Molecular and Phenotypic Associations in the Open Angle Glaucomas.

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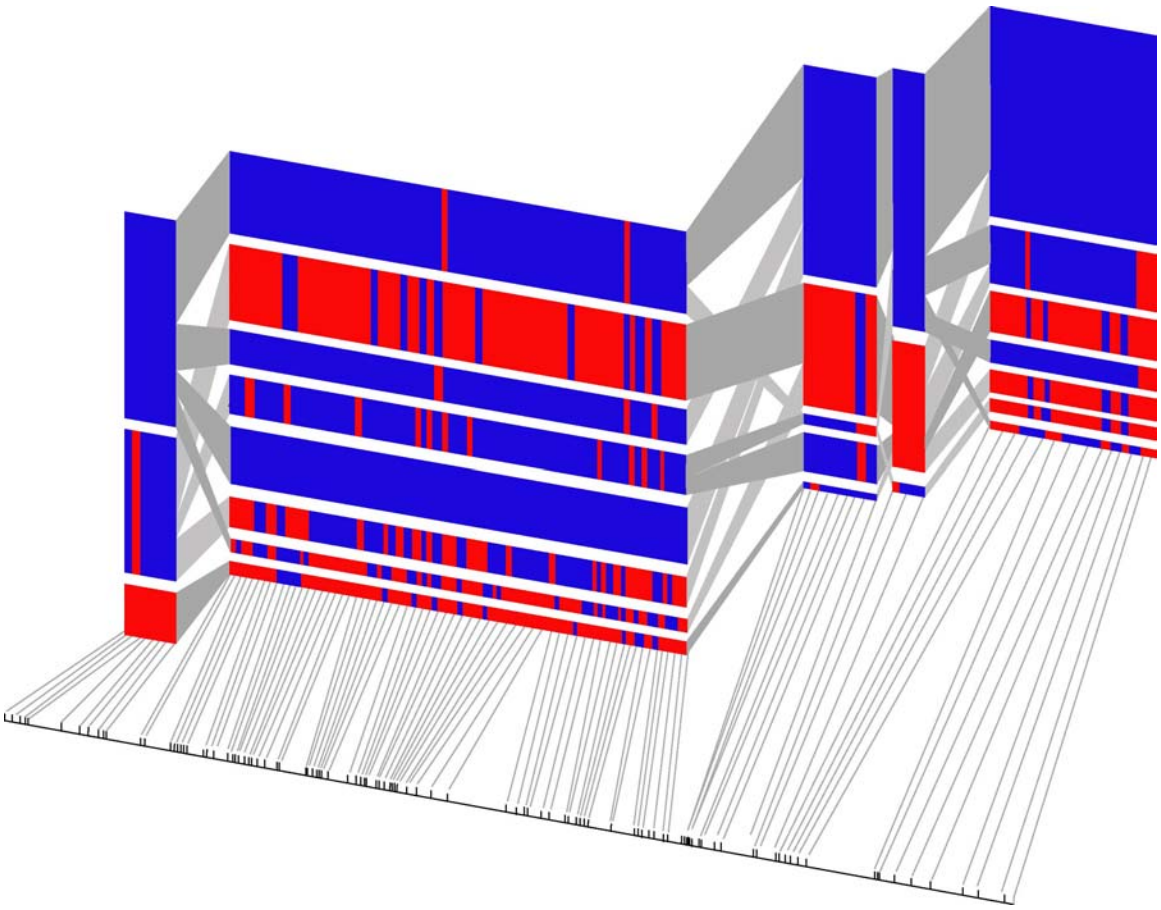

Haplotype Block Structure in the HapMap CEU population of the novel putative glaucoma locus on Xp25. Image based on the work of Dr Ben Fry, colours represent allelic variants and the z-offset emphasises the transition between blocks (see page 197).

