University of Newcastle upon Tyne
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An association study of *PITX2* polymorphism in a cohort of patients with primary open angle glaucoma and considerations on the genetics of glaucoma

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Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification for this or any other university or other institute of learning.

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ABSTRACT

Background: Glaucoma is a major cause of blindness world-wide. There is a need for methods to identify individuals at risk of developing glaucoma, so that early treatment can prevent visual loss. Genetic screening tests offer the prospect of pre-symptomatic diagnosis of at risk individuals. There is now strong evidence that a number of different genes are associated with glaucoma susceptibility. Mutations in the *PITX2* homeobox transcription factor gene disrupt normal development of the anterior segment and cause overt structural abnormalities. It is possible that, as yet undetected mutations/polymorphisms in *PITX2* may produce subtle and undetected abnormalities in anterior segment structure and function that could predispose to glaucoma.

Purpose: The aim of this thesis is two fold:
1. Screening for the presence of single nucleotide polymorphisms in PITX2 gene in a cohort of 100 unrelated primary open angle glaucoma/ ocular hypertension patients, 10 Posterior embryotoxon subjects and 100 age and ethnically matched controls to establish the mutation spectrum.
2. Identification, phenotyping and recruitment for genetic studies of primary open angle glaucoma patients with strong family history of glaucoma.

Materials and methods:
1. 100 primary open angle glaucoma patients and 60 age and ethnically matched controls were enrolled in the study. Patients and controls were phenotyped and a blood sample for DNA extraction collected. *PITX2* exon-specific primers were used to PCR amplify patient and control DNA. Direct sequencing was used to screen for sequence alterations in the entire coding sequence of *PITX2* gene. Concurrently, polymorphic sites reported in the *PITX2* gene were identified from the NCBI and Ensembl databases and the frequency of polymorphic sites was investigated. The SHEsis and UNPHASED software packages were used for statistical analysis.
2. Patients diagnosed with glaucoma and strong family history were identified from Glaucoma Unit at Sunderland Eye Infirmary, phenotyped and enrolled in the study. The pedigrees were constructed and interested relatives enrolled in the study and phenotyped. A sample of blood for DNA extraction was collected from all people enrolled in the study.

Results:

1. Direct sequencing did not identify any sequence variation in the coding region. 26 *PITX2* polymorphic sites were identified from the internet databases, including five in the coding sequence. Sixteen non coding SNPs were confirmed within our study group and SNP frequencies were examined. None of the coding sequence SNPs was identified in our cohort, demonstrating a high degree of sequence conservation. Also, none of the SNPs confirmed in this study group showed an increased frequency in the primary open angle glaucoma group compared with the control group.
2. Thirty-three pedigrees were identified with strong family of glaucoma during the time allowed for patient recruitment. Of these, twenty-two agreed to take part in the study. Thirteen pedigrees are presented in this study, mostly demonstrating autosomal dominant inheritance.

Conclusion: There is ample evidence to suggest that genetics play an important role in unravelling the pathogenesis of glaucoma. Identification and recruitment of patients for genetic studies is an essential step and the role of the clinician in this process is paramount. Also, developmental glaucoma genes are an important group of genes to be screened in primary open angle glaucoma/ocular hypertension patients, as they may play a role in the pathogenesis of this preventable blinding disease.

To my family

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Publications and presentations arising from the thesis

1. Idrees F, **Vaideanu D,** Fraser SG, Sowden JC, Khaw PT: A Review of Anterior Segment Dysgeneses. *Survey Ophthalmol* 51: 213-31, 2006

2. Idrees F, Bloch-Zupan A, Free SL, **Vaideanu D**, Thompson PJ, Ashley P, Brice G, Bitner-Glindzicz M, Khaw PT, Fraser SG, Sisodiya SM, Sowden JC: A Novel Homeobox Mutation in the *PITX2* Gene in a Family With Axenfeld-Rieger Syndrome Associated With Brain, Ocular, and Dental Phenotypes.
Am J Med Genet Part B (Neuropsychiatric Genetics) 141B:184–191, 2006

3. **Vaideanu D**, Sowden JC, Fraser SG: Prevalence of *PITX2* mutations/polymorphism in patients with primary open angle glaucoma. EVER 2004

4. **Vaideanu D**, Sowden JC, Fraser SG: Prevalence of *PITX2* polymorphism in patients with primary open angle glaucoma. EVER 2006

An association study of *PITX2* polymorphism in a cohort of patients with primary open angle glaucoma and considerations on the genetics of glaucoma

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Abbreviations

ADRB1 and ADRB2-Beta adrenergic receptors
AGTR2-Angiotensin II receptor, type 2
ALT = argon laser trabeculoplasty
ANF/NPPA – natriuretic peptide precursor A
APOE – apolipoprotein E
ARA/S – Axenfeld Rieger Anomaly/Syndrome
ASD – Anterior Segment Dysgeneses
BMP4 and 7 - bone morphogenetic protein
C2H2-150 – KRAB domain-containing C2H2 type zinc-finger protein
CCT – Central corneal Thickness
CD/CV –common disease/common variant
C/D – cup-disc ratio
CHED1-Congenital Hereditary endothelial dystrophy
CYP1B1 – cytochrome P450, subfamily 1, polypeptide 1
DKN1A-Cyclin-dependent kinase inhibitor 1A
DLX2 – distal–less homeobox 2
DNA –deoxyribonucleic acid

EGRα – early growth response alpha

EDNRA-Endothelin receptor, type A

EYA1-Drosophila eyes absent gene

FOXC1 and FOXC2-Winged-helix/fork head gene 1 and 2

FOXE3-Winged-helix/fork head gene 3

FGF8 – fibroblast growth factor 8

ER – endoplasmic reticulum

GJA1- gap junction protein, alpha 1

GSTM1-Glutathione S-transferase, mu1

GLC 3A-C-OMIM abbreviation for congenital glaucoma loci

GLC1A-N- OMIM abbreviation for glaucoma loci

HD-homeodomain

HESX1- homeobox gene expressed in ES cells

ICG – infantile congenital glaucoma

IH/IGDA – Iris Hypoplasia/ Iridogoniodysgenesis Anomaly

IGF2-Insulin-like growth factor II

IL1B and IL1ά-Interleukin 1-beta and Interleukin 1-alpha

IOP – intraocular pressure

JOAG –juvenile open angle glaucoma

LD –linkage disequilibrium

LHX3 – LIM homeobox gene

LMX1B-Lim homeobox transcription factor

MAF - Muscoloaponeurotic fibrosarcoma oncogene homolog

MTHFR-5,10-methylenetetrahydrofolate reductase

mRNA - mitochondrial ribonucleic acid

MYOC – myocilin

NOS3-Nitric oxide synthase 3

NPPA-Natriuretic peptide precursor A

NTG – normal tension glaucoma

OPTN-optineurin

PCOLCE2 – type I procollagen C-proteinase enhancer protein-like

OD-optic disc

OPTC –opticin

OCLM-Oculomedin

OPA-Optic atrophy 1

OHT – ocular hypertension

ON – optic nerve

OLFM2 –olfactomedin

PAX6-Paired box gene 6

PCR –polymerase chain reaction

PE –posterior embryotoxon

PEXG- psedoexfoliation glaucoma

PITX2-Pair-like homeodomain transcription factor 2

PITX3-Pair-like homeodomain transcription factor 3

POAG – primary open angle glaucoma

PLOD1 – procollagen lysyl hydroxylase

PROP1- prophet of PIT1

SEI –Sunderland Eye Infirmary

SNP –single nucleotide polymorphism

TAP1-Transporter, ATP-binding cassette, major histocompatibility complex, 1

TIGR- trabecular meshwork induced glucocorticoid response protein

TGFβ-Transforming growth factor beta super-family

TM – trabecular meshwork

TNF- α – tumour necrosis factor alpha

Tyr-Tyrosinase

UTR = untranslated region

VAMP 5 & 8 – vesicle associated membrane protein

WDR36- WD repeats containing protein 36

Chapter 1: INTRODUCTION

1.1 WHAT IS GLAUCOMA

The term glaucoma is first mentioned as a diagnosis by physicians in the Hippocratic Aphorisms. The word glaucoma is derived from the Greek glaukos meaning a dull, greyish green colour, which is descriptive of advanced glaucoma seen in those days (Kronfeld 1996).

Glaucoma is a family of diseases that share an acquired optic neuropathy characterised by excavation (or cupping) of optic nerve head and thinning of the neuro-retinal rim. When loss of optic nerve tissue is significant, patients develop visual field loss. Approximately 50% of fibres can be lost before visual field loss occurs (Sommer 1991b).

There are many classifications of glaucoma, e.g.: according to the anatomy of the anterior chamber (open angle, closed angle), age of onset (infantile, juvenile, and adult) and aetiology (primary, secondary). A definitive classification of glaucoma will be possible only when all of the etiological factors, including genetic loci and contributing factors, have been identified.

For the purpose of this work, glaucoma will be broadly classified into three main types, although there can be a considerable overlap between these categories. (Shields 1996)

- open angle
- closed angle
- developmental

The structures of the anterior segment of the eye (cornea, trabecular meshwork, iris as well as both the lens and ciliary body) play an integral part in the regulation of the intraocular pressure. The anterior chamber angle is formed by the junction of the iris posteriorly and the cornea anteriorly. Aqueous secreted by the ciliary body leaves the eye via the trabecular meshwork, which is located at the anterior chamber angle. Developmental abnormalities of the migration and differentiation of neural crest cells

that contribute to the development of these structures may lead to impaired aqueous outflow.

A procedure known as gonioscopy is used to differentiate between the different types of glaucoma. Gonioscopy involves examination of the anterior chamber with a lens that enables the observer to visualise the angle between the cornea-sclera and iris.

In closed angle glaucoma, the anterior chamber angle is narrowed to the extent that the peripheral iris blocks aqueous outflow through the trabecular meshwork. This occurs with and without pupillary block.

By contrast, primary open angle glaucoma (POAG) is a diagnosis of exclusion reached when the anterior segment appears normal and no cause for glaucoma can be identified. The disease has an adult onset (>40 years of age), is usually bilateral and has no noticeable symptoms in most patients until the later stages of the disease, when patients lose their central vision (European Glaucoma Society terminology and guideline for glaucoma 2008).

Developmental glaucoma occurs in the young as a result of a defect developed during gestation or at birth. An association of developmental glaucoma is with anterior segment dysgeneses of the eye.

Anterior segment dysgeneses (ASD) of the eye are a genetically heterogeneous spectrum of clinical disorders that result from malformation of neural crest derived endothelial tissues (Kupfer & Kaiser-Kupfer 1979, Shields 1983, Smelser & Ozanics 1971). One condition that belongs to this group and is important for this thesis is Axenfeld-Rieger anomaly and syndrome-ARA/S (mutations in the gene investigated in this project can result in ARA/S).

1.2 ANATOMY AND PHYSIOLOGY OF THE EYE RELEVANT TO GLAUCOMA

1.2.1 Ganglion cells and optic nerve anatomy

The optic nerve and the retina are an extension of the Central Nervous System. The 1.2 million axons of the ganglion cells of the retina converge towards the lamina cribrosa to form the optic nerve (ON) head. The distribution of these axons as they pass across the retina to enter the optic nerve head is the key to the interpretation of visual field loss in relation to optic nerve cupping in glaucoma. The nasal fibre enter nasally, the fibres temporal to the fovea follow an arcuate path around papillomacular bundle (fig 1.1) and the fibres arising from macula follow a straight course to the ON head, forming a spindle shaped area (papillomacular bundle).

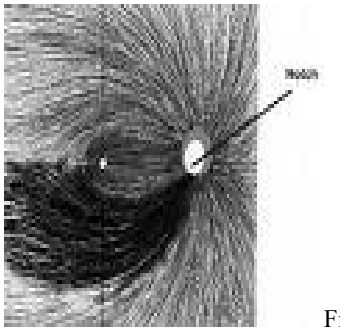


Figure 1.1: Diagrammatic representation of nerve fibres layer course at retinal level (from Madeiros 2002)

Ganglion cells axons exit from the eye through the lamina cribrosa of the scleral canal (fig 1.2), become myelinated posterior to the optic disc and form the optic nerve which travels towards the optic chiasma (Jakobiec 1982, Snell & Lemp 1998).

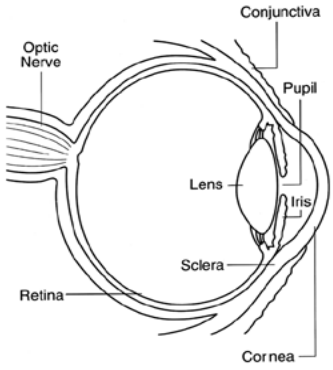


Figure 1.2: Diagrammatic sagittal section of the eye (adapted from www.nci.nih.gov/eyean/)

The optic cup is a small depression in the middle of the ON head which is not occupied by neural disc tissue. The neuroretinal rim is the tissue between the outer edge of the cup and the outer margin of the disc. A normal rim has a uniform width and a pink

colour. The retinal blood vessels enter the disc centrally and then course nasally following the edge of the cup (Figure 1.3).

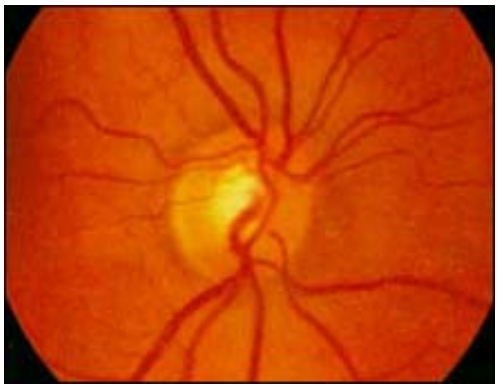


Figure 1.3: Colour photograph of a normal optic nerve head

The diameter of the cup can be expressed as a fraction of the diameter of the disc, both horizontally and vertically and is one the parameters used in diagnosing and monitoring progression of glaucoma. The cup – disc ratio is a partially inherited trait. This has been demonstrated in twin and family based cohort studies (Armaly 1967a, Schwartz et al 1975, Schwartz et al 1976, Klein et al 2004, Chang et al 2005, van Koolwijk et al 2007, Liu et al 2008). In these studies, the heritability of the cup disc ratio ranges from 0.48 to 0.8.

Normal cup-disc ratio in the horizontal meridian is 0.3 and it is larger than in the vertical meridian; only 2% of eye have a ratio more than 0.7 (Jonas et al 1988a).

The neuroretinal rim in the normal eyes shows a characteristic configuration. It is usually broadest in the inferior rim, followed by the superior and nasal rims, and thinnest in the temporal disc region. This pattern of rim is known as the ISNT rule and it was originally described by Jonas et al (1988a). The ISNT rule is a clinically useful method to aid in glaucoma diagnosis.

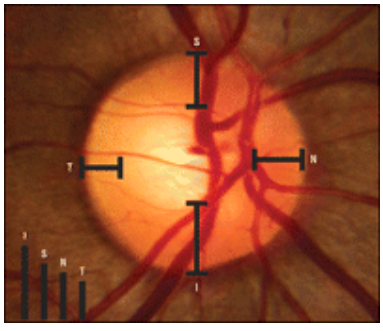


Figure 1.4: Diagram demonstrating the ISNT rule (from Harizman et al 2006)

1.2.2 Aqueous humour physiology

One of the strongest risk factors for POAG is the IOP, which is determined by the rate of aqueous humour secretion, the resistance in the outflow channels and the level of episcleral venous pressure.

The aqueous is actively secreted by the non-pigmented ciliary epithelium, accounting for 80% of the production, the remaining 20% being produced by passive secretion (ultrafiltration and diffusion). The passive secretion is dependent on the blood pressure in the ciliary capillaries, the plasma oncotic pressure and the level of intraocular pressure (Brubaker 1991).

This solution passes to the anterior chamber through the pupil and is drained through the trabecular meshwork, the uveoscleral route, and the iris (1-2%) to the episcleral veins (Figure 1.5). Direct measurements in human eyes have suggested that less than 15% is drained by the uveoscleral route. However, indirect calculations have given a value of about 35% in young adults with a dramatic reduction to 3% in persons more than 60 years of age (Nilsson 1997, Alm & Nilsson 2009, McLaren 2009).

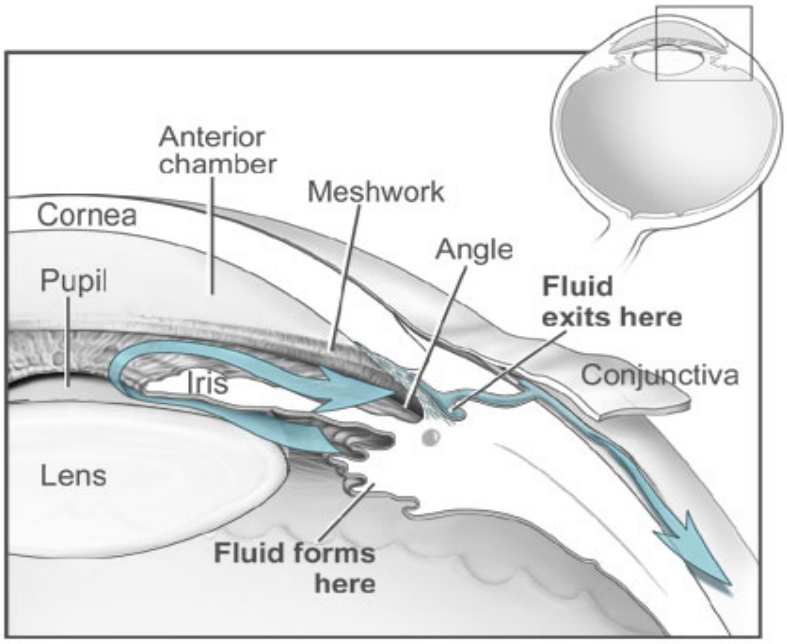


Figure 1.5: Diagram demonstrating the dynamic of the aqueous humour in the eye (adapted from www.nci.nih.gov/eyean/)

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 EPIDEMIOLOGY OF POAG

Glaucoma represents a significant public health problem. It is estimated that glaucoma is the second leading cause of visual loss in the world, with 60.5 million people having the disease in the year 2000 and 6.7 million bilaterally blind from it (Quigley 1996). As the elderly population grows disproportionately, the risk of developing glaucoma increases accordingly, with 8.4 million people estimated to be bilaterally blind from POAG in 2010, rising to 11.1 million by 2020 (Quigley and Broman 2006).
In England and Wales (prevalence estimated at 1.2% - Kroese et al 2002, Tuck and Crick 2003), the number of POAG cases is estimated to increase by a third by 2021, and then continue upwards to a similar pace to 2031.
In the developed countries, fewer than 50% of those with glaucoma are aware of their disease. In the developing world, the rate of known disease is even lower (Quigley 1996). POAG is the commonest type of glaucoma in European and African population and accounts for > 50% of all cases (McKusick 1994). Approximately 5% of people over 70 are affected and have variable ages of onset of the disease.

The prevalence of glaucoma varies with age and race and ranges from 0.43% to 8.8%.(Hollows and Graham 1966, Mason et al 1989, Tielsch et al 1991, Klein et al 1992a, Mitchell et al 1996, Tuck and Crick 1998, Wolfs et al 2000, Weih et al 2001, Varma et al 2004, Rudnicka et al 2006) Data on glaucoma incidence has been derived from age-specific prevalence. Leske et al, (1981) calculated an incidence of 0.08/1000 per year for those in their forties to 1.46/1000 per year for those in their eighties.

In 2007, Leske et al, published the nine year incidence of open angle glaucoma in "Barbados Eye Studies", which was found to be 4.4% or 0.5% per year, for definite glaucoma and 9.4% or 1% per year when the suspected/probable glaucoma cases were included in calculation. Quigley et al (1997), found the cumulative probability of POAG of 4.2% for white persons and 10.3% for black persons. Among those in whom POAG would ever develop, it would develop in 25% by age 64, 50% by age 72 and 75% by age 81 years for white population and about 10 years younger in each group for the black population.

Even mild visual impairment secondary to glaucoma may cause difficulty with mobility, driving and social interaction (Spaeth et al 2006, Ramulu 2009). The loss of

peripheral vision and contrast sensitivity associated with glaucoma can have a major effect on individual's life and the health service.

Because glaucoma is treatable and because the visual impairment from glaucoma is irreversible, early detection of the disease is critically important.

1.4 RISK FACTORS FOR DEVELOPING GLAUCOMA

Although POAG is the most studied of all the glaucomas, the mechanism of optic nerve damage has not been fully elucidated. However, there is a list of risk factors for developing glaucoma that have been suggested and can be classified in: demographic, genetic, systemic and ocular.

1.4.1 Demographic risk factors

1.4.1.1 Age

Prevalence and incidence of glaucoma show a sharp increase with age, usually in people over 40 years of age. The Collaborative Glaucoma Study identified age as the major independent predictor of incidence (Armaly et al 1980) and appears to be consistent across the studies (Hollows and Graham 1966, Leibowitz et al 1980, Tielsch et al 1991, Leske 1994, Mitchell et al 1996, Kroese et al 2002,, Varma et al 2004, Leske 2007b),

1.4.1.2 Gender

The gender as a risk factor for developing glaucoma is controversial, as some studies fail to show an association (Hollows and Graham 1966, Tielsch et al 1991, Klein et al 1992a, Coffey et al 1993,). However, other studies have shown a slight increased risk for females (Leibowitz et al 1980, Mitchell et al 1996), whereas The Barbados Eye Study and The Rotterdam Study show an increased risk for males (Leske et al 1994 and 2007b, Wolfs et al 2000). The Rudnicka et al (2006) performed a Bayesian meta-analysis looking at variation in POAG by age, gender and race. The analysis found an estimated prevalence to be 23% higher in men than women, which appears to be consistent with a very large study looking at the prevalence of glaucoma treatment in the UK (Owen et al 2006).

1.4.1.3 Ethnicity

The Rudnicka et al review (2006) quantifies the rate of change in the prevalence of POAG with age for different racial groups. Although the average prevalence at all ages was higher in black populations than in white or Asian populations, the rate of change of POAG prevalence with age appears highest in white populations (OR doubles per decade) - table1.1.

Table 1.1: Estimated POAG prevalence according to age and race (Rudnicka et al, 2006)

Age range(Y)	Predicted prevalence of OAG (95% CrI)		
	White	Black	Asian
30-39	-	1.8(1.2-2.7)	0.4(0.3-0.6)
40-49	0.4(0.3-0.6)	2.9 (1.9–4.4)	0.6 (0.4–1.0)
50-59	0.8 (0.5–1.2)	4.6 (3.1–6.8)	1.0 (0.6–1.6)
60-69	1.6 (1.1–2.5)	7.2 (4.9–10.6)	1.6 (1.0–2.4)
70-79	3.3 (2.2–4.9)	11.2 (7.6–16.1)	2.5 (1.6–3.8)
80-89	6.6 (4.4–9.7)	16.9 (11.7–23.8)	3.8 (2.3–5.9)
90-95	10.8 (7.2–15.8)	22.5 (15.7–31.2)	—

1.4.1.4 Socioeconomic factors

There is little work done regarding the role of these variables (Leske & Rosenthal 1979). The data on the role of occupational factors are conflicting, with some studies reporting an increased risk with outdoor exposure and other a decreased risk (Packer et al 1964, Bjornsson 1967, Morgan & Drance 1975).

Tielsch et al (1991) investigated the relation between the socioeconomic status and visual impairment and found an association between lower socioeconomic status and visual impairment. Fraser et al (2001) looked to identify socioeconomic risk factors for first presentation of advanced glaucomatous field defect and found that area and individual level deprivation were both associated with late presentation of glaucoma, thus increasing the risk of blindness from glaucoma.

1.4.2 Genetic risk factors

A positive family history of glaucoma is known to increase the risk of the disease (table1.2). Most POAG pedigrees do not show a simple Mendelian pattern of

inheritance; however both autosomal recessive and autosomal dominant transmission has been suggested (Leske 1983). The relatives of patients with glaucoma run an increased risk of developing the condition compared with general population, estimates of the prevalence range from 2.8% to 13.5% (Netland et al 1993).

In the Baltimore Eye Survey, first-degree relatives of patients with POAG had 2.9 times greater odds of having glaucoma than non-relatives (Tielsch et al 1994) and in The Rotterdam study, the life risk of glaucoma was 9.2 times higher in siblings and offspring of glaucoma patients than in sibling and offspring of controls (Hulsman et al 2002). The Beaver Dam Eye Study (Klein et al 2004) found a strong familial effect on risk indicators of glaucoma. To note in this study is the consistent and strong quantitative correlation of intraocular pressure and optic cup dimensions in siblings and in parent and children and the lack of correlation of these parameters in spouses.
A case control study on a Caucasian cohort of 175 POAG patients and 175 control subject showed that, for first degree relatives, a family history of POAG was a major risk factor with odds ratio of 7.67 (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 1991). With regards to ocular hypertension, 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 (Teikari 1987, Gottfredsdottir et al 1999). 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.

The increased susceptibility of glaucoma patients to corticosteroid induced rises in intraocular pressure and HLA typing of glaucoma patients were considered to be promising indicators, however the evidence is inconclusive (Leske 1983).

Some studies have shown a seven to eightfold higher frequency of maternal than paternal history of glaucoma or eye disease (Morgan & Drance 1975, Shin et al 1977), which still remains unexplained.

1.4.3 Systemic risk factors

1.4.3.1 Diabetes

Many studies have suggested that diabetes is associated with glaucoma (Armaly 1969, Becker 1971, Vacharat 1979). Katz & Sommer (1988) found a risk of 2.8 of having POAG if the subject was diabetic. Other large epidemiological studies failed to show an association between diabetes and glaucoma (Armaly et al 1980, Jonas & Gruntler 1998, Tielsch et al1991, Ellis et al 2000, Weih et al 2001, Vijaya et al 2007).

However, a significant association between diabetes and IOP was found in Framingham study, where the prevalence of diabetes was 2 to 3 times as high in persons with pressure > 21 mmHg compared with the persons with pressure less than 21mm Hg (Kahn & Milton 1980).

1.4.3.2 Blood Pressure

A large review of systemic findings in patients with POAG (Pache & Flammer 2006) examines the evidence for association of high and low blood pressure with POAG. The studies reporting on the association between arterial hypertension and POAG conclude a positive, negative or no relationships (, Leske et al 1995, Tielsch JM 1996, Bonomi et al 2000, Quigley et al 2001, Leske et al 2002, Mitchell et al 2004).
Regarding the association between progression of glaucomatous damage and arterial hypotension, there appears to be more evidence available for a positive relationship (Drance 1973, Kaiser & Flammer 1991, Gramer & Tausch 1995, Leske et al 1995, Bonomi et al 2000, Leske et al 2002). This evidence suggests that especially nocturnal drops in blood pressure may play a role in pathogenesis of glaucoma, possibly by reducing the ON head blood flow below a crucial level.
In The Barbados Eye Studies (Leske et al 2007a) a lower systolic blood pressure and especially lower ocular perfusion pressure, doubles the risk of POAG. Lower systolic perfusion pressure and lower systolic blood pressure were found to be positively

associated with glaucoma progression in the Early Manifest Glaucoma Trial (Leske et al 2007b). Similar results were published by the Rotterdam Study group (Hulsman et al 2007).

Table 1.2: 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 (, Klein et al 1992a, Sommer et al 1991a, Dielemans et al 1994, Congdon et al 2004,)
Black ethnicity	4 (Tielsch et al 1991)
First degree family history	2 to 4 (Tielsch et al 1994, Leske et al 1996,Wolfs et al 1998, Le et al 2003,)
IOP compared to 15mmHg	
19-21 mmHg	3 (Ekstrom 1993, Leske et al, 2001, Le et al 2003, Leske et al 2003a)
22-29 mmHg	13
>30 mmHg 40	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 (Tielsch et al 1995, Leske et al 1996, Bonomi et al 2000, Leske et al 2003b,)
FAIR EVIDENCE	
Large C/D ratio, diabetes mellitus, optic disc haemorrhage	
WEAK EVIDENCE	
Migraine (for NTG)	

1.4.4 Ocular risk factors

1.4.4.1 Intraocular pressure

Elevated intraocular pressure (IOP) is consistently reported as one of the primary risk factors for visual field loss. All large longitudinal glaucoma studies confirm that there is a direct relationship between the level of IOP and the progression of visual field loss (Drance et al 1981, Hovding & Aasved 1986, VanVeldhuisen et al 2000, Weih et al 2001, Gordon et al 2002, Mukesh et al 2002, Nouri-Mahdavi et al 2004, Bengtsson and Heijl 2005, de Voogd et al 2005, Bengtsson and Heijl 2007, Leske et al 2007b, Miglior et al 2007a,, Vijaya et al 2007,).

The commonly accepted range for normal IOP in general population is 10- 22 mm Hg. In their paper on IOP distribution in a healthy Iranian population, Hashemi et al (2005) summarise the results of different studies on IOP in various geographic areas and racial groups (table 1.3).

It is obvious from this table, that IOP distribution is different in different races and residents of different locations and may be a geographical or racial approach should be used for defining the normal IOP range.

Heritability of intraocular pressure has been investigated in family and sibling based studies (Armaly 1967b, Klein et al 2004, Duggal et al 2005) and it was estimated to be from 0.29 to 0.50. Several twin studies (Kalenak and Paydar 1995, Parssinen et al 2007, Carbonaro et al 2008, Zheng et al 2009) show a higher heritability (around 0.60) in part due to shared common environment that twins have, such as same age, intrauterine environment and family background. The high heritability of intraocular pressure could be used to detect linkage in a genome wide analysis.

Also, it is important to realize that the relative risk of glaucoma begins to rise from 16 mm Hg onwards and not from 22 mm Hg (Davenger et al 1991, Sommer et al 1991a).

The classical theory that increased IOP damages the optic nerve and produces characteristic changes in the visual field is not always true, especially in POAG. In some individuals, increased IOP does not cause any visual field loss, and this situation is termed – ocular hypertension (OHT). Also, in other individuals, glaucomatous cupping and visual field loss occur at pressure under 22mm Hg, and this group of open angle glaucoma is named normal tension glaucoma (NTG).

Table 1.3: Distribution of IOP and percentage of ocular hypertension in different studies (Hashemi et al, BJO 2005)								
Study	Country, year	Setting	Age/ years	Measurement tool for IOP	M/F	Group	IOP (mean (SD)*	OH (%)*
Shiose	Japan, 1988–9	Population based	⩾40	NCT†	3031/ 5095	M	13.1 (3.0)‡	1.8§
						F	13.4 (3.0)‡	1.3§
						Nl	13.3 (3.0)	NR
						T	13.4 (3.1)	1.4§
Weih 2001	Australia, 1992–6	Population based, cross sectional	⩾40	Tono-Pen (+ Goldmann)	2230/ 2514	Nl	14.5 (3.1)††	1.5
						T	14.7 (3.5)	2.8
Egna-Neumarkt (Bonomi 1998)	Italy, NR 1998	Cross sectional, epidemiological	>40	Goldmann	1882/ 2415	M	15.1 (2.8)	2.7
						F	14.9 (2.6)	1.6
						Nl	NR	NR
						T	NR	2.1
Lee 2002	Korea, 1997–2000	Healthy people examined at a health centre	20–84	NCT	6684/ 6528	M	16.1 (3.2)	6.1
						F	15.1 (2.9)	2.5
						Nl	15.5 (3.1)	4.3
Blue Mountains	Australia, 1992–4	Population based	49–97	Goldmann		M	NR	3.8
Mitchell 1996						F	NR	3.6
						T	16.1 (2.9)†	3.7
Baltimore	US, 1985–8	Population based	⩾40	Goldmann		White	17.2 (3.3)	NR
Sommer 1991a						Black	16.0 (4.2)	NR
Rotterdam Dielemans 1994	Netherland 1991–3	Population cohort	⩾55	Goldmann	1226/ 1836	T	14.6 (NR)	2.2
Beaver Dam	US, NR	Population based	43–84	Goldmann	2135/ 2721	M	15.3 (3.4)	4.4
Klein 1992a&b						F	15.5 (3.3)	4.7
						Nl	15.3 (3.2)	NR

Table 1.3: Distribution of IOP and percentage of ocular hypertension in different studies (Hashemi et al, BJO 2005)

Study	Country, year	Setting	Age (years)	Measurement tool for IOP	M/F	Group	IOP (mean (SD)*	OH (%)*
Barbados Leske 1997	Barbados, 1988–92	Population based	40–84			M‡	17.6 (3.4)	NR
						F‡	18.0 (3.6)	NR
						Black	18.1 (4.8)	12.8
Wallace 1969	Jamaica, 1965	Epidemio-logical study	35–74	Goldmann	232/ 342	M	16.8 (2.8)†‡	NR
						F	16.5 (2.9)†‡	NR
						T	NR	7.4
Rotchford 2002	South. Africa, NR	Population based, cross sectional	>40	Goldmann + Tono-Pen	280/ 725	Nl	13.9 (3.4)	3.5
Framingham	US, 1973–5	Population cohort	52–85	Goldmann	1030/	M	17.0 (4.0)	NR
Khan 1977					1415	F	17.1 (4.1)	NR
						T	17.0 (4.1)	NR
TES Hashemi 2005	Iran, 2002	Population based, cross sectional	≥10	Goldmann	1566/	M	14.4 (2.7)†‡¶	0.4**
					2267	F	14.5 (2.5)†‡¶	0.4**
						Nl	14.5 (2.6)†¶	0.4**

M/F, number of men/women; OH, ocular hypertension (IOP >21 mm Hg); M, male; F, female; Nl, healthy people/eyes; T, total sample; NCT, non-contact tonometer; NR, not reported; US, United States; TES, Tehran Eye Study (the current study).

*Figures were rounded to one decimal point; †Right eyes IOP reported; ‡IOP measurements on healthy eyes excluding glaucomatous eyes; §IOP >20 mm Hg; ††In healthy non-hypertensive eyes; ¶15.1 (2.9) mm Hg for normal men, women, or people ≥40 years; **0.9 for men, women, or people ≥40 years (and 1.8% IOP >20 mm Hg in men, women, or people ≥40 years).

However, as stated above, epidemiological studies show that as IOP increases, there is a corresponding increase in the risk of glaucomatous optic nerve damage and visual field loss (Armaly et al 1980, Drance et al 1981, Sommer et al 1991a, Gordon et al 2002, Nouri-Mahdavi et al 2004, Bengtsson et Heijl 2007, Leske et al 2007a & b, Vijaya et al 2007). Eye pressure more than 30 mm Hg commonly leads to the inference that the disease will soon be present. The Ocular Hypertension Treatment Study (OHTS) group calculated an estimated 5-year cumulative probability of developing POAG in ocular hypertension of 9.3%, and a 16.8% risk for the European Glaucoma Prevention Study placebo group (Gordon et al 2007).
Table 1.4 presents prevalence and relative risk of POAG associated with increasing IOP as presented in Shield's Textbook of glaucoma, 2005

Table 1.4: 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

Although it is evident that IOP is a strong risk factor for developing glaucoma, most of the new cases of field defects in a population cannot be attributed to this factor (Leske 1983).This infer that there must be other factors which contribute to certain people developing glaucoma.

1.4.4.2 Optic nerve head

In most individuals, the optic nerve and visual field changes seen in glaucoma are determined by both the level of IOP and the resistance of the optic nerve axons to pressure damage.
There are two main theories regarding the mechanism of optic nerve damage.

- The mechanical mechanism (IOP related), in which it is suggested that the pressure has a direct effect on the lamina cribrosa of the nerve (Fechtner & Weinreb 1994). The lamina cribrosa has been shown to be weaker superiorly and inferiorly and these are the areas at which glaucomatous damage first appears. 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 rats 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 that the interruption of axonal transport occurred at IOP levels of 40mmHg or even higher, which does not correlate to the lower IOP readings normally seen in patients with POAG

- The vasogenic mechanism, in which is felt that the damage is due to changes within the microcirculation of the optic disc capillaries (Fechtner & Weinreb 1994). 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 (Sossi and Anderson 1983, Ulrich et al 1986, Pillunat et al 1997). Animal studies involving rats have demonstrated that retinal ganglion cells death can occur with exogenous application of the vasoactive peptide endothelin-1, despite normal or low IOP (Chauhan et al 2004a). 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 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 normal tension glaucoma and high tension glaucoma 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)

In a study by Jonas et al (1988b) investigating the morphometric characteristics of optic disc in POAG and comparing them with normal, no significant difference in overall optic disc size and form existed between normal and glaucomatous eyes. In a major review of the optic disc size and glaucoma, Hoffmann et al (2007), advises that it is important to accurately assess the features of the optic disc (neuroretinal rim and cup area), as these are used in the diagnosis and monitoring of glaucoma. It also emphasizes that so far there is no evidence that disc size is an independent risk factor for glaucoma. As mentioned earlier, cup-disc ratio is used in monitoring the progression of glaucoma. Large horizontal cup-disc ratio was one of the five best predictor factors identified in the Collaborative Glaucoma Study (Armaly et al 1980). Other studies identified that vertical cup-disc ratio is a predictor factor for field loss (Armaly 1972, Gordon et al 2002, Miglior et al (EGPS) 2007b).

Optic disc haemorrhages have also been associated with an increased risk for progression of the visual field loss (relative risk (RR) =1.02) as shown by the Early Manifest Glaucoma Trial (Bengtsson et al 2008) and the Collaborative Normal Tension Glaucoma Study (RR = 2.72 - Drance et al 2001).

1.4.4.3 Central Corneal Thickness

Central corneal thickness (CCT) has only recently been described as a predictive factor for progression of OHT to glaucoma (Miglior et al 2007b, Gordon et al 2002, Leske et al 2007a & b, Medeiros et al 2005, Zeppieri et al 2005), greater severity of visual field damage (Herndon et al 2004, Hewitt et al 2005) and more rapid progression of established visual field loss (Madeiros et al 2003, Kim & Chen 2004). It has been demonstrated that CCT varies among racial and ethnic groups (Brandt et al 2001, La Rosa et al 2001, Shimmyo et al 2003) and may lead to misclassification of patients with NTG and OHT (Ehlers & Hansen 1974, Copt et al 1999). Several studies provided evidence that African-American, as a group, tend to have thinner corneas than

Caucasian counterparts and therefore have their IOP underestimated (Nemesure et al 2003, Shimmyo et al 2003).

It is possible that the predictive power of CCT may be due to its effect on IOP reading, however the possibility that the corneal thickness is related to other factor affecting susceptibility to glaucomatous damage cannot be excluded. (Gordon et al 2002). Pakravan et al (2007) reported that CCT is linked to disc size, thicker corneas being associated with smaller optic disc and Toh et al (2005) reported that CCT is one of the highly heritable aspects of ocular structure.

So far there is no validated correction algorithm for IOP in relation to CCT. The implication for the clinical practice is that pachymetry should be performed in patients diagnosed with glaucoma or glaucoma suspects and the corneas should be categorized in thin, average or thick. This would have implications on patient treatment, leading to discontinuation of treatment in many overtreated patients with ocular hypertension and intensifying the treatment in patients with thin cornea, where treatment may be inadequate (Brandt 2007).

1.4.4.4 Refractive status

Myopia or high myopia (> 5 dioptres) has been identified as a risk factor for developing glaucoma in some studies (Georgopoulos et al 1997, Mitchell P 1999, Perkins et al 1982, Ponte et al 1994, Ramakrishnan et al 2003, Xu et al 2007). However, most of the large epidemiological studies (The Barbados Eye Study, Ocular Hypertension Treatment Study and European Glaucoma Prevention Study) failed to identify myopia as a risk factor for developing glaucoma.
The problem with myopic fundus changes is that it can give rise to field defects that resemble those found in glaucoma and the appearance of the optic disc in myopic eyes can be misleading, making the diagnosis of glaucoma more difficult (Leske 1983).

1.4.5. Other proposed risk factors

Alcohol intake and cigarette smoking have been addressed in a number of studies. So far, the results are inconclusive. However, most studies fail to show an association

between POAG and smoking or alcohol intake (Kahn & Milton 1980, Katz & Sommer 1988, Klein et al 1993, Ponte et al 1994, Stewart et al 1994, Kang et al 2007).

All the above data support the concept that glaucoma is a complex disease affected by multiple genetic and environmental factors. Although the pathogenic mechanism is not elucidated, it is mostly accepted that irrespective of what causes the damage in glaucoma, the end-result is ganglion-cell death most probably due to apoptosis, because of lack of trophic factors (Quigley 1995). This theory is supported by research on animal models (Glovinsky et al 1991, Glovinsky et al 1993, Wygnanski et al 1995, Desatnik et al 1996, Weber et al 1998, Nickells 1999, Morgan et al 2000, Morisson et al 2005, Urcola et al 2006,,).

In addition, autoimmune reactions, increased concentration of nitric oxide, and raised concentration of glutamate may contribute to ganglion cell death in POAG (Dreyer & Lipton 1999, Yang et al 2001Ferreira et al 2004,, Gottanka et al 2004, Weinreb & Khaw 2004, Gherghel et al 2005). Figure 1.6 presents a diagram of the factors contributing to pathophysiology of glaucomatous neurodegeneration (adapted from Weinreb & Khaw 2004).

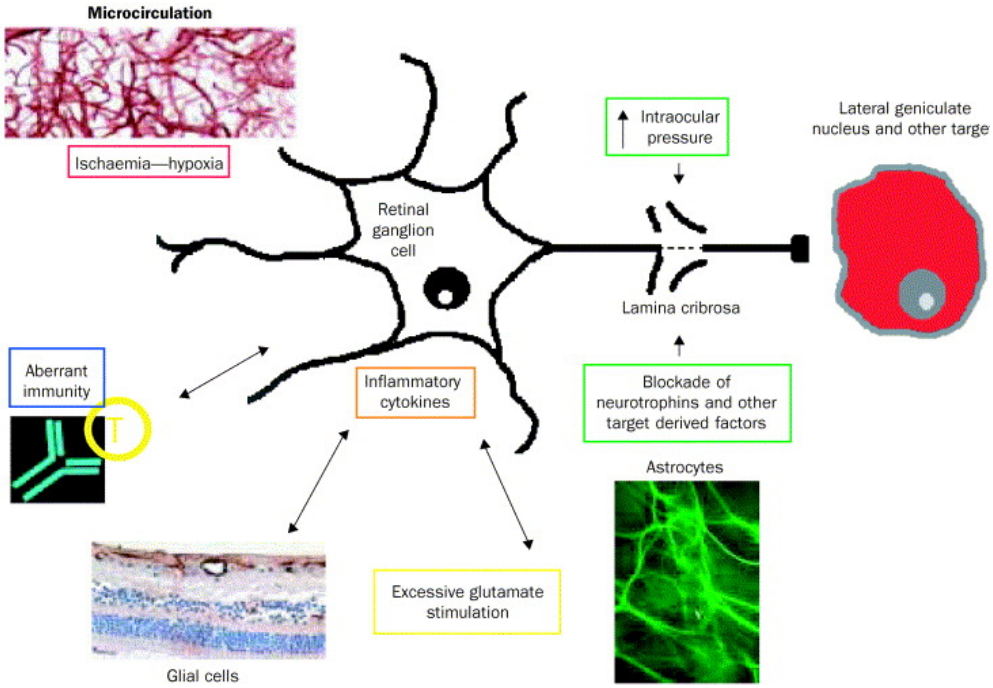


Figure 1.6: Diagrammatic representation of factors contributing to pathophysiology of glaucomatous neurodegeneration (from Weinreb & Khaw 2004)

Recently, Nickells (2007) suggested a theoretical sequence of events from ocular hypertension to ganglion cell death, which may explain many of the conflicting hypotheses of glaucoma pathogenesis. He hypothesizes that elevated IOP causes deleterious changes to glia in the optic nerve head (stage 1), which activates the autonomous self destruction of ganglion cell axons (stage 2), leading to loss of neurotrophic support and apoptotic death of ganglion cell somas in the retina (stage 3). In the initial wave of ganglion cell death, dying cells may adversely affect their neighbouring cells in a wave of secondary degeneration involving glutamate exposure (stage 4), which is followed by replacement of the lost neural tissue with a glial scar (stage 5)

1.5. CLINICAL DIAGNOSIS OF GLAUCOMA

Clinical diagnosis of glaucoma is based on: tonometry (measurements of IOP), gonioscopy (evaluation of the anterior chamber angle structures), examination of the optic disc and visual field testing (perimetry).

1.5.1 Tonometry

IOP is an important parameter for glaucoma evaluation, as it is one the strongest risk factor. Even when IOP is low and signs of glaucomatous neuropathy are present, reduction in IOP level has a beneficial effect on glaucoma progression (Collaborative Normal-Tension Glaucoma Study Group 1998, van Veldhuisen et al 2000, Leske et al 2003, Leske 2007 b).
The two main methods of measuring IOP are by applanation and indentation. The most widely used instrument in clinical practice is Goldmann tonometer. The accepted normal range for IOP is between 10 and 22 mm Hg in general population. However, as emphasised in the risk factor chapter, it is important that the IOP readings are interpreted in correlation with other ocular finding, for the diagnosis of glaucoma to be made.

1.5.2 Gonioscopy

The purposes of gonioscopy are to identify abnormal angle structures, estimate the width of the chamber angle and to visualize the angle during procedures like argon laser

trabeculoplasty and goniotomy. Figure 1.7 presents the view of the angle structures through a goniolens.

Figure1.7: View of the anterior chamber angle through a goniolens

The grading of the angle width is an essential part of the assessment of glaucomatous eyes. The aims are to evaluate the functional status of the angle, its degree of closure and the risk of future closure. There are three grading systems: Scheie (1957), Shaffer (1962) and Spaeth (1971). The most frequently used appear to be the Shaffer system (Figure 1.8), probably because it assigns a numerical grade to each angle with associated anatomical description, which allows for comparison of the width of different chamber angles.

1. Grade 4 (35-45°) is the widest angle in which the ciliary body is visualised with ease and it is impossible of closure.
2. Grade 3 (20-35°) is an open angle in which at least scleral spur can be identified and it is incapable of closure.
3. Grade 2 (20°) is a moderately narrow angle in which only the trabeculum can be identified. Angle closure is possible, but unlikely.
4. Grade 1 (10°) is a very narrow angle in which only Schwalbe's line and may be the top of trabeculum, can be identified. Angle closure is not inevitable, however the risk is high.
5. A slit angle is one in which there is no iridocorneal contact but no angle structures can be identified. This angle has the greatest danger of imminent closure.
6. Grade 0 (0°) is a closed angle resulting from iridocorneal contact. The apex of the corneal wedge cannot be identified.

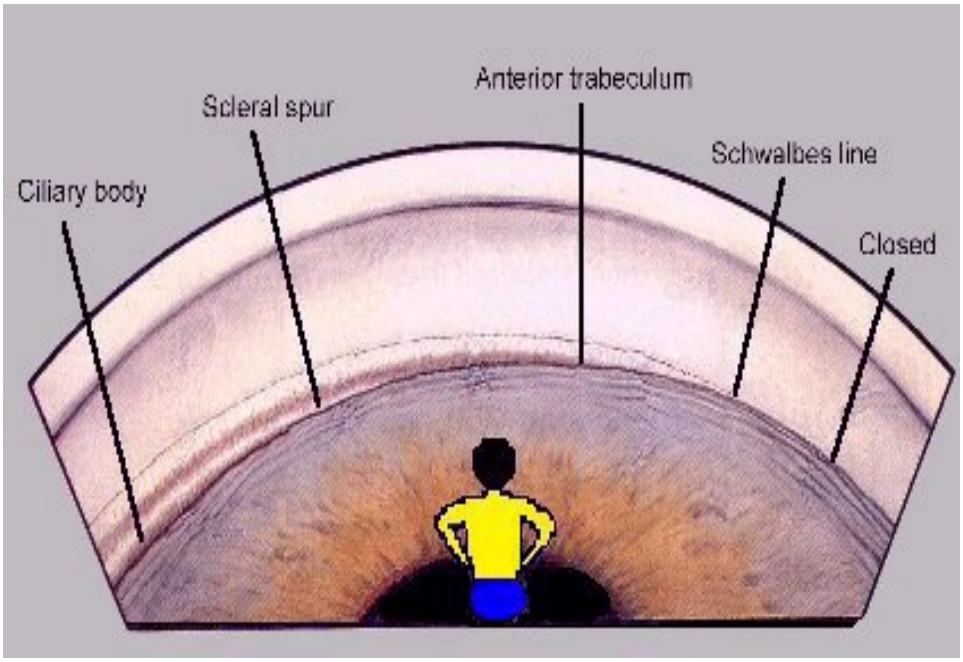


Figure 1.8: Shaffer grading system of angle structure (www.academy.org.uk/tutorials/gonio)

In recent years some other techniques of assessing the angle width and structure have been used (ultrasound biomicroscopy, anterior segment optical coherence tomography, scanning peripheral anterior chamber analysis). Friedman and He (2008) present a comprehensive account of all these techniques in their excellent review "Anterior chamber angle assessment techniques".

1.5.3 Optic disc examination

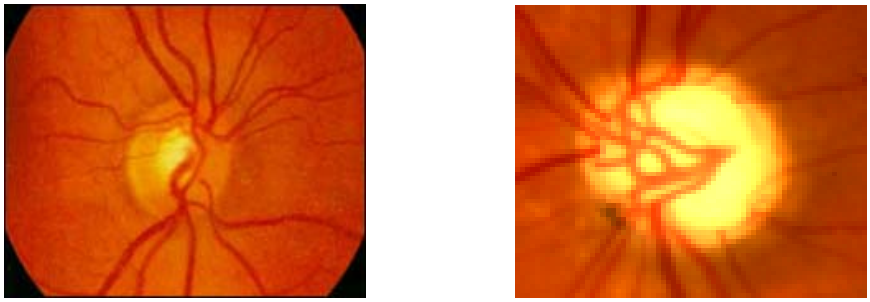
The optic nerve head can be clinically examined either with slit lamp biomicroscope with a condensing lens or with the direct ophthalmoscope through a dilated pupil. Slit lamp examination is superior, as it permits binocular stereoscopic view.

The appearance of the optic disc varies even in "normal" subjects, according to the overall dimension of the globe, the number of the optic nerve fibres present and the configuration of the blood vessels crossing the nerve.

Glaucoma causes a progressive loss of the retinal nerve fibres. The spectrum of disc changes in glaucoma ranges from highly localised tissue loss with notching of the neuroretinal rim to diffuse concentric enlargement of the cup.

1.5.3.1 Optic nerve characteristic in POAG

A standard method of evaluating glaucomatous damage is the cup-disc ratio (Quigley et al 1992). The risk of developing glaucomatous visual field defects increases significantly with cup-disc (C/D) ratio of greater than 0.5. Despite this, even a ratio of 0.8 can be normal, provided that the neuroretinal rim is healthy. Progressive increase of the C/D ratio is important because glaucomatous damage is superimposed on the amount of physiological cupping present before the onset of raised IOP (Figure 1.9.a & b).



a **b**

Figure 1.9: a: normal appearance of the optic disc; b: advanced cupping of the optic disc

Other characteristic changes in glaucoma are: splinter disc haemorrhages, focal (notching) and later generalised thinning of the neuroretinal rim, nasal displacement (nasalization) and change of the angle of the disc vessels (Jonas et al 1988a & b, Bengtsson 1989). Nerve fibre layer defects are detected on the retina corresponding to the area of damage of the disc. At the end stage the disc presents totally cupped and pale due to complete loss of the nerve fibres.

1.5.3.2 Optic nerve imaging

Traditionally, stereoscopic optic disc photographs are used most commonly for baseline assessment and follow up comparison. However, in recent years, imaging devices such as the confocal scanning laser ophthalmoscope, the retinal nerve fibre layer analyser (GDx) and optical coherence tomography (OC T) have been introduced to provide objective and reproducible analysis of optic disc morphometric parameters. They have higher reproducibility and lower interobserver variability compared with clinician

assessment of the optic disc and in addition they allow easy documentation and archiving of disc status, but they still require a degree of manual input (Chauhan 2004b).

1.5.4 Pachymetry

CCT variation is known to affect the accuracy of IOP readings on applanation tonometry, with thick corneas giving falsely high IOP readings and thin cornea falsely low readings (Whitacre et al 1993, Feltgen et al 2001). Although there is no generally accepted correction formula (Brandt 2004) nomograms, based on varying CCT exist for adjusting IOP readings (Ehlers et al 1975, Whitacre et al 1993, Stodtmeister 1998). Two recent studies that adjusted IOP for CCT found that the correction did not alter the diagnosis of high tension glaucoma or normal tension glaucoma (Miyazawa et al 2007), and did not affect the relationship between the prevalence of POAG and IOP respectively (Francis et al 2008). However, even if the diagnosis is not going to be altered, pachymetry should become part of the routine assessment of patients with glaucoma.

1.5.5 Perimetry

1.5.5.1 Principle of visual field testing

Perimetry is a method of evaluating the visual field. The visual field can be illustrated as an island (hill) with a central peak which corresponds to the macula. An area of the field that is not detected is called a scotoma. The optic disc produces a physiological scotoma which is detected only on testing.
The loss of ganglion cell axons in glaucoma results in retinal areas deprived of "output". As a consequence, these areas have reduced sensitivity to optical stimuli.
An efficient way to measure the degree of optic disc damage is by evaluating ones ability to perceive small light targets which are presented randomly on an illuminated background.

There are two main types of perimetry: static and kinetic.

Kinetic perimetry presents a contour map of the hill of vision at different levels while in static perimetry, vertical contours of the hill of vision are calculated along a selected meridian (Crick et al 1989).

Perimetry can be performed either manual or automated. From the manual type, only Goldmann perimeter is still used quite frequently nowadays, otherwise, the automated method is the preferred one.

The method of choice that is currently applied for glaucoma diagnosis and follow up is computerised static threshold technique (Agarwal et al 2000). Threshold is the level of brightness of a light target, which is detected 50% of the time. The values are saved and compared with data stored from normal subjects of matching age. Automated perimetry is fast and provides repeatable objective results (Asman and Heijl 1992) - Figure 1.10.

1.5.5.2 Visual field defects in glaucoma

Typical visual field defects in glaucoma are: nasal field loss, paracentral scotoma, arcuate field loss, and finally a central "tunnel" field of vision in the end stage.
As glaucoma is usually a slow progressive disease, serial annual perimetry used to be recommended to assess the stability of the disease (Macri et al 1998). However, more recent, Chauhan et al (2008) make the following recommendation regarding the frequency of visual field examination:

1. perform sufficient examinations to detect change;
2. six visual field examinations should be performed in the first 2 years, to rule out the presence of rapid progression and establish good baseline data;
3. measure the rate of visual field progression, which is invaluable for guiding therapeutic decisions and the likelihood of visual impairment during patients' life time; this may take several years;
4. use the same threshold test;
5. pay attention to examination quality.

The authors conclude that these recommendations should be used in context with other risk factors, such as baseline damage, age, IOP before a decision on patient management is made.

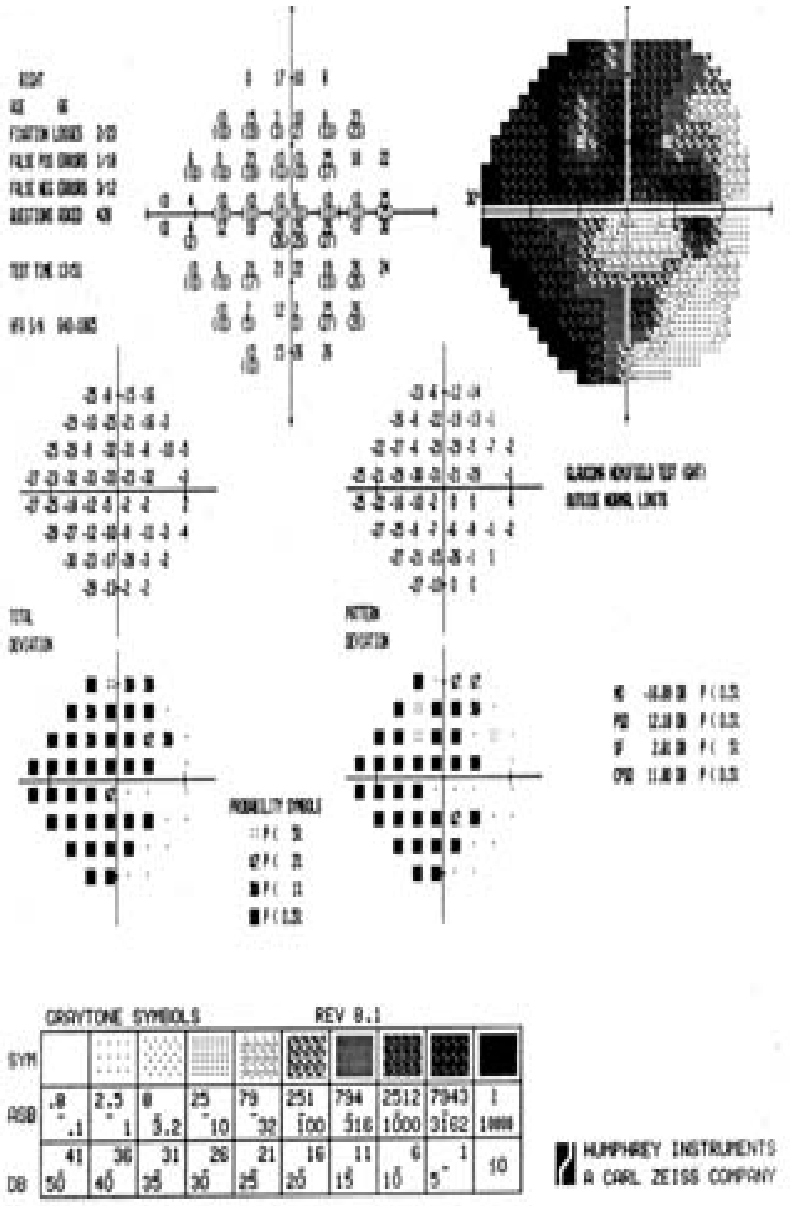


Figure 1.10: Automated perimetry (Humphrey visual field analyzer) demonstrating advanced glaucomatous optic neuropathy

A review from American Academy of Ophthalmology on automated perimetry (Delgado et al 2002), presents different types of technology available (short-wavelength automated perimetry –SWAP, frequency doubling technology – FDT, blue on yellow high pass resolution perimetry, motion automated perimetry and Swedish interactive threshold algorithm- SITA). The conclusion was that while these new techniques can provide additional or confirmatory evidence, they mainly remain in clinical research domain. One exception is SITA, in which studies show significant reduction in testing

time without affecting diagnostic accuracy, making it a more patient friendly test than standard threshold automated perimetry.

