriers before they exhibit symptoms of the disease.

Among the individuals carrying variants other than p.Gln368Ter, some had more severe disease with a younger age at glaucoma onset. In these families, we would expect cascade genetic testing to have similar positive outcomes if conducted at an early age, and we have previously discussed the benefits of a genetic testing approach for minors in these families.[23] Our numbers were too small to analyze this group separately in this study, but future studies should examine the clinical utility of genetic testing in individuals carrying *MYOC* variants associated with early glaucoma onset. Similarly, our findings could be extrapolated to other rarer, monogenic forms of the disease, such as *Optineurin* and *TBK1* glaucoma-associated variants. However, the utility of a genetic testing approach is currently less clear in the complex and more common forms of glaucoma that are the result of multiple genetic factors with small effect size.

Through our cascade testing program we make genetic testing available to all first-degree relatives, but we do not contact relatives directly, to promote autonomous and noncoercive decisions. This approach yields a response rate of 50%, which is similar to other adult-onset conditions with treatment options and high-penetrance genes, such as inherited cancers and cardiomyopathies.[24,25] Individuals with a family history are more likely to access screening for glaucoma.[26] However, in our cohort 77% (33/43) of individuals who presented clinically had a family history, including 67% (16/24) who presented with glaucoma. This suggests that family history may not be enough of a risk factor to diagnose at-risk individuals early. Additionally, we previously showed that the majority of newly identified *MYOC* carriers had never seen an eye specialist,[10] supporting genetic testing as an effective way to identify at-risk individuals in *MYOC* families in a more timely manner.

In this study, 25 individuals had no signs of glaucoma on examination following genetic results. These individuals were significantly younger than those identified as glaucoma suspects following genetic results. *MYOC* variants are highly penetrant: Age-related penetrance is complete at 50 years old for *MYOC* variants associated with an early age at onset[9,10,14,15] and almost complete at 75 years old for the common p.Gln368Ter variant.[13] Therefore, these unaffected individuals are expected to develop glaucoma at some stage. Interestingly, we are aware of 2 individuals who subsequently converted to glaucoma suspect in the Genetic group during follow-up. Long-term studies that follow at-risk asymptomatic individuals are still needed to assess clinical outcomes, the progression of the disease, and the best treatment strategies for *MYOC* carriers.

Cascade genetic screening for glaucoma is a promising avenue to prevent glaucoma blindness. A previous study demonstrated the acceptability of predictive genetic testing for *MYOC* glaucoma.[20] Data from the ANZRAG have recently shown that families perceived strong benefits to cascade testing, as it leads to the possibility of preventive measures.[21] We have previously shown that *MYOC* disease-causing variants are more prevalent in the advanced stages of glaucoma.[10] As a result, early diagnosis is important, as carriers may require earlier interventions and more aggressive management of their IOP. Our findings also confirm that *MYOC* carriers respond to IOP-lowering therapy. Personalized medicine using genetic information to predict disease development and to tailor preventative interventions for each patient is an evolving field.[27] Although current glaucoma therapies are effective in lowering IOP in patients with *MYOC* disease-causing variants, targeted therapies for *MYOC* glaucoma are emerging; studies have shown a reduction in the glaucomatous phenotype of *MYOC*-transgenic mice treated with topical ocular sodium 4-phenylbutyrate[28] and *MYOC*-transgenic mice with CRISPR-Cas9-mediated genome editing (Jain A, Zode G, Buge K, et al. CRISPR-Cas9 mediated genome editing of *Myocilin* in hereditary glaucoma. Presented at: ASHG Annual Meeting, October 7, 2015, Baltimore, MD). The identification of *MYOC* carriers will become even more important with the development of therapies targeted for *MYOC* glaucoma.

This study has some potential limitations. First, there might be a recruitment bias, as patients who are more likely to have undiagnosed glaucoma are also the ones who will not seek genetic testing and are less likely to be screened.[21] The ANZRAG recruits individuals with both advanced and nonadvanced POAG but has a recruitment bias toward more advanced disease, which could have resulted in an overestimation of the severity in the Clinical group. Second, this is a retrospective study, and clinical details at the time of initial diagnosis were missing for 25% of participants with a *MYOC* variant. Many of them had been diagnosed decades ago, and as such, records of the initial presenting details no longer existed or were irretrievable. However, a randomized clinical trial to study the efficacy of genetic testing for glaucoma leading to better visual outcome would be impossible to conduct. So although a retrospective study collecting clinical evidence has limitations, to our knowledge this is the first study to report such findings.

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Footnotes and Financial Disclosures

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Abbreviations and Acronyms:
ANZRAG = Australian and New Zealand Registry of Advanced Glaucoma; **BCVA** = best-corrected visual acuity; **CCT** = central corneal thickness; **CDR** = cup-to-disc ratio; **IOP** = intraocular pressure; **MD** = mean deviation; **POAG** = primary open-angle glaucoma.

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CHAPTER 3: GENETICS OF DEVELOPMENTAL GLAUCOMA

The different types of developmental glaucoma are all quite rare, but they are devastating conditions with major impact on patients and their families. The provision of a molecular diagnosis can greatly benefit the patients and their families in terms of understanding the natural course of the disease and monitoring potential complications, providing a mode of transmission, determining the risk for other family members and future children and providing access to potential reproductive options. The gap in knowledge resides in the lack of characterisation of some genes and the proportion of cases still unexplained by the known genes which limits the provision of genetic counselling. In the publications included in chapters 3-1 and 3-2, I contributed to the identification of two novel genes; *TMEM98* for nanophthalmos and *TEK* for PCG respectively. The identification of the *TMEM98* gene provides access to genetic testing and counselling in affected families and the identification of the *TEK* gene unravelled new information regarding the mode of transmission in these families with PCG. The publications included in chapters 3-3 and 3-4 summarise my original contribution to knowledge on the characterisation of known developmental glaucoma genes: the evaluation of potential other molecular mechanisms for *CYP1B1* leading to the disease in chapter 3-3 and the delineation of phenotypes and glaucoma prevalence associated with *FOXC1* and *PITX2* in chapter 3-4. These new findings have important implications in educating and supporting patients and their families with developmental glaucoma and greatly assist in the provision of genetic counselling.

3.1. Identification of *TMEM98* as the first gene for autosomal dominant nanophthalmos

In this study, I significantly contributed to the identification of the first gene associated with autosomal dominant nanophthalmos in a five generation Australian family from a British background using exome sequencing. Linkage localised the causative gene to chromosome 17p12-q12, overlapping with the NNO4 region previously identified in a Chinese pedigree. A missense deleterious variant was identified in the *TMEM98* gene segregating in 16 affected members and none of the 19 unaffected relatives, 285 controls or population reference databases. Six of the sixteen affected individuals were diagnosed with angle closure glaucoma, the majority of which had poor visual outcome. The association of *TMEM98* with nanophthalmos is strengthen by another study that identified *TMEM98* variants in two additional families with nanophthalmos. The replication of our finding sets *TMEM98* as a solid candidate gene for nanophthalmos and has direct translational implication for diagnostic purposes and genetic counselling. However, the function of *TMEM98* is still unknown and additional studies will be needed to determine its role in the pathogenesis of nanophthalmos and develop better therapeutic options for these families.

Contribution statement

Ms Souzeau was responsible for the recruitment of the family, the data collection and interpretation, the feedback of results, the provision of genetic counselling, and revised the manuscript. Dr Awadalla and A/Prof Burdon contributed to the study concept and design, data analysis and interpretation and drafted the manuscript. A/Prof Landers contributed to the study concept and design. A/Prof Hewitt contributed to the study concept and design and revised the manuscript. Dr Sharma contributed to the data interpretation and drafted the manuscript. Prof Craig contributed to the study concept and design, characterisation of the participants, data analysis and interpretation and drafted the manuscript.

The manuscript is freely available at:
https://jamanetwork.com/journals/jamaophthalmology/fullarticle/1873089

Mutation in *TMEM98* in a large white kindred with autosomal dominant nanophthalmos linked to 17p12-q12

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ABSTRACT

Importance: Nanophthalmos is a congenital disorder characterized by small eyes, with the main complications being severe hyperopia and angle-closure glaucoma.

Objective: To perform a clinical and genetic investigation of a large white family with autosomal dominant nanophthalmos.

Design, setting, and participants: Detailed clinical evaluation and genome-wide linkage scan was conducted in the family NNO-SA1. Linkage was evaluated with a 10K single-nucleotide polymorphism array, followed by whole exome sequencing, to identify novel segregating coding variants within the linked region. The candidate gene was screened for mutations in additional independent families by direct sequencing of the coding exons and intron/exon boundaries. The expression pattern of the candidate gene in ocular tissues was analyzed by reverse transcriptase-polymerase chain reaction. Participants were recruited through ophthalmology clinics at Flinders Medical Centre, Adelaide, South Australia, Australia Nanophthalmos was defined as an axial length less than 20.0 mm and/or refractive error greater than +7.00. Of the 35 available individuals from family NNO-SA1, 16 participants (46%) had a diagnosis of nanophthalmos, with mean refraction of +11.8 D and mean axial length of 17.6 mm. Unaffected unrelated individuals serving as controls were screened for the identified mutation. Additional independent families with clinically diagnosed nanophthalmos were also recruited.

Main outcomes and measures: Nanophthalmos status.

Results: Significant linkage was detected on chromosome 17 between single-nucleotide polymorphism markers rs2323659 and rs967293 with a maximum location score of 4.1. Exome sequencing identified a single novel segregating missense variant within the linkage region located in exon 8 of the transmembrane-98 (*TMEM98*) gene c.577G>C (p.Ala193Pro), which was absent in the Exome Variant Server database and among 285 local white individuals serving as controls. The *TMEM98* gene was expressed in all ocular tissues tested including sclera and optic nerve head.

Conclusions and relevance: A novel gene associated with nanophthalmos, *TMEM98,* most likely represents the cause of the disease in this family. To our knowledge, this represents the first gene identified causing autosomal dominant nanophthalmos.

Keywords: Nanophthalmos, Autosomal dominant, Genome-wide linkage exome sequencing

INTRODUCTION

Microphthalmia is a developmental eye disorder consisting of bilaterally small eyes. Posterior microphthalmia and nanophthalmos are 2 subtypes of the disorder. [1] Nanophthalmos is characterized by the axial length of the globe being more than 2 SDs smaller than the normal range (< 20 mm in adults), [2] and the cornea and lens are typically of normal size, [3] causing severe hyperopia (farsightedness) of +7.00 diopters (D) or more. The smaller dimensions of the anterior chamber depth cause the iridocorneal angle to be typically narrow. Abnormal thickening of the scleral connective tissue is often also observed. [3,4] The abnormal structure of the anterior chamber observed in nanophthalmos differs from that of posterior microphthalmia, a rare phenotype restricted to the posterior segment of the eye, where the anterior chamber is of normal dimensions. [5-7] A recent study [8] has revealed that eyes with posterior microphthalmia have corneal steepening proportional to the degree of the short axial length, suggesting that both nanophthalmos and posterior microphthalmia are not a distinct phonotype, but they represent a spectrum of high hyperopia. The prevalence of all microphthalmia in Australia is between 0.5 and 1.5 per 10 000 births. [9]

Nanophthalmos can be inherited in either an autosomal dominant or autosomal recessive mode. [2,10] Linkage studies in large families with autosomal dominant nanophthalmos have identified linkage to chromosome 11p in a family from the United States, [11] 2q11-q14 in a Chinese family, [12] and 17p12-q12 also in a Chinese pedigree. [13] To date, additional families showing linkage to these regions have not been reported and the causative genes in families with autosomal dominant nanophthalmos have not been identified.

We describe a large family of British ancestry with autosomal dominant nanophthalmos. We conducted genome-wide linkage analysis in this family, localizing the gene to a region of 16.9 Mb on chromosome 17 (overlapping with the linkage region in the Chinese family [13]) and investigated the genes in the linked region for causative mutations.

METHODS

Recruitment of Participants

Family NNO-SA1 (Figure 1) was identified following presentation of the proband (V:3) to Flinders Medical Centre, Adelaide, South Australia, Australia, for evaluation and treatment related to angle-closure glaucoma. The primary diagnosis of isolated nanophthalmos was made in that setting by one of the authors (J>E>C>). The family history of this patient was

obtained and the extended family was traced for 5 generations. Thirty-five family members were recruited into the study, 16 of whom were received a diagnosis of nanophthalmos. An additional 7 family members were reported to have the same phenotype, but were not available for study. The proband and her immediate family reside in Australia; however much of the extended family is living in the United Kingdom. Written informed consent was obtained from all the participants in accordance with the Declaration of Helsinki, and the study was approved by the Southern Adelaide Clinical Human Research Ethics Committee. The participants did not receive financial compensation.

The proband (V:3) received full ophthalmic examination included refraction, intraocular pressure, central corneal thickness measurement, slitlamp biomicroscopy, A-scan ultrasonography, and optic disc tomography. Angle-closure glaucoma status was assessed in all participants; however, it was not a part of the criteria used to determine affection status. The rest of the family members were classified as having nanophthalmos if they had an axial length less than 20.00 mm and/or refractive error at or greater than +7.00. Genomic DNA was extracted from either peripheral whole blood (QiaAmp DNA Blood Maxi Kit; Qiagen) or from saliva (Oragene DNA saliva collection kits; DNA Genotek) according to manufacturers' protocols.

Linkage analysis

All available family members were genotyped (GeneChip Xba 10K single-nucleotide polymorphism [SNP] arrays; Affymetrix Inc)) at the Australian Genome Research Facility, Melbourne, Australia. Data were provided in the form of linkage format files, and analyses were conducted in MERLIN, 1.1.2. [14] Because of computational limitations on the number of members of a pedigree, initial genome-wide linkage analysis was conducted using individuals from the proband's branch of the family (descendants of II:1 and II:2), excluding IV:1, V:1, V:2, and V:7, under a fully penetrant dominant model. The disease allele frequency was set to 0.0001 and the allele frequencies for each marker typed were obtained from the CEU collection (white Utah residents with Northern and Western European ancestry from the Centre d'Etude Polymorphism Humain) sample of the International HapMap project (http://hapmap.ncbi.nlm.nih.gov/). To confirm the findings, different individuals were excluded and analysis was repeated in the remaining branch of the family (descendants of II:6 and II:7). When the results were compared. The SNPs surrounding the linked region on chromosome 17 were extracted from the data set, and files were formatted with Mega2 [15] for linkage analysis on the entire pedigree in SimWalk2. [16] A fully penetrant dominant model

was used to calculate location scores (equivalent to multipoint logarithm of odds scores) and location scores vs chromosome location were plotted. Haplotypes were reconstructed in MERLIN [14] on subsections of the pedigree, with overlapping individuals included in each run to facilitate combining the data into the whole pedigree.

Exome sequencing

Sequencing was performed in 5 individuals selected to represent both main branches of the family: 4 affected individuals (IV:2, IV:5, IV:9, and V:9) and 1 unaffected member (IV:4). Enrichment for the exome was performed (TruSeq Exome Enrichment Kit; Illumina Inc) and enriched DNA was sequenced (HiSeq 200; Illumina Inc) by the Australian Genome Research Facility. Sequence alignment to hg19 was conducted with CASAVA, version 1.8.1 (https://support.illumina.com/downloads/casava_181.ilmn), and aligner module ELAND, version 2 (Illumina). Realignment and variant calls were made with Illumina Exome Script and variants annotated by ANNOVAR. [17] All bioinformatics was conducted by the sequencing service provider. The lists of single-nucleotide variants identified in each sample were filtered according to the following criteria: (1) not present in dbSNP131, (2) segregated in the 5 sequenced individuals, and (3) missense, stop, or splice variant.

Segregation in the family was assessed by Sanger sequencing using primer pair exon 8-1 (Supplementary [eTable 1]). Polymerase chain reaction (PCR) was performed for each available DNA sample with the following conditions: enzyme activation at 95ºC for 15 minutes, 30 cycles of denaturation at 95ºC for 30 seconds, annealing at 57ºC for 30 seconds, elongation at 72ºC for 30 seconds, and final elongation at 72ºC for 5 minutes. The PCR products were purified for sequencing using exonuclease I (20 U/µl) and USB Shrimp Alkaline Phosphatase (In Vitro Technologies) (1 U/µl), incubated at 37ºC for 60 minutes, and then inactivated at 80ºC for 20 minutes. [18] The product was sequenced (BigDye Terminator; Applied Biosystems) on an ABI 3100 DNA sequencer (Applied Biosystems). Chromatograms were compared with each other and the reference sequence (GenBank NM_001033504.1) (Sequencher 5.2.3. software, GeneCodes Corp).

The presence of the novel missense variant was tested in 285 individuals serving as controls using a restriction fragment length polymorphism. The cohort was ascertained from retirement villages in Adelaide. The mutation introduces a restriction site for Bsu36I (New England BioLabs Inc). Polymerase chain reaction was performed with the same primers used for sequencing above. A total of 10 µl of PCR product was digested with 2 U of Bsu36I enzyme in the presence of bovine serum albumin. The digested products were visualized

under UV light following electrophoresis on 1.4% agarose gel, stained with Gel Red (Biotium). The novel variant was searched against the Exome Variant Server database (http://evs.gs.washington.edu/EVS/), a large public data set of unrelated European-American individuals with exome sequence data available.

The functional significance of the mutation in transmembrane-98 (*TMEM98*; GenBank NM_001033504.1) was analyzed using PolyPhen-2, [19] SIFT using protein ID (ENSP00000261713), [20] and MutationTaster. [21] The conservation of normal TMEM98 protein was compared between species using data obtained from UniProtKB (http://www.uniprot.org/uniprot/) and aligned by ClustalW2. [22]

Gene screening in additional families with nanophthalmos

A total of 7 additional independent families with at 1 one affected member participated in the present study. Families were referred to the study from eye clinics in Australia. Extraction and sequencing of the DNA of probands were conducted using the same methods as described above with primers for each coding region of the gene, encompassing splice sites (Supplementary [eTable 1]).

Expression analysis

Ocular tissues were obtained from postmortem human eyes through the Eye Bank of South Australia according to the guidelines of the Southern Adelaide Clinical Human Research Ethics Committee. Total RNA was extracted from tissues using the RNeasy Micro Kit or Mini Kit (Qiagen). Primers were designed through National Center for Biotechnology Information/Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?) for each of the 2 known isoforms: isoform 1 (GenBank NM_015544) forward primer (5'-3') GCACCTGCCATCCTCTTCCCCA and reverse primer (5'-3') GCAGTCGTCCGTGCGTCCAG, and isoform 2 (GenBank NM_001033504) forward primer (5'-3') GGGAGCCACAGCCTGAGCTTT and reverse primer (5'-3') AGGAGCAGGGCAGTCGTCCG. First strand complementary DNA was synthesized using the SuperScript III reverse transcriptase (Invitrogen). Polymerase chain reaction was conducted with the following conditions: initialization at 95°C for 15 minutes, followed by denaturation at 95°c for 30 seconds, annealing at 62°C for 30 seconds, elongation at 72°C for 30 seconds for 30 cycles using complementary DNA from retina, optic nerve, optic nerve head, ciliary body, and iris, and 32 cycles for sclera, and then a final elongation at 72°C for 5 minutes. The PCR product was visualized on 1.4% agarose gel stained with Gel Red

(Biotium). Products were purified for sequencing as described above. The publicly available Illumina Human BodyMap, version 2.0, data were accessed on November 19, 2013, through the Ensembl Genome Browser (http://www.ensembl.org) to explore nonocular expression patterns.

RESULTS

Recruitment of Participants

Family NNO-SA1 (Figure 1) presented with an autosomal dominant nanophthalmos. Sixteen family members were classified as affected (Table). The mean (SD) refraction and axial length of the affected family members were +11.8 (2.5) D and 17.6 (0.6) mm, respectively. Best-corrected visual acuity in these patients ranged from no perception of light to 6/6. Angle-closure glaucoma was detected in 6 of the 16 patients, including the proband (V:3). Individual IV:2 demonstrated slightly elevated pressure but no sign of glaucoma at the time of recruitment. Other clinical features in affected family members included thick sclera with prominent scleral vessels and an increased frequency of optic disc drusen with some degree of increased tortuosity. There was also a tendency for aqueous misdirection to occur after intraocular surgery, as well as other complications (eg, macular edema and choroidal effusions), often leading to poor outcomes following intraocular surgery for cataract and glaucoma. Representative images of the ocular phenotype are shown in Figure 2.

Linkage analysis

Linkage analysis in the branch of the pedigree descended from II:1 and II:2 is shown in Figure 3A. Linkage to previously reported nanophthalmos regions on chromosomes 11p[11] and 2q[12] was excluded. Linkage was detected on chromosome 17p12-q12 between SNP markers rs2323659 and rs967293 with a maximum logarithm of adds score of 2.67, overlapping with the region previously identified in the Chinese pedigree. [13] The region was defined by recombination events in individuals III:3 (untyped) and IV:6 (Figure 1). The remaining branch of the family similarly showed linkage to this region on chromosome 17; however, no recombinants were observed that would further refine the linkage region. The linked region is approximately 16.9 megabases (Mb) in physical distance between 15.3 Mb and 32.7 Mb, encompassing the centromere of chromosome 17. Multipoint linkage analysis of this region in the entire family using SimWalk2 gave a location score of 4.1 (Figure 3B).

The 5' boundary marker rs2323659 is located on the p arm of the chromosome. The next

informative marker, rs1589464, is separated from the boundary marker by approximately 10 Mb, including the centromere. At the other end of the linked region, the recombination event in individual IV:11 occurred between rs952540 and rs967293. These markers are separated by approximately 370 kb, and this region contains minimal annotated genes. Thus, further fine mapping at this end of the linked region is unwarranted.

Exome sequencing

All detected variants not present in dbSNP131 are described in the Supplement (eTable 2). Only 3 variants show segregation in the 5 whole exome-sequenced individuals: rs118038927 at ubiquitin-specific peptidase 22 (*USP22* GenBank NM_015276.1), rs139539715 in the noncoding RNA *FOXO3B* (GenBank NR_026718.1), and a novel variant at *TMEM98*. Of these, rs118038927 is silent and has no predicted effect on protein function and rs139539715 is a common variant (minor allele frequency of 35%). Thus, the novel variant in *TMEM98* is the only variant meeting the criteria for a disease-causing variant. It is a substitution at position c.577G>C (NM_015544) leading to change of the amino acid alanine to proline at codon 193 (p.Ala193Pro) (Figure 4). This variant was shown by direct sequencing in the remainder of the family to segregate completely with disease. The mutation was present in all affected individuals and absent in all unaffected family members. The variant is not present in dbSNP v.137, and was not reported in the Exome Variant Server (as of March 31, 2014). The mutation introduced a restriction site for Bsu36I resulting in bands of 180 and 420 base pairs (bp) and the undigested wild-type product was 600 bp. The mutation was not present in 285 unaffected unrelated Australian white controls assessed with this restriction enzyme.

Polyphen-2, SIFT, and MutationTaster were used to predict the likely pathogenicity of this novel missense variant. MutationTaster predicted it to be a disease-causing variant; SIFT predicted the mutation to be damaging, with a score of 0.05; and Polyphen-2 predicted this mutation to be possibly damaging, with a score of approximately 60% (sensitivity 81% and specificity 83%) under the HumVar algorithm.[19]

Sequence alignment between multiple species showed a high level of conservation of the TMEM98 protein in the region of the mutation between mammals, amphibians, and fish. Conservation was less apparent with the one bird species accessed (zebra finch). The wild-type residue was found to be conserved among all vertebrates accessed as shown in the Supplement (eFigure 1).

Gene screening in additional families with nanophthalmos

Additional white families with nonsyndromic nanophthalmos were recruited. Of the 22 available family members from 7 families, 13 were affected.

Two families showed autosomal dominant inheritance and, in the remainder, appear to be autosomal recessive. All patients presented with short axial length with a mean of 18.8 (1.2) mm and severe hyperopia with a mean of +8.4 (4) D. No novel variants in *TMEM98* were detected in the probands. All polymorphic variants identified in *TMEM98* in the probands are presented in the Supplement (eTable 3).

Expression analysis

There are 2 reported transcript isoforms of *TMEM98* with different 5' untranslated regions; however, both isoforms encode the same protein. The expression of both transcripts of *TMEM98* in ocular tissues was assessed by reverse transcriptase-polymerase chain reaction (Supplement [eFigure 2]). Both transcripts were expressed in all eye tissues assessed (ie, corneal endothelium, iris, ciliary body, sclera, optic nerve, optic nerve head, and retina) and resulted in products of the expected size (576 bp for isoform 1 and 562 bp for isoform 2). Direct sequencing revealed complete alignment with the reference sequence, confirming the specificity of the products. Ocular expression was further confirmed using The Ocular Tissue Database (https://genome.uiowa.edu/otdb/), which showed the expression of *TMEM98* to be high in sclera, choroid RPE, iris, and ciliary body, which are believed to be involved in the pathogenesis of nanophthalmos. [23] According to the Illumina Human BodyMap data, *TMEM98* is expressed in all 16 tissues tested, including adrenal, adipose, brain, breast, colon, heart, kidney, liver, lung, lymph, ovary, prostate, skeletal muscle, testes, thyroid, and white blood cells.

DISCUSSION

In this study we evaluated a large pedigree of white background with autosomal dominant nanophthalmos, identifying a coding mutation in the *TMEM98* gene on chromosome 17 that likely accounts for the phenotype. The single novel segregating missense mutation, p.Ala193Pro in *TMEM98,* is predicted to be damaging. Given the lack of other segregating putatively functional variants, p.Ala193Pro is likely to be the causative mutation in family NNO-SA1. In addition, the mutation was not detected in 285 individuals serving as controls, and is it present in large public databases, strengthening the hypothesis that *TMEM98* is the

most likely cause of nanophthalmos in this family. However, the absence of pathogenic variants in additional families with nanophthalmos suggests that other genetic loci for this disorder are yet to be identified and replication of this finding in an independent family is yet to occur.

The *TMEM98* gene is expressed in all eye tissues that were assessed in the present study as well all the human tissues tested in the Illumina BodyMap project. This ubiquitous expression suggests a fundamental role in cellular processes; however, systemic effects of the mutation in family NNO-SA1 were not noted. Tissue-specific effects may develop through interaction with other ocular-specific transcripts during ocular development or through tissue-specific splicing and regulatory mechanisms. Both isoforms analyzed were expressed in the sclera. This is significant because sclera is abnormally thick in family NNO-SA1 and in patients with nanophthalmos in general. The gene was also expressed in tissues of the iridocorneal angle including iris and ciliary body. This expression indicates that *TMEM98* might be involved in causing angle-closure glaucoma in patients with nanophthalmos. Very little is known about the function of *TMEM98*. The encoded protein is 226 amino acids long, is leucine rich (13.3%), and is highly acidic, with a theoretical pI of 4.81[24]; it has been detected in most healthy tissues (localized to the nucleus and cytoplasm) as well as in cancers (http://www.proteinatlas.org/ENSG00000006042/tissue).

A similar linkage region, ranging from 14.1 Mb to 33.0 Mb on chromosome 17, has been reported[13] in autosomal dominant congenital simple microphthalmia in a Chinese family. The region reported in our study is entirely encompassed within the previously reported region. The overall phenotype appears similar to that described in the linked Chinese family,[25] although the white family has slightly worse refraction (mean, +11.8 D vs +8.0 D) and correspondingly slightly shorter axial length (mean, 17.6 mm vs 19.2 mm). Rates of glaucoma are similar between the 2 families. The distinction between nanophthalmia and microphthalmia is likely to be arbitrary; however, molecular genetics diagnosis may help better define such overlapping conditions. It is possible that the causative gene is different between the 2 families, but it is highly likely that both conditions are caused by a mutation in the same gene within the smaller region defined by the white family reported in this study.

CONCLUSIONS

To our knowledge, this is the first study to report mutations in *TMEM98* and to link this gene to a disease. Additional in-depth investigations are required to explore the involvement of *TMEM98* in normal eye development and determine its role in the pathogenesis of

nanophthalmos.

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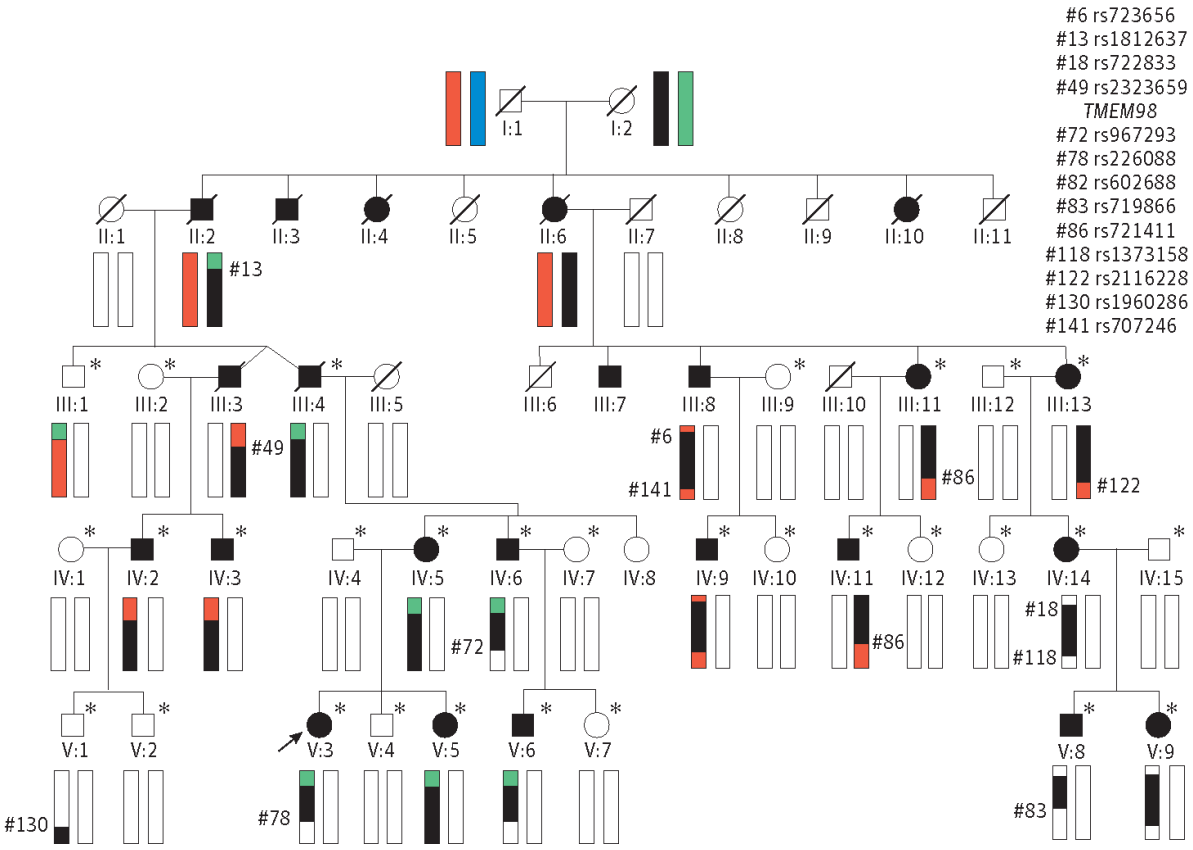
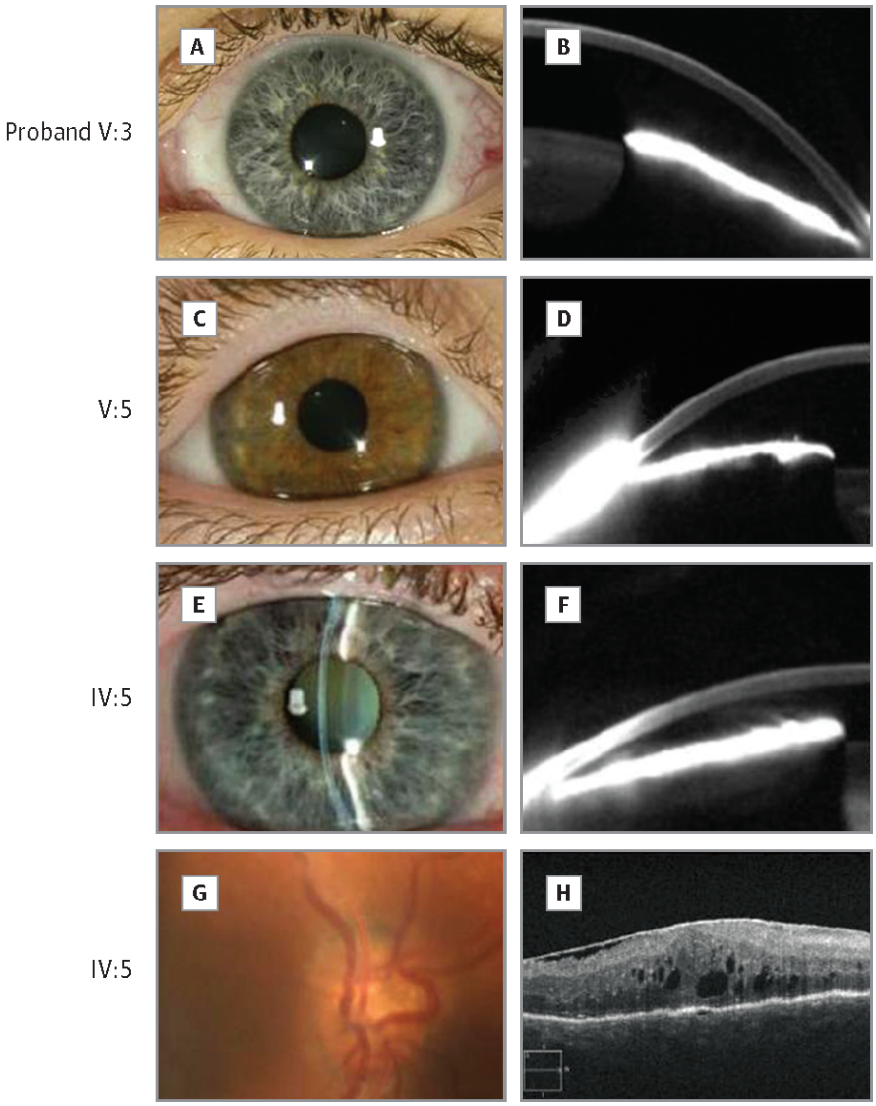
Figure 1. Family NNO-SA1 displaying autosomal dominant nanophthalmos.



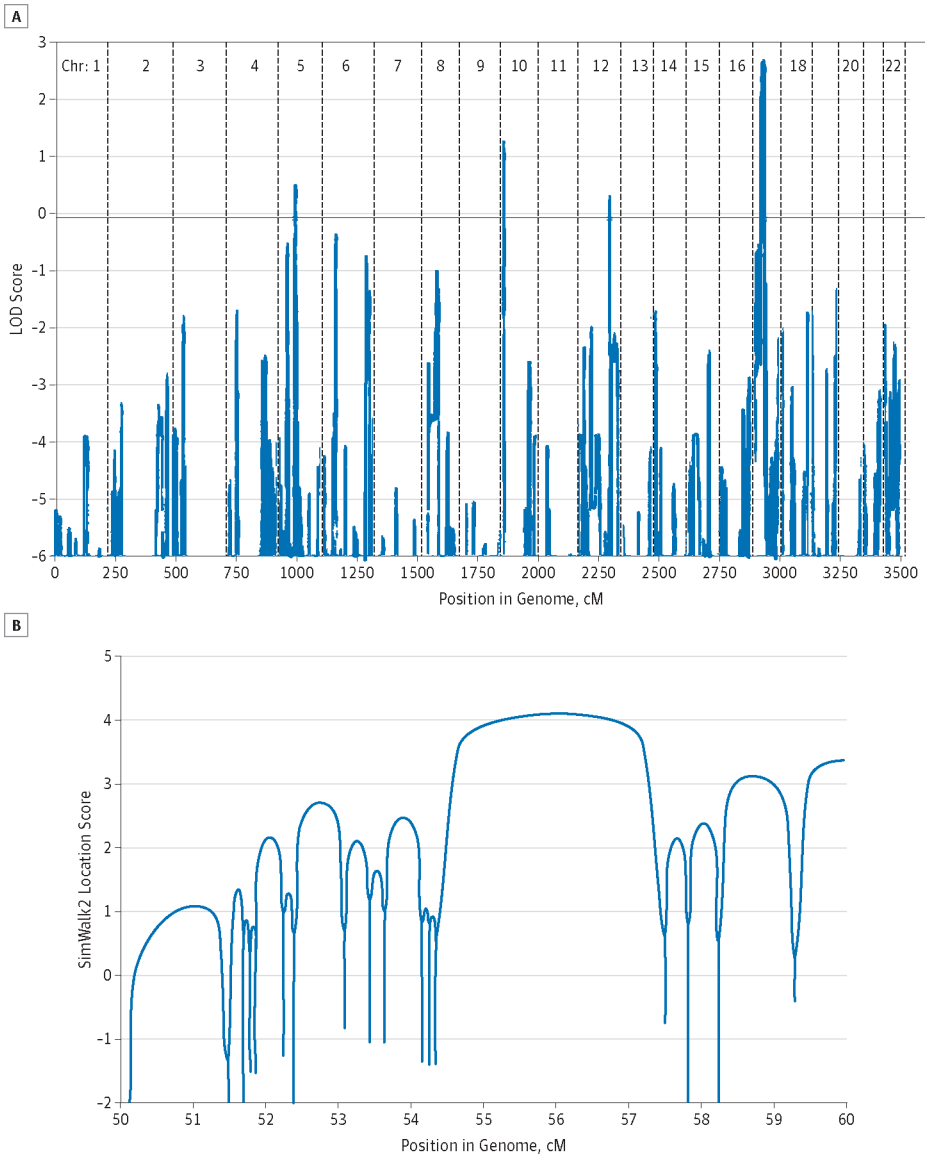
Squares indicate males; circles, females; slashes, deceased individuals and blackened symbols, affected individuals. The proband is indicated with an arrow. DNA was available from individuals marked with asterisks and was included in the linkage study. Haplotypes at the linked region of chromosome 17 are shown, and the segregating haplotype is black. The location of the mutated gene *TMEM98* relative to the numbered single-nucleotide polymorphism markers is given in the top right corner. The markers at the boundaries of recombination events are indicated and listed in the legend.

Figure 2. Clinical photos of the affected members in the Australian branch of family NNO-SA1.



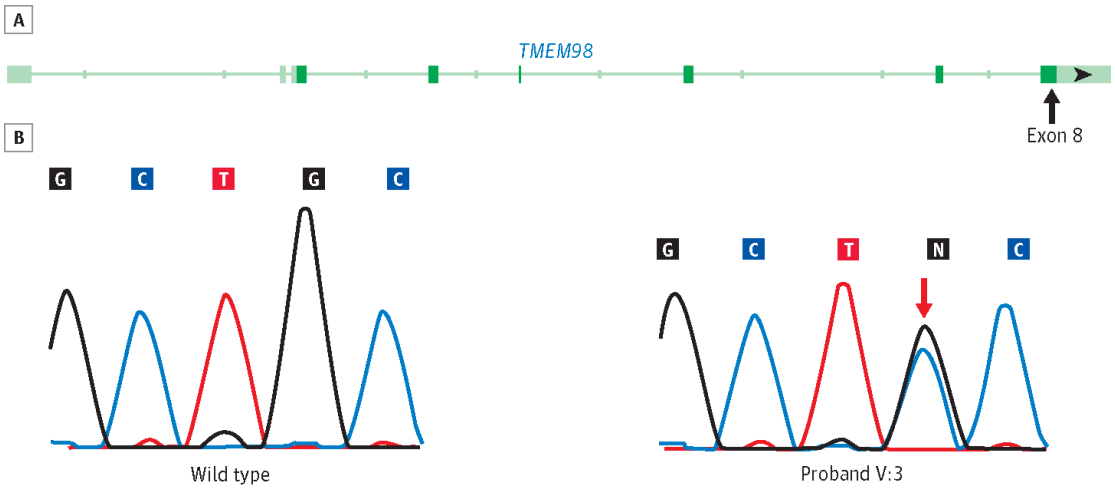
A, C, and E, External eye appearance of patients' nanophthalmos. B, D, and F, Corresponding images obtained with a rotating Scheimpflug camera system (Pentacam; Oculus). Narrow iridocorneal angles and shallow anterior chamber depth were present in all affected eyes. G, The optic disc with the presence of optic disc drusen. H, Optical coherence tomographic image of the macula showing postoperative cystoid macular edema with epiretinal membrane.

Figure 3. Linkage analysis of family NNO-SA1



A. Linkage plot showing logarithm of odds (LOD) scores across the whole genome for the descendants of II:1 and II:2. A suggestive peak was identified on chromosome (Chr) 17. B. Linkage plot showing multipoint LOD scores (SimWalk2 location scores) for the linked region of chromosome 17 (between 50 and 60 cM) in the entire family. A maximum score of 4.11 was noted at marker rs95281.

Figure 4. Mutation located in exon 8 of *TMEM98* gene.



A. Ideogram of *TMEM98.* B. Sequence chromatograms showing the wild type and the novel mutation in *TMEM98* in the proband V.3 of Family 1. The red arrow indicates mutation c.577G>C (p.Ala193Pro) in the proband.

Table. Clinical characteristics of the affected family members.

ID	Age (years)	BCVA		Refraction (D)		Axial length (mm)		IOP (mmHg)		ACG
		RE	LE	RE	LE	RE	LE	RE	LE	
III:4	91	na	na	+11.5	na	na	na	na	na	na
III:10	78	6/24	6/30	+15.00	+15.00	18.12	17.92	13	14	No
III:13	86	HM	CF	+9.50	na	18.02	na	16	17	ACG
III:15	84	na	na	+7.00	+9.50	17.43	17.31	na	na	ACG
IV:2	64	6/6	6/9	+11.00	+11.00	17.00	na	24	24	No
IV:3	49	6/9	6/12	+13.75	+12.87	na	na	18	18	na
IV:6	61	6/48	6/12	+14.00	+15.00	17.10	17.14	33	26	ACG
IV:7	63	na	na	+11.87	+12.37	na	na	18	19	No
IV:14	52	na	na	+9.00	+10.00	na	na	na	na	na
IV:16	49	na	na	+12.50	+12.00	na	na	na	na	na
IV:21	52	6/9	6/36	+14.25	+14.50	na	na	na	na	ACG
V:3	34	6/60	6/6	+9.75	+10.50	18.46	18.34	42	41	ACG
V:6	26	6/12	6/15	+15.50	+15.00	17.02	16.90	19	18	No
V:7	30	6/6	6/6	+13.25	+13.75	na	na	na	na	na
V:14	24	6/12	NLP	+7.50	na	18.42	na	40	na	ACG
V:15	26	na	na	+8.25	+8.50	na	na	11	11	No

ACG, angle closure glaucoma; BCVA, best-corrected visual acuity; CF, count fingers; D, dioptres; HM, hand movement; ID, identification; IOP, highest recorded intraocular pressure; LE, left eye; na, not available; NLP, no light perception; RE, right eye.

3.2. Identification of the *TEK* gene in PCG

Using whole exome sequencing (WES) technology, in this study we identified a novel gene, *TEK*, implicated in PCG. The Angiopoietin receptor TEK is a receptor tyrosine kinase that regulates vascular homeostasis. *TEK* hemizygous knock-out mice displayed developmental defects of the aqueous humor pathway and elevated IOP, suggesting that *TEK* gene dosage is important for the proper development of the eye.

Heterozygous variants were identified in 10 out of 189 unrelated PCG families. Although these results still require replication, our findings suggest that this novel gene might account for 5% of PCG cases. This would make *TEK* the second most common cause of PCG after *CYP1B1* and has implications for genetic counselling. The *TEK* variants failed to produce functional proteins by different cellular mechanisms and displayed an autosomal dominant transmission associated with high recurrence risk and risk for other family members. However, in 6 out of 8 families the variant was present in an unaffected parent, suggesting reduced penetrance. Additional studies on *TEK* are needed to assess the significance of the gene in PCG and to adequately counsel families about recurrence risks.

Contribution statement

Ms Souzeau was responsible for the recruitment of Australian participants, the feedback of results, the provision of genetic counselling and the genetic interpretation for Australian participants, and manuscript revision. A/Prof Souma and Dr Thomson were responsible for experimental studies, data analysis and manuscript drafting. Dr Tompson contributed to the genetic analysis, experimental studies, data analysis and manuscript drafting. Dr Siggs, Dr Limviphuvadh and Dr Mauer-Stroh contributed to data analysis and manuscript drafting. Dr Kizhatil, Prof John and A/Prof Jin contributed to experimental studies and manuscript drafting. Ms Whisenhunt contributed to experimental studies, data analysis and manuscript revision. Prof Yamaguchi, A/Prof Feng, Dr Kloss, Mr Tran-Viet and A/Prof Liu contributed to experimental studies and manuscript revision. A/Prof Yanovitch, Prof Kalaydjieva, Dr Azmanov, Dr Finzi, Dr Mauri, Ms Javadiyan, Dr Zhou, A/Prof Hewitt, A/Prof Burdon, Prof Mackey, Dr Allen, Dr Ruddle, Dr Lim and Dr Rozen recruited participants, contributed to genetic analysis and manuscript revision. Dr Wiggs contributed to patient material, data analysis and manuscript drafting. Dr Pasutto and Prof Craig contributed to patient material, data analysis and manuscript revision. Dr Quaggin and Prof Young were responsible for study design and conception, and manuscript drafting.

The Journal of Clinical Investigation RESEARCH ARTICLE

Angiopoietin receptor TEK mutations underlie primary congenital glaucoma with variable expressivity

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Primary congenital glaucoma (PCG) is a devastating eye disease and an important cause of childhood blindness worldwide. In PCG, defects in the anterior chamber aqueous humor outflow structures of the eye result in elevated intraocular pressure (IOP); however, the genes and molecular mechanisms involved in the etiology of these defects have not been fully characterized. Previously, we observed PCG-like phenotypes in transgenic mice that lack functional angiopoietin-TEK signaling. Herein, we identified rare *TEK* variants in 10 of 189 unrelated PCG families and demonstrated that each mutation results in haploinsufficiency due to protein loss of function. Multiple cellular mechanisms were responsible for the loss of protein function resulting from individual TEK variants, including an absence of normal protein production, protein aggregate formation, enhanced proteasomal degradation, altered subcellular localization, and reduced responsiveness to ligand stimulation. Further, in mice, hemizygosity for *Tek* led to the formation of severely hypomorphic Schlemm's canal and trabecular meshwork, as well as elevated IOP, demonstrating that anterior chamber vascular development is sensitive to *Tek* gene dosage and the resulting decrease in angiopoietin-TEK signaling. Collectively, these results identify *TEK* mutations in patients with PCG that likely underlie disease and are transmitted in an autosomal dominant pattern with variable expressivity.

Introduction

Glaucoma is a group of heterogeneous diseases that is characterized by a chronic degenerative optic neuropathy, affecting more than 60 million people worldwide (1–3). Primary congenital glaucoma (PCG) (OMIM 231300) is a severe form of the disease with unclear etiology and is characterized by infant/early-childhood ocular hypertension, enlarged eye globes (buphthalmos), and optic neuropathy, which can result in vision loss and blindness — often despite treatment (4–6). Indeed, PCG accounts for 18% of children enrolled in institutions for the blind worldwide (7). PCG occurs in all ethnic groups, but the disease incidence varies according to ethnic background, ranging from 1:1,250 in inbred populations to 1:30,000 in populations with heterogeneous ethnicity (6, 8–12). Families can exhibit autosomal recessive or dominant inheritance, although the majority of cases appear to be sporadic (5, 6, 10–14).

The molecular etiology of PCG is only partially understood, as only a few genes responsible for PCG have been identified (6, 10–14). Mutations in *CYP1B1*, which encodes a cytochrome P450 enzyme, is the most common cause of autosomal recessive PCG worldwide, accounting for up to 87% of familial cases in some inbred populations but only 25%–27% in populations with heterogeneous ethnicity (5, 9, 15–18). With *CYP1B1* mutations account-

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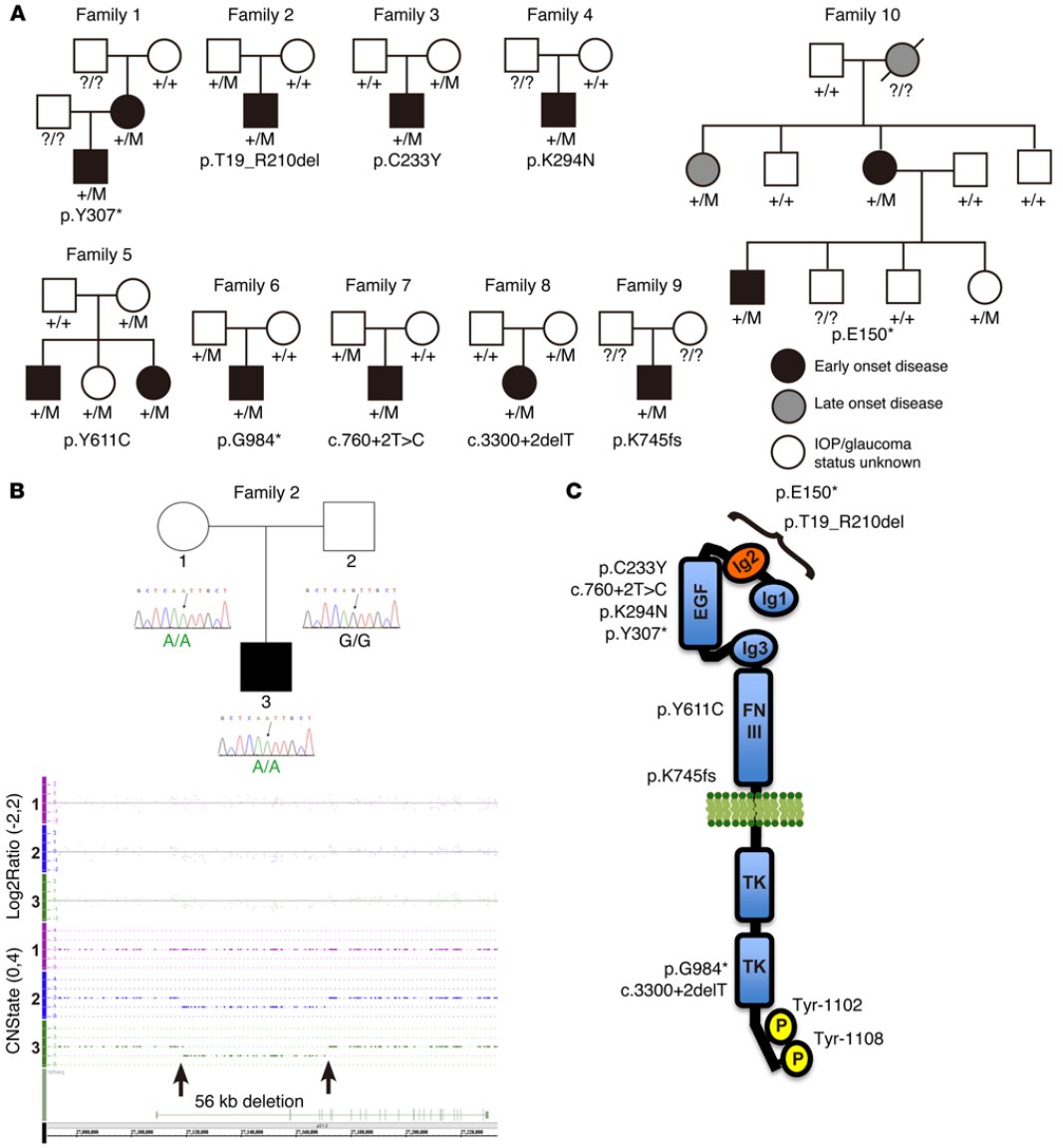

Figure 1. Pedigree structures and *TEK* mutations identified in 10 families. (**A**) Pedigrees of the 10 families with *TEK* mutations. Specific mutations in *TEK* are listed below the different pedigrees, with those family members annotated as +/M. Affected individuals are indicated by solid black/gray symbols (black for early-onset disease and gray for late-onset disease; see Table 1 for details). Note: White symbols do not exclude an undiagnosed late-onset disease. Unknown genotypes are marked as ?/?. (**B**) In family 2, a *TEK* SNP was inconsistent with father-to-son transmission (upper panel). Copy number variation analysis identified a 56-kb deletion that was passed from the unaffected father (annotated as 2; lower panel) to the unilaterally affected son (annotated as 3). The physical location of the *TEK* gene is shown, with arrows indicating the deletion breakpoints within introns 1 and 4. CN, copy number. (**C**) A schematic illustration of the TEK receptor with the locations of identified variants. Eight of 10 variants were identified in the ectodomain of the receptor. Tyr1102/Tyr1108 are the primary tyrosine phosphorylation sites in the C-terminal tail of the TEK receptor that initiate downstream signaling events (35, 36).

ing for PCG in less than a third of patients with heterogeneous ethnicity, alternate mechanisms must underlie the majority of these other cases. Furthermore, the mechanism of PCG due to mutations in *CYP1B1* is not clearly understood.