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

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Summary

Glaucoma is the commonest cause for irreversible optic neuropathy worldwide. Being a complex heterogeneous disease, Primary Open Angle Glaucoma (OAG) is likely to manifest due to the collision of germ-line, somatic, environmental and stochastic factors. This thesis explores both the phenotypic features and genetic mechanisms of the glaucomatous process.

Investigation of the *myocilin* gene, which has been unequivocally associated with OAG, demonstrated firm genotype-phenotype correlations. Possibly reflecting the association between *myocilin*-related glaucoma and elevated intraocular pressure, *myocilin* mutation carriers were found to have a lower prevalence of optic disc haemorrhages, compared to individuals with non-*myocilin* OAG. No structural differences of the optic nerve head were identified in young people known to carry *myocilin* mutations, but who do not have manifest glaucoma.

At the phenotypic level the role of OAG as a systemic disease and the biometric associations of advanced OAG were investigated. Using mortality data from over 27,000 people of whom 741 were known to have OAG, adjusted for gender and age at death, we identified a statistically significant association between death due to ischaemic heart disease and OAG. In a separate study investigating the systemic associations of OAG in 1,700 patients, a past history of migraine or presence of atherosclerosis was identified as being more common in patients with familial forms of OAG compared to people with sporadic disease. Biometric investigation of patients who had definitive end-stage glaucomatous visual field loss, confirmed that central corneal thickness was a significant risk factor for disease progression. Automatic optic disc imaging, which was performed on a subset of this end-stage cohort, revealed that the Stratus optical coherence tomography retinal nerve fibre

layer clock hour scan was most sensitive in detecting advanced disease. These findings may have important ramifications on phenotype-based screening programs.

At the genotypic level, the Asp658Gly variant in the *Winged Domain 40- repeat 36* gene was found, in a relatively small case-control study, to be a neutral variant in the Australian population and meta-analysis of the common *optineurin* Met98Lys, variant confirmed that its association with OAG, although weak, is highly statistically significant. Replicating previous work, two nonsynonymous variants in exon 1 of *lysyl oxidase–like 1* (Arg141Leu;Gly153Asp) were found to be strongly associated with pseudoexfoliative glaucoma. After validating a novel method of genome-wide association using equimolar DNA pools, where we were easily able to identify a strong association between markers at the *complement factor H* locus and age-related macular degeneration, genetic risk variants for OAG on chromosomes 3q21, 6p25, 14q13 and Xq25 were found. Nonetheless, further work is required before the association of variants at the novel OAG loci are definitively proven.

Accurate phenotypic descriptions, when compiled with relevant genetic information should enhance clinicians' understanding of the specific natural history of an individual patient's disease. Ongoing work investigating the clinical natural history and outcome to available therapy is required to correlate specific disease-causing variants with the phenotype, thereby bridging the clinician to the laboratory.

Acknowledgments

This body of knowledge is dedicated to Meggy who shared in the joys of discovery.

Marrying the clinician to the laboratory is an important endeavour, and I have certainly been fortunate to partake in the transfer of clinical questions generated at the slitlamp to the laboratory bench. The work that contributed to this thesis has provided the unique prospect of clinically phenotyping patients, collecting specimens from them, working in the laboratory and then returning to the clinic with useful molecular results. I am grateful to the many patients and study participants who are represented in every facet of this work.

It has been an immense privilege to have the opportunity to learn from Australia's prominent ophthalmic geneticists. Associate Professors Jamie Craig and David Mackey have been extremely supportive. Their refreshing approach to clinical-science differs positively; such that they complement each other well. They have certainly reinforced to me that many of the most pertinent questions relating to eye health arise in the clinic. Another important axiom that I learnt from them is that clinical research should be focused so as to ensure a translatable outcome. No doctorial candidate could seek more enthusiastic, driven or scientifically astute supervisors. I look forward to ongoing work with them.

In today's medical research environment, more than ever, significant contributions furthering the understanding into any discipline are being facilitated by coordinated teamwork. The time of solo authored scientific works presenting major findings has probably passed (see Nature. 2007 450:1165). Research and travel in different areas of medical-science ensured that many people contributed to this treatise. To reflect

this, I endeavoured in the body of this thesis to use the plural first person pronoun "we" rather than the singular form.

