An Investigation of Genetic Risk Factors in Primary Open-Angle Glaucoma

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DECLARATION

I, Soo Park, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Primary open-angle glaucoma (POAG) is a multifactorial disease with a strong genetic component. Notably however, few genes have been robustly associated with POAG in the general population. Genes in which mutation causes anterior segment angle anomalies, including *LMX1B* and *FOXC1* are associated with a high incidence of glaucoma to about 33-75% and are strong candidates for glaucoma susceptibility. In addition, growth factors including *TGFβ2* and *BMP4* act in concert to maintain a balance between extracellular matrix (ECM) deposition and degradation and may play a role in glaucoma pathogenesis through misregulation of ECM synthesis of the trabecular meshwork (TM). Furthermore, *OPTN* E50K mutation, a known genetic locus for POAG, has been shown to account for a high percentage of 13.5% of familial normal-tension glaucoma (NTG) in individuals of white British origin. In this study, the contribution of variation at the *LMX1B, FOXC1, TGFβ2* and *BMP4* loci to risk of glaucoma was investigated in a case-control genetic association study in a cohort of white British descent recruited in the North-East of England comprised of 272 patients with high-tension glaucoma (HTG), 37 patients with NTG, 58 patients with ocular hypertension (OHT), and 276 normal controls. The role of *OPTN* E50K mutation in these unrelated white British individuals with POAG was also examined. No significant associations were identified for *FOXC1, TGFβ2* and *BMP4*. The *OPTN* E50K mutation was also absent in this cohort. The study identified a significant under representation of two *LMX1B* haplotypes [ATG; P = 5.0E-4 (permutation P = 0.01), GCAGAC; P = 5.0E-4 (permutation P = 0.0150)] among the POAG individuals compared to the control population, consistent with a 0.3 fold decreased risk of developing POAG. A replication study involving a second cohort of 222 NTGs and 108 HTGs recruited in London showed a similar distribution of the ATG haplotype (P = 0.0047) but did not withstand permutation testing.

In conclusion, *LMX1B* haplotypes may influence susceptibility to develop POAG in the white British population, suggesting altered *LMX1B* function predisposes to glaucomatous damage.

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AGIS	Advanced glaucoma intervention study
ARM	Axenfeld-Rieger malformation
ARS	Axenfeld-Rieger Syndrome
ASD	Anterior segment dysgenesis
ATP	Adenosine tri-phosphate
BMP4	Bone morphogenic protein 4 gene
CCT	Central corneal thickness
C/D ratio	Cup-disc ratio
CNV	Copy number variation
CIGTS	Collaborative Initial Glaucoma Treatment Study
CNTGS	Collaborative Normal Tension Glaucoma Study
CYP1B1	Cytochrome P450, subfamily I, polypeptide 1 gene
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphate
ECM	Extracellular matrix
EM algorithm	Expectation-maximisation algorithm
EMGT	Early Manifest Glaucoma Trial
FHD	Forkhead domain
FOX	Forkhead box
FOXC1	Forkhead box protein C1 gene
HWE	Hardy-Weinberg equilibrium
IOP	Intraocular pressure
JOAG	Juvenile open angle glaucoma
LIM-HD	LIM-homedomain
LMX1B	LIM homeobox transcription factor 1, beta gene
LD	Linkage disequilibrium
MYOC	Myocillin gene
NTG	Normal tension glaucoma
OAG	Open angle glaucoma
OHT	Ocular hypertension
OHTS	Ocular Hypertension Treatment Study

OPTN	Optineurin gene
PCR	Polymerase Chain Reaction
PITX2	Paired-like homeodomain transcription factor 2 gene
POAG	Primary Open Angle Glaucoma
PPi	Pyrophosphate
RGC	Retinal ganglion cell
RO	Reverse osmosis
SAP	Shrimp alkaline phosphatase
SBE	Small base extension
SNPs	Single nucleotide polymorphisms
TAE	Tris acetic acid EDTA electrophoresis buffer
TGFβ2	Transforming growth factor-β
tSNP	Tagging SNP
TM	Trabecular meshwork
UTR	Untranslated region
WDR36	WD repeat domain 36 gene

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Chapter 1 Introduction

1.1 Definition of glaucoma

Glaucoma is a common and heterogeneous group of optic neuropathies which is broadly classified into three main types: open angle, closed angle, and developmental (Shields *et al.*, 1996). The aetiology of glaucoma is retinal ganglion cell death resulting in a characteristic visual field loss and a typical excavation of the optic nerve head. Glaucoma is frequently associated with raised intraocular pressure (IOP>21mmHg), which is recognised as one of the strongest known risk factors (Wilson and Martone, 1996). The statistical 'normal range' for the IOP is 11 to 21 mmHg (2 standard deviation on either side of the mean IOP of 16 mmHg). It is of note that this is a statistically derived figure and does not suggest that disease is present if the IOP level exceeds this value. Hence, although there appears to be no absolute cut-off level, any value above 21 mmHg should be considered as suspicious. The Baltimore Eye Survey showed that the risk of glaucomatous optic nerve damage increased with raised level of IOP, especially at levels above 22 mmHg (Sommer *et al.*, 1991). However, despite this proven risk factor, there seems to be no clearly defined level of "safe" IOP. Glaucomatous damage can occur even with an IOP of 12mmHg and, on the other hand, be absent in eyes with IOP of 30mmHg (Infeld and O'Shea, 1998).

This study investigates several developmental glaucoma genes and a known genetic locus for familial normal-tension glaucoma (NTG), as genetic risk factors for primary open-angle glaucoma (POAG) and ocular hypertension (OHT) (IOP > 21 mmHg) in a cohort of white British individuals.

1.2 Classification of glaucoma

Glaucoma can be classified into three basic subtypes:

- Open angle
- Closed angle
- Developmental

Differentiation of open angle glaucoma from closed angle glaucoma is considered crucial from a therapeutic point of view. In addition, glaucomas can be further categorized into primary or secondary glaucoma. Primary glaucomas such as POAG are not associated with known ocular or systemic disorders that cause increased resistance to aqueous outflow (American Academy of Ophthalmology, 2004b). On the other hand, secondary glaucomas are associated with either ocular or systemic disorders that lead to increased resistance to aqueous outflow. Examples of POAGs include high-tension glaucoma (HTG) and NTG, with OHT being an important risk factor for POAG.

1.2.1 Primary open angle glaucoma

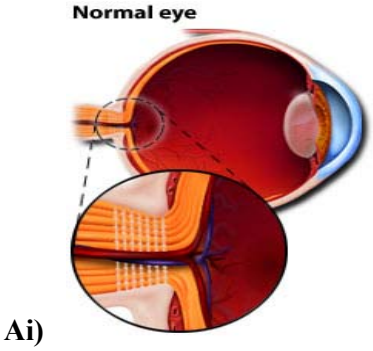
1.2.1.1 High tension glaucoma (HTG)

HTG is the commonest form of glaucoma with an estimated prevalence of 1.2% for the age group 40-89 years in the white population of the United Kingdom (Tuck and Crick, 1998). Its diagnosis is reached through exclusion of any identifiable cause (hence otherwise known as primary open angle glaucoma) and it is characterized by (Figure 1-1 and 1-2):

- Open anterior chamber angle on gonioscopic evaluation
- IOP > 21 mmHg at some stage during the course of disease
- Glaucomatous damage of the optic nerve head
- Characteristic visual field loss
- Adult onset (> 40 years of age)

1.2.1.2 Normal-tension glaucoma (NTG)

At the opposite end of the IOP spectrum is NTG, an important subtype of POAG. This is an optic neuropathy similar to HTG (i.e. open, normal appearance of anterior chamber angles, with presence of glaucomatous optic neuropathy) but with IOP levels within the statistically "normal range". Although by definition, IOP should never be above 21mmHg, it tends to be higher than those in the normal individuals (Gramer and Leydhecker, 1985). Whilst the bulk of the evidence has shown a significant effect of IOP on optic nerve damage in NTG (Araie *et al.*, 1994; Jonas *et al.*, 1998; Koseki *et al.*, 1997), some have shown no effect (Noureddin *et al.*, 1991). Hence, other factors may



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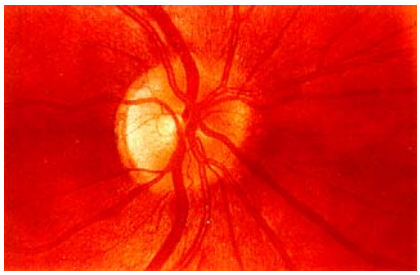

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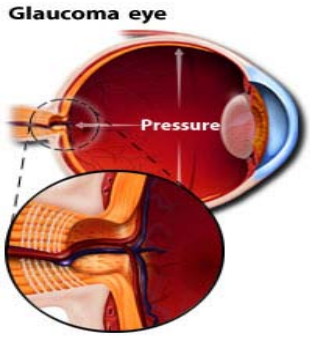

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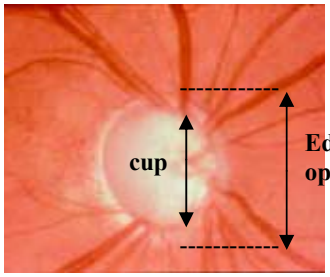

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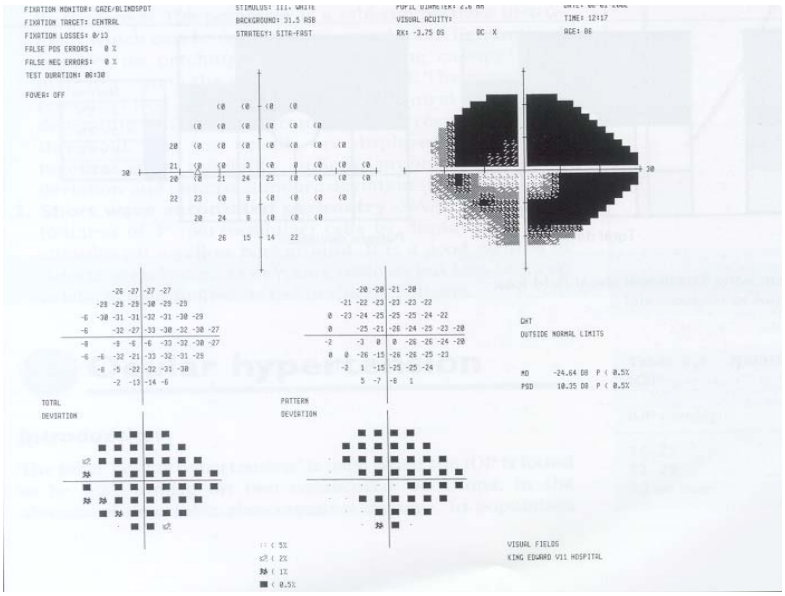


C)

Figure 1–1: Characteristic features of glaucoma.

(Ai) Cross section of the optic nerve in a normal eye and **(Aii)** the corresponding frontal view of the optic disc. **(Bi)** Cross section of the optic nerve in an eye with raised intraocular pressure and **(Bii)** the corresponding frontal view of the optic nerve showing end stage cupping of the optic head. **(C)** Humphrey's visual field (a form of a static perimetry which involves presentation of non-moving stimuli of varying illuminance) showing advanced visual field loss. Diagrams adapted from Clinical Ophthalmology, Kanski and from Merick Frosst Canada LTD.

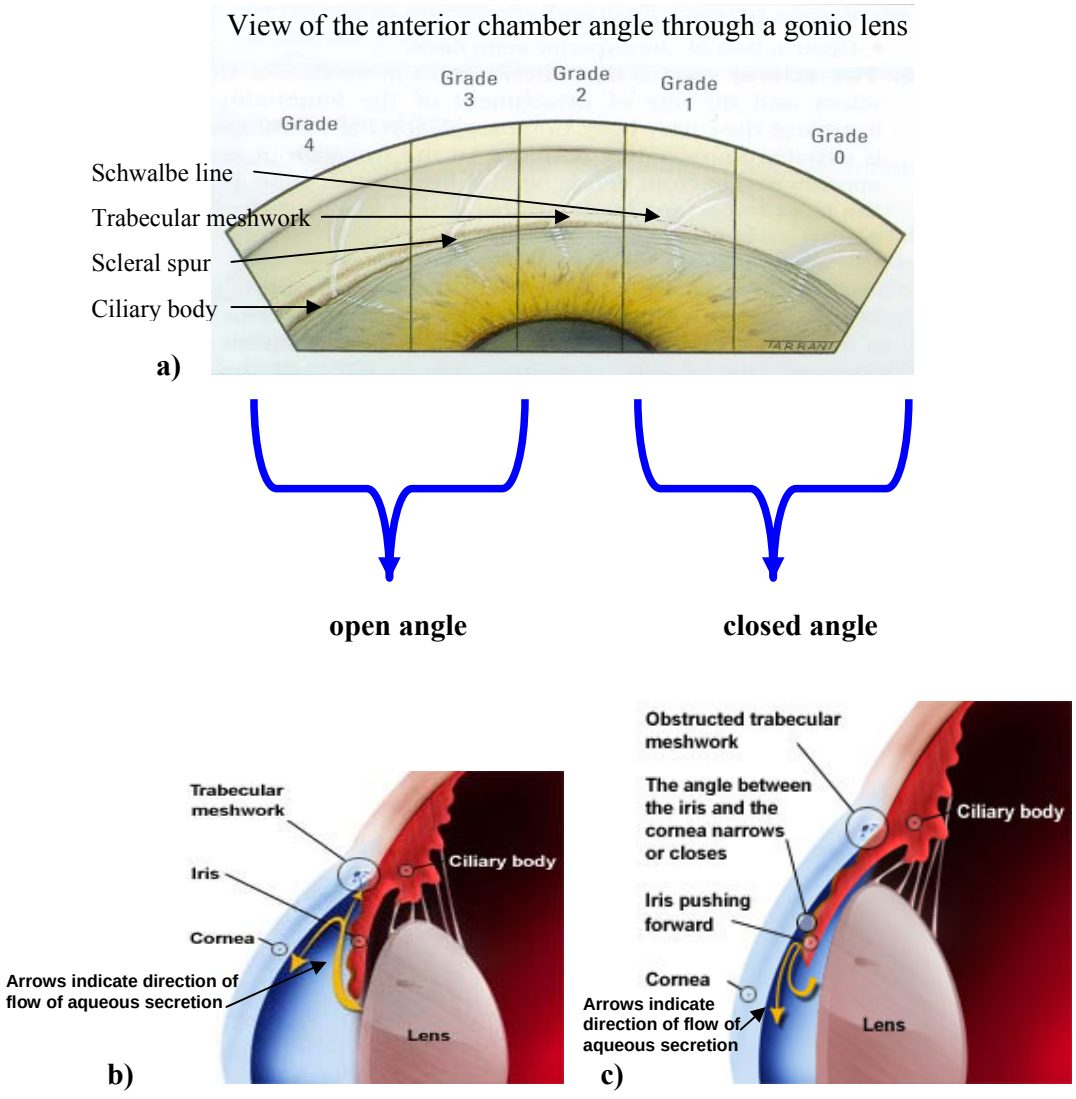


Figure 1–2: Width of the anterior chamber.

(a) Shaffer grading of anterior chamber angle width ranging from grade 4 being the widest (open) angle to grade 0 being the narrowest (closed) angle. **(b)** Cross section of the anterior segment with an open angle appearance which is typically found in POAG. **(c)** Cross section of the anterior segment with a closed angle appearance. Diagrams adapted from Clinical Ophthalmology, Kanski, and from Merick Frosst Canada LTD.

play an important role in NTG, most of which are still unknown. Studies have shown that individuals with NTG have higher prevalence of pressure independent associations such as abnormal ocular blood flow from vasospastic disorders (e.g. migraine and Raynaud's phenomenon (Corbett *et al.*, 1985; Phelps and Corbett, 1985), systemic hypotension (Bonomi *et al.*, 2000; Leske *et al.*, 2002; Leske *et al.*, 2008; Libby *et al.*, 2005) and coagulopathies (Drance *et al.*, 1973b)).

The prevalence of NTG in individuals over the age of 40 years is 0.2%, increasing up to 1.6% in those over 75 years of age (Klein *et al.*, 1992). It has been proposed that NTG has a predilection for females. Levene's review (Levene, 1980) showed an increased prevalence in females ranging from 6% to 75%. However, the Beaver Dam Eye Study found equal prevalence among sexes (Klein *et al.*, 1992).

The relation between NTG and HTG is debatable, with some supporting the idea that these two conditions are separate entities (Caprioli and Spaeth, 1984; Chumbley and Brubaker, 1976) whilst others support the idea that they are simply phenotypic variations of the same disease (Drance *et al.*, 1973a; Drance *et al.*, 1973b; Lewis *et al.*, 1983). In both NTG and HTG, the cupping of the optic disc, the visual field loss and the angle configuration are similar. On the other hand, shallower cupping, or saucerisation of the optic nerve head has been more commonly documented in NTG than HTG, with visual field defects in NTG appearing deeper and steeper. In addition, disc hemorrhages are more commonly encountered features of NTG (Sowka 2004).

Both HTG and NTG appear to represent a spectrum of glaucomas in which the mechanism of the glaucomatous optic disc damage shifts from predominantly elevated IOP in the former to additional IOP-independent factors in the latter, with a considerable overlap of causative factors. For the purpose of this study, NTG is considered as a subgroup of POAG.

1.2.2 Ocular hypertension

Ocular hypertension (OHT) is a term reserved for eyes in which the IOP is raised above the normal Caucasian population range of 21mmHg (i.e. two standard deviation above the mean IOP) (Leske, 1983). There are no clinical signs of a glaucomatous optic neuropathy (as evidenced by a normal appearance of the optic nerve and normal visual fields), and there is no ocular co-morbidity. OHT is an important risk factor for POAG, and the risk of POAG has been shown to increase with higher IOP (Gordon *et al.*, 2002). Due to its skewing of the normal distribution to the right, the prevalence of OHT is estimated to range from 4.5% to 9.4% for those who are >40 years old (Leske, 1983) rather than the expected 2.5% from a true normal distribution. The Ocular Hypertension Treatment Study (OHTS) has shown approximately 10% of individuals with OHT will convert to POAG over a ten year period (Kass *et al.*, 2002).

1.2.3 Angle closure glaucoma

Angle closure glaucomas comprise a diverse subgroup of glaucomas where the unifying theme is the presence of peripheral anterior synechiae (where the peripheral iris attaches anteriorly in the anterior chamber angle) and/or iridotrabecular apposition (American Academy of Ophthalmology, 2004a). As a result of this, there is reduced drainage of aqueous humor through the anterior chamber angle. The commonest cause of angle closure is a pupillary block (Figure 1-3). The flow of aqueous through the pupil from the posterior chamber is impeded. This leads to increase pressure in the posterior chamber causing the peripheral iris to bow forward against the trabecular meshwork (TM). For the purpose of this study which has assessed developmental genes and a known genetic locus for familial NTG as genetic risk factors for POAG, angle closure glaucomas have not been mentioned further.

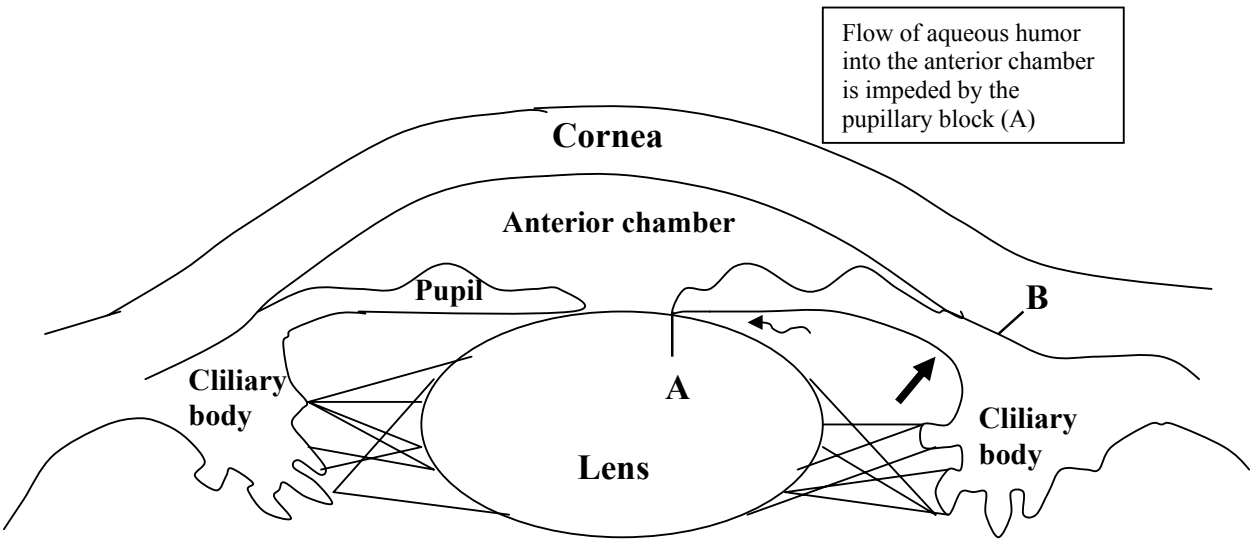


Figure 1–3: Pupillary block glaucoma.

A cross section view of the anterior chamber angle illustrating the presence of a functional block between the iris and the lens (A) causing a raised pressure in the posterior chamber (arrows) with a forward shift of the peripheral iris and closure of the anterior chamber angle (B).

1.2.4 Developmental glaucoma

Developmental glaucoma defines glaucomas that are associated with dysgenesis of the anterior segment. Anterior segment dysgenesis (ASD) are an ill-defined, genetically heterogeneous group of developmental abnormalities that share some common ocular features and a high prevalence of associated glaucoma (Gould and John, 2002). Since ASD may lead to an incomplete development or dysfunction of the structures that form the aqueous drainage pathway and are associated with glaucoma, they may provide important insights into one of the most common forms of glaucoma- POAG. The anomalies that make up the clinical spectrum of ASD including infantile congenital glaucoma, iris hypoplasia/iridogoniodysgenesis, Axenfeld and Rieger anomaly, Peters anomaly, congenital hereditary endothelial dystrophy, sclerocornea and megalocornea (Idrees *et al.*, 2006).

1.3 Ocular anatomy and physiology relevant to glaucoma

IOP is one of the strongest risk factors for the development of glaucoma (Wilson and Martone, 1996). Intraocular pressure is regulated by a balance between the secretion and drainage of aqueous humor. Hence IOP is affected by;

1) the rate of aqueous humor production by the ciliary body,
2) its drainage through the outflow channels of the angle of the anterior chamber angle,
3) the episcleral venous pressure.

Raised IOP is predominantly caused by increased resistance to drainage of aqueous humor (Gabelt and Kaufman, 2005; Tamm and Fuchshofer, 2007).

1.3.1 Aqueous humor secretion

Active secretion by the non-pigmented ciliary epithelium accounts for approximately 80% of aqueous production, whereas passive secretion (dependent on the level of blood pressure in the ciliary capillaries, plasma oncotic pressure and the level of IOP) contributes approximately 20% towards aqueous secretion (American Academy of Ophthalmology, 2008). Aqueous fluid flows from the posterior chamber (defined by the ciliary body, the lens and the posterior surface of the iris) into the anterior chamber (defined by the cornea, anterior surface of the iris and the iridocorneal junction) via the pupil (Figure 1-4).

1.3.2 Aqueous outflow physiology

Aqueous humor exits the anterior chamber into the venous circulation predominantly by two different routes: via the TM and independently through the uveoscleral outflow pathway (Figure 1-4).

The conventional route through the sieve-like TM accounts for the majority (90%) of aqueous outflow. Here, the aqueous flows through the trabeculum, and into the Schlemm canal. It exits the eye by draining into the episcleral veins (Kanski, 2003).

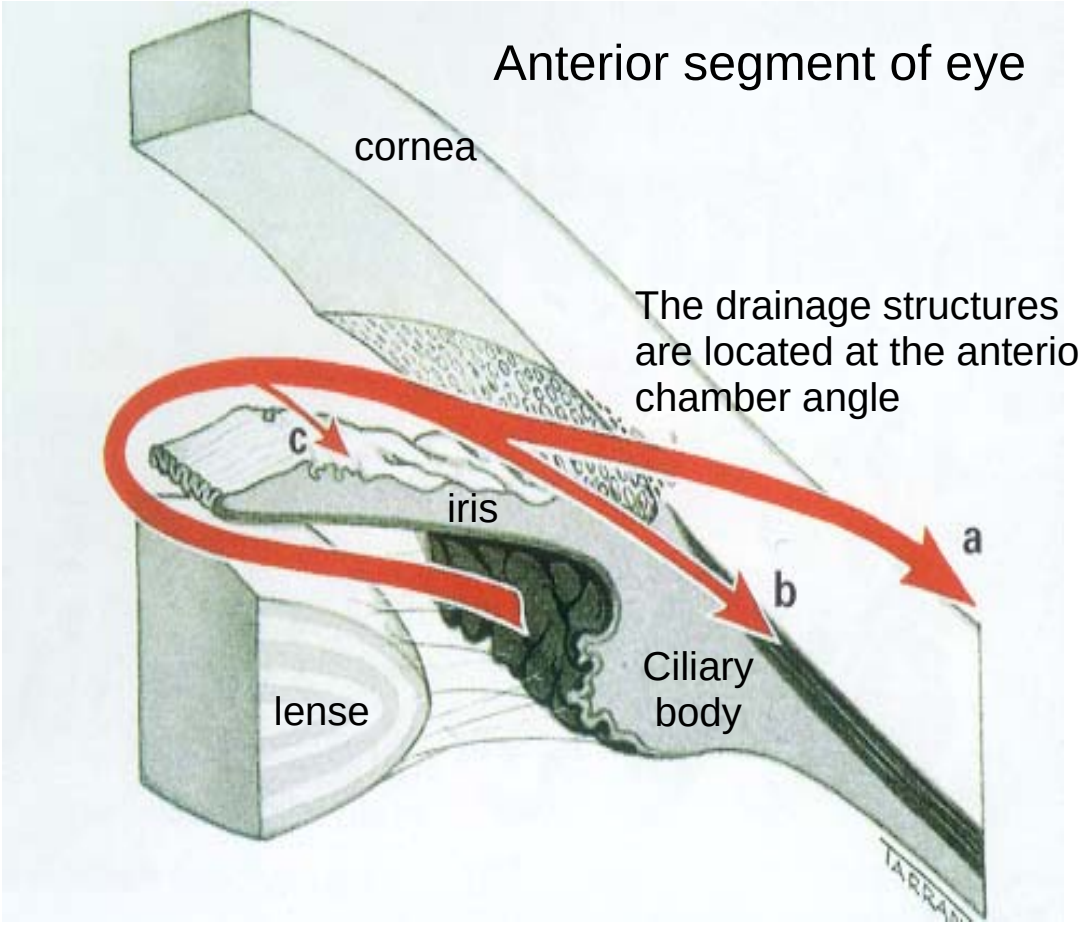


Figure 1-4: Cross section of the anterior chamber showing aqueous drainage pathways

(a) through the TM and; (b) through the uveoscleral pathway; and (c) through the iris. Adapted from Clinical Ophthalmology, Kanski.

The unconventional route through the uveoscleral drainage pathways (Figure 1-4) accounts for the remaining 10% of aqueous outflow. It flows across the face of the ciliary body into the suprachoroidal space. This drains into the venous circulation situated within the choroid and the sclera. A small minority of aqueous fluid is drained by the iris.

In most cases of glaucoma, there is an increased resistance to aqueous humor drainage through the trabecular pathway (Gabelt and Kaufman, 2005; Tamm and Fuchshofer, 2007).

1.3.3 Applied anatomy of the optic nerve head

Axons of retinal ganglion cells comprise the retinal nerve fibre layer, the innermost layer of the retina. These axons converge on the optic disc and form the optic nerve which contains about 1.2 million axons (Figure1-5). These ganglion cell fibres exit the eye by traversing through the lamina cribrosa (a series of perforated connective tissue sheets) and become myelinated and synapse in the lateral geniculate ganglion (Jakobiec, 1982; Kanski, 2003; Snell and Lemp, 1998). The distribution of these axons provide important clues for the interpretation of visual field loss in relation to glaucomatous optic nerve cupping since the axons' pattern of convergence dictates the pattern of visual field loss (Figure 1-6). Fibres arising from the macula travel a straight course to the optic nerve head forming the papillomacular bundle, with those arising from the nasal retina also following a relatively straight course to the optic nerve. Fibres situated temporal to the macula follow an arcuate path around the papillomacular bundle to reach the optic nerve. The optic cup is a central depression of the optic nerve devoid of any neural tissue. In contrast, the neuroretinal rim contains the exiting axonal fibres and is defined by the outer edge of the cup and the outer margin of the disc (Figure 1-7). The appearance of a normal rim has a uniform width and has a pink colour appearance. The degree of glaucomatous optic nerve head damage can be determined by the cup-disc (C/D) ratio. The diameter of the cup can be expressed as a fraction of the diameter of the disc, both in the vertical and horizontal meridians. The normal vertical C/D ratio is 0.3 or less and 2% of normal eyes have a ratio of more than 0.7 (Kanski, 2003).

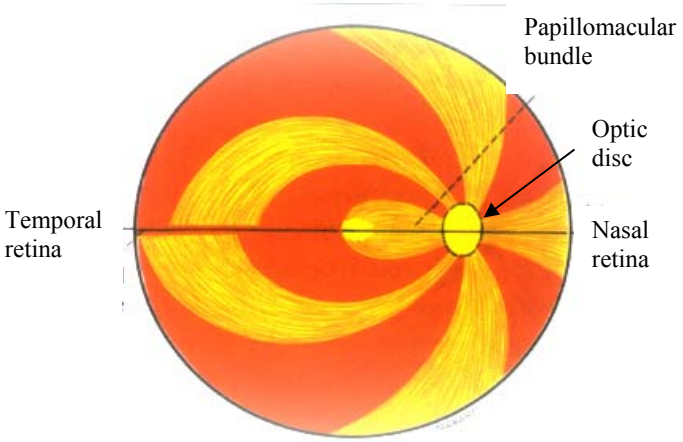


Figure 1–5: The anatomy of retinal fibres illustrating the pattern of convergence of ganglion cell fibres (depicted in yellow colour) towards the optic nerve.
Fibres arising from the macula travel a straight course to the optic nerve head forming the papillomacular bundle, with those arising from the nasal retina also following a relatively straight course to the optic nerve. Fibres situated temporal to the macula follow an arcuate path around the papillomacular bundle to reach the optic nerve. Adapted from Clinical Ophthalmology, Kanski.

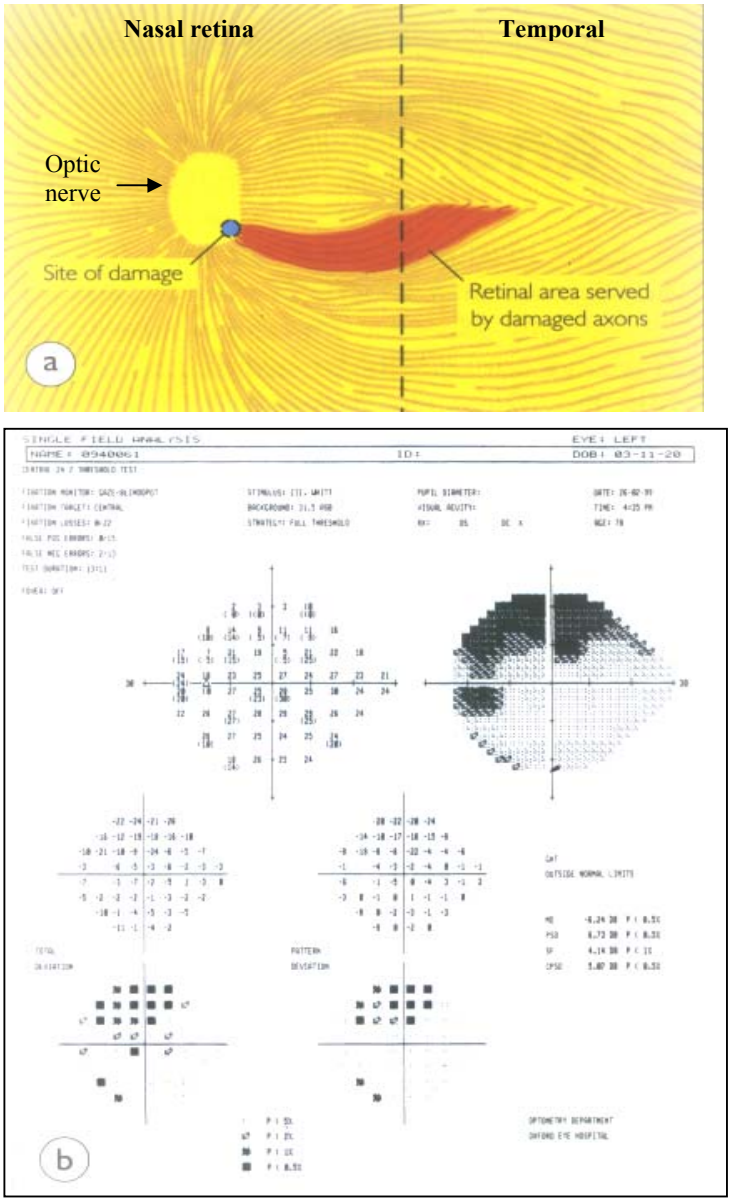


Figure 1–6: Diagrammatic illustration of the pattern of convergence of ganglion cell fibres on the optic disc, which dictates the pattern of visual field defect.

In this case, damage to the inferotemporal retinal nerve fibres (a) results in a superior arcuate field defect as shown in a Humphery's visual field (b). Adapted from Clinical Ophthalmology, Kanski.

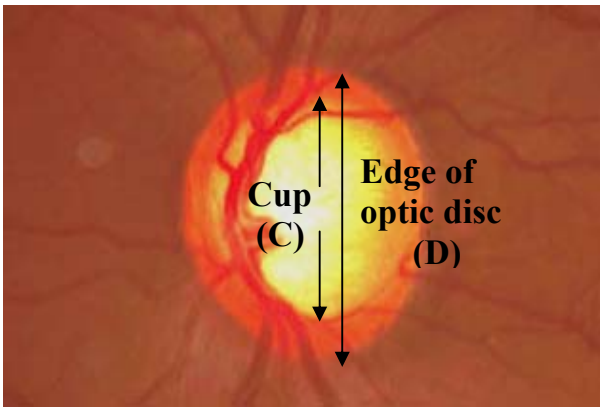


Figure 1–7: Cup-disc ratio of optic nerve head.

A photograph of the left optic disc with an enlarged cup-disc (C/D) ratio. Provided by Thomas Ressioniotis, University of Newcastle.

1.4 Epidemiology of glaucoma

Glaucoma presents a significant public health problem as it is the second leading cause of blindness world-wide. It is estimated that 67 million people are affected by this condition, with over 6.7 million people being bilaterally blind from glaucoma (Quigley, 1996). The prevalence of glaucoma has been studied extensively and has been shown to vary extensively between ethnic and racial groups (Table1-1). In addition, population-based studies have shown estimated prevalence rates varying greatly between different populations (Figure 1-8 and Table 1-1), ranging from 1-3% in Europe, 2-3% in Australia, 1-4% in Asia (Quigley and Broman, 2006; Rudnicka *et al.*, 2006), and high prevalence of 7-9%. among African-Caribbeans originating from West Africa (Leske *et al.*, 1994; Mason *et al.*, 1989). Within the African continent, prevalence varied from 1% in Nigeria (Murdoch *et al.*, 2001) to greater than 8% in Ghana (Ntim-Amponsah *et al.*, 2004). It is of note that these figures are confounded by differences in definitions and classifications of glaucoma. (Foster *et al.*, 2002).

Table 1–1: Population-based prevalence surveys of open angle glaucoma.
Adapted from Shield's Textbook of Glaucoma, Allingham.

Location	Age group (years)	Number of participants	Prevalence of POAG
Black			
Baltimore (USA, 1991)	>40	2396	4.7%
Barbados (1994)	40-84	4709	6.6%
Kongwa (Tanzania, 2000)	>40	3268	3.1%
St. Lucia (1989)	30-86	1679	8.8%
Temba (South Africa, 2003)	>40	839	2.9%
Hispanic Arizona (USA, 2001)	>40	4774	2.0%
Asian			
Andhra Pradesh (India, 2000)	>40	1399	2.6%
Japan (1991)	>40	8126	2.6%
Mongolia (1996)	>40	1000	2.2%
Singapore	40-79	1717	2.4%
White			
Baltimore (USA, 1991)	>40	2913	1.3%
Beaver Dam (USA, 1992)	43-84	4926	2.1%
Bedford (UK, 1968)	>30	5941	0.7%
Blue Mountains (Australia, 1996)	>49	3654	3%
Egna-Neumarket (Italy, 1998)	>40	5816	1.4%
Melbourne (Australia, 1998)	40-98	3271	1.7%
Rhonda Valley (UK, 196)6	40-74	4231	0.3%
Rotterdam (Netherlands, 1994)	>55	3062	3.1%
Reykjavik (Iceland, 2003)	>50	1045	4.0%

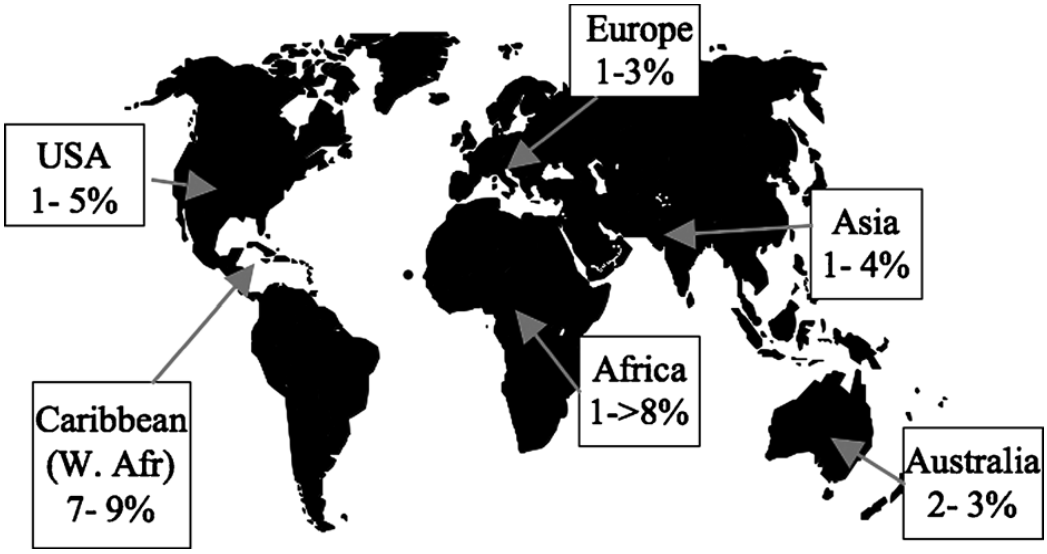


Figure 1–8: Estimates of global prevalence of open angle glaucoma in individuals >40 years of age.

Adapted from Leske M, 2007.

Primary open angle glaucoma is more prevalent in older individuals (Figure 1-9), and the number of people at risk of developing glaucoma is increasing rapidly as the elderly population grows disproportionately. Over 8.4 million people will be bilaterally blind from glaucoma in 2010, rising to 11.1 million by 2020 (Quigley and Broman, 2006). The danger of glaucoma is that substantial visual damage can occur before diagnosis since POAG is an asymptomatic disease. Indeed, late presentation is an important risk factor for subsequent blindness in glaucoma (Fraser *et al.*, 2001). It is estimated that approximately 50% of patients with POAG remain undiagnosed in most Western communities (Kroese *et al.*, 2002).

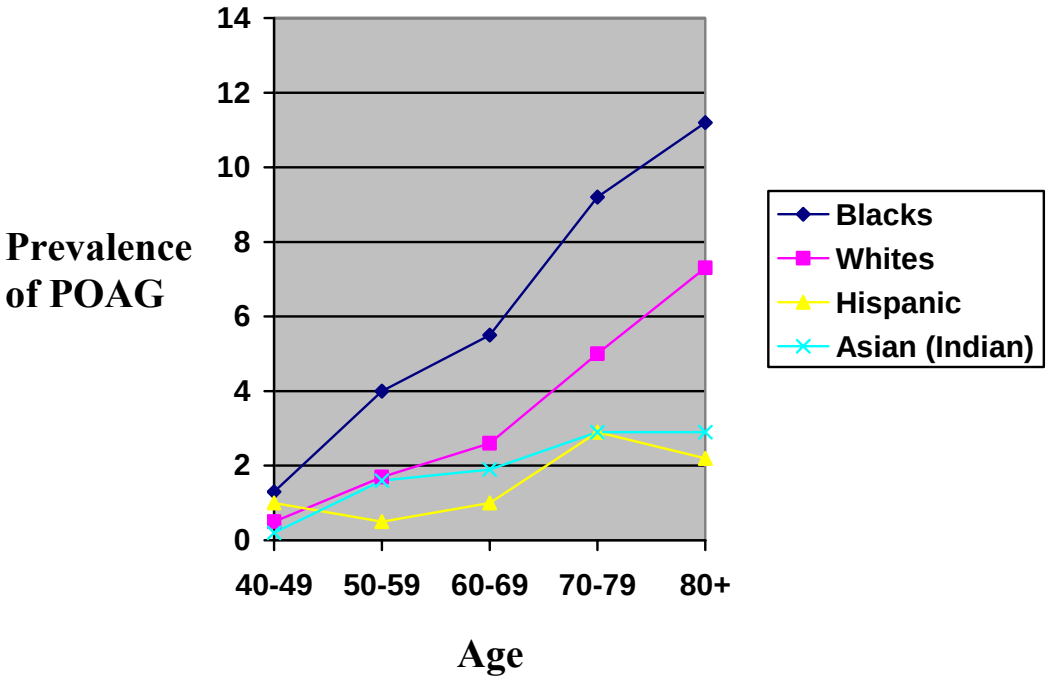


Figure 1–9: Age specific prevalence of POAG increases with age but varies between different ethnic groups.

Adapted from Shield's Textbook of Glaucoma, Allingham.

The sensitivity of current diagnostic technique is suboptimal and the diagnosis of glaucoma is usually made once irreversible damage has already occurred. As glaucomatous visual loss is preventable in the majority of cases, there is an urgent need for methods to identify individuals at risk of developing glaucoma, so that early treatment can prevent visual loss. Early disease detection will substantially reduce the burden on health care expenditures on glaucoma treatment and improve quality of life.

1.5 Management of glaucoma

The aim of treatment for patients with POAG is to prevent functional impairment of vision within the patient's lifetime and to avoid an adverse impact on quality of life. Currently the best method of achieving this goal is by lowering the IOP. Medical therapy is usually the first line treatment for POAG. These anti-glaucoma eye drops lower the IOP either by reducing aqueous production (e.g. beta-blockers, alpha$_2$-adrenergic agonists) or increase uveoscleral outflow (e.g. prostaglandin analogues, alpha$_2$-adrenergic agonists) or the trabecular outflow (e.g. parasympathomimetic (miotic) agents, prostaglandin analogues (e.g. bimatoprost)). Generally, drugs are initiated one at a time but subsequent addition of further drugs may be necessary if IOP remains unsatisfactorily high. Traditionally, beta-blockers were the preferred first option but prostaglandin analogues are now often favoured as they are as efficient with fewer side effects. For patients who do not respond to medical therapy, laser trabeculoplasty or incisional surgery are further methods that can be used to facilitate the aqueous outflow and lower the IOP (Lee and Higginbotham, 2005).

Topical therapy with anti-glaucoma drops or treatment with argon laser trabeculoplasty has been shown to significantly reduce progression in 50% of cases (Leskea *et al.*, 2004). However, despite the efficacy of medical and laser treatment for POAG, a study from the United States in a predominantly Caucasian population showed that the cumulative probability of undergoing filtration surgery in at least 1 eye was estimated to be 23% (Hattenhauer *et al.*, 1999). Furthermore, about 22% of individuals with POAG continued to develop blinding glaucomatous neuropathy over a 20-year period despite treatment (Hattenhauer *et al.*, 1998).

1.6 Clinical risk factors for glaucoma

Understanding the relative importance of and identifying risk factors for developing glaucoma is important as this will help to identify those at risk of developing glaucoma so that close medical supervision and early treatment can be initiated. Clinical risk factors are categorized into general risk factors, ocular risk factors, and systemic risk factors.

1.6.1 General risk factors

1.6.1.1 Age

Increased age is an important risk factor on the prevalence of POAG (Figure 1-9, Table 1-2, Table 1-4). In the Baltimore Eye Study (Tielsch *et al.*, 1991), the prevalence of glaucoma among blacks increased dramatically with age, rising above 11% in individuals who were 80 years or older. The Collaborative Initial Glaucoma Treatment Study (CIGTS) (Gillespie *et al.*, 2003) showed visual field defects were 7 times more likely to develop in patients who were 60 years old or older than those who were under 40 years of age. In the Early Manifest Glaucoma Trial (EMGT) (Leske *et al.*, 2003a), the relative risk for progression of early glaucoma was 1.5 for those of 68 years of age or older compared to those younger than 68 years. Hence age appears to be an important risk factor for developing glaucoma.

1.6.1.2 Race

On the whole, the prevalence of POAG is highest in black populations and lowest in northern Asian populations. The Baltimore Eye Survey found the prevalence of POAG was four times higher in blacks compared to whites (Tielsch *et al.*, 1991). A recent Meta-analysis study (Rudnicka *et al.*, 2006) confirmed that black populations had the highest POAG prevalence at all ages whilst white populations showed the steepest increase in POAG prevalence with age. In addition, the Advanced Glaucoma Intervention Study (AGIS) (AGIS Investigators, 2002) found that black race was a risk factor for progression (Wilson *et al.*, 1985). As shown by the Collaborative Normal Tension Glaucoma Study (CNTGS) (Drance *et al.*, 2001), Chinese individuals had a significantly reduced risk compared to white patients, of progression.

Table 1–2: Age specific prevalence of open angle glaucoma.
(Adapted from Shield's Textbook of Glaucoma, 2005)

	White		Blacks	Hispanic	Asian
Age (years)	Eye disease prevalence group (Friedman *et al.*, 2004)	Baltimore Eye Survey (Tielsch *et al.*, 1991)	Baltimore Eye Survey (Tielsch *et al.*, 1991)	Proyecto Ver (Arizona, USA) (Quigley *et al.*, 2001)	Tamil Nadu (India) (Ramakrishnan *et al.*, 2003)
40-49	0.6	0.9	1.2	0.5	0.3
50-59	1.5	0.4	4.1	0.6	1.6
60-69	2.7	0.9	5.5	1.7	1.8
70-79	5.1	2.9	9.2	5.7	2.9
80+	7.3	2.2	11.3	12.6	----
Overall	----	1.3	4.7	2.0	1.2

1.6.1.3 Family history

A positive family history is known to be an important risk factor for POAG (Table 1-4). Prevalence studies have shown a consistent association with an increased risk of POAG in individuals with a first degree relative with glaucoma. In the Baltimore Eye Survey, association between POAG and family history appears to be stronger when the affected relative is a sibling (odds ratio (OR) = 3.7) (see section 2.8.3 for further description of OR) rather than a parent (OR = 2.2) or child (OR = 1.1) (Tielsch *et al.*, 1994). Although this prevalence might not be high enough to justify a simple Mendelian pattern of inheritance, it suggests that a polygenic or multifactorial influence may contribute to the expression of the disease (Lichter 1994). A population-based study in Rotterdam examined familial members of individuals with and without POAG, and found that first degree relatives of an affected individual had a 22% risk of developing POAG compared to only 2.3% risk among familial members of controls (Wolfs *et al.*, 1998). This study also suggested that enlarged C/D ratio was the earliest and most prominent feature of familial aggregation. A case control study involving Caucasian individuals

with 175 POAG cases and 175 controls showed that, a family history of POAG was a major risk factor for first degree relatives (OR = 7.7) (Charliat *et al.*, 1994). More specifically, there appears to be a strong familial component to the development of NTG, where the presence of a positive family history is reported to be about 40 % (Geijssen H, 1991). With regards to OHT, a clinic based study involving 150 patients, showed 43% of patients with OHT had a positive family history (Shin *et al.*, 1977) despite family history of glaucoma not being identified as a risk factor for developing glaucoma in the OHTS study.

Further evidence that genetics is an important factor is supported by twin studies which have shown a higher degree of concordance among monozygotic twin (Gottfredsdottir *et al.*, 1999; Teikari, 1987). In particular, Gottfredsdottir et al showed the concordance of open angle glaucoma in monozygotic twin pairs was significantly higher at 98 % compared to their spouses (70%).

Taken together, these evidences strongly suggest that specific genetic defects contribute to the pathogenesis of POAG.

1.6.2 Ocular risk factors

1.6.2.1 IOP

Elevated intraocular pressure (IOP) has played an important role in the diagnosis and management of glaucoma and is currently the only treatable risk factor. There is sound evidence that IOP is a strong risk factor for glaucoma (Table 1-4). Numerous prevalence surveys (Table 1-3) and longitudinal studies have consistently shown a dose-response relationship between IOP and glaucoma (Le *et al.*, 2003; Leske *et al.*, 2002). Recent decisive results published from randomised clinical trials have shown that lowering IOP reduced incidence (OHTS) (Kass *et al.*, 2002) and progression of glaucoma (EMGT and CNTGS) (Collaborative Normal Tension Glaucoma Study Group, 1998; Heijl *et al.*, 2002) compared with no treatment. Baltimore Eye Survey showed prevalence of POAG increased with age, this being 40 times greater in those with an IOP of ≥ 35mmHg, compared to those with an IOP of ≤ 15 mmHg (Sommer *et al.*, 1991).

Table 1–3: Prevalence and relative risk of POAG with increasing IOP.
(Adapted from Shield's Textbook of Glaucoma, 2005)

IOP (mmHg)	Prevalence	Relative risk
<15	0.7	1.0
16-18	1.3	2.0
19-21	1.8	2.8
22-24	8.3	12.8
25-29	8.3	12.8
30-34	25.4	39.0
≥35	26.1	40.1

In the Barbados Eye Study, the incidence of POAG steadily increased with IOP (Leske *et al.*, 2001). More importantly, a reduction in the IOP by average of 23% decreased the incidence of POAG by 60% in the OHTS (Kass *et al.*, 2002). Both EMGT and CNTGS showed an IOP reduction of 25% and greater than 30% reduced the risk of progression by 33% and 50% respectively when compared to no treatment (Collaborative Normal Tension Glaucoma Study Group, 1998; Heijl *et al.*, 2002). Overall, in addition to raised IOP contributing to POAG, these data suggest that IOP may contribute towards the onset of glaucoma even in patients when the untreated IOP is in the normal range and that individual may benefit from IOP reduction.

1.6.2.2 Features of the optic nerve head

An enlarged optic disc has been generally considered as a risk factor for developing glaucoma (Armaly *et al.*, 1980; Hart, Jr. *et al.*, 1979; Yablonski *et al.*, 1980). Recent evidence from the OHTS has shown support for this association by showing that for every 0.1 unit increase in the baseline C/D ratio, there was a 1.4 fold increase in the incidence of POAG among OHT individuals.

Optic disc haemorrhages have also been associated with an increased risk for progression of visual field loss, as shown by the EMGT (relative risk (RR) =1.02;

relative risk is defined as incidence of disease rate in exposed divided by incidence rate in non-exposed group) and the CNTGS (RR = 2.72). Higher prevalence of glaucoma (10 fold increase) was found with disc haemorrhage, and disc haemorrhage was more commonly associated with NTG (25%) than HTG (8%) (Healey *et al.*, 1998).

1.6.2.3 Central corneal thickness

An association of the central corneal thickness (CCT) with POAG has been a recent finding and has shown to be a predictive factor for developing glaucoma. This association came to light in the Ocular Hypertension Treatment Study, where thinner CCT was a strong risk factor for developing POAG in individuals with OHT (Gordon *et al.*, 2002).

The average CCT is between 537 to 554 μm in normal individuals (Shah *et al.*, 1999; Wolfs *et al.*, 1997). Variation of the CCT in normal individuals can alter the measurement of IOP, with thicker corneas leading to falsely higher IOP reading and falsely lower measurements with thinner corneas. So far, there has been a lack of agreement for a standard algorithm to provide correction for IOP readings with varying CCT and the relationship between CCT and POAG remains unclear. The implication for clinical practice is that pachymetry should be performed on all newly diagnosed patients with OHT/POAG and the corneas should be categorized as thin, average or thick. This has implications on management of POAG/OHT since patients with POAG with thin corneas will require a lower threshold for starting treatment or intensifying treatment, whereas, patients with thicker corneas would require a higher threshold for either starting treatment or intensifying treatment (Brandt, 2007).

Of greater clinical significance, a thinner CCT has been identified as a risk factor in POAG, with the exact underlying mechanism yet to be determined. CCT has been found to be thicker in individuals with OHT (Brandt *et al.*, 2001; Herndon *et al.*, 1997) and thinner in individuals with NTG (Copt *et al.*, 1999). Recent results from a population-based study among individuals of African descent involving 3222 individuals in the 9 year follow-up in the Barbados Eye Studies showed thinner CCT as a risk factor in POAG (Leske *et al.*, 2008). Studies have further demonstrated that CCT varies between racial and ethnic groups (Nemesure *et al.*, 2003; Shimmyo *et al.*, 2003). In particular,

thinner corneas have been associated with African-Americans compared to Caucasians. Furthermore, CCT has been linked to other factors that may be associated with glaucoma, including disc size (Pakravan *et al.*, 2007), with thicker corneas associated with smaller disc, and another study involving twins suggesting that genetic factors were shown to be of major importance in CCT (Toh *et al.*, 2005). Interestingly, a recent study has suggested thinner CCT may have biomechanical properties such as increased elasticity, which in turn increases the susceptibility of the optic nerve to glaucomatous damage (Herndon *et al.*, 1997; Leske *et al.*, 2008).

1.6.2.4 Myopia

Numerous case series (Fong *et al.*, 1990; Perkins and Phelps, 1982), case reports (Daubs and Crick, 1981; Wilson *et al.*, 1987) and large population-based prevalence surveys (Weih *et al.*, 2001; Wong *et al.*, 2003) have shown an association of myopia with POAG. However, longitudinal studies such as OHTS or EMGT have failed to identify an association between myopia as a risk factor for POAG. The difficulty with myopia is that optic disc changes make accurate assessment of the C/D ratio difficult as well as giving rise to visual field defects resembling those seen in POAG (Leske, 1983).

1.6.3 Systemic risk factors

1.6.3.1 Diabetes mellitus

Studies have shown a higher prevalence of both elevated IOP and POAG among individuals with diabetes (Dielemans *et al.*, 1996; Klein *et al.*, 1994; Leske *et al.*, 2003a; Leske *et al.*, 1995; Mitchell *et al.*, 1997; Tielsch *et al.*, 1995b). However, despite the weight of this evidence in support of diabetes as a risk factor for glaucoma, this remains controversial due to inconsistent findings (ARMSTRONG *et al.*, 1960; Kahn *et al.*, 1977; Tielsch *et al.*, 1995b). In OHTS, diabetes was not associated with an increased risk of progression to glaucoma.

1.6.3.2 Blood pressure, migraine

There is evidence to suggest that a diastolic perfusion pressure of below 55mmHg is an risk factor for glaucoma (Bonomi *et al.*, 2000; Leske *et al.*, 2002; Tielsch *et al.*, 1995a). In addition, nocturnal arterial hypotension has been associated especially with NTG (Kaiser *et al.*, 1993; Meyer *et al.*, 1996), and the progression of NTG (Graham *et al.*, 1995). Recently, the Barbados Eye Studies and the EMGT have shown good evidence that a lower systolic BP was a long term risk factor for POAG (Leske *et al.*, 2008) and was associated with progression of POAG (Leske *et al.*, 2007).

Despite there being numerous studies associating migraine with NTG (Collaborative Normal Tension Glaucoma Study Group, 1998; Cursiefen *et al.*, 2000), this link remains inconclusive based on two large population-based studies (Klein *et al.*, 1993; Wang *et al.*, 1997).

1.7 Pathophysiology of glaucomatous damage of optic nerve

1.7.1 Apoptosis

Although the pathophysiology of glaucomatous neurodegeneration is not understood, current available data suggest that POAG is a multifactorial disease influenced by a complex interplay between environmental and genetic factors. This illustrates that glaucoma is a complex disease, where factors that regulate IOP and factors that determine ganglion cell viability interact to influence the final course of the disease. Irrespective of the cause of glaucoma, it is known that the common final pathway is ganglion cell death by apoptosis leading to glaucomatous optic neuropathy (Quigley, 1995). Animal studies have provided support for this biological mechanism (Garcia-Valenzuela *et al.*, 1995; Huang *et al.*, 2005; Nickells, 1999; Quigley *et al.*, 1995). For example, studies on monkey eyes have demonstrated morphologic features of apoptosis, including chromatin condensation and formation of apoptotic bodies within affected ganglion cells in glaucoma eyes, that was only rarely seen in control eyes (Quigley *et al.*, 1995). In another study, raised IOP in adult rats led to cell death by apoptosis, as demonstrated by DNA breaks in nuclei that were labelled in situ, using a method that specifically incorporated biotinylated deoxynucleotides by exogenous terminal deoxynucleotidyl transferase to the 3'-OH ends of DNA (TUNEL).

Table 1–4: Summary of clinical risk factors for POAG (Adapted from Shield's Textbook of Glaucoma, 2005)	
Good evidence	
Risk factor	**Relative risk**
Age (per decade over 40)	2 (Congdon *et al.*, 2004; Dielemans *et al.*, 1994; Klein *et al.*, 1992; Sommer *et al.*, 1991)
Black ethnicity	4 (Tielsch *et al.*, 1991)
First degree family history	2 to 4 (Le *et al.*, 2003; Leske *et al.*, 1996; Tielsch *et al.*, 1994; Wolfs *et al.*, 1998)
IOP compared to 15mmHg	
19-21 mmHg	3 (Ekstrom, 1993; Le *et al.*, 2003; Leske *et al.*, 2001; Leske *et al.*, 2003a)
22-29 mmHg	13
>30 mmHg	40
Myopia	1.5 to 3 (Ekstrom, 1993; Mitchell *et al.*, 1999; Weih *et al.*, 2001; Wong *et al.*, 2003)
Diastolic perfusion pressure (<55 mmHg)	3 (Bonomi *et al.*, 2000; Leske *et al.*, 1996; Leske *et al.*, 2003b; Tielsch *et al.*, 1995b)
Fair evidence	
Large C/D ratio, diabetes mellitus, optic disc haemorrhage	
Weak evidence	
Migraine (for NTG)	

The active nature of the death mechanism was shown by the reduction in numbers of biotin-labelled nuclei after administration of the protein synthesis inhibitor, cyclohexidine (Garcia-Valenzuela *et al.*, 1995). Similarly, elevated IOP in rat RGCs has been found to activate both caspases –8 and –9, initiating both the extrinsic and intrinsic apoptotic cascades. Activation of both caspases was demonstrated by the presence of cleaved forms of the caspases and the detection of cleaved Bid and PARP, downstream consequences of caspase activation (Huang *et al.*, 2005).

Apoptosis was also found be a mechanism of cell death in human eyes with POAG. Fixed, paraffin-embedded retinal sections were assayed by the TUNEL method to detect DNA fragmentation. A positive TUNEL reaction was observed among ganglion layer cells in 10 of 18 cases with glaucoma, compared with 1 of 11 control cases without confounding systemic disease. The frequency of TUNEL-positive cells in the ganglion cell layer in cases with POAG was 15.2 times greater than the control frequency (Kerrigan *et al.*, 1997). Similar immunohistological evidence of apoptosis was detected in the ganglion cells of 2 glaucomatous eyes with recent sight loss from POAG (Okisaka *et al.*, 1997).

Retinal ganglion cell apoptosis in POAG is thought to be triggered by several mechanisms including neurotrophic factor deprivation, hypoperfusion/ischaemia, glial cell activation, glutamate excitotoxicity, abnormal immune response and raised IOP (Kuehn *et al.*, 2005).

1.7.2 Mechanical and vascular theories of POAG

In order to provide an explanation for the development of glaucomatous optic nerve damage, two concepts on the pathogenesis of glaucomatous optic neuropathy were introduced in the mid-19th century. In 1858, Muler (Muler, 1858) suggested that elevated IOP resulted in direct compression and death of the neurons (the mechanical theory), whilst von Jaeger (von Jaeger, 1858) proposed that a vascular abnormality was responsible for optic nerve damage.

The mechanical theory suggests the underlying cause of optic nerve damage is as a result of raised IOP above the physiological level (as a result of increased resistance to

aqueous outflow), creating a pressure gradient across the lamina cribrosa and inducing mechanical stress and deformation of the axonal fibres at the lamina cribosa (Bellezza *et al.*, 2003). Experimental models of glaucoma have shown IOP induced interruption of axoplasmic flow at the lamina cribrosa (Weinreb *et al.*, 1997). In particular, research in animal models involving rat have proposed that impaired axonal neurotrophic factor delivery to the retina may contribute to retinal ganglion cell death (Quigley *et al.*, 2000). Injection of neurotrophic factor, in particular brain-derived neurotrophic factor (BDNF) into the vitreous cavity of rats with experimentally elevated IOP increased the survival rate of retinal ganglion cells compared with untreated eyes (Ko *et al.*, 2001). Contrary to these findings, another study involving a rat model of glaucoma with raised IOP showed apoptosis of retinal ganglion cells prior to axonal obstruction and alteration in neurotrophin levels (Johnson *et al.*, 2000). In addition, all of these studies have demonstrated the interruption of axonal transport occurred at IOP levels of 40mmHg or even higher, which however, does not correlate to the lower IOP readings normally seen in patients with POAG.

On the other hand the ischaemic theory stresses the importance of other factors independent of, or in addition to, IOP. Since the retina and the optic disc are dependent on local blood supply to replenish their high metabolic needs, local dysfunction of vascular autoregulation resulting in local ischaemia-hypoxia have been implicated to cause optic nerve damage (Pillunat *et al.*, 1997; Sossi and Anderson, 1983; Ulrich *et al.*, 1986). Animal studies involving rat have demonstrated that RGC death can occur with exogenous application of the vasoactive peptide endothelin-1, despite normal or low IOP (Chauhan *et al.*, 2004). An immunohistochemical study has shown that the expression of hypoxia-induced factor 1a (HIF-1a) is raised in the human glaucomatous retina and optic nerve head compared with healthy controls. This transcription factor is produced in response to low-cellular oxygen tension and induces activation of apoptotic genes whose functions are related to oxygen delivery and metabolic adaptation (Tezel and Wax, 2004). However, due to its difficulty in its access for experimental assessment, the exact role of the ischaemic theory remains difficult to establish.

Both theories have been recognised as a causative factor in the search for assessing the aetiology of glaucomatous optic nerve damage. For example, the observed differences in glaucomatous visual field defects in patients with NTG and HTG may suggest that

ischaemia may be a predominant factor in glaucomatous eyes with IOP in the lower end of the scale, whereas a more direct mechanical damage of the pressure may exist in patients with raised IOP (Caprioli and Spaeth, 1984) (Figure 1-10).

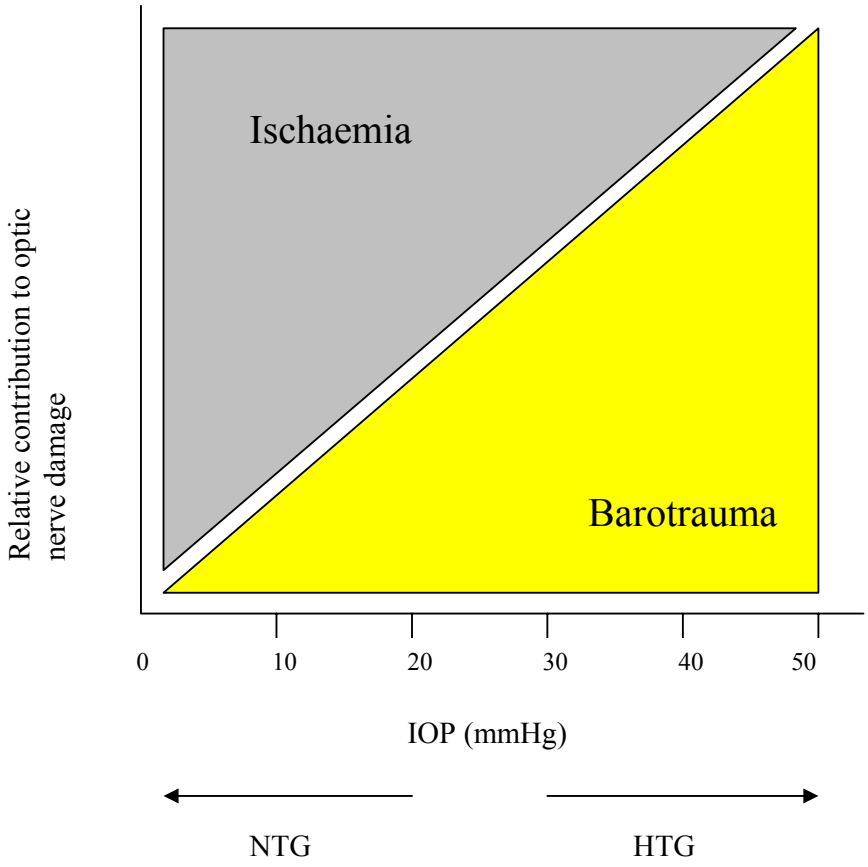


Figure 1–10: Theoretical representation of relative influence exerted by mechanical and vascular factors on the development of glaucomatous optic neuropathy at different levels of IOP.
Adapted from Caprioli *et al*., 1984.

1.7.3 Other factors contributing to glaucomatous optic nerve damage

Glial cell activation may also be contributing to RGC death in POAG. Glial cells support neuronal function by removing extracellular glutamate and synthesizing growth factors and metabolites. These cells appear to become activated in POAG. Glial cell activation as evidenced by an upregulation of glial fibrillary acidic protein is characteristic of central nervous system injury, and is significantly elevated in POAG (Wang *et al.*, 2000). In another animal study involving monkeys with elevated IOP showed increased elastin expression in astrocytes of the lamina cribrosa, and may alter the environment of the axons that is detrimental to the survival of healthy retinal ganglion cells (Pena *et al.*, 2001).

Furthermore, excessive stimulation of the glutaminergic system, in particular the N–methyl–D–aspartate subtypes, has been suggested to be involved in glaucomatous cell death (Dreyer *et al.*, 1996; Lipton, 2003; Yoles and Schwartz, 1998). However, it still needs to be decided whether excess glutamate has a positive or negative effect on RGC, and whether various types of cells respond differently to glutamate. Other proposed factors involved in ganglion cell death include oxidative stress and formation of free radicals, inflammatory cytokines (nitric oxide and tumour necrosis factor) (Liu and Neufeld, 2001; Yan *et al.*, 2000), and aberrant immunity (Schwartz, 2003; Tezel *et al.*, 1999) (Figure 1-11). Interestingly, the response towards the initial optic nerve injury in glaucoma may also affect the remaining surviving ganglion cells and their fibres leading to secondary neurodegenerative damage (Liu and Neufeld, 2001; Schwartz, 2003). According to this suggestion, the primary insult does not directly involve the RGC or its fibres, but causes changes in the neuronal environment which in turn increases the susceptibility of the remaining neurons towards ganglion cell death.

1.8 Genetics of POAG

The link to hereditary predisposition to glaucoma was first identified more than 150 years ago and subsequent epidemiological studies have ascertained genetic factors as a major role in the aetiology of glaucoma and these studies were discussed in section 1.6.1.3 (Klein *et al.*, 2004; Leske, 1983). Some forms of glaucoma exhibit a Mendelian pattern of inheritance consistent with the presence of a single gene defect

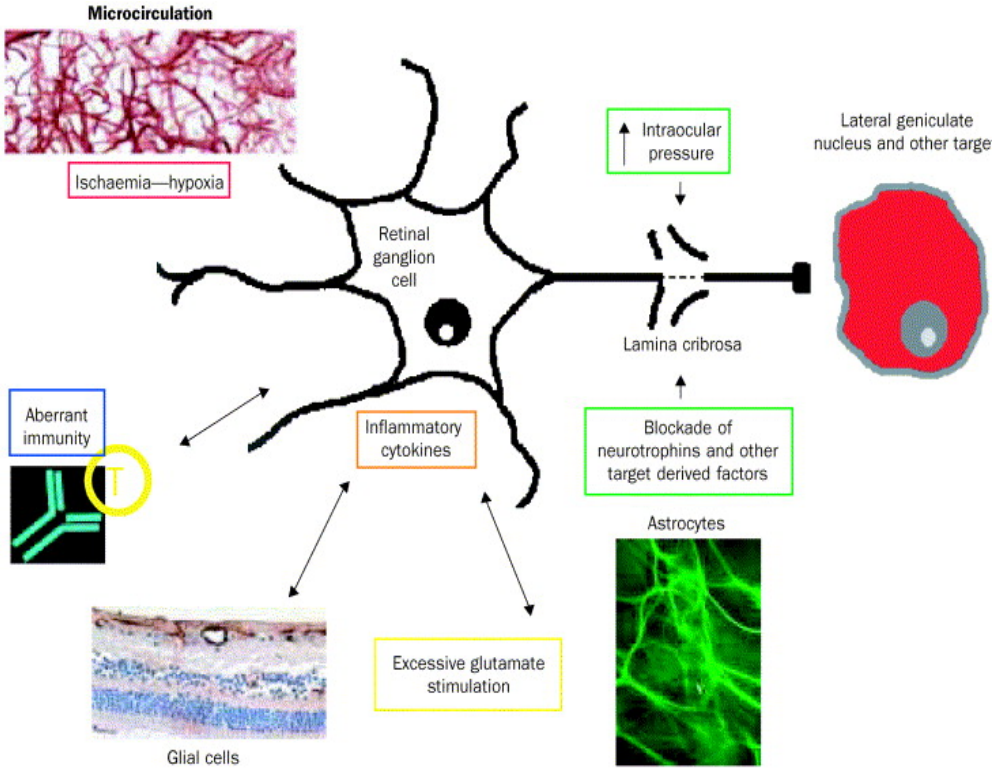


Figure 1–11: Diagrammatic representation of the possible factors contributing towards glaucomatous optic nerve damage.