It is well recognized that elevated intraocular pressure (IOP) is the key risk factor contributing to glaucoma, including PCG (1–3, 6, 19, 20). The elevated pressure in PCG is believed to result from defects in the aqueous humor outflow (AHO) pathway, rather than

Table 1. *TEK* variants identified in 10 PCG families

ID	Ethnicity	Sex	Eyes affected	Age at onset	Age of unaffected carrier(s)	Chromosome position	Exon(s)	Coding DNA mutation	Protein alteration	ExAC variant count	ExAC total alleles	ExAC ethnically matched alleles
1	Lat	F & M	U & U	Infant & 4 months	–	27180257	7	c.921C>A	NMD or p.Y307*	Novel	121,226	11,524
2	Rom	M	U	n/a	n/a	g.(27116823_27118707)_(27170308_27172125)del	2–4	c.53_628del	p.T19_R210del	-	-	-
3	Eur (Am)	M	U	Birth	25 yr	27172683	5	c.698G>A	p.C233Y	Novel	121,002	66,602
4	Afr (Am)	M	B	Birth	n/a	27173341	6	c.882G>C	p.K294N	12 (0 Afr)	121,284	10,406
5	Eur (Am)	M & F	U & n/a	2 yr & n/a	30 yr & 3 yr	27197520	12	c.1832A>G	p.Y611C	Novel	121,268	66,710
6	Eur (I)	M	U	5 mo	39 yr	27213554	18	c.2950G>T	NMD or p.G984*	Novel	121,294	66,698
7	Eur (Au)	M	B	Birth	54 yr	27172747	5	c.760+2T>C	NMD or p.A254Gfs*3	Novel	119,718	65,966
8	Eur (Au)	F	B	Birth	38 yr	27228305	22	c.3300+2delT	p.Y1068Pfs*3	Novel	120,580	66,356
9	Eur (Au)	M	B	Birth	n/a	27204931	14	c.2232dupG	NMD or p.K745Efs*76	Novel	121,392	66,726
10	Eur (Am)	F & M	B & B	2 mo & birth	25 yr	27168576	3	c.448G>T	NMD or p.E150*	Novel	115,554	63,256

Ethnicity: Lat, Latino; Rom, Romani; Eur, European; Afr, African; I, Italian; Am, American; Au, Australian. Sex: M, Male; F, Female. Eyes affected: U, unilateral; B, bilateral. n/a, data not available. NMD, nonsense-mediated decay of mRNA transcript predicted (no protein product). Chromosome position in accordance with GRCh37/hg19 assembly. *TEK* mRNA reference sequence NM_000459.4. TEK protein reference sequence NP_000450.2.

an increase in aqueous humor (AH) production (5, 6, 14). Ocular anterior chamber fluid (AH) is produced by the ciliary body and drained mainly through Schlemm's canal (SC) and uveoscleral pathways (21, 22). An imbalance in fluid homeostasis results in elevated IOP. In humans, 80% of fluid drainage is conducted through the conventional, or trabecular, AHO pathway (23). AH first passes through the trabecular meshwork (TM) before entering the lymphatic-like SC for drainage (22, 24, 25). The remaining 20% of fluid egresses via the unconventional uveoscleral tract pathway, which includes the extracellular spaces within the iris, ciliary muscle, and sclera (21).

The angiopoietin receptor TEK (tunica interna endothelial cell kinase, also known as Tie2) is a receptor tyrosine kinase and regulates vascular homeostasis through its auto- and transphosphorylation (26–29). The primary ligand, angiopoietin-1 (ANGPT1), is expressed by pericytes and other vascular supporting cells, and exerts its proangiogenic and vascular stabilizing effects through activating TEK. A second ligand, ANGPT2, is secreted by the endothelium and acts as a context-dependent partial agonist of TEK (26, 27, 29, 30).

In the eye, the TEK receptor is highly expressed in the SC endothelium (24, 25). Recently, we discovered that angiopoietin-TEK signaling is required for SC development in mice (31). Deletion of TEK or both major angiopoietin ligands after embryonic day 16.5 circumvents embryonic lethality and results in developmental loss of SC, extremely elevated IOP, rapid and complete retinal ganglion loss, and glaucoma (31). Despite the critical role of this signaling pathway for SC development in mice, the relevance of this pathway in human disease has not, to our knowledge, been previously reported.

Here, we describe human mutations in *TEK* identified in a PCG cohort of 189 families that do not carry mutations in the known disease-causing genes *CYP1B1* (16–18), *LTBP2* (32), *FOXC1* (33), and *MYOC* (13). We identified 10 heterozygous novel/rare *TEK* variants as disease-causing mutations and assessed their effects on TEK function. We also determined in mice whether haploinsufficiency for *Tek* is sufficient to result in abnormal SC development and elevation of IOP. In addition to the identification of a pathway for human glaucoma, we assign key functional roles to specific domains of the TEK receptor.

Results

TEK mutations are identified in PCG families with unknown etiology. To identify novel PCG causative genes, we applied whole exome sequencing to a multiethnic cohort of PCG families with unknown molecular etiology. We performed an initial exome sequencing analysis on 21 affected individuals from 20 families including 2 affected individuals in family 1 (Figure 1A) and 19 sporadic cases. Under a monogenic recessive inheritance model, filtering for novel nonsynonymous, homozygous, or compound heterozygous variants in a single gene identified no candidate genes. An analysis of 2 complete family trios also excluded the possibility of de novo mutations being causative for the phenotype in these 2 families. We initially focused our analysis on a family demonstrating direct parent-to-child transmission of the disease (family 1, Figure 1A), looking for novel shared heterozygous variants, and identified heterozygosity for 3 nonsense and 15 missense variants in common. One of the nonsense variants, p.Y307*, was present in *TEK* (Figure 1A and Table 1).

We reasoned that loss-of-function (LoF) mutations in *TEK* could be involved in the etiology of PCG, given our findings of a PCG-like phenotype in *Angpt1/Angpt2* and *Tek* cKO mice (31). Therefore, we sought additional novel/rare *TEK* variants in a collaborative multi-ethnic cohort totaling 209 individuals in 189 families. In family 2 (Figure 1A), we screened for *TEK* variants by Sanger sequencing and identified an inconsistency in father-to-son transmission for a known SNP (see Sanger sequencing traces in Figure 1B) that suggested the presence of a deletion. Copy number variation analysis was performed on the family trio using Affy-

Table 2. Results of the TEK splicing donor variants analysis

Splicing donor variant	Prediction algorithm	WT	Mutant	Variation (%)
c.760+2T>C	HSF	94.19	67.35	–28.5, WT site broken
	MaxEnt	10.45	2.7	–74.16
c.3300+2delT	HSF	99.05	49.22	–50.31, WT site broken
	MaxEnt	11	–2.48	–122.55

Functional prediction for splicing donor variants were performed using Human Splicing Finder version 3.0 (HSF3.0). A score of more than 65 for position weight matrices (HSF) and more than 3 for maximum entropy (MaxEnt) is predictive for a functional splice site. Moreover, if the variation score (between WT and mutant) is under –10% for HSF and –30% for MaxEnt, the tool considers that the mutation affects the splice site. See the predicted effects of the disruption of splice donor sites in Supplemental Figure 3.

metrix SNP 6.0 arrays, which identified a 56-kb deletion encompassing *TEK* exons 2–4 (p.T19_R210del) that was passed from the father (unknown affection status) to the unilaterally affected son (Figure 1B). In total in our cohort, whole exome and direct Sanger sequencing identified 10 novel/rare heterozygous *TEK* variants (Figure 1, A–C, and Table 1). Nine of these 10 mutations (p.Y307*, p.T19_R210del, p.C233Y, p.Y611C, p.E150*, p.K745fs, p.G984*, c.760+2T>C, and c.3300+2delT) were absent from publicly available exome databases (Exome Sequencing Project [NHLBI-ESP, http://evs.gs.washington.edu/EVS/], 6,500 exome sequences; Exome Aggregation Consortium [ExAC, http://exac.broadinstitute.org], 60,706 exome sequences) and 203 in-house control exomes (Table 1). The highest allele frequency observed for the rare variant (p.K294N) was 0.0003 in Latino populations (ExAC) (data not shown). In all 7 of these families for which we had whole exome data, the possibility of *TEK* gene copy number variants (CNVs) accounting for a second mutant allele was assessed using exome read depth coverage comparisons. Read depth data for each of the 23 *TEK* exons was extracted from the 7 PCG exomes and compared with the average read depth obtained from a number of unrelated same-batch exome-sequenced samples. In all cases, no evidence for additional exon-spanning duplications or deletions could be found (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI85830DS1).

Four mutations (p.E150*, p.Y307*, p.K745fs, and p.G984*) are predicted to be null alleles likely to result in mRNA degradation via nonsense-mediated decay or truncated proteins (see predicted truncated proteins in Supplemental Figure 2A; EnzymeX [http://nucleobytes.com/enzymex/] was used for predictions). Furthermore, the in silico predictive tool Human Splicing Finder (HSF 3.0, http://www.umd.be/HSF3/HSF.html) indicated that the two invariant splice donor mutations (c.760+2T>C and c.3300+2delT) affect splicing and result in functionally null proteins (Table 2 and Supplemental Figure 3). The strong enrichment for LoF mutations in our PCG cohort (6 of 189) compared with those observed in the ExAC database (2 of 60,706 individuals; $P = 2.3 \times 10^{-14}$; odds ratio = 995.2 [95% confidence interval = 199.5–4,963.0]; see Methods for details), supports a role for *TEK* mutations in PCG pathogenesis. Additionally, according to analysis of evolutionary conservation and in silico predictive tools (Polymorphism Phenotyping v2 [PolyPhen-2, http://genetics.bwh.harvard.edu/pph2/]; Sorting Intolerant from Tolerant [SIFT, http://sift.bii.a-star.edu.sg/]; and FoldX [http://foldx.crg.es/, http://foldxyasara.switchlab.org/]), 2 of the 3 missense mutations are also expected to affect protein function (Table 3 and Supplemental Figures 4–6).

TEK mutations identified in PCG patients exhibit LoF. We experimentally tested the functional impact of PCG-associated *TEK* variants in cell-based assays to determine their effect on TEK protein production, phosphorylation, and trafficking within the cell. As mutations were identified sequentially, we performed analysis of the first 5 mutations discovered in our primary cohort. In our system, WT TEK protein underwent normal baseline autophosphorylation when expressed in HEK293 cells (Figure 2A). As predicted, the p.Y307* mutant cDNA (identified in family 1) did not produce a full-length intact protein with C-terminal FLAG tag, as the introduced stop codon is upstream of the tag (Figure 2A and Supplemental Figure 7). To test the possibility that the predicted p.Y307 mutant protein is produced and secreted into the medium, we generated a TEK306-Fc–expressing vector. TEK306-Fc protein, which contains the Ig1, Ig2, EGF1, and EGF2 domains, was detected in the medium (Supplemental Figure 2B and Supplemental Figure 7).

The p.T19_R210del (ΔT_R, family 2) and p.C233Y (family 3) mutations resulted in distinctly reduced protein levels accompanied by negligible autophosphorylation, making them functional null alleles (Figure 2A). Interestingly, the p.K294N (fam-

Table 3. Functional prediction results for missense TEK variants

Prediction method	No. of representative sequences at positions 233, 294, & 611	Prediction result for TEK p.C233Y	Prediction result for TEK p.K294N	Prediction result for TEK p.Y611C
SIFT: TEK orthologs	64, 64, 63	Affect protein function, 0.0[A]	Affect protein function, 0.02[A]	Affect protein function, 0.0[A]
SIFT: TEK family	141, 139, 140	Affect protein function, 0.0	Tolerated, 0.11	Affect protein function, 0.0
SIFT: Blink[B]	102, 106, 172	Affect protein function, 0.0	Tolerated, 0.44	Affect protein function, 0.0
PolyPhen-2	n/a	Probably damaging, 1.0	Benign, 0.298	Probably damaging, 1.0
FoldX average ddG, Kcal/mol[C] (SD)	n/a	7.52 (3.48)[D]	0.93 (0.004)[D]	2.02 (0.02)[E]

[A]Sequences may not be diverse enough for confident prediction. [B]399 sequences were selected to be closely related to query sequence by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). [C]Average ddG (difference in free energies between mutant and WT protein) run over 5 times. [D]Average ddG from Protein Data Bank (http://www.rcsb.org/pdb/home/home.do) ID: 2GY5. [E]ddG = 2.02 from the structure model that was created using PDB ID: 4N68 as a template.

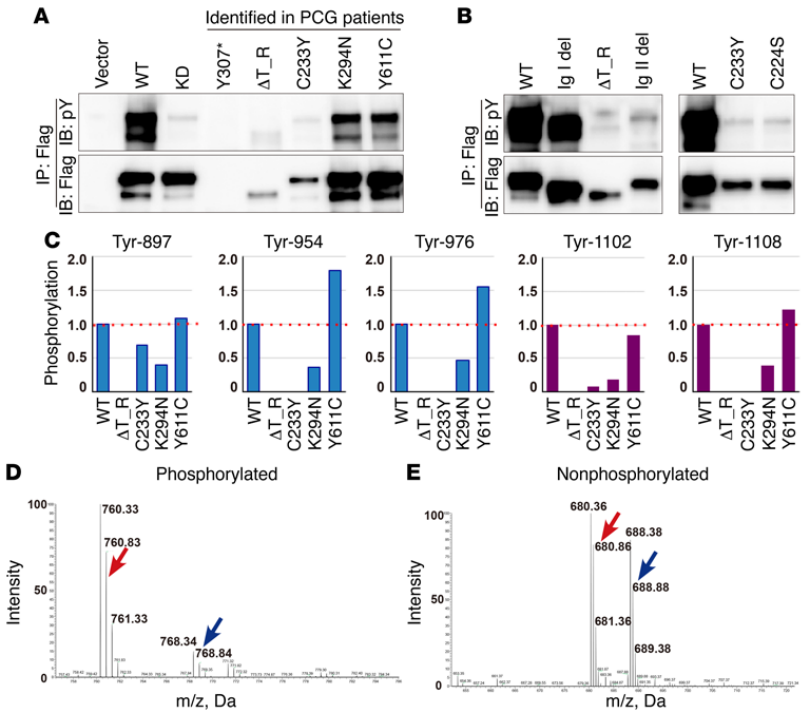

Figure 2. TEK variants lead to reduced autophosphorylation. (**A**) Western blotting of WT and variant TEK expressed in HEK293 cells, following immunoprecipitation with Flag antibody. (**B**) The effect of deleting immunoglobulin-like domain (left), and the effect of breaking the disulfide bond formed between C233 and C224 in the EGF I domain (right). (**C**) Quantitative phosphorylation analysis of each tyrosine residue in the kinase domain (Tyr897, Tyr954, and Tyr976) and C-tail (Tyr1102 and Tyr1108) using SILAC. (**D** and **E**) Representative MS1 spectrum of a peptide (KT**Y**VNTTL**Y**EK) derived from either WT (red, light-labeled) or p.K294N mutant (blue, heavy-labeled). Tyrosine residues in this peptide correspond to Tyr1102 and Tyr1108. The spectrum of phosphorylated peptides (KTpYVNTTLpYEK) is shown in **D**. The spectrum of nonphosphorylated peptides (KTYVNTTLYEK) is shown in **E**. Ig I del, natural variant lacking Ig1 domain; ΔT_R, PCG variant lacking Ig1 and Ig2 domain (p.T19_R210del); Ig II del, disease-associated variant lacking Ig2 domain (34); KD, an artificial kinase-dead mutant (D982A substitution, disrupting DFG motif of kinase domain). Note that both the Ig I del variant and the Ig II del mutant were not identified in PCG cohorts. All blotting data are representative of more than 3 biological replicates.

ily 4) and p.Y611C (family 5) mutations did not result in reduced protein expression or basal global autophosphorylation in this system (Figure 2A).

Deletion of exons 2–4 (p.T19_R210del, ΔT_R) results in loss of the terminal 4 aa of the signal peptide sequence (MDSLASLVLCG-VSLLLSG**TVEG**) and the entire Ig1 and Ig2 domains. To determine whether the loss of 4 aa from the signal peptide and/or loss of the Ig1 domain alone is sufficient to cause receptor degradation, we tested a natural splice variant (ENST00000519097) that lacks exon 2 — encoding the terminal 5 aa of the signal peptide as well as the first Ig domain (Ig1). This splice variant (Ig I del) was expressed and phosphorylated at normal levels (Figure 2B). Conversely, a form of TEK mutant carrying an in-frame somatic deletion of exon 3 and part of exon 4 (aa 122–165 of the Ig2 domain [Ig II del]) (34) was poorly expressed and phosphorylated, indicating that the Ig2 domain is important for TEK stability and function (Figure 2B, left panels).

As Cys233 forms a disulfide bridge with Cys224 in the EGF1 domain (Supplemental Figures 4 and 5), we hypothesized that the p.C233Y variant may cause misfolding of the protein, due to the disruption of the disulfide bond, followed by degradation. To investigate this possibility, we generated a p.C224S mutant and found that mutation of this paired cysteine led to a similar reduction in protein abundance (Figure 2B, right panels), confirming the critical importance of the disulfide bond for proper folding and subsequent expression.

Receptor tyrosine kinases mediate their specific signals through distinct tyrosine residues (28), and for TEK-mediated signaling the cytoplasmic C-terminal Tyr1102 and Tyr1108 residues play a central role (27, 35, 36). To investigate phosphorylation at individual tyrosine residues, we used mass spectrometry together with stable isotope labeling in cell culture (SILAC; Figure 2C and Supplemental Figure 8). While global phosphorylation of the p.K294N mutant was normal, SILAC revealed a 10-fold reduction in Tyr1102 phosphorylation (Figure 2, C–E). This site is known to mediate signaling through p85-PI3K (35, 36), suggesting that the p.K294N mutation results in reduced p85-PI3K signaling activity. Based on the critical importance of this site for TEK function (35), we propose that p.K294N is a LoF allele. In contrast, no effect on basal phosphorylation (i.e., ligand-independent phosphorylation) was observed in the p.Y611C mutant (Figure 2C).

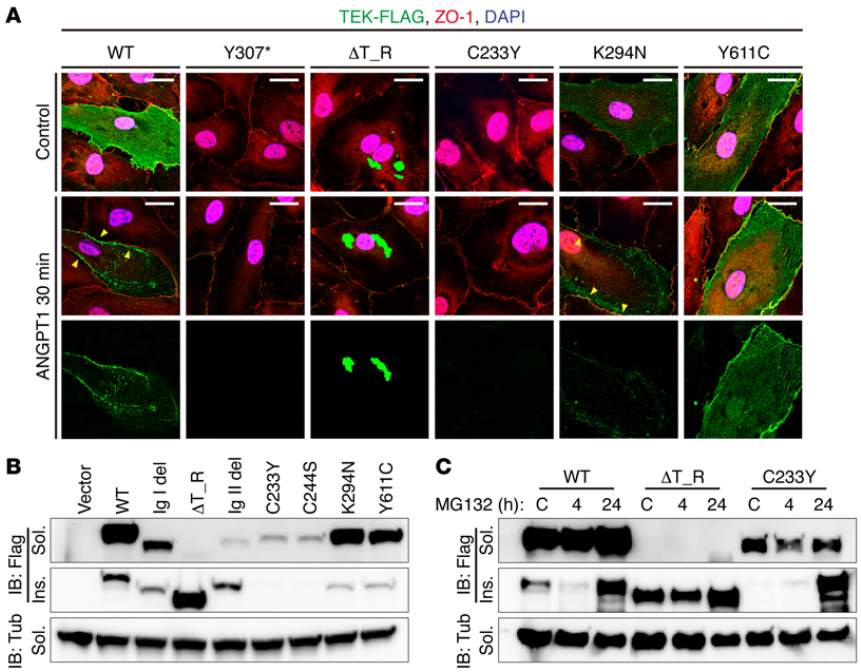

Figure 3. TEK variants lead to protein aggregate formation, reduced expression, and aberrant trafficking. (**A**) Expression pattern of WT and variant TEK in HUVECs. Transfected HUVECs were stained with anti-Flag (green) and anti–ZO-1 antibody (red). Nuclei were stained with DAPI (pink in merged images). Yellow arrowheads indicate the TEK receptor on cellular junctions. The Y307* mutant–transfected cells, in which Flag-tag is not expressed, were used as a mock negative control for imaging. Scale bars: 25 μm. (**B**) Solubility of TEK variants in HEK293 cells. Solubility of WT and each variant TEK protein was tested by fractionated samples. (**C**) Effect of proteasomal inhibition on TEK variant expression in HEK293 cells. Cells were treated with MG132, a proteasomal inhibitor, for the indicated times. C, control (DMSO-treated for 24 hours); Sol., soluble fraction; Ins., insoluble fraction; Tub, α-tubulin. All data are representative of more than 3 biological replicates.

As portions of the ectodomain are responsible for ligand binding (37, 38), subsequent multimerization (38), and subcellular localization (39–41), we predicted that the mutations in these regions could weaken or abolish ligand-mediated activation and signal transduction through the intracellular catalytic domain. Anticipating that TEK ectodomain mutations may affect ligand-mediated signaling, we expressed TEK variants in HUVECs and examined their subcellular localization after ligand stimulation. WT TEK was diffusely expressed on the membrane of resting cells (Figure 3A, control WT). When treated with ANGPT1, a major TEK agonist, WT TEK localized to cell-cell junctions and subsequently became internalized (Figure 3A and Supplemental Figure 9), as previously reported (39, 40). The p.K294N mutant also followed this dynamic receptor translocation. However, other mutants were unable to respond to ANGPT1 stimulus. p.T19_R210del formed intracellular aggregates, and p.C233Y was not found at the cell membrane (Figure 3A).

WT protein is typically detected within the soluble fraction of a cell lysate (Figure 3B), whereas mutant proteins can form aggregates inside cells and are found within the insoluble fraction. Consistent with subcellular localization studies, p.T19_R210del was found in the insoluble fraction (Figure 3B, ΔT_R). Our results clearly demonstrate that the deletion of Ig1 and Ig2 domains in ΔT_R reduces protein solubility, leading to protein aggregate formation, making this allele functionally null. The IgII deletion mutant was found in both fractions, and the p.C233Y mutant, although not insoluble, was less abundant, suggesting that solubility was not the cause of poor expression (Figure 3B). Other variants were largely found in the soluble fraction (Figure 3B).

To determine whether proteasomal degradation underlies the reduced levels of protein, we treated cells expressing the p.T19_R210del or p.C233Y mutant with the proteasomal inhibitor MG132. The inhibitor slightly increased WT protein expression in the soluble fraction. More prominently for WT and p.C233Y, there was a marked increase in insoluble protein following MG132 treatment (Figure 3C). These data suggest that TEK abundance is tightly regulated by proteasome-mediated degradation and that structural defects, such as p.C233Y, lead to enhanced protein degradation through this mechanism.

Interestingly, while the p.Y611C mutant is properly localized to the plasma membrane in resting cells, upon ANGPT1 stimulation it did not follow the normal course of dynamic clustering, junctional localization, and internalization, suggesting that this variant is unable to respond to ligand (Figure 3A, Y611C). In order to measure changes in ligand-mediated TEK phosphorylation, we cotransfected cells with protein tyrosine phosphatase recep-

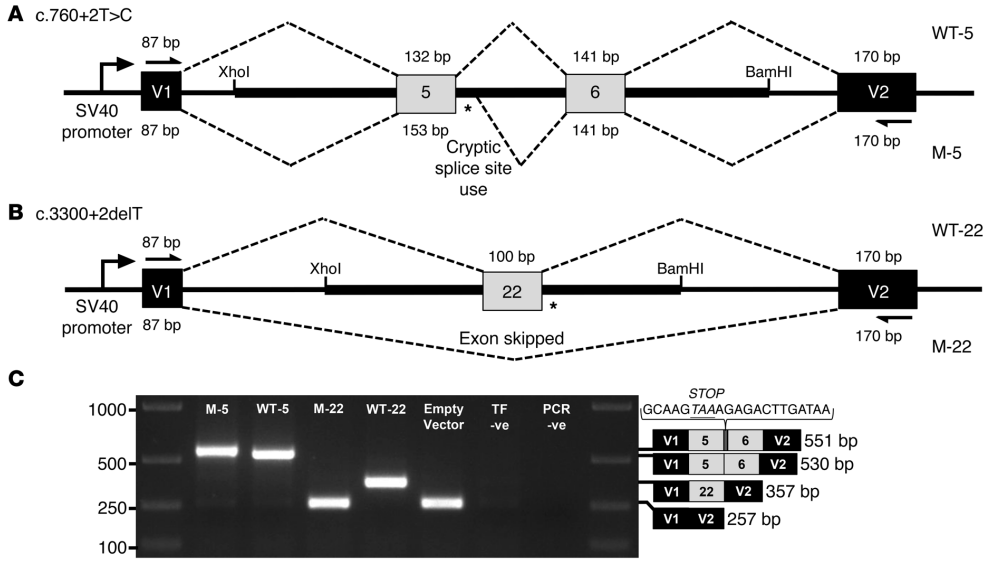

Figure 4. Splice donor site mutations lead to cryptic splice site use or exon skipping. (**A** and **B**) Splicing construct minigenes were generated by incorporating genomic regions of the *TEK* gene into the pSPL3 vector via XhoI and BamHI restriction sites. Vector exons, V1 and V2, are depicted as black boxes and *TEK* exons 5, 6, and 22 are in gray. WT and mutant splicing products, with included exon sizes in base pairs, are indicated by dashed lines above and below the construct, respectively. The locations of the splice site mutations are shown (*). (**A**) WT (WT-5) and mutant (M-5) genomic fragments containing *TEK* exons 5 and 6 were used to model the c.760+2T>C mutation from family 7. (**B**) WT (WT-22) and mutant (M-22) genomic fragments containing *TEK* exon 22 were used to model the c.760+2T>C mutation from family 8. (**C**) Gel electrophoresis of RT-PCR products from transfected Cos-7 cells. Vector exon-specific primers are indicated by arrows in **A** and **B**. TF -ve, cells transfected with PBS only; PCR -ve, PCR-negative control. WT and mutant transcript content, determined by Sanger sequencing, is depicted to the right of the gel image. The additional 21 bp of intron 5 sequence identified within the M5 transcript is shown incorporating a premature termination codon between exons 5 and 6.

tor type B (PTPRB) to reduce baseline phosphorylation of coexpressed TEK (42). ANGPT1 stimulated the phosphorylation of WT TEK but not p.Y611C, further suggesting a defect in ligand-mediated activation (Supplemental Figure 10).

We also experimentally tested the functional impact of the two splice donor site mutations, c.760+2T>C (family 7) and c.3300+2delT (family 8), in cell-based exon trapping splicing assays to determine their effect on mRNA splicing (Figure 4). In this system, the c.760+2T>C mutation eliminated the normal 5′ splice donor site of exon 5, which led to a cryptic splice site 21 bp into intron 5 (Figure 4, A and C). As the additional 21 bp sequence incorporated an in-frame termination codon into exon 5, it was expected to result in mRNA transcript destruction by the nonsense-mediated decay pathway or a severely truncated protein product (p.A254Gfs*3). In contrast, the c.3300+2delT splice donor site mutation resulted in complete skipping of exon 22, effectively removing 100 bp from the mRNA transcript and changing the codon reading frame by which the last exon would be translated (Figure 4, B and C). As a result, exon 23 would encode 2 different amino acid residues (Pro-Thr), followed by a termination codon instead of the normal 57 C-terminal residues (p.Y1068Pfs*3). Consequently, the mRNA would likely escape destruction by the nonsense-mediated decay pathway, and a protein lacking residues 1068–1124, which includes the C-terminal tyrosine phosphorylation sites (Tyr1102 and Tyr1108), would be produced. As these two tyrosine residues are critical for TEK receptor signaling function (35, 36), we propose that the c.3300+2delT mutation represents a LoF allele.

Collectively, our results have demonstrated that 7 of 10 *TEK* mutations identified in our PCG cohort lead to loss of functional protein by multiple cellular mechanisms — absence of intact protein production (p.Y307*, p.T19_R210del, c.760+2T>C [p.A254Gfs*3], and c.3300+2delT [p.Y1068Pfs*3]), enhanced proteasomal degradation (p.C233Y), impaired phosphorylation of key tyrosine residue (p.K249N), altered subcellular localization and reduced ligand responsiveness (p.Y611C), and protein aggregate formation (p.T19_R210del). We reason that the p.G984* (family 6), p.K745fs (family 9), and p.E150* (family 10) mutations are also likely LoF alleles, as their mRNA transcripts will either be destroyed by the nonsense-mediated decay pathway or encode proteins lacking at least a functional intracellular domain (See Supplemental Figure 2).

TEK haploinsufficiency results in abnormal AHO structure and elevated IOP. After proving that the heterozygous *TEK* mutations resulted in nonfunctional proteins, we hypothesized that TEK haploinsufficiency is sufficient to cause impaired SC development and ocular hypertension in mice. Using mice harboring a conditional by inversion allele (*Tek*^{COIN} mice) (43), we analyzed AHO drainage pathways in *Tek* hemizygous and conditional knockout mice. Confocal microscopy revealed that *Tek*-haploinsufficient mice developed a severely hypomorphic canal with convolutions and focal narrowing, while SC was normal in control mice (Figures 5–7). SC

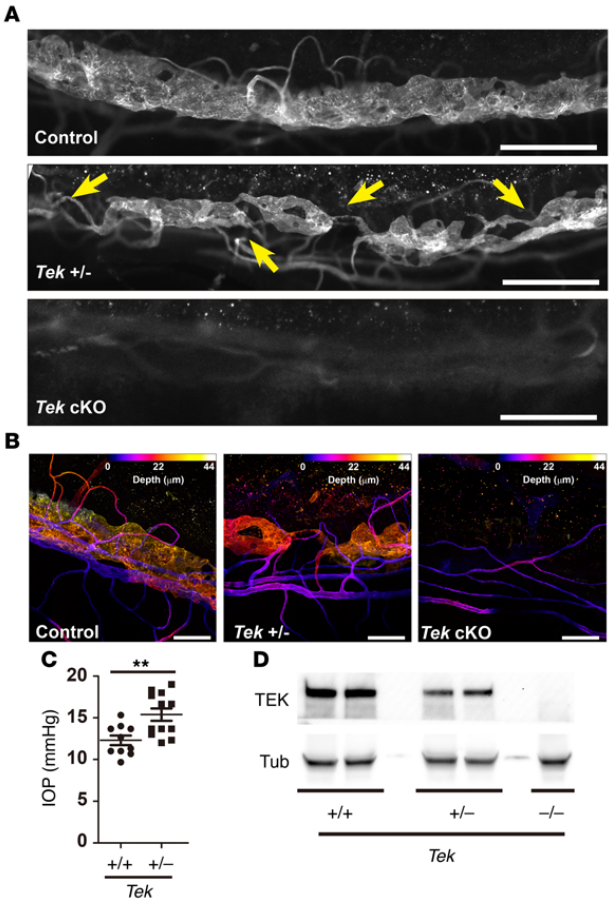

Figure 5. TEK-haploinsufficient mice exhibit hypomorphic SC with elevated IOP. (**A** and **B**) Confocal microscopic images of CD31 staining in WT (control), TEK-haploinsufficient (*Tek*$^{+/-}$), and TEK conditional knockout (*Tek* cKO) mice (10 weeks old). Yellow arrows indicate the convolutions and narrowing of SC. Z-stack (44 μm) and pseudo-colored depth projections are shown in **B**. Scale bars: 25 μm (**A**), 100 μm (**B**). (**C**) The effect of *Tek* genotype on IOP. Averaged IOP of both eyes from each mouse is shown. *n*= 8 and 9 for *Tek*$^{+/+}$ and *Tek*$^{+/-}$, respectively (25 weeks old). $^{**}P < 0.01$ (2-tailed Student's *t* test). Error bars indicate SEM. (**D**) TEK protein expression level of each genotype of mice using lung homogenate.

was completely absent in *Tek*-knockout mice, clearly demonstrating the absolute requirement and gene dosage sensitivity for TEK during canal development (Figure 5, A and B). Interestingly, quantitative analysis identified the variable degree of hypomorphism in *Tek*-hemizygous mice (Figure 6). We further analyzed the iridocorneal region using serial histologic sections. Whereas the control WT eyes showed well-developed SC and TM, eyes of *Tek*-hemizygous mice showed hypoplastic SC and TM, indicating that reduced TEK signaling is detrimental for formation of AHO structures (Figure 7).

To analyze the functional consequence of dysmorphic SC development and hypomorphic TM, we analyzed IOP levels by rebound tonometry. Measurement of IOP confirmed that haploinsufficient mice exhibited a 25% elevation in IOP (control 12.3 mmHg, *Tek*$^{+/-}$ 15.4 mmHg, $P = 0.0046$; Figure 5C and Supplemental Figure 11). The IOP levels were modestly correlated with the degree of SC hypomorphism ($r = 0.3703$, $P < 0.01$). These results demonstrate that reduced TEK signaling causes developmental defects of the AHO structure, a key feature of PCG, and correlates with elevated IOP.

Discussion

Ocular hypertension has been identified as the most important risk factor in human glaucoma, and current glaucoma therapy is focused on lowering IOP using pharmacological or surgical approaches (1–3, 20, 44, 45). While IOP is influenced by both production and drainage of the AH, defects in anterior chamber outflow have been identified as the source of ocular hypertension in glaucoma patients, including PCG patients (5, 46). Despite evidence that vascular growth factors are essential for SC development and AH outflow (25, 31, 47), links between these pathways and human glaucoma have not been identified. Herein, we describe 10 heterozygous novel/rare protein-altering mutations in *TEK* as a cause for PCG. Furthermore, our findings provide evidence that *TEK* gene dosage is critical for the proper development of AHO pathways, and ~50% reduced TEK signaling is sufficient to lead to defective SC development and compromised AH drainage in mice as well as in patients (Figure 8).

While PCG has clear genetic links, identification of causative alleles is complicated by variable disease penetrance and expressivity (5, 9, 12). Families identified in our patient cohort were no exception. *TEK* variants were heterozygous in all affected individuals, and the heterozygous disease allele was vertically transmitted from the affected parent to the affected child in two families (families 1 and 10), clearly indicating dominant transmission of the disease. Interestingly, the development of glaucoma in later decades in two mutation carriers in family 10 (one inferred) and the frequent unilateral involvement observed in mutation carriers (6 of 12 in our cohort with a *TEK* mutation versus 34 of 209 in our whole cohort) suggest that *TEK*-related disease can exhibit variable severity and age of onset (Figure 1 and Table 1). This highly variable expressivity may explain the high frequency of carriers without a typical early-onset PCG phenotype in family members. It was not possible to carefully examine many of the parents of the affected probands with PCG for evidence of glaucoma or raised IOP, and given the geographic and historical nature of some of the samples, it is not currently possible to gather this information. With the limited availability of human data, it is difficult to clearly distinguish variable expressivity from reduced penetrance. However, the biological plausibility is underscored by the mouse data clearly showing that haploinsufficiency for *Tek* is sufficient to lead to functional defects of the AHO pathway with variable expressivity. Therefore, we propose an autosomal dominant model with variable expressivity, consistent with other ocular disorders of developmental origin, such as those caused by mutations in *FOXC1*, *MYOC*, *PAX6*, and *OPA1* (6, 10, 48–52). Variable expressivity may be explained by stochastic developmental events

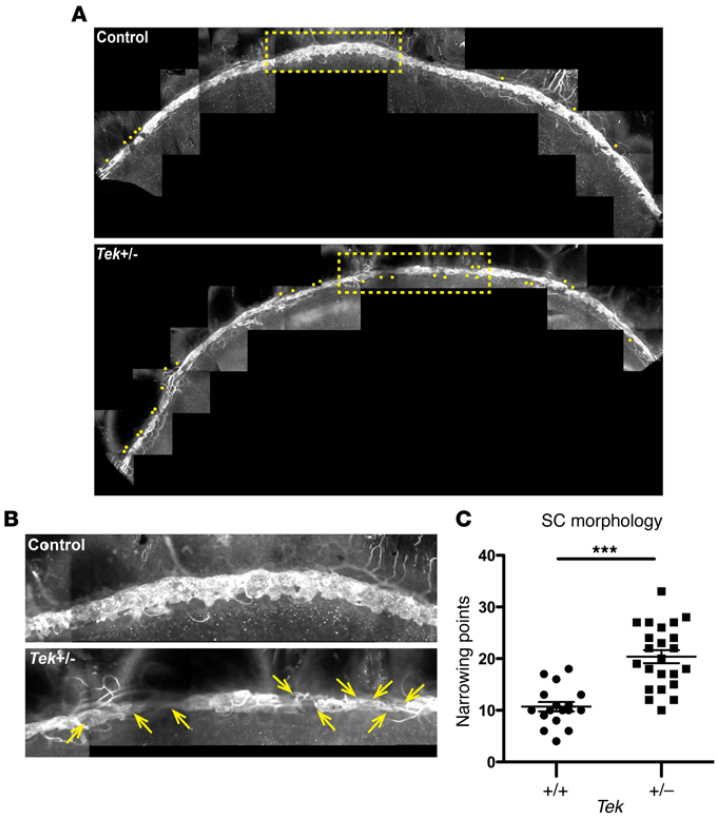

Figure 6. Quantitative analysis of SC morphology. (**A**) Confocal microscopic images of CD31 staining in control (*Tek*$^{+/+}$) and *Tek*-hemizygous (*Tek*$^{+/-}$) mice. Yellow dots indicate the points with SC narrowing. Original magnification, x20. (**B**) Higher magnification (x60) of the yellow dotted area in **A**. The arrows indicate the narrowing points. (**C**) Quantitative analysis of SC imaging. The number of narrowing points was compared between *Tek*$^{+/+}$ and *Tek*$^{+/-}$ mice (n = 17 eyes for *Tek*$^{+/+}$, n = 23 eyes for *Tek*$^{+/-}$; 10–25 weeks old, littermate controls were used). ***$P < 0.001$ (2-tailed Student's t test). Error bars indicate SEM.

in SC formation (25) or oligo-/digenic inheritance. Although we have screened 19 "candidate genes," including the signaling components of the ANGPT-TEK pathway (*TEK*, *ANGPT1*, *ANGPT2*, *TIE1*, *ANGPT4*, and *PTPRB*), genes expressed during SC development (*PROX1*, *FLT4*/*VEGFR3*, and *VEGFC*), and genes linked to various developmental glaucomas (*FOXC2*, *CYP1B1*, *MYOC*, *FOXC1*, *OPTN*, *ASB10*, *TBK1*, *WDR36*, *OPA1*, and *NTF4*), we did not identify possible pathogenic variants as a second allele for digenic inheritance. Our results also suggest that ascertaining future PCG subjects for genetic studies should incorporate thorough, longitudinal ophthalmic examinations of reportedly normal parents of affected probands, as they may have subclinical indicators of PCG/glaucoma.

The analysis of PCG mutations in *TEK* advances our understanding of structure-function relationships within the receptor. Interestingly, all the deletion and missense mutations identified in our PCG cohort were located in the ectodomain and are LoF. The location of PCG mutations is strikingly different from that of the gain-of-function (GoF) *TEK* mutations that have been linked with hereditary and sporadic venous malformations (VMs) (34, 53, 54). These VM mutations are located solely in the intracellular domain and result in enhanced kinase activity. Furthermore, our cell-based assays have identified new functional roles for various domains. The Ig2 domain, which functions as a ligand-binding domain (37, 38), is also critical for stable protein expression, as loss of this Ig domain in p.T19_R210del and IgII del leads to reduced protein levels. The mutation in the EGF1 domain highlights the critical role of cysteine residues, which are needed to disulfide cross-link the protein for stability (38, 55). Notably, the mutation in the membrane-proximal fibronectin type III (FN3) domain, which appears in ~40% of all receptor tyrosine kinases (ref. 28 and the Human Protein Reference Database [HPRD], http://www.hprd.org), sheds light on its novel role in ligand-mediated TEK receptor activation. The FN3 domain in the Eph family tyrosine kinase receptors directly stimulates intermolecular interactions to cluster the receptor into higher-order multimers at the plasma membrane (56). These Eph clusters act as efficient signaling centers to trigger cell responses and propagate phosphotyrosine signals. Although the TEK FN3 crystal structure is currently unavailable, the lack of membrane response following ANGPT1 stimulation as observed in HUVECs (Figure 3A) is likely due to impaired signaling activities.

GoF mutations in patients with VMs exhibit variable expressivity and led to the hypothesis that tight regulation of TEK activity is needed for vascular development (34, 54). Our findings that hemizygosity for *Tek* is sufficient to cause disease in mice and that heterozygosity for LoF mutations is associated with PCG of varying severity in patients suggest, for the first time to our knowledge, that there exists a dosage sensitivity for reduced activity of the receptor. It is reported that the conventional outflow path for AH drainage (i.e., SC) plays a less important role in mice than in humans, underscoring the significance of finding elevated IOP in the hemizygous mouse model and suggesting similar structural defects in SC would cause more severe disease in patients (57–59). Finally, our data indicate that TEK "dose" might be particularly important for proper development of the vasculature of the anterior chamber of the eye, suggesting that regulation of TEK signal strength may be a therapeutic option.

In summary, we have identified *TEK* mutations in patients with PCG that follow a dominant inheritance pattern with variable expressivity. Our experimental results further demonstrate that defects in the AHO pathway caused by reduced TEK signaling constitute a new mechanism of PCG disease in humans. It is likely

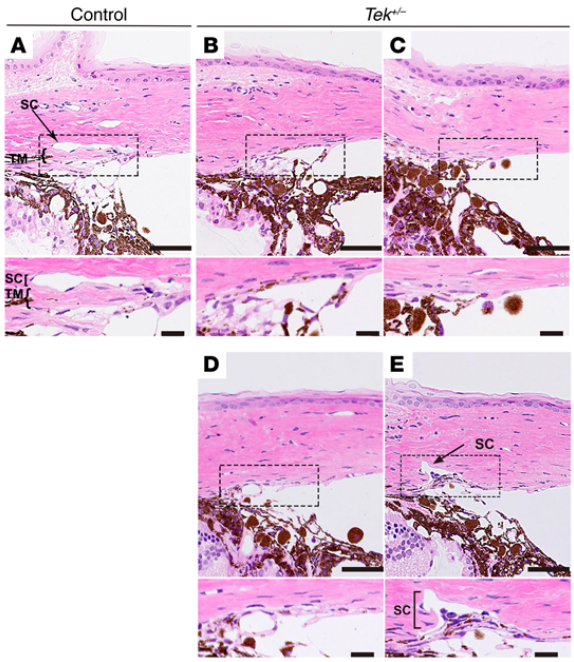

Figure 7. Formation of the SC and TM is defective in *Tek*-hemizygous mice. (**A**–**E**) Histological analysis of iridocorneal angle region from a control eye and *Tek*$^{+/-}$ mouse eyes (25 weeks old). Upper panels show the location of the SC and TM within the iridocorneal angle. Lower panels show magnified images of the dotted boxes in the upper panels. Note that the TM is hypoplastic and the SC is either absent (**B**–**D**) or severely reduced in size (**E**) in *Tek*$^{+/-}$ mice. Scale bars: 50 μm (upper panels), 10 μm (lower panels).

that investigating additional genes involved in the formation and maintenance of the AHO pathways will provide further insights to our understanding of this devastating eye disease and aid in the development of new therapeutic strategies for glaucoma.

Methods

Human study participants. PCG was defined by the following characteristics: (a) age at onset less than 3 years, (b) increased corneal diameter greater than 10 mm accompanied by corneal edema and/or Haab striae, and (c) IOP greater than 21 mmHg and/or optic nerve cupping greater than 0.4. Any patient with other ocular abnormalities or systemic conditions, other than iris stromal hypoplasia, was excluded from the study. Our studies adhered to the tenets of the Declaration of Helsinki and were compliant with the Health Insurance Portability and Accountability Act.

Whole exome sequencing. Exome sequencing of the initial PCG cohort of 20 families with at least one affected proband was performed using a Nimblegen EZ v3 capture kit (Roche) and 2 × 100 bp paired-end sequencing on a HiSeq2000 platform (Illumina). All exome variants were validated by Sanger sequencing. The *TEK* gene was Sanger sequencing screened in an additional 55 families in the primary cohort. An additional 114 families were also screened in a secondary international cohort (details are provided in the Supplemental Material). In total, 189 families containing 209 individuals affected by PCG participated in this study.

LoF gene-burden test. A LoF gene-burden test was performed by considering only the predicted LoF variants (i.e., stop gain, essential splice donor/acceptor, and frameshift insertion/deletion). While 7 of the 189 PCG probands carried heterozygous predicted LoF variants in *TEK* (p.E150*, p.Y307*, p.T19_R210del, p.K745fs, p.G984*, c.760+2T>C, and c.3300+2delT), only 2 heterozygous LoF *TEK* variants were present in 60,706 subjects in the ExAC cohort. Since large deletions (e.g., p.T19_R210del in family 2) would not have been called

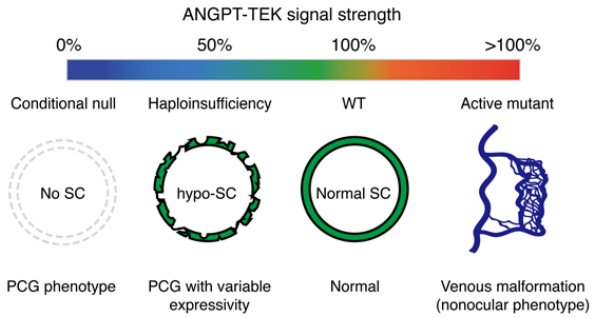

Figure 8. Model for ANGPT-TEK signal strength and human disease. Schematic model shows the importance of ANGPT-TEK signal strength as a critical determinant of ocular and nonocular vascular phenotypes. In the complete absence of ANGPT-TEK signaling after embryonic day 17.5, SC is not formed and conditional null mice exhibit a severe PCG-like phenotype (31). 50% reduction of the signal leads to severely hypomorphic SC formation with elevated IOP in mice, and PCG in humans with variable expressivity. In human patients, GoF mutations in *TEK* result in VMs in nonocular tissues (34, 53, 54). A frontal view of SC is illustrated in this figure.

by the VEP/LOFTEE pipeline used by ExAC (https://github.com/konradjk/loftee), we excluded this variant from our LoF gene-burden analysis. Thereby, we compared 6 of 189 PCG families with 2 of 60,706 individuals in ExAC.

In silico functional studies. PolyPhen-2 (60, 61) and SIFT (version 5.2.2) (62) were used to predict any functional effects that TEK missense variants may have on protein function. To analyze the effect of missense variants on protein structure, FoldX (63) analyses were performed using known crystal structures when available. For the two splice donor variants (c.760+2T>C, c.3300+2delT), we used HSF version 3.0 (64) and Automatic Analysis of SNP sites (AASsites) (65) to analyze whether the variants can cause changes in splice sites. Detailed protocols are available in the Supplemental Material.

Tissue culture and proteomics. TEK expression constructs were created by cloning full-length *TEK* cDNA and incorporating a 3′ Flag-tag sequence into the pcDNA3.1 vector. Site-directed mutagenesis and synthesized DNAs were used to generate variant TEK-Flag expression vectors. Total TEK phosphorylation was determined by Western blotting following immunoprecipitation. TEK solubility was analyzed by separating cell lysates into soluble and insoluble fractions. Protein localization studies were performed by transfecting HUVECs with the TEK-Flag vectors and immunostaining using standard protocols. For SILAC analysis, WT TEK-Flag was transfected into light isotope–labeled cells and PCG variants into heavy isotope–labeled cells. The heavy/light ratio of phosphorylated peptides identified by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) was calculated by quantification node, and the ratio was normalized by protein abundance. Detailed protocols are available in the Supplemental Material.

Exon trapping splicing assay. Genomic DNA fragments containing *TEK* exons 5–6 and exon 22 from affected individuals and a control subject were cloned into the exon trapping vector pSPL3. Total RNA from exon trapping construct–transfected Cos-7 cells were reverse-transcribed and PCR-amplified. The resulting RT-PCR products from WT and mutant minigenes were visualized by gel electrophoresis, and their sequence composition was determined by Sanger sequencing. Detailed protocols are provided in the Supplemental Material.

Mice and breeding. *Tek* WT ($Tek^{+/+}$), hemizygous ($Tek^{+/-}$), and homozygous knockout (*Tek* cKO) mice were generated by crossing Tek^{COIN} mice with *ROSA-rtTA*;*tetO-Cre* mice (Supplemental Figure 12). These mouse lines were described previously (30, 31). Doxycycline was added to the drinking water of pregnant dams from embryonic day 17.5 to induce *Tek* deletion. IOP was measured using a TONOLAB rebound tonometer (iCare). TEK expression in lung lysates was analyzed by standard blotting procedures. SC imaging was performed as previously described (31). Mice were maintained on a mixed background, and littermate controls were used for all analyses. Detailed protocols are available in the Supplemental Material.

Statistics. Comparisons of IOP between $Tek^{+/-}$ and WT mice were analyzed by unpaired 2-tailed Student's *t* test using GraphPad Prism software. The LoF gene-burden test was performed using JMP Pro 11 software, and a two-tailed Fisher's exact test was used for testing the statistical significance. A *P* value less than 0.05 was considered statistically significant.