1.5.6 Sensory tests

When a conversion from ocular hypertension to glaucoma occurs, the optic disc or retinal fibre layer changes often occur before the visual field defects (Sommer et al 1991 b, Kerrigan-Baumrind et al 2000). Therefore, in recent years, other sensory procedures psychophysical as well as electrophysiological, have been devised that may indicate glaucomatous damage earlier than perimetry (Korth 1997, Martus et al 1998, Martus et al 2000, Stroux et al 2003). These tests are: temporal contrast sensitivity test, spatiotemporal contrast sensitivity test, the peak time of a blue-on-yellow visual evoked potential and the amplitude of a pattern reversal electroretinogram.
However, it has to be noted that the conversion from OHT to glaucoma is relatively rare (1 to 2% per year- Lundberg et al 1987, Heijl et al 2002) and that most patients with glaucoma presenting to ophthalmologist already have established disease. For these patients, perimetry is probably still the most cost-effective way of monitoring the disease.

1.6. MANAGEMENT OF GLAUCOMA

1.6.1 Treatment algorithm for glaucoma

Presently, the goal of treatment for patients with glaucoma is to prevent further loss of functional vision during the remainder of the patient's life and to avoid an adverse impact on patient's quality of life. This goal can be difficult to achieve, as both the treatment for glaucoma (medical or surgical) and the visual loss from the disease affect patient's quality of life.

To reduce the rate of visual loss in glaucoma the aim is to lower the IOP to a level which is considered safe for the ganglion cells given the current amount of damage in the optic nerve head (target IOP). Once the target IOP has been achieved, the patient should be monitored at regular intervals. Progressive glaucomatous damage is determined by changes in the optic nerve head or in the visual field that are consistent with more ganglion cells loss. The development of new damage in the optic nerve head

or visual field requires reassessment of the target IOP and further lowering of pressure if possible (Coleman 1999).

Topically applied ocular medication is usually the initial therapy in the developed countries. Although most of glaucoma therapy is applied topically to the eye, systemic side effects can occur, and can affect adversely the patient's quality of life. Daily medical treatment is expensive and subject to poor compliance. Poor response to medical treatment or advanced glaucoma prompts surgical treatment.

The European Glaucoma Society (EGS) recommends the following steps in their flow chart on the management of POAG: first medication, followed by argon laser trabeculoplasty if IOP not lowered sufficiently with medical treatment and finally incisional surgery (trabeculectomy) if the first two steps were ineffective. If the IOP is still not controlled after observing the above algorithm, then drainage devices or cyclodestructive procedures can be used.

1.6.2 Neuroprotection in glaucoma

The role of neuroprotection in glaucoma is being actively investigated. Glaucoma neuroprotection offers potential as a complementary therapy to IOP-lowering for those patients in whom lowering of IOP does not adequately reduce the rate of disease progression. Also, offers potential as an alternative therapy for those patients in whom pressure lowering agents are not used, not tolerated or ineffective (Weinreb & Khaw 2004). An N-methyl-D-aspartate antagonist –memantine- which has been assessed in two large clinical trials, unfortunately was proved to be unsuccessful as neuroprotectant in glaucoma (Allergan press release Jan 2007).

1.6.3 Conclusion

In many cases, in spite of all efforts to maintain vision some patients will go blind. Ideally, we should be able to diagnose at risk individuals and prevent the onset of the disease.

Risk factors for blindness are:

- increasing age (Fraser et al 1999 and 2001)

- race (African- Caribbean and African subjects have increased risk because of an earlier onset and more aggressive disease and possibly reduced contact with medical services – Sommer et al 1999 c)
- social deprivation (Tielsch et al 1991, Fraser et al 1999 and 2001)
- educational level (Baltimore Eye Study found that years of schooling were inversely associated with the prevalence of blindness and visual impairment –Tielsch et al 1991)
- higher intraocular pressure at presentation (Fraser et al 1999 and 2001)
- longer interval since the subject has visited an optometrist (Fraser et al 1999 and 2001)

Because glaucoma is treatable, and because the visual impairment from glaucoma is irreversible, early detection of the disease is critically important.

The development of genetic diagnostic tests that identify at risk individuals who can be offered early treatment, prior to visual field loss is a promising route for the development of new glaucoma strategies. In the longer term, better understanding of the molecular mechanism underlying glaucoma pathogenesis should lead to new therapies to be used in combination with pre-symptomatic genetic testing.

Chapter 2: ANTERIOR SEGMENT DYSGENESES

2.1 INTRODUCTION

Abnormalities of the anterior segment that can lead to glaucoma have received attention (Gould & John 2002) as they often have large pedigrees and severe phenotypes. Although, relatively rare, they may provide important insights into one of the commonest forms of glaucoma –Open Angle Glaucoma (OAG).

The gene investigated in the pilot study and presented in this thesis (PITX2) has been first discovered by positional cloning in patients with Axenfeld-Rieger Syndrome by Semina et al (1996). Since then, mutations in the same gene have been found to cause different phenotypes diagnosed as ASD.

Therefore, in this chapter a brief description of anterior segment dysgeneses will be given, with Axenfeld-Rieger syndrome being more extensively described.

The Anterior Segment Dysgeneses (ASD), at present, is an ill-defined group of developmental abnormalities that share some common features and a high prevalence of associated glaucoma. Idrees et al (2006) reviewed anterior segment dysgeneses, suggesting a new classification system, which takes into account the embryological contribution to various eye structures affected.

The anterior segment of the eye comprises all the structures lying between the front surface of the cornea and the front surface of the vitreous, usually included within this definition are the eyelids. An Anterior Segment Dysgeneses is a developmental abnormality of the structures of the anterior segment.

2.2 EMBRYOLOGIC DEVELOPMENT OF 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.

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 24 day of gestation (Tripathi and Tripathi, 1989).

Cells derived from the neural crest are extremely important for ocular development in general and anterior segment development in particular (Bahn at al 1984, Beauchamp and Knepper 1984, Cook 1989, Kaiser-Kupfer 1989; Tsai and Grajewski 1994, Beebe & Coats 2000,)

An understanding of the migration and differentiation of neural crest cells (table1) and how these processes can go wrong is vital for an understanding of both the ocular and systemic features of the anterior segment dysgeneses and their association with glaucoma.

ECTODERM	
Surface ectoderm	Conjunctival epithelium
	Corneal epithelium
	Lens
Neuroectoderm	Sphincter and dilator muscles of iris
	Pigmented iris epithelium
	Pigmented ciliary epithelium
	Non-pigmented ciliary epithelium
	Zonules of the iris
Cranial neural crest cells	Corneal stroma and endothelium
	Sclera (except temporal portion)
	Trabecular meshwork
	Schlemm's Canal
	Chamber angle
	Thin layer of anterior iris stroma
	Ciliary muscles
	Ciliary stroma
	Uveal and epithelial melanocytes
MESODERM	
	Temporal portion of sclera
	Endothelial lining of blood vessels

Table 2.1: Embryonic derivatives that contribute to anterior segment formation (adapted from Idrees et al 2006)

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 (Figure 2.1). 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 & Lemp, 1998) - Figure 2.2.

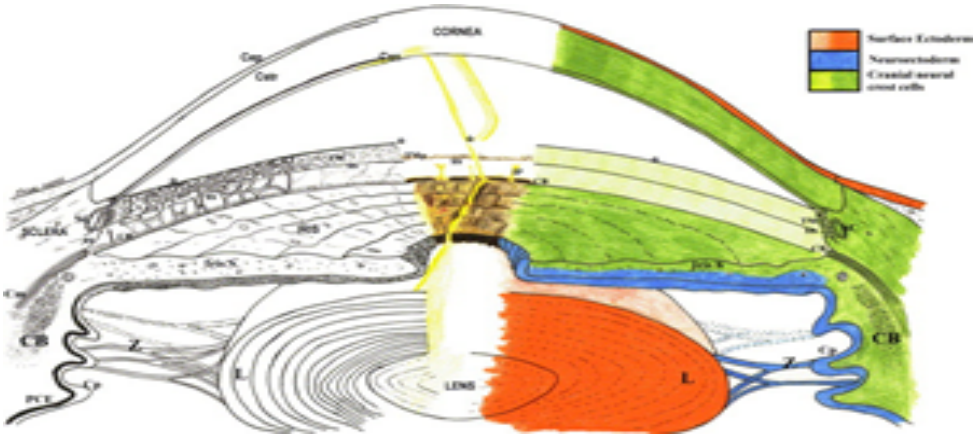


Figure 2.1 Cross section of a mature anterior segment chamber high-lighting important structures at the anterior chamber angle:
(left); in depth view of the angle showing the different layers of trabecular meshwork;
(center) gonioscopic view of a normal angle with a slit-lamp beam; (right) embryological 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)

Neural crest cells are neuroectodermal cells that migrate from the crest of the developing neural tube at about the 24 day of gestation. They migrate to many sites all over the developing embryo and give rise to many structures including the bone and cartilage of the skull, the meninges, the teeth, dermis, melanocytes, peripheral nerves, Schwann cells, the neuroendocrine system, cerebral and spinal ganglia (Bahn et al 1984; Tsai and Grajewski 1994) and of course many of the structures of the eye.

Johnston et al (1966) studied the fate of neural crest derived cells in the chick eye and found that they form the sclera, melanocytes, fibroblasts, muscles of the uveal tract and pericytes of the vascular system. In the developing anterior segment, neural crest cells migrate between the surface ectoderm and the periphery of the optic cup i.e. the developing anterior chamber. Once at the anterior chamber, the neural crest cells form the corneal endothelium and keratocytes, iris stroma cells (i.e. the anterior iris) (Beauchamp and Knepper 1984; Kaiser-Kupfer 1989; Tsai and Grajewski 1994) and melanocytes, trabecular meshwork and juxtacanalicular tissue.

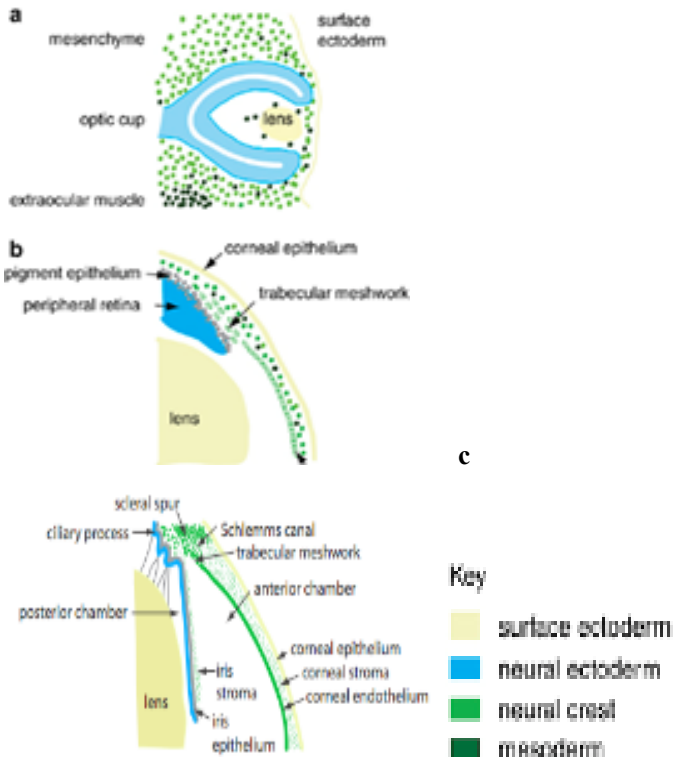


Figure 2.2: The development of the anterior segment of the embryonic and fetal eye. (a) At 5 weeks of gestation, the optic cup is surrounded by mesenchymal progenitor cells, mainly of neural crest origin. (b) Formation of the anterior segment is visible at 5 months of human gestation. (c) Fully developed 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)

At the 6 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 predominantly of neural crest origin (Figure 2.2a).

As early as the 12 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 exists 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.

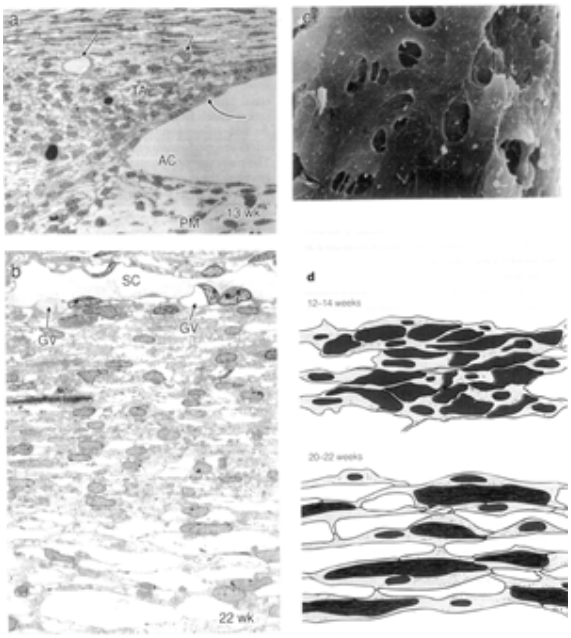


Figure 2.3: Embryonic development of the anterior chamber angle.
(a) Electron micrograph of the trabecular primordium in a 13 week-old fetus. Arrows point to two small capillaries on the deep aspect of the trabecular anlage (TA). AC, anterior chamber; PM, pupillary membrane. (b) Electron micrograph of 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 has 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)

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 2.3) (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.

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 are essential for anterior segment development (Kupfer & Ross 1971, Kupfer and Kaiser-Kupfer, 1979).

Up until 7 months gestation, neural crest cells form a continuous layer extending from the cornea to the trabecular meshwork and onto the anterior surface of the iris becoming continuous with the pupillary membrane and tunica vasculosa lentis (Keiser-Kupfer 1989). However, intercellular gaps between endothelial cells that cover the iridocorneal angle have been demonstrated by scanning laser microscopy (Maumenee 1960). This correlates with physiological evidence that some degree of aqueous drainage is effective by 17-18 weeks of fetal life, and gradually increases during development (Maumenee 1960).

At 7 months gestation, the trabecular meshwork is separated from the anterior chamber by the layer of neural crest cells and little aqueous can drain. It is thought that retraction of these cells allows exposure of the trabecular meshwork and therefore drainage of aqueous – this change correlates with the fact that aqueous outflow increases 0.09µl/min/mmHg at 7 months to 0.3µl/min/mmHg at 8 months.

Although many studies detail prenatal ocular development, few reports document postnatal development. Significant development of anterior chamber structure occurs in the postnatal period (Smith et al 2001, Baulmann et al 2002). This is especially true of the drainage structures that affect IOP and glaucoma. In the first 2-3 postnatal weeks, trabecular meshwork remodels to form a functional TM (Smith et al 2001). A major component of the functional TM is extracellular matrix (ECM) organized into a network of beams that are covered by trabecular cells. The ECM includes collagen, laminin, elastin, fibronectin and vitronectin (Yue 1996). Intratrabecular spaces, between the beams of functioning meshwork, allow aqueous humor to flow to endothelial wall of Schlemm's canal in drainage structures known as giant vacuoles. The lumen of Schlemm's canal connects to the venous system through collectors channels. Abnormal development of these iridocorneal angle structures can lead to elevated IOP and glaucoma (Gould et al 2004).

Since neural crest cells are a major contributing factor towards anterior segment development, one hypothesis for the underlying molecular mechanism involved in anterior segment dysgeneses 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 (Kupfer and Kaiser-Kupfer 1979, Kupfer and Kaiser- Kupfer 1978, Kaiser-Kupfer 1989). 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 (PITX2, FOXC1 and LMX1B) (Semina et al 1996, Pressman et al 2000, Gage et al 2005, Viera et al 2006) play an important role in normal morphogenesis of the anterior segment and provide important insight to the underlying genetic mechanism of glaucoma.

2.3 CLASSIFICATION

The Review of anterior segment dysgeneses paper by Idrees et al (2006), suggests that ASD should be classified in ASD-neural crest (ASD^{nc}), comprising conditions which affect only anterior segment structures derived from neural crest cell and ASD non-neural crest ($ASD^{non\text{-}nc}$), affecting structures which are mainly derived from non-neural crest cells. However, refining of the definition and difficulties in clinical diagnosis may only be resolved as the molecular genetics of these conditions becomes clearer.

The ASD^{nc} is a developmental abnormality of the cornea, angle or iris that can be recognised from a number of specific clinical signs. These occur with a number of other ocular or systemic abnormalities but may also occur in isolation in some patients. The abnormalities are likely to be caused by an underlying failure of neural crest cell migration or differentiation. These abnormalities are often associated with an increased risk of glaucoma.

Within the $ASD^{non\text{-}nc}$ type the authors have included other phenotypes affecting the anterior segment where the neural crest tissue is not thought to be the site of the primary

defect. These include aniridia, which is mainly a defect affecting the posterior iris, which develops from the periphery of the embryonic optic cup and is therefore of neuroectodermal origin (Ormestad et al 2002, Ramaesh et al 2003, Thut et al 2001). However, it has to be mentioned that the lens is the source of an evolutionarily conserved signal that instruct cells of the presumptive neural retina to express genes characteristic of the developing iris and ciliary body (Thut et al 2001). Therefore, it is considered that aniridia is due to gene mutations which act primarily in the lens. Other more recent identified conditions include Anterior Segment Mesenchymal Dysgeneses (Semina et al 1998). The causative gene mutations have been identified in these families and act primarily in the lens (which derives from surface ectoderm) with the defects found in the neural crest derived tissues of the angle probably occurring as a secondary consequence. Further characterisation of similar conditions may lead to greater refinement of the $ASD^{non\text{-}nc}$ classification to include ASD^{n} (ASD-neuroectoderm) and ASD^{se} (ASD-surface ectoderm) indicating the developmental origin of the affecting tissue.

There have been numerous classifications of the ASD's based on their clinical features. This classification remains useful at present but as the molecular genetics of the different conditions are unraveled it seems desirable that future classifications should reflect or incorporate the underlying genetic cause. However, the fact that different members of the same family (who share the same gene mutations) can have quite different phenotypes indicates the limitations of these classifications. Most of the anomalies can have an association with systemic disease.

The anomalies that make up the clinical spectrum of the ASD^{nc} are: (Waring III et al, 1975, de Louise et al, 1983, Shields et al, 1983, Shields et al, 1985)

- Infantile Congenital Glaucoma (ICG)
- Iris Hypoplasia
- Axenfeld anomaly
- Rieger anomaly
- Peters anomaly
- Congenital hereditary endothelial dystrophy (CHED).
- Sclerocornea
- Megalocornea
- Unclassified.

2.4 PHENOTYPES

In this chapter, a brief description of the above conditions and a flow chart suggesting genetic investigations is presented. The genetics of these conditions is discussed in the developmental glaucoma genetics chapter.

2.4.1 Infantile Congenital Glaucoma (ICG)

This is the commonest childhood glaucoma and usually presents within the first six months of life. Use of the term congenital suggests that it is present at birth; in fact although the angle abnormality may be present at birth, the intraocular pressure (IOP) rise may not occur until sometime after. Thus ICG should be reserved for those eyes without any other markers of ASD except in the angle. It is likely that ICG will be more fully understood when the genetics are fully elucidated.

Infantile congenital glaucoma (ICG) is a type of developmental glaucoma and it is inherited in an autosomal recessive fashion, with variable penetrance (Sarfarazi & Stoilov 2000).The incidence is high in the Gypsy population of Slovakia being 1:1250(Gencik 1989), and 1:2500 in the Middle East (Turacli et al 1992) whilst the incidence is less in Western countries ranging between 1:5000 and 1:10 000 (Francois 1985). Sarfarazi & Stoilov (2000) discuss the association of ICG with chromosomal abnormalities and suggests that the association is either secondary to the host of other abnormalities or is purely co-incidental.

The other features associated with ICG i.e. large eyes, thin sclera and breaks in Descement membrane (Haabs striae) are secondary to the rise in IOP rather than being an intrinsic abnormality of the disease itself.

The underlying reason for the block to aqueous drainage has not been fully elucidated - reflecting our incomplete knowledge of the development of the trabecular meshwork. What is known to be an essential step in the formation of the angle is the apparent movement of tissues relative to each other so that the ciliary body moves backwards to expose the developing trabecular meshwork. Anderson (1981) suggests that premature and/or excessive formation of collagenous beams within the trabecular meshwork prevents this normal posterior migration of the ciliary body.

Otto Barkan (1938) first described a membrane over the angle which he considered to be a thin membrane impeding aqueous outflow. This, however, has not been shown to exist with light or electron microscopy (Maumenee 1958, de Louise 1983). Gonioscopically a membrane is not always seen in these children but characteristically the iris inserts into the trabecular meshwork i.e. the iris root is seen to be very anterior (Maumenee 1958, Shields et al 1985). The role of the neural crest cells is not fully understood but is likely to play a role. Kaiser-Kupfer (1989) has suggested that persistence of neural crest cells lining the angle is responsible for the resistance to outflow. ICG is, perhaps, indicative of our poor understanding of the details of angle development and the limitations of clinical observations in understanding aetiology.

2.4.2 Iris Hypoplasia (IH)/Iridogoniodysgeneses anomaly (IGDA)

This was first described by Berg in 1932 in a family who had thin, featureless irides and a very high incidence of glaucoma at a relatively young age (16-43 years). He described the three key features of IGDA as iris hypoplasia, goniodysgeneses and early onset glaucoma. It may also be added to this that the cornea is essentially normal with no embryotoxon or iris adhesions.

There have been a number of pedigrees (Weatherhill & Hart 1969, Jerndal 1972, Waring III & Rodrigues 1980, Pearce et al 1982) described since then - all with the characteristic grey/brown iris colour that represents the pigmented iris epithelium showing through the hypoplastic iris stroma . The glaucoma that occurs is thought to be due to goniodysgeneses, although unlike true primary goniodysgeneses (i.e. ICG) the angle may look normal on gonioscopy and there is a poor response to goniotomy. One feature of the iris that is often overlooked in IH/IGDA is that the iris collarette is absent or small and peripheral and this can be a useful phenotypic marker for IH/IGDA. Idrees et al (2006) suggest that the term Iridogoniodysgeneses should be replaced by the term Iris Hypoplasia as goniodysgeneses has not been proven (Chisholm & Chudley 1983, Heon et al 1995, Walter et al 1996, Alward et al 1998).

2.4.3 Peter's anomaly

This anomaly is caused by the absence of corneal endothelium, Descemets membrane and posterior corneal stroma. Usually this occurs in the central cornea leading to a central corneal opacity – which is often present at birth. Classically iris synaechiae

extend from the collarette to this defect, as can strands from the lens. It may be unilateral or bilateral (Townsend 1974, Waring III et al 1975).
The Peters phenotype is very variable. The corneal opacity can vary from subtle (when it appears as a small indentation on the posterior corneal surface – some authors call this posterior keratoconus (Kenyon 1975, Waring III et al 1975, Krachner & Rodrigues 1978, Frydman et al 1991) to so dense that the diagnosis can only be made using ultrasound (Haddad et al 1997). The opacity may be the only abnormality, or it may be associated with adherent iris strands, or there may be full-blown lens and iris adhesion. The opacity can improve a little with time - perhaps due to improved endothelial function in cells surrounding the defect. Glaucoma occurs in about 50% of cases but is only rarely present at birth (Waring III et al 1975). Lowering of IOP can sometimes improve the corneal opacity itself.

Isolated Peter's anomaly has been described in some pedigrees as autosomal dominant (Hanson et al 1994), autosomal recessive and in others as sporadic (Warring III et al 1975). Peter's anomaly is phenotypically very variable, so much so that it has been suggested that it is a morphological abnormality likely to have a range of different causes (Townsend 1974). This is exemplified by the different syndromes in which it occurs. A number of the syndromes associated with Peter's anomaly have dominant or recessive inheritance whilst others are caused by chromosomal abnormalities. Peter's anomaly has also been described as part of the Foetal Alcohol Syndrome and Warfarin toxicity in pregnancy (Kerber et al 1968, Miller et al 1984) which further illustrates its multifactorial causes.

2.4.4 Congenital Hereditary Endothelial Dystrophy (CHED)

This is a complete or almost complete absence of the corneal endothelium and therefore presents with diffuse bilateral corneal oedema, which does not resolve or improve in the presence of a normal IOP. It has been classified within the ASD's (Churchill & Booth 1996, Waring III et al 1975) as it probably represents failure of migration/differentiation of neural crest cells.

Severity of disease and inheritance has led to two types of CHED being described. Type 1 CHED is less severe than Type 2 CHED; the latter has an earlier age of onset with worse vision and a higher likelihood of nystagmus (Maumenee 1960, Kirkness et al

1987). Type 1 may well not present until sometime after birth and can have a relatively good prognosis.

2.4.5 Sclerocornea

This is a non-progressive scleralisation (i.e. opacification and vascularisation) of the cornea, probably caused by abnormal neural crest migration (Axton et al 1997). It may result from the disordered second wave of neural crest cell migration, which normally passes between the corneal epithelium and endothelium to form the stroma. It is usually bilateral, varying from mild and peripheral to diffuse. Both sclerocornea and megalocornea can occur as isolated abnormalities (Bloch 1965, Howard & Abraham 1971) but are more often associated with other ocular (e.g. Peters) anomalies. Patients with cornea plana (flattening) usually have a degree of peripheral sclerocornea.

Like other anterior segment dysgeneses, sclerocornea has a higher than normal incidence of raised IOP and glaucoma.

2.4.6 Megalocornea

Megalocornea can occur without associated anterior segment abnormalities or in association with other entities such as Axenfeld/Rieger anomaly. True or primary megalocornea is a corneal diameter of greater than 12mm in a newborn or greater than 13mm at any age (Mackay et al 1991). This definition would also describe an eye with raised IOP in a neonate such as those with PCG. To differentiate primary megalocornea from raised IOP, it must be non-progressive, have an IOP within the normal range and have no breaks in Descement membrane or optic disc cupping. Corneal topography shows a normal cornea centrally; therefore the enlargement occurs at the limbus. Specular microscopy shows normal endothelial densities and morphology – unlike in PCG (Skuta et al 1983).

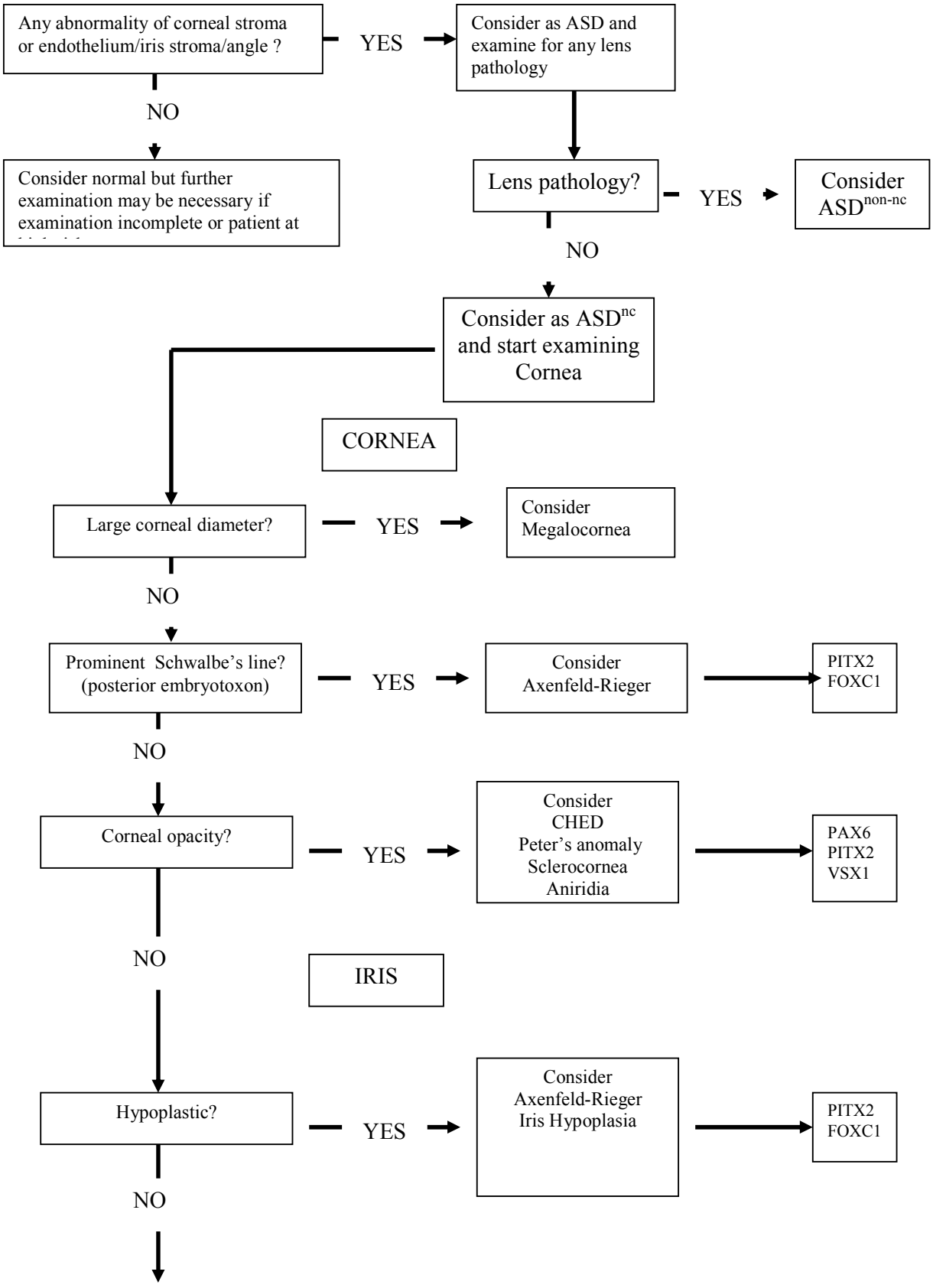
The classification is complicated by the use of the term megalocornea in a description of the buphthalmic eye; this is really a secondary megalocornea. It is probably better to restrict the term megalocornea to the primary type only and to use buphthalmos to describe large eyes secondary to raised intraocular pressure (IOP) from any cause.

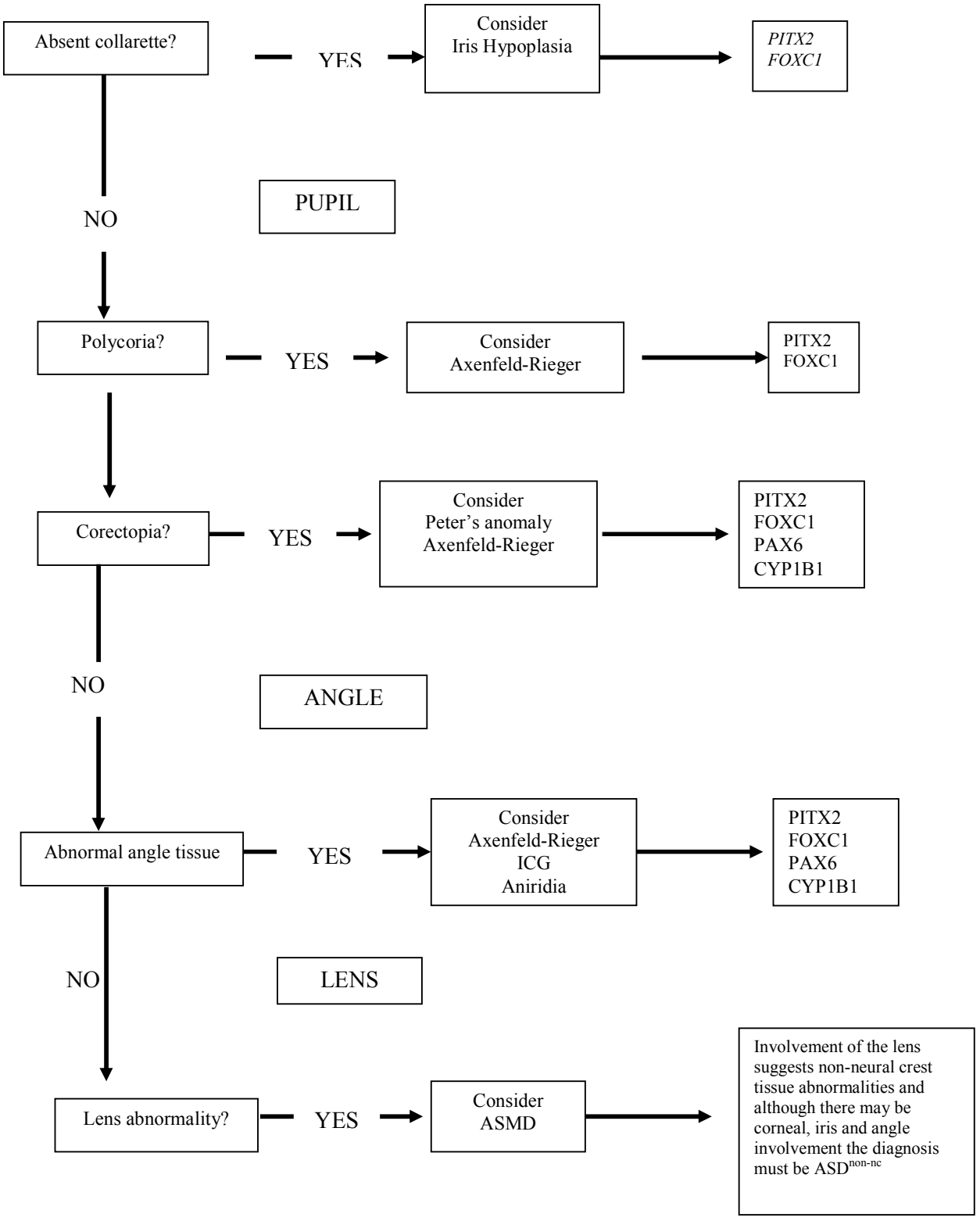
2.4.7 Unclassified

Even those who are familiar with the ASD's often find that certain patients do not fit into the categories described but do seem to have some sort of dysgeneses of their anterior segment often with a secondary glaucoma. From a clinical point of view often the only solution is to describe them (usually by looking for the characteristic features of ASD's) and follow their glaucoma expectantly. From a diagnostic point of view it again seems likely that in the future, molecular genetics will hold the key to classification of these anomalies.

The relative rarity of the anomalies described above can make diagnosis difficult and trying to remember the differing features can be confusing. In fact, their diagnostic features are quite limited and systematic examination of the anterior segment can often allow a logical diagnosis to be made. As with any anterior segment examination, description begins with the cornea, then the iris and if possible the angle. (figure 2.4: flow chart for the diagnosis of ASD/ adapted from Idrees et al 2006).

Figure 2.4: Flow chart of the diagnosis of ASD




Absent collarette?
YES
Consider
Iris Hypoplasia
PITX2
FOXC1
NO
PUPIL
Polycoria?
YES
Consider
Axenfeld-Rieger
PITX2
FOXC1
Corectopia?
YES
Consider
Peter's anomaly
Axenfeld-Rieger
PITX2
FOXC1
PAX6
CYP1B1
NO
ANGLE
Abnormal angle tissue
YES
Consider
Axenfeld-Rieger
ICG
Aniridia
PITX2
FOXC1
PAX6
CYP1B1
NO
LENS
Lens abnormality?
YES
Consider
ASMD
Involvement of the lens suggests non-neural crest tissue abnormalities and although there may be corneal, iris and angle involvement the diagnosis must be ASD non-nc

2.5 AXENFELD RIEGER ANOMALY/SYNDROME (ARA/S)

As mutations in the gene investigated in this study cause mainly ARA/S, the clinical findings in this entity will be presented in this sub-chapter. However, the genetics of this condition is exhaustively discussed in both the genetics of developmental glaucoma and *PITX2* gene chapter and will not be discussed here.

This condition was originally described by Axenfeld in 1920 as a bilateral, white line on the posterior aspect of the cornea with strands from the peripheral iris to this line (Axenfeld T 1920). In 1934, Rieger described the same changes but with the addition of changes in the iris structure such as pulling of the iris towards part of the angle (corectopia), thinning of the iris and if this is severe, hole formation in the iris , sometime mimicking multiple pupils (polycoria)- fig 2.5 I-II.

It has been noted that the severity of both anomalies can vary quite considerably, with a 'severe' Axenfeld little different from a 'mild' Rieger and for this reason the spectrum of abnormalities is often called Axenfeld-Rieger anomaly (ARA - Shields et al 1985). For the moment, it may still be useful to attempt to separate classical Axenfeld type changes (i.e. only peripheral iris involvement) from classical Rieger type changes as it may be that they represent different mutations of the same gene (Alward 2000, Kozlowski and Walter 2000, Lines et al 2002, Lines et al 2004). When they are associated with systemic anomalies they are given the suffix -syndrome i.e. Axenfeld-Rieger syndrome (ARS).

Around 50% of those with Axenfeld-Rieger anomaly develop glaucoma and a rise in IOP is most likely to occur later in childhood. The risk of developing glaucoma does not seem to be related to the severity of the phenotype (Shields 1983, Shields et al 1985). It can however, present at any time from birth to adulthood and patients need lifelong follow-up. If the rise in IOP occurs in the first two years of life it is important to differentiate ARA from ICG (either from the family history or if possible from examination) as the former tends to respond less favourably to goniotomy (McPherson and Berry 1983, Shields 1983).

Various systemic abnormalities have been described in relation to Axenfeld-Rieger anomaly and the combination is called Axenfeld-Rieger syndrome. It has classically

been thought that the presence of systemic features indicated Rieger syndrome but in fact, in keeping with the spectrum of anterior segment anomalies, systemic features can occur in those with an Axenfeld or even IH/IGDA phenotype (Walter et al 1996).

Whilst some authors use certain systemic features as criteria for the syndrome, Idrees et al (2006) suggest that Axenfeld-Rieger anomaly in association with any of the well-described systemic abnormalities should be called Axenfeld-Rieger syndrome. Future information from molecular genetic studies should confirm features that are consistently part of the syndrome and those that are chance or incompletely penetrant associations.

The ocular findings in ARA/S are varied and comprise:

- Posterior embryotoxon (prominent, centrally displaced Schwalbe's line – figure 2.5.I)
- Peripheral anterior synaechiae (iridocorneal adhesions)
- Hypoplastic iris, often disrupted, with displaced pupil (corectopia) or "multiple pupils" (polycoria)-figure 2.5.II
- Cornea can be thicker or thinner then average (mutations in *PITX2* appear to cause reduced corneal thickness –Asai-Coakwell et al 2006, whereas mutations in *FOXC1* appear to have thicker corneas – Lehmann et al 2003); if in addition to being thicker it also is cloudy and large (megalocornea), may be indicative of increased intraocular pressure.
- Glaucoma – present in about 50% of the patients, is a major consequence (Reese and Ellsworth 1966). It can develop in infancy, but more frequent in adolescence or early adulthood.

I **II**

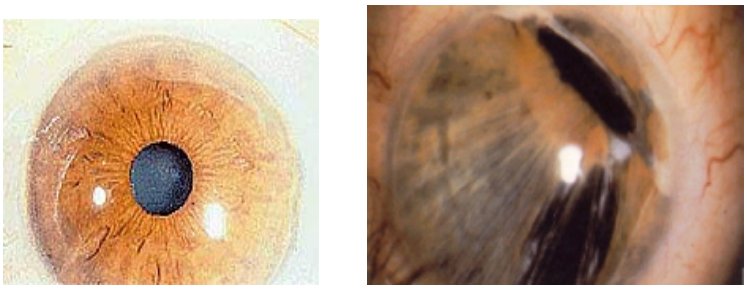


Figure 2.5: I: posterior embryotoxon; II: iris hypoplasia with polycoria and corectopia

Well-described systemic associations in ARS

- Facial and dental anomalies are probably the commonest systemic feature and often occur together (Rieger's (1934) original description was of malformation of the anterior chamber and dental abnormalities).

- Hypertelorism is a wide separation of the two orbits with an increased interpupillary distance, it occurs when the axes of the orbits do not complete the shift from the 180-degree alignment to the normal angle of 71 degrees at birth. The clinical appearance of hypertelorism often includes broadening of the nasal bridge and pushing upward and forward of the frontal region to form a prominent forehead. Both of these have been described in Axenfeld-Rieger syndrome (Steinsapir et al 1990).

- While hypertelorism implies an increased interpupillary distance, it should not be confused with telecanthus, which is defined as an increased distance between the two medial canthal angles with a normal interpupillary distance and is also described in Axenfeld-Rieger syndrome.

- Maxillary hypoplasia is well described and consists of flattening of the mid-face and a short upper lip/philtrum (i.e. relatively protruding lower lip because the mandible is unaffected). Closely associated with these maxillary abnormalities is dental hypoplasia. Other dental anomalies described are: small teeth (microdontia), a decreased number of evenly spaced teeth (hypodontia), focal absence of teeth (oligodontia or anodontia/ Drum et al 1985, Shields et al 1985), characteristic conical shaped teeth, a single set of teeth and abnormalities in enamel resulting in large numbers of dental caries at a relatively young age.

- Varying degrees of umbilical abnormalities have been described in relation to Axenfeld-Rieger anomaly (Friedman 1985) and these appear to represent a failure of involution of the periumbilical skin (Jorgenson et al 1978) rather than a true umbilical hernia – although a number of patients have been found to have true umbilical herniation. Toppare et al (1995) measured the peri-umbilical skin

in neonates and suggested that if the upper umbilical skin (i.e. the cranial side) was greater than 18.82mm this was consistent with Rieger syndrome.

- Hypospadias has been described a number of times as has inguinal herniation in association with Axenfeld-Rieger type anomalies (Jorgenson et al 1978, Chisholm and Chudley 1983).

- Anal stenosis appears to occur with some frequency (Crawford 1967, Brailey 1890).

- Pituitary abnormalities have also been described in different forms. Primary empty sella syndrome (Gould et al 1997), isolated growth hormone deficiency and short stature (Kleinmann et al 1981) and parasellar arachnoid cyst (Shields et al 1985). A recent study (Meyer-Marcotty et al 2008) also found sella turcica bridge combined with a prominent posterior clinoid process followed by a steep clivus and an elongated sella turcica in a family with four members affected.

Syndromes and rare associations with Axenfeld-Rieger anomaly

Axenfeld-Rieger anomaly appears to be part of a number of syndromes and the majority of these are listed below.

- **SHORT** syndrome (Brodsky et al 1996) was described in 1975 and consists of **S**hort stature, **H**yperextensible joints, **H**ernia, **O**cular depression, **R**ieger anomaly and **T**eething delay. Eight separate cases have been described, seven had Rieger anomaly, and it is interesting to note that the other family member had megalocornea.

- Atrial septal defect and sensorineural hearing loss (Cunningham et al 1998) and other cardiac abnormalities have been reported (Tsai and Grajewski 1994).

- Busch et al (1960) found an association between myotonic dystrophy and Rieger anomaly.

- De Hauwere et al (1973) proposed Rieger anomaly with hypertelorism and psychomotor retardation as a separate syndrome. Learning difficulties have been described as a separate association.

- Brooks et al (1989) suggested a new syndrome called 'short-FRAME' consisting of short stature, facial abnormalities (underdevelopment of maxilla and mandible), Rieger anomaly, mid-line anomalies (microcondyles, choanal atresia, anal atresia, scoliosis and kyphosis) and enamel defects.

2.5.1 Posterior Embryotoxon

This is the name given to a very prominent Schwalbe's line seen on the peripheral posterior cornea. Although its origin is uncertain it does seem to be a hallmark of certain ASD's. It has, however, been found to be present in 8-15% of, apparently, otherwise normal eyes (Waring III 1975).
It can vary morphologically from a subtle thin strand on the posterior surface of the cornea to a very thick white band and can even be partially detached and hang from the cornea. It is seen three times more commonly temporally than nasally and is often not seen superiorly and inferiorly as the sclera extends further forward here. In the presence of other anterior segment anomalies the presence of a posterior embryotoxon indicates that the diagnosis is Axenfeld-Rieger anomaly.

Posterior embryotoxon (PE) is a feature of ARA/S, but also found as an incidental finding in the normal population (Waring III 1975, Ozeki et al 1997, Rennie et al 2005). Interestingly, in the papers mentioned above some of the patients who were found to have PE on examination, were also found to have POAG.

Chapter 3: GENETICS OF GLAUCOMA

The current understanding of the pathogenesis of glaucoma is limited and this creates problems for both diagnosis and management. Various risk factors have been identified and among these, raised IOP (over 21 mm Hg) is recognised as one of the most important (Sommer et al 1991a, Tielsch et al 1991, De Voogd et al 2005, Miglior et al 2007a&b, Leske et al 2007b). Hereditary predisposition to glaucoma was first suggested more than 150 years ago and subsequent epidemiological studies established that genetic factors play a major role in the aetiology of the glaucoma's (Leske 1983, Klein et al 2004,). In this chapter an overview of the genetics of open angle glaucoma and developmental glaucoma will be presented.

3.1. PRIMARY OPEN ANGLE GLAUCOMA

3.1.1 Introduction

POAG is considered a heterogeneous, multifactorial disease, in which probably multiple genetic and non-genetic factors are involved (Shin et al 1977, Charliat et al 1994, Hulsman et al 2002). However, it is difficult to discriminate whether familial occurrence of a multifactorial disease is the result of shared genetic or environmental factors.

So far, no environmental factor has consistently been associated with the risk of POAG; either in clinic-based case controlled studies (Charliat et al 1994) or in population based studies (Leske 1983, Katz and Sommer 1988). A twin study in Iceland showed concordance of POAG in twins, but not in spouses, suggesting the involvement of genetic rather than environmental factors (Gottfredsdottir et al 1999). The same study also shows that several of the specific ocular parameters associated with glaucoma are also heritable. These include IOP, optic nerve cup-disc ratio, outflow facility responsiveness to steroids and ocular dimensions such as axial length and anterior chamber depth.

Some forms of glaucoma exhibit a Mendelian pattern of inheritance consistent with the presence of a single gene defect (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, PITX2), as well as primary congenital glaucoma caused by recessive mutation of the cytochrome mono-oxygenase, CYP1B1 (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 (Nemesure et al 1996, Wolfs et al 1998, McNaught et al 2000, Nemesure et al 2001). This is supported by twin studies which have shown a higher degree of concordance among monozygotic twin, as mentioned earlier (Teikari 1987, Gottfredsdottir et al 1999).

Racial differences in prevalence of POAG exist, further supporting a genetic predisposition for glaucoma. The prevalence in Africans is estimated to be six time as high in certain age groups, as that in Caucasians (Hewitt et al 2006). Similar findings in Africans and African Americans lessen the likelihood that such differences are primarily due to external societal or environment specific confounders (Tielsch et al 1991).

In the majority of POAG cases it is likely that more than one genetic predisposition is required to manifest the disease.

The search for the underlying pathogenic mechanism for POAG has been challenging due to the following factors:

- the limited understanding of the biochemical and cellular mechanism involved in the IOP control and retinal ganglion cell function;
- the complex genetics of POAG;
- each causal gene is presumed to only make a small contribution to overall phenotype.

So far, two types of approaches for gene mapping in POAG have been widely applied:

- genome-wide linkage studies

- association studies using either a candidate-gene approach or a genome-wide association approach (figure 3.1)

However, with the advance in technology, microarray gene studies have recently become one of the most promising methods to search for POAG genes.

3.1.1.1 Genome wide studies

When the elementary pathophysiology of a disease is unavailable, genome-wide studies can be applied, in order to identify the genetic profile of the disorder. This method detects genetic markers that segregate with the disease in relatives more often than expected. The advantage of this approach is a comprehensive search across the genome, which has been successful in many monogenic Mendelian disorders (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).

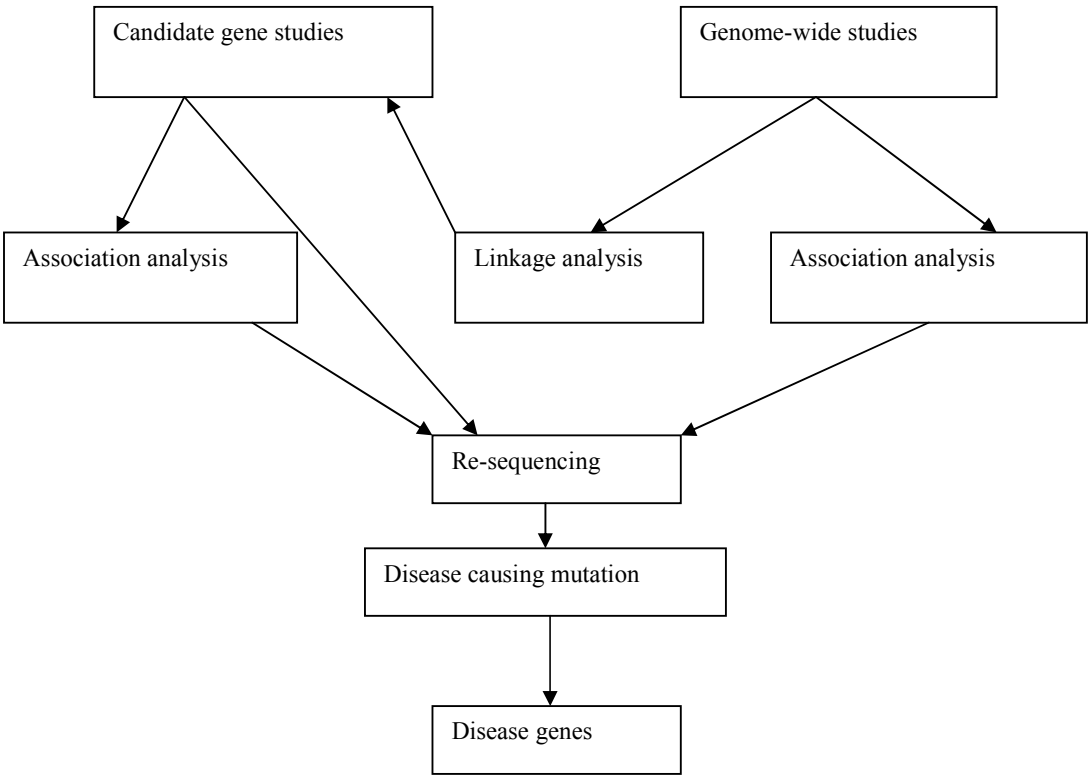


Figure 3.1; Genome-wide and candidate gene approaches towards gene mapping (adapted from Fan et al, 2006).

Linkage analysis has been less successful for complex diseases, probably because of its limited power to detect the modest effects of common genetic variants on disease.
In the case of POAG, pedigree linkage studies had good power for detecting uncommon genes with major effect (*MYOC* and *OPTN*). However, identification of large pedigrees with many affected subjects and living relatives has proved to be hard to find. In their excellent review of "Complex genetics of complex traits: the case of primary open angle glaucoma", Hewitt et al (2006) have identified some reasons why gene identification by linkage has encountered complications like: phenocopy and intrapedigree genetic heterogeneity (Sack et al 1996, Craig et al 2001), loss of statistical power introduced by obscurity of clinical diagnosis in early POAG, high age of functional impairment or manifestation of POAG. Also, it is possible that high false negative results were obtained in linkage studies of POAG pedigrees and sibling pairs using micro-satellites markers, the studies having insufficient power to detect true loci. This might be worth reinvestigating using SNP base platforms, as the high SNP density allows loci to be defined more precisely (John et al 2004).

While a pedigree linkage analysis may be hindered by the high age at functional impairment or manifestation of the disease, it may improve the power of an association study, through conserved LD regions (non-random association of alleles at different loci in haplotypes– Lewontin 1964). For diseases with late age of functional impairment, evolutionary, may not have been under negative selection pressure, and therefore, founder haplotype, as has been identified for *MYOC*, may exist (Baird et al 2003).

Compared with traditional linkage studies, association studies based on LD have two major advantages: only unrelated individuals need to be genotyped, making it possible to study large number of individuals and because LD reflects a large number of historical recombination events, rather than just those in a pedigree, it is possible to fine map disease causing mutations.

Its disadvantage is that they are much more expensive, as they need larger samples and more consumables (Hirschhorn and Daly 2005). This method is increasingly applied to elucidate genetic mechanisms in complex disease (Edwards et al 2005).

Recently, the genome-wide association study by genotyping single nucleotide polymorphisms (SNPs) in case-control studies has become a more powerful tool to

identify genetic causes, especially for complex diseases. This approach surveys most of the genome for the casual variants (Zhang et al 2004). 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.

3.1.1.2 Candidate gene studies

This approach assumes that there is a known candidate gene based on biological hypotheses or location within known POAG loci. Association studies or re-sequencing approaches can be used, to confirm or reject the hypotheses. Association studies are simpler and cheaper than complete re-sequencing of candidate genes, and have been suggested as a powerful method to identify the common genetic variants that underlie multifactorial diseases (Jimenez-Sanchez et al 2001).

3.1.1.3 Microarray gene expression studies

Microarray gene expression studies have recently become one of the most promising methods to search for new POAG genes. Instead of studying one gene at the time, many tens of thousands of genes can be studied simultaneously by using high density gene chip. This global approach has created many new opportunities to study complex genetic diseases like POAG (Leung & Cavalieri 2003a, Leung et al 2003b).
DNA microarrays allow the comprehensive genetic analysis of an organism or a sample. They are based on probes, which are immobilized in an ordered two-dimensional pattern on substrates, such as nylon membranes or glass slides. Probes are either spotted cDNAs or oligonucleotide and are designed to be specific for an organism, a gene, a genetic variant (mutation or polymorphism), or intergenic regions. Thus, they can be used for example for genotyping, expression analysis, or studies of protein-DNA interactions and in the biomedical field they allow the detection of pathogens, antibiotic resistances, gene mutations and polymorphisms, and pathogenic states and can guide therapy. Microarrays, which cover the whole genome of an organism, are available as

well as those which are focussed on genes related to a certain diagnostic application (Bilitewski 2009).

The complementary use of genome wide DNA scanning by linkage/ association analysis and RNA level scanning by microarray technology and functional studies should enhance the identification of candidate genes for POAG (Fan et al 2006).

3.1.1.4 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 and they 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), 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 (Lander 1996, Risch and Merikangas 1996, Collins et al 1997). 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. SNPs are preferred to other genetic markers, because of their high abundance, relatively low mutation rate and easy adaptability to automatic genotyping.

As mentioned above, SNPs can occur in gene coding regions as well as the intronic regions. So far, the majority of disease gene research has concentrated on coding region and to a lesser extent, the promoter region of the genes. However, recent evidence

suggests that intronic regions might be functionally more important than previously assumed (Hewitt et al 2006). Also, to note is relatively few genes are found in the human genome compared with other species (Collins et al 2004) and it has become clear that many genes can produce more than one protein (through alternate splicing), which can have dramatically different functional roles (Hewitt et al 2006).

As already mentioned, genetic association studies can take two forms, termed direct and indirect. In direct association studies, one tests the hypothesis that a given SNP is a causative factor in a disease, by examining whether its frequency is increased in affected individuals. In the indirect approach of LD studies, one uses multiple SNPs to search for evidence for an ancestral haplotype that is enriched in affected individuals. The latter approach, does not require the discovery of each disease causing allele, but does require an extremely dense SNP map and significant increase genotyping capacity (Cargill et al 1999).

The allele frequency of non-synonymous coding SNPs has implications for the sample size need it to perform disease association. Power calculations (Risch and Merikangas 1996) show that detecting association with alleles having frequencies <5% will typically require thousands of patients (except in the rare cases in which the relative risk attributable to the allele is large). Therefore, association studies involving most non-synonymous coding SNPs (cSNP) would require larger populations than are currently available for most phenotypes, as well as higher throughput genotyping technologies. In the near term, direct association with missense cSNPs may be the most practical and promising approach, as SNPs that affect the aminoacid sequence of a protein are likely to explain a significant fraction of disease variation (Cargill et al 1999).

SNP association studies are valuable tools in identification of significant disease associated alleles, as they would be able to detect significant non-coding or silent sequencing changes within gene regulatory sequence, which may alter the levels of functional proteins and cause disease. These alleles may be identified by SNP associated studies, but would not be identified by standard mutation screening of coding sequence. However, interpreting the consequences of non-coding sequence variants is more complicated, as the relationship between promoter and intragenic sequence variation, gene expression level and trait phenotype is less well understood than the relationship between coding DNA sequence and protein function (Glazier et al 2002).

It is believed that SNPs maps will enable scientists to identify the multiple genes associated with complex diseases as cancer, diabetes, vascular disease, age related macular degeneration, glaucoma. These associations are difficult to establish with the conventional gene hunting methods, because a single altered gene may make only a small contribution to the disease phenotype.

As emphasized above, single nucleotide polymorphism (SNP) is the most common type of genetic variant in human genome. Haplotype, defined as a specific set of alleles observed on a single chromosome, or a part of a chromosome, has been an integral part of human genetics for decades. Some studies demonstrated that the analyses of haplotype defined by the grouping and interaction of several variants rather than any individual SNP correlated with complex phenotypes (Judson et al 2000, Morris & Kaplan 2002). Indeed, SNPs based strategies for complex diseases have had recent success, with the identification of a single risk allele for age related macular degeneration in the *complement factor H* gene being achieved through a focused fine SNP mapping (Klein et al 2005, Edwards et al 2005, Hageman et al 2005, Haines et al 2005).

As human population has relatively limited genetic diversity, most of the heterozygosity in the population is attributable to common alleles (that is those that are present at a frequency of >1% in the general population). The infrequent variants include the primary cause of rare, Mendelian diseases, with these allele being usually recent mutations and highly penetrant. By contrast, it has been hypothesized, that the common variants may contribute significantly to genetic risk for common diseases (Cargill et al 1999).

It has been suggested that the haplotype has greater power than any individual SNP to track an unobserved, but evolutionarily linked, variable site. The study of genetic variation will promote our understanding of the differential predisposition to common diseases and variation in drug responses of individuals and ethnic populations (Ouyang & Krontiris 2006). In the case of glaucoma, some studies indicate the presence of disease causing founder haplotypes, especially for *MYOC* gene (Baird et al 2003, Fan et al 2005) and this is an avenue which receives a lot of attention in the research of complex diseases at present.

3.1.1.5 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).

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 (the square of correlation co-efficient). Both measures are calculated, firstly by estimating pairwise haplotype frequencies through expectation-maximisation (EM) (Long et al 1995), 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: $D = f(A1B1) - f(A1)\,f(B1)$ (Lewontin 1964). A and B refer to two genetic markers and f is their frequency. D' is obtained from D/Dmax 2 and does not take into account allele frequencies. R 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 = 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 threshold of > 0.8 would allow a selected tSNP to improve the correlation and resolve >80% of all existing haplotypes.

Most studies use r > 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.

3.1.1.6 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 (2002) was implemented by the HapMap project to constructed LD blocks. This is a methodical 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.