My initial interest in glaucoma was spawned by Dr Richard Cooper, who is certainly one of the most astute clinicians I have met. For example, two years prior to the publication of Estermann and colleagues (J Ocul Pharmacol Ther. 2006; 22:62-67), Richard mentioned his observation that donepezil lowers intraocular pressure. Being a modern day Priestly Smith, Richard's openness about the complexity of the glaucomas is humbling.

Over the course of this doctorial candidature I have had the opportunity to work in many clinical and research Departments. I am indebted to many people at the Clinical Genetics Unit at the Centre for Eye Research Australia, University of Melbourne and the Royal Victorian Eye and Ear Hospital where I spent my first year of study, in particular Lisa Kearns, as well as Drs Sonya Bennett, Johan Poulson, and Jon Ruddle. Maree Ring from the Department of Ophthalmology at the University of Tasmania performed much of the background genealogy included in Chapter 2 and Chapter 3. I am also very appreciative of the constructive, grammatical comments provided by Lori Bonertz on the manuscripts arising from this thesis. Many additional people contributed over the previous decade to the phenotyping and recruitment of participants for the Glaucoma Inheritance Study in Tasmania and the Twins Eye Study in Tasmania. In particular I am grateful to Drs Catherine Green and Johnny Wu.

I am also grateful for the support provided by many people from the Department of Ophthalmology at Flinders University and Flinders Medical Centre, where my final

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Dr Tim Chataway and Amy McCormick from the Department of Human Physiology at Flinders University introduced me to the evolving world of proteomics. Tarun Kakaday assisted with finite element modelling of proteomic biomarkers for glaucoma.

Being involved in the establishment of the *Myocilin* gene screening service possibly represents the most positive, clinically relevant outcome undertaken during my PhD candidature. As such, I am particularly appreciative for the work undertaken by Associate Professor Pamela Sykes and Drs Scott Grist and Andrew Dubowsky from the Department of Genetic Pathology at Flinders Medical Centre in facilitating this service.

Dr Pat Toohey was fundamental in the website development, which was the primary translation of the research described in Chapter 2. The haplotype analysis of Thr377Met *myocilin* families, presented in Chapter 2, was kindly performed in the laboratory of Associate Professor Mary Wirtz. Paul Sanfilippo assisted with much of the disease coding as described in Chapter 3. Dr John Linacre and Associate Professor Konrad Pesudovs provided assistance with the WINSTEPS programming utilized in Chapter 6.

Much of the content of the last section of Chapter 6 arose from discussions with numerous ophthalmic experts including: Drs Wido Budde; John Fingert; Paul Foster; David Garway-Heath; Catherine Green; Christopher Hammond; William Morgan; and Professors Wallace Alward; Sohan Hayreh; Jost Jonas; Paul Kaufman; Neil Miller; Nancy Newman; Harry Quigley; John Samples; and George Spaeth. It was thoroughly enjoyable to openly discuss broad topics, ranging for example from the architecture of the optic nerve head or means to develop a unifying theory for glaucomas, to the possible demise of Tasmanian Gondwanian forests. I also enjoyed immensely the discussion with Professor Don Melrose from the Department of

Theoretical Physics at the University of Sydney regarding the dimensionality of biological systems introduced briefly in the concluding chapter.

Funding for this work has been obtained from many sources and it has certainly been a privilege to be paid for undertaking such an enjoyable hobby. There can be no better community endorsement for research than the provision of funds to junior investigators. Financially I have been supported by a Medical Postgraduate Scholarship from the National Health and Medical Research Council (NHMRC) and a travel award from the NHMRC allowed me to undertake the laboratory work at the QIMR which contributed predominately to Chapter 5. The Australia-China Special Funding scheme of the International Science Linkages programme allowed travel the Zhongshan Ophthalmic Centre, Guangzhou, People's Republic of China, to visit the exciting Ophthalmic twin project established by Associate Professor Mingguang He. Travel fellowships from the Association for Research in Vision and Ophthalmology (ARVO) and the Singapore Eye Research Institute/ARVO permitted some of the results from this thesis to be presented. The majority of this work has also been sustained by project grants from the NHMRC, the Ophthalmic Research Institute of Australia, the American Health Assistance Foundation as well as an NHMRC Enabling Grant.