Study approval. Subjects and their families were recruited from multiple international centers, each of which had received study approval from their respective institutional review boards: University of Wisconsin-Madison Health Sciences Institutional Review Board; Duke University Health System Institutional Review Board for Clinical Investigations; Children's Hospital of Philadelphia Institutional Review Board; Massachusetts Eye and Ear Infirmary Human Studies Committee; Human Research Ethics Committee of the University of Western Australia; Southern Adelaide Clinical Human Research Ethics Committee, Flinders Medical Centre, Bedford Park, South Australia, Australia; Tasmanian Health and Medical Human Research Ethics Committee, Hobart, Tasmania, Australia; Human Research and Ethics Committee of the Royal Victorian Eye and Ear Hospital; Ethical Review Board of the Medical Faculty of the Friedrich-Alexander University Erlangen-Nürnberg; Ethical Review Board of the Medical Faculty of Niguarda Ca'Granda Hospital; Commission of Ethics for Analysis of Research Projects — Clinical Board of Hospital Clinics and the Faculty of Medicine, University of São Paulo. Written informed consent for study participation was obtained from the subjects or subjects' parents. All animal experiments were approved by the Animal Care Committee of Northwestern University.

Author contributions

SEQ and TLY conceived, designed, and supervised the study, and wrote the manuscript. TS designed and performed proteomic, cell biology, and comparative biology experiments, analyzed data, and wrote the manuscript. SWT performed and coordinated the human genetic analyses, designed and performed exon trapping experiments, analyzed data, and wrote the manuscript. JJ designed and supervised the proteomics and cell biology study, and wrote the manuscript. JLW supplied patient material, performed human genetic analyses, and wrote the manuscript. FP and JEC supplied patient material and performed human genetic analyses. BRT designed and performed comparative biology experiments, analyzed data, and wrote the manuscript. OMS performed human genetic analyses and wrote the manuscript. VL and SMS performed in silico analyses and wrote a portion of the manuscript. KNW analyzed genetic data and performed experiments. KK and SJ performed experiments and wrote the manuscript. SY, LF, BK, KNTV, and XL performed experiments. LK, DNA, SF, LM, ES, TZ, AWH, KPB, DAM, KFA, JBR, SHL, and SR contributed human subjects, performed genetic analyses, and contributed to writing the manuscript. All authors contributed to the review and approval of the manuscript.

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3.3. *CYP1B1* copy number variations do not contribute strongly to PCG

CYP1B1 deleterious variants are transmitted in an autosomal recessive manner. PCG cases heterozygous for *CYP1B1* variants have been identified and a few isolated reports of *CYP1B1* deletions have been published. However, there was a gap in knowledge regarding the extent to which *CYP1B1* deletions might contribute to PCG and explain cases heterozygous for *CYP1B1* variants. In this study, I directly investigated whether *CYP1B1* CNVs account for PCG using multiplex ligation-dependent probe amplification (MLPA). No deletion or duplication of part of the gene or covering the entire gene were detected among 50 PCG cases previously negative or heterozygous for *CYP1B1*. The outcome of this study showed that *CYP1B1* CNVs are not a major contributor of PCG and does not support a clinical utility of testing for *CYP1B1* CNVs as a diagnostic procedure.

Contribution statement

Ms Souzeau was responsible for the study conception and design, data collection, analysis and interpretation, drafting and submitting the manuscript as corresponding author. Dr Hayes contributed to the genetic studies, data analysis and revised the manuscript. Dr Ruddle, Prof Elder, Ms Staffieri, Mrs Kearns and Prof Mackey contributed to the data collection and revised the manuscript. Dr Zhou contributed to data analysis and revised the manuscript. Ms Ridge contributed to the recruitment of participants, data collection and revised the manuscript. A/Prof Burdon contributed to data interpretation and revised the manuscript. Dr Dubowsky contributed to the study conception, genetic studies, data analysis and interpretation and revised the manuscript. Prof Craig contributed to the study design and conception, data collection and interpretation and revised the manuscript.

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CYP1B1 copy number variation is not a major contributor to primary congenital glaucoma

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Purpose: To evaluate the prevalence and the diagnostic utility of testing for *CYP1B1* copy number variation (CNV) in primary congenital glaucoma (PCG) cases unexplained by *CYP1B1* point mutations in The Australian and New Zealand Registry of Advanced Glaucoma.
Methods: In total, 50 PCG cases either heterozygous for disease-causing variants or with no *CYP1B1* sequence variants were included in the study. *CYP1B1* CNV was analyzed by Multiplex Ligation-dependent Probe Amplification (MLPA).
Results: No deletions or duplications were found in any of the cases.
Conclusion: This is the first study to report on *CYP1B1* CNV in PCG cases. Our findings show that this mechanism is not a major contributor to the phenotype and is of limited diagnostic utility.

Primary congenital glaucoma (PCG, OMIM 231300) is an important cause of glaucoma blindness in children and results from a developmental defect of the aqueous outflow system. It is characterized by increased intraocular pressure (IOP), buphthalmos, corneal clouding, and Haab's striae, and it generally manifests in the neonatal or early infantile period [1]. The level of incidence varies across different ethnic groups. A high incidence has been found among some populations (Slovakian Gypsies 1/1250 [2], Saudi Arabian 1/2500 [3]), but it is usually lower in Western countries (1/22,000–1/23,000) [2,4]. In Australia, PCG has an incidence of 1/30,000 births [5].

CYP1B1 (GLC3A, OMIM 601771) on chromosome 2p21 was the first gene discovered to cause PCG [6]. Pathogenic variants in *CYP1B1* have been identified among different populations [7-13], and they have been reported to occur in as few as 15% (in an American cohort) [12] and as high as 92% (in a Saudi Arabian cohort) [3] of PCG patients. In Australia, *CYP1B1* variants occur in 22% of PCG cases [14]. Four other PCG loci have been described (GLC3B-E), and *LTBP2* has been identified on chromosome 14q24 (GLC3C, OMIM 602091) [15]. However, a more recent study suggested that *LTBP2* variants might be responsible for primary congenital megalocornea with secondary glaucoma [16].

CYP1B1 is a member of the cytochrome P450 superfamily and is composed of three exons, of which only the last two are coding. The protein is responsible for the metabolism of a wide range of diverse substrates, both endogenous and exogenous [17]. Functional studies have demonstrated that *CYP1B1* pathogenic variants reduce the enzymatic activity or stability of the enzyme [18,19]. The mechanism by which they cause glaucoma is not fully understood, but it is hypothesized that the expressions of genes important to the development of the eye may be altered by the level of key regulatory molecules or the presence of normally eliminated metabolites [17].

CYP1B1 pathogenic variants are inherited in an autosomal recessive manner. However, individuals with PCG harboring only one pathogenic variant in *CYP1B1* have been reported [7-9,13,14]. These patients might have a second variant in the promoter, a non-coding region, or a deletion of a part of or the entire gene on their other allele, explaining their phenotype. No study has evaluated the prevalence of *CYP1B1* copy number variation (CNV) among PCG cases. In this report, we investigated whether *CYP1B1* gene CNV accounts for PCG cases heterozygous for *CYP1B1* mutations or with no pathogenic sequence variant, as identified through gene sequencing, and we explored the diagnostic use of testing for *CYP1B1* CNV.

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METHODS

Recruitment of participants: Ethics approval was obtained from the Southern Adelaide Clinical Human Research Ethics Committee. The study was conducted in accordance with the revised Declaration of Helsinki and following the National Health and Medical Research Council statement of ethical conduct in research involving humans.

Individuals with PCG were recruited through the Australian and New Zealand Registry of Advanced Glaucoma by referral from their eye practitioner [20]. Informed written consent and a blood sample for DNA extraction purposes were obtained. Clinical information was collected by the patient's usual clinical ophthalmologist. The diagnosis of PCG was based on combinations of corneal enlargement and buphthalmos, loss of corneal transparency, photophobia, raised IOP, or optic disc cupping.

Genetic testing: All PCG cases had bidirectional sequencing of the two *CYP1B1* coding exons and the respective intron–exon boundaries. Cases with two confirmed pathogenic sequence variants identified were excluded from the current analysis. Our cohort consisted of PCG patients with only one pathogenic sequence variant, with sequence variants of unknown clinical significance, or with no pathogenic sequence variants identified in the *CYP1B1* gene (n = 50).

CYP1B1 was analyzed for copy number abnormalities by Multiplex Ligation-dependent Probe Amplification (MLPA) using the SALSA MLPA P128 Cytochrome P450 probemix (MRC Holland, Amsterdam, The Netherlands) according to the manufacturer's instructions. MLPA allows for the detection of small copy number changes to the DNA sequence of a gene [21]. The probes consisted of two oligonucleotides complementary to adjacent sequences of the target DNA. The two probe sequences were joined by DNA ligase. Perfect homology between the two probes with the target at this junction is required for this step. The probes were then denatured from the template and amplified in a multiplex PCR reaction with the use of a single primer pair. Each probe generated an amplification product of a unique length, allowing for the separation of products by capillary electrophoresis. An indirect measurement of the copy number present in the original DNA specimen was determined from the relative amplitude of each amplicon product detected using the ABI 3130*xl* Genetic Analyzer (Life Technologies, CA), as well as analyzed using the Peak Scanner Software v1.0 (Life Technologies).

The P128 Cytochrome P450 probemix contained internal controls. The kit included probes targeting copy number changes of 14 Cytochrome P450 and Glutathione S-transferase genes. In addition, 12 reference probes allowed for the detection of several different autosomal chromosomal locations and probes to the X and Y chromosome were included.

RESULTS

We studied 50 individuals with PCG, with 20 being female (40%) and 30 being male (60%). The majority were Caucasian (78%), while the rest were of African (8%), Asian (6%), Melanesian (2%), and Middle Eastern (6%) backgrounds. Most cases were diagnosed before the age of 3 years (92%). A family history of PCG was present in four patients; three had affected siblings and one had an affected parent. The mean highest IOP was 29.5±9.3 mmHg and the mean cup-to-disc ratio was 0.6±0.3.

Prior to this study, all cases had been directly sequenced for *CYP1B1* variants. Three of the included cases were heterozygous for previously reported variants (R444Q, P513_K514del, and E229K [14,22]). An MLPA analysis was successful in all 50 patients. Internal controls detected several copy number changes in some patients in regions that are known to differ in copy number within the normal population (e.g., *GSTM1* and *GSTT1* genes). Moreover, we were able to correctly identify the sex of the individuals tested with probes to both the X and Y chromosomes. No *CYP1B1* CNV of part of or the entire gene was detected in any cases.

Table 1. *CYP1B1* deletions in primary congenital glaucoma previously reported (Reference genome GRCh37).

Location (Chr2)	Size	Deletion	Genotype	Ethnicity	Family history	Ref
Intragenic	Intron 2 - exon 3	Partial	Homozygous	Turkish	Segregated in 3 affected siblings	[5,23]
38,222,086 – 38,368,231	146 kb	Whole gene	Homozygous	Cypriot	Negative	[25]
38,191,823 – 38,385,253	193 kb	Whole gene	Homozygous	Caucasian	Segregated in 2 affected siblings	[26]
38,187,289 –38,349,505	162 kb	Whole gene	Compound heterozygous (T404fs*30)	Spanish	Negative	[24]

DISCUSSION

CYP1B1 is the only known gene to cause PCG in our population to date. Its prevalence among PCG cases varies across different populations, but overall, it accounts for approximately one in five cases in Caucasians [9,12-14]. Cases heterozygous for known pathogenic variants have been frequently reported [7-9,11,13,14]. In these situations, the other allele could display a variant in the promoter, in a non-coding region, or a deletion of part of or the entire gene. Whole gene deletions or duplications have been identified to be causative in other glaucoma-associated genes, such as *PITX2*, *FOXC1*, or *TBK1* [23,24]. Moreover, a few isolated cases of partial or whole gene *CYP1B1* deletions have been previously reported (Table 1) [6, 25–28]. In this study, we screened 50 PCG cases, in which *CYP1B1* sequencing results did not fully explain the phenotype, for *CYP1B1* CNV. An MLPA analysis did not detect any intragenic deletions or duplications in this cohort.

The intragenic deletion reported by Stoilov et al. was identified through the sequencing of the coding exons [6,25]. Other *CYP1B1* whole gene deletions previously reported were identified because of suspicious sequencing results: two studies reported a homozygous whole-gene deletion following repeated failures to amplify the *CYP1B1* coding exons through sequencing [27,28]. A third study found an apparently homozygous pathogenic variant in a patient that was present in the heterozygous state in the mother, but not in the father. After excluding non-paternity and maternal disomy, a gene dosage assessment revealed a heterozygous deletion on the other allele in the patient [26]. Although some deletions would be suspected by gene sequencing, others would still be missed. The four cases of *CYP1B1* CNV previously reported were all detected by chance through direct sequencing. This is the first study to assess directly the prevalence of *CYP1B1* CNV and to explore whether such a test would be of diagnostic use to complement *CYP1B1* gene sequencing. Although we cannot deny that this mechanism does play a role in rare PCG, the prevalence of such mutations in Caucasian PCG cases is expected to be less than 1%. As the probes used cover exons 1 and 3 only, we cannot completely exclude the possibility of an intragenic exon 2 CNV. However, the three whole gene deletions and the intragenic deletion previously published would have been detected with the probes used. Further, as we did not detect any CNV in this gene, the diagnostic potential of such a test is not high.

In conclusion, we did not identify any *CYP1B1* CNV among 50 PCG cases recruited through the Australian and New Zealand Registry of Advanced Glaucoma. To the best of our knowledge, this is the first study to report MLPA results for the *CYP1B1* gene in PCG cases and to assess its clinical utility. *CYP1B1* CNV is not a major contributor to PCG in our cohort of cases CYP1B1-negative or heterozygous through sequencing. It would be interesting to assess the prevalence of *CYP1B1* CNV in other populations, especially in those where *CYP1B1* mutations are more prevalent. This finding does not exclude this mechanism occurring rarely, but it means that research efforts should be focused on identifying further causative genes that when mutated cause PCG, and that testing for *CYP1B1* CNV as a diagnostic procedure in PCG is likely to have a low yield.

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3.4. Glaucoma spectrum and prevalence in *FOXC1* and *PITX2* carriers

FOXC1 and *PITX2* variants are associated with Axenfeld-Rieger anomaly and syndrome and increase an individual's risk of developing glaucoma. However, there is a gap in knowledge regarding the spectrum and prevalence of glaucoma in mutation carriers. In this study, I delineated the glaucoma prevalence and phenotype associated with *FOXC1* and *PITX2* variants in a cohort of patients diagnosed with ARM and their affected family members. I collected data on 53 individuals from 24 families, comprising 30 *FOXC1* carriers and 23 *PITX2* carriers. Glaucoma was present in 58.5% and did not differ between *FOXC1* carriers (53.3%) and *PITX2* carriers (60.9%, $p = 0.59$). However, the median age at diagnosis was significantly younger in *FOXC1* carriers (6.0 ± 13.0 years) compared with *PITX2* carriers (18.0 ± 10.6 years). Moreover, the penetrance at 10 years was significantly higher among *FOXC1* carriers (42.9%) compared with *PITX2* carriers (13.0%, $p = 0.03$) whereas the penetrance at 25 years did not differ between both genes (*FOXC1* 57.7% versus *PITX2* 71.4%, $p = 0.38$). These findings have implications when counselling individuals and their family members about their risk of developing glaucoma based on genetic testing results and should be considered in glaucoma monitoring.

Additionally, I identified one individual initially diagnosed as PCG and five as POAG. These findings suggested that *FOXC1* and *PITX2* may be involved in the genetic architecture of other glaucoma subtypes. In these families, the molecular diagnosis helped refine the clinical diagnosis and has important genetic counselling implications in terms of the mode of inheritance involved in the families, the availability of predictive genetic testing for at-risk family members and the proper management of potential systemic features.

Contribution statement

Ms Souzeau was primarily responsible for the design and conception of the study, the feedback of results, the provision of genetic counselling, the data collection, analysis and interpretation as well as the drafting and submission of the manuscript. Dr Siggs and Dr Zhou contributed to data analysis and revised the manuscript. Dr Galanopoulos, Dr Hodson, Dr Taranath, Prof Mills, A/Prof Landers, Dr Pater, Dr Smith, Dr Elder, A/Prof Rait, Dr Giles, Dr Phakey, Prof Mackey, A/Prof Hewitt and Dr Ruddle contributed to the characterisation of participants and revised the manuscript. Mrs Staffieri and Ms Kearns contributed to data collection and revised the manuscript. Dr Dubowsky contributed to genetic and data analysis and revised the manuscript. A/Prof Burdon contributed to data analysis and revised the

manuscript. Prof Craig contributed to the conception, design and supervision of the study, characterisation of participants, data analysis and revised the manuscript.

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ARTICLE

Glaucoma spectrum and age-related prevalence of individuals with *FOXC1* and *PITX2* variants

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Variation in *FOXC1* and *PITX2* is associated with Axenfeld-Rieger syndrome, characterised by structural defects of the anterior chamber of the eye and a range of systemic features. Approximately half of all affected individuals will develop glaucoma, but the age at diagnosis and the phenotypic spectrum have not been well defined. As phenotypic heterogeneity is common, we aimed to delineate the age-related penetrance and the full phenotypic spectrum of glaucoma in *FOXC1* or *PITX2* carriers recruited through a national disease registry. All coding exons of *FOXC1* and *PITX2* were directly sequenced and multiplex ligation-dependent probe amplification was performed to detect copy number variation. The cohort included 53 individuals from 24 families with disease-associated *FOXC1* or *PITX2* variants, including one individual diagnosed with primary congenital glaucoma and five with primary open-angle glaucoma. The overall prevalence of glaucoma was 58.5% and was similar for both genes (53.3% for *FOXC1 vs* 60.9% for *PITX2*, $P=0.59$), however, the median age at glaucoma diagnosis was significantly lower in *FOXC1* (6.0 ± 13.0 years) compared with *PITX2* carriers (18.0 ± 10.6 years, $P=0.04$). The penetrance at 10 years old was significantly lower in *PITX2* than *FOXC1* carriers (13.0% *vs* 42.9%, $P=0.03$) but became comparable at 25 years old (71.4% *vs* 57.7%, $P=0.38$). These findings have important implications for the genetic counselling of families affected by Axenfeld-Rieger syndrome, and also suggest that *FOXC1* and *PITX2* contribute to the genetic architecture of primary glaucoma subtypes.
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INTRODUCTION

Anterior segment dysgenesis is a heterogeneous group of developmental disorders affecting the anterior structures of the eye.[1] Axenfeld-Rieger malformation (ARM) represents a subgroup of anterior segment dysgenesis and refers to congenital ocular features including abnormal angle tissue, iris stromal hypoplasia, pseudopolycoria (additional pupillary opening in the iris), corectopia (displaced pupil), posterior embryotoxon (thickened and centrally displaced Schwalbe's line) and/or peripheral anterior synechiae (irido-corneal adhesions).[2,3] A range of systemic features can also be present as part of the Axenfeld-Rieger syndrome (ARS), with the most common including facial dysmorphism, dental anomalies, periumbilical skin, cardiac defects and hearing loss.[3,4] The presence of anomalies in the anterior chamber angle and drainage structures of the eye contribute to a lifetime risk of developing glaucoma and can lead to irreversible blindness,[5] although the severity of the anomalies does not seem to correlate well with the age at diagnosis, the severity or the progression of glaucoma.[5] It is usually reported that ~50% of individuals with ARM will develop glaucoma, with an age of onset ranging from birth to late in adulthood.[5] Guidelines for appropriate follow-up strategies for affected individuals without glaucoma lack a clear evidence base.

ARS is genetically heterogeneous. Deleterious sequence variants in the *FOXC1* (6p25.3, MIM 601090)[6,7] and *PITX2* genes (4q25, MIM 601542),[8] as well as two additional loci (RIEG2 at 13q14 and 16q24),[9,10] have been associated with ARS. In addition, several copy number variants and chromosomal rearrangements disrupting *FOXC1* or *PITX2* have been reported in association with ARS.[11–14] Deleterious variants in both genes account for approximately 40% of individuals with ARS,[11,15] with inter- and intra-familial variable expressivity frequently reported.[16,17] Although the ARM ocular phenotype associated with variants in *FOXC1* and *PIXT2* is thought to be highly penetrant and undistinguishable between both genes, there is a variable expressivity of systemic features. Variants in *PITX2* are more often associated with dental and/or umbilical anomalies, whereas individuals with *FOXC1* variants often have the ARM phenotype alone, or display a range of other systemic anomalies including heart anomalies, hearing defects, developmental delay and/or growth delay.[11,15,18] In addition, *FOXC1* has recently been identified as a

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primary open-angle glaucoma (POAG) susceptibility locus[19] and an essential regulator of lymphangiogenesis.[20]

FOXC1 encodes a forkhead transcription factor encoded by a single exon, whereas *PITX2* is a member of a paired class of homeodomain transcription factors and encodes four alternative transcripts (*PITX2A*, *B*, *C* and *D*). Both are expressed during embryonic development and regulate downstream genes important for cell differentiation and migration via DNA binding. *FOXC1* and *PITX2* are co-expressed in the periocular mesenchyme and can physically interact with each other via their C-terminal domain and their homeobox domain, respectively.[21] *PITX2* can inhibit the transactivation activity of *FOXC1*, which is lost in the presence of *PITX2* loss-of-function variants.[21] The transcriptional activity of both genes requires tight regulation during embryogenesis for proper development of anterior segment tissues.

Although the morphological ocular anomalies associated with *FOXC1* and *PITX2* variants have been well described, the spectrum and prevalence of glaucoma in mutation carriers have not been well characterised. In this study, we delineated the glaucoma prevalence and phenotype in a cohort of patients diagnosed with ARM and their family members with *FOXC1* and *PITX2* variants.

METHODS

Participants' recruitment

Ethics approval was obtained from the Southern Adelaide Clinical Human Research Ethics Committee. The study was conducted in accordance with the revised Declaration of Helsinki. Individuals were recruited through the Australian and New Zealand Registry of Advanced Glaucoma (ANZRAG) as described previously.[22] Informed written consent and a blood or saliva sample for DNA extraction purposes were obtained. Clinical information was collected by the patient's usual treating ophthalmologist. The feedback of results and genetic counselling was provided to the participants.

Individuals with ARM recruited in the ANZRAG were tested for the *FOXC1* and *PITX2* genes. Family members of individuals with variants in these genes were offered genetic testing. The cohort included all ARM probands and their family members identified as having a deleterious variant in the *FOXC1* or the *PITX2* genes. The diagnoses of ARM and glaucoma were made by the treating specialist who referred the patient to the ANZRAG. Individuals with ARM were recruited in the ANZRAG regardless of their glaucoma status. Advanced glaucoma was defined as visual field loss in the worse eye related to glaucoma with at least two out of the four central fixation squares having a pattern standard deviation $<0.5\%$ on a reliable Humphrey 24-2 field, or a mean deviation of <-22 dB. Ocular hypertension was defined by an intraocular pressure $\geqslant 21$ mm Hg.

Genetic testing

FOXC1 and *PITX2* genetic testing was performed through the NATA (National Association of Testing Authorities) accredited laboratories of SA Pathology at the Flinders Medical Centre (Bedford Park, SA, Australia). Venous blood specimens were collected into EDTA tubes; genomic DNA was prepared from a 200 µl sample of blood and extracted by a QIAcube automated system using QIAamp DNA Blood Mini kit (Qiagen, Chadstone, VIC, Australia) reagents according to the manufacturer protocols. Saliva specimen were collected into an Oragene•DNA Self-Collection Kit (DNA Genotek Inc., Ottawa, ON, Canada) and the DNA was isolated from 500 µl of sample and extracted as described in the manufacturer's instructions. The entire coding sequence and intron exon boundaries of *FOXC1* and *PITX2* were amplified as overlapping fragments in separate reactions with primer pairs (Supplementary Table 1).

Each polymerase chain reaction (PCR) was prepared using 100 ng of purified genomic DNA as template, 0.5 µM of each primer and a 1× concentration of AmpliTaq Gold 360 Master Mix (Thermo Fisher Scientific, Scoresby, VIC, Australia); 2.5 µl of 360 GC Enhancer (Thermo Fisher Scientific) was added to each *PITX2* reaction mix, and 5 µl to each *FOXC1* reaction mix. All reactions were adjusted to a final volume of 25 µl with deionised water. Gene template targets were amplified in a Veriti thermal cycler (Thermo Fisher Scientific) using separate conditions for each gene; *FOXC1*: Step 1, 95 °C for 10 min; Step 2, 95°C for 30 s, 55 °C for 30 s, 72 °C for 30 s, repeated for 40 cycles; Step 3, 72 °C for 7 min. *PITX2*: Step 1, 94 °C for 5 min; Step 2, 94 °C for 30 s, 61 °C for 30 s (decreasing after five cycles by 1 °C every five cycles), 72 °C for 1 min, repeated for 15 cycles; Step 3, 94 °C for 30 sec, 58 °C for 50 s, 72 °C for 1 min, repeated for 35 cycles; Step 4, 72 °C for 10 min.

PCR amplified products were prepared for DNA sequencing by the 'ExoSAP' method using a 10 µl sample of each PCR reaction treated with 5 U of Exonuclease I (Biolabs, Ipswich, MA, USA) and 1 U of shrimp alkaline phosphatase (USB Corporation, Cleveland, OH, USA) to remove residual primers and dNTPs. Bi-directional BigDye Terminator Cycle Sequencing (Thermo Fisher Scientific) reactions of the appropriate template and *FOXC1* and *PITX2* PCR primers were resolved and base called on an Applied Biosystems 3130*xl* Genetic Analyser (Thermo Fisher Scientific).

Detection of sequence variants was performed with the aid of the Mutation Surveyor v4.0 (SoftGenetics LLC, State College, PA, USA) programme; all forward and reverse sequence trace files were assembled by the programme against the *FOXC1* (NM_001453.2) and *PITX2* (NM_153427.2) GenBank reference. Significant differences in the relative peak heights of the sequence traces observed between that of the patient sample and a normal control were automatically called as a sequence variant by Mutation Surveyor; all such calls were visually inspected for confirmation. The Sorting Intolerant From Tolerant (SIFT; http://sift.jcvi.org) and PolyPhen-2 HumVar (http://genetics.bwh.harvard.edu/pph2) software programmes were used to predict the potential impact of amino acid substitutions on the protein. Homologene (www.ncbi.nlm.nih.gov/homologene) was used to assess the conservation among mammalian species. Allelic frequencies were compared with the population frequencies from the Exome Variant Server (EVS, http://evs.gs.washington.edu/EVS) and the Exome Aggregation Consortium v0.3.1 (ExAC, http://exac.broadinstitute.org). All variants are publically available at the ClinVar database (www.ncbi.nlm.nih.gov/clinvar/; accession numbers SCV000494251-SCV000494276).

FOXC1 and *PITX2* were analysed for copy number variation by multiplex ligation-dependent probe amplification (MLPA) using the SALSA MLPA P054-B2 FOXL2-TWIST1 probemix (MRC Holland, Amsterdam, The Netherlands) according to the manufacturer's instructions. The copy number present in the original DNA specimen was determined from the relative amplitude of each amplicon product detected using the ABI 3130*xl* Genetic Analyzer, and the data analysed using Peak Scanner v2.0 (Thermo Fisher Scientific). Breakpoints of the genomic deletions and duplications that have been further characterised in Clinical Genetics using SNP arrays have been added in Tables 1 and 2.

Statistical analysis

PASW Statistics, Rel. 18.0.1.2009 (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. Data are presented as median ± standard deviation. Fisher's exact test and Mann–Whitney *U*-test were used for the assessment of differences in non-parametric data.

RESULTS

The cohort consisted of 53 individuals from 24 families with heterozygous *FOXC1* or *PITX2* variants. The pedigrees are depicted in Figure 1. There were 25 males (47.2%) and 28 females (52.8%). The majority were Caucasian (94.3%). The mean age of the participants was 35.7 ± 20.4 years. Twenty probands (83%) had a family history of ARM and/or glaucoma.

FOXC1 variants were present in 30 individuals (56.6%) compared with 23 (43.4%) in *PITX2*. We identified 19 sequence variants across both genes (Tables 1 and 2 and Figure 2), of which 15 were considered novel (not reported in the literature and not present in the EVS or the ExAC database). Four nonsense, eight frameshift and one splice site sequence variants were considered to affect function. Among six missense sequence variants identified, *PITX2* p.(Pro64Leu) has previously been reported among different families as deleterious.[23–26] The other five missense sequence variants have not been reported before and were absent from the EVS and the ExAC database. Sequence

Table 1 Summary of *FOXC1* variants identified and associated phenotype

ID	*Age*	*Sex*	*Genomic location (NC_000006.12, hg38)*	*Nucleotide change (NM_001453.2)*	*Amino acid change (NP_001444.2)*	*Ocular features*	*Systemic features*	*Glc*	*Age glc dx*
1A	70	M	g.1610545_1610554del	c.100_109del10	p.(Gly34ThrfsTer8)	PAS, PE	Nil	Yes	3
1B	41	F	g.1610545_1610554del	c.100_109del10	p.(Gly34ThrfsTer8)	Haab's striae, mild PAS	Nil	Yes	4
2A	25	M	g.1610561_1610568del	c.116_123del8	p.(Ala39GlyfsTer41)	PE, IH, corectopia	Nil	No	—
2B	34	M	g.1610561_1610568del	c.116_123del8	p.(Ala39GlyfsTer41)	IH, PE, PAS, corectopia	Nil	No	—
3A	37	M	g.1610701C>T	c.256C>T	p.(Leu86Phe)	Corectopia	Nil	Yes	33
3B	6	F	g.1610701C>T	c.256C>T	p.(Leu86Phe)	Corectopia	Nil	OHT	5
4	56	F	g.1610713G>A	c.268G>A	p.(Ala90Thr)	PE, PAS, ectropion uvea	Nil	Yes	33
5A	41	F	g.1610761C>T	c.316C>T	p.(Gln106Ter)	IH, corectopia, Haab's striae	Nil	No	—
5B	13	F	g.1610761C>T	c.316C>T	p.(Gln106Ter)	PE, PAS	Pulmonary stenosis	No	—
5C	42	M	g.1610761C>T	c.316C>T	p.(Gln106Ter)	IH, PE, PAS	Nil	Yes	37
5D	10	F	g.1610761C>T	c.316C>T	p.(Gln106Ter)	PE, PAS	Nil	OHT	6
6A	37	F	g.1610902A>C	c.457A>C	p.(Thr153Pro)	Haab's striae, mild corectopia, cataracts	Short stature	Yes	0
6B	54	F	g.1610902A>C	c.457A>C	p.(Thr153Pro)	PE, very mild PAS	Hearing Loss	Yes	12
6C	4	M	g.1610902A>C	c.457A>C	p.(Thr153Pro)	Corneal haze, PE, mild corectopia	Nil	No	—
6D	17	F	g.1610902A>C	c.457A>C	p.(Thr153Pro)	PE, PAS, mild corectopia	Intellectual disability, heart defect, hearing Loss, short stature, hydrocephalus	No	—
7	26	F	g.1611044_1611062del	c.599_617del19	p.(Gln200ArgfsTer109)	PE, PAS, corneal oedema, ectropion uveae	Nil	Yes	0
8A	1	M	g.1611111_1611126del	c.666_681del16	p.(Ile223ProfsTer87)	PE	Heart defect	Yes	0
8B	28	M	g.1611111_1611126del	c.666_681del16	p.(Ile223ProfsTer87)	NA	Nil	Yes	8
9A	46	M	g.1611370_1611394del	c.925_949del25	p.(Ser309CysfsTer84)	Corectopia, pseudopolycoria, ectropion uveae, Haab's striae	Nil	Yes	1
9B	44	M	g.1611370_1611394del	c.925_949del25	p.(Ser309CysfsTer84)	PE, IH, corectopia	Nil	No	—
9C	69	F	g.1611370_1611394del	c.925_949del25	p.(Ser309CysfsTer84)	PE, amblyopia	Club foot	No	—
9D	76	M	g.1611370_1611394del	c.925_949del25	p.(Ser309CysfsTer84)	IH, ectropion uveae	Club foot	No	—
9E	50	F	g.1611370_1611394del	c.925_949del25	p.(Ser309CysfsTer84)	NA	Nil	No	—
10A	29	M	g.1611710C>A	c.1265C>A	p.(Ser422Ter)	PE, corectopia, megalocornea	Nil	OHT	2
10B	33	M	g.1611710C>A	c.1265C>A	p.(Ser422Ter)	NA	Nil	Yes	8
11A	41	F	g.1611936C>G	c.1491C>G	p.(Tyr497Ter)	Mild PAS, PE	Hearing Loss	Yes	15
11B	6	M	g.1611936C>G	c.1491C>G	p.(Tyr497Ter)	PE, PAS	Nil	No	—
12	7	F	g.(?_165632)_(3549058_35532219)del	c.(?_-1)_(*1_?)del	—	PE, PAS, corectopia	Fine motor skills delay	Yes	2
13A	43	M	g.?_?insNC_000006.12:g.(947938_951144)_(1832702_1833922)dup	c.(?_-1)_(*1_?)dup	—	PAS	Nil	Yes	18
13B	64	F	g.?_?insNC_000006.12:g.(947938_951144)_(1832702_1833922)dup	c.(?_-1)_(*1_?)dup	—	Nil	Nil	Yes	34

Abbreviations: Glc, glaucoma; IH, iris stromal hypoplasia; OHT, ocular hypertension; PAS, peripheral anterior synechiae; PE, posterior embryotoxon; NA, not available.

Table 2 Summary of *PITX2* variants identified and associated phenotype

ID	*Age*	*Sex*	*Genomic location (NC_000004.12, hg38)*	*Nucleotide change (NM_153427.2)*	*Amino acid change (NP_00316.2)*	*Ocular features*	*Systemic features*	*Glc*	*Age glc/ OHT*
14A	38	M	g.110621211_110621232del	c.184_205del22	p.(Arg62AlafsTer86)	Corectopia	Dental, periumbilical skin, WPW syndrome	Yes	14
14B	66	F	g.110621211_110621232del	c.184_205del22	p.(Arg62AlafsTer86)	PAS, IH	Dental, periumbilical skin	Yes	12
15A	82	M	g.110621225G>A	c.191C>T	p.(Pro64Leu)	Nil	Dental, periumbilical skin	Yes	48
15B	55	F	g.110621225G>A	c.191C>T	p.(Pro64Leu)	IH, PE, PAS	Dental, umbilical hernia	Yes	25
15C	42	M	g.110621225G>A	c.191C>T	p.(Pro64Leu)	Mild IH	Dental, periumbilical skin	OHT	20
15D	20	F	g.110621225G>A	c.191C>T	p.(Pro64Leu)	IH, PE	Dental, periumbilical skin	OHT	18
16	42	M	g.110621163C>T	c.252+1G>A	—	IH	Dental, umbilical hernia	Yes	20
17A	20	M	g.110618670G>C	c.271C>G	p.(Arg91Gly)	PE, PAS, pseudopolycoria	Dental, periumbilical skin	Yes	9
17B	70	M	g.110618670G>C	c.271C>G	p.(Arg91Gly)	PAS, pseudopolycoria, corectopia	Dental, umbilical hernia, absent left vas deferens	Yes	28
17C	26	F	g.110618670G>C	c.271C>G	p.(Arg91Gly)	PE, PAS, corectopia	Dental, periumbilical skin	Yes	26
18A	49	F	g.110618667C>G	c.274G>C	p.(Ala92Pro)	Corectopia, pseudopolycoria, PAS	Dental, periumbilical skin, epilepsy	Yes	19
18B	22	F	g.110618667C>G	c.274G>C	p.(Ala92Pro)	Corectopia, pseudopolycoria, PAS, PE, IH	Dental, umbilical hernia	Yes	21
18C	16	F	g.110618667C>G	c.274G>C	p.(Ala92Pro)	Peters, PAS, IH, cataract	Dental, umbilical hernia	OHT	15
19	7	M	g.110618315_110618316del	c.487_488delTC	p.(Ser163HisfsTer35)	Rieger anomaly	Dental, periumbilical skin, cleft palate	Yes	1
20A	59	F	g.110618365_110618386del	c.555_576del22	p.(Thr186SerfsTer4)	IH, PE	Dental, periumbilical skin	Yes	26
20B	37	F	g.110618365_110618386del	c.555_576del22	p.(Thr186SerfsTer4)	IH, PAS, PE	Dental, periumbilical skin	No	—
21A	24	M	g.110618293A>T	c.648T>A	p.(Cys216Ter)	PAS, PE, IH, ectropion uvea, pseudopolycoria	Dental, periumbilical skin	Yes	3
21B	18	F	g.110618293A>T	c.648T>A	p.(Cys216Ter)	PE	Dental, periumbilical skin	No	—
22A	45	F	g.(?_110618049)_(110622472_?)del	c.(?_47-1103)_(*76_?)del	—	PE, PAS, IH	Dental, periumbilical skin	No	—
22B	20	M	g.(?_110618049)_(110622472_?)del	c.(?_47-1103)_(*76_?)del	—	PE, IH, corectopia, pseudopolycoria	Dental, periumbilical skin, fine motor skills delay	No	—
22C	17	F	g.(?_110618049)_(110622472_?)del	c.(?_47-1103)_(*76_?)del	—	PE, IH	Dental, periumbilical skin, imperforate anus	OHT	17
23	36	M	g.(111426357_111528916)_(111888401_111990971)del	c.(?_47-1103)_(*76_?)del	—	PAS, corectopia, pseudopolycoria	Dental, umbilical hernia	Yes	13
24	28	F	g.(?_110618124)_(110632999_?)del	c.(?_1)_(*1_?)del	—	PE, PAS, IH, corectopia	Dental, periumbilical skin, epilepsy	OHT	6

Abbreviations: Glc, glaucoma; IH, iris stromal hypoplasia; OHT, ocular hypertension; PAS, peripheral anterior synechiae; PE, posterior embryotoxon; WPW, Wolff–Parkinson-White.

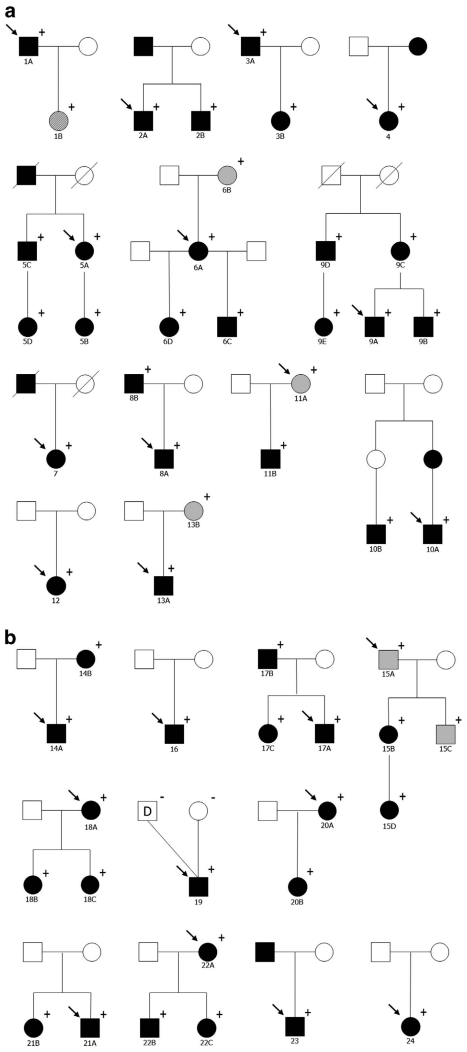

Figure 1 Pedigrees of the families. Round symbols indicate females; square symbols, males; black symbols, Axenfeld-Rieger Syndrome; grey symbols; primary open-angle glaucoma, dashed symbols; primary congenital glaucoma, unfilled symbols, unaffected; diagonal line, deceased; arrow, proband; D, sperm donor; +/−, presence/absence of the gene variant. (**a**) Families with *FOXC1* variants. (**b**) Families with *PITX2* variants.

variants p.(Leu86Phe), p.(Ala90Thr) and p.(Thr153Pro) are located in the forkhead domain of the *FOXC1* gene which is important for DNA binding. Both Polyphen-2 and SIFT predicted these sequence variants to be damaging to the protein, with all three residues highly conserved across different species (Supplementary Figure 1). Similarly, *PITX2* variants p.(Arg91Gly) and p.(Ala92Pro) are located in the homeobox domain, which is essential for binding to *FOXC1*,[21] with both residues highly conserved across different species (Supplementary Figure 1) and predicted deleterious by Polyphen-2 and SIFT algorithms. As a result, these five missense variants were also considered likely to be disease-causing. Finally, we identified five copy number variants: one deletion and one duplication of the *FOXC1* gene, and three deletions of the *PITX2* gene. All encompassed the entire gene, apart from one *PITX2* deletion, which spanned the last two coding exons.

In this cohort, 31 individuals (58.5%) had glaucoma, 6 (11.3%) had ocular hypertension and 16 (30.2%) showed no sign of glaucoma (Tables 1 and 2 and Supplementary Tables 2 and 3). Among the individuals with glaucoma, 48.4% (15/31) had advanced glaucoma as defined by the ANZRAG visual field criteria. The prevalence of glaucoma did not differ between *FOXC1* (16/30, 53.3%) and *PITX2* carriers (14/23, 60.9%, $P=0.59$), with the median age of glaucoma diagnosis at 13.5 ± 12.2 years (range 0–48 years) for the entire cohort. Median age at diagnosis was significantly lower in *FOXC1* compared with *PITX2* carriers (6.0 ± 13.0 years (range 0–37) *vs* 18.0 ± 10.6 years (range 1–48), $P=0.04$). The penetrance at 10 years of age was 29.4% for the whole cohort and was significantly higher for *FOXC1* carriers (42.9%) than *PITX2* carriers (13.0%, $P=0.03$). Penetrance at 25 years was 63.8% for the whole cohort and was similar between both genes (57.7% for *FOXC1* carriers *vs* 71.4% for *PITX2* carriers, $P=0.38$). The median age of individuals who did not have glaucoma was 29.5 ± 21.2 years (range 5–77 years) and was not significantly different between *FOXC1* (34.0 ± 24.2 years, range 5–77) and *PITX2* carriers (20.0 ± 13.1 years, range 17–46, $P=0.74$).

As shown in Tables 1 and 2, there was inter- and intra-familial phenotypic variability in ocular morphology and glaucoma type, although glaucoma prevalence was not different between loss-of-function variants, missense variants and copy number variants ($P=0.72$). Among the 53 individuals included in this study, 47 (88.7%) had classic ocular features of ARM (Figure 3). One family member of an individual with ARM was diagnosed with primary congenital glaucoma (PCG) and had mild features of ARM on re-examination after genetic testing (1B). Five family members of individuals with ARM had been diagnosed with POAG and had only mild or no features of ARM on re-examination after genetic testing (Figure 4). Four of the POAG cases had systemic features consistent with ASD, three carried *FOXC1* variants (11A, 6B, 13B) and two had *PITX2* variants (15A, 15C).

All individuals with *PITX2* variants had dental and umbilical anomalies including microdontia, hypodontia, redundant periumbilical skin or umbilical hernia. Additional systemic features were present in seven individuals and included Wolff–Parkinson–White syndrome, cleft palate, imperforate anus, fine motor skill delay, unilateral vas deferens and epilepsy. In comparison with *PITX2*, the majority of *FOXC1* carriers (21/30, 70.0%, $P<0.001$) had no systemic features. Among the *FOXC1* carriers who did have systemic features, a range of abnormalities were reported including hearing loss (3/30, 10.0%), heart anomalies (3/30, 10.0%), short stature (2/30, 6.7%), club foot (2/30, 6.7%), hydrocephalus (1/30, 3.3%), intellectual disability (1/30, 3.3%) and fine motor skills delay (1/30, 3.3%).

DISCUSSION

In this study, we investigated the clinical phenotype of 53 ARM probands and their family members with variants in *FOXC1* or *PITX2*. Glaucoma was present in 59% and ocular hypertension in an additional 11%. Shields[5] reported glaucoma in 58% of individuals with a purely clinical diagnosis of ARM (with or without systemic features), and glaucoma prevalence among ARS patients with *FOXC1* or *PITX2* variants has previously been estimated at between 35% and

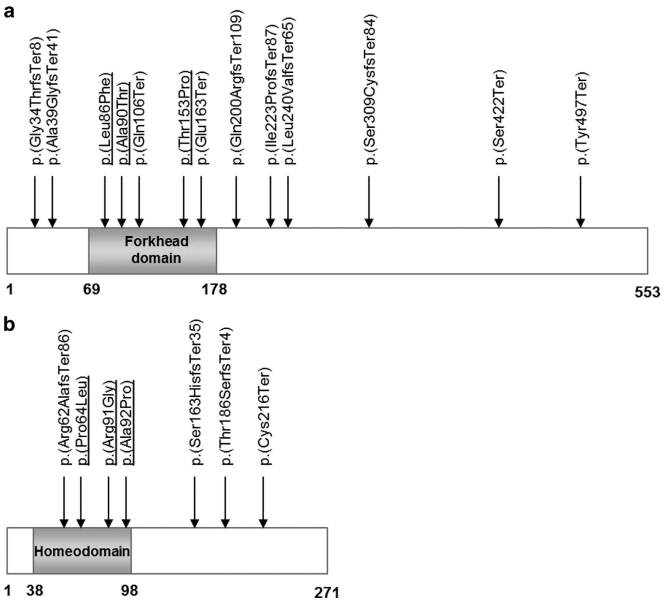

Figure 2 Distribution of the identified variants in the FOXC1 (**a**) and PITX2 proteins (**b**). Missense variants are underlined.

75%.[11,15,18] This is the first study to investigate the age-related glaucoma prevalence of each gene, along with the associated glaucoma phenotype.

We found a similar prevalence of glaucoma between *FOXC1* and *PITX2* carriers. Previous studies found a higher prevalence of glaucoma among *FOXC1* carriers than for *PITX2* carriers.[11,15,18] These differences can be explained by the age at diagnosis of affected individuals and the age of unaffected carriers reported. Although we have identified similar rates of glaucoma between both genes, the median age at diagnosis was significantly younger for *FOXC1* carriers than for *PITX2* carriers. Moreover, the prevalence at 25 years old was similar between both genes but the prevalence at 10 years old was significantly higher for *FOXC1* carriers than for *PITX2* carriers. Our results suggest that young unaffected individuals can still develop glaucoma at a later age. As a result, previous studies that were skewed toward *PITX2* carriers[15] or had young unaffected individuals[11,15] had a lower glaucoma prevalence than studies including a high proportion of *FOXC1* carriers.[18] Our findings showed that although the prevalence of glaucoma is similar between both genes, patients with *FOXC1* variants are likely to develop glaucoma at a younger age than *PITX2* carriers. In addition, our cohort included all available family members who were found to carry the suspected deleterious variant, which reduced the risk of recruitment bias.

In our study, one individual was initially diagnosed as PCG and five as POAG by their referring ophthalmologist. On re-examination, the individual with PCG and two individuals diagnosed with POAG had mild irido-corneal adhesions, whereas one individual with POAG had mild iris stromal hypoplasia. Two of the four *FOXC1* carriers had hearing loss and the other two *PITX2* carriers had dental and/or umbilical anomalies, none of which had been recorded before the molecular diagnosis. Individuals with variants in *FOXC1* or *PITX2* but without ocular features of ARS have been reported before,[15,27,28] and in some individuals ARM can be so mild that it results in a clinical diagnosis of PCG or POAG. The variable expressivity associated with *FOXC1* and *PITX2* variants can make clinical diagnosis of ARS challenging, especially in the absence of ARM. However, all individuals were part of families with ARM and four out of six had systemic features consistent with ARS, emphasising the importance of delineating the systemic features associated with each gene to assist clinicians in reaching a differential diagnosis. Our findings confirmed that *PITX2* variants are strongly associated with dental and umbilical anomalies, whereas the majority (70%) of *FOXC1* carriers lack systemic features; however, our cohort is biased toward participants with ocular features.

In ARS, glaucoma is often challenging to treat and the intraocular pressure difficult to control, often requiring incisional surgery (trabeculectomy or glaucoma drainage implant) and in some cases repeated surgical procedures.[5,18] Standard medications and surgical procedures often have a lower success rate than in non-ARS glaucoma patients.[29] In our study, 48% of individuals with glaucoma had advanced visual field loss and all had incisional surgery. This highlights the importance of early molecular diagnosis for effective monitoring and treatment options.

Although the exact mechanism by which ARS causes glaucoma is not fully understood, it is hypothesised that an arrested development of the anterior chamber angle structures during gestation (characterised by an incomplete maturation of the trabecular meshwork, an absent or poorly developed Schlemm's canal and/or a high insertion of the iris) alters the aqueous humour flow, thereby increasing intraocular pressure and resulting in glaucoma.[5] Broad phenotypic variability associated with *FOXC1* and *PITX2* variants has been reported before,[11,14,17,18] as it has in this study, although Shields[5] previously reported that the severity of ocular defects did not correlate with the

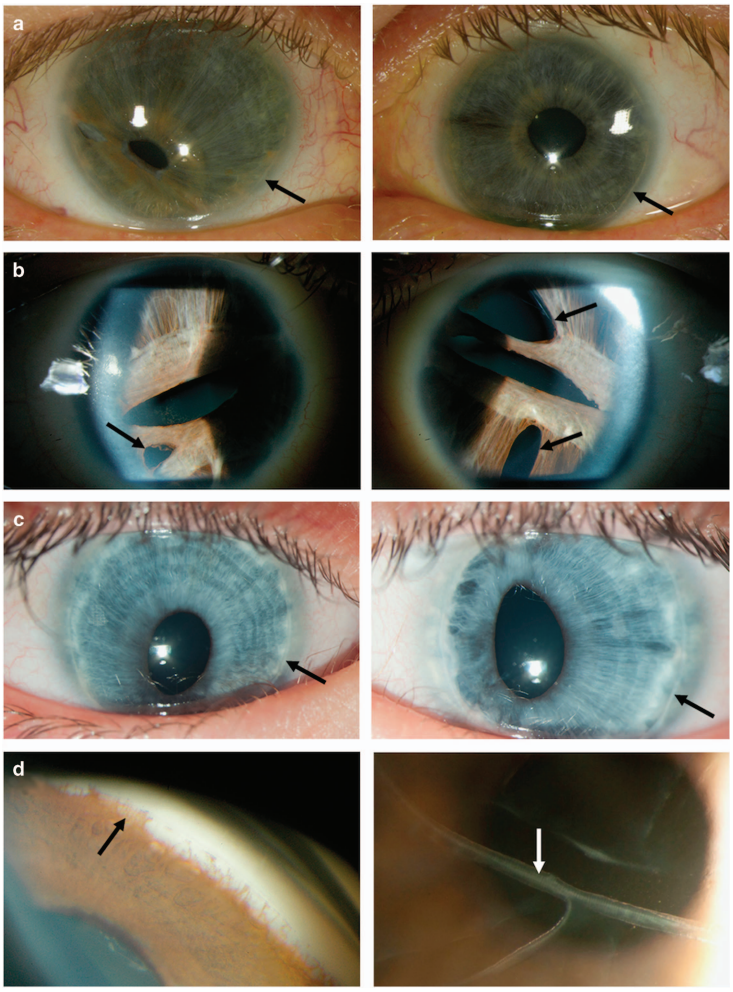

Figure 3 Clinical photographs of individuals with ocular features of Axenfeld-Rieger Malformation. Photographs in (**a**–**c**) showing the right eye (left panel) and the left eye (right panel). (**a**) Slit lamp photos showing corectopia in the left panel, iris stromal hypoplasia in both eyes and posterior embryotoxon (black arrows) in both eyes (individual 9B). (**b**) Slit lamp photos showing corectopia, pseudopolycoria (black arrows) and iris stromal hypoplasia in both eyes (individual 18B). (**c**) Slit lamp photos showing corectopia and posterior embryotoxon (black arrows) in both eyes (individual 12). (**d**) Gonioscopy showing irido-corneal adhesions (black arrow, left panel) and photo showing the presence of breaks in the Descemet's membrane (Haab's striae, white arrow, right panel; individual 5A).

development of glaucoma. *FOXC1* and *PITX2* both encode developmental transcription factors expressed in a tightly regulated temporal and spatial manner during development. Therefore, it is likely that disruption to the protein expression or activity level might not be well tolerated. However, inter- and intra-familial variability is often reported,[16,17,30] and is reflected by the variability in glaucoma phenotype within the families described here. Clinical heterogeneity of *FOXC1* and *PITX2* variants is likely to be explained by genetic modifiers, as suggested by the differing ocular phenotypes of *Foxc1*-deficient mice on different genetic backgrounds.[31] However, these modifiers remain to be identified.