3.2 POAG LOCI (*GLC1A-N*)

Although not common, a number of POAG pedigrees do demonstrate a Mendelian pattern of inheritance. Pedigrees with autosomal recessive inheritance have been described and it has been suggested that this may be the commonest type of Mendelian inheritance in POAG (Booth et al 1997).
Autosomal dominant pedigrees with penetrance varying from 60% to 100% and extremely rare X- linked inheritance have also been described (Stokes 1940, Fleck & Cullen1986).
In the rare families that show dominantly inherited glaucoma, 13 different gene loci have been identified. (Libby et al 2005, Baird et al 2005, Pang et al 2006, Fan et al 2007) and are presented in the table 3.1. However, so far only 3 genes which are involved in POAG have been identified.

3.2.1 *GLC1A*

The *GLC1A* gene encodes a trabecular meshwork protein, *TIGR* (this nomenclature has now been changed to myocilin and abbreviated as *MYOC*) and so far, screening suggests that mutations in *GLC1A* causes 3% of cases of POAG (Lichter 1994, Raymond 1997, Stone et al 1997, Polansky and Nguyen 1998) and over 10% of the Juvenile Open Angle Glaucoma (Fingert et al 1999, Aldred et al 2004).

In the 1960's studies were done to investigate the relation between the IOP response to steroids and glaucoma. These studies led to the suggestion that not only was the observed IOP response to topical steroids inherited, but also that the genes controlling this response were also closely related to the inheritance of POAG (Armaly & Becker 1965, Becker 1965, Armaly 1967c).

The first gene to be associated with POAG was the same gene which was associated with Juvenile Open Angle Glaucoma (JOAG - open angle glaucoma with the age of onset before 40 years of age). JOAG was a logical start point for studying genetics of open angle glaucoma because it shows a strong autosomal dominant inheritance pattern, occurs at an early age, has obvious phenotypic signs and it is found in multiple generations. In 1997, Stone et al identified the *GLC1A* locus for juvenile onset open angle glaucoma and the mutant gene for this locus was found to be the previously described *TIGR* gene (trabecular meshwork induced glucocorticoid response).

The *TIGR* gene has been found to be the same as the *MYOC* gene, described by the Kubota et al (1997), and in 1998 the Human Genome Organisation- Genome Database Nomenclature Committee assigned the gene the name myocilin *(MYOC*). The myocilin protein is associated with the cytoskeleton in the retina and it is present in a variety of ocular and non-ocular tissues (Fingert 1998, Fingert et al 2002). In the eye, it can be produced in greater amounts in time of stress. This stress protein role seems to be of protecting vital cellular proteins or enzymes (Kubota et al 1997). Some of the stress proteins are thought to bind to important cellular proteins, preventing them from unfolding or denaturating under stress - so called "molecular chaperones" (Ellis & van der Vies 1991, Wang & Spector 1995). Liu & Vollrath (2004) shows that mutant *MYOC* molecules induce endoplasmic reticulum (ER) stress and cell death in the trabecular meshwork (TM), due to accumulation of misfolded proteins. An increased susceptibility of TM cells raises the possibility that protein folding abnormalities and ER stress may contribute to glaucoma caused by other genes and that treatment which promote protein folding and secretion may have beneficial effects for IOP (Libby et al 2005).
Also, because ER stress often causes apoptosis (Rao et al 2004), antiapoptotic treatment may also be beneficial by reducing cell death.
As mentioned, myocilin protein is located in many ocular tissues relevant to glaucoma, including aqueous humour, trabecular meshwork, ciliary body and retinal ganglion cell

(Tamm 2002). The protein has an amino terminal signal sequence, a myosin like domain and an olfactomedin domain. Most of the pathogenic mutations occur in the olfactomedin domain (Tamm 2002).
Libby et al (2005) look at the evidence for the role of MYOC protein in aqueous humour physiology and conclude that MYOC does not appear to be necessary for normal IOP homeostasis and that the pathogenesis of *MYOC* mutations is not due to loss of function. On the contrary, it would appear that myocilin associated glaucoma is due to gain of function (Morissette et al 1998, Wiggs & Vollrath 2001). Consistent with clinical findings, MYOC-knockout mice grow normally with normal IOP and ocular morphology (Kim 2001), suggesting that total absence of myocilin is harmless to IOP and visual field. In the trabecular meshwork, *MYOC* has been revealed to principally interact with optomedin, an olphactomedin related protein (Torrado et al 2002), as well as binding with flotin-1, a lipid raft protein (Joe et al 2005). Genes interacting with *MYOC* are potentially good candidate genes for future POAG investigation.

The phenotypic expression of this gene appears to be variable (Fingert et al 2002). That is demonstrated in the case of two genetically identical twins who inherited the same copy of chromosome 1 from the affected father. One twin had severe glaucoma and the other one just minimal change (Wiggs et al 1995). This suggests that other genes or environmental factors might contribute to the full expression of POAG.

So far, more than 50 mutations in *MYOC* gene sequence have been described, all associated with either JOAG or POAG (Fingert et al 2002, Funayama et al 2006), with the Gln368STOP mutation being the most common known individual glaucoma causing variant worldwide (Fingert et al 1999).
To note is that individuals homozygous for some *MYOC* mutations do not manifest severe disease unlike their heterozygous counterparts (Morissette et al 1998, Hewitt et al 2006). This unusual situation may indicate specific biochemical interactions between the mutant and non-mutant *MYOC* subunits (Hewitt et al 2006).

3.2.2 *GLC1B*

Since the discovery of *GLC1A*, several other loci have been identified.

GLC1B locus on chromosome 2cen-q13, seem to possibly be implicated in the pathogenesis of normal tension glaucoma, as well as in those cases of POAG associated with only a modest rise in IOP (Stoilova et al 1996).

Brinkmann et al (2005) investigated the role of *VAMP5* and *VAMP8* as potential candidate genes for POAG, on the basis of the genetic position (*GLC1B* locus), expression in the optic nerve and biochemical function (target membrane transport processes). The study was done on 90 POAG patients and 60 controls and the results indicate that it is unlikely that these 2 genes are involved in glaucoma.

Table 3.1: Genes and loci involved in POAG

Locus name	Chromosomal location	Gene Identified	Contribution to familial glaucoma	References
GLC1A	1q21-q31	*MYO C*	4.4% (Stone et al 1997, Suzuki et al 1997)	Sheffield et al 1993, Richards et al 1994, Graff et al 1995, Morissette et al 1995, Stone et al 1997, Suzuki et al 1997
GLC1B	2cen-q12			Stoilova et al 1996
GLC1C	3q21-q24			Wirtz et al 1997, Samples et al 2004
GLC1D	8q23			Trifan et al 1998
GLC1E	10p15-14	*OPTN*	16.7% of familial NTG (when all variants of OPTN are combined) (Rezaie et al 2002)	Sarfarazi et al 1998, Rezaie et al 2002
GLC1F	7q35			Wirtz et al 1999
GLC1G	5q22.1	*WDR36*	6.92% (Monemi et al 2005)	Monemi et al 2005
GLC1H	2p15- p16			Suriyapperuma et al 2007
GLC1I	15q11-q13			Allingham et al 2005
GLC1J	9q22			Wiggs et al 2004
GLC1K	20p12			Wiggs et al 2004
GLC1L	3p21-22			Baird et al 2005
GLC1M	5q22			Pang et al 2006, Fan et al 2007
GLC1N	5q23-24			Wang et al 2006a

3.2.3 *GLC1C*

The *GLC1C* locus has been described on chromosome 3, 3q21-q24 (Kitsos et al 2001, Samples et al 2004, Wirtz et al 1997). The *type I procollagen C-proteinase enhancer protein-like (PCOLCE2)* gene as a candidate gene for this locus was studied, but no coding sequence mutation were detected.

3.2.4 *GLC1D*

The fourth locus for POAG, *GLC1D* has been assigned to chromosome 8q23. Trifan et al (1998) who discovered the *GLC1D* locus have suggested two possible candidate genes at this site.

First candidate gene, *Syndecan 2*, belongs to a family of integral membrane proteoglycans that participate in cell matrix interactions and the modulations of growth factor response. The authors speculate that a mutation of the *SYND2* gene would alter the adherence properties of adult oligodendrocytes, leading to a demyelinisation of the optic nerve fibres, rendering them more sensitive to other stress factors, such as elevated IOP.

Another positional candidate gene is *EGR*α (*early growth response alpha*) gene. Functional analysis of this protein demonstrates that it could bind to, and stimulate transcription from, a basic transcription element consensus DNA sequence. Basic transcription element is a naturally occurring binding sequence in the promoter region of some cytochrome P450 members and function as transcription modulators. If *EGR*-α is the physiological regulator of the *CYP1B* gene, which has been shown to be responsible for primary congenital glaucoma (Sarfarazi et al 1995) than it is possible that mutations in *EGR*-α may also cause chronic open angle glaucoma.

3.2.5 *GLC1E*

The *GLC1E* locus has been assigned to chromosome 10p14-p15 (Sarfarazi et al 1998) and is associated with mild to moderately raised eye pressures in individuals with optic nerve damage.

Optineurin (OPTN) gene has been linked to the *GLC1E* locus on chromosome 10. The study suggests that mutations in this gene may be responsible for 16.7% of families with normal tension glaucoma, with an additional attributable risk factor of 13.6% in both familial and sporadic cases (Rezaie et al 2002).

The most common mutation is E50K and it was identified in a family with British descent. It has been suggested that a founder effect accounts for this mutation frequency in British patients and therefore estimates of *OPTN* mutation frequency in glaucoma should not be extrapolated from the original study (Alward et al 2003). Other extensive studies (Aung et al 2003, Baird et al 2004, Leung et al 2003a, Toda et al 2004) found similar mutation distributions in patients and controls. When all the studies are considered, *OPTN* mutations do not appear to be a common cause of glaucoma (only 0.1% of unselected POAG cases –Alward et al 2003, Aung et al 2003). One possibility is that *OPTN* mutations do not induce glaucoma unless the genetic context is permissive, and that explain why mutations are also present in controls (Libby et al 2005).

OPTN is a 577 amino acid protein that appears to be secreted. *Optineurin* is expressed in trabecular meshwork, nonpigmented ciliary epithelium, retina and brain. Its expression in the glial cells of the retina and optic nerve indicates that it can affect retinal ganglion cells survival (Rezaie et al 2002). The authors speculate that the wild-type *Optineurin*, operates through *TNF*-α (tumour necrosis factor alpha) signalling pathway and plays a neuro-protective role in the eye and optic nerve. However, when defective, it produces visual loss and optic neuropathy as typically seen in normal and high pressure glaucoma. Components of *TNF*-α signalling pathway can shift the equilibrium towards induction of apoptosis (Rezaie et al 2002). A Japanese study (Funayama et al 2004) found a possible interaction between polymorphisms in the *OPTN* and the *TNF-ά* genes that would increase the risk for glaucoma, which further supports the above hypothesis.

3.2.6 *GLC1F*

The *GLC1F* locus has been assigned to chromosome 7q35-q36 (Wirtz et al 1999). Two candidate genes have been identified by the authors for this locus. First one is *C2H2-150*, which is a KRAB domain-containing C2H2 type zinc-finger protein. C2H2-

type zinc-finger proteins are transcription factors, and the KRAB domain may repress gene transcription (Becker et al 1997).
Nitric Oxide Synthase also maps to this region (Janssens et al 1992). Nitric oxide synthase is an oxidative stress protein and a cytochrome P-450-type hemoprotein (White & Marletta 1992) and an association of a polymorphism in the 5' region of *Nitric Oxide Synthase* in patients with familial POAG has been reported (Tunny et al 1998).

3.2.7 *GLC1G*

The *GLC1G* locus was mapped to 5q22.1 by Monemi et al (2005). The candidate gene for this locus was identified as *WDR36*, which is a novel gene with 23 exons, encoding 951 amino acids and a protein with multiple G-beta WD40 repeats. The gene is expressed in multiple organs throughout the body (heart, placenta, liver, skeletal muscle, kidney, pancreas and eye). In the eye, the gene is expressed in lens, iris, sclera, ciliary muscle, ciliary body, trabecular meshwork, retina and optic nerve (Monemi et al 2005).

The G proteins are a family of membrane associated proteins that act as intermediaries in transduction of the signals generated by trans-membrane receptors. The G-beta subunit is required for membrane anchoring and receptor recognition. Therefore, mutations affecting the structure of the WD40 repeats may interfere with interaction of WDR36 with other proteins. In the original study, a mutation rate of 5.02% was found for this gene (Monemi et al 2005). Subsequent studies, failed to find an association between *WDR36* variants and POAG and suggest that may be *WDR36* is a glaucoma modifier gene (Fingert et al 2007, Hauser et al 2006, Weisschuh et al 2007).

3.2.8 *GLC1H*

GLC1H locus was assigned to chromosome 2p15-p16 by Suriyapperuma et al (2007) and promising genes have been identified and are being investigated at present. However, screening of 35 of these genes has not yielded a causative mutation.

3.2.9 *GLC1I*

GLC1I locus has been identified by Allingham et al (2005) and has been allocated to chromosome 15q11-q13.Variation in sequence in candidate genes in this region would appear to be associated with older age at diagnosis of POAG. This locus contains genes for autism, Prader-Willi and Angelman syndrome and is considered highly complex in genomic function and stability (Nicholls & Knepper 2001). The candidate genes identified by the authors are being investigated at present.

3.2.10 *GLC1J* and *GLC1K*

Wiggs JL et al (2004) identified two novel loci for early onset open angle glaucoma. These are: *GLC1J* located on chromosome 9q22 and *GLC1K* located on chromosome 20p12. The authors compiled a list of genes for each locus which have significant ocular expression (15 genes for chromosome 9 and 23 genes for chromosome 20) and are being investigated at present. One gene the authors single out is the *VSX1* gene, located on chromosome 20. Mutations in this gene have been shown to be responsible for some cases of posterior polymorphous dystrophy, a disease which can be associated with early onset glaucoma (Heon et al 2002).

3.2.11 *GLC1L*

Evidence for a new glaucoma locus –*GLC1L*- located on 3p21-22 has been published by Baird et al (2005) and a positive association between this region and Q368STOP mutation of *myocilin* has been demonstrated in affected individuals.

3.2.12 *GLC1M*

Finally, Pang et al (2006) and Fan et al (2007) have mapped a new locus for POAG, located on long arm of chromosome 5 at 5q22.1-q32 in a large autosomal dominant JOAG family from Philippines and it was designated as *GLC1M*. The same group, have identified another locus, designated *GLC1N* located at 15q22-q24 (Wang et al 2006a). Candidate genes at both loci are being investigated, but no results as yet.

The study carried out by Acharya et al (2007) on 200 patients and 100 controls, examines the *Opticin* (*OPTC*) gene, located on chromosome 1q31-32 as a candidate gene for glaucoma because of its expression in the trabecular meshwork (Friedman et al 2002).

Their results suggest that the "silent" change they identified is associated with glaucomatous phenotype. However, as this mutation was found in only one glaucoma patient out of 200, further investigation is warranted, before any firm conclusion can be drawn. A study published in 2002 (Friedman et al), screened for *OPTC* mutations in 97 patients with sporadic POAG and although Leu268Pro variation was identified in 7 patients, 8/55 controls had the same mutation. These results indicate that so far *OPTC* cannot be associated with glaucomatous phenotype.

3.3 POAG ASSOCIATION STUDIES

So far, the actual genes identified as causative for glaucoma, account for only about 10% of all POAG cases. More information about the genetics of glaucoma comes from association studies. The reported genes and studies to date are presented in table 3.2. Most of the genes investigated are only reported in one or two studies although there are a few genes that have been the subject of multiple association studies.

3.3.1 *Apolipoprotein E*

Apolipoprotein E (APOE) has been reported to be a potent modifier gene for POAG (Copin et al 2002). The same positive association was found by Vickers et al (2002) on a group of 70 Tasmanian patients, by Mabuchi et al (2005) in a study of 310 of NTG Japanese patients and by Junemann et al (2004) on a group of 150 POAG German patients. However, 3 other studies (Lake et al 2004, Ressiniotis et al 2004, Zetterberg et al 2007) found no association on 137 (UK), 155 (UK) and 242 (Sweden) POAG patients respectively.

Apolipoprotein E (APOE) is a lipid transporting protein produced in the liver and brain and it is unique among apolipoproteins through its relevance to nervous tissue. It is involved in mobilisation and redistribution of cholesterol in repair, growth and

maintenance of myelin and neuronal membranes during development or after injury (Junemann et al 2004).
So far it is not obvious how the APOE alleles may be a source of genetic risk for glaucoma and it needs further investigation.

3.3.2 *OPA1*

Another gene which has received more attention in POAG patients is *OPA1* gene, mutations in this gene being a cause of autosomal dominant optic atrophy (ADOA). ADOA is another condition that is characterised by progressive optic nerve damage, attributable to primary degeneration of retinal ganglion cells, followed by ascending atrophy of the optic nerve. The *OPA1* gene has been shown to be expressed in the retinal ganglion cells and the optic nerve (Alexander et al 2000).

Since different mutations in the same gene can cause widely different phenotypes, as demonstrated in glaucoma by *FOXC1, MYOC, PITX2*, Aung et al (2002) hypothesised that the similarities between glaucoma and ADOA make *OPA1* a good candidate gene to examine in glaucoma patients. The study has found that polymorphisms in the *OPA1* gene is associated with glaucoma and may be a marker for the disease (Aung et al 2002). Another study by Powel et al (2003) has found the same results as the initial study. However, 2 studies on ethnically different population (Woo et al 2004 –Korean population and Yao et al 2006 – African Caribbean population) did not find an association between polymorphism in *OPA1* gene and POAG.

3.3.3 *p53 gene*

As suggested earlier, glaucomatous neuropathy appears to be a type of cell death by apoptosis (Nickells et al 1999, Osborne et al 1999). Therefore, some researchers have studied the association between *p53* codon polymorphism and POAG (Acharya et al 2002, Lin et al 2002, Ressiniotis et al 2004) as *p53* genes is one of the regulatory genes of apoptosis (Smith et al 2003).

The first study (Lin et al 2002) has found that the proline form of *p53* gene codon 72 appears to be a significant risk factor in the development of POAG in Chinese patients. However, another study published same year (Acharya et al 2002), could not find an

association between the *p53* codon polymorphism and POAG in a cohort of Indian POAG patients. Ressiniotis et al (2004) have investigated the *p53* haplotype and found a significant difference in distribution between cases and controls. This suggests that none of the polymorphisms investigated can account for the increased risk of POAG, but the true causative mechanism may be in linkage disequilibrium with the haplotype.

3.3.4 *TNF-alpha*

Few studies investigated the association between *TNFα* and glaucoma, on the basis that OPTN is induced by *TNFα* and interacts with several proteins to regulate apoptosis, inflammation and vasoconstriction (Funayama et al 2004). Also, Vittitow and Boras (2002) demonstrated that sustained elevated IOP, *TNFα* exposure and prolonged dexamethasone treatment significantly upregulated *OPTN* expression in the trabecular meshwork.

Lin et al (2003 b) has found and association between the *TNFα* -308 polymorphism and POAG in a cohort of 60 Chinese patients. The association could not be demonstrated on a cohort of 114 Caucasian patients (Mossbock et al 2006b). However, Funayama et al (2004) have found and increased frequency of *TNFα*-857T and *OPTN*/412 carriers in a group of 194 POAG patients and suggests that their findings show a possible interaction between polymorphisms in the OPTN and the *TNFα* genes that would increase the risk for glaucoma.

3.3.5 *MTHFR* gene

On the basis that moderate increase of levels of homocysteine have been found in patients with POAG, and that homocysteine induces apoptotic cell death of retinal ganglion cells and also exerts gliotoxic effect, thus representing a hypothetical risk factor for POAG, a common polymorphism of methylenetetrahydrofolatereductase (MTHFR) has been investigated to test for an association with POAG. The C677T variant of MTHFR gene has been investigated for association in both POAG and psedoexfoliation glaucoma (PEXG) by Junemann et al (2005) and a positive association with POAG has been demonstrated. Also a positive association has been found between NTG and 677C/T MTHFR polymorphism in a Korean study (Woo et al 2009).

However, a Japanese study (Mabuchi et al 2006) and a Caucasian study (Mossbock et al 2006a) failed to show a positive association.

3.3.6 *ADRB1 & 2* genes

A couple of studies investigated the role of beta-adrenergic receptors polymorphisms in open angle glaucoma. The beta-adrenergic receptors (*ADRB 1 and 2)* genes are expressed in human ciliary body and trabecular meshwork and are also found in the human optic nerve and the wall of the microvessels of the optic nerve (Inagaki et al 2006). Also the antagonists of the ADRBs (beta antagonists) are widely used as topical medication to lower intraocular pressure in patients with ocular hypertension and glaucoma.

Gungor at al (2003) reported no differences in the common *ADRB2* allele frequencies in both congenital glaucoma and open angle glaucoma in a Turkish population. However, Inagaki et al (2006) found that some alleles are associated with a younger age at diagnosis and a higher IOP at diagnosis in Japanese patients and that these *ADRB2* polymorphisms may influence the pathophysiology of glaucoma in this ethnic group. The most recent study of β2-adrenergic receptor gene in glaucoma looked at 2 different ethnic population (white Caucasians and Black- African) and found no difference in *ADRB2* alleles and haplotypes between POAG group and control group in either population (McLaren et al 2007). However, the role of *ADRB2* haplotypes in glaucoma as a risk factor of intraocular pressure fluctuation and variation in intraocular pressure response to β-blockers is still to be evaluated.

3.3.7 Gene – gene interactions in POAG

It is obvious from the above review of genetics of POAG that only a small portion of POAG follows the classical Mendelian pattern of inheritance and that a considerable fraction of POAG results from a large number of variants in several genes, each contributing a small effect.

The exact mechanism of the genes implicated in susceptibility of POAG is still unknown. There are reports of gene-gene interactions (Copin et al 2002, Fan et al 2005,

Funayama et al 2004, Funayama et al 2006, Vincent et al 2002), that suggest that the inheritance pattern of glaucoma is polygenic.

In 2002, Vincent et al published the results of a study which suggests that *MYOC* and *CYP1B1* might interact through a common pathway and that the inheritance of glaucoma might be digenic in some cases. Funayama et al (2004) suggest that a possible gene-gene interaction between *OPTN* and *TNF-ά* increases the risk of POAG in Japanese patients. The same research group in a paper published in 2006, identifies a mutation in *noelin2 (OLFM2)* gene as a possible disease causing mutation in Japanese patients with POAG and also suggest that *OLFM2, MYOC* and *OPTN* may contribute interactively to clinical features in patients with POAG, indicating a polygenic aetiology.

APOE polymorphism has been reported to interact with *MYOC* polymorphism to increase IOP in POAG patients (Copin et al 2002).
Fan et al (2005) in their study on 400 POAG patients found possible gene-gene interaction between *MYOC, OPTN* and *APOE*, bringing further evidence that all these genes may interactively contribute to POAG, indicating a polygenic aetiology.

3.3.8 Mitochondrial abnormalities in POAG

Some research has been conducted in the role of mitochondrial DNA abnormalities in POAG. Although the pathogenesis of glaucoma is far from being unravelled, it is believed that ganglion cell apoptosis may play a role (Nickells 1999, Quigley 1999). It has also been described that some neurodegenerative diseases and some optic neuropathies (Leber's hereditary optic neuropathy) are associated with abnormalities of the mitochondrial DNA (Sadun 2002, Howell 2003, Autere et al 2004,).

Abu-Amero et al (2006) has identified mitochondrial abnormalities in a group of 27 POAG patients. In a later study, the same group investigated the distribution of mitochondrial haplogroups in different types of glaucoma in an Arabic population and found that there is an association between a specific haplogroup and primary angle closure glaucoma; these patients were at higher risk of developing the disease (Abu-Amero et al 2008).

Table 3.2: POAG association studies genes			
Gene symbol	Gene name	Chromosomal location	Studies
AGTR2	Angiotensin II receptor, type 2	Xq22-q23	Hashizume et al 2005
APOE	Apolipoprotein E	19q13.2	Copin et al 2002; Vickers et al 2002, Lake et al 2004, Ressiniotis et al 2004, Mabuchi et al 2005, Lam et al 2006, Zetterberg et al 2007
CDKN1A	Cyclin-dependent kinase inhibitor 1A	6p21.2	Tsai et al 2004, Ressiniotis et al 2005
CYP1B1	Cytochrome P450, subfamily 1, polypeptide 1	2p22-p21	Vincent et al 2002
EDNRA	Endothelin receptor, type A	4q31.2	Ishikawa et al 2005, Kim et al 2006
GSTM1	Glutathione S-transferase, mu1	1p13.3	Juronen et al 2000
IGF2	Insulin-like growth factor II	11p15.5	Tsai et al 2003
IL1B and IL1ά	Interleukin 1-beta and Interleukin 1-alpha	2p14	Lin et al 2003 a, Wang et al 2007a &b, How et al 2007, Wang et al 2006b,
MTHFR	5,10-methylenetetrahydrofolate reductase	1p36.3	Junemann et al 2005, Mossbock et al 2006a, Mabuchi et al 2006, Woo et al 2009
NOS3	Nitric oxide synthase 3	7q36	Tunny et al 1998, Logan et al 2005, Pang et al 2005
NPPA	Natriuretic peptide precursor A	1p36.2	Tunny et al 1996, Jeoung et al 2007
OCLM	Oculomedin	1q31.1	Fujiwara et al 2003, Leung et al 2000, Jansson et al 2003
OPA1	Optic atrophy 1	3q28-q29	Aung et al 2002, Powell et al 2003, Woo et al 2004,

			Yao et al 2006, Mabuchi et al 2007
TAP1	Transporter, ATP-binding cassette, major histocompatibility complex, 1	6p21.3	Lin et al 2004
TNF	Tumour necrosis factor	6p21.3	Lin et al 2003 b, Funayama et al 2004, Mossbock et al 2006b
ADRB1 and ADRB2	Beta adrenergic receptors	10q24-q26 and 5q32-q34	Inakagi et al 2006, McLaren et al 2007

However, the group investigated was small and the findings would have to be replicated in a larger cohort and different racial groups for any conclusion to be drawn. A study by Andrews et al (2006), found no association between mitochondrial haplogroups and POAG in a cohort of 140 POAG patients and 75 controls.

A review by Kong et al (2009) provides evidence that age-associated mitochondrial dysfunction renders retinal ganglion cells susceptible to glaucomatous injury by reducing the energy available for repair processes and predisposing the ganglion cells to apoptosis. Eliciting the role of mitochondria in glaucoma pathogenesis may provide a different avenue for therapeutic targets to protect the optic nerve.

3.4 SECONDARY OPEN ANGLE GLAUCOMA

3.4.1 Psedoexfoliation glaucoma

Exfoliation syndrome is an age-related, generalized disorder of the extracellular matrix characterized by the production and progressive accumulation of a fibrillar extracellular material in many ocular tissues (Ritch & Schlotzer-Schrehardt 2001).

It is now understood to be the most common identifiable cause of open angle glaucoma world wide (Ritch 1994). The risk of developing glaucoma is 5 to 10 times more common in eyes with psedoexfoliation than in those without it. Patients with pseudoexfoliation are twice as likely to convert from having ocular hypertension to having glaucoma and when glaucoma is present to progress more rapidly (Grodum et al 2005). Also, an increasing number of systemic associations have been reported more recently (Ritch 2008).

In his review of Genetics of pseudoexfoliation syndrome, Challa (2009) discusses the inheritance patterns and recent genetic advances in the study of this disorder. His conclusion is that pseudoexfoliation syndrome is a major cause for glaucoma that has a strong familial association. Recent studies confirmed that *LOXL1* is a major gene associated with both pseudoexfoliation syndrome and pseudoexfoliation glaucoma, however the exact mechanism by which this gene leads to the development of these conditions has not been identified. He concludes that further studies are needed to answer this question.

3.4.2 Pigmentary glaucoma

Pigment dispersion syndrome and pigmentary glaucoma are characterised by a disruption of the iris pigment epithelium and deposition of the dispersed pigment throughout the anterior segment (Ritch 1996). The classic diagnostic triad consists of corneal pigmentation (Krukenberg spindle); slit like, radial, midperipheral iris transillumination defects and dense trabecular pigmentation. The iris insertion is typically posterior, and the peripheral iris tends to have a concave configuration. There is evidence to suggest that pigment dispersion syndrome is inherited in an autosomal dominant fashion, with phenotypic onset probably beginning in most people in the mid 20s (McDermott et al 1987). That Caucasians are almost exclusively affected is also consistent with a genetic origin.

The frequency with which pigment dispersion syndrome converts to glaucoma has been estimated to about 50% (Ritch et al 1993). However, a study by Siddiqui et al (2003), suggests that the probability of converting to glaucoma was 10% at 5 years and 15% at 15 years.

Pigment dispersion syndrome has been mapped to the 7q35-q36 locus by linkage analysis, but the candidate gene has not been identified yet (Andersen et al 1997).

As pseudoexfoliation syndrome and pigment dispersion syndrome are two common disorders that can produce secondary glaucoma through trabecular blockage, the involvement of the three psedoexfoliation syndrome and glaucoma associated *LOXL1* polymorphisms were studied in two cohort of pigment dispersion and pigmentary

glaucoma patients (Rao et al 2008, Gramer et al 2009). However no significant association was found.

Studies on mice indicate that mutations in genes encoding melanosomal proteins (Anderson et al 2002, Anderson et al 2006) can contribute to developing pigment dispersion syndrome and glaucoma. However, Lynch et al (2002) investigated DNA sequence variants in the tyrosinase-related protein 1(*TYRP1*) gene in a group of patients with pigment dispersion glaucoma and found no association. As with any other types of glaucoma, further studies are needed to help unravel the genetic contribution to this disease.

3.5 CONCLUSION GENETICS OF OPEN ANGLE GLAUCOMA

Notably so far, 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). Fingert et al (1999) 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 2006). Hence it is highly likely that other unidentified loci or genes still exist.

It is obvious from the information presented in this chapter that a great deal of research goes into the genetics contribution to open angle glaucoma. Although, we are still far from unravelling its secrets further studies will add to the body of knowledge and get us a step closer to understanding the disease.

3.6 DEVELOPMENTAL GLAUCOMA GENETICS

3.6. 1 Introduction

Developmental glaucomas are associated with widespread malformations of neural crest derived tissues in the anterior segment of the eye (anterior segment dysgeneses) or systemic anomalies, but glaucoma may not manifest until the second or third decade (Lichter 1994).

These forms of glaucoma often exhibit a Mendelian pattern of inheritance consistent with the presence of a single gene defect. The associated glaucoma may arise from the malformations seen from birth or may arise from continued expression of the gene product throughout life.

Transcription factors expressed in the periocular mesenchyme (*PITX2, FOXC1* and *LMX1B*) (Semina et al 1996, Pressman et al 2000, Gage et al 2005, Viera et al 2006) play an important role in normal morphogenesis of the anterior segment and provide important insight to the underlying genetic mechanism of glaucoma.

Therefore the genes causing developmental glaucoma are an obvious group to test as genetic susceptibility factors for POAG. These genes have not been analysed in any association studies at the time the pilot study presented in this paper was set up, except for *CYP1B1*.
CYP1B1 causes primary congenital glaucoma. It is also involved in cases of juvenile open angle glaucoma (JOAG) (Vincent et al 2002, Acharya et al 2006,) and a recent study identified a *CYP1B1* polymorphism as a susceptibility factor for POAG (Bhattacharjee et al 2008, Melki et al 2005).

The genes and gene loci involved in anterior segment development and in which mutations cause anterior segment dysgeneses are presented in table 2.3.

3.6.2 *CYP1B1* gene

Three loci have been identified, designated *GLC3A* (Sarfarazi et al 1995), *GLC3B* (Akarsu et al1996) and *GLC3C* (Stoilov & Sarfarazi; IOVS 43: E abstract 3015; 2002b).

GLC3A has been mapped to region 21 on the short arm of chromosome 2 i.e. 2p21 whilst *GLC3B* has been mapped to 1p36. Stoilov *et al* (1997) have discovered mutations in the *CYP1B1* gene in the *GLC3A* linked families.

The *CYP1B1* gene codes for a member of cytochrome P450 enzymes, which is a multigene superfamily responsible for phase 1 metabolism of a wide range of structurally diverse substrates (Sarfarazi & Stoilov 2000). It has been suggested that *CYP1B1* gene is involved in the normal development and function of the eye by metabolising essential endogenous and exogenous molecules, including steroids and retinoids (Murray 2001).

Table 2.3: Genes involved in anterior segment development

Gene Symbol/locus	Gene name		Chromosomal location	ASD humans	Reference
TGFβ	Transforming growth factor beta super-family	BMP4 and 7 (bone morphogenetic protein)	14q22-q23 for BMP4 Chr 20 locus for BMP7		Chang et al 2001, Gould et al 2004, Wordinger et al 2002, Wordinger et al 2007
		TGFβ activin/nodal branch	1q41		
PAX6	Paired box gene 6		11p13	Aniridia ARA/S Peters IH	Hanson et al 1994, Axton et al 1997, Hanson et al 1999 Riise et al 2001, Baulmann et al 2002, Nanjo et al 2004 Chavaria-Soley et al 2006a Dansault et al 2007
FOXC1 and FOXC2	Winged-helix/fork head gene 1 and 2		6p25 /FOXC1 16q24.3/ FOXC2	IGDA/ ARA/S Peters ICG	Walter et al 1996 Jordan et al 1997 Mirzayans et al 1997 Nishimura et al 1998 Ekong et al 2004
FOXE3	Winged-helix/fork head gene 3		1p32	ASD Cataract	Semina et al 2001, Valleix et al 2006

PITX2	Pair-like homeodomain transcription factor 2	4q24	IGDA AXRA/S Peters	Heon et al 1995, Semina et al 1996, Alward et al 1998
PITX3	Pair-like homeodomain transcription factor 3	10q25	ASD Posterior polar cataract	Semina et al 1998, Addison et al 2005 Sakazume et al 2007
LMX1B	Lim homeobox transcription factor	9q34.1	Nail-patella syndrome	Lichter et al 1997, Mimiwati et al 2006, Milla et al 2007
CYP1B1 (GLC3A)	Cytochrome P450, family 1, subfamily b, polypeptide 1	2p22-21	ICG Peters	Sarfarazi et al 1995, Stoilov et al 1997 Colomb et al 2003 Coca-Prados & Escribano 2007
Tyr	Tyrosinase	11q14-q21		Libby et al 2003, Bidinost et al 2006
GLC3B		1p36	ICG	Akarasu et al 1996
GLC3C		14q24.3	ICG	Stoilov et al 2002b
EYA1	Drosophila eyes absent gene	8q13.3	Peters ASD	Azuma et al 2000
CHED1	Congenital Hereditary endothelial dystrophy	21p13	CHED	Toma et al 1995
MAF	Muscoloaponeurotic fibrosarcoma oncogene homolog	16q23.2	Cataract Peters	Jamieson et al 2002

Since 1997 (Stoilov et al), numerous mutations of *CYP1B1* have been characterized in ICG patients in different populations (Michels-Rautenstrauss et al 2001, Panicker et al 2002, Belmouden et al 2002, Stoilov et al 2002, Colomb et al 2003, Sitorus et al 2003, Hollander et al 2006, Reddy et al 2004, Alfadhli et al 2006, El- Ashry et al 2007, Messina-Baas et al 2007). Chavarria-Soley et al (2006 b) investigated some *CYP1B1* mutations which appeared more often associated with ICG and established that eight *CYP1B1* mutations were found more than once, each of them presenting one identical haplotype in different individuals, confirming a founder effect.

Interestingly, studies by Bejjani et al (2002) and Doshi et al (2006) analysing the expression patterns of Cyp1b1 in mice found that it was not expressed in the trabecular

meshwork but is confined anteriorly to the ciliary body and posteriorly to the retinal neuroepithelium and around the optic nerve, but not in the nerve itself.

Coca-Prados and Escribano (2007) review the evidence for the *CYP1B1* expression in the eye and the pathogenesis of *CYP1B1* associated glaucoma. They observe that *CYP1B1* has a higher expression in the fetal eyes than in the adult eyes, suggesting that the ciliary epithelium has a role in the regulation of normal development of the anterior chamber of the eye, particularly TM, and in maintaining some critical function in the adult eye. Therefore, this would infer that abnormalities of the development of the TM in glaucoma associated with *CYP1B1* mutation may result from the diminished or absent metabolism of important endogenous molecules (morphogens) in the ciliary epithelium due to non-functional *CYP1B1* enzyme (Doshi et al 2006).

Mutations in *CYP1B1* cause structural and functional abnormalities of the protein. They produce loss of enzyme function by various mechanisms: promoting an incorrect insertion of the protein in the ER membrane; impairing correct folding and protein stability; modifying substrate binding and damaging the active centre. The loss of function *CYP1B1* mutations can produce ICG, POAG (Melki et al 2004, Acharya et al 2006, Lopez-Garrido et al 2006) and even Peter's anomaly (Vincent et al 2001).

Coca-Prados and Escribano (2007) speculate that homozygous or compound heterozygous carriers of *CYP1B1* mutations could suffer during development a dramatic reduction in the overall *CYP1B1* activity (below a normal threshold) in the tissues of anterior segment of the eye, which would produce severe malformation in the drainage angle of the eye, leading to ICG. However, heterozygous carriers of hypomorphic *CYP1B1* alleles, in which the total enzymatic activity is close to being below a normal threshold, will end up with clinically undetectable developmental abnormalities of the ocular drainage structures. These abnormalities may contribute to an elevated IOP over time, leading to POAG. From this reasoning it follows that it is possible that heterozygous carriers of low inactive mutations might maintain the enzymatic activity above the critical threshold to give rise to a normal phenotype. The threshold hypothesis has been suggested as a general mechanism to explain the difficulties in correlating mutations with phenotypes in Mendelian disorders (Dipple and McCabe 2000).

Also to note is that *CYP1B1* oxidises all-trans-retinol to all-trans-retinal, the rate-limiting step for retinoic acid biosynthesis. The reason this is of interest is that retinoic acid receptor mutations causes anterior segment dysgeneses (Chen et al 2000) and mutations in the gene investigated in this study –*PITX2*- is an important cause of Axenfeld Rieger Anomaly/Syndrome (ARA/S), another ASD entity. In addition, mutations in *CYP1B1* have been identified in patients with Peter's anomaly, which is another condition that belongs to anterior segment dysgenesis (Vincent et al 2001).

As mentioned in the POAG chapter, in 2002, Vincent et al, showed that there is a strong variability of expression and allelic heterogeneity for *MYOC* and *CYP1B1* mutations and that congenital glaucoma and juvenile glaucoma are allelic variants, at least in some cases. They suggest that *MYOC* and *CYP1B1* may interact through a common pathway and that the inheritance of glaucoma may be multiallelic in some cases. Also to note is the identification of *MYOC* mutations in 3 ICG patients, who did not harbour any *CYP1B1* mutation (Kaur et al 2005).

Revealing the identity of the *CYP1B1* substrate is one of the most interesting questions in glaucoma research, since it can direct us to as yet unknown biochemical cascade(s) controlling the terminal stages of anterior chamber angle development.
To date, the genes responsible for *GLC3B* and *GLC3C* have not been identified.

Although ICG is often considered to be fully penetrant, the disease shows only 50% penetrance in some Saudi Arabian families (Bejjani et al 2000). A higher prevalence has been observed in genetically inbred population and in certain ethnic and religious groups in which parental consanguinity, especially cousin-cousin marriages, is common. ICG has been reported to occur more frequently in males than females and is reported to be bilateral in 70% to 80% of cases. Familial cases tend to have an equal sex distribution (Papadopoulos et al 2007). The familial segregation of the nonpenetrance suggests a genetic modifier.

Prompted by these observations, mice were used to identify a modifier gene that alters the phenotype in *Cyp1b1* mutant mice. *Cyp1b1* mutant mice have focal angle abnormalities similar to the ICG patients, but do not develop increased IOP and glaucoma. This suggests that modifier genes interact with *Cyp1b1* to exacerbate the angle anomalies and enhance the likelihood of glaucoma. *Tyrosinase* (*Tyr* – a rate

limiting enzyme in the pigment production pathway) has been identified as a potential modifier gene. Although, angle abnormalities were more severe in mice with mutant *Cyp1b1* and mutant *Tyr*, increased IOP was still not present. When mice with mutations in *Cyp1b1*, *Tyr* and *Foxc1* have been engineered, raised IOP has been noticed in some of the mice (Libby et al 2003, Gould et al 2004). The authors speculate that mutations in multiple genes that contribute to developmental glaucoma affect L-dopa levels. L-dopa levels may be altered in neural crest cells from which the angle structures and iris stroma derive. Another possibility is that mutations in glaucoma genes affect the activity of tyrosine hydroxylase (TH) which produce L-dopa from tyrosine. Some of the genes that cause anterior segment dysgenesis and/or developmental glaucoma can promote either TH expression or the proliferation of TH expressing neural crest cells during the development of other tissues (*PITX2* and *PITX3* induce TH expression – Lebel et al 2001, Gould et al 2004). The diagram in figure 3.2 suggests some of the pathways in which these genes can affect the L-dopa levels.

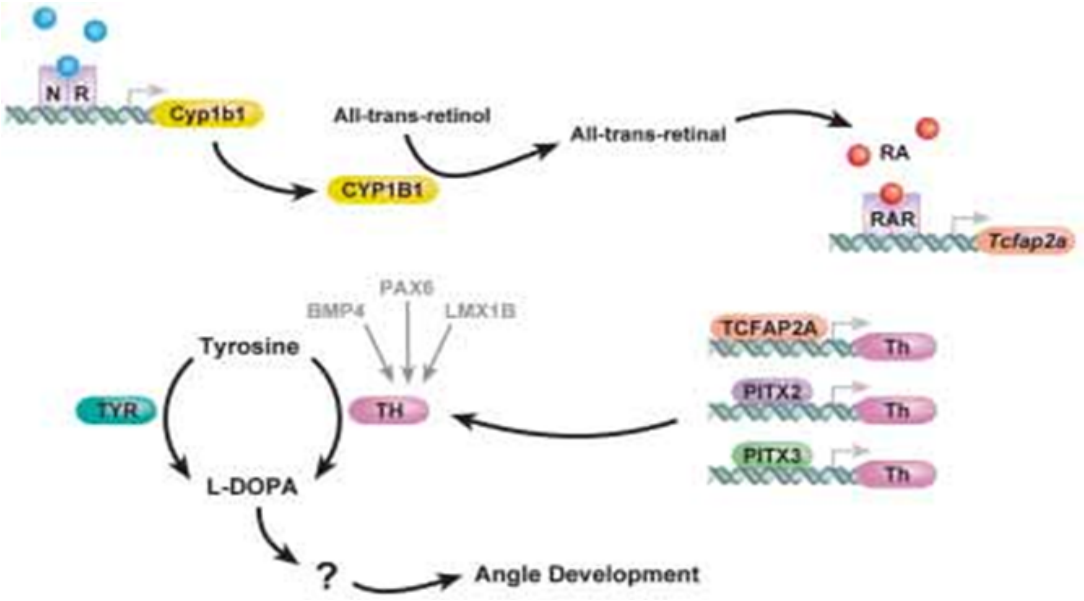


Fig. 3.2: Multiple genes implicated in anterior segment development and glaucoma may modulate L-dopa levels. Many of the genes implicated in anterior segment dysgenesis, elevated IOP and glaucoma may affect L-dopa levels. Most can be linked to L-dopa through tyrosine hydroxylase (TH, as discussed in the text). The dark arrows represent known direct relationships. *TCFAP2*, *PITX2* and *PITX3* can all directly bind to the tyrosine hydroxylase promoter. The fainter arrows and text indicate that the represented genes affects on TH and L-dopa may not be direct. *BMP4, PAX6* and *LMX1B* can promote either tyrosine hydroxylase expression or the number of TH expressing neural crest cells during the development of other tissues, but how they do so is not known. How L-dopa modulates angle development also is not known. It is possible that either L-dopa itself or a catecholamine metabolite(s) of L-Dopa mediates an important signalling event(s) (adapted from Gould et al 2004).

ASD and congenital glaucoma has been reported in some humans with albinism (Catalano et al 1988), but so far has been considered a coincidence. These findings suggest that *Tyr* may play a role in congenital glaucoma in humans, by affecting L-dopa levels and can open new avenues for a possible therapy for reducing the incidence of glaucoma in certain high risk families.
However, when the effect of *TYR* as a modifier gene in patients with ICG with a known *CYP1B1* mutation was studied, this hypothesis could not be proven (Bidinost et al 2006). But because the mode of interaction between *CYP1B1* and *TYR* is not known, a modifier effect of *TYR* on other *CYP1B1* human mutations cannot be excluded.

3.6.3 *FOXC1* gene

FOXC1 gene encodes a forkhead/winged helix transcription factor which belongs to a family of genes with a characteristic 100-aminoacid motif and it was originally identified in Drosophila (Pierrou et al 1994). Forkhead transcription factors have a characteristic forkhead and winged helix DNA- binding domain and are required for various developmental processes. *FOXC1* and *FOXC2* are expressed in the mesenchyme from which the ocular drainage structures derive (Smith et al 2000); also, Fox factors are important in establishment of the body axis and the development of tissues from all three germ layers.

FOXC1 gene is sensitive to altered gene dosage, increase or decrease in the number of copies resulting in ocular anterior segment developmental defects, demonstrating that precise dosage is critical for normal eye development (Lehmann et al 2003a).

FOXC1 shares coordinated function and overlapping tissue expression with *FOXC2* with 97% aminoacid identity across their forkhead (DNA binding) domains. *FOXC2* causes lymphoedema-distichiasis in human, while mutations in *Foxc2* in mice cause iris, trabecular meshwork and iridocorneal angle anomalies (Smith et al 2000). This raises the possibility of an unrecognised role for *FOXC2* in iris and trabecular meshwork development (Lehmann et al 2003a).

Also it has been suggested that forkhead proteins might have one function during embryonic development and organogenesis, and a completely distinct function in adult, differentiated tissues (Lehmann et al 2003a)

The importance of the locus 6p25 for anterior segment dysgeneses can not be underestimated and although the *FOXC1* gene has been linked to a number of diseases which may merely represent allelic disorders it are possible that there may be other candidate genes close to this locus. This is supported by Davies et al (1999), discovering a child with microphthalmia and corneal clouding and a number of other dysmorphic features, including hypertelorism, micrognathia, and limb, ear and heart defects. Although this child had an interstitial deletion of 6p25-p24, it did not include *FOXC1* but did include *AP2-ά* which they suggest as a possible candidate gene.
The chromosome 6p25 region has also been implicated in ICG as well as other ASD's (Vincent et al 2001). The Iris Hypoplasia/IGDA locus was mapped to *6p25* by Walter et al (1996) and the linkage of IGDA to *6p25* was confirmed by Mirzayans et al (1997).

It is interesting to observe that Axenfeld-Rieger anomaly (Fuse et al 2007, Gould et al 1997, Lehmann et al 2002, Mortermousque B 2004, Panicker et al 2002) and Iris Hypoplasia/IGDA have all been mapped to *6p25* which has been linked to the *FOXC1* gene (Nishimura et al 1998) who also report 2 ICG patients with chromosomal anomalies involving *6p25.*

Deletions in 6p were identified in an aniridic family and a Peter's anomaly family (Levin et al 1986). In fact Axenfeld-Rieger anomaly, Iris Hypoplasia/IGDA may merely represent allelic disorders arising from mutations of the *FOXC1* gene. In addition duplications and deletions of the 6p25 region including the *FOXC1* gene have been identified with the clinical phenotypes (Lehmann et al 2000, Lehmann et al 2002).

Cella et al (2006) assessed the frequency of *PITX2, FOXC1, CYP1B1* and *GJA1* genes in patients with AXRS with glaucoma and identified a polymorphism in *GJA1* (gap junction protein, alpha 1) in a patient with *FOXC1* mutation and therefore suggest the possibility of its participation as a modifier gene.
Another gene belonging to the forkhead box family is *FOXE3*, located on chromosome 1p32. Mutations in this gene have been found in a family with anterior segment dysgenesis and cataracts (Semina et al 2001) and also in congenital primary aphakia (Valleix et al 2006).

3.6.4 *PITX2* gene

Heon et al (1995)mapped IH/IGDS to *4q25*, which is the same locus for Axenfeld-Rieger syndrome and mutations were found by Alward et al (1998) in these patients affecting the same gene (*PITX2*) implicated in Axenfeld-Rieger syndrome. Walter et al (1996) studied the IGDS patients reported by Chisholm and Chudley (1983) and established that in the family with the extraocular abnormalities shared by Axenfeld-Rieger Syndrome (maxillary hypoplasia with dental anomalies, inguinal hernia, redundant periumbilical skin and hypospadias in males) linkage was to the *4q25* region, the locus for the *PITX2* gene. They also demonstrated that in the family without extraocular abnormalities reported by Pearce et al (1982) and Pearce et al (1983) there was no linkage to *4q25*. Mutations in *PITX2* gene have also been reported by Kulak et al (1998).

The progress in identifying the genetic changes underlying these conditions has made it clear that the clinical phenotypes of Axenfeld-Rieger and Iris Hypoplasia/IGDA syndrome result from the same underlying developmental abnormality. The range of variable features between these conditions probably reflects differences in genetic backgrounds between individuals and the effect of specific gene mutations within a given gene. In the future it is very likely that this situation will become more complicated as other loci are found and candidate genes within these regions are identified.
PITX2 gene will be discussed in detail in chapter 4.

3.6.5 *PAX6* gene

Hanson et al (1994) described a mutation of the PAX6 gene, on chromosome 11p13, in a family with Peter's anomaly. *PAX6* is a homeobox developmental gene expressed in different parts of the body including the developing eye and known to be the cause of many cases of aniridia (Axton et al 1997, Prosser and van Heyningen 1998, van Heyningen and Williamson 2002).

PAX6 plays an important role in ocular development by regulating the expression of genes that are involved in embryogenesis of the eye. The level of protein production from both normal copies of a gene is required by some cellular processes. *PAX6* gene is

one such paired-box gene, disruption of one copy of this gene reduces the protein product, termed 'haploinsufficiency', resulting in abnormal development possibly by altering the expression of other genes (Tang et al 1997). As shown above mutations in the same gene (*PAX6*) can produce extensive phenotypic variability (variable expressivity- Sale et al 2002, Gehring & Kazuho 1999).

Mutant phenotypes therefore include Axenfeld-Rieger, Peter's anomaly and Aniridia from amongst the ASDs (Hanson et al 1994, Prosser & van Heyningen 1998, Riise et al 2001). Within the same family, Hanson et al (1994) describe patients with *PAX6* mutations but with variable phenotypes including a Rieger type-ASD. Doward et al (1999) reported a mutation in *PITX2* gene producing Peter's anomaly and a number of extraocular associations, including failure of involution of the umbilical skin and signs of abnormal dental development and Dansault et al (2007) describe new mutations in *PAX6* gene causing ophthalmic phenotype associated with neurodevelopmental abnormalities. Also, Edward et al (2004) report mutations in *CYP1B1* which cause Peters anomaly in Saudi Arabia population.

Zhou and Kochhar (2003), suggest that inhibition of retinoic acid (RA) signaling pathway by RA receptor antagonist influence the expression of AP-2 transcription factors (a family of RA responsive proteins), thus missregulating the neural crest cell differentiation by increased apoptosis in the developing eye, in vitamin A deficiency model. This can result in the absence of endothelium, accompanied by other anomalies of iris and anterior chamber, consistent with Peters Anomaly.

3.6.6 *LMX1B* gene

In a recent study, Milla E et al (2007) identified a mutation in the *LMX1B* gene in a family with nail-pattela syndrome with variable expression of OAG. LMX1B protein is a homeodomain protein, which are a family of transcription factors frequently involved in pattern formation during embryonic formation. Most of the mutation reported in this gene lead to the absence or inactivation of the homeodomain, resulting in a protein unable to recognize its target genes.

The LMX1B transcription factor protein is involved in normal patterning of the dorsoventral axis of the embryo during development and early morphogenesis of

glomerular basement membrane. Further molecular and immunochemical studies showed the involvement of *lmx1b* in collagen regulation (Milla et al 2007). Therefore, the authors speculate that the collagen defects in the eye could be responsible for the presence of glaucoma. The *LMX1B* gene is also expressed in the TM and many other ocular anomalies have been sporadically associated with nail-pattela syndrome (microcornea, sclerocornea, congenital cataracts, iris processes and pigmentation of inner margin of the iris). However, none of these are a specific trait of this syndrome (Milla et al 2007).

A study carried out by Park et al (2009) on 272 POAG patients, 37 NTG, 58 OHT and 276 controls shows that *LMX1B* haplotypes influence susceptibility to glaucoma in the general population, suggesting altered *LMX1B* function predisposes to glaucomatous damage and that this role may be independent of raised intraocular pressure.

3.6.7 *TGFβ* super-family genes

Another group of genes implicated in anterior segment morphogenesis is the *TGFβ* superfamily of secreted molecules that influence a spectrum of biological processes, including pattern determination, cell proliferation and differentiation, cell death, bone morphogenesis and wound repair (Graham et al 1994, Mabie et al 1999). The *TGFβ* super-family is divided in two similar but separate branches: The *BMP/GDF* (bone morphogenetic protein/growth and differentiation factor) branch and the *TGFβ/activin/nodal* branch.

Each branch works through extracellular ligand binding to a membrane bound receptor complex consisting of Type I and Type II serine/threonine kinase receptors. Ligand binding initiates cytoplasmic signal cascade that activate SMAD (also called MADH) proteins. SMAD proteins enter the nucleus where they associate with transcription factors and participate in transcriptional regulation of target genes (Chang et al 2001, Miyazono et al 2001).

BMPs proteins are a large subclass (more than 20 members) and have been found to be expressed in multiple tissues during embryonic development, including heart, lung, kidney, brain and eye (Hogan 1996). In the eye, *Bmp4* expression is first identified in the distal optic vesicle and overlying surface ectoderm (Furuta & Hogan 1998).

Subsequently, expression was found in iris, ciliary body, and retinal pigment epithelium, endothelial cells of conjunctiva, retina and orbit (Chang et al 2001). Although no expression of *Bmp4* was demonstrated in TM and Schlemm canal, haploinsufficiency of *Bmp4* in mice, causes iridocorneal angle abnormality (small or absent Schlemm canal, hypoplastic or absent TM and iris attachments to peripheral cornea, hypoplastic or malformed iris and occasionally ciliary body; peripheral corneal thinning with neovascularisation, anterior and posterior subcapsular cataract, persistence of anterior hyaloids vessels, increased IOP, retinal abnormalities). Although the above study did not demonstrate expression of *Bmp4* in the TM, a study by Wordinger et al (2002), demonstrates expression of BMPs proteins (2,4,5,7), all three BMP receptors and inhibitory BMP associated proteins in cultured human TM and ONH cells.

Changes demonstrated in *Bmp4* haploinsufficient mice are similar to the anterior segment dysgenesis observed in humans and it may be a good candidate gene to be screened in AXRS patients. It appears that *BMP4* influences TM morphogenesis by regulating extracellular matrix features (including composition and signalling) that are important for mesenchymal migration, differentiation and/or remodelling (Gould et al 2004).

A recent study by Fuchshofer et al (2007), demonstrates that *BMP-7* strongly antagonises in vitro the *TGFβ* induced expression of a broad panel of molecules, which would result in accumulation of TM ECM in situ and it is reasonable to hypothesize that it has the same effect in vivo. Therefore, pharmacological modulation of *BMP-7* signalling in the TM might be a promising strategy to treat POAG.

TGFβ2 is a signalling ligand involved in anterior segment development. The cornea of mice with *TGFβ2-/-* have reduced accumulation of ECM and are thin with densely packed keratocytes; the corneal endothelium fails to differentiates and an anterior chamber never forms (Saika et al 2001).

Miyazono et al (2001) have demonstrated that the *BMP4* and *TGF β2* pathways converge and overlap. Members of the *TGF β* signalling superfamily are important for normal ocular development and therefore, any gene that influence the balance of *TGFβ/BMP* signalling are candidates to affect anterior segment development and glaucoma phenotype. Also, some studies on the effect of *TGF β2* on human trabecular

meshwork suggests that *TGF β2* reduce outflow facility by increasing ECM in TM and producing alteration in the cytoskelatal proteins of these cells in glaucomatous eyes (Fuchshofer et al 2003, Gottanka et al 2004, Zhao et al 2004, Wordinger et al 2007). Increased levels of *TGF β2* increases the expression of *αB crystalline* (Welge-Lussen et al 1999) and *MYOC* (Tamm et al 1999), and in the astrocytes of the optic nerve increases expression of fibronectin, type I collagen, tissue transglutaminase and plasminogen activator inhibitor as well as collagen type IV, which correspond with changes seen in glaucomatous eyes (Fuchshofer et al 2005). However, a study screening for *TGFβ1* polymorphism in Indian patients with POAG have not found an association (Sripriya et al 2007).

3.6.8 CHED genetics

Congenital Hereditary Endothelial Dystrophy (CHED) has been described as having autosomal dominant (CHED 1) and autosomal recessive (CHED 2) forms – with the recessive form being more severe and having an earlier age of presentation. Kirkness et al (1987) and Pearce et al (1969) reported large pedigrees with AD inheritance. One of these families underwent linkage and a locus was found on chromosome 20. Interestingly, Toma et al (1995) analysed the evidence on cytogenetic location markers used in the mapping CHED and Posterior polymorphous corneal dystrophy and concluded that both loci were in the pericentric region of chromosome 20 i.e. 20p11.2-q11.2. CHED1 has been mapped to 20p13.In 2004, Mintz-Hittner et al described a mutation in *VSX1* (*RINX*) gene, located at 20p11.2 in a family with craniofacial anomalies, empty sella, corneal endothelial changes (CHED 1) and abnormal retinal and auditory bipolar cells.

3.6.9 Axenfeld Rieger Syndrome genetics

Autosomal dominant inheritance has been identified in Axenfeld-Rieger anomaly and syndrome for many years (Semina et al 1996). There is considerable genetic heterogeneity of Axenfeld-Rieger as suggested by affected individuals having a variety of chromosomal abnormalities, which include deletions of chromosomes 4, (Flomen et al 1998) and chromosome 13 (Phillips et al 1996) and the failure to find linkage to 4q25 in one pedigree by Legius et al (1994). Deletion of 13q14 was described in 2 cases (Akazawa et al 1981 and Stathacopoulos et al 1987).

It is thought that there may be a number of genes that can cause Axenfeld-Rieger syndrome as a variety of chromosomal abnormalities have been reported to give rise to the classic features in addition to the 4q25 (sometimes referred to as Rieger syndrome Type 1 or RIEG1) and 13q14 (sometimes referred in the literature as RIEG2 or Rieger syndrome Type 2) loci (Gould et al 1997).
Using linkage analysis, loci have been found for Axenfeld-Rieger on chromosomes 4q25 (Murray et al 1992, Sarfarazi et al 1997), 6p25 (Gould et al 1997, Honkanen et al 2003, Saleem et al 2003), 13q14(Philips et l 1996) and another potential locus for a candidate gene at 16q24 is being investigated (Ferguson & Hicks 1987, Smith et al 2000).