Raised intraocular pressure can cause blockade cribrosa of axonal protein transport at the lamina, causing neuronal retinal ganglion cell death by trophic insufficiency. Other factors implicated in ganglion cell death include local ischaemia-hypoxia, aberrant immunity, excessive stimulation of the glutamatergic system, and alterations in glial cells or astrocytes. Diagram obtained from Weinreb & Khaw, 2004.

(Raymond, 1997). Although these are a minority of the total glaucoma cases, studies on such rarer glaucomas offer new insight into glaucoma pathology. These include the developmental glaucomas, caused by dominant mutations in genes encoding transcription factors (for example, *FOXC1*), as well as primary congenital glaucoma caused by recessive mutation of the cytochrome mono-oxygenase, *CYP1B1* (reviewed by Gould *et al.,* 2004). POAG, the most common form of glaucoma is more complex and does not usually show Mendelian inheritance (Netland *et al.*, 1993) but does exhibit a significant heritability. A family history has been known to be an important risk factor for POAG, implying the involvement of genetic factors towards the pathogenesis of POAG. It has been shown that 20-60% of patients with POAG have a family history, and under-reporting of a family history is a documented feature in glaucoma (McNaught *et al.*, 2000; Nemesure *et al.*, 1996; Nemesure *et al.*, 2001; Wolfs *et al.*, 1998). This is supported by twin studies which have shown a higher degree of concordance among monozygotic twin, as mentioned earlier (Gottfredsdottir *et al.*, 1999; Teikari, 1987).

The search for the underlying pathogenic mechanism for POAG has been challenging because of: 1) the limited understanding of the biochemical and cellular mechanism involved in the IOP control and retinal ganglion cell function; 2) the complex genetics of POAG; and 3) because each causal gene is presumed to only make a small contribution to overall phenotype. POAG is considered to be a complex, heterogeneous, multifactorial disease, resulting from interactions between multiple genetic factors.

The two main methods used to identify susceptibility genes for POAG have been (Figure 1-12):

1. Genome-wide linkage studies.
2. Association studies using either a candidate-gene association approach or a genome-wide association approach.

1.8.1 Genome-wide linkage studies

In genome-wide linkage analysis, genes are mapped by typing genetic markers in families to identify regions that segregate with the disease or trait within pedigrees more

than is expected by chance, since such linked regions are more likely to have a causal genetic variant. The advantage of this method is that it provides a comprehensive, unbiased search across the genome for the causal gene and has been successful in identifying many monogenic Mendelian diseases (Jimenez-Sanchez *et al.*, 2001). It has been applied to the study of eye disease and has lead to the discovery of several disease genes including RP31 for autosomal dominant retinitis pigmentosa (Papaioannou *et al.*, 2005).

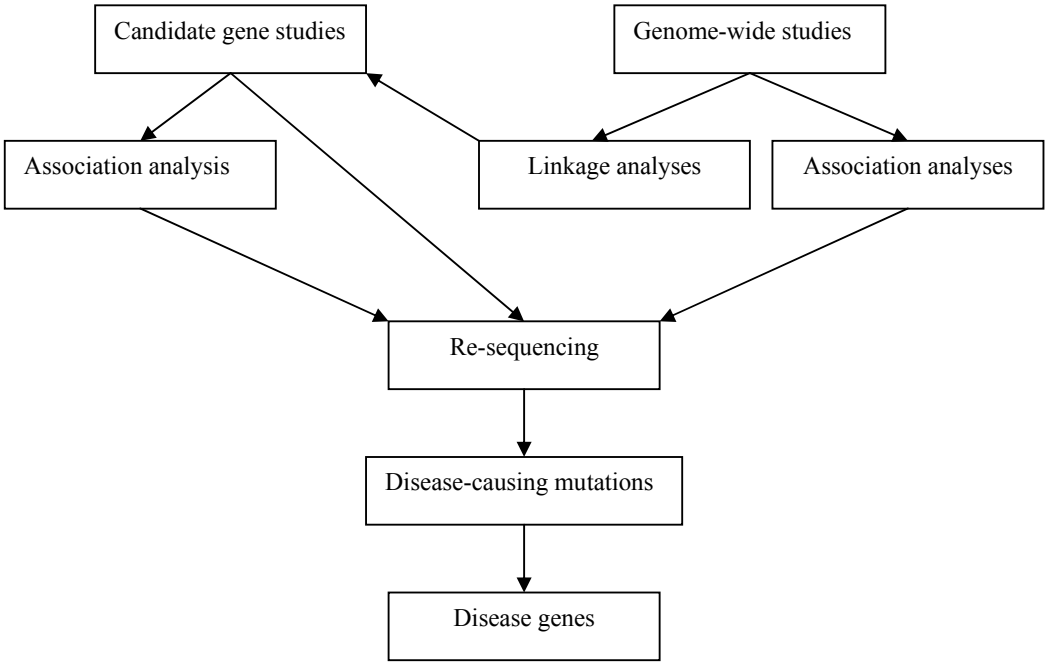


Figure 1–12: Genome-wide and candidate gene approaches towards gene mapping. Adapted from Fan *et al*., 2006.

Genome-wide linkage analysis has also been utilized for many common but complex diseases and quantitative traits, for which it has been less successful. This has been because of its reduced power to detect common genetic variants that have modest effects on disease. However, such methods have identified 9 genetic loci for POAG in families showing clear Mendelian patterns of inheritance (GLC1A-H) (Table 1-5) (Monemi *et al.*, 2005; Sarfarazi *et al.*, 1998; Sheffield *et al.*, 1993; Stoilova *et al.*, 1996; Suriyapperuma *et al.*, 2007; Trifan *et al.*, 1998; Wirtz *et al.*, 1999; Wirtz *et al.*, 1997), including POAG candidate genes *MYOC* (GLC1A), *OPTN* (GLC1E) and *WDR36* (GLC1G). Notably however, few genes have been robustly associated with POAG in the general population. Among the three identified genes, only *MYOC* is established as directly causative, while due to conflicting results, the exact roles of *OPTN* and *WDR36* in POAG remain uncertain. Mutations in *MYOC* account for a small proportion of approximately 2-4% of POAG (Alward *et al.*, 1998; Alward *et al.*, 2002; Fingert *et al.*, 1999; Stone *et al.*, 1997; Suzuki *et al.*, 1997). For example, Alward et al. screened 716 patients with primary open-angle glaucoma and 596 control subjects for sequence changes in the *MYOC* gene and found 4.6% of cases had mutations. Fingert et al. on the other hand screened 1703 patients from five different populations (including Caucasians, African Americans and Asian patients from Japan) and despite different frequencies of specific *MYOC* mutations being found in each of the five populations, the overall frequency of MYOC mutations was similar (~ 2-4%) in all populations. The combined contribution of *MYOC, OPTN* and *WDR36* to POAG add up to only 10% (Fan *et al.*, 2006b). Hence it is highly likely that other unidentified loci or genes still exist.

1.8.2 Association studies

While linkage studies have been the predominant method used to identify POAG loci, with advances in genome technology and bioinformatics it has become feasible to undertake studies to unravel the complex genetics of glaucoma. In particular, association studies using single nucleotide polymorphisms (SNPs) offer a powerful method for assessing candidate genes with small effects (Hirschhorn and Daly, 2005) and are increasingly being applied to elucidate genetic mechanisms in complex disease

(Edwards *et al.*, 2005). Even small cohorts have proved to be remarkably powerful (Klein *et al.*, 2005).

1.8.2.1 Single nucleotide polymorphism (SNP)

A SNP is defined as a polymorphic variation at a single nucleotide that occurs in at least 1% of the population. They make up about 90% of all human genetic variation and occur every 100 to 300 bases along the 3 billion base human genome (International HapMap Project; http://www.hapmap.org/whatishapmap.html.en). SNPs can occur in both coding and non-coding regions of the genome. SNPs are examples of the newest generation of genetic markers.

One approach, which has proved useful in other complex diseases including association between variation in the gene encoding apolipoprotein E (APOE) and Alzheimer's disease (Corder *et al.*, 1993), and recently was used to demonstrate the role of the complement factor H gene in age-related macular degeneration (Edwards *et al.*, 2005; Ennis *et al.*, 2008) is to use SNP genotyping. This method can be used as a means to test candidate genes as genetic susceptibility factors (candidate genetic studies) or search across a region of the genome where markers segregate with the disease or trait, for susceptibility genes (genome-wide studies) (Hirschhorn and Daly, 2005). Because the majority of heterozygosity in the human population is attributable to common variants and because the evolutionary history of common human diseases is not yet known, one promising approach is to assess common genetic variations (such as SNPs) for association to medical conditions (Collins *et al.*, 1997; Lander, 1996; Risch and Merikangas, 1996). This approach is feasible because 4 million (Sachidanandam *et al.*, 2001; Venter *et al.*, 2001) of the estimated 10 million SNPs (Kruglyak and Nickerson, 2001) are already known.

This approach relies on selection of SNPs to tag disease-associated alleles (Johnson *et al.*, 2001). In essence, this method compares the frequency of alleles or genotypes of a particular variant between cases and controls. If a particular SNP is more common among individuals with glaucoma, that SNP could be used as a pointer to locate and identify the gene involved in the disease. However, testing all of the 10 million common SNPs present in a patient's chromosome is extremely expensive (International HapMap

Project). The problem is finding which SNPs are biologically significant, as most have no effect. Commonly, for reasons of limited resources, only a few representative SNPs on the chromosomes of interest are genotyped. The hope is that by determining the pattern of bases at a few selected positions, these can infer values of bases at intermediate SNPs which are not being measured. The premise of this approach is that mutations will have occurred on a background of mutations that are already present, and over small evolutionary distances, the rate at which this background pattern is disrupted will be fairly low. As genetic variants that are near each other tend to be inherited together (on average genotypes at SNPs which are < 10 kb apart tend to be correlated) (Carlson *et al.*, 2004), one can capture the majority of the diversity within a region using a subset of SNPs. This is called haplotype tagging and relies on selecting a sufficiently representative set of tagging SNPs (tSNPs) covering the region of the genome under study (Figure 1-13). The number of tSNPs that are informative of the remaining SNPs is estimated to be about 300,000 to 600,000 which is far fewer than the 10 million common SNPs in the genome (International HapMap Project; http://www.hapmap.org/whatishapmap.html.en).

1.8.2.2 Haplotype and Linkage disequilibrium

In interpreting genetic association studies, it is essential to understand the structure of haplotypes in the human genome. A haplotype is defined as a combination of alleles at multiple loci that are transmitted together on the same chromosome and yields information about recombination. When a new mutation occurs, it is located on a specific haplotype. The association between each mutant allele and its ancestral haplotype is disrupted in subsequent generation by recombination. It should thus be possible to locate disease-causing mutations through linkage methods using information on recombination. More specifically, statistical associations are assessed between two SNPs in the genome and this is known as linkage disequilibrium (LD) (defined as the non-random association of alleles at two or more loci).

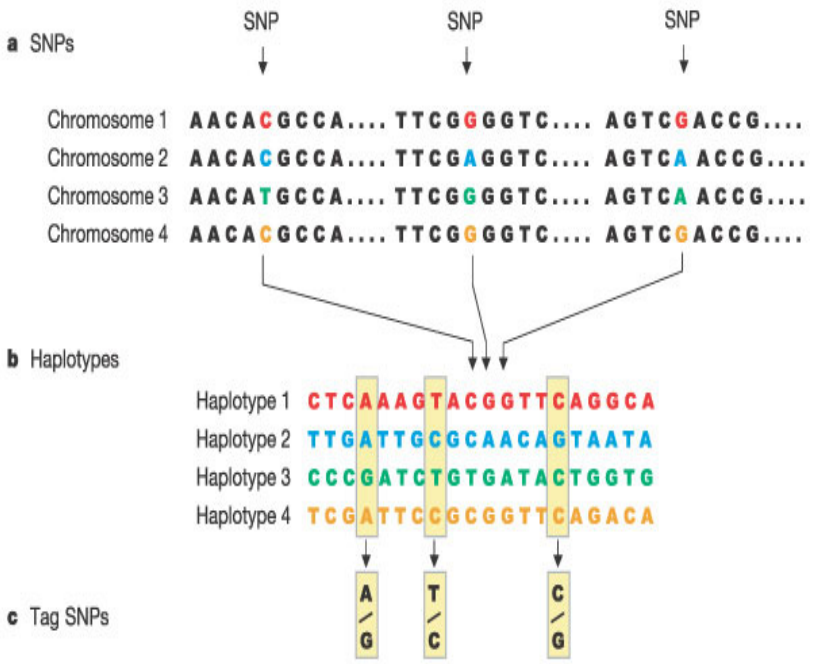


Figure 1–13: tagging SNP.

(a) SNPs are identified in DNA samples from multiple individuals. **(b)** Adjacent SNPs that are inherited together are compiled into "haplotypes." **(c)** "Tag" SNPs within haplotypes are chosen that uniquely identify those haplotypes. Diagram obtained from the International HapMap Project (http://www.hapmap.org).

There are basically two measures of LD: *D'* (a measure of how much two loci are associated together i.e. co-variance between loci) and r^2 (the square of correlation co-efficient). Both measures are calculated, firstly by estimating pairwise haplotype frequencies through expectation-maximisation (EM) (see section 1.8.2.3 below for more details on EM), then by assessing the statistical strength of association through a likelihood ratio test, by comparing the EM frequencies with haplotype frequencies estimated assuming no LD. Both measures of LD are based upon D, the basic pairwise disequilibrium coefficient, the difference between the probabilities of observing the alleles independently in the population: $D = f(A_1B_{1)} - f(A_1)f(B1)$ (Lewontin, 1964). A and B refer to two genetic markers and *f* is their frequency. *D'* is obtained from D/D_{max} and does not take into account allele frequencies. r^2 has a more strict interpretation than *D'* as it takes into account both LD and allele frequency. However since *D'* is more accurate with larger sample sizes and higher allele frequencies, if the population group is large enough and rarer SNPs are excluded then *D'* is utilised. *D'* varies from 0 (complete equilibrium) to 1 (complete disequilibrium). When *D'* = 0, typing one SNP provides no information on the other SNP. When $r^2 = 1$, two SNPs are in perfect LD (Carlson *et al.*, 2004; Pritchard and Przeworski, 2001) and allele frequencies are identical from both SNPs, and typing one SNP provides complete information on other untyped SNPs that are in LD. A relatively stringent r^2 threshold of > 0.8 would allow a selected tSNP to improve the correlation and resolve >80% of all existing haplotypes. Most studies use $r^2 > 0.8$ when deciding on their study design but when viewing haplotype blocks, common SNPs are of value and these are represented more clearly with *D'* which calculates a fairly accurate LD among such SNPs. The disadvantage of using *D'* is that it is less sensitive to marginal allele frequencies.

1.8.2.3 Statistical inference using Haploview

To obtain information on haplotypes from the genotype data, phase information (the relation between alleles at two linked loci) is required. However, from case-control data, it is not possible to infer the phase of an individual's genotypes (i.e. on which of the two chromosomes a series of markers lie for a given individual) and therefore the haplotypes, and it is necessary to resort to estimation. There are several statistical methods (e.g. Arlequin, Clark, HAPLO, Haplotyper and PHASE) for inferring unknown haplotypes from genotype data. These are all ultimately based on the idea that among many of possible haplotypes, few will actually be observed in any population. Thus, phase resolution is achieved by looking for the haplotype pair that is most commonly occurring in the population among those pairs consistent with the genotype. Some of the programs used for inferring phase (based on the maximum likelihood method) employ EM algorithms (Long *et al.*, 1995). This is a class of algorithm that allows estimation of the optimal set of parameters to a hypothesised model. This algorithm is easily generalised and can be applied to any situation where a set of data is available for which a model with unknown parameters is estimated. More specifically, EM algorithms set out to estimate a minimum number of haplotypes consistent with the data by first identifying the known haplotypes (individuals who are homozygous for all sites and individuals who are heterozygous for only one site) and then searching for genotype combinations that are consistent with the known haplotype.

1.8.2.4 International HapMap Project

The international HapMap project is a freely-available public resource to increase the power and efficiency of genetic association studies to medical traits. It provides information on SNP/haplotype frequencies and linkage disequilibrium within several population samples, including one with northern European ancestry (CEPH). The principle behind the method utilized by Gabriel *et al.*, ((Gabriel *et al.*, 2002) was implemented by the HapMap project to constructed LD blocks. This is a methological and quantitative way of constructing a haplotype map of the human genome by using common SNP markers to capture the majority of the diversity within a region of interest.

1.8.2.5 Candidate gene studies

Candidate gene studies rely on having correctly predicted the identity of a disease gene based on biological hypothesis or its location within a known POAG locus. This approach, which utilises common genetic variants, is simpler and cheaper than complete re-sequencing of candidate genes, and offers a proficient way of identifying common genetic variants that underlie complex disease.

1.8.2.6 Genome-wide association studies

By contrast, genome-wide association approaches survey most of the genome for the causal variants. In a sense, the entire genome is tested for a relationship between a marker and a phenotype. This approach has the advantage of making no assumptions about the location of the causal variants, and represents an unbiased as well as a comprehensive method, when there is no pre-existing evidence about the underlying biological mechanism of the disease. Because segments of LD are analysed using tens of thousands of bases, this approach has a greater power to identify the effects of common variants. However, as more markers are required, this approach is a more expensive method.

1.8.3 Classification of genetic loci for POAG

For the purposes of genetic nomenclature, glaucoma loci have been classified into three groups: GLC1 refers to POAG, GLC2 to Primary Closed Angle Glaucoma and GLC3 to Congenital Glaucomas. An alphabetical capital letter is added after the GLC prefix for every novel locus (For example, the first genetic locus for POAG is GLC1A). All of the 9 genetic loci for adult onset POAG show an autosomal dominant inheritance pattern (Table 1-5).

Table 1–5: Genetic loci for adult onset POAG

Chromosomal location	Name of locus	Identified gene	Inheritance pattern	Phenotype	Original study	Contribution to familial POAG	Contribution to POAG in general population
1q21-q31	GLC1A	*MYOC*	AD	HTG	(Sheffield *et al.*, 1993; Stone *et al.*, 1997)	4.4% (Stone *et al.*, 1997; Suzuki *et al.*, 1997)	2 - 4.6% (Alward *et al.*, 1998; Alward *et al.*, 2002; Fingert *et al.*, 1999)
2p16.3-p15	GLC1H	-	AD	HTG	(Suriyapperuma *et al.*, 2007)		
2cen-q13	GLC1B	-	AD	HTG	(Stoilova *et al.*, 1996)		
3q21-q24	GLC1C	-	AD	HTG	(Wirtz *et al.*, 1997)		
5q22.1	GLC1G	*WDR36*	AD: complex	HTG + NTG	(Monemi *et al.*, 2005)	6.92% (Monemi *et al.*, 2005)	1.1% (Monemi *et al.*, 2005) Association study; 3.7% in POAG and 0.2% in controls (P = 0.0005) (Pasutto *et al.*, 2008) Association study; 3.6% in POAG and 1.6% in controls (no significant association; P = 0.24) (Fingert *et al.*, 2007)
7q35-q36	GLC1F	-	AD	HTG	(Wirtz *et al.*, 1999)		
8q23	GLC1D	-	AD	HTG	(Trifan *et al.*, 1998)		
10p15-p14	GLC1E	*OPTN*	AD	HTG + NTG	(Sarfarazi *et al.*, 1998)	16.7% of familial NTGs (when all variants of *OPTN* are combined) (Rezaie *et al.*, 2002)	1.5% for E50K for NTG but none among controls (Aung *et al.*, 2003) 0.1% (1 NTG with family history out of 1048 individuals with POAG; cohort consisted of 2 different ethnic origins- Asian and Caucasian) (Alward *et al.*, 2003)
15q11-q13	GLC1I	-	AD		(Allingham *et al.*, 2005)		

1.8.3.1 *GLC1A*- Myocilin (*MYOC*; OMIM 601652)

The candidate gene for the *GLC1A* locus, *MYOC* (also known as the TM inducible glucocorticoid response (TIGR) gene) was the first POAG gene to be identified (Sheffield *et al.*, 1993; Stone *et al.*, 1997). The GLC1A locus was mapped to chromosome 1 band q21-q31 through linkage study involving a single large Caucasian family in America, in which 22 out of 37 family members had juvenile open angle glaucoma (JOAG), a more severe form of POAG that has an earlier age of onset of 35 years or below (c.f. age of onset is after 35 years it is considered as POAG) (Wiggs *et al.*, 1998). Subsequent studies involving several different populations, including French Canadian (Morissette *et al.*, 1995), American (Richards *et al.*, 1994; Wiggs *et al.*, 1995), Danish (Graff *et al.*, 1995), French (Meyer *et al.*, 1994) and Panamanian (Lichter *et al.*, 1997a) have consolidated this finding. In Caucasians, *MYOC* mutations accounts for up to 36% in JOAG individuals (Shimizu *et al.*, 2000) and a smaller proportion of 4 % of POAG individuals (Fingert *et al.*, 1999). The protein encoded by this gene is located in several glaucoma relevant ocular tissues including the TM, aqueous humor, ciliary body, and retinal ganglion cells (Tamm, 2002). It consists of 501 amino acids, a myocilin like domain, a leucine zipper domain, and an olfactomedian domain where the majority of the known mutations occur. Studies have shown that mutant MYOC molecules result in accumulation of misfolded proteins, which induce stress of the endoplasmic reticulum (Liu and Vollrath, 2004), which in turn induces apoptosis (Rao *et al.*, 2004).

1.8.3.2 *GLC1B*

Stoilova et al. identified the GLC1B locus through linkage analysis involving 6 Caucasian families in the UK within a region of 11.2 cM flanked by markers D2S2161 and D2S176 at chromosome 2cen-q13. Patients who had POAG within these families had phenotypes of low to moderately raised IOP, age of disease onset towards the late 40s and good response rate to topical anti-glaucoma medication.

1.8.3.3 *GLC1C*

The third locus for POAG (*GLC1C*) was identified within a region of 11.1 cM flanked by markers D3S3637 and D3S1744 at chromosome 3q21-q24 (Wirtz *et al.*, 1997) following a linkage study on a single large Caucasian pedigree with POAG from America. Clinical phenotypes of these individuals included adult-onset disease with an average onset of over 40 years, moderate to high IOP and abnormal cup disc ratio.

1.8.3.4 *GLC1D*

The *GLC1D* locus was mapped within a 6.3 cM region on chromosome 8q23, flanked by markers D8S1830 and D8S592 from a linkage study involving a North American family composed of 4 generations (Trifan *et al.*, 1998). This family showed a variable phenotype with glaucomatous visual field defects developing in the younger age group of third or fourth decade, followed by mild to modest increase in IOP with disease progression in the older age group.

1.8.3.5 GLC1E- Optineurin (OPTN; OMIM 602432*)*

The candidate gene for the *GLC1E* locus, *OPTN* was identified from a linkage study performed on a large study involving 54 families of British origin, where 15 out of 46 family members had NTG. From this study, the *GLC1E* locus was mapped within a region of 21 cM (and subsequently reduced to 5 cM), on chromosome 10p15-p14, flanked by markers D10S1729 and D10S1664 (Sarfarazi *et al.*, 1998).

1.8.3.5.1 Molecular structure of OPTN

There are 16 exons within the *OPTN* gene covering 38 kb of genomic sequence. Translation initiates in the 4th exon and alternative splicing of the first three non-coding exons produces at least three different isoforms. All, however, encode for a 577 amino acid protein with a molecular weight of approximately 66 kDa. Optineurin is a cytoplasmic protein that is preferentially expressed in RGCs (De *et al.*, 2006; Kroeber *et al.*, 2006). This protein has been shown to interact with several proteins with diverse functions such as Rab8, Huntington, adenovirus E3 14.7-kDa protein, myosin VI, and transcription factor IIIA (Anborgh *et al.*, 2005; Hattula and Peranen, 2000; Li *et al.*,

1998; Sahlender *et al.*, 2005; Schwamborn *et al.*, 2000). Expression of optineurin mRNA and protein is induced by treatment of cells with tumor necrosis factor α and interferons (Hattula and Peranen, 2000; Li *et al.*, 1998).

1.8.3.5.2 *OPTN* mutations

Three sequence variants of *OPTN* have been considered as disease causing (Figure 1-14) (Rezaie *et al.*, 2002; Sarfarazi *et al.*, 1998): E50K in exon 4, c.691-692insAG located in exon 6, and R545Q located in exon 16. M98K was suggested to confer increase susceptibility to glaucoma (Rezaie *et al.*, 2002). Amongst these, the missense mutation E50K (c.458G>A transition in which glutamic acid in position 50 is replaced by lysine) (Figure 1-15) is the most frequent *OPTN* mutation in familial adult onset POAG (Rezaie *et al.*, 2002).

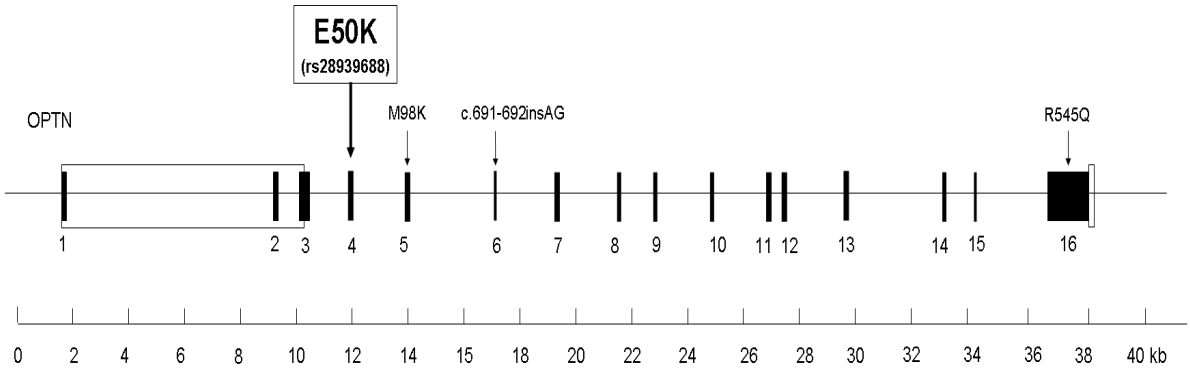


Figure 1–14: Diagrammatic representation of the 3 disease causing *OPTN* mutations (E50K, c.691-692insAG, and R545Q) and one proposed risk factor (M98K) for POAG.

E50K mutation is located in exon 4, M98K in exon 5, c.691-692insAG in exon 6, and R545Q in exon 16. Exons are indicated as solid boxes and numbered accordingly. Untranslated exons are shown as open box.

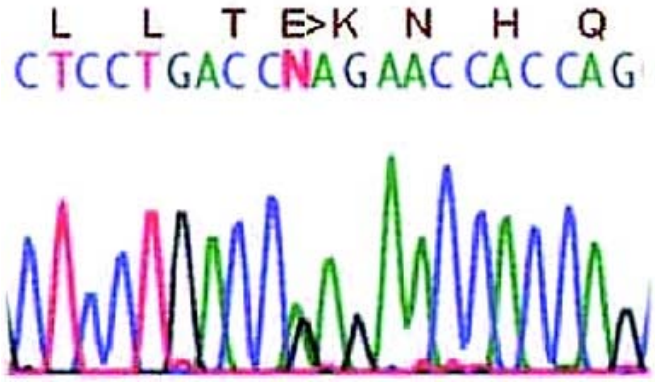


Figure 1–15: A diagrammatic representation (from DNA sequence analysis) of the *OPTN* E50K mutation as a result of c.458G>A transition.
Adapted from Rezaie *et al*., 2002.

However, little is known about the molecular mechanisms responsible for the pathogenesis of glaucoma caused by such mutations.

1.8.3.5.3 NTG as the phenotype of *OPTN*

OPTN mutations accounted for 16.7% of hereditary NTG cases (Rezaie *et al.*, 2002). In particular, Rezaie *et al*. found that seven out of 52 families with POAG (13.5%) harboured a Glu50Lys (E50K) sequence variation, 18.4% of which had elevated IOP and the remaining individuals having normal IOP. The mean age of diagnosis was 44 years (ranging from 23 to 65 years). In subsequent studies when the prevalence of E50K mutation was assessed among sporadic POAG cases in the general population, a much smaller prevalence was observed (Table 1-6). One study including more than 1000 POAG patients containing a mixture of participants of various ethnicity implicated E50K mutation with POAG in only one patient (Alward *et al.*, 2003). This patient had the same E50K mutation that accounted for most of the POAG in the initial study (Rezaie *et al.*, 2002). Interestingly, the patient with the E50K mutation and all of the patients from the original study were of British descent and had familial type glaucoma. This suggests that perhaps a founder effect may be responsible for the presence of

Table 1–6: Prevalence of *OPTN* E50K mutation within the general population of different ethnic origins.

Prevalence rate varied from 1.5% from a UK study involving 2 British individuals (Aung *et al.*, 2003), 1.5% (Hauser *et al.*, 2006) and 0.4% (Alward *et al.*, 2003) from 2 separate US studies, each involving a single patient with a family history of glaucoma, and 3.0% from a US study involving a single Caucasian Hispanic individual (Ayala-Lugo *et al.*, 2007).

Reference	Phenotype	Number of patients according to their ethnicity	Number of patients with *OPTN* E50K mutation	Presence of family history of glaucoma	Prevalence
(Ayala-Lugo *et al.*, 2007)	NTG	1 Hispanic 4 Africans (US., Ghana, Nigeria, Caribbean) 2 Asians (Korea, China, Philippines) 26 Caucasians (Europe and Middle East)	1 from Hispanic origin	Yes (autosomal inheritance)	1 out of 33 POAG = 3.0% (note: the total number of participants consists of individuals of different ethnicity)
	HTG	7 Hispanics 63 Africans 159 Caucasians (Europe and Middle East) 1 Asians (Korea, China, Philippines)	----		
	control	50 Hispanics 116 Caucasians (Europe and Middle East) 117 Asians (Korea, China, Philippines) 88 Africans (US, Ghana, Nigeria, Caribbean)	----		
(Hauser *et al.*, 2006)	NTG	59 US Caucasians 8 Asians 3 African-Americans	1 from US Caucasian origin	Yes	1 out of 67 = 1.5% (note: the total number of participants consists of individuals of different ethnicity)
	HTG	76 US Caucasians 9 African-Americans 2 Asians	----		
	controls	90 US Caucasians 2 African-Americans 8 Asians	----		
(Aung *et al.*, 2003)	NTG	132	2 from UK Caucasian origin	No	1.5%
	HTG	183			----
	control	95			----
(Alward *et al.*, 2003)	NTG	29 US Caucasians with family history of glaucoma 247 Japanese	1 US Caucasian origin	Yes-(father's side of the family was of British origin)	1 out of 276 = 0.4% (note: the total number of participants consists of individuals of different

					ethnicity)
	HTG	441 US Caucasians 84 Australians	----		
	control	162 US Caucasians 89 Japanese	----		

E50K mutation in *OPTN* amongst British individuals (Alward *et al.*, 2003). Since then, E50K mutation has been found in 3 further studies (Table 1-6) where the prevalence rate varied from 1.5% from a UK study involving 2 British individuals (Aung *et al.*, 2003), 1.5% (Hauser *et al.*, 2006) from a US study, and 3.0% from a US study involving a Caucasian Hispanic individual (Ayala-Lugo *et al.*, 2007). The first study involved sporadic NTG cases, whereas the latter two involved familial NTG cases. All of these studies showed an absence of E50K mutation among the HTG cases. A similar result was shown by an additional US study involving familial HTGs (Wiggs *et al.*, 2003) as well as by other studies involving different ethnic populations of sporadic HTG or NTG cases (Table 1-7) including German (Weisschuh *et al.*, 2005), Japanese (Funayama *et al.*, 2004; Fuse *et al.*, 2004; Tang *et al.*, 2003; Toda *et al.*, 2004; Umeda *et al.*, 2004), Australian (Baird *et al.*, 2005), Chinese (Leung *et al.*, 2003), Spanish (Lopez-Martinez *et al.*, 2007), Italian (Ariani *et al.*, 2006) and Indian (Sripriya *et al.*, 2006). Hence, there appears to be an absence of correlation between E50K mutation and HTG, and with NTG individuals of various ethnic origins other than British and US ethnicity. Hence the E50K mutation should be considered mainly in the context of British and to a lesser extent in US subjects.

1.8.3.5.4 Expression and role of *OPTN*

OPTN is expressed in various ocular tissues including the human TM, Schlemm canal, non-pigmented ciliary epithelium, optic nerve and ganglion cells (De *et al.*, 2006; Kroeber *et al.*, 2006; Obazawa *et al.*, 2004; Rezaie *et al.*, 2005; Rezaie *et al.*, 2002; Rezaie and Sarfarazi, 2005; Sarfarazi and Rezaie, 2003). As it is expressed in the neuronal and the glial cells of the optic nerve and the retina, it has been speculated that optineurin has a neuroprotective role in the eye and optic nerve (Rezaie *et al.*, 2002). In addition, *OPTN* expression was also found in aqueous humor samples obtained from humans suggesting it is a secreted protein (Rezaie *et al.*, 2002).

Table 1–7: Absence of E50K mutation among populations of various non British ethnic origins

Reference	Ethnicity	Phenotype	Number of patients	Presence of familial glaucoma	Presence of E50K mutation
(Lopez-Martinez *et al.*, 2007)	Spanish	HTG OHT Control	110 40 98	nil	**nil**
(Sripriya *et al.*, 2006)	Indian	HTG NTG Control	170 50 100	nil	**nil**
(Ariani *et al.*, 2006)	Italian	HTG NTG Control	35 (familial) 18 sporadic --- ---	yes	**nil**
(Weisschuh *et al.*, 2005)	German	HTG NTG Control	--- 112 ---	nil	**nil**
(Toda *et al.*, 2004)	Japanese	HTG NTG Control	165 148 196	nil	**nil**
(Umeda *et al.*, 2004)	Japanese	HTG NTG Control	55 28 58	nil	**nil**
(Fuse *et al.*, 2004)	Japanese	HTG NTG Control	89 65 100	nil	**nil**
(Funayama *et al.*, 2004)	Japanese	HTG NTG Control	194 217 218	nil	**nil**
(Baird *et al.*, 2004)	Australian	HTG NTG Control	18 27 108	nil	**nil**
(Tang *et al.*, 2003)	Japanese	HTG NTG Control	165 148 196	nil	**nil**
(Leung *et al.*, 2003)	Chinese	POAG Control	199 126	nil	**nil**
(Wiggs *et al.*, 2003)	US	HTG NTG Control	86 (familial) --- 80	yes	**nil**

1.8.3.5.5 Role of *OPTN*

The function of *OPTN* is still unknown, but the protein interacts with the tumour necrosis factor α (TNFα) signalling pathway. Studies have shown that it may directly regulate cell death (Chen *et al.*, 1998; Li *et al.*, 1998) by interacting with proteins involved in apoptosis and by inducing TNFα. It has been shown that expression of optineurin in cells infected with adenovirus E3-14.7K results in protection from tumor necrosis factor induced cytolysis and that interferon and TNF can induce a *de novo* expression of optineurin (Schwamborn *et al.*, 2000). TNFα increases the severity of damage of optic nerve heads of POAG individuals (both HTG and NTG) (Danson, 1988; Haramaki *et al.*, 1997). Hence wild-type optineurin may act via the TNFα pathway and provide a neuroprotective role in the optic nerve, and when defective, causes glaucomatous optic neuropathy.

Recently, Park et al (Park *et al.*, 2006) showed that over expression of E50K in human TM and retinal pigment epithelial cells resulted in Golgi fragmentation and cell death. In addition, a study which explored functional features of *OPTN* showed that proteins with the E50K mutation acquired the ability to selectively induce cell death in retinal ganglion cells which was mediated by oxidative stress (Chalasani *et al.*, 2007). Furthermore, *OPTN* has shown to provide a protective role within the TM. Vittitow and Borras (Vittitow and Borras, 2002) assessed the effect of glaucomatous insults from raised IOP, tumor necrosis factor-alpha, and dexamethasone on the expression of *OPTN* in human TM maintained in organ culture. All of these 3 factors resulted in an up regulation of *OPTN* expression.

1.8.3.5.6 Conclusion

When combining all of the available results on *OPTN*, *OPTN* mutations may be involved in the aetiology of familial glaucoma and its E50K mutation appears to be a cause of NTG in individuals of British and US origin. In addition, there appears to be no association between E50K mutation and unrelated cases of HTG.

1.8.3.6 *GLC1F*

Through a genome wide study of a large POAG family, *GLC1F* locus was localised within a region of 5.3 cM on chromosome 7q35-q36, flanked by markers D7S2442 and D7S483 (Wirtz *et al.*, 1999). All of the 10 members of the family had IOPs of more than 22 mmHg, a vertical C/D ratio of 0.6 or more, with 5 individuals having glaucomatous visual field defects.

1.8.3.7 *GLC1G*- WD repeat domain 36 (*WDR36*; OMIM 609669)

The third candidate gene for POAG for the *GLC1G* locus, *WDR36*, was localized on chromosome 5q22.1, in a region of 2 Mb between D5S1466 and D5S2051 (Monemi *et al.*, 2005). Four mutations (N355S, A449T, R529Q and D658G) were identified in 17 (5-7%) unrelated POAG subjects, 11 of which were HTGs and six were NTGs. However, Fingert et al (Fingert *et al.*, 2007) were unable to confirm this association in 2 large cohorts of POAG individuals.

WDR36 gene is composed of 23 exons and its protein is expressed in a number of ocular tissues including TM, ciliary body, ciliary muscles, retina, optic nerve, lens, iris, and sclera (Monemi *et al.*, 2005).

Studies have shown that *WDR36* may be involved in T-cell activation (Mao *et al.*, 2004) and recently, it was hypothesized that T-cell-mediated responses may participate in glaucomatous optic neuropathy (Mao *et al.*, 2004). However, the exact role in glaucoma pathogenesis remains unclear.

1.8.3.8 *GLC1H*

Suriyapperuma et al. (2007) mapped a new genetic locus for adult-onset primary open-angle glaucoma (POAG) by using families previously unlinked to *GLC1A-GLC1F*. Through performing a genome-wide study in 322 individuals with POAG from 91 unrelated families, a consistent linkage to chromosome 2p16-p15 in 7 families was found. Analysis in 1 large family with 12 affected members revealed a common haplotype within a region of approximately 11 Mb flanked by markers D2S123 to D2S2165. All affected members of the remaining 6 families shared similar haplotypes

that overlapped with that of the first large family, but with no founder effect. Combined analysis of the 7 families produced the highest lod score of 9.30 with marker D2S2320. Age of diagnosis ranged from 32 to 78 years with an average age at onset of 64 years. IOPs varied from 24 mmHg to 60 mmHg with C/D ratio ranging from 0.6 to 0.9.

1.8.3.9 *GLC1I*

Using ordered subset analysis on 86 POAG families in which age of onset was used as covariate, Allingham et al. (2005) identified an 11 cM linkage interval in chromosome 15q11-q13 in a subset of 15 families (Allingham *et al.*, 2005). The mean age of diagnosis of the affected families was 44 years compared to the mean age of diagnosis of 61 years for the complementary group.

1.8.3.10 Summary

Based on the current available evidence, mutations in *MYOC* account for a small proportion of 2 to 4.6 % of non-familial POAG, whereas variations in *OPTN* and *WDR36* show varying evidence for association with POAG in the general population depending on the ethnicity. The emerging view is that POAG is a complex, multifactorial disease resulting from interactions between several genetic factors, and it is highly likely that other unidentified loci or genes still exists. It would be interesting to assess the role of other loci once the genes have been identified.

1.8.4 Genes associated with POAG

The development of genetic screening programmes to identify high risk glaucoma gene variants in populations is a realistic future goal made possible with the current pace of technological advances in high throughput genotyping using association studies. To achieve this goal, association studies need to first prove the role of susceptibility genes in large well-defined cohorts. This field is in its infancy and so far, studies have focused on analysis of genes that are suggested to increase susceptibility to glaucomatous damage (retinal ganglion cell death). Often using relatively small cohorts, some studies have found associations between polymorphisms in genes thought to affect ganglion cell survival, including *p53, APOE, OPA1, IGF2* and *TNFα,* (see Table 1-8 for other

genes associated with POAG). The majority of these genes have been assessed in single studies, whilst some have some conflicting results, and none has been definitely shown to be a major factor (Libby *et al.*, 2005).

To date, at least 22 genes have been associated with POAG (Table 1-8).

Table 1–8: Reported genes associated with POAG.

Cohort size which is ≥ 200, $P \leq 0.05$ and studies with adjustment for multiple testing are high-lighted in bold.

Gene symbol	Chromosomal location	Function	Size of cohort	Association with POAG	Reference	Adjusted for multiple testing or 2nd cohort study performed
AGTR2	Xq22–q23	Regulation of aqueous humor dynamics	190 POAG, **268 NTG, 240 controls**	P = 0.0095 for female NTG but not for male NTG	(Hashizume *et al.*, 2005)	nil
ANP	1p36.2	Involved in the homeostatic control of body water, sodium, potassium and fat	53 familial POAG, 60 controls	No association	(Tunny *et al.*, 1996)	nil
APOE	19q13.2	Essential protein in lipid transport-notably in the metabolism of neuronal membrane	**242 POAG**, 187 controls	No association	(Zetterberg *et al.*, 2007a)	nil
			294 POAG, 106 NTG, **300 controls**	***P* = 0.008** for NTG (protective effect)	(Lam *et al.*, 2006)	nil
			310 POAG, 179 controls	***P* = 0.018**	(Mabuchi *et al.*, 2005)	Adjusted for age
			140 POAG, 73 controls	No association	(Ressiniotis *et al.*, 2004c)	nil
			155 NTG, 349 controls	No association	(Lake *et al.*, 2004)	nil
			137 POAG, 75 controls	No association	(Ressiniotis *et al.*, 2004a)	Logistic regression analysis used
P21	6p21.2	An important protective component of the apoptotic	140 POAG, 73 controls	No association	(Ressiniotis *et al.*, 2005)	nil
						nil

		pathway, regulating cellular arrest in the presence of DNA damage	58 POAG, 59 controls	***P* = 0.0078**	(Tsai *et al.*, 2004)	
CDH-1	16q22.1	Cell adhesion molecule	60 POAG 103 controls	***P* < 0.000**	(Lin *et al.*, 2006)	nil
CYP1B1	2p22–p21	1) Modifier of *MYOC* 2) Causative gene for primary congenital glaucoma	**264 POAG**, 95 controls 134 POAG (30 of which are JOAG), **200 controls** **224 POAG**, 47 controls	**P = 0.0001** ***P* = 0.006** ***P* = 0.0036** (protective effect)	(Bhattacharjee *et al.*, 2008) (Chakrabarti *et al.*, 2007) (Melki *et al.*, 2005)	nil nil **Bonferroni correction made**
EDNRA	4q31.2	Endothelin-1 (EDN1), a potent physiologic vasoconstrictors	67 NTG, 100 controls **250 NTG**, 176 POAG	***P* = 0.028** No association (but genotype associated with worse visual field defects in NTG patients (***P*** = 0.014))	(Kim *et al.*, 2006) (Ishikawa *et al.*, 2005)	nil Adjusted for age
GSTM1	1p13.3	Multifunctional detoxification enzyme	**250 POAG, 202 controls** 144 POAG, 121 controls (Turkish ethnicity) **200 POAG, 200 controls**	***P* = 0.002** ***P* < 0.001** No association	(Juronen *et al.*, 2000) (Unal *et al.*, 2007) (Jansson *et al.*, 2003a)	**Bonferroni correction made** nil nil
HSPA1A	6p21.3	Heat-shock proteins expressed in response to heat shock and a variety of other stressful stimuli, including oxidative free radicals	**211 POAG, 290 NTG 241 controls**	***P* = 0.007** (POAG vs controls) ***P* = 0.032** (NTG vs controls)	(Tosaka *et al.*, 2007)	nil
IGF2	11p15	Neurotrophic agent	60 POAG, 104 controls	***P* = 0.010**	(Tsai *et al.*, 2003)	nil
IL1β	2q14	Immune response mediator	58 POAG, 105 controls (Chinese) 231 NTG, 245 controls (Chinese)	***P* = 0.035** No association	(Lin *et al.*, 2003b) (Wang *et al.*, 2007)	nil nil
MTHFR	1p36.3	Regulates homocystein	76 POAG, 71 controls	***P* = 0.01**	(Junemann *et al.*, 2005)	nil

		metabolism, reduced activity of which causes hyperhomocysteinaemia (risk factor for vascular disease)	78 NTG, 100 controls	***P* = 0.006**	(Woo *et al.*, 2007)	nil
			243 POAG, 187 controls	No association	(Zetterberg *et al.*, 2007b)	nil
			133 POAG, 131 NTG, 106 controls	No association	(Mabuchi *et al.*, 2006)	nil
			204 POAG, 138 controls	No association	(Mossbock *et al.*, 2006a)	nil
NOS3	7q36	Regulation of microcirculation (produces nitric oxide which causes vasoconstriction)	56 POAG, 100 controls	***P* < 0.01**	(Tunny *et al.*, 1998)	nil
			58 POAG, 76 NTG, 30 controls	***P* = 0.06** ***P* < 0.001 (in** POAG patients with migraine)	(Logan *et al.*, 2005)	nil
			66 POAG, 100 controls	No association	(Lin *et al.*, 2005)	nil
NPPA	1p36.2	Regulation of intraocular fluid homeostasis and IOP	67 NTG, 100 controls	No association	(Jeoung *et al.*, 2007)	nil
			53 familial POAG, 60 controls	No association	(Tunny *et al.*, 1996)	nil
OCLM	1q31.1	Mechanical stretch response gene in TM	75 POAG, 29 NTG, 5 OHT, 50 controls	2/75 POAG had AA substitution	(Fujiwara *et al.*, 2003)	nil
			200 POAG, 200 controls	No association	(Jansson *et al.*, 2003b)	nil
OLFM2	19p13.2	Myocilin-related proteins	**215 POAG, 277 NTG, 240 controls**	Combination of OLFM2 and OPTN associated with raised IOP ***P* = 0.018**	(Funayama *et al.*, 2006)	**Adjusted for multiple testing**
OPA1	3q28–q29	Component of the mitochondrial network, mutation of which causes an autosomal dominant optic atrophy	83 NTG, 100 controls	**P = 0.005** (1st cohort)	(Aung *et al.*, 2002b)	**Confirmed in 2nd cohort**
			80 NTG, 86 controls (2nd cohort)	***P* = 0.002** (2nd cohort)		
				***P* = 0.00001** (when both cohorts combined)		**Adjusted for multiple testing**
			90 HTG, 186 controls	No association	(Aung *et al.*, 2002a)	nil
			61 NTG, 168 controls	***P* = 0.006** (prior to	(Powell *et al.*,	**Remains**

				bonferroni correction)	2003)	**significant after Bonferroni correction**
			65 NTG, 101 controls (Korean ethnicity)	No association	(Woo *et al.*, 2004)	nil
			48 POAG, 61 NTG, 48 controls (Barbados ethnicity)	No association	(Yao *et al.*, 2006)	**Bonferroni correction made**
			194 NTG, 191 HTG, 185 controls	***P* = 0.004** for NTG	(Mabuchi *et al.*, 2007)	Adjusted for age, gender, but not for multiple testing.
			Caucasian (**279 POAG, 227controls),** African American (193 POAG, 97 controls), Ghanaian (170 POAG, 138 controls)	No association	(Liu *et al.*, 2007)	nil
PON1	7q21.3	Antioxidant	**243 POAG, 312 NTG, 284 controls**	No association	(Inagaki *et al.*, 2006)	**Bonferroni correction made**
TAP1	6p21.3	Delivers peptide to the HLA class I molecule	66 POAG, 105 controls	***P* = 0.01**	(Lin *et al.*, 2004)	nil
TLR4	9q32-q33	Toll-like receptor 4 mediates immune responses and interacts with heat shock proteins	**255 NTG, 318 controls**	***P* = 0.0095**	(Shibuya *et al.*, 2008)	**Adjusted for multiple testing**
TNF	6p21.3	A major immunomodulator and proinflammatory cytokine	POAG 114, **228 controls**	No associations	(Mossbock *et al.*, 2006b)	nil
			60 POAG, 103 controls	***P* = 0.00016**	(Lin *et al.*, 2003a)	As above
TP53	17p13.1	Regulator of apoptosis	**282 HTG**, 62 NTG, 178 controls	No associations	(Dimasi *et al.*, 2005)	nil
			140 HTG, 73 controls	***P* < 0.0001** for haplotype distribution	(Ressiniotis *et al.*, 2004b)	nil
			67 HTG, 112 controls (Asian ethnicity)	No association	(Acharya *et al.*, 2002)	nil
			58 POAG, 59 controls	***P* = 0.00782**	(Lin *et al.*, 2002)	nil

To date, 52 association studies have been performed on POAG, 23 of which have shown no association. Of the ones that have shown association, only 7 genes have showed significant association in more than 1 study with POAG (*APOE, CYP1B1, GSTM1, MTHFR, NOS3, OPA1,* and *TP53*) using sample sizes in the majority of cases of less than 200 with the exception of 5 studies (involving *APOE, CYPB1, GSTM1)* which have used sample sizes ranging from 224 to 310. When adjusted for multiple testing which is considered a standard statistical requirement for this type of analysis (Miller, 1981), only 6 genes (*CYP1B1, GSTM1, OLFM2, OPA1, PON1* and *TLR4*) have produced significant associations. Among these, only one gene (*OPA1*) has shown reproducible association in a 2nd independent cohort. Based on these finding, *OPA1, CYP1B1* and *GSTM1* have demonstrated the best association with POAG.

1.8.5 Conclusion

The exact mechanism of these genes in POAG susceptibility remains unknown. It is still controversial as to whether the inheritance pattern of POAG is a simple monogenic inheritance or a complex polygenic inheritance. It could well be that variable expressivity, reduced penetrance or even phenotypic alteration by genetic modifiers (where certain mutations may only cause disease when present in a susceptible genetic context as in *OPTN* mutations) may confound genotypic-to-phenotypic associations and make it difficult to ascertain a monogenic inheritance. On the other hand, there are several pieces of evidence to suggest that glaucoma may be affected by multiple interacting genes. For example *APOE* has been shown to interact with the *MYOC* to increase IOP (Copin *et al.*, 2002). Another study showed interaction between *MYOC* and *CYP1B1* through a common pathway with the inheritance of glaucoma being digenic in some cases (Vincent *et al.*, 2002). In addition, a possible interaction between *OPTN* and *TNF* was identified to increase the risk of glaucoma (Funayama *et al.*, 2004). More recently, possible gene–gene interactions between *MYOC, OPTN* and *APOE* was identified which collectively contributed to POAG (Fan *et al.*, 2005). Evidence to further support the polygenetic inheritance of POAG include type II diabetes mellitus and steroid responsiveness from endogenous steroids (i.e. stress) and pharmacologic steroids increasing the risk of glaucoma (Pasquale *et al.*, 2006; Zhang *et al.*, 2005).

Current evidence reveals only a small proportion of POAG exhibit Mendelian inheritance as evidenced by the known POAG genes (*MYOC, OPTN, WDR36*) accounting together for 10% of all POAG patients. It remains to be proven whether variants of several genes, each contributing small effects in a given individual increase the risk for developing POAG (Fan *et al.*, 2005), or whether there exist a single locus of large effect. In addition, *OPTN* mutations, in particular E50K mutation, appear to be a suitable candidate gene to further assess its role in POAG within a cohort of individuals of British descent.

For the purpose of this study, from this point onwards, POAG has been considered as a complex polygenic disease.

1.9 Developmental glaucoma genes

An obvious group of genes to test as genetic susceptibility factors for POAG are the developmental glaucoma genes which have not been analysed in any association studies to date with the exception of the CY1B1 gene. *CYP1B1* causes primary congenital glaucoma. It is also involved in cases of juvenile open angle glaucoma (JOAG) (Acharya *et al.*, 2006; Vincent *et al.*, 2002) and a recent study identified a *CYP1B1* polymorphism as a susceptibility factor for POAG (Bhattacharjee *et al.*, 2008; Melki *et al.*, 2005). Developmental glaucoma defines glaucomas that are associated with dysgenesis of anterior segment. Anterior segment dysgenesis (ASD) are an ill-defined, genetically heterogeneous, autosomal-dominant group of developmental abnormalities that share some common ocular features and a high prevalence of associated glaucoma (Gould and John, 2002). Clinical features of ASD includes combinations of congenital abnormalities affecting the cornea (e.g. posterior embryotoxon, corneal opacities), iris (polycoria, corectopia, iris hypoplasia), lens and anterior chamber angle (peripheral anterior synechiae)

1.9.1 Development of eye and anterior chamber

An understanding of the embryonic development of the anterior segment of the eye is essential to understand the association of ASD with glaucoma. The anterior segment of

the eye consists of the cornea, iris, lens, ciliary body and the ocular drainage structures, which contains the TM and the Schlemm's canal. The drainage structures are located at the iridocorneal angle where the cornea and the iris meet. These structures are formed by coordination of events involving induction and differentiation of three primary embryonic tissues: surface ectoderm, neural ectoderm and the periocular mesenchyme (Snell and Michael, 1998) (Figure 1-16 and 1-17). Through the course of ocular development the surface ectoderm will form the corneal epithelium and the lens while the neural ectoderm will form the retina and epithelia of both the iris and ciliary body (Kaufman, 1995). The corneal stroma, corneal endothelium, sclera, iris stroma, ciliary muscle, ciliary stroma and TM are all derived from periocular mesenchyme, which consists of neural crest cells. These cells are neuroectodermal cells that migrate from the crest of the developing neural tube at about the 24th day of gestation (Tripathi and Tripathi, 1989). Schlemm's canal is formed by remodeling of vasculature in the corneoscleral transition zone (Hamanaka *et al.*, 1992) and is also derived from periocular mesenchyme. Hence, cells that are derived from the neural crest, and the accurate specification and differentiation of mesenchymal progenitor cells, is essential for anterior segment development (Kupfer and Kaiser-Kupfer, 1979).

At the 6th week of embryonic development, a bi-layered embryonic optic cup from the forebrain neuroectoderm has formed, whilst the lens vesicle has formed through invagination and separation from the overlying surface ectoderm. The primordial eye is now surrounded by mesenchymal progenitor cells which are of predominantly of neural crest origin (Figure 1-16a).

As early as the 12th week a roughly wedged-shaped distinctive mass of mesenchyme, the trabecular primordium, is detected at the junction of the pupillary membrane (a thin central portion of the iridopupillary lamina occluding the pupil in fetal life which exist as a source of blood supply to the lens) and lateral margins of the cornea i.e. the anterior chamber angle (McMenamin, 1989a). The trabecular primordium consists of a dense collection of stellate mesenchymal cells and some loosely arranged extracellular matrix. The deep aspect of the wedge-shaped primordium is characterized by a row of small capillaries and is lined by mesoderm-derived vascular endothelial cells.

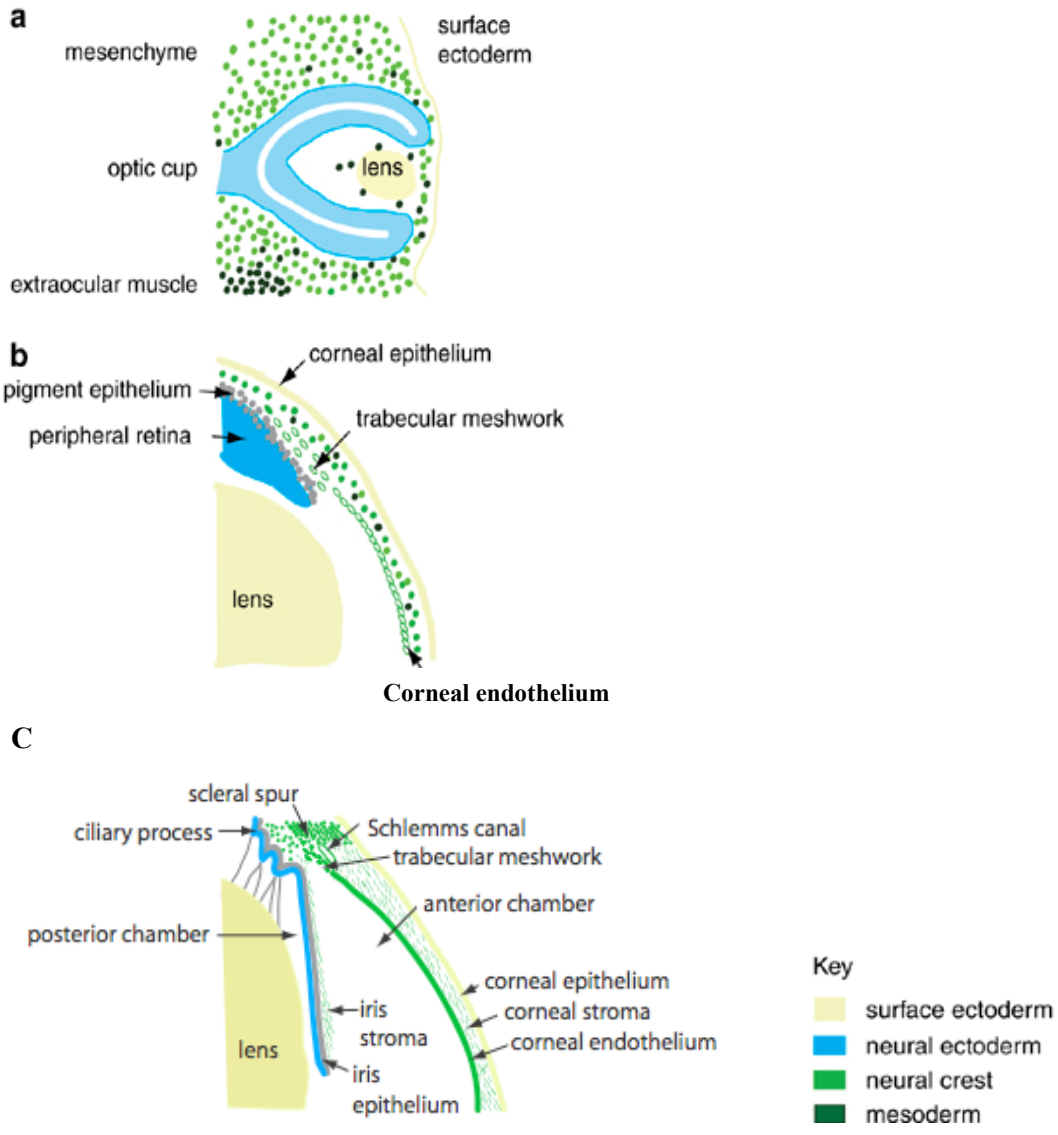


Figure 1–16: The development of the anterior segment of the embryonic and fetal eye.

(a) At 5 weeks of gestation, the optic cup is surrounded by mesenchymal progenitor cells, mainly of neural crest origin. (b) Formation of the anterior segment is visible at 5 months of human gestation. (c) Fully developed anterior segment depicting the lens, iris, irido-corneal angle, and the cornea. The diagrams are colour coded to represent the embryonic origin of the anterior segment structures. Adapted from Sowden, 2007.

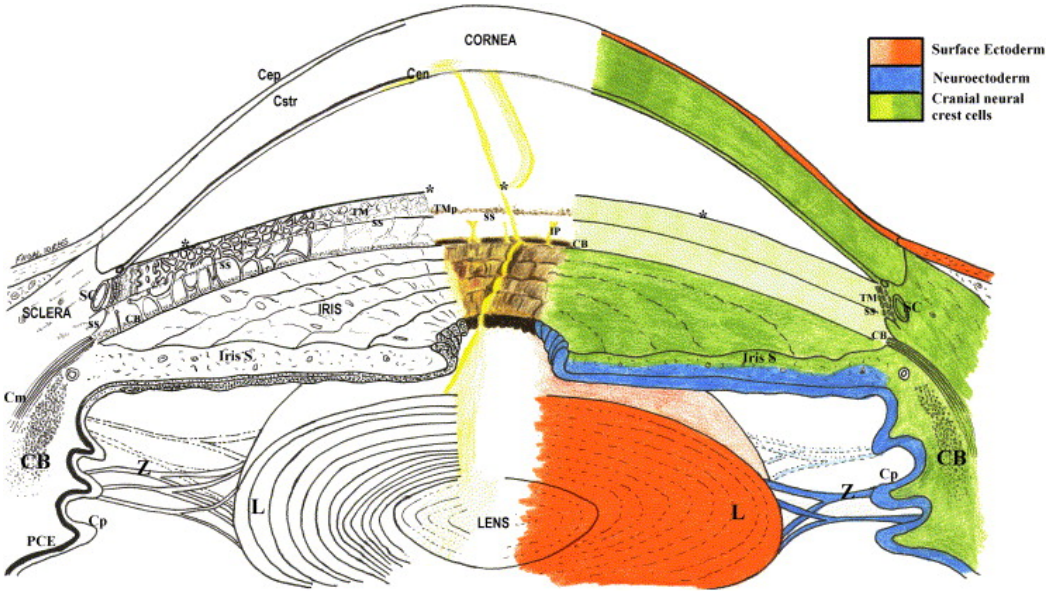


Figure 1–17 Cross section of a mature anterior segment chamber high-lighting important structures at the anterior chamber angle:

(*left*); in depth view of the angle showing the different layers of trabecular meshwork; (*center*) gonioscopic view of a normal angle with a slit-lamp beam; (*right*) embryological derivatives forming the structures of the anterior chamber (red = surface ectoderm; blue = neuroectoderm; green = cranial neural crest cells).

Cep = corneal epithelium; Cen = corneal endothelium; Cstr = corneal stroma; * = Schwalbe's line; ss = scleral spur; CB = ciliary body; Cp = Ciliary process; Cm = ciliary muscle; PCE = pigmented ciliary epithelium; SC = Schlemm's canal; TM = trabecular meshwork; IP = iris process; Iris S = iris stroma; L = lens; Z = zonule/suspensory ligament of lens. Adapted from Idrees *et al*., 2006.

By 20-22 weeks of gestation, the anterior chamber is formed and well demarcated. The trabecular primordium consists of flattened endothelial-lined sheets and cords (early trabeculae) separated by intervening spaces (Figure 1-18) (McMenamin, 1989b; McMenamin, 1991; McMenamin and Krause, 1993). On the deep aspect of the undeveloped TM, the collection of small capillaries fuses to form a single elongated canal of Schlemm lined by endothelial cells derived from mesoderm. The giant vacuoles within the endothelium which are responsible for the passage of aqueous across the canal, appear around 18-20 weeks of gestation. At 28 weeks (7 months) the TM is separated from the anterior chamber by the layer of neural crest cells and is capable of aqueous drainage to a small degree (Kupfer and Ross, 1971). During the later stages of fetal development the meshwork becomes further specialized into three morphogenically distinct layers- cord like inner uveal trabeculae, intermediate layers of lamellar corneoscleral trabeculae, and a deep loosely arranged cribriform meshwork.

1.9.2 *FOXC1* and *LMX1B* as genetic risk factors in POAG

Since neural crest cells are a major contributing factor towards anterior segment development, one hypothesis for the underlying molecular mechanism involved in anterior segment dysgenesis is that defects in migration and/or differentiation of neural crest derived periocular mesenchyme could lead to abnormal anterior segment development and subsequently, raised IOP and glaucoma. Evidence in support of this proposal has been shown by fate mapping of cranial neural crest cells using quail-chick chimeras (Kaiser-Kupfer, 1989; Kupfer and Kaiser-Kupfer, 1979; Kupfer and Kaiser-Kupfer, 1978). These have lead to the creation of the model which has suggested that the majority of the anterior segment is derived from neural crest cells and that the developmental arrest of these cells causes ASD in humans. Indeed, transcription factors expressed in the periocular mesenchyme (*FOXC1* and *LMX1B*) (Gage *et al.*, 2005; Pressman *et al.*, 2000) play an important role in normal morphogenesis of the anterior segment and provide important insight to the underlying genetic mechanism of glaucoma.

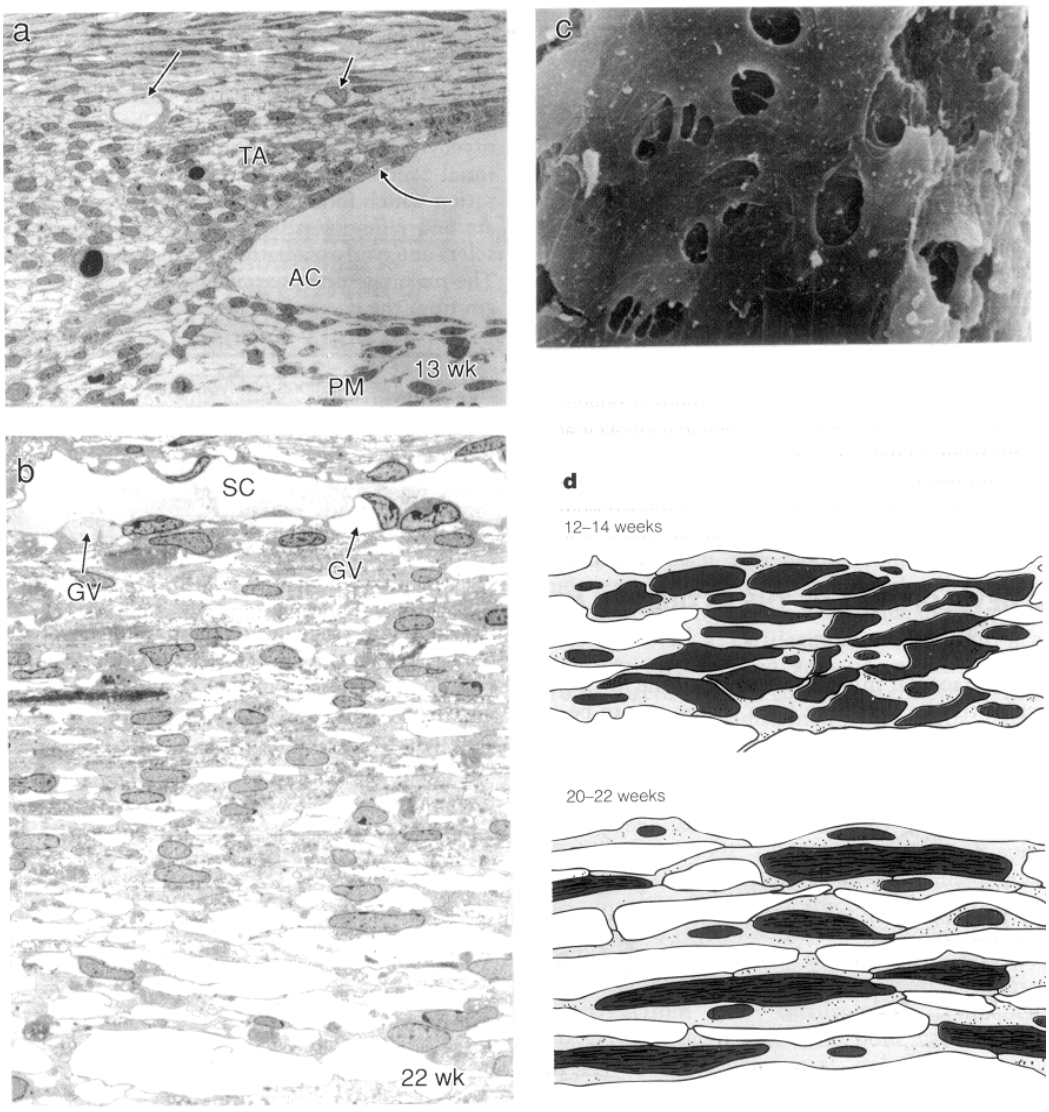


Figure 1–18: Embryonic development of the anterior chamber angle.

(a) Electron micrograph of the trabecular primordium in a 13 week-old fetus. Arrows point to two small capillaries on the deep aspect of the trabecular anlage (TA). AC, anterior chamber; PM, pupillary membrane. **(b)** Electron micrograph of the TM in a 22-week-old human fetus. Note the intratrabecular spaces separated by connective tissue trabeculae and the Schlemm's canal (SC) which posses giant vacuoles (GV) in its inner wall. **(c)** Scanning electron micrograph of the inner surface of the trabecular anlage in a 13-week-old human fetus showing numerous perforations to allow communication between the anterior chamber and the developing meshwork. **(d)** Summary of the morphogenetic changes that occurs during remodeling of the loose mesenchyme of the trabecular anlage to form the TM. Adapted from McMenamin, 1989.

1.9.2.1 FOXC1 (OMIM; 601090)

1.9.2.1.1 General role

Transcription factors of the forkhead box (FOX) family were first identified as a region of homology between the *Drosophila ogaster melan* protein forkhead and rat hepatocyte nuclear factor 3 proteins (Weigel and Jackle, 1990). These transcription factors have been shown to be key regulators of diverse cellular functions including development of many organ systems, oncogenesis and energy homeostasis (Carlsson and Mahlapuu, 2002; Lehmann *et al.*, 2003a). In addition, FOX proteins have been identified as important gene regulators within a broad range of species from yeast to human and have been involved in a variety of biological processes including tumorigenesis and even in language acquisition (Lai *et al.*, 2001).

1.9.2.1.2 Structure of forkhead domain and FOXC1

These transcription factors consist of a well conserved monomeric 110 amino acid DNA-binding domain, known as the forkhead domain (FHD). The DNA binding motif is a variant of the helix-turn-helix, and consists of three α-helices, two β-sheets and two large loops that form 'wing-like' structure (Figure 1-19) (Clark *et al.*, 1993). This domain binds to DNA and localizes FOXC1 to the nucleus of the cell, and is likely to be involved in protein-protein interaction as evidenced by missense mutations in the FHD reducing the ability for FOXC1 to transactivate, and affecting protein stability and DNA binding (Saleem *et al.*, 2001; Saleem *et al.*, 2003b; Saleem *et al.*, 2003a). The *FOXC1* gene (formerly known as *FKHL7)* is located at chromosome 6 at p25 and was first shown to cause ASD in 1998 (Mears *et al.*, 1998; Nishimura *et al.*, 1998). It is a member of the winged-helix family of the transcription factors as it contains the conserved FHD (Larsson *et al.*, 1995; Mears *et al.*, 1998; Nishimura *et al.*, 1998) and is comprised of 1 exon of 1.6 kb which encodes a 533 amino acid protein (Larsson *et al.*, 1995; Mears *et al.*, 1998; Nishimura *et al.*, 1998).