A limitation of the study is that not every family member was tested, although all first-degree relatives of mutation carriers were invited to participate. Therefore, it is possible that individuals with milder phenotypes exist in the population but were not included. In addition, recruitment was somewhat biased towards glaucoma since participants were part of an advanced glaucoma registry, although the registry includes participants with ARM irrespective of their glaucoma status. Finally, diagnoses were made by different treating specialists, which may have introduced some variation in the phenotypic descriptions.

In conclusion, 59% of *FOXC1* and *PITX2* carriers in our cohort had glaucoma. Variants in both genes were associated with a similar risk of

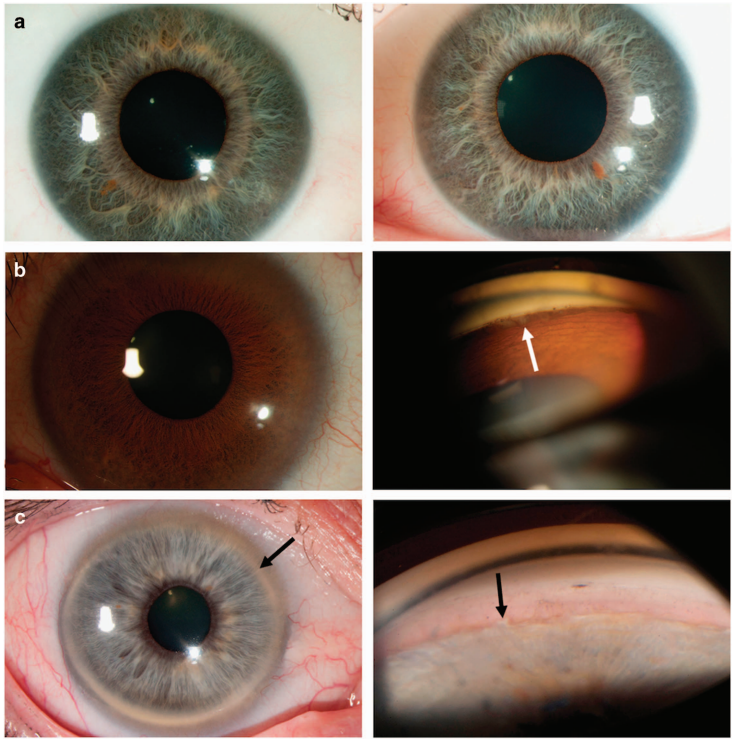

Figure 4 Clinical photographs of individuals with glaucoma and no or mild ocular features of Axenfeld-Rieger malformation after re-examination. Photographs in **a** showing the right eye (left panel) and the left eye (right panel). (**a**) Slit lamp photos showing iris stromal hypoplasia and diffuse posterior embryotoxon in both eyes (individual 15D). (**b**) Slit lamp photo showing the absence of iris anomalies and diffuse posterior embryotoxon in the left panel and gonioscopy showing mild irido-corneal adhesions (white arrow) in the right panel (individual 11A). (**c**) Slit lamp photo showing posterior embryotoxon (black arrow) in the left panel and gonioscopy showing mild irido-corneal adhesions (black arrow) in the right panel (individual 6B).

glaucoma, with *FOXC1* carriers displaying an earlier age of onset than *PITX2* carriers. These findings have implications when counselling individuals and their family members about their risk of developing glaucoma following genetic testing results. Furthermore, one family member was diagnosed with PCG and five with POAG, suggesting that variants in *FOXC1* and *PITX2* may also contribute to the genetic architecture of POAG and PCG. Further sequencing of large patient cohorts will be needed to determine the contribution of these genes to other glaucoma subtypes.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on European Journal of Human Genetics website (http://www.nature.com/ejhg)

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A.
Human (NP_001444.2)                        82  SYIALITMAIQNAPDKK          145  KPGKGSYWTLDPDSYNM
Chimpanzee (XP_003311061.2)                    ----|---|--------              ---------|--------
Mouse (NP_032618.2)                        82  SYIALITMAIQNAPDKK          145  KPGKGSYWTLDPDSYNM
Norway Rat (NP_599165.1)                   82  SYIALITMAIQNAPDKK          145  KPGKGSYWTLDPDSYNM
Zebrafish (NP_571803.1)                    78  SYIALITMAIQNSPDKK          141  KPGKGSYWTLDPDSYNM
Western clawed frog(NP_001007864.1)        83  SYIALITMAIQNAPEKK          146  KPGKGSYWTLDPDSYNM

B.
Human (NP_00316.2)                         56  ATFQRNRYPDMSTREEI          83   VRVWFKNRRAKWRKRERN
Chimpanzee (XP_001141234.1)                56  ATFQRNRYPDMSTREEI          83   VRVWFKNRRAKWRKRERN
Mouse (NP_001035967.1)                     56  ATFQRNRYPDMSTREEI          83   VRVWFKNRRAKWRKRERN
Norway Rat (NP_599165.1)                   56  ATFQRNRYPDMSTREEI          83   VRVWFKNRRAKWRKRERN
Zebrafish (NP_571803.1)                    46  ATFQRNRYPDMSTREEI          83   VRVWFKNRRAKWRKRERN
Western clawed frog  (NP_001007864.1)      77  ATFQRNRYPDMSTREEI          83   VRVWFKNRRAKWRKRERN
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Supplementary Figure 1: Alignment of protein sequences of FOXC1 between residues 82-98 and 145-161, and of PITX2 between residues 56-72 and 83-100. The residues of interest are highlighted for A. *FOXC1* variants p.(Leu86Phe), p.(Ala90Thr) and p.(Thr153Pro) and B. *PITX2* variants p.(Pro64Leu), p.(Arg91Gly) and p.(Ala92Pro). Reference sequences IDs of the species aligned are shown in brackets.

Supplementary Table 1: Primers used for amplification of the *FOXC1* and *PITX2* genes.

Gene	Exons	Primer sequence (forward/reverse)	Size (bp)
FOXC1	1.1	5'-CTTTCTTTTTGTCTGCTTTCC-3' 5'-GTCCAGTAGCTGCCCTT-3'	783
	1.2	5'-GACAAGAAGATCACCCTGAA-3' 5'-AGCGACGTCATGATGTTGT-3'	655
	1.3	5'-AGGACATCAAGACCGAGAAC-3' 5'-TTCAGGTACCACGAGGTGA-3'	757
	1.4	5'-CAACCTGCAAGCCATGA-3' 5'-GTTTCGATTTTGCCTTGATG-3'	554
PITX2	3	5'-TAGTCTCATCTGAGCCCTGC-3' 5'-CACTGGCGATTTGGTTCTGA-3'	282
	4	5'-CAGCTCTTCCACGGCTTCT-3' 5'-TTCTCTCCTGGTCTACTTGG-3'	374
	5	5'-GTTGGCCTCCGATGGAAGT-3' 5'-CTTCTAGCATAATTCCCAGTCTTTC-3'	719

Supplementary Table 2: Glaucoma phenotype of individuals with *FOXC1* variants

ID	BCVA	Max IOP (mmHg)	CDR	Treatment
1A	PL/NPL	56/67	0.9/0.9	Trab BE
1B	20/40:20/25	40/19	0.95/0.6	Trab RE, goniotomy BE, cat ex RE, drainage implant RE
2A	20/30:20/30	20/20	0.4/0.4	Nil
2B	20/30:20/30	20/16	0.3/0.2	Nil
3A	20/20:20/50	35/24	0.9/0.7	Drops BE
3B	20/50:20/30	30/30	0.5/0.3	Drops BE
4	20/20:20/30	28/28	0.85/0.9	Trab LE, cat ex BE
5A	20/20:20/50	14/14	0.4/0.4	Nil
5B	20/20:20/20	14/12	0.3/0.4	Nil
5C	20/40:20/100	22/35	0.4/0.95	drainage implant LE, drops LE
5D	20/20:20/20	32/37	0.5/0.5	Drops BE
6A	20/40:20/60	48/40	0.6/0.8	Trab BE, cat ex BE, corneal grafts BE
6B	20/16:20/20	22/20	0.9/0.6	Drops BE
6C	na:20/40	17/19	0.4/0.4	Nil
6D	20/30:20/40	10/15	0.4/0.4	Nil
7	20/25:NPL	30/45	0.9/na	Trab BE, cyclodiode laser LE
8A	20/60:na	32/24	0.5/0.5	Goniotomy BE
8B	na	na	na	Trab BE
9A	20/32:20/125	18/23	0.2/0.3	Trab LE
9B	20/40:20/20	8/10	na	Nil
9C	CF:20/32	16/21	0.3/0.3	Cat ex BE
9D	20/40:20/30	14/14	0.3/0.3	Nil
9E	20/20:20/16	16/10	0.4/0.5	Nil
10	na	25/24	0.3/0.3	Drops BE
11A	20/60:20/25	50/50	0.99/0.99	Trab BE
11B	20/20:20/20	18/17	0.2/0.2	Nil
12	20/25:20/30	31/26	0.7/0.7	Drops BE
13A	20:30:20/30	34/28	0.8/0.8	Trab BE
13B	20/30:20/40	30/27	0.95/0.95	Trab BE

BCVA: best corrected visual acuity, BE: both eyes, Cat ex: cataract extraction, CDR: cup-disc ratio, CF: count finger, IOP: intraocular pressure, LE: left eye, NPL: non-penetrating light, PL: penetrating light, RE: right eye, Trab: trabeculectomy, na: not available

Supplementary Table 3: Glaucoma phenotype of individuals with *PITX2* variants

ID	BCVA	Max IOP (mmHg)	CDR	Treatment
14A	20/20:20/30	14/52	0.8/0.9	Trab BE
14B	20/200:NPL	40/60	1.0/1.0	Trab BE, cat ex RE, corneal graft RE, drainage implant RE
15A	20/30:NPL	15/55	0.75/1.0	Trab BE
15B	20/20:20/25	28/19	0.85/0.6	Trab BE
15C	20/20:20/20	40/44	0.2/0.4	Laser BE, cat ex BE, Trab BE
15D	20/20:20/25	23/23	0.6/0.4	Nil
16	20/20:NPL	35/40	0.7/0.99	Trab BE
17A	20/30:20/30	18/19	0.85/0.9	Trab BE
17B	NPL:20/720	na/52	0.9/0.9	Trab LE, cat ex BE, drainage implant LE, corneal graft LE
17C	20/25:20/25	25/20	na	Drops BE
18A	CF:HM	na	0.8/0.9	Trab BE, cat ex BE, corneal graft LE
18B	20/60:20/60	34/25	0.85/0.9	Trab RE
18C	PL:20/400	24/27	na/0.1	Corneal graft LE
19A	20/40:20/60	58/47	0.65/0.85	Trab BE
19B	20/16:20/16	16/17	0.2/0.2	Nil
20	20/200:20/200	58/<21	na	Goniotomy RE, Trab BE, Cat ex LE
21A	20/30: HM	35/26	0.3/0.95	Iridectomy LE, Trab LE, drops RE
21B	na	14/15	0.2/0.2	Nil
22A	20/30:20/30	12/12	0.3/0.3	Nil
22B	20/40:20/200	15/15	0.6/0.6	Nil
22C	20/25:20/20	25/22	0.0/0.0	Nil
23	20/60:CF	45/23	0.95/na	Trab LE, drainage implant RE, cat ex RE
24	20/120:20/25	41/29	0.3/0.2	Trab RE, drops LE

BCVA: best corrected visual acuity, BE: both eyes, Cat ex: cataract extraction, CDR: cup-disc ratio, CF: count finger, HM: hand movement, IOP: intraocular pressure, LE: left eye, NPL: non-penetrating light, PL: penetrating light, RE: right eye, Trab: trabeculectomy, na: not available.

CHAPTER 4: ETHICAL AND PSYCHOLOGICAL IMPLICATIONS OF GENETIC COUNSELLING FOR GLAUCOMA

The publications incorporated in this chapter address the gap in knowledge of two important dimensions of genetic testing in translational research: the psychological implications and the ethical implications of genetic testing. The psychological implications of predictive genetic testing and patients' experience have been well studied for other genetic conditions of adult-onset, but they have been mainly overlooked for glaucoma, which affects the effectiveness of genetic counselling. Chapters 4-1 and 4-2 include my original contribution to knowledge on patients' experience of *MYOC* predictive genetic testing for glaucoma and on the benefits and families' experience of predictive genetic testing in minors for *MYOC* variants associated with a childhood onset of glaucoma. These studies were critically needed in genetic counselling for glaucoma to better support patients and their families undergoing genetic testing. Similarly, the ethical implications that arose from the use of testing technologies well implemented in genetic research have not been well described in the context of glaucoma. Technologies such as genotyping arrays or high throughput sequencing have the ability to identify incidental or unexpected findings as shown in chapter 4-3. In chapter 4-4, I reviewed the ethical implications of identifying incidental findings in genetic research and the importance of implementing adequate frameworks. Patients with glaucoma and their families can greatly benefit from the provision of genetic counselling and the evaluation of the psychological and ethical implications for glaucoma testing is essential in providing a framework for genetic counselling in glaucoma.

4.1. Participants' experience of predictive genetic testing for *MYOC*

Although cascade genetic testing for *MYOC* has been available for years, little is known about the decision-making process and the impact of testing relatives for a treatable eye condition such as glaucoma. In this study, I reported for the first time the experience of individuals who underwent predictive genetic testing for *MYOC* glaucoma. Forty individuals from 17 families returned a questionnaire about their motivations, feelings and concerns following genetic testing. The main perceived benefits of being tested included the availability of monitoring and treatment and the removal of uncertainty. Previous studies have shown that perceived health benefits (detection, prevention, control) and perceived emotional benefits (reassurance, reduction of uncertainty and emotional preparation) were strong motivators for predictive testing [377]. The majority of people who opted to get tested had a high perceived risk of carrying the familial variant and all were satisfied with their choice of being tested. Individuals who choose predictive genetic testing may represent a selected group of people more likely to be able to cope with the results. The main concerns were related to loss of vision, potential impact on insurance policies and transmission to children. These findings can assist health professionals involved in the genetic testing process to provide better counselling to patients interesting in genetic testing for glaucoma.

Contribution statement

Ms Souzeau was responsible for the design and conception of the study, the data collection, analysis and interpretation, drafting and submitting the manuscript as corresponding author. Dr Glading, Dr Keane and Dr Zhou contributed to data analysis and revised the manuscript. Ms Ridge, A/Prof Burdon and Prof Craig contributed to the design and conception of the study, data analysis and revised the manuscript.

This is the peer reviewed version of the following article: Souzeau E, Glading J, Keane M, Ridge B, Zhou T, Burdon KP, Craig JE. (2014) Predictive genetic testing experience for Myocilin primary open angle glaucoma using the Australian and New Zealand Registry of Advanced Glaucoma. Genetics in Medicine. 16(7): 558-63 which has been published in final form at http://dx.doi.org/10.1038/gim.2013.196.

Predictive genetic testing experience for myocilin primary open-angle glaucoma using the Australian and New Zealand Registry of Advanced Glaucoma

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Short running title: *MYOC* predictive testing experience

The authors declare no conflict of interest.

ABSTRACT

Purpose: Predictive genetic testing of relatives of known myocilin (*MYOC*) gene mutation carriers is an appropriate strategy to identify individuals at risk for glaucoma. It is likely to prevent irreversible blindness in this high-risk group because this treatable condition might otherwise be diagnosed late. The Australian and New Zealand Registry of Advanced Glaucoma has established genetic testing protocols for known glaucoma genes, including *MYOC*.

Methods: Through the Australian and New Zealand Registry of Advanced Glaucoma, we investigated the experience of 40 unaffected individuals who had undergone predictive genetic testing for *MYOC* mutations through questionnaires.

Results: The main motivations for being tested were (i) to make appropriate interventions and (ii) to reduce uncertainty. All our respondents perceived strong benefits, either medical or emotional, in being tested. However, different concerns were raised by the respondents that need to be addressed during counseling. Greater family awareness was reported by the majority of the respondents, and the ability to provide information to children was a strong motivation for being tested.

Conclusion: This study provides valuable information on the personal and familial impacts of having predictive genetic testing for glaucoma, which will help health professionals to better address the issues faced by patients and provide them adequate support.

Key Words: genetic counseling; glaucoma; *MYOC*; predictive genetic testing; POAG

INTRODUCTION

With the development of new technologies and a better understanding of the genetics of diseases, genetic testing is becoming increasingly available. Predictive genetic testing is now possible for treatable conditions such as glaucoma. Glaucoma is a progressive optic neuropathy that when untreated may cause irreversible blindness; it affects 60 million people worldwide.[1] There are strong medical benefits in favor of predictive genetic testing for primary open-angle glaucoma (POAG; OMIM no. 137760), the most common type of glaucoma. Half of all cases go undiagnosed[2,3] because the early stages of the condition are often asymptomatic, and appropriate therapeutic interventions can prevent or minimize glaucoma-induced blindness.[4-6] As a result, predictive genetic testing is an attractive goal to identify presymptomatic at-risk individuals, which allows them to be educated about their risks and options before the onset of the condition and to receive appropriate management to prevent or at least minimize the vision loss that would have otherwise occurred.

Mutations in the myocilin gene (*MYOC,* OMIM no. 601652), which are strongly associated with POAG, are transmitted in an autosomal dominant fashion and cause glaucoma at a younger age than is seen in the general population.[7,8] The most common mutation, Gln368X, has a mean age at diagnosis in the early 50s,[8,9] whereas other mutations, such as Pro370Leu, can be associated with an age at diagnosis as early as the teens.[10,11] Detecting a mutation in an unaffected individual does not predict the age of onset, the severity, or the progression of the condition but puts the person at a very high risk of developing glaucoma in his/her lifetime.

Because POAG is a treatable condition, predictive genetic testing is usually well accepted.[12] Although the medical benefits have been well studied, little is known about the internal motivations and the experience of individuals undergoing predictive testing for *MYOC* mutations. Decisions for undergoing genetic testing are usually driven by social and personal factors. These can range from personal experience with glaucoma in the family and knowledge of glaucoma and related treatment options to personality- and health-related locus of control. Know

ledge about individuals' experiences of predictive genetic testing is essential to provide adequate counseling and support to people who have been tested or who are considering being tested. In this study, we examined the motivations, the perceived benefits, and the feelings and concerns of individuals who had undergone predictive genetic testing for *MYOC* mutations.

MATERIALS AND METHODS

Recruitment

Participants were recruited through the Australian and New Zealand Registry of Advanced Glaucoma (ANZRAG)[13]. Ethics approval was obtained from the Southern Adelaide Clinical Human Research Ethics Committee. In an antecedent study, potential participants with a definite diagnosis of glaucoma referred by their eye specialist were initially screened for *MYOC* mutations[8]. Genetic testing was then made available to all adult first-degree relatives of individuals confirmed as carrying a *MYOC* mutation. Because *MYOC* mutations display very high penetrance but some inter- and intrafamilial variability, we recommended that all at-risk adult relatives be offered genetic testing regardless of their age.[14] To promote autonomous and voluntary decisions, relatives interested in being genetically tested had to contact the ANZRAG registry of their own volition. At the initial contact, a trained genetic counselor (E.S.) reviewed the testing process and the implications with the individual. Written informed consent was obtained, and DNA was extracted from a blood sample. The test results were provided directly to the participants and, if nominated, an eye specialist. When the familial mutation was identified, a referral to a local ophthalmologist was facilitated.

Our cohort comprised family members of individuals carrying a *MYOC* mutation, aged 18 years and older, who had consented to genetic testing for glaucoma and had already obtained their test result. A questionnaire was posted to each of them, regardless of the outcome of the test. After 1 month, individuals who did not return their questionnaire were contacted as a reminder and were given the opportunity to complete it over the phone.

Questionnaire

The questionnaire collected data relating to sociodemographic variables, perceived risks and feelings before being tested, perceived benefits and disadvantages of being tested, personal impact of the result, and familial impact of the results. Sociodemographic questions included gender, age, marital status, number and age of children, and education level. For analysis, we chose to categorize respondents into age groups, comparing those aged 40 years and younger with those older than 40 years. The cutoff point of 40 years was selected based on the knowledge that those older than 40 years have a higher likelihood of developing glaucoma and therefore may have different motivations for undergoing genetic testing than younger respondents. The perceived lifetime risk of developing glaucoma and the perceived

risk of carrying the familial mutation were measured using four alternatives choices (highly unlikely, unlikely, likely, and highly likely). Responses of (i) highly unlikely and unlikely and (ii) likely and highly likely were then combined to create a dichotomous variable for analysis. The perceived severity of glaucoma was assessed on a scale of 1-5, with 1 being considered not severe and 5 being considered very severe. The motivations for engaging in genetic testing were explored through multiple-choice responses. In free-response questions, participants were asked about the perceived benefits and disadvantages of the test, their concerns and fears with regard to glaucoma before and after the test, their initial reaction after finding out their test result, their positive and negative feelings associated with the result, and their level of satisfaction with the testing. Finally, respondents were asked about the experience within their family, including communication and disclosure patterns to children and other relatives, and any wider impact that their testing had on the family as a whole.

Statistical Analyses

Statistical analyses were conducted on all quantitative data using the Statistical Package for the Social Sciences, version 19 (SPSS, Chicago, IL). Significance was set at $P < 0.05$. Comparisons across demographic groups were made using χ^2 tests for independence. Analysis of qualitative responses was undertaken in Microsoft Excel, with similar responses categorized together in frequency tables.

RESULTS

Demographic Data

We have previously shown that *MYOC* mutations account for 4.2% of advanced POAG patients.[8] In this study, we evaluated 18 *MYOC*-positive families, which comprised 82 at-risk relatives. The questionnaire was sent to the 52 (63%) participants who had requested to be tested and had received their *MYOC* test result; it was completed and returned by 43 respondents (83%) from 17 families. Three respondents were excluded from our analysis because they had already been diagnosed with glaucoma before being tested and thus their motivations and emotional reactions to their results were likely to differ from those who did not have a glaucoma diagnosis. The demographics of nonresponders and those excluded due to previous diagnosis did not differ significantly from those of the included participants on any demographic measures (all $P > 0.70$).

The demographic data of the 40 included respondents are shown in Table 1. The average age of the respondents was 46.6 ± 16.1 years old (range 18-87). Twenty-two respondents (55%) had tested positive for the *MYOC* gene. The *MYOC* mutations observed among the 18 families approached (Gln368X, Trp286Arg, Trp373X, and Thr377Met) were all of comparable severity. Three at-risk individuals younger than 40 years displayed a combination of two mutations (Gln368X and Thr377Met) associated with a more severe phenotype in one family.[15]

Risk perception and intentions regarding genetic testing

Before being tested, half (20/40) of the respondents perceived their risk of developing glaucoma as being likely, or highly likely, and almost three quarters (28/40, 70%) perceived their risk of carrying the familial mutation as being likely or highly likely. Respondents believed glaucoma to be a moderately severe disorder, giving it an average severity score (on a scale of 1-5) of 3.6 ± 1.2. The perceived severity of glaucoma and the perceived risk of developing glaucoma or of carrying the familial mutation were not influenced by gender, age, education, carrier status, or the tested *MYOC* mutation ($P > 0.20$ for all).

The motivations for individuals to undergo testing are summarized in Table 2. A significant interaction between respondent age group and motivations for having the genetic testing was found. Those older than 40 years of age reported that they had testing in order to provide information to their children about their risk of developing glaucoma significantly more often than younger respondents ($\chi^2 = 4.263$, $P = 0.039$). However, this difference was no longer significant when data for just those respondents with children was analyzed ($P = 0.287$) because older respondents had children more often than younger ones. Neither gender nor education nor the tested *MYOC* mutation influenced respondents' motivations for being tested ($P > 0.10$ for all motivations).

Perceived benefits and disadvantages of genetic testing

All respondents considered predictive genetic testing for glaucoma useful. They described advantages of predictive testing for glaucoma, on the whole, more often than disadvantages. The main benefit reported by the respondents was the availability of monitoring for early detection and prevention of glaucoma-induced visual loss. The only disadvantage mentioned was that if identified as a carrier, they would have to live with the knowledge of being at increased risk of developing glaucoma.

Respondents' reactions and feelings

The main initial reaction of noncarriers was happiness and relief, whereas carriers experienced a range of different emotions (Table 3). Positive feelings expressed by carriers were the awareness and the accompanying ability to act and therefore help reduce the impact of glaucoma, in addition to the possibility of providing better information to their children. Negative feelings and concerns of carriers were various and are summarized in Table 4. Three carriers expressed feelings of guilt. One noncarrier expressed mixed feelings because a sibling was found to have the familial mutation when she did not. Regardless of their test results, all respondents were satisfied with their decision to be tested.

Impact on family

Almost all of the respondents had discussed having genetic testing with their family (36/40, 90%) and had discussed their genetic result with them (38/40, 95%). The majority of the respondents who had children had discussed their result with them (18/31, 58%). Respondents were significantly less likely to discuss positive results with their children if they were younger than 18 years old ($\chi^2 = 4.74$, $P = 0.029$). Almost all respondents with adult children (13/14, 93%) communicated their results to them, whereas a minority with minor children did so (5/17, 29%). However, 67% (8/12) of carriers who did not discuss their results with their minor children had selected the provision of information to children as a motivation for being tested. Finally, the majority of respondents (30/40, 75%) reported increased awareness in the family regarding glaucoma risks and genetic testing.

DISCUSSION

Genetic testing for POAG has been available since the discovery of the *MYOC* gene in 1997.[16] Even though *MYOC* mutations account only for 3-4% of all POAG cases,[7,8] relatives of *MYOC* carriers have had the opportunity to be screened and become educated about their glaucoma risk; moreover, they have been able to benefit from early prevention and management. However, there is a paucity of literature on the decision-making process and the impact of predictive genetic testing on individuals with treatable eye conditions such as glaucoma. In comparison, studies on inherited cancers have thoroughly evaluated patients' motivations, family communication, and experience with predictive testing[17-20]. Inherited cancers differ from glaucoma in that they are life-threatening and require invasive interventions.[17,21] However, both inherited cancers and glaucoma can be of juvenile or adult onset, have treatment options, and have an incomplete but strong penetrance; moreover,

associated genetic testing has proven to have clinical validity for both conditions. We therefore used the literature on inherited cancers to draw parallels with our results.

Several theoretical models have been created in attempting to predict health behaviors. The Health Belief Model[22] postulates that the higher the perceived susceptibility to and the perceived severity of the condition, and the higher the perceived effectiveness in taking actions, the more the person will engage in health behaviors.[21,23] Our findings show that the majority of individuals who chose to be tested had a high perceived risk of having the familial mutation before being tested and considered glaucoma to be a serious medical condition. Some previous studies on inherited cancers have shown that individuals are more likely to be tested if their perceived risk, not their actual risk, of cancer was high.[17,18,24]

A previous study on inherited cancers found that the perceived benefits component was the most powerful variable in explaining interest in predictive testing.[21] Another study identified two clusters of motives: one included perceived health benefits (early detection, prevention, and control), and the other included perceived emotional benefits (reassurance, reduction of uncertainty, and emotional preparation).[23] Similarly, in our study, taking appropriate medical interventions and the reduction of uncertainty were the two most-often-selected motivations for undertaking genetic testing, and the main reported benefit was monitoring for early detection.

A range of emotions were expressed by carriers of *MYOC* mutations after genetic results communication. These individuals were concerned about losing their vision, the potential impact on insurance, the transmission of the mutation to children, and the efficacy of interventions in treating glaucoma. Three carriers and one noncarrier also reported feelings of guilt. It is valuable to understand these concerns in order to better address them during counseling. Our findings show that people who undertake predictive genetic testing for *MYOC* mutations have no regrets with regard to being tested and are satisfied with their decision, regardless of their result. Healey *et al.* had previously reported the acceptability of genetic testing for *MYOC* glaucoma among the members of one very large affected Australian family.[12]

Greater family awareness following genetic testing was reported by the majority of the respondents. Recommendation by a family member was a major motivator for being tested. Almost all respondents disclosed their results to their family, regardless of their genetic result, and most of them even talked about the testing process before knowing their results, consistent with studies on hereditary cancers.[25,26] All respondents with adult children, except

one, disclosed their genetic result. The individual who did not was a noncarrier, and we postulate that the person did not think it useful to discuss the result with her children because there was no increased risk of developing glaucoma. Respondents with minor children were less likely to communicate their genetic result to them. However, the majority of the respondents who tested positive but did not share their results with their children had indicated that providing their children with information was a motivation for them to be tested, and so it is likely they will pass on this information when the children are older. Previous studies on families with inherited breast and ovarian cancers showed that the majority of parents (70-80%) discussed their genetic result with children of adult age, regardless of their carrier status[27,28] and the age of the children was positively associated with communication. Most parents who did not disclose their result did so because they thought their children were too young or immature. Predictive testing is not offered to individuals younger than 18 years, unless the family age of onset is known to be at less than 18 years and there is an immediate medical benefit to test.[29] However, without offering genetic testing, young children can still benefit from learning the family's carrier situation and therefore their potential risk. Genetic counselors can help parents in providing information to children while respecting their decisions and family dynamics.

There are some limitations to our study. Our sample is relatively small and our results might be skewed because our cohort reflects the motivations and concerns of individuals who decided to be tested. Further research in larger cohorts is required on the long-term perceived benefits and satisfaction of tested individuals, in addition to research on the at-risk relatives in our investigated families who did not request predictive *MYOC* testing. It also appears that people 40 years and younger who had received a negative result were less likely to respond to our invitation to participate and therefore our data were skewed to include an overrepresentation of individuals in this age group who had *MYOC* gene mutations. Although we do not make contact with the relatives' clinicians before the test, we cannot exclude that some relatives talked to their clinician before contacting us and that this might have influenced their decision to be tested or not. The fact that some individuals come from the same family could be another bias as it might create some familial clustering effects. The *MYOC* mutations identified among our respondents were of comparable severity and did not seem to have affected the responses. However, one family displayed two *MYOC* mutations and a more severe phenotype, and we acknowledge that this has the potential to have skewed the results with regard to motivations and psychological topics. Finally, this was a retrospective study asking participants to recall their feelings before genetic testing. Recall of events can be biased and may be influenced by the length of time elapsed

between disclosure of results and administration of the questionnaire, as well as being affected by the test results. A two-part questionnaire gathering data both before and after respondents receive their results would control for this. However, our analysis showed no association between the carrier status and the perceived risks or the different motivations for being tested, suggesting that recall bias did not have had a large impact on the results.

Despite these limitations, this study provides valuable preliminary findings on the motivators of asymptomatic individuals toward predictive genetic testing for POAG, and the personal and familial impacts of such testing. The acceptability of such an approach had been reported previously in an Australian family,[12] but, to our knowledge, this is the first study to address the motivations, feelings, and concerns of individuals as applied to a whole population rather than within a single large family. We demonstrate that the testing process increases awareness about glaucoma among relatives, especially children of adult age, of those tested. This is important because these individuals are at risk of having inherited the familial mutation and can greatly benefit from preventive measures. We show that, similar to individuals who chose to have predictive testing for inherited cancers[20], individuals who chose to have predictive testing for glaucoma perceived strong benefits, either medical or emotional, in being tested and may represent a selected group of individuals more likely to be able to cope with genetic results. Our cohort expressed strong satisfaction with their choice, and few people reported concerns or fears associated with genetic testing. However, one should not conclude from these findings that these individuals do not need support. Pretest genetic counseling needs to address, among other things, participants' motivations for testing, perceived risks and benefits, potential concerns, and family dynamics. Posttest genetic counseling may need to focus more on associated feelings of guilt, regardless of the genetic result.

Our findings are valuable for health professionals involved in the genetic testing process and the management of carriers; these health professionals need to be sensitive to the differences in personal concerns and intentions toward predictive testing. Such results will help them in providing better support and in addressing the relevant medical, psychological, and familial issues with patients undergoing predictive genetic testing for POAG.

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Table 1. Descriptive characteristics of the respondents

	n (%)
Age (years)	
18-40	15 (37.5)
>40	25 (62.5)
Gender	
Male	19 (47.5)
Female	21 (52.5)
Marital status	
Single	8 (20.0)
Married/de facto	28 (70.0)
Divorced/separated	2 (5.0)
Widowed	2 (5.0)
Children	
No	9 (22.5)
Yes	31 (77.5)
<18 years old	17 (54.8)
≥18 years old	14 (45.2)
Education level	
Primary School	6 (15.0)
High school	10 (25.0)
Technical College	5 (12.5)
University	18 (45.0)
Not specified	1 (2.5)

Table 2. Individuals' motivations for being tested.

	n (%)
Motivations for being tested	
Take appropriate interventions	32 (80.0)
Remove uncertainty	27 (67.5)
Family's recommendation	26 (65.0)
Provide information to children	23 (57.5)
Respondents with children	22 (71.0)
Provide information to relatives	14 (35.0)
Doctor's recommendation	2 (5.0)

Respondents could choose more than one answer from the listed suggested motivations.

Table 3. Main reaction after testing according to genetic result.

Emotional response	*n* (%)
Carriers	
Sad/disappointed	5 (22.7)
Anxious	3 (13.6)
Surprised	3 (13.6)
Not surprised	4 (18.2)
Upset	4 (18.2)
Proactive	3 (13.6)
Non-carriers	
Happy/relieved	18 (100.0)

This was a free-response question and similar responses were categorized together in frequency tables.

Table 4. Concerns relating to positive genetic test result.

Concern	n (%)
Loss of vision	10 (45.5)
Impact on health insurance	5 (22.7)
Transmission to children	3 (13.6)
Efficacy of glaucoma treatments	2 (9.1)
Travelling distance to clinic when living in rural area	1 (4.5)

This was a free-response question and similar responses were categorized together in frequency tables

4.2. Predictive genetic testing for *MYOC* in minors

Predictive genetic testing for *MYOC* is well established. However, some *MYOC* deleterious variants are associated with a glaucoma onset before 18 years old and the implications of testing minors in these families have not been addressed. In this study, I explored the benefits of predictive genetic testing in minors for *MYOC* variants associated with childhood onset glaucoma. Six at-risk children from three families with *MYOC* variants associated with a disease onset before 18 years old were offered predictive genetic testing. Testing was pursued for three children and none carried the familial variant. Factors influencing the decision to test children included the age of glaucoma onset and the severity in the family and the age of the child. My results suggest that families with *MYOC* associated childhood onset glaucoma could benefit from genetic counselling to discuss genetic testing options.

Contribution statement

Ms Souzeau was responsible for study design and conception, genetic counselling provision, data collection, analysis and interpretation, manuscript drafting and submission as corresponding author. Dr Glading contributed to study design and manuscript revision. Ms Ridge contributed to participants' recruitment and manuscript revision. Dr Wechsler and Dr Chehade contributed to participants' characterisation and manuscript revision. Dr Dubowsky contributed to genetic analysis and interpretation and manuscript revision. A/Prof Burdon contributed to data analysis and manuscript revision. Prof Craig contributed to study design and conception, data analysis and manuscript revision.

Predictive genetic testing in minors for Myocilin juvenile onset open angle glaucoma

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ABSTRACT

Myocilin glaucoma is an autosomal dominant disorder leading to irreversible blindness, but early intervention can minimize vision loss and delay disease progression. The purpose of this study was to discuss the benefits of predictive genetic testing in minors for *Myocilin* mutations associated with childhood onset glaucoma. Three families with *Myocilin* mutations associated with an age of onset before 18 years and six unaffected at-risk children were identified. Predictive genetic testing was discussed with the parents and offered for at-risk minors. Parents opted for genetic testing in half of the cases. None carried the familial mutation. The age of disease onset in the family, the severity of the condition, and the age of the child are all factors that appear to influence the decision of the parent to test their children. Predictive genetic testing for early onset *Myocilin* glaucoma can facilitate early detection of disease or discharge from routine ophthalmic examinations.

Key words: genetic counseling, glaucoma, minor, myocilin, predictive genetic testing

Conflict of interest

The authors declare no conflicts of interest.

INTRODUCTION

Glaucoma is a leading cause of irreversible blindness worldwide, affecting 3% of the population over the age of 50 (1). The most common type is primary open angle glaucoma (POAG, MIM 137760) characterized by optic nerve damage with open drainage angles (2). Juvenile open angle glaucoma (JOAG) is defined arbitrarily as diagnosis prior to age 40 and is usually associated with a more severe phenotype (3). The early stages are asymptomatic, and irreversible blindness may occur if left untreated. Therapeutic and/or surgical interventions aim at controlling intraocular pressure (IOP) and are usually effective in minimizing loss of visual function and delaying progression of the disease (4, 5).

Pathogenic variants in the *Myocilin* gene (MIM 601652) are the commonest cause of inherited POAG. We have previously reported a prevalence of 4% in POAG and 17% in JOAG in the Australian population (6). *Myocilin* glaucoma is inherited in an autosomal dominant manner and is usually associated with high IOP and earlier age of onset than other unselected glaucoma cases (6). Genotype-phenotype correlations exist with some mutations associated with a very young age of onset, as early as the teens or childhood (6-8).

Considering that effective treatments which minimize vision loss are available, it becomes important to identify at-risk individuals before any irreversible damage occurs. Adequate identification of families with onset of glaucoma in childhood is essential as children can benefit from early genetic testing. In this study, we report on three families with known pathogenic *Myocilin* variants associated with very early age of onset for which predictive genetic testing for unaffected minors was discussed.

MATERIALS AND METHODS

This study was approved by the Southern Adelaide Clinical Human Research Ethics Committee and was conducted in accordance with the National Health and Medical Research Council statement of ethical conduct in research involving humans.

Patients were recruited through the Australian and New Zealand Registry of Advanced Glaucoma (ANZRAG) via referral from their treating specialist as previously reported (9). *Myocilin* sequencing was performed through an accredited laboratory as described previously (6). When a pathogenic variant was identified, genetic counselling was offered and *Myocilin* genetic testing was made available to family members. Contact was made only through the proband referring relatives to the ANZRAG in order to promote a voluntary

decision. In cases with an age at diagnosis of less than 18 years reported in the family or in the literature for the sequence variant in question, predictive genetic testing of unaffected minors was discussed with the children's parents and their treating specialist. Genetic counselling was provided, written informed consent was obtained from the parents and where possible children gave assent, and a blood sample was provided for the purpose of predictive genetic testing.

RESULTS

In the ANZRAG, *Myocilin* mutations are present in 4% of our POAG cases (50/1248). Among these, three families with *Myocilin* mutations associated with an age of onset before 18 years and six first-degree at-risk minors were identified (Fig. 1). Predictive genetic testing was discussed with the parents, and three children from two families were tested. The clinical details of the three families are presented in Table 1.

The proband from Family A (III-1) was diagnosed at 11 years old. At the age of 17, she required trabeculectomy to control her glaucoma. She had a very strong family history of severe JOAG with her daughter, father, sister, as well as a grandmother, aunt and cousin on the paternal side affected. All affected relatives were diagnosed in their childhood or teens, and all required glaucoma surgery.

Myocilin sequencing in the proband identified the previously reported mutation p.T438I (c.1313C>T). The mutation segregated with the condition in four affected family members. The proband's son (IV-1), aged 17 had normal IOP. Her nephew (IV-3), aged 3 is reported to be unaffected but has not been clinically examined. *Myocilin* predictive genetic testing of both children was requested by their respective parents. Neither child carried the familial mutation.

In Family B, the proband (III-2) was noted to have increased IOP and optic nerve changes consistent with JOAG at age 13. He presented at age 17 with a left ischemic central retinal vein occlusion which left the eye blind from retinal ischemia and uncontrolled glaucoma. Despite regular ophthalmic follow-up and topical therapy, there were periods of non-compliance, and at age 34 with uncontrolled IOP and worsening visual fields, he underwent a right trabeculectomy. His most recent visual field test is displayed in Fig. 2. The patient had a significant family history of glaucoma with his father and paternal grandfather diagnosed with glaucoma in their teens.

Myocilin sequencing identified the previously reported mutation p.G367R (c.1099G>A) in the proband and his father (II-3). The proband's 10-year-old son (IV-3) had normal IOP and optic nerves. Testing was requested by his father, and results were negative for the familial mutation.

The proband from Family C (III-1) was diagnosed at 20 years old with significantly elevated IOP. She had laser trabeculoplasty and was on maximal medical therapy to control her IOP. Visual fields were largely intact, but there was possibly an early defect on the right side (Fig. 2). There is a positive family history with a sister, father and paternal grandfather diagnosed with JOAG and requiring glaucoma filtration surgery to control their IOP.

The p.G367R (c.1099G>A) *Myocilin* mutation was identified in the proband and her sister. The proband's daughter (IV-1) aged 3, and her sister's sons (IV-3, IV-4), aged 1 and 3 have not been examined but were said to be unaffected. Predictive genetic testing of the three children was discussed with the parents, but they decided to defer testing until the children were older.

DISCUSSION

While predictive genetic testing for adult or late onset conditions is accepted in adults, the international consensus is that such testing in minors should be deferred until they are old enough to make an informed decision (10). However, genetic testing for diseases that have childhood onset, where established early surveillance or treatment may alter the course of the disease, is generally supported (10, 11). This approach is mainly endorsed by the immediate and direct medical benefits for the child. *Myocilin* POAG is typically an adult-onset condition; however, some mutations are known to be associated with a juvenile-onset (6-8). In these situations, predictive genetic testing of minors should be considered, particularly as POAG is difficult to clinically diagnose in its early stages, and earlier detection and intervention lead to better visual outcomes.

Based on these considerations, through the ANZRAG, we identified three families with *Myocilin* mutations associated with very early age of onset POAG that could benefit from predictive genetic testing of at-risk minors. These families displayed two previously reported *Myocilin* mutations: p.T438I and p.G367R. Both variants have previously been reported in individuals displaying an age at diagnosis in childhood or early adulthood, significantly elevated IOP, and glaucoma surgery required for most individuals (7, 8, 12-15). Family A carrying p.T438I, exhibited a similar phenotype. In Family B, the two affected individuals

carrying p.G367R were diagnosed in their mid-teen years, and progressed to severe visual impairment by their mid-20s. In contrast, the two affected individuals from Family C carrying the same mutation presented in their early twenties and their glaucoma was not as advanced.

Among all three families, six unaffected children were identified as being at risk of carrying the familial *Myocilin* mutation. The parents of three of the children from two families (A and B) decided to opt for genetic testing and these three children did not carry the familial mutations. The third family (C) decided to defer testing. We previously showed that individuals who have a higher perceived severity of glaucoma and/or a higher perceived risk of developing glaucoma are more likely to act upon it (16). The two families who decided on testing their children had strong family histories of glaucoma developing during childhood or early teens, with some individuals having severe glaucoma and two of the children tested were getting near the age of onset of the familial mutations. Family C had so far experienced less severe glaucoma and vision loss than the affected members of Family B carrying the same mutation (Fig. 2), had a later onset and the at-risk children were of younger age, which could account for their decision of deferring testing.

When counselling parents on deciding whether to test or not, the benefits and potential harms need to be reviewed and the best interests of the child should always be central to any decision. Medical benefits include close monitoring and surveillance to detect glaucoma signs at the earliest possible time point, and early intervention and/or treatment to prevent or minimize damage to the optic nerve and associated vision loss in the case of a positive genetic result. If results are negative, the advantages are reduced number of visits and eye exams required, including potential examination under anesthesia in the cases of very young children. Psychosocial benefits include the removal of uncertainty and the opportunity for adjusting life plans. Potential harms for the child are mainly psychosocial in nature, including the alteration of self-image and self-esteem, increased anxiety, negatively altered life choices and potential for discrimination and stigmatization (10, 17). Distortion of parental perception and education of the child, parental anxiety and a potentially negative impact on the extended family dynamics are other potential harms to be considered.

The timing of testing for childhood onset conditions that have therapeutic options is an important question (11). A reasonable approach for *Myocilin* JOAG would be to offer testing near the age considered adequate for starting medical surveillance and/or interventions. This age could vary depending on the familial variant but should be based on the age at

diagnosis within the family, and the reported age of onset for that variant in the literature.

The acceptability and the psychosocial experience of adults undergoing predictive genetic testing for glaucoma have been previously documented (16, 18). However, there are few studies on the psychosocial impact of genetic testing on children in general. A recent review of the literature did not suggest that a child's emotional state, self-perception or social wellbeing were significantly affected by predictive genetic testing (17). Future research will be needed to understand the potential impacts of predictive genetic testing for glaucoma in minors to better counsel the families.

In conclusion, we identified three families with *Myocilin* JOAG who could benefit from predictive genetic testing of at-risk minors in view of the potential immediate medical benefits for the children. Three children were found not to carry the familial mutation, removing the need for unnecessary regular ophthalmic examinations. The decision for testing seemed to be influenced by the personal experience of the family in question with glaucoma. Knowing how to better counsel parents during the decision-making process, the best age to undergo testing, and the potential impact of the testing are key areas of future focus which will lead to better outcomes in families affected with early age of onset *Myocilin* glaucoma.

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Figure 1. Pedigree of the families. Round symbols indicate females; square symbols, males; fully filled symbols, primary open-angle glaucoma; unfilled symbols, unaffected; diagonal line, deceased; arrow, proband; P, pregnancy; wt: wild-type allele. The age of the at-risk children is displayed at the bottom.

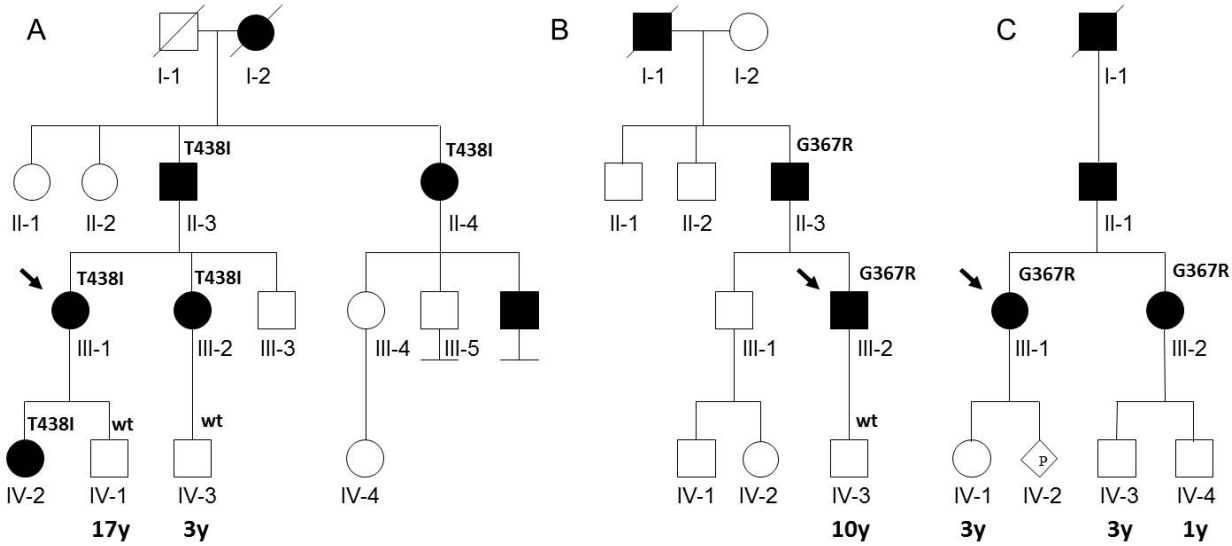

Figure 2. Visual Fields of the proband from Family B (III-2) and Family C (III-1) with the p.G367R *Myocilin* mutation. As Individual III-2 from Family B is blind in his left eye, visual fields of both individuals are only displayed for the right eye. RE, right eye.

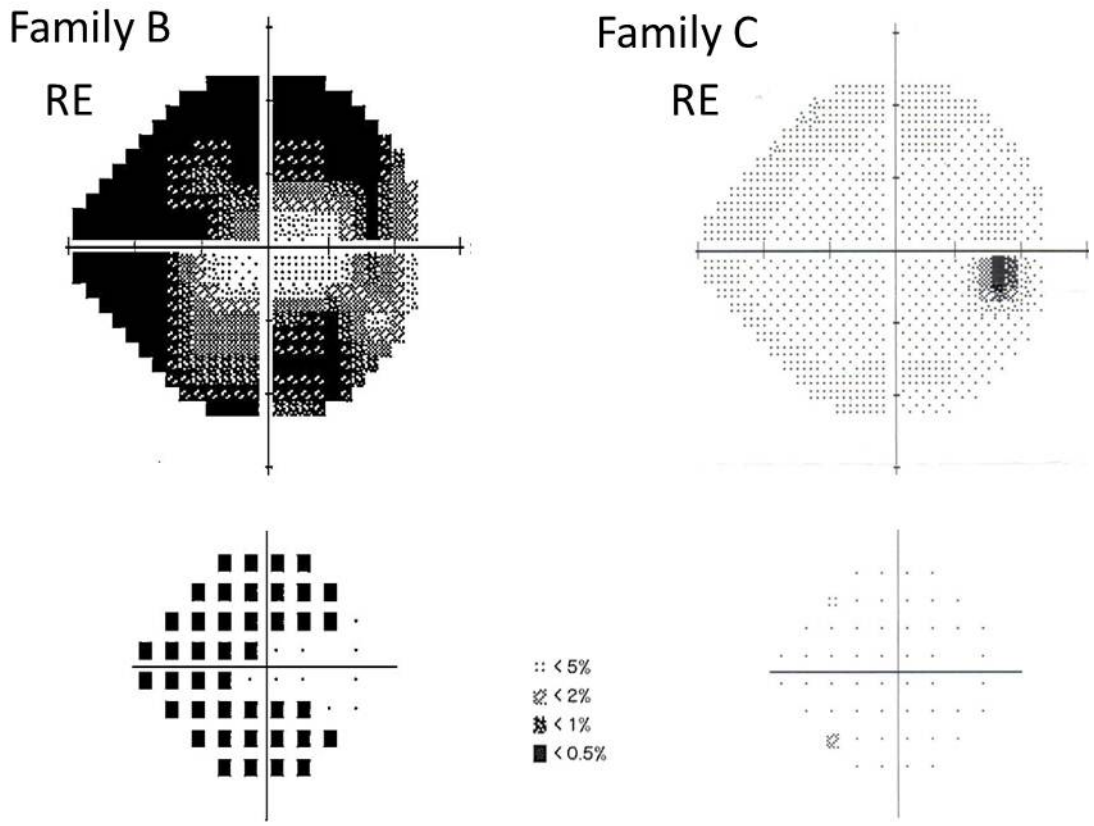

Table 1. Clinical details and genetic results of the recruited individuals

Patient	Age at examination (years)	Phenotype	Myocilin mutation	Age at diagnosis (years)	Maximum IOP (mmHg, RE/LE)	BCVA (RE:LE)	CDR (RE/LE)	Glaucoma surgery
Family A								
II-3	70	JOAG	T438I	14	na	20/40:20/30	na	Yes
II-4	75	JOAG	T438I	11	25/20	20/20:20/20	0.8/0.6	Yes
III-1[a]	43	JOAG	T438I	11	16/31	20/25:20/25	0.4/1.0	Yes
III-2	42	JOAG	T438I	7	na	na	na	Yes
IV-1	18	JOAG	T438I	17	44/46	na	0.7/0.7	No
IV-2	17	Unaffected	wt		16/17	na	na	No
IV-3	3	Unaffected	wt		na	na	na	No
Family B								
II-3	62	JOAG	G367R	14	na	20/16:20/30	0.6/0.99	Yes
III-2[a]	34	JOAG	G367R	13	40/40	20/40:NLP	0.9/1.0	Yes
IV-3	10	Unaffected	wt		13/13	20/25:20/25	0.3/0.3	No
Family C								
III-1[a]	31	JOAG	G367R	20	35/35	20/16:20/16	0.3/0.4	No
III-2	33	JOAG	G367R	23	32/33	20/20:20/20	0.7/0.6	Yes

BCVA, best corrected visual acuity; CDR, cup to disc ratio; IOP, intraocular pressure; JOAG, juvenile open-angle glaucoma; LE, left eye; na, not available; RE, right eye; wt, wild-type allele.