Semina et al (1996) cloned the *PITX2* gene (originally called RIEG1 gene) from the 4q25 region and identified six mutations in affected individuals. They also report a strong homology between the human (*PITX2*) and mouse (*Pitx2*) gene. This is important as mouse Pitx2 mRNA has been localised in the periocular mesenchyme, maxillary and mandibular epithelia, umbilicus, Rathke pouch and limb mesenchyme of the mouse - consistent with the sites of some of the abnormalities in ARS. The *PITX2* gene is a member of the homeobox transcription gene family and appears to play an important role in embryogenesis. Homeobox genes are conserved throughout the animal kingdom and play a fundamental role in the genetic control of body patterning and development.

PITX2 and *DLX2* are transcription markers observed during early tooth development. Espinoza et al (2002) and Cox et al (2002) demonstrated that *PITX2* binds to bicoid and bicoid-like elements in the *DLX2* promoter and activates this promoter 30 fold in hamster ovary cells. They have shown that different *PITX2* mutations have varying abilities to transactivate the *DLX2* promoter and therefore different mutations can lead to residual activity of the mutant. The result is that certain ARS phenotypes may be less severe as *DLX2* expression is required for tooth and craniofacial development.

Physical or functional haploinsufficiency of *PITX2* has been shown by Flomen et al (1998) as being the pathogenic mechanism for AXRS. They demonstrated that the syndrome can result from the haploid, whole gene deletion of *PITX2* but also from a translocation break upstream from the gene. They suggest that there may be key regulatory region upstream important for the homeobox gene expression.

Kozlowski et al (2000) and Walter et al (2003) with recombinant techniques and transactivation studies demonstrated reduced activity of certain *PITX2* mutations. They were able to show that those mutant *PITX2* proteins that retained partial function result in milder anterior segment abnormalities where as non-functional mutant proteins produced ARS. Increasing use of mutational and functional analyses is yielding a greater insight into the pathogenesis of *PITX2* and as more loci are investigated, the potential of discovering new causative genes or regulatory factors becomes a real possibility (Priston et al 2001, Saadi et al 2003).

As described above the *FOXC1* gene, a member of the forkhead/winged-helix transcription factor family, at 6p25 is particularly linked with Axenfeld-Rieger Anomaly (ARA) and it had been considered that mutations at 6p25 were not associated with extraocular phenotypes in the way that *PITX2* is. Mirzayans et al (2000) have shown that ARS can result from mutations of *FOXC1*. Mutations in *FOXC1*, ranging from single missense mutations to null defects have been identified in patients suggesting haploinsufficiency accounts for this phenotype as well (Mears et al 1997, Mirzayans et al 1997, Nishimura et al 1998, Lehmann et al 2002, Komatireddy et al 2003, Saleem et al 2003b).

Mears et al (1997) excluded *FOXC1* mutations in two families with ARA but with linkage to 6p25, which could suggest another gene at this locus. However, Lehmann et al (2000 and 2002) and Nishimura et al (1998 and 2001) have reported chromosomal duplication of the 6p25 region in patients as a possible alternative explanation to another gene at that locus.

It seems likely that *PITX2* and *FOXC1* play equal important roles in the development of the eye, given that mutations of either gene produce virtually identical phenotypes (Lines et al 2002).

Also, to note is that Riise et al (2001) reported an 8 year old girl with ARS who was found to have a small deletion of *PAX6.*

Linkage analysis and chromosomal rearrangements indicate the presence of additional Axenfeld-Rieger malformation loci at 13q14 and 16q24 (Lines et al 2002), that remain to be cloned (Ferguson et al 1987, Phillips et al 1996).

FOXC2 has been considered as a candidate gene; however mutations in this gene appear to produce lymphoedema- distichiasis syndrome rather than Axenfeld-Rieger malformations (Fang et al 2000).

Also, a second relevant candidate gene has been identified at 16q. Mutation of the *MAF* transcription factor gene on 16q23.2 causes cataract, corneal opacity and microcornea, iris coloboma and anterior segment dysgenesis (Jamieson et al 2002). *MAF* is expressed in surface ectodermal component of the embryonic eye (lens placode and vesicle), suggesting a lens specific signalling defect blocking anterior chamber formation and may account for a proportion of AR malformation.

Azuma et al (2000) have reported three missense mutations of the *EYA1* (drosophila eyes absent) gene in patients who had congenital cataract and ocular anterior segment anomalies and the authors suggest that this gene, which initially was thought not to be involved in eye morphogenesis in humans warrants further investigation in patients with anterior segment anomalies.

3.6.10 Summary of literature review

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 is evidence to suggest that glaucoma may be affected by multiple interacting genes (*APOE, MYOC, CYP1B1, TNF, OPTN*) and that has been extensively discussed in chapter 3.3.7. 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 (Zhang et al 2005, Pasquale et al 2006).

Current evidence reveals only a small proportion of POAG exhibit Mendelian inheritance as demonstrated 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.

Genes in which mutations causes anterior segment anomalies and glaucoma are strong candidates for glaucoma susceptibility genes in a wider population. As emphasised above, several gene loci for these developmental glaucoma have been identified (*PITX2, FOXC1, PAX6, LMX1B, 6p25, 13q14*). These genes 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 (Sheldrick et al 1994), compared with 33-75% of patients who have mutations in the developmental glaucoma genes (Strungaru et al 2007), suggests that these genes are very good candidate risk factors. Mutational and functional analysis of their function promises to shed light on the underlying developmental mechanisms and may help in the understanding of pathogenesis of the prevalent adult onset glaucoma.

Identification of the gene defects which cause glaucoma will provide improved understanding of glaucoma pathogenesis. Research into genotype-phenotype relations in families will be necessary even after the identification of disease causing genetic defects, to study possible modifier genes or environmental factors influencing their expression. It may also advance the development of genetic screening as a diagnostic test for at risk individuals. POAG is asymptomatic until significant, irreversible sight loss has occurred, so reliable testing and early treatment are of paramount importance if blindness from the disease is to be reduced.

Chapter 4: PITX2 GENE

4.1 INTRODUCTION

Paired like homeodomain transcription factor 2 (*PITX2)* mutation causes a range of anterior segment dysgeneses some of which are associated with dental and umbilical anomalies (collectively termed Axenfeld- Rieger syndrome), in which 50% of patients develop glaucoma (Alward 2000, Amendt et al 2000).

An important part of this project focuses on the *PITX2* which belongs to homeobox gene family.

4.2 HOMEOBOX GENES STRUCTURE

Homeobox genes belong to a gene family encoding products with large, highly conserved domains. A homeodomain is an evolutionarily conserved region found in many DNA-binding transcription factors that control biological processes such as cell type specification, embryonic pattern formation and determination of cell fate (McGinnis & Krumlauf 1992, Krumlauf 1992, Gehring et al 1994).

The homeobox encodes a 60 amino acid residue polypeptide, called the homeodomain that represents the DNA-binding domain of the respective proteins. The homeodomain proteins have been also found in metazoan, fungi and plants and in the course of evolution, the amino acid sequence of the homeodomain has been conserved to a high degree, indicating a strong evolutionary pressure to preserve the amino acid sequence of the homeodomain (Gehring et al 1994).

The homeodomain is responsible for recognising specific DNA sequences to bring the transcription factors to proper target genes.

The three dimensional structure of the homeodomain has been elucidated by Gehring et al (1994).The 60-amino acid domain is composed of three helices and a flexible amino-terminal arm (figure 4.1). The helices are folded into a tight globular structure: helix I is preceded by a flexible N-terminal arm and separated by a loop from helix II, which

forms with helix III a helix-turn-helix motif, which appears to be highly conserved among otherwise different species.

The DNA - binding specificity of a homeodomain is determined primarily by its third helix, called the recognition helix, which inserts itself into the major groove of the recognition site. In addition the flexible amino-terminal arm wraps around DNA and makes specific contacts in the minor groove. The second helix of the homeodomain also makes backbone contacts, further contributing to specific homeodomain – DNA interactions.

The recognition sites for most homeodomains have a common 5'-TAAT-3' core, which is followed by two residues that confer differential binding specificity. It has been proposed that the 9^{th} position of the recognition helix (the 50^{th} position of the homeodomain) plays a critical role in differential DNA recognition (Treisman et al 1989, Treisman et al 1992).

Genetic experiments indicate that the N-terminal arm of the homeodomain contributes significantly to the functional specificity of the homeodomain proteins (Gehring et al 1994). Also, the DNA binding specificity and functional activity of homeodomain proteins depends upon combinatorial interactions with other transcription factors. These interactions may occur between free proteins in solutions or alternatively when the proteins are bound to the DNA (Gehring et al 1994).

4.3 *PITX2* GENE TISSUE EXPRESSION AND FUNCTION

PITX2 gene (OMIM 601542) was isolated from within a cosmid contig (the goal of physical mapping is to produce a set of overlapping clones that encompass an entire chromosome or an entire genome. Sequence-tagged sites are particularly useful in aligning overlapping cosmids (vectors) into contigs (contiguous DNA material). As more clones are characterized, contigs grow to the size of entire chromosomes/http://www.ncbi.nlm.nih.gov/books/) encompassing two Rieger syndrome translocations at chromosome 4q25. The initial name designated by Semina et al (1996) was *RIEG1*, and the protein it encodes was called solurshin.

Solurshin is a 271 amino acid protein (33 kDa) showing sequence identity with the bicoid class of homeodomain proteins. These genes encode transcription factors which regulate tissue specific gene expression by binding to regulatory DNA sequences via their 60 amino acid homeodomain (Gehring et al 1994, Semina et al 1996).

By in situ hybridization experiments, Semina et al (1996) showed that in mouse embryos Rieg/Pitx2 mRNA localizes in the periocular mesenchyme, maxillary and mandibular epithelia and umbilicus, which is consistent with the anomalies characteristic of Rieger syndrome. Expression persist in the mesenchyme and in the presumptive corneal stroma at E13.5 and by E18.5 *Pitx2* expression is restricted to iris and iridocorneal angle (Hjalt et al 2000).This expression pattern is very similar to expression patterns of *Foxc1* and *Foxc2*. Homozygous null mutant *Pitx2* mice die by E15.5 (Lin et al 1999). At this stage, these mice have thickened, undifferentiated corneas that do not develop corneal endothelium or anterior chambers (Gage et al 1999, Lu et al 1999) similar to the eyes of Tgfb or Foxc1 knockout mice. Similar to humans with *PITX2* mutations, heterozygote *Pitx2* mice have variable degrees of ASD (Gage et al 1999).

Also, the gene was expressed in the Rathke pouch, vitelline vessels and limb mesenchyme. Because of expression of the gene in Rathke pouch, it was suggested that the gene might play a role in anterior pituitary gland development.

Further studies on mice by Gage and Camper (1997) proved the importance of the gene in differentiation and proliferation of the thyrotrope, somatotrope and lactotrope lineages, and designated the gene as *Pitx2.*

Subsequently, it has been demonstrated that *Pitx2* is asymmetrically expressed in lateral plate mesoderm and appears to exert critical roles in left-right situs (Kitamura et al 1999, Logan et al 1998, Piedra et al 1998, Ryan et al 1998, Yoshioka et al 1998, Hjalt et al 2000).

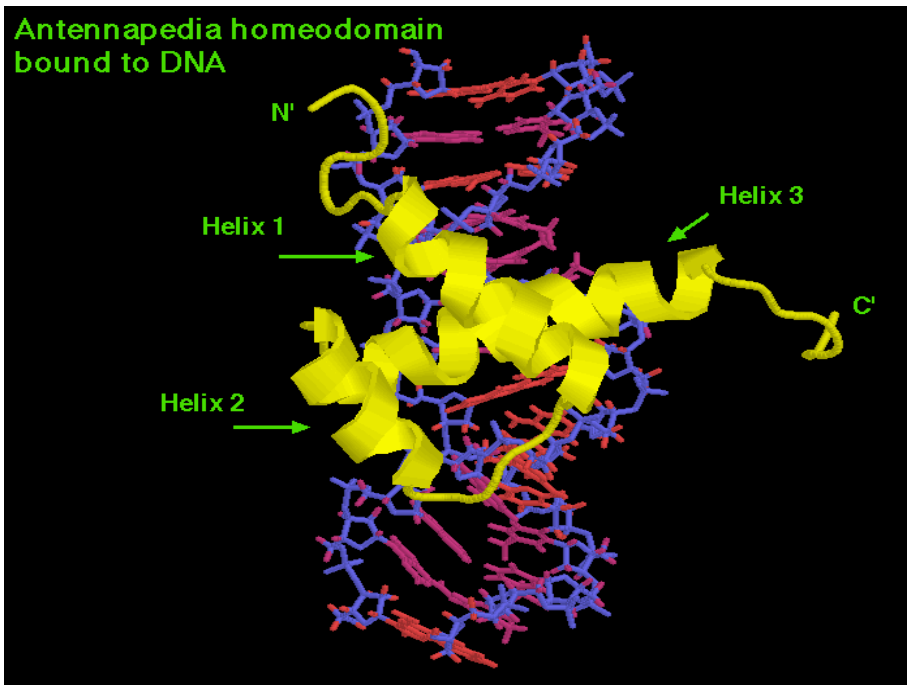


Figure 4.1: Three dimensional structure of the homeodomain (adapted from: www.cbt.ki.se/groups/tbu/homeo/antp2)

Franco and Campione (2003) suggest that altered left-right signalling due to impaired function of *PITX2* underlies the aetiologies of several common congenital cardiac malformations. Also, *Pitx2* has been found to be expressed in terminally differentiated neurons of the zona limitans intrathalamica and mammillary region and in gama-aminobutyric acid (GABA) – producing neurons of the zona incerta and superior colliculus (Martin et al 2002 and 2004).

Viera et al (2006) and Evans & Gage (2005) demonstrated that *PITX2* is expressed in the human embryonic and foetal periocular mesenchyme at early developmental stages and it is well established that periocular mesenchyme makes a major contribution to the development of ocular anterior segment structures.

Furthermore, significant *PITX2* expression in the human developing ciliary body, ciliary processes, iridocorneal angle and corneal endothelium has been demonstrated by the same group and this provides a strong molecular basis for explaining the frequent occurrence of glaucoma in patients with ARS. Also, transfection of a dominant negative RhoA (Rhodopsin A protein, which belongs to the GTP-ase superfamily) mutant into perfused human anterior segment cultures increases aqueous outflow facility,

suggesting that *PITX2* may be essential for intraocular pressure homeostasis in the prevention of glaucoma (Vittitow et al 2002).

Gage et al (1999) have assessed the requirements or *Pitx2* for the development of multiple organs and established that *Pitx2* is required in a dosage-sensitive manner. Also, Diehl et al (2006) have demonstrated that *Pitx2* gene dose regulates both morphogenesis and gene expression in developing extraocular muscles. The expression of key muscle specific transcription factor genes is regulated by *Pitx2* gene dose, suggesting that sufficient levels of *PITX2* protein are essential for early initiation of the myogenic regulatory cascade in extraocular muscles.

The fact that other transcription factors (*Foxc1, Tgfb*) act in a dosage sensitive manner, demonstrates the importance of a narrow range of activity of various transcription factors for normal anterior segment development and reflects a delicate balance of signalling by interacting pathways (Gould et al 2004).

There is evidence that *Pitx2* is at least partly controlled by *TGFβ* superfamily signalling. Multiple studies show that *TGFβ* super-family member, NODAL, can induce *Pitx2* expression (Logan et al 1998, Piedra et al 1998). *Pitx2* has a NODAL response element in its promoter and mice deficient for NODAL receptor have phenotypes very similar to mice deficient for *Pitx2* (Lin et 1999, Lu et al 1999).

Procollagen lysyl hydroxylase (*Plod*) genes are potential downstream targets for *PITX2*. The promoter regions of *PLOD1* and *Plod2* have multiple *PITX2* binding sites. *PLOD1* and *PLOD2* belong to a family of enzymes responsible for hydroxylating lysine residues in collagens. Hydroxylysine residues provide stability to intermolecular collagen cross-links (Kiviriko and Myllyla 1985). Therefore, disturbances of ECM stability and function may underline *PITX2* phenotypes (Gould et al 2004)

4.4 *PITX2* STRUCTURE

The *PITX2* gene consists of six exons and so far there are four major isoforms identified - PITX2A to D- figure 3.2 (Semina et al 1996, Gage and Camper 1997, Kitamura et al 1999, Cox et al 2002).

PITX2A and *PITX2B* are generated by alternative splicing mechanism and *PITX2C* and D uses an alternative promoter located upstream of exon 4. All isoforms contain dissimilar N-terminal domains, whereas the homeodomain and C-terminal domains are identical. The C-terminal domain contains a highly conserved 14-amino acid region described in the homeobox genes Otp, aristaless and rax which is called the OAR (Otp, aristaless, rax) domain.

Cox et al (2002), who identified the fourth (*PITX2D)* isoform, have investigated the regulation of gene expression by *PITX2* isoforms.

This study, provide evidence that *PITX2* isoforms differentially activate genes involved in development (*Procollagen lysyl hydroxylase -PLOD1, Dlx2 and Prolactin Promoters*) and that *PITX2A, B and C* isoforms have different transcriptional activities that are promoter-dependent. *PITX2D* isoform is generated by the *PITX2C* alternative promoter and differential splicing, and appears to be present only in humans. The *PITX2D* isoform acts to down regulate the transcriptional activities of *PITX2A* and *PITX2C*. Because the major *PITX2* isoforms only differentiate in their N termini, they speculate that the N terminus must play a role in the differential transcriptional activities of these isoforms.

Protein kinase C has been found to regulate the transactivating capacity of *PITX2*, and a C-terminal mutation affects phosphorilation (Espinoza et al 2005). There are several downstream transcriptional target genes suggested for *PITX2* in different organs (*PRL, PROP1, HESX1* and *LHX3* in pituitary,*PLOD1* in eye, *TRIO* in HeLa cells, *DLX2, FGF8,BMP4* in tooth, *ANF/NPPA* in heart and *CYCD2* in pituitary and heart – Hjalt and Semina 2005).

Amendt et al (1999) investigated the role of *Pitx2* homeodomain protein C-terminal tail and concluded that the C-terminal tail intrinsically inhibits the *Pitx2* protein and that inhibition can be overcome by interaction with other transcription factors to allow activation during development. Work done by Kioussi et al (2002) and Wei et al (2002), places *PITX2* in a signalling cascade that appears to modulate cell proliferation, differentiation and morphogenesis. Also, a study by Footz et al (2009), analysing the functional role of the C-terminal region of *PITX2* and assessing the effect of previously

uncharacterized *PITX2* missense mutations concludes that stringent control of *PITX2* is required for normal ocular development and function.

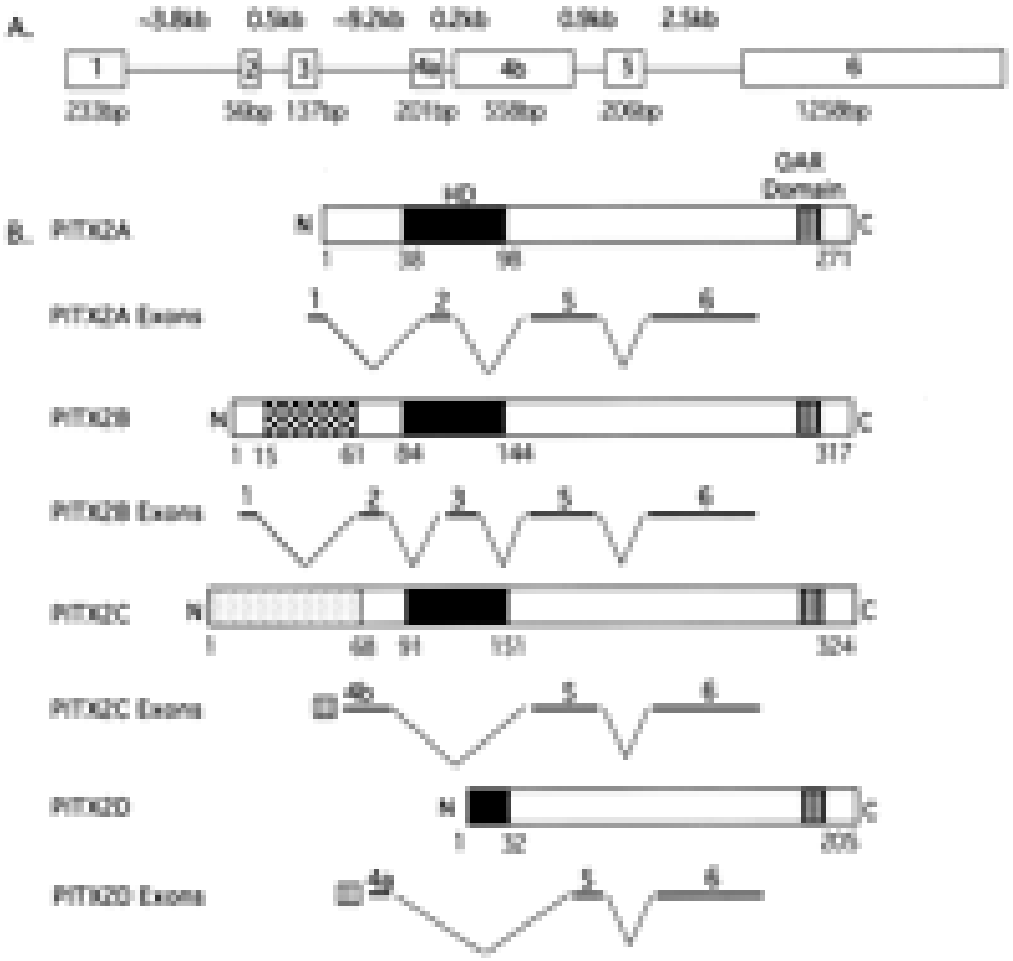


Figure 4.2: *PITX2* major isoforms found in humans (adapted from Cox et al, 2002).

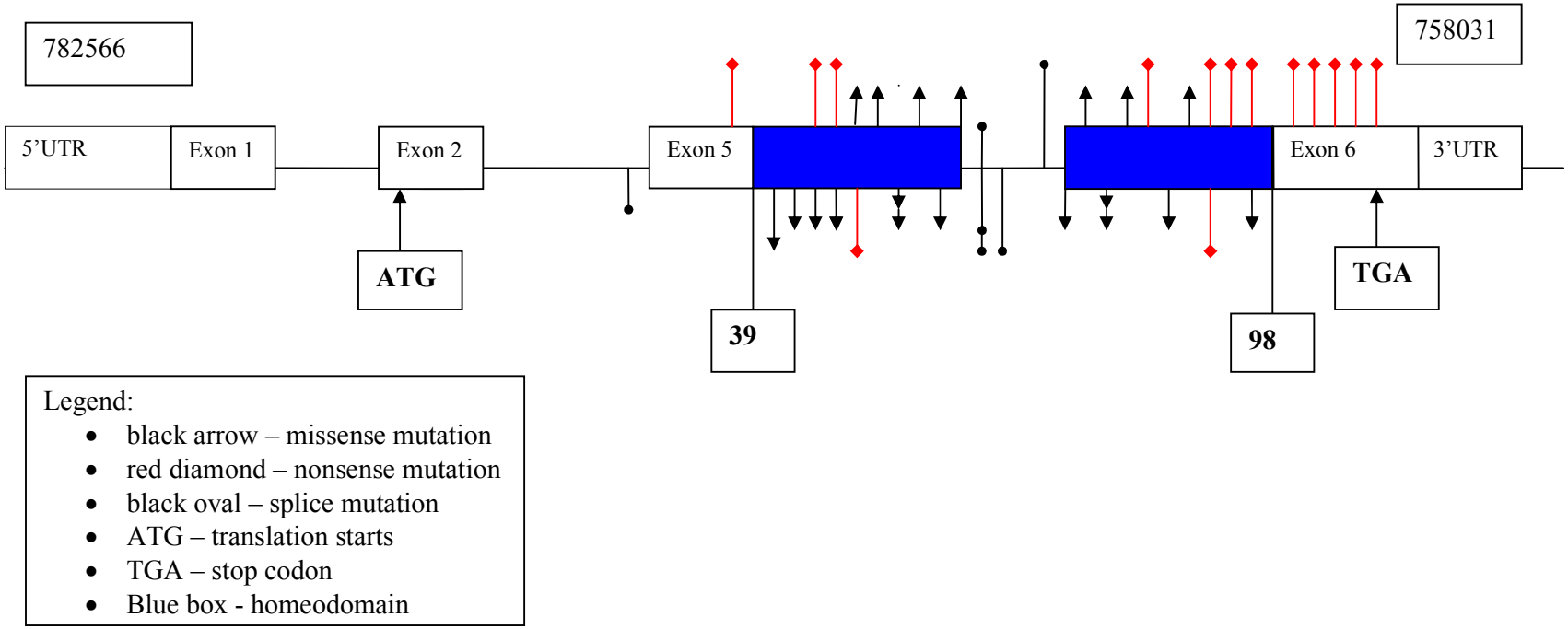
4.5 *PITX2* MUTATIONS

Numerous mutations have been reported in *PITX2*, producing a continuum of clinical phenotypes, including ARS, IGD and IH, as well as rarer cases of Peters' like anomaly (table 4.1 and figure 4.3).

Table 4.1: Mutation identified in *PITX2* gene causing ocular phenotypes			
Nucleotide & protein change	Effect on protein function	Phenotype	Reference
Missense mutations			
Arg53Pro/ R53P		ARS	Kozlowski and Walter 2000
C744T>A/ L54Q (1^{st} helix of HD)	Unable to bind DNA and deficient transactivation	ARS	Semina et al 1996, Amendt et al 1998
C785A>C/ T68P (2^{nd} helix HD)	Unable to bind DNA and deficient transactivation. Pitx2 cannot transactivate dlx2 promoter leading to abnormal tooth development	ARS	Semina et al 1996, Amendt et al 1998 Kozlowski and Walter 2000
C855G>C/ R91P (3^{rd} helix HD)		ARS	Semina et al 1996
Arg46Trp C>T/ R84W/ HD	Reduced DNA binding and transactivation. Dlx2 promoter can be activated	IH	Alward et al 1998, Kozlowski and Walter 2000
789G>A/ R70H (position 31HD/ 2^{nd} helix)	Reduced DNA binding activity	IGD	Kulak et al 1998
Arg31His/ R31H HD		IGDS	Kozlowski and Walter 2000
Leu16Gln/ L16Q HD		ARS	Kozlowski and Walter 2000
Lys88Glu/ K88E HD	Defective DNA binding and transactivation, but has a dominant negative effect on wild-type protein	ARS	Perveen et al 2000, Saadi et al 2001
851C>T/ R90C/HD		ARS	Perveen et al 2000
c.852G>C/ R90P			Phillips et al 2002
c.896C>G/ L105V			Phillips et al 2002
c.906A>C/ N108T			Phillips et al 2002
c.744C>T/ P64L		ARS+ sella turcica anomaly	Phillips et al 2002, Weisschuh et al 2006 Meyer-Marcotty et al 2008
c.710C>T/R43W		ARS	Idrees et al 2006b
c.744C>G/P64R		ARS	Weisschuh et al 2006
Gly137Val		ARS+ Fuchs' ED	Kniestedt et al 2006
753C>G/F58L		ARS	Vieira et al 2006
742C>G/E55X		ARS	Vieira et al 2006
942C>A/Y121X		ARS	Vieira et al 2006
649C>A/Pro217Thr		Peters & PHPV	Arikawa et al 2009
Nonsense			
C981G>A W133X		ARS	Semina et al 1996
c.830G>C/ V83L		ARS	Priston et al 2001
IVS3(-1) G>T -1 position of the 3' ss associated with exon 4		ARS	Lines et al 2004, Maciolek et al 2006
W133Stop	Gain of function: increased DNA binding, transactivation and dimerization	ARS	Saadi et al 2006
Splice-site mutations			
IVS3(-2)A>C		Peters	Doward et al 1999

IVS3 (-11)A>G 3rd intron (additional acceptor site, 5' from normal one)splice site	Protein with truncated HD is expressed at same levels as wild type protein	ARS	Semina et al 1996, Borges et al 2002
IVS3(+5) G>C/ conserved position +5 of the 5' splice site 3rd intron	Poorly expressed truncated protein	ARS	Semina et al 1996, Maciolek et al 2006
IVS3(-2)/ A>T		ARS	Perveen et al 2000
IVS2(-1)/ G>C		ARS	Perveen et al 2000
Deletions/insertions			
c.1272 del G 1-bp del exon 3/pos690/ Premature termination of PITX2 protein		ARS	Borges et al 2002
g.del 114/ exon 3/c697delG/ Frameshift(/large nonsense peptide 114 aa encoded)		ARS	Lines et al 2004
c.del 416/ 998delC/ frameshift from T139 onwards, encoding 15 mutant residues followed by premature stop codon		ARS	Lines et al 2004
c.del 366/959delC	Frameshift(change in codon 122) D122FS	ARS	Saadi et al 2006
c.717-720delACTT			Wang et al 2003
c.1261delT			Brooks et al 2004
1251 ins (CGA CTC CT)		ARS	Vieira et al 2006
g.20913G>T/G137V		ARS	Kniestedt et al 2006
Intragenic del/18,183 to 21,242 del/end exon5, intron 6, part exon 6/ 144 nc lost from HB			de la Houssaye 2006
C ins 1083		ARS	Perveen et al 2000
A del 939		ARS	Perveen et al 2000
1235-1236 TA>AAG		ARS	Perveen et al 2000
AA del 868-869		ARS	Perveen et al 2000
7aaDup/c713-733dupl/7 amino acids duplication of residue 6-12 Hd		ARS	Priston et al 2001

Figure 4.3: Diagram of the *PITX2A* gene demonstrating all ocular phenotypes causing mutations in human reported to date (Vaideanu 2007)



Although some mutations results in gain of function (Priston et al 2001, Saadi et al 2001, Holmberg et al 2004, Saadi et al 2006), the majority of mutations cause deficiency of the normal *PITX2* protein (haploinsufficiency). This is supported by the presence of large deletions that include *PITX2* in some ARA/S patients and functional studies of proteins derived from mutant alleles (Maciolek et al 2006). Holmberg et al (2004), suggest a model for gain-or-loss-of-function in ARS (figure 4.4)

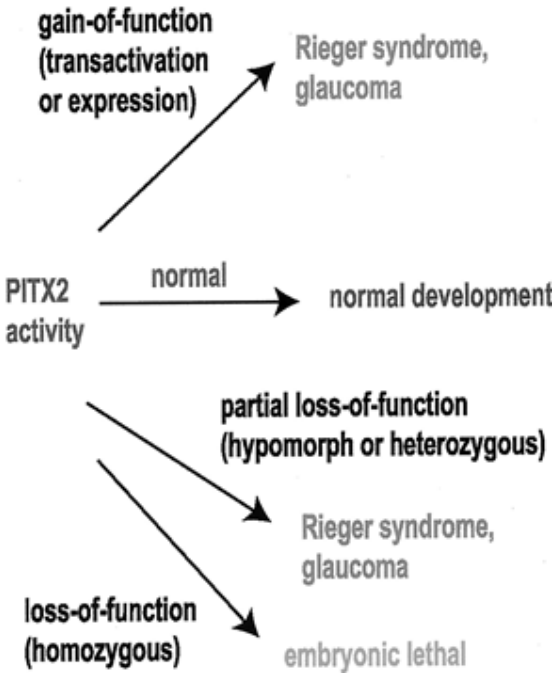


Figure 4.4: Model for gain-or loss-of-function in Rieger syndrome

Known heterozygous defects in the *PITX2* gene can cause eye disorders. The human and mouse homozygotes are early embryonic lethal. These mice develop hypertrophic corneas. Heterozygous mice develop phenotypes resembling human patients, even the hypomorphic variants, with *PITX2* expression levels between heterozygous and normal. The Rieger-syndrome-causing mutation V45L elevates *PITX2* transactivating activity. The *Pitx2* mutant mice overexpress *PITX2* in the cornea and iris, resulting in hypertrophic corneas like the mouse Pitx2 homozygotes, and Rieger syndrome-like effects including irido-corneal adhesions and retinal degeneration/glaucoma (adapted from Holmberg et al, 2004)

A correlation between the dosage of normal *PITX2* protein and the severity of phenotype has been noted (Kozlowski and Walter 2000, Priston et al 2001, Saadi et al 2001, Espinoza et al 2002). It can be observed that most of the human *PITX2* mutations described thus far affect the region encoding the homeodomain or C-terminal domains, with only a few intronic mutations reported (table 4.1).

4.6 GENE – GENE INTERACTION

PITX2 has been reported to function as a negative regulator for *FOXC1* transactivation activity with its homeodomain (Berry et al 2006). It appears that the amounts of the *PITX2* and *FOXC1* genes expressed are crucial for triggering developmental or oncogenic abnormalities.
It may be that protein interactions may explain the strict dosage sensitivity of *PITX2* and *FOXC1*: *PITX2-PITX2* and *PITX2-FOXC1* complexes may form in a concentration and/or cofactor dependent fashion and changes in the expression of either gene may alter the relative abundance of either complex.

Depending on the cellular context, genetic alterations of *FOXC1* or *PITX2* could result in the loss of transcriptional activation or the loss of transcriptional repression of target gene activity (Berry et al 2006). Identification of target genes regulated by *PITX*2 and *FOXC1* will enable further understanding of the underlying ARA/S pathology.

In their review of anterior segment development relevant to glaucoma, Gould et al (2004), suggest a model for multiple genes interaction implicated in anterior segment development (figure 3.2). The diagram has been presented in chapter 3.6.2.

4.7 PHENOTYPES CAUSED BY *PITX2* MUTATIONS

The eye phenotypes caused by mutations in *PITX2* range from iris hypoplasia/ IGDA to ARA/S and have been discussed in previous chapters (Anterior segment dysgeneses and Developmental glaucoma genetics chapters) and therefore will not be discussed here again.

However, it has to be mentioned that it was noted that in general ARS patients who display defects in other organ systems, such as teeth and umbilicus, have mutations of

the *PITX2* gene, while in patients with isolated ASD, mainly *FOXC1* mutations are detected. So far, no obvious correlation has been noted between the localization of a mutation in the *PITX2* gene and the severity of the phenotype. Tumer and Bach-Holm (2009) in their excellent review of Axenfeld Rieger and spectrum of *PITX2* and *FOXC1* mutation give the example of two consecutive missense mutations within HD, which lead to ARS and IH respectively. Subsequent functional studies demonstrated that one mutation was gain of function (resulting in a milder phenotype) and the other was loss of function, resulting in a more severe phenotype.

Also, in a study published in 2007, Strungaru et al, examined genotype-phenotype correlations in 126 patients with ARS and found that 75% of the patients in this study had glaucoma that had developed in adolescence or early adulthood. Glaucoma in only 18% of the patients with either *PITX2* or *FOXC1* genetic defects responded to medical or surgical treatment (used solely or in combination). They concluded that patients with *FOXC1* mutations have the mildest prognosis for glaucoma development, whereas patients with *PITX2* defects and patients with *FOXC1* duplication have a more severe prognosis for glaucoma development. In this study, current medical therapies do not successfully lower intraocular pressure or prevent progression of glaucoma in patients with ARS who have *FOXC1* or *PITX2* alterations.

4.8 PHENOTYPIC FEATURES OF *Pitx2* KNOCKOUT MICE

Homozygous null mutant mice of *Pitx2*, exhibit septal and valve defects, single atrium, abnormal cardiac positioning, pulmonary isomerism, omphalocele, early arrest in pituitary development, defect in tooth organogenesis, defective development of the mandibular and maxillary facial prominence and multiple eye defects (Gage et al 1999, Lin et al 1999, Lu et al 1999).

Heterozygote *Pitx2* mice have thinning of the ventral body, small body size, eye and tooth defects as described in some studies (Gage et al 1999, Lin et al 1999), whereas in other studies they do not show obvious haploinsufficiency phenotypes (Lu). Over expression of *Pitx2* in mouse corneal mesenchyme and iris results in corneal opacification, corneal hypertrophy, irido-corneal adhesions and severely degenerated retina (Holmberg et al 2004).

4.9 MOLECULAR PATHWAY ARS

Local control of cell signalling activity and integration of inputs from multiple signalling pathways are central for normal development but the underlying mechanisms remain poorly understood.
In their review of molecular understanding of ARS, Hjalt and Semina (2005), suggest an upstream regulatory pathway for *PITX2*, demonstrated in figure 4.5.

As seen in figure 4.5, there are at least two major regulatory pathways identified upstream of *PITX2*: the Wnt-β-catenin-Pitx2-CyclinD2 and the Nodal-Shh-Lefty2-Pitx2 pathways.

Also, multiple direct downstream target genes of *Pitx2* have been identified from many different functional groups: transcription factors, cell-cycle control proteins, growth factors, morphogens and modifying enzymes of extracellular matrix proteins. It is yet not known how nor if these pathways interact or if there are additional regulatory pathways upstream of *Pitx2*.

However, it is almost certain that continuing research is going to add new levels of complexity to what we already know about *PITX2* gene.

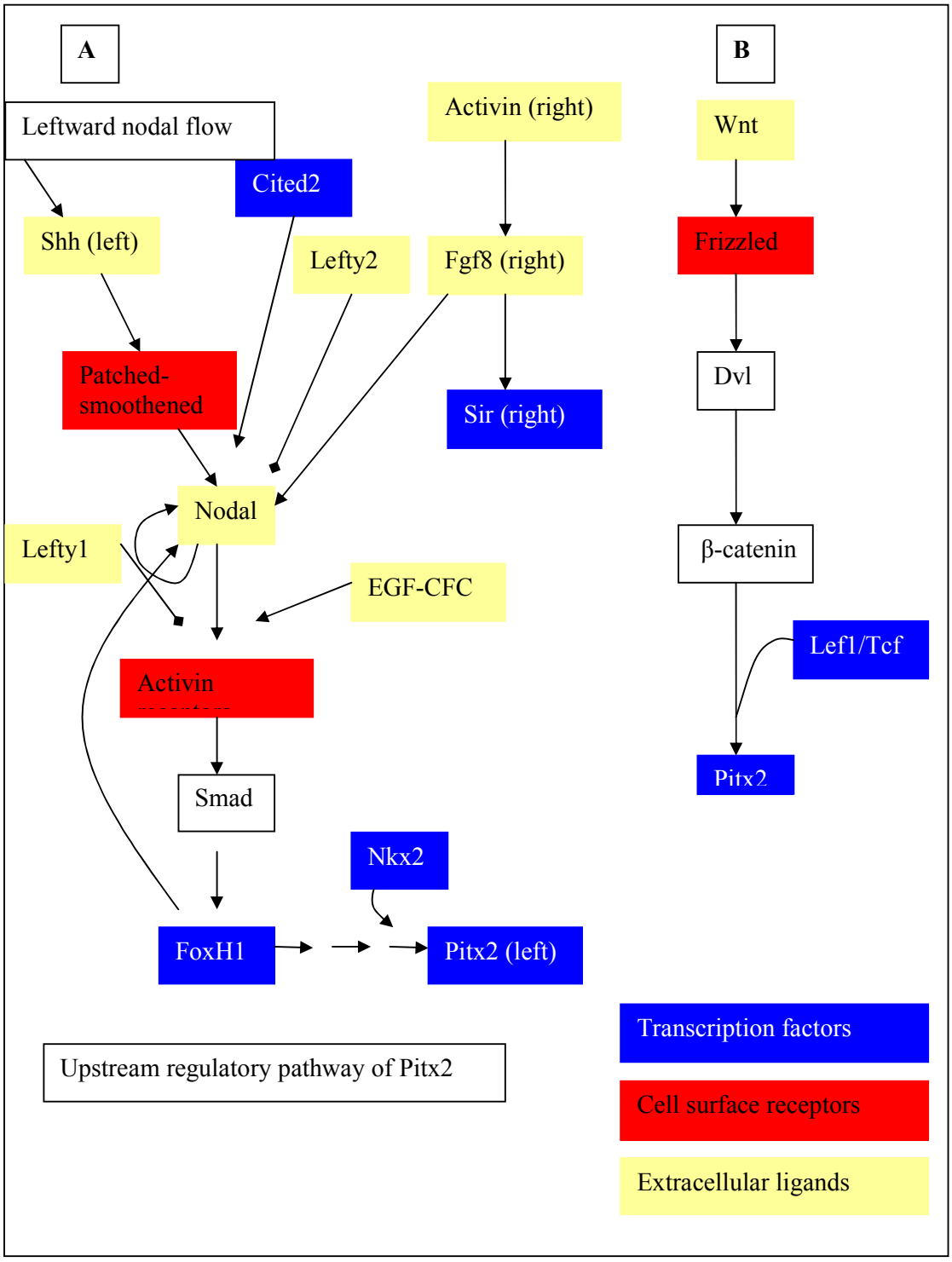


Figure 4.5: Upstream regulatory pathway for Pitx2 (adapted from Hjalt and Semina: Current molecular understanding of Axenfeld-Rieger syndrome (2005)/ Expert Rev Mol Med) Abbreviations: Dvl: dishevelled 1; EGF-CFC: epidermal growth factor-cripto; Fgf: fibroblastic growth factor; FoxH1: forkhead box H1; Lef1/Tcf: lymphoid enhancer-binding protein/T-cell specific transcription factor; Nkx2: NK2 transcription factor related; *Pitx2*: pituitary homeobox 2; Shh: Sonic hedgehog; Smads: mother against decantaplegic genes; lefty: left right determination factor; Cited2: CBP/p300- interacting transactivator with Clu/ASP-rich C-terminal domain 2; Wnt: wingless type MMTV integration site family

4.10 SUMMARY- *PITX2* AS A GENETIC RISK FACTOR FOR POAG

Taking into account the essential role of *PITX2* for the development of the anterior segment implies that *PITX2* is crucial for the normal development of drainage structures and preservation of normal IOP. This is emphasized by the observation that glaucoma occurs in a high percentage in ARS patients and that patients with *PITX2* defects have a more severe prognosis for glaucoma development.

Therefore, these features of *PITX2* provide a strong basis to consider *PITX2* as a candidate for the more common adult-onset POAG through hypothesizing that sub-clinical mutations/polymorphisms in *PITX2* 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.

Chapter 5.
HYPOTHESIS AND OBJECTIVES OF THE STUDY

5.1 HYPOTHESIS PILOT STUDY

Subclinical mutations/polymorphisms in *PITX2* gene may produce subtle and undetected age related abnormalities in anterior segment structure and function, which predisposes to glaucomatous optic neuropathy and may be a significant susceptibility factor for the development of POAG. The arguments for choosing genes involved in ASD and why *PITX2* in particular has been presented in Glaucoma Genetics (chapter 3) and PITX2 chapter (chapter 4) and will not be reiterated again here.

5.2 OBJECTIVES OF THE PILOT STUDY

1. To identify, phenotype and recruit 100 patients with POAG and 100 matched controls for genetic studies, using explicit inclusion/exclusion criteria.

2. To recruit 10 patients with posterior embryotoxon for genetic studies.

3. To genotype the coding sequence of *PITX2* gene in patients and controls and the frequency of any polymorphism identified calculated. If a difference in allele frequency was identified, significant association was to be tested by calculating the Odds Ratio. An Odds Ratio more than 1 would suggest an increased susceptibility to glaucoma is associated with the variant allele.

4. To select appropriate *PITX2* single nucleotide polymorphisms published in electronic databases and to ascertain if these allele predispose to POAG in general population composed of British subjects by performing a case-control genetic association study to compare the prevalence of *PITX2* SNPs in a group of POAG patients with IOP at presentation > 30 mmHg and normal control group.

5.3 HYPOTHESIS AND OBJECTIVES OF FAMILY HISTORY OF GLAUCOMA STUDY

Hypothesis:

Recruiting families (ideally large pedigrees with living relatives in different generations) with multiple members affected of a disease is important for genetic studies as it allows mapping the disease loci by linkage analysis.
Linkage analysis methods attempt to identify a region of the genome that is transmitted within families with the disease phenotype of interest and has been extremely useful in the identification of genes responsible for diseases with simple Mendelian inheritance.

Therefore, second part of my project involved continuing to recruit POAG patients with family history of glaucoma from North East of England.

The objectives for this part of the study were:

1. Identify POAG patients with family history of glaucoma with more than 2 family members affected.

2. Phenotype the POAG patients

3. Construct the pedigrees

4. Enrol relatives for the genetic studies

5. Collect samples (blood or buccal mucosa) for DNA extraction

6. Forward samples and clinical information to London, with a view to be sent on to Prof Sarfarazi laboratory for genetic studies

Chapter 6.
AN ASSOCIATION STUDY OF PITX2 POLYMORPHISM IN A COHORT OF UNRELATED POAG/OHT PATIENTS AND MATCHED CONTROLS – A PILOT STUDY

6.1 INTRODUCTION

6.1.1 Hypothesis

The characteristic ocular anterior segment abnormalities seen in Axenfeld-Rieger syndrome patients indicate that the *PITX2* protein plays an essential role in the development of the cornea, iris and trabecular meshwork. The associated glaucoma may arise from malformations seen from birth or may arise from continued expression of the gene product throughout life.

It is possible that varying amounts of residual *PITX2* activity underlies the severity of the ocular phenotypes. It has been demonstrated that there is reduced activity of iris hypoplasia and iridogoniodysgenesis mutant *PITX2* proteins, whereas the ARS mutant *PITX2* proteins proved to be non-functional. These results are consistent with the hypothesis that mutant *PITX2* proteins that retain partial function result in milder anterior segment aberrations (Kozlowski et al 2000).

While mutations of *PITX2* have been detected that disrupt normal development of the anterior segment and cause an overt structural abnormality, it is also possible that as yet undetected mutations/polymorphism in *PITX2* may produce subtle and undetected abnormalities in anterior segment structure and function which predispose to glaucoma. This *PITX2* allele may for example represent a significant susceptibility factor in patients currently assigned a diagnosis of POAG.

Therefore, a pilot study was designed to address the following research question: Do *PITX2* alleles predispose to glaucoma in the wider population?

As the glaucoma associated with AXRS presents with very high IOP, we hypothesised that *PITX2* variations would be more likely to be present in POAG/OHT patients with very high IOP at presentation.

The identification, recruiting, phenotyping of patients and controls and genetic studies execution and results are presented.

6.1.2 Introduction to study design

SNPs, already introduced in the genetics of g;laucoma chapter, are defined as a polymorphic variation at a single nucleotide that occurs in at least 1% of the population and make up about 90% of all human genetic variation. The human genome contains approximately three billion nucleotide and the SNPs as mentioned before occur approximately every 100 to 300 bases (Collins et al, 2004), making them more suited to high resolution genotyping (Nowotny et al 2001).

Complex diseases are generally caused by intricate interactions of multiple genes and environmental factors, and although family based studies still have a place in the study of population association (in addition to linkage), this kind of research has more in common with classical epidemiological studies.

It follows, that issues of study design and analysis have more in common with the rest of epidemiology. Although controversial, population based, case control study has been popular for genetic association analysis due to its cost-efficiency in collecting the data (Ioannidis 2001, Evangelou 2006).

To avoid bias, ideally, cases and controls should be identical samples from a single population, except for diagnosis.

This pilot study was designed as a case-control study to assess the presence of *PITX2* variants in general population and to test for a significant association with glaucoma in patients with POAG/OHT.

Significant association was to be tested by calculating an Odds ratio with 95% confidence intervals: a ratio greater than 1 would suggest an increased susceptibility to glaucoma is associated with a variant allele.

In addition, investigation of *PITX2* sequence variance was to be carried out on a panel of 10 individuals with posterior embryotoxon, but no glaucoma.

Approval for the study was granted by the local research ethics committee (research ethic committee number: 06/Q0508/8). The research followed the tenets of the Declaration of Helsinki.

6.1.3 Definition of cases and controls for *PITX2* screening

Cases were defined as patients with POAG or OHT and the inclusion criteria were:

- Presentation of the disease over the 40 years of age.
- Highest IOP at presentation over 30 mm Hg. As the IOP in AXR patients associated with glaucoma is very high, it has been hypothesised that it is more likely to find *PITX2* variations in a subset of patients with POAG/OHT with very high IOP at presentation and we chose 30 mm Hg as a cut off point.
- Cupping of the OD and progressive VF loss in POAG patients.
- Absence of glaucomatous optic disc cupping and detectable glaucomatous field loss in OHT patients.
- Open drainage angles (at least grade 3 according to Shaffer's classification on gonioscopy).

Exclusion criteria for the study were:
Secondary types of glaucoma (psedoexfoliative, pigment dispersion syndrome, traumatic or steroid induced).

- Non-glaucomatous field loss.

Controls were defined as individuals who fulfilled the following criteria:

- More than 40 years of age.
- No glaucoma on ophthalmologic examination.
- No family history of glaucoma.
- Intraocular pressure on examination less than 22 mm Hg.

6.1.4 Sample size

As this was a pilot study of genetic association between the POAG and developmental glaucoma genes, it was considered that a sample size of 100 patients and 100 controls, matched for age, sex and ethnicity would be appropriate for the purpose of the study.

Considering that the frequency of a particular allele in general population (controls) is 10%, for a cohort of 100 POAG/OHT patients and 100 controls, the study would have 80% power to detect the difference between allele frequency of 10% to 26% in the two groups and 92.5% power to detect a difference of 10% to 30 % in the two groups, at a significance level of = 0.05.

6.2 IDENTIFICATION OF PATIENTS AND CONTROLS FOR *PITX2* SCREENING

6.2.1. Identification of POAG/OHT patients

The patients enrolled in the study were from the Glaucoma Unit at Sunderland Eye Infirmary (SEI), attended by 6,000 glaucoma patients per year.

The protocol for the *PITX2* study was as follows:
Identification of the patients with glaucoma/OHT with IOP > 30mmHg from the patients who attended out-patient clinics or the nurse led glaucoma review clinics from clinical notes. All the patients were previously phenotyped by Mr Fraser, Consultant Ophthalmic Surgeon, with vast experience in glaucoma.
Suitable patients were provided with a study information sheet (appendix 1), when checking in at reception
Either the nurse or the doctor discussed the study with the patient
If patient communicated willingness to assist, an appointment was organised with me, for recruitment to the study.
At the appointment the following steps were followed:

1. Discussion of the study.
2. Written consent for taking part in the study was obtained.
3. Clinical examination.
4. A sample of venous blood was collected.

The blood samples were stored at -80°C at the Institute of Human Genetics, until the laboratory work started in October 2002.
Any result specific to an individual would be relayed from laboratory to Mr Fraser for genetic counselling purposes.

The examination protocol was standardised and it comprised:

- Best corrected Snellen visual acuity assessment
- Anterior segment examination with slit lamp
- Goldman applanation tonometry
- Gonioscopy
- Fundus examination and optic disc assessment with a 90D condensing lens
- All the patients had at least two reproducible visual field tests in the immediate past (Humphrey automated perimetry, 24-2 Carl Zeiss Meditec AG, Jena, Germany - a form of static perimetry which involves presentation of non- moving stimuli of varying illuminance).
- All patients recruited from SEI had optic discs photographs.

Details about other systemic and ocular conditions and glaucoma treatment as well as age at diagnosis were also recorded (clinical sheet – appendix 2).

291 POAG/OHT patients were identified from the notes and were provided with study information sheet. Of these, 105 patients were enrolled in the study.

6.2.2 Identification of controls

Controls were identified from spouses of patients with POAG/OHT, accompanying the patient to their clinic appointment at SEI (30). The spouses of patients with POAG/OHT, who decided to take part in the study upon reading the information sheet (appendix 3), had an appointment organised with the Research Fellow. The controls enrolment from SEI followed the same protocol as the patient's enrolment.
The rest of the controls enrolled in the study (30) came from healthy volunteers enrolled at Royal Victoria Infirmary (RVI), Newcastle upon Tyne (collaboration).

6.2.3 Identification of subjects with posterior Embryotoxon

The subjects with posterior embryotoxon were identified by Mr Fraser (Consultant Ophthalmic Surgeon at SEI) from the patients attending his outpatient clinics. The inclusion criteria for the study were:

- presence of posterior embryotoxon on examination.
- no evidence of glaucoma.
- no evidence of anterior segment dysgenesis.

If the patients agreed to participation in the study, the contact details of the patient were provided to Dr Vaideanu who contacted the patient and organised an appointment.

6.3. STUDY EXECUTION

6.3.1. Patients recruitment for *PITX2* screening and phenotyping

Once the patients and controls were identified and expressed interest in taking part in the study, an appointment was organised with Dr Vaideanu. The recruitment of patients took place between October 2001 and October 2002.

105 POAG/OHT patients and 6 PE subjects were enrolled in the study. All the subjects with POAG/OHT and PE were obtained from patients attending SEI and peripheral clinics in area covered by Sunderland NHS Trust.

At the appointment with Dr Vaideanu, the study was discussed, any questions the patients had were answered and consent for participating in the study was obtained. This was followed by a standardised ophthalmologic examination as described in chapter 6.2.1. Figure 6.1 presents a flow chart of the recruitment process for the patients.

The VF data (Humphrey 24:2) and past ophthalmic and medical history was obtained and recorded in the clinical sheet (appendix 2) from the clinical notes. Also, family history of glaucoma was recorded. Following ophthalmic examination, 20 ml of venous blood for DNA extraction was collected, from the arm, by venupuncture, with a 20 ml syringe, after the area was sterilized with alcohol wipes. The blood was then distributed in four 5 ml EDTA tubes, labelled with the name, date of birth of the patient and date blood extracted. The blood was then stored in -80°C freezer at the Institute of Human Genetics, Centre for Life, Newcastle upon Tyne, until the laboratory work started.

6.3.2 Controls recruitment for *PITX2* screening and phenotyping

Before the enrolment of controls can be commenced, the local ethics committee had to be informed by letter. When the initial application for the study was submitted and approved (before I became involved with the study) it was focused in enrolling patients with POAG and strong family history of glaucoma. As this study was designed as a

case-control study, the chairman's approval for enrolling controls had to be granted first. The delay in starting enrolling controls had a bearing on the number of controls finally recruited (60 instead of 100).

30 controls were obtained from the spouses of patients with glaucoma, who were accompanying the patients to the SEI glaucoma nurse led clinic. Spouses accompanying the patient to the clinic were provided with an information sheet (appendix 3) for controls. Upon reading the information sheet, spouses who agreed to participated were given an appointment with Dr Vaideanu.

At the appointment with Dr Vaideanu, the same steps for enrollement of POAG/OHT patients were followed.

The other 30 controls were enrolled from healthy volunteers at Royal Victoria Infirmary (RVI), Newcastle upon Tyne (collaboration). The controls enrolled at RVI underwent the same standardised ophthalmic examination and in addition had their refractive status and VF assessed. None of the controls has any family history of glaucoma.

All the clinical information of the patients and controls has been imputed in a database.

All patients and controls recruited for the study were Caucasians, other ethnic groups being uncommon in the North-East of England.

The demographics and clinical characteristics of the patients recruited for the association study, as well as treatment and associated ocular pathology are presented in the table 6.1.a-e.

6.3.3 PE subjects recruitment for *PITX2* screening and phenotyping

PE subjects identified by Mr Fraser were contacted by Dr Vaideanu to organise an appointment for recruitment to the study, when they expressed interest in taking part in the study.
Of the 8 patients identified during the recruitment period (October 2001-2002), 6 agree to take part in the study.
Of the 6 PE subjects, 1 also had glaucoma. Although, this was an exclusion criterion, I decided to include the patient in the study; any *PITX2* variation found in this patient but

not in any of the other POAG/OHT subjects would add more support to our hypothesis. Demographic and clinical details of the PE subjects are presented in table 6.1.f.