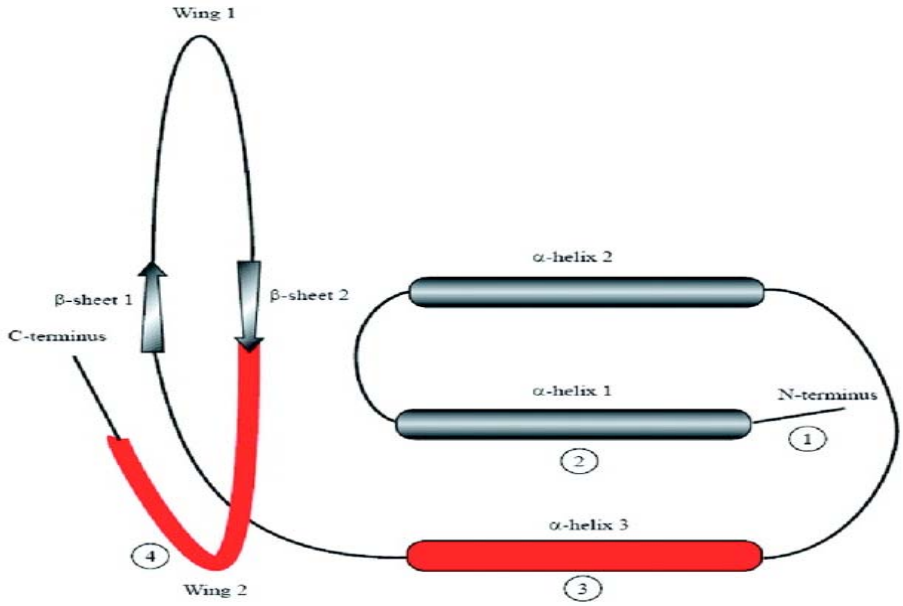


Figure 1–19: Diagrammatic representation of the sub-domains of the FOXC1 forkhead domain.

Region 1 and 2 represent the N-terminal portion of FHD. α-helix 1 permits organization of FHD for transactivation, DNA binding and nuclear localization. Region 3 represents α-helix 3 which is involved in DNA binding specificity of FHD, and allows organization of the FHD with respect to nuclear localization and binding. Region 4 represents the wing 2 region which allows organization of FHD for transactivation and DNA binding. Adapted from Saleem *et al.*, 2004.

1.9.2.1.3 Phenotypes of *FOXC1* mutations (Axenfeld-Reiger malformation and Axenfeld-Reiger syndrome)

ASD comprises a spectrum of inherited mainly autosomal-dominant diseases that result from maldevelopment of the anterior segment of the eye, with the development of glaucoma as one of the serious ophthalmic consequences, in which only 18% responds to non-invasive medical treatment (Strungaru *et al.*, 2007). Within the broad disease spectrum of maldevelopment of the anterior segment caused by *FOXC1* mutation, iris hypoplasia falls towards the mild end of the spectrum, with iridogoniodysgenesis (iris hypoplasia and goniodysgenesis with excess tissue in the angle and anomalous angle vascularity (Mears *et al.*, 1996)) being at the intermediate end of the spectrum, and Axenfeld-Rieger malformations (ARM) of the eye (iris hypoplasia, correctopia, goniodysgenesis, prominent and displaced Schwalbe's line (posterior embryotoxon) and peripheral anterior synechiae)) situated at the most severe end of the disease spectrum (Walter, 2003).

A recent study involving 126 patients with ARM showed up to 75% of such individuals developed glaucoma (Strungaru *et al.*, 2007). In addition, non-ocular manifestations such as facial malformations (hypoplasia of the maxillary bones), dental dysgenesis (hypodontia and microdontia), umbilical anomalies, sensorineural hearing loss, congenital cardiac defects and even pituitary abnormalities can occur in individuals with *FOXC1* mutations with incomplete penetrance and varying in expressivity (Shields *et al.*, 1985; Swiderski *et al.*, 1999; Winnier *et al.*, 1999). ARM of the eye in association with extraocular malformations is known as Axenfeld-Rieger Syndrome (ARS). This is a rare syndrome with the incidence estimated to be around 1:200,000 (Gorlin *et al.*, 1976). There are four known genetic loci associated with ARM/ARS, including *FOXC1* and *PITX2,* which are located in chromosome 6p25 and 4q25 respectively (Nishimura *et al.*, 1998; Semina *et al.*, 1996). The third locus has been mapped to chromosome 13q14 and the gene is yet to be identified (Phillips *et al.*, 1996). Interestingly, an isolated case of ARM has shown to carry a deletion in the *PAX6* gene (Riise *et al.*, 2001). The broad range of clinical features suggests that *FOXC1* has an extensive role in the developmental process.

1.9.2.1.4 Expression of *FOXC1*

FOXC1 is expressed in the human fetus as well as in human adults (Mears *et al.*, 1998; Nishimura *et al.*, 1998; Pierrou *et al.*, 1994). Analysis of the function of FOXC1 in animal models has provided a comprehensive insight into the embryonic origin of the malformation. In the developing mouse eye, *Foxc1* is expressed in the periocular mesenchyme, and mesenchymal cells that have migrated into the eye (Kidson *et al.*, 1999; Kume *et al.*, 1998), TM (Kidson *et al.*, 1999), and in the developing skeleton and organ structures including the heart and kidney (Hiemisch *et al.*, 1998; Kume *et al.*, 2000; Kume *et al.*, 1998). Since *Focx1* is expressed in the developing embryonic TM, mutations or altered expression of *FOXC1* could interfere with normal function of the tissue and lead to increased risk of glaucoma. Although expression of *FOXC1* is yet to be studied in adults, it is highly possible that continued expression of the abnormal gene product (from age-related, subclinical mutations) throughout life, or altered levels of expression of *FOXC1* could interfere with normal function of the TM, thereby leading to increased risk of glaucoma through the effects of raised IOP. This notion is supported by the fact that glaucoma associated with mutations in the developmental glaucoma genes can present at any time from birth to adulthood (Cella *et al.*, 2006; Idrees *et al.*, 2006), and in some instances above 70 years of age (Shields, 1983).

Mice with homozygous loss of function of *Foxc1* die at birth. In addition, they have profound failure of the normal development of the anterior segment as well as absence of anterior chamber (Hong *et al.*, 1999; Kidson *et al.*, 1999; Kume *et al.*, 1998). Similar studies on mice with heterozygote mutations show milder anterior segment defects, similar to those found in humans with *FOXC1* mutations. These include iris malformations, iridocorneal adhesions, hypoplastic TM, small or absent Schlemm's canal (Smith *et al.*, 2000). Notably, the iridocorneal angle structures showed paucity of extracellular matrix (ECM) including collagen and elastic tissues (Smith *et al.*, 2000). Considering the important developmental contribution of ECM to the formation of the anterior segment and the TM (reviewed by(Acott and Kelley, 2008; Gould *et al.*, 2004), altered ECM regulation as a result of mutations in *FOXC1* may predispose to glaucoma.

Furthermore, the penetrance of these anterior segment defects has been shown to vary with genetic backgrounds. For example, although subtle iris and corneal abnormalities

occur which are not clinically obvious in certain mice with heterozygote mutations on some genetic backgrounds, all of the mice have histologically abnormal TM and Schlemm's canal (Smith *et al.*, 2000). This suggests genetic modifiers have a differential effect on the development of iris and cornea in comparison to the development of iridocorneal angle, and possibly suggest that the development of the latter is more sensitive to levels of *FOXC1*.

1.9.2.1.5 Molecular mechanisms underlying ASD

Numerous mutations have been identified in *FOXC1*, including missense mutations insertions, deletions, duplications, and translocations (Komatireddy *et al.*, 2003; Lehmann *et al.*, 2002; Lehmann *et al.*, 2000; Mears *et al.*, 1998; Mirzayans *et al.*, 2000; Nishimura *et al.*, 2001; Swiderski *et al.*, 1999). These mutations lead to ARS via 2 main mechanisms: 1) by affecting the ability of the transcription factor protein to bind to DNA and either increasing or decreasing the trans-activation activity through mutations causing gain, or loss, of transcription factor function (Kozlowski and Walter, 2000; Priston *et al.*, 2001); and 2) via alteration in the level of functional protein through chromosomal deletions or duplication. Both mutations and duplications of *FOXC1* have been shown to produce a similar Axenfeld-Rieger phenotype (Ekong *et al.*, 2004; Lehmann *et al.*, 2000; Nishimura *et al.*, 2001). Furthermore, missense or frameshift mutations as well as segmental and telomeric deletions of *FOXC1* produce a spectrum of ARM phenotype (Kawase *et al.*, 2001; Lines *et al.*, 2002; Mears *et al.*, 1996; Mirzayans *et al.*, 2000; Murphy *et al.*, 2004; Nishimura *et al.*, 2001; Nishimura *et al.*, 1998; Saleem *et al.*, 2001; Saleem *et al.*, 2003b; Saleem *et al.*, 2003a; Suzuki *et al.*, 2001; Swiderski *et al.*, 1999), whilst duplication of the 6p subtelomeric region results in iridogoniodysgenesis (Ekong *et al.*, 2004; Saleem *et al.*, 2003a). Hence, when taking account of these results as a whole, both a lower and an upper threshold may exist for normal *FOXC1* activity, where ARM can result from loss-of-function mutation where *FOXC1* expression is below 80% of the normal FOXC1 activity, or from increased expression of *FOXC1* above 150% of the wild type activity (*FOXC1* duplication) (Ekong *et al.*, 2004; Saleem *et al.*, 2001). However, the level of *FOXC1* activity does not correlate with the severity of ASD (Walter, 2003). A recent finding that FOXC1 and PITX2 proteins interact with each other to negatively regulate the activity of *FOXC1* may provide an explanation for this not straight forward observation (Walter, 2003). *In*

vitro assays demonstrate that in cells expressing both *FOXC1* and *PITX2,* PITX2 binds to FOXC1 and inhibits FOXC1 target genes. Loss of function of *PITX2* leads to inappropriate activation of *FOXC1*. Interestingly, this may be equivalent to a duplication of a *FOXC1* gene giving rise to increased levels of FOXC1 protein. Identifying the downstream target genes of FOXC1 would provide a more comprehensive understanding of the molecular pathogenesis of ASD. To date, the known downstream targets of FOXC1 protein are FGF19 (a member of the fibroblast growth factor family, the reduced activity of which give rise to ASD in zebrafish) (Tamimi *et al.*, 2006) and FOXO1A (a key protein involved in the cellular stress response and apoptotic pathways) (Berry *et al.*, 2008).

Furthermore, a recent study has shown that *FOXC1* mutations have a milder and hence a better prognosis for the development of glaucoma, whereas individuals with *FOXC1* duplications have a more severe and worse prognosis for glaucoma development (Strungaru *et al.*, 2007). More specifically, patients with *FOXC1* duplication had iridogoniodysgenesis and a higher incidence of raised IOP and glaucoma compared with those with *FOXC1* mutations. On the contrary, individuals with *FOXC1* mutations had a diverse clinical presentation, including iris hypoplasia, correctopia, posterior embryotoxon and anterior synechiae. Iris malformation can range from normal to severe defects which are clinically obvious (polycoria and correctopia) (Smith *et al.*, 2000). Similar changes have been found in the TM. This suggests that patients with *FOXC1* mutations appeared to be more diverse phenotype than in the patients with *FOXC1* duplication, and the relatively milder phenotype is as a result from changed levels of expression of (and not from mutant) protein through duplication. In some affected family members with glaucoma, the anterior segment malformation may be very subtle, and easily missed in clinical examination (Lehmann *et al.*, 2002; Shields, 1983), a feature more in keeping with POAG. In addition, the risk of developing glaucoma is not related to the severity of the phenotype (Shields *et al.*, 1985; Shields, 1983), suggesting that subtle dysfunction of the angle drainage structures may be contributing towards glaucoma (Chisholm and Chudley, 1983; Heon *et al.*, 1995).

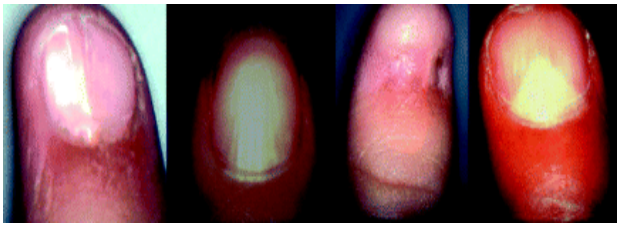
1.9.2.1.6 Summary- *FOXC1* as a genetic risk factor for POAG

Taking into account the essential role of *FOXC1* for the development of the anterior segment and in the development of TM implies that *FOXC1* is crucial for the normal development of drainage structures and preservation of normal IOP. This is evidenced by targeted heterozygous mutation in animal models resulting in malformation of the drainage structures with a high incidence of glaucoma to about 75% or above. Therefore, these features of *FOXC1* provide a strong basis to consider *FOXC1* as a candidate for the more common adult-onset POAG through hypothesizing that sub-clinical mutations/polymorphisms in *FOXC1* gene may produce subtle and undetected abnormalities in anterior segment structure and function through age related changes, which predispose to glaucoma and may be a significant susceptibility factor for the development of POAG.

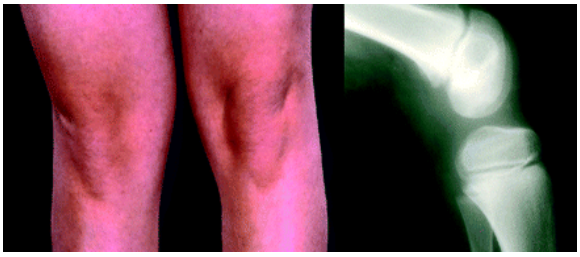
1.9.2.2 *LMX1B* and Nail-patella syndrome (OMIM 161200)

Nail-patella syndrome (NPS; OMIM # 161200), also known as hereditary osteo-onychodysplasia, Fong disease, Turner-Keiser syndrome, and Osterreicher-Turner syndrome, is a rare autosomal dominant (Lucas and Wiffler, 1966; Turner, 1933), pleiotropic disorder with an estimated incidence of 1 in 50,000 (Sweeney *et al.*, 2003). NPS was initially recognized in 1883 as a familial patellar abnormality (Pye-Smith, 1883) and as a familial nail abnormality in 1897 (Little, 1897). NPS is characterized by (Dreyer *et al.*, 1998) dysplastic nails (Guidera *et al.*, 1991) (98%), hypoplastic or absent patella (92%), elbow/arm abnormalities (90%) (Figure 1-20), iliac horns (70%) and nephropathy (38%) (HAWKINS and SMITH, 1950), epilepsy (6%) and neurological/vasomotor abnormalities of the peripheral limbs (25%) (Sweeney *et al.*, 2003). Glaucoma is now recognized as part of the syndrome (9.6-31%). Clinical manifestations of NPS vary widely in both frequency and severity, as well as showing inter- and also intrafamilial heterogeneity (Beals and Eckhardt, 1969; Turner, 1933). On one hand, clinical features can be very mild and not associated with any disabilities, and on the other hand, skeletal malformation can be severe, even at birth (Morita *et al.*, 1973). In addition, individuals may be more severely affected by one component of the illness and exhibit a less severe form elsewhere. Hence, despite diagnosis being made at birth (Guidera *et al.*, 1991) it is more common for individuals

A



B



C

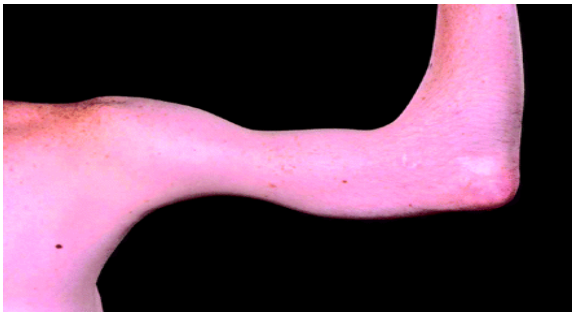


Figure 1–20: Clinical features of NPS; (A) dysplastic changes of the nails, (B) absent or hypoplastic patellae (absent patellae on x-ray), and (C) arm/elbow abnormality (underdeveloped triceps, flexion contracture of elbow).
Adapted from Mimiwati *et al.,* 2007.

as well as families to remain undiagnosed for generations despite having been reviewed by several doctors of various disciplines (Lucas and Wiffler, 1966).

1.9.2.2.1 Linkage of NPS

One of the first linkages established in humans was between NPS and the blood ABO group (RENWICK and LAWLER, 1955). Further refinement of the location of NPS was made to a 1-2 cM interval on 9q34.1 (Campeau *et al.*, 1995). Involvement of Lmx1b in the dorso-ventral patterning of limb during vertebrae development was first established from studies assessing limb development in chick embryos (Riddle *et al.*, 1995). Knock-out mice lacking *Lmx1b* were then created to investigate this further (Dreyer *et al.*, 1998). Findings from phenotypic abnormalities in these Lmx1b null mice with absence of nails and patellae (Dreyer *et al.*, 1998) and independently through positional cloning (Vollrath *et al.*, 1998), revealed that the NPS gene encoded the LIM-homeodomain transcription factor LMX1B.

1.9.2.2.2 The role of LMX1B in development

LMX1B is a member of the LIM-homedomain (LIM-HD) family of transcription factors involved in:

1) Body pattern formation during embryonic development in invertebrates and vertebrates (Riddle *et al.*, 1995; Vogel *et al.*, 1995).
2) Directing the initial trajectory of motor axonal fibres in developing limb (Kania *et al.*, 2000).
3) Regulation of the development of the anterior segment of murine eyes (Pressman *et al.*, 2000).
4) Formation of podocytes in the kidney (Rohr *et al.*, 2002).
5) Regulation of expression of type IV collagen (Morello *et al.*, 2001).

1.9.2.2.3 *LMX1B*, the NPS gene

The *LMX1B* gene is composed of 8 exons, giving rise to a ~7kb mRNA when spliced (Dreyer *et al.*, 1998; Vollrath *et al.*, 1998) (Figure 1-21 A). Two isoforms exist (372 and 379 amino acids) as a result of alterative splicing of exon 7 at the 3' end

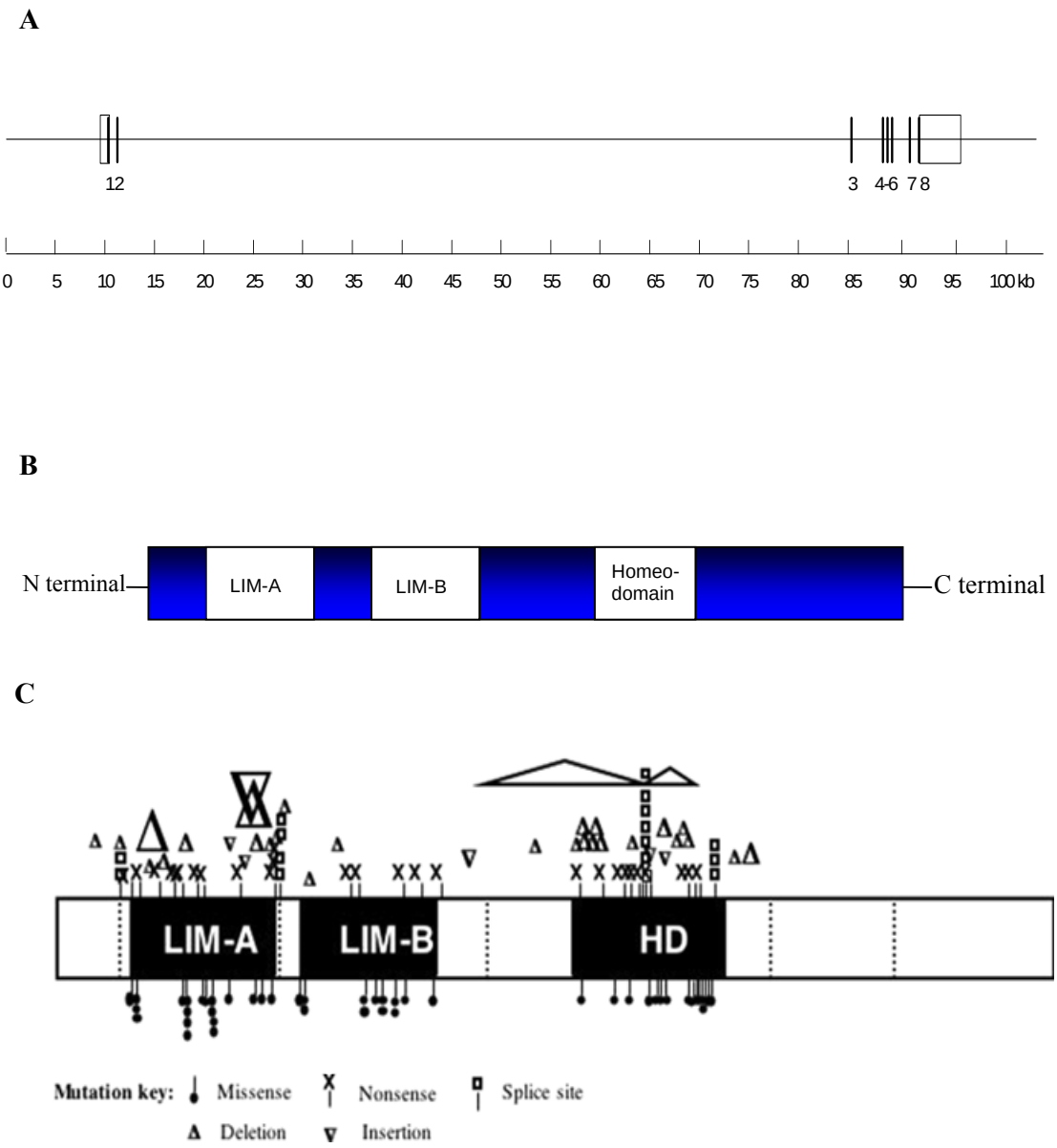


Figure 1–21: (A) *LMX1B* gene structure. 8 coding exons are indicated as solid boxes and numbered accordingly. Untranslated exons are shown as open boxes (B) Schematic of the LMX1B protein showing LIM domains (encoded by exons 2 and 3) and homeodomain (encoded by exons 4, 5, and 6). (C) Location of mutations in LMX1B protein.

Adapted from McIntosh et *al.,* 2005).

(Seri *et al.*, 1999). The functional differences between the two spliced isoforms are still unknown. Detailed analysis of the structure of *LMX1B* gene revealed the ~7kb transcript was composed of a long 1.3 kb 5'-untranslated region (UTR), 4.6 kb 3'UTR, and a coding region of 1206 nucleotides (Dunston *et al.*, 2004). The basal promoter activity extended 112 bp upstream. An interesting feature of note is the long non-coding intron 2, where 5 regions of longer than 250 bp with greater than 85% of sequence conservation were observed between mouse and human, a feature suggestive of a functional role possibly as a regulatory region involved in gene expression (Dunston *et al.*, 2004).

The *LMX1B* gene encodes a transcription factor protein that contains cysteine-rich LIM A and LIM B domains (each binding 2 zinc ions) at the N-terminal followed by a homeodomain at the C-terminal end (Figure 1-21 B). The LIM domain is named from 3 proteins found with this domain: *C. elegans* **L**in-11 (Freyd *et al.*, 1990), rat **I**sl-1 (Karlsson *et al.*, 1990), and *C. elegans* **M**ec-3 (Way and Chalfie, 1988). LIM domains are involved in protein-protein interaction, whereas, the homeodomain is a 60 amino acid domain necessary for DNA binding and transcriptional activation (reviewed in (Curtiss and Heilig, 1998; Dawid *et al.*, 1995). LIM domains regulate LIM-homeodomain activity in a positive manner by encouraging protein-protein interaction. This enables cooperative binding to regulatory regions within tissue-specific promoters.

Most mutations in *LMX1B* occur in the LIM domain or homeodomain protein (Bongers *et al.*, 2005; Clough *et al.*, 1999; Hamlington *et al.*, 2001) (Figure 1-21 C) and NPS is the result of haploinsufficiency of *LMX1B* (Dreyer *et al.*, 1998; Vollrath *et al.*, 1998). Whereas the majority of missense mutations are found in the homeodomain and in the canonical residues of the LIM domains, frameshift and nonsense mutations are found more widely spread within the coding regions. Large deletions of the coding sequence have been shown by analysis of intragenic variations and through Southern blotting (Clough *et al.*, 1999). Deletions involving various parts of the gene, including a whole gene deletion, were found in 9 of 28 unrelated families (Dunston *et al.*, 2004). In particular, one deletion extended from 100kb upstream to at least exon 4 in a sporadic case of NPS, with a 50% probability of extension into the 3' flanking region. Deletions of all or part of *LMX1B* represented ~5% of NPS mutations (Bongers *et al.*, 2005;

Dunston *et al.*, 2004). In addition, balanced translocations have been reported in NPS, resulting in defective *LMX1B* (Duba *et al.*, 1998; Hamlington *et al.*, 2001).

To date, 137 mutations (excluding large deletions) within the coding sequence have been identified in *LMX1B* among NPS. Interestingly, no missense mutations have been identified downstream of the homeodomain, possibly suggesting that sequence alteration of this region could result in a phenotype separate from NPS (Bongers *et al.*, 2005; Dunston *et al.*, 2004). Co-transfection of the wild type with mutant *LMX1B* construct showed NPS mutations failing to act in a dominant-negative manner on wild-type LMX1B (Dreyer *et al.*, 2000). In addition, no variations in the severity of clinical features were noted between patients with nonsense, missense, splice or frameshift mutations as well as those with large deletions. This evidence suggests and support haploinsufficiency as the underlying mechanism of NPS (Clough *et al.*, 1999; Dunston *et al.*, 2004; McIntosh *et al.*, 1998).

1.9.2.2.4 *LMX1B* as genetic risk factor for POAG

More importantly, NPS has been shown to cosegregate with raised IOP and POAG in humans (Lichter *et al.*, 1997b; McIntosh *et al.*, 1998; Vollrath *et al.*, 1998). In particular, POAG exhibited an age-dependant penetrance (Vollrath *et al.*, 1998). One study involving 29 families showed that POAG was present in 31% of cases (McIntosh *et al.*, 1997). A large review evaluating 123 NPS patients revealed 9.6% of NPS had glaucoma and an additional 7.2% had ocular hypertension (Sweeney *et al.*, 2003). These figures rose to 16.7% and 11.9% when individuals under the age of 40 were excluded. The mean age of diagnosis of OHT or glaucoma was 47.9 years (range 23-75).

Another study which involved 106 subjects from 32 NPS families found glaucoma and OHT were present in 35.3% (Bongers *et al.*, 2005). Open angle glaucoma was present in 3.9%, whilst OHT was present at 3.9%, and surprisingly, NTG was present at a higher percentage of 7.8%. The mean age of diagnosis of OHT was 63.4 years (range 55-72 years). Over the age of 50 years, a higher percentage of 13.3% had open angle glaucoma, 26.7% had NTG, and 6.7% had OHT.

Interestingly, a higher frequency of 11.1% of open angle glaucoma and 11.1% of OHT was shown by a recent study involving 52 subjects with NPS (Mimiwati *et al.*, 2006). This figure increased to 33% of glaucoma cases when individuals over the age of 40 years were considered. The mean age at diagnosis for glaucoma was 48 years and for OHT 24 years. Of note was the presence of one NPS case with NTG. This patient was diagnosed with NTG at 63 years and had advanced cupping.

In addition to determining dorsal limb patterning during vertebrate development (Dreyer *et al.*, 2000), the transcription factor *LMX1B* regulates programs of gene expression essential for normal morphogenesis and cell differentiation of the anterior segment (Pressman *et al.*, 2000). Targeted homozygous mutation in animal models resulted in eye phenotypes comprised of iris and ciliary body hypoplasia, and corneal stromal defects in which defective collagen fibrillogenesis was observed.

Notably, *Lmx1b* is expressed in the TM and ciliary body, the sites of aqueous drainage and production respectively (Pressman *et al.*, 2000). Although, mutations in the *LMX1B* gene can cause a spectrum of ocular phenotypes that vary in severity including rare anterior segment anomalies of iris processes, ptosis, hypertelorism, epicanthal folds, bilateral sclerocornea, microcornea, keratoconus, congenital cataracts, mircophakia (Bongers *et al.*, 2005; Lichter *et al.*, 1997b; Spitalny and Fenske, 1970), developmental abnormalities of the anterior segment are not always clinically detectable in patients carrying *LMX1B* mutation in the majority of cases (Mimiwati *et al.*, 2006; Sweeney *et al.*, 2003), a feature consistent with POAG.

Furthermore, animal models have also shown *LMX1B* plays an important role in the expression of other ECM. It directly regulates expression of the basement membrane collagens *Col4a3* and *Col4a4*, and in the formation of podocytes in the kidney (Morello *et al.*, 2001; Rohr *et al.*, 2002). Expression of *Col4a4* is reduced in the glomerular basement in Lmx1b$^{-/-}$ mice. Considering the important developmental contribution of ECM to the formation of the TM (reviewed by (Acott and Kelley, 2008; Gould *et al.*, 2004), in particular, towards its structural and physiological role, altered ECM regulation as a result of mutations in *LMX1B* may predispose to glaucoma. Though there is not yet evidence that *Lmx1b* regulates *Col4a1*, mutations in the collagen *Col4a1*

gene resulted in ASD with optic nerve hypoplasia, with raised as well as low IOP (Gould *et al.*, 2007).

Thus, taking all the evidence into consideration, *LMX1B* appears to be a strong candidate gene for adult onset POAG through the hypotheses that subclinical mutations/polymorphisms in *LMX1B* gene may result in subtle and undetected abnormalities in the anterior segment structure and function, and predispose to glaucoma through its effect on the regulation of ECM of the TM.

1.9.3 *TGFβ2* and *BMP4* as genetic risk factors for POAG

There is increasing evidence to suggest that growth factors may be important in maintaining normal homeostasis in the ocular tissues that are involved in the pathogenesis of glaucoma (Fleenor *et al.*, 2006; Gottanka *et al.*, 2004; Lutjen-Drecoll, 2005; Pena *et al.*, 1999; Tamm and Fuchshofer, 2007; Wordinger *et al.*, 1998; Wordinger *et al.*, 2002; Wordinger *et al.*, 2007; Wordinger and Clark, 2007). The TM expresses a range of growth factors and growth factor receptors including BMPs (as well as BMP4), BMP receptors, and TGFβ2 (Tripathi *et al.*, 1993a; Tripathi *et al.*, 1993b; Tripathi *et al.*, 1994c; Tripathi *et al.*, 1994a; Wordinger *et al.*, 1998; Wordinger *et al.*, 1999; Wordinger *et al.*, 2007). *TGFβ2* and *BMP4* are also expressed in the human optic nerve head (Pena *et al.*, 1999; Wordinger *et al.*, 1998; Wordinger *et al.*, 2002). In addition, the aqueous humor contains a variety of growth factors including FGF2, EGF, HGF and TGFβ (Hu and Ritch, 2001; Tripathi *et al.*, 1992; Wordinger *et al.*, 2007). More specifically, elevated levels of TGFβ2 have been found in POAG patients. In addition, studies have shown that raised IOP in POAG is as a result of increased resistance to aqueous outflow (Grant, 1963) and this is associated with biochemical and morphological changes in the TM (Rohen, 1983). There is an accumulation of ECM in the TM of glaucoma patients, and this may be as a result of disruption of the balance between ECM deposition and degradation (Lutjen-Drecoll, 2005; Rohen, 1983). Hence, growth factors such as BMP4 and TGFβ2 appears to be strong candidate genes that may be involved in glaucoma pathogenesis through its influence on the normal development or function of the TM and optic nerve head.

1.9.3.1 TGFβ2 and BMP4 signaling pathways

TGFβ2 and BMP4 are secreted signaling molecules that constitute the TGFβ super-family. They are multifunctional growth factor proteins and exhibit a spectrum of biological processes which includes inflammation, wound repair, ECM accumulation, tissue development, cell differentiation, cell proliferation, cell death, and bone morphogenesis (Barnard *et al.*, 1990; Graham *et al.*, 1994; Hogan, 1996; Hogan, 1999; Janssens *et al.*, 2005; Mabie *et al.*, 1999). In keeping with its diverse role, abnormal TGFβ signalling pathway results in a variety of clinical disease processes (Miyazono *et al.*, 2001). TGFβ molecules activate the Smad family of signal transducers, which form complexes with specific DNA-binding proteins to regulate gene expression (Massague, 2000; Whitman, 1998). Two similar but discrete Smad-dependent signaling pathways have been identified: TGF, Activin and Nodal signal via the Smad2 (or Smad3)–Smad4 complex, whereas BMP signals via the Smad1–Smad4 complex (Massague, 2000; Whitman, 1998). Activation of each signaling pathway involves an extracellular ligand binding to a membrane bound Type I and Type II serine/threonine kinase receptors. This initiates a cascade of cytoplasmic signaling that activates SMAD proteins. These activated SMAD proteins enter the nucleus and associate with transcription factors and take part in transcriptional regulation of target genes (Miyazono *et al.*, 2001).

1.9.3.2 TGFβ2 (OMIM; 190220)

1.9.3.2.1 Function of TGFβ2 in ocular development

Studies on animal models have shown that *TGFβ2* is important in anterior segment development. The corneas of mice with homozygous mutations have less deposition of ECM and appear thin (Saika *et al.*, 2001). In addition, the corneal endothelium fails to differentiate whilst there is absence of anterior chamber development. The TM in mice is yet to be assessed. Interestingly, a study not directly involving *TGFβ2* but rather involving inactivation of TGFβ signalling in neural crest cells showed that normal formation of the ciliary body and TM requires TGFβ signalling in these cells, and the phenotypic abnormalities found in *Tgfbr2*- mutant mice were characteristic of those found in ARM (Ittner *et al.*, 2005).

1.9.3.2.2 Function of TGFβ2 in adult ocular development

In POAG, the resistance to aqueous outflow in the trabecular outflow is abnormally raised (Gabelt and Kaufman, 2005; Tamm and Fuchshofer, 2007). Despite there being inconclusive evidence for the underlying mechanism responsible for the increase in outflow resistance in POAG, there is growing evidence that changes in the ECM of the TM may play an important role (Fleenor *et al.*, 2006; Lutjen-Drecoll, 2005; Tamm and Fuchshofer, 2007; Wordinger *et al.*, 2007).

The structural changes characteristically found in the juxtacanalicular tissue of the TM in individuals with POAG is an accumulation of plaque-like material (derived from thickened sheaths of elastic fibres within the juxtacanalicular tissue) in addition to an increase in ECM (Rohen and Witmer, 1972). Although the underlying regulatory mechanism of ECM turnover in the normal TM and its increase in POAG is still poorly understood, *TGFβ2* appears to be a strong candidate involved in the pathogenic structural changes in TM. In addition to *TGFβ2* being expressed in human TM (Wordinger *et al.*, 1998), several independent studies have shown presence of higher concentrations of TGFβ2 in the aqueous humor of individuals with POAG (Inatani *et al.*, 2001; Ochiai and Ochiai, 2002; Picht *et al.*, 2001; Tripathi *et al.*, 1994b). These initial reports have been followed by in vitro studies showing that TGFβ2 acts via several pathways on human TM to increase ECM deposition and cross linkage that hinders degradation. More specifically, exogenous treatment of *TGFβ2*:

1) Increases in vitro synthesis of ECM molecules in cultured human TM cells (Welge-Lussen *et al.*, 2000), and within the perfused anterior segment organ culture model (Gottanka *et al.*, 2004).
2) Increases expression of plasminogen activator inhibitor which inhibits activation of matrix metalloproteinases via tissue plasminogen activator and/or urokinase (Fuchshofer *et al.*, 2003). This, in turn results in an increase in ECM in TM of individuals with POAG.
3) Increases irreversible cross-linkage of ECM components (fibronectin) in TM cells via tissue transglutaminase (Welge-Lussen *et al.*, 2000), the effects of which are inhibited by BMP4 (Wordinger *et al.*, 2007). In perfused anterior segment culture model, *TGFβ2* induced focal accumulation of fine fibrillar ECM in the TM as well

as an increase in fibronectin, both of which were correlated with a reduction in outflow facility (Fleenor *et al.*, 2006; Gottanka *et al.*, 2004). Interestingly, a correlation was found between the amount of ECM underneath the inner wall of Schlemm's canal and the degree of axonal loss within the glaucomatous optic nerve head (Gottanka *et al.*, 1997).

4) Inhibits TM cell proliferation (Gottanka *et al.*, 2004; Wordinger *et al.*, 1998).

In addition to its effect on TM, the effects of TGFβ2 have also been assessed on the optic nerve head. Support for the involvement of TGFβ2 in glaucomatous optic nerve heads was provided by a study which showed expression of *TGFβ2* was elevated 70-100 fold more in the astrocytes of the human glaucomatous optic nerve heads (Pena *et al.*, 1999) compared to controls. When astrocytes from optic nerve head were treated with TGFβ2 in the same concentration used for TM cells, this resulted in an increased expression of fibronectin, type I as well as type IV collagen, tissue transglutaminase and plasminogen activator inhibitor (Fuchshofer *et al.*, 2005).

1.9.3.3 BMP4 (OMIM; 112262)

BMP proteins are phylogenetically conserved signalling molecules that constitute the largest subfamily within the TGFβ superfamily of growth factors (Attisano and Wrana, 1996; Canalis *et al.*, 2003; Massague and Chen, 2000; Reddi, 2001). Although BMP proteins were originally identified as osteoinductive cytokines that regulated bone and cartilage formation (Reddi, 2000; Wozney *et al.*, 1988), subsequent studies have now shown that BMPs have a more diverse role including body patterning and morphogenesis (Hogan, 1996; von and Cho, 2001). In addition, BMPs have important roles in adults in the pathophysiology of several diseases, including osteoporosis, cancer, kidney diseases, pulmonary hypertension and cerebrovascular disease (Chang *et al.*, 2003; Howe *et al.*, 2001; Hruska *et al.*, 2004; Klahr, 2003; Miyazono *et al.*, 2001; Morse *et al.*, 2001; Reddi, 2000; Wang, 1993; Zeisberg *et al.*, 2004).

1.9.3.3.1 Function of BMP4 in ocular development

Several studies have shown that BMP4 is important in ocular development, in particular, for the development in the anterior segment. All mutant mice with heterozygous mutations for *BMP4* showed severely malformed anterior segment with raised IOP (Chang *et al.*, 2001). The phenotype was similar to ASD in humans, and was variable as it depended on specific genetic background. In particular, all of the tissues within the anterior segment, including the TM and Schlemm's canal, were affected. The TM appeared hypoplastic with reduced ECM. In addition, abnormalities of the optic nerve were frequently present, with even absence of the optic nerve on certain genetic background. Furthermore, BMP signalling has shown to be required for the development of the ciliary body. Noggin, a BMP antagonist, blocked BMP signalling in mouse eye, and this resulted in the failure of the formation of the ciliary process (Zhao *et al.*, 2002).

Although BMP4 has no known targets in the periocular mesenchyme, BMP4 receptors are present in mesenchyme (Furuta and Hogan, 1998) and mesenchyme-derived tissues are malformed in mice with heterozygous mutations in *BMP4*. It is therefore a possibility that BMP4 may directly affect the mesenchyme. In such mice with heterozygous loss of *BMP4*, the mesenchyme-derived TM show significant deficiencies of ECM. It is known that ECM is developmentally important as it regulates developmental processes such as proper migration and differentiation (Adams and Watt, 1993; Lin and Bissell, 1993; Perris, 1997). Hence BMP4 may influence the morphogenesis of the TM by regulating the compositions of ECM that are crucial for mesenchymal migration, differentiation and remodelling.

1.9.3.3.2 Function of BMP4 in adult ocular tissue

Studies involving cultured human TM cells have shown that BMP4 is capable of inhibiting the effects of TGFβ2 on the induction of fibronectin and thereby modulating the effects of TGFβ2 in TM cells (Wordinger *et al.*, 2007). Furthermore, gremlin, a BMP antagonist which is expressed in TM cells, is able to block the inhibitory effects of BMP4 on TGFβ2 (Figure 1-22).

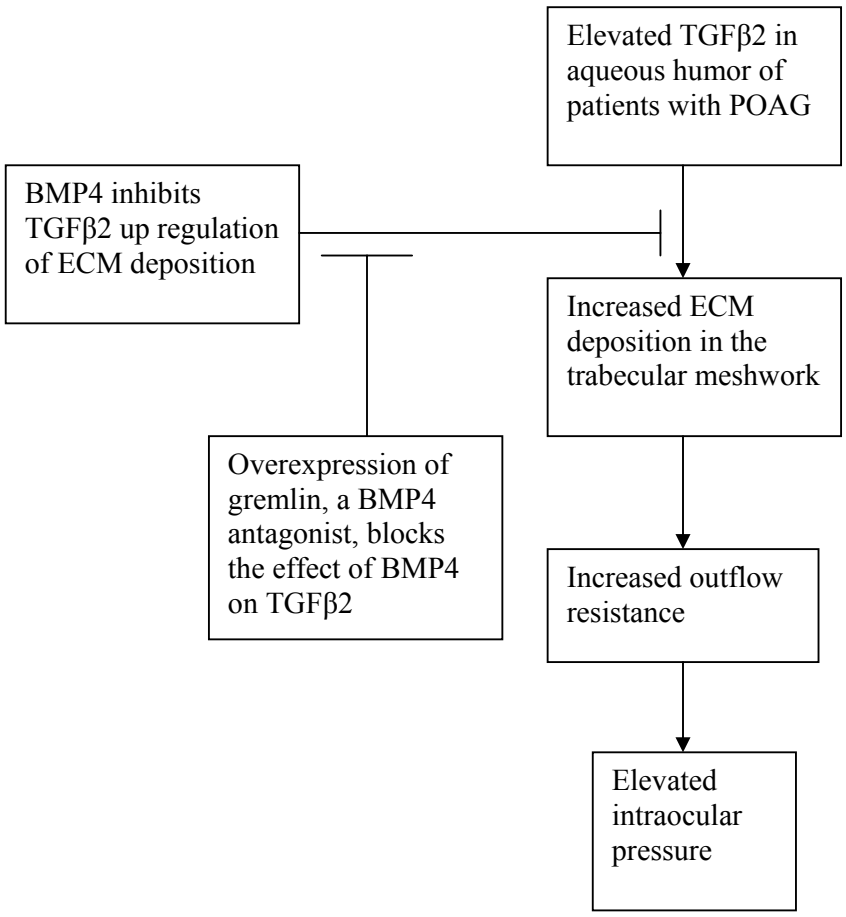


Figure 1–22: Diagrammatic representation of the interactions of TGFß2, BMP4, and Gremlin within the TM.

Elevated TGFß2 are found in the aqueous humor of individuals with POAG, and this may lead to increased deposition of ECM proteins in the TM. Increased ECM deposition results in increased resistance to aqueous humor outflow and raised IOP. BMP4 inhibits TGFß2 upregulation of ECM deposition and maintains a normal balance of ECM synthesis, deposition, and degradation within the human TM. The overexpression of Gremlin, a secreted BMP antagonist, in glaucomatous TM, inhibits the effect of BMP4 and leads to an imbalance in TGFß2 action. Adapted from Wordinger *et al* ., 2007.

1.10 Summary of literature review

Taken together, these reports suggest that both *TGFβ2* and *BMP4* are strong candidate genes that act in concert to maintain a balance between ECM deposition and degradation, and may play an important role in glaucoma pathogenesis through mis-regulation of ECM synthesis and cross-linkage of ECM components of the TM and in the optic nerve head (Table 1-9).

Genes in which mutation causes anterior segment angle anomalies and glaucoma are strong candidates for glaucoma susceptibility and may contribute to glaucoma more frequently than expected, and possibly play an important role in the common forms of POAG. The fact that around 1-2% of individuals who are ≥40 years of age in the general population develop POAG (Fraser, 2004), compared with 33-75% of patients who have mutations in the developmental glaucoma genes *FOXC1* and *LMX1B* suggests that these genes are very good candidate risk factors (Table 1-9).

In addition, the E50K *OPTN* mutation is a candidate susceptibility gene for POAG in patients of white British origin (Table 1-9).

1.11 Hypotheses and aims of study

1.11.1 Hypotheses

1) Sub-clinical mutations/polymorphisms in *LMX1B, FOXC1, TGFβ2* and *BMP4* may produce subtle and undetected age-related abnormalities in anterior segment structure and function, which predispose to glaucomatous optic neuropathy and may be a significant susceptibility factor for the development of OHT and POAG.
2) The *OPTN* E50K mutation may be a significant POAG risk factor for the white British acting via a defective neuroprotection on the optic nerve head.

Table 1–9: Summary of supporting evidence for *LMX1B, FOXC1, TGFβ2, BMP4* and *OPTN* as candidate genes for POAG

Developmental glaucoma genes	Phenotype	Raised IOP	Expression in trabecular meshwork +/- optic nerve head	Malformed drainage structures	Additional supporting features as candidate genes for glaucoma
LMX1B	Nail-Patella Syndrome (Dreyer *et al.*, 1998)	In 33% of NPS (Mimiwati *et al.*, 2007)	In developing TM of mice and post-natally (Pressman *et al.*, 1999)	*Lmx1b*$^{-/-}$ mutant mice showed ciliary body hypoplasia (Pressman *et al.*, 1999)	NTG present in 26.7% (Bongers et al., 2005) OHT present in 11.9% (Sweeney et al., 2003)
FOXC1	ARS (Smith *et al.*, 2000)	In ~50-75% of ASD (Strungaru *et al.*, 2007)	In developing TM of mice (Smith et al, 2000)	*Foxc1*$^{+/-}$ showed hypoplastic trabecular meshwork with paucity of ECM (*Smith et al.*, 2000)	Age range from birth to above 70 years (Shields et al., 1983)
TGFß2	Peters' like anomaly (Saike *et al.*, 2001)	Elevated levels of *TGFß2 in* POAG patients (Tripathi et al, 1994)	In adult human TM and optic nerve head (Wordinger *et al.*, 1998, 2007)	*Tgfß2*$^{-/-}$ mutant mice showed absent anterior chamber (Saike *et al.*, 2001). The TM in mice is yet to be assessed. TGFß signal inactivation in NC cells resulted in absent Tm and ciliary body (Ittner *et al.*, 2005)	Elevated in aqueous humor of POAG patients (Tripathi et al, 1994) Increased ECM deposition (Fuchshofer et al, 2003) and cross linkage that hinders degradation in cultured TM cells (Welge-Lussen *et al.*, 2000), with reduced aqueous outflow (Fleenor *et al.*, 2006).
BMP4	ARS (Chang *et al.*, 2001)	In ~40% (mice) (Chang *et al.*, 2001)	In adult human TM (Wordinger *et al.*, 2007)	*Bmp4* $^{+/-}$ showed hypoplastic trabecular meshwork with raised IOP, and absence of optic nerve (Chang *et al.*, 2001)	BMP4 inhibited TGFß2 upregulation of ECM deposition in adult TM cell lines (Wordinger *et al.*, 2007)
OPTN	NTG (Rezaie *et al.*, 2002)	In 0.1-16.7% individuals with NTG (predominantly found in individuals from British descent)	In adult human TM and aqueous humor (Rezaie *et al.*, 2002) (and monkey's optic nerve) (Rezaie et al., 2005)	Yet to be assesed	Mean age of NTG patients; 44 years (ranging from 23 to 65 years) (Rezaie *et al.*, 2002)

1.11.2 Aims of the study

The aims of the study are as follows:

1) To recruit adequate numbers of matched POAG patient/control cohorts to achieve a study power of 80%, using explicit inclusion/exclusion criteria.
2) The selection of appropriate tagging single nucleotide polymorphisms (tSNPs) using the HapMap resource, or in the case of *OPTN,* selecting the SNP responsible for E50K mutation.
3) To ascertain whether alleles of *LMX1B, FOXC1, TGFβ2, BMP4* predispose to POAG in the general population composed of white British subjects by performing a case-control genetic association study to compare the prevalence of tSNPs of *LMX1B, FOXC1, TGFβ2, BMP4* in four groups, HTG, NTG, OHT and a normal control group.
4) To validate any significant findings in a 2nd independent cohort study.
5) To assess the prevalence of *OPTN* E50K mutation in unrelated glaucoma patients of white British origin comprising of HTG, OHT, NTG and normal controls, in addition to investigating the clinical features of individuals with the *OPTN* E50K mutation in order to assess whether this mutation imparts a characteristic glaucoma phenotype.
6) To validate the prevalence of *OPTN* E50K mutation using an independent sequencing method.

In the short term, this study aims to offer an opportunity to unravel the complex genetics of POAG and hence provide a better understanding of the aetiology of glaucoma in the UK population. In the long term, it may contribute towards development of screening tests. This will offer the prospect of pre-symptomatic diagnosis of at risk individuals to allow early implementation of treatment to prevent visual loss. This will substantially relieve health care expenditures on glaucoma treatment and more importantly, improve quality of life. Identification of the genes involved in glaucoma pathology will also provide new targets for the development of new molecular based therapies in the future. In addition, the panel of DNA samples from this study will be useful for assessing the role of other genes in glaucoma. Glaucoma genetic screening is an important tool for reducing the incidence of blindness

and vision loss from a predominantly asymptomatic disease and is now a feasible goal made possible with recent scientific and technological advances.

Chapter 2 Methods and Materials

2.1 Overview of study design

Recruitment of 367 cases and 276 controls

- 272 HTG
- 58 OHT
- 37 NTG

Inclusion criteria for cases:

- Adult individuals (≥ 40 years) of white British descent with a diagnosis of HTG, NTG or OHT after the age of 40 years.
- Presence of glaucomatous optic neuropathy (defined by loss of neuroretinal rim) with compatible and reproducible visual field loss for HTG and NTG, and absence of a detectable glaucomatous damage or field loss for OHT cases.
- Open drainage angles on gonioscopy.
- IOP consistently ≥ 22mmHg on diurnal testing for HTG and OHT, and ≤ 21 mmHg for NTG
- Absence of a secondary cause for glaucomatous optic neuropathy;
- Absence of non-glaucomatous field losses and disc changes (i.e. high myopia).

1) Assessment of *LMX1B, FOXC1, BMP4* and *TGFβ2* as candidate genes for HTG, NTG and OHT
2) Evaluation of prevalence of *OPTN* E50K mutation among HTG, NTG and OHT

- DNA extraction from peripheral blood mononuclear cells.
- Selection of tSNPs using the HapMap resource (minor allele frequency ≥ 0.1, minimum r^2=0.8).
- Application of a) Pyrosequencing/real time PCR to obtain preliminary results
  b) Sequenom MassARRAY™ System to allow high-throughput SNP genotyping.
- Evaluation of the prevalence of each tSNP for *LMX1B, FOXC1*, *BMP4, TGFβ2*, and E50K mutation for *OPTN*, in cases and controls.
- Validation of the prevalence of *OPTN* E50K mutation using an independent sequencing method.

Replication study for any significant association with an independent ethnically matched cohort

- 108 HTG
- 222 NTG

2.2 Recruitment of patients

2.2.1 First cohort

All of the participating subjects for the first cohort (cohort 1) were recruited from glaucoma outpatient clinics between February 2005 and October 2005 at the Sunderland Eye Infirmary in the North-East of England, UK, a secondary ophthalmology referral centre. The research followed the tenets of the Declaration of Helsinki. Informed consent was obtained from all participants after the nature and possible consequences of the study were explained (Appendix I and II). The study had Local Research Ethics Committee approval (research ethics committee number: 06/Q0508/8). A cohort of HTG cases (*n*=272), and unrelated controls (n=276) matched for ethnicity, were recruited to the study. Cases with NTG (*n*= 37) and OHT (*n*= 58) were also collected. All cases (n=367) and controls were of white British descent. Of these, I recruited, collected and performed ophthalmic examination on 483 individuals. Dr Daniela Vaideanu (Specialist Ophthalmic Registrar based in the Northern Deanery) recruited and examined a further 160 subjects using the same inclusion/exclusion criteria. All clinical examination and diagnosis were performed under the supervision of Mr Scott Fraser who is a Consultant Ophthalmologist with a special interest in glaucoma.

Control participants (*n*=276), either accompanying spouses or friends of individuals with glaucoma, were recruited randomly. All controls underwent a complete ophthalmic examination in order to exclude individuals with glaucoma from the control group, and were confirmed to have no visual complaints and IOP of < 22mmHg with a normal disc appearance. Control individuals with a family history of glaucoma were excluded.

All case subjects underwent a complete ophthalmic examination including best visual acuity, and visual field testing using a Humphrey SITA standard 24-2 perimetry (Carl Zeiss Meditec AG, Jena, Germany) (a form of a static perimetry which involves presentation of non-moving stimuli of varying illuminance), slit lamp examination of the anterior segment (including gonioscopy using a single mirror goniolens: this is applied to the surface of the eye, thereby preventing total internal reflection at surfaces within the eye, notably at the cornea:air interface and hence allows visualization of the anterior chamber angle), measurement of IOP by Goldmann applanation tonometer (this

is applied to the cornea with sufficient force to produce a standard area of contact of a diameter of 3.06mm: such applanation tonometry is based on the Imbert-Fick principle, which states that for an ideal, dry, thin-walled sphere, the pressure inside the sphere (*P*) equals the force necessary to flatten its surface (*F*) divided by the area of flattening (*A*) i.e. $P = F/A$), posterior segment examination of the retina and optic disc following pupil dilation and measurement of the C/D ratio. An arbitrary assessment of the severity of the visual field defect was not recorded, nor was the CCT measured, as a pachymeter was not available. However, all the visual field tests showed reproducible field defects, and were ensured to have a satisfactory reliability scores of ≤20% fixation loss, false positive of ≤33% and/or false negative of ≤33%.

Adult individuals with a diagnosis of HTG, NTG or OHT from the age of 40 years were enrolled based on the following clinical criteria:

i) Presence of glaucomatous optic neuropathy (defined by loss of neuroretinal rim) with compatible and reproducible visual field loss for HTG and NTG, and absence of detectable glaucomatous damage or field loss for OHT;
ii) Open drainage angles on gonioscopy;
iii) IOP consistently ≥ 22mmHg on diurnal testing for HTG and OHT, and ≤ 21 mmHg for NTG;
iv) Absence of a secondary cause for glaucomatous optic neuropathy;
v) Absence of non-glaucomatous field losses and disc changes (i.e. high myopia).

Clinical details of all the participants were recorded onto a clinical sheet as shown in Figure 2-1.

2.2.2 Second cohort

In order to assess whether any of the significant findings could be replicated, a second ethnically matched cohort consisting of HTG cases (n = 108) and NTG cases (n = 222) was provided from a tertiary referral clinic at Moorfields Eye Hospital (London). These white/Caucasian individuals which may represent a more diverse ethnicity, were already recruited and were previously investigated for the prevalence of *OPTN* and *OPA1* mutation (Aung *et al.*, 2003; Aung *et al.*, 2002b). Glaucoma was defined by a similar

Name…….. Date of examination……..

Age…….
Sex……..

A. Diagnosis

1. Ocular hypertension (i.e. IOP ≥ 22mmHg and absence of detectable glaucomatous optic nerve damage or field loss) Yes ☐ No ☐ R ☐ L ☐

2. Primary open angle glaucoma

a) High tension glaucoma- IOP ≥ 22mmHg Yes ☐ No ☐ R ☐ L ☐

b) Normal tension glaucoma- IOP < 22mmHg Yes ☐ No ☐ R ☐ L ☐

3. Secondary glaucoma (e.g. pseudoexfoliation, pigmentary, others)

Yes ☐ No ☐ R ☐ L ☐

Date of diagnosis of OHT or POAG……..

B. Risk factors

a) Family history ☐

b) Myopia ☐

c) Afro-Caribbean origin ☐

C. Ocular findings

Visual acuity…… R 6/…… L 6/……
IOP- most recent……...…… R…….. L……. Instrument used……..
Highest- Date…… R…….. L……. Instrument used……....
Angle appearance…… R…….. L…….
Optic disc appearance
Cup/disc ratio ……... R 0. …… L 0……....
Presence of reproducible visual field defect… R ☐ ……... L ☐ …… Nil ☐
Instrument used……
Character of field loss……... R…… L……
Evidence of deterioration…….

D. Therapy

a. Medication R…….. L…….. Nil ☐
b. Laser R…….. L…….. Nil ☐
c. Surgery R…….. L…….. Nil ☐

Signature……... Name……... Date……

Figure 2–1: An example of the clinical sheet used to gather the details of the participants.

strict inclusion/exclusion criteria:

i) Presence of typical glaucomatous optic neuropathy with compatible visual field loss;
ii) Open drainage angles on gonioscopy;
iii) Patients with NTG had a mean IOP without treatment that was consistently 21 mmHg or less on diurnal testing;
iv) Patients with HTG had a mean IOP consistently above 21 mm Hg;
v) Absence of a secondary cause for glaucomatous optic neuropathy such as a previously raised IOP after trauma, a period of steroid administration, or uveitis;
vi) Patients showed no evidence of high myopia or congenital abnormality.

The same control individuals used in cohort one was re-utilised for cohort two.

2.3 DNA extraction

20 ml of venous blood was extracted from the participant's arm and distributed into 4 5ml ethylenediaminetetraacetic acid (EDTA) tubes. This was stored in a -80ºC freezer at the Institute of Child Health until the laboratory work started.

Genomic DNA was extracted from peripheral blood leucocytes using a standard salting-out procedure. At the start of each experiment, two 500ml bottles of reverse osmosis (RO) water and one bottle of 0.1% Igepal solution were placed in the fridge to cool. 3ml of thawed blood sample was transferred into a labeled 15ml Falcon tube. Empty EDTA blood tubes were discarded into 300ml of 10% Chloros (sodium hypochlorite) for 2 hours for disposal. Ice-cold RO water was poured into each Falcon tube to produce a final volume of 15ml. This was inverted to mix and lyse red blood cells, followed by centrifuging for 20 minutes at 4°C and 2900 revolutions per minute (rpm) (1800 times gravity (*g*)) in a Sorvall® Legend RT centrifuge. Supernatant was dispensed into 10% Chloros. Ice-cold 0.1% Igepal was poured onto the white cell pellet and the volume was made up to 6ml for cell membrane degradation. The pellet was resuspended by vortexing, followed by spinning for 20 minutes at 4°C and 2900 rpm in the same centrifuge. Supernatant was discarded into 10% Chloros and 1ml of Nuclei Lysis Buffer (containing 10Mm of Tris at pH 8.2; 400mM of sodium chloride; 2mM of EDTA) was added to each 15ml Falcon tube for nuclei lysis. The sample was vortexed to re-suspend

the pellet completely. For protein degradation, 67µl of 10% SDS and 200µl Proteinase K solution were added to each 15ml Falcon Tube. The sample was mixed by gentle inversion and incubated in a water bath at 60°C for 1.5 hours (or overnight at 37°C). Following incubation, 0.3ml of saturated ammonium acetate was poured into each 15ml Falcon tube. Each tube was shaken vigorously for 15 seconds and left to stand at room temperature for 10-15 minutes. Each tube was then spun for 20 minutes at 3300 rpm and at 24°C. Meanwhile, for each sample, another 15ml Falcon and a 1.5ml eppendorf was labeled identically. The supernatant was poured into the corresponding new Falcon tube. Onto the supernatant, two volumes of absolute ethanol (approximately 2ml) were poured and the DNA was precipitated by gentle inversion. The tip of a glass pasteur pipettes (unplugged: Fisherbrand) was gently melted using a flame from a Bunsen burner and shaped into a loop. This was then used to scoop and lift the DNA out into the corresponding new eppendorf containing 500µl of Tris EDTA (TE) and was submerged for dissolving.

2.4 Agarose gel electrophoresis

A 1 % (w/v) agarose gel was prepared by heating 1 g of agarose (Flowgen Bioscience, Nottingham, UK) in 100 ml TAE electrophoresis buffer (containing 40mM Tris at pH 7.2; 0.114% (v/v) glacial acetic acid; 1mM EDTA) for 2 minutes in a 900 W microwave oven. One microlitre per milliliter of 0.0025% (v/v) ethidium bromide was added to aid DNA detection; it intercalates between DNA molecules and fluoresces under UV light. The liquid gel was left to slightly cool for approximately 30 minutes and poured into a gel tray containing appropriate sized combs. The gel was allowed to solidify at room temperature for at least 30 minutes. It was then placed into an electrophoresis tank, Horizon®11.14 (Life Technologies, Gaithersburg, MD) and completely submerged with TAE buffer and the comb was then removed. 2µl of 5x Orange G loading buffer was added and mixed into each 8 µl of diluted DNA and loaded into the corresponding well. 5 µl of 1 kb DNA ladder, Hyperladder^{TM} IV (Bioloine, London) was loaded to reference the product size. The gel was run at 100 mV at 80-100 mA for at least 40 minutes. Gels were then imaged using UV light with a camera capture system (Uvidoc system, Uvitec).

The quality of the DNA was assessed by examining for the presence of; a) any smearing of the fluorescent bands to detect for any DNA fragmentation, or; b) for faint fluorescent bands that signify the presence of any contamination.

2.5 Spectrophotometric determination of the amount of DNA

Nanodrop (ND-1000 Spectrophotometer V3.2, NanoDrop Technologies, Inc.) was used to quantitate the amount of DNA by loading 1 µl of the diluted DNA onto the spectrophotometer. Readings were taken at wavelengths of 260 nm and 280 nm. The reading at 260 nm allowed calculation of the concentration of nucleic acid in the sample. A reading of 1 optical density at 260 nm corresponds to 50 µg/ml of double stranded DNA. The ratio between readings at 260 nm and 280 nm provided an estimate of the purity of the nucleic acid. Pure preparations of DNA had ratio of 1.8.

2.6 HapMap resource and selection of SNPs

The HapMap resource (www.hapmap.org) was used to guide selection of SNPs from candidate genes. A tSNP approach was employed to screen *FOXC1, BMP4, TGFβ2,* and *LMX1B* including 10 kb of upstream and downstream flanking sequence in patient and control groups (HapMap Data Release #22/Phase II Apr 2007). By using the algorithm tagger which was implemented in Haploview v.4.0 (Carlson *et al.*, 2004) (www.broad.mit.edu /mpg/haploview) to select tSNPs that capture untyped SNPs with a minimum r^2 value of 0.8, 4 SNPs for *FOXC1* (Figure 2-2, Table 2-1), 5 *S*NPs for *BMP4* (Figure 2-3, Table 2-2), 20 SNPs for *TGFβ2* (Figure 2-4, Table 2-3), and 26 SNPs for *LMX1B* (Figure 2-5, Table 2-4), were analysed. Selection was restricted to SNPs with a minor allele frequency (MAF) of ≥10% (i.e. SNPs with a high degree of polymorphism) as relatively abundant SNPs rather than rare mutations were more likely to contribute to complex traits and improve the chances of detecting associations. Selection was also focused on identifying polymorphic SNPs which caused amino acid substitutions as they were most likely to impact gene function and may represent functional variants rather than indirect markers of functional variation. Individually chosen possible functional SNPs included rs1091993 for *FOXC1* (5 SNPs in total when combined with SNPs selected from HapMap), rs28939692 for *LMX1B* (27 SNPs in total when combined with SNPs selected from HapMap), and rs28939688 for *OPTN*

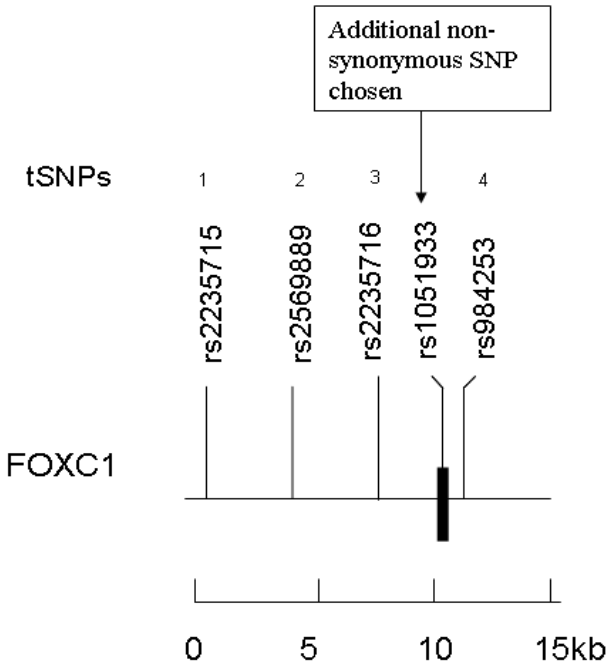


Figure 2–2: The relative position of 5 SNPs for *FOXC1*, on chromosome 6p25.
The relative position of the 4 tSNPs chosen from Haploview v.4.0 with the additional non-synonymous SNP (chosen manually) is shown. The coding exon is indicated as a solid box.

Table 2–1: Selected SNPs for *FOXC1*

tSNP no	Reference ID	Position	Type	Polymorphism	Amino acid change
1	rs2235715	1543941	upstream (intergenic)	C/T	
2	rs2569889	1548686	upstream (intergenic)	G/C	
3	rs2235716	1552115	upstream	C/T	
Manually chosen	rs1051933	1556955 (c.1276G>A)	exon (non-synonymous)	G/A	aspartic acid to asparagine
4	rs984253	1558528	3' UTR	A/T	

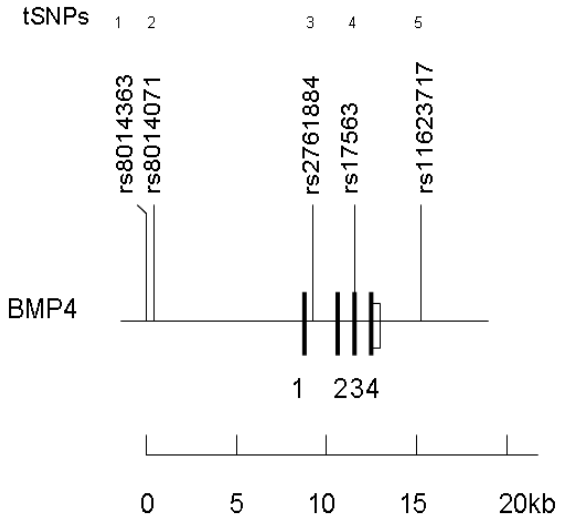


Figure 2–3: The relative position of 5 tSNPs for *BMP4*, on chromosome 14q21.
The relative position of the 5 tSNPs is shown. 4 coding exons are indicated as solid boxes and numbered accordingly. Untranslated exons are shown as open boxes.

Table 2–2: Selected tSNPs for *BMP4*

tSNP no	Reference ID	Position	Type	Polymorphism	Amino acid change
1	rs8014363	53501325	promoter	C/T	
2	rs8014071	53501250	promoter	A/G	
3	rs2761884	53490802	intron 1	G/T	
4	rs17563	53487272 (c.455T>C)	exon 3 (non-synonymous)	T/C	valine to alanine
5	rs11623717	53483882	downstream (intergenic)	A/G	

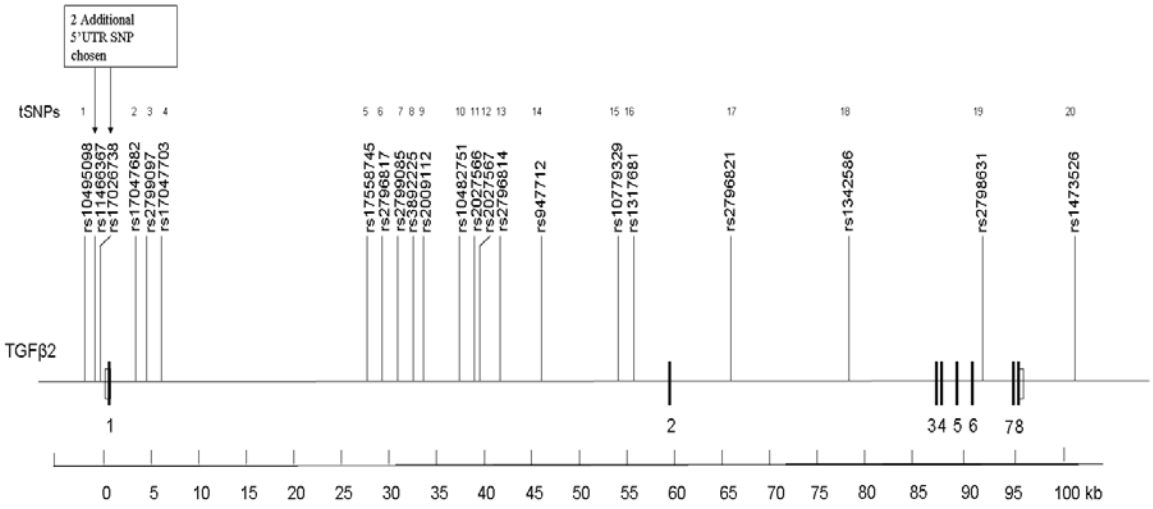


Figure 2–4: The relative position of 22 SNPs for *TGFβ2*, on chromosome 1q41.

The relative position of the 20 tSNPs with 2 additional 5' UTR SNPs (chosen manually) is shown. 8 coding exons are indicated as solid boxes and numbered accordingly. Untranslated exons are shown as open boxes.