[a]: proband

4.3. Imputation of *MYOC* deleterious variant

Genotyping arrays are designed to detect common variants; however, deleterious *MYOC* variants are rare. In this study, we used arrays and imputation methods to reliably detect *MYOC* p.Gln368Ter variant (rs74315329), the most common deleterious variant reported in this gene. The variant was successfully imputed among 1155 POAG cases and 1992 controls and showed a strong associated risk (odds ratio = 15.53). Samples were further sequenced to confirm the presence of the *MYOC* variant. The sensibility between imputation and sequencing was 100%, the specificity 99.9%, the positive predictive value 95.7%, and the negative predictive value 100%. Our findings indicated that rare variants can be imputed with high accuracy using dense SNP arrays with appropriate reference populations and might provide an alternative method to sequencing for the detection of clinically relevant genetic variants. These findings have genetic counselling implications. GWAS results are not commonly reported to research participants because of an inability to interpret data at an individual level. However, a proportion of controls from these studies that are suspected to carry deleterious *MYOC* variants will be at risk of developing preventable glaucoma. This raises ethical issues for researchers as to if and how to return medically relevant results to participants.

Contribution statement

Ms Souzeau was responsible for the study design, data collection and interpretation, genetic counselling, and revision of the manuscript. Dr Gharahkhani contributed to the data analysis and interpretation, and drafted the manuscript. A/Prof Burdon and A/Prof Hewitt contributed to the study design, data interpretation and revised the manuscript. Dr Law contributed to data analysis and interpretation and revised the manuscript. A/Prof Radford-Smith contributed to the recruitment of participants and revised the manuscript. Prof Montgomery contributed control data and revised the manuscript. Prof Mackey and Prof Craig contributed to the recruitment of participants, the study design, data interpretation and revised the manuscript. A/Prof MacGregor contributed to the study design, provided control data, performed data analysis and interpretation and revised the manuscript.

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Genetics

Accurate Imputation-Based Screening of Gln368Ter Myocilin Variant in Primary Open-Angle Glaucoma

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Purpose. Myocilin (*MYOC*) is a well-established primary open-angle glaucoma (POAG) risk gene, with rare variants known to have high penetrance. The most common clinically relevant risk variant, Gln368Ter, has an allele frequency of 0.1% to 0.3% in populations of European ancestry. Detection of rare *MYOC* variants has traditionally been conducted using Sanger sequencing. Here we report the use of genotyping arrays and imputation to assess whether rare variants including Gln368Ter can be reliably detected.

Methods. A total of 1155 cases with advanced POAG and 1992 unscreened controls genotyped on common variant arrays participated in this study. Accuracy of imputation of Gln368Ter variants was compared with direct sequencing. A genome-wide association study was performed using additive model adjusted for sex and the first six principal components.

Results. We found that although the arrays we used were designed to tag common variants, we could reliably impute the Gln368Ter variant (rs74315329). When tested in 1155 POAG cases and 1992 controls, rs74315329 was strongly associated with risk (odds ratio = 15.53, $P = 1.07 \times 10^{-9}$). All POAG samples underwent full sequencing of the *MYOC* gene, and we found a sensitivity of 100%, specificity of 99.91%, positive predictive value of 95.65%, and negative predictive value of 100% between imputation and sequencing. Gln368Ter was also accurately imputed in a further set of 1801 individuals without POAG. Among the total set of 3793 (1992 + 1801) individuals without POAG, six were predicted (probability > 95%) to carry the risk variant.

Conclusions. We demonstrate that some clinically important rare variants can be reliably detected using arrays and imputation. These results have important implications for the detection of clinically relevant incidental findings in ongoing and future studies using arrays.

Keywords: primary open-angle glaucoma, *MYOC*, Gln368Ter, rare variants, imputation

Glaucoma is a major cause of blindness worldwide. Primary open-angle glaucoma (POAG; Online Mendelian Inheritance in Man [OMIM] 137760) is the most common subtype of glaucoma, which is characterized by a progressive loss of peripheral vision, although patients may remain undiagnosed until central vision is affected.[1,2] Treatment to lower intraocular pressure delays the progression of visual field loss. Several genetic loci have been associated with POAG in linkage and genome-wide association studies (GWAS).[3–9] Mutations in the myocilin (*MYOC*) gene (OMIM 601652) have been reported in different populations and found to account for 2% to 5% of unselected POAG patients.[10] Gln368Ter is the most common mutation in populations of European ancestry and confers a high risk of POAG.[3,11,12] The Gln368Ter mutation has been observed across multiple populations,[11] and was shown to be associated with an average onset of POAG in the fifth and sixth decades.[13] Previous studies in 15 Australian families, a large French Canadian family, and two unrelated French Canadian families suggested that this mutation has derived from a common ancestor, showing a founder effect.[14,15] Detection of the Gln368Ter mutation is clinically important as it allows for early diagnosis and intervention. However, the risk allele has a frequency of approximately 0.09% to 0.1% among multiple ethnicities (http://www.ncbi.nlm.nih.gov/clinvar/variation/7949/, http://exac.broadinstitute.org/variant/1-171605478-G-A [both in the public domain]), 0.1% in the European American population (http://evs.gs.washington.edu/EVS/ [in the public domain]), and 0.26% in the 1000 Genomes phase 1 European population. This is remarkably similar to the Gln368Ter frequency of 0.09% found in the Blue Mountains Eye Study consisting predominantly of European Australians.[16] Sanger

sequencing is traditionally used to detect this mutation, and it is not directly genotyped on commonly used genotyping arrays.

Genome-wide association studies have identified thousands of common variants (i.e., variants with a minor allele frequency [MAF] > 5%) associated with human complex diseases[17] (http://www.genome.gov/gwastudies/ [in the public domain]). Together the GWAS hits from common variants explain little genetic variance of complex traits, resulting in the "missing heritability" problem.[18–20] The heritability of POAG and its endophenotypes including intraocular pressure and vertical cup-to-disc ratio was estimated at 0.81, 0.42, and 0.66, respectively, in a previous study from our group.[21] Since the identified genetic variants contributing to the risk of POAG and its endophenotypes explain a small proportion of the genetic variance, missing heritability is an important issue for POAG as it is for many other complex traits. Part of the missing heritability may be due to excluding the rare variants (MAF < 5%) from the standard GWAS.[18–20] Although next-generation sequencing technologies have enabled efficient identification of rare variants,[22] the cost of sequencing is high, limiting sample size in many situations and leading to low statistical power to identify rare variants associated with complex traits.[23]

Genotype imputation is a less expensive approach to impute genotypes of untyped genetic variants. However, one study showed that the proportion of well-imputed single nucleotide polymorphisms (SNPs) (imputation quality score [INFO] > 0.4) was only 69%, 60%, and 49% for SNPs with MAF from 0.3% to 5% for individuals genotyped on Omini1M, HumanHap 610, and Illumina 317k arrays, respectively, where 1000 Genomes pilot was used as the reference panel for imputation.[24] However, none of the very rare variants (MAF < 0.3%) were well imputed.[24] In addition, given that statistical power is proportional to allele frequency and imputation quality, standard GWAS may be underpowered to test low-frequency imputed variants. Methods for association analysis of imputed rare variants are mainly based on combining information across the rare variants within a gene or pathway while accounting for genotype uncertainty due to the imputation.[25,26] Thus, it remains unclear whether variants with MAF < 0.3% can be accurately imputed and used in GWAS.

We previously performed a GWAS for POAG using the variants with MAF > 1% imputed to the 1000 Genomes phase 1,[7] and in this present study, we explicitly considered the accuracy of imputing rare variants (MAF < 1%) including the Gln368Ter mutation using common variants captured on genotyping arrays. We then investigated whether it is possible to detect the previously established association of the Gln368Ter mutation with POAG from imputed data using a standard GWAS, and whether we can detect other GWAS hits for POAG using imputed rare variants.

Methods

Study Design

In total, 1155 cases with advanced POAG from the Australian and New Zealand Registry of Advanced Glaucoma (ANZRAG) were available for this study, of whom 618 were genotyped on Illumina Omni1M and 537 were genotyped on Illumina OmniExpress array. Controls included 1992 individuals drawn from the Australian Cancer Study (225 esophageal cancer cases, 317 Barrett's esophagus cases, and 552 controls genotyped on Illumina HumanOmni1-Quad) or from a study of inflammatory bowel diseases (303 cases and 595 controls genotyped on Illumina HumanOmniExpressExome). The cohort detail and diagnostic criteria have been previously published.[7,27] The data from cases and controls were merged and cleaned (see details below), and the overlapping SNPs between the arrays were used as the basis of imputation to the 1000 Genomes phase 1 reference panel and subsequent GWAS. The research followed the tenets of the Declaration of Helsinki. All participants provided written informed consent. Approval was obtained from the Human Research Ethics Committees of Southern Adelaide Health Service/Flinders University, University of Tasmania, QIMR Berghofer Institute of Medical Research (Queensland Institute of Medical Research), and the Royal Victorian Eye and Ear Hospital.

Quality Control (QC)

The QC was performed in PLINK[28] (http://pngu.mgh.harvard.edu/~purcell/plink/ [in the public domain]) by removing individuals with more than 3% missing genotypes, SNPs with call rate < 97%, MAF < 1%, and Hardy-Weinberg equilibrium $P < 0.0001$ in controls and $P < 5 \times 10^{-10}$ in cases. The same QC protocol was used before merging the cases and controls to avoid mismatches between the merged datasets. Following merging, the genotypes for 569,249 SNPs common to the arrays were used for subsequent analyses. The autosomal markers were used to compute identity by descent in PLINK, with one of each pair of individuals with relatedness of >0.2 removed. The smartpca package from EIGENSOFT software (http://www.hsph.harvard.edu/alkes-price/software/ [in the public domain]) was used to compute principal components for all participants and reference samples of known northern/western European ancestry (1000 Genomes British, CEU [Utah Residents with Northern and Western European Ancestry], Finland participants).[29,30] Ancestry outliers with PC1 or PC2 values > 6 standard deviations from the known northern/western European ancestry group were excluded.

Imputation

We used IMPUTE2[31] to perform imputation with the 1000 Genomes phase 1[32] (March 2012 release) as the reference panel. The worldwide reference panel was used, with SNPs with a MAF < 0.1% in Europeans filtered out. Imputation was performed in 1-Mb sections with the recommended settings for IMPUTE2 including a 250-kb buffer flanking imputation sections and the effective size of the sampled population as 20,000.[31] Imputation quality can be objectively assessed by the average concordance between input SNPs genotypes and their "best guess" genotypes imputed from the surrounding SNPs; we achieved a very acceptable ≥0.95 across the genome. Single nucleotide polymorphisms with an INFO < 0.4 were discarded. The imputation maximum posterior probability was used to assign the best guess imputed genotypes for rs74315329 and two SNPs in linkage disequilibrium (LD) with rs74315329 (measurement of the degree to which alleles at two genetic loci are associated, where $r^2 = 0$ indicates independent alleles and $r^2 = 1$ indicates completely correlated alleles), rs187423359, and rs182384379 ($r^2 = 0.5$ with rs74315329), with setting the threshold of calling genotypes to 0.6.

Statistical Analysis

Of the SNPs with INFO > 0.4 from imputation, only very well-imputed SNPs (INFO > 0.8) were carried forward for association analysis. SNPTEST[33,34] was used to perform association testing on the imputed data using additive model (-frequentist 1) and full dosage scores (-method expected)

adjusting for sex and the first six principal components. Genomic inflation factor lambda (λ) was calculated to investigate the presence of population stratification and inflation. The *P* values were corrected for genomic inflation factor λ (1.06) by dividing the χ^2 values by 1.06.

Sequencing

We screened the POAG cases in this study for the Gln368Ter mutation using direct sequencing as previously described.[12,35,36]

Genotyping and Imputation for the Twin 610K Study

To assess imputation quality using the HumanHap610 array we examined 1801 unrelated individuals genotyped on the Illumina HumanHap610 array from the Brisbane Adolescent Twin Study.[37,38] Following cleaning, 504,071 SNPs were available for imputation. Imputation was performed as for the POAG cohorts above.

Results

Genotype data from 1155 individuals with advanced POAG from the ANZRAG and 1992 controls were combined and cleaned, and 569,249 SNPs were used as the base of imputation to the 1000 Genomes phase 1 reference panel. A panethnicity reference panel was used, with SNPs with a MAF $< 0.1\%$ in European 1000 Genomes samples filtered out to exclude the singleton or monomorphic SNPs. In total, 5,537,665 SNPs were imputed with MAF $< 1\%$, of which 3,260,097 (59%) were imputed with an acceptable imputation quality (INFO > 0.4). This ratio of SNPs with INFO > 0.4 drops marginally to 53% (466,199 from 876,619 SNPs) for the SNPs with MAF $< 0.1\%$ (allele frequencies reported here are in the imputed samples). The proportion of well-imputed SNPs with INFO > 0.8 was lower at 11% (615,714 SNPs) for SNPs with MAF $< 1\%$, and 4% (35,512 SNPs) for the SNPs with MAF $< 0.1\%$. These data suggest that a high proportion of acceptable quality SNPs were imputed in this study, even for SNPs with MAF $< 0.1\%$.

The Gln368Ter (rs74315329) rare variant (MAF $= 0.1\%$ in our controls, MAF $= 1.2\%$ in our cases estimated from the imputation data) had a high imputation quality (INFO $= 0.93$). The imputation maximum posterior probability (posterior probabilities for each of the three genotypes of a SNP in the population, i.e., homozygous for wild-type allele, heterozygous, and homozygous for mutant allele) was used to assign the best guess imputed genotypes for the Gln368Ter variant (the threshold of calling genotypes was set to 0.6). The best guess imputed genotypes were then compared with the genotypes obtained from direct sequencing to investigate the concordance between the genotypes obtained from imputation and sequencing. None of the individuals were homozygous for the mutant allele (*A* allele) (imputation posterior probability was zero for the *A/A* genotype in all the individuals). Table 1 shows the imputation probabilities and results of direct sequencing for individuals with posterior probabilities > 0 for the heterozygous genotype (*A/G*). Of the 37 individuals in Table 1, 30 (all case samples) had also the genotypes available from sequencing. Of the 30, 28 (93.3%) were confirmed by sequencing to be carriers (Table 1). In addition, four POAG unaffected individuals were also carriers of the mutation based on the imputation results, three of them with high confidence (imputation posterior probabilities > 0.95). The other POAG cases in this study who are not included in Table 1 ($n = 1124$) were not carriers of the mutation as confirmed by both imputation and sequencing. Overall, we found a sensitivity of 96.29%, specificity of 99.91%, positive predictive value of 96.29%, and negative predictive value of 99.91% for imputation of the Gln368Ter variant compared with direct sequencing. When only individuals with high imputation posterior probabilities (>0.9) are included to reduce the uncertainty for the best guess genotypes obtained from imputed data, the accuracy is higher at a sensitivity of 100%, specificity of 99.91%, positive predictive value of 95.65%, and negative predictive value of 100% (Table 2).

Table 1 also shows the best guess imputed genotypes and imputation posterior probabilities for the heterozygous genotypes of rs187423359 and rs182384379 (the proxy rare variants in $r^2 = 0.5$ with rs74315329). Although the imputation results for rs187423359 and rs182384379 were consistent with the imputation results for rs74315329, the results were not identical because those SNPs are not in complete LD with rs74315329 ($r^2 = 0.5$).

We also investigated whether rs74315329 can be imputed accurately using the other commonly used genotyping arrays. rs74315329 was well imputed with INFO $= 0.83$ in 1801 individuals of European descent (all unscreened for POAG) genotyped on the Illumina HumanHap610 array, with 1000 Genomes phase 1 as the reference panel. Three individuals in this dataset were carriers of the risk allele with high confidence (imputation posterior probability of 100%). The lower imputation accuracy for rs74315329 in that study may be due in part to a lower frequency of the risk allele in individuals without POAG (frequency of 0.1% estimated from the imputed data in this dataset compared with 0.5% in the ANZRAG dataset), or may be due to lower SNP coverage on the HumanHap610 array. However, while lower, INFO 0.83 still represents high-quality imputation, and suggests that the HumanHap610 arrays, in addition to Omni1M, OmniExpress HumanOmni1- Quad, and HumanOmniExpressExome arrays, can be used for imputation of rs74315329 Gln368Ter.

The other POAG-associated *MYOC* variants (http://www.omim.org/entry/601652 [in the public domain]) either were monomorphic in 1000 Genomes phase 1 European population and were filtered out during imputation or were not present in the reference panel. Thus, this study could not investigate the imputation of other POAG-associated *MYOC* variants.

Association analysis for the imputed variants was performed using an additive model adjusted for the sex and the first six principal components. The genomic inflation factor λ was 1.06 after including sex and the first six principal components as covariates. The *P* values obtained from the association analysis were corrected for the genomic inflation factor λ. In addition to the common variants previously reported,[7] the only genome-wide significant rare variant (MAF $< 1\%$) associated with POAG in this study was rs74315329, the Gln368Ter mutation (odds ratio $= 15.53$ and $P = 1.07 \times 10^{-9}$).

Discussion

In this study we report the accurate imputation of a rare variant (Gln368Ter mutation in the myocilin gene [*MYOC*] with MAF $= 0.5\%$ in our study population) imputed to the 1000 Genomes phase 1 reference panel. The imputed variant was successfully used in a GWAS to detect an association with POAG using standard allelic association analysis. This study suggests that rare variants can be accurately imputed using dense reference panels such as the 1000 Genomes project data

Table 1. Imputation Maximum Posterior Probability, Best Guess Imputed Genotypes, and Genotypes From Direct Sequencing for rs74315329, rs187423359, and rs182384379

Individual ID	POAG	rs74315329* Best Guess Imputed Genotypes†	rs74315329 *G/A* Probabilities‡	rs74315329 Genotypes From Direct Sequencing	rs187423359 Best Guess Imputed Genotypes†	rs187423359 *C/T* Probabilities‡	rs182384379 Best Guess Imputed Genotypes†	rs182384379 *G/A* Probabilities‡
AG-107	Yes	*G/A*	1	*G/A*	*C/T*	0.999	*G/A*	1
AG1176	Yes	*G/A*	1	*G/A*	NA	0.436	*G/A*	1
AG1335	Yes	*G/A*	1	*G/A*	*C/T*	1	*G/A*	1
AG-136	Yes	*G/A*	1	*G/A*	*C/T*	0.999	*G/A*	1
AG1408	Yes	*G/A*	1	*G/A*	NA	0.436	*G/A*	1
AG1432	Yes	*G/A*	1	*G/A*	*C/T*	0.999	*G/A*	1
AG-301	Yes	*G/A*	1	*G/A*	*C/T*	0.999	*G/A*	1
AG-315	Yes	*G/A*	1	*G/A*	*C/T*	0.999	*G/A*	1
AG-542	Yes	*G/A*	1	*G/A*	*C/T*	0.999	*G/A*	1
AG-697	Yes	*G/A*	1	*G/A*	*C/T*	1	*G/A*	1
AG-720	Yes	*G/A*	1	*G/A*	*C/T*	0.999	*G/A*	1
GTas2-21	Yes	*G/A*	1	*G/A*	NA	0.437	*G/A*	1
GTas229-2	Yes	*G/A*	1	*G/A*	*C/T*	0.999	*G/A*	1
GTas2-68	Yes	*G/A*	1	*G/A*	NA	0.436	*G/A*	1
GTas337-4	Yes	*G/A*	1	*G/A*	*C/T*	0.999	*G/A*	1
GTas440-1	Yes	*G/A*	1	*G/A*	*C/T*	0.957	*G/A*	0.976
GTas447-1	Yes	*G/A*	1	*G/A*	*C/T*	0.881	*G/A*	1
Gvic117-1b	Yes	*G/A*	1	*G/A*	*C/T*	0.993	*G/A*	1
Gvic122-1	Yes	*G/A*	1	*G/A*	*C/T*	0.999	*G/A*	1
Gvic139-1	Yes	*G/A*	1	*G/G*	*C/T*	0.999	*G/A*	1
AG-633	Yes	*G/A*	0.999	*G/A*	*C/T*	0.998	*G/A*	0.997
AG-021	Yes	*G/A*	0.998	*G/A*	*C/T*	0.87	*G/A*	0.999
AG-242	Yes	*G/A*	0.956	*G/A*	*C/T*	0.749	*G/A*	0.956
Gvic124-1b	Yes	*G/A*	0.839	*G/A*	*C/T*	0.689	*G/A*	0.79
AG0857	Yes	*G/A*	0.762	*G/A*	*C/T*	0.749	*G/A*	0.698
AG1315	Yes	*G/A*	0.734	*G/A*	*C/T*	0.711	*G/G*	0.191
AG-093	Yes	*G/A*	0.6	*G/A*	NA	0.501	NA	0.496
AG0792	Yes	*G/G*	0.193	*G/A*	NA	0.49	*G/G*	<0.1
AG1383	Yes	*G/G*	0.157	*G/G*	*C/C*	<0.1	*G/G*	<0.1
AG0730	Yes	*G/G*	0.127	*G/G*	*C/C*	0.332	*G/G*	<0.1
GTas0-1358b	Yes	*G/A*	1	NA	*C/T*	0.998	*G/A*	0.998
171401	No	*G/A*	1	NA	*C/T*	0.999	*G/A*	1
251442	No	*G/A*	1	NA	*C/T*	0.999	*G/A*	1
687.001	No	*G/A*	0.951	NA	*C/T*	0.742	*G/A*	0.9
251270	No	*G/A*	0.857	NA	*C/T*	0.853	*G/A*	0.851
CON_3972	No	*G/G*	0.39	NA	*C/C*	<0.1	*G/A*	0.396
FMC_758.001	No	*G/G*	0.156	NA	*C/C*	0.113	*G/G*	0.155

This table shows the imputation maximum posterior probability for the individuals with posterior probability of more than 0 for the heterozygous genotypes of rs74315329 and two other proxy SNPs (rs187423359 and rs182384379). The best guess imputed genotypes have been also shown for these SNPs (calling threshold was set to 0.6). The genotypes of rs74315329 obtained through direct sequencing are also presented. Allele *G* is the wild-type allele, and *A* is the POAG risk allele for rs74315329. NA, not available.

* The Gln368Ter variant.

† Best guess imputed genotypes obtained from the imputation maximum posterior probabilities with the threshold of calling genotypes set to 0.6.

‡ Imputation maximum posterior probabilities for the heterozygous genotypes of the respective variants.

Table 2. Accuracy of Imputation Compared With Direct Sequencing for the Individuals With Imputation Posterior Probabilities > 0.9 for Gln368Ter Variant

Sequencing / Imputation	Carrier	Noncarrier
Carrier	True positive, TP = 22	False positive, FP = 1
Noncarrier	False negative, FN = 0	True negative, TN = 1124

%Sensitivity = 100*(TP/TP + FN) = 100*(22/22) = 100%. %Specificity = 100*(TN/TN + FP) = 100*(1124/1125) = 99.91%. %Positive predictive value = 100*(TP/TP + FP) = 100*(22/23) = 95.65%. %Negative predictive value = 100*(TN/TN + FN) = 100*(1124/1124) = 100%.

and high-coverage microarrays such as HumanHap610, OmniExpress, Omni1M, HumanOmniExpressExome, and HumanOmni1-Quad. Imputation of rare variants is currently far more cost-effective than sequencing methods for genotyping a large numbers of variants. This in its own right is important given the prohibitive costs of whole-genome sequencing and the resultant small sample sizes, which are poorly powered to detect an association with complex traits.[23] Although detecting associations of imputed rare variants by single variant tests in standard GWAS may not be powerful due to the low allele frequency and low imputation accuracy,[25] the results of this study suggest that some clinically important rare variants can be imputed with high accuracy to detect an association with complex traits in standard GWAS.

This study used a mix of arrays with the sets genotyped separately; they were combined and thinned to a common set of SNPs with appropriate QC. We were able to accurately impute Gln368Ter with SNPs from the intersection of a number of arrays, suggesting that the method to impute Gln368Ter might be robust to array choice.

Could this approach be used for screening other pathogenic variants with lower MAF? Imputation effectiveness is dependent on the existence of a haplotype that tags the target SNP, that haplotype being properly captured/characterized in the reference panel, and the genotyping array containing SNPs in that haplotype. Thus, while imputation difficulty is inversely proportional to MAF (as linkage disequilibrium is limited by the relative allele frequency difference, and rarer SNPs have a smaller range of allele frequencies that can tag them), there isn't a simple cut off. Also the rarer the target SNP, the larger the reference panel required to capture the correct haplotypes to impute it, should such a haplotype exist. Accordingly, the lower limit of this approach is proportional to the MAF, the size of the reference panel, and the SNP array coverage.

It was demonstrated previously that using dense genotyping arrays (such as Illumina Omin1M and HumanHap 610 arrays) and dense reference panels (such as 1000 Genomes) will increase the accuracy of imputation for common and rare variants.[24] While none of the rare variants with MAF $< 0.3\%$ were well imputed (INFO > 0.4) in that study, we could accurately impute 53% of rare variants with MAF $< 0.1\%$ (INFO > 0.4). The likely reason for this poor imputation of rare variants with MAF $< 0.3\%$ could be the smaller sample size used for imputation (153 individuals versus 3147 individuals used in the ANZRAG dataset) as well as the greater coverage in the 1000 Genomes phase 1 release. These data suggest that using dense reference panels and genotyping arrays along with a large number of people for imputation can improve the imputation accuracy of rare variants.

HapMap-based imputation has a higher proportion of well-imputed rare SNPs than 1000 Genomes pilot (not phase 1) imputation.[24] This may be due to the larger number of rare variants (including very rare variants with MAF $< 0.3\%$) in the 1000 Genomes panel compared to the HapMap panel, which in turn may result in an overall reduction in the proportion of well-imputed rare variants. On the other hand, the 1000 Genomes pilot reference panel contains a relatively small population (60 CEU [Utah Residents with Northern and Western European Ancestry] individuals, 62 Han Chinese in Bejing + Japanese in Tokyo (CHB+JPT) individuals, and 59 Yoruba in Ibadan, Nigeria (YRI) individuals) compared to the following release (phase 1) of the 1000 Genomes data. Moreover, since genotypes in 1000 Genomes have been derived using low pass sequencing, the genotyping quality of the reference panel may be low for very rare SNPs. However, 1000 Genomes may be a better source for imputation of rare variants compared to HapMap due to the increased density and inclusion of a larger number of rare variants.[24]

Screening rare variants associated with complex traits can be clinically important for prediction of risk and diagnosis and treatment. Here, we could accurately screen the Gln368Ter mutation in the *MYOC* gene, which is associated with POAG, by imputing this mutation using genotypes available on common genotyping arrays. The penetrance of the Gln368Ter mutation is high and increases with aging.[39–41] The frequency of this mutation has been estimated to be 0.1% to 0.3% in the European population, which means that at least 2 people in every 1000 are expected to be carriers. As such this represents a relatively high number of people at risk who can be accurately screened for the mutation using a relatively cheap array typing. We have found that detecting the Gln368Ter *MYOC* mutation using imputation can accurately identify people at high risk of developing POAG. This can in turn result in early diagnosis and timely treatment, thereby preventing the development of irreversible blindness. Of the total of 3793 individuals without POAG in this study, 6 people were found with high confidence (imputation posterior probability > 0.95) to carry the Gln368Ter mutation. These people are at high risk of developing POAG later in their life. Similarly, a large number of individuals have had their genome scanned using arrays (e.g., almost a million 23andMe customers); being able to predict which of those individuals carry a high-risk *MYOC* mutation would be of considerable significance as it would lead directly to many individuals seeking appropriate clinical advice.

One limitation of this study is that we did not use other reference panels or imputation tools to compare the results and investigate whether high accuracy of imputation will also be obtained using those panels and tools. In addition, although we validated the imputation results for the Gln368Ter variant using Sanger sequencing in the 1155 POAG cases, we did not have DNA available for the controls and hence did not Sanger sequence controls to verify any controls that were carriers.

In summary, we showed that imputation using common SNPs directly genotyped on genotyping arrays could be an accurate and less expensive (compared to direct sequencing) approach for detecting some clinically important rare variants such as Gln368Ter. These results are clinically important in terms of early detection and treatment of patients at high risk.

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Study (BEAGESS) sponsored the genotyping of cases with esophageal cancer and Barrett's esophagus, which were used as unscreened controls in the ANZRAG cohort. BEAGESS was funded by Grant R01 CA136725 from the US National Cancer Institute. SM is supported by an Australian Research Council Future Fellowship. GWM, KPB, and JEC are supported by Australian NHMRC Fellowships. GR-S was funded by NHMRC during the period of this study. The Australian Twin Registry was supported by an NHMRC enabling grant (2004–2009).

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4.4. Management of incidental findings in research

In this paper, I reviewed the implications of incidental findings in genetic research. Whole genome sequencing (WGS) and WES are being increasingly used in research for their effectiveness in identifying novel genes and mechanisms in genetic diseases. Their ability to detect any genomic variants means that they also have the potential to detect incidental findings, e.g. variants of clinical significance that are not related to the indication of the test. Although guidelines for handling incidental findings in a clinical setting are well defined, recommendations in research lack clear guidance. Here I discussed some of the issues related to the return of incidental findings in research and the need for researchers to establish a framework for the disclosure (or nondisclosure) of incidental findings and the context of disclosure.

Contribution statement

Ms Souzeau was responsible for the study conception, design and research, drafted and submitted the manuscript as corresponding author. A/Prof Burdon, Prof Mackey, A/Prof Hewitt, Prof Savarirayan, Prof Otlowski, and Prof Craig contributed to the study conception and design and revised the manuscript.

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Perspective

Ethical Considerations for the Return of Incidental Findings in Ophthalmic Genomic Research

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Whole genome and whole exome sequencing technologies are being increasingly used in research. However, they have the potential to identify incidental findings (IF), findings not related to the indication of the test, raising questions regarding researchers' responsibilities toward the return of this information to participants. In this study we discuss the ethical considerations related to the return of IF to research participants, emphasizing that the type of the study matters and describing the current practice standards. There are currently no legal obligations for researchers to return IF to participants, but some viewpoints consider that researchers might have an ethical one to return IF of clinical validity and clinical utility and that are actionable. The reality is that most IF are complex to interpret, especially since they were not the indication of the test. The clinical utility often depends on the participants' preferences, which can be challenging to conciliate and relies on participants' understanding. In summary, in the context of a lack of clear guidance, researchers need to have a clear plan for the disclosure or nondisclosure of IF from genomic research, balancing their research goals and resources with the participants' rights and their duty not to harm.

Introduction

Landmark advances in science are often accompanied by ethical challenges. During the past decade, new methods for massively parallel sequencing have been developed, computational approaches have advanced, and there has been an increased availability of large public sequencing datasets. As a result, whole genome and whole exome sequencing (WGS/WES) technologies have emerged as useful tools in both research and clinical molecular diagnostics. WGS/WES facilitates the sequencing of large regions of the genome with a timely turnaround, and they are increasingly affordable.[1] The potential uses for WGS/WES in medical genomics research are rapidly expanding. In the past few years, the technology has allowed the discovery of new genes and new mechanisms, unraveling the genetic cause of single gene and complex disorders where conventional sequencing methods have failed in the past.[2–5] This is an exciting time for ophthalmic genomic research as these techniques are now becoming increasingly used. However, because WGS/WES are less targeted than conventional genetic testing, they generate a vast amount of genomic data well beyond what has been generated by traditional targeted genetic approaches, including the potential for incidental findings (IF). As a result, complex ethical questions arise and challenge the researchers' responsibilities regarding disclosure of these data to research participants. Although not the topic of this paper, the same ethical issues apply to genome-wide association studies, which also have the potential to identify IF.[6] In this paper, we discuss the elements to consider when debating the return of genomic IF generated from WGS/WES in a research setting.

Table 1. Ethical Principles in Favor or Against the Disclosure of Incidental Findings to Research Participants

For	Against
Availability of results	Availability of results outside of research
Principle of beneficence	Principle of nonmaleficence (do not harm)
Leads to positive health outcome	Promotes therapeutic misconception
Respects participant autonomy	Risk for social discrimination/stigmatization
Respects the right to know	Respects the right not to know
Increases trust in research	Burden on research infrastructure
Principle of reciprocity	Emotional harm
Duty to rescue	

Findings from WGS/WES can be broadly classified in two categories: (1) the pertinent or primary findings that are results relevant to the indication for which the test was ordered and (2) unsolicited, secondary or incidental findings that are results that are not related to the primary indication of the test and may or may not be relevant to the patient's health (for example a variant related to cancer identified through the conduct of a study on the genetic causes of congenital glaucoma). There is presently a great deal of controversy over how IF should be handled in research: which IF, if any, should be returned to participants and how they should be returned.[7–13] This debate has been described by Wolf et al.[14] as a problem of translational research, when findings from research have some potential clinical utility and impact on clinical management.

The arguments in favor and against the return of IF to research participants are outlined in Table 1.[9,13,15–18] When evaluating whether IF should be returned to participants, researchers need to consider the type of the study, the practice standards and ethical approvals in place, the analytical and clinical relevance of the findings, and the participant's preferences in relation to return of results with specific reference to the research consent documents.

Research Versus Clinical Settings

The context in which the return of individual genomic research results is discussed does matter.[19,20] The distinction between research and clinical care is important because the underlying key principles are different. The goal in research is to generate data for a communal benefit, whereas in clinical care the individual patient's needs and benefits prevail. As a result, the rights and duties of the individuals implicated are different.[21–23] However, the boundary between clinical and research settings can be blurred, especially when the research participants are patients and when the researcher could also be their clinician, making it harder to distinguish the responsibilities of each person.[16,22] This is a complex area, and the distinctions are often poorly understood by patients and health care workers generally. Even within the research context, there are nuances, depending on the circumstances, including the type of WGS/WES performed, and the social context in which they take place.[19] Researchers' obligations toward participants are defined by the consent form and the protocol approved by institutional review boards (IRBs) or their equivalents, the overriding duty to protect participants from harm, and the respect of privacy and confidentiality.[24] It is suggested that rather than a one-size fits all, a case-by-case (or disease-by-disease) approach is required regarding factors such as degree of vulnerability of the study cohort, depth of researcher/participant relationship, and degree of participant dependence.[20]

Existing Recommendations

Several recommendations have been published regarding the return of IF in both clinical and research settings (Table 2). In the clinical setting, on one side of the spectrum, the American College of Medical Genetics and Genomics (ACMG) published a statement advocating for opportunistic screening and recommended that variants from a list of 56 genes associated with 24 disorders with high penetrance and clinical actionability be actively looked for and returned, regardless of the age of the patient.[25,26] In the wake of vocal criticism of its position, the ACMG revised its recommendations to allow patients to opt out of the analysis of medically actionable genes when undergoing WGS/WES.[26] On the other end of the spectrum, the European Society of Human Genetics, the Canadian College of

Table 2. Published Guidelines for the Report of Genomic Results in a Clinical and a Research Setting

Organization	Recommendations	Reference
Clinical setting		
American College of Medical Genetics and Genomics (USA, 2013)	• Laboratories need to actively search for the specified types of mutations in 56 genes associated with 24 conditions with high probability of severe adverse outcome and report them to the clinician. Variants to be reported need to be known pathogenic or expected pathogenic. • This is done regardless of the indication of the test and the age of the patient, but patients can opt out of the analysis of the genes during the consent process.	25, 26
European Society of Human Genetics (Europe, 2013)	• The use of a targeted approach to avoid IF is recommended, and variants with limited clinical utility should be filtered out. The use of WGS/WES requires a justification of necessity and proportionality. • The detection of IF of serious health problems that are actionable should be reported.	27
Royal College of Pathologists of Australasia (Australia, 2014)	• Genomic testing should have a sound evidence base, and targeted analysis is recommended. • Clinicians should use standard practices in deciding whether to return IF as long as the policy is clearly provided to the patient and the patient has agreed to it.	28
Canadian College of Medical Geneticists (Canada, 2015)	• Genome-wide sequencing should only be considered when proved useful in the evaluation process and a selective filtering process is recommended. • Should IF be detected, the patient should be given the option to receive them or not prior to testing.	29
Research setting		
National Heart Lung and Blood Institute (USA, 2010)	• Genetic research results should be offered if the findings have important health implications, are actionable and analytically valid, comply with all applicable laws, and the study participant has opted to receive them. • Genetic research results may be returned if the potential benefits outweigh the risks from the participant's perspective, the IRB has given approval, the findings are analytically valid, they comply with all applicable laws, and the study participant has opted to receive them (includes variants related to reproductive risks, personal meaning or utility).	30
Tri-Council Policy Statement (Canada, 2010)	• Researchers have an obligation to disclose to the participants any material IF discovered during the course of the research defined as having significant welfare implications for the participant, as long as the participant consented and the disclosure plan has been approved by an IRB. • Exception to the obligation to disclose can be requested based on the impracticability or impossibility of disclosure (undue hardship or onerousness jeopardizing the conduct of the research).	31

Table 2. Continued.

Organization	Recommendations	Reference
Presidential Commission for the Study of Bioethical Issues (USA, 2012, 2013)	• Researchers should develop a plan to manage IF, which should be approved by an IRB. Participants should be informed of whether and how they might opt out of receiving IF. Researchers do not have a duty to look for IF.	35, 65
Public Health Genomics Foundation (UK, 2013)	• Research findings that are validated, scientifically relevant, clinically significant, severely or moderately life threatening, and clinically actionable should be returned with the participant's consent.	32
Network of Applied Genetic Medicine (Canada, 2013)	• IF should be offered when they are scientifically and clinically valid, have clinical utility, exceptions and considerations related to the research context have been weighted, IRB approval has been obtained, participant has consented, and the result has been confirmed. • IF may be offered if they are scientifically and clinically valid, the benefits of return surpass the risks, IRB approval has been obtained, participant has consented, and the result has been confirmed.	33
Clinical Sequencing Exploratory Research Consortium/Electronic Medical Records and Genomics Network (USA, 2014)	• Analytically and clinically IF that are actionable should be offered to research participants if they agreed to the return of results. Participants have the right to refuse any results that are offered. • Researchers do not have a duty to look for actionable IF.	34
National Health and Medical Research Council (Australia, 2015)	• When the return of IF is feasible and the results are adequately validated, participant should have the autonomy to decide whether or not to request the return of IF.	24

Medical Geneticists, and the Royal College of Pathologists of Australasia encouraged caution and recommended a targeted approach to the clinical question to avoid the detection of IF.[27–29] In the research setting, the guidelines vary from defining which IF should or may be returned[30–34] to recommendations that do not advocate for or refrain from looking for IF, but frames how IF should be returned if feasible.[24,35,36]

Return of Incidental Genetic Findings in the Context of Eye Diseases

Although still disputed, there is a viewpoint that even if researchers have no legal obligation, they could have an ethical obligation to return genomic variants that are of clinical validity (the variant is known to be associated with a particular disease), have clinical utility (the likelihood of a positive health outcome), and are actionable (medical actions can be taken to decrease the risk).[8,13,30,34,37,38] As an example, clinical validity would be low for genetic variants associated with macular degeneration because of the weak correlation between specific genotypes and visual outcome,[39] but would it be higher for disease-causing variants in the *MYOC* gene associated with glaucoma and high penetrance.[40] Similarly, retinitis pigmentosa (RP) disease-causing variants would currently be of limited clinical utility due to the lack of available treatments. This may change with the advent of gene therapy for retinal dystrophies. Predictive genetic testing for RP family members is a controversial topic.[41] There is some evidence that taking high doses of vitamin A supplements may slow the progression of RP.[42] A patient may be symptomatic of RP, having nyctalopia, but not be diagnosed with RP. Genetic testing

would then alert them to their symptoms to justify further diagnostic testing with visual fields, an electroretinogram, and dark adaptation. Finally, diagnosis of RP in young adults helps ensures safety with driving and allows reproductive choices before they have children. Genetic variants known to cause retinoblastoma or choroidal melanoma would have a stronger clinical utility based on their actionability and the importance of early diagnosis. Information related to reproductive or personal utility has received much less consensus for disclosure. Overall, the consensus reached refers to situations where the potential benefits outweigh the potential harm for the participant and the findings reach a relevant threshold of validity and medical significance.[43]

Despite the endorsement of clinical validity, clinical utility, and actionability for the return of IF, the definition of each criterion has been relatively inconsistent and is based on a range of different interpretations.[43] The reality is that many IF are actually of unknown or dubious significance and therefore not interpretable. Additionally, the meaning of a pathogenic variant can differ between different family members. Across the world it is generally accepted that children should not be tested for adult-onset conditions unless there is an immediate medical benefit. When research involving children discovers results related to adult predisposition conditions that can be clinically relevant to the parents well before it will have a clinical impact on the child, the question has been raised whether these results should be disclosed to the parents.[44] Finally, an area that has received little discussion is the lack of empirical evidence regarding the clinical utility of most IF in routine testing. Most data on disease-causing variants have been collected using cohorts of affected individuals, which can result in an overestimation of the penetrance and expressivity[45] and limit the extrapolation to low-risk populations. The sensitivity and specificity of any genetic test is only as strong as the indication for the test. Along the same lines, the US Preventive Services Task Force has recommended against routine genetic testing for BRCA-related cancer.[46] Overall, researchers need to think about how the information can be used for patients' better health and the potential to do more clinical harm than good.

Prevalence of IF

Undoubtedly, WGS/WES will discover clinically actionable variants in research participants. The ACMG statement anticipated medically relevant IF in 1% of sequencing reports.[25] Based on a mathematical model and using the ACMG list, Ding et al.[47] predicted IF in 2.7% of screened participants. Two recent studies reported pathogenic variants from the ACMG list among 0.9% to 1.7% of individuals,[48,49] while others have reported prevalence of up to 12% for variants of various clinical utility.[50–53] The difference between the studies can be attributed to the cohort selection, the pathogenicity classification criteria of variants, and the inclusion of conditions and genes based on the definition of clinical utility. When including variants associated with carrier status of newborn diseases, risk factors for macular degeneration, and drug response, Tabor et al.[54] demonstrated that every exome would contain variants of potential clinical utility. Furthermore, the prevalence of clinically actionable findings is expected to increase in the future with the improved accuracy of variant annotation of genomic databases, better understanding of the genetics of diseases, and development of therapies.

Practical Considerations in the Return of Research Results

Additional factors for the potential return of IF to research participants must be considered. Most research laboratories are not accredited to report findings that could be used in clinical management. The analytical validity of genetic variants identified through WGS/WES in a research setting is not reliable or robust enough to be reported. Validation in an accredited laboratory and assessment for clinical validity and significance by competent and accredited professionals has been strongly advocated for disclosure.[24,30,32–34] Researchers often have a lack of expertise for results or conditions that are outside the scope of their research. As a result, posttest counseling and medical follow-up needs to be provided by trained professionals. Many have argued that the requirements for the return of IF take substantial time, effort, and resources that would put an unsustainable burden on the research enterprise and move resources away from the primary research.[15,16,55,56] Substantial resources are required for each of these steps, and current research funding is typically not allocated to conduct this activity. One study suggested a framework by which the clinical setting would take care of those steps, ensuring the distinction between research and clinical care re-

mains.[57] However, this option would move the burden to the clinical setting, which would equally struggle to sustain this workload.

Participants' Perspectives and Understanding

All guidelines recognize that the participants' preferences need to be taken into account. Whenever possible, participants should be informed of the possibility of return of IF and the potential risks and benefits, and they should be able to opt out of its return.[8,24,30,33,34,58] Participants' familial, cultural, and religious beliefs also need to be acknowledged. Different models of consent[59,60] and dynamic return of results[61,62] have been proposed to address the complexity of the return of IF. To give informed consent for every eventuality is impossible, and studies have shown that categorizing the results potentially returned facilitates the process.[38,63]

Respecting participants' preferences can also pose some challenges. In some situations, further investigations of the participant and his or her family, necessitating recontact, can be required to ascertain the pathogenicity of a variant, making it difficult to respect an individual's wishes to learn only about clinically significant variants.[64] Historically, IF were not always addressed properly in consent forms, which creates issues for disclosure. Published guidelines have discussed whether the absence of reference to IF disclosure in the consent form would prevent their return and to what extent researchers can respect participants' wishes of not knowing IF of clinical significance.[24,30,34,65] Consultation with IRBs has been advised in these situations.

Most studies evaluating the intention to receive results among research participants[66–71] or the general public in hypothetical scenarios[18,67,72–74] have shown that the majority wish to receive results, regardless of the clinical validity and utility. However, previous studies have often shown that patients who expressed interest in obtaining results do not always get tested, and even though the uptake of genetic testing is higher for conditions with preventive measures, it is still lower than expected based on intentions.[75–77] Moreover, individuals make different choices depending on what is at stake and on the framing of the options, emphasizing the difficulty of explaining the complexity and uncertainty of research findings.[78] The issues surrounding IF are complex and take time to explain and process. Tabor et al. evaluated a protocol for obtaining informed consent for WGS in two families that was nine pages long and took 2 to 3 hours.[79] Although both families complained about the length of time and the complexity of the process, they both recognized the extent of the scope of information that needed to be covered in order for them to make informed decisions regarding the return of IF. Few studies have reported what patients really understood of the actual impact of reporting or evaluated their experience of receiving IF and the potential psychological harm.[80] More empirical data are needed on the actual benefits or harm of receiving IF and the true understanding of participants in regard to IF.

Researchers' Perspectives

Genetic professionals and researchers are generally supportive of the disclosure of actionable IF but are usually less so with results pertaining to untreatable conditions, adult-onset conditions for pediatric participants, or variants with lower clinical validity and utility.[56,74,81–84] Surveys among researchers showed that although the majority are in favor of returning highly penetrant, clinically actionable results, they also feel that it would be a burden on researchers.[74,82]

Integrating the opinions of both stakeholders and participants is vital in developing an effective plan for the return of IF, but the discrepancies between what results researchers and participants believe should be disclosed might pose a challenge in balancing the integrity of participant autonomy with researcher's decisions. Increasingly, particularly in light of the growing discourse supporting disclosure, there is need to ensure that participants' expectations are carefully managed during the informed consent process and through clear information in the information sheet and consent form as to what, if any, results will or may be returned.

Incidental Genetic Findings: A Duty to Find and Recontact?

If there is a duty for researchers to report IF, some have questioned whether there could also be a duty to actively look for IF since researchers have access to the genomic data. Studies so far have concluded that researchers do not have an obligation to look for IF.[34,56,65] The rationale is that it would blur the

distinction between research and clinical care, create clinical responsibilities for researchers, and accentuate therapeutic misconception—the notion that research will benefit individuals.[22] Similarly, Gliwa et al.[55] concluded that at present, although there could be benefits for participants, and researchers are in a unique position to access these data, the burden on the research is too extensive for researchers to actively look for IF. However, they argued that in the future, if the analysis process becomes more efficient and if WGS/WES are not yet implemented as a standard of care in clinical care, researchers could face an obligation to look for IF.

Similarly, knowledge about disease associations will evolve over time, and variants are likely to be interpreted differently.[85] This raises the issue of a potential duty to recontact research participants in the light of new information. The question of recontact could also apply to IF related to adult-onset conditions identified in children. Most guidelines recommend that researchers do not have to return IF beyond the termination of research funding.[30,34] Indeed, even in the clinical setting it is recognized that there must be limits on the duty to recontact in the context of WGS/WES given the vast amount of data potentially available.[23] The preferable approach is to explain to patients the fast-moving nature of this area and put the onus on them to recontact in the future if they want to find out if any new information has come to light.

The Importance of Implementing a Disclosure Plan

In the context of a lack of clear policies, researchers need to implement a plan for managing genomic data.[30,31,33,34,65] The plan should describe the type of results that could be disclosed, the modalities of communication (who would disclose results, to whom, when, how), and what should be discussed during the consent process. Different frameworks for the return of results have been proposed in the literature: policy of no disclosure, disclosure of IF of clinical utility and actionability only, disclosure of all IF, return of all genomic data without interpretation, and participant decides which IF would be returned.[8,13,16,32,37] Obviously, the frameworks providing more autonomy to participants also put additional burden on the research infrastructure. Another suggested approach has been to apply filters during the analysis stage to hide unwanted results to minimize the potential for IF.[27,86] This strategy has the benefit of limiting IF of potential clinical utility and minimizing the burden on the research infrastructure. Ultimately, the feasibility, cost, and consequences of each approach need to be balanced. Finally, IRBs oversee research involving human subjects. They are in a unique position to provide valuable insight in reviewing the disclosure plan to research participants and participate in the development of policies and guidelines.[8,30,87]

Conclusion

In summary, there is a lack of definite guidance regarding the return of personal genomic research results. At present, there is no legal obligation for researchers to return IF from WGS/WES, but the emerging view is that there might be an ethical one. However, many have raised concerns about the impact such obligation would have, and the feasibility of such return is debated, with many arguing that the burden on the research infrastructure would be too significant. In any case, adopting a plan for the return of IF needs to take into account the nature of the research, the relationship between the researcher and the participants, the nature of the informed consent, and the duty to do no harm. Ultimately, even in the case of an ethical obligation, the decision is at the researcher's discretion, with the support of IRBs, recognizing that the participants' rights need to be balanced with the research goals. There is an evolving need to develop stronger frameworks and guidance to assist researchers in clarifying their responsibilities toward the management and return of IF, particularly in the view that the genetic landscape is continuously expanding.