Manuscripts published during the Doctoral candidature (those marked with an asterisk contributed directly to this thesis):

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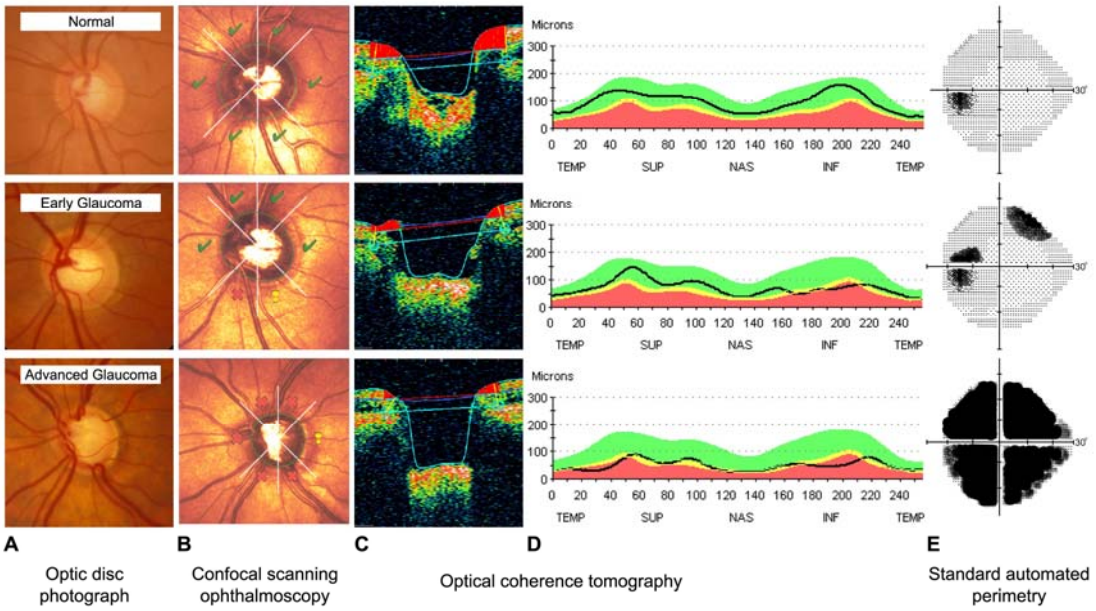
Chapter 1 – INTRODUCTION: The significance and pathoaetiology of the glaucomas.

The glaucomas are the principal cause for optic nerve degeneration and one of the leading causes for irreversible blindness worldwide (Quigley 1996; Resnikoff et al., 2004). They are a heterogeneous group of disorders, of which primary open-angle glaucoma (OAG) is the most common subset. Although the definition of OAG has not been consistent across studies, it is generally referred to as a progressive excavation of the optic disc with corresponding loss of visual field (Foster et al., 2002). OAG is often, but not invariably, associated with an elevated intraocular pressure (IOP) (Hollows and Graham, 1966). In over 20% of cases, IOP elevation is absent and a diagnosis of normal tension glaucoma (NTG) can be made (Hollows and Graham, 1966; Kamal and Hitchings, 1998). Although it may be erroneous to sub-classify OAG as high-tension glaucoma (HTG) on the basis of an IOP greater than 21mmHg, there is evidence that even in NTG therapeutic lowering of IOP may slow further loss of visual field (van der Valk et al., 2005). Further to this, both the NTG and HTG groups could be further subcategorized (e.g. a NTG cohort may comprise people with principally vascular risk factors for OAG, neurodegenerative OAG, or misclassified HTG, i.e. people with erroneously low applanation IOP readings). It is difficult to diagnose OAG in the early stages because of the subtlety of its clinical features (Figure 1-1).