Table 2–3: Selected SNPs for *TGFβ2*				
tSNP no	**Reference ID**	**Position**	**Type**	**Polymorphism**
1	rs10495098	216582933	Upstream	G/T
Manually chosen	rs114663679	216586555	5'UTR	C/A
Manually chosen	rs17026738	216586556	5'UTR	A/C
2	rs17047682	216589648	Intron 1	A/G
3	rs2799097	216591255	Intron 1	A/G
4	rs17047703	216592211	Intron 1	C/A
5	rs17558745	216615144	Intron 1	C/T
6	rs2796817	216617631	Intron 1	T/G
7	rs2799085	216619078	Intron 1	A/C
8	rs3892225	216619920	Intron 1	A/G
9	rs2009112	216620152	Intron 1	C/T
10	rs10482751	216622920	Intron 1	C/T
11	rs2027566	216624950	Intron 1	C/A
12	rs2027567	216625211	Intron 1	G/A
13	rs2796814	216628204	Intron 1	C/G
14	rs947712	216631504	Intron 1	C/T
15	rs10779329	216640364	Intron 1	T/C
16	rs1317681	216641825	Intron 1	G/A
17	rs2796821	216652444	Intron 2	C/T
18	rs1342586	216664482	Intron 2	C/T
19	rs2798631	216678501	Intron 6	A/G
20	rs1473526	216687370	Downstream	T/C

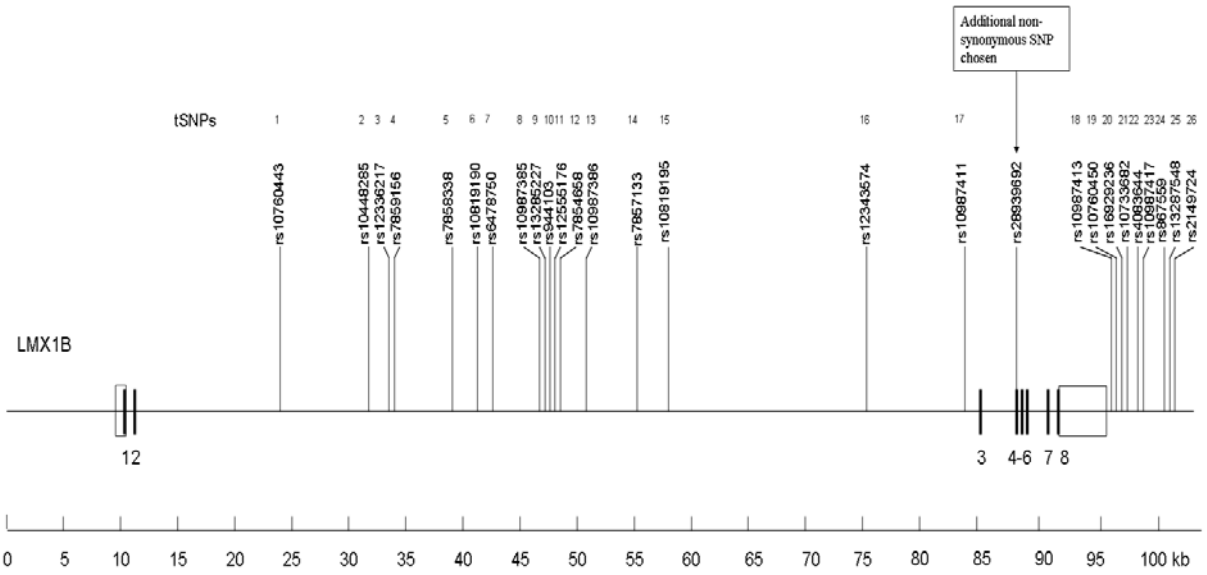


Figure 2–5: The relative position of 27 SNPs for *LMX1B*, on chromosome 9q32-q34.1.

The relative position of the 26 tSNPs with the additional non-synonymous SNP (chosen manually) is shown. 8 coding exons are indicated as solid boxes and numbered accordingly. Untranslated exons are shown as open boxes.

Table 2–4: Selected SNPs for *LMX1B*

tSNP no	Reference ID	Position	Type	Polymorphism	Amino acid change
1	rs10760443	128435167	Intron 2	C/T	
2	rs10448285	128436835	Intron 2	C/T	
3	rs12336217	128439691	Intron 2	A/G	
4	rs7859156	128439849	Intron 2	C/T	
5	rs7858338	128446465	Intron 2	T/C	
6	rs10819190	128448334	Intron 2	G/A	
7	rs6478750	128449019	Intron 2	C/T	
8	rs10987385	128453030	Intron 2	G/A	
9	rs13285227	128453119	Intron 2	C/T	
10	rs944103	128453311	Intron 2	C/T	
11	rs12555176	128454124	Intron 2	G/T	
12	rs7854658	128454759	Intron 2	G/A	
13	rs10987386	128456138	Intron 2	C/T	
14	rs7857133	128460457	Intron 2	G/A	
15	rs10819195	128462604	Intron 2	G/A	
16	rs12343574	128480661	Intron 2	A/C	
17	rs10987411	128490260	Intron 2	C/T	
Manually chosen	rs28939692 (c.599G>A)	128495350	Exon 4 (non synonymous)	G/A	arginine to glutamine
18	rs10987413	128499259	Downstream	G/A	
19	rs10760450	128499449	Downstream	C/T	
20	rs16929236	128499806	Downstream	A/G	
21	rs10733682	128500735	Downstream	A/G	
22	rs4083644	128501535	Downstream	C/T	
23	rs10987417	128502322	Downstream	G/T	
24	rs867559	128505146	Downstream	A/G	
25	rs13287548	128506277	Downstream	C/T	
26	rs2149724	128506589	Downstream	C/G	

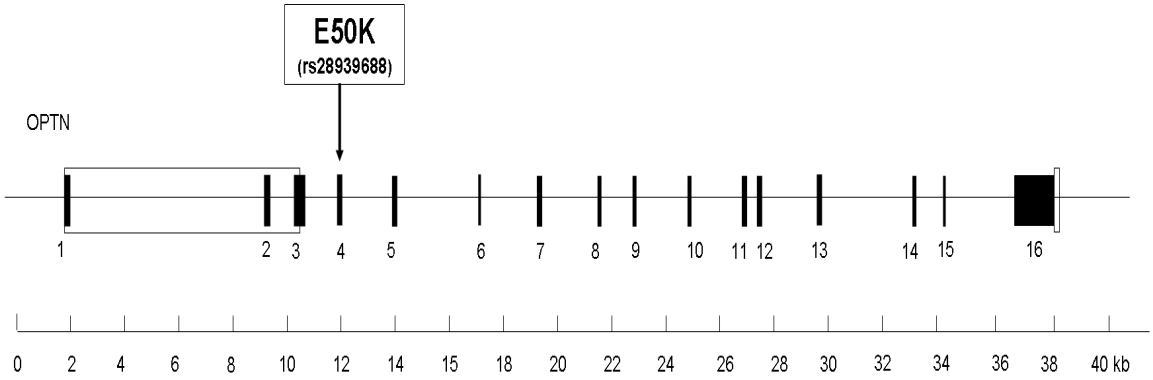


Figure 2–6: The relative position of the chosen SNP (rs28939688) in *OPTN* in exon 4 (on chromosome 10p15-p14) which causes a c.458G>A transition (otherwise known as E50K mutation).

16 coding exons are indicated as solid boxes and numbered accordingly. Untranslated exons are shown as open boxes.

Table 2–5: Selected SNP for *OPTN*

tSNP no	Reference ID	Position	Type	Polymorphism	Amino acid change
1	rs28939688	28495350 (c.148G>A)	exon 4 (non-synonymous)	G/A	glutamic acid to lysine

(Figure 2-6, Table 2-5). For *TGFβ2,* two 5' UTR SNPs rs114663679 and rs17026738 were chosen (22 SNPs in total when combined with SNPs selected from HapMap). The 5' UTR is a particular section of messenger RNA (mRNA) and precedes the translation initiation site of the gene, bounded on the 5' end by the start of transcription and on the 3' end by the start codon. Several regulatory sequences may be found in the 5' UTR, such as binding sites for proteins that may affect the mRNA's stability or translation, and sequences that promote the initiation of translation. In addition, as genes were likely have long range regulatory sequences (Flomen *et al.*, 1998), *in silico* sequence alignment analysis using zPicture software programme (available at zPicture.decode.org) was performed on 100kb of flanking sequences in addition to introns, and polymorphic SNPs within the identified evolutionarily conserved regions between chick/mouse/human genome sequences were sought. However, common SNPs were not present within these regions and were not selected for any of the candidate genes. A total number of 58 SNPs were analysed in cohort 1.

For the replication study using cohort 2, out of the initial 26 tSNPs chosen for *LMX1B*, 11 tSNPs (rs12336217, rs7859156, rs10819190, rs10987385, rs13285227, rs944103, rs12555176, rs7854658, rs10987386, rs16929236, and rs867559) were analysed.

2.7 Application of Pyrosequencer, real time PCR and Sequenom iPLEX™ Assay MassARRAY®

Due to the ease of access of an onsite pyrosequencer, SNPs were genotyped initially using a Pyrosequencer **(**PyroMark Q96 MD, Qiagen; Hilden, Germany) to obtain preliminary results. However, the pyrosequencing technique proved to be a costly and laborious method and was subsequently discontinued from further SNP genotyping. Instead the one-step TaqMan assays with an ABI 7500 Fast Real-Time PCR System (Applied Biosystems; Foster City, CA) was utilized. Subsequently, to achieve high-throughput genotyping, Sequenom iPLEX™ assay MassARRAY® was utilized through a commercially available service based at the Wellcome Trust Centre for Human Genetics in Oxford (http://www.well.ox.ac.uk/).

A pilot study previously performed by Mr Fraser and Dr Daniela Vaideanu (Sunderland Eye Infirmary) investigated the prevalence of *PITX2* polymorphisms in a small cohort

of 105 glaucoma patients. Their preliminary analysis suggested a significantly increased prevalence in the disease population of a single polymorphic SNP rs2739202 in the untranslated region of *PITX2*. To explore this observation in a larger cohort, by using the algorithm tagger in Haploview v.4.0, 7 tSNPs in the *PITX2* gene with a MAF of ≥10% and a minimum r^2 value of 0.8 were selected (Table 2.6). However, analysis of 139 HTG subjects found no evidence for variation of the putative rs2739202 SNP and further analysis of this gene was not carried out. Overall, 4 to 5 months of laboratory work was spent on pyrosequencing and determining the best genotyping methodology.

2.7.1 Pyrosequencer

Pyrosequencing is a direct DNA sequencing technique which enables high capacity genetic analysis. Each nucleotide is dispensed and tested individually for its incorporation into the emerging DNA template. Short stretches of sequence are examined adjacent to a defined start point. By using a cocktail of enzymes, it performs DNA sequencing through a synthesis approach, where by, it couples the release of pyrophosphate that occurs when deoxyribonucleotide triphosphate (dNTP) is added to a growing DNA chain, to light emission (Figure 2-7). It is catalysed by four enzymes, DNA polymerase, ATP sulfurylase, luciferase, and apyrase. For every base incorporated, there is a quantitative release of light where the peak height represents the amount of nucleotide incorporated, and this is represented as a pyrogram. Nucleotide sequence is determined from the order of which the nucleotide is dispensed and peak height in the program.

In this study, the template DNA required for pyrosequencing was obtained from polymerase chain reaction (PCR), which amplified the region of the genomic DNA of interest, to a significant amount to allow for sequencing. Three primers were utilised for this reaction; a) 2 primers for PCR, one of which was biotinylated, to allow separation of double stranded PCR product into a single stranded DNA and; b) a sequencing primer to enable pyrosequencing (Figure 2-8).

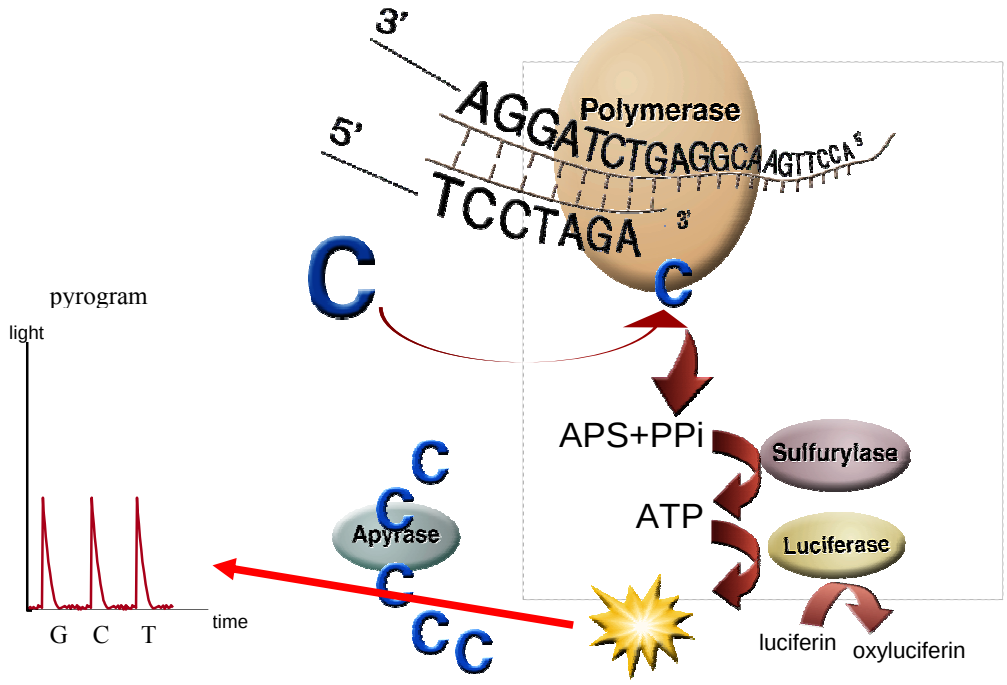


Figure 2–7: Summary of pyrosequencing reaction.

Sequencing primer hybridises to PCR amplified single standed DNA template. Pyrosequencer adds enzymes (DNA polymerase, ATP sulfurylase, luciferase and apyrase) and substrates (adenosine 5'phosphosulphate, luciferin). Individual bases are added one at a time. Each incorporation event is accompanied by release of pyrophosphate (PPi) in a quantity equimolar to the amount of incorporated nucleotide. Sulfurylase converts PPi to ATP. ATP drives luciferin to oxyluciferase which generates visible light in amounts that are proportional to the amount of incorporated nucleotide. Unincorporated dNTPs and ATP are degraded by apyrase. Next base is added once all previous dNTPs and ATPs have been removed. The complementary DNA strand is built up and the nucleotide sequence is determined from the order of which the nucleotide is dispensed and the light peak in the program. Adapted from http://www.pyrosequencing.com/DynPage.aspx?id=8726&mn1=1366.

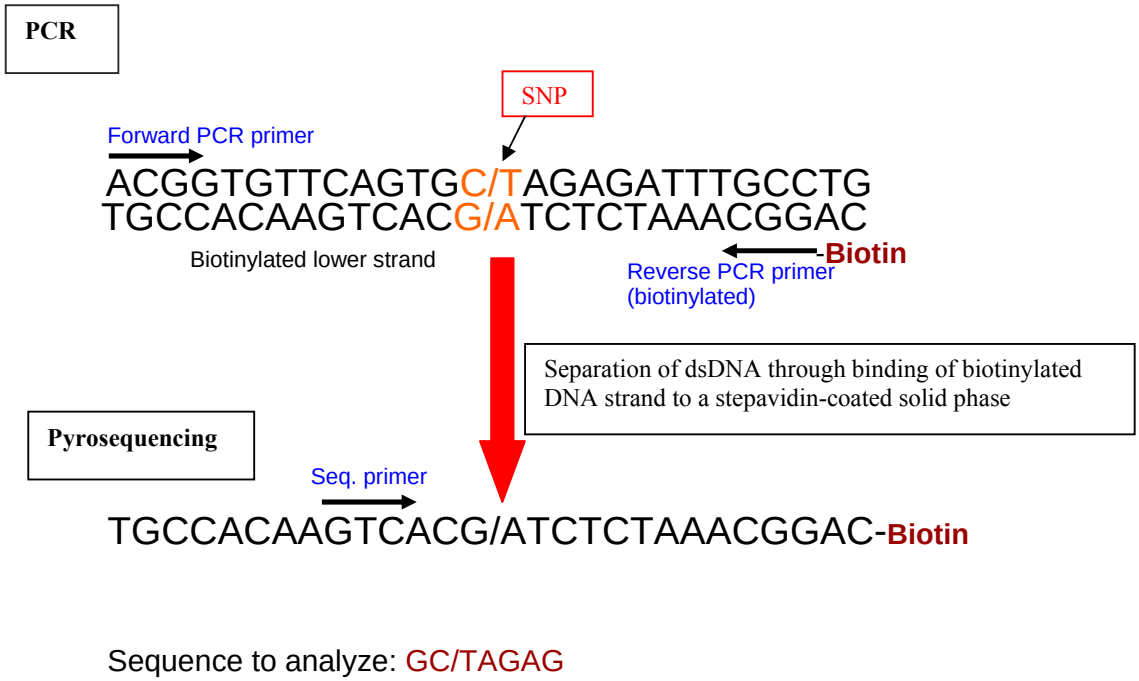


Figure 2–8: Primers for PCR/Pyrosequencing.

A forward primer and a biotinylated reverse primer is used for PCR. Separation of double stranded DNA template to allow pyrosequencing is achieved through binding of biotinylated DNA strand to a stepavidin-coated solid phase. A separate sequencing primer is used for pyrosequencing.

2.7.1.1 Primers

Primer sequences were designed using the Biotage assay design software version 1.0.6 (Table 2-6) and the primers were obtained from Operon. Each primer set consisted of a high performance liquid chromatography (HPLC) purified, biotinylated forward primer, and a standard reverse primer for the PCR, and a HPLC purified sequencing primer (S) for pyrosequencing reaction. Biotinylation was required for separation of double stranded PCR product into a single stranded DNA for pyrosequencing. This was achieved by transferring it through a strepavidin-coated solid phase. These primers were 15 to 23 base pairs long. The amplified target fragments were 87-198 bp in length. The primers contained a relatively balanced GC-AT content (33.3-66.7% GC).

Prior to usage, primer stock solutions were prepared at 100μM with TE followed by further dilution by 10 times to 10μM with sterile Milli-Q® water.

2.7.1.2 PCR

A master mix (Table 2-7) was prepared on ice prior to aliquoting into separate tubes. This was to ensure reproducibility of dispensing the same mixture of reagents into each tube. All of the reagents were obtained from Roche except for the primers, which were obtained from Operon. The PCR machine utilised for this study was a PCR-100™ Programmable Thermal Controller (MJ Research). For each PCR reaction, a positive control and at least two negative controls (i.e. every thing except the DNA) were included.

The PCR consisted of a series of cycles of three successive reactions as shown in table 2-8. The annealing temperature and the number of cycles were optimized empirically (Table 2-6) by running a PCR reaction on a temperature gradient (annealing temperature varying between 50 to 65 ° C) and varying the number of cycles from 40 to 50 cycles.

Table 2–6: Primers for *PITX2* SNPs				
SNP ID	Primer sequences (5'-3')	Product (bp)	Cycles	Tm (°C)
rs976568	(F) AAT AGC TTTG GCC CAG TGG A (R) CGC GTG TGA AGG TGT TGT C (S) AAG TCG CTG GTG TGC	134	40	55
rs1982361	(F) TTC TGC TAC GAG CGC CTC TTA A (R) AGT TAC AGG ACA CCG GAA AGT GC (S) TGA GTA ATG GGC TAA TAA TC	156	40	55
rs2278782	(F) CGG CCT GGT TGC GAG ATT A (R) TCG TGA GAT CGC GGG ATG (S) GCC TCG GAG AGG GAA	112	40	60
rs2595110	(F) TGG ACT TTA ATG GCT CAA GAC AG (R) TGC CAA ATA ACC AAT TTC AAG GA (S) CAG CCT TGA GCT CAC	87	40	50
rs2739202	(F) GCA TGT TAT ACA GAA GCG ATT AGG (R) CGA AAT GTT ATA CAC AGG GTA GGT (S) CAT CAT TGC ATC CAC C	198	40	55
rs4834295	(F) ACC CCC TTA AAA AAA AAA ACT CG (R) CCA GGT TCC CAA ACA TTT CAT TA (S) ACC ATT TTC TCA AGT TCA	70	40	55
rs7668322	(F) AGG AGG GGG CAT TTG CTA A (R) ACC TAG GAA AAC TGT GGC AGT AA (S) CAG CAT GTT CGT TGA GT	87	40	55

Table 2–7: Reagents for PCR		
Reagent	**1×50 µl reaction**	**Master mix of 7 reactions**
	volume (µl)	**volume (µl)**
10µM primer (forward) (Operon)	1	----
10µM primer (reverse) (Operon)	1	----
FastStart Taq DNA polymerase (5U/µl)	0.2	----
$MgCl_2$ (25mM)	5	35
dNTPs (2.5mM)	5	35
Buffer	5	35
Sterile Milli-Q® water	31.8	222.6
Genomic DNA (20ng/µl)	1	----
Total volume	**50**	**327.6**

Table 2–8: Program for PCR cycles				
Reaction step		**Temperature (°C)**	**Duration (mm:ss)**	**Nature of reaction**
Cycle repeated for 39 times	1a	95	04:00	Denaturation
	1b	95	00:30	Separation of DNA strand
	2	50-60	00:30	Primer annealing
	3a	72	00:30	DNA synthesis (transcription)
	3b	72	05:00	DNA synthesis (elongation)
	3c	4	24:00	DNA preservation following end of reaction

2.7.1.3 Agarose gel electrophoresis

The PCR products were run on a 1.5% agarose gel electrophoresis containing 1µl/ml of 0.0025% ethidium bromide. The same method as described in section 2.4 was used to prepare the agarose gel and to analyse the PCR products. The PCR product sizes were ensured that they were of correct size and the quality of the DNA was assessed by choosing an annealing temperature as high as possible (to increase specificity of annealing) that produced a single bright band.

2.7.1.4 Pyrosequencing preparation

2.7.1.4.1 Preparation of strepavidin-coated sepharose beads

Strepavidin-coated sepharose beads (from Amersham, catalogue number 17-5113-01) was resuspended to obtain a homogenous solution by shaking the the 5ml bottle. 500 µl of this was transferred into a sterile eppendorf containing 500 µl of binding buffer (10 mM Tris-HCL; 2M NaCl; 1 mM EDTA; 0.1 % Tween^{TM} 20). This was then spun at 1000 rpm (1300 x *g*) in a Biofuge Pico Heraeus centrifuge. The supernatant was then carefully discarded with a pipette. These steps were repeated once more to provide a total of 2 washes. The beads were then resuspended in a 500 µl of binding buffer and were stored at 4°C.

2.7.1.4.2 Strand separation

The prepared streptavidin Sepharose beads were shaken until homogenous solution was obtained. 2 µl of bead solution was pipetted into an eppendorf containing 38 µl of binding buffer (per sample). This was then mixed to obtain a homogenous solution and was added into a skirted 96 well plate (Abgene, catalogue number AB800) containing 10 µl of PCR product. The 96 well plate was placed onto a Monoshaker at maximum setting for at least 5 minutes to maintain dispersion of beads. During this period, a mastermix of 0.3 µM sequencing primer in 15 µl of annealing buffer (pH 7.6 (20mM Tris-Acetate; 5 mM $MgAc_2$)) was prepared and 15 µl of this was pipetted into the wells of the white pyrosequencing 96 well reaction plate. The streptavidin-coated Sepharose bead and PCR mixture was transferred to a filter plate (Amersham Biosciences) and the

binding buffer was removed by vacuum. The biotinylated DNA attached to the streptavidin-coated Sepharose beads was denaturated in 120 ml of denaturation buffer (0.2 M NaOH) for 1 minute. The denaturation buffer was then removed by vacuum and the DNA was washed in 120 ml of wash Buffer (pH 7.6, 10 mM Tris-Acetate). The DNA is resuspended into the white pyrosequencing reaction plate containing the 15 ul of annealing buffer.

2.7.1.4.3 Primer annealing

This plate was covered with an adhesive film and was left on a pyrosequencing heat plate set at 80°C for 2 minutes, which allowed the sequencing primer to anneal to the target complementary DNA sequence. This was then left to cool for 5 minutes at room temperature.

2.7.1.4.4 Sequencing reaction

The enzyme (DNA polymerase, ATP sulfurylase, luciferase and apyrase) and substrate (adenosine 5'phosphate and luciferin) was dissolved in 620 µl of 18.2 mΩ double distilled water and swirled gently to mix solution. The reagents were left at room temperature for 5 – 10 minutes before loading into the corresponding pyrosequencing cartridge. The dNTPs were diluted to 50% using double distilled water and were also loaded into the corresponding cartridge. Sequencing of the DNA was commenced once the plate was placed on the Pyrosequencer.

2.7.2 Real time PCR and Sequenom iPLEX™ Assay MassARRAY®

Due to the simplicity and ease of access of an onsite real time PCR, 4 SNPs in *FOXC1* (rs2235715, rs2569889, rs1051933, and rs984253), and one SNP in each of the following genes, *LMX1B* (rs28939692), *BMP4* (rs17563), and *OPTN* (rs28939688) were initially genotyped using TaqMan™ assays (Table 2-9) with the remaining 51 SNPs analysed using Sequenom iPLEX™ Assay. The reason for choosing the latter method was due to its increased cost effectiveness and high-through-put nature for SNP genotyping. Array-based technologies such as Affymetrix chips or the Illumina bead arrays were not chosen for this study since such costly methods provide efficient SNP genotyping for thousands of SNPs, which was not the aim for this study. Solution-based

one-step procedures such as TaqMan assays using real-time fluorescent detection, or MALDI mass spectrometry-based procedures are particularly cost-effective in studies where up to 50 SNPs require typing.

Table 2–9: ABI Assay ID for SNPs genotyped by real time PCR
(https://products.appliedbiosystems.com/ab/en/US/adirect/ab?cmd=catNavigate2&catID=601283)

SNP no	SNP ID	Gene	ABI assay ID
1	rs28939692	*LMX1B*	C_27541341_10
2	rs2235715	*FOXC1*	C_15952927_10
3	rs2569889	*FOXC1*	C_16045002_10
4	rs1051933	*FOXC1*	C_8770138_10
5	rs984253	*FOXC1*	C_11660541_20
6	rs17563	*BMP4*	C_9597660_20
7	rs28939688	*OPTN*	C_27860341_10

2.7.2.1 Real time PCR

The ease of handling a one-step reaction lasting for less than 2 hours provided a suitable platform to initiate small scale SNP genotyping.

2.7.2.1.1 Principles of allelic discrimination with TaqMan SNP genotyping assays

The TaqMan SNP genotyping assay is a multiplexed, end point assay that detected variants of a single nucleic acid sequence. Each assay consists of two primers for amplifying the target sequence and two TaqMan minor groove binding (MGB) probes for the detection of alleles (Figure 2-9). The presence of two primer/probe pairs in each reaction enables analysis of two possible alleles at a SNP site in a target template sequence. Each TaqMan MGB probe contained a reporter dye (either VIC for allele 1 or FAM for allele 2) at the 5' end of each probe, a minor groove binder (which produced.

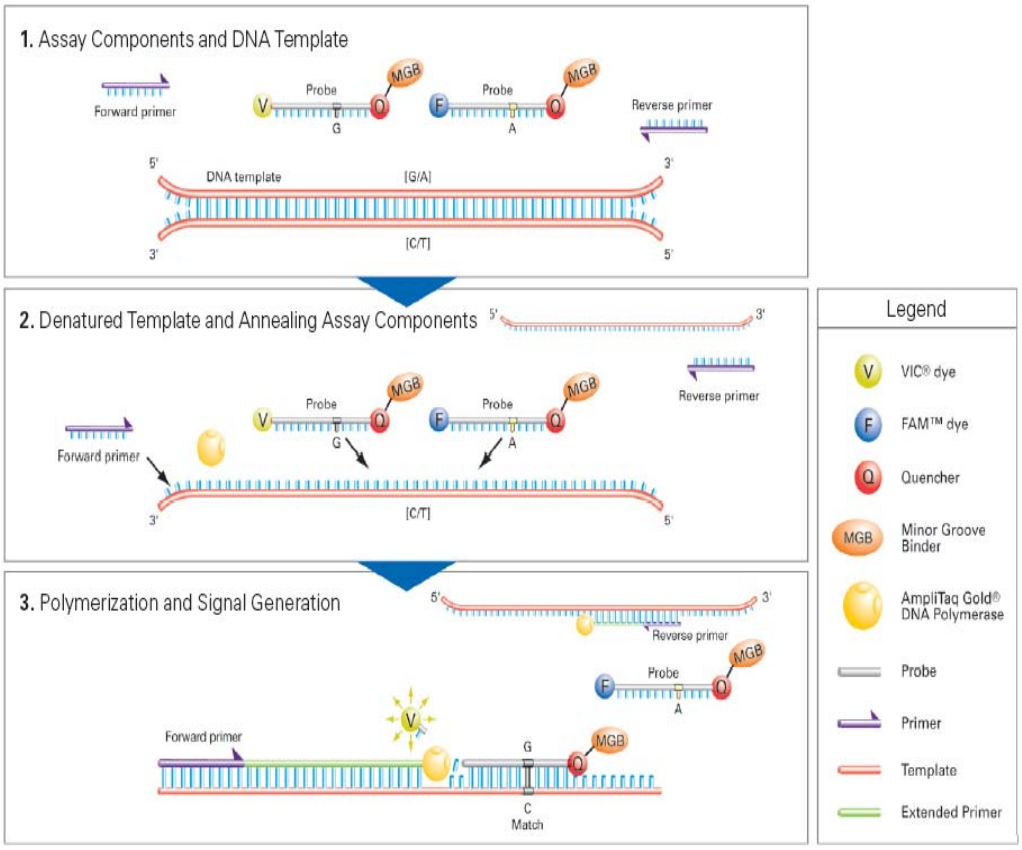


Figure 2–9: Allelic discrimination with TaqMan SNP genotyping assays.

The presence of 2 MGB probes allows allelic discrimination through selective annealing of probes labeled with either a VIC or a FAM reporter dye at the 5' end. 1) The assay components consists of 2 MGB probes containing either VIC for allele C or FAM for allele T, a forward and a reverse primer, AmpliTaq Gold, and a double stranded DNA template. 2) Denaturing of the double stranded DNA template allows annealing of assay components. 3) Polymerisation of the MGB probe for allele C results in subsequent cleavage of the reporter dye VIC by AmpliTaq Gold. Note the presence of a non-fluorescent quencher at the 3' end which suppresses fluorescence of the reporter dye FAM by its close proximity when the probe is not annealed to the complementary DNA. Adapted from Applied Biosystems.

accurate allelic discrimination by increasing the melting temperature (Tm) without increasing the probe length), and a non-fluorescent quencher (NFQ) at the 3' end of the probe When the probe was not hybridised to its complementary sequence and remained intact, the quencher dye prevented the reporter dye from fluorescence due to their proximity. However, probes that were hybridized to their target sequence were cleaved by AmpliTaq Gold® and as a result separated the reporter dye from the quencher dye. This allowed fluorescence by the reporter. Hence fluorescence signaling occurred when the target sequence was complementary to the probe, and allelic differentiation was indicated by whether the fluorescent signal was a FAM or a VIC dye.

2.7.2.1.2 SNP genotyping for *FOXC1, BMP4, LMX1B* and *OPTN* with Applied Biosystems 7500 fast real time PCR

Four SNPs located in *FOXC1* (rs2235715 (intronic), rs2569889 (intronic), rs1051933 (exonic; non synonymous), and rs984253 (intronic)), and one SNP located in *BMP4* (rs17563; exonic), *LMX1B* (rs28939692; exonic), and *OPTN* (rs28939688; exonic) were initially evaluated.

The following components were used for the reaction plate:

- 2× TaqMan Universal PCR Master Mix, No AmpErase UNG
- 20× SNP Genotyping Assay Mix
- 1-20 ng of genomic DNA in TE solution

The final volume of the reaction mix was 20 μL. The volume of the components for each well on a 96 well reaction plate is shown in Table 2-10. Each well contained 20 ng genomic DNA, 1 μL SNP Genotyping Assay mix (containing probes and primers), 10 μL TaqMan Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems; Foster City, CA), and 9 μL water. Two "no template" controls and a known genomic DNA control were included for each reaction plate. This was run on a ABI 7500 Fast system with the program outlined in Table 2-11. The DNA was amplified with the cycle settings as follows: 95ºC for 10 min, followed by 40 cycles of 92ºC for 15 sec and 60ºC for 1 min. Fluorescent signals were detected and analysed for allelic discrimination using the Sequence Detection System version 1.3.1 software (Applied Biosystems).

Table 2–10: Real time PCR reaction components	
Component	**Volume (μL/reaction)**
2× TaqMan Universal PCR Master Mix, No AmpErase UNG	10
2× SNP Genotyping Assay Mix	1
Genomic DNA (1-20 ng)	9
Total	20

Table 2–11: Program for real time PCR cycles				
Reaction step		Temperature (°C)	Duration (mm:ss)	Nature of reaction
	1	95	10:00	AmpliTaq Gold® DNA polymerase activation
Cycle repeated for 40 times	2a	92	00:15	Separation of DNA strand
	2b	60	01:00	Primer annealing/DNA synthesis

2.7.2.2 Sequenom iPLEX™ Assay MassARRAY®

The remaining tSNPs, including one tSNP in *FOXC1,* 26 SNPs in *LMX1B,* 4 tSNPs in *BMP4* and 22 SNPs in *TGFβ2*, were genotyped using Sequenom iPLEX™ Assay MassARRAY technology through a commercially available service based at the Wellcome Trust Centre for Human Genetics in Oxford (http://www.well.ox.ac.uk/). For the replication study on cohort two, Sequenom Mass Array technology was also utilized to genotype 11 tSNPs in *LMX1B* (rs12336217, rs7859156, rs10819190, rs10987385, rs13285227, rs944103, rs12555176, rs7854658, rs10987386, rs16929236, and rs867559).

2.7.2.2.1 The principles of Sequenom iPLEX Assay MassARRAY

The main differences with Sequenom MassARRAY from that of TaqMan SNP genotyping assays is that no labeling and clustering of signals are required for Sequenom, and instead the detection is based on a physical property (the mass to charge ratio) of target sequences by using the matrix-assisted laser desorption-ionisation time-flight mass spectrometry (MALDI-TOF MS) based technology. This allows a direct detection of the end product and the speed and accuracy of MALDI-TOF MS offers high-throughput genotyping. In addition, Sequenom is capable of performing multiple PCR and iPLEX reactions in a single well (multiplexing) which allows further increase in throughput and reduced cost per genotyping reaction.

The iPLEX assay is based on the annealing of a specific oligonucleotide primer adjacent to the SNP of interest (Figure 2-10). The addition of a mixture of a DNA polymerase with terminator nucleotides allows the extension of the primer into the SNP site and generates an allele of specific mass, which is terminated after a single base extension. Each allele-specific extension product has a unique molecular mass which is then analysed by MALDI-TOF MS and a genotype is assigned in real time. Since the mass separation of small base extension (SBE) is small, this prohibits the routine use of high throughput SNP genotyping. The iPLEX assay alleviates this problem by incorporating mass-modified terminators which increases the mass differences of SBE products. This

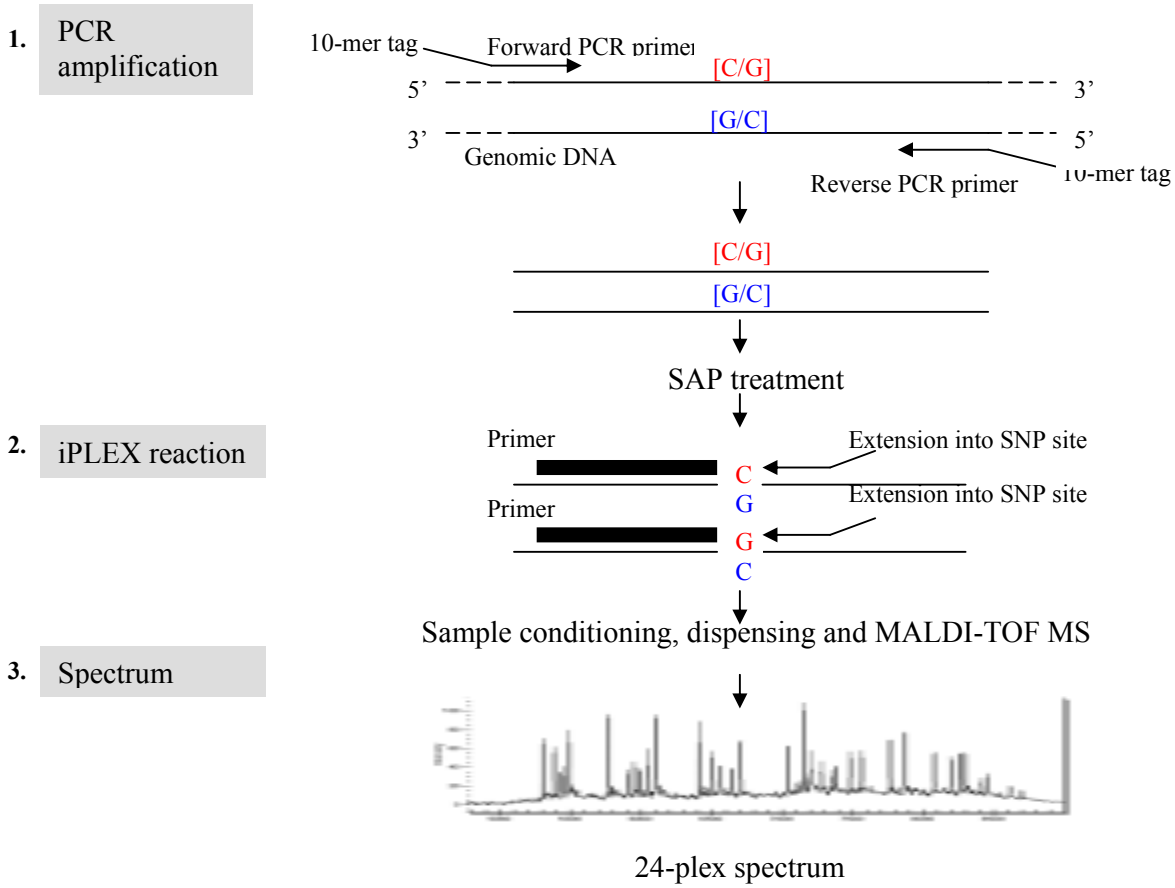


Figure 2–10: Sequenom iPLEX Assay MassARRAY

SNP genotyping with iPLEX assays is divided into 3 parts: 1) primary multiplexing PCR amplification of a small region of genomic DNA containing the SNP with a forward and a reverse primer; 2) iPLEX reaction to genotype SNP with a single primer; and 3) the final spectrum analysis. Genomic DNA is amplified using PCR primers tagged to a 10-mer (5'-ACGTTGGATG-3'). The tags increase the masses of unused PCR primers, and by doing so falls outside the mass range of analytic peaks. Shrimp alkaline phosphatase (SAP) enzyme is utilized to dephosphorylate unincorporated dNTPs from the end product of PCR. The iPLEX assay is based on the annealing of an oligonucleotide primer adjacent to the SNP of interest. The addition of a mixture of a DNA polymerase with terminator nucleotides allows the extension of the primer into the SNP site and generates allele and mass specific products and is terminated after a single base extension. Each allele-specific extension product has a unique molecular mass which is then analysed by MALDI- TOF MS and a genotype is assigned in real time. A nanodispenser is used to dispense the reaction products onto a SpectroCHIP bioarray, where upon it is analysed.

ensures no two alleles are within 15Da of each other. However, if DNA samples are contaminated or not properly desalted by either sodium (22Da) and potassium (38Da), this can reduce the allelic discriminative ability between A/C (24Da) and C/G (40Da). Even in such circumstances, the peaks generated by these contaminates are relatively much smaller to their parent signal and this property can be used to distinguish between them.

Essentially, SNP genotyping with Sequenom MassARRAY involved three steps, requiring 3 primers for each SNP:

1. Designing assays.
2. Carrying out a primary multiplexing PCR reaction to amplify regions of genomic DNA containing the SNPs of interest. This involved the presence of several forward and reverse primers to cover all the SNPs in each multiplex group (Table 2-12 and 2-13).
3. Performing a multiplexing iPLEX reaction to genotype SNPs using several single primers of distinct mass each specific for one genotype.

2.7.2.2.2 Assay design

PCR and iPLEX assays (including the primers for each SNP) were created by using Assay Design 3.0 software (Table 2-14) when the upstream and downstream flanking sequences were available. For these assays, a quality control measure was applied, whereby, the selected primer assays were tested on CEPH DNAs and the genotypes compared with that of the HapMap data. This allows us to assess whether the results were either concordant or disconcordant with the HapMap data, with any assays producing disconcordant results being excluded from the study.

Table 2–12: Two sets of multiplex reactions (Plex 1 and 2) for the first cohort as recommended by the MassARRAY Assay Design 3.0 software.

SNPs are colour coded according to the gene they are representing: colour green represents SNPs for *TGFβ2*; yellow represents SNPs for *LMX1B*; red represents SNPs for *BMP4*; and purple represents SNPs for *FOXC1*.

Multiplex reaction	SNP ID
Plex 1	rs10448285, rs10819195, rs2027567, rs10733682, rs10987386, rs1473526, rs10987411, rs13287548, rs17047703, rs13285227, rs12343574, rs2796817, rs16929236, rs944103, rs2009112, rs2149724, rs10482751, rs2799085, rs6478750, rs10495098, rs2796821, rs7854658,, rs2798631, rs8014363, rs7858338, rs3892225, rs2761884, rs7859156, rs2027566, rs11623717, rs867559, rs947712, rs2235716
Plex 2	rs10760443, rs10760450, rs10779329, rs10819190, rs10987385, rs1317681, rs10987417, rs12336217, rs1342586, rs10987413, rs7857133, rs2799097, rs4083644, rs17047682, rs2796814, rs12555176, rs17558745, rs8014071

Table 2–13: One set of multiplex reactions for the replication study on *LMX1B* as recommended by the MassARRAY Assay Design 3.0 software.

Multiplex reaction	SNP ID
Plex 1	rs12336217, rs7859156, rs10819190, rs10987385, rs13285227, rs944103, rs12555176, rs7854658, rs10987386, rs16929236, rs867559

Table 2–14: Forward, reverse, and extension primers for the selected SNPs in *FOXC1, BMP4, TGFβ2* and *LMX1B* for the PCR/iPLEX assays.

SNP ID	Forward and reverse primer for PCR amplification	Amplification Length (bp)	Extension primer for iPLEX reaction
rs3892225	**F:**ACGTTGGATGGTGCCTGGAGTATATGAAGC **R:**ACGTTGGATGTCCACAGTTGGTATTTAGTC	98	AGCACCGCATCGGTA
rs7859156	**F:**ACGTTGGATGAGAGCCAGCCCAGGTTTCG **R:**ACGTTGGATGCAGTCACCCATCATCACCC	100	CGGCGGGAATTTCCT
rs13287548	**F:**ACGTTGGATGAGTGCAGTGGCAGGTCAATG **R:**ACGTTGGATGACCCAGCAGCCTTCTCCCAA	100	AGGGTGGTCAGCTGG
rs867559	**F:**ACGTTGGATGGAAAGCCCCATTAATCCTCC **R:**ACGTTGGATGCACTGAGGCTCAGAGAAATC	92	GCTGTGCAGTCTGGG
rs10733682	**F:**ACGTTGGATGTGTGTCCCCTTGTTCAGGTG **R:**ACGTTGGATGAGGCAGACTTTAGGGTGGTG	100	GGTGCTCACAACCCT
rs16929236	**F:**ACGTTGGATGCAGGGTGAATCTCTAAGGTC **R:**ACGTTGGATGTGGCACCCTTCCTCTGTTAA	91	GGCCAAATCATTGTCTT
rs2009112	**F:**ACGTTGGATGTACCTATCAAAGTCGCTGCC **R:**ACGTTGGATGAGGGACACATGATTGGTCAC	100	CTCGCATTCATTGGAAG
rs944103	**F:**ACGTTGGATGTCTCCCTCCTTCTCTCCGTG **R:**ACGTTGGATGTTATTTATGCCCAGGAGCAG	97	ACTGATCCTCCTCCATCC
rs10987386	**F:**ACGTTGGATGCAGGCTTGGTCTTGCCAATC **R:**ACGTTGGATGTATCCTAAGAATGGTGGAGC	96	TGCCATACCCCGATC
rs1473526	**F:**ACGTTGGATGAGAGGGTTTCCAACTCCAGG **R:**ACGTTGGATGCAACCCAACTGAAAGAACAC	100	TCGGTGATGTCACCTG
rs2235716	**F:**ACGTTGGATGGAAAAAAGAGAGCTCCTTC **R:**ACGTTGGATGTGTAGGAATGTGTGTGCTGG	99	AGAGCTCCTTCTGGCTCC
rs10819195	**F:**ACGTTGGATGAGAGGGTAAGTGTCTTGTCC **R:**ACGTTGGATGTGTGGATGCTGCAGCCAGGT	100	TGTCTTGTCCAAAGTCTCA
rs13285227	**F:**ACGTTGGATGGATAAAGATAAACGGAATTG **R:**ACGTTGGATGGAGTCCTTCCCCATCACCG	99	GTAATTGAGTCTGCTAAGG
rs7854658	**F:**ACGTTGGATGAAGCAGTGCCCAGTCCAGTC **R:**ACGTTGGATGGCACAGAGTACATCTGCCC	97	CAGATCTGAGTCCCTTCTTT
rs10987411	**F:**ACGTTGGATGCTTCTCCAAAGGCTCAGTGC **R:**ACGTTGGATGACAGATGATACCCTGAGTGG	83	TCAGTGCCGCCATTAC
rs2027567	**F:**ACGTTGGATGCTGTCCACATATTCCAGAGG **R:**ACGTTGGATGGAAGGGTAGAGTAGAATTAG	99	CCAGAGGTCATTCAGAAA
rs2761884	**F:**ACGTTGGATGCTCCGGGCAGATTCAGAAAG **R:**ACGTTGGATGCTCGGCCACATTAAGATCTG	94	TTCAGAAAGAGGCGTC
rs2149724	**F:**ACGTTGGATGGTTGCTCTGAAGTCAAAGCC **R:**ACGTTGGATGTGACTCTATGCCAGGCAGAC	100	CCATGAACAGAGTCCCC
rs6478750	**F:**ACGTTGGATGCGACCCCTTGAACCTTAATG **R:**ACGTTGGATGAGGTCACAGTCTAACAAGGG	98	CTTAATGCTAGAGCACCT
rs10482751	**F:**ACGTTGGATGGGTGGTGTTTCTCTTTTGGC **R:**ACGTTGGATGAAAACAATCTTCTTACCCC	93	TCTCTTTTGGCTACTCATC
rs10495098	**F:**ACGTTGGATGCATTTACGGTTACCAGGGAG **R:**ACGTTGGATGCACCTCTCAAATGATCAGTTC	96	GACAGCTCTAGCAATCTTGCC

rs17047703	**F:**ACGTTGGATGCTTCCGCTATCTTGATGTAG **R:**ACGTTGGATGGCAAGCACATCTGGCAAATC	98	GATGTAGCATTTCCGGTA
rs2796817	**F:**ACGTTGGATGACTCTTCTGCTGGTCGCCTG **R:**ACGTTGGATGATAGGCCACGACTGGACTTC	100	CTGGTCGCCTGCCCCTATGT
rs2799085	**F:**ACGTTGGATGTAAAAGCAGACCGAGAACAC **R:**ACGTTGGATGGAGACTCCGTCTCTAAAAAAG	98	GACCGAGAACACATGGTAC
rs947712	**F:**ACGTTGGATGGTGAGTTTTGGGGATTGAATG **R:**ACGTTGGATGTAAATGATACTGCAGGGCAC	100	CATCAACGATTCTTCATTTTATTA
rs11623717	**F:**ACGTTGGATGGTTTCGGCATTTACTATTT **R:**ACGTTGGATGAAAGCAACGCCGAGAATCAC	99	GCATTTACTATTTTTGCCTAATA
rs7858338	**F:**ACGTTGGATGCTATTGATCGACCGACTGGC **R:**ACGTTGGATGTGAGCCTGACTCCACCATC	99	CCAAGTCAGCATTTAAGCTTTAA
rs2796821	**F:**ACGTTGGATGTGAAACAGGAGTGGGTTTGG **R:**ACGTTGGATGAGTACGCACACTGAAACACG	100	GGTTAATTGGTATGCATAGTATTA
rs10448285	**F:**ACGTTGGATGCTACTGTCCAGGTTGTTGTA **R:**ACGTTGGATGTCTTCCAGGTACACTCGCAG	100	TGTTGTAGGGATTAATTTTGTAG
rs2798631	**F:**ACGTTGGATGGCCAAACTCAAGAGTTGATA **R:**ACGTTGGATGGAGAAACGAATTTTCAAGTC	90	AACTCAAGAGTTGATATGAAGTAC
rs2027566	**F:**ACGTTGGATGGAAGGGTTCACTCTGGTTTC **R:**ACGTTGGATGCGATGCCACAATGATGTCAC	99	ATAATAATACGAGCCATCTGCC
rs12343574	**F:**ACGTTGGATGAGGATGGGTGGTTTTCAATG **R:**ACGTTGGATGGCATGTTTTTATCACCTGCC	100	AATGTTAACAAAATTACCAAAGTTTA
rs8014363	**F:**ACGTTGGATGGGCCTAAGAATCTCTAACAT **R:**ACGTTGGATGGGTAAATAAAAGTGATTGTTG	100	AGATTAACATTTAATTTAAATACAACAA
rs4083644	**F:**ACGTTGGATGTCACAAACCCCCGAATCCAG **R:**ACGTTGGATGAACAGTCTGTGGGTCACAAG	99	ACCCCATGCCAGGAG
rs1342586	**F:**ACGTTGGATGCTTCTACATCACCTGGGAAC **R:**ACGTTGGATGCCCCAGAGCCTCTGATTTAA	100	CAGATTCTTGGGCCTC
rs10779329	**F:**ACGTTGGATGTTGTGCTCACTTGGGCATGG **R:**ACGTTGGATGAGTTTTAGGGCAAGGGTCTC	99	GTGACAGGAGACTTTCA
rs7857133	**F:**ACGTTGGATGACTAGCTCTGGATCCCTTTG **R:**ACGTTGGATGGGGGAGGGCCAGCCGAAT	98	GGGTAACAGCAAGCCA
rs12555176	**F:**ACGTTGGATGTCAAGGGTCCTGGCTGTTGG **R:**ACGTTGGATGGTAGTGGCCAGAGATGGGG	100	CCCCAAGCCTTGGAGTAT
rs12336217	**F:**ACGTTGGATGGCCATTGTGGGCTAATGTGT **R:**ACGTTGGATGTCCATACATCACACCCGCAG	100	GGGCTAATGTGTCAGGGC
rs10760443	**F:**ACGTTGGATGGAGTAGGGAGAGTTTTCCAG **R:**ACGTTGGATGTACTCAGGCTGTGGTGGTTG	100	AGAAGAAAGGATGATGGA
rs2796814	**F:**ACGTTGGATGACTGCCTTGGACAGGCAATC **R:**ACGTTGGATGAGCAAAGGGATCAACTGGAG	93	CAGCCTAAACACAGACAGA
rs10987385	**F:**ACGTTGGATGTGCAGAACCCAGCTTTTATG **R:**ACGTTGGATGCTAAATTGGCTACATGACAC	96	CGGGGCACACTTAGA
rs3796902	**F:**ACGTTGGATGAAACTACCCTTTCCGGGTTC **R:**ACGTTGGATGAAAGACCACCTCTTCGTCAG	97	GAGTTTAATATGCCAAGCTC
rs10760450	**F:**ACGTTGGATGCTCTGTTGTCTGGTCTGATG **R:**ACGTTGGATGACCAACTCTAGAGGCAGATG	80	TGGTCTGATGCTACCT
rs17558745	**F:**ACGTTGGATGGGGCCTCTATGTTTCTTTTG **R:**ACGTTGGATGCCAAAGGTTGACATCTTCAC	98	TCTTTTGCTTTGTATAACAGA
rs10987413	**F:**ACGTTGGATGAGAAGGGACAGGGATGCTCA **R:**ACGTTGGATGGGGTCCCCTGCCTGGCCT	93	CTGCCTTGTCAGAAAGA

rs10987417	**F:**ACGTTGGATGCAAGAGTCAGACCAAGGTGC **R:**ACGTTGGATGTCCTAAGATGCCCAGCCAC	100	ACCAAGGTGCCAGCACA
rs10819190	**F:**ACGTTGGATGTCAGGCACACCTTCCTGCTT **R:**ACGTTGGATGTATGAAGTGACATGTCAGGG	100	ACACCTTCCTGCTTTTTTAGATA
rs1317681	**F:**ACGTTGGATGTCCCGACTGGGCATCAAGG **R:**ACGTTGGATGTTTGACCCTGCCTTTACTTC	100	CATCAAGGGGCCTTTTTG
rs8014071	**F:**ACGTTGGATGGATCCAGTTTATTTCCCCAG **R:**ACGTTGGATGCTTAGTCCCTTAATCACCTG	100	GTTTTAGACGAAATTCAGATAGA
rs17047682	**F:**ACGTTGGATGCCACATTGCTAAAAATATG **R:**ACGTTGGATGTTACAGATTGCTGATAGCCC	89	TATGTAGTCTGAACTTTATAAAATG
rs2799097	**F:**ACGTTGGATGGCTTTATGTAGTAAGCATTT **R:**ACGTTGGATGTATCTCATACACCCACATGC	99	TTACTATTTTATTTTTTAGGATTCTCT

2.7.2.2.3 PCR amplification

Prior to the iPLEX reaction, the genomic DNA was amplified in 384 well plates using PCR primers tagged with a 10-mer (5'-ACGTTGGATG-3'). The tags increased the masses of unused PCR primers, and by doing so fell outside the mass range of analytical peaks. The SNPs were grouped (by the Assay Design 3.0 software to allow for multiplex reaction) in such a way that the combination of the assays used in each multiplex reaction was compatible with each other. Separate PCR reactions were performed for each multiplex group; there were two separate multiplex groups for cohort 1 (Plex 1 and 2) (Table 2-12) and one multiplex group for the subsequent cohort 2 (Table 2-13). Quality control was provided by randomly including a non-DNA containing well. The various reagents used for a multiplex PCR reaction are shown in Table 2-15.

Table 2–15: PCR reaction components for Sequenom

Reagent	Concentration in 5μL	Volume (μl) (1 reaction)	Volume (μl) (384 reactions)
Nanopure H2O PCR Buffer with	NA	3.70	1776
$MgCl_2$ (10x)	1.25x	1.24	600
$MgCl_2$ (25mM)	1.625mM	0.65	312
dNTP mix (25mM)	500μM	0.20	96
Primer mix (500nM each)	100nM	2.00	960
Genomic DNA (5-10ng/μL)	5-10ng/reaction	2.00	960
Hotstar Taq® (5U/μL)	0.5U/reaction	0.20	96
Total		10.00	4800

PCR reactions were run with the program outlined in Table 2-16 using a final reaction volume of 10µl in each well. 5µl of the PCR product was run on a 2% agarose gel to ensure that the PCR product was of good quality. The remaining 5µl was utilized for SNP genotyping (iPLEX reaction in section 2.7.2.2.4)

Table 2–16: Program for PCR amplification cycles for Sequenom

Reaction step		Temperature (°C)	Duration (mm:ss)	Nature of reaction
Cycle repeated for 45 times	1a	94	15:00	Denaturation
	1b	94	00:20	Separation of DNA strand
	2	56	00:30	Primer annealing
	3a	72	01:00	DNA synthesis (transcription)
	3b	72	03:00	DNA synthesis (elongation)
	3c	4	24:00	DNA preservation following end of reaction

Shrimp alkaline phosphatase (SAP) enzyme was utilized to dephosphorylate unincorporated dNTPs from the end product of PCR. This was achieved by adding 2µL of the SAP mix to each 10µL PCR reaction (Table 2.17), then gently vortexing the mixed samples, followed by incubation in a standard thermocycler with the temperature settings as follows:

- 37°C for 20 minutes
- 85°C for 5 minutes
- 4°C until retrieval

Table 2–17: SAP treatment

SAP Mix Reagent	Volume (µL) (1 reaction)	Volume (µL) (384 reactions)
Nanopure H2O	1.530	734.4
10x SAP buffer	0.170	81.6
SAP enzyme (1U/µL)	0.300	144.0
Total	2.000	960.0

2.7.2.2.4 iPLEX reaction

In a similar fashion to the PCR reaction, iPLEX amplification was performed in 2 separate multiplex reactions for cohort 1 (Table 2-12) and in 1 multiplex reaction for cohort 2 (Table 2-13) in a 384 well plate.

The iPLEX amplification involved aliquoting 5μL of the PCR product into a 384 well plate and adding 2μL of the corresponding multiplex reaction mix (Table 2-18) followed by gentle vortexing. This was then subjected to a two-step 200 short cycle program in a standard thermocycler (Table 2-19).

Table 2–18: iPLEX reaction mix

Reagent (384rxns)	**Concentration in 9μL**	**Volume (1rxn)** (μL)	**Volume** (μL)
Nanopure H2O	NA	0.755	362.40
iPLEX Buffer (10x)	0.222X	0.200	96.00
iPLEX termination mix	1X	0.200	96.00
Primer mix (7μM: 14μM- double the concentration for high mass primers)	0.625uM (for low mass primers) 1.25uM (for high mass primers)	0.804	385.92
iPLEX enzyme	1X	0.041	19.68
Total		2.000	960.00

Table 2–19: Program for PCR cycles for iPLEX reaction				
Reaction step		**Temperature (°C)**	**Duration (mm:ss)**	**Nature of reaction**
Cycle repeated for: 40 times	1	94	00:30	Denaturation
	2	94	00:05	Separation of DNA strand
5 times	3	52	00:05	Primer annealing
	4	80	00:05	DNA synthesis (transcription)
	5	72	03:00	DNA synthesis (elongation)
	6	4	24:00	DNA preservation following end of reaction

During the iPLEX reaction, the reaction mix was denatured at 94° C. Primers were annealed at 52° C for 5 seconds and extended at 80° C for 5 seconds. The annealing and extension cycle was repeated four more times for a total of fives cycles and then looped back to the denaturation step at 94° C for 5 seconds and proceeded sequentially through annealing and extension loop again. This cycle was repeated an additional 39 times to complete a total of 40 cycles. This in addition to the 5 cycles of annealing and extension steps added up to a total of 200 cycles (5×40). A final DNA extension at 72° C for three minutes was performed and then cooled to 4° C.

2.7.2.2.5 Desalting of iPLEX reaction product and dispension onto SpectroCHIP® Bioarrays

The iPLEX reaction products were desalted by diluting with 25µL of water containing 6mg of resin, to optimize for mass spectrometric analysis. This was left on a rotator for half an hour to ensure good mixture of the resins into the reaction mix followed by

centrifuging at 4500rpm for 5 minutes to allow the resin to settle to the bottom of the well.

A nanodispenser was used to dispense 15nL of the reaction products onto a 384-element SpectroCHIP bioarry. MassARRAY Workstation version 3.3 software was used to process and analyse iPLEX SpectroCHIP bioarrays (Figure 2-11).

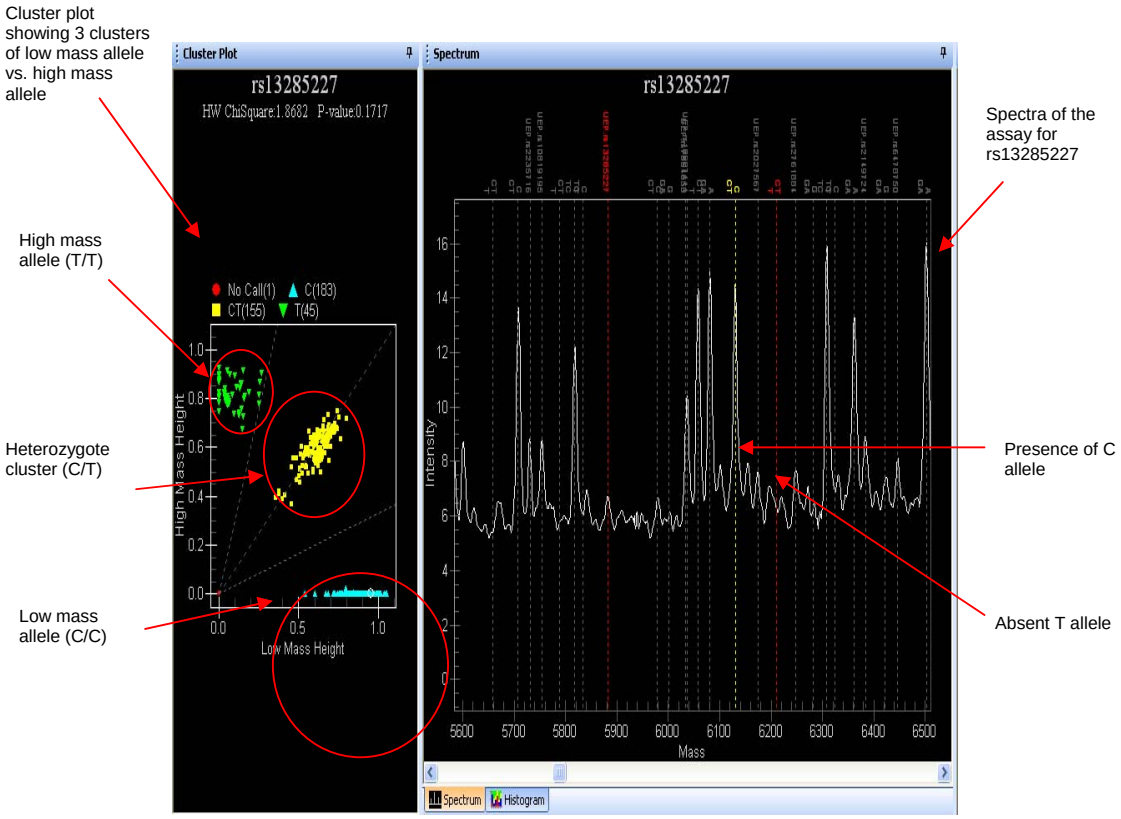


Figure 2–11: An example of the results displayed in Sequenom for rs13285227 demonstrating a cluster plot (left side of the illustration) and a spectrum plot (on the right side of the illustration).

The cluster plot demonstrates 3 clusters of low mass allele (C/C) and a high mass allele (T/T) and an intermediate mass allele (C/T). The spectrum plot shows the presence of a C allele and an absent of a T allele.

2.8 Sequencing of *OPTN* E50K mutation

To validate the results of the prevalence of *OPTN* E50K mutation obtained from using TagMan SNP genotyping assay, the region of the genomic DNA containing the E50K mutation was sequenced using an Applied Biosystems 3730*xl* DNA Analyzer. Essentially, this method involved amplifying the region around the E50K mutation by PCR. Sequencing was achieved using a single primer (either a forward or a reverse primer) using Sanger dideoxy terminator method. All reagents for the PCR reaction were bought from Bioline other than the primers which were obtained from Invitrogen. The reagents used for the sequencing reaction were obtained from Applied Biosystems.

Primer sequences were designed using Primer 3 software [available as a WWW interface (http://frodo.wi.mit.edu/)] (Table 2-20). The amplified target fragment was 358 bp in length.

2.8.1 PCR reaction for sequencing

A master mix for the PCR reactions was prepared on ice prior to aliquoting into separate tubes (Table 2-21). This was to ensure reproducibility of dispensing the same mixture of reagents into each MicroAmp™ 8- tube strip (Applied Biosystems). For each PCR reaction, the master mix consisted of 2.5 µl of 10x NH_4 PCR buffer, 2.5 µl of dNTP mix (2mM), 0.75 µl of $MgCl_2$ (50mM), 0.1 µl of forward and reverse primer (100µM), 17.95 µl of sterile Milli-Q® water, 0.1 µl of Biotaq™ polymerase (5U/µl), and 5-25ng of genomic DNA. The Applied Biosystems Veriti™ 96-Well Thermal Cycler PCR machine was utilised to carry out the PCR reactions**.** Quality control was provided for each set of PCR reactions by including a positive control and a negative blank control.

Table 2–20: Forward and reverse primers for PCR and sequencing reactions for E50K mutation		
SNP ID	Forward and reverse primer	Amplification length (bp)
rs28939688	**F:** GGGACAGCTCTATTTTCAACA **R**: CAAATGCTAAATCCTGTGCTT	358

Table 2–21: PCR reaction components		
Reagent	**Volume (µl) (1 reaction)**	**Volume (µl) (10 reactions)**
10x NH_4 PCR buffer	2.5	25
dNTP mix (2mM)	2.5	25
$MgCl_2$ (50mM)	0.75	7.5
Forward Primer (100µM)	0.1	1.0
Reverse Primer (100µM)	0.1	1.0
Sterile Milli-Q® water	17.95	179.5
Biotaq™ polymerase (5U/µL)	0.1	1.0
Genomic DNA (5-25ng/µL)	1.0	960
Total	25	250

PCR reactions were run with the program outlined in Table 2-22, with a final reaction volume of 25µl. The PCR consisted of a series of cycles of three successive steps as shown in table 2-22. The annealing temperature was optimized empirically (Figure 2-10) by running PCR reactions on a temperature gradient (annealing temperature varying between 53 to 60 ° C). An annealing temperature of 57° C was chosen as this produced the brightest band on 2.0% agarose gel electrophoresis (Figure 2-12).

Table 2–22: Program for PCR amplification cycles for sequencing				
Reaction step		**Temperature (°C)**	**Duration (mm:ss)**	**Nature of reaction**
Cycle repeated for 35 times	1a	95	02:00	Denaturation
	1b	95	00:20	Separation of DNA strand
	2	57	00:20	Primer annealing
	3	72	00:30	DNA synthesis

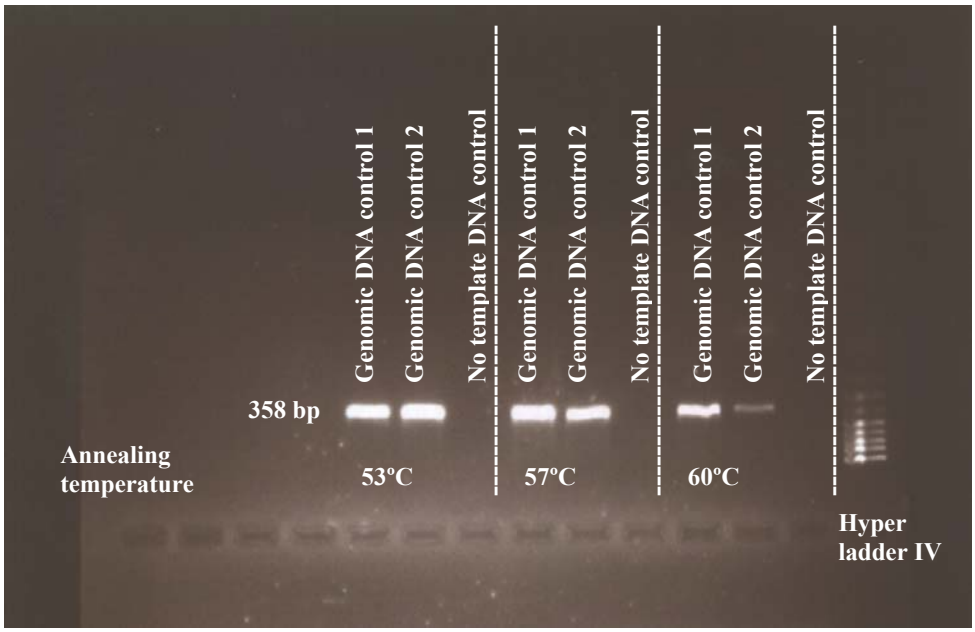


Figure 2–12: Optimisation of annealing temperature.

Gel electrophoresis showing three PCR products (each containing 2 genomic DNA controls and a no template DNA control) for the annealing temperatures 53 ºC, 57 ºC and 60 ºC. The annealing temperature of 57 ºC produced the brightest band.

2.8.2 Post PCR clean-up

Shrimp Alkaline Phosphatase (SAP) (Amersham Biosciences) and Exonuclease I (EXO I) (New England BioLabs) was used to prepare the PCR products for sequencing. Exonuclease I digests the single stranded PCR primers whilst SAP removes the phosphate groups from the excess dNTPs left over from the PCR. A master mix was prepared consisting of 1µl of the SAP, 0.3µl of EXO I, 0.9µl of SAP dilution buffer, 0.3µl of sterile Milli-Q® water (Table 2.23). 2.5 µl of the master mix was then pipetted into 5µl of PCR reaction product. This was gently vortexed and incubated in a standard thermocycler with the temperature settings as follows:

- 37°C for 30 minutes
- 80°C for 5 minutes
- 4°C until retrieval

Table 2–23: Reagents used for cleaning of PCR products for sequencing.

Components	Volume (µl/reaction)
Exonuclease I (20,000 U/µl)	0.3
Shrimp Alkaline Phosphatase (SAP) (1U/µl)	1.0
10x SAP Dilution Buffer	0.9
Sterile Milli-Q® water	0.3
Total	2.5

2.8.3 Automated cycle sequencing

Sequencing was performed by Sanger dideoxy Terminator Method using BigDye® v1.1 chemistry on an Applied Biosystems Veriti™ 96-Well Thermal Cycler PCR machine.

2.8.3.1 Cycle sequencing reaction by Sanger Dideoxy terminator method

The principle of automated cycle sequencing involves utilisation of dideoxynucleotides (ddNTPs) which lack the hydroxyl (OH) at the third carbon atom of the nucleotide base (3'). This OH group is essential for DNA chain extension since the 5' phosphate group of dNTPs forms a covalent bond with the 3' OH group, thus linking nucleotide bases. The lack of this OH group prevents the bonding of dNTPs to an incorporated ddNTP, resulting in the chain being terminated at such sites.

During sequencing, the DNA template is denatured into a single strand. This, along with a single primer, four dNTPs, DNA polymerase and fluorescently labelled ddNTPs are added into the reaction mix. The primer anneals to its complementary region of the DNA and is extended by incorporating dNTPs, the reaction being driven by DNA polymerase. Random incorporation of ddNTPs terminates the sequencing reaction such that complementary strands of varying lengths are formed. At the end of the cycle, numerous fragments are generated, each having different lengths and a tagged nucleotide at the end. The reaction components ensure that the fragments of every possible length are generated.

All the reagents for the cycle sequencing reactions were obtained from Applied Biosystems. Two master mixes was prepared, each consisting of 1µl of either the forward and reverse primer respectively (stock solution prepared at 100µM with TE followed by further dilution by 20 times to 5µM with sterile Milli-Q® water). Both primers were the same primers used for the prior PCR reactions (section 2.8.1). Each master mix also contained 0.5µl of BigDye® Terminator v1.1 Cycle Sequencing Ready Reaction Mix, 1.5µl of 5x BigDye® Terminator v1.1 sequencing buffer, and 2µl of ddH_2O (Table 2-24). 5µl of the master mix was then pipetted into another MicroAmp™

8- tube strip (Applied Biosystems) containing 3µl of the PCR product. Using the same PCR machine, cycle sequencing reactions were carried out with the parameters as shown in Table 2-25.