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CHAPTER 5: DISCUSSION

Genetic counselling is a relatively young profession, but since its creation, it has constantly been evolving in response to new genetic knowledge and advances in technologies. The profession is now facing some interesting challenges relevant to translational research with the integration of genomics into healthcare. At the same time, genetic counselling is emerging in a number of subspecialties, including ophthalmogenetics [15], and is experiencing a shift toward common diseases [16]. Genetic counselling is highly relevant to glaucoma: glaucoma has a high heritability, is genetically and phenotypically heterogeneous and affects all age groups each with their own psychological and ethical implications.

Glaucoma is the most common cause of irreversible blindness worldwide [86] and has a major impact on the quality of life of affected individuals [26-28]. Around 5-15% of glaucoma patients will lose vision to the point that they cannot hold a driver's licence [116-119]. Early diagnosis of glaucoma enables the implementation of treatment before irreversible vision loss occurs and can significantly diminish the impact on the quality of life of patients. However, the current screening programmes are ineffective in detecting glaucoma early and many patients still present with significant vision loss. The identification of deleterious variants allows cascade genetic testing in families, which is an effective screening strategy to detect individuals at high risk of developing glaucoma. The understanding of the genetics of glaucoma is important in developing novel therapeutic options and screening strategies. The publications that led to this thesis contributed to translational research toward identifying novels genes in glaucoma, defining the natural history of glaucoma and the genotype/phenotype correlations, and evaluating the implications of genetic testing for glaucoma to improve clinical care and provide optimised genetic counselling to patients and their families.

5.1. Contribution toward better understanding of the genetics of glaucoma

5.1.1. Interpretation of genetic variants

The interpretation of genetic findings or results is an integral part of genetic counselling and the evaluation of the information provided to patients. A molecular diagnosis is only as good as the interpretation of the genetic findings and should always be interpreted in the context of the clinical background (medical and familial history). Genetic results used for diagnosis and treatment need to be validated in certified clinical laboratories following recognised international standards and applying a stringent set of criteria for the classification of genetic variants. This is of utmost importance in the context of the recent implementation of WGS/WES in clinical diagnosis and the constant discovery of new associations of genes with diseases. Although known glaucoma genes

accounts for a minority of glaucoma cases, an accurate molecular diagnosis has a major impact on the patient and their family. Therefore, it is critical to interpret correctly genetic variants in glaucoma genes and establish the likelihood of pathogenicity.

The evaluation of variants' pathogenicity is a qualitative assessment and accurately interpreting the clinical significance of a variant is often challenging. Guidelines have been developed by the Royal College of Pathologists of Australasia [378], the Association for Clinical Genetic Science in the UK [379] and the American College of Medical Genetics and Genomics with the Association for Molecular Pathology [380] to assist with the interpretation of sequence variants. Testing laboratories often use a tiered classification system for variant interpretation, for instance the American College of Medical Genetics and Genomics developed a five-tiered classification scheme (benign, likely benign, uncertain significance, likely pathogenic and pathogenic). The classification of a genetic variant is evidence-based and uses a number of criteria to weigh the evidence described below.

Firstly, the recurrence of a variant in the same gene in unrelated individuals with a similar phenotype provides strong support for pathogenicity. For that reason, data sharing related to variants is becoming increasingly essential. A number of freely accessible public disease databases aggregate variants in association with phenotypes and they are constantly growing and evolving. Such databases include ClinVar (www.ncbi.nlm.nih.gov/clinvar), the Human Gene Mutation Database (HGMD; www.hgmd.org) and OMIM (www.omim.org) for genetic variants, and DECIPHER (http://decipher.sanger.ac.uk) for CNVs. Additionally, gene/disease specific databases are usually curated for a single gene or a set of genes implicated in a disease. For example, the *PAX6* database (http://pax6.hgu.mrc.ac.uk) reports all sequence variants in *PAX6* and the *MYOC* database (www.myocilin.com) established in 2008 by Prof Jamie Craig and A/Prof Alex Hewitt is a comprehensive online database listing all reported *MYOC* genetic variants with detailed phenotypic information [169]. These databases are useful resources for medical professionals interpreting the clinical significance of variants and translating results to the patients. However, they should be used with caution. Sources and studies should be carefully analysed, and variants should be considered deleterious in the clinical testing setting only if they have been reported as such in more than one study.

Support for pathogenicity is stronger when variants are either absent or present in very low frequencies in unaffected individuals. Population databases are useful references for the frequency of variants in very large populations. However, individuals from these cohorts have often not been screened for the disease studied, therefore they do contain deleterious variants. A minor allele frequency of 1% is the cut-off often used for common benign variants. A frequency threshold can also be based on the disease prevalence, the mode of inheritance and/or the age of onset. The

1000 Genomes Project (http://browser.1000genomes.org), Exome Variant Server (EVS; http://evs.gs.washington.edu/evs), dbSNP (www.ncbi.nlm.nih.gov/snp) and the Exome Aggregation Consortium (ExAC; http://exac.broadinstitute.org) are good examples of population databases for genetic variants, as well as dbVar for CNVs (www.ncbi.nlm.nih.gov/dbvar).

Segregation studies, when possible, provide additional evidence for or against variant pathogenicity. However, they can be limited, especially for conditions like glaucoma that can have phenocopies or a late age of onset with most affected individuals deceased and therefore not available for studies. Moreover, segregation of a variant with the phenotype can also be due to linkage disequilibrium, meaning that the alleles of different genes are inherited together more often than would be expected by chance.

Information about protein function and tissue expression, evolutionary conservation of the variant among species, location within the protein sequence, biochemical impact of the amino acid change, and pathogenicity prediction add to the line of evidence. Certain types of variants (nonsense, frameshift, splice site and large indels) are often considered deleterious, especially when they result in a truncated protein, absence of the protein product or nonsense-mediated decay. However it is important to consider the known mechanism of the disease in the context of the mode of inheritance, the localisation of the variant in the gene and the presence of alternate gene transcripts before drawing conclusions [380]. A loss of function variant in a gene associated with a disease not caused by this mechanism might not be deleterious. In the case of missense variants or splicing variants, computational predictive programs using in-silico algorithm are useful tools in determining the effect of a sequence variant on the protein structure or function. Such software include Sorting Intolerant From Tolerant (SIFT; http://sift.jcvi.org), Polyphen-2 (http://genetics.bwh.harvard.edu/pph2) and Mutation Taster (www.mutationtaster.org). Finally, functional studies and animal models can provide further evidence in the assessment of genetic variants.

Ultimately, variant classification provides a qualitative assertion of the likelihood of a variant being deleterious or benign based on the weight of evidence and the current knowledge. The diligence used for variant interpretation and classification is necessary when genetic results are used in genetic counselling to predict the mode of inheritance, the risk for other family members, the prognosis or to alter the treatment or the surveillance of a patient. Discordance in variant calling has been reported between laboratories evaluating the same variants, highlighting the complexity of the process [381,382]. Additionally, variant classification is likely to evolve over time as new knowledge and new technologies arise. For example, *MYOC* variants considered deleterious have been reclassified as benign and some benign *MYOC* variants have been reclassified as

deleterious based on new evidence [383]. Although the interpretation of genetic variants is essential for genetic counselling, the elucidation and characterisation of the different genes involved in a particular disease is the base for translational genetic research.

5.1.2. Delineation of known glaucoma genes

Glaucoma is a genetically heterogeneous disorder with multiple genes involved in different types of glaucoma. The publications leading to this thesis helped characterise some of these genes, assess the pathogenicity of novel variants for diagnosis purposes, establish genotype-phenotype correlations, and demonstrate the involvement of some genes in more than one type of glaucoma.

5.1.2.1. MYOC

The *MYOC* gene is the most studied gene for glaucoma. However, the contribution of *MYOC* variants to different degrees of disease severity has not been investigated. In the publication included in chapter 2-2, I used samples from individuals with advanced POAG to demonstrate that *MYOC* deleterious variants are significantly more prevalent in advanced disease (4.2%) than non-advanced disease (1.6%) [375]. Moreover, my findings showed that the prevalence of *MYOC* variants increased with younger age of onset, higher IOPs and a positive family history of glaucoma. The fact that *MYOC* variants may lead to severe disease if left untreated advocates for early identification of asymptomatic individuals.

As discussed above, the interpretation of novel genetic variants is essential in genetic counselling. Garber et al. recently reported that novel variants were more likely to be classified as pathogenic or likely pathogenic when they were found in genes with a well-established role with disease or in genes with several pathogenic variants reported [382]. This emphasises the importance of accurately interpreting novel variants and reporting them to ultimately increase our body of knowledge and our ability to classify variants in any particular gene. As part of the ANZRAG, I have contributed to characterise a number of novel *MYOC* variants (publications included in chapters 2-2 and 2-3) that I have added to the *MYOC* database (www.myocilin.com). These included p.(Trp373Ter), p.(Pro254Leu), p.(Ala447Thr), p.(Asp395Asn), p.(Leu303Ile), p.(Arg296His), and p.(Pro481Thr). The p.(Trp373Ter) variant is a novel nonsense variant considered deleterious and associated with a phenotype similar to the common p.Gln368Ter variant [384]. The p.(Pro254Leu) variant was identified in a sporadic case of JOAG with very early age of onset and was considered deleterious [385]. p.(Pro481Ser) and p.(Arg296His) have been currently classified as likely pathogenic while p.(Ala447Thr), p.(Asp395Asn) and p.(Leu303Ile) have been currently classified as likely benign based on the available evidence. The interpretation of the variants has been critical to accurately counsel patients and their family about the risk of recurrence and the availability of genetic testing for at-risk family members.

Homozygous or compound heterozygous variants in autosomal dominant disorders are rare and often results in a more severe phenotype (such as familial hypercholesterolemia) [386], or a lethal outcome (such as achondroplasia). However, in the case of *MYOC*, both the absence of the disease and a more severe phenotype have been reported in association with biallelic deleterious variants [177,387]. Therefore, it is essential to characterise and report novel occurrence of double variants in *MYOC* for genetic counselling purposes. Hewitt et al. previously reported an individual homozygous for p.Gln368Ter showing no evidence of glaucoma at age 49 [177]. Homozygosity for p.(Lys423Glu) was reported in 4 individuals aged 43-50 years old who similarly did not exhibit glaucoma [388]. Variant p.(Lys423Glu) age-related penetrance at age 40 was 90% in this family whereas p.Gln368Ter has an age-related penetrance of 50% at 50 years old. These individuals would be expected to be affected if the presence of the double variants was causing a more severe phenotype. In contrast, two cases homozygous for p.Thr377Met have been reported, one diagnosed with glaucoma at age 16, the other with severe glaucoma at age 68 although the age of onset was unknown [387]. Variant p.Thr377Met is usually associated with an onset in the forties, which suggests a more severe disease in homozygotes. In the paper by Young et al. included in this thesis (chapter 2-3), I reported the first co-occurrence of the two most common *MYOC* deleterious variants (p.Gln368Ter and p.Thr377Met) associated with a more severe phenotype than each variant alone [389]. Three of the four carriers of both variants aged 26-35 years old had ocular hypertension or glaucoma. *MYOC* deleterious variants cause the protein to aggregate and form dimers, acting through a gain of function mechanism. It is possible that in some cases, the mutant proteins are able to interact together, resulting in the absence of the disease (or at least a less severe phenotype), whereas in others, they create further aggregation, leading to a more severe disease than expected. These publications highlight the importance of characterising new variants or novel association of variants for the provision of accurate genetic counselling to patients and their families regarding their risk of developing the disease.

5.1.2.2. TBK1

In the paper by Awadalla et al. included in this thesis (chapter 2-4) [390], we reported *TBK1* results on the largest cohort of NTG cases and the first cohort of individuals with advanced glaucoma. Fingert et al. initially reported a duplication encompassing the *TBK1* gene in 1.3% of patients with NTG [159]. This finding was replicated in 0.4% of NTG patients from Japan [373] and 1.0% of NTG patients from USA [374]. In the Australian population, *TBK1* CNVs were detected in 4/334 (1.2%) of patients with severe NTG. Similar to *MYOC* [375], these findings indicate that *TBK1* CNV can lead to severe glaucoma. Additionally, the first cohort of HTG patients was tested and we showed that *TBK1* does not contribute to glaucoma with elevated IOP. Characterisation of the phenotype associated with *TBK1* CNVs can assist in identifying which families would benefit from genetic testing.

Our study was the first report of a *TBK1* triplication. Retesting the families reported by Fingert et al. further confirmed a second case of *TBK1* triplication in one of their families [159]. The breakpoints of the duplications and triplications we and the paper by Fingert et al. reported were different for all cases, apart from two. This refutes a founder effect and may suggest a potential hotspot for chromosomal rearrangements. The two families with the triplication had an average age at diagnosis of 32.4 ± 13.0 years compared with 37.6 ± 7.4 years for the individuals carrying *TBK1* duplication. This might suggest an earlier onset with extra copies of *TBK1*. Triplications associated with a more severe phenotype than duplications can be explained by the increase in gene dosage. This phenomenon has been reported for other genetic conditions such as Charcot-Marie-Tooth Type 1A caused by *PMP22* CNVs [391], Pelizaeus-Merzbacher syndrome caused by *PLP1* CNVs [392] and a syndrome including mental retardation, a severe expressive language delay, behavioural problems and dysmorphism associated with CNVs of the 7q11.23 chromosomal region [393]. In the case of Charcot-Marie-Tooth Type 1A, a recent study demonstrated that *PMP22* triplications arose *de novo* from transmitted duplication during the meiosis and that their prevalence might be underestimated [391]. It would be interesting to assess whether *TBK1* triplications could arise from duplications and whether *TBK1* triplications could be associated with a more severe phenotype than *TBK1* duplications as suggested by our findings. The duplication-to-triplication event and the more severe phenotype associated with triplication can imitate genetic anticipation and has implications for genetic counselling and testing.

5.1.2.3. CYP1B1

Deleterious variants in *CYP1B1* were first linked to PCG and are transmitted in an autosomal recessive manner [394]. However, PCG cases heterozygous for *CYP1B1* variants have been reported [299,395-398] and it is currently unknown whether these patients carry a genetic defect affecting the *CYP1B1* gene on the other allele. Isolated reports of *CYP1B1* deletions have been reported [394,399] but no study assessed their contribution to the phenotype. In the study included in chapter 3-3, I investigated the role of CNVs in *CYP1B1* using MLPA for the first time [400]. No deletions were identified among 50 PCG cases negative or heterozygous for *CYP1B1*. Further potential mechanisms include the presence of variants in the promoter or in non-coding regions affecting the expression of *CYP1B1* or a digenic inheritance implicating other genes yet to be discovered. My findings suggested that *CYP1B1* deletions are not a major contributor of the disease and did not support a diagnostic utility of CNV testing for *CYP1B1*.

The presence of one or two *CYP1B1* variants has also been reported in several cohorts of JOAG cases with variable frequencies [168,304,399,401-403]. These studies were limited by the different definitions of inclusion criteria for JOAG (age at diagnosis and IOP) and the small sample size of most of the cohorts. In the paper included in chapter 2-5, I reported the first cohort of individuals

with severe JOAG screened for *CYP1B1* variants, regardless of their IOP or family history [404]. I reported that heterozygosity for *CYP1B1* variants was significantly higher in cases than controls, supporting the contribution of *CYP1B1* variants in JOAG. *CYP1B1* carriers had a younger age at diagnosis, worst mean deviation on visual field tests and required trabeculectomy more often than individuals who were *CYP1B1* negative. However, the contribution of *CYP1B1* heterozygous variants is complicated by the absence of glaucoma phenotype in carrier parents of a child with PCG and *CYP1B1* variants. Although gene modifiers might explain the phenotypic variability encountered by individuals carrying the same *CYP1B1* variants, *CYP1B1* could also act as a modifier gene or as part of a digenic inheritance in JOAG.

My findings established a significant contribution of *CYP1B1* homozygous or compound heterozygous deleterious variants among JOAG cases diagnosed before 25 years (11%) [404]. Genetic counselling for *CYP1B1* is complicated by the incomplete penetrance observed in some families with unaffected individuals at the age of 50 years [70,220] and the occurrence of both PCG and JOAG among siblings carrying the same *CYP1B1* variants [302,303]. The findings from my research and the current knowledge suggest that *CYP1B1* variants are associated with a variable expressivity, causing PCG in the majority of cases, sometimes associated with a later age of onset of JOAG/POAG, and rarely associated with an absence of symptoms. The occurrence of different phenotypes associated with *CYP1B1* variants need to be discussed with patients in genetic counselling.

5.1.2.4. *FOXC1* & *PITX2*

FOXC1 and *PITX2* variants are associated with Axenfeld-Rieger syndrome and a 35-75% risk of developing glaucoma [289,290,342]. However, no study has been properly designed to assess the prevalence of glaucoma among carriers and there is a gap in knowledge regarding the possible age of glaucoma onset associated with both genes. In the paper included in chapter 3-4 of this thesis, I reported on the glaucoma prevalence among *FOXC1* and *PITX2* carriers [405]. All family members who tested positive for these two genes were included to minimise the risk of recruitment bias. The prevalence of glaucoma was 58.5% and was not different between *FOXC1* and *PITX2* carriers. Interestingly, the age at diagnosis was significantly younger for *FOXC1* carriers than for *PITX2* carriers. The findings from my research might explain the discrepancies between previous studies reporting different rates for both genes with higher glaucoma prevalence for *FOXC1* compared to *PITX2* variants. Studies including a majority of *PITX2* carriers or young unaffected will likely underestimate the prevalence of glaucoma whereas studies comprising a majority of *FOXC1* carriers are more likely to report higher glaucoma rates, especially if it is a young cohort. The different age of glaucoma onset associated with both genes should be addressed in genetic counselling and taken into consideration for patient management and follow-up. Although both

FOXC1 and *PITX2* carriers need a lifetime glaucoma monitoring, the translational outcomes of my research have direct impact on patients' healthcare, indicating that *FOXC1* carriers would benefit from closer glaucoma monitoring during childhood.

Interestingly, in this study I reported variants in *FOXC1* or *PITX2* in *s*ome individuals initially diagnosed with PCG or POAG [405] and I subsequently reported *FOXC1* variants in additional patients with PCG (manuscript under preparation). These patients had either no ocular features characteristic of ARS or very mild signs upon re-examination. These findings suggest that *FOXC1* and *PITX2* might contribute to the architecture of PCG or POAG. Isolated reports of individuals with *FOXC1* or *PITX2* variants not displaying the classic ocular features of ARS have been reported before [290,406,407]. The variable expressivity of these two genes means that the full ocular spectrum of symptoms is not always obvious and can make clinical diagnosis of ASD challenging. *FOXC1* and *PITX2* often have associated systemic features and a targeted medical history as well as a family history assessment of ARS symptoms can prompt clinicians to test for ARS associated genes. These findings have important implications for the patient and its family and for the provision of genetic counselling since a refined molecular diagnosis of ARS will affect patient management and monitoring of associated symptoms.

5.1.3. Identification of novel glaucoma genes

5.1.3.1. TMEM98

In the paper by Awadalla et al. included in chapter 3-1 of this thesis, we identified a novel gene (*Transmembrane Protein 98* (*TMEM98)*, 17q11.2, MIM 615949) in a five generation Australian family from British background with autosomal dominant nanophthalmos [408]. The *TMEM98* missense variant, p.(Ala193Pro), segregated in 16 affected members and none of the 19 unaffected relatives, 285 controls or population reference databases (dbSNP and EVS). In-silico analysis predicted the variant to be deleterious, and the residue was highly conserved among species. *TMEM98* is the first gene linked to autosomal dominant nanophthalmos and could correspond to the NNO4 locus (MIM 615972) that was previously linked to a similar interval on chromosome 17 in a Chinese family with autosomal dominant microphthalmia [275]. A subsequent study reported additional *TMEM98* variants segregating in two families of German and Micronesian heritage [80]. The identification of *TMEM98* variants in different families with nanophthalmos from different ethnic background provides strong evidence for an association of this gene with nanophthalmos and improves confidence in our findings. Confirmation of our results has important implications for families with nanophthalmos: It allowed the development of genetic testing for the first gene linked to autosomal dominant nanophthalmos, and allows families or even sporadic cases to benefit from the testing and genetic counselling.

TMEM98 encodes two different isoforms differing only by the 5' untranslated region. The protein is expressed in ocular tissues such as the sclera, choroid, retinal pigment epithelium and iris that may be implicated in the pathogenesis of nanophthalmos. Yu et al. described disrupted collagen fibers and coil-like amorphic materials in the sclera of affected individuals which was three times thicker than normal [409]. The function of the protein encoded by *TMEM98* is unknown but Khorram et al. suggested an involvement in sclera thickness and secondary glaucoma based on its expression in the sclera and tissues of the outflow pathway [80]. In our study, 37.5% of individuals with *TMEM98* variants developed secondary ACG. Additional studies investigating the pathophysiology associated with *TMEM98* variants can assist in understanding their involvement in glaucoma and developing better therapeutic options or novel targeted therapies for affected and at-risk individuals.

5.1.3.2. TEK

In the paper by Souma et al. included in chapter 3-2 of this thesis, we identified 10 heterozygous novel/rare variants considered deleterious in the *Tyrosine Kinase, Endothelial* gene (*TEK*, 9p21.2, MIM 600221) among 189 unrelated PCG cases (5.3%) [410]. Among them, a deletion of 56kb removing exons 2-4 of the gene was identified, causing decreased protein solubility and protein aggregation. Three nonsense (p.(Glu150Ter), p.(Tyr307Ter), p.(Gly984Ter)) and one frameshift (p.(Lys745fs)) variants were expected to result in a truncated protein or mRNA degradation by nonsense-mediated decay. Two splice site variants (c.(760+2T>C), c.(3300+2delT)) were expected to affect splicing and to result in functionally null proteins. Three missense variants were considered deleterious: p.(Cys233Tyr) caused misfolding of the protein and enhanced proteasomal degradation, p(Lys294Asn) impaired the phosphorylation of key tyrosine residue and p.(Tyr611Cys) altered the protein localisation and impaired ligand responsiveness. The *TEK* variants were transmitted in an autosomal dominant manner. The variant was transmitted from an affected parent to affected children in two families. However, in six families, the carrier parent did not have glaucoma. The glaucoma was unilateral in 6/12 (50.0%) of carriers compared to 34/209 (16.3%) of the whole cohort. The unilateral involvement and the lack of symptoms that we observed among some carriers suggest variable expressivity and/or reduced penetrance.

Variants in *TEK* have also been involved in hereditary [411] and sporadic [412] venous malformations. The variants identified led to ligand-independent hyperphosphorylation, suggesting a gain-of-function mechanism [413]. The localisation of the loss-of-function variants associated with PCG differs from that of the gain-of-function variants linked to venous malformations: the former are located in the ectodomain whereas the latter are located in the intracellular domain [410]. These findings have implications for genetic counselling as it means that patients with loss-of-function variants in *TEK* are not at increased risk of venous malformations.

TEK encodes the TEK receptor tyrosine kinase involved in angiogenesis. Its ligands are the angiogenic growth factors angiopoietin 1 and 2 (encoded by *ANGPT1* and *ANGPT2*). TEK is expressed almost exclusively in endothelial cells and in the eye it is highly expressed in the Schlemm's canal endothelium [414]. The angiopoietin-TEK signalling is essential for the development of the Schlemm's canal in mice. Eyes of *Tek*-hemizygous mice had hypoplastic Schlemm's canal and trabecular meshwork [410] and a 25% increase in IOP. Deletion of *TEK* or both angiopoietin ligands is lethal in mice, but deletion after day 16.5 resulted in extremely increased IOP, buphthalmos, and rapid and complete loss of retinal ganglion cells [415]. Our findings suggest that *TEK* gene dosage is essential for the proper development of the aqueous humour outflow pathway and disruption can result in PCG. However, the presence of unaffected carriers also suggests that *TEK* variants are not sufficient to cause glaucoma. It is possible that TEK is tightly regulated during development and that the genetic variants might affect the development of the eye differently in different individuals carrying the same variant. Additionally, it is possible that additional *TEK* variants not detected by sequencing (in the promoter, introns or enhancers for example) or variants in other genes interacting with *TEK* are present in these families and are involved in the phenotype. The clinical utility of predictive or prenatal genetic testing for *TEK* is currently limited by our incomplete comprehension of the association between the genetic variants and the phenotype. Elucidating how *TEK* variants can lead to PCG will be important to translate these research outcomes into accurate genetic counselling.

5.1.4. Overlapping phenotypes and genetic heterogeneity

Through the publications included in this thesis, I showed that together, *MYOC* and *CYP1B1* deleterious variants explain a third of individuals with severe JOAG diagnosed before 25 years old [375,404]. *FOXC1* and *PITX2* defects are associated with JOAG in a minority of cases [405]. These four genes are also implicated in POAG but to a lesser extent, and in addition to *TBK1* and *OPTN*, these genes together account for approximately 10% of advanced POAG. A Mendelian inheritance is often encountered in glaucoma patients with an early age of onset such as JOAG, but POAG displays the characteristics of a multifactorial disease with both Mendelian inheritance and common genetic variants of lesser effect implicated. The characterisation and discovery of genes involved in JOAG and POAG has direct translational research outcomes in terms of clinical diagnosis and genetic testing.

The molecular aetiology of PCG is still mainly uncharacterised although a number of genes have now been implicated. Deleterious variants in *CYP1B1* are the most common cause of PCG at present, explaining around 20% of cases in Caucasian populations [299]. Similarly, deleterious variants in *TEK* account for 5% of individuals diagnosed with PCG (chapter 3-2) [410] and deleterious variants in *FOXC1* might explain 5% of cases with congenital glaucoma with or without features of

ARS (manuscript under preparation). In the ANZRAG, additional genes such as *LTBP2*, *CHRDL1* (MIM 300350, X-linked megalocornea), *SLC4A11* (MIM 610206, Congenital hereditary endothelial dystrophy 2 or CHED2) and *COL18A1* (MIM 120328, Knobloch syndrome) have each been identified in a few cases (unpublished results). These genes are associated with other conditions that can cause congenital glaucoma. They might represent cases of PCG that were misdiagnosed or mimicked PCG because they presented with no or mild systemic features of the associated condition. It is becoming clear that PCG is a heterogeneous group of diseases with multiple molecular causes and that the glaucoma might not always be primary as initially thought. Molecular diagnosis in PCG can greatly assist genetic counselling by refining the clinical diagnosis, identifying the mode of transmission and allowing for reproductive options.

The publications included in this thesis emphasise the phenotypic heterogeneity of the different genes and the genetic heterogeneity of each type of glaucoma. The phenotypic overlap of the different glaucoma associated genes emphasise the difficulty to maintain clear clinical diagnosis boundaries and complicate genetic testing for glaucoma. A better understanding of the different genes involved in each type of glaucoma and their associated phenotypes allows appropriate genetic counselling with regards to the natural history of the disease and the potential mode of inheritance.

5.2. Counselling families about the mode of inheritance

5.2.1. Counselling in the context of genetic results

Variants in genes that have a Mendelian inheritance usually follow a particular mode of transmission. However, different genetic mechanisms can lead to unusual transmission of these variants. In the publications from chapter 2-3, I presented two families in which additional testing of family members allowed to properly identify at-risk individuals in families and counsel individuals about their risk of developing the condition.

MYOC variants are transmitted in an autosomal dominant manner. The majority of *MYOC* carriers report a positive family history of glaucoma with one parent affected. However, findings from my research suggested that 20% of the carriers did not have affected relatives [375]. Although this could be due to individuals being undiagnosed, deceased before developing symptoms, or probands not being aware of the diagnosis of their relative, *de novo* occurrence is another potential explanation. In the paper included in chapter 2-3, I reported the second occurrence of a molecular proven *de novo* occurrence of *MYOC* variant in an individual with JOAG [385]. The occurrence of *de novo* *MYOC* variants is currently unknown because parents of carriers are often not available for genetic testing. This study showed that this mechanism does exist and that *MYOC* testing should not be

excluded in sporadic cases with JOAG or POAG. A molecular diagnosis of *MYOC* is even more important in an individual with no family history as it can prompt genetic testing of other at-risk individuals who would not have been tested otherwise.

MYOC double mutation carriers have been identified in a few families, as reported in the paper by Young et al. in chapter 2-3 [389]. For these individuals, the counselling regarding at-risk family members is different, and the implications for the children are significant. In a family with a single *MYOC* variant, individuals are counselled that the risk of recurrence for children is 50% and that the variant might be inherited from one parent, putting one side of the family at increased risk of glaucoma. The presence of two *MYOC* variants on different alleles in an individual means that all their children, and potentially both parents, carry one variant and are at increased risk of developing glaucoma. If both parents carry deleterious variants, their first-degree relatives also become at risk of having the familial variant and of developing glaucoma. In that regard, segregation studies are important to properly counsel family members regarding their risk of developing the disease.

5.2.2. Novel association of genes with glaucoma subtypes

Counselling patients about the risk of occurrence or recurrence is often complicated for individuals with sporadic condition. Previous studies on retinitis pigmentosa and congenital cataracts showed that sporadic cases can represent undiagnosed autosomal dominant or X-linked forms of the condition [416,417]. In glaucoma, JOAG is usually considered an autosomal dominant disorder based on the numerous pedigrees that have been reported with several generations affected, whereas PCG is most often considered a sporadic or an autosomal recessive disorder because of the lack of affected relatives or the presence of the disease in siblings only. Additionally, the first genes discovered (and the main genes associated with each disease so far) are transmitted in an autosomal dominant manner for JOAG (*MYOC*) and an autosomal recessive manner for PCG (*CYP1B1*). However, these associations are being challenged as new knowledge arises and new genes are discovered.

In the case of individuals with severe JOAG diagnosed before the age of 25, I recently showed that, although 20% of cases are explained by deleterious variants in *MYOC* transmitted in an autosomal dominant manner, up to 10% can be explained by biallelic deleterious variants in *CYP1B1* transmitted in an autosomal recessive manner [404]. This is supported by previous reports of families in which sib pairs carrying the same two *CYP1B1* variants presented with PCG in one and JOAG or POAG in the other [302,303]. Patients with JOAG who carry biallelic *CYP1B1* variants often have no family history of the disease, which supports an autosomal recessive inheritance. My research highlights the importance of considering *CYP1B1* when conducting genetic testing for

individuals with JOAG, especially in the absence of a family history of glaucoma. Unlike *MYOC*, the presence of biallelic *CYP1B1* variants in an individual is not associated with a 50% risk of inheriting the variants for first-degree relatives: siblings are at 25% risk of carrying the familial variants, offspring and parents are unlikely to carry two variants and develop the disease. The identification of *CYP1B1* variants in an individual with JOAG has critical implications for genetic counselling and genetic testing of family members.

Similarly, my findings indicate that PCG is not always an autosomal recessive disease, and that deleterious variants transmitted in an autosomal dominant manner may account for more cases than previously recognised. In chapter 3-2, we showed that *TEK* may account for up to 5% of PCG cases [410]. Interestingly, *TEK* is a novel gene associated with PCG and the presence of unaffected carriers may suggest a more complex picture such as incomplete penetrance, or a digenic inheritance. Similarly, in chapter 3-4, my results suggest an association between *FOXC1* variants and PCG [405]. I have further evidence indicating that *FOXC1* may explain up to 5% of PCG cases (manuscript under preparation). *FOXC1* variants are transmitted in an autosomal dominant manner. Families with *FOXC1* or *TEK* variants need to be counselled about the risk not only for siblings but for all first-degree relatives, including children and parents (especially in the case of *FOXC1* where variants are associated with a lifetime risk of developing glaucoma). These genes should not be ruled out in the absence of affected relatives. *FOXC1* variants can occur *de novo* in some individuals [347,406] who often have no family history of the condition. Unlike *CYP1B1*, the offspring of these individuals have a 50% risk of inheriting the genetic variant. The identification of autosomal dominant deleterious variants in PCG cases alters recurrence risks and reproductive options for the families and the provision of genetic counselling for PCG.

These novel genes or associations of genes with glaucoma subtypes allow for improved diagnosis accuracy and can alter clinical management. Equally important is the fact that patients and their families can benefit from genetic counselling about the mode of inheritance, risk assessment for family members or future children, and discussions about screening options such as predictive genetic testing for at-risk relatives or reproductive options such as prenatal and preimplantation diagnosis in the case of severe and devastating conditions.

5.2.3. Genetic counselling in the absence of molecular diagnosis

Patients can still benefit from genetic counselling in the absence of an identified genetic cause. The analysis of the family history can assist in determining if the condition is more likely transmitted in an autosomal dominant or recessive manner, although self-reporting medical history should be incorporated with caution and the possible occurrence of phenocopies in glaucoma needs to be considered. New knowledge on the different mode of inheritance associated with each type of

glaucoma need to be integrated in genetic counselling. The work included in this thesis emphasises the importance of discussing the different scenarios, including the possibility of autosomal dominant and autosomal recessive transmissions with different risk estimates for family members in both individuals with PCG or JOAG of unexplained diagnosis. Additionally, empirical risks can provide useful information to families. Previous figures have shown a risk nine times higher for first-degree relatives of individuals with POAG [137]. This prevalence did not consider the proportion of glaucoma explained by Mendelian genes. Further studies are needed to examine the risk to first-degree relatives once monogenic forms of glaucoma have been ruled out through genetic testing. This is currently being achieved with the Targeting At-Risk Relatives of Glaucoma patients for Early diagnosis and Treatment study that excluded patients with variants in known glaucoma genes. Our pilot data identified a 55% risk of developing glaucoma among first-degree relatives of 100 patients with severe glaucoma (manuscript under preparation). A second phase involving 1200 patients in currently underway and will provide additional information for family member of individuals with advanced glaucoma. Genetic counsellors can assist patients in evaluating who among their relatives might have higher risks of developing glaucoma and how prevention can be implemented for these relatives through appropriate regular eye screening.

5.3. Identifying at-risk individuals

5.3.1. Predictive genetic testing

The National Health and Medical Research Council recommendations support a glaucoma screening approach that targets individuals at higher risk of developing glaucoma rather than the general population [418]. First-degree relatives of individuals diagnosed with glaucoma are considered at high risk of developing glaucoma [137]. It is recommended that they receive regular eye health check 5-10 years earlier than the age of onset of glaucoma in their affected relatives and that they receive ongoing monitoring for the development of glaucoma [418]. However, this approach does not specifically target at-risk individuals based on their genotype, and at-risk individuals without a family history of glaucoma or unaware of their family history will not benefit from this screening program.

The provision of a molecular diagnosis allows family members to access genetic testing and risk prediction for the development of glaucoma. Early detection can help preserve visual acuity and quality of life in at-risk individuals. Cascade genetic testing is an efficient way to identify at-risk individuals and implement adequate glaucoma monitoring and treatment. Predictive genetic testing can determine whether or not the deleterious variant responsible for the condition in the family has been inherited and determine an individual's risk of developing glaucoma. This type of testing has important implications that need to be addressed with patients. Standard of practice for predictive

genetic testing includes pre-test genetic counselling, informed consent, genetic testing conducted in an accredited laboratory facility and post-test genetic counselling [419]. Additionally, the counselling needs to address the risk and benefits that apply to each type of glaucoma. Through the ANZRAG, I have developed genetic testing protocols for known glaucoma genes in collaboration with the NATA accredited laboratory of SA Pathology which allow the feedback of results to research participants [420].

Deleterious variants in *MYOC* and *OPTN* and *TBK1* CNVs are transmitted in an autosomal dominant manner which means that first-degree relatives of a carrier are at 50% risk of having the same variant and are at high risk of developing POAG. The relevance of genetic testing is emphasised by the fact that the onset of the disease is often earlier in these families than in other glaucoma patients. The identification of a deleterious variant in an asymptomatic individual does not predict the age of onset or the progression of the disease but significantly increases the risk of developing glaucoma in the person's lifetime and should prompt close glaucoma monitoring. In comparison, the absence of a familial deleterious variant indicates that the risk of developing glaucoma is potentially similar to the general population risk (providing that no other close relatives have glaucoma due to unexplained genetic causes). Those individuals do not need to be monitored closely and can follow the standard recommendations for the population. Healey et al. previously demonstrated the acceptability of predictive genetic testing for *MYOC* in an Australian family [376]. However, there is a gap in knowledge regarding the clinical utility of predictive genetic testing for glaucoma. Based on data from the ANZRAG included in chapter 2-6, I confirmed that cascade predictive genetic testing was effective in identifying *MYOC* carriers before they exhibit signs of glaucoma [421]. Individuals who had an eye exam following their genetic results were younger at presentation, and for the majority asymptomatic, compared with individuals who had their eyes checked following a referral to an ophthalmologist. The latter had early signs of glaucoma in half of the cases or had already developed glaucoma in the other half. All individuals undergoing predictive genetic testing have a family history, which should have prompted them to have regular eye examinations for glaucoma. However, my data suggest that this is often not the case and that genetic testing for glaucoma is a better strategy to identify those individuals at high risk of developing glaucoma before the onset of the disease. *MYOC* variants are associated with high IOPs and IOP lowering therapies are effective in reducing IOP in *MYOC* carriers [421]. However, long-term longitudinal studies will be needed to evaluate the effectiveness of regular glaucoma treatments in preventing or decreasing glaucoma progression and confirm clinical utility of genetic testing.

In the study included in chapter 4-1, I investigated the motivations, feelings and concerns of individuals undergoing predictive genetic testing for *MYOC* to address the gap in knowledge in that

matter [422]. The main motivations for being tested included the availability of preventative measures and the removal of uncertainty. Previous studies have shown that perceived health benefits (detection, prevention, control) and perceived emotional benefits (reassurance, reduction of uncertainty and emotional preparation) were strong motivators for predictive testing [377]. The majority of people who opted to get tested had a high perceived risk of carrying the familial variant and all were satisfied with their choice of being tested. Individuals who choose predictive genetic testing may represent a selected group of people more likely to be able to cope with the results. It would be interesting to investigate the perceived risks and concerns from individuals who are not interested in being tested or who declined testing. However, because of their decision not to get tested, these individuals are usually not accessible for such studies. Individuals who underwent testing still expressed a range of concerns after genetic testing feedback. The findings from this study provided important insights into patients' experience of predictive genetic testing and can greatly assist genetic counsellors in addressing them during counselling to support patients who have been tested or who are considering being tested.

Predictive genetic testing in minors and children is usually deferred until the individual is old enough to make an informed decision about being tested unless there are immediate and direct medical benefits for the child that can alter the course of the disease [423]. In the majority of cases, the onset of *MYOC* glaucoma is during adulthood but some deleterious variants are associated with an onset during childhood. In the paper included in chapter 4-2, I investigated the benefits of predictive genetic testing for *MYOC* in minors in families with *MYOC* variants associated with a glaucoma onset before the age of 18 years [174]. The findings from three families carrying *MYOC* variants associated with childhood glaucoma onset indicated that the decision to test children was influenced by the age of glaucoma onset and the severity within the family as well as the age of the child. The timing of testing can be addressed in genetic counselling with the families. Whenever possible the child should be included in the discussion. Further research will be needed to evaluate the potential psychosocial impact of glaucoma genetic testing in minors but the findings from my work suggest that predictive genetic testing in minors for *MYOC* glaucoma can be discussed with parents in genetic counselling.

Unlike the adult-onset POAG genes mentioned above (*MYOC*, *OPTN* and *TBK1*), other genes such as *CYP1B1*, *FOXC1* and *PITX2* are associated with different types of glaucoma as shown in some of the publications included in this thesis, which complicates genetic counselling and risk predictions of glaucoma for family members. Biallelic *CYP1B1* variants usually lead to PCG but incomplete penetrance is common, and variants can be associated with JOAG or POAG. In the paper included in chapter 2-5, I demonstrated that biallelic *CYP1B1* variants were involved in advanced JOAG cases and could explain up to 10% of the cases diagnosed before the age of 25

years [404]. Because the condition is transmitted in an autosomal recessive manner and because of the lack of common deleterious variants, genotype/phenotype correlations are difficult to establish. Considering that late glaucoma onset is possible, predictive genetic testing for all siblings of an individual with *CYP1B1* variants need to be discussed in genetic counselling, regardless of the siblings' age. First-degree relatives of an affected individual can also benefit from knowing their carrier status. Partners of *CYP1B1* carriers can then be offered carrier testing and couples carrying *CYP1B1* variants can be counselled about reproductive risks and options such as prenatal diagnosis and preimplantation diagnosis.

Similarly, in chapter 3-4 I have shown that deleterious variants in *FOXC1* and *PITX2* not only result in ARS with variable expressivity but that they also contributed to the phenotype in some individuals diagnosed with POAG or PCG [405]. This illustrates how molecular diagnosis can help refine the clinical diagnosis and provide new information about the mode of inheritance and the natural history of the condition. Because these two genes are transmitted in an autosomal dominant manner, first-degree relatives of *FOXC1* and *PITX2* carriers can benefit from genetic counselling and predictive genetic testing. Additionally, both genes can also be associated with systemic features or complications that might require additional screening and management such as hearing loss or heart defects. Cerebral small-vessel disease has also been reported in carriers of variants in these two genes [343] and additional studies are needed to understand the clinical significance of this finding. As a result, a molecular diagnosis of ARS has substantial implications for patients as it allows appropriate management and interventions for associated symptoms and access to genetic testing and counselling among at-risk family members.

In the paper included in chapter 3-1, I contributed to the identification of a novel nanophthalmos gene, *TMEM98*, which was the first gene identified for autosomal dominant nanophthalmos [408]. Together with *MFRP* and *PRSS56*, these genes are associated with early onset secondary glaucoma due to the presence of developmental ocular defects. The glaucoma management can be complicated in patients with nanophthalmos and the identification of variants in these genes allows for the testing of at-risk family members and the implementation of glaucoma monitoring and treatment strategies targeted to the condition.

It is important to recognise that the interpretation of genetic results from predictive genetic testing can be limited in situations where the inheritance and penetrance of a gene are not fully understood. For example, in the paper included in chapter 3-2, I contributed to the identification of variants in the *TEK* gene associated with PCG and transmitted in an autosomal dominant manner [410]. However, the mechanisms by which *TEK* variants lead to glaucoma are still unknown. Unaffected adult carriers were identified and the penetrance of the gene variants has not been

characterised yet. Considering that variants in the *CYP1B1* and the *FOXC1* genes have been associated with a spectrum of glaucoma from PCG to POAG, it is not impossible that *TEK* variants could be associated with a later onset of glaucoma. Additional studies are needed on the penetrance, expressivity and function of the gene in order to provide adequate genetic counselling to families. In the meantime, genetic testing for family members of individuals with *TEK* variants might be of limited benefit.

In summary, the discovery of new genes or new associations of genes to different types of glaucoma allows family members to benefit from optimised genetic counselling, genetic testing to predict their risk of developing glaucoma and potentially novel targeted therapies. Early diagnosis is the key, especially in the context of proven effective treatment strategies and the fact that late diagnosis leads to poorer visual outcome which can significantly affect the quality of life of patients. Unaffected at-risk relatives can benefit from predictive genetic testing through increased surveillance and early interventions to prevent or minimise irreversible vision loss. Alternatively, individuals who do not carry the deleterious variant(s) can avoid unnecessary surveillance, which often includes examination of babies and young children under anaesthesia. The identification of genes associated with childhood-onset and developmental glaucoma that are often associated with poorer visual outcome allows families to benefit from prenatal diagnosis and preimplantation diagnosis or early postnatal diagnosis. Finally, the identification of the genes and the characterisation of the molecular mechanisms leading to glaucoma enable the development of novel therapeutic approaches.

5.3.2. Precision medicine

At present, all glaucoma treatments are symptomatic and target a reduction of IOP, whether medically or surgically. Although standards of care are based on the best current evidence, this one-size-fits-all approach is not effective for all patients. There is a need for more targeted and effective treatments to prevent irreversible glaucoma blindness. Precision medicine is an innovative approach that tailors prevention and treatment to the specific characteristics of an individual. A number of recent advances and discoveries raise the possibility of effective individual-centered preventative treatment and therapies to restore vision in glaucoma.

Functional characterisation of different gene defects has already resulted in the development of therapeutic strategies aimed at correcting the molecular defect or the resulting impact on the phenotype. For example, *MYOC* glaucoma is a protein-misfolding disease [179]. This prompted some researchers to investigate whether chemical chaperones could correct MYOC misfolding. Zode et al showed that topical administration of sodium 4-phenylbutarate PBA (a chemical chaperone known to reduce protein mislocalisation and endoplasmic reticulum stress) in a transgenic *MYOC*

mouse model reduced elevated IOP, improved the secretion of MYOC in the aqueous humor, and reduced MYOC accumulation and endoplasmic reticulum stress in the trabecular meshwork [424]. Jia et al. reported improved solubility of *MYOC* mutants in transfected human trabecular meshwork cells treated with a chemical chaperone, trimethylamine N-oxide (TMAO) [425]. Similarly, an inhibitor of TBK1 (BX795) has been shown to improve the insolubility of the *OPTN* p.Glu50Lys variant in vitro [197]. Additionally, therapeutic strategies targeting different functional groups of deleterious variants are being investigated for other conditions such as cystic fibrosis and Duchenne muscular dystrophy. Drugs that promote the read-through of premature stop codons to restore protein synthesis [426,427] or that promote exon skipping to rescue protein expression [427] are currently in development or in clinical trial for both conditions. These strategies could be applied to other diseases with similar molecular defects. Clinical trials involving humans may be implemented in the near future to test the safety and efficacy of these drugs.

iPSCs have the ability to differentiate into any cell type of the body and to provide an unlimited supply of relevant cells that are otherwise largely inaccessible [428]. Most structures of the eye cannot be sampled from living individuals, which makes it challenging to study their involvement in eye diseases. With iPSC technology, fibroblasts from skin biopsy can be returned to a pluripotent state and transformed into trabecular meshwork or retinal ganglion cells [429,430]. iPSCs represent a unique opportunity to study the mechanisms of cellular damage in glaucoma [183,199], an important tool for experimental drug screening and discovery [431,432], and a potential approach to patient-centered treatments [428,433]. Additionally, patient-specific iPSCs can help investigate newly-discovered disease-causing genes or novel genetic variants of unknown pathogenicity [431,434,435]. iPSCs have already been developed for tissues implicated in glaucoma such as the trabecular meshwork [436] and retinal ganglion cells [199,437], and for different glaucoma models including *MYOC* variants [183], *TBK1* duplications [199] and *OPTN* variant p.Glu50Lys associated with POAG [197]. Patient-induced iPSCs can be transplanted to replace damaged tissues and restore cellular function. In a recent paper, Zhu et al. successfully injected iPSCs-derived trabecular meshwork cells in a transgenic *MYOC* mouse model of glaucoma and demonstrated significantly reduced IOP and improved aqueous humour outflow facility up to 9 week after treatment [183]. Their findings also showed a proliferative response of endogenous trabecular meshwork cells indicating that regeneration of the trabecular meshwork after the onset of glaucoma is possible [183].

The recent development of gene editing technology allows researchers to target and correct specific genetic defects. Gene editing can be tailored to each individual genetic defect and is therefore an attractive therapeutic approach for conditions with strong genetic heterogeneity such as glaucoma. The combination of iPSC with gene editing opens the way to create patient-specific genetically corrected iPSCs to replace deficient or damaged cells. The ability to use the patient's

own cells eliminates the risk of rejection and the need for immunosuppression therapy. There is potential for both *in vitro* and *in vivo* approaches: Patient-specific iPSCs could be differentiated and gene-corrected *in vitro* before being transplanted for functional restoration of defective cells, or direct *in vivo* editing of somatic cells could be designed [181]. The eye is an ideal target for *in vivo* gene editing because of its compartmentation, its ease of access, and its immune privilege [181]. Clinical applications for humans are not ready yet but studies using animal models are showing promising results. Hung et al recently demonstrated the efficacy of retinal gene modification *in vivo* in mice [438]. Another recent study showed that *in vivo* gene editing of the retina could prevent retinal degeneration and improve visual outcome in a rat model of retinitis pigmentosa [439]. These studies present promising early results for gene therapy in ophthalmology.

In summary, the elucidation of the pathophysiology of glaucoma and the functional characterisation of gene defects is leading to the development of innovative therapeutic options, whether they aim to replace damaged cells and tissues or to correct the causative molecular defect or a particular class of molecular defect. Clinical trials targeted to the molecular diagnosis of individuals are likely to become available in the near future. Disease registries such as the ANZRAG have in important role to play in future therapies approaches through their ability to facilitate patients' recruitment in appropriate clinical trials. The emergence of precision medicine in glaucoma emphasises the need for better genetic testing strategies and higher detection rates of causative genetic defects to identify individuals carrying gene variants who could benefit from future clinical trials.

5.4. The future of genetic testing for glaucoma

Genetic testing for glaucoma is still largely based on the evaluation of the phenotype of the patient and the family history. It involves a detailed review of the symptoms and the mode of inheritance to determine the most likely causative gene involved. Although genetic heterogeneity exists for all types of glaucoma, some genes account for a larger proportion of cases than others. As such, genes can be prioritised for molecular testing purposes. For example, as part of the ANZRAG, *CYP1B1* is initially sequenced in a patient with PCG because *CYP1B1* variants are identified in 20% of Caucasian cases and an even higher proportion of some non-Caucasian populations. A patient presenting with features consistent with ARM, or with glaucoma and systemic features of ARS, or with glaucoma and a family history of ARS, is initially tested for *FOXC1* and *PITX2* variants. A patient with POAG and HTG is preferentially sequenced for *MYOC*, whereas *OPTN* and *TBK1* are tested first in the case of NTG. Moreover, novel identified genes can be added to the list of genes tested. However, clinical diagnosis is often unable to differentiate patients with molecular causes in different genes leading to similar phenotypes. Systemic features that could assist clinical diagnosis are not always present at birth in children with congenital glaucoma (ex: hearing loss,

teeth anomalies). Additionally, the genetic heterogeneity of glaucoma and the overlap in the phenotypic spectrum associated with most genes can even make molecular diagnosis challenging and genetic testing can become quickly time-consuming and uneconomical. The complexity of the genetics of glaucoma calls for more efficient genetic screening approaches.

WGS and WES allow for rapid sequencing and parallel testing of numerous genes at once, which reduces greatly the cost of genetic testing. The ability to analyse multiple genes at once can achieve a higher detection rate at a quicker pace and reduce the costs of sequential genetic testing [440]. However, the transition of WGS/WES from research to clinical diagnosis needs to be supported by technical accuracy of variant calling. Accurate prediction of variants calling depends on a number of factors including the genomic region, the class of variant and the informatics tools used (capture method, sequencing platform, alignment, coverage and variant filtering and calling methods) [441]. Professional standards and guidelines have recently been developed by the American College of Medical Genetics and genomics [442], the Royal College of Pathologists of Australasia [378], the Canadian College of Medical Geneticists [443] and the European Society of Human Genetics [444] to assist testing laboratories implement WGS and WES in healthcare with adequate accreditation. Additionally, clinical data needs to guide the selection of genes screened because genetic results need to be interpreted in the medical and familial context of the patient. The ability of WES to establish molecular diagnosis has already been proven for a number of genetically heterogeneous conditions including retinal dystrophies [445,446], congenital cataracts [417], intellectual disability [447,448], hereditary cancers [449] and cardiomyopathies [450]. The genetic heterogeneity and the phenotypic overlapping in glaucoma support a targeted analysis of genes using a WGS/WES approach as an effective strategy to confirm or redefine clinical diagnosis. Sequencing using WGS/WES can be targeted to the genes involved with the condition by either sequencing these genes only (gene testing panels) or by sequencing all known disease genes (clinical exome) and applying targeted bioinformatics analysis. Detection rates will improve with the discovery of new genes and these can quickly be added to the targeted gene testing panel. Targeted exome/genome sequencing is likely to quickly become a front line tool in molecular diagnosis of POAG and developmental glaucoma as a complement to clinical diagnosis to improve detection rates and genetic counselling provision.