Figure 6.1: Flow chart of the POAG/OHT patient recruitment to the pilot study

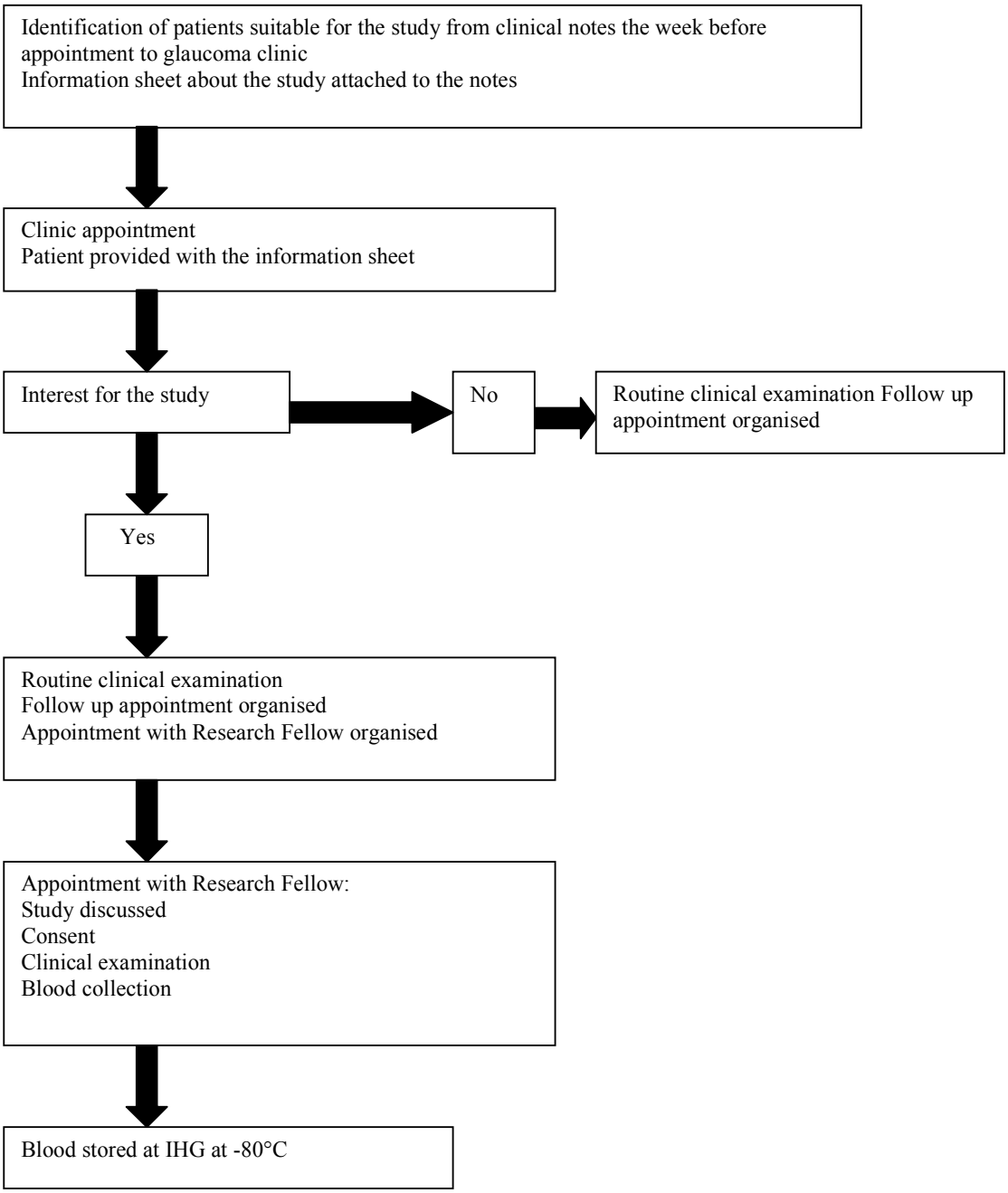


Table 6.1.a: Demographic and clinical details POAG patients

ID	sex	age	Age dg	FHx	IOP RE	IOP LE	CD RE	CD LE	VF RE	VF LE	Treatment	Associated pathology
30	M	74	65	no	31	28	0.3	0.4	inferior loss	nil	Med	
79	M	62	51	no	36	33	0.9	0.7	altitudinal defect	nil	Med + Trb BE	Cornea guttata
6	M	82	76	no	30	32	0.8	0.8	arcuate superior >90	arcuate superior <90	Med	
5	F	71	61	yes	56	42	0.9	0.7	concentric peripheral loss	sup- temporal arcuate	Trb BE	
78	M	71	67	no	39	29	0.9	0.6	concentric peripheral loss	nil	Med	
89	F	70	66	no	30	30	0.9	0.6	dense nasal defect	nil	Med	
96	M	66	60	no	30	29	0.7	0.5	early arcuate	nil	Med	
95	M	75	72	no	23	37	0.8	1	early arcuate	total loss	Med	
10	M	52	50	no	36	30	0.6	0.4	early nasal step	early nasal step	Med	
59	M	41	41	no	34	34	0.7	0.7	early peripheral loss	early peripheral loss	Med	
93	F	79	51	no	30	26	0.8	0.7	early peripheral loss	nil	Med	
26	M	71	67	no	32	18	0.7	0.3	early superior loss	nil	Med	
28	M	74	65	no	33	33	0.8	0.8	early superior scotoma	nil	Med	
103	M	82	71	no	56	56	0.7	1	extensive loss	complete VF loss	Trb BE	CRVO LE
86	M	83	75	no	30	30	0.4	0.4	fluctuating	nil	Med	
67	M	55	51	no	50	26	0.8	0.3	inferior arcuate	nil	Med	
66	M	72	66	yes	30	24	0.9	0.8	inf arcuate + sup nasal step	small superior loss	Trb BE	
43	M	87	73	no	30	28	0.8	0.6	inferior loss	inferior loss	Med	
74	M	65	48	no	30	30	0.9	0.8	inferior loss 90	inferior loss 90	Med	
40	F	77	77	no	34	24	0.4	0.8	inferior	low reliability	Med	
92	M	67	61	yes	32	30	0.5	0.6	nasal step	nasal step	Med	
25	M	67	66	no	39	22	0.6	0.4	nasal step	nil	Med	
100	F	73	58	no	34	28	0.7	0.6	nasal step	nil	Med + ALT	
24	M	80	70	no	20	30	0.6	0.6	nil	amblyopia	Med	Amblyopia LE

Table 6.1.b: Demographic and clinical detail POAG patients												
ID	sex	Age	Age dg	FHx	IOP RE	IOP LE	CD RE	CD LE	VF RE	VF LE	Treatment	Associated pathology
72	M	69	62	no	19	54	0.4	0.9	nil	complete loss	Trb LE	
99	M	55	55	no	26	50	0.3	0.8	nil	Severe loss	Med	
88	M	67	64	no	21	34	0.5	0.9	nil	early inferior loss	Med	
16	F	71	71	no	34	38	0.5	0.6	nil	early sup nasal step	Med	
17	M	59	59	no	30	38	0.5	0.5	nil	early inferior nasal step	Med	
42	F	62	54	no	30	22	0.3	0.5	nil	inferior nasal loss	Med	
35	F	70	60	yes	33	32	0.8	0.7	nil	nil	Med	
41	F	55	55	no	30	27	0.8	0.6	nil	nil	Med	
52	M	68	64	no	30	29	0.4	0.8	nil	nil	Med	
70	F	68	55	no	30	23	0.6	0.4	nil	nil	Med + Trb RE	
94	M	74	66	no	30	28	0.7	0.6	nil	nil	Med	
62	M	60	46	yes	24	40	0.3	0.6	nil	sup arcuate	Med	
64	F	57	57	yes	24	36	0.3	0.7	nil	sup arcuate	Med	
105	M	43	43	yes	22	46	0.5	0.9	nil	sup arcuate	Med + Trb LE	
98	M	77	71	yes	24	42	0.5	0.8	nil	sup loss	Med	
61	F	78	75	no	28	36	0.3	0.3	nil	sup loss	Med	
3	M	70	63	no	24	35	0.3	0.9	nil	temporal defect	Med	
37	M	83	83	no	18	32	0.2	0.7	nil	superior nasal step	Med	
34	M	64	61	no	30	26	0.8	0.5	Peripheral loss	nil	Med	
44	M	87	81	no	24	31	0.6	0.7	Peripheral loss	Peripheral loss	Med	
47	M	54	47	no	38	27	0.8	0.7	Peripheral loss	Peripheral loss	Med	
32	F	77	66	no	36	65	0.9	1	Peripheral loss	total loss	Med + Trb BE	

Table 6.1.c: Demographic and clinical details POAG patients												
ID	sex	Age	Age dg	FHx	IOP RE	IOP LE	CD RE	CD LE	VF RE	VF LE	Treatment	Associated pathology
12	F	53	40	no	52	26	0.9	0.5	peripheral loss 270	nil	Med + Trb RE	
39	F	56	51	no	30	35	0.9	0.8	severe loss	severe loss/post staphyloma	Med	High myopia
29	M	75	66	no	32	31	0.8	0.9	severe loss 180	severe 180	Med + Trb BE	
55	F	74	64	yes	32	32	0.6	0.7	small peripheral def	nil	Med	
71	F	55	51	yes	29	31	0.3	0.5	small sup defect	nil	Med	
91	M	72	72	no	26	42	0.5	0.8	small sup scotoma	small sup scotoma	Med	
51	M	55	47	no	29	29	0.5	0.5	sup + inferior arcuate	sup + inferior arcuate	Med	
87	M	81	59	no	28	30	0.9	0.9	sup +inferior mod def	inferior def 90	Trb BE	
57	F	62	59	no	31	30	0.8	0.8	sup arcuate	Hemifield sup loss	Med	
33	F	51	48	yes	38	31	0.9	0.1	sup arcuate	nil	Med	
69	M	78	68	yes	35	29	0.6	0.3	sup arcuate	sup arcuate	Trb BE	
76	M	73	70	no	32	26	0.9	0.7	sup loss	early arcuate	Med	
22	M	66	63	no	38	35	0.9	0.9	sup loss 180	sup-temp loss 120	Med	
60	M	68	60	no	30	30	0.7	0.7	sup nasal step	early peripheral loss	Med + Trb RE	
97	M	67	78	no	30	29	0.3	0.2	sup scotoma	nil	Med	
65	F	77	62	no	34	28	0.8	0.6	sup temp	sup temp loss	Med + ALT + Trb BE	
63	M	82	55	no	49	53	0.8	0.9	sup temp wedge	severe concentric loss	Trb BE	
56	F	68	55	no	29	33	0.8	0.9	sup VF loss 180	concentric VF loss	Trb BE	
27	M	80	55	no	30	32	0.8	0.8	nasal step	Nasal step	Med + Trb BE	

Table 6.1.d: Demographic and clinical details Ocular Hypertension patients											
ID	sex	Age	Age diagnosis	FHx	IOP RE	IOP LE	CD RE	CD LE	VF RE+LE	Treatment	Associated pathology
1	F	84	84	no	45	40	0.4	0.2	nil	Med	
4	M	78	69	no	48	48	0.2	0.2	nil	Med	
7	M	69	58	no	34	27	0.5	0.4	nil	Med	
9	M	40	40	no	28	32	0.4	0.5	nil	Med	
11	M	74	74	no	26	31	0.2	0.4	nil	Med	
13	M	75	69	no	32	29	0.6	0.5	nil	Med	
14	M	73	67	no	35	34	0.3	0.3	nil	Med	
15	M	72	58	yes	26	38	0.4	0.4	nil	Med	Asteroid hyalosis
18	M	68	65	yes	30	29	0.4	0.2	nil	Med	Corneal scar
19	M	54	50	no	30	32	0.5	0.4	nil	Med	
20	F	69	59	no	34	35	0.5	0.5	nil	Med	
21	F	61	61	no	24	33	0.5	0.6	nil	Med	
31	M	60	40	no	30	28	0.7	0.6	nil	Med	
46	F	75	67	no	32	34	0.5	0.6	nil	Med	
48	F	71	68	no	30	36	0.4	0.7	nil	Med	
49	F	61	50	yes	34	34	0.3	0.4	nil	Med	
50	F	61	55	no	30	29	0.4	0.3	nil	Stopped Med	
53	M	61	61	no	32	22	0.4	0.3	nil	Med	
54	M	63	61	yes	27	30	0.7	0.8	nil	Med	
58	F	76	76	no	31	29	0.2	0.3	nil	Med	
73	M	76	62	yes	34	31	0.3	0.3	nil	nil	Med
75	M	77	50	no	32	30	0.3	0.3	nil	nil	Med

Table 6.1.e: Demographic and clinical details Ocular Hypertension patients											
ID	sex	Age	Age diagnosis	FHx	IOP RE	IOP LE	CD RE	CD LE	VF RE+LE	Treatment	Associated pathology
68	M	74	64	yes	33	34	0.5	0.6	nil	Trb BE	
77	F	73	59	yes	29	30	0.7	0.8	nil	Med	
81	M	74	66	no	31	28	0.8	0.8	nil	Med	
82	F	72	62	no	33	30	0.5	0.4	nil	Med	CRVO RE
83	F	64	51	yes	29	30	0.6	0.6	nil	Med	
84	M	59	48	no	30	30	0.6	0.8	nil	Trb BE	
90	M	62	62	no	30	26	0.5	0.3	nil	None	
101	M	69	51	no	30	38	0.3	0.4	nil	Med	
102	F	63	63	no	32	30	0.6	0.5	nil	Med	
104	M	71	61	no	30	30	0.6	0.6	nil	Med	
23	M	65	65	no	26	30	0.6	0.7	nil	Med	
36	M	71	54	no	30	32	0.6	0.6	nil	Med +Trb BE	
38	M	73	79	no	26	30	0.2	0.2	nil	Med	

Table 6.1.f: Demographic and clinical details Posterior Embryotoxon subjects											
ID	sex	Age	Age diagnosis	FHx	IOP RE	IOP LE	CD RE	CD LE	VF RE+LE	Treatment	Associated pathology
1	M	32	32	Yes	16	16	0.4	0.2	nil	nil	
2	F	72	72	No	15	16	0.3	0.5	nil	nil	
3	F	66	66	No	13	16	0.2	0.2	nil	nil	
4	F	55	55	Yes	17	16	0.7	0.7	Sup field loss	Med	
5	M	65	65	Yes	18	18	0.7	0.7	nil	nil	
6	M	42	42	Yes	18	18	0.2	0.2	nil	nil	

Fig 6.2.a and 6.2.b shows the highest IOP and the worst CD ratio distribution in patients and controls. The demographics of the patients enrolled in the study are presented in the results chapter.

Figure 6.2.a and b: Graphic representation for the distribution of highest IOP (a) and worst CD ratio (b) in patients and controls.

Figure 6.2.a

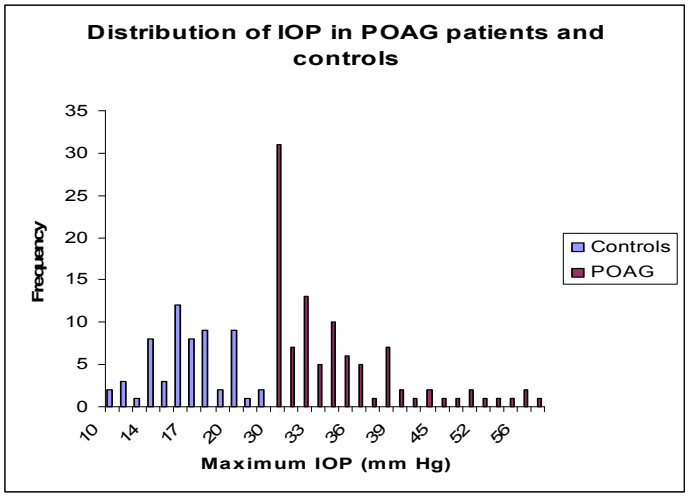
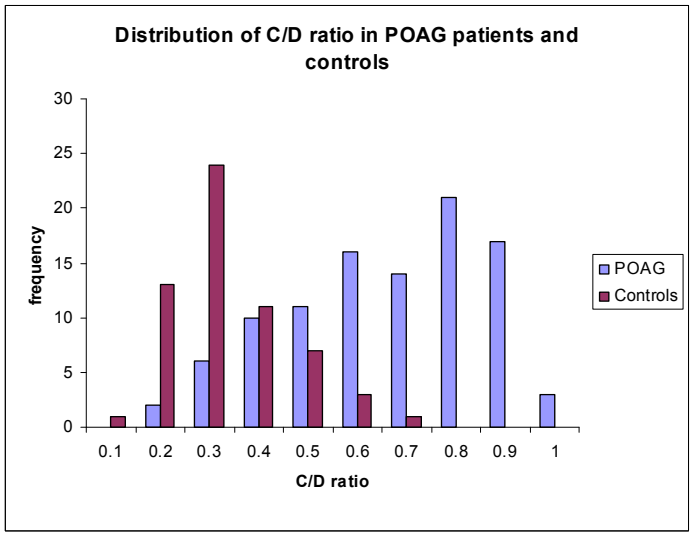


Figure 6.2.b



6.4. GENETIC SCREENING – METHODS AND RESULTS

6.4.1. Genomic DNA extraction

Genomic DNA was isolated from participants' blood, using the Nucleon BACC2/3 kit (Tepnel Life Sciences Plc), according to the protocol (appendix 4a).
The presence and quality of high molecular weight DNA was assessed by running it on a 0.8% agarose gel electrophoresis (figure 6.3)

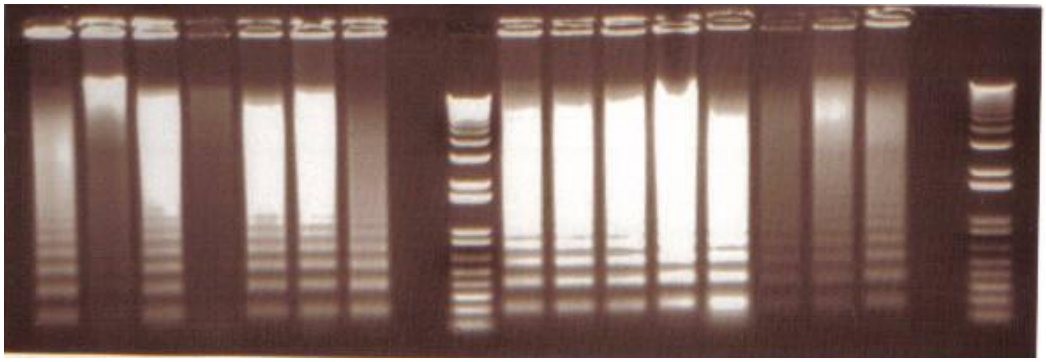


Figure 6.3: Agarose gel demonstrating the presence of high molecular weight DNA

The gel was examined for the presence of:

- smearing of the fluorescent bands to detect DNA fragmentation
- faint fluorescent bands that signify the presence of contamination

If smearing or faint fluorescent bands were present, the DNA extraction was repeated.

6.4.2 Polymerase chain reaction (PCR amplification) and sequencing

As most of the significant mutations have been documented in the homeobox (fig 4.3), for the first stage of the study, the sequencing screening was done on the homeobox region. Second stage of the sequencing screening included the rest of the coding sequence.

Four primer sets were designed to PCR amplify the isoform A coding sequence from four exons of the *PITX2*, including the exon-intron boundaries (fig 6.4). The primers were obtained from MWG (www.mwg-biotech.com) – table 6.2

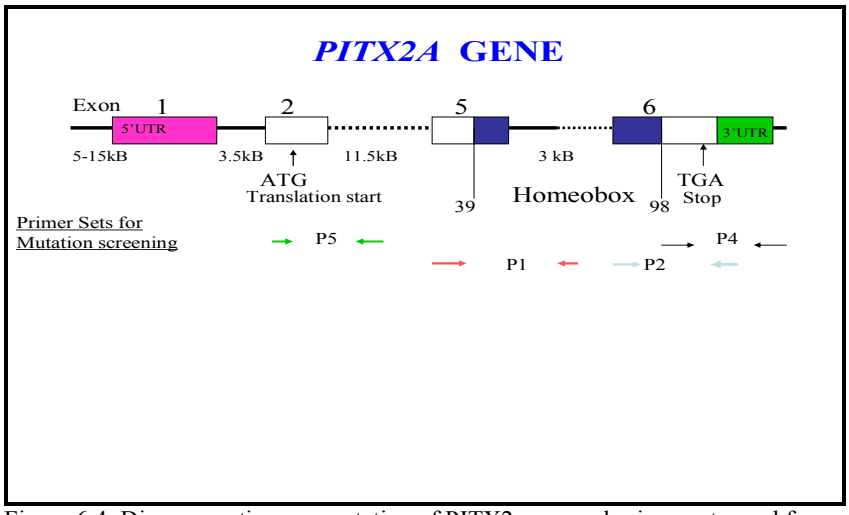


Figure 6.4: Diagrammatic representation of PITX2 gene and primer sets used for mutation screening

Primer set 1 was designed to cover the homeobox, with the other three primer sets covering the rest of the coding sequence.

Table 6.2: Primer sequence, annealing temperature and product size

Primer	Oligonucleotide sequence	Annealing temperature	PCR cycles	PCR product size (bp)
1aF **1R**	5'-CAG CTC TTC CAC GGC TTC T-3' 5'-CTC CCT TGA CAT TAG AGC GT-3'	**60°C**	33	**472**
2F **3R**	5'-GTA ATC TGC ACT GTG GCA TC-3' 5'-CCC CTT TTG TAC GAG ACA CT-3'	**62°C**	33	**298**
4F **4R**	5'-CTA TCC ACC AAG AGC TTC-3' 5'-GAC TCC TGT AGG GAA ACT-3'	**60°C**	33	**621**
5F **5AR**	5'- CTG AAG CCT AGC ACA CAG TA-3' 5'-GCA GGA GAA GGG GGT TCT TA-3'	**62°C**	33	**191**

A 25 µl PCR reaction was used for each sample. The composition of the PCR mix is presented in table 6.3 and the protocol for PCR in table 6.4.

Table 6.3: PCR reagents used for sequencing		
Reagent		1 x 25µl reaction (volume - µl)
Forward primer		1
Reverse primer		1
Master mix	Promega Taq DNA polymerase	0.25
	$MgCl_2$ (1.5mM)	1.5
	dNTPs (2.5mM	2.5
	Promega buffer	2.5
Genomic DNA (20ng/ µl)		1
Water (distilled and autoclaved)		15.25

Table 6.4: PCR protocol for direct sequencing				
Reaction step	Temperature (°C)	Duration (min:sec)	Nature of reaction	Number of cycles
1	95	03:00	Denaturation	1
2	95	00:30	Separation of DNA strand	33
3	Tm (60-62)	00:30	Primer annealing	
4	72	00:30	DNA synthesis (transcription)	
5	72	10:00	DNA synthesis (elongation)	1

The presence of the PCR products for each primer set was assessed with a 1% agarose gel (figure 6.5).

PCR product was purified using ExoSAP protocol (appendix 4b) and quantification of DNA present was done by running a 1.5% agarose gel (figure 6.6). Direct sequencing (protocol - appendix 4c) of the single stranded DNA was carried out, using the automated sequencer facilities (MegaBACE DNA Analysis System/Amersham Pharmacia).

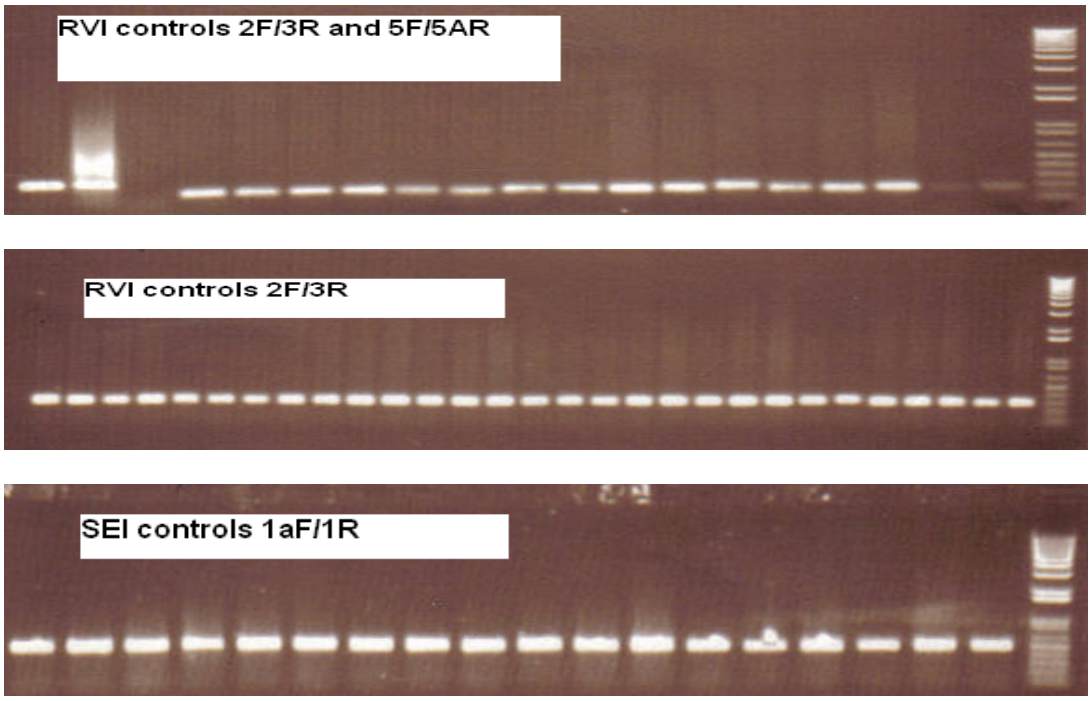


Figure 6.5: 1% agarose gel images demonstrating the presence of PCR product to be used for sequencing.

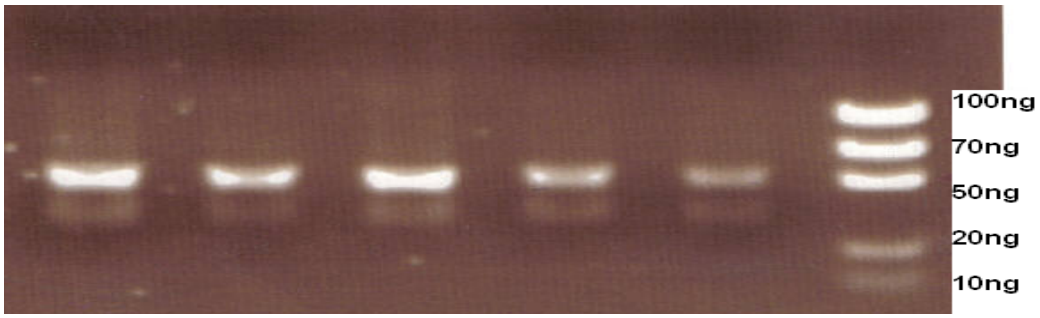


Figure 6.6: 1.5% agarose gel, demonstrating quantification of DNA present after ExoSap purification

6.4.3 Sequencing analysis and results

Sequencing results were analysed using BioEdit program – http://www.mbio.ncsu.edu/BioEdit/bioedit. (Hall 1999) and Sequencher – http://www.sequencher.com/ (Nishimura 2000) and Clustal W http://www.ebi.ac.uk/Tools/clustalw/ (Higgins and Sharp 1988) program was used for sequence alignment.

6.4.3.1 Sequencing analysis programs

Programs to import, manage and align sequences and to analyse the properties of DNA, RNA and proteins are essential for every biological laboratory. The most frequent used programs and the ones used to analyse the results of this work are a freeware BioEdit for MS Windows and a commercial program Sequencher for MS Windows and Apple MacOS. BioEdit and Sequencher offer functions such as sequence alignment and editing plus reading of sequence trace files. While Sequencher impresses with a very user-friendly interface and easy- to-use tools, BioEdit offers the largest and most customisable variety of tools. Sequences can easily be analysed for composition with a graphical output and BioEdit uses ClustalW for multiple sequence alignment (Tippmann, 2004)

6.4.3.2 Multiple sequence alignment programs

One of the cornerstones of modern bioinformatics is the comparison or alignment of protein sequences. With the aid of multiple sequence alignments, biologists are able to study the sequence patterns conserved through evolution and the ancestral relationships between different organisms. Sequences can be aligned across their entire length (global alignment) or only in certain regions (local alignment). The most widely used programs for global multiple sequence alignment are from the Clustal series of programs. The first Clustal program was written by Higgins and Sharp (1988) and was designed specifically to work efficiently on personal computers. It combined a memory-efficient dynamic programming algorithm (Myers and Miller 1988) with the progressive alignment strategy developed by Feng and Doolittle (1987) and Taylor (1988). The multiple alignments are built up progressively by a series of pairwise alignments, following the branching order in a guide tree. The initial pre-comparison used a rapid word-based alignment algorithm (Wilbur and Lipman 1983) and the guide tree was constructed using the UPGMA method (Unweighted Pair Group Method with Arithmetic Mean is a sequential clustering algorithm, in which local topological relationships are identified in order of similarity, and the phylogenetic tree is build in a stepwise manner - Sneath and Sokal 1973). The third generation of the series, ClustalW (Thompson et al 1994), incorporated a number of improvements to the alignment algorithm. The rationale behind the development of the Clustal series has been to provide robust, portable programs that are capable of providing good, biologically accurate alignments within a reasonable time limit.

6.4.3.3 Results

105 patients with POAG and 60 controls were enrolled in the study. Of the 105 POAG patients, 5 were excluded, as the age when disease presented and were recruited for the study was below 40 years of age. For the posterior embryotoxon group, six subjects were recruited for the study.
Demographics of the patients and controls are presented in the table 6.5.

Table 6.5: Demographics of the patients and controls			
	PATIENTS	CONTROLS	Posterior embryotoxon
Female	33/100 (33%)	38/60 (63.3%)	3/6(50%)
Male	67/100 (67%)	22/60 (36.7%)	3/6 (50%)
Mean age + SDV	61.3 ± 9.6 (40-84)	73.8 ± 9.6 (41-90)	55.1 ± 15.8
Median	61.5	75	60
IOP	34.9 ± 6.91 (30-65)	16.6 ± 2.7 (10-22)	16.3 ± 1.8
CD ratio	0.66 ± 0.2 (0.2-1)	0.33 ± 0.1 (0.1-0.7)	0.45 ± 0.22

In the POAG group, 20% of patients enrolled had family history of glaucoma and 60% had a visual field defect. In the PE group, 66.6% (4/6) individuals had a family history of glaucoma and 1 patient had glaucoma.

The work on this project has been carried on the *PITX2A* isoform and a diagram with the primer sets and SNPs published in the internet database (http//www.ncbi.nih.gov/SNP and www.ensembl.org) is presented in Fig 6.7.

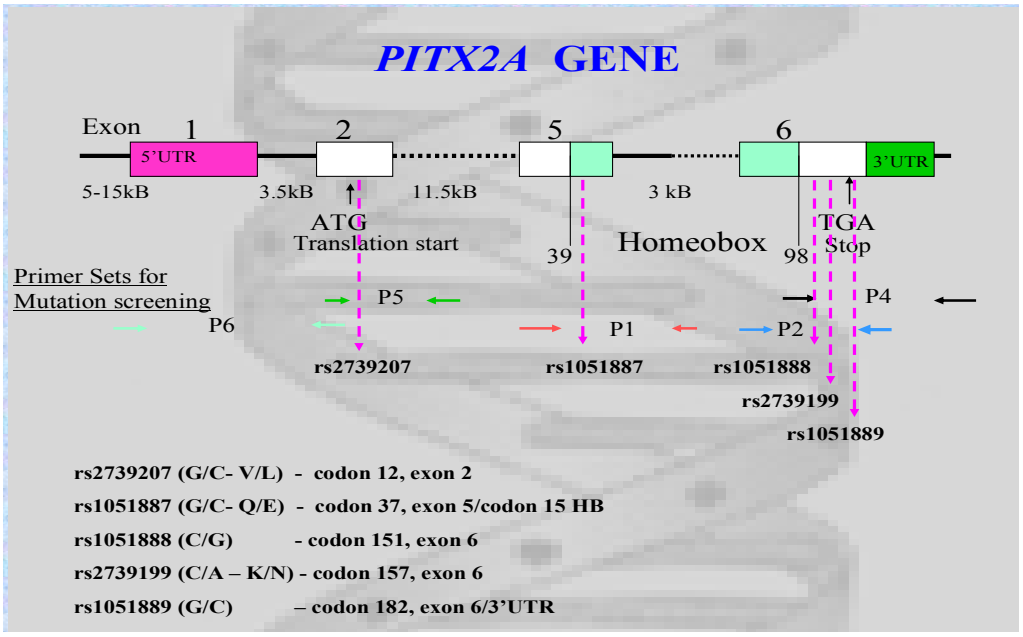


Figure 6.7: Diagram of the *PITX2A* isoform, indicating the position of the SNPs recorded in NCBI (http//www.ncbi.nih.gov/SNP) database and the primer sets used for PCR amplification.

The sequence of the coding region, together with the position of the primers used for sequencing and position of the SNPs published in the NCBI (http//www.ncbi.nih.gov/SNP) database is presented below.

SNPs in the coding regions of genes or in regulatory regions are more likely to cause functional differences then SNPs elsewhere. Therefore, in the first stage, the homeobox sequencing has been prioritised (pink, underlined region), as any variation found in this region would be a very significant finding. The sequencing reactions were carried out for 105 patients and 60 controls, as well as 6 posterior embryotoxon patients, using primer set 1.

Analysis of the traces did not identify any sequence variation in this cohort. This indicates a high level of sequence conservation in the homeobox region of *PITX2* gene.

A sample of sequence alignment for the primer set 1 (homeobox region) is presented. This sample is centred on the site of SNP 1051887, and it demonstrates that there is no sequence variation present in this cohort of patients.

Alignment of sequences (1R) using Clustal W, demonstrating the site of SNP1051887

```
IRSEI63 GCGGTTCCTCTGGAAAGTGGCCTCCAGCTCCTGGAGCTGCTGGCTGGTAA-AGTGAGTCC 276
IRSEI9  GCGGTTCCTCTGGAAAGTGGCCTCCAGCTCCTGGAGCTGCTGGCTGGTAA-AGTGAGTCC 270
IRSEI62 GCGGTTCCTCTGGAAAGTGGCCTCCAGCTCCTGGAGCTGCTGGCTGGTAA-AGTGAGTCC 271
IRSEI70 GCGGTTCCTCTGGAAAGTGGCCTCCAGCTCCTGGAGCTGCTGGCTGGTAA-AGTGAGTCC 277
IRSEI65 GCGGTTCCTCTGGAAAGTGGCCTCCAGCTCCTGGAGCTGCTGGCTGGTAA-AGTGAGTCC 272
IRSEI66 GCGGTTCCTCTGGAAAGTGGCCTCCAGCTCCTGGAGCTGCTGGCTGGTAA-AGTGAGTCC 273
IRSEI72 GCGGTTCCTCTGGAAAGTGGCCTCCAGCTCCTGGAGCTGCTGGCTGGTAA-AGTGAGTCC 276
IRSEI71 GCGGTTCCTCTGGAAAGTGGCCTCCAGCTCCTGGAGCTGCTGGCTGGTAA-AGTGAGTCC 271
IRSEI75   CGGTTCCTCTGGAAAGTGGCCTCCAGCTCCTGGAGCTGCTGGCTGGTAA-AGTGAGTCC 275
1Rw     GCGGTTCCTCTGGAAAGTGGCCTCCAGCTCCTGGAGCTGCTGGCTGGTAA-AGTGAGTCC 347
IRSEI79   CGGTTCCTCTGGAAAGTGGCCTCCAGCTCCTGGAGCTGCTGGCTGGTAA-AGTGAGTCC 273
IRSEI67     GGTTCCTCTGGAAAGTGGCCTCCAGCTCCTGGAGCTGCTGGCTGGTAA-AGTGAGTCC 269
IRSEI73   CGGTTCCTCTGGAAAGTGGCCTCCAGCTCCTGGAGCTGCTGGCTGGTAA-AGTGAGTC 280
IRSEI78   CGGTTCCTCTGGAAAGTGGCCTCCAGCTCCTGGAGCTGCTGGCTGGTAA-AGTGAGTCC 274
IRSEI77   CGGTTCCTCTGGAAAGTGGCCTCCAGCTCCTGGAGCTGCTGGCTGGTAA-AGTGAGTCC 271
```

PITX2A

Exon 1

6F: 5'-TTC TGG TCC TCC TTT CCA CA-3'
TGGGAGTCCGTGCTCCTGCTCCTCGGTTGGCTCCTAAGTGCCCCGCCAGGTCCCCTCTCCTTTCGCTCTC
CCGGCTCCGGCTCCCGACTCTTCGGCC**C**GCTGGCATCTGCTTCCCTCCCCTGCCTCGTTTCTCGTCGCCC
CTGCTCGCTCCCCCCGGCGCTCGCCCGGGCGCTGTGCTCGCTCCTGGATCGCCAGCCGCGCAGCCGGGC
TCGGCCGGCCGCCCGCGCGCCACTGTGCAGTGGAGTTTGGTGGAATCTCTGCTGACGTCACGTCACTCC
CCACACGGAGTAGGAGCAGAGGGAAGAGAGAGGG**ATGA**GAGGGAGGGAGAGGA3'-
GAGAGAGTGCGAGACCGAGC-5'(7R-SNP 2739200)
GAGAAAGCTGGAGAGGAGCAGAAAGAAACTGCCAGTGGCGGCTAGATTTCGGAGGCCCCAGTGCACC
CGTGGACTCCTTCGGAACTTGGCACCCTCAGGAGCCCTGCAGTCCTCTCAGGCCCGGCTTTCGGGCGCT
TGCCGTGCAGCCGGAGGCTCGGCTCGCTGGAAATCGCCCCGGGAAGCAGTGGGACGCGGAGACAGCAG
CTCTCTCCCGG**TAG**CCG**gt**
6R:5'-CCA GAC TCG CAT TAT CTC AC-3'

Exon 2

5F:5'-CTG AAG CCT AGC ACA CAG TA-3'

agATAACGGGGA**ATG**GAGACCAACTGCCGCAAACTGGTGTCGGCGTGT***G***TGCAATTAG**gt**
SNP2739207 (G/C-V/L non synonymous change)

5AR: 5'-GCA GGA GAA GCG GGT TCT TA-3'

Exon 5

1aF: 5'-CAG CTC TTC CAC GGC TTC T-3'

AGAAAGATAAAAGCCAGCAGGGGAAGAATGAGGACGTGGGCGCCGAGGACCCGTCTAAGAAGAAGCG
GCAAAGGCGGCAGCGGACTCACTTTACCAGCCAGCAGCTCCAG***G***AGCTGGAGGCCACTTTCCAGAGGA
ACCGCTACCCGGACATGTCCACACGCGAAGAAATCGCTGTGTGGACCAACCTTACGGAAGCCCGAGTC
CGGGTTTGG
SNP 1051887(Q/E-G/C-nonsynonymous change))
1R: 5'-GAG GGA ACT GTA ATC TCG CA-3'

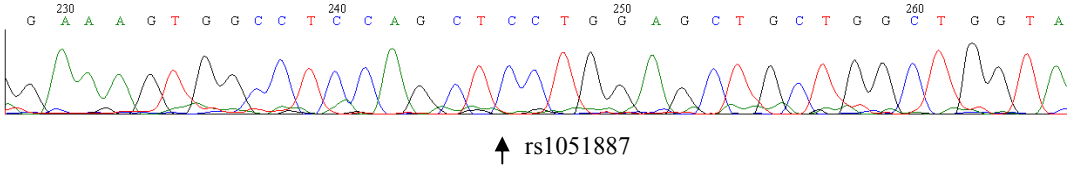
Exon 6

2F:5'-GTA ATC TGC ACT GTG GCA TC-3'
TTCAAGAATCGTCGGGCCAAATGGAGAAAGAGGGAGCGCAACCAGCAGGCCGAGCTATGCAAGAATG
GCTTCGGGCCGCAGTTCAATGGGCTCATGCAGCCCTACGACGACATGTACCCAGGCTATTCCTACAACA
ACTGGGCCGCCAAGGGCCTTACATCCGCCTCC 5'CTA TCC ACC AAG AGC TTC 3'**(4F)**
CCCTTCTTCAACTCTATGAACGTCAACCCCCTGTCA3'TCA CAG AGC ATG TTT TCC CC-5'**(3R)**
ACCCAACTCTATCTCGTCCATGAGCATGTCGTCCAGCATGGTGCCCTCAGCAGTGACAGGCGTCCCGGG
CTCCAGTCTCAACAGCCTGAATAACTTGAACAACCTGAGTAGCCCGTCGCTGAATTCCGCGGTGCCGAC
GCCTGCCTGTCCTTACGCGCCGCCGACTCCTCCGTATGTTTATAGGGACACGTGTAACTCGAGCCTGGC
CAGCCTGAGACTGAAAGCAAAGCAGCACTCCAGCTTCGG***C***TACGCCAGCGTGCAGAA***C***CCGGCCTCCA
ACCTGAGTGCTTGCCAGTATGCAGTGGAC
SNP1051888 (synonymous change C/G) rs2739199 (nonsynonymous change C/A-K/N)

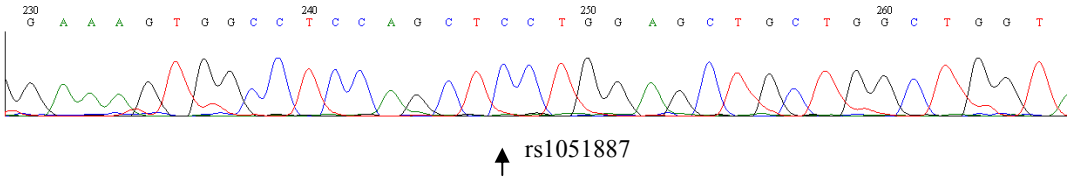
CGGCCCGTG**TGA**GCCGCACCCACAGCGCCGGGATC***C***TAGGACCTTGCCGGATGGGGCAACTCCGCCCT
TGAAAGAC
SNP1051889 (G/C-UTR)
TGGGAATTATGCTAGAAGGTCGTGGGCACTAAAGAAAGGGAGAGAAAGAGAAGCTATATAGAGAAAA
GGAAACCACTGAATCAAAGAGAGAGCTCCTTTGATTTCAAAGGGATGTCCTCAGTGTCTGACATCTTTC
ACTACAAGTATTTCTAACAGTTGCAAGGACACATACACAAACAAATGTTTGACTGGATATGACATTTTA
ACATTACTATAAGCTTGTTATTTTTTAAGTTTAGCATTGTTAACATTTAAATGACTGAAAGGATGTATAT
ATATCGAAATGTC*AAATTAA*TTTTATAAAAGCAGTTGTTAGTAATATCACAACAGTGTTTTTAAAGGTTA
GGCTTTAA*AATAA*AGCATGTTATACAGAAGCGATTAGGATTTTTCGCTTGCGAGCAAGGGAGTGTATAT
ACTAAATGCCACACTGTATGTTTCTAACATATTATTATTATTATAAAAAATGTGTGAATATCAGTTTTAG
AATAGTTTCTCTGGTGGATGCAATGATGTTTCTGAAACTGCTATGTACAACCTACCCTGTGTATAACATT
TCGTACAATATTATTGTTTTACTTTTCAGCAAATATGAAACAAATGTGTTTTATTTCATGGGAGTAAAAT
ATACTGCATACAAA
4R: 5'-CTG AGG ACA TCC CTT TGA-3'

Figure 6.8: Section of chromatograms for patient and control, demonstrating identical trace in patient and control at the site of SNP 1051887

Control (1R)



Patient (1R)



The next stage consisted in sequencing the rest of the coding region of the *PITX2* gene, using primer sets 2, 4 and 5. Some segments of the gene sequenced better than other and therefore the results are not complete for the entire coding sequence in all the patients.

Analysis of traces, failed to identify any sequence variation in the samples in which good traces were obtained.

Figure 6.9 presents an example of sequence alignment, using the Clustal W (http://srs.ebi.ac.uk) program. It highlights the sequence conservation for three of the SNPs sites published in the NCBI (http//www.ncbi.nih.gov/SNP) database within the coding sequence of the *PITX2* in the region of primer set 4 (exon 6).

Results

- 4F/4R

SNPrs1051888(C/G)

- F_w CTGAGACTGAAAGCAAAGCAGCACTCCAG*CTTCGG*CTACGC 356
- FI_2 CTGAGACTGAAAGCAAAGCAGCACTCCAGCTTCGGCTACGC 299
- FI_4 CTGAGACTGAAAGCAAAGCAGCACTCCAGCTTCGGCTACGC 298

SNPrs2739199(C/A-K/N)

- F_w CA*GCGTGCAGAA* CCCGGCCTCCAACCTG-AGTGCTT-411
- FI_2 CAGCGTGCAGAACCCGGCCTCCAACCTG-AGTGCTT- 354
- FI_4 CAGCGTGCAGAACCCGGCCTCCAACCTG-AGTGCTT- 353

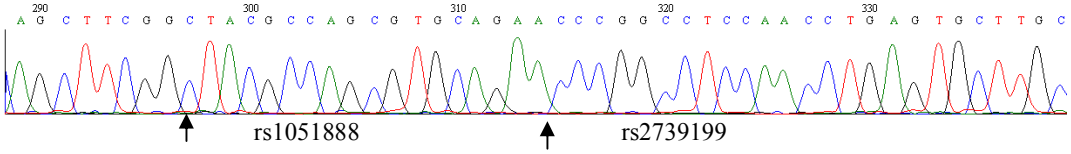
SNPrs1051889(G/C)-UTR

- F_w CCGCACCCACAGCGCC*GGGATCC*TAGGACCTTGCCGGATGGG 465
- FI_2 CCGCACCCACAGCGCCGGGATCCTAGGACCTTGCCGGATGGG 408
- FI_4 CCGCACCCACAGCGCCGGGATCCTAGGACCTTGCCGGATGGG 407

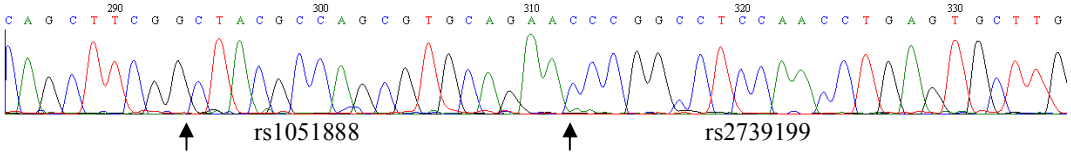
Figure 6.9: Example of sequence alignment for the region of primer set 4/exon 6 (Clustal W)

Figure 6.10: Section of chromatograms for patient and control, demonstrating sequence conservation at SNPs site: rs1051888, rs2739199, rs1051889 in the region of primer set 4 (exon 6) and rs2739207 in the region of primer set 5 (exon 2).

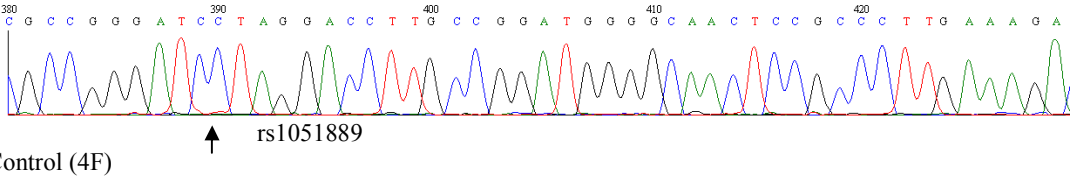
Patient (4F)



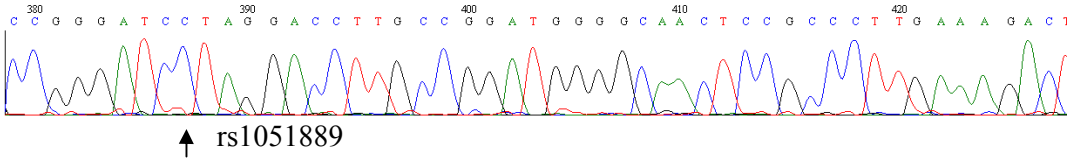
Control (4F)



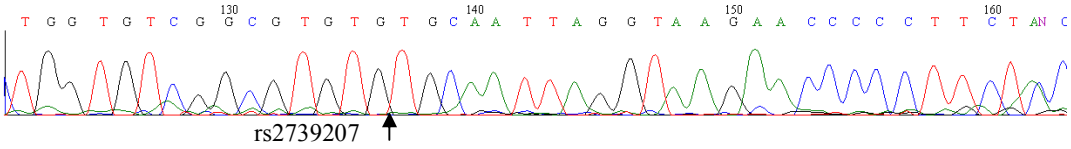
Patient (4F)



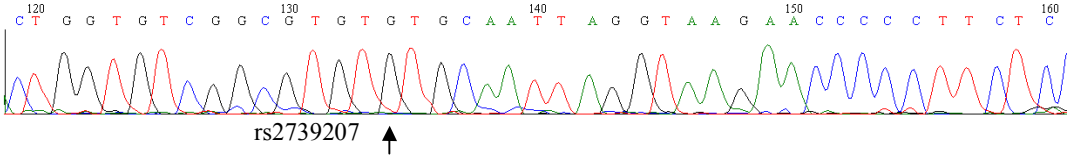
Control (4F)



Patient (5F)



Control (5F)



6.4.3.4 Sequencing protocol review

Problems with sequencing have been encountered, and that prompted a sequencing protocol review.
Table 6.6 presents the number of sequencing reactions carried out for each primer set and the number of traces which could be analysed.

Table 6.6: Sequencing reactions and output

Primer sets	Sequencing reactions	Good traces	%patients & controls sequenced
1aF1R	358	170 (47.4%)	98%patients/93.3%controls
2F3R	323	129 (39.9%)	88%patients/45%controls
4F4R	90	16 (17.7%)	10%patients/25%controls
5AF5R	257	116 (45.1%)	45%patients/61.6%controls

It is obvious from the above table, that the sequencing results were not completed for all primer sets and all samples.
When sequencing reactions are processed, a control reaction is set up and the role of this is to confirm the performance of the sequencing premix under specified and tested conditions. Although the control reaction in 99% of the cases would work well, the patients and control sequencing reactions did not always work.
In the attempt to identify a reason why sequencing was not working, all the protocols were revised.

Possible reasons for failure of sequencing are:

1. Quantity and quality of the template (DNA) present in the reaction
2. primer considerations (amount, design)
3. contamination of reagents
4. cycling conditions
5. human error

Every step in leading to the sequencing of the DNA template has been revised as a result of sequencing failure in quite a lot of the sample at the beginning of the experiments.

The double stranded DNA has been checked on an agarose gel and then has been checked by spectrometry, to asses the purity. If protein and phenols are present, then the product needs cleaning up.

Issues of quantity of DNA used for PCR reaction was dealt with by repeating reactions using various dilution (1:10; 1:5; 1:2) and an EZ Load Precision Molecular Mass Ruler (Bio-Rad ladder) has been used for quantification of the PCR product on an 1.4% agarose gel.

With regard to the primer, the sequence of the primer has been revised (length, base composition) and it was found that the rules for designing a primer have been adhered to, so no change was necessary.
Reactions with various concentrations and amounts of primer have been carried out, to optimise the amount which gives the best result. Again, in the end, there was no need to change the amount suggested in the protocol.

Another reason for sequencing failure is contamination of reagents. Therefore, all reagents (primers, dNTPs, working DNA stock, etc) have been renewed.

Finally, cycling conditions (numbers of cycles, annealing temperatures) have been revised as well; however no change to the protocol ensued, as no better results were obtained with different temperatures or more cycles.

Due to all the problems with sequencing, this method was not cost and time effective in producing results. After discussion with all involved in the project, the decision to stop direct sequencing has been reached and other methods of high throughput screening were examined.

One suggestion was to consider heteroduplex analysis using the Mega BASE; this method has been successfully used at ICH, UCL. However, when investigation on methodology was made, we were informed that the method used at ICH has not been published and therefore could not be used elsewhere until published.

Around the time the problems with sequencing were encountered, the IHG acquired a Sequenom machine, which is capable of performing high throughput SNP screening. After studying the method and realising its potential, the decision to use this opportunity for screening SNPs in the *PITX2* gene was made.

However, although it is a very cost effective method for screening a large number of SNPs, it is still expensive. Therefore, I had to prepare a proposal for extra funds in order to be able to continue the project. Securing the funds allowed the second part of the pilot study to take place.

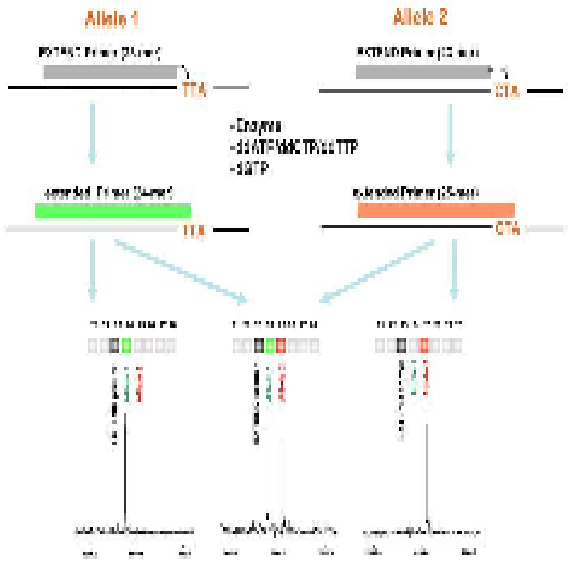
The second part of the study consists of genotyping and haplotype analysis of selected SNPs published in the NCBI (http//www.ncbi.nih.gov/SNP) and Ensembl (www.ensembl.org) databases.

6.4.4 High throughput SNP screening using the Sequenom™

The Sequenom ™ uses the MassARRAY homogenous MassEXTEND™ (hME) Assay technology (www.SEQUENOM.COM).

The hME assay is based upon annealing of the MassEXTEND primer adjacent to the polymorphic site of interest. The addition of a DNA polymerase, plus a cocktail mixture of nucleotides and terminators, allows extension of the primer through the polymorphic site, and generates a unique mass product. The resultant mass of the primer extension product is then analysed and used to determine the sequence of the nucleotides at the polymorphic sites. Through the application of matrix assisted laser desorption time-of-flight (MALDI-TOFF) mass spectrometry to the analysis of primer extension products, SNPs can be accurately determined (Fig 6.11)

Figure 6.11: Diagram of MassEXTEND genotyping assay (www.SEQUENOM.COM)



The recommended protocol for the Sequenom use at Institute of Human Genetics (IHG), Centre for Life, Newcastle upon Tyne, is as follow:

- Select SNPs within gene of interest
- Submit SNP sequences to technician for assay/primer design
- Assay design
- Order primers
- Amplify DNA by PCR
- Provide technician with PCR products, assay and sample information
- Genotyping by technician
- Generation of report by technician and e-mailing of results

6.4.4.1 SNP selection for *PITX2* gene

The NCBI and Ensembl SNP database (http//www.ncbi.nih.gov/SNP and www.ensembl.org) was searched for polymorphic sites published for *PITX2*.

The criteria for SNP selection were:

- Position of the SNP in the coding sequence, untranslated region (UTR) and intron-exon boundary
- SNPs validation status.
- Priority given to SNPs which cause an aminoacid change

26 SNPs (table 6.7) were selected from the database, including 5 in the coding area, 12 in UTR and the rest in the intronic region of the coding sequence. The SNPs ids, position in the gene, base change, allele frequency and validation status are presented in the table 6.7.

Table 6.7: List of SNPs screened

SNP no	SNP ID	Base Position	Gene Position	Polymorphism/ base change	Allele frequency/ validation
1	rs2739202	111758171	3UTR	C/G	n/v
2	rs1051889	111758706	3UTR	C/G	n/v
3	rs2739199	111758781	Exon6/Nonsynon Change	A/C;N/K	n/v
4	rs1051888	111758799	Exon6/Syn Change	C/G	n/v
5	rs3796902	111759917	Intron	C/T	0.399 v
6	rs2278783	111761573	Intron	C/T	0.416 v
7	rs2278782	111761603	Intron	C/T	0.354 v
8	rs1051887	111761864	Exon5/Nonsynon Change	C/G; E/Q	n/v
9	rs2595110	111764772	Intron	C/T	v
10	rs994978	111771847	Intron	A/G	n/v
11	rs2595105	111772210	Intron	A/G	n/v
12	rs2559104	111772582	Intron	A/C	n/v
13	rs1530717	111773452	Intron	C/T	n/v
14	rs2739207	111773567	Exon2/Nonsynon Change	C/G;V/L	n/v
15	rs2276966	111773643	5'UTR	C/T	n/v
16	rs2595103	111773948	5'UTR	C/T	v
17	rs2739206	111774158	5'UTR	C/G	0.490 v
18	rs2723321	111774448	5'UTR	A/G	0.494 v
19	rs3796898	111774643	5'UTR	C/G	0.493 v
20	rs2595101	111775872	5'UTR	A/G	v
21	rs2595100	111775879	5'UTR	A/G	v
22	rs976568	111776170	5'UTR	A/C	n/v
23	rs2723324	111776469	5'UTR	A/G	0.493 v
24	rs2739204	111776709	5'UTR	A/T	0.493 v
25	rs2739200	111777860	5UTR	C/G	O.493 v
26	rs1982361	111779872	Intron	A/G	0.493 v

Abbreviations: UTR = untranslated region; A = adenine; C = cytosine; G = guanine;

T = Thymine; N = aspargine; K = lysine; E = glutamic acid; Q = glutamine;

V = valine; L = leucine; v = validated; n/v = non validated

The primers were designed using MassARRAY Designer (Sequenom assay design software –table 6.8)

Table 6.8: Forward, reverse and extension primers for each SNP

SNP_ID	Forward, reverse primer	Extension primer
rs2595105	**F**:ACGTTGGATGTTCCCTCCTCCATCCCTTAC **R**:ACGTTGGATGAAAAGTCTCCAACCTACGCC	GCCTTTCCTGAATTGAA
rs2739200	**F**:ACGTTGGATGTCCCCTCTCCTTTCGCTCTC **R**:CGTTGGATGGACGAGAAACGAGGCAGGG	GGGAAGCAGATGCCAGC
rs2595101	**F**:ACGTTGGATGCCAAGGACTCTTGTTTTGGC **R**:ACGTTGGATGTGCTTTGCCATGGTTCCTCG	CATGGTTCCTCGTCCTCC
rs1051889	**F**:ACGTTGGATGTCTTTCAAGGGCGGAGTTGC **R**:ACGTTGGATGTTGCCAGTATGCAGTGGACC	GCACCCACAGCGCCGGGATC
rs2276966	**F**:ACGTTGGATGAACGTAGTCTCATCTGAGCC **R**:CGTTGGATGAACGTAGTCTCATCTGAGCC	AGAAGAGAAGGAAGGCTGTA
rs994978	**F**:ACGTTGGATGAGCACTGGATCGCTTTAGAC **R**:ACGTTGGATGGTCTTGCATTGGAGTTTCCC	GTGAATCTGAGAGAAAATGATC
rs1530717	**F**:ACGTTGGATGAAGAGGGCACTGGCCTGCG **R**:ACGTTGGATGTTCTGATTTCCTTCCTGCCC	CAGGCTGGCCCCTCGGG
rs976568	**F**:ACGTTGGATGCTGAAAGTGTTCGACCCAAG **R**:ACGTTGGATGTGCCACAACGGGCCATGGAG	CCAGAGGGAGGAAGTCT
rs2595110	**F**:ACGTTGGATGCCTGCTTGGACTTTAATGGC **R**:ACGTTGGATGGCCAAATAACCAATTTCAAGG	CAGTCATTTTCTTTTCCC
rs2595104	**F**:ACGTTGGATGTCGCTCGTTATATCCCGCTG **R**:ACGTTGGATGATGAACCGCGCACCCACAAG	GGCGTGGCTGAGAGCCTGAG
rs2278783	**F**:ACGTTGGATGAGCTCGTGAGATCGCGGGATG **R**:ACGTTGGATGTTCCCTCTCCGAGGCCGACA	GAGGCCGACAGGACTGC
rs2723324	**F**:ACGTTGGATGTGCTTATGTGCTTTGTTTTC **R**:ACGTTGGATGCACACAAAATATCATTTTGGC	TCATTTTGGCAGAAATCCA
rs2595100	**F**:ACGTTGGATGTTGCCTGCCTGCTTTGCCAT **R**:ACGTTGGATGGGACTCTTGTTTTGGCCAAC	ACTACACGGCTAACGGAGGA
rs1051888	**F**:ACGTTGGATGGACTGAAAGCAAAGCAGCAC **R**:ACGTTGGATGCATACTGGCAAGCACTCAGG	TTCTGCACGCTGGCGTA
rs2278782	**F**:ACGTTGGATGAGCTTTGCAAAGGCAGAAGG **R**:ACGTTGGATGAGTCCTGTCGGCCTCGGAGA	GCCTCGGAGAGGGAACT
rs2739202	**F**:ACGTTGGATGTGTGTGAATATCAGTTTTAG **R**:ACGTTGGATGCAGGGTAGGTTGTACATAGC	ACATCATTGCATCCACCA
rs1051887	**F**:ACGTTGGATGCTAAGAAGAAGCGGCAAAGG **R**:ACGTTGGATGAGCGGTTCCTCTGGAAAGTG	CTGGAAAGTGGCCTCCAGCT
rs2739206	**F**:ACGTTGGATGATTCTGAAACGAACGGGCTC **R**:ACGTTGGATGAAAGTGGCTCCTGATGGATG	TGATGGATGAGAGGCCCGAA
rs3796902	**F**:ACGTTGGATGAAACTACCCTTTCCGGGTTC **R**:ACGTTGGATGAGCAAAAGACCACCTCTTCG	TTCGTCAGCTCGTTAACTCCA
rs3796898	**F**:ACGTTGGATGCCTCTCTCCACGCTAACCAG **R**:ACGTTGGATGACAACGCTTGCTCCCACCC	TGGGCCCTCCGGTCGTC
rs2739199	**F**:ACGTTGGATGGACTGAAAGCAAAGCAGCAC **R**:ACGTTGGATGCATACTGGCAAGCACTCAGG	CTCAGGTTGGAGGCCGG
rs2723321	**F**:ACGTTGGATGGCGCGTTAAATGCTTGGCTC **R**:ACGTTGGATGTAACTGCTACCCAGTTCTCC	CTTTTTCACGTTTTCAACC
rs2739207	**F**:ACGTTGGATGATGACCCGGGCAGGAGAAG **R**:ACGTTGGATGGAAATGGAGACCAACTGCCG	GCAAACTGGTGTCGGCGTGT
rs2595103	**F**:ACGTTGGATGAGAGAAAAGTCCTCTCCACC **R**:ACGTTGGATGCTTTCAAGAATCTCGGTGCC	AACTCATCTGAACGCAATGGC
rs1982361	**F**:ACGTTGGATGTTTTTGCAGCCTCCCCATTC **R**:ACGTTGGATGGAGCGCCTCTTAATCTCTAC	TCTACAAAATGAAAAGAAAAAAAG

The extension primer was designed so that the 3' end is immediately adjacent to the polymorphic site; the extension primer size is 16-25 nucleotide long (4800 to 7500 Da), with a melting temperature of 60°C or higher.