Table 2–24: Cycle sequencing reaction components

Component	Volume (µL/reaction)
BigDye® Terminator v1.1 Cycle Sequencing Ready Reaction Mix	0.5
5× BigDye® Terminator v1.1 Cycle Sequencing Buffer	1.5
Sterile Milli-Q® water	2.0
Primer (5µM)	1.0
Total	5.0

Table 2–25: Parameters for sequencing

Reaction step		Temperature (°C)	Duration (mm:ss)	Nature of reaction
Cycle repeated for 35 times	1a	96	02:00	Hold
	1b	96	00:15	Denaturation
	2	55	00:10	Primer annealing
	3	60	04:00	Extension

2.8.3.2 Post-sequencing reaction clean-up

Excess primers, dNTPs and tagged ddNTPs were removed from the reaction products to allow for sequencing. To achieve this, the PCR products were loaded into a MicroAmp Fast Optical 96 well reaction plate (Applied Biosystems) and 2 µl of 3M sodium acetate and 50 µl of 100% ethanol were added. This was left for 30 minutes at room temperature and was spun at 3000 x *g* using a Heraeus® Labofuge® 400 Centrifuge (Thermo Scientific). The 96 well plate was gently turned upside down to remove the supernatant. 50 µl of 70% ethanol was then added and spun at 3000 x *g* for 15 minutes. The PCR plate was again turned upside down to remove the supernatant and was left to dry for 10 minutes. 20 µl of 0.1 x TE was added to resuspend the PCR product, following which it was then loaded into the 3730*xl* DNA Analyzer for sequence analysis.

2.8.3.3 Fragment separation by capillary electrophoresis on ABI 96-capillary 3730 *xl* Sequencher

The Applied Biosystems 3730*xl* DNA Analyzer is an automated, high-throughput, capillary electrophoresis system which utilises thin capillaries filled with polymer matrix. The samples are electrokinetically injected into the array of capillaries. The labelled DNA sequence fragments are separated by size as they migrate through the polymer-filled capillary array (electrophoresis) toward the anode, with the smallest fragments moving the fastest. As they reach the detection window, the laser beam excites the dye molecules and causes them to fluoresce. The fluorescence emissions are collected and displayed as an electropherogram.

2.8.3.4 Data analysis

The sequence data was analysed using the Sequencher® 4.9 DNA software (by Gencodes; www.genecodes.com).

2.9 Statistical analysis

2.9.1 Chi-square test (χ^2)

χ^2 is a statistical test commonly used between two or more proportions (when there is a large number of observations) to compare observed data with the expected data obtained according to a specific hypothesis. An example to explain this more clearly would be as follows:

According to Mendel's law, half of 20 (i.e. 10) offspring would be male. But if the observed number of males was 8, then one would want to know about the "goodness to fit" between the observed and expected. Were the deviations (differences between observed and the expected) as a result of chance, or were they due to other factors. The χ^2 tests the null hypothesis, which states that there is no significant difference between the expected and observed result. More specifically, χ^2 is the sum of the squared difference between observed and the expected data, divided by the expected data:

$$\chi^2 = \frac{(\text{observed-expected})^2}{\text{expected}}$$

(χ^2 utilises numerical values, and not percentages or ratios.)

Using the chi-square distribution table (Table 2-26), the significance of the value is then determined (i.e. $P <$ or > 0.05). In order to calculate this, the degrees of freedom (n–1, where n = number of variables) is determined and by finding the location of where this row meets the χ^2 value, and by moving up this column, will provide the P value.

For this study χ^2 goodness-of-fit test was used to test for the following significance test:

1. Whether allele frequencies for each SNP conformed to Hardy-Weinberg equilibrium (HWE) (see below).
2. For differences in the distribution of sex and individuals with a family history of glaucoma between cases and controls using SPSS (version 15).

3. For any significant differences in allele and haplotype frequencies between cases and controls using Haploview v.4.0.

Table 2–26: χ^2 distribution table

Degrees of Freedom *(df)*	**Probability (*p*)**										
	0.95	**0.90**	**0.80**	**0.70**	**0.50**	**0.30**	**0.20**	**0.10**	**0.05**	**0.01**	**0.001**
1	0.004	0.02	0.06	0.15	0.46	1.07	1.64	2.71	3.84	6.64	10.83
2	0.10	0.21	0.45	0.71	1.39	2.41	3.22	4.60	5.99	9.21	13.82
3	0.35	0.58	1.01	1.42	2.37	3.66	4.64	6.25	7.82	11.34	16.27
4	0.71	1.06	1.65	2.20	3.36	4.88	5.99	7.78	9.49	13.28	18.47
5	1.14	1.61	2.34	3.00	4.35	6.06	7.29	9.24	11.07	15.09	20.52
6	1.63	2.20	3.07	3.83	5.35	7.23	8.56	10.64	12.59	16.81	22.46
7	2.17	2.83	3.82	4.67	6.35	8.38	9.80	12.02	14.07	18.48	24.32
8	2.73	3.49	4.59	5.53	7.34	9.52	11.03	13.36	15.51	20.09	26.12
9	3.32	4.17	5.38	6.39	8.34	10.66	12.24	14.68	16.92	21.67	27.88
10	3.94	4.86	6.18	7.27	9.34	11.78	13.44	15.99	18.31	23.21	29.59
	Nonsignificant								**Significant**		

2.9.2 Hardy-Weinberg equilibrium (HWE)

HWE is used to better understand the genetic characteristics of populations. Hardy–Weinberg principle states that both allele and genotype frequencies in a population remain constant, or are in equilibrium from generation to generation, unless specific disturbing influences are introduced. Those disturbing influences include non-random mating, mutations, selection, limited population size, random genetic drift and gene flow. Using this principle, population allele frequencies are used to calculate the equilibrium-expected genotypic proportions (Hardy, 1908; Weinberg, 1908). If p is the frequency of one allele (A) and q is the frequency of the alternative allele (a) for a biallelic locus, then the HWE-expected frequency will be p^2 for the AA genotype, $2pq$

for the *Aa* genotype, and q^2 for the *aa* genotype. The three genotypic proportions should sum to 1, as should the allele frequency (Hardy, 1908; Weinberg, 1908).

$$p^2 + 2pq + q^2 = 1$$

The most common method to assess HWE is through a goodness-of-fit χ^2 test (Weir, 2008). The null hypothesis is that alleles are chosen randomly, and the genotypic proportions thus follow HWE-expected proportions (i.e. p^2, $2pq$, and q^2). In genetic association studies, testing for HWE is commonly used for quality control of large-scale genotyping and is one of the few ways to identify systematic genotyping errors in unrelated individuals (i.e. if $P = \leq 0.05$ for HWE) (Gomes *et al.*, 1999; Hosking *et al.*, 2004; Weir, 2008).

For this study, allele frequencies for each SNP were tested for agreement with Hardy-Weinberg expectations (P>0.05) using a chi-square (χ^2) goodness-of-fit test. This calculation was performed using a software package (SHEsis) obtained from http://analysis.bio-x.cn/myAnalysis.php.

2.9.3 Odds ratio

The odds ratio (OR) is a way of comparing whether the probability of a certain event is the same for two groups. As case control studies begin with a selection of individuals with the disease (case) and another group without the disease (control), there is no way of knowing disease rates because these groups are not determined by nature but by the investigators' selection criteria. Instead, OR is used. OR is defined as the odds that a case is exposed divided by the odds that a control is exposed.

$$\text{Odds ratio} = \frac{\text{exposure rate to risk factor in cases}}{\text{exposure rate to risk factor in controls}}$$

An OR of 1 implies that the event is equally likely in both groups. An OR greater than one implies that the event is more likely to occur in the case group compared to the control group. An odds ratio less than one implies that the event is less likely to occur in the case group compared to the control group. As it is based on a sample collected from a population, it is essentially only an estimate. For this reason it is conventional to

calculate the 95% confidence interval (CI) (defined as a 95% chance for the true OR of the population to lie within the interval) for the OR.

2.9.4 Adjustment for multiple testing

Multiple testing corrections adjust *P* values derived from multiple statistical tests to correct for occurrence of false positives. This is considered a stringent and useful method for validation of association study data (Miller, 1981). The incidence of false positives is proportional to the number of tests performed and the significance level. For example, when a single test is performed at the usual 5% significance level, there is only a 5% chance of incorrectly rejecting the null hypothesis (this is equivalent to a false negative error). However, when performing 100 tests where all the null hypotheses are true, the chances of rejecting at least one null hypothesis is relatively much higher (the expected number would be 100 × 5% which is exactly 5). The problem of multiple comparisons can be described as the potential increase in false positive error that occurs when statistical tests are used repeatedly. If *n* independent comparisons are made, the overall significance level α is given by:

$$\alpha = 1\text{-}\ (1 - \alpha_{\text{per comparison}})^{\text{number of comparison}}$$

and it increases as the number of comparisons increases (Miller, 1981).

A number of mathematical techniques have been developed to control the false positive error rate associated with making multiple statistical comparisons. One such technique used for this research study is the permutation test (Westfall and Young, 1993). The advantage of this method is that it is not as conservative as the Bonferroni correction (which is calculated as the corrected *P* value = *P* × number of SNPs being tested) and takes into account the dependence structure between genes, by permuting all the genes at the same time. The following steps are applied during a permutation test:

1) *P* values are calculated for each gene based on the original data set and ranked.
2) The permutation method creates a pseudo-data set by dividing the data into artificial patient and control groups.
3) *P* values for all genes are computed on the pseudo-data set.

4) The successive minima of the new *P* values are retained and compared to the original ones.
5) This process is repeated a large number of times (usually 1000 times), and the proportion of resampled data sets where the minimum pseudo-*P* value is less than the original *P* value is the adjusted *P* value.

For this study, permutation testing was performed using Haploview v.4.0, with 1,000 simulations to calculate corrected *P* values for multiple testing.

2.9.5 Thesias

Since haplotypes as a whole may have an impact towards functionality of a variant, it has become clear that, in order to assess the role of a candidate gene, the full haplotypic information should be evaluated (Hodge *et al.*, 1999; Rieder *et al.*, 1999). Most available methods used to estimate haplotype frequencies from the genotype data based on statistical inference (Chiano and Clayton, 1998; Clark, 1990; Clark *et al.*, 1998; Excoffier and Slatkin, 1995; Stephens *et al.*, 2001), estimate haplotype frequencies from genotype data without allowing assessment of interaction between two or more loci, which are essential data when dealing with complex diseases. Thesias on the other hand (obtained from www.genecanvas.org), is capable of investigating haplotype-phenotype associations including haplotype-covariate interactions, in addition to haplotype frequencies, through utilizing the maximum likelihood method of inference as described in Tregouet et al (Tregouet *et al.*, 2002). Furthermore, since the LD observed between SNPs and different functional effects of the haplotypes are potential sources of bias when studying multiple SNPs, Thesias overcomes this problem by performing haplotype background analysis, where it assesses the effects of the individual SNPs among the multiple SNPs found within a haplotype.

2.9.6 Summary of the statistical methods used

1. By using the algorithm tagger which was implemented in Haploview v.4.0, tSNPs that captured untyped SNPs with a minimum r^2 value of 0.8 were chosen. Selection was restricted to SNPs with a minor allele frequency (MAF) of ≥10% (i.e. SNPs with a high degree of polymorphism).

2. Haplotypes were constructed from genotype data in the full-size case-control panel within LD blocks by using an accelerated EM algorithm method in Haoloview v.4.0 (www.broad.mit.edu /mpg/haploview) (Barrett *et al.*, 2005). Since 3-5 haplotypes in general capture >90% of all chromosomes, in each haplotype block, common haplotypes with frequencies ≥1% were inferred since this would account for >98% of the chromosomes.
3. Haplotypes identified within cases and controls were inferred using Haploview v.4.0. The method of Gabriel *et al.*, (Gabriel *et al.*, 2002), uses Haploview to construct LD blocks from tSNPs with MAF ≥ 10%. LD between tSNPs was measured by the pairwise *D*' statistic and the LD structure was examined using the 80% confidence bounds of *D*' to define sites of historical recombination between tSNPs.
4. Associations between tSNPs/haplotypes and POAG were investigated using Haploview v.4.0. Differences in allele and haplotype frequencies between cases and controls were determined using a χ^2 distribution with 2 degrees of freedom. Only SNPs conforming to HWE as tested in SHEsis were analysed.
5. SNP and haplotype effects identified from cases and controls were investigated with and without adjustment for age and sex covariates using Thesias v.3.1 (www.genecanvas.org). ORs were calculated using Thesias v.3.1 with 95% confidence intervals (CIs) for each genotype with the respective wild type as the reference. Covariate-adjusted haplotype–phenotype association was calculated by comparison to the most frequent haplotype. Haplotype background analysis of the effects of the individual SNPs within a given haplotype was performed to correct for the potential source of bias created by the presence of the multiple SNPs.
6. χ^2 test was used to calculate for significant differences in the distribution of sex and individuals with a family history of glaucoma between cases and controls (using SPSS version 15), whereas independent samples t test was used to calculate for significant differences in the distribution of age, number of individuals with a family history of glaucoma, mean C/D ratio, IOP and individuals with glaucoma filtration surgery (using SPSS version 15). The latter method is a significance test for quantitative (continuous) data distributed between two distinct groups. When using SPSS for the independent samples t test, the case status (i.e. case or control) was set as group 1 (case) and 2 (control). The clinical variable (e.g. age, mean C/D ratio, IOP etc) was set as a test variable.

7. Multivariate logistic regression analysis was performed using SPSS under the guidance of Dr Yalda Jamshidi (Senior Lecturer in Human Genetics at St George's Hospital). The case status was set as a binary dependant variable (HTG, OHT or NTG = 1, control = 0), and age, IOP and tSNP as a covariate, followed by selecting the binary logistic regression for analysis. The logistic regression makes use of several predictor variables that may be either numerical or categorical (in this case IOP, age and tSNP), for predicting the probability of occurrence of an event (i.e. the case status).

2.9.7 Power calculation

The sample size of 276 controls and 367 cases was used in a power calculator (StataCorp. 2003. Stata Statistical Software: Release 8.0 College Station, TX: Stata Corporation) and showed a power of 80% to identify a difference in genotype and allele frequency between 10-18% at a significance level of $P < 0.05$ between the controls and cases.

Chapter 3 Analysis of phenotypic variables of cases and controls

3.1 Introduction

In this chapter, a comprehensive assessment of the phenotypic variables (including age, sex, family history of glaucoma, mean cup-disc ratio (C/D ratio), mean of the highest IOP and glaucoma surgery) of HTG, OHT and NTG cases as well as normal controls has been performed. The HTG group is characterised by glaucomatous damage, and raised IOP, with the other two patient groups OHT and NTG, each having only one of these characteristics, either glaucomatous damage without raised IOP (NTG), or raised IOP without glaucomatous damage (OHT). Since HTG+OHT and HTG+NTG shared the same phenotypic variable of raised IOP or optic nerve damage respectively, they have been grouped together according to these variables (i.e. HTG and OHT into group 1 with raised IOP, and HTG and NTG into group 2 with glaucomatous damage, irrespective of IOP).

3.2 Results

A total of 367 cases and 276 unrelated controls of white British descent were enrolled in this study. Recruitment of cases and controls took 6 months to achieve. Among the cases, 272 (74.1%) were classified as HTG, 37 (10.1%) as NTG, and 58 (15.8%) as OHT (Table 3-1). The phenotypic characteristics of the controls and the HTG, OHT and NTG patients are depicted in Table 8-1, 8-2, 8-3 and 8-4, all of which are found in Appendix III, IV, V and VI respectively.

3.2.1 Age distribution in cases and controls

Within the case group, the mean age was as follows (Table 3-1, Figure 3-1):

- 71.17 years for the HTG group (± SD 10.448, range 40-93)
- 65.19 years for the OHT group (± SD 11.494, range 41-92)
- 77.51 years for the NTG group (± SD 8.849, range 60-92)

- 70.12 years for the combined HTG and OHT group (± SD 10.863, range 40-93)
- And 71.93 years for the combined HTG and NTG group (± SD 10.462, range 40-93)

For the normal control group, the mean age was 70.76 years (± SD 9.313, range 40-90).

There was a significant difference between the mean age of the control group and the OHT group (Table 3-1).

Table 3–1: Comparison of age, age at diagnosis, sex, number of individuals with a family history of glaucoma, mean CDR, mean of the highest recorded IOP and number of individuals with glaucoma surgery between HTG, OHT, NTG, HTG+OHT group (with raised IOP), HTG+NTG group (with glaucomatous optic neuropathy) and the control group.

* indicates significant difference ($P > 0.05$) between controls and the separate case groups (HTG, NTG, OHT). P^1 and P^2 indicate significant difference between controls and the HTG+OHT group, or the HTG+NTG group, respectively. P values were calculated using independent samples t test, except a χ^2 test was used for sex, using SPSS, version 15 (SPSS Inc., Chicago, IL, USA).

	HTG	OHT	NTG	Controls	Cases combined (HTG+OHT)	P^1	Cases combined (HTG+NTG)	P^2
n	272	58	37	276	330	----------	309	----------
Mean age (SD)	71.17 (10.448)	*65.19 (11.494)	77.51 (8.849)	70.76 (9.313)	70.12 (10.863)	0.437	71.93 (10.462)	0.158
Mean age at diagnosis (SD)	60.97 (10.686)	58.57 (10.679)	72.35 (9.364)	----------	62.71 (10.836)	----------	64.61 (10.888)	----------
Sex % Male	*54.4	41.4	*27.0	42.8	52.1	0.022	51.1	0.047
Number of individuals with a family history of glaucoma (%)	*35.7	*46.6	*67.6	0	36.6	< 0.0001	39.5	< 0.0001
Mean C/D ratio (SD)	*0.72 (0.182)	0.36 (0.138)	*0.74 (0.138)	0.21 (0.231)	0.66 (0.221)	< 0.0001	0.7204 (0.177)	< 0.0001
Mean of the highest recorded IOP (SD)	*29.13 (5.506)	*27.16 (4.021)	17.64 (2.090)	15.45 (2.352)	28.79 (5.326)	< 0.0001	27.75 (6.415)	< 0.0001
Number of individuals with glaucoma filtration surgery (%)	25.7	5.2	5.4	0	22.1	< 0.0001	23.3	< 0.0001

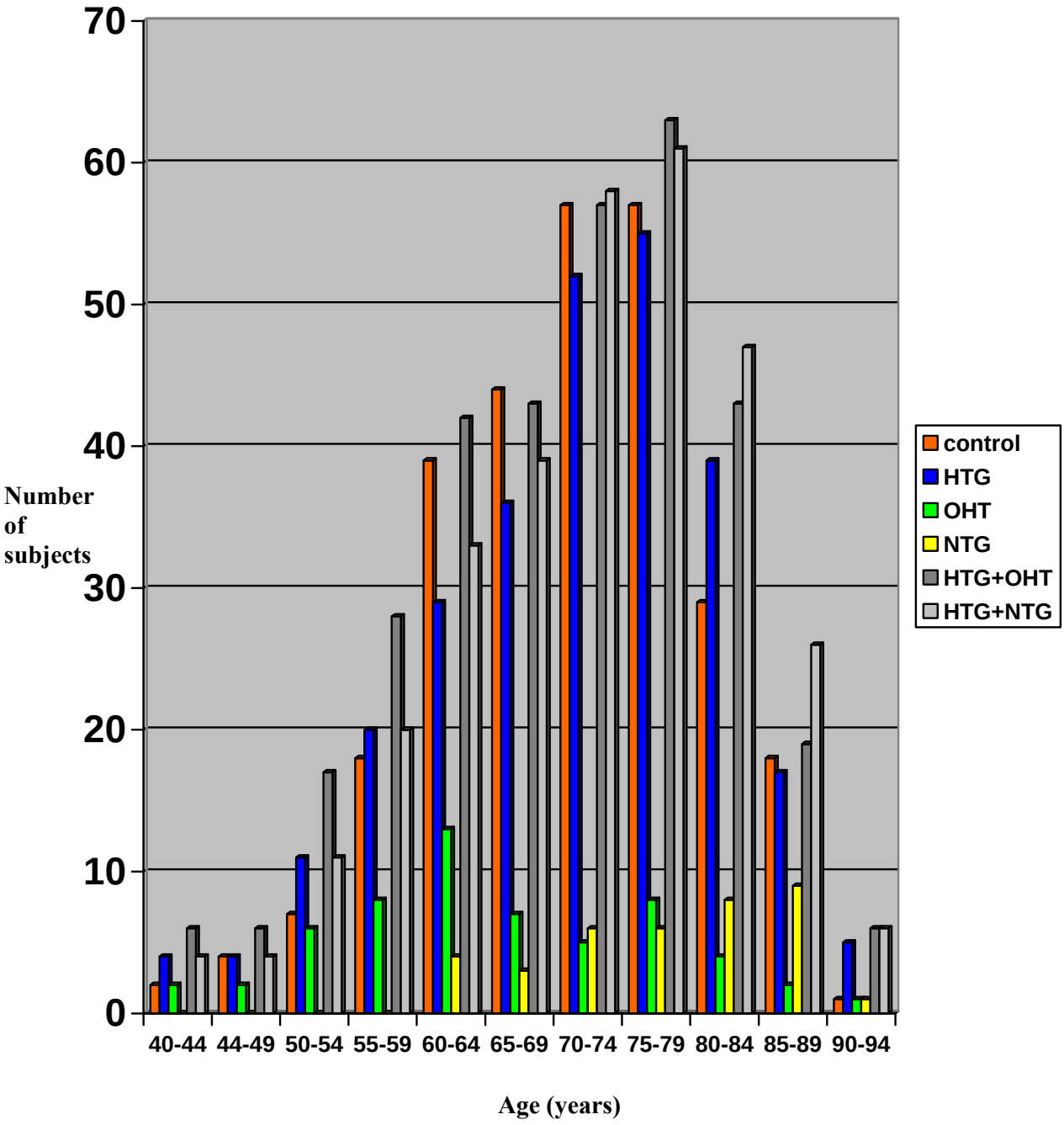


Figure 3–1: Distribution of age among the control group and 5 different case groups; HTG, OHT, NTG, HTG+OHT and HTG+NTG.

The mean age for the NTG group (77.51 years) was the highest among the case groups with the OHT group having the lowest mean age (65.19 years). Each group is coded by different colours.

On the whole, the mean age and the mean age at diagnosis for the NTG group (77.51 and 72.35 years respectively) was the highest among the cases groups, with the OHT group having the lowest mean age and the mean age at diagnosis (65.19 and 58.57 years respectively). All of the case groups have shown a mean age that is well above the age of 40 years (i.e. the age at which it is considered to be adult-onset glaucoma). This made certain that all of the cases were of adult-onset glaucoma in addition to excluding normal controls that may have (as yet undeveloped) glaucoma.

3.2.2 Sex distribution in cases and controls

Within the cases, the percentage of male was as follows (Table 3-1, Figure 3-2):

- 54.4% in the HTG group.
- 41.4% in the OHT group.
- 27% in the NTG group.
- 52.1% in the combined HTG and OHT group.
- 51.1% in the combined HTG and NTG group.

Within the normal control group, 42.8% of individuals were male.

On the whole, the percentage of males was the highest in the HTG group at just above 50%. As expected, the percentage of male was significantly lower in the NTG group (27%) (where the prevalence of female is known to be higher) (Levene, 1980) when compared to the control group. In addition, other case groups (including HTG, HTG+OHT and HTG+NTG) also showed significant difference in sex distribution when compared to the normal controls.

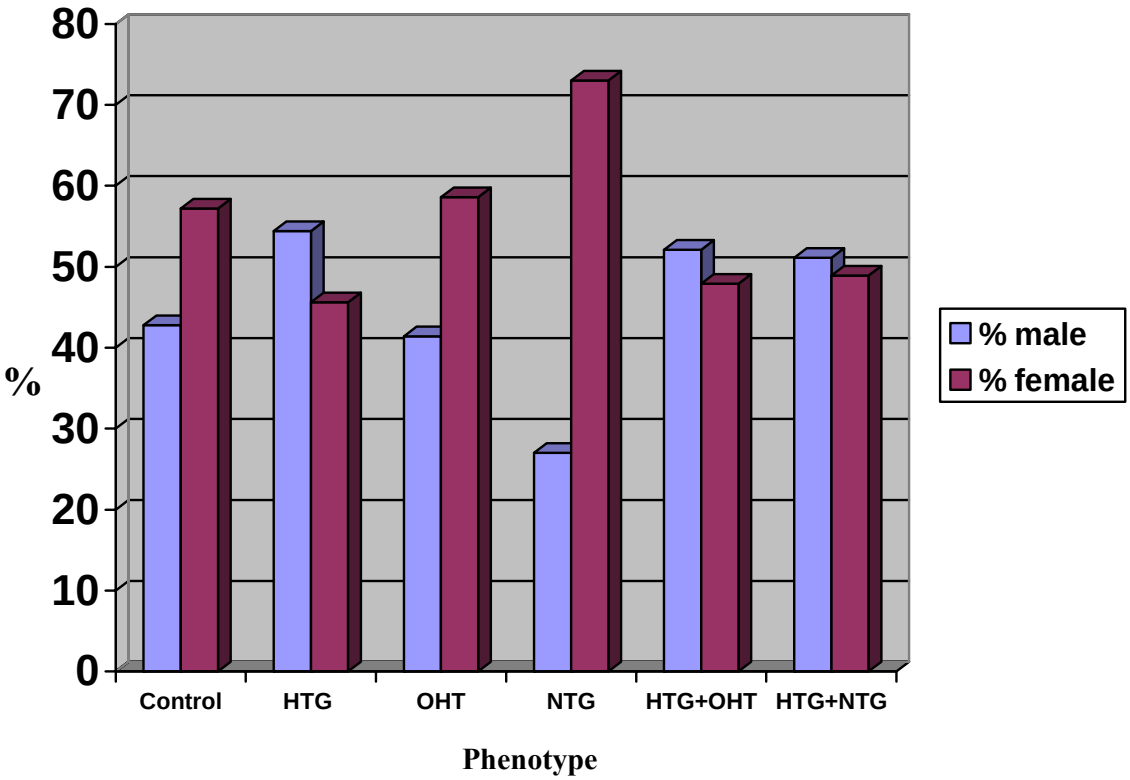


Figure 3–2: Distribution of sex within the normal control group and 5 different case groups; HTG, OHT, NTG, HTG+OHT and HTG+NTG.

The percentage of males was significantly different between the cases groups NTG, HTG, HTG+OHT, HTG+NTG and the control group.

3.2.3 Distribution of individuals with family history of glaucoma

Within the cases, the percentage of individuals with a family history of glaucoma was as follows (Table 3-1, Figure 3-3):

- 35.7% in the HTG group
- 46.6% in the OHT group
- 67.6% in the NTG group
- 37.6% in the HTG+OHT group
- 39.5% in the HTG+NTG group

Control individuals with a family history of glaucoma were excluded from this study.

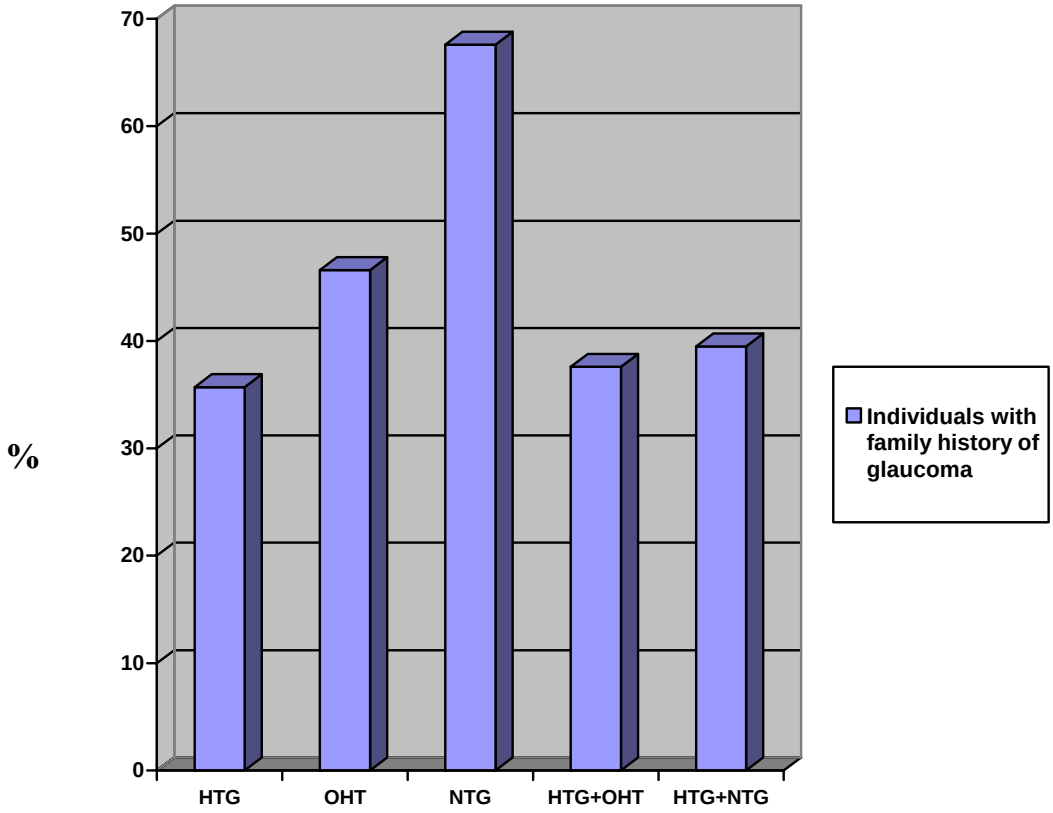


Figure 3–3: The distribution of individuals with a family history of glaucoma among the case groups HTG, OHT, NTG, HTG+OHT and HTG+NTG.

The NTG group had the highest percentage of individuals with a family history of glaucoma at 67.6%, with the HTG group having the lowest percentage at 35.7%.

The NTG group had the highest percentage of individuals with a family history of glaucoma at 67.6% whilst the HTG group had the lowest percentage at 35.7%. When the phenotypic variables of the individuals with a family history of glaucoma was further analysed (Table 3-2), the percentage of females was higher in all groups with a family history of glaucoma when compared with those without a family history of glaucoma, except for the NTG group where there was a higher percentage of male. Only the combined groups HTG+OHT and HTG+HTG showed a significant difference in sex distribution between the individuals with and without a family member of glaucoma. In addition, there was a higher prevalence of glaucoma surgery for all of the case groups (HTG, OHT, NTG, HTG+OHT and HTG+NTG) with a positive family history of glaucoma, when compared with those without a family history of glaucoma. However, these were not significantly different when tested by a χ^2 analysis (Table 3-2).

Table 3–2: Comparison of age at diagnosis, sex, mean CDR and of the highest recorded IOP, and number of individuals with glaucoma surgery between cases with a family history of glaucoma and cases without a family history of glaucoma

* indicates significant difference ($P > 0.05$) in gender between individuals with a family history of glaucoma and individuals without a family history of glaucoma within each case group. *P* values were calculated using independent samples t test, except a χ^2 test was used for sex and family history of glaucoma, using SPSS, version 15 (SPSS Inc., Chicago, IL, USA).

	Family history of glaucoma	Mean age at diagnosis (SD)	Sex % Male	Mean of the highest recorded IOP (SD)	Mean C/D ratio (SD)	Number of individuals with glaucoma filtration surgery (%)
HTG	**No**	63.65 (10.549)	58.3	31.87 (7.055)	0.74 (0.173)	23.4
	Yes	63.49 (10.970)	47.4	30.14 (5.362)	0.74 (0.159)	29.9
OHT	**No**	59.10 (10.594)	48.4	28.81 (3.027)	0.33 (0.113)	0.0
	Yes	57.95 (10.946)	33.3	28.82 (5.114)	0.31 (0.120)	11.0
NTG	**No**	74.58 (9.662)	25.0	16.5 (2.576)	0.75 (0.131)	0.0
	Yes	71.28 (9.221)	28.0	18.28 (19.90)	0.75 (0.112)	8.0
HTG+OHT	**No**	62.96 (10.665)	*56.8	31.26 (6.565)	0.66 (0.227)	19.9
	Yes	62.29 (11.160)	*44.4	29.83 (5.312)	0.64 (0.232)	25.8
HTG+NTG	**No**	64.53 (10.976)	*56.1	30.40 (8.162)	0.74 (0.169)	21.9
	Yes	64.73 (10.794)	*43.4	27.39 (6.940)	0.74 (0.154)	25.4

3.2.4 Distribution of cup-disc ratio in cases and controls

Within the cases, the mean C/D ratio was as follows (Table 3-1, Figure 3-4):

- 0.72 (± SD 0.182, range 0.4 - 1.0) for the HTG group.
- 0.36 (± SD 0.138, range 0.1 – 0.5) for the OHT group.
- 0.74 (± SD 0.138, range 0.5 – 0.9) for the NTG group.
- 0.66 (± SD 0.221, range 0.1 – 1.0) for the HTG+OHT group.
- 0.72 (± SD 0.177, range 0.4 – 1.0) for the HTG+NTG group.

For the control group, the mean C/D ratio was 0.21 (± SD 0.231, range 0.0 – 0.5).

The mean C/D ratio was the lowest among the OHT group (C/D ratio 0.36) and the controls (C/D ratio 0.21), between which the C/D ratio was not significantly distributed. This was as expected since there was absence of glaucomatous optic nerve damage within these two groups. The group with the worst mean C/D ratio was the NTG group (C/D ratio 0.74) followed by the HTG group (C/D ratio 0.72).

3.2.5 IOP distribution in cases and controls

Within the cases, the mean of the highest recorded IOP was as follows (Table 3-1, Figure 3-5):

- 29.13 mmHg (± SD 5.51, range 24 - 65) among the HTG group
- 27.16 mmHg (± SD 4.02, range 24 - 41) among the OHT group
- 17.64 mmHg (± SD 2.09, range 10 - 20) among the NTG group
- 28.79 mmHg (± SD 5.33, range 24 – 65) among the HTG+OHT group
- 27.75 mmHg (± SD 6.42, range 10 - 65) among the HTG+NTG group

For the control group, the mean of the highest recorded IOP was 15.45 mmHg (± SD 2.35, range 9 - 20).

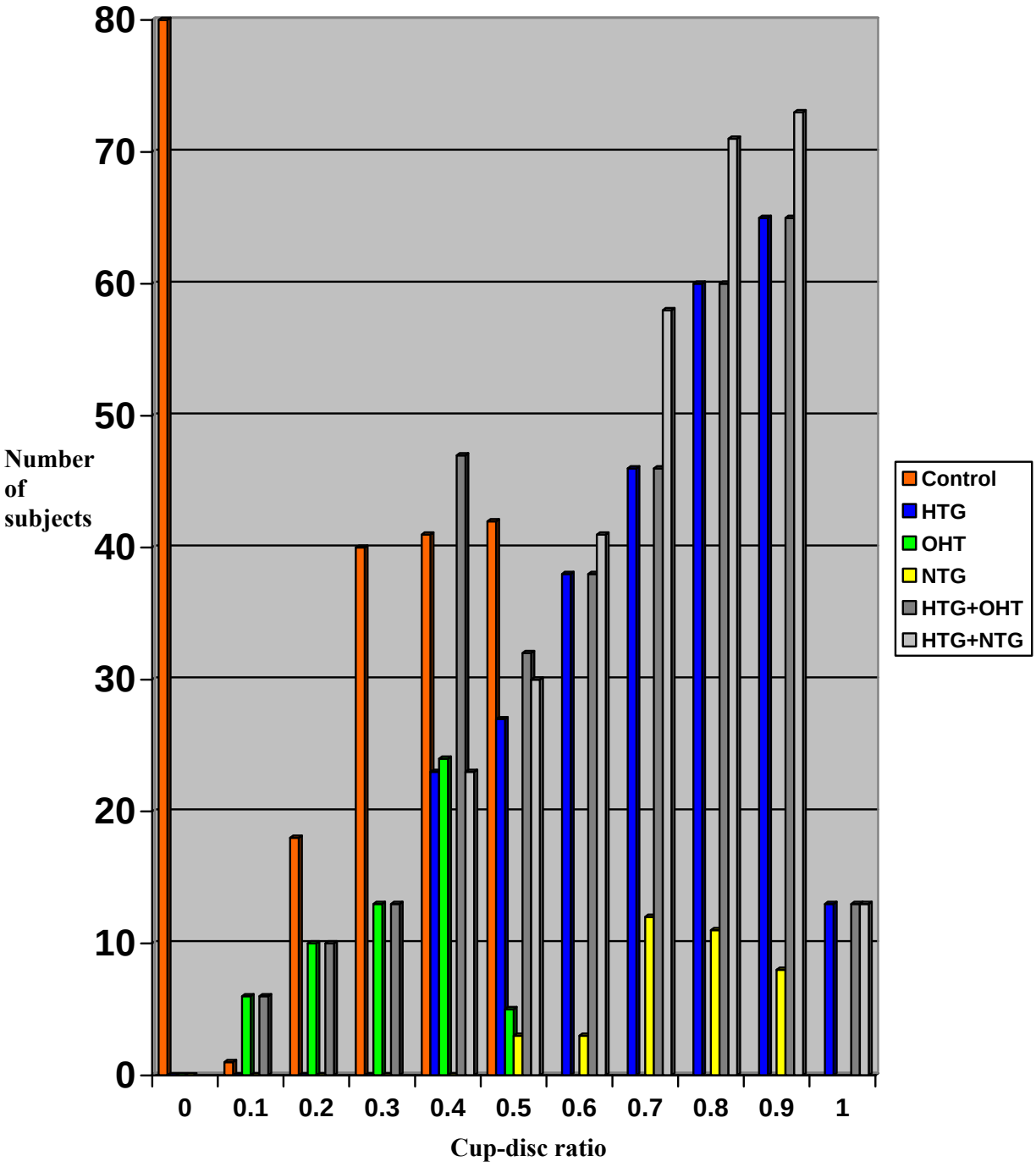


Figure 3–4: Distribution of cup-disc ratio within the control group and 5 different case groups; HTG, OHT, NTG, HTG+OHT and HTG+NTG.

The controls and the OHT subjects are distributed in the lower end of spectrum of the cup-disc ratio, where as the HTG, NTG and HTG+NTG cases are distributed in the higher end of the spectrum. Each group is coded by different colours.

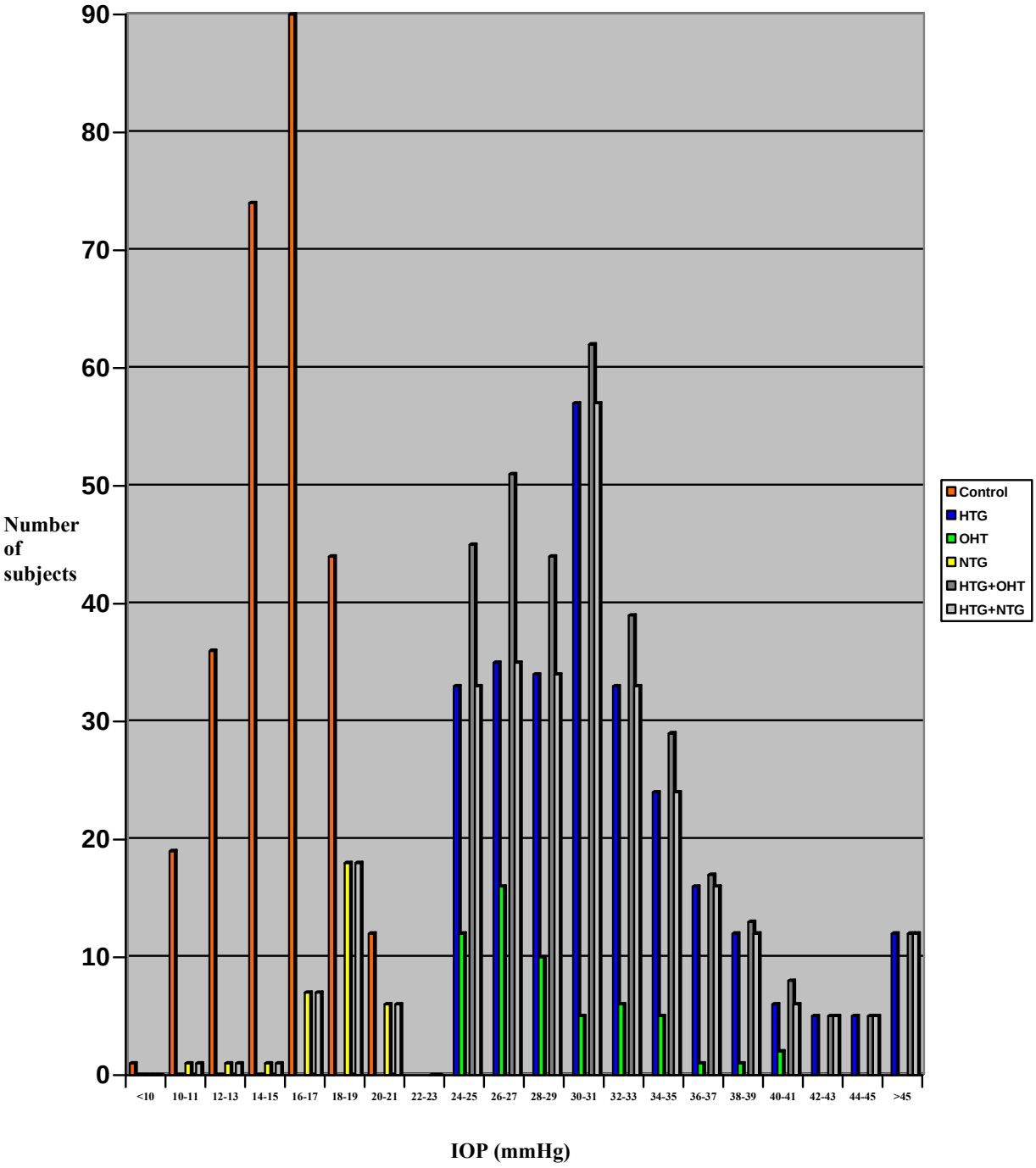


Figure 3–5: Bimodal distribution of IOP of the control group and 5 different case groups; HTG, OHT, NTG, HTG+OHT and HTG+NTG.

The first peak consisted mainly of control individuals and NTG cases and the second peak comprised mainly of HTG, OHT, HTG+OHT and HTG+NTG cases. Each group is coded by different colours.

As expected, the IOP in the normal controls (range 9 – 20 mmHg) and the NTG group (range 10 – 20 mmHg) was consistently within the statistically "normal range" with the mean of the highest recorded IOP for the control group being 15.45 mmHg and for the NTG group 17.64 mmHg. The IOP was not significantly distributed between these two groups. The case group with the highest recorded mean IOP was the HTG group (29.13 mmHg). Figure 3-4 shows a bimodal distribution of the IOP with the first peak consisting mainly of control individuals and NTG cases and the second peak consisting mainly of HTGs, OHTs, HTGs+OHTs and HTGs+NTGs.

3.2.6 Percentage of individuals with glaucoma surgery

The percentage of individuals who had glaucoma filtration surgery among the cases was as follows (Table 3-1, Figure 3-6):

- 25.7% in the HTG group
- 5.2% in the OHT group
- 5.4% in the NTG group
- 22.1% in the HTG+OHT group
- 23.3% in the HTG+NTG group

The case group with the highest percentage of individuals with glaucoma surgery was the HTG group (25.7%), with the OHT group having the lowest percentage of individuals with glaucoma surgery (5.2%).

3.3 Discussion

Overall, there were 643 participants recruited for this study, 367 of whom were unrelated POAG/OHT cases and the remaining 276 of whom were unrelated normal controls. The cases were composed of three homogenous cohorts of HTGs (n = 272), OHTs (n = 58) and NTGs (n = 37). All cases and controls were of white British descent and were ethnically matched.

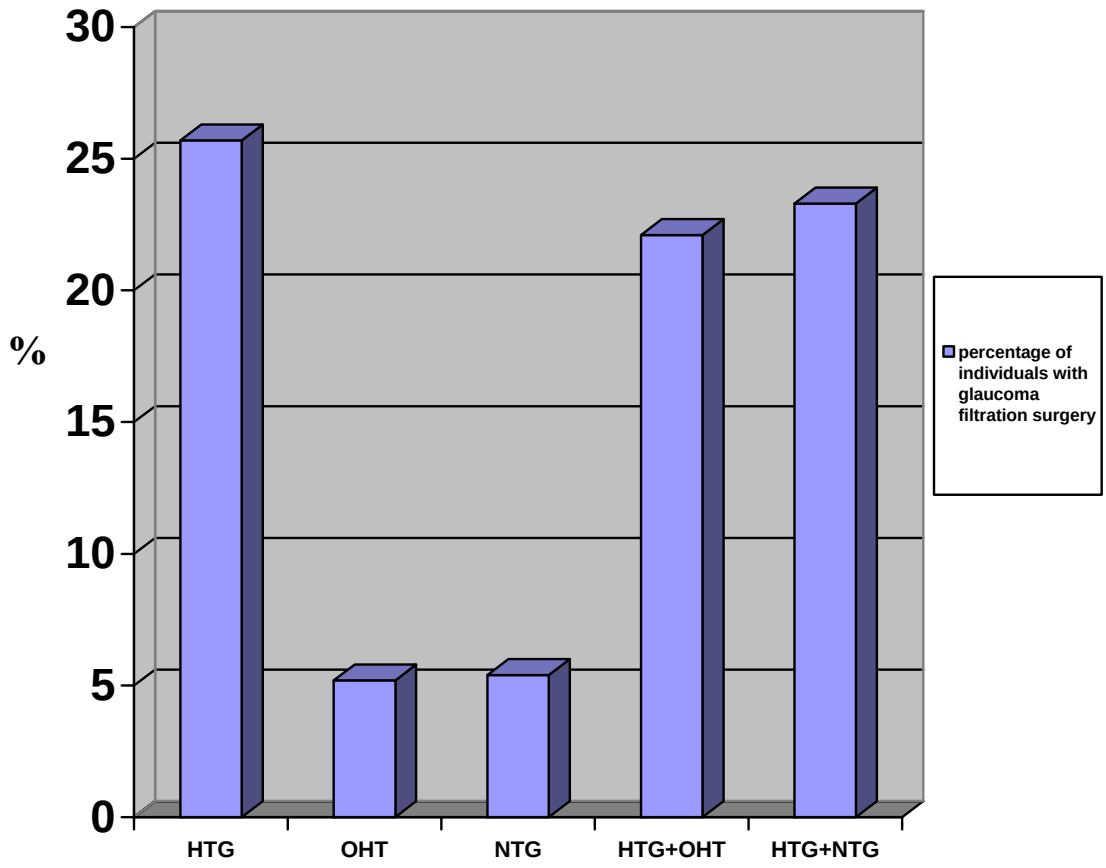


Figure 3–6: The percentage of individuals with glaucoma filtration surgery within the case groups HTG, OHT, NTG, HTG+OHT and HTG+NTG.

The HTG group had the highest percentage of individuals with glaucoma surgery and the OHT group had the lowest percentage individuals with glaucoma surgery.

Six phenotypic variables of the cases and controls were assessed (age, sex, number of individuals with a family history of glaucoma, mean CDR, highest recorded mean IOP and number of cases with glaucoma surgery). In order to ensure that the cases and controls were comparable, age and sex distribution between these groups were examined to see whether they were equally distributed. On examining the mean age between cases and controls, the OHT group had a mean age of 65.19 years, which was significantly lower than the mean age of the controls (70.76 years) ($P = < 0.05$). In addition, when the sex distribution was assessed, the percentage of males was significantly different ($P = < 0.05$) between the HTG, NTG, HTG+OHT, HTG+NTG and the controls. The uneven distribution of age and sex may produce confounding bias and distort any potential association between cases and the controls. Hence, to address this problem, calculations for *P* values in subsequent chapters have adjusted for this when assessing the prevalence of genetic variants between these specific groups.

On assessing the individuals with family history of glaucoma, the case group with the largest percentage of such individuals was the NTG group at 67.6 % compared to the HTG and the OHT group at 35.7% and 46.6% respectively. Despite this being a crude assessment because of the small number of participants representing the NTG group, based on this result, it can be concluded that there may be a larger contribution of genetic factors towards the pathogenesis of NTG within the cohort. This is in line with a case control study involving Caucasian individuals of 175 POAG cases and 175 controls which showed a strong familial component to the development of NTG (Charliat *et al.*, 1994). With this in mind, it would be interesting to assess the prevalence of *OPTN* mutation (a dominant NTG gene within British familial cases and to a lesser extent in sporadic POAG cases) (Alward *et al.*, 2003; Aung *et al.*, 2003; Ayala-Lugo *et al.*, 2007; Hauser *et al.*, 2006; Rezaie *et al.*, 2002), in particular the E50K mutation within the cohort of NTG cases as well as the HTG/OHT cases (see Chapter 4).

In addition, when the phenotypic variables of the group of individuals with a family history of glaucoma were further analysed, a higher prevalence of females were present for all of the case groups except for the NTG group, where the percentage of males were higher. This was significantly different only in the combined groups HTG+OHT and

HTG+NTG. In addition, a higher prevalence of glaucoma surgery was observed for all case groups (albeit not being significantly different) compared to those without a family history of glaucoma. This is in agreement with the Canadian Glaucoma Study (Chauhan *et al.*, 2008) which showed female gender being a risk factor for progression of POAG. However, conflicting results was shown by the AGIS (AGIS Investigators, 2002) in which male gender was a risk factor.

With regards to the distribution of mean IOP within cases and controls, there was a bimodal distribution with the first peak consisting mainly of control individuals and NTG cases and the second peak consisting of HTG, OHT, HTG+OHT and HTG+NTG cases (Figure 3-5). Since HTG and OHT shared the same normal distribution on the upper end of the IOP spectrum, HTG and OHT were combined as one group (see Chapter 5 and 6 for the assessment of IOP as a covariate). In addition, individuals with borderline IOPs (21–23 mmHg) (43 subjects in total) were excluded from this study. This was to ensure that the participants were correctly assigned to the appropriate groups depending on whether the IOP was above the statistically normal upper limit (HTG or OHT) or within the statistically normal range (NTG or controls).

When analysing the percentage of individuals with glaucoma surgery, the case group with the largest percentage of surgery was the HTG group at 25.7% compared to the OHT and the NTG groups at 5.2% and 5.4% respectively. This suggests that the HTG group had the highest percentage of the progressive form of POAG.

Other phenotypic characteristics that could have been analysed in order to provide a more comprehensive assessment of the study group would be an objective measurement of the degree of visual field loss using global indices (mean deviation, pattern standard deviation, corrected pattern standard deviation), recording of risk factors associated with POAG (e.g. CCT, systolic blood pressure, cardiovascular history, refractive status) as well as clinical features associated with POAG (e.g. migraine and NTG).

In summary, a systematic assessment of the phenotypic variables of the study group has been made.

Chapter 4 The role of *OPTN* E50K mutation in unrelated cases of OHT and POAG

4.1 Introduction

Here, the contribution of *OPTN* E50K mutation to unrelated NTG cases is investigated. In addition, given the phenotypic variability of E50K mutation with some affected individuals having HTG whilst others having NTG (Rezaie *et al.*, 2002), the role of E50K mutation in unrelated HTG and OHT cases is also assessed. Specifically, the prevalence of E50K mutation is examined in unrelated glaucoma patients comprised of HTG (n = 272), OHT (n = 58), NTG (n = 37) and a normal control group of white British origin (n = 276), using TaqMan SNP genotyping assays. The results are then validated using cycle sequencing reaction by Sanger's dideoxy Terminator Method.

OPTN E50K mutation has not been previously identified in unrelated HTG and OHT case within the general POAG population, and after detailed analysis, this report confirms the absence of the E50K mutation in OHT subjects (a major risk factor for HTG), HTG individuals, in addition to NTG individuals.

4.2 Results

4.2.1 Assessment of prevalence of *OPTN* E50K mutation in unrelated OHT/POAG individuals

4.2.1.1 Using TaqMan SNP genotyping assay

Overall, when analysing the results of the automated allelic discrimination plot of the TaqMan assay (Figure 4-1), the E50K mutation was identified in one out of 37 NTG individuals (patient ID number 522p) (2.7%), and none in the control group. In addition, this mutation was found in 3 out of 272 HTG subjects (patient ID numbers 432p, 520p, 525p) (1.1%) as well as among 1 out of 58 OHT individuals (patient ID number 527p) (1.7%). However, given that *OPTN* E50K mutation is a rare allele (Ariani *et al.*, 2006; Baird *et al.*, 2004; Funayama *et al.*, 2004; Fuse *et al.*, 2004; Leung *et al.*, 2003; Lopez-Martinez *et al.*, 2007; Sripriya *et al.*, 2006; Tang *et al.*, 2003; Toda *et al.*, 2004; Umeda

et al., 2004; Wiggs *et al.*, 2003) and that there may be a tendency for the allelic discrimination plot to miscall genotypes (Callegaro *et al.*, 2006), the amplification plot of the individual wells were further analysed despite the cluster plot showing distinct clusters (Figure 4-1). And surprisingly, there was an absence of the E50K mutation among the five individuals (Figure 4-2., 4-3, 4-4, 4-5 and 4-6) who were suspected to have the mutation as shown by the cluster plot.

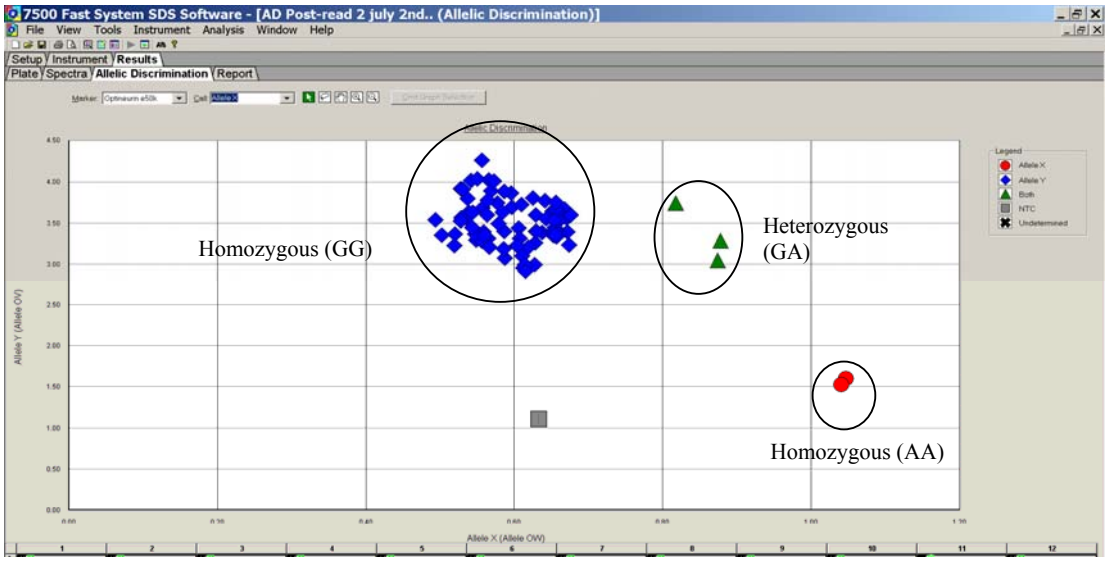


Figure 4–1: A cluster plot from a Fast 7500 Real-Time PCR using TaqMan SNP genotyping assay showing allelic discrimination of the SNP rs28939688 responsible for *OPTN* E50K mutation.

There are 2 signals for AA homozygous (2 copies of mutant allele), 2 signals for GA heterozygous (1 copy of mutant A allele) with the majority being GG homozygous (absence of E50K mutation). Note the signal for the "no template" control which is indicated by a grey box.

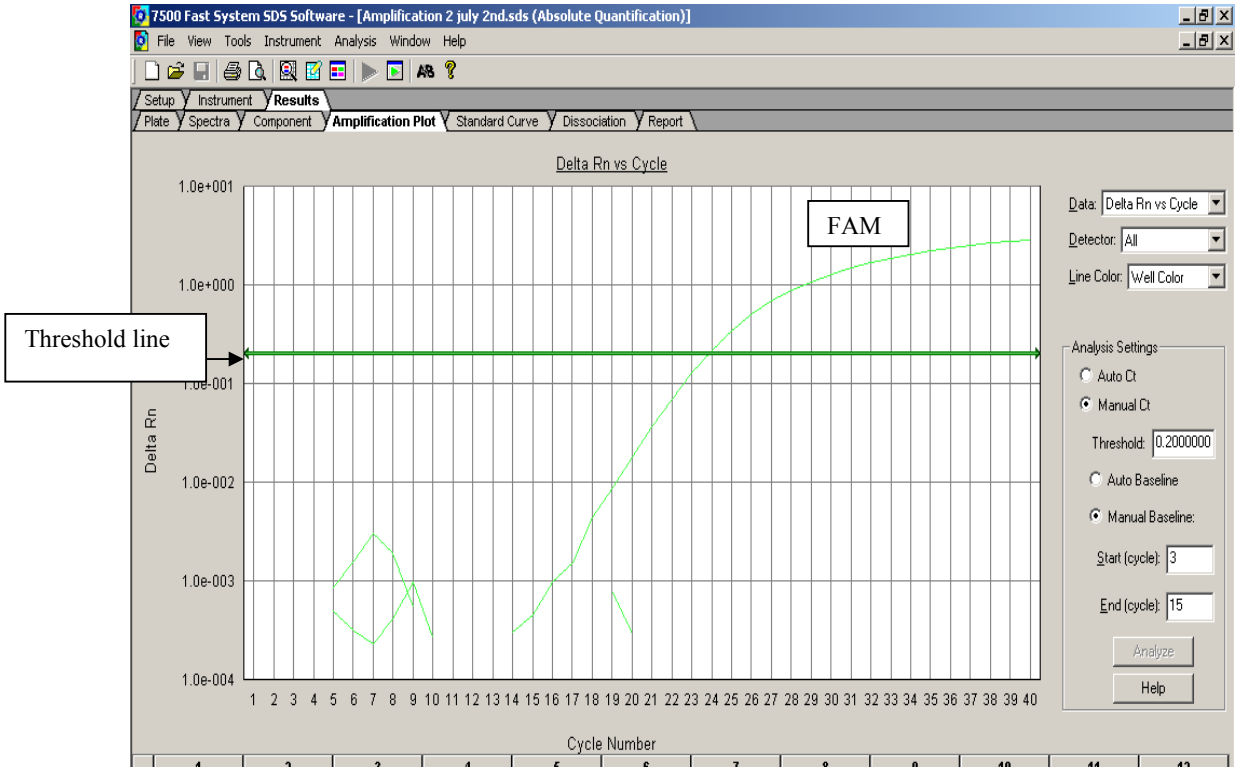


Figure 4–2: Amplification plots of delta Rn versus cycle number of an individual with NTG (patient ID number 522p) showing an increase in FAM fluorescence only and absence of VIC fluorescence.

When the fluorescent reporter signal increases to a detectable level, it is captured and displayed as an amplification plot as shown here. As the reporter dye FAM represents the wild type allele G and the VIC dye the mutant allele A, the amplification plot which shows an increase in FAM fluorescence only hence shows a GG genotype. Rn is the normalized reporter signal, where as delta Rn is defined as the magnitude of the fluorescence signal generated during the PCR at each time point. The threshold line is the point at which the level of fluorescence is above the background intensity

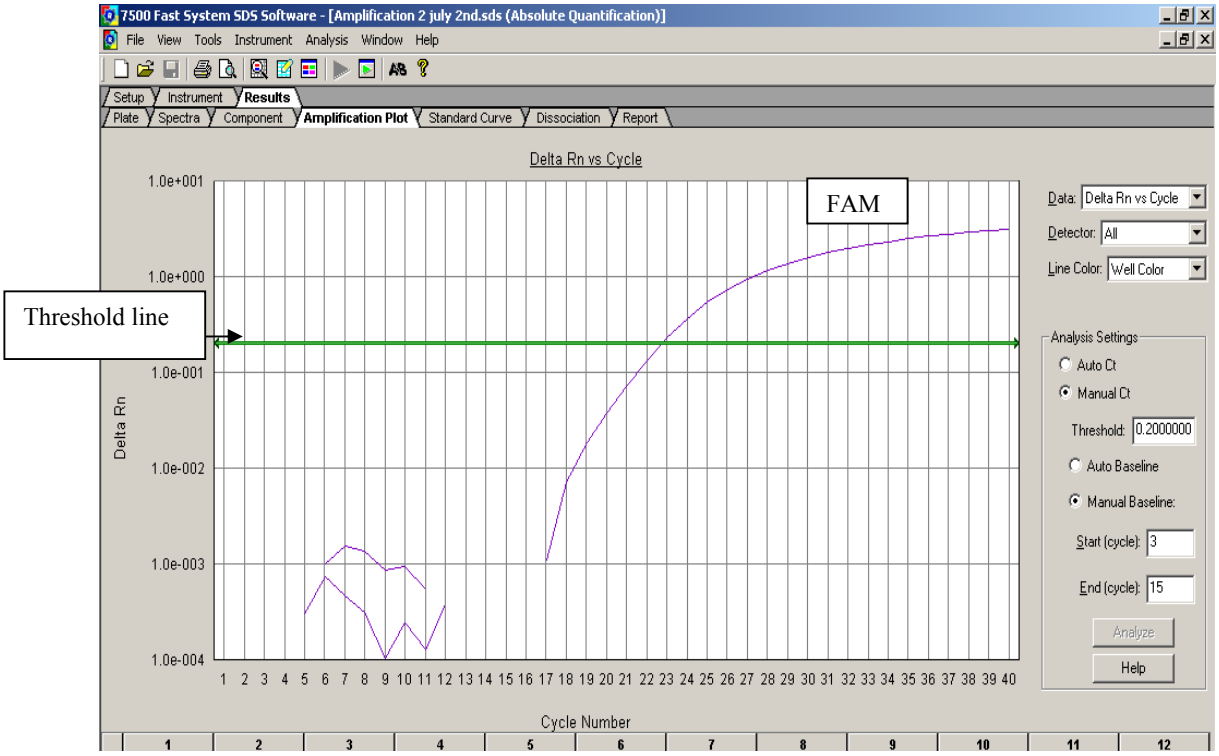


Figure 4–3: Amplification plots of delta Rn versus cycle number of an individual with HTG (patient ID number 432p) showing an increase in FAM fluorescence and absence of VIC fluorescence.

As the reporter dye FAM represents the wild type allele G and the VIC dye the mutant allele A, the component plot shows a GG genotype. Rn is the normalized reporter signal, where as delta Rn is defined as the magnitude of the fluorescence signal generated during the PCR at each time point. The threshold line is the point at which the level of fluorescence is above the background intensity.

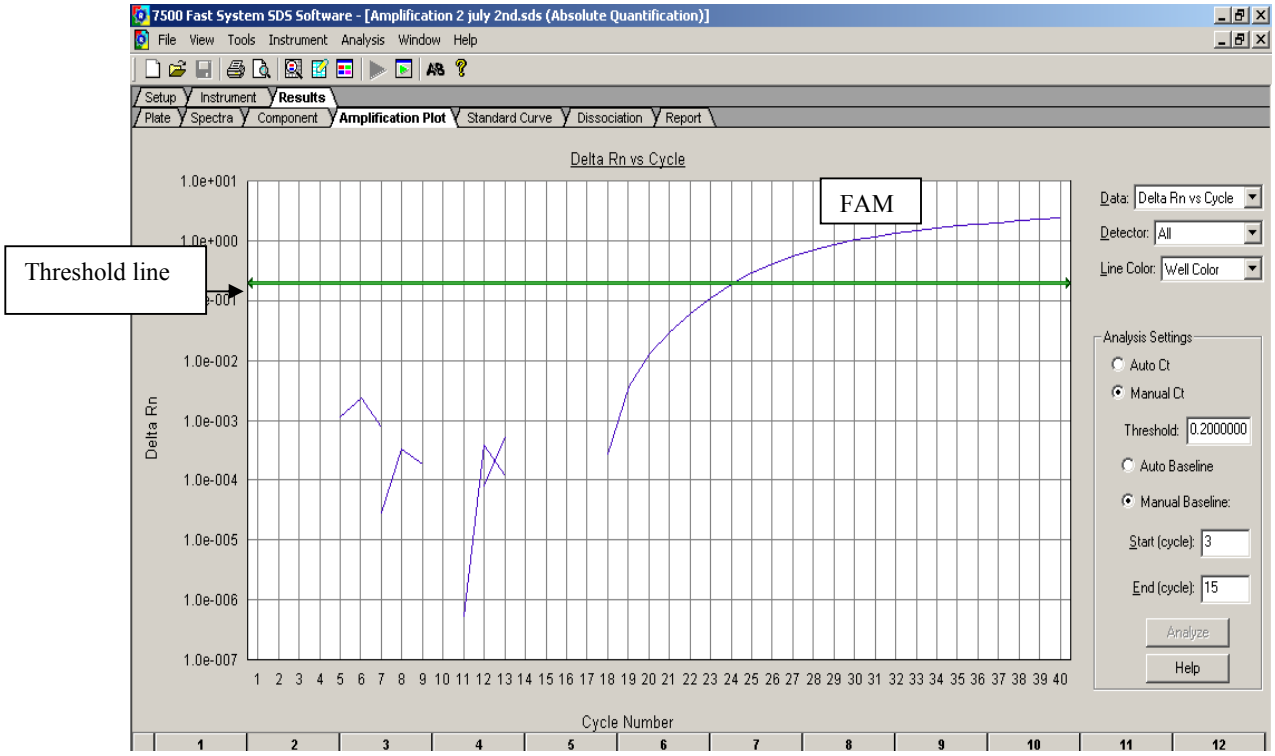


Figure 4–4: Component plots of delta Rn versus cycle number of an individual with HTG (patient ID number 520p) showing an increase in FAM fluorescence and absence of VIC fluorescence.

As the reporter dye FAM represents the wild type allele G and the VIC dye the mutant allele A, the component plot shows a GG genotype. Rn is the normalized reporter signal, where as delta Rn is defined as the magnitude of the fluorescence signal generated during the PCR at each time point. The threshold line is the point at which the level of fluorescence is above the background intensity.

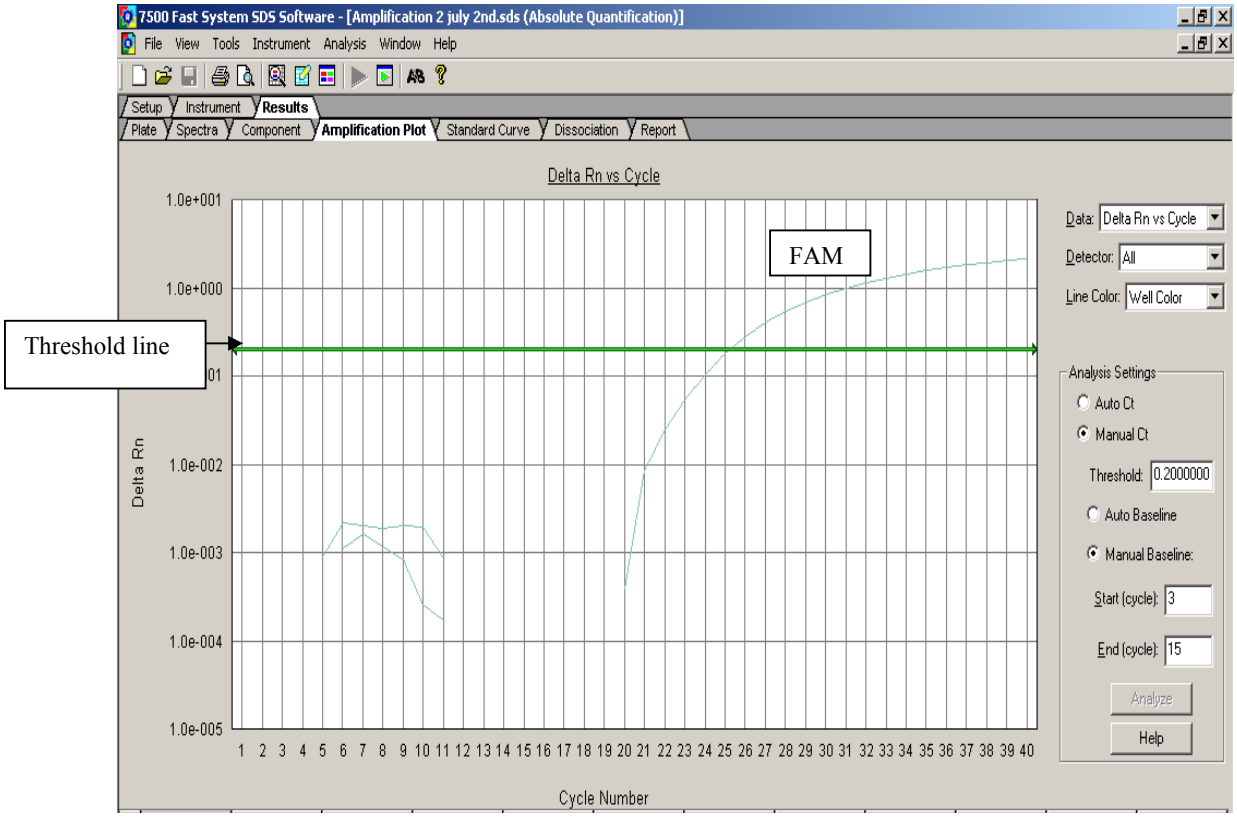


Figure 4–5: Component plots of delta Rn versus cycle number of an individual with HTG (patient ID number 525p) showing an increase in FAM fluorescence and absence of VIC fluorescence.

As the reporter dye FAM represents the wild type allele G and the VIC dye the mutant allele A, the component plot shows a GG genotype. Rn is the normalized reporter signal, where as delta Rn is defined as the magnitude of the fluorescence signal generated during the PCR at each time point. The threshold line is the point at which the level of fluorescence is above the background intensity.