Significant discoveries have been made in the identification of genetic risk factors associated with POAG. With its unique extreme phenotype cohort of glaucoma patients, the ANZRAG has led to several loci identification for POAG using GWAS [229,232,233,239,451-453]. I have been involved in three studies that led to publications included in the appendices of this thesis. In the study by Burdon et al., we identified two candidate genes (*TMCO1* and *CDKN2B-AS1*) in association with advanced POAG. Carriers of one or more risk alleles at these two loci had a threefold increase in risk [451]. In

the study by Gharahkhani et al., we reported three new risk loci for POAG (*ABCA1*, *AFAP1* and *GMDS*) [452]. Finally in the study by Springelkamp et al., we identified 18 loci for CDR, including 8 previously reported and 10 novel [453]. These 10 new loci together increased the risk of POAG by 2.5 times. The genes at or near these loci are implicated in different molecular pathways that play a role in POAG or can provide insight in the pathophysiology of POAG or its endophenotypes: *RPAP3* [454], *CHEK2* [455], *TMCO1* [456], *ABCA1* [457] and *CARD10* [458] are involved in cell cycle regulation and apoptosis, *ADAMTS8* [459] plays a role in the formation and turnover of the extracellular matrix, *DUSP1* [460] and *HSF2* [461] are involved in cellular stress response, *RERE* [462] regulates retinoid acid signalling, *PLCE1* [463] plays a role in lipid metabolism, *TMTC2* [464] plays a role in calcium homeostasis, *SALL1* [465], *ATOH7* [466] and *SIX6* [467] are involved in ocular development, *AFAP1* [468] is involved in actin cytoskeleton-modulating signals, and *CDKN2B-AS1* [469], *BMP2* and *CDC7/TGFBR3* belong to the transforming growth factor beta family involved in cell proliferation.

These findings raise the question as to whether - and how - genetic risk factors for glaucoma could be implemented in patients' risk assessment for glaucoma. GWAS aims at detecting common genetic variants in association to a disease. These genetic variants are usually present in both affected individuals and healthy individuals but they are more often found in cases than controls. They do not cause the disease on their own like Mendelian genes do but rather confer a susceptibility to the disease and are of modest effect. However, risk loci could be used to calculate a genetic risk score that would determine the cumulative genetic risk of an individual to develop glaucoma, progress or respond to some treatment. These predictive models are quite complex to develop because their performance is affected by several factors including phenotypic data, demographic data, environmental factors and genetic factors. Polygenic risk score models have been tested for age-related macular degeneration [470] and breast cancer [471]. The clinical utility of a polygenic risk score for glaucoma using common risk alleles is currently unknown but it could form the basis for detection individuals at risk of developing the condition. It is currently estimated that collectively the common variants explain around 10% of the overall familial relative risk, which is still much lower than in AMD, for which we showed that known loci explain almost 50% of the disease heritability [472]. Further studies are needed to characterise the effect of each gene/variant on the overall risk and determine their applicability in risk prediction models for glaucoma patients.

Additionally, common genetic risk variants could help further refine the risk associated with highly penetrant variants and variants of reduced penetrance. The common *MYOC* deleterious variant p.Gln368Ter is highly penetrant but is associated with a variable age of onset from 30's to 80's [173] that needs to be taken into account in patients' monitoring. Variants in novel PCG gene *TEK* are associated with incomplete or even low penetrance [410], complicating the provision of genetic counselling and risk of recurrence predictions. The identification and the potential implementation

in genetic testing of gene modifiers for glaucoma could have important implications for the clinical management and genetic counselling of patients. Genetic modifiers have already been identified that alter the age of onset of neurological symptoms in Huntington's disease [473], the severity of lung disease in cystic fibrosis [474] or the cardiac complications [475] and loss of ambulation onset [476] in Duchenne muscular dystrophy. In *BRCA*-associated hereditary cancers, gene modifiers incurred large differences in the absolute risk of developing breast cancer among *BRCA1* carriers: the lifetime risk was 28-50% for the 5% at lower risk compared to 81-100% for the 5% at higher risk when including the known genetic risk factors [477]. More accurate predictions based on glaucoma genetic modifiers could alter the timing of management and interventions to improve the visual outcome and could improve risk predictions in families and genetic counselling provision.

5.5. Implications of new testing technologies

The field of genetics is constantly evolving with the implementation of new technologies to improve gene identification and genetic testing. For example, the new WGS and WES technologies have some major advantage over traditional Sanger-based sequencing because of their ability to screen for several genes at once, replacing serial genetic testing strategies that can be both expensive and time consuming. For patients with no causative variants identified in known genes through WGS/WES, the search can be extended to other genes/exons, especially in families with cooperative individuals or additional affected family members available for segregation studies. However, WGS and WES also have some limitations and can result in important ethical implications for patients. The majority of variants in the genome are still largely uncharacterised in terms of disease association and population prevalence which has implications for their interpretation and potential use in patients' healthcare. Additionally, the technologies have the ability to identify relevant information not always related to the indication of the test. These two issues were addressed in the publications included in chapters 4-3 and 4-4 and are further discussed below.

The penetrance of known deleterious variants in low-risk individuals and in the general population is not well characterised. In the paper by Gharahkhani et al included in chapter 4-3, we reported the accurate imputation of the p.Gln368Ter *MYOC* variant using high-coverage genotyping arrays and reference panels with a well characterised haplotype for the variant of interest [478]. The sensitivity was 100% and specificity 99.9% between imputation and direct sequencing. This study demonstrated the ability to detect rare clinically significant deleterious genetic variants with high accuracy using low-cost technology such as GWAS. In this study, we also identified 6 individuals with the *MYOC* p.Gln368Ter variant in a group of controls not screened for glaucoma. It is possible that these individuals already have signs of glaucoma but have not been diagnosed or that they

have not developed glaucoma yet because *MYOC* variants are associated with an age-related penetrance. These findings may have clinical implications for these individuals, as well as for the millions of individuals who had their genome screened using arrays via companies offering direct-to-consumer services such as 23andMe and who could have rare deleterious variants accurately imputed in the same manner. The *MYOC* p.Gln368Ter variant is associated with a strong penetrance of developing glaucoma increasing with age in family-based studies; the penetrance is 50% at 50 years old and close to 100% at 75 years old in affected patients and their families (www.myocilin.com). However, a recent study imputing the *MYOC* p.Gln368Ter variant found a much lower glaucoma penetrance of 20-33% at 70 years old in two population-based studies [479]. The difference in prevalence between case cohorts and population-based cohorts can be explained both by the presence of other genetic or environmental factors that might segregate in families with affected individuals and by a possible under-representation of affected individuals from "healthy" population-based cohorts.

As more unaffected individuals get tested, we are gaining a better understanding of the clinical significance and the penetrance of variants in individuals with or without a family history of the associated condition and we will be in a position to implement surveillance programs adequate for each individual. A recent study reported on adult individuals with variants associated with severe early-onset diseases and annotated as being fully penetrant who were unaffected [480]. These individuals have been referred to as "resilient individuals" [481]. Similar to *MYOC*, studies on *BRCA1/2* and the risk of developing breast cancer have shown a lower penetrance of deleterious variants in moderate-risk groups compared with high-risk groups [482]. Subsequently in 2014, the US Preventive Services Task Force recommended against routine *BRCA* genetic testing for women whose family history is not associated with an increased risk for deleterious variants in *BRCA1/2* because of the uncertainty that the harms might outweigh the benefits [483]. The evidence accumulated on *MYOC* suggests that individuals carrying deleterious variants are at risk of developing glaucoma. However, the degree of risk might be less than initially thought and caution is needed when interpreting variants and counselling healthy individuals. The interpretation of the pathogenicity of genomic variants should always be conducted in their biological context and in the medical and familial context of the individual. Additionally, the identification of resilient individuals and the analysis of their genomes might assist in the search for gene modifiers that are protective against developing the disease and provide insights into disease mechanisms that could lead to novel treatment options [481].

WES/WGS are promising technologies for gene discovery but they also come with ethical implications. The ability to screen the entire exome or genome of an individual yields the potential to identify genetic findings not related to the indication of the test. These findings are referred to as

secondary findings, unsolicited findings or incidental findings. They may or may not have clinical implications for the patient and they raise ethical considerations regarding their management and report. These considerations were the focus of the paper included in chapter 4-4 of this thesis [484]. In this paper, I discussed how the context in which incidental findings are identified (clinical or research) impacts on their management. In both contexts, the disclosure of findings that are of limited clinical utility or non actionable is usually not supported [485-487]. The international guidelines agree on the feedback of incidental findings to patients in a clinical context as long as they are associated with serious health problems and are actionable, and as long as the patient consented and received genetic counselling [378,443,488,489]. In a research setting, the return of incidental findings is strongly debated between different guidelines because research is not centered on the individual's preferences [490-493]. There is however an emerging viewpoint considering that researchers might have an ethical responsibility to return incidental findings of clinical utility [485-487]. The difficulty with this prospect lies in the fact that the confirmation of the incidental findings and the provision of genetic counselling necessitate resources and expertise not always available in research and would put a significant burden on the research enterprise. Collaboration between clinic and research could lessen the issue ensuring that the clinic has sufficient resources to deal with the volume of incidental findings resulting from research [494]. In the current context of a lack of clear guidance regarding the management and return of incidental findings, researchers should establish a plan for the return of incidental findings that takes into account the nature of their research, the consent and relationship with the participants and their resources. Stronger frameworks need to be developed to assist clinicians and researchers with the implications related to new technologies and to assist genetic counsellors involved with research participants.

These publications emphasise the potential ethical implications of new genetic technologies and the importance of addressing them in translational research. Genetic counsellors have been evolving in the constantly changing environment of genetics and keeping up with new scientific knowledge and technological developments. They are well suited for liaising between genetic research and its translation into patients' healthcare. Zierhut et al. discussed the value of genetic counsellors in translational research, both for the multidisciplinary research team and for research participants [495]. Genetic counsellors can assist research teams in addressing the ethical challenges of research projects, manage participants' recruitment and large data. Similarly, genetic counsellors have the skill set to review the risks, benefits, limitations and implications of new genetic testing technologies with research participants, obtain informed consent, explain complex genetic results, explore their impact on individuals and help them cope with the new information. In an introduction commentary to special issue on genetic counsellors development, Callanan rightly stated that "becoming a genetic counsellor is clearly not a fixed destination, but rather an iterative

and “non-linear” developmental journey" [496]. In the current landscape of the integration of genetics into healthcare, genetic counsellors will likely become an integral part to multidisciplinary teams in translational research.

5.6. Conclusions

Genetic counselling is becoming increasingly relevant to subspecialties such as ophthalmology but there is a gap in knowledge of the benefits of genetic testing and counselling for glaucoma. This thesis aimed at addressing how we can provide the best genetic counselling and genetic testing options to individuals with glaucoma and their families using the most up to date and valid information from translational research. Through the publications included in this thesis, my original contribution to knowledge covers the phenotypic delineation of glaucoma genes, genotype-phenotype correlations, novel genes identification, the understanding of patients’ experience of genetic testing and the ethical implications related to new testing technologies in glaucoma. These translational research outcomes can assist clinicians and health professionals in the provision of personalised genetic counselling for patients and their families in regard to the mode of inheritance, the natural history of the condition, the risk of recurrence for future children and the risk of occurrence for other family members.

The delineation of the phenotypes associated with genetic variants in glaucoma-associated genes and the identification of novel genes has multiple benefits for the patients and their families. The characterisation of the genetics of glaucoma provides access to predictive genetic testing for family members of individuals with variants in glaucoma-associated genes. Unaffected individuals at risk of developing glaucoma can benefit from close monitoring, early diagnosis and therapeutic options to prevent glaucoma blindness. Similarly, the identification of genetic risk factors contributes to elucidating the mechanism leading to glaucoma and could be integrated into polygenic risk scores. Recent published studies suggest that preventative or restorative therapies for glaucoma that may target patients’ genotypes as part of precision medicine may be developed in the near future. This emphasises the importance of improving detection rates and genetic testing technologies to identify at-risk individuals who would benefit from these patient-centered therapies.

The identification of novel genes or novel association of genes with developmental glaucoma allows accurate genetic counselling in regard to the mode of inheritance, the need to monitor for possible associated symptoms or complications, the risk of recurrence in families and potential access to reproductive options.

The emergence of new technologies such as WGS/WES has already great potential for gene identification and genetic testing. However, they raise important ethical concerns that need to be

discussed and guidelines need to be developed. The potential application and clinical utility of WES/WGS for targeted genetic testing for glaucoma, patients' experience with new testing technologies and the impact these will have on genetic counselling will need to be assessed.

The goals of translational research are to close the gap between basic science and patient clinical care by using research knowledge to develop new prevention, diagnosis or treatment approaches and to transfer new knowledge into clinical practice to improve patients' healthcare [497]. Translational genetic research comes with some specific challenges that genetic counsellors are best suited to tackle [495]. The publications that led to this thesis summarise the importance of genetic counsellors in translational research using glaucoma as a model.

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APPENDIX 1

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Genome-wide association study identifies susceptibility loci for open angle glaucoma at *TMCO1* and *CDKN2B-AS1*

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We report a genome-wide association study for open-angle glaucoma (OAG) blindness using a discovery cohort of 590 individuals with severe visual field loss (cases) and 3,956 controls. We identified associated loci at *TMCO1* (rs4656461[G] odds ratio (OR) = 1.68, $P = 6.1 \times 10^{-10}$) and *CDKN2B-AS1* (rs4977756[A] OR = 1.50, $P = 4.7 \times 10^{-9}$). We replicated these associations in an independent cohort of cases with advanced OAG (rs4656461 $P = 0.010$; rs4977756 $P = 0.042$) and two additional cohorts of less severe OAG (rs4656461 combined discovery and replication $P = 6.00 \times 10^{-14}$, OR = 1.51, 95% CI 1.35–1.68; rs4977756 combined $P = 1.35 \times 10^{-14}$, OR = 1.39, 95% CI 1.28–1.51). We show retinal expression of genes at both loci in human ocular tissues. We also show that *CDKN2A* and *CDKN2B* are upregulated in the retina of a rat model of glaucoma.

Glaucoma is a group of neurodegenerative ocular diseases united by a clinically characteristic optic neuropathy. It is the second leading cause of blindness worldwide[1]. Primary OAG is the commonest glaucoma subtype[1]. OAG pathogenesis and factors determining disease progression are poorly understood. Early intervention with measures to reduce intraocular pressure retards visual loss in most individuals[2], but many cases of glaucoma remain undiagnosed until after irreversible vision loss. Elucidation of SNPs associated with severe outcomes could enable better targeting of expensive lifelong treatments, with associated morbidity, to individuals with the highest risk of blindness. Linkage and candidate gene studies have identified several genes likely to be involved in OAG including *MYOC* (encoding myocilin)[3] and *NTF4* (ref. 4), although for the latter, findings have varied in different populations[5]. A recent genome-wide association study (GWAS) using Icelandic cases with OAG of unselected severity identified association with variants near *CAV1* (ref. 6). To identify genes predisposing individuals to OAG blindness, we performed a GWAS in Australians of European descent with advanced OAG (individuals with OAG who have progressed to severe visual field loss or blindness).

We selected cases with advanced OAG ($N = 590$ after data cleaning) from the Australian and New Zealand Registry of Advanced Glaucoma (ANZRAG) and the Glaucoma Inheritance Study in Tasmania (GIST)[7,8]. We used two previously described Australian samples as controls ($N = 1,801$ and $N = 2,155$, for a total combined $N = 3,956$)[9]. Cohort demographics are given in **Table 1**, and recruitment and disease definitions are listed in the **Supplementary Note**. We typed samples on Illumina arrays (we typed cases using Omni1 and controls using HumanHap610 or HumanHap660). We combined cases and controls into a single dataset for cleaning and imputation. All participants were Australians of European descent.

After cleaning, 298,778 SNPs were available for association testing. The genomic inflation factor (λ) in the discovery cohort was 1.06 (quantile-quantile plots uncorrected and corrected for λ are shown in **Supplementary Fig. 1a**,**b**). The λ reduced to 1.04 when we included the first ten principal components as covariates. The association results across the genome are displayed in **Figure 1**; results are presented corrected for $\lambda = 1.06$ without correction for principal components. Results with correction for principal components were similar (data not shown). Two regions clearly reached genome-wide significance (defined as $P < 5 \times 10^{-8}$; **Table 2**), with $P = 6.1 \times 10^{-10}$ at rs4656461[G] near *TMCO1* on chromosome 1q24 and $P = 4.7 \times 10^{-9}$ at rs4977756[A] in *CDKN2B-AS1* on chromosome 9p21. Association results at these loci for both genotyped and imputed SNPs are shown in **Figure 2**. Imputation of SNPs from the 1000 Genomes Project did not reveal any SNPs with substantially

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Table 1 Demographic features of the cohorts

	n		Age			% female		
Cohort	Case	Control	Case	Control	*P*	Case	Control	*P*
Discovery	615	3,956	76.6 ± 13.9	43.4 ± 11.5	$<1.0 \times 10^{-6}$	52.1	78.9[a]	$<1.0 \times 10^{-6}$
First replication, advanced glaucoma	334	434	74.9 ± 11.7	78.7 ± 9.1	2.0×10^{-6}	55.6	59.1	0.35
Second replication, less severe glaucoma	465	1,436	71.8 ± 12.6	52.0 ± 0.0	$<1.0 \times 10^{-6}$	61.4	49.7	1.2×10^{-5}
Third replication, Blue Mountains Eye Study	93	2,712	76.5 ± 9.4	70.1 ± 10.1	$<1.0 \times 10^{-6}$	8.5	45.4	$<1.0 \times 10^{-6}$
Combined replication studies	892	4,582	72.0 ± 13.0	64.9 ± 12.4	$<1.0 \times 10^{-6}$	51.1	47.4	0.050

[a]One of the two control cohorts was entirely female, as discussed in the main text.

stronger association than the top genotyped SNPs (**Fig. 2**) or identify additional genome-wide significant loci. At both loci, the most associated SNP is supported by concordant results for other SNPs in moderate or high linkage disequilibrium.

We drew three replication cohorts from the Australian population, and all subjects were of European descent (**Table 1**). The advanced glaucoma replication cohort consisted of 334 additional cases with advanced OAG and 434 controls over 60 years of age (mean age 78.7 years). The less severe cohort consisted of 465 cases with OAG and 1,436 controls from the Wellcome Trust Case Control Cohort 1958 Birth Cohort (WTCCC 58BC). The third cohort was a population-based study, the Blue Mountains Eye Study, containing 93 cases with glaucoma and 2,712 examined controls. The most-associated SNPs at each locus from the discovery cohort clearly replicated in all replication cohorts (**Table 2**). Other SNPs in both of these regions were also associated in the replication cohorts (**Supplementary Table 1a**). Combining all raw data from all replication cohorts in an association analysis gave $P = 7.56 \times 10^{-6}$ (OR = 1.39, 95% CI 1.20–1.61) for rs4656461 near *TMCO1* and $P = 4.19 \times 10^{-7}$ (OR 1.33, 95% CI 1.19–1.48) at rs4977756 in *CDKN2B-AS1* (**Supplementary Table 1b**). These SNPs of interest were also still significantly associated with OAG following adjustments for age and sex in a logistic regression analysis (**Supplementary Table 1b**), indicating that the observed associations are independent of these parameters despite the differences between the case and control cohorts. We combined all available controls to enable a comparison of ORs for the risk alleles at both loci between the advanced OAG cohorts and the less severe OAG cases (**Supplementary Table 1c**). We observed stronger ORs in the advanced cases, and these results support the hypothesis that the risk alleles identified are associated with OAG in general but are more strongly associated with cases which progress to advanced disease. Alternatively, higher diagnostic certainty in severe disease could account for this observation.

Combining raw data from the discovery and all replication cohorts in a study-wide association analysis generated an overall OR = 1.51 (95% CI 1.35–1.68) and $P = 6.0 \times 10^{-14}$ for rs4656461 and OR = 1.39 (95% CI 1.28–1.51) and $P = 1.35 \times 10^{-14}$ for rs4977756 (**Table 2**). Haplotype analyses indicated three common haplotypes around *TMCO1* and two at *CDKN2B-AS1*. The overall *P* values for association were $P = 6.56 \times 10^{-12}$ around *TMCO1* and $P = 2.59 \times 10^{-9}$ at the *CDKN2B-AS1* locus (**Supplementary Table 2**). In both cases, the risk alleles detected in the single SNP analysis are present on a single common haplotype that shows significant association with OAG. The haplotype with the alternative allele at each location appears to be protective against developing OAG. We sequenced 12 individuals with OAG who were homozygous for the risk allele at rs4656461 at all coding exons of *TMCO1* and the 3′ untranslated region (UTR). We found several common SNPs in the 3′ UTR to be present on the risk haplotype, although the functionality of these SNPs is not known (**Supplementary Table 3**). The lack of identified coding variants suggests the true causative variants are likely to be located in a regulatory region of *TMCO1*.

We used two control cohorts in this study; one population sample based on parents of twins and the other a sample of individuals with endometriosis. We subjected cases and controls to the same cleaning regime to ensure a well-matched dataset. The male to female ratio was similar between the case cohort and the twin-based controls, but the endometriosis controls were all female. We repeated the association analysis excluding the endometriosis controls, and this generated *P* values at rs4656461 and rs4977756 of $P = 5.3 \times 10^{-9}$ and $P = 1.1 \times 10^{-7}$, respectively. The reduced significance level of this association can be explained by the smaller sample size, as allele frequencies were very similar between control cohorts (**Supplementary Table 4a**). In addition, we utilized the WTCCC 58BC data as an alternative control cohort for the discovery analysis. The top SNPs at both loci were associated with genome-wide significance levels in this analysis, indicating that our findings do not represent an artifact of the historic controls used (**Supplementary Table 4b** and **Supplementary Fig. 1c**).

To obtain an unbiased estimate of risk for advanced glaucoma, we focused on the first replication cohort[10]. Taking the cases with advanced glaucoma ($N = 334$), the matched examined elderly controls ($N = 434$) and similar age-matched controls from the Blue Mountains Eye Study cohort ($N = 502$), we fitted rs4977756 and rs4656461 into a logistic regression model. Assuming an additive model, individuals carrying four risk alleles (two at each locus) had a 4.50-fold (95% CI 1.84–11.01) higher risk of advanced OAG relative to non-carriers. Grouping individuals with one or two risk alleles together at both loci (dominant model) gave a 3.03-fold (95% CI 1.52–6.07) increased risk. Eighteen percent of the population are in this risk category.

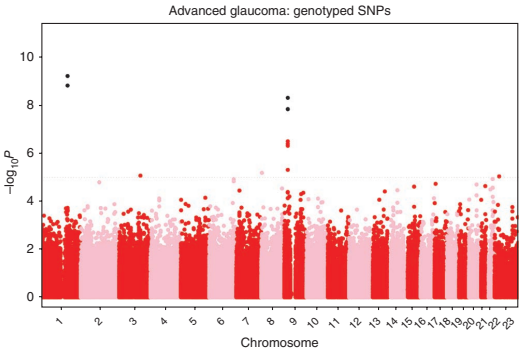

Figure 1 Association results for genotyped SNPs. SNPs with *P* values reaching genome-wide significance ($P < 5 \times 10^{-8}$) are shown in black. Results are corrected for $\lambda = 1.06$. Chromosome 23 refers to the X chromosome.

Table 2 Association results for genome-wide significant genotyped SNPs in the discovery cohort and three replication cohorts and all cases and controls combined (combined studies)

SNP: risk allele	Chr.	Position[a]	Discovery cohort			First replication, advanced glaucoma			Second replication, less severe glaucoma			Third replication, Blue Mountains Eye Study			Combined discovery and replication studies		
			Frequency[b]	*P*[c]	OR (95% CI)	Frequency[b]	*P*	OR (95% CI)	Frequency[b]	*P*	OR (95% CI)	Frequency[b]	*P*	OR (95% CI)	Frequency[b]	*P*	OR (95% CI)
rs4656461:G	1	163,953,829	0.19/0.12	6.1×10^{-10}	1.68 (1.43–1.98)	0.17/0.12	0.010	1.47 (1.09–1.97)	0.15/0.12	0.026	1.28 (1.03–1.59)	0.17/0.12	0.022	1.57 (1.07–2.32)	0.17/0.12	6.0×10^{-14}	1.51 (1.35–1.68)
rs7518099:C	1	164,003,504	0.18/0.12	4.7×10^{-10}	1.67 (1.42–1.96)	0.16/0.12	0.032	1.38 (1.03–1.86)	0.15/0.12	0.022	1.29 (1.04–1.61)	0.18/0.12	0.007	1.68 (1.15–2.46)	0.17/0.12	4.0×10^{-13}	1.49 (1.34–1.66)
rs4977756:A	9	22,058,652	0.69/0.60	4.7×10^{-9}	1.50 (1.31–1.70)	0.69/0.63	0.042	1.25 (1.01–1.56)	0.64/0.58	0.013	1.21 (1.04–1.41)	0.68/0.60	0.015	1.48 (1.08–2.04)	0.67/0.60	1.4×10^{-14}	1.39 (1.28–1.51)
rs10120688:A	9	22,046,499	0.58/0.48	1.4×10^{-8}	1.44 (1.28–1.63)	0.56/0.52	0.153	1.16 (0.95–1.43)	0.51/0.46	0.003	1.27 (1.08–1.48)	0.57/0.48	0.025	1.40 (1.04–1.88)	0.55/0.48	9.1×10^{-12}	1.32 (1.22–1.43)

The frequency of the risk allele in cases and controls is given. All tests were performed under an allelic model.
[a]Position in Build 36. [b]Frequency in cases/controls. [c]Corrected for lambda of 1.06. Chr., chromosome.

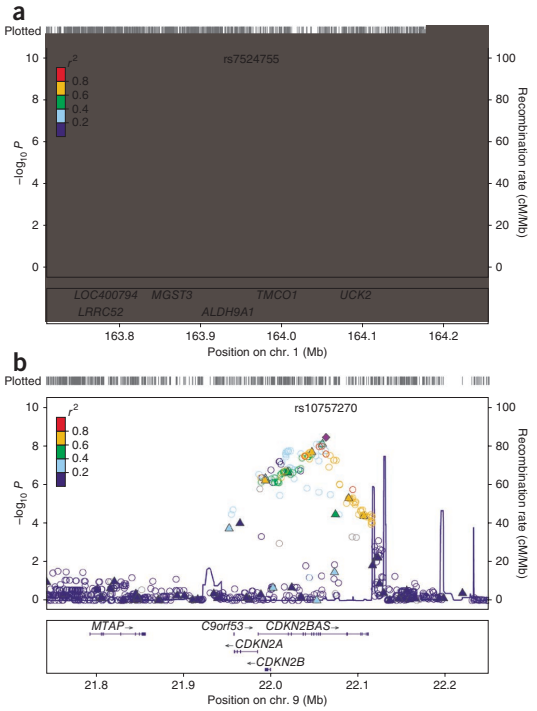

Figure 2 Association results for SNPs at the genome-wide significant loci corrected for $\lambda = 1.06$. Genotyped SNPs are indicated by solid triangles, and imputed SNPs are indicated by hollow circles. The top ranked SNP at each locus is shown as a solid diamond. Imputation *P* values for all SNPs are plotted. The color scheme indicates linkage disequilibrium between the top ranked SNP and other SNPs in the region. Note that the imputed and genotyped *P* values for genotyped SNPs differ slightly because for the imputed result, the analysis was based on dosage scores, whereas with genotyped SNPs, the hard genotype calls are used. (**a**) Chromosome 1q24 region. The imputation *P* value was $P = 1.0 \times 10^{-9}$ for the top SNP, rs7524755, with the top genotyped SNP, rs4656461, the fourth best SNP after imputation, with $P = 1.6 \times 10^{-9}$. (**b**) Chromosome 9p21 region. The imputation *P* value was $P = 3.7 \times 10^{-9}$ for the top SNP, rs10757270, with the top genotyped SNP, rs4977756, the second best SNP after imputation, with $P = 8.1 \times 10^{-9}$.

rs4656461 at the 1q24 locus is ~6.5 kb downstream of *TMCO1*. rs4977756 at the 9p21 locus is located within the antisense RNA gene *CDKN2B-AS1*. This region also harbors the tumor suppressor genes *CDKN2A* and *CDKN2B* and is adjacent to *MTAP*. *CDKN2A* also encodes an alternate open reading frame, known as ARF. We analyzed expression of these genes in human ocular tissues by RT-PCR. All the genes are expressed in the iris, ciliary body, retina and optic nerve, but the expression levels varied among the tissues analyzed (**Fig. 3a**). Furthermore, we determined which of the *CDKN2B-AS1* splice variants were expressed in the retina, the tissue that is ultimately compromised in glaucoma. RT-PCR revealed expression of three splice variants of this gene in the human retina (**Fig. 3b**). This is consistent with expression of more than one *CDKN2B-AS1* splice variants in a tissue or cell line[11,12]. We used well-characterized antibodies directed against Cdkn2a, Cdkn2b, Mtap and Tmco1 to explore the distribution of these proteins in rat retina. Cdkn2a and Cdkn2b were expressed in retinal ganglion

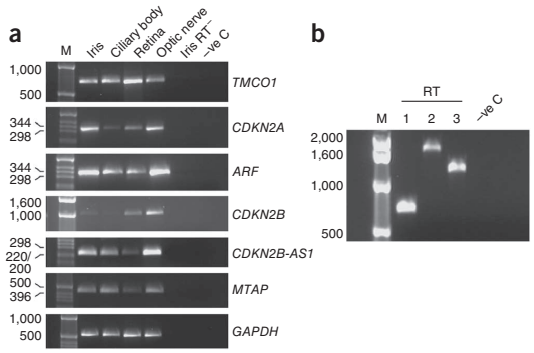

Figure 3 Ocular expression of the genes at the glaucoma-associated loci. (**a**) Analysis of the expression of *TMCO1*, *CDKN2A/ARF*, *CDKN2B*, *CDKN2B-AS1* and *MTAP* in various human eye tissues by RT-PCR using gene-specific primers (**Supplementary Table 5**). We amplified *GAPDH* to control for the amount of complementary DNA (cDNA) template used from each tissue for PCR. The expected size of each PCR product is indicated in **Supplementary Table 5**. (**b**) Expression of *CDKN2B-AS1* splice variants in human retina. We performed RT-PCR with gene-specific primers in exon 1 and 19 of *CDKN2B-AS1* (**Supplementary Table 5c**). Lanes 1, 2 and 3 correspond to the splice variants amplified upon primer annealing at 52 °C, 54 °C and 56 °C, respectively. The variant in lane 1 resulted from the splicing of exons 1-5-6-7-19, in lane 2 from the splicing of exons 1-5-6-7-10-11-13-14-15-16-17-18-19 and in lane 3 from the splicing of exons 1-5-6-7-15-16-17-18-19. These variants are different to previously reported *CDKN2B-AS1* variants[11,12]. The full-length variant (DQ485453) and alternatively spliced variants (DQ485454 and GQ495924)[11,12] were undetectable in human retina (data not shown). M, molecular weight markers in base pairs; RT⁻, reverse transcription negative control; –ve C, PCR negative control.

cells (RGC) and other retinal cell types with nuclear patterns of localization, similar to the patterns reported in other tissues (**Supplementary Fig. 2**). Tmco1 was also associated with all retinal cells, but we observed the strongest expression in RGC. Mtap was expressed at low levels in retinal astrocytes (data not shown). To determine whether these genes are candidates for involvement in the pathogenesis of glaucoma, we performed real-time PCR analysis of their expression levels in a validated rat model of glaucoma[13] (**Fig. 4**). We observed strong upregulation of expression of *Cdkn2a* and *Cdkn2b*, but not *Tmco1*, in the retina one week after induction of ocular hypertension, a time point corresponding to ongoing RGC death, as indicated by axonal cytoskeleton damage in the optic nerve of the animals studied.

Recessive mutations in *TMCO1* cause a syndrome consisting of craniofacial dysmorphism, skeletal anomalies and mental retardation[14]. The gene encodes a transmembrane protein with a coiled-coil domain that may localize to the Golgi apparatus and endoplasmic reticulum[15] or to the mitochondria[16] in different cell types. In humans, the gene is ubiquitously expressed in developing and adult tissues[14]. The protein sequence is completely conserved among many mammalian species[14]. Although requiring experimental confirmation, researchers in a previous study proposed a role for TMCO1 in apoptosis[16]. This may suggest a mechanism for the association with glaucoma, which is characterized by excessive RGC apoptosis. It is also possible that other genes adjacent to *TMCO1*, such as *ALDH9A1*, could be responsible for the glaucoma association observed in this study.

CDKN2B-AS1 resides in the 9p21 region that has been clearly associated with cardiovascular disease[17], diabetes[18], intracranial aneurysm[19] and glioma[20]. The antisense RNA encoded by *CDKN2B-AS1* regulates neighboring genes at 9p21, particularly *CDKN2B*, with which its expression levels are reciprocally related[21]. CDKN2B and CDKN2A activate the retinoblastoma tumor suppressor pathway, whereas ARF activates the p53 tumor suppressor pathway. The 9p21 locus is activated in response to oncogenic stimuli[22]. *CAV1*, recently reported to be associated with OAG[6], regulates mitogenic signaling and acts synergistically with *CDKN2A* (ref. 23). Although the *CAV1* SNP (rs4236601) did not reach statistical significance in this GWAS (P = 0.17 for a one-sided test), the observed odds ratio of 1.07 is consistent with that previously reported in larger European cohorts, as are the allele frequencies (cases, 0.290; controls, 0.276 for the A allele). It should be noted that many of the cases in the current study are included in the previously reported Australian replication cohort[6]. Genes at the 9p21 locus are known to play a role in aberrant cell division, and we propose that the 9p21 OAG risk variants may predispose RGCs to gradual apoptosis. This hypothesis is supported by observations that the opposite risk alleles in *CDKN2B-AS1* are associated with glaucoma and glioma. For example, at rs4977756 and rs1063192, the G and C alleles, respectively, are protective for glaucoma but are the risk alleles for glioma[20]. The direction of association is the same for glaucoma as for cardiovascular disease[17] and diabetes[18], but further work is required to determine whether the same causative variant(s) underlie these different disease associations.

Recently, rs1063192 in *CDKN2B* was reported to be associated at genome-wide significance with optic cup to disc ratio in healthy individuals[24]. Nominal association of this SNP with glaucoma in a small case series was also reported[24]. In our study, this particular SNP had $P = 3.9 \times 10^{-7}$ in the discovery cohort, and the nearby SNPs in *CDKN2B-AS1* reached genome-wide significance. Thus, we provide further compelling evidence that the 9p21 region is a strong genetic risk factor for OAG in support of the previous suggestive association with OAG at this locus.

This study shows evidence of association of two candidate genes, *TMCO1* and *CDKN2B-AS1*, with advanced OAG, imparting a threefold increase in risk for carriers of one or more risk alleles at the two loci.

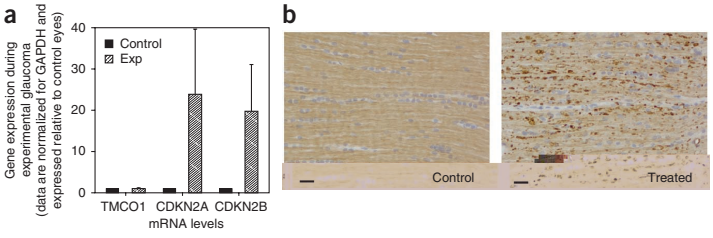

Figure 4 Expression of genes at the glaucoma-associated loci in a rat model of glaucoma. (**a**) Expression of *TMCO1*, *CDKN2A* and *CDKN2B* mRNAs in rat retina 7 days after induction of experimental glaucoma (mean intraocular pressure at time of death of 32 ± 3.7 mm Hg) as determined by quantitative real-time RT-PCR, where $n = 4$. Error bars, s.e.m. (**b**) Axonal degeneration in the distal optic nerve of one representative animal as evaluated by immunolabeling for non-phosphorylated neurofilament heavy protein. Numerous axonal swellings and abnormalities are visible in the optic nerve of the treated eye (right panel) compared with the control optic nerve (left panel), which appears normal. Scale bars in **b**, 25 µm.

In addition, we have shown strong upregulation of *CDKN2A* and *CDKN2B* in response to elevated intraocular pressure, further indicating that this region is important in the molecular pathways leading to glaucoma development. This discovery was made utilizing an approach of selecting cases with severe blinding OAG for the GWAS, but as expected, the risk alleles are also associated with less severe cases, showing the efficacy of using extreme cases to identify genes for a common disease. OAG can be difficult to diagnose in the early stages, and these findings may be useful in the future to prioritize treatment effectively for individuals with possible but not definite early glaucoma (glaucoma suspects), for whom it is often difficult to decide upon the timing of treatment initiation. As treatment for glaucoma is proven to slow disease progression[2], timely initiation of conventional treatment in those individuals at the highest risk could reduce glaucoma blindness. In addition, we have highlighted biochemical pathways involved in this disease, which could lead to more targeted OAG treatment regimes aiming to protect RGC in ways other than lowering intraocular pressure which has until now formed the cornerstone of treatment.

URLs. EIGENSOFT, http://genepath.med.harvard.edu/~reich/Software.htm; MACH2, http://www.sph.umich.edu/csg/abecasis/MACH/index.html; 1000 Genomes, http://www.1000genomes.org; PLINK, http://pngu.mgh.harvard.edu/~purcell/plink/; LocusZoom, http://csg.sph.umich.edu/locuszoom/; Australian & New Zealand Registry of Advanced Glaucoma, www.anzrag.com; European Genome-phenome Archive,

ONLINE METHODS

Cohort descriptions. See **Supplementary Note**.

Genotyping and data quality control. Following DNA extraction, Australian twin and endometriosis sample controls were genotyped at deCODE Genetics (Reykjavik, Iceland) on Illumina HumanHap 610W Quad and Illumina
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(PVDF) membrane. The blot was blocked with 5% skimmed milk and Tris buffered saline containing 0.1% Tween 20, probed with antibodies to actin or TMCO1 followed by appropriate secondary antibodies conjugated to biotin and then streptavidin-peroxidase conjugate. The blot was developed with a 0.016% solution of 3-amino-9-ethylcarbazole in 50 mM sodium acetate (pH 5) containing 0.05% Tween-20 and 0.03% H_2O_2.

Evaluation of gene expression levels in a rat model of glaucoma. Sprague-Dawley rats were anesthetized with an intraperitoneal injection of 100 mg/kg ketamine and 10 mg/kg xylazine, and local anesthetic drops were applied to the eye. Ocular hypertension was induced in the right eye of each animal by laser photocoagulation of the trabecular meshwork as previously described[13]. Intraocular pressures were measured in both eyes at baseline, at 8 h and at days 1, 3 and 7 using a rebound tonometer calibrated for use in rats. All rats were killed by transcardial perfusion with physiological saline under deep anesthesia. The retinas were dissected for RT-PCR, and the chiasm from each rat was taken for immunohistochemistry to verify that the procedure had induced an appropriate injury response using the same method detailed above. Total RNA was isolated from each retina, and first strand cDNA was synthesized from 2 µg DNase-treated RNA. Duplicate real-time PCR reactions were carried out using the cDNA equivalent of 20 ng total RNA for each sample in a total volume of 25 µl containing 1× SYBR Green PCR master mix (BioRad) in an IQ5 icycler (Bio-Rad). The primer sets used are detailed in **Supplementary Table 5**. After the final cycle of the PCR, primer specificity was checked by the dissociation (melting) curve method. The relative expression in each sample was calculated using *Gapdh* as reference mRNA as previously described[31].

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Common variants near *ABCA1*, *AFAP1* and *GMDS* confer risk of primary open-angle glaucoma

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Primary open-angle glaucoma (POAG) is a major cause of irreversible blindness worldwide. We performed a genome-wide association study in an Australian discovery cohort comprising 1,155 cases with advanced POAG and 1,992 controls. We investigated the association of the top SNPs from the discovery stage in two Australian replication cohorts (932 cases and 6,862 controls total) and two US replication cohorts (2,616 cases and 2,634 controls total). Meta-analysis of all cohorts identified three loci newly associated with development of POAG. These loci are located upstream of *ABCA1* (rs2472493[G], odds ratio (OR) = 1.31, $P = 2.1 \times 10^{-19}$), within *AFAP1* (rs4619890[G], OR = 1.20, $P = 7.0 \times 10^{-10}$) and within *GMDS* (rs11969985[G], OR = 1.31, $P = 7.7 \times 10^{-10}$). Using RT-PCR and immunolabeling, we show that these genes are expressed within human retina, optic nerve and trabecular meshwork and that ABCA1 and AFAP1 are also expressed in retinal ganglion cells.

POAG, the most common subtype of glaucoma, is characterized by a progressive loss of peripheral vision, but cases may remain undiagnosed until central vision is affected[1,2]. The etiology and pathogenesis of POAG are poorly understood. Linkage studies, candidate gene studies and genome-wide association studies (GWAS) have identified several loci reproducibly associated with development of POAG[3–7]. Our previous GWAS of advanced POAG identified two loci at *TMCO1* and *CDKN2B-AS1* (ref. 6), with studies of non-advanced POAG also having implicated *CAV1* (ref. 5), *SIX6* and a region at 8q22 (ref. 7). Here we used a three-stage GWAS to identify additional genetic loci associated with POAG in participants of European descent.

The stage 1 discovery cohort comprised 1,155 cases with advanced glaucoma from the Australian and New Zealand Registry of Advanced Glaucoma (ANZRAG) and 1,992 controls genotyped on Illumina Omni1M or OmniExpress arrays (**Supplementary Note** and **Supplementary Table 1**). We combined and cleaned the genotype data from cases and controls and used 569,249 SNPs as the base of imputation against the 1000 Genomes phase 1 European-ethnicity data set. We successfully imputed 7,594,768 SNPs with minor allele frequency (MAF) >0.01 and imputation quality score >0.8.

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We performed association analysis using an additive model adjusted for sex and six principal components. We corrected the *P* values from the association analysis for the estimated genomic inflation factor, λ, of 1.06 (the quantile-quantile plot is shown in **Supplementary Fig. 1**).

The stage 1 association results across the genome are shown in **Supplementary Figure 2**, and the association results for all SNPs with $P < 1 \times 10^{-7}$ are shown in **Supplementary Table 2**. Two previously unreported regions reached genome-wide significance ($P < 5 \times 10^{-8}$) in the stage 1 discovery cohort, with a further previously unreported region showing association at close to genome-wide significance (**Table 1**). The top newly associated SNPs were rs2472493[G] upstream of *ABCA1* (encoding ATP-binding cassette, subfamily A, member 1) on chromosome 9 (OR = 1.43, $P = 2.0 \times 10^{-10}$), rs11827818[G] close to *ARHGEF12* (encoding Rho guanine nucleotide exchange factor 12) (OR = 1.52, $P = 9.2 \times 10^{-9}$) on chromosome 11 and rs114096562[A] in *GMDS* (encoding GDP-mannose 4,6-dehydratase) (OR = 1.55, $P = 7.0 \times 10^{-8}$) on chromosome 6. The regional association results for these three SNPs are shown in **Figure 1**. We also performed the analysis after removing controls affected by other diseases (**Supplementary Note**) and found that the effect sizes were similar (**Supplementary Table 3**).

Table 1 Association results for the top SNPs in previously unreported regions with $P < 1 \times 10^{-7}$ in the discovery cohort

Chr.	SNP	Position[a]	Gene	Risk allele	*P*[b]	OR	s.e.m.	Frequency[c]
9	rs2472493	107695848	*ABCA1*[d]	G	2.0×10^{-10}	1.43	0.05	0.51/0.43
11	rs11827818	120198728	*ARHGEF12*[d]	G	9.2×10^{-9}	1.52	0.07	0.20/0.14
6	rs114096562	1984385	*GMDS*	A	7.0×10^{-8}	1.55	0.08	0.88/0.83

[a]Position in build 37. [b]*P* corrected for the genomic inflation factor ($\lambda = 1.06$). [c]Allele frequency in cases/controls. [d]The corresponding SNP is not in the indicated gene; instead, characterized genes located near these SNPs are shown. Chr., chromosome.

We then investigated the associations of top SNPs in the discovery cohort in a stage 2 set comprising two Australian replication data sets (the ANZRAG and Blue Mountains Eye Study (BMES) data sets, totaling 932 cases and 6,862 controls; **Supplementary Note** and **Supplementary Table 1**). All replication cohort participants were of European descent. To make maximum valid use of our cohorts, for replication we focused on SNPs directly genotyped on the Illumina Human610 and Human670 arrays; we used proxy genotyped SNPs

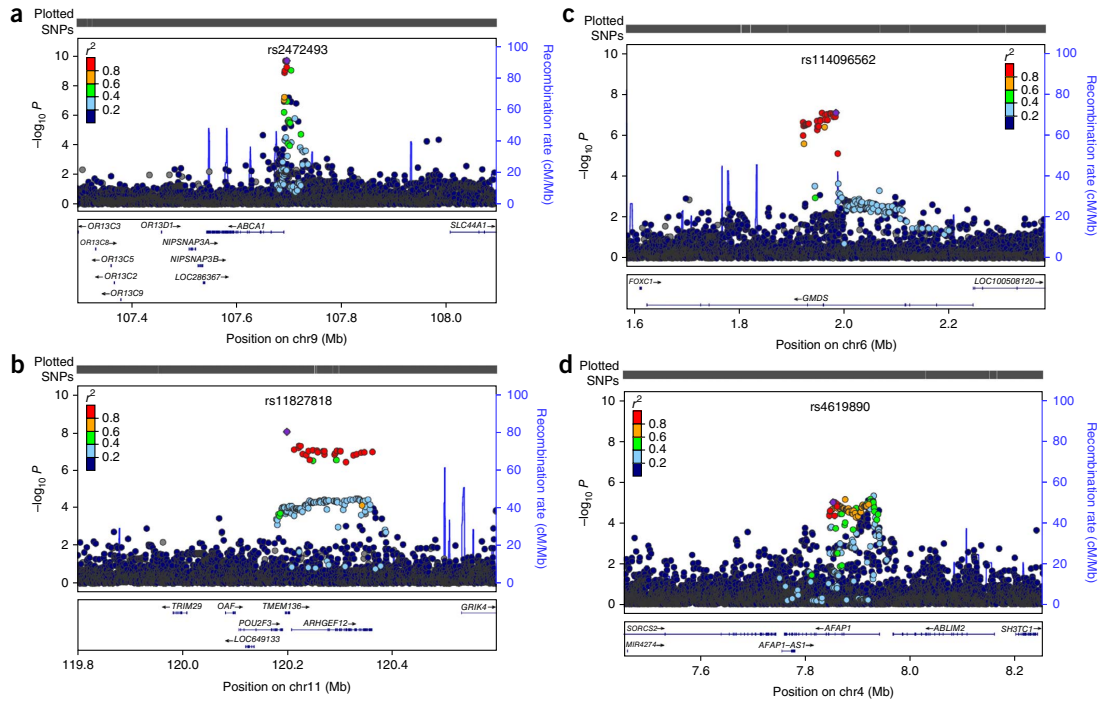

Figure 1 Association results for the regions reaching genome-wide significance. These plots show the regional association (using logistic regression with sex and the first six principal components fitted as covariates) and recombination rates for the top SNPs in the discovery data set (1,155 cases with advanced POAG and 1,992 controls). In each plot, the solid diamond indicates the top-ranked SNP in the region based on two-sided *P* values. The colored box at the right or left corner of each plot indicates the pairwise correlation (r^2) between the top SNP and the other SNPs in the region. The blue spikes show the estimated recombination rates. The box underneath each plot shows the gene annotations in the region. Each plot was created using LocusZoom (http://csg.sph.umich.edu/locuszoom/) for the top-ranked SNP in each region with a 400-kb region surrounding it. (**a**) The top-ranked SNP for this plot is rs2472493 on chromosome 9 upstream of *ABCA1* with $P = 2.0 \times 10^{-10}$. (**b**) The top-ranked SNP for this plot is rs11827818 on chromosome 11 near *ARHGEF12* with $P = 9.2 \times 10^{-9}$. (**c**) The top-ranked SNP for this plot is rs114096562 on chromosome 6 in *GMDS* with $P = 7.0 \times 10^{-8}$. (**d**) This plot is centered on rs4619890 on chromosome 4 in *AFAP1* with $P = 9.7 \times 10^{-6}$. This SNP clearly reached genome-wide significance ($P = 7.0 \times 10^{-10}$) in the meta-analysis of the results between the discovery and replication cohorts.

Table 2 Association and meta-analysis of the discovery and replication cohorts for the top-ranked loci

						ANZRAG (discovery)		ANZRAG (replication)		BMES		NEIGHBOR		MEEI		Meta-analysis			
Chr.	SNP	Position[a]	A1[b]	A2	Gene	OR	P^c	OR	P	OR	P^c	OR	P	OR	P	OR	P	Het $I^{2,d}$	Het P^e
3	rs2710323	52815905	T	C	*ITIH1*	1.25	9.16×10^{-5}	1.14	0.005	1.44	0.01	1.06	0.25	0.87	0.31	1.14	4.53×10^{-6}	64.6	0.02
4	rs4619890	7853160	G	A	*AFAP1*	1.26	9.76×10^{-6}	1.20	0.0004	1.07	0.62	1.14	0.008	1.13	0.38	1.20	7.03×10^{-10}	0	0.57
4	rs4478172	7902003	C	A	*AFAP1*	1.29	2.73×10^{-5}	1.15	0.02	1.21	0.26	1.16	0.005	1.11	0.47	1.19	2.19×10^{-8}	0	0.64
6	rs11969985	1922907	G	A	*GMDS*	1.53	3.18×10^{-7}	1.23	0.009	0.92	0.71	1.28	0.001	1.28	0.24	1.31	7.70×10^{-10}	46.4	0.11
6	rs2761233	1949101	T	C	*GMDS*	1.53	3.35×10^{-7}	1.19	0.02	0.94	0.79	1.28	0.001	1.28	0.23	1.29	2.17×10^{-9}	48.5	0.10
9	rs2472493	107695848	G	A	*ABCA1*[f]	1.43	2.08×10^{-10}	1.26	4.84×10^{-6}	1.44	0.01	1.26	7.05×10^{-6}	0.99	0.89	1.31	2.16×10^{-19}	53.6	0.07
11	rs2276035	120346360	A	G	*ARHGEF12*	1.47	1.13×10^{-7}	1.08	0.29	1.05	0.81	1.09	0.15	NA[g]	NA[g]	1.18	7.83×10^{-6}	77.1	0.004

Association results for three loci that reached genome-wide significance in the discovery cohort, as well as other top-ranked loci showing replication. Proxy SNPs are presented where imputed data were not available for the replication cohorts.
[a]Position in build 37. [b]Effect allele in all the cohorts. [c]*P* value corrected for the genomic inflation factor. [d]I^2 statistic measuring heterogeneity (Het) on a scale of 0% to 100%. [e]*P* values for the heterogeneity test. [f]The corresponding SNP is not in the indicated gene; instead, a characterized gene located near the SNP is shown. [g]rs2276035 SNP was not available (NA) in the MEEI cohort. Chr., chromosome.

where imputed data were not available for the replication cohorts (Online Methods).