For each SNP an appropriate termination mix is chosen; the extension primer and termination mix combination should terminate at the SNP site for one allele (extension primer of just one nucleotide = the terminator) and as soon thereafter for the alternative allele (extension primer of two nucleotide, including the terminator). The mass of the extended primer should be within the mass range of 5000 to 8500 Da.
The amplification primer optimal size, containing the SNP site is 70 to 150bp, and its mass should be different from the extension primer and its extension product.

PCR amplification was performed in multiplex reactions as presented in table 6.9.

Table 6.9: Multiplex reactions suggested by the MassARRAY Designer

Multiplex reaction	SNPs
1 (6 plex)	SNPrs1051888 + SNPrs2278782 +SNPrs2739202 + SNPrs1051887 + SNPrs2739205 + SNPrs379602
2 (6 plex)	SNPrs3796898 + SNPrs2739199 + SNPrs2723321 + SNPrs2739207 + SNPrs2595103 + SNPrs1982361
3 (6 plex)	SNPrs2595105 + SNPrs2739200 + SNPrs2595101 + SNPrs1051889 + SNPrs2276966 + SNPrs994978
4 (4 plex)	SNPrs1530717 + SNPrs976568 + SNPrs2595110 + SNPrs2559104
5 (3 plex)	SNPrs2278783 + SNPrs2723324 + SNPrs2595100
6 (uniplex)	SNPrs2739204

6.4.4.2 Sequenom genotyping

The following steps for Sequenom genotyping (http://www.ncl.ac.uk/ihg/research/facilities/sequenom) were followed:

- The PCR reactions were performed in 96 well plates at final volumes of 10µl (the starting volume of PCR product required for the Sequenom system is 5µl per well in a 384 well plate)
- 5µl is then transferred to a 384 well plate (ABgene 384 well plates to be used, sealed with ABgene adhesive PCR film) and the remainder was tested on 1% agarose gel.
- Primers used were ordered (http://www.metabion.com/) in lyophilised state. The mass of the extension primer is critical to the assay working successfully and therefore,

extension primers were HPLC purified and MALDI TOF checked. PCR primers were at desalted purity.

- Extension primers for hME SNP genotyping were resuspended at 100µM before being given to the technician (PCR primers contain an identical 10bp tail, which increases their mass to over 9000Da so they don't interfere with the extension peaks. They also help to standardise the primers so that they will work more efficiently in multiplex).
- Termination mixes have been chosen for each SNP. The termination mix brings about primer termination after 1 base addition for one allele, and 2/3 bases for the alternative allele.
- When multiplexing, SNPs are grouped by termination mix it needs to be ensured that all possible products differ by at least 50Da.
- The Sequenom touchdown PCR protocol (table 4.8) was followed.

DNA amplification by PCR was performed for all samples following the protocol set for the Sequenom. Tables 6.10 and 6.11 present the reagents and the protocol for PCR. PCR reactions were checked on a 2% agarose gel, prior to continuing with the SNP genotyping. Quality control and quality assurance were provided by randomly including non-DNA containing well in the chip.

Table 6.10: PCR reagents used for Sequenom reactions

Reagent	**Initial concentration**	**Volume (µl)**	**Final concentration**
ddH20	-	6.76	-
Qiagen Hot Star 10x buffer	10 x (& 15mM $MgCl_2$)	1.0	1
Qiagen Hot Star $MgCl_2$	25mM	0.4	2.5mM
dNTPs	6.25mM each	0.32	0.2mM each
Forward primer 1 to 6	25µM	0.08 each	0.2µM
Reverse primer 1 to 6	25µM	0.08 each	0.2µM
Qiagen Hot Star Taq	5U/µl	0.04	0.2 units
DNA	2.5ng/µl	1	2.5ng
Total volume (µl)		**10**	

Table 6.11: PCR touchdown protocol for Sequenom

Reaction step	Temperature (°C)	Duration (min:sec)	Nature of reaction	Number of cycles
1	95˚C	15 minutes	Denaturation	1 cycle
2-4	95˚C	20 seconds	Separation of DNA strand	
	56˚C	30 seconds	Primers annealing	45 cycles
	72˚C	1 minute	DNA synthesis (transcription	
5	72˚C	3 minutes	DNA synthesis (elongation)	1 cycle
	4˚C	Forever		

The report generated by the Sequenom technician has been entered in a database, which includes both the clinical details and the genotypes for all sites analysed.

The figures 6.12 a-c presents various Sequenom screens with assays results. The first screen shows the results of a multiplex (six in this case) assay, which worked well. The second screen shows the image obtained when there is a machine failure (no extension primer visible) and the third screen shows a negative control (no DNA present), which is also the appearance of a failed PCR (no product in the well to be extended).

Figure 6.12.a: Sequenom screen demonstrating a sample spectrum from sixplex genotyping assay obtained with MassEXTEND, with ID information displayed

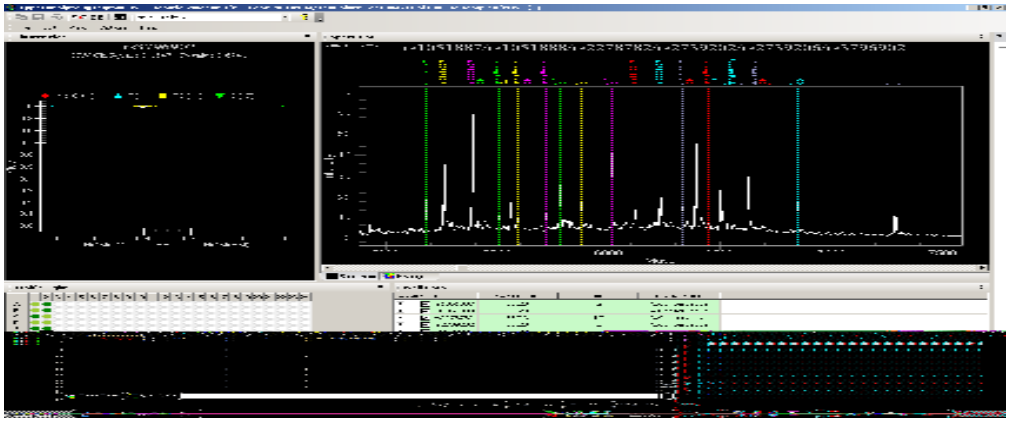


Figure 6.12.b: Sequenom screen demonstrating machine failure (no extension primer present)

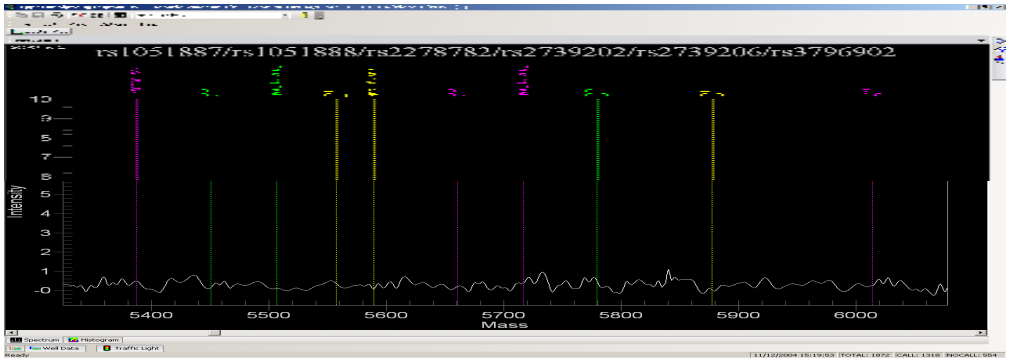
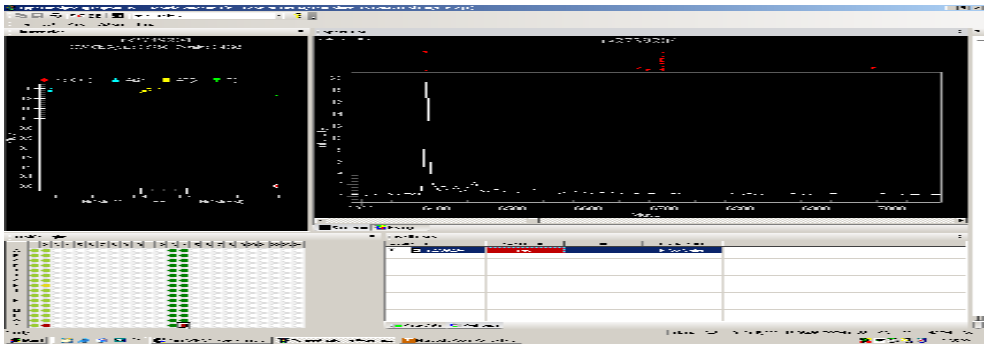


Figure 6.12.c: Sequenom screen demonstrating negative control or absence of DNA



6.4.5 Statistical methods for analysing Sequenom genotyping results

6.4.5.1 Chi-square

Chi –square test 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:

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

χ^2 utilizes numerical values, and not percentages or ratios.

Using the chi-square distribution table (Table 6.12), the significance of the value is then determined (i.e. $P < \text{or} > 0.05$). 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 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).
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 SHEsis (http://analysis.bio-x.cn/myAnalysis.php) and UNPHASED (http://www.mrc-bsu.cam.ac.uk/personal/frank/software/unphased).

Table 6.12: Chi square distribution table											
Degree of freedom	Probability										
	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	3.07	4.35	6.06	7.29	9.24	11.07	15.09	20.52
6	1.63	2.20	3.07	3.82	5.35	7.23	8.56	10.64	12.59	16.81	22.46
7	2.17	2.83	3.82	4.59	6.35	8.38	9.80	12.02	14.07	18.48	24.32
8	2.73	3.49	4.59	5.38	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
	Non- significant								Significant		

6.4.5.2 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 for the AA genotype, 2pq for the Aa genotype, and q 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 test (Weir 2008). The null hypothesis is that alleles are chosen randomly, and the genotypic proportions thus follow HWE-expected proportions (i.e. p, 2pq, and q). 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 = =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 (p>0.05) goodness-of-fit test. This calculation was performed using a software package SHEsis and UNPHASED as above.

6.4.5.3 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 imply 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.

6.4.5.4 Fisher's exact test

Fisher's exact test is a statistical significance test used in the analysis of contingency tables where sample sizes are small. This is one of a class of exact tests, so called because the significance of the deviation from null hypothesis can be calculated exactly, rather than relying on an approximation that becomes exact in the limit as the sample size grows to infinity, as many statistical tests. Most uses of the Fisher test involve a 2x2 contingency table. The p value from this test is computed as if the margins of the table are fixed. With larger samples, a chi-square test can be used in this situation (see

above). However the principle of the test can be extended to the general case of an mxn table and some statistical packages provide a calculation for the more general case

6.4.5.5 Summary of statistical methods used for analysing Sequenom genotyping and haplotyping results

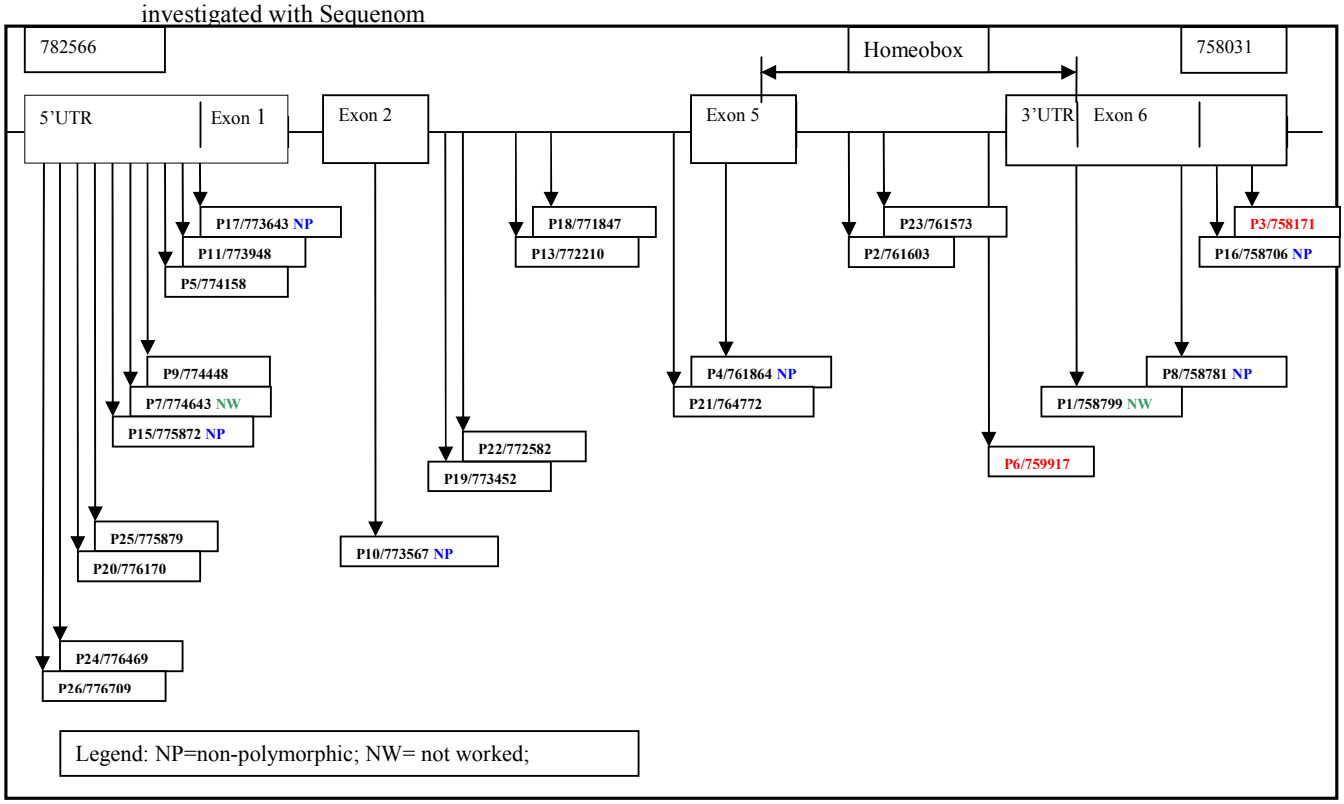
For the statistical analysis of the genotype (association tests for individual SNPs) and haplotype analysis the SHEsis (http://analysis.bio-x.cn/myAnalysis.php), UNPHASED version 3.0.12 - Dudbridge F 2003, (http://www.mrc-bsu.cam.ac.uk/personal/frank/software/unphased) and SPSS version 15.0 (www.spss.com) software packages were used. The SHEsis and UNPHASED programs were used for performing Hardy-Weinberg equilibrium, Fisher's exact test, odds ratio, confidence intervals, chi-square tests and haplotype analysis. The SPSS was used for performing frequencies analysis.

6.4.6 Genotype and haplotype analysis of the SNP screening with Sequenom™

6.4.6.1 Genotype analysis

Second part of genetic investigations in this cohort of patients concerned screening of the PITX2 gene for SNPs already published in internet databases NCBI (http//www.ncbi.nih.gov/SNP) and Ensembl (www.ensembl.org).Figure 6.13 present the position of the SNPs chosen for high throughput genotyping with the Sequenom. The method of selection of SNPs from NCBI and Ensembl database has been presented above.

Figure 6.13: Diagram of PITX2A gene SNPs site, from NCBI (http//www.ncbi.nih.gov/SNP) database, investigated with Sequenom



The SHEsis (http://analysis.bio-x.cn/myAnalysis.php) and UNPHASED (http://www.mrc-bsu.cam.ac.uk/personal/frank/software/unphased/) programs were used to test every investigated SNP for association with POAG (Hardy-Weinberg equilibrium, Fisher's exact test, odds ratio, confidence intervals and chi-square test). The results of the analysis are presented in the table 6.12 a-c.

Table 6.13.a: Results of single site association analysis					
Polymorphism	Genotype/Allele	OAG /OHT probands		Controls	
		n	%	n	%
RS3796902/p6	CC	57	75	29	69
	TT	1	1.3	1	2.4
	CT	18	23.7	12	28.6
	C	132	86.8	70	83.3
	T	20	13.2	14	16.7
	OR= 1.3	CI:0.6-2.7	P=0.4	X2=0.5	
		HW/P=0.7		HW/p=0.8	
RS2278783/p23	CC	66	73.3	34	73.9
	TT	0	0	0	0
	CT	24	26.7	12	26.1
	C	156	86.7	80	87
	T	24	13.3	12	13
	OR=0.97	CI(0.4-2)	P=0.9	X2=0.005	
		HW/P=0.1		HW/P=0.3	
RS2278782/p2	CC	52	78.8	21	75
	TT	1	1.5	0	0
	CT	13	19.7	7	25
	C	117	88.6	49	87.5
	T	15	11.4	7	12.5
	OR=1.1	CI(0.4-2.9)	P=0.8	X2=0.049	
		HW/P=0.8		HW/P=0.4	
RS2595110/p21	CC	6	6.2	5	8.3
	TT	50	51.5	31	51.7
	CT	41	42.3	24	40
	C	53	27.3	34	28.3
	T	141	72.7	86	71.7
	OR=0.95	CI(0.5-1.5)	P=0.8	X2=0.038	
		HW/P=0.5		HW/P=0.9	
RS994978/p18	AA	10	10.2	8	13.3
	GG	42	42.9	27	45
	AG	46	46.9	25	41.7
	A	66	33.7	41	34.2
	G	130	66.3	79	65.8
	OR=0.97	CI(0.6-1.5)	P=0.9	X2=0.008	
		HW/P=0.6		HW/P=0.5	
RS2595105/p13	AA	5	7.2	7	13
	GG	25	36.2	27	50
	AG	39	56.5	20	37
	A	49	35.5	34	31.5
	G	89	64.5	74	68.5
	OR=1.2	CI(0.7-2.04)	P=0.5	X2=1.12	
		HW/P=0.05		HW/P=0.2	

Table 6.13.b: Results of single site association analysis					
Polymorphism	Genotype/Allele	OAG /OHT probands		Controls	
		n	%	n	%
RS2559104/p22	AA	9	9.2	8	13.3
	CC	41	41.8	28	46.7
	AC	48	49	24	40
	A	66	33.7	40	33.3
	C	130	66.3	80	66.7
	OR=1.01	CI(0.6-1.6)	P=0.9	X2=0.0038	
		HW/P=0.3		HW/P=0.4	
RS1530717/p19	CC	97	99	57	100
	TT	0	0	0	0
	CT	1	1	0	0
	C	195	99.5	114	100
	T	1	0.5	0	0
	X2=0.58	P=0.4			
		HW/P=0.9		HW/P=1	
RS2595103/p11	CC	4	4.8	5	8.6
	TT	45	53.6	35	60.3
	CT	35	41.7	18	31
	C	43	25.6	28	24.1
	T	125	74.4	88	76.9
	OR=1	CI(0.6-1.8)	P=0.7	X2=0.07	
		HW/P=0.3		HW/P=0.2	
RS2739206/p5	CC	7	9.6	6	16.2
	GG	29	39.7	14	37.8
	CG	37	50.7	17	45.9
	C	51	34.9	29	39.2
	G	95	65.1	45	60.8
	OR=0.8	CI(0.4-1.4)	P=0.5	X2=0.38	
		HW/P=0.8		HW/P=0.3	
RS2723321/p9	AA	9	10	8	13.5
	GG	37	41.1	27	45.8
	AG	44	48.9	24	40.7
	A	62	34.4	40	33.9
	G	118	65.6	78	66.1
	OR=1.02	CI(0.6-1.6)	P=0.9	X2=0.009	
		HW/P=0.4		HW/P=0.4	
RS2595100/p25	AA	9	9.1	8	14.3
	GG	41	41.4	27	48.2
	AG	49	49.5	21	37.5
	A	67	33.8	37	33
	G	131	66.2	75	67
	OR=1.03	CI(0.6-1.6)	P=0.8	X2=0.02	
		HW/P=0.29		HW/P=0.2	

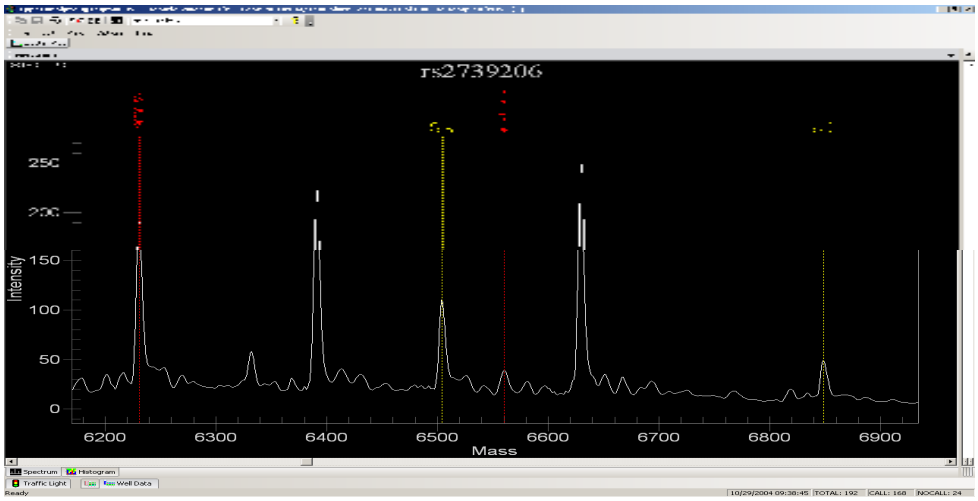
Table 6.13.c: Results of single site association analysis					
Polymorphism	Genotype/Allele	OAG /OHT probands		Controls	
		n	%	n	%
RS976568/p20	AA	35	35.7	24	40
	CC	11	11.2	9	15
	AC	52	53.1	27	45
	A	122	62.2	75	62.2
	C	74	37.8	45	37.5
	OR=0.9	CI(0.6-1.5)	P=0.9	X2=0.002	
		HW/P=0.2		HW/P=0.7	
RS2723324/p24	AA	9	9.9	7	15.2
	GG	38	41.8	22	47.8
	AG	44	48.4	17	37
	A	62	34.1	31	33.7
	G	120	65.9	61	66.3
	OR=1.01	CI(0.5-1.7)	P=0.9	X2=0.003	
		HW/P=0.4		HW/P=0.2	
RS2739204/p26	AA	51	52	36	60
	TT	5	5.1	5	8.3
	AT	42	42.9	19	31.7
	A	144	73.5	91	75.8
	T	52	26.5	29	24.2
	OR=0.8	CI(0.5-1.4)	P=0.6	X2=0.2	
		HW/P=0.3		HW/P=0.29	
RS1982361/p12	AA	38	84.4	27	75
	GG	4	8.9	5	13.9
	AG	3	6.7	4	11.1
	A	79	87.8	58	80.6
	G	11	12.2	14	19.4
	OR=1.7	CI(0.7-4)	P=0.2	X2=1.59	
		HW/P=0.0001		HW/P=3.8e-006	
RS2739200/p14	CC	9	11	8	14.3
	GG	42	51.2	28	50
	CG	31	37.8	20	35.7
	C	49	29.9	36	32.1
	G	115	70.1	76	67.9
	OR=0.8	CI(0.5-1.5)	P=0.6	X2=0.1	
		HW/P=0.3		HW/P=0.1	

SNP rs1982361 was not in Hardy-Weinberg equilibrium either in the patients or controls and therefore has been discarded. SNPs rs105889, rs2739199, rs1051887, rs2739207, rs2739202, rs2276966 and rs2595101 were not polymorphic in this cohort and 2 SNPs (rs1051888 and rs3796898) did not work. None of the SNPs are associated with POAG in this cohort of patients. All the sites which were not polymorphic in this cohort are the sites within the coding sequence (4 sites) or in the 3'UTR (2 sites) and 5'UTR (1 site). The SNP rs1051888 which did not work in this assay was actually

covered by the sequencing of the gene with primer set 1 and the polymorphism was not present in this cohort.

Figure 6.14 demonstrates the appearance of a Sequenom screen in a heterozygous sample for SNP rs3796902 (CG), showing peaks at both sites (C and G).

Figure 6.14: Sample spectrum from genotyping obtained with MassEXTEND showing a heterozygous assay (peaks at both the C and G site)



6.4.6.2 Haplotype analysis

Recently, many researchers investigating SNPs association in complex diseases have found that haplotype analysis can be more powerful when disease susceptibility might be conferred by a haplotype (Judson et al 2000).

Haplotype analysis has been performed on this cohort of patients and controls, using the SHEsis and UNPHASED platforms as above. Initially, an analysis of all the sites found polymorphic and in Hardy-Weinberg equilibrium in this cohort was carried out. Subsequently, various haplotypes were constructed and analysed.

Figure 6.15 demonstrates the r^2 value, measure of linkage disequilibrium between the SNP sites investigated (r^2 is a measure of LD and r^2=1 can be described as complete LD because allelic association can be as strong as possible. Genotypes at SNPs which are < 10kb apart tend to be correlated and a stringent r^2 threshold of >0.8 would resolve > 80% of existing haplotype of the SNPs tested).

From the diagram it is evident that there is a block of SNPs (p9-18, p20, p22, p24-26) which are in linkage and the haplotype analysis has been concentrated on this block.

Table 6.14: Results of haplotype analysis block p9-p18, p20,p22,p24-p26

P9	p11	p13	p14	p18	p20	p22	p24	p25	p26	Case freq	%	control freq	%	X2	p	OR	CI
A	C	A	C	A	C	A	A	A	T	20.9	26.8	19	25	0.06	0.7	1.1	0.5-2.2
A	T	A	C	A	C	A	A	A	A	2.96	3.8	5	6.6	0.6	0.4	0.5	0.1-2.4
G	T	G	G	A	A	C	G	G	A	0	0	1	1.3	1.03	0.3		
G	T	G	G	G	A	C	G	G	A	45.8	58.8	47	61.8	0.14	0.7	0.88	0.4-1.6
G	T	G	G	G	C	C	G	G	A	5.1	6.5	4	5.3	0.11	0.7	1.2	0.3-4.8
A	C	A	C	A	A	A	A	A	T	1.07	1.4	0	0	1.04	0.3		
A	T	A	C	A	A	A	A	A	A	1.04	1.3	0	0	1.01	0.3		
G	C	G	G	G	A	C	G	G	A	1	1.3	0	0	0.9	0.3		
GLOBAL X2 = 118.1, df=6, p= 0.00e+000																	

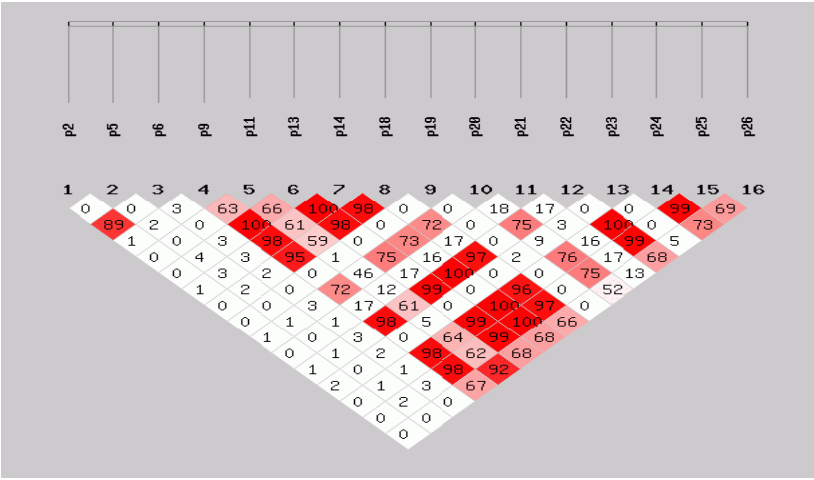
Table 6.15: Results of haplotype analysis p2 & p6

P2	P6	case	%	control freq	%	X2	p	OR	CI
C	C	112	87.5	49	87.5	0.01	0.8	1.06	0.5-2.7
T	C	0	0	1	1.8	2.2	0.1		
T	T	15	11.7	6	10.7	0.04	0.8	1.1	0.4-3.04
C	T	1	0.8	0	0	0.14	0.7	0.88	0.4-1.6
GLOBAL X2 = 135.5, df=1, p= 0.00e+000									

Haplotype analysis also failed to identify any significant association of a haplotype with POAG/OHT in this cohort.

The implications of sequencing results, SNP screening and haplotype analysis will be discussed.

Figure 6.15: Graphic representation demonstrating the r2 values (measure of linkage disequilibrium) between the SNP sites investigated in this study (obtained with SHEsis software platform)



6.5 DISCUSSION PILOT STUDY

Open angle glaucoma is the major primary type of glaucoma in most population world wide and one of the main causes of blindness if left untreated. It is more prevalent in black than in white populations (Leske et al 1994, Tielsch et al 1991,) and a Chinese population based cross-sectional study showed that glaucoma is the major cause of blindness and that POAG is an important form of the disease (Foster et al 2000). Demographic (age, gender, ethnicity, socioeconomic), genetic, systemic (diabetes, high or low blood pressure), ocular (elevated IOP, optic nerve head status, central corneal thickness, refractive status) as well as some other risk factors (smoking and alcohol intake) have been described, however, the pathogenesis of the disease is still unclear.

It is generally accepted that POAG in most cases is inherited as a complex trait, resulting from interaction of multiple genes and environmental factors (Fan et al 2006b, Sieving and Collins 2007). This complex disorder results from diverse pathological process, not limited to involving only the aqueous outflow, but the RGC, the optic nerve and even cerebrospinal fluid, as suggested by Berdahl et al, 2008.

6.5.1 Summary of the study

So far, genetic studies only identified three POAG genes (MYOC, OPTN and WDR36). Mutations in these genes account for only 10% of all POAG patients. Therefore it is very likely that other unidentified loci and genes still exist (Fan et al 2006). An obvious group of genes to test as genetic susceptibility factors for POAG are the developmental glaucoma genes, which at the conception of this pilot study have not been analysed in any association study, with the exception of the *CYP1B1* gene.

The genes involved in anterior segment dysgeneses, play an important role in normal morphogenesis of the eye and especially anterior segment and can provide insight into the underlying genetic mechanism of glaucoma. Since these genes cause malformations of the drainage structure of the eye and elevation of IOP, patients with ASD are at an increase risk of developing glaucoma. Therefore, it is possible that these genes can contribute to age related glaucoma by causing subtle changes, which are not clinically visible, but which cause dysfunction with age. Also, in the mean time, an association

between *LMX1B* and POAG patients has been demonstrated (Park et al 2009). This study also used the cohort from the pilot study presented in this thesis.

This pilot study screened for *PITX2* gene variations in a cohort of 100 patients with POAG/OHT, six patients with posterior embryotoxon and 60 matched controls, and tested for a significant association with glaucoma in these patients.

The patients with POAG/OHT were identified and enrolled in the study according to strict criteria. All patients were phenotyped by experienced glaucoma specialist and as such it represents a homogenous, well defined group. Genetic studies were carried out by Dr Vaideanu, at the Institute of Human Genetics, Centre for Life, Newcastle upon Tyne.

First part of the genetic study consists of sequencing analysis of the coding region in *PITX2* gene. Analysis of the traces did not identify any sequence variation in this cohort. This indicates a high level of sequence conservation in the homeobox region of *PITX2* gene.

Second part of the genetic study consists of high throughput screening of SNPs published in the internet databases. Genotype analysis demonstrates that none of the SNPs screened are associated with POAG in these patients. Haplotype analysis also failed to identify any significant association of a haplotype with POAG/OHT in this cohort.

In this particular cohort, a large effect of *PITX2* polymorphism in POAG has not been demonstrated. However, as mentioned above, a recent paper by Park et al (2009), demonstrates that certain LMX1B haplotype influence susceptibility to glaucoma, predisposing to glaucomatous damage independent of intraocular pressure. This finding, lends further support to the hypothesis that genes associated with anterior segment dysgeneses may predispose to glaucoma. Therefore, further studies into the role of developmental glaucoma genes in the pathogenesis of POAG are needed, as smaller, more subtle effect and /or possible gene-gene interactions might be demonstrated.

6.5.2 Patient and control identification and recruiting discussion

As this study was set up as a pilot study, a sample of 100 POAG/OHT patients, 10 posterior embryotoxon subjects and 100 matched controls was considered to be an appropriate size for answering the study question.

Identification of the patients has been straightforward, as the details were checked in the notes and if the criteria for inclusion were met, the patient was offered the opportunity to take part in the study. Most patients, were very positive about taking part and about 1in 3 accepted to be part of the study.

The controls sample size was meant to be the same as the patient sample. However, this aim was not achieved. Two factors contributed to this situation.

While, the ethical committee approval was already obtained for the POAG patient's involvement in genetic studies, that was not the case for the controls. To get the ethical committee approval, a letter had to be written, explaining why the controls were needed. Therefore, the recruiting of the controls has been delayed until the approval was received. By that stage, about half of the POAG/OHT patients were already enrolled in the study. That made it more difficult to find spouses of POAG/OHT patients willing to take part in the study. The motivation to take part was not the same for relatives of patients who were not part of the study.

The second factor which played a part in achieving a smaller number of controls was a misunderstanding on my part regarding the collaboration set up with Royal Victoria Infirmary. The collaboration was set up so that we would exchange DNA control sample, to help with boosting up the numbers. Once this has been agreed, my understanding was that I would give them a DNA sample of the 30 controls I already enrolled, and this would be reciprocated by getting the rest of the sample to make up to a hundred for me. This was not the case and the exchange was only sample for sample. This emphasises the naïveté of the clinician when getting involved with laboratory research. It was also a learning point for me in making sure everything is well understood and clear when these informal agreements are made. By the time all this was

resolved, I have already started the laboratory work. Time and being physically away from the recruiting centre made it difficult to start recruiting again. Therefore, I have settled on the number of control samples I already had, which was 60.

Also, the aimed number of 10 subjects with posterior embryotoxon was not achieved. The reason was that only eight subjects with posterior embryotoxon came through Mr Fraser's clinic during the recruiting period. Of these, six agreed to take part in the study.

6.5.3 Genetic studies results discussion

6.5.3.1 Sequencing results discussion

As already stated, sequencing analysis of the coding region of *PITX2* gene in this cohort has not identified any sequence variation in this cohort. The sequencing reaction worked well and on almost all patients and controls only on primer set 1, which covered mainly the homeobox. No sequence variation has been identified in this region in the cohort presented and this indicates a high level of conservation of this region. Considering the important role of the homeobox in cell type specification, embryonic pattern formation and determination of cell fate (Gehring et al 1994), and the fact that mutations in this region cause obvious structural abnormalities (Axenfeld- Rieger anomaly/syndrome, Peters), may be this finding is to be expected. The rest of the *PITX2* coding region has been sequenced only partially and therefore a final statement about sequencing analysis cannot be made in this case. The reasons for sequencing not being complete have been discussed in chapter 6.4.3.1 and it will not be reiterated here.

It could be argued, that if variations in the sequence which cause subtle structural changes were investigated, the search should have concentrated on the intron-exon boundaries and the UTR region of the gene. With hindsight, may be this would have been a better strategy, however, I think that the main coding region had to be screened regardless. Also, most of the UTR region was covered by the primer set 4. Unfortunately, this is the primer set the sequencing analysis was least successful in this study.

6.5.3.2 High throughput screening results discussion

When this study was set up (2001), the hypothesis allowed for a fairly large effect caused by a sequence variation. Since then, technological advances (high throughput screening methods, development of internet SNPs database) and better understanding of genetics of complex diseases, allowed the scientists to understand that most likely a smaller effect to the phenotypic expression is caused by any given sequence variation.

The advantages of using SNPs for association studies have been explained in the introduction to the study design (chapter 6.1.2).Therefore, it was a logical step to take this study further, and screen the SNPs published in the internet database for an association with POAG/OHT in this cohort of patients and controls.

As mentioned above, the fact that sequencing analysis did not work well in all samples and primer sets and technological advances during the duration of the study, allowed for further genetic studies in the form of high throughput SNPs screening using the Sequenom. The selection of the SNPs from the internet databases has been presented in the chapter 6.4.4.1. As, it is obvious from the table 6.7, there is a large number of SNPs selected and screened. Most likely, a smaller number of SNPs would have been enough to be screened, to get robust information for statistical analysis and this is explained next.

When this study has been started, The HapMap project was in its beginnings and the tagSNPs program (www.hapmap.org) was not available. As the pace of technological progress in this area is very fast, I became aware of it after doing the SNP analysis. As mentioned previously, SNPs are a newest generation of marker to locate genes in DNA sequences. The problem with using SNPs as markers is their abundance (10 million common SNPs), which makes a direct association study unfeasible. Therefore, it is important to find biologically significant SNPs, as most have no effect. At present, only a few representative SNPs on the chromosomes of interest are genotyped. This is done in the hope 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, as genetic variants that are near each other tend to be inherited together. This is called haplotype tagging and relies on sufficiently representative set of tagging SNPs covering region of

genome under study (figure 7.6). The number of tag SNPs that are informative of the remaining SNPs is estimated to be about 300,000 to 600,000 which is much less than 10 million common SNPs. R^2 is a measure of LD and $r^2 = 1$ can be described as complete LD because allelic association is as strong as possible. Genotypes at SNPs which are < 10kb apart tend to be correlated. A relatively stringent threshold of > 0.8 would allow a selected tagSNPs to improve correlation and resolve >80% of all existing haplotype.

Therefore, the program has been used to identify if any of the *PITX2* SNPs selected previously would be picked up. Indeed, 5 SNPs (out of 26 selected) were picked up by the program, suggesting that the SNP selection and analysis of the *PITX2* gene is robust. The SNPs selected are: rs2739202, rs2278782, rs2595110, rs976568 and rs1982361.

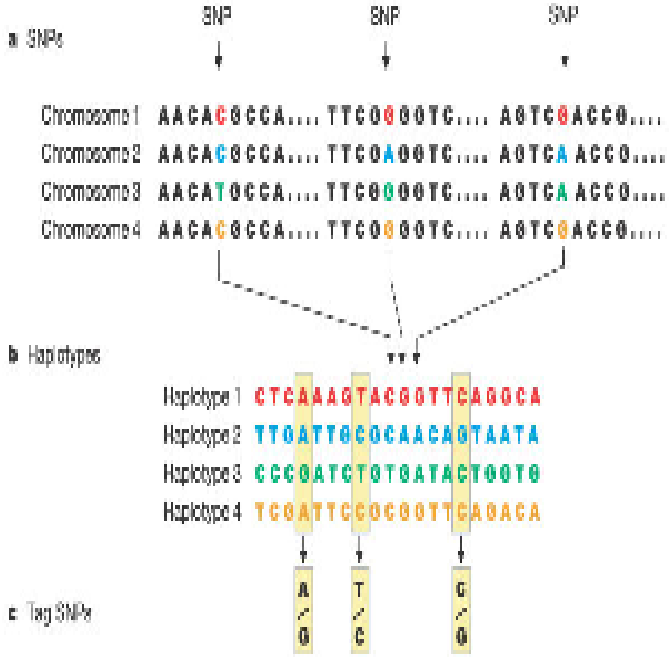


Figure 6.16 (a) SNPs are identified in DNA samples from multiple individuals. (b) Adjacent SNPs that are inherited together are compiled into "haplotypes." (c) "Tag" SNPs within haplotypes are chosen that uniquely identify those haplotypes (Diagram obtained from the HapMap Project – http//www.hapmap.org/)

6.5.4 Assessment of study design and data quality

How can case-control genetic association studies be evaluated? The following criteria are suggested by Silverman and Palmer (2000) - table 7.5.

Table 6.16: Evaluation of candidate gene case-control association studies (adapted from Silverman and Palmer, 2000)

Issue	Key questions	Possible solutions
Selection of candidate gene polymorphism	Is candidate gene biologically reasonable?	Demonstration of biologically functional effect
	Is the candidate gene a positional candidate?	Within linked region in man or syntenic from animal model
Population stratification	Are cases and control matched?	Matching on ethnicity
		Family based association designs
		Negative results with multiple unlinked markers
Hardy-Weinberg (H-W) equilibrium	Is control group in H-W equilibrium?	Calculation of H-W equilibrium with goodness of fit test (2 alleles) or simulation (multiple alleles)
Multiple comparisons	How many alleles were tested?	Bonferroni correction
	How many genetic loci were tested?	Estimation of empirical p values

So, how does the current study evaluate against the above criteria?

6.5.4.1 Selection of candidate gene polymorphism

It has been demonstrated above why developmental glaucoma genes are good candidate genes to be studied for susceptibility to POAG The role of *PITX2* in anterior segment morphogenesis and its function has been extensibly described both under Genetics of developmental glaucoma and *PITX2* gene chapters as well as in this chapter. 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, adding further support to the biological plausibility of these genes as candidate genes for POAG

6.5.4.2 Population stratification

The cases and controls have been carefully matched for age and ethnicity, so type I error is not a concern with this study (type I error occurs when the population sampled consists of several sub-populations that do not intermix and can lead to spurious false positives results, if the cases and controls are not well matched for ancestry and if disease rates and marker allele frequencies are different between ancestral populations).

The patients and controls enrolled in this study are of Caucasian origin and no significant population migration occurred in the North-East of England. It could be argued that this cohort is a sub-population of glaucoma patients, due to the strict inclusion criteria of IOP at presentation > 30 mm Hg. However, I have presented the reason why this particular IOP value has been chosen. If a positive association would have been demonstrated in this group, then the cohort would have to be enlarged and possibly patients with glaucoma with IOP < 30 mm Hg at presentation included in the study, to ascertain if the results can be reproduced.

6.5.4.3 Is control group in H-W equilibrium?

The control group is in H-W equilibrium for all the markers tested and the calculation is presented in table 7.2. SNPs which were not in H-W equilibrium have been discarded (SNP rs1982361). Also, as described above, the selection of markers is robust, allowing for a good coverage of the gene and resolving most of the haplotypes.

6.5.4.4 Statistical considerations

As first stage of the study was to sequence the coding region of the *PITX2* gene and identify any polymorphism, determine the frequency in the controls and then test for association with POAG, it was considered that a cohort of 100 POAG/OHT patients and 100 matched controls would be sufficient for the purpose.

To have an association with the disease, a polymorphism would have to have a frequency of at least > 1% in the general population, or if the frequency is < than 1% in the general population, than the effect of the polymorphism has to be quite large for an association with the disease to be detected.

This is known as common disease, common variant (CD/CV) hypothesis, which questions why these diseases are so common and why are they maintained in the population at such high frequency. This hypothesis states, “the genetic risk for common diseases will often be due to disease-producing alleles found at relatively high frequencies (>1%)”-Becker 2004. The CD/CV hypothesis addresses the question of why these disorders are so common; because the underlying disease influencing alleles are common. Polymorphisms in the *APOE* gene in the context of late onset Alzheimer’s disease have been suggested as prototype for this hypothesis, due to high population frequency of *APOE4* allele. Late onset Alzheimer’s disease is a common disorder

affecting up to 60% of the population after the age of 75. APOE4 is present in 15% of the population and is found in up to 50% of individuals affected with late onset Alzheimer's (Cargill and Daley 2000).

Considering that the frequency of a particular allele in general population (controls) is 10%, for a cohort of 100 POAG/OHT patients and 100 controls, the study would have 80% power to detect a 10 to 26% difference between the groups and 92.5% power to detect a difference of 10 to 30 % difference between the groups, significance level of 0.05.

However, for this study, only 60 controls were enrolled. Therefore, assuming the same 10% frequency in general population, this study has 80% power to detect 10 to 30% difference between the groups, (p=0.05).

It has to be mentioned that choosing to do calculations for an estimated SNP frequency of 10% is may be being too optimistic about the findings and also, not doing my background research regarding SNPs frequencies adequately. More realistic is to consider a 1% frequency as being quite a high value for the context of complex diseases.

So, if the calculation is redone for an assumed allele frequency in the general population of 1%, then this study has 80% power to detect a difference from 1 to 11,5 % and 99% power to detect a difference from 1 to 22%, (p=0.05).

From the above calculations it is evident that the study has enough power to detect a large effect, even if the frequency of the polymorphism in general population is 1%. However, as mentioned, in this cohort no association was found between *PITX2* polymorphism and POAG/OHT.

It also has to be mentioned of 26 alleles chosen for this study from the published database only 10 had a frequency published, of which the highest was 0.494%. If any of these alleles would have shown an association that would indicate a very large effect. So, it may be isn't a surprise that no association was shown for this size cohort and could be a good idea to repeat this study in a much larger cohort.

6.5.4.5 Adjustment for multiple testing

The incidence of false positive error is proportional to the number of significance tests performed and multiple testing can lead to a potential false positive error. This has been addressed in the algorithm of the program used for analysis and also by using two different programs to do the same analysis (SHEsis and UNPHASED).

6.6 LIMITATIONS OF THE PILOT STUDY

6.6.1 Size of the cohort

The main limitation of this study is the size of the cohort, which, despite being adequate to produce reliable results could include more samples. However, this would have not been feasible within the time framework of this project.

When this project was set up, the initial aim was to genotype the whole coding sequence, screen for SNPs and then calculate the frequency in the control and patient group and look for association with the disease. The sample size was thought to be appropriate for this aim, and it was demonstrated above that the study has enough power to detect a large effect from the gene variation, should one be present.

After founding that no variation was present in the coding region and due to the development of the SNP database and rapid technological advances of high throughput genotyping during the course of the project, it become possible to perform SNP screening of the gene of inters in this cohort.

Statistical work regarding genetic association case-control studies has received a lot of attention in last five years. If genotype frequencies would be known a priori, then standard statistical methods and software could be used to calculate power (Ambrosius 2004).

Although a lot of statistical work has been done for family-based tests of association, there was not much work about unrelated case control genetic association studies. This received more attention when positive findings in the initial study could not be

replicated in subsequent studies. In their review of "Genetic association studies", Cordell and Clayton (2005) emphasize that the standards of statistical proof that become acceptable in the general biomedical literature are not appropriate for genetic association studies and they suggest that Bayesian methods might be more appropriate. This might be resolved with the implementation of microarray studies.

For this study, delay with getting ethical committee approval for control enrolment, further hindered the number of controls enrolled, and the initial objective of 100 controls has not been achieved. Work at the Institute of Child Health, UCL has addressed issues of cohort size and sub-group stratification for both cases and controls and an association between POAG and LMX1B1 has been demonstrated (Park et al, 2009).

6.6.2 Visual field recording

Another limiting factor for the study is the lack of objective assessment of the severity of Humphrey visual field using global indices (mean deviation, pattern standard deviation, corrected pattern standard deviation). On the plus side, all the visual fields tests showed reproducible fields 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 satisfactory reliability score of <20% fixation loss, false positive of < 33% and/or false negative of <33%.

6.6.3 Central corneal thickness

The third limiting factor of this study is the absence of CCT measurements. CCT variation is known to affect the accuracy of IOP readings on applanation tonometry, with thick corneas giving falsely high IOP readings and thin cornea falsely low readings (Feltgen et al, 2001, Whitacre et al, 1993). In the current study the IOP measurements were checked by a Tono-Pen which is less affected by CCT (Bahn et al 2002) in addition to performing applanation tonometry. Although nomograms, 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). Two recent studies that adjusted IOP for CCT found that the correction did not alter the diagnosis of high tension glaucoma or normal tension glaucoma (Miyazawa et al 2007),

and did not affect the relationship between the prevalence of POAG and IOP respectively (Francis et al 2008). Even if a correction formula 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 change would equate to a 100-150 μm change in the CCT, which is a considerable amount. Therefore, it is highly unlikely that the individuals with IOPs of 20 mmHg or below or IOPs of 24mmHg 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), these subjects would require having either abnormally thin corneas or 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 from PITX2 mutation (Asai-Coakwell et al 2006).

CCT data was not collected for the cases and controls in this study, as a pachymeter was not available at the time when this study was carried out. However, the POAG/OHT patients enrolled in this study have an IOP > 30mmHg (mean 34.9 ± 6.9), so even allowing for IOP overestimation, the IOP would still be very high. However, the role of IOP in POAG/OHT and the interactions between the IOP and genetic factors needs more clarification.

6.6.4 C/D ratio recording

A fourth limiting factor of the study is the lack of objective measurement of the C/D ratio as a measure for the severity of the disease, as this can be quite subjective, depending on the observer. Subsequent to the collection data for this work, newer imaging methods of the optic nerve have been developed, in the form of Optical Coherence Tomography and GDx nerve fibre analyser test. These are superior diagnostic tools, which will allow earlier detection of structural damage, and will provide more objective data for research purposes. However, the patients included in this study 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.

6.7 FUTURE WORK PERTAINING TO THE PILOT STUDY

1. Enlarging sample size for patients and controls as well as identifying and recruiting for genetic studies patients with strong family history of glaucoma

2. Set objective criteria for phenotype description

2. Extending the panel of ASD genes to be screened in POAG patients

3. Any demonstrated association should be reproduced in an independent cohort

4. If an association is demonstrated, then the future aim will be to isolate the causative variant(s). This can be achieved by identifying potentially functional genetic variations at the locus researched by sequencing all the exons, splice sites and conserved non-coding sequences in the cohort studied.

5. Functional analysis of any coding 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. Secondly, in order to assess whether non-coding variants are associated with altered expression levels of mRNA and protein levels of the gene investigated and downstream target genes, cadaver tissue and other appropriate cell lines can be analyzed. 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 analyzed 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.

6.8 CONCLUSION

Accurate standardization of the POAG/OHT cohort is very important, however still not 100% objective. Despite the fact that the diagnosis of POAG /OHT on cases was definite, the effect of genetic factors on the progress of the disease on the existing cohort cannot be studied.

The definition for POAG should be ideally based on objective criteria and not only on clinical examination. Digital photographs of the optic discs, together with imaging reports of sophisticated instruments such as the nerve fibre analyser or the OCT, could be assessed by experts in reference centres, to assure firm and unbiased diagnosis.

Automated perimetry examination must be uniform, performed by the same observer on a reliable machine, such as the Humphrey analyser, using the same software programme. This will limit the inter-observer and intra-observer variability, especially on parameters such as the C/D ratio, which is proven to be a very "soft" diagnostic and research tool.

To note is that the cohort studied is solid, from the same population of the North East of England, with strict documentation and quality POAG/OHT and control subjects, carefully examined by a Glaucoma Specialist Consultant Ophthalmologist vast experience in the field. The laboratory and statistical analysis were rigorous, allowing accurate results. Reproducibility remains an issue, denoting the necessity for subsequent work that needs to be undertaken in order to unravel the enigma of the POAG aetiology and which for this study has been addressed, by continuing the work on a cohort of 400 patients and 400 controls at the ICH, UCL, London.

As mentioned above, this work has now been concluded and it demonstrated an association between *LMX1B* and glaucoma (Park et al 2009). This is the first study to demonstrate an association between a gene involved in anterior segment dysgeneses and glaucoma.

Chapter 7:
IDENTIFICATION, PHENOTYPING AND RECRUITMENT FOR GENETIC STUDIES OF POAG PATIENTS WITH STRONG FAMILY HISTORY OF GLAUCOMA

7.1 INTRODUCTION

It is generally accepted that a family history of glaucoma increases the risk of the disease, with a life risk of glaucoma of 9.2 times higher in siblings and offspring of glaucoma patients than in siblings and offspring of controls (Hulsman et al 2002).

Identifying a family history of glaucoma is important for screening programmes, as it would appear that familial glaucoma is more aggressive and has a younger age of onset than sporadic glaucoma (Wu et al 2006) and therefore a screening programme will be more successful if it is developed with appropriate weighting towards those with a positive family history of the disease.

Recruiting families (ideally large pedigrees with living relatives in different generations) with multiple members affected of a disease is important for genetic studies, as it allows mapping the disease loci by linkage analysis. Linkage analysis methods attempt to identify a region of the genome that is transmitted within families with the disease phenotype of interest and has been extremely useful in the identification of genes responsible for diseases with simple Mendelian inheritance.

So far, 14 POAG loci have been mapped by linkage analysis and genome wide scanning in families with autosomal dominant mode of inheritance and age dependent penetrance.

Ideally, when recruiting families, one would like to have a large number of affected members; a figure of 11 or more is considered to be ideal for conducting linkage analysis.

The patient population at Sunderland Eye Infirmary (SEI) has not had the opportunity to take part in these studies. Collaboration between Mr Scott Fraser at SEI, the Genetics Department at St George's Hospital, London and Professor Sarfarazi, USA has been set up with a view of identifying glaucoma pedigrees and recruiting them for genetic studies.

Part of my project was to continue with recruitment of POAG patients with family history of glaucoma from North East of England, construct the pedigrees, liaise with relatives of the proband, collect samples and forward the samples and clinical information to London. From London, the samples were sent to Professor Sarfarazi laboratory for the genetic studies.

In the next chapter, some of the pedigrees identified from glaucoma patients in North-East of England and the phenotyping details are presented, together with an update on the genetic work carried out on these patients so far.

7.2 IDENTIFICATION OF PATIENTS WITH FAMILY HISTORY OF GLAUCOMA, RECRUITING TO THE STUDY AND PHENOTYPING

The protocol for the glaucoma family history study was as follows:

- Identification of the patients with glaucoma who had a family history of the disease from the patients who attended out-patient clinics or the nurse led glaucoma review clinics
- Suitable patients (at least two family members affected with glaucoma) to be provided with a study information sheet (appendix 1), by the consultant or the nurse
- Either the nurse or the doctor will discuss the study with the patient
- If the patient communicates willingness to assist, the details were passed on to the Research Fellow, to arrange an appointment for recruitment to the study
- Upon receipt of the patient details, the patient was contacted and an appointment was organised with myself to:

1. discuss the study
2. obtain written consent for taking part in the study
3. construct the pedigree and obtain as much information as possible about the relatives
4. perform a clinical examination
5. collect a sample of venous blood

- Upon receipt of information from the proband about relatives who are interested in taking part in the study, the relatives were contacted and appointments for clinical examination and sample collection was organised
- Where the relatives were unable to come to the hospital, the blood collection was organised to the GP practice or at home
- If clinical examination could not be done, information was requested from the optician, with consent from the family member involved or from ophthalmologists in the cases of affected relatives
- The blood samples were sent to London, and were forwarded on to Professor Sarfarazi for genetic studies
- Any result specific to an individual will be relayed from laboratory to Mr Fraser for genetic counselling purposes

The examination protocol was standardised and it comprised:

- Best corrected Snellen visual acuity assessment
- Anterior segment examination with slit lamp
- Goldman applanation tonometry
- Gonioscopy
- Fundus examination and optic disc assessment with a 90D condensing lens
- All the probands and individuals with definite POAG diagnosis had at least two reproducible visual field tests in the immediate past (Humphrey automated perimetry, 24-2). Also, all the glaucoma patients recruited from the SEI had optic discs photographs
- For the relatives participating in the study and unaffected, due to staff shortage and financial constraints, visual fields were not carried out. However, for the majority, copies of the visual fields were obtained from the optician

Details about other systemic and ocular conditions and glaucoma treatment as well as age at diagnosis were also recorded. The study has local ethical committee approval and the GP's of the individuals participating in the study were kept informed. Figure 7.1 A and B presents flow charts for recruitment of patients and relatives to the study.

POAG diagnostic criteria were:

- Characteristic glaucomatous cupping of optic disc
- Characteristic glaucomatous visual field defect, consistent with optic disc appearance
- open angles on gonioscopy (grade 3/4 Shaffer classification)

If 1 or 2 are present, with open angles on gonioscopy, depending on IOP values patients were diagnosed with either high tension glaucoma (IOP > 21 mmHg) or normal tension glaucoma (IOP < 21 mmHg).
OHT diagnostic criteria:

1. IOP > 21 mmHg
2. non-glaucomatous appearance of optic disc
3. normal visual filed
4. open angles on gonioscopy (grade 3/4 Shaffer classification)

Open angle glaucoma suspect diagnostic criteria:

1. IOP < 22mmHg
2. suspicious optic disc appearance (cup disc ratio >0.5)
3. asymmetry of cup/disc ratio of > 0.2 between the 2 eyes
4. normal visual field
5. open angles on gonioscopy

No corrections were made for corneal thickness, because this information was not routinely collected at the time of the patient ascertainment.