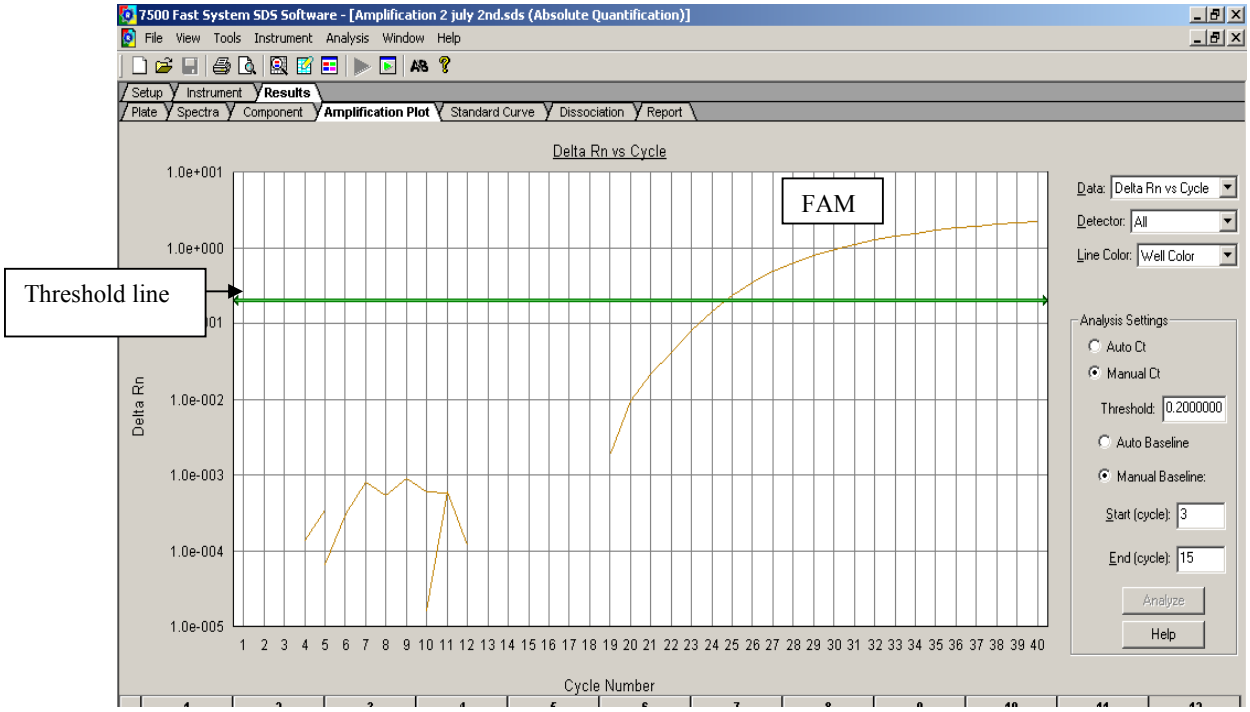


Figure 4–6: Component plots of delta Rn versus cycle number of an individual with OHT (patient ID number 527p) showing an increase in FAM fluorescence and absence of VIC fluorescence.

As the reporter dye FAM represents the wild type allele G and the VIC dye the mutant allele A, the component plot shows a GG genotype. Rn is the normalized reporter signal, where as delta Rn is defined as the magnitude of the fluorescence signal generated during the PCR at each time point. The threshold line is the point at which the level of fluorescence is above the background intensity.

4.2.1.2 Using Sanger dideoxy Terminator Method

To validate the genotyping results obtained using TaqMan SNP assay by automated cycle sequencing, the genomic DNA of the five individuals plus a further 20 controls samples were sequenced.

4.2.1.2.1 Gel electrophoresis of PCR products for cycle sequencing

Using the annealing temperature as 57 ºC and the DNAs from 25 individuals [5 cases (patient ID number 522p, 432p, 520p, 525p, 527p) and 20 controls (patient ID number 361, 362, 363, 402, 404_VB, 405, 408, 409, 442, 445, 446, 450, 451, 452, 455, 456, 457, 462, 465, 466)], in addition to 1 known genomic DNA and 2 no template DNA controls, good PCR products were obtained as shown in Figure 4-7 and 4-8.

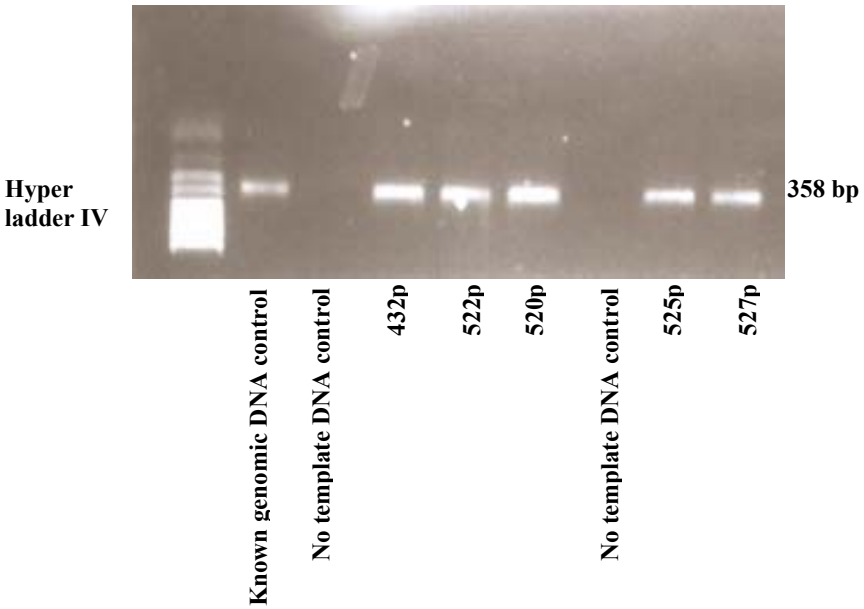


Figure 4–7: Gel electrophoresis of PCR products of genomic DNA from 5 patients with the *OPTN* E50K mutation (patient ID number 432p, 522p, 520p, 525p and 527p), as well as from 1 known genomic DNA and 2 no template DNA controls.

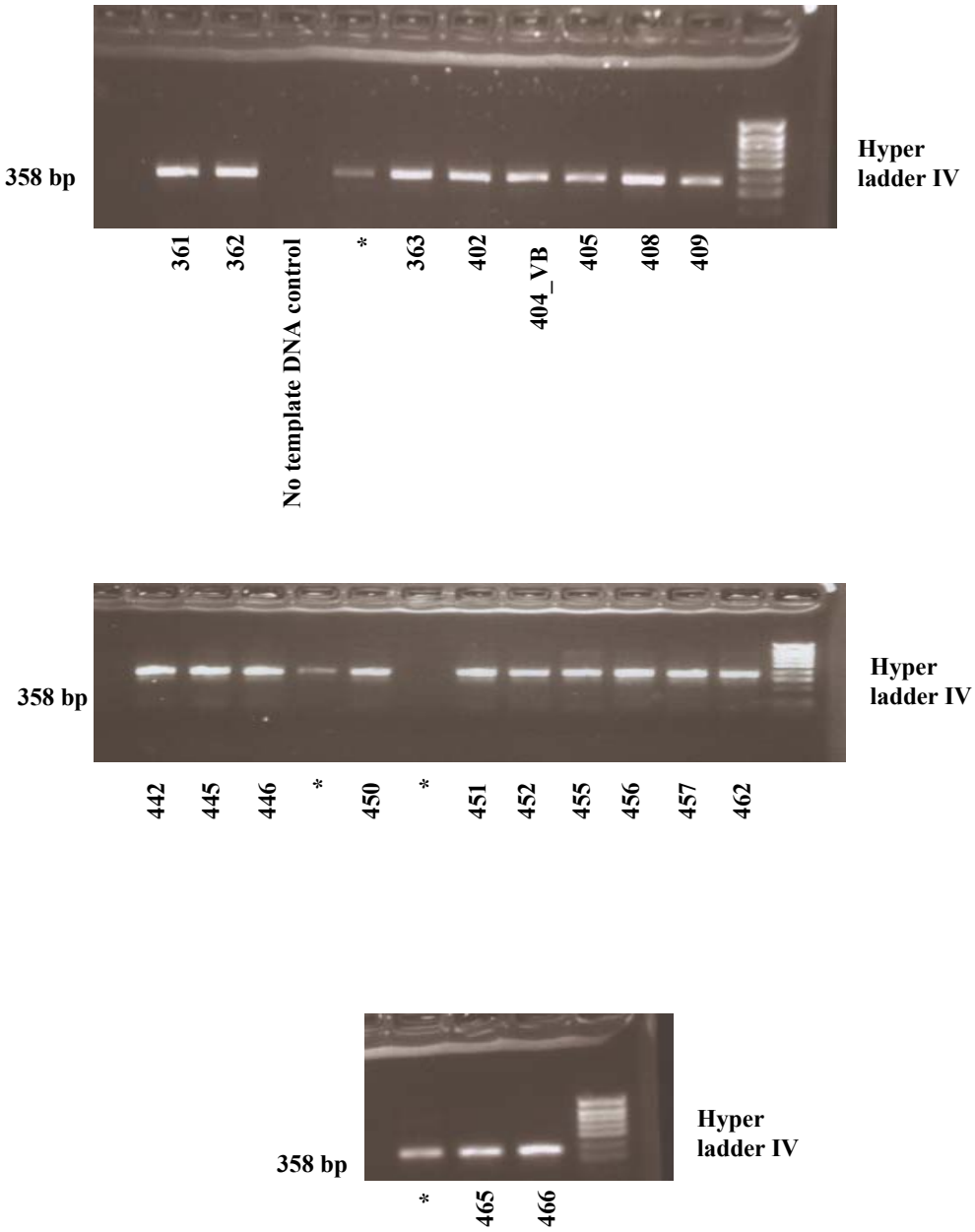


Figure 4–8: Three gel electrophoresis of PCR products of genomic DNA from 20 control individuals (patient ID number 361, 362, 363, 402, 404_VB, 405, 408, 409, 442, 445, 446, 450, 451, 452, 455, 456, 457, 462, 465, 466).

Lanes labeled with * were excluded from subsequent cycle sequencing due to poor PCR product.

4.2.1.2.2 Cycle sequencing

All five individuals were confirmed to have the absence of the mutant A allele and were all GG homozygous (Figure 4-9). A further 20 control DNA samples were sequenced and confirmed the absence of the E50K mutation.

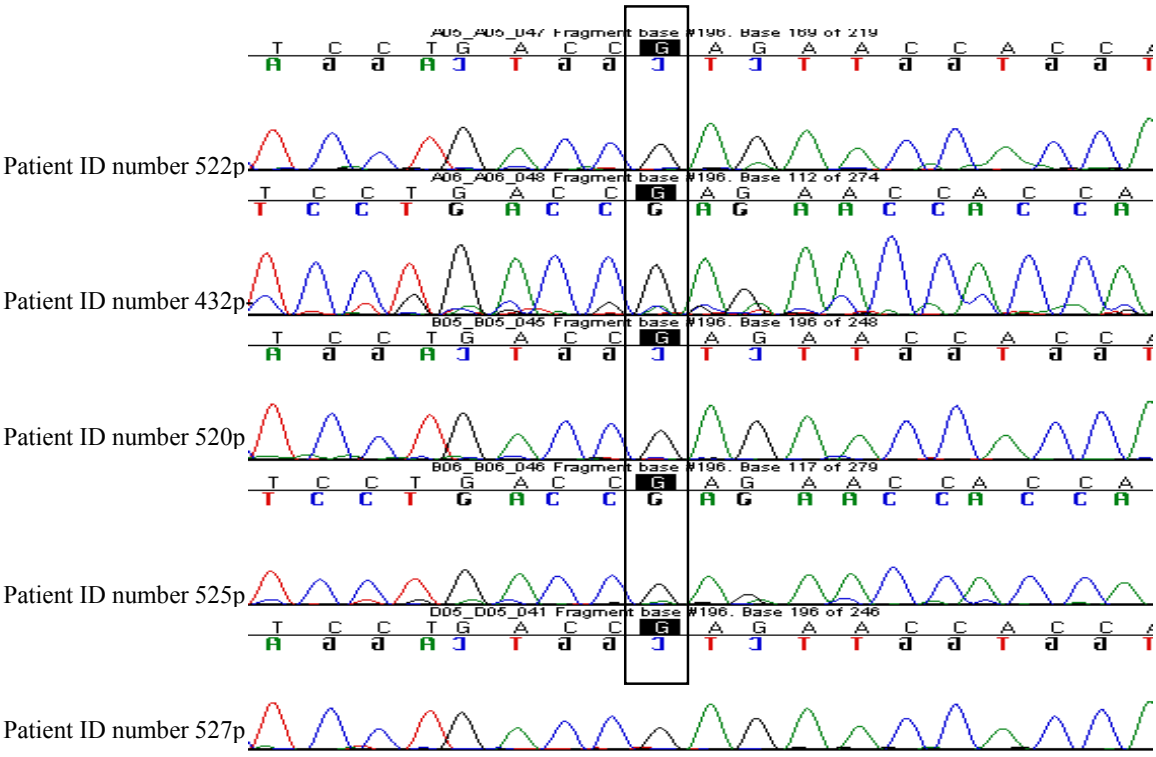


Figure 4–9: Electropherogram of 5 individuals (patient ID number 522p, 432p, 520p, 525p, 527p) showing absence of the mutant A allele.

These data confirmed the discrepancy between the results shown by the automated allelic discrimination plot (IQ software; Bio-Rad Laboratories, Hercules, CA) and the individual spectral contribution of the reporter dyes of the TaqMan assay. Hence, in conclusion, there is absence of E50K mutation among the recruited cohort.

4.3 Discussion

OPTN, the second POAG gene, was first identified by Rezaie *et al* (Rezaie *et al.*, 2002) who discovered that variations in *OPTN* were associated with NTG. The most frequent *OPTN* mutation, Glu50→Lys (E50K) was present in 7 out of 52 families (prevalence rate of 13.5%) with dominantly inherited POAG (Rezaie *et al.*, 2002). The E50K mutation is caused by a c.458G>A transition, so normal individuals without this mutation would be homozygous (GG) at this position, and affected individuals with this mutation would be either heterozygous (GA) or homozygous (AA). More specifically, this mutation converts the DNA codon for glutamic acid (GAA) into that for lysine (AAA). Within the 7 affected families, 16 out of the 124 members were asymptomatic carriers of the E50K mutation. When this prevalence is compared to a smaller percentage of 2-4% of sporadic POAG patients carrying mutations in MYOC gene (Fingert *et al.*, 1999), *OPTN* appeared to be a promising gene, making it a considerable risk associated genetic susceptibility factor for glaucoma. However, in subsequent studies when the assessment of *OPTN* mutation among sporadic NTG was carried out, a much smaller prevalence of E50K mutation was observed, and only in specific ethnic groups (Table 1-6 in Chapter 1). Prevalence rates varied from 1.5% from a UK study involving 2 British individuals out of 132 NTG cases (Aung *et al.*, 2003), 1.5% (1 out of 67 NTG cases) (Hauser *et al.*, 2006) and 0.4% (1 out of 276 NTG cases) (Alward *et al.*, 2003) from 2 separate US studies, each involving a single patient with a family history of glaucoma, and 3.0% from a US study involving a single Caucasian Hispanic individual (1 out of 33 NTG cases) (Ayala-Lugo *et al.*, 2007). Interestingly, the E50K mutation has not been identified among NTG individuals of Japanese origin where the prevalence of NTG is higher (Funayama *et al.*, 2004; Fuse *et al.*, 2004; Tang *et al.*, 2003; Toda *et al.*, 2004; Umeda *et al.*, 2004). The high prevalence of the E50K mutation from the original study (approximately 10 fold higher) was thought to be due to the enrichment of the mutation within the autosomal dominant NTG pedigrees.

Furthermore, there has been absence of correlation between E50K mutation and HTG (Table 1-7 in Chapter 1) (Ariani *et al.*, 2006; Baird *et al.*, 2004; Funayama *et al.*, 2004; Fuse *et al.*, 2004; Leung *et al.*, 2003; Lopez-Martinez *et al.*, 2007; Sripriya *et al.*, 2006; Tang *et al.*, 2003; Toda *et al.*, 2004; Umeda *et al.*, 2004; Wiggs *et al.*, 2003). In line with the results of these studies, this report has confirmed the absence of E50K mutation among unrelated NTG cases, in addition to HTG and OHT individuals. More specifically, the miscalling of the genotype by the automated allelic discrimination software which produced spurious results came into light through detailed assessment of the amplification plot and the absence of the E50K mutation was consolidated through sequencing.

An important requirement of a SNP genotyping method is to be able to distinguish clearly between the allelic variants of homozygous and heterozygous forms. The TaqMan assay which was originally designed for quantitative real time PCR, is a homogenous assay i.e. a single-phase assay without separation steps, allowing continual monitoring during amplification. The TaqMan SNP genotyping assay utilizes the 5'-exonuclease activity of AmpliTaq Gold DNA polymerase to cleave the MGB probe that is hybridized to their target sequence. Cleavage separates a reporter dye (either VIC for allele 1 or FAM for allele 2) at the 5' end from the 3' quencher, leading to a detectable fluorescent signal. Allelic discrimination is determined by the relative amount of intensities of the two fluorescent probes at the end of amplification. Thus, instead of using the full set of real-time PCR data as in quantitative studies, only the end-point data are used. The results of the assay are then automatically determined by a genotyping software provided with real time PCR (e.g. Sequence Detection Software of Applied Biosystems). As this program utilises autoscaling for generating the allelic discrimination plot, caution should be taken when analyzing data for rare alleles as it may produce spurious genotypes (Callegaro *et al.*, 2006). For example, when minor alleles are absent in the run, the software can miscall genotypes either as heterozygous or homozygous. When considering the results of the TaqMan assay in this study, it can be thus concluded that the SNP responsible for the E50K mutation (rs28938688) is indeed rare and absent among the recruited white British cohort. This is in line with the HapMap database where the minor allele frequency of rs28938688 is still unknown. As a result of its infrequent prevalence, it has resulted in the miscall of the genotypes and thus generating the spurious results indicating the presence of E50K mutation among

POAG cases when in fact there is no mutation as shown by the individual amplification plot.

There is evidence to suggest that *OPTN* may play a neuroprotective role by reducing susceptibility of RGC to apoptosis. The E50K mutation is located within a putative leucine zipper-containing bZIP motif, which is conserved in the mouse, bovine and macaque genomes and is thought to have a dominant-negative effect (Rezaie *et al.*, 2002). The bZIP motif is a transcription factor domain that is normally involved in DNA binding and protein dimerization. One identified mutation in *OPTN* is a 2 base pair "AG" insertion that truncates the protein by 76% and may lead to loss of function or haploinsuffiency (Rezaie *et al.*, 2002). By contrast, Chalasani *et al* has shown that proteins with the E50K mutation acquired the ability to selectively induce cell death in retinal ganglion cells which was mediated by oxidative stress (Chalasani *et al.*, 2007). *OPTN* interacts with several proteins, including Rab8 which is a small GTPase that has been implicated in various processes, including protein trafficking (Hattula and Peranen, 2000). De Marco *et al* (2006) have shown that in response to apoptotic stimuli through high oxidative stress, OPTN (a cytoplasmic protein) translocates from the Golgi apparatus to the nucleus in a Rab8-dependent manner. Overexpression of *OPTN* blocks cytochrome c release from mitochondria and protects cells from hydrogen peroxide-induced cell death (Figure 4-10). The *OPTN* E50K mutation inhibits translocation to the nucleus. The Rab8 interaction domain (amino acids 58-209) is situated in the vicinity of the E50K mutation. It is speculated that a conformational change induced by E50K mutation may alter its interaction with Rab8 and is hence unable to translocate to the nucleus to provide protection from oxidative stress (De *et al.*, 2006). Whether the loss of interaction with Rab8 is responsible for RGC death is still to be proven.

In studies that have examined the cellular role of *OPTN*, Zhu *et al* showed that *OPTN* negatively regulated TNFα-induced NF-κB activation (Figure 4-11A) (Zhu *et al.*, 2007). TNFα- stimulated NFκB-dependent gene transcription was greatly enhanced when the level of *OPTN* expression was reduced, which in turn lowered the apoptotic threshold. Other studies have revealed that *OPTN* E50K mutation increased its binding to TANK-binding kinase 1(TBK1), which formed a complex that regulated TNFα and its pro-apoptotic effects (Morton *et al.*, 2008). It has been suggested that the *OPTN* E50K

mutant may cause aberrant activation of TBK1 which may be the cause of the increased susceptibility to ganglion cell loss in subjects with familial NTG.

Hence, the exact function of *OPTN* in relation to glaucoma pathogenesis is still unclear, but the emerging picture is that OPTN protein interacts with the TNF*α* signalling pathway and that it may regulate cell death (Chen *et al.*, 1998; Li *et al.*, 1998) by interacting with proteins involved in apoptosis as well as by inducing TNFα. TNFα increases the severity of damage of optic nerve heads of POAG individuals (Danson, 1988; Haramaki *et al.*, 1997). Hence, it is postulated that the wild-type optineurin may act via the TNFα pathway and provide a neuroprotective role in the optic nerve, and when defective, causes glaucomatous optic neuropathy. Furthermore, sustained elevation of IOP has been shown to result in an upregulation of *OPTN* expression (Vittitow and Borras, 2002) in human TM maintained in organ culture and it is speculated whether *OPTN* may provide a protective effect on TM during raised IOP by possibly influencing ECM remodeling and facilitating aqueous humor outflow. In another study where the relationship between *OPTN* and *MYOC* was assessed, *OPTN* overexpression upregulated endogenous MYOC (which is responsible for 4% of HTG) in human TM cells (Park *et al.*, 2007) by prolonging the turnover rate of MYOC mRNA. Taken together, these reports suggest that *OPTN* may provide a defence mechanism within TM against raised IOP by facilitating aqueous outflow in the anterior segment (and possibly through exerting a regulatory role on MYOC expression) (Figure 11B), in addition to a neuroprotective role in the optic nerve. *OPTN* may therefore have a potential role in the underlying genetic cause of HTG and OHT, in addition to NTG. Thus, taking all the evidence into consideration, *OPTN* appears to be a strong candidate for glaucoma susceptibility. However, in this study, there was an absence of the E50K mutation among NTG, HTG and OHT cases.

In conclusion, this study confirms the absence of E50K mutation among unrelated POAG individuals from the North-East of England.

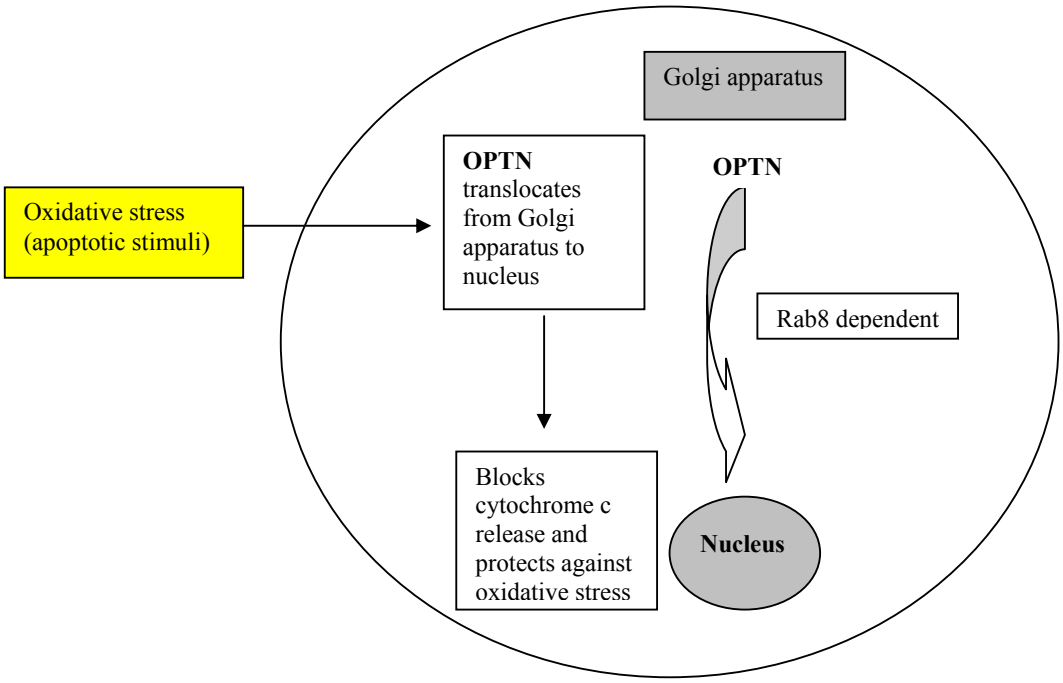
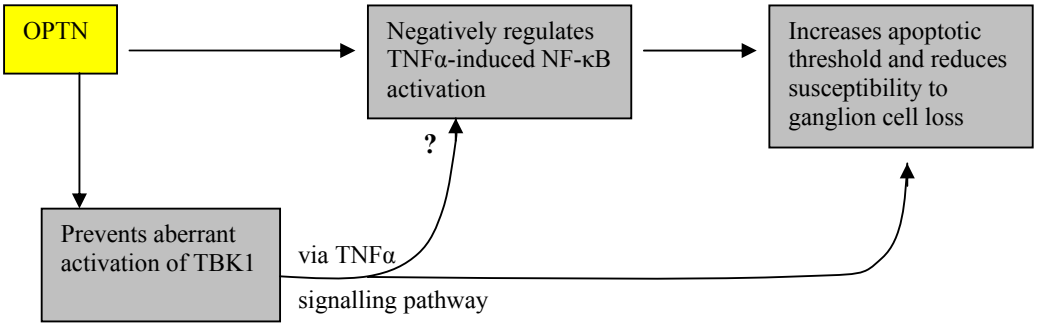


Figure 4–10: Overview of OPTN response to oxidative stress.

In response to oxidative stress OPTN translocates from Golgi apparatus to the nucleus in a Rab8-dependent manner and prevents release of cytochrome c from mitochondria. In doing so, it protects cells from hydrogen-peroxide induced cell death (De *et al.*, 2006).

A. Posterior segment



B. Anterior segment

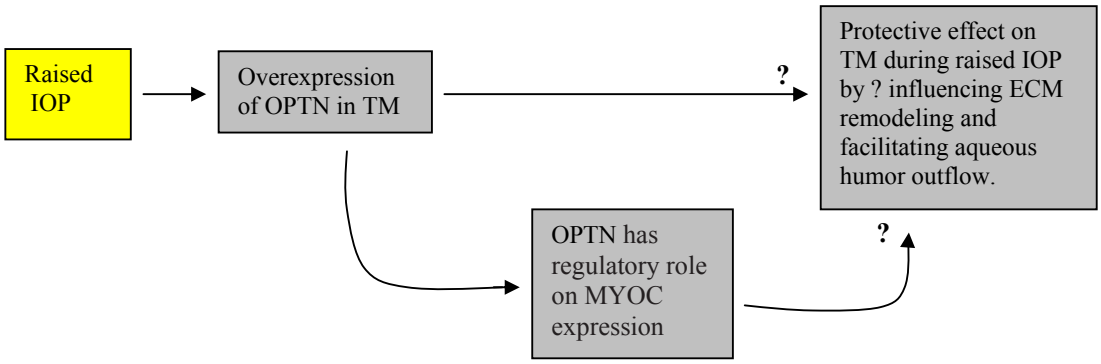


Figure 4–11: Summary of the potential cellular role of OPTN in the posterior segment (A) and in the anterior segment (B).

(A) *OPTN* negatively regulates TNFα-induced NF-κB activation and prevents its pro-apoptotic effects. *OPTN* also prevents aberrant activation of TBK1, which in turn regulates TNFα and its pro-apoptotic effects (B) Sustained elevation of IOP results in an upregulation of *OPTN* expression in human TM maintained in organ culture. It is speculated whether *OPTN* may provide a protective effect on the TM during raised IOP by possibly influencing ECM remodeling and facilitating aqueous humor outflow and this may involve exerting a regulatory role on *MYOC* expression.

Chapter 5 The role of transcription factor gene *LMX1B* in OHT and POAG

5.1 Introduction

This chapter investigates whether variant alleles of *LMX1B* play a role in POAG susceptibility in the general population.

SNP association studies, in particular, genome wide association methods, provide a powerful tool for identifying genes for common diseases and complex traits (Hirschhorn and Daly, 2005) as evidenced by the mapping of genes for age-related macular degeneration (Edwards *et al.*, 2005), myocardial infarction (Ozaki *et al.*, 2002), and breast cancer (Kammerer *et al.*, 2005). This has been mainly due to the advent of LD maps of SNPs across the whole genome and the development of high-throughput technologies for SNP genotyping. Association studies are particularly valuable as they should be able to detect significant variations across wide regions of the genomes e.g. non-coding changes, or within gene regulatory sequence, that may alter levels of functional protein and cause disease. These types of disease-associated alleles may be identified by SNP association studies but would not be identified by standard mutation screening of coding sequences.

A case-control genetic association study was performed to compare the prevalence of *LMX1B* SNPs in four groups, HTG (n = 272), OHT (n = 58), NTG (n = 37), and a normal control group (n = 276). *LMX1B* haplotypes were identified and their prevalence assessed in patients with glaucomatous optic neuropathy (HTG and NTG patients), and in patients with raised IOP (HTG and OHT patients). In order to determine whether the initial positive findings could be replicated, a separately ascertained second cohort consisting of ethnically matched HTG (n = 108) and NTG (n = 222) cases were assessed with the same control group.

LMX1B has not been analysed in any association studies to date and here protective *LMX1B* haplotype associations for both glaucoma and raised IOP were identified.

5.2 Results from the first cohort of HTG, OHT and NTG

By using the algorithm tagger which was implemented in Haploview v.4.0 to select tSNPs that captured untyped SNPs with a minimum r^2 value of 0.8, 26 *S*NPs for *LMX1B* were chosen. Selection was restricted to SNPs with a minor allele frequency (MAF) of ≥10% (i.e. SNPs with a high degree of polymorphism). Selection was also focused on identifying non-synonymous SNPs and this included rs28939692 (27 SNPs in total when combined with SNPs selected from HapMap). Due to poor performing TaqMan assays, as evidence by indistinct cluster plots, the SNP assays of rs10819195, rs785713, and rs7858338 were excluded from further analysis. Hence a total of 23 tSNPs and a manually chosen non-synonymous SNP, all of which spanned a region of 102,314 bp (including the 82,314 bp *LMX1B* gene) were assessed in all individuals (Figure 5-1) using Sequenom iPLEX^{TM} Assay MassARRAY technology.

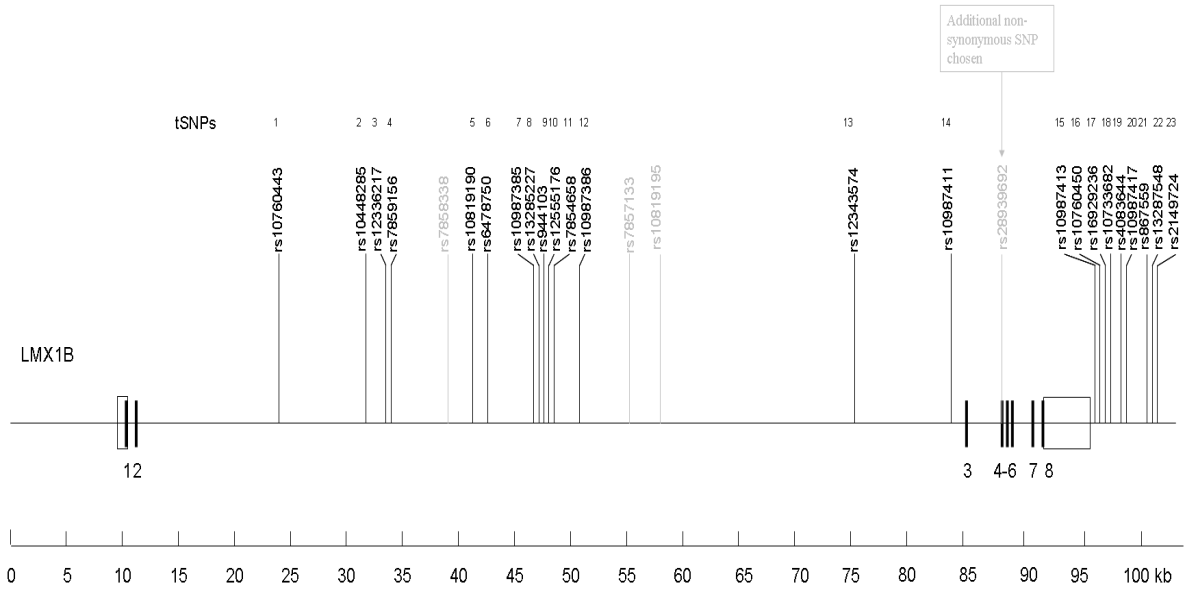
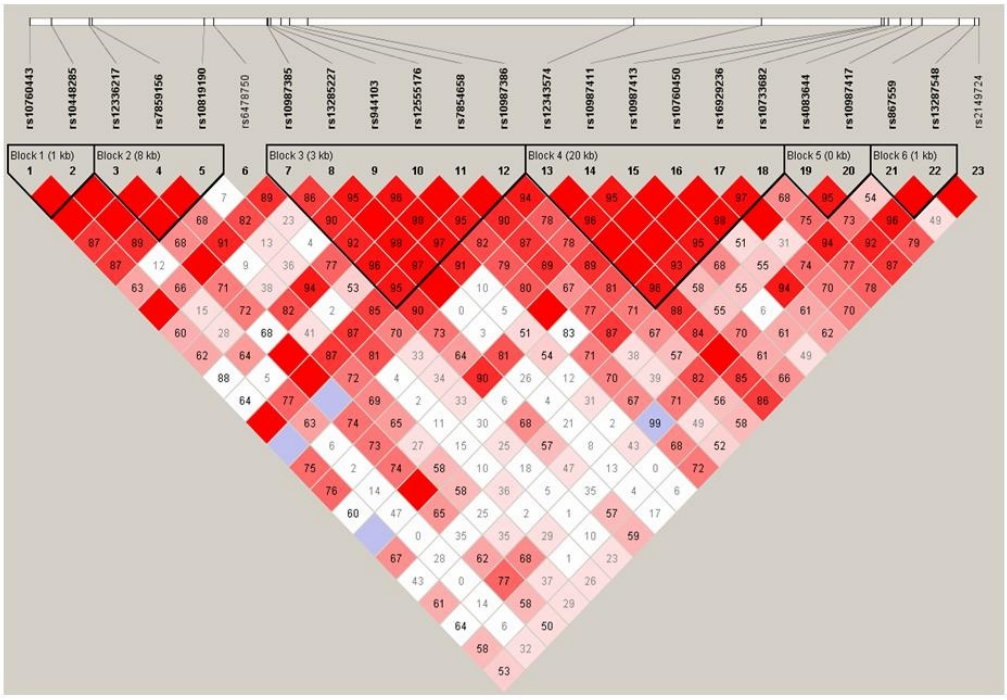


Figure 5–1: The relative position of the 23 tSNPs (labeled above with the respective haplotype) in *LMX1B* are shown after having excluded tSNPs rs10819195, rs785713, and rs7858338 due to its poor performing assays, and rs28939692 as it deviated from HWE (shown in light grey font colour).

8 coding exons are indicated as solid boxes and numbered accordingly. Untranslated exons are shown as open boxes.

A



B

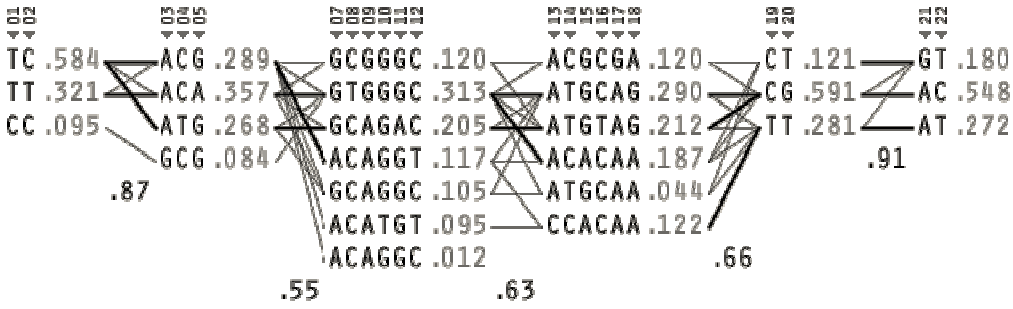


Figure 5–2 (A) Diagram of block structure of *LMX1B* generated using Haploview v.4.0.

LD plots were identified by strong LD. Depth of red/pink colour indicates the computed pairwise *D*' value; deeper pink indicates a higher *D*' value. (B) The selected tSNPs and estimated haplotype frequencies in the six major haplotype blocks (1-6) are shown. Marker numbers and arrows above the haplotypes indicate tSNPs. The frequency of each haplotype within a block is given to the right of the haplotype. The thickness of the lines connecting the haplotypes across blocks represents the relative frequency [i.e., high (*thick*) versus low (*thin*)] with which a given haplotype is associated with the haplotype in the neigbouring block.

Genotyping success rate varied from 92.0 % and 99.0% for the first cohort of HTG, OHT and NTG patients and controls. The genotype distributions of these 23 SNPs in the controls did not differ from those expected from HWE except for the non-synonymous SNP rs28939692 and this was subsequently excluded from further analysis. The pairwise LD matrix generated in Haploview v.4.0 of the entire patient and control cohort revealed 6 main haplotype blocks (Figure 5-2).

5.2.1 Associations between individual SNPs and glaucoma

The allele frequencies of the 23 tSNPs between the 3 separate case groups (HTG, NTG, OHT) as well as among 2 combined groups (HTG+OHT, HTG+NTG), and controls were assessed using Haploview v.4.0 and tSNPs showing significant effects are shown in Table 5-1.

In the HTG group, 4 tSNPs (all situated in intron 2) had allele frequencies that differed significantly between patients and controls. Protective allele frequencies of rs7859156, rs6478750 and rs7854658 were higher in controls than in HTG patients (P = 0.0015, 0.0258, 0.0057 respectively), with ORs of 0.64, 0.72 and 0.65 respectively after adjustment for sex. By contrast the risk allele frequency of rs10987385 was higher in the HTG patients than in controls (P = 0.0180), with an OR of 1.43 after adjustment for sex.

In the OHT group, 2 of these tSNPs (both situated in intron 2) had allele frequencies that differed significantly between patients and controls. Protective allele frequencies of rs7859156 and rs6478750 were higher in controls than in OHT patients (P = 0.0482 and 0.0309 respectively), both with ORs of 0.59 after adjustment for age.

In the NTG group 5 tSNPs had allele frequencies that differed significantly between NTG patients and controls, but only one of these alleles (rs7854658) was in common with the significant HTG tSNPS. The protective allele frequency of rs7854658 was higher in the controls than in NTG patients (P = 0.0041) with an OR of 0.30. In addition, risk allele frequencies of rs944103, rs16929236, rs10733682 and rs867559

were higher in NTG patients than in controls (P = 0.0488, 0.0064, 0.0489 and 0.0295 respectively), with ORs of 1.71, 2.30, 1.75 or 1.82 respectively, after adjustment for sex.

Table 5–1: Distribution of *LMX1B* tSNPs showing significant effects between HTG, OHT, NTG, HTG+OHT and HTG+NTG, compared to the wild type control group. tSNPs significantly distributed after 1000 permutation tests are highlighted in bold. Odds ratios were calculated using Thesias v.3.1 whereas the remaining columns (including case and controls counts, chi-square, *P* value, permutation *P*) were generated using Haploview v.4.0.

Phenotype	tSNP	SNP ID	Allele	Case counts (%)	Control counts (%)	Chi-square	*P* value	Permutation *P*	Odds ratio (95% confidence interval)
HTG	**4**	**rs7859156**	**T**	**121 (23.1)**	**171 (31.8)**	**10.06**	**0.0015**	**0.0376**	**0.64 (0.49-0.85)**
	6	rs6478750	C	193 (36.3)	236 (42.9)	4.97	0.0258	NS	0.72 (0.55-0.93)
	7	rs10987385	A	147 (27.7)	118 (21.5)	5.59	0.0180	NS	1.43 (1.07-1.91)
	11	rs7854658	A	95 (18.0)	137 (24.9)	7.63	0.0057	NS	0.65 (0.49-0.89)
OHT	4	rs7859156	T	24 (22.2)	171 (31.8)	3.90	0.0482	NS	0.59 (0.34-1.01)
	6	rs6478750	C	35 (31.8)	236 (42.9)	4.66	0.0309	NS	0.59 (0.35-0.97)
NTG	9	rs944103	G	41 (55.4)	238 (43.3)	3.88	0.0488	NS	1.71 (1.06-2.77)
	11	rs7854658	A	7 (9.7)	137 (24.9)	8.25	0.0041	NS	0.30 (0.14-0.64)
	17	rs16929236	G	17 (23.0)	64 (11.6)	7.42	0.0064	NS	2.30 (1.30-4.05)
	18	rs10733682	A	44 (59.5)	260 (47.3)	3.88	0.0489	NS	1.75 (1.05-2.90)
	21	rs867559	G	22 (29.7)	104 (18.9)	4.74	0.0295	NS	1.82 (1.09-3.05)
HTG + OHT	**4**	**rs7859156**	**T**	**145 (22.9)**	**171 (31.8)**	**11.52**	**7.0E-4**	**0.0120**	**0.63 (0.48-0.82)**
	6	rs6478750	C	228 (35.5)	236 (42.9)	6.81	0.0090	NS	0.69 (0.54-0.89)
	7	rs10987385	A	175 (27.3)	118 (21.5)	5.37	0.0205	NS	1.39 (1.05-1.84)
	11	rs7854658	A	116 (18.2)	137 (24.9)	7.98	0.0047	NS	0.66 (0.50-0.88)
HTG + NTG	**4**	**rs7859156**	**T**	**138 (23.2)**	**171 (31.8)**	**10.40**	**0.0013**	**0.0360**	**0.64 (0.49-0.84)**
	6	rs6478750	C	220 (36.3)	236 (42.9)	5.27	0.0217	NS	0.73 (0.57-0.94)
	7	rs10987385	A	162 (26.8)	118 (21.5)	4.37	0.0366	NS	1.35 (1.02-1.79)
	11	**rs7854658**	**A**	**102 (17.0**	**137 (24.9)**	**10.90**	**0.0010**	**0.0270**	**0.62 (0.46-0.83)**

rs7854658 and rs944103 are situated in intron 2, whereas rs16929236, rs10733682 and rs867559 are situated in the 3' region of *LMX1B*. After permutation testing, all *P* values for these differences between patients in each group and controls increased beyond the significant level of 0.05, except for rs7859156 which had a permutated *P* value of 0.0376 after 1,000 tests in the HTG group.

Next the allele frequencies of the 23 tSNPs in the combined groups of all patients with raised IOP (HTG and OHT), or all patients with glaucomatous optic neuropathy i.e. all the POAG patients (HTG and NTG) were compared with the control group. In both groups, HTG+OHT and HTG+NTG, the same four tSNPs had an allele frequency that differed significantly between patients and controls (Table 5-1). Protective allele frequencies of rs7859156, rs6478750 and rs7854658 were higher in controls than cases, and risk allele frequencies of rs10987385 were higher in patients than in controls. In both combined groups, tSNP rs7859156, remained significantly different after 1,000 permutation tests (P = <0.05). In addition, in the combined POAG case group (HTG+NTG) a second tSNP rs7854658 remained significantly different after permutation testing.

Of note are the combined effects of age, IOP and tSNP rs7854658, which was capable of correctly guessing the case status 93.8 % of the time in multivariate regression analysis. In another words, after adjusting for the effects of IOP and age, rs7854658 was not significantly associated with the case status, thus revealing that the influence of rs7854658 may be partially explained by its relationship with IOP and age.

5.2.2 Association between haplotypes and HTG

The difference in the distribution of all common haplotypes between individuals with HTG and controls (Table 5-2) was assessed and a significant halotype effect of block 2 and block 3 was found. Haplotypes ATG and ACG (defined by tSNPs rs12336217, rs7859156, rs10819190) were significantly distributed between patients and controls, with the haplotype ATG exhibiting the strongest effect. This was less prevalent among

Table 5–2: Distribution of *LMX1B* haplotypes between HTG cases and controls.
Haplotypes significantly distributed after 1000 permutation tests are highlighted. *P* values after adjustment for sex are provided only for haplotypes that are significantly distributed. Odds ratios were calculated using Thesias v.3.1 whereas the remaining columns (including haplotype, all subject counts, case and controls counts, chi-square, *P* value, permutation *P*) were generated using Haploview v.4.0.

Block	Haplotype	All subjects (%)	Case counts (%)	Control counts (%)	Chi-square	*P* value	Permutation *P*	Odds ratio (95% confidence interval) *P* value after adjustment for sex
2 (tSNP 3 to 5)	ACA	35.0	34.8	35.2	0.023	0.8804	1.0000	
	ACG	28.7	32.2	25.4	6.185	0.0129	0.2720	1.39 (1.02-1.90) *P* = 0.0377
	ATG	**27.4**	**22.8**	**31.7**	**10.775**	**0.0010**	**0.0300**	**0.75 (0.54-1.03) *P* = 0.0750**
	GCG	8.6	9.9	7.4	2.057	0.1515	0.9770	
3 (tSNP 7 to 12)	GTGGGC	31.8	31.8	31.8	0.00	0.9653	1.0000	
	GCAGAC	21.1	17.5	24.7	8.15	0.0043	0.1040	0.73 (0.52-1.02) *P* = 0.0648
	ACAGGT	11.7	14.2	9.4	6.19	0.0128	0.2720	1.64 (1.09-2.48) *P* = 0.0189
	GCGGGC	11.0	12.3	9.8	1.78	0.1818	0.9890	
	GCAGGC	10.1	9.5	10.6	0.49	0.4851	1.0000	
	ACATGT	9.2	9.2	9.2	0.00	0.9892	1.0000	
	ACAGGC	1.4	1.4	1.3	0.01	0.9327	1.0000	

the patients compared with the controls (22.8% versus 31.7%; P = 0.0010) and remained significant after permutation testing.

Individuals with haplotype ATG were at a reduced risk of developing HTG compared with non-carriers (OR 0.75) but this did not remain significant after adjustment for sex (P = 0.0750).

Within block 3, 2 haplotypes, (GCAGAC, ACAGGT; defined by rs10987385, rs13285227, rs944103, rs12555176, rs7854658, and 10987386) were differentially distributed in patients and controls, but these associations did not withstand permutation testing (Table 5-2).

5.2.3 Associations between haplotypes and raised IOP

Here, the difference in frequency distribution of all common haplotypes between the combined raised IOP patient group (HTG+OHT) and the controls were compared (Table 5-3) and a significant haplotype effect of block 2 and block 3 was found. Within block 2, two haplotypes (ATG and ACG) were differentially distributed in patients and controls (Table 5-3). Of these haplotype ATG was most significant and was less prevalent among patients compared with controls (22.7% versus 31.7%; $P = 0.0005$) and remained significant after permutation testing.

Table 5–3: Distribution of *LMX1B* haplotypes between HTG+OHT cases and controls.
Haplotypes significantly distributed after 1000 permutation tests are highlighted. *P* values after adjustment for sex are provided only for haplotypes that are significantly distributed. Odds ratios were calculated using Thesias v.3.1 whereas the remaining columns (including haplotype, all subject counts, case and controls counts, chi-square, *P* value, permutation *P*) were generated using Haploview v.4.0.

Block	Haplotype	All subjects (%)	Case counts (%)	Control counts (%)	Chi-square	*P* value	Permutation *P*	Odds ratio (95% confidence interval) *P* value after adjustment for sex
2 (tSNP 3 to 5)	ACA	35.6	35.8	35.2	0.048	0.8269	1.0000	
	ACG	28.8	31.7	25.4	5.847	0.0156	0.3020	1.33 (0.99-1.78) *P* = 0.0591
	ATG	**26.9**	**22.7**	**31.7**	**12.223**	**5.0E-4**	**0.0100**	**0.72 (0.53-0.97) *P* = 0.0330**
	GCG	8.5	9.4	7.4	1.565	0.2110	0.9987	
3 (tSNP 7 to 12)	GTGGGC	31.6	31.4	31.8	0.02	0.8865	1.0000	
	GCAGAC	21.0	17.8	24.7	8.517	0.0035	0.0710	0.75 (0.54-1.03) *P* = 0.0770
	ACAGGT	11.8	14.0	9.4	6.005	0.0143	0.2770	1.61 (.08-2.41) *P* = 0.0190
	GCGGGC	11.2	12.4	9.8	1.999	0.1574	0.9875	
	GCAGGC	10.2	9.8	10.6	0.185	0.6668	0.9875	
	ACATGT	9.5	9.8	9.2	0.095	0.7580	1.0000	
	ACAGGC	1.2	1.2	1.3	0.083	0.7729	1.0000	

Haplotype ATG carriers were at a decreased risk of developing raised IOP compared with non-carriers (OR 0.72) and this remained significant after adjustment for sex (P = 0.0330). The LD observed between SNPs and different functional effects of the haplotypes are potential sources of bias when studying multiple SNPs. Therefore, haplotype background analysis was performed in Thesias v.3.1 and although rs12336217, rs7859156 and rs10819190 are in LD, the rs7859156 polymorphism exerted an independent decreased risk to glaucoma (OR = 0.54; 95% CI 0.39 - 0.75; P =0.0003).

Within block 3, two haplotypes (GCAGAC, ACAGGT) were differentially distributed in patients and controls, although these associations did not withstand permutation testing (Table 5-3, 5-4).

These data suggest that *LMX1B* haplotypes, or perhaps an unknown nearby functional variant in LD, affect IOP and consequently predispose to glaucoma.

5.2.4 Associations between haplotypes and glaucoma (HTG+NTG)

Examination of the difference in frequency distribution of all common haplotypes between the combined POAG patient group (HTG+NTG) and the controls (Table 5-4) found a significant effect of the same block 2 and block 3 haplotypes on glaucoma. Within block 2, the ACG and ATG haplotypes were differentially distributed in patients and controls with haplotype ATG showing the strongest effect. ATG was significantly less prevalent among patients compared with controls (22.9% versus 31.7%; P = 0.0008 and remained significant after permutation testing). ATG carriers were at a decreased risk of developing glaucoma compared with non-carriers (OR 0.73) and this remained significant after adjustment for sex (P = 0.0450). Haplotype background analysis was performed to see if any of the tSNPs within the haplotype block 2 had an independent effect on glaucoma risk. Although rs12336217, rs7859156 and rs10819190 are in LD, rs7859156 polymorphism exerted an independent increased risk (OR = 0.54; 95% CI 0.39-0.75; P =0.0002).

Table 5–4: Distribution of *LMX1B* haplotypes between HTG+NTG cases and controls. Haplotypes significantly distributed after 1000 permutation tests are highlighted. *P* values after adjustment for sex are provided only for haplotypes that are significantly distributed. Odds ratios were calculated using Thesias v.3.1 whereas the remaining columns (including haplotype, all subject counts, case and controls counts, chi-square, *P* value, permutation *P*) were generated using Haploview v.4.0.

Block	Haplotype	All subjects (%)	Case Counts (%)	Control counts (%)	Chi-square	*P* value	Permutation *P*	Odds ratio (95% confidence interval) *P* value after adjustment for sex
2 (tSNP 3 to 5)	ACA	35.3	35.3	35.2	0.0010	0.9718	1.0000	
	ACG	28.9	32.2	25.4	6.464	0.0110	0.2350	1.36 (1.01-1.83) *P* = 0.0437
	ATG	**27.1**	**22.9**	**31.7**	**11.303**	**8.0E-4**	**0.0220**	**0.73 (0.54-0.99) *P* = 0.0450**
	GCG	8.4	9.3	7.4	1.351	0.2452	1.0000	
3 (tSNP 7 to 12)	GTGGGC	31.6	31.4	31.9	0.024	0.8768	1.0000	
	GCAGAC	**20.4**	**16.5**	**24.7**	**11.949**	**5.0E-4**	**0.0150**	**0.70 (0.51-0.98) *P* = 0.0360**
	GCGGGC	11.9	13.9	9.8	4.444	0.0350	0.5760	1.51 (1.003-2.28) *P* = 0.0480
	ACAGGT	11.7	13.8	9.4	5.392	0.0202	0.3980	1.6 (1.09-2.46) *P* = 0.0170
	GCAGGC	10.5	10.3	10.8	0.086	0.7689	1.0000	
	ACATGT	9.1	9.1	9.3	0.015	0.9033	1.0000	
	ACAGGC	1.3	1.2	1.3	0.027	0.8697	1.0000	

Within block 3, three haplotypes (GCAGAC, GCGGGC, ACAGGT defined by rs10987385, rs13285227, rs944103, rs12555176, rs7854658, and 10987386) were differentially distributed in patients and controls and one of these GCAGAC, was significant after permutation testing. Haplotype GCAGAC was significantly less prevalent among patients compared with controls (16.5% versus 24.7%; $P = 0.0005$ and turned into $P = 0.0150$ after 1,000 permutation tests). GCAGAC carriers were at a decreased risk of developing glaucoma compared with non carriers (OR, 0.70; $P = 0.0360$) after adjustment for sex in multivariate logistic regression analysis.

Crucially, the relation between haplotypes in block 2 and 3 and glaucoma risk did not persist after adjusting for CDR in Thesias. CDR is significantly increased in both NTG and HTG compared with controls (Table 5-1). Thus, the influence of these *LMX1B*

haplotypes or a nearby functional variant in LD, on glaucoma risk may be partially explained by their relation with CDR.

5.2.5 Results from the second cohort

A smaller number of 11 tSNPs were analysed for the replication study using cohort 2 comprised of HTG, NTG and controls (Figure 5-3). The association with the OHT group was not assessed in the replication study as DNA samples of OHT individuals were not available. These tSNPs were chosen either because the individual tSNPs (rs7859156, rs10987385, rs944103, rs7854658, rs16929236, rs867559) or groups of tSNPs comprising haplotype blocks 2 and 3 showed significant distribution between the cases and controls. Genotyping success rate for the second cohort was 99.0%. The haplotype block 2 and 3 from the initial study are now depicted as block 1 and 2 (Figure 5-4) in the replication study.

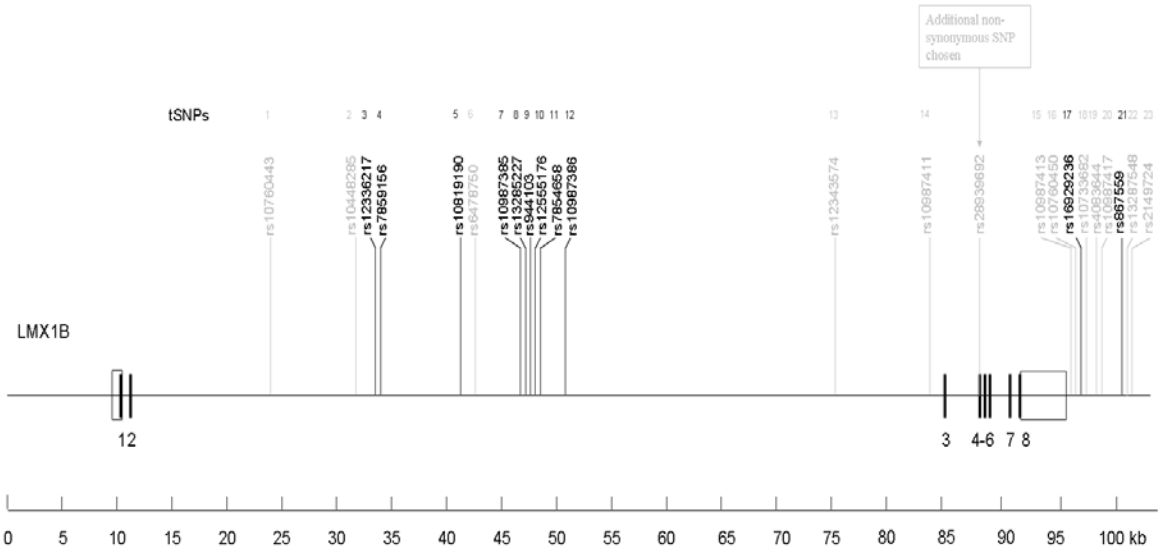
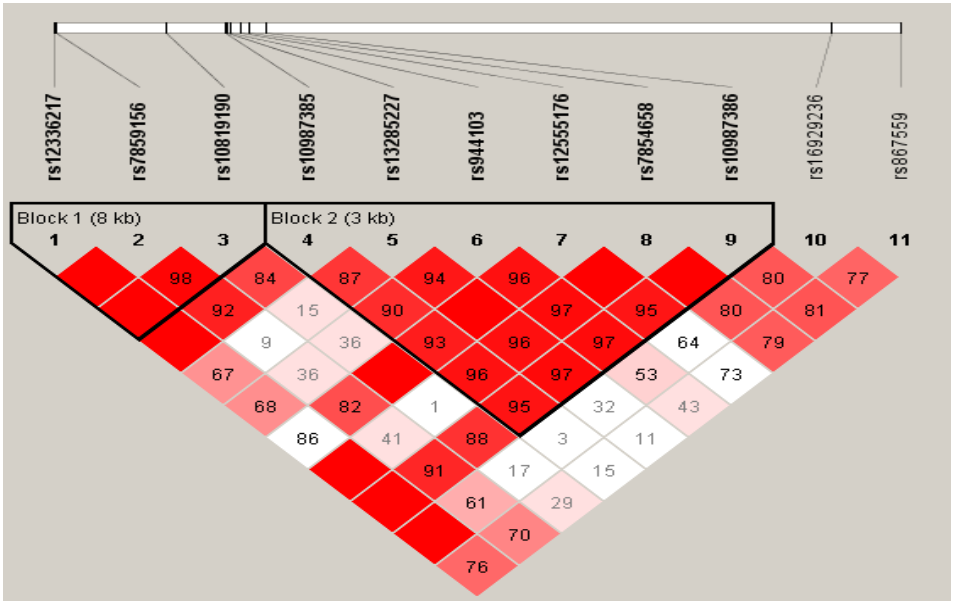


Figure 5–3: The relative position of the 11 tSNPs (labeled above with the respective haplotype) in *LMX1B* for the replication study in relation to the original sets of 24 tSNPs shown in light grey font colour.

A



B

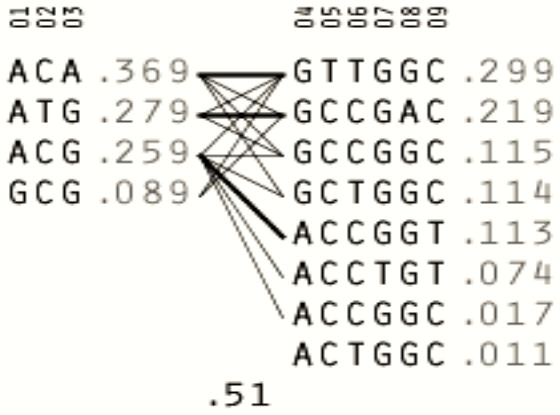


Figure 5–4: (A) Diagram of block structure of *LMX1B* generated for the replication study using the Haploview v.4.0 from 11 tSNPs only.

LD plots were identified by strong LD. Depth of red/pink colour indicates the computed pairwise *D*' value; deeper pink indicates a higher *D*' value. (B) The selected tSNPs and estimated haplotype frequencies in the two major haplotype blocks (1 and 2) are shown. Marker numbers and arrows above the haplotypes indicate tSNPs. The frequency of each haplotype within a block is given to the right of the haplotype. The thickness of the lines connecting the haplotypes across blocks represents the relative frequency [i.e., high (*thick*) versus low (*thin*)] with which a given haplotype is associated with the haplotype in the neighbouring block.

5.2.5.1 Associations between individual SNPs and glaucoma

A similar pattern of tSNP association was observed in the second cohort (Table 5-5) with the protective allele frequency of rs7859156 being significantly associated with HTG, NTG and HTG+NTG (P = 0.0387, 0.0470, and 0.0274 respectively), but relatively less when compared to those obtained from the first cohort. In addition, a tSNP rs12555176 (situated in intron 2) which was not associated with any of the case groups in the initial study, had an allele frequency that showed a stronger association (in comparison to tSNP rs7859156) with the NTG group (P = 0.0089) as well as the HTG+NTG group (P = 0.0274), when compared with the controls.

Table 5–5: **Association of *LMX1B* tSNPs in the replicate study showing a comparable pattern of distribution between HTG, NTG, and HTG+NTG, compared to the wild type control group but did not withstand permutation testing.**

Odds ratios were calculated using Thesias v.3.1 whereas the remaining columns (including case and controls counts, chi-square, *P* value, permutation *P*) were generated using Haploview v.4.0.

Phenotype	tSNP	SNP ID	Allele	Case counts (%)	Control counts (%)	Chi-square	*P* value	Permutation *P*	Odds ratio (95% confidence interval)
HTG	4	rs7859156	T	49 (24.0)	171 (31.8)	4.28	0.0387	NS	0.68 (0.48-0.98)
NTG	4	rs7859156	T	111 (25.9)	171 (31.8)	3.95	0.0470	NS	0.76 (0.58-1.00)
	10	rs12555176	T	23 (5.2)	52 (9.7)	6.84	0.0089	NS	0.48 (0.29-0.82)
HTG + NTG	4	rs7859156	T	160 (25.3)	171 (31.8)	5.99	0.0144	NS	0.74 (0.57-0.95)
	10	rs12555176	T	41 (6.2)	52 (9.7)	4.87	0.0274	NS	0.59 (0.38-0.93)

5.2.5.2 Association between haplotypes and HTG, NTG and HTG+NTG

When the difference in the frequency distribution of all common haplotypes between the cases (including HTG, NTG, HTG+NTG) and the controls was examined within block 1 (Table 5-6) of the second cohort, the protective haplotype ATG that was found significantly distributed in the initial study, continued to show differential distribution in all of the case group (HTG, NTG, HTG+NTG) compared to the controls (P = 0.0150, 0.0241. 0.0047 respectively). Among the case group, haplotype ATG had a strongest

Table 5–6: Distribution of *LMX1B* haplotypes between HTG, NTG, HTG+NTG cases and controls.

Odds ratios were calculated using Thesias v.3.1 whereas the remaining columns (including haplotype, all subject counts, case and controls counts, chi-square, *P* value, permutation *P*) were generated using Haploview v.4.0.

Block	Haplotype	Case group	All subjects (%)	Case counts (%)	Control counts (%)	Chi-square	*P* value	Permutation *P*	Odds ratio (95% confidence interval)
1 (tSNP 1 to 3)	ATG	HTG	29.3	22.9	31.8	5.92	0.0150	0.1610	0.67 (0.44-1.01)
		NTG	29.1	25.4	32.0	5.09	0.0241	0.2360	0.79 (0.57-1.08)
		HTG+NTG	27.9	24.6	31.9	7.99	0.0047	0.0650	0.75 (0.56-1.00)
2 (tSNP 4 to 9)	ACAGGT	HTG+NTG	11.3	13.0	9.2	4.38	0.0363	0.3690	1.4 (0.97-2.21)
	ACATGT	NTG	7.3	5.0	9.2	6.53	0.0106	0.1210	0.52 (0.28-0.93)
		HTG+NTG	7.4	5.9	9.2	4.76	0.0292	0.3130	0.65 (0.39-1.07)

effect on the combined glaucoma group (HTG+NTG) (OR 0.75) and was less prevalent among the patients compared with controls (24.6% versus 31.9%).

Within block 2, haplotype ACAGGT which was differentially distributed in the first cohort in all of the case groups, showed a similar pattern of association in the HTG+NTG group (P = 0.0363). In addition, haplotype frequency of ACATGT which did not differ significantly between the cases and the controls in the initial cohort, was significantly distributed within both NTG and HTG+NTG groups (P = 0.0106 and 0.0292 respectively). The remaining haplotypes (GCAGAC, GCGGC) which produced significant effects in the first cohort did not maintain its noteworthy effects in the second cohort.

Despite the fact that none of the associations between the haplotypes and the case groups withstood permutation testing, the replicate study on an independent cohort has shown a comparable pattern of SNP and haplotype distribution which provides further evidence to support the initial findings of an association between alleles in *LMX1B* and

POAG. It should also be noted that cohort two is considerably smaller than cohort one and therefore has less statistical power.

5.2.6 Discussion

Around 1-2% of individuals ≥40 years of age in the general population develop POAG, compared with 33% of patients with the rare condition, NPS (MIM161200) caused by dominant mutations in the *LMX1B* gene. In this study *LMX1B* has been investigated as a candidate genetic risk factor for the most common form of glaucoma and significant associations were identified between *LMX1B* alleles and haplotypes and POAG.

In the large HTG cohort, the frequency of the protective tSNP rs7859156 in intron 2 was significantly reduced compared with the controls and this effect withstood stringent permutation testing. A similar pattern of result was reproduced in the smaller HTG group in the replication study. Interestingly, in the first cohort, a number of weak associations (with tSNPS rs6478750, rs10987385 and rs7854658) among the HTG group were replicated in the smaller OHT and NTG groups.

The HTG group is characterised by glaucomatous damage, and raised IOP. The other two patient groups each have only one of these characteristics, either glaucomatous damage without raised IOP (NTG), or raised IOP without glaucomatous damage (OHT). In order to test the relationship between the variant alleles of *LMX1B* and these two characteristics patients have been grouped into those with raised IOP (HTG and OHT), or those with glaucomatous damage irrespective of IOP (HTG and NTG). Notably, in both of the combined groups the significant association with the protective tSNP rs7859156 in intron 2 was replicated and enhanced. Again, a similar pattern of association was reproduced and enhanced in the replication study between tSNP rs7859156 with the combined group HTG and NTG. Moreover, tSNPs rs7854658, identified in the separate NTG and HTG groups was highly significant and withstood permutation testing in the combined NTG+HTG group. This was an interesting result and was confirmed by haplotype analysis suggesting that the effect of *LMX1B* on glaucoma susceptibility may involve functions that are broader than effects on IOP. As the HTG cases are highly represented in the combined groups, the replication study investigated a larger group of NTG cases and replicated the associations with the tSNPs

and haplotypes seen in the initial cohort, but with weaker correlations. Of note is the tSNP rs12555176 which emerged to be significantly distributed with the larger NTG group, but not with the smaller cohort of the initial study.

Recent studies have shown that 26.7 % of individuals with NPS above the age of 50 develop NTG (Bongers *et al.*, 2005), whereas 11.9% of individuals develop OHT over the age of 40 (Sweeney *et al.*, 2003). These data are also consistent with the proposal that the role of *LMX1B* in glaucoma pathology may be complex.

Six main haplotype blocks were identified across the *LMX1B* gene. Haplotypes within two of these blocks containing the significantly varying tSNPs were found to show significantly altered distributions in the two combined patient groups, POAG (HTG+NTG) and raised IOP±HTG (HTG+OHT). The block 2 ATG haplotype is associated with both groups and with HTG alone, and a similar pattern of results were confirmed with the HTG+NTG in the replication study. Whereas the block 3 GCAGAC haplotype shows the strongest association with the HTG+NTG group in the initial study but interestingly not in the replication study in which a different haplotype ACATGT emerged as having the best association. The block 2 haplotype (spanning a region of ~6700 bp) and the block 3 haplotype (spanning a region of ~28000 bp) are both located within the large intron 2 of *LMX1B*. This region shows stretches of greater than 85% conservation between the sequences of humans and mouse, and a functional role, such as gene expression regulatory regions, has been suggested (Dunston *et al.*, 2004). Significantly, the block 2 haplotype is in complete LD with the promoter and 5' upstream regions (Dunston *et al.*, 2004). Therefore, despite being an intronic sequence, this haplotype may tag important conserved regions involved in gene regulation.

The proposal that levels of *LMX1B* expression are relevant to risk of elevated IOP and glaucoma is in line with the well documented sensitivity of ocular development to gene dosage. For example, variations in the copy number of *FOXC1* (Lehmann *et al.*, 2002) and *PAX6* (Dora *et al.*, 2008) which are presumed to alter levels of gene expression, or mutations in *PITX2* that increase or decrease levels of transcription factor function (Kozlowski and Walter, 2000) cause anterior segment anomalies and glaucoma. These developmental glaucoma genes are obvious candidates for further association studies for POAG, and it is possible that the combined inheritance of multiple loci may give a

greater association with HTG and/or OHT and may serve as important prognostic indicators in the general population.

Despite the progress in identifying genes that are involved in POAG, understanding of the underlying pathogenic mechanism is still limited. This is partly because if each causal gene only makes a small contribution to the overall phenotype, then unravelling the role each plays in the biochemical and cellular pathways involved in IOP control and retinal ganglion cell function is complex. New insights into a possible *Lmx1b* pathway relevant to glaucoma pathology was provided by a report showing mutations in the collagen *Col4a1* gene resulted in ASD with optic nerve hypoplasia, and raised, as well as low IOP (Gould *et al.*, 2007). *Lmx1b* is known to directly regulate expression of *Col4a3* and *Col4a4* in the kidney (Morello *et al.*, 2001; Rohr *et al.*, 2002) and *Lmx1b*$^{-/-}$ mice showed defective collagen fibrillogenesis in the eye, as well as ciliary body hypoplasia (Pressman *et al.*, 2000) though there is not yet evidence that *Lmx1b* regulates *Col4a1*. Considered together the data from this study and other studies suggest that altered LMX1B activity levels cause abnormalities and dysfunction in the IOP-regulating anterior segment structures that are age-related and are clinically undetectable, resulting in POAG. While JOAG can be a consequence of *LMX1B* mutation in NPS (Mimiwati *et al.*, 2006; Sweeney *et al.*, 2003), the mean age at which open angle glaucoma or OHT is detected in NPS has been above the age of 40 at 63.4 (range 55- 72) (Bongers *et al.*, 2005), and 47.9 years (range 23-78) (Sweeney *et al.*, 2003) a feature more in consistent with POAG.

Summary This is the first study to show an association between variants of *LMX1B* and POAG. These data indicate altered *LMX1B* function may be associated with the common forms of POAG in the general population and provides a platform for further investigation of *LMX1B* as a genetic risk factor for adult-onset glaucoma.

Chapter 6 The role of signaling molecules *TGFβ2*, *BMP4* and transcription factor gene *FOXC1* in OHT and POAG

6.1 Introduction

In this chapter, the contribution of variations at the *TGFβ2, BMP4* and *FOXC1* loci to risk of OHT and POAG was assessed within the general population. A case-control genetic association study was performed to compare the prevalence of *TGFβ2, BMP4* and *FOXC1* SNPs in four groups, HTG (n = 272), OHT (n = 58), NTG (n = 37), and a normal control group (n = 276). *TGFβ2, BMP4* and *FOXC1* haplotypes were identified and their prevalence assessed in patients with glaucomatous optic neuropathy (HTG and NTG patients), and in patients with raised IOP (HTG and OHT patients).

This is the first association study to assess *TGFβ2, BMP4* and *FOXC1* as candidate genetic risk factors for OHT/POAG, and here no associations were identified with both glaucoma and raised IOP.