Examining all autosomal SNPs with $P < 1 \times 10^{-4}$ in stage 1 (24 SNPs with the best *P* values were used as the lead SNPs; **Supplementary Table 4**), four regions showed nominal evidence ($P < 0.05$ for seven SNPs in or near *ABCA1*, *GMDS*, *ITIH1* and *AFAP1*) for replication in the ANZRAG replication samples (**Supplementary Table 4**). When we combined stages 1 and 2, SNPs near *ABCA1* and in *AFAP1* exceeded genome-wide significance ($P < 5 \times 10^{-8}$ for rs2472493 and rs4619890, respectively) with consistent effect sizes and directions of effects among the cohorts (**Table 2** and **Supplementary Table 4**).

In the stage 3 replication, we examined the newly identified top SNPs from stage 2 in data available from two additional replication cohorts (**Supplementary Note** and **Supplementary Table 1**): the National Eye Institute Glaucoma Human Genetics Collaboration (NEIGHBOR) and the Massachusetts Eye and Ear Infirmary (MEEI) (totaling 2,616 cases and 2,634 controls). We also performed a meta-analysis of the results for these SNPs between all cohorts (the discovery stage and all four replication cohorts) using the effect sizes and their standard errors. In the meta-analysis results, SNPs in or near *ABCA1*, *AFAP1* and *GMDS* clearly reached genome-wide significance ($P < 5 \times 10^{-8}$) (**Table 2**).

The top SNP within *ARHGEF12* (rs2276035) did not reach the significance level ($P < 5 \times 10^{-8}$) in our standard meta-analysis (**Table 2**), primarily because of heterogeneity between stage 1 and stages 2 and 3. This heterogeneity could be explained by the difference in glaucoma status in these cohorts, the 'winner's curse' effect that leads to inflated OR estimates in GWAS or chance. The top SNP within *ITIH1* (rs2710323) was not genome-wide significant in our meta-analysis (**Table 2**).

At each of the newly discovered loci, the effect size was larger in the discovery cohort than in the replication cohorts (**Table 2**). The discovery cohort comprises cases with advanced POAG only, whereas the replication cohorts contained cases with POAG representing a range of disease severity. One cannot directly infer, however, that the true effect size is largest in advanced POAG. A winner's curse effect in the ANZRAG discovery cohort would inflate the OR estimates. Furthermore, there may have been greater diagnostic certainty in the cases with advanced POAG. To investigate further whether the newly discovered loci conferred higher risk in advanced compared to non-advanced POAG, we performed a subanalysis on the ANZRAG replication cohort. We found no consistent difference between the ORs for the cases with non-advanced (n = 605) and advanced (n = 220) POAG separately (**Supplementary Table 5**). This subanalysis, together with the significant results in the replication cohorts taken alone, suggest that the newly discovered loci in this study are associated with POAG in general (and not advanced POAG only), indicating the generalizability of our findings.

Intraocular pressure (IOP) was not a criterion in the definition of POAG in this study because patients with POAG may have either normal or elevated IOP[8]. Thus, the new loci identified in this study are associated with POAG in general regardless of IOP levels. However, we had peak IOP measures available for 1,039 of the 1,155 cases in the ANZRAG discovery cohort. Of these cases, 330 (31.8%) had normal-tension glaucoma (NTG) (IOP ≤ 21 mm Hg) and 709 (68.2%) had high-tension glaucoma (HTG) (IOP >21 mm Hg). We investigated the association of the new loci identified in this study in 330 cases with NTG and 709 cases with HTG compared to 1,992 population controls in the discovery cohort (**Supplementary Table 6**). The direction and magnitude of effects of the risk alleles were similar for NTG, HTG and all POAG (**Table 2** and **Supplementary Table 6**). However, the

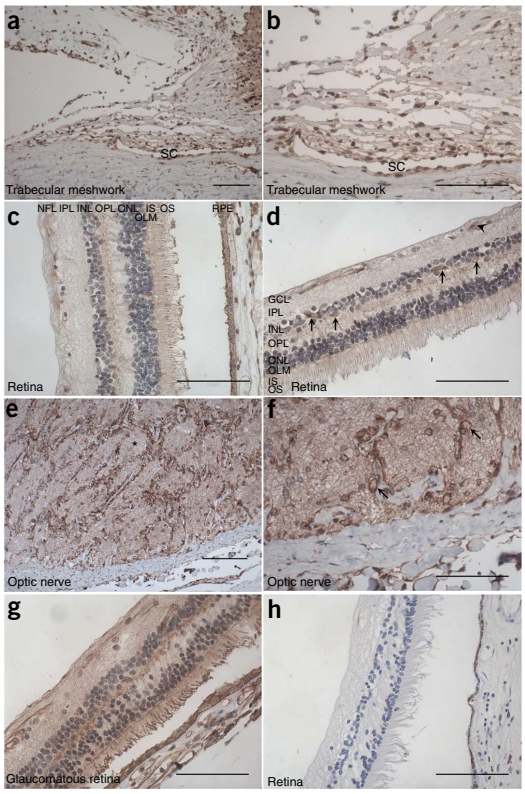

Figure 2 Distribution of the ABCA1 protein in human ocular tissues. (**a**–**f**) Sections of a normal human eye were immunolabeled with the ABCA1-specific antibody (brown) and counterstained with haematoxylin to visualize nuclei (blue). Positive immunolabeling was detected in the trabecular meshwork (**a**,**b**), throughout the retina (**c**,**d**) and in the optic nerve (**e**,**f**). In the retina (**c**), comparatively pronounced ABCA1 immunolabeling was observed at the tips of photoreceptors and in the outer limiting membrane (OLM), outer plexiform layer (OPL) and nerve fiber layer (NFL). (**d**) Labeling was also pronounced in some cells in the inner nuclear layer (INL, arrows), in retinal ganglion cells in the ganglion cell layer (GCL, arrowhead) and in the retinal blood vessel wall (not shown). In the optic nerve (**e**,**f**), the protein was distributed in the nerve fiber bundles (**e**, asterisk) and at the cell boundary of astrocytes in the glial columns (**f**, arrows). (**g**,**h**) In sections of a glaucomatous eye (data not shown), including in the retina (**g**), similar distribution of the protein to that in the normal eye was observed. The experiment was repeated once for reproducibility. (**h**) Section hybridized with the secondary detection reagent alone as a negative control. SC, Schlemm's canal; RPE, retinal pigment epithelium; OS, outer segment; IS, inner segment; ONL, outer nuclear layer; IPL, inner plexiform layer. Scale bars, 100 μm.

analysis for NTG and HTG was less powerful compared to that for POAG because of the smaller sample sizes of the subgroups.

None of our newly identified POAG loci overlapped with the previously published loci associated with POAG subphenotypes, including IOP and vertical cup-disk ratio[9–11]. We also investigated the association of the new loci identified in this study with peak measured IOP in 1,039 cases with POAG with available data in the ANZRAG discovery cohort. The new loci were not associated with peak IOP in the ANZRAG discovery cohort (**Supplementary Table 7**), although the *ABCA1* SNP showed a trend toward significance ($P = 0.0675$, two-sided test). The *ABCA1* glaucoma risk–increasing allele acts in the expected direction on IOP (the allele increases IOP), resulting in a P value of 0.034 in a one-sided test. Larger sample sizes and further meta-analyses of multiple studies will unambiguously determine whether the new loci identified in this study are associated with subphenotypes such as IOP.

We also investigated previously reported GWAS hits identified in other studies[5–7] in the meta-analysis of results between our discovery and replication cohorts (**Supplementary Table 8**). The *TMCO1*, *CDKN2B-AS1* and *SIX6* loci were clearly genome-wide significant ($P < 5 \times 10^{-8}$), whereas *CAV1-CAV2* and the locus on chromosome 8 were associated with POAG but not at a genome-wide significance level. SNP rs11669977 at *NTF4* was not associated with POAG in our meta-analysis.

We used ENCODE project data[12] and the Genevar database[13] (expression quantitative trait locus (eQTL) database) to predict the possible functional effects of the top SNPs identified in this study. The top SNP rs2472493 located upstream of *ABCA1* is an eQTL in lymphoblastoid cell lines (Genevar database) and may alter the sequence of motifs for proteins such as FOXJ2 and SIX5 (HaploReg v2)[14]. One of the SNPs in high linkage disequilibrium (LD, $r^2 > 0.8$) with the top SNP near *ABCA1* (rs2472494) alters the regulatory motif for binding of PAX6 (HaploReg v2). *PAX6* is an established master control gene in eye development[15]. A SNP (rs28495790) in high LD ($r^2 > 0.8$) with the best SNP in *AFAP1* (rs4619890) is likely to affect the binding of proteins (score 2b in RegulomeDB)[16] such as CTCF and RAD21 in a variety of cell lines, including WERI-Rb-1 (retinoblastoma). rs28495790 alters the sequence of regulatory motifs for binding of several proteins, including PAX6 (HaploReg v2). This may suggest a regulatory role for this SNP in gene expression in a pathway similar to that of rs2472494 near *ABCA1*. In *GMDS*, rs3046543 (in high LD, $r^2 = 0.8$, with the top imputed SNP rs114096562) alters the sequence of the regulatory motif for binding of SIX6; *SIX6* variants confer glaucoma risk[7]. SNPs close to *SIX6* also clearly reached genome-wide significance in our meta-analysis (top SNP rs10483727[T], OR = 1.32, $P = 1.56 \times 10^{-17}$). These data suggest that the top SNPs identified in this study may have important regulatory roles.

ABCA1 is a membrane-bound receptor involved in phospholipid and cholesterol efflux from cells. In monkey retinas, *ABCA1* is expressed in retinal ganglion cells[17], the cells that undergo apoptosis in glaucoma. We analyzed the expression of *ABCA1* mRNA in human ocular tissues by RT-PCR and found that the iris, ciliary body, retina, optic nerve head, optic nerve and trabecular meshwork cell lines derived from normal and glaucomatous eyes expressed the main transcript that encodes the full-length protein (**Supplementary Fig. 3a**). We also detected an alternative transcript in the ocular tissues (**Supplementary Fig. 3a**) that had unknown function[18,19]. Immunolabeling of sections of normal human eye with ABCA1-specific antibody (**Supplementary Fig. 4**) showed a distribution of the protein in the trabecular meshwork, all layers of the retina (including retinal ganglion cells) and the optic nerve (**Fig. 2**). We observed similar ABCA1 labeling in a glaucomatous eye, including in the layers of the retina (**Fig. 2g**). *ABCA1* has been reported to regulate neuroinflammation and neurodegeneration through coordinated activity in various cell types in mouse brains[20], and it may be involved in glaucoma through a similar function in the retina.

AFAP1 encodes a protein that binds to actin filaments and allows their crosslinking[21,22]. Actin cytoskeleton-modulating signals have been shown to be involved in the regulation of aqueous outflow and

intraocular pressure[23–25], which are important parts of glaucoma pathogenesis. *AFAP1* encodes two isoforms, the neuronal cell–specific A isoform and the ubiquitously expressed B isoform. By RT-PCR, we detected expression of both the A and B isoforms in human retina (**Supplementary Fig. 3c**) and expression of the B isoform in other ocular tissues, including the iris, ciliary body, lens, optic nerve and optic nerve head, as well as in cultured trabecular meshwork cells (**Supplementary Fig. 3b**). Consistent with the mRNA expression data, we observed AFAP1-positive immunolabeling in the trabecular meshwork, retina (including retinal ganglion cells) and optic nerve of normal human eye (**Supplementary Fig. 5**) using AFAP1-specific antibody (**Supplementary Fig. 6**). We observed similar AFAP1 labeling in a glaucomatous eye, including in the retina (**Supplementary Fig. 5g,h**). These data indicate that the function of AFAP1 in the trabecular meshwork and retina may be relevant in the pathogenesis of glaucoma.

GMDS encodes a protein that is required for the first step in *de novo* synthesis of fucose[26]. Fucose is required for diverse biological functions, including growth factor receptor signaling[27]. Several studies have suggested effects of growth factors on the development of glaucoma[23,28–32]. *GMDS* expresses two variant transcripts, 1 and 2. We detected expression of the variant 1 transcript in human ocular tissues and cultured trabecular meshwork cells by RT-PCR (**Supplementary Fig. 3d**), which indicates ubiquitous expression of the gene in the eye.

In this study we identified three new risk loci for POAG and highlighted related candidate genes and pathways that might be involved in developing POAG. These new loci, in addition to the previously known risk loci, will improve risk profiling for glaucoma, with better opportunities for the management of high-risk individuals. Currently, many cases of glaucoma remain undiagnosed until severe visual loss occurs; early detection and treatment can slow disease progression and prevent blindness[33]. Further dissection of these new POAG risk loci will likely lead to insights into the etiology of this common, irreversible cause of blindness.

URLs. PLINK, http://pngu.mgh.harvard.edu/~purcell/plink; R Project, http://www.r-project.org; LocusZoom, http://csg.sph.umich.edu/locuszoom; Ensembl, http://www.ensembl.org/index.html; NCBI, http://www.ncbi.nlm.nih.gov; UCSC Genome Bioinformatics, http://genome.ucsc.edu; GeneCards, http://www.genecards.org; UniprotKB, http://www.uniprot.org; ENCODE project, http://www.genome.gov/10005107; RegulomeDB, http://regulome.stanford.edu; HaploReg v2, http://www.broadinstitute.org/mammals/haploreg/haploreg.php; Genevar, http://www.sanger.ac.uk/resources/software/genevar.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

K.P.B., S. MacGregor and J.E.C. were involved in designing the study. A.W.H., K.C., L.R.P., M.A.H., A.C.V., P. McGuffin, F.T., P.J.F., J.J.W., G.W.M., N.G.M., G.R.-S., D.C.W., M.A.B., J.L.W., D.A.M., P. Mitchell and J.E.C. were involved in participant recruitment, sample collection or genotyping. Analysis was performed by P.G., R.F., K.P.B., S.S., M.H.L., J.N.C.B., S.J.L., L.R.P., J.L.H., J.L.W. and S. MacGregor. Designing and conducting the laboratory experiments were performed by K.P.B., S.S., S. Martin and R.F. Clinician assessments were performed by S.L.G., R.J.C., M.C., A.J.W., T.Z., E.S., J.L., J.T.F., S.K., J.B.R., I.G., P.R.H., R.A.M., D.A.M. and J.E.C. The initial draft was written by P.G., K.P.B., S.S. and S. MacGregor.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Study design. In total, 1,155 cases with glaucoma and 1,992 controls, genotyped on Illumina Omni1M or OmniExpress arrays and imputed to the 1000 Genomes phase 1 Europeans panel, were used as the discovery cohort in this study to perform a GWAS for POAG (stage 1). The association results for the top SNPs from the discovery cohort were replicated in stage 2 and 3 replication cohorts. The cohort details, genotyping platforms for each cohort and diagnostic criteria are listed in the **Supplementary Note**. In addition, we performed a meta-analysis for the top SNPs in the discovery and replication cohorts. In this methods section, we describe the methods used for imputation and statistical analyses in the discovery cohort. Methods used for each replication cohort are included in the **Supplementary Note**.

Quality control. The quality-control (QC) methods for the discovery cohort were performed in PLINK[34] by removing individuals with more than 3% missing genotypes, SNPs with call rate <97%, MAF < 0.01 and Hardy-Weinberg equilibrium $P < 0.0001$ in controls and $P < 5 \times 10^{-10}$ in cases. We used the same QC protocol before merging the cases and controls in our discovery cohort to avoid mismatches between the merged data sets. After merging, the genotypes for 569,249 SNPs common to the arrays were taken forward for analysis. Identity by descent was computed in PLINK based on autosomal markers, with one of each pair of individuals with relatedness >0.2 removed. Principal components were computed for all participants and reference samples of known northern European ancestry (1000G British, CEU and Finland participants) using the smartpca package from EIGENSOFT software[35,36]. Participants with principal component 1 (PC1) or PC2 values >6 s.d. from the known northern European ancestry group were excluded.

Imputation. Imputation was conducted using IMPUTE2 (ref. 37) in 1-Mb sections, with the 1000 Genomes phase 1 Europeans (March 2012 release) used as the reference panel[38]. Genotyped SNPs that were strand ambiguous (for example, A/T or C/G) were dropped from the input genotype panel before imputation; given that these are deliberately under-represented on Illumina arrays, this step has limited effects on the ability to impute data but gives greater confidence in the imputation's quality. Imputation was performed with the recommended settings for IMPUTE2, including a 250-kb buffer flanking the imputation sections and an effective size of the sampled population of 20,000 (ref. 37). Reference panel SNPs with MAF < 0.001 in Europeans were not imputed. SNPs with imputation quality score (INFO) >0.8 and MAF > 0.01 were carried forward for analysis.

Statistical analyses. Association testing on the imputed data was performed in SNPTEST[39,40] using an additive model (-frequentist 1) and full dosage scores (-method expected) with sex and the first six principal components fitted as covariates. The genomic inflation factor λ was calculated to investigate the presence of population stratification and inflation. The *P* values were corrected for the genomic inflation factor λ. Quantile-quantile and Manhattan plots were created in R[41]. Regional association plots for the regions reaching genome-wide significance were created using LocusZoom[42].

To investigate whether any hits identified in the discovery cohort were driven by a subset of controls affected by the other diseases (i.e., esophageal cancer, Barrett's esophagus or inflammatory bowel diseases), we also performed a genome-wide association analysis after removing the controls that were affected by these other diseases (the structure of controls in the discovery cohort is provided in the **Supplementary Note**). This analysis included 1,155 cases with glaucoma and 1,147 controls.

Associations of the top autosomal SNPs in the discovery cohort ($P < 1 \times 10^{-4}$) (stage 1) were investigated in the replication cohorts (stages 2 and 3) (the structure of the replication cohorts, QC protocols and statistical analyses for each cohort are provided in the **Supplementary Note**). Stage 2 included two Australian replication data sets (total of 932 cases and 6,862 controls), and stage 3 included two US cohorts (total of 2,616 cases and 2,634 controls). For replication in stage 2, 24 SNPs with the best *P* values in the discovery cohort were used as the lead SNPs for the autosomal regions with $P < 1 \times 10^{-4}$ (**Supplementary Table 4**). The SNPs that were nominally replicated in stage 2 ($P < 0.05$) were taken forward for replication in stage 3. To make maximum valid use of our cohorts, for replication we focused on SNPs directly genotyped on the Illumina Human610 and Human670 arrays. Because some of the cases in stage 2 were genotyped on a non–genome wide platform (Sequenom), we could not accurately evaluate the imputed SNPs from stage 1. Hence, the most-associated SNP upstream of *ABCA1* (rs2472493) and SNPs in high LD with the most-associated SNP near *ARHGEF12* (rs11217878 and rs2276035, $r^2 = 1$ and $r^2 = 0.94$, respectively, with rs11827818) were used in the replication studies. Similarly, SNPs in high LD with the most-associated SNP in *GMDS* (rs2761233 and rs11969985, $r^2 = 0.93$ and $r^2 = 0.87$, respectively, with rs114096562) were used for replication studies.

Fixed-effects meta-analysis for the top SNPs was performed between the discovery and replication cohorts in METAL[43] using the effect sizes and their standard errors for the risk alleles. The presence of heterogeneity between cohorts for the effect sizes of risk alleles was investigated using the I^2 statistic as implemented in METAL.

Identifying candidate genes. Candidate genes in the regions of association were selected on the basis of the location and function of the genes, the pathways that the genes are involved in, the tissue location of expression of the gene and whether similar phenotypes have been reported to be caused by mutations in these genes. This information was found in Ensembl[44], NCBI, UCSC genome Bioinformatics[45], Genecards[46] and UniprotKB[47], as well as in available published data. To predict functional effects of the top POAG-associated SNPs identified in this study, we used the ENCODE project data[12] and the associated databases RegulomeDB[16] and HaploReg v2 (ref. 14). We used the Genevar database[48] to investigate eQTLs within genetic regions of interest.

Expression analysis of genes at associated loci in ocular tissues and cells. Ocular tissues from post-mortem human eyes were obtained through the Eye Bank of South Australia according to guidelines of the Southern Adelaide Clinical Human Research Ethics Committee. Normal and glaucomatous trabecular meshwork cell lines, NTM-5 and GTM-3, respectively, were a kind gift from C. Abbot (Alcon Research Ltd.). Both the cell lines tested negative for mycoplasma contamination. Total RNA was extracted using the RNeasy Mini Kit (Qiagen Pty Ltd.). First-strand cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen, Life Technologies Australia Pty Ltd.) and random hexamers. PCR was performed using Hot Star Taq Plus polymerase (Qiagen) and gene-specific primers (**Supplementary Table 9**). PCR was performed at the conditions specified in **Supplementary Table 9**. The enzyme was activated at 95 °C for 5 min, denaturation was at 95 °C for 30 s and elongation was at 72 °C. Additional elongation at 72 °C for 5 min was allowed after completion of the amplification cycles. The specificity of each amplified product was confirmed by sequencing.

Immunohistochemical labeling. Eye tissue was fixed in neutral buffered formalin and embedded in paraffin. For immunolabeling, 4-µm sections were blocked with 5% normal goat serum and incubated with the mouse anti-ABCA1 (1:2,000, Ab66217, Sapphire Biosciences) or anti-AFAP (1:1,000, 610200, BD Transduction Laboratories) primary antibody at 4 °C overnight. Primary antibody binding was detected with the Novolink Polymer detection kit (Leica Microsystems) and Chromogen substrate coloration (Dako). Sections were counterstained with haematoxylin and mounted in dePeX (Merck KGaA). Light microscopy was performed on an Olympus BX50 brightfield upright microscope with a Q-Imaging color charge-coupled device (CCD) camera attached; images were taken using the QCapture software (Q-Imaging Corporate).

Western blotting. For western blotting, proteins from NTM-5 and GTM-3 human trabecular meshwork cells established from a normal individual and an individual with glaucoma, respectively, were extracted in RIPA buffer, analyzed by SDS-PAGE using the mini-PROTEAN TGX gel and transferred onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories Pty. Ltd.). Western blotting was performed using the mouse anti-ABCA1 (1:500, Ab66217, Sapphire Biosciences) or anti-AFAP (1:250, 610200, BD Transduction Laboratories) primary antibody followed by hybridization with the hydrogen peroxide–conjugated goat anti-mouse IgG secondary antibody (1:1,000, 115-035-003, Jackson ImmunoResearch Laboratories Inc.). ABCA1 antibody binding was detected using the Pierce SuperSignal West Pico (Jackson ImmunoResearch

Laboratories Inc.), and AFAP1 antibody binding was detected using ECL Prime (GE Healthcare Australia and New Zealand) chemiluminescence reagents.

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Meta-analysis of genome-wide association studies identifies novel loci that influence cupping and the glaucomatous process

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Glaucoma is characterized by irreversible optic nerve degeneration and is the most frequent cause of irreversible blindness worldwide. Here, the International Glaucoma Genetics Consortium conducts a meta-analysis of genome-wide association studies of vertical cup-disc ratio (VCDR), an important disease-related optic nerve parameter. In 21,094 individuals of European ancestry and 6,784 individuals of Asian ancestry, we identify 10 new loci associated with variation in VCDR. In a separate risk-score analysis of five case-control studies, Caucasians in the highest quintile have a 2.5-fold increased risk of primary open-angle glaucoma as compared with those in the lowest quintile. This study has more than doubled the known loci associated with optic disc cupping and will allow greater understanding of mechanisms involved in this common blinding condition.

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Optic nerve degeneration caused by glaucoma is the most common cause of irreversible blindness worldwide[1]. Glaucomatous optic neuropathy is recognized by changes in the morphology of the optic nerve head, or optic disc, caused by loss of retinal ganglion cells and thinning of the retinal nerve fibre layer. In glaucoma, the nerve fibre layer typically thins in the superior and inferior regions of the nerve creating a vertically elongated depression (the cup). The ratio of the cup to the overall nerve size (the disc), called the vertical cup-disc ratio (VCDR), is a key factor in the clinical assessment and follow-up of patients with glaucoma. VCDR has been shown to be heritable with h^2 scores ranging between 0.48 and 0.66[2–7]. At least seven loci have been associated with VCDR in previous genome-wide association studies (GWAS) and three of these were subsequently implicated in primary open-angle glaucoma (POAG)[8–11]. So far, the explained variance of open-angle glaucoma by age, sex, intraocular pressure and established POAG genes is still small (4–6%)[12]. As with other complex diseases, large sample sizes are needed to ensure sufficient power to fully define the underlying genetic architecture.

Here, we report the largest genome-wide meta-analysis for VCDR, with data from 14 studies from Europe, the United States, Australia and Asia, as part of the International Glaucoma Genetics Consortium. The aim of the study is to identify loci associated with VCDR, and to determine whether these variants are also associated with glaucoma.

We perform the meta-analysis in four stages. In the first stage, we meta-analyse summary data from 10 populations of European ancestry comprising 21,094 individuals. In the second stage, we test the cross-ancestry transferability of the statistically genome-wide-significant associations from the first stage in 6,784 individuals from four Asian cohorts. In the third stage, we examine whether the associations are independent of disc area and/or spherical equivalent. We also combine the genome-wide-significant effects into a genetic risk score and associate this score with the POAG risk in five populations. Finally, we perform gene-based tests and pathway analysis.

We find 10 new loci associated with VCDR, which together increase the risk on POAG 2.5 times. Our findings will help us to unravel the pathogenesis of glaucoma.

Results

Meta-analysis of GWAS. In stage 1, we analysed ~2.5 million HapMap stage 2 single-nucleotide polymorphisms (SNPs)—either directly genotyped or imputed in 21,094 subjects of European ancestry (Supplementary Fig. 1; Supplementary Table 1; Supplementary Methods). The inflation factors (λ) varied between 0.98 and 1.12, implying adequate within-study control of population substructure (Supplementary Table 2; Supplementary Figs 2 and 3). The overall λ was 1.05. This analysis yielded 440 genome-wide-significant SNPs ($P<5.0\times10^{-8}$) located across 15 chromosomal regions (Table 1; Supplementary Fig. 4a). In stage 2, we investigated the SNP with the strongest association at each region in the Asian populations and found that eight were nominally significant ($P<0.05$) with an effect in the same direction and generally the same order of magnitude (Table 1; Supplementary Fig. 4b). Five of the seven loci that did not reach nominal significance in those of Asian descent had a similar effect in the same direction. Supplementary Table 3 shows the most significant SNPs in Asians within 100,000 base pairs from the most significant associated SNP in Europeans. Meta-analysis of only the Asian populations did not result in new genome-wide-significant findings. The combined analysis of the European and Asian populations resulted in three additional genome-wide-significant associations on chromosomes 1, 6 and 22 (Table 1; Fig. 1). The level of heterogeneity across the samples are shown in Table 1. Of the 18 genome-wide-significant loci, 10 are novel for the VCDR outcome (*COL8A1*, *DUSP1*, *EXOC2*, *PLCE1*, *ADAMTS8*, *RPAP3*, *SALL1*, *BMP2*, *HSF2* and *CARD10*) (Supplementary Fig. 5). There were no significant differences in terms of allele frequencies across the different cohorts (Supplementary Table 4). The effect estimates from the participating cohorts appear not to be influenced by main demographic characteristics, such as mean age and sex ratio (Supplementary Fig. 6).

Adjustment for disc area and spherical equivalent. Four of the 18 genome-wide-significant loci have been previously associated with optic disc area (*CDC7/TGFBR3*, *ATOH7*, *SALL1* and *CARD10*)[10,13]. Because the size of the optic nerve varies between individuals and is correlated to the VCDR[14], we adjusted the association to VCDR for optic nerve (disc) area. This resulted in a reduced effect size and significance ($P=3.48\times10^{-11}$ to $P=9.00\times10^{-3}$) at the *CDC7–TGFBR3* locus, suggesting the VCDR association at this locus is explained primarily by its known association with disc area (Supplementary Table 5a–c). A similar reduction in effect was seen for *ATOH7*. However, for this locus there remains a significant disc-area-independent effect ($P=7.28\times10^{-9}$). There was no change in association significance for any of the 10 new loci reported here, suggesting they do not act primarily on disc area.

It is of interest that two genes (*SIX6* and *BMP2*) overlap with those implicated in myopia[15], an important risk factor for POAG[16]. The correlation between VCDR and spherical equivalent is low (Supplementary Table 6), and adjusting for spherical equivalent did not lead to any major changes in the effects for these or other loci in European populations (Supplementary Table 7a), suggesting a joint genetic aetiology for POAG and myopia. In Asian cohorts, the direction of effect on VCDR at the chromosome 11 locus (*MIR612-SSSCA1* region) was not consistent with the European populations (Supplementary Table 7b). However, after adjusting for spherical equivalent the direction of effect on VCDR was similar to both populations. At the *BMP2* myopia locus, we observed a large difference in allele frequency between those of European and Asian ancestry (Table 1), which may explain the difference in effect direction.

Risk for POAG. The 18 loci, together with age and sex, explain 5.1–5.9% of the VCDR phenotypic variability in Europeans (measured in the Rotterdam Study I, II and III), of which 1.6–1.8% is explained by the new loci. The phenotypic variability explained by all common SNPs is 41–53% in these cohorts, which is in line with the heritability estimates from family-based studies. In addition to confirming the previously published *CDKN2BAS* and *SIX1/6* POAG risk loci, we found nominally significant ($P<0.05$) associations with POAG for six newly identified genetic variants ($P=8.1\times10^{-5}$ from binomial test for chance of seeing six or more such nominally significant associations in 16 tests) (Supplementary Table 8), with odds ratios varying between 0.73 and 1.20. In the combined case-control studies, we found that the sum of all effects of these genes increased the risk of POAG 2.5-fold (Supplementary Table 9) for those in the highest quintile compared with those in the lowest quintile.

Gene-based test. To identify new loci not previously found through individual SNP-based tests, we performed gene-based tests using VEGAS software[17]. Because of the smaller number of tests (17,872 genes tested), our gene-based significance threshold is $P_{\text{gene-based}}<0.05/17{,}872=2.80\times10^{-6}$. In addition to the SNPs

Table 1 | Summary of the results of the meta-analyses of genome-wide association studies.

						Caucasians (n = 21,094)					Asians (n = 6,784)					Combined (n = 27,878)				
SNP	Chr	Position	Nearest Gene	Annotation	A1 / A2	MAF	β	s.e.	P value	P value hetero-geneity	MAF *	β	s.e.	P value	P value hetero-geneity	β	s.e.	P value	P value hetero-geneity	I^2
rs4658101	1	91849997	*CDC7/TGFBR3*	intergenic	a/g	0.18	0.015	0.002	8.80E–14	9.34E–02	0.14	0.016	0.005	3.13E–03	4.26E–01	0.015	0.002	1.06E–15	1.68E–01	0.54
rs2623325	3	100614445	*COL8A1*	intergenic	a/c	0.13	0.018	0.003	7.05E–09	5.62E–02	0.16	0.011	0.005	1.46E–02	3.43E–01	0.016	0.003	6.61E–10	7.01E–02	0.42
rs17658229	5	172123657	*DUSP1*	intergenic	c/t	0.05	–0.020	0.004	8.06E–09	5.95E–01	0.00	–0.086	0.133	5.17E–01	**	–0.020	0.004	8.06E–09	5.95E–01	0
rs17756712	6	570071	*EXOC2*	intronic	g/a	0.18	0.010	0.002	1.98E–08	6.74E–01	0.14	0.011	0.005	1.76E–02	4.05E–01	0.010	0.002	1.13E–09	7.23E–01	0
rs7865618	9	22021005	*CDKN2BAS*	intronic	g/a	0.43	–0.013	0.001	2.80E–20	8.93E–01	0.15	–0.021	0.005	8.11E–06	3.31E–01	–0.013	0.001	4.97E–24	6.97E–01	0
rs1900005	10	69668061	*ATOH7*	intergenic	a/c	0.23	–0.019	0.002	7.21E–31	2.96E–04	0.32	–0.010	0.004	2.08E–02	1.58E–01	–0.018	0.002	5.51E–31	8.54E–05	0.69
rs7072574	10	96026296	*PLCE1*	intronic	a/g	0.33	0.009	0.002	6.17E–09	1.09E–01	0.38	0.007	0.003	4.80E–02	8.18E–01	0.009	0.001	1.02E–09	2.56E–01	0.18
rs1346	11	65093827	*SSSCA1*	5upstream	t/a	0.19	–0.014	0.002	2.54E–15	7.49E–01	0.16	0.003	0.005	5.23E–01	7.19E–01	–0.012	0.002	4.89E–13	1.51E–01	0.28
rs4936099	11	129785935	*ADAMTS8*	intronic	c/a	0.42	–0.009	0.002	6.38E–09	8.31E–01	0.09	–0.007	0.009	4.15E–01	1.14E–01	–0.009	0.002	4.61E–09	6.79E–01	0
rs11168187	12	46330278	*RPAP3*	intergenic	g/a	0.16	–0.009	0.002	2.96E–08	1.00E+00	0.18	–0.005	0.004	2.80E–01	6.19E–01	–0.009	0.002	2.96E–08	9.98E–01	0
rs10862688	12	82447043	*TMTC2*	intergenic	g/a	0.45	0.008	0.001	1.24E–11	4.80E–02	0.56	0.004	0.003	2.48E–01	1.20E–01	0.008	0.001	1.49E–11	2.61E–02	0.44
rs4901977	14	59858929	*SIX1/6*	intergenic	t/c	0.31	0.010	0.002	1.98E–11	7.86E–01	0.53	0.017	0.003	2.64E–07	3.82E–02	0.011	0.001	2.13E–16	2.02E–01	0.22
rs1345467	16	50039822	*SALL1*	intergenic	g/a	0.27	0.010	0.002	2.70E–12	1.68E–01	0.13	0.011	0.006	5.53E–02	4.13E–01	0.010	0.001	4.19E–13	2.48E–01	0.18
rs6054374	20	6526556	*BMP2*	intergenic	t/c	0.42	–0.009	0.002	1.79E–08	1.26E–01	0.72	0.001	0.004	8.66E–01	5.99E–01	–0.007	0.001	1.69E–07	8.19E–02	0.37
rs1547014	22	27430711	*CHEK2*	intronic	t/c	0.30	–0.013	0.001	2.98E–18	1.93E–01	0.17	–0.013	0.004	4.26E–03	8.11E–01	–0.013	0.001	4.77E–20	3.90E–01	0.06
rs301801	1	8418532	*RERE*	intronic	c/t	0.33	0.008	0.001	1.61E–07	2.46E–02	0.13	0.012	0.005	2.59E–02	5.38E–01	0.008	0.001	1.66E–08	5.23E–02	0.39
rs868153	6	122431654	*HSF2*	intergenic	g/t	0.36	–0.007	0.001	5.08E–06	9.27E–01	0.39	–0.013	0.003	1.44E–04	4.96E–01	–0.007	0.001	1.39E–08	7.96E–01	0
rs5756813	22	36505423	*CARD10*	intergenic	g/t	0.39	0.006	0.001	1.60E–05	8.22E–01	0.32	0.017	0.004	1.71E–06	1.84E–01	0.008	0.001	7.73E–09	1.98E–01	0.22

Chr., chromosome; MAF, minor allele frequency; SNP, single-nucleotide polymorphism.
Summary of SNPs that showed genome-wide-significant ($P<5\times10^{-8}$) association with vertical cup-disc ratio (VCDR) in subjects of European ancestry (stage 1), with results of replication in Asians (stage 2) and the additional SNPs that showed genome-wide-significant ($P<5\times10^{-8}$) association in the combined analysis (stage 3) (*P* values were calculated by using the *z*-statistic). We tested for heterogeneous effects between the Asian and European ancestry samples, for which *P* values are shown (Cochran's *Q*-test). Nearest gene, reference NCBI build 37; A1, reference allele; A2, other allele; MAF, average minor allele frequency; β, effect size on VCDR based on allele A1; s.e., s.e. of the effect size. The last three rows indicate the SNPs that reached genome-wide significance in the combined analysis, but not in stage 1 or stage 2.
*Note that, for the sake of keeping the same reference allele, MAF values may be >0.50 in the Asian populations.
**For this SNP, only one Asian study is contributing to the meta-analysis, so the *P* value for heterogeneity could not be calculated for this SNP in stage 2.

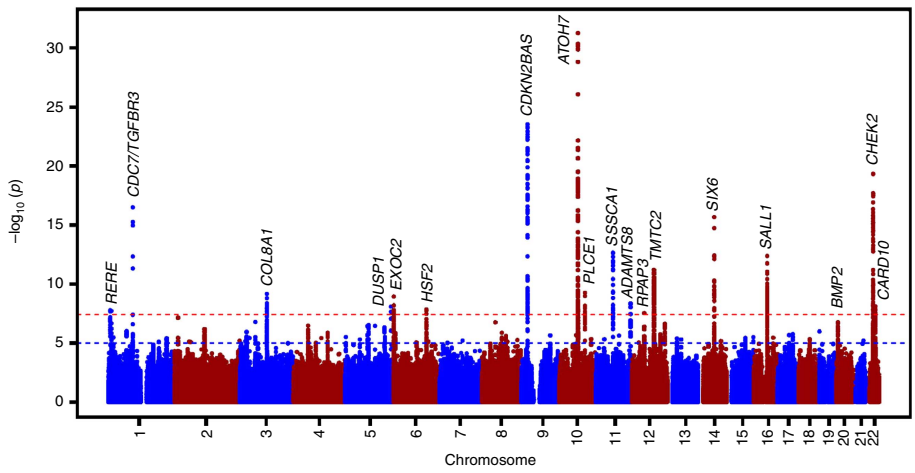

Figure 1 | Manhattan plot of the GWAS meta-analysis for vertical cup-disc ratio in the combined analysis (n = 27,878). The plot shows $-\log_{10}$-transformed *P* values for all SNPs (*z*-statistic). The red-dotted horizontal line represents the genome-wide significance threshold of $P<5.0\times10^{-8}$; the blue-dotted line indicates *P* value of 1×10^{-5}.

identified as significant ($P<5\times10^{-8}$) in a SNP-based test, we also found two new genes significantly associated with VCDR using the VEGAS gene-based test (Supplementary Table 10). These were *REEP5* ($P=7.48\times10^{-7}$) and *PITPNB* ($P=4.89\times10^{-7}$). *PITPNB* is ~800 kb from another gene with a significant SNP association (*CHEK2*, rs1547014) (Supplementary Fig. 7). Although the association signal centred over *CHEK2* extends a long distance towards *PITPNB*, a separate association peak over *PITPNB* can be observed, which is unrelated (no linkage disequilibrium (LD)) to the *CHEK2* peak. The results we obtained using the specified definition of the gene unit were substantially the same when alternative cutoff points from the transcription initiation and end sites were used (Supplementary Table 11). The *REEP5* gene showed no association with POAG

(Supplementary Table 12). The *PITPNB* gene showed evidence for association with POAG in Australian & New Zealand Registry of Advanced Glaucoma (ANZRAG) ($P=0.03$) in the gene-based test, with a best single SNP P value of 0.003, but this was not confirmed in two other studies.

Pathway analysis. To test whether gene-based statistics identified were enriched in 4,628 pre-specified Gene Ontology pathways, we performed pathway analysis using Pathway-VEGAS[18]. We used a pathway-wide significance threshold to be 1.08×10^{-5} (0.05/4,628). The only pathway exceeding the pathway-wide significance level was 'negative regulation of cyclin-dependent protein kinase activity' (Supplementary Table 13). The second top-pathway 'negative regulation of epithelial cell proliferation' is related to the top pathway, both suggesting retardation of cell growth. The 'negative regulation of cyclin-dependent protein kinase activity' finding was driven not only by the result at the *CDKN2A* locus but also by the result at *APC*, a gene close to *REEP5*.

Regulatory elements and expression data. Six of the 18 most associated SNPs are located in DNase I hypersensitivity sites (Supplementary Table 14). The retinal pigment epithelium has the highest signal of all 125 available cell lines in one of these DNase I hypersensitivity sites. Thus, these results are suggesting that some of the SNPs may have their effect on VCDR by altering regulatory functions. We investigated the expression of the genes implicated in VCDR by these analyses in human ocular gene expression databases or the published literature. Most of these genes are expressed in eye tissues, including the optic nerve (Supplementary Tables 15 and 16).

Discussion

This study reports 10 novel loci associated with VCDR, with an additional two loci identified using gene-based testing. Pathway analysis suggests retardation of cell growth as a major biological mechanism. The results for the most associated pathways 'negative regulation of cyclin-dependent protein kinase activity' and 'negative regulation of epithelial cell proliferation' are primarily driven by the *CDKN2A* and *CDKN2B* genes, respectively, but in both pathways the gene-based result at *APC* ($P=7.20\times10^{-5}$ in Caucasians and $P=8.80\times10^{-3}$ in Asians) also contributes to the pathway result. The *APC* gene has previously been reported to be a critical gene regulating retinal pigment epithelium proliferation and development[19]. These results add to our earlier findings on the role of growth and the transforming growth factor beta (TGFB) pathways in VCDR[10]. Various new genes fall into these pathways. The protein encoded by the *BMP2* (bone morphogenetic protein 2) gene on chromosome 20 belongs to the TGFB super-family. Two other new genes regulate apoptosis: *RPAP3* (RNA polymerase II-associated protein 3) on chromosome 12[20] and *CARD10*, a gene that was previously found to be associated with disc area[13]. Another new VCDR association previously associated with disc area is *SALL1*[10]. This gene is implicated in ocular development.

Our findings offer new insights in the aetiology of optic nerve degeneration. *COL8A1* (collagen, type VIII, alpha 1) is part of a collagen pathway recently implicated in corneal thickness[18], an ocular trait also associated with glaucoma risk. Missense mutations in *COL8A2* (collagen, type VIII, alpha2) were found in POAG patients with a very thin central corneal thickness (CCT)[21]. The collagen SNP (rs2623325) was not significantly associated with CCT (in Caucasians: $\beta=-0.044$, $P=0.19$; in Asians: $\beta=0.007$, $P=0.89$) or intraocular pressure (in Caucasians and Asians combined: $\beta=-0.02$, $P=0.73$) in largely the same cohorts[18,22], suggesting that the collagen involvement in VCDR is not due to the influence by CCT or intraocular pressure. We also found several genes involved in cellular stress response. *DUSP1* (dual specificity phosphatase 1) is the nearest gene to the most strongly associated SNP on chromosome 5. This gene, inducible by oxidative stress and heat shock, may play a role in environmental stress response[23], and may also participate in the negative regulation of cellular proliferation. *HSF2* (heat shock transcription factor 2), one of the genes at the chromosome 6 locus, also is part of the cellular stress response pathway. Deficiency of this factor causes various central nervous system defects in mice[24,25]. Another pathway emerging in this study is that of exocytosis. The SNP on the other chromosome 6 locus is located in *EXOC2* (exocyst complex component 2). The encoded protein is one of the eight proteins of the exocyst complex[26]. This multi-protein complex is important for directing exocytic vesicles to the plasma membrane, a mechanism that also has been implicated in neuronal degeneration in the brain[27]. Lipid metabolism emerges as another pathway. The gene on chromosome 10, *PLCE1* (phospholipase C, epsilon 1), belongs to the phospholipase C family, which plays a role in the generation of second messengers[28]. Various processes affecting cell growth, differentiation and gene expression are regulated by these second messengers. From a clinical perspective, the findings on *ADAMTS8* are of interest. ADAMTS enzymes have different functions, including the formation and turnover of the extracellular matrix[29]. Strikingly, a variant in *ADAMTS10* has been linked to a form of glaucoma in dogs[30,31].

In summary, we have now identified 10 novel loci associated with cupping of the optic nerve, a key determinant of glaucoma. Together, these genetic risk variants increased the risk of POAG in case-control validation studies. Pathway analysis implicated negative regulation of cell growth and cellular response to environmental stress as key pathological pathways in glaucoma, and that novel therapies targeting these pathways may be neuro-protective in glaucoma.

Methods

Study design. We performed a meta-analysis on directly genotyped and imputed SNPs from individuals of European ancestry in 10 studies, with a total of 21,094 individuals. Subsequently, we evaluated significantly associated SNPs in 6,784 subjects of Asian origin including four different studies and performed a meta-analysis on all studies combined.

Subjects and phenotyping. All studies included in this meta-analysis are part of the International Glaucoma Genetics Consortium. The ophthalmological examination of each study included an assessment of the optic nerve head to measure the VCDR (Supplementary Table 17a). Unreliable optic nerve data were excluded.

The meta-analysis of stage 1 was based on 10 studies of European ancestry: Brisbane Adolescent Twin Study, Blue Mountains Eye Study, Erasmus Rucphen Family Study, Gutenberg Health Study (GHS I/GHS II), Glaucoma Genes and Environment (controls only), National Eye Institute Glaucoma Human Genetics Collaboration (NEIGHBOR; controls only), Raine Study, Rotterdam Study (RS-I/RS-II/RS-III), Twins Eye Study in Tasmania and TwinsUK. Stage 2 comprised four Asian studies: Beijing Eye Study, Singapore Chinese Eye Study, Singapore Malay Eye Study and Singapore Indian Eye Study. For each SNP with the strongest association at each locus the association with POAG was tested in five case-control studies: ANZRAG, deCODE, Massachusetts Eye and Ear Infirmary, NEIGHBOR and Southampton.

Information on general methods, demographics, phenotyping and genotyping methods of the study cohorts can be found in Supplementary Tables 1 and 17 and the Supplementary Note. All studies were performed with the approval of their local medical ethics committee, and written informed consent was obtained from all participants in accordance with the Declaration of Helsinki.

Genotyping and imputation. Information on genotyping in each cohort and the particular platforms used to perform genotyping can be found in more detail in Supplementary Table 17b. To produce consistent data sets and enable a meta-analysis of studies across different genotyping platforms, the studies performed

genomic imputation on available HapMap Phase 2 genotypes with MACH[32] or IMPUTE[33], using the appropriate ancestry groups as templates.

Each study applied stringent quality control procedures before imputation, including minor allele frequency cutoffs, Hardy–Weinberg equilibrium, genotypic success rate, mendelian inconsistencies, exclusion of individuals with >5% shared ancestry (exception made for family-based cohorts in which due adjustment for family relationship was made) and removal of all individuals whose ancestry as determined through genetic analysis did not match the prevailing ancestry group of the corresponding cohort (Supplementary Note). SNPs with low imputation quality were filtered using metrics specific to the imputation method and thresholds used in previous GWAS analyses. For each cohort, only SNPs with imputation quality scores >0.6 (proper-info of IMPUTE) or R2>0.6 (MACH) were included into the meta-analysis.

Statistical analysis. In subjects drawn from their respective populations in which the prevalence of glaucomatous changes is relatively low, the correlation between left and right eye is high[34]. Therefore, we used the mean VCDR of both eyes. In cases of missing or unreliable data for one eye, data of the other eye was taken. Each individual study did a linear regression model between the VCDR and the SNPs under the assumption of an additive model for the effect of the risk allele. Analyses were adjusted for age, sex and the first two principal components (for population-based studies) or family structure (for family-based studies). Secondary analyses were done with adjustments for disc area or spherical equivalent. In the Rotterdam Studies, we calculated the phenotypic variability explained by the new loci, and explained by all common SNPs using the 'Genome-wide Complex Trait Analysis' tool[35,36].

We performed an inverse variance weighted fixed-effect meta-analysis. This was performed with METAL software[37]. *P* values for the association results were calculated by using the *z*-statistic. *P* values for heterogeneity were calculated by using the Cochran's *Q*-test for heterogeneity. In addition to this, I^2 values were calculated to assess heterogeneity[38]. F_{st} values were calculated to assess the genetic variation due to subdivision of populations. All study effect estimates were corrected using genomic control and were oriented to the positive strand of the NCBI Build 36 reference sequence of the human genome, which was the genomic build on which most available genotyping platforms were based. Coordinates and further annotations for the SNPs were converted into Build 37, the most recent version of the available builds at the time of this study.

In stage 1, a *P* value $<5.0\times10^{-8}$ (the genome-wide threshold of association) was considered significant. In stage 2, a *P* value <0.05 was considered significant. Manhattan, regional and forest plots were made using *R*[39], LocusZoom[40] and Stata/SE 12.0 (StataCorp LP, College Station, TX, USA).

Risk-score models. In five case-control studies, a weighted genetic risk score per individual was calculated. Standardized regression coefficients were used as weighting factor. The weighted risk scores were divided into quintiles. Odds ratios were calculated for each quintile, using the first quintile as a reference.

Gene-based test using VEGAS. There are different gene-based tests of which VEGAS is one of the most powerful tests[41]. We therefore performed gene-based testing using VEGAS software[17], which combines the test statistics of all SNPs present within and 50 kb upstream/downstream of each gene. LD between the markers is accounted for through simulations from the multivariate normal distribution, based on estimates of LD from reference populations. Since Asian and European ancestry populations show different LD patterns, we performed separate gene-based tests for each population. Hapmap 2 CEU population was used as a reference to calculate LD for European ancestry data, whereas Hapmap 2 JPT and CHB combined population was used as a reference for Asian ancestry data. After calculation of gene-based test statistics for Asian and European ancestry populations separately, meta-analysis was conducted using Fisher's method for combining *P* values. VEGAS was applied to the summary data from the full VCDR analysis (as in Table 1) and to three of the POAG data sets; ANZRAG, Massachusetts Eye and Ear Infirmary glaucoma clinic and Glaucoma Genes and Environment (Supplementary Note).

Pathway-analysis using pathway-VEGAS. Pre-specified pathways from the Gene Ontology database with size ranging in 5–500 genes were used to perform pathway analysis. Pathway-VEGAS combines VEGAS gene-based test statistics based on pre-specified biological pathways[18]. Pathway *P* values were computed by summing χ^2-test statistics derived from VEGAS *P* values. Empirical 'VEGAS-pathway' *P* values for each pathway were computed by comparing the real-data-summed χ^2-test statistics with 500,000 simulations where the relevant number (as per size of pathway) of randomly drawn χ^2-test statistics was summed. To ensure clusters of genes did not adversely affect results, within each pathway, gene sets were pruned such that each gene was >500 kb from all other genes in the pathway. Where required, all but one of the clustered genes was dropped at random when genes were clustered. Pathway-VEGAS was performed separately for European and Asian ancestry data sets. Meta-analysis was conducted using Fisher's method for combining *P* values.

Regulatory functions. We used the ENCyclopedia Of DNA Elements[42] data in the UCSC Genome Browser[43] to look at DNase I hypersensitivity sites and other functional elements.

Gene expression in human eye tissue. We examined the expression of genes that reached significance in the individual SNP-based test or gene-based test. We used published literature or human ocular gene expression databases (Supplementary Tables 15 and 16).

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