Figure 7.1A: Flow chart demonstrating the patient recruitment to the genetic studies in the family history study

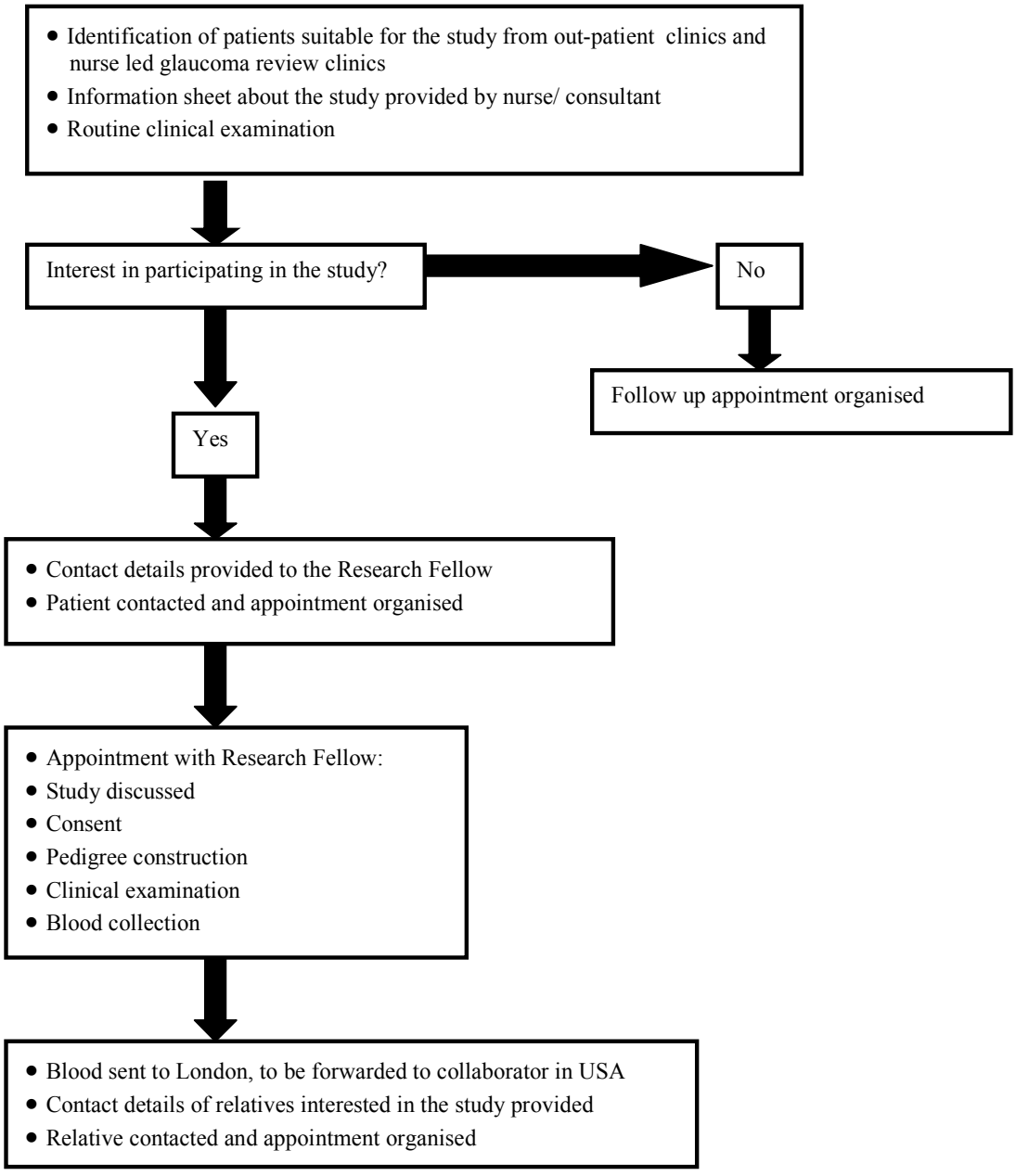
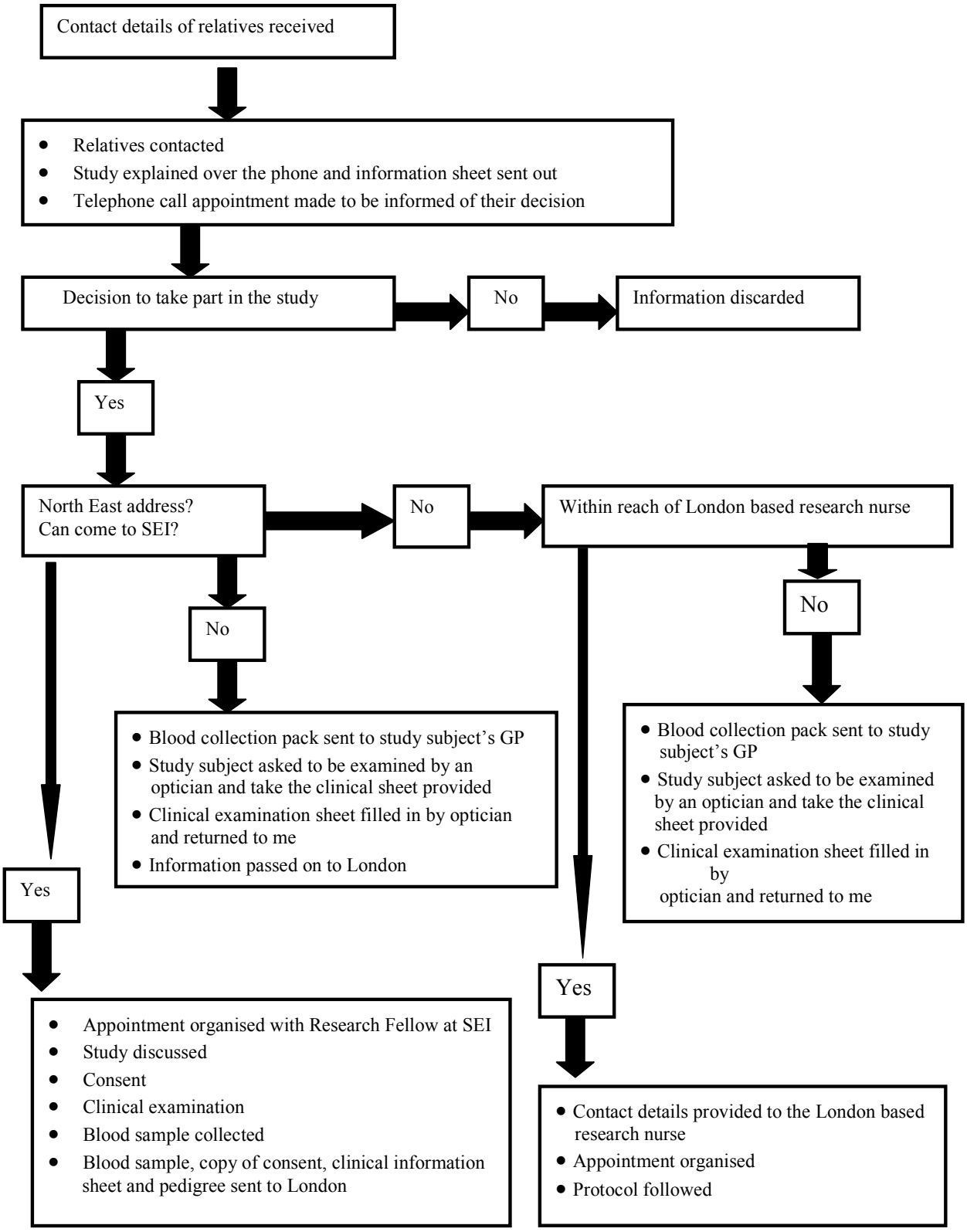


Figure 7.1B: Flow chart demonstrating relatives' recruitment to genetic studies in the family history study



The recruitment of patients for the study took place between October 2001 and October 2002. During this period, 33 patients with strong family history (at least 2 members affected) of glaucoma were identified. Of these, 22 agreed to take part in the study. Eight patients couldn't get any of the relatives to take part in the study, or most of the affected relatives were dead. However, a blood sample has been collected from them and the pedigree was constructed.

13 of the pedigrees and the clinical details of the participants are presented in this chapter. Most of the pedigrees present an autosomal dominant inheritance, with variable penetrance. Apart from pedigrees GL1718/SEI 11 and GL1803/SEI 12, in which the age at diagnosis is lower than 40 years of age (JOAG), all the other have age at diagnosis over 40 years of age. Also, it appears that the IOP at presentation in majority of the affected subjects is high, frequently over 30 mm Hg.

There are few pedigrees in which the type of inheritance is uncertain GL 1200/SEI2, GL 1199/SEI3, GL 1424/SEI4, GL 1719/SEI 7 and GL 1449/SEI9). The proband in the pedigree GL 1424/SEI4, has both parents affected, and unless relatives from both sides of the family are taking part in the study, it is difficult to know from which parent the gene is inherited. In this case, the parents could not be recruited for the study, but some maternal relatives, one of which has glaucoma accepted to take part. This situation has been highlighted by Sack et al (1996) in the Glaucoma Inheritance Study in Tasmania (GIST).

The GL 1719/SEI 7 pedigree is the only one with the diagnosis of normal tension glaucoma in this cohort.

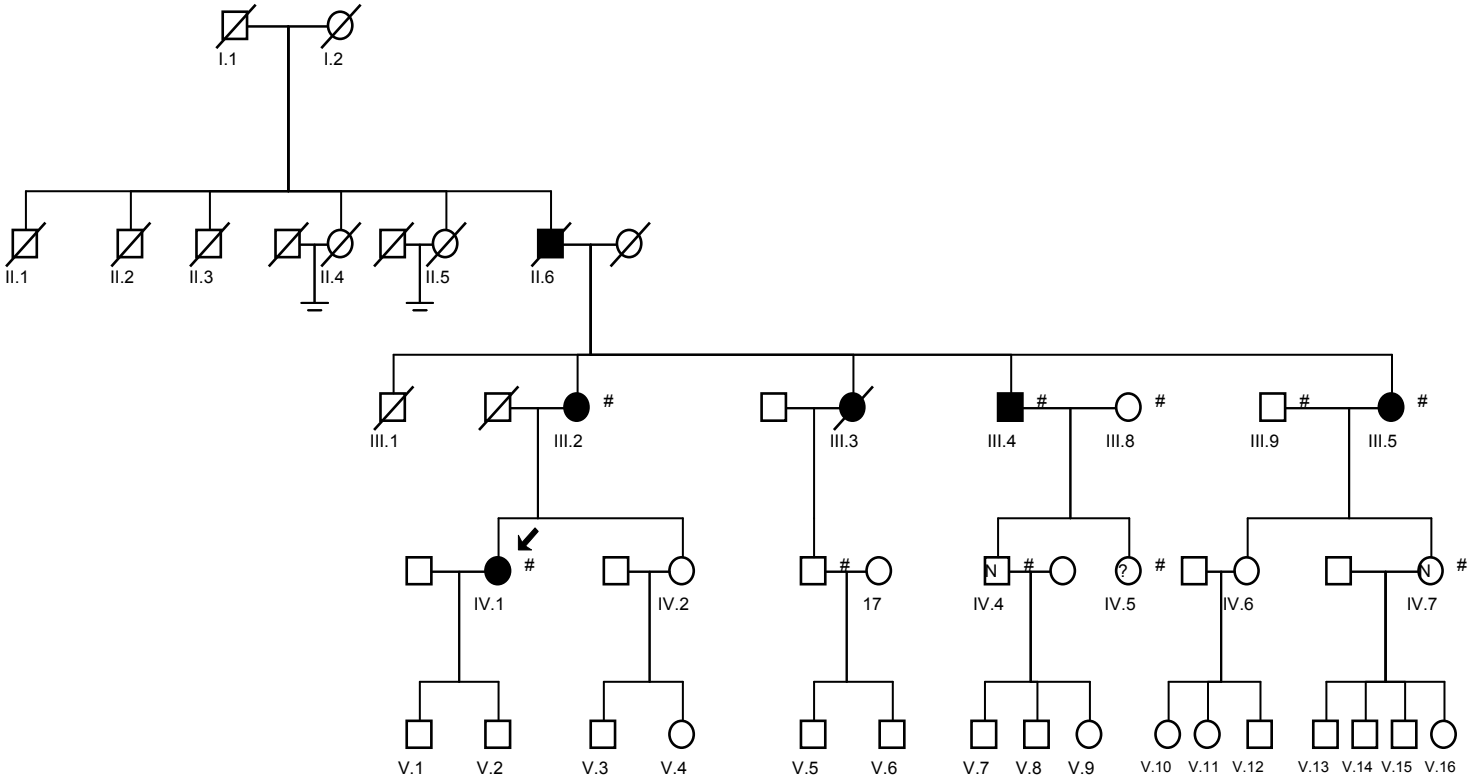
For the pedigrees with the adult onset OAG (SEI 1-6), the IOP at presentation for the individuals diagnosed with glaucoma was 31.5 ±10.2 mm Hg and the CD ratio was 0.68 ± 0.166. The mean age at diagnosis was 61.27 ± 12.9 (range 42 to 81). Of the 11 individuals diagnosed with glaucoma in the 7 pedigrees, 4 were males and 7 females. It is interesting to observe that the presenting IOP in the affected individuals is very high (> 30 mm Hg).

Also 2 pedigrees (GL 1423/SEI 1 and GL 1200/SEI 2) present family members with both glaucoma and OHT. Presumably, the individuals with OHT were diagnosed in the

light of the family history, rather by chance alone. It is likely that a proportion of these patients will develop glaucoma, however, because they are know to have OHT, the early signs of glaucoma, will hopefully be observed in time and adequate action taken which in turn will slow the progression of the disease. This emphasises the importance of family history in diagnosing and management of glaucoma.

7.3 PEDIGREES AND PHENOTYPES

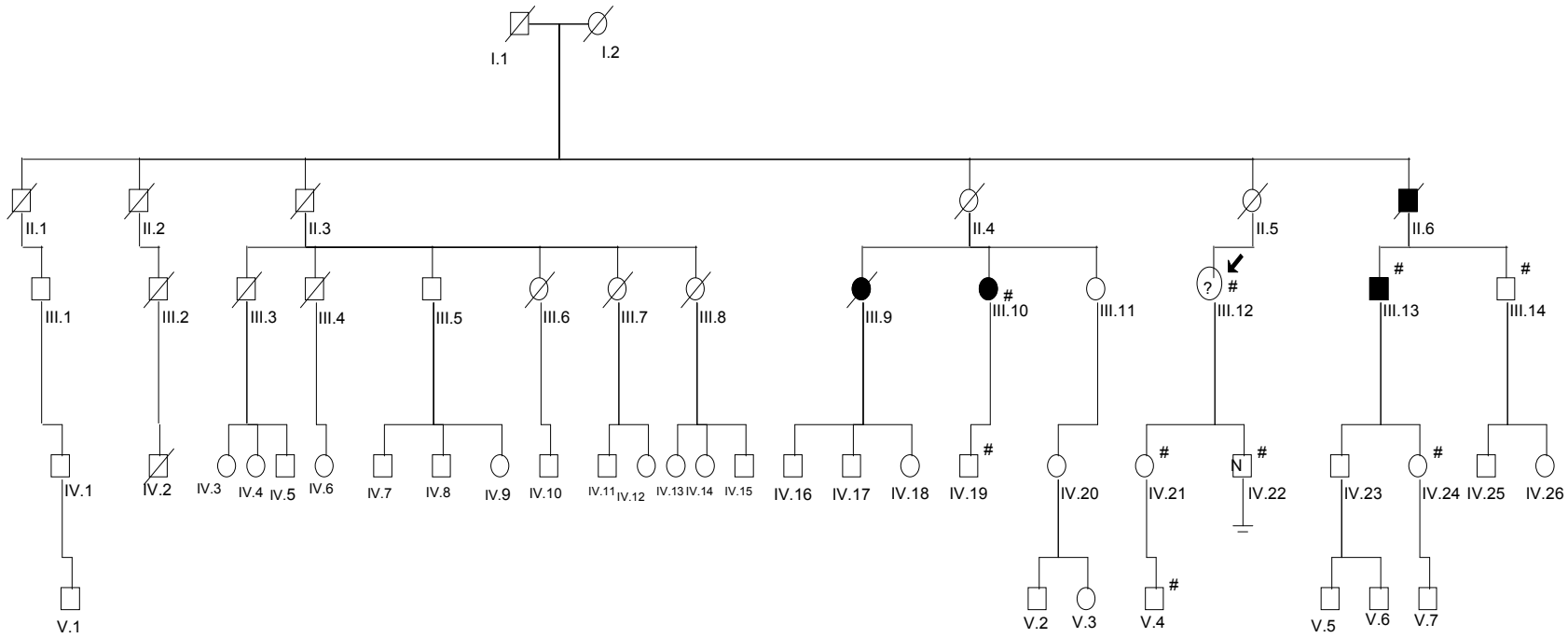
GL 1423/ SEI 1



GL 1423/SEI 1: autosomal dominant															
ID no	Pedigree ID	Sex	Age	Age at diagnosis	VF RE	VF LE	CD ratio RE	CD ratio LE	VA RE	VA LE	Highest IOP RE	Highest IOP LE	Therapy RE	Therapy LE	Diagnosis
1	III.4	M	71	58	N/A	N/A	0.7	0.7			32	35	Trb	Trb	POAG
2	III.8	F	71	n/a									n/a	n/a	Spouse III.4
3	IV.4	M	47	n/a	N/A	N/A	0.5	0.5			18	18	nil	nil	Unaffected
4	IV.5	F	42	41	Inf-temporal loss	Nil	0.4	0.5	6/5	6/5	25	21	Med	Med	POAG
5	IV.7	F	44	n/a	N/A	N/A	0.15	0.15	6/6	6/6	21	21	nil	nil	Unaffected
6	III.2	F	79	70	Superior arcuate	Superior + inferior arcuate	0.5	0.45	6/18	6/9	23	22	Med	Med	POAG
7	IV.3	M	41	n/a	Nil	Nil	0.2	0.2	6/5	6/5	19	19	nil	nil	unaffected
8	III.9	M	69	n/a				.					n/a	n/a	Spouse III.5
9	III.5	F	65	50	Nil	Nil	0.3	0.4	6/6	6/6	NK	NK	Trb	Trb	OHT
10	IV.1	F	52	52	Nil	Nil	0.3	0.3	6/5	6/5	29	21	Med	Med	OHT

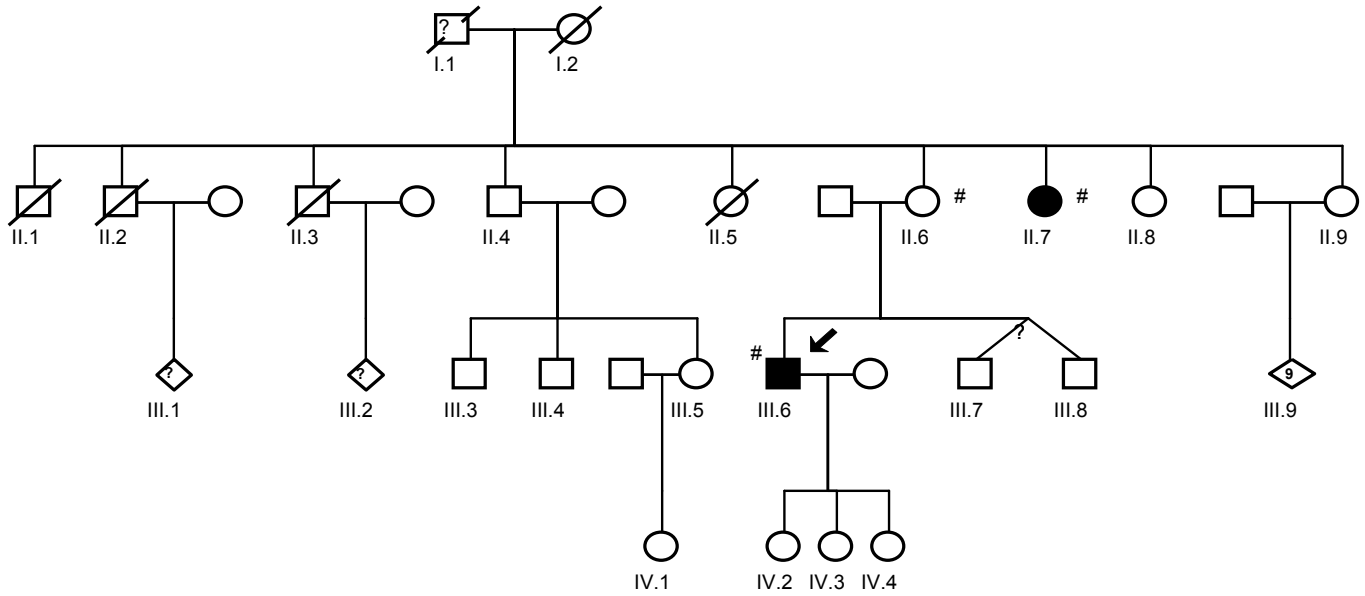
Legend of abbreviations: VF = visual field; RE = right eye; LE = left eye; BE = both eyes; VA = visual acuity; sup = superior; inf = inferior; conc = concentric; periph = peripheral; Med = medical; Trb = trabeculectomy; ALT = argon laser trabeculoplasty; n/a = not applicable; NK = not known; MMC =mitomycin C

GL 1200/ SEI 2


I.1
I.2
II.1
II.2
II.3
II.4
II.5
II.6
III.1
III.2
III.3
III.4
III.5
III.6
III.7
III.8
III.9
III.10
III.11
III.12
III.13
III.14
IV.1
IV.2
IV.3
IV.4
IV.5
IV.6
IV.7
IV.8
IV.9
IV.10
IV.11
IV.12
IV.13
IV.14
IV.15
IV.16
IV.17
IV.18
IV.19
IV.20
IV.21
IV.22
IV.23
IV.24
IV.25
IV.26
V.1
V.2
V.3
V.4
V.5
V.6
V.7
?
N
#

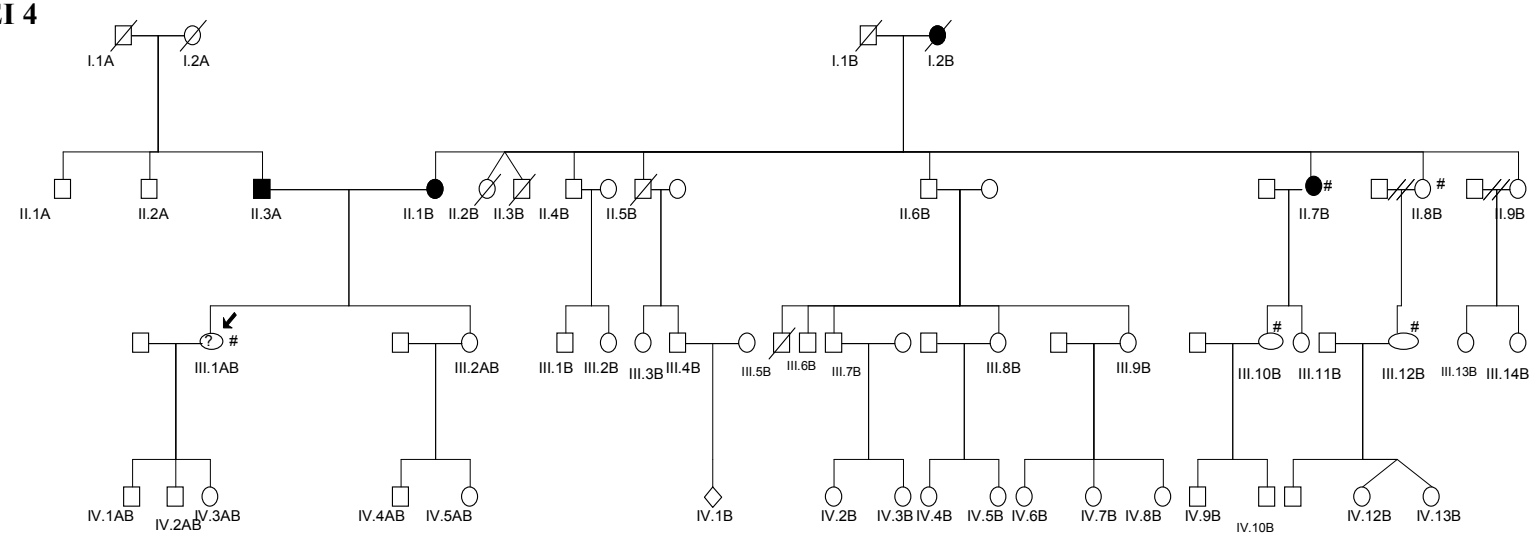
GL 1200/SEI 2: inheritance uncertain															
ID	Pedigree ID	Sex	Age	Age Dg	VA RE	VA LE	VF RE	VF LE	CD RE	CD LE	IOP RE	IOP LE	Therapy RE	Therapy LE	Diagnosis
1	III.12	F	69	68	6/6	6/7.5	NIL	NIL	0.6	0.6	19	17			Suspect
2	IV.21	F	48		6/6	6/6	NIL	Nil	0.3	0.3	18	18			Unaffected
3	V.4	M	20		6/5	6/5	NIL	Nil	0.3	0.3	19	19			Unaffected
4	IV.19	M	46		6/6	6/6	Not done	not done	0.2	0.2	20	20			Unaffected
5	III.13	M	64	47	6/5	6/5	Sup + inf nasal step	Sup + inf nasal step	0.75	0.8	23	28	Trb+ MMC	Trb + Med	POAG
6	III.14	M	62	60	6/4	6/4	NIL	Nil	0.2	0.2	28	28			OHT
7	III.10	F	74	68	6/6	6/36	Sup + inf arcuate scotoma	Sup loss	0.7	0.7	25	27	Med	Med	POAG
8	IV.24		31		6/5	6/5	NIL	Nil	0.2	0.2	20	21			Unaffected
9	IV.22	M	47		6/18	6/4	not done	not done	0.1	0.1	18	17			Unaffected

GL 1199/ SEI 3



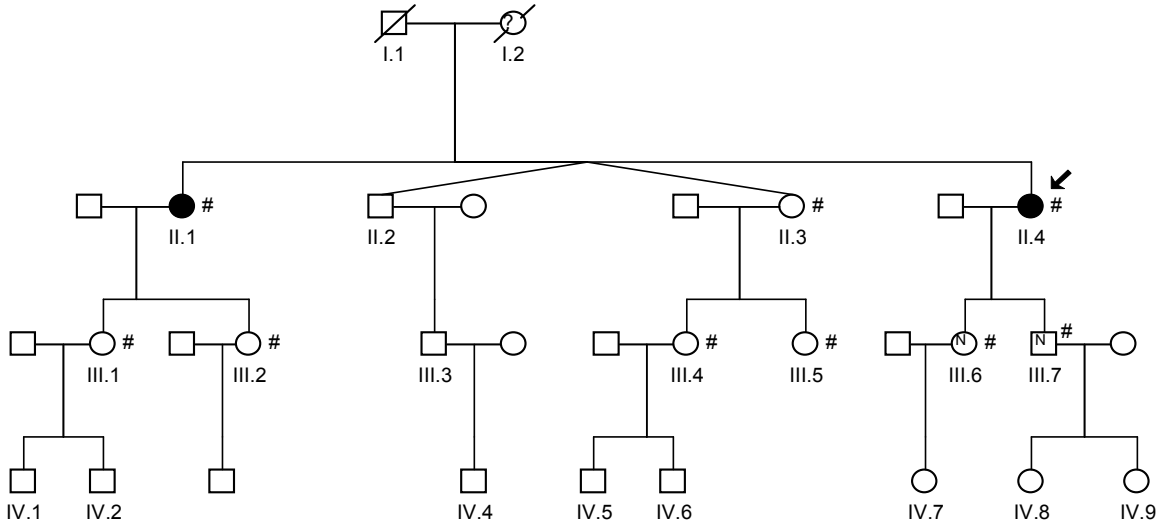
GL 1199/SEI 3:inheritance uncertain															
ID	Pedigree ID	SEX	AGE	AGE Dg	VA RE	VA LE	VF RE	VF LE	CD RE	CD LE	IOP RE	IOP LE	Therapy RE	Therapy LE	Diagnosis
1	II.6	F	68		6/6	6/6	NIL	NIL	0.2	0.4	18	17			Unaffected
2	II.7	F	71	61	6/9	6/12	Concentric peripheral loss	Sup arcuate	0.9	0.7	56	42	Trb	Trb	POAG
3	III.6	M	43	42	6/6	6/12	NIL	Sup arcuate	0.5	0.9	22	46	Med	Med + Trb	POAG

GL 1424/ SEI 4



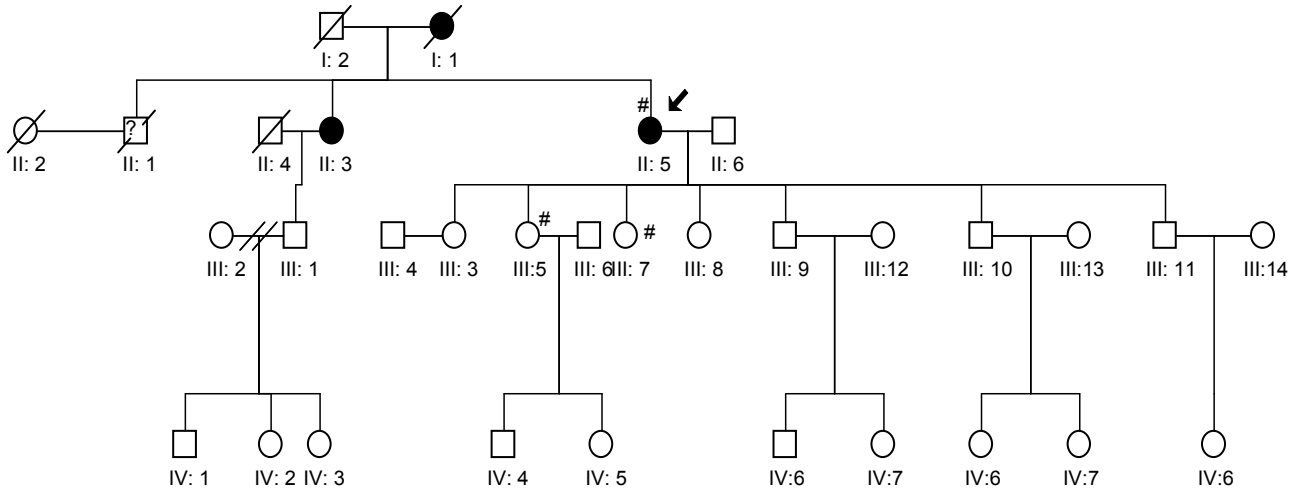
GL 1424/SEI 4: inheritance uncertain															
ID	Pedigree ID	Sex	Age	Age Dg	VA RE	VA LE	VF RE	VF LE	CD RE	CD LE	IOP RE	IOP LE	Therapy RE	Therapy LE	Diagnosis
1	III.11B	F	44		6/5	6/5	nil	nil	0.2	0.2	16	16			unaffected
2	II.7B	F	75	68	6/9	6/12	sup arcuate	nil	0.6	0.5	32	24	Med	Med	POAG
3	III.10B	F	46		6/5	6/5	nil	nil	0.4	0.3	20	20			unaffected
4	II.8B	F	66		6/6	6/6	nil	nil	0.4	0.4	18	18			Unaffected
5	III.12B	F	40		6/4	6/4	nil	nil	0.2	0.2	17	17			unaffected
6	III.1AB/P	F	41	40	6/6	6/6	nil	nil	0.6	0.6	20	21	nil	nil	suspect

GL 1143/ SEI 5



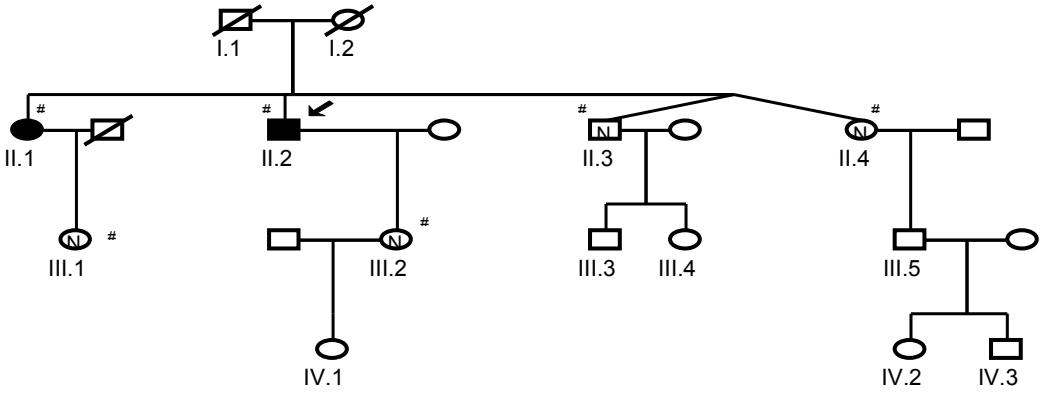
GL 1143/SEI 5: autosomal dominant															
ID	Pedigree ID	Sex	Age	Age Dg	VA RE	VA LE	VF RE	VF LE	CD RE	CD LE	IOP RE	IOP LE	Therapy RE	Therapy LE	Diagnosis
1	II.4	F	71	69	6/6	6/12	nil	paracentral scotoma	0.4	0.4	25	25	Med	Med	POAG
2	III.6	F	45	n/a	6/6	6/5	nil	nil	0.1	0.1	20	20	nil	n/a	Unaffected
3	III.7	M	41	n/a	6/5	6/6	nil	nil	0.1	0.1	18	20	nil	n/a	Unaffected

GL 1427/ SEI 6



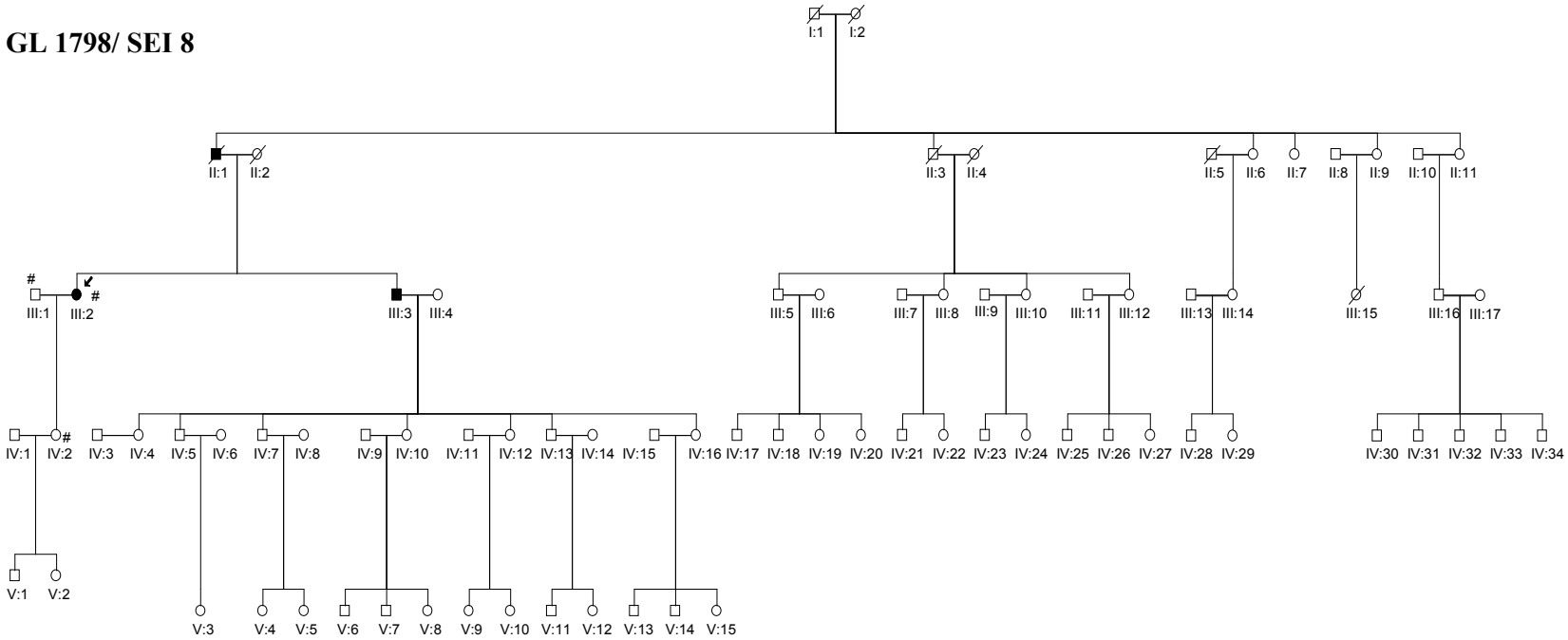
GL 1427/SEI 6: autosomal dominant															
ID	Pedigree ID	Sex	Age	Age Dg	VA RE	VA LE	VF RE	VF LE	CD RE	CD LE	IOP RE	IOP LE	Therapy RE	Therapy LE	Diagnosis
1	III.7	F	40		6/5	6/5	nil	nil	0.2	0.2	15	15			Unaffected
2	III.5	F	43		6/6	6/6	nil	nil	0.3	0.3	20	20			Unaffected
3	II.5	F	69	69	6/6	6/5	early nasal step	early nasal step	0.6	0.7	21	27	Med	Med	POAG

GL 1719/ SEI 7



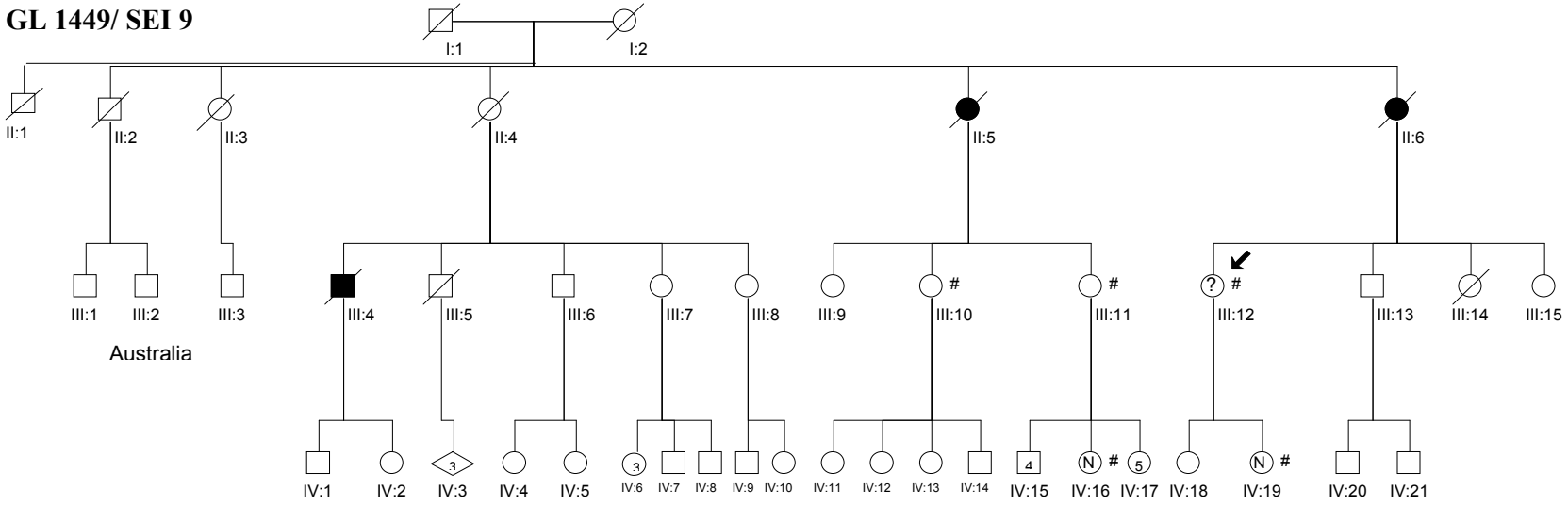
GL 1719/SEI 7: autosomal dominant															
ID	Pedigree ID	Sex	Age	Age Dg	VA RE	VA LE	VF RE	VF LE	CD RE	CD LE	IOP RE	IOP LE	Therapy RE	Therapy LE	Diagnosis
1	II.2	M	74	72	6/6	6/6	inf arcuate	sup arcuate	0.6	0.7	20	20	Med	Med	NTG
2	II.1	F	87	75	6/9	6/9	Nasal step	nil	0.7	0.5	21	19	Med	Med	NTG
3	II.3	M	68		6/6	6/6	nil	nil	0.3	0.4	18	20			Unaffected
4	II.4	F	68		6/6	6/6		not done	0.2	0.2	20	18			Unaffected
5	III.1	F	53		6/6	6/6		nil	0.4	0.5	16	17			Unaffected
6	III.2	F	49		6/6	6/6		not done	0.2	0.3	20	20			Unaffected

GL 1798/ SEI 8



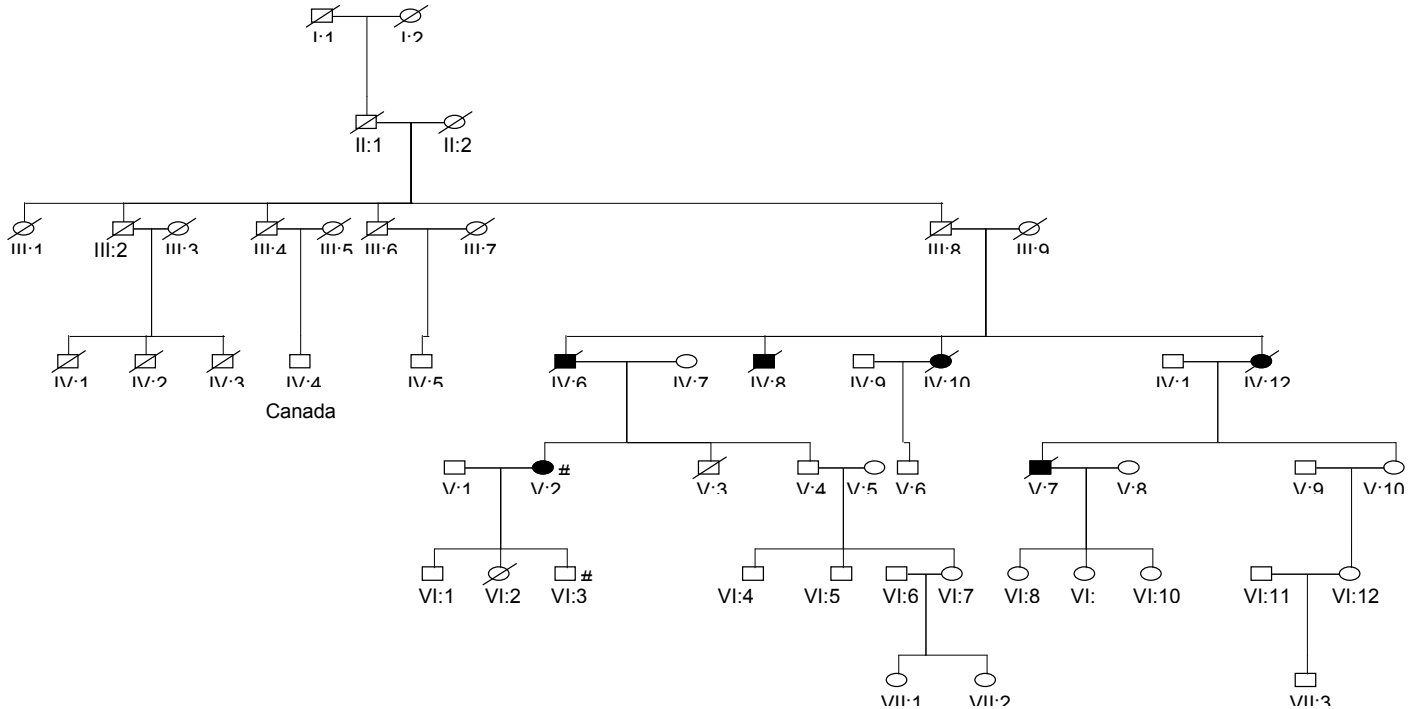
GL 1798/SEI 8: autosomal dominant															
ID	Pedigree ID	Sex	Age	Age Dg	VA RE	VA LE	VF RE	VF LE	CD RE	CD LE	IOP RE	IOP LE	Therapy RE	Therapy LE	Diagnosis
1	III.2	F	66	61	6/6	6/6	nil	nil	0.7	0.6	26	20	Med	Med	OHT
2	III.1	M	72		6/5	6/5	nil	nil	0.2	0.2	14	16			Unaffected
3	IV.2	F	44		6/5	6/5	nil	nil	0.1	0.1	21	19			Unaffected

GL 1449/ SEI 9



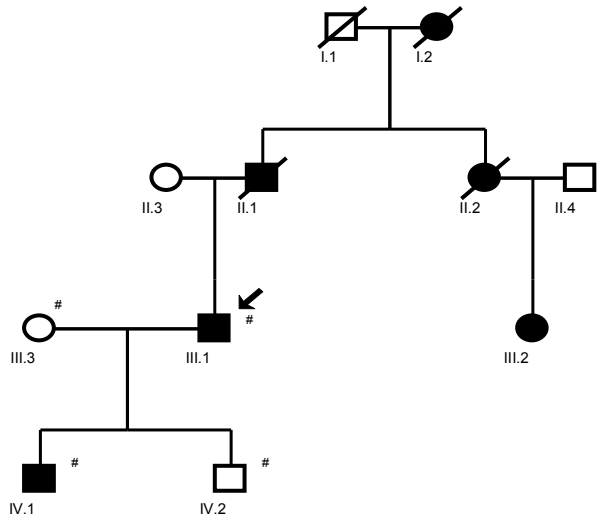
GL 1449/SEI 9: inheritance uncertain															
ID	Pedigree ID	Sex	Age	Age Dg	VA RE	VA LE	VF RE	VF LE	CD RE	CD LE	IOP RE	IOP LE	Therapy RE	Therapy LE	Diagnosis
1	IV.16	F	37		6/5	6/4	not done		0.2	0.2	18	L17			Unaffected
2	III.10	F	75		6/6	6/6	nil		0.3	0.3	20	19			Unaffected
3	III.11	F	69		6/6	6/6	not done		0.2	0.2	23	23			unaffected
4	IV.19	F	40		6/5	6/5	not done		0.2	0.2	15	15			unaffected
5	III.12	F	61	61	6/6	6/9	nil		0.2	0.3	24	21	nil	nil	OHT

GL 1450/ SEI 10



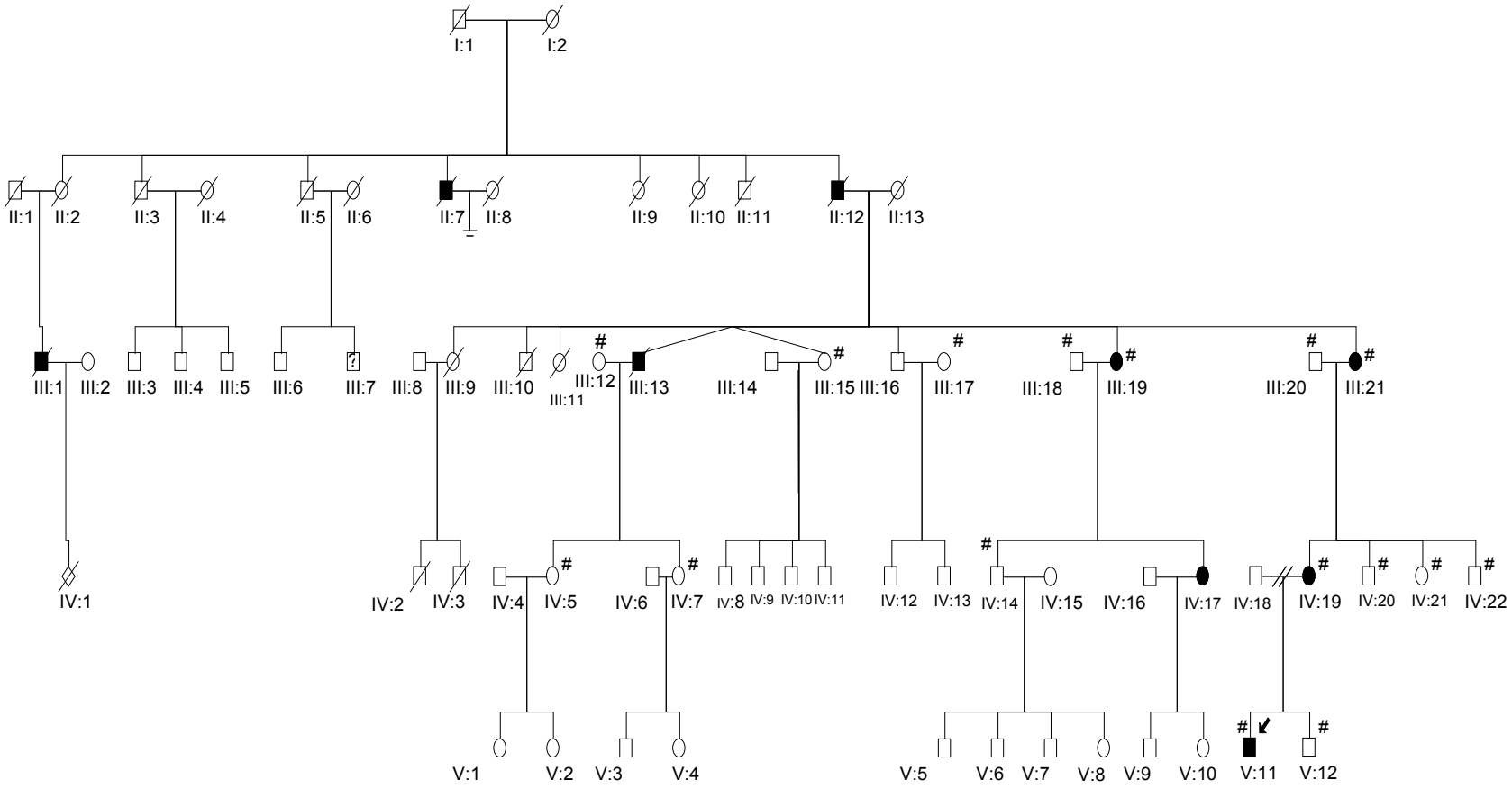
GL 1450/SEI 10: autosomal dominant															
ID	Pedigree ID	Sex	Age	Age Dg	VA RE	VA LE	VF RE	VF LE	CD RE	CD LE	IOP RE	IOP LE	Therapy RE	Therapy LE	Diagnosis
1	V.2	F	71	70	6/6	6/6	nil	nil	0.6	0.6	25	23	nil	nil	OHT
2	VI.3	M	45		6/4	6/5	nil	nil	0.3	0.2	19	22			Unaffected

GL 1718/ SEI 11



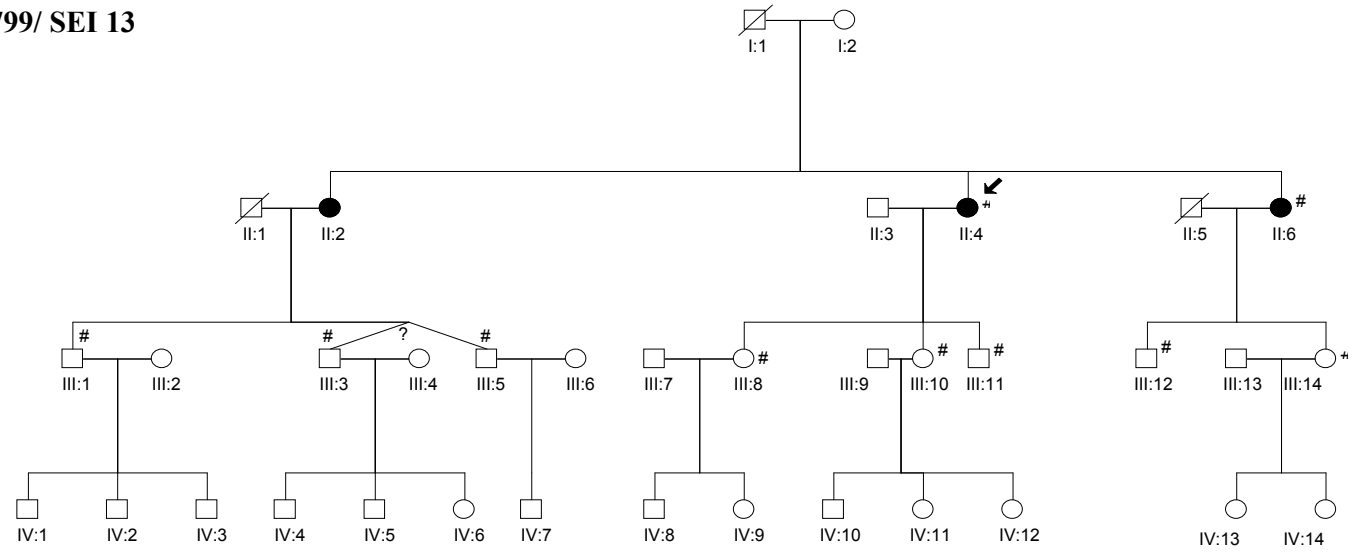
GL 1718/SEI 11: autosomal dominant															
ID	Pedigree ID	Sex	Age	Age dg	VA RE	VA LE	IOP RE	IOP LE	VF RE	VF LE	CD RE	CD LE	Therapy RE	Therapy LE	Diagnosis
1	III.1	M	61	38	6/6	6/6	39	35	inf nasal	inf arcuate	0.7	0.9	Trb	Trb+ Med	POAG
2	IV.1	M	36	35	6/60	6/5	48	43	v.poor	upper partial arcuate +nasal step	0.9	0.6	Med/ refused surgery	Med	POAG
3	IV.2	M	31	N/A	6/5	6/5	20	19	Nil	Nil	0.1	0.1	N/A		Unaffected
4	III.3	F	60										N/A		Spouse III.1

GL 1803/ SEI 12



GL 1803/SEI 12: autosomal dominant														
ID	pedigree ID	Sex	Age	Age at diagnosis	VF RE	VF LE	CD RE	CD LE	VA RE	VA LE	Highest IOP RE	Highest IOP LE	Therapy	Diagnosis
1	V.11	M	26	26	Arcuate sup	Nil	0.6	0.5	6/5	6/6	25	23	Med	POAG
2	IV.19	F	50	39	Borderline	Nil	0 .5	0.4	6/5	6/5	26	22	Med +Trb +ALT RE	POAG
3	III.21	F	76	51	Arcuate sup	Nasal step	0.6	0.8	6/9	9/9	25	26	Med	POAG
4	III.20	M	79	n/a	Nil	Nil	0.2	0.2	6/9	6/9	20	21	n/a	Unaffected
5	III.19	F	75	66	Total loss	Advanced loss	0.99	0.9	HM	6/9	25	35	Med	POAG
6	IV.14	M	46	n/a	Nil	Nil	0.2	0.2	6/5	CF	21	21	n/a	Unaffected
7	III.18	F	81	n/a	Nil	Nil	0.1	0.1	6/9	6/9	14	13	nil	Spouse III.13, POAG/ deceased
8	IV.5	F	46	n/a	Nil	Nil	0.3	0.3.	6/9	6/5	27	24	n/a	OHT

GL 1799/ SEI 13



GL 1799/SEI 13: autosomal dominant														
ID	pedigree ID	Sex	Age	Age at diagnosis	VF RE	VF LE	CD ratio RE	CD ratio LE	VA RE	VA LE	Therapy	Highest IOP RE	Highest IOP LE	Diagnosis
1	II.4	F	74	74	Sup + inf scotoma	Early inf nasal loss	0.8	0.6	6/6	6/6	Med	26	26	POAG
2	III.8	F	52	n/a	Nil	Nil	Disc drusen	Disc drusen			n/a			Unaffected
3	III.11	M	38	n/a	Nil	Nil	0.3	0.3	6/6	6/6	n/a	17	15	Unaffected
4	II.6	F	70	66	Nasal step	Nil	0.4	0.5	6/9	6/6	Med	28	24	POAG
5	III.14	F	45	n/a	Nil	Nil	0.3	0.3	6/6	6/6	n/a	18	18	Unaffected
6	II.3	M		n/a	Nil	Nil	0.1	0.1	6/5	6/5	n/a	15	15	Unaffected

7.4 GENETIC SCREENING RESULTS OF THE FAMILY HISTORY STUDY PATIENTS

Our collaborator, Professor Sarfarazi, has communicated some available information regarding the screening of the patients from the above families.

All SEI pedigrees have been screened for mutations in MYOC and WDR36, and no mutations have been found in these patients.

M98K variation in OTN (10p13) was identified in GL 1143/SEI 5 and GL 1719/SEI7. This is a risk associated DNA variation and not a disease causing mutation. This is probably to be expected for the pedigree GL 1719/SEI 7 as the diagnosis is NTG, however, for the GL 1142/SEI 5 pedigree the highest IOP in the proband was 25 mm Hg.

The GLC1D locus on chromosome 8q23 was screened in the following pedigrees: GL 1143/SEI 5, 1200/SEI2, 1423/SEI 1, 1719/SEI 7, 1803/ SEI 12 but no concluding results have been obtained as yet. The work is still in progress.

For the GLC1B locus on 2cen-q13 region and another unpublished locus on 2p25, the following candidate genes (NCK2, SLC5A7, SOX11, BX089860, TSSC1, MTCBP-1) have been screened in the above families, but no conclusive mutations have been identified.

The GL 1423/SEI 1 and GL 1803/ SEI 12 have been screened for GLC1N locus on chromosome 15q22.1-q25.1, but no conclusion was reached.

A number of single affected subjects from the above panel of families have been selected for a new genome-wide association study (using SNP markers), but the results will not be available for another year. Unrelated affected subjects were randomly selected from the families and included in a total of 750 unrelated POAG subjects for this particular study.

Also, these families were included in a classical genome –wide screening study (using short tandem repeat polymorphisms –STRP - markers) that consisted of mainly the affected sibs.

However, due to the small size of the families and the limited number of affected sibs per family, many hits have been observed all over the genome. These regions have been systematically checked, and some extremely promising genes were sequenced, but so far all is proven non-conclusive and the work is still continuing.

It is evident from the above information that so far no conclusive findings were made, despite intensive research. One of the reasons is that the size of the families is small and also the number of affected sibs per family, available for the study is small too. At the same time, it also, emphasizes the challenges posed to the genetic researchers into complex diseases and the importance of the clinician in identifying as large as possible pedigrees with affected relatives who are alive.

7.5 DISCUSSION

7.5.1 Identifying POAG pedigrees

As mentioned previously, it is generally accepted that POAG has a strong genetic component. Family history is important clinically because the risk for POAG among first degree relatives of a POAG patient is 7-10 times higher than that of the general population (Tielsch et al 1991, Wilson et al 1987) and surveillance targeting these individuals is indicated for early detection and treatment for POAG.

As early as 1949, Possner and Schlossman discuss the difficulties of pedigrees description for adult onset POAG in their excellent paper “Role of inheritance in glaucoma”. They observe that while pedigrees for JOAG and combination of POAG and JOAG can trace glaucoma back in six generations; this is not the case for pedigrees with only POAG, where there are very few pedigrees which have been followed for even 3 generations. This is explained by the difficulty in following hereditary disease later in life and also by the fact that some patients with potential glaucoma do not live long enough to reach the age at which they would have the disease. Of course, this poses the question whether there is any real difference between JOAG and POAG, as the main criteria for separating these entities is an arbitrarily chosen age of onset of the disease. However, the age of onset of the disease is difficult to pinpoint as the disease can run for many years with no symptoms, and

therefore what is actually used in classifying these diseases is the age at diagnosis. Also, it has been shown that the mutation in GLC1A gene is responsible for causing both JOAG and POAG (Morissette et al 1995, Vincent et al 2002), suggesting that these two entities are a continuum of the same disease.