6.2 Results

By using the algorithm tagger which was implemented in Haploview v.4.0 to select tSNPs that captured untyped SNPs with a minimum r^2 value of 0.8, 5 *S*NPs for *BMP4* (Figure 6-1), 22 SNPs for *TGFβ2* (Figure 6-3), and 4 SNPs for *FOXC1* (Figure 6-5) were chosen and their prevalence analysed. Selection was restricted to SNPs with a MAF of ≥10%. Selection was also focused on identifying functional SNPs and these included two 5'UTR SNPs for *TGFβ2* (rs11466367 and rs17026738), and one non-synonymous SNP for *FOXC1* (rs1051933). However, either due to the presence of a deletion adjacent to a SNP (-/CAAA for rs11466367 and -/AAAC for rs17026738, both in *TGFβ2*) or due to poor performing assays, as shown by indistinct cluster plots (rs2799085 in *TGFβ*), these SNPs were excluded from further analysis. In addition, as one SNP in *FOXC1* (rs1051933) differed from that expected from HWE within the control group, this was excluded from further analysis. Hence a total of 5 tSNPs for *BMP4* spanning a region of 15,272 bp (including the 4,814 bp *BMP4* gene), 19 tSNPs

for *TGFβ2,* spanning a region of 98,075 bp (including the 95,108 bp *TGFβ2* gene), and 4 tSNPs for *FOXC1* spanning a region of 12,012 bp (including the 1,661 bp *FOXC1* gene) were assessed in all individuals (Figure 6-1, 6-3 and 6-5 respectively). Using Sequenom iPLEX^{TM} Assay MassARRAY technology [except for 4 SNPs in *FOXC1* (rs2235715, rs2569889, rs1051933, and rs984253), and one SNP in *BMP4* (rs17563), which were genotyped using TaqMan SNP genotyping assays], the genotyping success rate varied from 92.0 % and 99.0% for all genes. For TaqMan SNP genotyping assays, the genotyping success rate varied from 95.0% to 98%. The pairwise LD matrix revealed 2 main haplotype blocks for *BMP4* (Figure 6-2) and 4 haplotypes blocks for *TGFβ2* (Figure 6.4). Haplotype analysis of *FOXC1* revealed no common haplotype between patients and variants of *FOXC1* (Figure 6-6) due to lack of LD between SNPs.

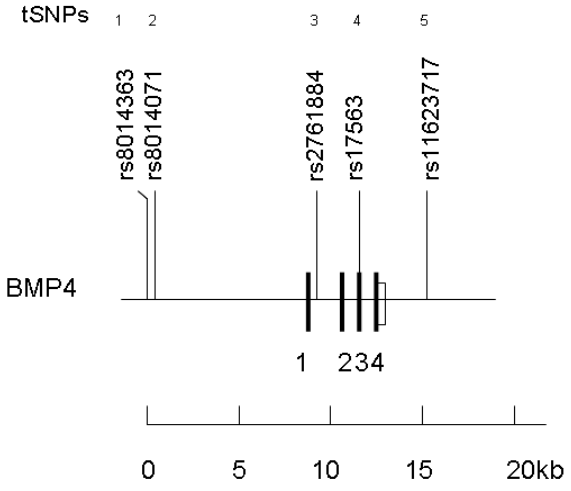
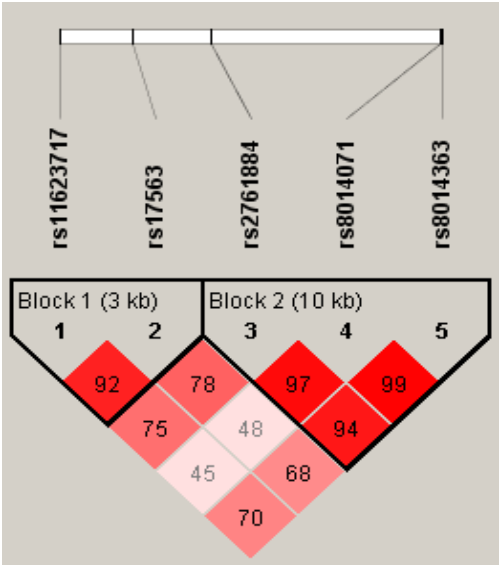


Figure 6–1: The relative position of the 5 tSNPs in *BMP4* spanning a region of 15,272 bp.

4 coding exons are indicated as solid boxes and numbered accordingly. Untranslated exons are shown as open boxes.

A



B

Figure 6–2: (A) Diagram of block structure of *BMP4* generated using Haploview v.4.0.

LD plots were identified by strong LD. Depth of red/pink colour indicates the computed pairwise *D*' value; deeper pink indicates a higher *D*' value. (B) The selected tSNPs and estimated haplotype frequencies in the two major haplotype blocks are shown. Marker numbers and arrows above the haplotypes indicate tSNPs. The frequency of each haplotype within a block is given to the right of the haplotype. The thickness of the lines connecting the haplotypes across blocks represents the relative frequency [i.e., high (*thick*) versus low (*thin*)] with which a given haplotype is associated with the haplotype in the neighboring block.

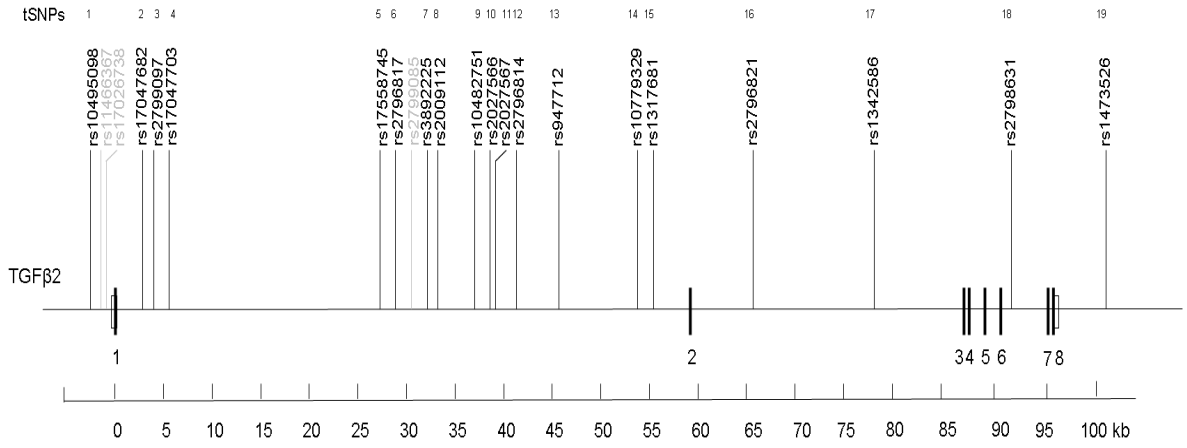
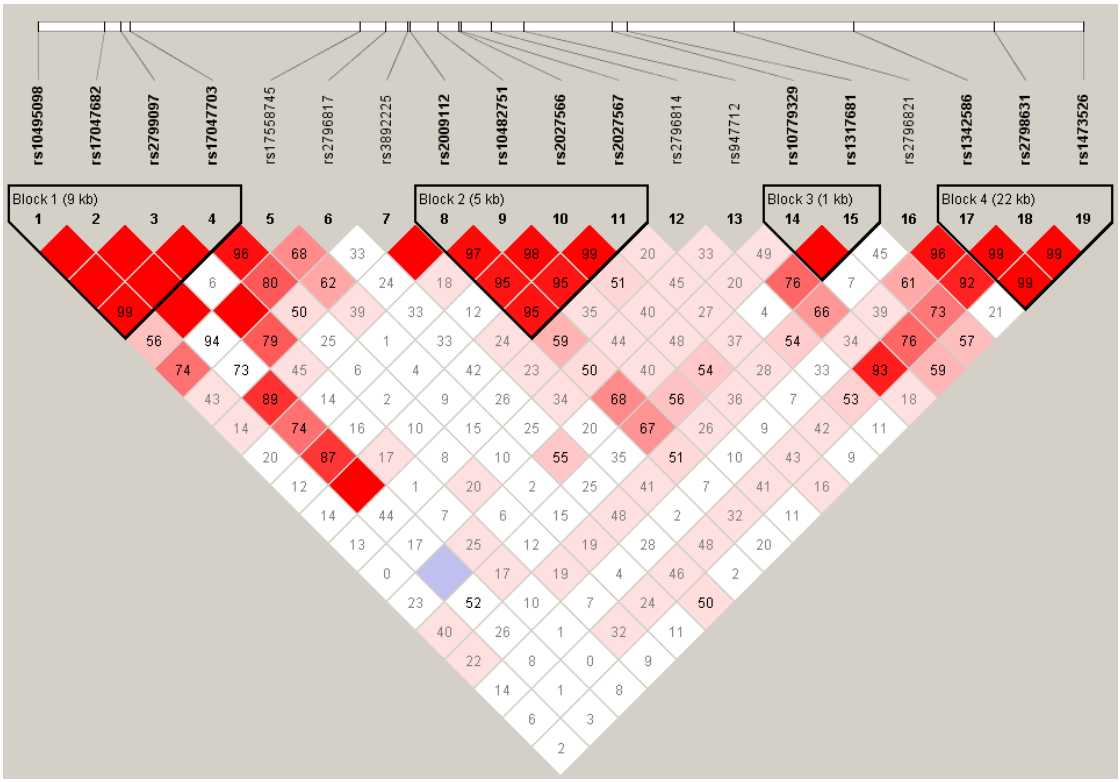


Figure 6–3: The relative position of the remaining 19 tSNPs in *TGFβ2* (labeled above with the respective haplotype) spanning a region of 98,075 bp after excluding tSNPs rs11466367 and rs17026738 due to a presence of a deletion adjacent to the SNPs, and rs2799085 due to poor performing assays.

Excluded SNPs are shown in light grey font colour. 8 coding exons are indicated as solid boxes and numbered accordingly. Untranslated exons are shown as open boxes.

A



B

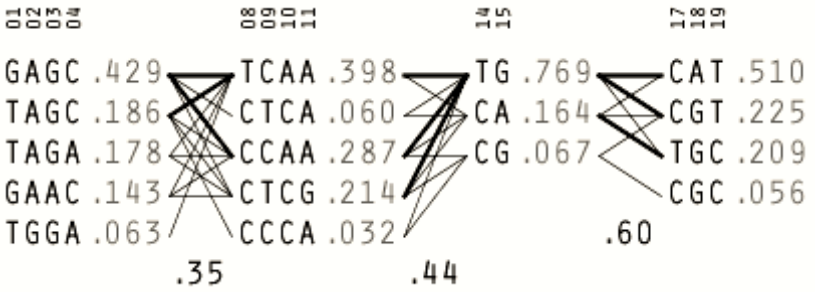


Figure 6–4 (A) Diagram of block structure of *TGFβ2* generated using Haploview v.4.0.

LD plots were identified by strong LD. Depth of red/pink colour indicates the computed pairwise *D*' value; deeper pink indicates a higher *D*' value. (B) The selected tSNPs and estimated haplotype frequencies in the four major haplotype blocks (1-4) are shown. Marker numbers above the haplotypes indicate tSNPs. The frequency of each haplotype within a block is given to the right of the haplotype. The thickness of the lines connecting the haplotypes across blocks represents the relative frequency [i.e., high (*thick*) versus low (*thin*)] with which a given haplotype is associated with the haplotype in the neighbouring block.

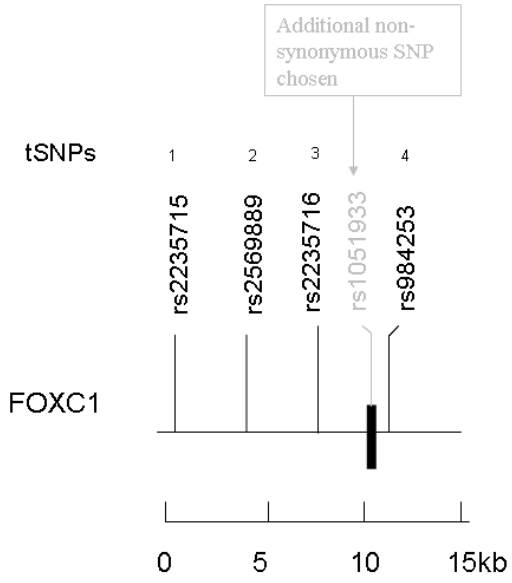


Figure 6–5: The relative position of the remaining 4 tSNPs (labeled above with the respective haplotype) spanning a region of 12,012 bp in *FOXC1*, after excluding rs1051933 (shown in light grey font colour) as it differed from HWE.

The coding exon is indicated as a solid box.

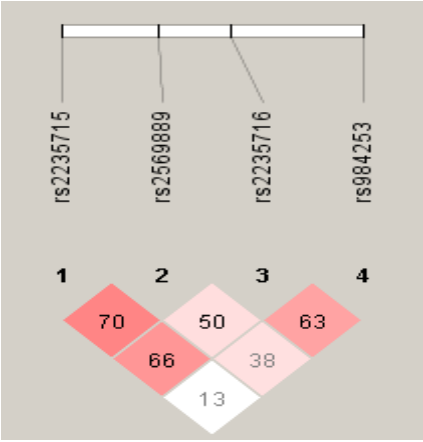


Figure 6–6: Diagram of block structure of *FOXC1* generated using Haploview v.4.0 showing absence of common haplotype due to low LD between tSNPs.

Depth of red/pink colour indicates the computed pairwise *D*' value; deeper pink indicates a higher *D*' value.

6.2.1 Lack of association between SNPs in *TGFβ*, *BMP4* and *FOXC1* and POAG

The allele frequencies of the 19 tSNPs in *TGFβ2*, 5 tSNPs in *BMP4* and 4 tSNPs in *FOXC1* between the 5 separate case groups (HTG, NTG, OHT, HTG+OHT, HTG+NTG) and the controls were assessed. No significant associations were found (Table 6-1, 6-2 and 6-3) between *TGFβ2, BMP4,* and *FOXC1* and glaucoma, except between the combined group HTG+OHT and *BMP4* where there was a weak association which did not withstand permutation testing (uncorrected P = 0.040, corrected P = 0.1320, OR 1.26).

Table 6–1: Distribution of *TGFβ2* tSNPs showing no significant associations between HTG, OHT, NTG, HTG+OHT and HTG+NTG, compared to the wild type control group.

All of the columns were generated using Haploview v.4.0.

Phenotype	tSNP	SNP ID	Allele	Case Counts (%)	Control counts (%)	Chi-square	Uncorrected *P* value
HTG	1	rs10495098	T	232 (43.8)	237 (43.2)	0.03	0.8619
	2	rs17047682	G	39 (7.4)	31 (5.7)	1.32	0.2502
	3	rs2799097	A	69 (13.3)	80 (15.0)	0.67	0.4108
	4	rs17047703	A	140 (26.8)	127 (23.2)	1.89	0.1685
	5	rs17558745	T	191 (36.6)	169 (31.2)	3.47	0.0623
	6	rs2796817	C	70 (13.2)	83 (15.1)	0.78	0.3749
	7	rs3892225	G	119 (22.5)	106 (19.3)	1.74	0.1873
	8	rs2009112	T	215 (40.6)	222 (40.4)	0.01	0.946
	9	rs10482751	C	145 (27.6)	153 (27.8)	0.01	0.9265
	10	rs2027566	C	167 (31.5)	172 (31.4)	0.00	0.9654
	11	rs2027567	G	116 (22.1)	124 (22.5)	0.04	0.8463
	12	rs2796814	G	132 (25.1)	118 (21.6)	1.82	0.1776
	13	rs947712	T	185 (35.6)	191 (35.1)	0.03	0.8735
	14	rs10779329	C	124 (23.6)	119 (21.7)	0.53	0.4667
	15	rs1317681	A	84 (15.9)	89 (16.4)	0.04	0.8409
	16	rs2796821	T	147 (27.8)	144 (26.2)	0.38	0.5396
	17	rs1342586	C	106 (20.0)	125 (22.8)	1.26	0.2609
	18	rs2798631	G	255 (48.7)	267 (48.9)	0.01	0.9382
	19	rs1473526	C	138 (26.1)	150 (27.3)	0.18	0.6734
OHT	1	rs10495098	G	72 (65.5)	311 (56.8)	2.85	0.0912
	2	rs17047682	A	105 (95.5)	517 (94.3)	0.22	0.6399
	3	rs2799097	A	17 (16.0)	80 (15.0)	0.07	0.7934
	4	rs17047703	C	89 (0.809)	421 (76.8)	0.88	0.3491
	5	rs17558745	C	78 (72.2)	373 (68.8)	0.49	0.4835
	6	rs2796817	G	19 (17.3)	83 (15.1)	0.33	0.5633
	7	rs3892225	A	90 (81.8)	444 (80.7)	0.07	0.7904
	8	rs2009112	C	68 (61.8)	328 (59.6)	0.18	0.6698
	9	rs10482751	C	80 (72.7)	397 (72.2)	0.01	0.9071
	10	rs2027566	C	35 (31.8)	172 (31.4)	0.01	0.9292
	11	rs2027567	A	88 (80.0)	426 (77.5)	0.35	0.5571

	12	rs2796814	G	26 (23.6)	118 (21.6)	0.22	0.6398
	13	rs947712	T	41 (37.3)	191 (35.1)	0.19	0.6655
	14	rs10779329	C	31 (28.2)	119 (21.7)	2.18	0.1401
	15	rs1317681	A	20 (18.5)	89 (16.4)	0.30	0.5830
	16	rs2796821	T	33 (30.0)	144 (26,2)	0.68	0.4093
	17	rs1342586	C	91 (82.7)	423 (77.2)	1.64	0.1999
	18	rs2798631	G	55 (50.0)	267 (48.9)	0.04	0.8334
	19	rs1473526	T	84 (76.4)	400 (72.7)	0.62	0.4311
NTG	1	rs10495098	G	42 (60.0)	311 (56.8)	0.28	0.6051
	2	rs17047682	G	6 (8.3)	31 (5.7)	0.81	0.3674
	3	rs2799097	A	12 (16.7)	80 (15.0)	0.13	0.7181
	4	rs17047703	A	21 (30.0)	127 (23.2)	1.59	0.2077
	5	rs17558745	T	25 (35.7)	169 (31.2)	0.59	0.4430
	6	rs2796817	G	14 (19.4)	83 (15.1)	0.92	0.3383
	7	rs3892225	A	61 (84.7)	444 (80.7)	0.67	0.4147
	8	rs2009112	C	44 (62.9)	328 (59.6)	0.27	0.6044
	9	rs10482751	C	52 (72.2)	397 (72.2)	0.00	0.9943
	10	rs2027566	C	23 (31.9)	172 (31.4)	0.01	0.9237
	11	rs2027567	A	59 (81.9)	426 (77.5)	0.75	0.3873
	12	rs2796814	G	20 (27.8)	118 (21.6)	1.39	0.2377
	13	rs947712	C	49 (70.0)	353 (64.9)	0.72	0.3973
	14	rs10779329	T	57 (79.2)	429 (78.3)	0.03	0.8643
	15	rs1317681	G	63 (87.5)	455 (83.6)	0.71	0.4000
	16	rs2796821	C	56 (77.8)	406 (73.8)	0.52	0.4698
	17	rs1342586	C	55 (78.6)	423 (77.2)	0.07	0.7948
	18	rs2798631	A	37 (51.4)	279 (51.1)	0.00	0.9631
	19	rs1473526	C	20 (27.8)	150 (27.3)	0.01	0.9280
HTG+OHT	1	rs10495098	G	370 (57.8)	311 (56.8)	0.14	0.7125
	2	rs17047682	G	44 (6.9)	31 (5.7)	0.77	0.3819
	3	rs2799097	G	540 (86.3)	452 (85.0)	0.40	0.5294
	4	rs17047703	A	161 (25.5)	127 (23.2)	0.84	0.3591
	5	rs17558745	T	221 (35.1)	169 (31.2)	1.99	0.1579
	6	rs2796817	G	89 (13.9)	83 (15.1)	0.34	0.5623
	7	rs3892225	G	139 (21.8)	106 (19.3)	1.14	0.2855
	8	rs2009112	C	257 (40.2)	222 (40.4)	0.01	0.9420
	9	rs10482751	C	175 (27.5)	153 (27.8)	0.01	0.9076
	10	rs2027566	C	202 (31.6)	172 (31.4)	0.00	0.9482
	11	rs2027567	G	138 (21.7)	124 (22.5)	0.12	0.7258
	12	rs2796814	G	158 (24.8)	118 (21.6)	1.71	0.1905
	13	rs947712	T	226 (35.9)	191 (35.1)	0.07	0.7854
	14	rs10779329	C	155 (24.4)	119 (21.7)	1.17	0.2800
	15	rs1317681	G	104 (16.4)	89 (16.4)	0.00	0.9970
	16	rs2796821	T	180 (28.2)	144 (26.2)	0.61	0.4331
	17	rs1342586	C	125 (19.5)	125 (22.8)	1.91	0.1669
	18	rs2798631	G	310 (48.9)	267 (48.9)	0.00	0.9986
	19	rs1473526	C	64 (25.7)	150 (27.3)	0.37	0.5413
HTG+NTG	1	rs10495098	T	260 (43.3)	237 (43.2)	0.00	0.9768
	2	rs17047682	G	45 (7.5)	31 (5.7)	1.57	0.2097
	3	rs2799097	A	81 (13.7)	80 (15.0)	0.42	0.5173
	4	rs17047703	A	161 (27.2)	127 (23.2)	2.43	0.1185
	5	rs17558745	T	216 (36.5)	169 (31.2)	3.55	0.0595
	6	rs2796817	C	84 (14)	83 (15.1)	0.30	0.5839
	7	rs3892225	G	130 (21.7)	106 (19.3)	1.01	0.3153
	8	rs2009112	T	241 (40.2)	222 (40.4)	0.01	0.9458
	9	rs10482751	T	165 (27.6)	153 (27.8)	0.01	0.9318
	10	rs2027566	C	190 (31.6)	172 (31.4)	0.01	0.9492
	11	rs2027567	G	129 (21.6)	124 (22.5)	0.16	0.6910
	12	rs2796814	G	152 (25.4)	118 (21.6)	2.29	0.1299
	13	rs947712	T	206 (34.9)	191 (35.1)	0.01	0.9452
	14	rs10779329	C	139 (23.2)	119 (21.7)	0.38	0.5359
	15	rs1317681	A	93 (15.5)	89 (16.4)	0.16	0.6912
	16	rs2796821	T	163 (27.2)	144 (26.2)	0.14	0.7061
	17	rs1342586	T	121 (20.2)	125 (22.8)	1.19	0.2756
	18	rs2798631	G	290 (48.7)	267 (48.9)	0.01	0.9345
	19	rs1473526	C	158 (26.3)	150 (27.3)	0.13	0.7193

Table 6–2: Distribution of *BMP4* tSNPs between HTG, OHT, NTG, HTG+OHT and HTG+NTG, compared to the wild type control group.

tSNPs that are significantly distributed are high lighted in bold. All of the columns were generated using Haploview v.4.0.

Phenotype	tSNP	SNP ID	Allele	Case Counts (%)	Control counts (%)	Chi-square	Uncorrected *P* value
HTG	1	rs11623717	A	197 (62.4)	219 (60.0)	0.63	0.4264
	2	rs17563	A	315 (61.5)	298 (58.0)	1.34	0.2467
	3	rs2761884	T	265 (50.2)	243 (44.3)	3.69	0.0548
	4	rs8014071	C	342 (64.8)	344 (63.0)	0.36	0.5463
	5	rs8014363	A	248 (49.6)	235 (47.6)	0.41	0.5222
OHT	1	rs11623717	A	68 (61.8)	329 (60.0)	0.12	0.7274
	2	rs17563	A	60 (60.0)	298 (58.0)	0.14	0.7073
	3	rs2761884	T	56 (50.9)	243 (44.3)	1.59	0.2069
	4	rs8014071	C	74 (67.3)	344 (63.0)	0.72	0.3956
	5	rs8014363	A	52 (48.1)	235 (47.6)	0.01	0.9134
NTG	1	rs11623717	G	33 (45.8)	219 (40.0)	0.91	0.3404
	2	rs17563	G	29 (42.6)	216 (42.0)	0.01	0.9220
	3	rs2761884	T	35 (48.6)	243 (44.3)	0.47	0.4936
	4	rs8014071	T	30 (41.7)	202 (37.0)	0.59	0.4417
	5	rs8014363	G	34 (53.1)	259 (52.4)	0.01	0.9165
HTG+OHT	1	rs11623717	A	395 (62.3)	329 (60.0)	0.64	0.4251
	2	rs17563	A	375 (61.3)	298 (58.0)	1.26	0.2610
	3	**rs2761884**	**T**	**321 (50.3)**	**243 (44.3)**	**4.21**	**0.0401**
	4	rs8014071	C	416 (65.2)	344 (63.0)	0.62	0.4312
	5	rs8014363	A	300 (49.3)	235 (47.6)	0.34	0.5585
HTG+NTG	1	rs11623717	A	366 (61.4)	329 (60.0)	0.23	0.6347
	2	rs17563	A	354 (61.0)	298 (58.0)	1.06	0.3036
	3	rs2761884	T	300 (50.0)	243 (44.3)	3.68	0.0552
	4	rs8014071	C	384 (64.0)	344 (63.0)	0.12	0.7264
	5	rs8014363	A	278 (49.3)	235 (47.6)	0.31	0.5765

Table 6–3: Distribution of *FOXC1* tSNPs showing no significant effects between HTG, OHT, NTG, HTG+OHT and HTG+NTG, compared to the wild type control group.

All of the columns were generated using Haploview v.4.0.

Phenotype	tSNP	SNP ID	Allele	Case Counts (%)	Control counts (%)	Chi-square	*P* value
HTG	1	rs2235715	T	192 (88.9)	320 (83.3)	3.41	0.0648
	2	rs2569889	C	260 (49.6)	259 (48.7)	0.09	0.7614
	3	rs2235716	T	363 (69.3)	350 (65.1)	2.14	0.1433
	4	rs984253	T	185 (35.7)	183 (34.4)	0.20	0.6550
OHT	1	rs2235715	C	14 (21.2)	64 (16.7)	0.81	0.3675
	2	rs2569889	G	59 (52.7)	273 (51.3)	0.07	0.7931
	3	rs2235716	C	41 (37.3)	188 (34.9)	0.22	0.6416
	4	rs984253	T	39 (34.8)	183 (34.4)	0.01	0.9318
NTG	1	rs2235715	C	11 (21.2)	64 (16.7)	0.65	0.4210
	2	rs2569889	G	37 (54.4)	273 (51.3)	0.23	0.6305
	3	rs2235716	C	28 (40.0)	188 (34.9)	0.69	0.4057
	4	rs984253	A	46 (65.7)	349 (65.6)	0.00	0.9851
HTG+OHT	1	rs2235715	T	244 (86.5)	320 (83.3)	1.28	0.2585
	2	rs2569889	C	313 (49.2)	259 (48.7)	0.03	0.8569
	3	rs2235716	T	432 (68.1)	350 (65.1)	1.25	0.2643
	4	rs984253	T	224 (35.6)	183 (34.4)	0.17	0.6804
HTG+NTG	1	rs2235715	T	233 (86.9)	320 (83.3)	1.60	0.2067
	2	rs2569889	C	291 (49.2)	259 (48.7)	0.03	0.8746
	3	rs2235716	T	405 (68.2)	350 (65.1)	1.24	0.2651
	4	rs984253	T	209 (35.5)	183 (34.4)	0.16	0.6881

6.2.2 Absence of association between haplotypes in *TGFβ2* and *BMP4* and HTG

The difference in the distribution of all common haplotypes in *TGFβ2* and *BMP4* between individuals with HTG and controls (Table 6-4 and 6-5) was assessed (but not for *FOXC1* as common haplotypes were not present) and no significant haplotype associations were identified for each haplotype blocks. The absence of common haplotypes between patients and *FOXC1* can be explained by the small size of the FOXC1 gene (3,447 bp) which makes it less likely for the presence of any haplotype blocks in such a small region.

Table 6–4: Distribution of *TGFβ2* haplotypes showing no significant effects between HTG cases and controls.

All of the columns were generated using Haploview v.4.0.

Block	Haplotype	All subjects (%)	Case (%)	Control (%)	Chi-square	*P* value
1 (tSNPs 1 to 4)	GAGC	42.2	42.9	41.6	0.19	0.6657
	TAGC	18.8	17.3	20.2	1.56	0.2112
	TAGA	18.3	19.1	17.5	0.49	0.4841
	GAAC	14.2	13.4	15.1	0.67	0.4123
	TGGA	6.5	7.4	5.6	1.39	0.2379
2 (tSNPs 8 to 11)	TCAA	40.1	40.1	40.1	0.00	0.9891
	CCAA	28.4	28.3	28.5	0.01	0.9386
	CTCG	21.5	21.2	21.9	0.08	0.7746
	CTCA	6.0	6.3	5.7	0.20	0.6592
	CCCA	3.0	2.9	3.2	0.10	0.7473
3 (tSNPs 14 to 15)	TG	77.4	76.5	78.3	0.47	0.4950
	CA	16.2	16.0	16.5	0.05	0.8175
	CG	6.3	7.5	5.2	2.32	0.1280
4 (tSNPs 17 to 19)	CAT	51.1	51.1	51.1	0.00	0.9927
	CGT	22.1	22.7	21.5	0.21	0.6462
	TGC	21.2	19.8	22.6	1.28	0.2581
	CGC	5.5	6.2	4.7	1.16	0.2809

Table 6–5: Distribution of *BMP4* haplotypes showing no significant effects between HTG cases and controls.

All of the columns were generated using Haploview v.4.0.

Block	Haplotype	All subjects (%)	Case (%)	Control (%)	Chi-square	*P* Value
1 (tSNP 1 to 2)	AA	57.9	59.1	56.9	0.53	0.4652
	GG	37.1	35.3	38.9	1.47	0.2255
	AG	3.2	3.2	3.2	0.00	0.9555
	GA	1.7	2.4	1.1	2.73	0.0985
2 (tSNP 3 to 5)	TCA	46.1	48.0	44.3	1.55	0.2131
	GTG	35.6	34.2	36.9	0.87	0.3506
	GCG	14.9	13.8	5.9	0.92	0.3389
	GCA	2.2	1.6	2.8	1.86	0.1722

6.2.3 Absence of association between haplotypes in *TGFβ2* and *BMP4,* and raised IOP (HTG+OHT)

Here, assessment for possible common haplotype associations between combined raised IOP patient group (HTG+OHT) and the controls were performed for *TGFβ2* (Table 6-6) and *BMP4* (Table 6-7) (but not for *FOXC1* as common haplotypes were not present) and no significant haplotype effects for each haplotype blocks were found.

Table 6–6: Distribution of *TGFβ2* haplotypes showing no significant effects between HTG+OHT cases and controls.

All of the columns were generated using Haploview v.4.0.

Block	Haplotype	All subjects (%)	Case (%)	Control (%)	Chi-square	*P* Value
1 (tSNPs 1 to 4)	GAGC	42.9	44.0	41.6	0.70	0.4035
	TAGC	18.6	17.1	20.3	1.92	0.1664
	TAGA	17.8	18.1	17.4	0.10	0.7500
	GAAC	14.3	13.7	15.1	0.48	0.4866
	TGGA	6.3	6.9	5.6	0.82	0.3659
2 (tSNPs 8 to 11)	TCAA	39.8	39.6	40.1	0.03	0.8680
	CCAA	28.7	28.8	28.5	0.01	0.9220
	CTCG	21.4	20.9	21.9	0.15	0.6992
	CTCA	6.0	6.3	5.7	0.22	0.6401
	CCCA	3.2	3.1	3.2	0.00	0.9491
3 (tSNPs 14 to 15)	TG	76.9	75.7	78.3	1.08	0.2979
	CA	16.4	16.4	16.5	0.00	0.9485
	CG	6.7	7.9	5.2	3.44	0.0636
4 (tSNPs 17 to 19)	CAT	51.0	50.9	51.1	0.01	0.9415
	CGT	22.5	23.3	21.5	0.54	0.4606
	TGC	20.9	19.4	22.6	1.89	0.1691
	CGC	5.6	6.3	4.7	1.29	0.2556

Table 6–7: Distribution of *BMP4* haplotypes showing no significant effects between HTG+OHT cases and controls.

All of the columns were generated using Haploview v.4.0.

Block	Haplotype	All subjects (%)	Case (%)	Control (%)	Chi Square	*P* Value
1 (tSNP 1 to 2)	AA	58.1	59.2	56.9	0.63	0.4270
	GG	37.0	35.5	38.9	1.47	0.2252
	AG	3.1	3.1	3.2	0.01	0.9151
	GA	1.7	2.3	1.1	2.62	0.1055
2 (tSNP 3 to 5)	TCA	46.2	47.9	44.2	1.58	0.2084
	GTG	35.2	33.8	36.9	1.29	0.2565
	GCG	15.0	14.3	15.9	0.62	0.4322
	GCA	2.1	1.5	2.8	2.54	0.1112

6.2.4 Absence of associations between haplotypes in *TGFβ2* and *BMP4*, and glaucoma (HTG+NTG)

Finally, none of the common haplotypes within each haplotype block showed any significant difference in distribution between the combined POAG patients group (HTG+NTG) and the controls for *TGFβ2* (Table 6-8) and *BMP4* (Table 6-9).

Table 6–8: Distribution of *TGFβ2* haplotypes showing no significant effects between HTG+NTG cases and controls.

All of the columns were generated using Haploview v.4.0.

Block	Haplotype	All subjects (%)	Case (%)	Control (%)	Chi Square	*P* Value
1 (tSNPs 1 to 4)	GAGC	42.3	42.9	41.6	0.21	0.6473
	TAGA	18.5	19.4	17.5	0.71	0.3986
	TAGC	18.3	16.4	20.2	2.78	0.0955
	GAAC	14.4	13.8	15.1	0.43	0.5147
	TGGA	6.6	7.5	5.6	1.65	0.1993
2 (tSNPs 8 to 11)	TCAA	40.0	39.8	40.1	0.01	0.9284
	CCAA	28.5	28.6	28.5	0.00	0.9873
	CTCG	21.3	20.8	21.9	0.20	0.6516
	CTCA	6.2	6.7	5.7	0.54	0.4620
	CCCA	3.1	3.0	3.2	0.04	0.8517

3 (tSNPs 14 to 15)	TG	77.5	76.9	78.3	0.34	0.5629
	CA	16.0	15.6	16.5	0.19	0.6662
	CG	6.4	7.6	5.2	2.65	0.1037
4 (tSNPs 17 to 19)	CAT	51.1	51.1	51.1	0.00	0.9979
	CGT	22.0	22.5	21.5	0.15	0.7006
	TGC	21.3	20.2	22.6	1.01	0.3150
	CGC	5.4	6.1	4.7	0.97	0.3260

Table 6–9: Distribution of *BMP4* haplotypes showing no significant effects between HTG+NTG cases and controls.

All of the columns were generated using Haploview v.4.0.

Block	Haplotype	All subjects (%)	Case (%)	Control (%)	Chi Square	*P* Value
1 (tSNP 1 to 2)	AA	57.7	58.5	56.9	0.30	0.5857
	GG	37.6	36.4	38.9	0.77	0.3815
	AG	3.0	2.8	3.2	0.09	0.7592
	GA	1.7	2.3	1.1	2.54	0.1112
2 (tSNP 3 to 5)	TCA	46.2	47.9	44.2	1.55	0.2126
	GTG	36.0	35.1	36.9	0.40	0.5256
	GCG	14.4	13.1	15.9	1.78	0.1827
	GCA	2.1	1.6	2.8	2.10	0.1474

6.3 Discussion

Despite recent progress in identifying genes associated with glaucoma, the pathogenesis of POAG continues to remain unclear. Given the relatively high prevalence of POAG within the normal population, and the fact that it is amendable to treatment when detected early, identification of genetic risk factors would offer the prospect of early POAG diagnosis and a significant reduction in the health care expenditure. However, such screening programmes are currently limited by the paucity of the identified causative genes (Fan *et al.*, 2005) and identification of the most significant disease-associated alleles in different populations is of paramount importance.

Genes that cause developmental glaucoma (Gould *et al.*, 2004), with the exception of the *CYP1B1* gene (Bhattacharjee *et al.*, 2008; Hattula and Peranen, 2000; Melki *et al.*,

2005) and LMX1B (see Chapter 5), have yet to be assessed as genetic susceptibility factors for POAG. Existing studies indicate that *TGFβ2* and *BMP4* act in concert to maintain a balance between extracellular matrix (ECM) deposition and degradation (Wordinger *et al.*, 2007), and may play an role in glaucoma pathogenesis through mis-regulation of ECM synthesis and cross-linkage of ECM components of the TM. All of these 3 genes (*TGFβ2, BMP4* and *Foxc1*) are expressed in the TM and cause developmental malformation of the anterior segment, and *FOXC1* in particular, is associated with a high incidence of glaucoma to about 75% or above (Strungaru *et al.*, 2007). Hence, taking these into account, *TGFβ2, BMP4* and *FOXC1* appear to be strong candidates for glaucoma susceptibility.

The most prevalent subgroup of POAG, the HTG group, is characterised by glaucomatous damage, and raised IOP. The other two patient groups each have only one of these characteristics, either glaucomatous damage without raised IOP (NTG), or raised IOP without glaucomatous damage (OHT). In order to test the relationship between the variant alleles of *TGFβ2, BMP4* and *FOXC1* and these two characteristics the patients have been grouped into those with raised IOP (HTG and OHT), or those with glaucomatous damage irrespective of IOP (HTG and NTG).

In this study, no significant associations were identified between *TGFβ2, BMP4* and *FOXC1* alleles and haplotypes and POAG. This represents the first association analysis of *TGFβ2, BMP4* and *FOXC1* and the lack of association of common polymorphism suggests that these genes do not have a significant genetic role in the pathogenesis of glaucoma among white British subjects.

A negative association, however, should be interpreted with caution unless proven by a replication study or by investigating a substantially larger sample of the population. This is because of the small possibility of such results being caused by a false-negative error. To illustrate this further, both a study power of 80% and a r^2 threshold of > 0.8 would allow detection of an existing association in 80% of cases (i.e. there is a 20% chance of missing a true association) and the selected tSNP to resolve >80% (but not 100%) of all existing haplotypes respectively. Hence the possibility cannot be excluded, albeit being small, of missing a true association despite careful attention to study design. In addition, the results obtained from this study reflect only one ethnic group (in this

case white British adults) and not other ethnicity and it would be interesting to see if other ethnic groups showed the same negative results.

In summary, this study is unable to demonstrate any significant allelic or haplotype associations between *TGFβ2, BMP4* and *FOXC1* and OHT/POAG. It is hence unlikely that *TGFβ2, BMP4* and *FOXC1* play a role in the genetic aetiology of POAG.

Chapter 7 General discussion

7.1 Highlights of the work presented

It has been proposed that POAG in the majority of cases, is inherited as a complex trait, where the disease results from the interaction of multiple genes and environmental factors (Fan *et al.*, 2006b; Sieving and Collins, 2007). It is a complex disorder which results from diverse pathological process that is not just limited to involving the aqueous humor outflow, but the RGC, the optic nerve and even as suggested recently, the cerebrospinal fluid (Berdahl *et al.*, 2008).

Mutations in the three known POAG genes (*MYOC, OPTN* and *WDR36*) account for no more than 10% of all POAG patients and hence it is highly likely that other unidentified loci or genes still exist (Fan *et al.*, 2006b). An obvious group of genes to test as genetic susceptibility factors for POAG are the developmental glaucoma genes which have not been analysed in any association studies to date with the exception of the *CYP1B1* gene. They play an important role in normal morphogenesis of the anterior segment and provide important insight into the underlying genetic mechanism of glaucoma. Since ASD leads to malformation of the drainage structures and elevation of IOP, patients with ASD are consequently at an increased risk for developing glaucoma in 33-75% of cases. It is possible that some of the developmental glaucoma genes contribute to age-related open angle glaucoma, where the ocular drainage structures have abnormalities that are not clinically visible but which cause dysfunction with age. The present study has examined the hypothesis that sub-clinical mutations/polymorphisms in *LMX1B, FOXC1, TGFβ2* and *BMP4* may produce subtle and undetected abnormalities in anterior segment structure and function, which predispose to glaucomatous optic neuropathy through the effects of raised IOP and may be a significant susceptibility factor for the development of OHT and POAG. In addition, the cohort of white British individuals recruited for this study has provided an ideal opportunity to assess further the role of *OPTN* E50K mutation in unrelated POAG/OHT cases.

7.1.1 Assessment of study design and data quality

One fundamental problem with a genetic association study is the ability to distinguish causal from spurious association. To help distinguish this, tight standards for statistical significance were established for this study (Silverman and Palmer, 2000).

7.1.1.1 Biological plausibility of candidate genes

Developmental glaucoma genes *LMX1B, TGFβ2, BMP4* and *FOXC1* provide a unique entry point for studying POAG as they have demonstrated that they are biologically plausible as candidate genes for POAG. In addition, the associated glaucoma can present at any time from birth to adulthood and in some affected family members with developmental glaucoma, the phenotype of the anterior segment malformation can be very subtle and easily missed, both features of which further support the biological plausibility of these gene as candidates for POAG.

7.1.1.2 Classification of cases and controls

Mis-classification of recruited individuals can markedly reduce study power and bias the results towards lack of association. Hence, to avoid this from occurring, all participants (affected as well as controls) underwent a complete ophthalmic examination; the clinical diagnosis was made by the same consultant with a special interest in glaucoma and experience in anterior segment phenotyping. This ensured exclusion of individuals with glaucoma from the control group and made certain that cases were correctly classified either as HTG, OHT and NTG.

In addition, individuals with borderline IOPs whose diagnosis would be uncertain were excluded from this study.

7.1.1.3 Confounding factors; population stratification, age and sex

Since population stratification is known to have major implications for the validity of genetic association studies (Hoggart *et al.*, 2003), the cases and controls were carefully matched for ethnicity. Population stratification refers to differences in allele frequencies between cases and controls due to systematic differences in ancestry rather than

association of genes with the disease, hence giving rise to a false positive association. The patients and controls enrolled in this study were of Caucasian origin and from the same geographical region from the North-East of England. However, cases and controls were not matched for sex (for all groups except between OHTs and controls) and age (for the OHTs and controls). This study has addressed this problem when assessing the prevalence of genetic variants between these specific groups by calculating *P* values that have been adjusted either for sex and age.

7.1.1.4 Selection of informative tSNPs and assessment of Hardy-Weinberg equilibrium

In order to select a good subset of tSNPs from an extensive SNP set, tSNPs should ideally have a high degree of polymorphism and be able to capture the majority of the diversity within a region without having to genotype all the markers. Hence for this study, SNPs with a MAF of ≥10% with a minimum r^2 of 0.8 were chosen. This allowed for the maximization of information content and minimization of the sample size without losing the power to detect genetic association. In addition, since deviation of allele frequencies from those expected from HWE can be as a result of systematic genotyping errors, such SNPs were excluded from this study.

7.1.1.5 Low genotyping errors with good quality control

Genotyping errors, especially if they occur differentially between cases and controls, are an important cause of spurious associations. This study has shown a good genotyping success rate varying from 92% and 99%. In addition, a quality control measure was applied, whereby, selected primer assays were tested on CEPH DNAs and the genotypes compared with that of the HapMap data. In doing so, it was possible to assess whether the results were either concordant or disconcordant with the HapMap data, with any assays producing disconcordant results being excluded from the study.

7.1.1.6 Adjustment for multiple testing

Since the incidence of false positive error is proportional to the number of significance tests performed, multiple testing can lead to a potential increase in false-positive error.

Hence, in order to correct for any spurious association that can arise as a result of this, permutation testing was performed with 1,000 simulations for any results with a significant *P* value.

7.1.2 Absence of *OPTN* E50K mutation among HTG and OHT, in addition to NTG

This study has investigated the contribution of *OPTN* E50K mutation to unrelated POAG/OHT cases of white British origin. Based on the current literature on *OPTN* E50K mutation, it can be concluded that OPTN may have a protective role in the anterior segment during raised IOP through its effect on the TM (Vittitow and Borras, 2002) and its interaction with *MYOC* (Park *et al.*, 2007), as well as a neuroprotective role in the posterior segment through affecting the TGFα signaling pathway. However, this study has demonstrated an absence of the E50K mutation in line with existing reports (Ariani *et al.*, 2006; Baird *et al.*, 2004; Funayama *et al.*, 2004; Fuse *et al.*, 2004; Leung *et al.*, 2003; Lopez-Martinez *et al.*, 2007; Sripriya *et al.*, 2006; Tang *et al.*, 2003; Toda *et al.*, 2004; Umeda *et al.*, 2004; Wiggs *et al.*, 2003).

7.1.3 Genetic risk for primary open angle glaucoma determined by *LMX1B* haplotypes

This study has assessed whether variants of *LMX1B* play a role in POAG susceptibility in the general population and has identified significant associations between *LMX1B* alleles and haplotypes in intron 2 and POAG. Pathogenic mutations within *LMX1B* cause a rare autosomal condition, NPS, in which the prevalence of OAG, OHT and NTG were 11.1% (Mimiwati *et al.*, 2006), 11.1% (Mimiwati *et al.*, 2006) and 7.8% (Bongers *et al.*, 2005) respectively. Moreover, the mean age of NPS cases with OAG or OHT was 63.4 (range 55-72) (Bongers *et al.*, 2005) and 47.9 years (range 23-78) respectively (Sweeney *et al.*, 2003), a feature more consistent with POAG. In this case-control genetic association study of adult onset POAG patients in the general population, there was a significant under representation of two *LMX1B* haplotypes among individuals with POAG compared to a control population, consistent with a 0.3 fold decreased risk of developing POAG in individuals carrying these haplotypes. In

mice, *Lmx1b* is expressed in the TM and ciliary body, the sites of aqueous drainage and production respectively from embryonic day 10.5 and continues to be expressed at high levels post-natally (Pressman *et al.*, 2000). Functional polymorphic variation within the gene may therefore play a role in influencing IOP. Together these data suggest that the developmental homeobox gene transcription factor *LMX1B* may influence the risk of POAG in the general population and is in agreement with the hypothesis that subtle abnormalities in the anterior segment structure and function may predispose to POAG. In addition, the effects of *LMX1B* on glaucoma susceptibility may involve functions that are broader than effects on IOP.

With regards to the downstream targets of LMX1B, the current understanding is that *LMX1B* may have a role in POAG pathogenesis through its effect on the regulation of ECM. Recently Gould et al provided new insight into the possible role of *Lmx1b* in glaucoma pathology by showing mutations in the collagen *Col4a1* gene resulted in ASD with optic nerve hypoplasia, with raised as well as low IOP (Gould *et al.*, 2007). Although there is not yet evidence that *Lmx1b* regulates *Col4a1*, LMX1B has shown to play an important role in the expression of basement membrane collagens *Col4α3* and *Col4α4* (Morello *et al.*, 2001; Rohr *et al.*, 2002), a major constituent of ECM. Considering the important developmental contribution of ECM to the formation of the TM (reviewed by (Acott and Kelley, 2008; Gould *et al.*, 2004), altered ECM regulation within the TM as a result of mutations in *LMX1B* may predispose to glaucoma.

7.1.4 Variants of *TGFβ2*, *BMP4* and *FOXC1* do not confer susceptibility to POAG

In this study, variants of *TGFβ2*, *BMP4* and *FOXC1* were assessed to see if they conferred susceptibility to POAG, but no significant associations were identified. Existing studies indicate that *TGFβ2*, *BMP4* and *FOXC1* are strong candidate genes for POAG susceptibility. In particular, *TGFβ2* and *BMP4* act in concert to maintain a balance between ECM deposition and degradation (Wordinger *et al.*, 2007), and may play an role in glaucoma pathogenesis through mis-regulation of ECM synthesis and cross-linkage of ECM components of the TM. All of these 3 genes (*TGFβ2, BMP4* and *Foxc1*) are expressed in the TM and cause developmental malformation of the anterior segment, and *FOXC1* in particular, is associated with a high incidence of glaucoma to

about 75% or above (Strungaru *et al.*, 2007). However, despite this evidence in favour of these genes as candidates for POAG, no significant allelic or haplotype associations with POAG were demonstrated by this study.

7.2 Limitations of study

7.2.1 Under representation of NTG

The key determinant of quality in an association study is the sample size since the power to detect an association depends partly on this. For this study, with a sample size of 276 controls and 367 cases, an adequate study power of 80% was achieved if a difference in genotype and allele frequency was 10-18% between controls and cases at a significance level of $P < 0.05$. However, if the individual subgroups are considered, the NTG group ($n = 37$) was clearly under-represented, despite being adequate to produce a robust result as being part of the whole cohort. Hence, to improve the chances of detecting genetic variants that are associated with the NTG cases, the sample size of the relatively small NTG group should be increased, and this was achieved in the replication study where the number of NTG cases was increased to a total of 222 individuals.

7.2.2 Absence of central corneal thickness measurement

Another limitation of this study is the absence of CCT measurement. CCT variation is known to affect the accuracy of IOP readings on applanation tonometry, with thick corneas giving falsely high IOP readings and thin corneas giving falsely low readings (Feltgen *et al.*, 2001; Whitacre *et al.*, 1993). In the current study, IOP measurements were checked by a Tono-Pen which is less affected by CCT (Bhan *et al.*, 2002), in addition to performing applanation tonometry. Although normagrams, based on varying CCT, exist for adjusting IOP readings (Ehlers *et al.*, 1975; Stodtmeister, 1998; Whitacre *et al.*, 1993), as yet, there is no generally accepted correction formula (Brandt, 2004). However, two recent studies that adjusted IOP for CCT found that the correction did not alter the diagnosis of HTG or NTG (Miyazawa *et al.*, 2007), and did not affect the relationship between the prevalence of POAG and IOP respectively (Francis *et al.*, 2008). Furthermore, to be certain that the participants were correctly assigned to the

appropriate case groups, individuals with borderline IOPs (21–23 mmHg) were excluded from this study. Even if a correction formulae were to be applied with a 10 μm change in the corneal thickness inducing a 0.2 mmHg change in IOP reading (Doughty and Zaman, 2000), a 2-3 mmHg IOP change (which would include the excluded individuals within the borderline IOP) would induce a 100 - 150 μm change in the CCT, which is a considerable amount. Hence it is still highly unlikely that the individuals with IOPs of 20 mmHg or below or IOPs of 24 mmHg or higher would have their diagnosis altered (assuming that the average CCT is approximately 537 – 550 μm) (Shah *et al.*, 1999; Wolfs *et al.*, 1997) since these subjects would be require to have either an abnormally thin corneas or an unusually thick corneas.

Several studies have shown that increased CCT may occur in certain types of dominantly inherited developmental glaucoma, including iris hypoplasia (*FOXC1* duplication) (Lehmann *et al.*, 2003b), aniridia (*PAX6/Pax6*) (Brandt *et al.*, 2004; Ramaesh *et al.*, 2003) and dysgenic lens (*Foxe3*) (Lehmann *et al.*, 2003b). Conversely, a reduced corneal thickness was associated with Axenfeld-Rieger malformation of the anterior segment from *PITX2* mutation (Asai-Coakwell *et al.*, 2006). A thinner CCT was also found to be a risk factor for developing POAG in individuals with OHT (Gordon *et al.*, 2002). CCT data was not collected for the cases and controls in the current study since a pachymeter was not available at the time when this study was carried out. However, the analysis of the relationship between *LMX1B* haplotypes and CCT will be important in future work, as like these other developmental glaucoma genes, *LMX1B* may affect CCT.

7.2.3 Arbitrary assessment of visual field defect

The third limiting factor of this study is the lack of objective assessment of the severity of Humphrey visual fields using global indices (mean deviation, pattern standard deviation, corrected pattern standard deviation). However, all of the visual field tests showed reproducible field defects that were compatible with the degree of glaucomatous cupping of the optic nerve head (defined by loss of neuroretinal rim), and were ensured to have a satisfactory reliability score of ≤20% fixation loss, false positive of ≤33% and/or false negative of ≤33%.

7.2.4 Arbitrary assessment of cup-disc ratio

The final limiting factor of this study is the lack of objective measurement of the C/D ratio as a measure of the severity of the disease, as clinical assessment of the C/D ratio can be quite subjective depending on the observer. Subsequent to the collection of data for this study, newer imaging methods of the optic nerve have been developed, in the form of Ocular Coherence Tomography and the Zeiss GDx nerve fibre analyser test. These are superior diagnostic tools, which allow earlier detection of structural damage, and provide a more objective measurement of the C/D ratio for research purposes. However, all of the recruited patients had photographs of the optic disc, which provides some measure of objectivity, as well as being carefully examined by a consultant ophthalmologist with a special interest in glaucoma and experience in glaucoma phenotyping.

The definition for POAG should be ideally based on an objective criterion and not on clinical assessment only. Digital photographs of the optic discs, together with imaging reports of sophisticated instruments such as the nerve fibre analyser or the OCT, should be assessed by experts in reference centres, and ideally by the same observer, to assure firm and unbiased diagnosis as well as limiting inter-observer variability.

7.3 Current and future status in glaucoma research

With recent advances in high through-put genotyping, it is now possible to genotype one million SNPs on large datasets containing thousands of samples within a few days. Through this approach, common disease-associated genetic variants in chronic disorders including diabetes mellitus, cancer and heart disease have been identified (Feero *et al.*, 2008). In Ophthalmology, common genetic variants that predispose to pseudoexfoliation syndrome (*LOXL1)* have been recently identified by genome-wide association studies (Thorleifsson *et al.*, 2007).

Currently, only a small percentage of POAG is accounted for by genetic mutations. On the whole, there is still much to learn of the complex the genetics of POAG and even less insight of the underlying cellular biology. Linkage and association studies in large families have been essential and have accelerated genetic research into POAG. To

further increase the chances of detection of glaucoma gene, animal experiments and genomics approaches are crucial components to human studies.

7.3.1 Human genetics

The polygenic complexity of POAG has potential to confound genetic association studies when assessing candidate genes. This can be minimized by implementing the full array of genetic approaches, including linkage analysis, candidate gene analysis and well-designed association studies. Given the likelihood of multifactorial interaction, it will be important to evaluate the roles of multiple genes or loci even when a single mutant gene is identified. Assessment of a large cohort of POAG individuals will provide greater statistical power and will assist detection of multifactorial interactions. It will be crucial to take note of environmental history and lifestyle data, in addition to family history of glaucoma and medical information. Whilst protecting individual's confidentiality, it will be important to gather the genotype and any relevant data into a large shared database. This will aid progress by firstly allowing further assessment by other researchers using different techniques, and secondly by increasing the power of future human genetic analyses.

7.3.2 Animal genetics

Due to the complex nature of POAG in which there are no clear pattern of Mendelian inheritance animal experiments will be an essential complement to human studies. Animal studies offer the ability to study the effects of specific alleles in defined genetic backgrounds and controlled environments and will be extremely valuable (John *et al.*, 1999). To date, the mouse has been the best suited mammalian model for assessing the complex genetics of POAG (John *et al.*, 1999). Although mouse studies have only marginally played a role towards the understanding of POAG, they will become an essential aspect of glaucoma research in the future. Mouse models with potential relevance to POAG include the *Col1a1* mutant mouse and the C57BL/6J mouse. The *Col1a1* mutant mouse has a mutation for the α1 subunit of collagen 1, a component of outflow pathway ECM, and has developed elevated IOP with almost 25% loss of their RGC (Mabuchi *et al.*, 2004). The C57BL/6J mouse strain has extensive RGC loss in the presence of normal IOP and may be useful for studying NTG (Danias *et al.*, 2003).

7.3.3 Genomics

Genomics can be applied to human tissue samples in addition to tissues from various model systems that are used to assess specific aspects of glaucomatous pathophysiology. Difference in the expression patterns between patients and controls will help to prioritise the assessment of candidate genes. For example, microarray analysis is being utilised to identify the molecular pathways involved in raised IOP in cultured TM cells and to identify changes in gene expression in response to raised IOP (Borras, 2003; Ishibashi *et al.*, 2002). Identifying differences in expression patterns for specific genes will be helpful, especially when these genes are located within the implicated chromosomal regions. Genomics methods are also utilised to assess neuronal responses to elevated IOP in rats and primates (Ahmed *et al.*, 2004; Miyahara *et al.*, 2003).

7.4 Concluding remarks

Genes that contribute to POAG may influence the regulation of IOP or ganglion cell viability, or even both as suggested by the results of this study on *LMX1B.* Specifically, this study showed that variants of *LMX1B* are associated with both NTG and HTG and are correlated with a 0.3 fold decreased risk of developing POAG in the first cohort. This is in agreement with the idea that these two conditions HTG and NTG may simply be phenotypic variations of the same disease, rather than separate entities. However, these findings were not replicated in the smaller and ethnically more diverse cohort and further investigations are needed to fully understand the role of *LMX1B* in POAG.

Clearly, the current understanding of the underlying genetic architecture of a complex inherited disorder such as POAG continues to be limited, with the genetic basis of the majority of glaucoma cases still largely unknown. It is highly probable, as in many complex inherited disorders, the condition result from the independent actions of multiple genes as well as from interactions of multiple genes. As shown by this study, with the implementation of SNP-based technologies, a substantial increase in research and understanding into the molecular basis of complex inherited disorders is underway. The ultimate goal would be to discover a complete panel of genes that contribute to glaucoma and acquire diagnostic and prognostic correlates for the mutations.

7.5 Glaucoma screening

One of the goals of identifying disease gene(s) is the development of predictive diagnostic tests. For a disease such as POAG, where early treatment is essential, diagnostic tests aimed at identifying individuals at risk of developing POAG will be valuable. Currently, identification of new glaucoma cases is achieved by routine screening which involves evaluation of the optic disc, IOP measurement and assessment of visual field deficit. However, such methods have not been shown to be cost effective (Tuck and Crick, 1997). Furthermore, these methods have provided at best a 'snap shot' where a negative result applies to a single time point. Hence many people are repeatedly reviewed unnecessarily, the majority of whom may never develop the disease of concern, and with a high likelihood of missing incident disease. A screening regimen should be highly sensitive and specific so that it only detects potentially serious disease and not so called 'pseudo-disease' (Harris, 2005). Since POAG is initially an asymptomatic disease, effective screening techniques should identify at risk individuals so that early treatment could be initiated before irreversible optic nerve damage occurs.

Establishment of a genetic screening program offers many advantages and these are as follows (Pang, 1998):

1) It provides an unequivocal test of high specificity,
2) It enables identification of non-symptomatic carriers, who are at risk of developing POAG,
3) And it provides information for genetic counseling.

However, to establish a molecular diagnostic service, the disease-causing mutations must be identified. Furthermore:

1) the clinical information about the onset of disease, course of disease and response to therapy needs to be collected,
2) the prevalence of the mutations in the population must be known to obtain a predictive value of the genetic testing,
3) and the pattern of inheritance of these mutations must be clarified in order for the genetic data to be used for genetic counseling (Pang, 1998).

Current genetic screening for glaucoma genes is limited to genes that are already recognized to be associated with POAG (mainly *MYOC*) (Fan *et al.*, 2006a) and is primarily diagnostic, rather than prognostic. With respect to *OPTN* E50K mutation, since this mutation has never been detected in unaffected controls, diagnostic testing of sporadic NTG cases for E50K mutation may be of value in the UK population. However, the striking difference in the prevalence between familial and sporadic cases should favor the implementation of targeted diagnostic testing for individuals with a family history of NTG.

Commercially available kits that have been utilized to screen for MYOC mutation (such as Ocugene test from InSite Vision) have also yielded low sensitivity as it tests for only 3 out of the many identified disease causing variations (Alward *et al.*, 2002), and demonstrates the current drawbacks of genetic screening for POAG. However, once it reaches a sufficient level of accuracy, genetic screening would provide a focused delivery of medical resources to a smaller proportion of at risk populations.

7.6 Future work

The main finding from this study is that variants of *LMX1B* influence the genetic risk for POAG. All data presented here will form the basis for future studies for further assessment of the association between *LMX1B* and POAG.

Now that a potential new gene, *LMX1B*, is shown to be associated with POAG, the future aim will be to isolate the causative variant(s) by identifying all of the functional SNP(s) that influence the risk of developing POAG through fine mapping the *LMX1B* locus. This can be achieved by identifying potentially functional genetic variation at the *LMX1B* locus by sequencing all of the exons, splice sites and in particular, conserved non-coding sequences, including the proximal promoter region, as well as within and around the gene. Approximately 2.7kb upstream of the start of transcription of *LMX1B* has been shown to contain elements involved in the regulation of gene expression, in addition to several regions of high conservation (greater than 85% sequence identity) between human and mouse within intron 2, suggestive of a role in gene regulation (Dunston *et al.*, 2004). In order to assess how many patients are required to achieve sufficient power to identify the majority of the polymorphic SNP(s), it is estimated that

sequencing a panel of 24 POAG patients should be able to achieve a power of >90% to detect all of the SNPs with a MAF of 5%. In doing so, there should be sufficient power to identify all SNPs with sufficient power to detect a significant association. In order to ascertain the resultant new haplotype structure that will include these additional polymorphic SNP(s), any novel variants that are identified will be combined together with those previously genotyped SNPs. Following this, the additional SNPs that have not already been genotyped and which are required to capture all of the remaining variation will be genotyped in the remaining cohort of 343 cases and all of 276 controls to test for genetic association. This should assist the rapid detection of all potential functional variants underlying the risk/protective haplotype.

Functional analysis of these associated coding and/or non-coding SNPs could then be performed in order to isolate the causative variant(s) affecting the risk of developing POAG. This can be achieved in two ways. Firstly, identified non-synonymous amino acid variants can be investigated using DNA binding assays (e.g. electrophoretic mobility shift assay: this is an electrophoretic separation of a protein-DNA or protein-RNA mixture on a polyacrylamide or agarose gel. It is a common affinity electrophoresis technique used to study protein-DNA or protein-RNA interactions) and reporter assays to test differences between protein variants to activate transcription of artificial reporter genes and putative biological targets such as *COL4A3* and *COL4A4*. Secondly, in order to assess whether non-coding variants are associated with altered expression levels of mRNA and protein levels of *LMX1B* and downstream target genes such as *COL4A4*, cadaver tissue and other appropriate cell lines can be analysed. RNA and protein can be extracted from genotyped tissue samples. This will be followed by application of real time quantitative PCR to assess for mRNA expression levels. Extracted proteins can be analysed by Western blotting for assessment of protein expression levels. In doing so, allelic differences in the expression levels of mRNA and proteins could be identified and therefore ascertain the responsible non-coding variants.

Chapter 8 Appendices

8.1 Appendix I: Patient information sheet for control subjects

Mr Soo Park **Tel: 0191 565 6256** **Version 5.0**
Mr Scott Fraser
Dr Jane Sowden

PATIENT INFORMATION SHEET

Searching for Genes for Glaucoma- control subjects

Within the department of Ophthalmology we are carrying out research in order to discover the underlying cause of glaucoma.

As part of this study we require a group of patients who do not have glaucoma who are willing to assist us. This group would act as a 'control' sample for comparison with those diagnosed with glaucoma.

Before you decide, it is important for you to understand how the research is being done and what it will involve. Please take time to read the following information carefully. Ask us if there is anything which is not clear or if you would like more information. Take time to discuss this with your relatives and family doctor if you wish, to decide whether or not to take part. If you decide you do not wish to take part please contact us either by phone, email or letter and we will destroy any sample you have donated. We will acknowledge in writing that we have received your notification that you no longer wish to be part of the study. Thank you for reading this information sheet.

What is the purpose of the study?
Glaucoma is the third commonest cause of visual loss in the world. The most recent approach to preventing visual loss is to discover the underlying genes responsible in families and individuals with glaucoma. In order to confirm that a newly discovered gene causes glaucoma we need to compare the findings in patients who do not have glaucoma. Those who do not have glaucoma should not have the same abnormality in the gene.

Why have I been chosen?
You have been asked to help because you have been screened for glaucoma and found to be unaffected. Approximately 400 other people will be asked to assist in this study.

Do I have to take part?
This decision is up to you entirely. If you do decide to take part, please keep this information sheet. You will be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving any reason. This will not affect the standard of care you receive.

What will happen to me if I take part?
A brief summary of your eye findings will be prepared by your ophthalmologist for inclusion in the study. We will ask for a small blood sample taken from an arm by an experienced doctor. This blood sample will be sent to the Institute of Child Health where DNA will be extracted from this sample, and only the genes for glaucoma will be studied. This study may take us 2-3 years, since there are many genes involved.

Are there any side effects of giving a blood sample?
This procedure involves a momentary pain, like a mosquito bite, and may leave a small bruise.

What are the possible benefits of taking part?
We hope to find the cause of glaucoma in families in which there is a genetic cause. This should lead to preservation of vision in a more efficient way than was possible in the past. We also hope that by discovering the underlying genes causing glaucoma, we can use this new knowledge to find a more specific treatment or a screening test to identify those at risk of developing glaucoma. At present, we simply lower the eye pressure; we do not know why the visual loss occurs in the first place.
It is unlikely that you will benefit directly from this research as you do not have glaucoma.

Will my taking part in this study be kept confidential?
We request your permission to restricted access to your medical records and the information collected about you in the course of the study. All information which is collected about you will be kept strictly confidential. Any information about you which is included in a research publication will have all identifying features removed, so that you cannot be recognised from it.

What will happen to the results of the research study?
Once we find a new gene which causes glaucoma, the results will be published in a scientific journal dealing with such information. In this way, it will be made available internationally, and other investigators can look at their glaucoma families to see if they have the same gene. However, the results will not be made available to you unless you have specifically requested it.

You will not be identified in the publication.

Contact for further information?
Please contact your ophthalmologist or a member of the genetics team on the number shown on page 1 for further information. Thank you for taking part in this study. This is your copy of the information sheet.

8.2 Appendix II: Patient information sheet for cases

Mr Soo Park **Tel: 0191 565 6256** **Version 5.0**
Mr Scott Fraser
Dr Jane Sowden

PATIENT INFORMATION SHEET

Searching for Genes for Glaucoma

Within the department of Ophthalmology we are carrying out research in order to discover the underlying cause of glaucoma. Before you decide, it is important for you to understand how the research is being done and what it will involve. Please take time to read the following information carefully. Ask us if there is anything which is not clear or if you would like more information. Take time to discuss this with your relatives and family doctor if you wish, to decide whether or not to take part. If you decide you do not wish to take part please contact us either by phone, email or letter and we will destroy any sample you have donated. We will acknowledge in writing that we have received your notification that you no longer wish to be part of the study. Thank you for reading this information sheet.

What is the purpose of the study?
Glaucoma is the third commonest cause of visual loss in the world. The most recent approach to preventing visual loss is to discover the underlying genes responsible in families and individuals with glaucoma, so that we may test the at-risk individual for visual loss, and treat at the earliest possible moment to prevent such loss. So far, through studying large family members who have inherited glaucoma through generations, 3 inherited genes have been identified which may be involved in glaucoma. However, up to date, several studies have shown that these are rare causes of glaucoma and their role in the underlying cause of glaucoma still remains uncertain. We request your help in discovering the remaining genes.

Why have I been chosen?
Your ophthalmologist has selected you because of your diagnosis of glaucoma. Approximately 400 other people will be asked to assist in this study.

Do I have to take part?
This decision is up to you entirely. If you do decide to take part, please keep this information sheet. You will be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving any reason. This will not affect the standard of care you receive.

What will happen to me if I take part?
A brief summary of your eye findings will be prepared by your ophthalmologist for inclusion in the study. We will ask for a small blood sample taken from an arm by an experienced doctor. This blood sample will be sent to the Institute of Child Health where DNA will be extracted from this sample, and only the genes for glaucoma will be studied. This study may take us 2-3 years, since there are many genes involved.

Are there any side effects of giving a blood sample?
This procedure involves a momentary pain, like a mosquito bite, and may leave a small bruise.

What are the possible benefits of taking part?
We hope to find the cause of glaucoma in families in which there is a genetic cause. This should lead to preservation of vision in a more efficient way than was possible in the past. We also hope that by discovering the underlying genes causing glaucoma, we can use this new knowledge to find a more specific treatment or a screening test to identify those at risk of developing glaucoma. At present, we simply lower the eye pressure; we do not know why the visual loss occurs in the first place.

Will my taking part in this study be kept confidential?
We request your permission to restricted access to your medical records and the information collected about you in the course of the study. All information which is collected about you will be kept strictly confidential. Any information about you which is included in a research publication will have all identifying features removed, so that you cannot be recognised from it.

What will happen to the results of the research study?
Once we find a new gene which causes glaucoma, the results will be published in a scientific journal dealing with such information. In this way, it will be made available internationally, and other investigators can look at their glaucoma families to see if they have the same gene. However, the results will not be made available to you unless you have specifically requested it.

You will not be identified in the publication.

Who has reviewed the study?
This study has been reviewed by your hospital Research Ethics Committee.

Contact for further information?
Please contact your ophthalmologist or a member of the genetics team on the number shown on page 1 for further information. Thank you for taking part in this study. This is your copy of the information sheet.

8.3 Appendix III: Demographic and phenotypic features of control individuals

Table 8–1: Demographic and phenotypic features of control individuals (*n* = 276)

Patient ID	Sex	Age	Family history of glaucoma	Best visual acuity		Highest recorded IOP		Anterior chamber angle	Cup-disc ratio		Surgery
				Right	Left	Right	Left		Right	Left	
1	female	55	nil	6/6	6/6	15	15	open	0.4	0.4	nil
2	female	79	nil	6/9	6/9	15	15	open	0	0	nil
4	female	78	nil	6/9	6/9	10	11	open	0	0	nil
5	female	80	nil	6/9	6/9	16	13	open	0	0	nil
7	female	74	nil	6/9	6/9	19	19	open	0	0	nil
8	male	66	nil	6/6	6/6	13	13	open	0.4	0.4	nil
9	male	67	nil	6/9	6/9	11	11	open	0.5	0.5	nil
10	male	74	nil	6/9	6/9	15	15	open	0	0	nil
11	male	69	nil	6/9	6/9	15	15	open	0	0	nil
12	female	64	nil	6/9	6/9	17	17	open	0	0	nil
14	female	77	nil	6/9	6/6	16	16	open	0.3	0.3	nil
15	female	85	nil	6/9	6/9	17	17	open	0	0	nil
16	female	76	nil	6/9	6/9	15	15	open	0	0	nil
17	female	51	nil	6/12	6/9	17	17	open	0	0	nil
18	female	80	nil	6/9	6/9	16	14	open	0.3	0.3	nil
21	female	71	nil	6/9	6/9	17	16	open	0	0	nil
40	male	78	nil	6/9	6/9	16	15	open	0.5	0.5	nil
41	female	73	nil	6/9	6/9	16	16	open	0.4	0.4	nil
43	female	75	nil	6/6	6/6	16	16	open	0.5	0.5	nil
44	female	72	nil	6/9	6/9	13	13	open	0.5	0.4	nil
45	female	83	nil	6/9	6/9	17	17	open	0	0	nil
47	male	81	nil	6/9	6/9	11	10	open	0.4	0.4	nil
48	male	77	nil	6/9	6/9	14	14	open	0	0	nil
49	female	59	nil	6/9	6/6	17	17	open	0	0	nil
50	male	63	nil	6/9	6/9	14	14	open	0.4	0.4	nil
51_LG	female	72	nil	6/9	6/9	15	15	open	0	0	nil
51_SH	female	61	nil	6/5	6/5	19	19	open	0	0	nil
52	male	74	nil	6/6	6/6	19	18	open	0.5	0.5	nil
53	male	70	nil	6/9	6/9	19	19	open	0	0	nil
54	female	77	nil	6/9	6/9	16	16	open	0	0	nil
55	male	60	nil	6/6	6/6	15	15	open	0.4	0.5	nil

56	male	69	nil	6/5	6/5	14	11	open	0	0	nil
57	female	72	nil	6/9	6/9	19	18	open	0	0	nil
58	male	70	nil	6/9	6/9	19	18	open	0.5	0.5	nil
59	female	65	nil	6/6	6/6	13	13	open	0.4	0.4	nil
60	female	67	nil	6/6	6/6	15	11	open	0.4	0.4	nil
61	male	87	nil	6/9	6/9	14	14	open	0	0	nil
62	male	75	nil	6/9	6/9	12	12	open	0	0	nil
63	female	61	nil	6/9	6/9	17	16	open	0	0	nil
64	male	66	nil	6/9	6/6	13	13	open	0	0	nil
65	male	56	nil	6/5	6/6	18	18	open	0.2	0.2	nil
66	female	84	nil	6/9	6/9	14	14	open	0	0	nil
67	female	68	nil	6/9	6/9	16	16	open	0	0	nil
68	male	73	nil	6/9	6/9	14	14	open	0.5	0.5	nil
69	female	66	nil	6/9	6/9	16	16	open	0	0	nil
70	male	69	nil	6/9	6/6	14	14	open	0.4	0.4	nil
71	male	72	nil	6/9	6/9	16	16	open	0	0	nil
72	female	75	nil	6/9	6/9	12	12	open	0	0	nil
73	male	74	nil	6/5	6/5	13	12	open	0.3	0.3	nil
74	male	76	nil	6/6	6/6	16	17	open	0	0	nil
76_D	male	80	nil	6/9	6/9	16	18	open	0.5	0.5	nil
76_J	male	58	nil	6/9	6/9	14	13	open	0.3	0.3	nil
77	female	76	nil	6/6	6/6	17	16	open	0.2	0.2	nil
78	male	77	nil	6/5	6/5	16	16	open	0	0	nil
79	female	74	nil	6/9	6/9	16	18	open	0.4	0.4	nil
80	female	64	nil	6/9	6/9	20	20	open	0.5	0.5	nil
81_NG	male	73	nil	6/6	6/6	13	13	open	0	0	nil
82	male	81	nil	6/6	6/6	14	14	open	0	0	nil
83	female	65	nil	6/9	6/9	17	13	open	0	0	nil
85	male	74	nil	6/9	6/9	14	14	open	0	0	nil
86	female	85	nil	6/9	6/9	18	18	open	0	0	nil
87	female	58	nil	6/9	6/6	12	12	open	0	0	nil
88	male	57	nil	6/4	6/5	17	17	open	0	0	nil
89	male	64	nil	6/9	6/9	19	19	open	0	0	nil
90	female	72	nil	6/9	6/9	19	18	open	0	0	nil
91	female	61	nil	6/6	6/6	14	14	open	0	0	nil
92	female	84	nil	6/6	6/6	15	15	open	0	0	nil
93	male	67	nil	6/9	6/9	20	18	open	0	0	nil
94	male	72	nil	6/9	6/9	14	14	open	0.4	0.4	nil
95	female	89	nil	6/9	6/9	14	14	open	0.2	0.2	nil
96	female	69	nil	6/9	6/6	18	18	open	0.3	0.3	nil
97	female	65	nil	6/6	6/5	11	13	open	0.4	0.4	nil
98	female	53	nil	6/6	6/6	14	14	open	0.2	0.2	nil
99	female	75	nil	6/9	6/9	13	14	open	0	0	nil

100	male	69	nil	6/9	6/6	17	17	open	0.4	0.4	nil
100_MH	female	68	nil	6/9	6/9	14	19	open	0	0	nil
101	female	76	nil	6/9	6/9	17	19	open	0	0	nil
102	male	67	nil	6/12	6/9	15	17	open	0	0	nil
103	female	87	nil	6/9	6/12	15	18	open	0	0	nil
104	male	73	nil	6/9	6/9	18	18	open	0.4	0.4	nil
105	male	78	nil	6/9	6/9	12	12	open	0	0	nil
106	female	68	nil	6/9	6/9	17	15	open	0.4	0.4	nil
107	male	70	nil	6/9	6/9	13	13	open	0	0	nil
108	male	66	nil	6/6	6/6	10	18	open	0	0	nil
109	female	84	nil	6/9	6/9	10	10	open	0	0	nil
110	female	71	nil	6/9	6/9	17	18	open	0	0	nil
111	female	82	nil	6/9	6/9	18	16	open	0.2	0.2	nil
112	male	68	nil	6/9	6/9	18	14	open	0	0	nil
113	male	71	nil	6/9	6/6	18	18	open	0	0	nil
114	female	68	nil	6/9	6/9	16	16	open	0.4	0.4	nil
115	female	85	nil	6/12	6/9	18	18	open	0.4	0.4	nil
116	male	70	nil	6/9	6/9	13	12	open	0	0	nil
117	female	70	nil	6/9	6/9	15	15	open	0.3	0.3	nil
118	female	70	nil	6/9	6/9	16	19	open	0.3	0.3	nil
119	female	61	nil	6/9	6/6	15	15	open	0	0	nil
120	female	58	nil	6/5	6/6	16	14	open	0.5	0.5	nil
122	female	52	nil	6/9	6/9	18	18	open	0.3	0.3	nil
150p	male	83	nil	6/9	6/9	12	12	open	0.5	0.5	nil
351	female	78	nil	6/9	6/9	15	15	open	0	0	nil
352	male	75	nil	6/9	6/9	15	7	open	0	0	nil
353	female	75	nil	6/9	6/6	20	20	open	0	0	nil
354	female	85	nil	6/9	6/9	16	17	open	0	0	nil
355	male	72	nil	6/9	6/9	10	14	open	0	0	nil
356	female	60	nil	6/6	6/6	16	16	open	0.5	0.5	nil
357	female	76	nil	6/9	6/9	14	14	open	0	0	nil
358	male	62	nil	6/9	6/9	13	14	open	0	0	nil
359	female	82	nil	6/9	6/9	15	15	open	0	0	nil
360	male	63	nil	6/9	6/9	16	16	open	0.5	0.5	nil
361	female	82	nil	6/9	6/6	17	14	open	0	0	nil
363	male	73	nil	6/9	6/9	13	14	open	0	0	nil
364	male	76	nil	6/9	6/9	14	14	open	0	0	nil
365	male	65	nil	6/6	6/6	15	15	open	0.4	0.4	nil
366	male	52	nil	6/4	6/5	11	11	open	0.4	0.4	nil
367	male	81	nil	6/4	6/4	14	14	open	0	0	nil
368	female	40	nil	6/5	6/6	13	14	open	0	0	nil
369	male	46	nil	6/4	6/4	16	18	open	0	0	nil
370	male	82	nil	6/9	6/9	16	15	open	0	0	nil

371	female	73	nil	6/9	6/9	15	16	open	0	0	nil
372	female	67	nil	6/9	6/6	15	14	open	0	0	nil
373	female	63	nil	6/5	6/6	20	19	open	0	0	nil
374	female	82	nil	6/9	6/9	15	16	open	0	0	nil
375	female	56	nil	6/9	6/9	17	17	open	0	0	nil
376	female	72	nil	6/9	6/9	15	16	open	0	0	nil
377	male	76	nil	6/9	6/9	12	12	open	0	0	nil
379	male	61	nil	6/9	6/6	19	19	open	0.3	0.3	nil
380	male	72	nil	6/9	6/9	17	17	open	0	0	nil
381	female	62	nil	6/9	6/9	15	15	open	0	0	nil
382	female	76	nil	6/6	6/6	16	16	open	0	0	nil
383	female	61	nil	6/9	6/9	20	21	open	0.3	0.3	nil
384	male	60	nil	6/9	6/9	16	16	open	0	0	nil
385	female	56	nil	6/9	6/9	16	16	open	0	0	nil
386	male	62	nil	6/9	6/9	18	17	open	0.5	0.5	nil
387	female	83	nil	6/9	6/6	17	18	open	0	0	nil
388	female	77	nil	6/9	6/9	15	15	open	0	0	nil
391	male	78	nil	6/9	6/9	10	11	open	0.5	0.5	nil
392	male	69	nil	6/6	6/6	16	16	open	0	0	nil
394	male	59	nil	6/4	6/5	10	11	open	0	0	nil
396	female	60	nil	6/6	6/5	18	18	open	0	0	nil
397	female	68	nil	6/5	6/6	16	15	open	0.5	0.5	nil
398	female	57	nil	6/4	6/4	15	15	open	0	0	nil
399	female	69	nil	6/9	6/9	18	17	open	0.3	0.3	nil
400	female	73	nil	6/9	6/6	17	16	open	0.4	0.4	nil
400_MM	female	73	nil	6/5	6/6	13	13	open	0	0.4	nil
400_P	female	74	nil	6/9	6/9	14	19	open	0.4	0	nil
401_JM	female	59	nil	6/9	6/9	16	17	open	0	0	nil
402	female	63	nil	6/9	6/9	19	17	open	0.5	0.5	nil
402_VS	female	53	nil	6/9	6/9	13	14	open	0	0	nil
403_GM	female	86	nil	6/9	6/6	19	19	open	0	0	nil
404_VB	female	60	nil	6/9	6/9	18	18	open	0	0	nil
405	female	72	nil	6/9	6/9	19	19	open	0	0	nil
408	male	78	nil	6/6	6/6	15	15	open	0.4	0.4	nil
409	male	75	nil	6/9	6/9	18	17	open	0	0	nil
410	female	71	nil	6/9	6/9	19	18	open	0	0	nil
411	male	75	nil	6/9	6/9	11	13	open	0	0	nil
412	male	70	nil	6/9	6/9	15	18	open	0	0	nil
415	female	67	nil	6/9	6/6	17	16	open	0	0	nil
416	female	67	nil	6/9	6/9	16	17	open	0	0	nil
417	male	70	nil	6/9	6/9	16	18	open	0.3	0.3	nil
418	male	88	nil	6/6	6/6	18	16	open	0.5	0.5	nil
419	male	68	nil	6/6	6/5	16	16	open	0.5	0.5	nil

421	male	68	nil	6/6	6/5	16	16	open	0.4	0.4	nil
422	female	70	nil	6/5	6/6	18	18	open	0.5	0.5	nil
423	female	70	nil	6/6	6/9	17	18	open	0	0	nil
424	male	70	nil	6/9	6/9	12	12	open	0.4	0.4	nil
425	female	72	nil	6/9	6/9	17	17	open	0	0	nil
426_JS	female	77	nil	6/9	6/9	12	12	open	0	0	nil
427	male	76	nil	6/9	6/6	13	14	open	0.3	0.3	nil
428	female	76	nil	6/9	6/9	17	17	open	0	0	nil
429	male	72	nil	6/9	6/12	17	19	open	0	0	nil
430	male	84	nil	6/9	6/9	14	14	open	0.5	0.5	nil
431	female	73	nil	6/9	6/9	12	12	open	0	0	nil
432	female	61	nil	6/9	6/9	20	19	open	0	0	nil
433	male	81	nil	6/9	6/6	12	12	open	0.5	0.5	nil
434	female	64	nil	6/6	6/6	13	13	open	0	0	nil
435	female	80	nil	6/9	6/9	14	13	open	0	0	nil
436	female	71	nil	6/9	6/9	17	17	open	0	0	nil
437	female	79	nil	6/9	6/9	19	19	open	0.5	0.5	nil
438	male	59	nil	6/9	6/9	15	15	open	0	0	nil
439	male	60	nil	6/9	6/6	14	14	open	0	0	nil
441	male	63	nil	6/9	6/9	17	16	open	0	0	nil
442	female	45	nil	6/9	6/9	16	17	open	0	0	nil
443	male	62	nil	6/6	6/6	14	13	open	0.5	0.5	nil
444	male	59	nil	6/9	6/9	15	14	open	0.3	0.3	nil
445	female	76	nil	6/9	6/9	16	18	open	0.5	0.5	nil
446	male	49	nil	6/6	6/6	9	9	open	0	0	nil
447	male	63	nil	6/9	6/9	16	17	open	0.5	0.5	nil
448	male	66	nil	6/9	6/6	16	14	open	0	0	nil
449	male	76	nil	6/9	6/9	17	17	open	0.4	0.4	nil
450	male	76	nil	6/9	6/9	13	13	open	0	0	nil
451	female	69	nil	6/6	6/6	15	15	open	0	0	nil
452	female	65	nil	6/5	6/5	15	16	open	0	0	nil
453	female	53	nil	6/6	6/4	14	15	open	0	0	nil
454	female	75	nil	6/5	6/6	14	15	open	0	0	nil
455	female	78	nil	6/6	6/9	19	19	open	0	0	nil
456	male	61	nil	6/9	6/9	16	18	open	0	0	nil
457	female	64	nil	6/6	6/5	14	11	open	0	0	nil
458	male	48	nil	6/5	6/6	17	18	open	0	0	nil
460	male	75	nil	6/6	6/9	20	20	open	0.5	0.5	nil
461	female	69	nil	6/9	6/9	16	15	open	0	0	nil
462	female	71	nil	6/9	6/9	14	14	open	0	0	nil
463	male	75	nil	6/9	6/9	16	16	open	0.4	0.4	nil
464	female	68	nil	6/9	6/6	16	15	open	0	0	nil
465	male	68	nil	6/9	6/9	20	20	open	0	0	nil

466	male	72	nil	6/9	6/12	14	14	open	0.5	0.5	nil
467	male	85	nil	6/9	6/9	18	18	open	0.4	0.4	nil
468p	female	77	nil	6/9	6/9	11	13	open	0	0	nil
468	male	55	nil	6/9	6/9	11	11	open	0.4	0.4	nil
469	male	85	nil	6/9	6/6	11	14	open	0	0	nil
470	male	60	nil	6/6	6/6	10	15	open	0	0	nil
471	male	68	nil	6/9	6/9	16	16	open	0	0	nil
473	female	66	nil	6/9	6/9	11	11	open	0.5	0.5	nil
474	female	73	nil	6/9	6/9	15	15	open	0.5	0.5	nil
475	male	64	nil	6/9	6/9	16	16	open	0.5	0.5	nil
476	female	76	nil	6/9	6/6	14	14	open	0.5	0.5	nil
478	male	62	nil	6/9	6/9	18	16	open	0.3	0.3	nil
479	female	63	nil	6/9	6/9	15	15	open	0.3	0.3	nil
C1	female	66	nil	6/6	6/6	15	15	open	0.2	0.2	nil
C2	female	80	nil	6/9	6/9	16	15	open	0.3	0.3	nil
C3	male	54	nil	6/9	6/9	14	14	open	0.4	0.4	nil
C4	female	58	nil	6/6	6/6	11	10	open	0.2	0.2	nil
C5	female	73	nil	6/9	6/9	16	16	open	0.4	0.4	nil
C6	female	69	nil	6/9	6/6	15	15	open	0.5	0.5	nil
C7	female	66	nil	6/9	6/9	17	16	open	0.3	0.3	nil
C8	male	64	nil	6/9	6/9	14	14	open	0.3	0.3	nil
C9	female	57	nil	6/6	6/6	13	13	open	0.2	0.2	nil
C10	female	71	nil	6/5	6/5	12	11	open	0.4	0.4	nil
C11	male	63	nil	6/6	6/4	16	16	open	0.3	0.3	nil
C12	female	83	nil	6/5	6/6	13	12	open	0.2	0.2	nil
C13	male	67	nil	6/6	6/9	17	17	open	0.2	0.2	nil
C14	male	64	nil	6/9	6/9	16	16	open	0.4	0.4	nil
C15	female	59	nil	6/6	6/6	18	18	open	0.5	0.5	nil
C16	female	63	nil	6/9	6/9	15	14	open	0.3	0.3	nil
C17	male	83	nil	6/9	6/6	14	14	open	0.3	0.3	nil
C18	male	68	nil	6/9	6/9	13	13	open	0.4	0.4	nil
C19	female	41	nil	6/5	6/4	11	11	open	0.2	0.2	nil
C20	female	64	nil	6/9	6/9	18	18	open	0.2	0.2	nil
C21	female	72	nil	6/9	6/9	16	15	open	0.2	0.2	nil
C22	female	71	nil	6/6	6/6	17	17	open	0.3	0.3	nil
C23	female	72	nil	6/9	6/9	13	13	open	0.3	0.3	nil
C24	female	61	nil	6/9	6/6	17	15	open	0.1	0.1	nil
C25	female	73	nil	6/9	6/9	18	18	open	0.2	0.2	nil
C26	male	73	nil	6/9	6/9	14	14	open	0.4	0.4	nil
C27	male	75	nil	6/6	6/6	13	13	open	0.3	0.3	nil
C28	female	72	nil	6/9	6/5	17	17	open	0.4	0.4	nil
C29	female	72	nil	6/9	6/9	13	13	open	0.3	0.3	nil
C30	male	77	nil	6/9	6/9	13	13	open	0.2	0.2	nil

RVI87	male	77	nil	6/9	6/9	20	20	open	0.3	0.3	nil
RVI89	male	83	nil	6/9	6/9	19	20	open	0.2	0.2	nil
RVI90	female	89	nil	6/9	6/6	16	16	open	0.3	0.3	nil
RVI92	female	75	nil	6/9	6/9	17	17	open	0.3	0.3	nil
RVI107	female	76	nil	6/9	6/9	16	16	open	0.5	0.5	nil
RVI108	female	75	nil	6/6	6/6	16	16	open	0.3	0.3	nil
RVI109	female	83	nil	6/9	6/9	14	14	open	0.2	0.2	nil
RVI111	male	68	nil	6/9	6/9	18	18	open	0.3	0.2	nil
RVI115	female	75	nil	6/6	6/6	19	19	open	0.4	0.4	nil
RVI116	female	75	nil	6/9	6/9	20	20	open	0.4	0.3	nil
RVI117	female	78	nil	6/9	6/6	16	16	open	0.5	0.5	nil
RVI119	female	80	nil	6/9	6/9	14	13	open	0.3	0.3	nil
RVI120	female	87	nil	6/9	6/9	14	14	open	0.3	0.3	nil
RVI124	female	85	nil	6/6	6/6	20	19	open	0.5	0.5	nil
RVI125	female	80	nil	6/5	6/5	18	18	open	0.3	0.3	nil
RVI126	female	89	nil	6/6	6/4	16	16	open	0.3	0.3	nil
RVI128	male	87	nil	6/5	6/6	17	17	open	0.5	0.5	nil
RVI130	male	78	nil	6/6	6/9	20	19	open	0.3	0.3	nil
RVI131	female	77	nil	6/9	6/9	19	20	open	0.4	0.3	nil
RVI145	male	82	nil	6/6	6/6	10	10	open	0.5	0.5	nil
RVI177	male	90	nil	6/12	6/9	14	14	open	0.2	0.2	nil
RVI220	female	70	nil	6/9	6/6	17	17	open	0.3	0.3	nil
RVI221	female	81	nil	6/9	6/9	18	18	open	0.4	0.4	nil
RVI228	female	87	nil	6/9	6/6	12	12	open	0.3	0.3	nil
RVI239	male	75	nil	6/9	6/9	16	16	open	0.3	0.3	nil
RVI240	male	77	nil	6/9	6/9	15	16	open	0.3	0.3	nil
RVI241	male	87	nil	6/6	6/6	15	15	open	0.4	0.4	nil
RVI242	female	78	nil	6/9	6/5	18	18	open	0.5	0.5	nil
RVI243	male	77	nil	6/6	6/9	18	18	open	0.5	0.5	nil
RVI244	male	78	nil	6/9	6/9	16	16	open	0.4	0.4	nil

8.4 Appendix IV: Demographic and phenotypic features of HTG individuals

Table 8–2: Demographic and phenotypic features of HTG individuals (*n* = 272)

Patient ID	Sex	Age	Age at diagnosis	Family history of glaucoma	Best visual acquity		Highest recorded IOP		Anterio chambe angle	Cup-disc ratio		Visual field defect		Topical medication	Laser	Surgery
					Right	Left	Right	Left		Right	Left	Right	Left			
1p	female	87	83	nil	6/9	6/9	25	25	open	0.7	0.8	advanced	advanced	yes	nil	nil
2a	female	63	55	yes	6/9	6/9	25	24	open	0.6	0.5	arcuate	arcuate	yes	nil	nil
2p	female	80	76	nil	6/9	6/9	24	21	open	0.7	0.6	nasal step	early nasal step	yes	nil	nil
3p	male	82	78	nil	6/9	6/9	26	20	open	0.8	0.7	nasal step	nasal step	yes	nil	nil
6p	female	71	59	nil	6/9	6/6	29	28	open	0.5	0	paracentral	nil	yes	nil	nil
7p	male	71	71	yes (sister)	6/9	6/9	27	21	open	0.6	0.4	arcuate	nil	yes	nil	nil
8p	female	89	80	yes (sister and father)	6/9	6/9	22	28	open	0.4	0.7	paracentral	advanced paracentral	yes	nil	nil
9p	female	85	70	nil	6/6	6/6	25	24	open	0.8	0.8	paracentral	paracentral, nasal step	yes	nil	nil
10p	male	75	67	yes (sister)	6/9	6/9	24	19	open	0.9	0.5	advanced	nasal step	yes	nil	nil
11p	male	66	57	nil	6/9	6/9	54	42	open	0.9	0.8	advanced	nasal step	yes	nil	right trab
12p	female	86	80	yes (father and sister)	6/6	6/6	29	21	open	0.9	0.6	advanced	arcuate	yes	nil	nil
14p	male	68	66	yes (uncle and aunt-maternal side)	6/9	6/9	26	26	open	0.3	0.4	nil	paracentral	yes	nil	nil
15p	male	60	54	yes (father and brother)	6/9	6/6	35	36	open	0.7	0.7	nasal step	nasal step	yes	nil	nil
16p	female	63	50	yes	6/9	6/9	24	25	open	0.8	0.7	advanced nasal step	arcuate	nil	nil	bilateral trab
17p	female	71	70	yes	6/9	6/9	26	44	open	0.8	0.8	paracentral and nasal step	nasal step	yes	nil	nil
18p	female	76	68	nil	6/6	6/6	25	25	open	0.7	0.6	paracentral	nasal step	yes	nil	nil
19p	female	66	65	yes	6/5	6/5	18	25	open	0.5	0.7	nil	nasal step	yes	nil	nil
20p	female	52	49	nil	6/6	6/4	44	45	open	0.9	0.8	advanced	advanced	yes	nil	right trab
21p	female	84	57	nil	6/5	6/6	26	29	open	0.7	0.8	arcuate	advanced	yes	nil	left trab
22p	male	77	62	yes (cousin-	6/6	6/9	36	44	open	0.6	0.9	nasal step	advanced	yes	nil	bilateral trab

				paternal side)												
23p	male	76	68	yes (brother)	6/9	6/9	32	26	open	1	1	advanced arcuate	advanced	yes	nil	bilateral trab
24p	male	83	78	yes (son)	6/6	6/6	31	35	open	0.5	1	nil	arcuate and advanced nasal	yes	nil	left trab
25p	female	82	76	nil	6/9	6/9	26	22	open	0.4	0.2	arcuate	nil	yes	nil	nil
26p	female	85	81	nil	6/9	6/6	32	24	open	0.5	0.4	arcuate	nasal step	yes	nil	nil
27p	male	66	66	yes (mother)	6/9	6/9	28	15	open	0.8	0.6	nasal step	nil	yes	nil	nil
28p	male	76	70	nil	6/5	6/4	27	36	open	0.5	1	nil	advanced	yes	nil	nil
29p	male	66	62	nil	6/9	6/9	30	38	open	0.5	0.7	nil	arcuate	yes	nil	nil
30p	female	85	80	yes	6/9	6/9	31	28	open	0.7	0.4	nasal step	nil	yes	nil	nil
43p RT	female	83	81	yes (grandmother and 2 nieces)	6/6	6/6	20	25	open	0.4	0.7	arcuate	nasal and arcuate	yes	nil	nil
44p	male	73	66	nil	6/9	6/9	34	36	open	0.3	0.6	nil	nasal	yes	nil	nil
45p	female	72	61	nil	6/9	6/6	28	23	open	0.7	0.5	arcuate	nil	yes	nil	right trab
46p	male	82	77	nil	6/9	6/9	29	20	open	1	0.8	advanced	nasal and arcuate	yes	nil	nil
47p	male	64	61	yes (father)	6/9	6/9	18	28	open	0.3	0.7	nil	arcuate and paracentral	yes	nil	nil
50p	female	56	45	nil	6/6	6/6	29	17	open	0.8	0.6	nasal step	nasal step	yes	nil	nil
51p mr	female	70	69	nil	6/9	6/5	25	22	open	0.5	0.3	paracentral	nil	yes	nil	nil
52p	female	80	69	nil	6/9	6/9	25	31	open	0.2	0.5	nil	arcuate	yes	nil	nil
52p ht	male	72	44	nil	6/9	6/9	28	36	open	0.5	0.6	nil	paracentral scotoma	yes	nil	nil
55p	male	77	65	yes (mother and son)	6/9	6/9	28	26	open	0.8	0.7	nasal and arcuate	paracentral	yes	nil	nil
59p	female	93	85	nil	6/6	6/6	29	30	open	0.4	0.7	paracentral	paracentral and arcuate	yes	nil	nil
60p	male	64	61	nil	6/9	6/9	27	27	open	0.7	0.7	nasal step	arcuate	yes	nil	nil
61p jcah	male	61	56	nil	6/9	6/6	29	29	open	0.7	0.7	nasal step	nasal step	yes	nil	nil
62p bug	male	61	58	nil	6/9	6/9	27	27	open	0.7	0.7	paracentral	arcuate	yes	nil	nil
62p ws	male	74	62	yes (father)	6/5	6/6	25	29	open	0.6	0.7	arcuate	arcuate	yes	nil	bilateral trab
65p	male	76	74	yes (brother)	6/9	6/9	34	27	open	0.6	0.3	nasal step	nil	yes	nil	nil
67p	female	88	58	nil	6/9	6/9	30	30	open	0.9	0.9	advanced	advanced	yes	nil	bilateral trab
70p	male	72	71	yes (aunt and uncle-paternal side)	6/6	6/6	25	16	open	0.8	0.7	nasal step	arcuate	yes	nil	nil
71p	male	63	63	nil	6/9	6/9	44	14	open	1	0.4	advanced	nil	yes	nil	right trab
71p jc	female	65	52	yes (mother, uncle and grandfather-maternal side)	6/9	6/6	30	29	open	0.5	0.5	paracentral	nasal	yes	nil	nil
72p	male	84	81	nil	6/9	6/9	15	50	open	0.5	0.6	nil	nasal step	yes	nil	nil
72p rg	female	77	64	yes (sister)	6/9	6/9	26	33	open	0.8	0.9	arcuate	advanced arcuate	yes	nil	left trab
76p	male	82	69	nil	6/6	6/6	24	28	open	0.9	1.0	advanced arcuate and paracentral	advanced	yes	nil	left trab
80p	female	79	78	nil	6/9	6/5	26	22	open	0.8	0.5	nasal step	nil	yes	nil	nil
81p	male	76	69	yes (father and	6/9	6/9	23	27	open	0.7	0.9	nasal step and	advanced	yes	nil	right trab

				brother)								arcuate				
82p	male	75	59	yes (brother and sister)	6/9	6/9	29	28	open	0.6	0.5	nasal and arcuate	arcuate	yes	nil	nil
83p	male	76	67	nil	6/6	6/9	20	36	open	0.5	0.6	nil	nasal step	yes	nil	nil
84p	male	45	42	nil	6/4	6/5	34	34	open	0.7	0.6	arcuate	nasal step	yes	nil	nil
86p	female	85	74	nil	6/9	6/9	27	24	open	0.8	0.5	arcuate	arcuate	nil	nil	bilateral trab
90p	male	49	48	nil	6/5	6/4	28	30	open	0.9	0.9	paracentral	paracentral	yes	nil	right trab
97p	female	77	66	yes (sister)	6/9	6/9	28	27	open	0.8	0.6	arcuate	nasal step	yes	nil	nil
99p	male	82	71	nil	6/9	6/6	20	28	open	0.5	0.7	nil	nasal and paracentral	yes	nil	nil
104p	female	67	58	nil	6/9	6/9	24	25	open	0.7	0.7	nasal step	arcuate	yes	nil	nil
106p	female	81	70	nil	6/5	6/6	31	32	open	0.8	0.9	paracentral	arcuate	nil	nil	bilateral trab
110p	male	56	53	nil	6/9	6/9	34	22	open	0.7	0.4	nasal step	nil	yes	nil	nil
113p	male	83	79	nil	6/9	6/9	25	20	open	0.8	0.3	arcuate	nil	yes	nil	nil
114	male	50	41	yes (father)	6/6	6/6	22	28	open	0.4	0.8	nil	nasal step and arcuate	yes	nil	nil
116p	female	75	74	nil	6/9	6/9	31	33	open	0.8	0.8	nasal	arcuate	yes	nil	nil
123p	male	81	65	nil	6/9	6/6	24	22	open	0.8	0.9	arcuate and nasal	temp wedge	yes	nil	nil
124p	female	75	53	yes (grandmother-paternal side)	6/9	6/9	34	28	open	0.7	0.6	paracentral	nil	yes	nil	bilateral trab
125p	male	67	59	nil	6/9	6/6	23	27	open	0.2	0.4	nil	nasal step	yes	nil	nil
129p	female	59	51	yes (mother and father)	6/9	6/9	29	31	open	0.3	0.5	nil	arcuate	yes	nil	nil
134p	female	72	53	yes (father)	6/5	6/6	25	26	open	0.4	0.4	nasal step	nasal step	yes	nil	nil
135p	male	79	75	nil	6/9	6/9	26	26	open	0.8	0.9	paracentral	arcuate and nasal step	yes	nil	nil
136p	male	85	83	nil	6/9	6/9	34	34	open	0.5	0.6	temp wedge and nasal step	temp wedge and nasal step	yes	nil	right trab
137p	female	68	59	yes (father)	6/6	6/6	25	22	open	0.6	0.4	arcuate	nil	yes	nil	nil
140p	female	75	74	nil	6/9	6/9	20	26	open	0.3	0.8	nil	nasal	yes	nil	nil
142p	female	79	64	nil	6/9	6/9	23	25	open	0.4	0.9	nil	advanced	yes	nil	bilateral trab
145p	female	76	47	nil	6/5	6/4	30	30	open	0.6	0.8	nil	nasal step	yes	nil	nil
146p	female	57	51	yes (aunt- maternal side)	6/9	6/9	34	41	open	0.5	0.8	nasal	arcuate	yes	nil	nil
147p	male	78	63	yes (brother)	6/9	6/6	26	30	open	0.5	0.9	nil	advanced	yes	nil	left trab
149p	female	80	61	yes (grandfather)	6/9	6/9	26	28	open	0.9	0.9	nasal	advanced arcuate	yes	nil	nil
152p	female	57	44	yes (mother, father, uncle (paternal side) and aunt (maternal side)	6/5	6/6	24	36	open	0.3	0.6	nil	paracentral	yes	nil	nil
153p	male	69	62	nil	6/9	6/9	18	26	open	0.8	0.9	nasal step	arcuate	nil	nil	bilateral trab
157p	female	76	75	nil	6/9	6/9	25	33	open	0.6	0.6	arcuate	arcuate	yes	nil	nil
158p	female	77	77	yes (brother)	6/6	6/6	17	25	open	0.4	0.8	nil	arcuate	yes	nil	nil
160p	female	63	57	yes (father and	6/9	6/9	26	26	open	0.9	0.7	arcuate and	nasal	yes	nil	nil

				sister)								paracentral				
350p	male	85	76	nil	6/9	6/6	25	38	open	0.9	0.9	advanced nasal step	advanced	yes	nil	bilateral trab
393	female	59	58	nil	6/6	6/9	22	30	open	0.4	0.7	nil	arcuate and nasal step	yes	nil	nil
401p	male	67	58	nil	6/9	6/6	27	22	open	0.9	0.8	nasal step and paracentral	nil	yes	nil	nil
403	female	77	72	nil	6/9	6/9	24	20	open	0.5	0.5	advanced	nasal step	yes	nil	nil
405p	male	81	75	yes (brother)	6/5	6/6	31	29	open	0.7	0.3	arcuate	nil	yes	nil	nil
408p	male	56	53	yes (grandmother-maternal side)	6/9	6/9	36	40	open	0.8	0.9	arcuate	arcuate	yes	nil	bilateral trab
409p	female	79	55	yes (father)	6/9	6/9	29	34	open	0.9	0.9	advanced arcuate	arcuate	yes	nil	bilateral trab
411p	male	68	62	nil	6/6	6/6	30	26	open	0.8	0.5	paracentral and nasal step	nil	yes	nil	nil
412p	female	69	52	yes (father and uncle-paternal side)	6/9	6/9	27	28	open	0.4	0.6	nil	nasal step	yes	nil	nil
413p	female	82	72	nil	6/9	6/6	41	40	open	0.5	0.4	nasal step	nasal step	nil	nil	bilateral trab
414p	female	57	55	nil	6/9	6/9	28	26	open	0.9	0.4	advanced arcuate	nil	yes	nil	right trab
415p	female	69	64	yes (father)	6/9	6/6	29	26	open	0.8	0.8	paracentral and arcuate	nasal step	yes	nil	right trab
417p	male	57	54	nil	6/9	6/9	30	30	open	0.9	0.9	arcuate and paracentral	paracentral	yes	nil	nil
418p	female	73	64	nil	6/5	6/4	28	26	open	0.4	0.3	nasal step	paracentral	yes	nil	nil
419p	female	77	71	nil	6/9	6/9	24	18	open	0.8	0.7	nasal step	nil	yes	nil	nil
420P	male	82	75	yes (grandmother-maternal side)	6/9	6/9	28	24	open	0.9	0.3	advanced arcuate	nil	yes	nil	nil
421p	male	78	63	nil	6/6	6/6	35	31	open	0.8	0.8	advanced	advanced	yes	nil	left trab
423p	female	82	79	yes (mother and daughter)	6/12	6/9	26	26	open	0.8	0.8	nasal step	arcuate	yes	nil	nil
425p	female	82	76	nil	6/9	6/6	27	28	open	0.7	0.8	arcuate	advanced arcuate	yes	nil	nil
427p	male	86	79	nil	6/9	6/9	30	30	open	0.7	0.6	nasal step	paracentral and nasal step	yes	nil	nil
428p	female	76	70	nil	6/9	6/9	26	24	open	0.9	0.8	arcuate	arcuate and nasal	yes	nil	nil
430p	female	63	60	yes (father)	6/6	6/6	25	22	open	0.9	0.6	advanced nasal step	temporal wedge	yes	nil	nil
431p	male	46	44	yes (father)	6/9	6/5	26	26	open	0.8	0.8	advanced	advanced	yes	nil	nil
432p	male	79	72	yes (mother and sister)	6/9	6/9	34	36	open	0.9	0.8	advanced	advanced	yes	nil	right trab
433p	female	72	62	yes (grandfather)	6/9	6/9	36	34	open	0.7	0.5	arcuate	nasal step	yes	nil	nil
434p	female	80	76	nil	6/9	6/9	25	26	open	0.4	0.4	nasal step	nasal step	yes	nil	nil
436p	female	53	53	yes (mother)	6/6	6/6	25	26	open	0.3	0.5	nil	nasal step	yes	nil	nil
437p	female	89	86	nil	6/9	6/9	28	26	open	0.4	0.8	nasal	arcuate	yes	nil	nil
438p	male	92	83	yes (sister)	6/9	6/6	20	24	open	0.5	0.7	nasal step	paracentral	yes	nil	nil

439p	female	90	84	nil	6/9	6/9	23	24	open	0.5	0.9	nasal step	advanced	yes	nil	nil
440p	male	77	64	yes (mother, sister and brother)	6/6	6/6	24	26	open	0.6	0.8	nil	nasal step	yes	nil	nil
441p	female	45	44	yes (uncle- maternal side)	6/9	6/9	38	42	open	0.8	1	nasal step	advanced nasal step and paracentral scotoma	yes	nil	left trab
442p	male	72	63	nil	6/9	6/9	22	33	open	0.5	0.9	nasal step	advanced	yes	nil	left trab
443p	male	57	56	nil	6/6	6/6	28	42	open	0.5	0.9	nil	arcuate and nasal	yes	nil	nil
444p	female	74	74	yes (sister and uncle- maternal side)	6/9	6/9	23	27	open	0.6	0.9	nasal step	paracentral and arcuate	yes	nil	nil
445p	male	76	58	yes (brother and 2 sisters)	6/9	6/6	32	32	open	0.3	0.7	nil	nasal step	yes	nil	nil
447p	female	76	68	yes (aunt, aunt's daughter, uncle's son- all paternal side)	6/9	6/9	30	26	open	1	0.9	arcuate	paracentral	yes	nil	nil
448p	male	51	50	yes (father)	6/4	6/5	20	29	open	0.2	0.9	nil	advanced	yes	nil	left trab
449p	female	70	53	nil	6/9	6/9	30	38	open	0.9	0.8	advanced arcuate	nasal step	yes	right ALT	bilateral trab
451p	male	71	67	yes (father and sister)	6/9	6/9	22	26	open	0.5	0.6	nil	nasal step	yes	nil	nil
452p	female	81	74	yes (mother)	6/6	6/6	26	26	open	0.9	0.8	advanced arcuate	arcuate	yes	nil	nil
453p	male	86	70	nil	6/9	6/9	26	30	open	0.5	1	nil	advanced	nil	left ALT	left trab
458p	female	68	47	yes (aunt, aunt's daughter- paternal side)	6/6	6/6	30	36	open	0.9	0.9	advanced	advanced	yes	nil	bilateral trab
462p	male	80	72	nil	6/12	6/9	29	20	open	0.6	0.3	arcuate	nil	yes	nil	right trab
465p	female	76	74	yes (sister)	6/9	6/9	26	25	open	0.7	0.8	nasal step	nasal step	yes	nil	nil
467p	male	59	55	nil	6/9	6/9	32	36	open	0.8	0.5	arcuate	nil	yes	nil	nil
469p	male	76	74	yes (grandfather)	6/6	6/6	20	27	open	0.2	0.5	nil	arcuate	yes	nil	nil
470p	female	83	81	yes (2 cousin each from 2 separate aunt-paternal side)	6/9	6/5	20	24	open	0.4	0.7	arcuate	nasal and arcuate	yes	nil	nil
473p	female	71	61	nil	6/9	6/9	30	28	open	0.4	0.4	paracentral	nil	yes	nil	right trab
474p	male	73	66	nil	6/9	6/9	16	24	open	0.6	0.8	nil	paracentral	yes	nil	nil
477p	male	73	72	yes (aunt)	6/9	6/9	28	22	open	0.6	0.4	paracentral	nil	yes	nil	nil
478p	female	71	58	yes (sister and mother)	6/6	6/6	40	36	open	0.9	0.9	advanced	advanced	yes	nil	bilateral trab
479p	female	82	63	yes (mother and daughter)	6/9	6/9	30	35	open	0.8	0.7	paracentral	paracentral	yes	nil	nil
480p	female	79	71	nil	6/9	6/6	30	24	open	1	0.8	advanced	nasal step	yes	nil	nil
482p	female	65	49	yes (mother)	6/9	6/9	26	26	open	0.6	0.3	arcuate	paracentral	yes	nil	nil

483p	male	67	41	nil	6/5	6/6	25	25	open	0.8	0.9	nil	paracentral	yes	nil	bilateral trab
484p	male	60	56	nil	6/6	6/9	25	20	open	0.9	0.9	advanced	nasal step	yes	nil	nil
485p	female	81	78	nil	6/9	6/9	26	30	open	0.9	0.9	paracentral	nil	yes	nil	nil
488p	female	87	69	yes (father and grandfather- paternal side)	6/9	6/9	29	29	open	0.7	0.9	paracentral	paracentral	nil	nil	bilateral trab
489p	male	74	56	yes (cousin-paternal side)	6/6	6/6	28	33	open	0.3	0.5	nasal	arcuate	yes	nil	nil
491p	male	78	65	nil	6/9	6/9	18	40	open	0.4	0.9	nasal	paracentral and nasal	nil	nil	left trab
492p	female	72	63	yes (brother)	6/9	6/6	28	20	open	0.4	0.2	nasal	nil	yes	nil	nil
493p	female	78	62	nil	6/9	6/9	31	27	open	0.7	0.5	nasal	nil	yes	nil	nil
494p	female	92	79	nil	6/12	6/12	24	23	open	0.9	0.7	advanced arcuate	arcuate	nil	nil	bilateral trab
495p	male	71	70	nil	6/9	6/9	32	32	open	0.8	0.3	arcuate	nil	yes	nil	nil
496p	female	87	70	nil	6/9	6/6	24	20	open	0.5	0.6	nasal step	nasal step	nil	nil	left trab
498p	male	79	73	nil	6/9	6/9	32	32	open	0.4	0.4	arcuate	paracentral	yes	nil	nil
500p	female	61	54	yes (grandfather, uncle and aunt- all maternal side)	6/5	6/4	23	24	open	0.5	0.6	nasal step	nasal step	yes	nil	nil
501p	female	75	74	yes (mother, 2 aunts- maternal side)	6/9	6/9	21	30	open	0.7	0.9	arcuate	advanced arcuate	yes	nil	nil
502p	male	82	81	yes (grandmother- paternal side)	6/9	6/9	14	24	open	0.3	0.7	nil	nasal step	yes	nil	nil
503p	male	72	69	nil	6/6	6/6	29	24	open	0.4	0.3	nasal step	nil	yes	nil	nil
504p	male	44	44	nil	6/9	6/9	42	28	open	0.9	0.8	advanced	arcuate	yes	nil	nil
505p	male	54	53	yes (mother and brother)	6/9	6/6	23	30	open	0.5	0.7	nil	nasal step	yes	nil	nil
506p	female	62	53	nil	6/9	6/5	25	26	open	0.5	0.9	nil	advanced arcuate	yes	nil	nil
507p	male	60	50	nil	6/9	6/9	32	30	open	0.6	0.8	nil	nasal	yes	nil	nil
508p	male	78	65	yes (mother)	6/6	6/6	33	34	open	0.4	0.6	nil	arcuate	nil	nil	bilateral trab
512p	female	90	89	yes (brother)	6/9	6/5	21	27	open	0.7	0.8	nil	nasal step	yes	nil	nil
516p	male	81	81	nil	6/9	6/9	23	25	open	0.7	0.8	nasal step	arcuate	yes	nil	nil
517p	female	67	64	yes (sister and sister's son)	6/9	6/9	26	28	open	0.3	0.5	nasal step	nil	yes	nil	nil
520p	male	52	47	nil	6/6	6/5	26	29	open	0.9	0.9	advanced arcuate	early arcuate	yes	nil	right trab
521p	male	86	75	nil	6/6	6/6	24	28	open	0.7	0.8	arcuate	paracentral	yes	nil	nil
523p	female	74	58	nil	6/9	6/9	26	26	open	0.4	0.6	nil	arcuate	yes	nil	nil
525p	female	79	73	nil	6/9	6/6	23	37	open	0.9	1	nasal step	advanced	yes	nil	nil
530p	female	70	60	yes (sister, brother, father, grandfather, 2 aunts –all paternal side)	6/9	6/9	26	27	open	0.7	0.6	nasal step	nasal step	yes	nil	nil
531p	male	62	56	nil	6/5	6/6	26	26	open	0.8	0.7	arcuate	paracentral	yes	nil	nil

532p	male	81	79	yes	6/9	6/9	24	25	open	0.6	0.6	nasal step	nasal step	yes	nil	nil
533p	female	72	63	nil	6/9	6/9	27	27	open	0.7	0.7	arcuate	arcuate	yes	nil	nil
sei1	female	84	69	nil	6/6	6/6	45	40	open	0.4	0.2	arcuate	nil	yes	nil	nil
sei3	male	70	61	nil	6/9	6/9	24	35	open	0.3	0.9	nil	temporal wedge	yes	nil	nil
sei4	male	78	58	nil	6/9	6/6	48	48	open	0.4	0.5	nasal step	nasal step	yes	nil	nil
sei5	female	71	76	yes	6/9	6/9	56	42	open	0.9	0.7	advanced	arcuate	nil	nil	bilateral trab
sei6	male	82	50	nil	6/6	6/6	30	32	open	0.8	0.8	arcuate	arcuate	yes	nil	nil
sei7	male	69	40	nil	6/9	6/9	34	27	open	0.5	0.4	arcuate	nil	yes	nil	nil
sei9	male	40	74	nil	6/9	6/6	28	32	open	0.4	0.5	nil	nasal step	yes	nil	nil
sei10	male	52	40	nil	6/9	6/9	36	30	open	0.6	0.4	nasal step	nasal step	yes	nil	nil
sei11	male	74	69	nil	6/5	6/4	26	31	open	0.2	0.4	nil	nasal step	yes	nil	nil
sei12	female	53	71	nil	6/9	6/9	52	26	open	0.9	0.5	advanced	nil	yes	nil	right trab
sei13	male	75	67	nil	6/9	6/9	32	29	open	0.6	0.5	temporal wedge	nasal step	yes	nil	nil
sei14	male	73	58	nil	6/6	6/6	35	34	open	0.4	0.3	paracentral	nil	yes	nil	nil
sei15	male	72	65	yes	6/9	6/9	26	38	open	0.4	0.5	nil	arcuate	yes	nil	nil
sei16	female	71	59	nil	6/9	6/6	34	38	open	0.5	0.6	nil	nasal step	yes	nil	nil
sei17	male	59	63	nil	6/9	6/9	30	38	open	0.5	0.5	nil	nasal step	yes	nil	nil
sei18	male	68	50	yes	6/9	6/9	30	29	open	0.4	0.2	paracentral	nil	yes	nil	nil
sei19	male	54	59	nil	6/6	6/6	30	32	open	0.5	0.5	arcuate	temporal wedge	yes	nil	nil
sei20	female	69	61	nil	6/9	6/5	34	35	open	0.5	0.5	nasal step	nasal step	yes	nil	nil
sei21	female	61	40	nil	6/9	6/9	24	33	open	0.5	0.6	paracentral	nasal step	yes	nil	nil
sei22	male	66	70	nil	6/9	6/9	38	35	open	0.9	0.9	advanced	advanced	yes	nil	nil
sei23	male	65	54	nil	6/9	6/9	26	30	open	0.6	0.7	nil	arcuate	yes	nil	nil
sei24	male	80	66	nil	6/6	6/6	20	30	open	0.5	0.6	nil	paracentral	yes	nil	nil
sei25	male	67	67	nil	6/9	6/9	39	22	open	0.6	0.4	nasal step	nil	yes	nil	nil
sei26	male	71	55	nil	6/9	6/6	32	18	open	0.7	0.3	arcuate	nil	yes	nil	nil
sei27	male	80	65	nil	6/9	6/9	30	32	open	0.8	0.8	nasal step	nasal step	yes	nil	bilateral trab
sei28	male	74	66	nil	6/5	6/6	33	33	open	0.8	0.8	paracentral	arcuate	yes	nil	nil
sei29	male	75	65	nil	6/9	6/9	32	31	open	0.8	0.9	advanced	advanced	yes	nil	bilateral trab
sei30	male	74	66	nil	6/9	6/9	31	28	open	0.4	0.3	arcuate	nil	yes	nil	nil
sei31	male	60	67	nil	6/6	6/6	30	28	open	0.7	0.6	nasal step	nil	yes	nil	nil
sei32	female	77	48	nil	6/9	6/9	36	65	open	0.9	1	advanced	advanced	yes	nil	bilateral trab
sei33	female	51	61	yes	6/9	6/6	38	31	open	0.9	0.1	arcuate	nil	yes	nil	nil
sei34	male	64	60	nil	6/9	6/9	30	26	open	0.8	0.5	arcuate	nil	yes	nil	nil
sei35	female	70	83	yes	6/9	6/9	33	32	open	0.8	0.7	paracentral	nil	yes	nil	nil
sei36	male	71	79	nil	6/6	6/6	30	32	open	0.6	0.6	nasal step	nasal step	yes	nil	bilateral trab
sei37	male	83	51	nil	6/9	6/5	18	32	open	0.2	0.7	nil	nasal step	yes	nil	nil
sei38	male	79	73	nil	6/9	6/9	26	30	open	0.4	0.5	nil	paracentral	yes	nil	nil
sei39	female	56	77	nil	6/9	6/9	30	35	open	0.9	0.8	advanced	advanced	yes	nil	nil
sei40	female	77	55	nil	6/6	6/9	24	34	open	0.4	0.8	nasal step	arcuate	yes	nil	nil
sei41	female	55	54	nil	6/6	6/6	30	27	open	0.8	0.6	arcuate	nil	yes	nil	nil
sei42	female	62	73	nil	6/9	6/9	30	22	open	0.3	0.5	nil	nasal step	yes	nil	nil

sei43	male	87	81	nil	6/9	6/6	30	28	open	0.8	0.6	arcuate	arcuate	yes	nil	nil
sei44	male	87	47	nil	6/9	6/9	24	31	open	0.6	0.7	temporal wedge	arcuate	yes	nil	nil
sei46	female	75	68	nil	6/5	6/6	32	34	open	0.5	0.6	nil	nasal step	yes	nil	nil
sei47	male	54	47	nil	6/6	6/9	38	27	open	0.8	0.7	arcuate	nasal step	yes	nil	nil
sei48	female	71	50	nil	6/9	6/9	30	36	open	0.4	0.7	nil	arcuate	yes	nil	nil
sei49	female	61	55	yes	6/9	6/9	34	34	open	0.3	0.4	nil	nasal step	yes	nil	nil
sei50	female	61	61	nil	6/6	6/6	30	29	open	0.5	0.3	paracentral	nil	yes	nil	nil
sei51	male	55	64	nil	6/9	6/9	29	29	open	0.5	0.5	arcuate	arcuate	yes	nil	nil
sei52	male	68	64	nil	6/9	6/6	30	29	open	0.4	0.8	nil	nasal step	yes	nil	nil
sei53	male	61	61	nil	6/9	6/9	32	22	open	0.4	0.3	paracentral	nil	yes	nil	nil
sei54	male	63	76	yes	6/12	6/9	27	30	open	0.7	0.8	nil	arcuate	yes	nil	nil
sei55	female	74	55	yes	6/9	6/9	32	32	open	0.6	0.7	nasal step	paracentral	yes	nil	nil
sei56	female	68	59	nil	6/9	6/6	29	33	open	0.8	0.9	advanced arcuate	advanced	nil	nil	bilateral tab
sei57	female	62	41	nil	6/6	6/9	31	30	open	0.8	0.8	arcuate	arcuate	yes	nil	nil
sei58	female	76	64	nil	6/6	6/6	31	29	open	0.4	0.3	nasal step	nil	yes	nil	nil
sei59	male	41	60	nil	6/6	6/9	34	34	open	0.7	0.7	temporal wedge	nasal step	yes	nil	nil
sei60	male	68	75	nil	6/9	6/9	30	30	open	0.7	0.7	nasal step	nasal step	yes	nil	right trab
sei61	female	78	46	nil	6/9	6/6	28	36	open	0.3	0.5	nil	arcuate	yes	nil	nil
sei62	male	60	55	yes	6/9	6/9	24	40	open	0.3	0.6	nil	arcuate	yes	nil	nil
sei63	male	82	57	nil	6/5	6/9	49	53	open	0.8	0.9	temp wedge	advanced	nil	nil	bilateral trab
sei64	female	57	62	yes	6/9	6/9	24	36	open	0.3	0.7	nil	arcuate	yes	nil	nil
sei65	female	77	66	nil	6/9	6/9	34	28	open	0.8	0.6	arcuate	temporal wedge	yes	ALT	bilateral trab
sei66	male	72	51	yes	6/6	6/6	30	24	open	0.9	0.8	arcuate and nasal step	arcuate	nil	nil	bilateral trab
sei67	male	55	68	nil	6/9	6/9	50	26	open	0.8	0.3	arcuate	nil	yes	nil	nil
sei68	male	74	59	yes	6/9	6/6	33	34	open	0.5	0.6	nasal step	arcuate	nil	nil	bilateral trab
sei69	male	78	55	yes	6/9	6/9	35	29	open	0.6	0.3	arcuate	arcuate	nil	nil	bilateral trab
sei70	female	68	51	nil	6/9	6/9	30	23	open	0.6	0.4	paracentral	nil	yes	nil	right trab
sei71	female	55	62	yes	6/6	6/6	29	31	open	0.3	0.5	nil	arcuate	yes	nil	nil
sei72	male	69	62	nil	6/9	6/5	19	54	open	0.4	0.9	nil	advanced	yes	nil	left trab
sei73	male	76	48	yes	6/9	6/9	34	31	open	0.4	0.3	nasal step	nil	yes	nil	nil
sei74	male	65	50	nil	6/9	6/9	30	30	open	0.9	0.8	advanced	arcuate	yes	nil	nil
sei75	male	77	70	nil	6/9	6/9	32	30	open	0.4	0.3	paracentral	nil	yes	nil	nil
sei76	male	73	67	nil	6/6	6/6	32	26	open	0.9	0.7	advanced	arcuate	yes	nil	nil
sei77	female	73	66	nil	6/9	6/9	29	30	open	0.7	0.8	nil	nasal step	yes	nil	nil
sei78	male	71	51	nil	6/9	6/6	39	29	open	0.9	0.6	advanced	nasal step	yes	nil	nil
sei79	male	62	75	nil	6/9	6/9	36	33	open	0.9	0.7	advanced	paracentral	yes	nil	bilateral trab
sei81	male	74	62	nil	6/5	6/6	31	28	open	0.8	0.8	nasal step	arcuate	yes	nil	nil
sei82	female	72	51	nil	6/9	6/9	33	30	open	0.5	0.4	paracentral	nasal step	yes	nil	nil
sei83	female	64	48	yes	6/9	6/9	29	30	open	0.6	0.6	nasal step	arcuate	yes	nil	nil
sei84	male	59	62	nil	6/6	6/6	30	30	open	0.6	0.8	nil	temporal wedge	nil	nil	bilateral trab
sei86	male	83	59	nil	6/9	6/9	30	30	open	0.4	0.4	nasal step	nasal step	yes	nil	nil

sei87	male	81	64	nil	6/9	6/9	28	30	open	0.9	0.9	advanced	advanced	nil	nil	bilateral trab
sei88	male	67	66	nil	6/9	6/9	21	34	open	0.5	0.9	nil	arcuate	yes	nil	nil
sei89	female	70	72	nil	6/9	6/9	30	30	open	0.9	0.6	advanced nasal step	nil	yes	nil	nil
sei90	male	62	51	nil	6/6	6/6	30	26	open	0.5	0.3	arcuate	nil	yes	nil	nil
sei91	male	72	61	nil	6/9	6/9	26	42	open	0.5	0.8	paracentral scotoma	arcuate	yes	nil	nil
sei92	male	67	51	yes	6/9	6/6	32	30	open	0.5	0.6	nasal step	nasal step	yes	nil	nil
sei93	female	79	66	nil	6/9	6/9	30	26	open	0.8	0.7	arcuate	paracentral	yes	nil	nil
sei94	male	74	72	nil	6/9	6/9	30	28	open	0.7	0.6	nasal step	nil	yes	nil	nil
sei95	male	75	60	nil	6/6	6/6	23	37	open	0.8	1	arcuate	advanced	yes	nil	nil
sei96	male	66	78	nil	6/9	6/5	30	29	open	0.7	0.5	arcuate	nil	yes	nil	nil
sei97	male	67	71	nil	6/9	6/9	30	29	open	0.4	0.2	paracentral	nil	yes	nil	nil
sei98	male	77	55	yes	6/9	6/9	24	42	open	0.5	0.8	nil	arcuate	yes	nil	nil
sei99	male	55	58	nil	6/9	6/9	26	50	open	0.3	0.8	nil	arcuate	yes	nil	nil
sei100	female	73	71	nil	6/6	6/6	34	28	open	0.7	0.6	nasal step	nil	yes	nil	nil
sei101	male	69	63	nil	6/9	6/9	30	38	open	0.3	0.4	nil	nasal step	yes	nil	nil
sei102	female	63	61	nil	6/9	6/6	32	30	open	0.6	0.5	paracentral	nil	yes	nil	nil
sei103	male	82	43	nil	6/9	6/9	56	56	open	0.7	1	arcuate	advanced	nil	nil	bilateral trab
sei104	male	71	65	nil	6/6	6/9	30	30	open	0.5	0.6	nil	nasal step	yes	nil	nil
sei105	male	43	84	yes	6/6	6/5	22	46	open	0.5	0.9	nil	arcuate	yes	nil	left trab

8.5 Appendix V: Demographic and phenotypic features of OHT individuals

Table 8–3: Demographic and phenotypic features of OHT individuals (*n* = 58)

Patient ID	Sex	Age	Age at diagnosis	Family history of glaucoma	Best visual acuity		Highest recorded IOP		Anterior chamber angle	Cup/disc ratio		Visual field defect		Medication	Surgery
							Right	Left		Right	Left	Right	Left		
5p	male	66	66	no	6/6	6/5	25	26	open	0.3	0.3	nil	nil	yes	nil
13p	female	66	58	no	6/9	6/6	28	28	open	0.4	0.4	nil	nil	yes	nil
42p	male	76	74	yes (mother)	6/9	6/9	20	27	open	0.2	0.2	nil	nil	yes	nil
49p	female	86	69	yes (aunt- maternal side)	6/12	6/9	28	30	open	0.3	0.4	nil	nil	yes	nil
53p	male	61	61	no	6/6	6/5	28	33	open	0.3	0.3	nil	nil	yes	nil
54p	female	73	66	no	6/9	6/9	27	27	open	0.2	0.2	nil	nil	yes	nil
56p	female	45	45	yes (2 uncle- paternal side)	6/5	6/6	24	25	open	0.2	0.2	nil	nil	nil	nil
66p	male	53	74	yes (2 uncle, 1 aunt, 1 cousin- all paternal side)	6/9	6/9	25	22	open	0.4	0.4	nil	nil	yes	nil
73p	female	57	40	yes (father and grandfather-paternal side)	6/6	6/6	25	25	open	0.4	0.4	nil	nil	yes	nil
75p	male	67	59	yes (brother)	6/9	6/5	26	25	open	0.5 (healthy NRR)	0.5 (healthy NRR)	nil	nil	yes	nil
79p	female	52	52	yes (father and grandmother-paternal side)	6/5	6/6	36	37	open	0.3	0.3	nil	nil	yes	nil
85p	female	41	40	no	6/4	6/5	28	28	open	0.5 (healthy NRR)	0.5 (healthy NRR)	nil	nil	yes	nil
87p	female	70	61	yes (sister)	6/9	6/9	26	26	open	0.4	0.4	nil	nil	yes	nil
91p	female	75	61	no	6/6	6/6	28	28	open	0.5 (healthy NRR)	0.5 (healthy NRR)	nil	nil	yes	nil
94p	male	53	40	no	6/6	6/5	30	27	open	0.4	0.4	nil	nil	yes	nil
98p	female	68	52	no	6/9	6/6	26	26	open	0.5 (healthy NRR)	0.5 (healthy NRR)	nil	nil	yes	nil
101p	male	78	64	yes (two sisters)	6/9	6/9	37	41	open	0.4	0.4	nil	nil	yes	nil
105p	male	59	58	no	6/5	6/6	26	21	open	0.4	0.4	nil	nil	yes	nil
108p	female	75	70	yes (grandmother-maternal side)	6/9	6/9	25	26	open	0.5 (healthy NRR)	0.5 (healthy NRR)	nil	nil	yes	nil

111p	male	80	63	no	6/9	6/9	34	31	open	0.3	0.3	nil	nil	yes	nil
117p	female	74	67	no	6/6	6/6	28	19	open	0.3	0.3	nil	nil	yes	nil
118p	male	79	73	no	6/9	6/9	34	20	open	0.2	0.2	nil	nil	yes	nil
121p	female	57	56	no	6/9	6/6	27	27	open	0.4	0.4	nil	nil	yes	nil
128p	female	68	61	no	6/9	6/9	23	26	open	0.4	0.4	nil	nil	yes	nil
130p	female	68	58	yes (2 uncle and 1 aunt- all maternal side)	6/9	6/9	27	27	open	0.2	0.2	nil	nil	yes	nil
132p	female	83	83	yes (aunt- maternal side)	6/6	6/6	24	24	open	0.2	0.2	nil	nil	yes	nil
133p	female	41	41	no	6/5	6/5	25	25	open	0.3	0.3	nil	nil	nil	nil
141p	female	64	64	yes (cousin)	6/9	6/9	23	26	open	0.4	0.4	nil	nil	yes	nil
143p	male	72	60	no	6/9	6/9	29	29	open	0.1	0.1	nil	nil	yes	nil
154p	male	63	61	no	6/6	6/9	32	29	open	0.4	0.4	nil	nil	yes	nil
155p	female	78	56	yes (aunt and cousin)	6/6	6/6	32	24	open	0.1	0.1	nil	nil	yes	nil
156p	male	60	54	no	6/9	6/9	34	30	open	0.4	0.4	nil	nil	yes	nil
404p	male	60	43	yes (sister)	6/9	6/6	26	28	open	0.1	0.1	nil	nil	yes	nil
406p	female	58	52	yes (father)	6/9	6/9	30	25	open	0.3	0.3	nil	nil	yes	nil
410p	female	76	76	yes (brother, sister, mother, uncle, aunt- maternal side)	6/5	6/6	24	24	open	0.4	0.4	nil	nil	yes	nil
416p	female	87	75	no	6/6	6/9	25	24	open	0.2	0.2	nil	nil	nil	nil
429p	male	61	48	no	6/9	6/9	32	24	open	0.3	0.3	nil	nil	yes	nil
455p	male	52	52	yes (grandfather- maternal side)	6/9	6/9	25	24	open	0.2	0.2	nil	nil	nil	nil
457p	female	59	54	yes (grandfather, cousin- paternal side)	6/6	6/6	25	24	open	0.3	0.3	nil	nil	nil	nil
459p	female	80	79	no	6/9	6/9	34	35	open	0.1	0.1	nil	nil	yes	nil
461p	female	51	49	no	6/6	6/6	24	26	open	0.4	0.4	nil	nil	yes	nil
463p	female	60	54	yes (mother, sister, nephew)	6/5	6/9	20	26	open	0.4	0.4	nil	nil	yes	nil
464p	male	61	59	no	6/9	6/9	22	27	open	0.3	0.3	nil	nil	yes	nil
475p	female	59	50	yes (grandmother- maternal side)	6/9	6/9	30	22	open	0.4	0.4	nil	nil	yes	nil
476p	female	59	56	no	6/9	6/9	28	29	open	0.3	0.3	nil	nil	yes	nil
481p	male	53	47	no	6/9	6/9	25	24	open	0.4	0.4	nil	nil	yes	nil
486p	female	60	58	yes (sister)	6/6	6/6	24	19	open	0.1	0.1	nil	nil	nil	nil
487p	male	81	51	no	6/9	6/9	32	30	open	0.3	0.3	nil	nil	yes	nil
497p	male	79	69	no	6/6	6/6	28	23	open	0.3	0.3	nil	nil	yes	nil
509p	male	48	47	no	6/6	6/6	30	30	open	0.4	0.4	nil	nil	yes	nil
513p	female	60	58	yes (father)	6/9	6/9	20	38	open	0.4	0.4	nil	nil	yes	nil
514p	female	92	83	no	6/9	6/9	32	25	open	0.2	0.2	nil	nil	yes	nil
515p	female	58	55	yes (2 aunts- paternal side)	6/5	6/6	25	17	open	0.4	0.4	nil	nil	yes	nil
518p	male	70	54	yes (sister, 2 cousins-	6/6	6/9	40	38	open	0.4	0.4	nil	nil	yes	nil

				maternal side)											
524p	male	60	55	no	6/5	6/6	28	29	open	0.1	0.1	nil	nil	yes	nil
526p	female	65	65	no	6/4	6/5	26	23	open	0.4	0.4	nil	nil	yes	nil
527p	male	48	42	yes (mother)	6/4	6/5	34	34	open	0.2	0.2	nil	nil	yes	nil
528p	male	63	59	no	6/6	6/6	26	28	open	0.4	0.4	nil	nil	yes	nil

8.6 Appendix VI: Demographic and phenotypic features of NTG individuals

Table 8–4: Demographic and phenotypic features of NTG individuals (*n* = 37)

Patient ID	Sex	Age	Age at diagnosis	Family history of glaucoma	Best visual acquity		Highest recorded IOP		Anterior chamber angle	Cup/disc ratio		Visual field defect		Medication	Laser	Surgery
							Right	Left		Right	Left	Right	Left			
41p	male	80	75	no	6/9	6/5	18	19	open	0.7	0.8	nasal and paracentral	nasal step	yes	nil	nil
57p	female	79	75	no	6/9	6/9	17	17	open	0.8	0.7	arcuate	nasal step	yes	nil	nil
58p	male	75	72	yes (mother)	6/9	6/9	19	19	open	0.6	0.8	nil	nasal step and paracentral	yes	nil	nil
63p	female	83	83	yes (male)	6/6	6/9	18	19	open	0.7	0.8	paracentral	nasal step	yes	nil	nil
64p	female	83	83	yes (sister)	6/6	6/6	18	18	open	0.8	0.9	nasal step	nasal step	yes	nil	nil
68p	female	60	56	no	6/9	6/9	17	17	open	0.5	0.6	nil	nasal step and paracentral scotoma	yes	nil	nil
74p	female	80	74	no	6/9	6/6	19	17	open	0.6	0.4	arcuate and nasal step	nil	yes	nil	nil
88p_51	male	60	58	yes (father)	6/9	6/9	16	16	open	0.5	0.5	paracentral	nasal step	yes	nil	nil
88p_72	female	63	49		6/5	6/6	18	18	open	0.8	0.8	advanced	advanced	yes	nil	yes
89p	female	67	67	yes (father)	6/6	6/9	20	19	open	0.5	0.6	nasal step	arcuate	yes	nil	nil
95p	male	85	85	no	6/9	6/9	14	15	open	0.8	0.8	arcuate	nasal	yes	nil	nil
96p	male	75	67	yes (mother)	6/9	6/9	17	19	open	0.7	0.7	paracentral	paracentral	yes	nil	nil
100p	female	87	82	yes (brother)	6/6	6/6	20	19	open	0.8	0.6	arcuate	nasal step	yes	nil	nil
102p	female	73	70	yes (brother)	6/9	6/9	14	14	open	0.8	0.7	paracentral and nasal step	nasal step	yes	nil	nil
109p	male	70	65	yes (brother and nephew)	6/9	6/6	19	15	open	0.8	0.6	paracentral	nasal step	yes	nil	nil
112p	female	89	87	no	6/9	6/9	16	17	open	0.5	0.7	nil	arcuate and nasal step	yes	nil	nil
115p	male	72	66	yes (father)	6/12	6/9	20	19	open	0.8	0.8	paracentral and nasal step	arcuate and nasal step	yes	nil	nil
119p_ah	female	88	84	no	6/9	6/9	15	15	open	0.8	0.8	advanced	advanced	yes	nil	nil
126p	female	85	85	yes (sister)	6/9	6/6	15	15	open	0.4	0.5	arcuate	paracentral	yes	nil	nil
131p	female	84	77	no	6/6	6/9	10	10	open	0.9	0.9	advanced	advanced	yes	nil	nil
138p	female	71	64	yes (uncle-maternal side, and nephew)	6/6	6/6	20	18	open	0.9	0.8	arcuate	nasal step	yes	nil	nil
139p	female	88	69	no	6/6	6/9	19	20	open	0.8	0.9	arcuate and paracentral	arcuate	yes	nil	nil
148p	female	77	75	yes (sister)	6/9	6/9	19	19	open	0.7	0.7	nasal step and arcuate	arcuate	yes	yes	nil
151p	female	64	59	yes (mother)	6/9	6/6	18	18	open	0.7	0.6	nasal step	paracentral	yes	nil	nil
161p	female	75	70	yes (mother)	6/9	6/9	19	17	open	0.3	0.5	nil	arcuate	yes	nil	nil
402p	female	83	73	yes (2 aunts-maternal side)	6/5	6/9	16	16	open	0.7	0.4	nasal step and arcuate	nasal step	yes	nil	nil

424p	male	66	61	yes (5 aunts-paternal side)	6/9	6/9	19	19	open	0.9	0.9	advanced	advanced	yes	nil	nil
426p	female	82	82	yes (father and aunt-paternal side)	6/9	6/9	15	18	open	0.5	0.7	paracentral	arcuate	yes	nil	nil
435p	female	92	88	yes (mother)	6/6	6/6	14	19	open	0.9	0.9	advanced nasal step	advanced arcuate	yes	nil	nil
454p	female	65	65	yes (mother and sister)	6/9	6/9	16	19	open	0.6	0.7	nil	nasal step	yes	nil	nil
456p	male	74	74	yes, (father)	6/9	6/6	19	18	open	0.6	0.7	nil	arcuate	yes	nil	nil
460p	female	88	77	no	6/9	6/9	17	17	open	0.6	0.7	nasal step	nasal step and arcuate	yes	nil	nil
472p	female	83	78	yes (brother)	6/12	6/9	10	12	open	0.4	0.7	nil	paracentral	yes	nil	nil
490p	male	73	71	yes (uncle-maternal side)	6/9	6/9	15	20	open	0.5	0.7	nasal step	paracentral	yes	nil	nil
511p	female	77	66	yes (aunt-maternal side)	6/9	6/9	19	19	open	0.7	0.7	nasal step	nasal step	yes	nil	nil
519p	female	84	67	yes (sister and mother)	6/6	6/9	19	18	open	0.8	0.9	nasal step and arcuate	nasal step and arcuate	yes	nil	yes
522p	female	86	78	no	6/9	6/6	16	14	open	0.8	0.9	advanced	advanced	yes	nil	nil

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