It is also important to note that due to the low penetrance of the disease it is possible that some sporadic cases are hereditary. To elicit that would necessitate genetic investigation and it becomes obvious that this would be very cost ineffective to be done on a large scale.

Gong et al (2007) suggest a new classification of POAG according to the degree of familial aggregation into inherited, familial and sporadic categories. Inherited POAG is defined when three or more relatives inclusive of the proband are documented with POAG in two or more consecutive generations, one of which has to be a first degree relative of the other two. Familial POAG involves two or more first and/or second degree relatives and does not meet the criteria for inherited POAG. Sporadic POAG involves a single patient without affected first or second degree relatives. According to the authors, these classes of POAG appear to be distinct not only in inheritance pattern and the degree of familial aggregation but also in methodology and outcome in mapping POAG.

Identification of large glaucoma pedigrees has been instrumental in discovery of glaucoma loci and genes. For most cases, the pedigrees described are for JOAG, and it helped identify most of the glaucoma loci (GLC1A-M), apart from 2 loci – GLC1B and E, which were discovered in NTG phenotype pedigrees (table 3.1). The methods used were positional cloning (GLC1A to I), genome wide screening (GLC1J and K) and Markov chain Monte Carlo multipoint estimations of identity by descent sharing and allele-sharing methods for GLC1L. Since the discovery of first POAG locus on chromosome 1, many more pedigrees, either JOAG or POAG have been described and patients screened for mutations in the known glaucoma genes.

7.5.1.1 Identification of POAG pedigrees from SEI patients

Patients included in this study were referred from two sources: the glaucoma consultants (Mr Fraser and Mr Phelan) and the nurses from nurse led glaucoma clinic was one source and referral from consultants with other subspecialty interest was the second source.

In order to make clinical staff aware of this study, a presentation at in house teaching and postgraduate regional teaching about the study was made by both Mr Fraser and Dr Vaideanu. The inclusion criteria were particularly emphasised and an appeal for everyone to help with referral was made. The first source provided a steady stream of referrals; however, the second source provided only sporadic referrals. One reason for that could be that the consultants with other subspecialty interest do not see quite as many glaucoma patients. However, to make sure they are reminded about the study, I have placed typed reminders in all clinical rooms. These were placed on the wall, above the desk, at eye level.

Also, the study has been presented at two of the 3 monthly Glaucoma Support Group meeting, again by Mr Fraser and Dr Vaideanu. The aim of these presentations was to make patients aware of the risk of having family history of glaucoma, present the findings of genetic studies so far and encourage them to agree to take part in the study if requested.

The nurses from the glaucoma unit at SEI had an important role in helping with referrals and organising appointments. Therefore, I provided them with a timetable for organising appointments.

As mentioned above, 33 pedigrees with at least 2 family members affected were identified during the recruiting period. However, only 22 patients agreed to take part in the study and only 13 patients managed to get the relatives to take part. These pedigrees are presented in this thesis.

According to Gong et al (2007) classification, the pedigrees GL 1423 (SEI 1), 1200 (SEI 2), 1424 (SEI4), 1427 (SEI6), 1798 (SEI 8), 1449 (SEI 9), 1450 (SEI10), 1718 (SEI11), 1803 (SEI12) are inherited and pedigrees GL 1199 (SEI 3), 1143 (SEI 5), 1719 (SEI 7), 1799 (SEI 13) are familial. Pedigree 1143 (SEI 5) could possibly be inherited, however the

information we have is incomplete and for the time being makes it familial. This situation is highlighted in the Gong et al paper, emphasising that some of the pedigrees which appear familial due to lack of information may actually be inherited.

7.5.2 Pedigree construction

The relatively late age of onset of most forms of POAG has complicated the efforts to identify the mode of inheritance and the genes involved, but segregation analysis in several adult onset pedigrees has shown autosomal dominant inheritance to be the predominant mode.

Therefore, the pedigrees published in literature fall in three categories:

- those in which all the affected members have juvenile glaucoma
- those in which, owing to anticipation, the parents and grandparents have adult glaucoma, while in succeeding generations the affected members have juvenile glaucoma
- those in which all the affected members have adult glaucoma

As mentioned above, most of the pedigrees published in literature demonstrate an autosomal dominant mode of inheritance for POAG. Regarding the role of autosomal recessive mode of inheritance in POAG, there is not sufficient data available to justify any conclusion, although it has been suggested that the autosomal recessive may be predominant (Booth 1997). In recessive heredity, the gene may become widespread in the population without manifesting the disease except may be as a result of chance mating of carriers, and that may be one explanation for the appearance of some of the sporadic cases. So far, there is one paper, by Yoon et al (1999) describing a Korean case of apparent autosomal recessive inheritance of JOAG.

Most of the pedigrees identified from patients attending SEI and presented in this chapter appear to have an autosomal dominant mode of inheritance and therefore similar to the published pedigrees. However, the SEI pedigrees are of relatively small size and only in few we have managed to phenotype and collect samples in more than 5 members (SEI 1, 2,

4, 7, 9, 12 and 13). Also, the pedigrees identified from the North East population only have 2 or 3 affected members, who are still alive; the only pedigree with 5 members affected alive being GL1803/SEI 12. Still, 5 of this group of pedigrees have at least 5or 6 members affected, with pedigree GL 1803 (SEI 12) having 9 member affected. Unfortunately, for the SEI pedigrees the clinical information from the deceased POAG patients is no longer available.

As discussed above, large pedigrees (at least 11 members affected) which exhibit Mendelian inheritance patterns can help in location of the genes associated with glaucoma, by using the standard linkage analysis.

Although most of the pedigrees which helped in discovering the POAG/JOAG genes were very large, mutations in these genes, as well as identification of new glaucoma loci has been achieved with the help of smaller pedigrees (Wiggs et al 2004, Mackey et all 2003, Bruttini et al 2003, Forsman et al 2003). Also, a known mutation in a small pedigree can help characterize the phenotype associated with the specific mutation (Mackay et al 2003). For that reason, collecting pedigrees, even with only 3 members affected, it still provides a valuable resource for genetic research.

However, the common types of glaucoma, including POAG, typically do not exhibit Mendelian inheritance patterns. Wiggs (2007) emphasises that the common age related ocular disorders do have a significant heritability, but the genetic contributions to these disorders are complex, resulting from interaction of multiple factors, and are susceptible to the influence of environmental exposures.

7.5.2.1 Pedigree construction in SEI POAG patients

Once the POAG patients with family history of glaucoma were identified and agreed to take part in the study, constructing the pedigree did not pose too much of a challenge. All the participants were very helpful at providing the information they knew. The patients with relatives, who did not want to take part in the study, were very prompt at informing me about the situation. A great deal of effort was made by some patients to find out

information about relatives who lived away (different part of the country, different country or continent) to help provide as complete a picture as possible.

Invaluable help in both constructing the phenotype and contacting and collecting blood samples from relatives living in South East of England was offered by my liaison in London, Glen Brice (genetics research nurse at St George's Hospital, London). He also, forwarded the clinical information and blood sample to Professor Sarfarazi.

Communication with him was very important from logistic point of view (timing of sending the samples, storage, and feedback about other members of the family recruited to the study).

The blood samples collected, had to be stored between few hours to a day before being sent to London. A fridge in the glaucoma unit which was being used for storing medication has been agreed as temporary storage, after discussion with all relevant people.

7.5.3 Phenotyping

Without sufficient knowledge of clinical aspects of the disease of interest (phenotype definition), genetic studies would be useless. Different forms and sub-phenotypes of glaucoma have been recognised, which helps with genetic studies. Within the sub-phenotypes of POAG, different clinical manifestations such as myopia, disc haemorrhage, reduced corneal thickness might represent different genetic bases (Gong et al 2004).

Rapid advances in genetics, provides us with the tools to look at a disease from "inside out", by detecting the underlying genetic defect first and then their pathophysiological consequences. However, this approach is still dependable on accurate identification of the disease phenotype. Therefore, the role of the clinician is invaluable in inquiring about the family history in all patients, identifying individuals and families with a genetic trait, constructing the pedigree and then liaising with an investigator/laboratory that is performing or would be willing to perform molecular genetic studies.

It is generally accepted that in the future, genetic testing will become common and that the clinician will be likely to obtain a genomic profile on an individual and determine the risk of that person for developing simple Mendelian or complex inherited diseases. Identification of at risk individuals will make primary prevention possible for the first time. And while for complex diseases, the underlying genetic causes are still being unravelled, the identification of pedigrees and phenotyping of the patients remains very important in providing data for the genetic studies.

The clinician has a crucial role in the success of all genetic linkage studies. Constructing the clinical features that define the affected status of a disease for linkage studies has to be done carefully and consistently by all clinicians involved in a study. Objective criteria for affected, suspect and unaffected must be clearly defined, this resulting in as homogeneous a phenotype as possible, therefore simplifying linkage analysis.

Kroese and Burton (2003) discuss the problems provided by the lack of a consensus case definition for POAG, emphasising that there is considerable uncertainty about what is diagnosed and treated as POAG. This impact on the person diagnosed with POAG, on the commissioning and provision of clinical healthcare services, and on a firm basis for research into the condition.

7.5.3.1 Phenotyping of the proband and relatives in SEI pedigrees

The proband in the pedigrees collected from SEI patients, have been diagnosed by Consultants Ophthalmologist with vast experience in the field. Also, in most of the other pedigree members affected by glaucoma and included in the study, we have collected objective clinical information (visual fields, optic disc appearance, IOP values) which supports the diagnosis of glaucoma.

For the unaffected subjects, only IOP, C/D ratio and anterior chamber angle details were collected. Some of them, had visual fields at the optician, and where possible we collected these. Otherwise, due to financial and time constraints, visual fields were not routinely done on unaffected members.

7.5.4 Genetic studies

In the majority of POAG cases it is likely that more than one genetic predisposition is required to manifest disease and it is now generally accepted that POAG is a complex trait (Hewitt et al 2006).

As mentioned earlier, investigating the genetics of pedigrees with late age of onset is difficult. The parents of POAG cases are often deceased (the case with many of SEI pedigrees), while the patients' children are frequently too young to manifest the disease. Adding to the confusion, POAG can be discordant in time, differing in age of onset for some related cases and there is often considerable overlap between glaucoma families (Sack et al 1996). Despite these issues, large glaucoma pedigrees have been genetically linked and numerous loci have been identified (table 3.1).

Once the disease has been linked to a locus and a gene has been identified, the gene can be sequenced and mutations identified. It is critical to ensure that a mutation is real (not just a polymorphism) by demonstrating it cosegragates with affected individuals and is not present in unaffected individuals and in population controls.

The next step is to investigate the effect of the mutation on the protein, by expression studies and by using animal models. Once this has been understood, it may lead to understanding the pathogenesis of the disease and then to developing diagnostic tests and possibly gene/drug therapies with the final result of either disease prevention or cure (Damji and Allingham 1997).

Once a mutation in a gene causing the disease has been identified, clinical information about the onset of the disease, course of the disease and response to therapy need to be collected (Wiggs 2007).

The genotype-phenotype studies will answer the following questions:

- What is the range of phenotypic variation of a given mutation (can one predict the prognosis of the disease if the mutation is known?)
- Are certain mutations associated with particular aspects of the disease phenotype?

- Are certain mutations necessary, but not sufficient to cause the disease? Such a mutation would require other additional genetic defects or environmental factors to be fully manifest.

By going back and analysing the clinical findings in a particular group of patients where a mutation has been identified, we can try and ascertain if there are certain characteristics of the group (i.e. higher IOP at presentation, certain characteristics of the CD ratio, etc). One such example is the study by Mackey et al (2003), where glaucoma phenotype in pedigrees with Myocillin Thr377Met mutation was analysed. The authors concluded, that this mutation is associated with POAG that, in the pedigrees studied, had a younger age at onset and higher peak intraocular pressure than in pedigrees with the more common Gln368STOP mutation, and that this group was more likely to have undergone glaucoma drainage surgery. This emphasises, that certain mutation produces more aggressive disease, and therefore, identifying and monitoring at risk individuals might catch the disease early and prevent morbidity.

Factors which contribute to the challenge of discovering complex disease genes are the underlying molecular heterogeneity, imprecise definition of phenotypes, inadequately powered study designs and the inability of standard sets of microsatellite markers to extract complete information about inheritance.

Because genetic models cannot be defined, methods to identify genes responsible for these conditions are more complex than those used for Mendelian disorders. Genome scans and model free analysis have been performed using families demonstrating clustering of complex diseases (largely sibpairs), as well as families affected with rare forms showing apparent Mendelian inheritance (Wiggs 2007).

One method to address genetic heterogeneity and strengthen linkage findings is to incorporate phenotypic sub-setting of the data. Most phenotypic stratification approaches require that subsets be identified before linkage studies. An alternative to traditional stratification approaches for incorporating a trait related covariate is ordered subset analysis. This method provides maximum evidence of linkage, by using the covariate without a priori specification of families to a subset. With this approach, families are

ordered by a phenotypic variable (e.g., age of onset, cup/disc ratio, IOP). Linkage analysis with a specific marker set is performed on the first family in the list and then repeated, adding one family each time. In this way, the subset of the families with greatest evidence of linkage can be determined. Subsets with evidence of increased linkage can be used for candidate gene analysis. This approach has been successfully used to confirm and narrow area of proposed linkage, as well as to define novel region of linkage in several complex traits, including diabetes, Alzheimer disease, macular degeneration (Allingham et al 2005).

Adult onset glaucoma frequently occurs in multiple family members (familial aggregation), but does not usually follow a clear Mendelian inheritance pattern, suggesting that inherited risk factors can result in a susceptibility to the disease, but is not necessarily causative. Multiple risk factors and/or environmental factors may be responsible for this disease in older individuals.

Although 14 loci have been identified for POAG, so far only three genes have been discovered (*MYOC, OTN, WDR36)* and each of these genes is only responsible for a small fraction of cases with POAG, reflecting the small percentage of POAG inherited as a Mendelian trait rather than a complex trait.

In the North East of England POAG family history cohort, none of the patients had a disease causing mutation in the above genes and only a risk association DNA variation was identified in 2 pedigrees (GL 1143/SEI 5 and GL 1719/SEI 7).

7.5.5 From bench to slit lamp

Translating the genetic research into clinical settings still has large gaps. Glaucoma is a model disease to investigate, as genetic knowledge of the disease may be allowing the treating ophthalmologist the ability to detect and treat a disease with potentially blinding complications.

Mapping, cloning and identification of novel mutations involved in the aetiology of glaucoma provide a significant opportunity for pre-symptomatic diagnosis, improved prognosis and better understanding of the aetiology of this blinding condition.

The development of genotype – phenotype databases for glaucoma genes will be an important step toward clinically useful DNA-based diagnostic testing for glaucoma.

However, before achieving this goal, more research into unravelling the pathogenesis of glaucoma is necessary. The current state suggests that multi-staged design that encompasses data from linkage, association and molecular experiments when feasible is to be desired. In future, ocular genetic studies may rely on multicenter and community collaboration for mapping experiments (Iyengar SK 2007).

Before incorporating genetic tests into the diagnosis algorithm, it is important to determine the significance of a disease variant. Understanding the sensitivity spectrum, prevalence and penetrance of gene sequence changes ensures that patients can be adequately informed of the likely implications of carrying such a gene. For all this to be achieved community based longitudinal studies, which incorporate both molecular and environmental components are required.

Hewitt et al (2006) suggest a cascade screening protocol, where once a POAG patient is identified as having a disease causing gene mutation, all of their first degree relatives can be tested for the same mutation. If they carry the mutation, then they are followed closely for early clinical signs of glaucoma and their first degree relatives are also tested.

This process moves on in a stepwise direction from the index case until all relatives harbouring the mutation have been identified (this process has been successfully applied in cancer genetics). The perceptions of family members involved in cascade screening with MYOC glaucoma in a large Australian pedigree have been evaluated and found them to be positive (Healey et al 2004).

With regards to this study, identification of POAG pedigrees, as large as possible and accurate phenotyping with the methods available at the time of patient enrolment represents a very valuable resource for further genetic studies, especially in the setting of rapid technological advances. By setting up collaborations with research groups nationally and

internationally, the large number of patients with a complex disease needed to draw statistically meaningful results are easier to add up.

7.5.6 Conclusion

The ability to identify individuals with a genetic predisposition for developing glaucoma, would allow efficient, cost-effective population based screening programs to be designed. When POAG is detected early and appropriate therapeutic intervention is initiated, blindness from glaucoma is preventable.

Therefore, efforts should be made in collecting, phenotyping and enrolling in genetic studies POAG pedigrees. Although, so far the SEI pedigrees have not yielded any results, they represent a valuable resource for further studies.

Chapter 8. GENERAL DISCUSSION

8.1 INTRODUCTION

As emphasised all the way through this thesis, open angle glaucoma is the major primary type of glaucoma in most population world wide and one of the main causes of blindness if left untreated. Various risk factor have been described, however, the pathogenesis of the disease is still unclear.

To date, ophthalmic based screening systems for POAG have proven cost ineffective for a community (Tuck and Crick 1997). Strategies to eliminate the blinding toll of glaucoma must be aimed at identifying at-risk individuals and therefore, screening regimen must be highly sensitive and specific to detect potentially serious disease (Harris 2005). As POAG is initially asymptomatic, effective screening techniques should identify people with no obvious signs or symptoms of the disease, allowing early diagnosis and management.

8.2 OVERVIEW OF THE WORK PRESENTED

It is generally accepted 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 retinal ganglion cells, the optic nerve and even as suggested recently, the cerebrospinal fluid (Berdahl et al 2008).

Genetic studies have identified some of the genes involved in different types of glaucoma presented in the genetics of glaucoma chapter (chapter 3) which also present different type of studies employed in genetic investigation. Paramount to the ability to do these studies is identification, phenotyping and recruitment of patients willing to help.

This work presents two aspects of glaucoma genetics studies.

The first part of the study is a pilot study which was set up to test the hypothesis that genes involved in anterior segment dysgeneses may have a role to play in predisposing to OAG development. 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 (Strungaru et al 2007). 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.

For this pilot study the gene chosen was *PITX2* as sub-clinical mutations/polymorphisms in this gene 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.

The discussion chapter of the pilot study deals with issues of patients and controls identification and recruitment, phenotyping and the challenges of the genetic work itself. This study did not identify a relationship between POAG and *PITX2* polymorphism and the possible reasons for this find are presented in the pilot study discussion chapter (chapter 6). Also, possible future studies stemming from this pilot studies are also presented.

The second part of the thesis presents identification, phenotyping and recruitment to genetic studies as well as pedigree construction of patients with family history of glaucoma, with at least two members affected. The discussion at the end of family history chapter (chapter7) emphasises the challenges encountered especially in identification of the pedigrees with an adequate number of affected members and how that affects the amount of genetic information that can be obtained.

In this final chapter after a short reflection on the work presented, considerations on the genetics of complex traits (mainly pertaining to glaucoma) and current and future status of glaucoma genetics will be discussed.

8.2.1 Reflection on the work presented

So, what are the lessons learned from this work and what did it bring to genetic research of glaucoma?

1. Get your statistics rights

If I were to start over again, the first thing I would do after I worked out the idea for the project would be to consult a statistician. As emphasized many times in this work, the statistics of genetic research are complex and the fact that I have not consulted a statistician at the beginning of the study showed my inexperience and naïveté. I have trusted the idea of the project as it was presented to me and not questioned the numbers of patients considered to be needed. That may be excused in a small proportion by the fact that the initial idea of the study (looking for polymorphisms in the *PITX2* gene in general population and comparing it with findings in POAG/OHT population) varied slightly during the study and as a result made the statistics more difficult to explain.

2. Get the numbers stated in the protocol

Time constraints are always a factor when doing research for a limited period and there is a timetable to keep to. However, in retrospective, I wished that I sacrificed some of the lab time to finish the recruitment of the controls. I could have continued with recruitment of patients as well, as the DNA resource created in this way could always represent a valuable resource for further genetic studies.

3. Get the finances sorted out

May be one of the more important lessons was the financing of genetic research. I had no idea before starting in the lab how expensive lab equipment and especially consumables were. Although, an initial budget was made and funds were allocated accordingly, as I have stated before I have run out of funds midway through the project. This creates discontinuity and puts strain on ones work at the time. More time has to be spent finding more funds and

it can jeopardize the entire project if not found. A contingency fund should always be allowed for as unexpected spending will occur.

In retrospective, this work has been started with very little money for consumables. One need to make sure has adequate advice regarding how much things cost and have a back up plan if things do not go according to the protocol.

4. Continuity in the lab

As during the research period I was also doing some clinical sessions, the work in the lab did not have continuity. Potentially this could be a reason for having so many difficulties with the sequencing work. One solution for this would be to contract the work out to commercial labs. This however was not an option for me, as I already spent the money on buying consumables to do the work myself and also, the cost per reaction was I considered quite expensive at the time.

May be if I did not find out about the Sequenom and still continued to have problems with the sequencing, I might have gone down that route. Would that have made a difference to the overall results? Probably not, as it has been shown by high throughput SNP screening that the homeobox (the region sequenced) is highly conserved which infers that mutations in this region are more likely to produce overt structural abnormalities (ARS type).

5. Speedy writing up of the thesis

Writing up ones results as soon as possible after the work is finished is very important. It places the work in the context at the time and allows for further work to be done, building on the results of the initial report.

In this case, the writing up of the thesis took much longer than expected and therefore makes the writing up of the pilot study results more difficult and may be somehow redundant. Also, in the mean time the work has been continued on an extended panel of DNA samples and multiple ASD genes and the results of that work has already been published.

So, is this study worth doing again? The answer is yes, only I would apply all the above points and get the numbers of patients and controls required for a statistical significant result for this type of study. Also, I would take advantage of the technological advantages and do high throughput screening straight from the beginning. The validity of the hypothesis has already been proven for the *LMX1B* gene (Park et al 2009) on work carried out on DNA samples which include the patients and controls from the pilot study too. An attempt to sequence *PITX2* gene on the extended POAG DNA panel has been made, however that proved difficult and as it didn't yield enough data has been abandoned.

I have stated above some of the lessons learned from doing work, pertaining mainly to the pilot study. With regards to the family history part of the study, which is important for any type of genetic research, the importance of patients and control identification, standardization of phenotyping and recruitment has been emphasised many times. May be when setting up a genetic study on families afflicted by a disease it is important to have very strict recruiting criteria and only recruit patients with family with a certain number the members affected, if the intention is to do linkage studies.

So, did this study bring anything to the genetic research of glaucoma?

Firstly, the DNA samples collected for both the pilot study and the family history study and the clinical data-base represent a valuable resource to be used in further genetic studies.

Secondly, the hypothesis for the pilot study, for which ample support has been presented throughout this thesis, has been an original idea for the first time tested with this study.

On a personal level, although at times things did not go as smoothly as desired, it provided me with the opportunity of learning about genetic research and what it means to be a scientist. Should the opportunity arise in the future, I would like to use this knowledge.

8.3 CONSIDERATIONS ON THE GENETICS OF COMPLEX TRAITS

The remarkable achievements in human genetics over the years have been due to technological advances in gene mapping and in statistical methods that relate genetic variants to disease. The vast majority of Mendelian genetic disorders have now been mapped to a specific gene or set of genes, but these discoveries have been limited to high-risk variant alleles that segregate in rare families. With a working draft of the human genome now completed, the availability of high-throughput genotyping, a plethora of genetic markers and the development of new analytical methods, more and more scientists turn their attention to common complex disorders like diabetes, hypertension, Alzheimer disease and in ophthalmology open angle glaucoma and age related macular degeneration.

Most researcher believe that complex disorders are oligogenic, the cumulative result of variants in several genes, or polygenic, resulting from a large number of genetic variants, each contributing a small effect. Other, have proposed that these disorders result from an interaction between one or more genetic variants and environmental or non genetic risk factors.

The difficulty facing researchers who work on complex genetic disorders is in designing the appropriate studies to merge the richness of modern genome science with vast potential of population based, epidemiological research.

While linkage analysis is considered the most powerful method for identification of rare, high risk alleles in Mendelian disease, many consider genetic association analysis to be the best method for identifying genetic variants related to common complex diseases. Association analysis is generally model free, or non-parametric, so the researcher does not have to assume a mode of inheritance is unknown. Unlike linkage analysis, where markers are identified, association studies determine whether or not a specific allele within a marker is associated with the disease.

Association studies also have limitations. Because linkage disequilibrium, cosegragation of a series of genetic markers or alleles, is sustained only over a short chromosomal segment, a large number of loci need to be tested to cover a region. This increases the possibility of

false positive findings. With each test, the possibility of false positive results increases, requiring the need either for replication in an independent study or computer simulation (Mayeux 2005).

Study design and interpretations of results must include appropriate statistical thresholds that take multiple hypotheses testing into account. Balancing the need for power to detect modest effects with cost of genotyping large number of markers will probably require a multistage design.

Genome-wide studies have the potential to identify many genes for common diseases and quantitative traits, however care will be required with their design, performance, analysis and interpretation, and well conceived pilot studies might be valuable for understanding and minimising the pitfalls of this approach (Hirschhorn & Daly 2005).

8.3.1 Epistasis

For many Mendelian conditions, genotype-phenotype correlations remain inadequately correlated and the inability to predict the phenotype on the basis of genotype is now apparent in many disorders (Dipple and McCabe 2000). Broad variability in phenotypic expression within and between families with the same mutation characterizes many monogenic conditions -relevant to this thesis the case of ARS (Summers 1996).

Additionally, gene mutations can lead to absence of a recognised phenotypic effect because of reduced penetrance (van Heyningen and Yeyati 2004). Also, it has been recognised that for many monogenic conditions, there may be important genes at other loci or environmental modifiers that change expression.

The genes of an individual do not operate in isolation, but are functioning in a common cellular environment. Therefore, it is expected that interactions between genes (epistasis) may occur. Conditions showing complex pattern of inheritance often involve multiple types of genetic mechanisms, like modifier genes, triallelic inheritance, digenic inheritance, opposite transcripts (transcribed from the same genomic locus as their target but from the opposite DNA strand and form perfect pairs –Wang et al 2005)

The effect of modifier genes may include reduced penetrance, dominance modification, expressivity and phenotypic pleiotropy. These effects are commonly seen when mice with single gene mutations are crossed onto different genetic backgrounds (Gropman and Adams 2007). Modifier genes also alter the onset, the range of symptoms and the severity of disease in humans. Modifier genes have become increasingly recognised as an important source of phenotypic variation that may explain the relationship between phenotype and genotype. They can also have small quantitative effects on the expression level of another gene. The products of modifier genes may affect splicing, transcription, translation, protein trafficking, glycosylation, protein expression, degradation or secretion.

Modifier genes differ from susceptibility genes as follows: modifier genes are genetic variants that affect the clinical manifestation of disease (as opposed to liability), whereas susceptibility genes or loci affect genetic liability, generally for complex disorders (Gropman and Adams 2007).

In the case of POAG, the exact mechanism of interaction of the identified associated genes (table 2.2) in susceptibility of the diseases remains unknown. The *APOE* polymorphism -491A>T has been reported to interact with the *MYOC* polymorphism *MYOC.mt1*, to increase IOP in POAG patients (Copin et al 2002). Another study suggests that *MYOC* and *CYP1B1* might interact through a common pathway and that the inheritance of glaucoma might be digenic in some cases (Vincent et al 2002). Also a possible gene-gene interaction between *OPTN* and *TNF* was also identified to increase the POAG risk (Funayama et al 2004). In a large association study by Fan et al (2005), possible gene interactions were reported between *MYOC, OPTN* and *APOE*.

In the case of anterior segment dysgeneses associated with glaucoma, Cella et al (2006) assessed the frequency of *PITX2, FOXC1, CYP1B1* and *GJA1* genes in patients with AXRS with glaucoma and identified a polymorphism in *GJA1* (gap junction protein, alpha 1) in a patient with *FOXC1* mutation and therefore suggest the possibility of its participation as a modifier gene.

Also, when the effect of *TYR* as a modifier gene in patients with ICG with a known *CYP1B1* mutation was studied, this hypothesis could not be proven (Bidinost et al 2006). But because the mode of interaction between *CYP1B1* and *TYR* is not known, a modifier effect of *TYR* on other *CYP1B1* human mutations cannot be excluded.

Other potential modifiers of gene expression that affect complex disorders are single nucleotide polymorphisms. These are single base pair positions in the human genome at which variation exists in the nucleotide sequence that gives rise to alternative alleles in normal individuals. As emphasised previously, it is believed that SNPs maps will enable scientists to identify the multiple genes associated with complex diseases as cancer, diabetes, vascular disease and age related ocular diseases like AMD and glaucoma. These associations are difficult to establish with the conventional gene hunting methods, because a single altered gene may make only a small contribution to the disease phenotype (Nowotny et al 2001).

8.3.1.1 Other determinants of susceptibility

Genetic susceptibility factors are important in determining whether a person develops glaucoma. In addition to factors already implicated in glaucoma through genetic studies, other factors that affect IOP and RGC survival are likely important. In their paper on the complex genetics of glaucoma Libby et al (2005) provide evidence for how genetic changes affecting aqueous production, outflow ocular development, outflow extracellular matrix and outflow-cellular stress and survival may alter susceptibility to IOP elevation in glaucoma. Also, they discuss the neural susceptibility to glaucoma, emphasizing its complexity and although some genes (*APOE, OPA1, IGF2*, and *TNFα)* have been suggested to increase this susceptibility, none has been definitely shown to be a major factor as yet.

8.3.2 Quantitative traits analysis

Another way to progress with unravelling the genetics of glaucoma is to investigate POAG constitutional anatomical or pathophysiological components. The study of intermediate phenotypes might be more powerful than just ascertaining whether disease is present or

absent and such a strategy has been implemented for IOP and CD ratios (Charlesworth et al 2005).

Quantitative trait linkage analysis of precursors of glaucoma, such as IOP and CD ratio, may have simpler genetic architecture than diagnosis of glaucoma, making it easier to map causative loci (Williams and Blangero 1999 a) and also it is particularly powerful in large families (Williams and Blangero 1999 b).

Heritability of IOP and vertical CD ratio has been estimated by the Beaver Dam Eye Study investigators, providing evidence for the genetic determinants of these factors (Klein et al 2004) and commingling analysis of IOP and glaucoma, suggested the existence of a major gene accounting for 18% of the variance of IOP in the Blue Mountains Eye Study population (Viswanathan et al 2004). The Beaver Dam Eye Study investigators found two possible linkage regions for IOP on chromosome 6 and 13 (Duggal et al 2005). Charlesworth et al (2005), revealed significant linkage for IOP to the long arm of chromosome 10 and suggestive linkage for vertical CD ratio on short arm of chromosome 1.

The heritability of IOP and OD morphology has also been recently confirmed by a large Dutch study (Koolwijk et al 2007), which estimated that heritability for IOP at 0.35, 0.48 for retinal nerve fibre layer thickness and 0.39 for neuroretinal rim area and that non genetic factors account for only a small proportion of the variance (< 0.13) in all three traits. Therefore they conclude that early, continuous markers of POAG are strongly determined by additive genetic effects.

8.3.3 Twin studies

With regards to the heritability and identification of disease causing gene, twin studies represent a major tool. Twin studies allow for well controlled association studies, as well as the study of genetic versus environmental contributions to the traits. Comparison between covariance of monozygotic and dizygotic twin pairs allow estimation of the genetic and environmental contributions to the trait in question and it can be broken down into

dominant versus additive genetic components and shared versus non-shared environmental elements (Hewitt et al 2006).

Once the components of a trait have been modelled and the effect of human differences has been determined, it is possible to locate the precise location of these genes, by using discordant sibling pair analysis of the dizygotic twins. The twin studies have been used successfully to demonstrate the role of heritability in the morphology of optic disc (Schwartz et al 1976, Teikari et al 1992, Hewitt et al 2007) and intraocular pressure (Kalenak & Paydar 1995, Parssinen et al 2007).

8.3.4 Statistical analysis challenges

The rapid growth of human genetics created countless opportunities for studies of disease association. However, given the number of potentially identifiable markers and the multitude of clinical outcomes to which these may be linked, the testing and validation of statistical hypotheses in genetic epidemiology presents a task of unprecedented scale.

One way this problem can be solved is to use meta-analysis, which provides a quantitative approach for combining the results of various studies on the same topic, and for estimating and explaining their diversity.

An evaluation of 370 studies (addressing 36 genetic associations for various outcomes of disease) by meta-analysis demonstrated that significant difference between studies heterogeneity is frequent and that the result of the first study correlates only modestly with subsequent research on the same association (Ioannidis et al 2001).

The first study frequently suggests a stronger genetic effect than is found by subsequent studies. Bias and genuine population diversity might explain why early association studies tend to overestimate the genetic component, however, systematic meta-analysis may assist in estimating population-wide effects of genetic risk factors in human disease.

Also, contributing to this problem is publication bias and time-lag bias with small studies with “negative” statistically non-significant results taking longer to be published than

"positive" statistically significant studies (Easterbrook et al 1991, Ioannidis 1998). Estimates of the size of a genetic effect may be inflated, if only based on a single study with impressive results. Also, in some cases there may be large statistical uncertainty in the first study. Often, genetic associations of disease are of modest magnitude (OR >0.5 and < 2) and single studies are underpowered to detect them.

Isolated statistical significance does not guarantee a genetic association, and lack of formal statistical significance does not exclude the possibility of an association (Ioannidis et al 2001).

8.3.5 Current status and future directions of glaucoma genetic research

8.3.5.1 Current status of glaucoma genetics

Presently only a small proportion of glaucoma has been genetically accounted for. So far there is little understanding of the genetics of POAG and the cell biology underlying it. Linkage and association studies in human families have been essential in identifying loci and genes and these efforts need to be continued and combined with genomics and proteomics approaches to increase the rate of glaucoma gene identification. However, even with the genes identified, the situation is not as simple as first hoped. There appears to be phenotypic alteration by genetic modifiers and some mutations may only cause disease in a susceptible genetic context. This situation confounds the genotype-phenotype association and makes it even more difficult to characterize disease causing alleles in more complex cases of POAG in which multiple genes and environmental factors are likely to affect phenotype and the genetic changes may be subtle. For this reason animal studies are needed as an important complement to human studies.

The ability to study effects of specific alleles in a defined genetic background and controlled environment makes mouse studies particularly valuable. So far mouse studies only marginally contributed to our understanding of glaucoma, but they will become more important in the future. Mouse models with potential relevance to glaucoma include the C57BL/6J mouse (strain of mouse which loses 50% of its RGG in the presence of normal IOP- important for studying NTG, Gould & John 2002), Colla1 mutant mice (this strain has

a mutation for the a1 subunit of collagen 1, a component of outflow pathway ECM, and has developed elevated IOP with almost 25% loss of their RGC (Marneros & Olsen 2003) and DBA/2J mice important for studying pigmentary glaucoma (Anderson et al 2002).

The future combination of mouse approaches includes candidate gene mutation, addition of human genes and mutations, modifier genes characterization and sensitized mutagenesis screens.

Although ASD phenotypes often exhibit dominant inheritance, variable expressivity and incomplete penetrance point to a multifactorial etiology. To date, most human ASD genes that are associated with glaucoma encode transcription factors and although the pathogenic mechanisms are not established, ocular tissue appear to be exquisitely sensitive to deviant activity levels of these transcription factors, suggesting that their levels are rate limiting for target gene modulation.

Identification of new human genes based on linkage, association and candidate genes analysis will continue to produce results. Also, mouse studies will provide an important experimental system for dissecting developmental glaucomas. Genomics approaches such as microarray analysis and chromatin immunoprecipitation, to identify important developmental genes and how they are affected by specific mutations will also prove powerful.

8.3.5.2 Future directions human genetics

Human studies are essential and have identified a number of glaucoma genes. Genome-wide studies suggest the existence of even more glaucoma loci and the identification of new genes will continue. However, it has been stated many times that glaucoma is a complex disease and therefore will be significantly more difficult to unravel (probably involving heterogeneous causes, genes of more modest effect and relatively subtle mutations in multiple genes). In family linkage studies, the number of affected individuals typically will be limiting. The multifactorial complexity has the potential to confound familial and non-familial studies even when assessing candidate genes. Distinguishing a truly causative gene

from a closely linked gene of no consequence can also be problematic. So, what do we do next?

1. The full array of genetic approaches should be used, including linkage studies, candidate genes analysis and sophisticated association studies. Also, given the likelihood of multifactorial interactions, it will be important to asses the role of multiple genes or loci on individual patients and not stop when a single mutant gene has been identified. As it has been mentioned above, there are some studies that strongly support the existence of modifier genes.

2. Continue to gather DNA samples (affected individuals and non-affected family members, as more cases develop with age). Analysis of large samples will facilitate detection of multifactorial interactions and will provide greater statistical power.

3. Collecting as much family history, medical information, environmental history and lifestyle data as possible to accompany these samples it is also very important.

4. As much as it is possible, analysis techniques, information gathering, and diagnostic criteria should be standardized and new epidemiological studies should be conducted.

5. While safeguarding patient protection and privacy, it would be useful for genotype and all other relevant data to be incorporated in large scale shared databases. This would facilitate progress by enabling firstly interrogation by the research community using different approaches and secondly would increase the power of future human genetic analyses.

8.3.5.3 Animal genetics

Animal studies complement human studies and can provide important insight into genetic etiology and molecular mechanism for further assessment in people. Animal models and cell culture systems for studying glaucoma are well established and will continue to make important contributions to understanding glaucoma. At present, the mouse is the best suited mammalian model for deciphering complex genetic interaction that underlines glaucoma

susceptibility. The effect of genetic background effect on gene's pathogenicity can be studied and help with identifying modifier genes. Such studies may help explain incomplete penetrance and currently discrepant genotype-phenotype relationships. Also, they will identify new molecular pathways that may contribute to glaucoma in patients. And finally, mutagenesis screens in mice will also contribute to elucidating genes and mechanisms that may interact to cause glaucoma in patients.

8.3.5.4 Genomics and proteomics

Genomics can be applied to human samples and to tissues from the many model systems that are used to study specific aspects of glaucomatous pathophysiology. Different expression patterns between treatments or between patients and controls will help prioritize the assessment of candidate genes (e.g.: microarray analysis is used to determine the molecular pathway that underlie elevated IOP in cultured TM cells and to determine changes in gene expression in response to elevated IOP). Identifying expression differences for specific genes will be especially useful when these genes or other members of the pathways they mediate reside in implicated chromosomal regions. Genomics tools are also used in examining neural responses to elevated IOP using a variety of models, including primates. These techniques can be used to ask precise questions about individual cell populations responses to glaucomatous insults.

Proteomics is further said to contribute greatly to the understanding of gene function in the post-genomic era. Proteomics can be divided into three main areas:

1. protein micro-characterization for large scale identification of proteins and their post-translational modifications;

2. "differential display" proteomics for comparison of protein levels with potential application in a wide range of diseases;

3. studies of protein-protein interactions using techniques such as mass spectrometry or the yeast two-hybrid system. Because it is often difficult to predict the function of a protein based on homology to other proteins or even their three-dimensional structure,

determination of the components of a protein complex or of a cellular structure is central in functional analysis (Pandey et al 2000).

8.4 CONCLUDING REMARKS

The progress in understanding glaucoma has been great in the last few decades; however, an efficient strategy for detecting patients with glaucoma or at risk of developing glaucoma is still missing. Even in developing countries, half of people with POAG remain undiagnosed (Hollows & Graham 1966, Mitchell et al 1996). Strategies to reduce glaucoma blindness must be aimed at identifying individuals at risk.

Identification of risk factors allows early diagnosis and treatment prior to loss of visual function. Cross sectional studies have suggested that more than 50% of all glaucoma is familial and a family history of glaucoma conveys up to 3 fold increase in the risk of developing POAG (Tielsch et al 1994, Weih et al 1972).

Also, as previously emphasized, there is an increased prevalence of POAG in first degree relatives of patients with the disease, and a recent study demonstrates that the severity of the disease is greater in familial glaucoma than in sporadic disease (Wu et al 2006).This study and another recently published study on the clinical and cost effectiveness of screening for open angle glaucoma (Burr et al 2007), suggest that the screening programs should target high risk groups (i.e. family history of the disease, black ethnicity).

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. 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 normal tension glaucoma cases for E50K mutation may be of value in the UK population. 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 normal tension glaucoma.

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. Nonetheless, 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.

Some strategies for current and futures studies have been outlined above, emphasizing the role of both the clinician and researcher. These strategies discuss mainly the future of genetic studies in glaucoma; however, the role of environmental factors in the pathogenesis of the disease should not be minimized. For a late onset disease it is likely that the genetically determined features are more sensitive to environmental influences because of disruption of normal physiologic homeostatic mechanisms. Therefore, future studies should include both evaluation of environmental factors that may be associated with glaucoma and investigations into specific gene-environment interactions in patients with POAG.

With regards to this study, identification of POAG pedigrees, as large as possible and accurate phenotyping with the methods available at the time of patient enrolment represents a very valuable resource for further genetic studies, especially in the setting of rapid technological advances. By setting up collaborations with research groups nationally and internationally, the large number of patients with a complex disease need it to draw statistically meaningful results are easier to add up.

In the case of the second cohort of POAG/OHT patients enrolled in this study, the criteria for inclusion were very strict and therefore, again, they represent a valuable resource for studying the disease in a homogeneous, well defined population. In this particular cohort, a large effect of *PITX2* polymorphism in POAG has not been demonstrated. This should be the basis for further studies into the role of developmental glaucoma genes in the pathogenesis of POAG, as smaller, more subtle effect and /or possible gene-gene interactions might be demonstrated.

Chapter 9: CONCLUSION

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.

Victorian physician Sir William Osler wrote in 1892: “If it were not for the great variability among individuals, medicine might be a science not an art”. More than a century later, analysis of SNPs raises the prospect of understanding the factors driving this variability and tailoring treatment to the patient.

The work done for this thesis offered me a fascinating insight into the world of molecular genetics, especially for someone with a clinical background. It also, emphasized the importance of the clinical aspect of setting up a genetic study. I was in the privileged situation of identifying, recruiting, phenotyping two different cohorts (the family history cohort and the high IOP POAG/OHT cohort) and then followed on with the genetic study of the high IOP POAG/OHT cohort. This situation allowed me to appreciate the importance of collaboration between clinicians and researchers, in the setting up and running of genetic studies, and it left me with huge enthusiasm for being part of future studies.

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APPENDICES

Appendix 1

Tel: 0191 565 6256 **Version 4.0**

Miss Daniela Vaideanu

Mr Scott Fraser

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.

Appendix 2

Department of Cardiological Sciences - GLAUCOMA DATABASE

Cranmer Terrace
London SW17 ORE
Direct Line 020 8725 2591
Fax 020 8725 2653
Aircall 04325 107332
E-mail g.brice@sghms.ac.uk

Re: ..... (.....) Our ref. – GL Date: .....

Date of most recent examination.....

A./ Diagnosis.

1. Ocular hypertension (i.e. IOP ≥22mmHg and no other signs suggestive of glaucoma) Yes☐ No☐ R☐ L☐

2. Primary open angle glaucoma (POAG i.e. optic nerve damage)

a/ IOP ≥22mmHg - (CSG - chronic simple glaucoma) Yes☐ No☐ R☐ L☐

b/ IOP <22mmHg (NPG - normal pressure glaucoma) Yes☐ No☐ R☐ L☐

3. Secondary glaucoma

a/ pseudoexfoliation R☐ L☐

b/ pigmentary R☐ L☐

c/ other (please specify) ..... R☐ L☐

4. Date of detection of POAG or OH R..... L.....

B/ Risk Factors (if known)

a/ Afro-Carribean origin ☐ b/ Myopia ☐ g/ Family history ☐

c/ Diabetes ☐ d/ Raynauds symptoms ☐

e/ Migraine ☐ f/ Arterial hypertension ☐

C/ Ocular Findings

Visual acuity..... R 6/..... L 6/.....

Intraocular pressure - Most recent R ☐ L ☐ Instrument used.....

Highest - Date..... R ☐ L ☐ Instrument used.....

Optic disc appearance- (e.g. pallor of rim, notching) .....

Cup/disc ratio. R 0. L 0.

Visual field defects R☐ L☐ Nil ☐

- Instrument used..... Character of field loss - R..... L.....

- Evidence of deterioration.....Degree of field loss R..... L.....

Angle appearance R..... L .....

Please enclose copies of most recent visual fields if at all possible

D/ Therapy a/ Medication R.....L..... Nil ☐

b/ Laser R.....L..... Nil ☐

c/ Surgery R.....L..... Nil ☐

Signature..... Name ..... Date.....

Appendix 3

Tel: 0191 565 6256 **Version 4.0**

Miss Daniela Vaideanu

Mr Scott Fraser

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.

Appendix 4

Protocols

a. DNA extraction

Genomic DNA was extracted from peripheral blood leucocytes, using a Nucleon BACC2/3 kit (Tepnel Life Sciences PLC).

Blood was collected in 5 ml sodium EDTA tubes.

The main steps in the Nucleon protocol are as follow:

1. cell lysis
2. deproteinisation with sodium perchlorate
3. extraction with chlorophorm and Nucleon TM resin
4. DNA recovery
5. DNA washing

Cell preparation from the whole blood:

1. to 5 ml of blood add 20 ml of reagent A (4x the volume of blood) in a 50 ml Falcon tube
2. shake at room temperature for 5 minutes
3. spin at 1300g for 30 min
4. discard supernatant
5. warm reagent B at 37°C
6. add 4ml reagent A then vortex briefly to resuspend pellet
7. spin at 1300g for 10 minutes

Cell Lysis

1. add 2ml reagent B then vortex briefly to resuspend pellet
2. incubate at 37°C for 10 minutes
3. transfer to a 10ml Falcon tube

Deproteinisation

1. add 500µl Na perchlorate and mix by inverting at least 7 times

DNA extraction

1. add 2ml chlorophorm and mix by inverting at least 7 times (emulsifies phases)
2. add 300µl nucleon resin and without mixing phases, spin at 1300g for 5 minutes
3. transfer upper layer to a new 10 ml tube

4. add 2x the volume of cold 100% ethanol (approx 5 ml) and invert several times until the DNA is precipitated
5. transfer precipitate to Eppendorph containing 1ml of sterile H_2O

b. PCR clean up for automated sequencing (ExoSAP protocol)

ExoSAP protocol may be used for clean up of the PCR product prior to automated sequencing.

Exonuclease I (E. coli)/New England BioLabs Inc. degrades excess single stranded primer oligonucleotide (in the 3'to 5' direction) from a reaction mixture containing double stranded extension products.

SAP (Shrimp Alkaline Phosphatase)/USB Corporation desphophorylation removes 5'-phosphates from double stranded DNA

A 10µl PCR volume is mixed with 2µl Exo I at 1U/ µl and 2µl SAP at 10U/ µl by gentle vortexing, then briefly centrifuged. This is then incubated at 37°C for 20 minutes in a thermal cycler. This is followed by the inactivation of the ExoSAP by heating to 80°C for 20 minutes as step two in the thermal cycler.

c. Sequencing protocol DYEnamic ET (Dye terminator cycle sequencing kit for MegaBACE DNA analysis systems/ Amersham Biosciences)

96 well microtiter plate (Eppendorph) was used
Primer diluted at a concentration of 5pmol/µl

Sequencing reaction:	
Template DNA	4 µl
Primer	1 µl
Water	7 µl
Sequencing reagent premix	8 µl
Total volume	20 µl

Control reaction	
M13mp18 control template	1 µl
Control primer	2.5 µl
Water	8.5 µl
Sequencing reagent premix	8 µl
Total Volume	20 µl

The plate was briefly centrifuged at 700 rcf, for 1 minute.
The plate was then placed in the thermal cycler with the following cycling program running: 95°C for 20seconds, 60°C for 1minute (the 50°C for 15 seconds step has been omitted as the melting temperature for these samples was >60°C) for 25 cycles. Following cycling, the plate was briefly centrifuged, so that the reaction mixture was collected at the bottom of the wells.

Post-reaction clean up using the isopropanol precipitation

To the 20 µl volume in the wells, 80 µl of 80% isopropanol was added to each well. The plate was then spanned at 2800rcf, 15°C for 30 minutes (SH-3000/Sorval Legend RT)
The plate was inverted on paper and spanned again at 700rcf for 1 minute, to remove the supernatant.
The DNA pellet was washed with 70% ethanol and centrifuged briefly. The supernatant was removed by a brief inverted spin and then air-dried and submitted for sequencing.

d. Agarose gel electrophoresis and visualisation

DNA fragments were separated by electrophoresis on an agarose gel, with ethidium bromide as an intercalating dye. DNA was electrophoresed in 0.8% to 2% (w/v) agarose 1x TAE gel. Agarose was dissolved in 1x TAE buffer containing 0.4ng ethidium bromide (Sigma), heated in a microwave and allowed to cool. Molten agarose was then poured into a horizontal gel apparatus (Scotlab) and allowed to polymerise. Samples and Hyperladder IV (Bioline) were mixed with TAE loading buffer and electrophoresed at a constant 100V in a 1x TAE buffer. DNA was visualised by exposure to UV using an Alphamager™ 2200 (Alpha Innotech).

MATERIALS

Chemicals and reagents

All chemicals were of molecular biology grade and solutions were prepared with autoclaved (120°C, 1.8bar, 20mins) nanopure water (anH_2O).

DNA extraction, purification and precipitation

Nucleon DNA Extraction Kit	BACC2/3	Tepnel Life Sciences PLC
Isopropanol		Sigma
Phenol		Sigma
Chloroform		Sigma
Isoamyl alcohol		Sigma
Sodium Acetate		Sigma
Glycogen		Sigma
Ethanol AnalaR		Sigma
MilliQ ultrapure water		Millipore
Wizard^{R} DNA clean-up system		Promega

Polymerase Chain Reaction

Hotstar^{TM} *Taq* Polymerase	Promega
- Supplied with 10x Reaction Buffer	
dNTPS (dATP, dTTP, dCTP and dGTP)	Fermentas
Oligonucleotide primers	Promega, MWG Biotech
PCR Master Mix	Promega

PCR clean up for automated sequencing

Exonuclease I (E. coli)	BioLabs
Shrimp Alkaline Phosphatase	USB

Sequencing

DYEnamic ET Dye Terminator Cycle Sequencing Kit for MegaBACE DNA Analysis Systems	Amersham Biosciences
Primers for Sequenom	Metabion

Agarose gel electrophoresis

Agarose	Helena Biosciences
Ethidium Bromide	Sigma
Eppendorf TAE (50x)	Helena Biosciences
Hyperladder IV	Bioline
1Kb Plus DNA Ladder	Invitrogen
EZ Load™ Molecular Rulers	BioRad

Loading Buffers

Orange G	Bioline

EQUIPMENT AND CONSUMABLES

Equipment

Alphamager™ 2200	Alpha Innotech
Bench top microfuge	Eppendorf
Gilson Pipetteman P2	Gilson
Gilson Pipetteman P20	Gilson
Gilson Pipetteman P200	Gilson
Gilson Pipetteman P1000	Gilson
Horizontal gel electrophoresis:	
Model E132	Consort
Model E802	Consort
Model HU10	Scotlab
Model HU13	Scotlab
Incubation Oven	Cell Path
MasterCycler™	Eppendorf
MilliQ ultrapure, water purification system	Millipore
VortexGenie™	Scientific Industries
MegaBACE DNA Analysis System	Amersham Pharmacia
Sequenom™	Sequenom®

Consumables

Eppendorf 0.2ml tubes	Eppendorf

Eppendorf 0.5ml tubes	Eppendorf
Eppendorf 1.5ml tubes	Eppendorf
96 well plates	ABgene
384 well plates	ABgene
Falcon tube 15ml	BD Biosciences
Falcon tube 50ml	BD Biosciences
Gilson Tips	Eppendorf
Parafilm	Jencon
Adhesive PCR film	ABgene

Solutions and Buffers

DNA loading buffers ;	
-0.25% (w/v) Orange G	Sigma
-30% (v/v) Glycerol	Sigma
Electrophoresis Buffer;	
-40ml 50xTAE in 2L of MilliQ ultrapure water	Eppendorf
-80µl Ethidium bromide	Sigma

COMPUTER PROGRAMS AND ONLINE RESOURCES

Genetic analysis

BioEdit v 7.0.0 (http://www.mbio.ncsu.edu/BioEdit/)

Sequencher (http://www.sequencher.com)

Clustal W (http://www.ebi.ac.uk/Tools/clustalw)

SHEsis (http://analysis.bio-x.cn/myAnalysis.php)

UNPHASED v 3.0.12

(http://www.mrc-bsu.cam.ac.uk/personal/frank/software/unphased)

Online resources

NCBI (http://www.ncbi.nlm.nih.gov/)

Ensembl (www.ensembl.org)

Institute of Human Genetics, Centre for Life, Newcastle upon Tyne

(http://www.ncl.ac.uk/ihg/research/facilities/sequenom)

HapMap (www.hapmap.org)

SPSS v. 15.0 (https://ras.ncl.ac.uk/Citrix/MetaFrame/default/default.aspx)

MAJOR REVIEW

A Review of Anterior Segment Dysgeneses

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Abstract. The anterior segment dysgeneses are an ill-defined group of ocular developmental abnormalities that share some common features and have a high prevalence of glaucoma. Current classification of what are and what are not anterior segment dysgeneses seems to vary and our knowledge of them is incomplete. As the limits of classical clinical medicine based on evaluation of signs and symptoms are reached, further advancements increasingly will come from molecular medicine and genetics. In this article we review the normal and abnormal development of the anterior
segment (concentrating primarily upon neural crest derived dysgeneses), describe the various clinical entities produced and their diagnosis, and discuss the current knowledge of the genetics of these disorders. We also suggest a new approach to the classification of anterior segment dysgeneses, based upon the embryological contribution to the formation of the anterior segment of the eye. (Surv Ophthalmol 51:213--231, 2006. _ 2006 Elsevier Inc. All rights reserved.)
Key words. anterior segment dysgeneses _ developmental glaucoma _ genetics _ glaucoma _ neural crest

I. Introduction The anterior segment dysgeneses, at present, are an ill-defined group of developmental abnormalities that share some common features and a high prevalence of associated glaucoma. Classifications of what is and what is not an anterior segment dysgeneses seem to vary and our knowledge of them is incomplete. Throughout the whole of medicine a revolution is occurring as the limits of classical clinical medicine based on the evaluation of signs and symptoms is reached and further advances are increasingly coming from the fields of molecular medicine and molecular genetics. These new disciplines are undoubtedly going to allow us a far greater understanding of many diseases including the anterior segment dysgeneses. However, if we are to understand how alterations in genetic code cause functional abnormalities we must be sure that we can differentiate between what is normal and abnormal. Just as we systematically discover the nucleotide sequence of a particular gene we must be sure that we have systematically described and categorized the possible phenotypic changes that the gene could produce. Abnormalities of the anterior segment that can lead to glaucoma have recently received attention
[45] as they often have large pedigrees and severe phenotypes. Although relatively rare, they may provide important insights into one of the most common forms of glaucoma—chronic open-angle glaucoma (COAG). This article reviews the normal and abnormal development of the anterior segment concentrating primarily upon neural crest derived dysgeneses. It describes the various clinical entities

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A Novel Homeobox Mutation in the PITX2 Gene in a Family With Axenfeld-Rieger Syndrome Associated With Brain, Ocular, and Dental Phenotypes

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Axenfeld-Rieger Syndrome (ARS) is a genetically heterogeneous birth defect characterized by mal-formation of the anterior segment of the eye associated with glaucoma. Mutation of the PITX2 homeobox gene has been identified as a cause of ARS. We report a novel Arg5Trpmissensemutation in the PITX2 homeodomain, which is associated with brain abnormalities. One patient had a small sella turcica likely to reflect hypoplasia of the pituitary gland and consistent with the critical role identified for Pitx2in pituitary development in mice. Two patients had an enlarged cisterna magna, one with a malformed cerebellum, and two had executive skills deficits one in isolation and one in association with a below average intellectual capacity. The mutation caused a typical ARS ocular phenotype. All affected had iris hypoplasia, anterior iris to corneal adhesions, and corectopia. The ocular phenotype varied significantly in severity and showed some asymmetry. All affected also had redundant peri-umbilical skin, a hypoplastic maxilla, microdontia, and hypodontia missing between 20 and 27 teeth with an unusual pattern of tooth loss. Dental phenotypes were documented as they are often poorly characterized in ARS patients. All affected individuals showed an absence of first permanent molars with variable absence of other rarely absent teeth: the permanent upper central incisors, maxillary and mandibular first and second molars, and the mandibular canines. Based on the distinctive dental anomalies, we suggest that the dental phenotype can assist in predicting the presence of a PITX2 mutation and the possibility of brain abnormalities. _ 2006 Wiley-Liss, Inc.

KEY WORDS: PITX2; glaucoma; tooth hypodontia; cisterna magna; cerebellar vermis hypoplasia

INTRODUCTION

Malformation of the anterior segment of the eye with dental hypoplasia (hypodontia) was first identified as a dominantly inherited disorder by Rieger (MIM 180500). The PITX2 homeobox gene was identified as the Rieger syndrome type 1 gene on chromosome 4q25; two further loci have been mapped to 13q14 (Rieger syndrome type 2; MIM 601499) and 16q24 [Phillips et al., 1996; Semina et al., 1996; Smith et al., 2000].Clinical reports of Rieger syndrome describe a range of associated features including maxillary hypoplasia, anal stenosis, hypospadias, growth hormone deficiency, congenital heart defects, hearing defects, hydrocephaly, and psychomotor retardation (MIM 180500, MIM 601499, and MIM180500). Semina et al. [1996] noted that families with PITX2 mutation typically present with ocular and dental features together with failure of involution of periumbilical skin. However, other clinical features have not been associated with PITX2 mutation. Deletion of the homeobox gene, PAX6, was identified in a single case of Rieger syndrome [Riise et al., 2001]. Mutation of the forkhead gene FOXC1 has also been reported in families with features resembling Rieger syndrome [Mirzayans et al., 2000; Riise et al., 2001; Honkanen et al., 2003]. PAX6 mutation is more commonly associated with aniridia and FOXC1 mutation with anterior segment defects without other systemic features [Hanson et al., 1993] MIM106210; [Mears et al., 1998; Nishimura et al., 1998] MIM 601090. As there is considerable overlap in the anterior segment phenotypes caused by mutations in the PITX2, FOXC1, and PAX6 genes [Alward et al., 1998; Kulak et al., 1998; Mears et al., 1998; Nishimura et al., 1998, 2001; Lehmann et al., 2000; Mirzayans et al., 2000] the term, Axenfeld-Rieger syndrome, ARS, has been proposed to encompass this genetically heterogenous clinical spectrum [Alward, 2000]. The ocular features of ARS include iris hypoplasia, anteriorly displaced and prominent Schwalbe's line (posterior embryotoxon),

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