Figure 1-1

Spectrum of glaucomatous disease. (A) Optic nerve photography: small central cup in healthy eye; enlargement of cup and loss of neuroretinal rim in glaucomatous eye. (B) Confocal scanning laser ophthalmoscopy: neuroretinal rim area within normal limits (ticks) in healthy eyes, but reduced in glaucomatous eyes (crosses). (C) Optical coherence tomography: cross section of optic disc displaying deep large cup in glaucomatous eye with thin neuroretinal rim (red). (D) Optical coherence tomography: retinal nerve fibre layer thickness in each sector is normal (black line within green region) in healthy eyes, yet is markedly reduced in advanced glaucoma (black line dipping into red region). (E) Standard automated perimetry: normal blind spot in healthy eyes, with progressive loss of sight in advancing glaucoma severity.



Large population-based epidemiological studies have revealed that the prevalence of OAG in Australia is between 2.3 and 4.4 % in people aged greater than 49 years (Mitchell et al., 1996; Wensor et al., 1998). Definite OAG in Caucasians aged more than 40 years, has an overall 5-year incidence between 0.5% and 0.62%, with the incidence increasing with age (de Voogd et al., 2005; Mukesh et al., 2002). Alarmingly, in the general community more than half of the people with OAG remain undiagnosed – a statistic that has not improved over the past 40 years, providing further support for the notion that current screening algorithms are failing (Hollows and Graham, 1966; Mitchell et al., 1996; Wensor et al., 1998).

To date, ophthalmic-based screening systems for OAG have specifically incorporated assessment of the optic disc, IOP measurement and investigation for visual field deficit. Given the high likelihood of missing incident disease and the fact that many people are repeatedly reviewed unnecessarily, such methods are not cost-effective for a community (Tuck and Crick, 1997). Strategies to eliminate the blinding toll of glaucoma must be aimed at identifying at-risk individuals. The corollary of this is that a screening regimen must be highly sensitive and specific so as to only detect potentially serious disease not pseudo-disease (Harris 2005). Because OAG is initially asymptomatic, effective screening techniques should identify people with no obvious signs or symptoms of the disease, allowing early diagnosis and management.

Glaucoma is a model disease for evaluation of genetic screening in a complex disease. The evidence for success of OAG treatment, the mainstay of which is IOP reduction, is expanding (van der Valk et al., 2005). Increased clinical screening of genetically at-risk individuals would allow early therapeutic intervention prior to the

loss of visual function. Despite OAG being identified as a clinical entity almost as soon as the ophthalmoscope was developed, the precise pathogenesis remains elusive (von Graefe 1857). Our current understanding of the disease mechanisms at the molecular level is relatively poor.

The field of genetics is pivotal in understanding underlying molecular mechanisms and pathways. The advances in methods for genetic screening continually add to the clinician's diagnostic armoury. It must be recognized that some individuals have a misplaced fear that draconian intervention is required when a genetic predisposition is recognized however; this is generally not the case. For example, the identification of the genetic predisposition to phenylketonuria has allowed thousands of at-risk individuals to avoid dietary stressors, with a subsequence avoidance of mental retardation (Lenke and Levy, 1980).

This chapter will summarise the current understanding of the genetics of OAG, clearly delineating it as a complex trait, and then briefly explore potential avenues for future breakthroughs. This review will not discuss developmental or congenital glaucoma for which much genetic progress has been made (Mackey and Craig, 2003; Sarfarazi et al., 2003).

Current understandings of the genetics of OAG:

Prior to embarking on a full-scale probe for the genes involved in OAG, it is necessary to first consider the evidence supporting the fact that glaucoma is a "genetic disease." Over the past century there has been a paradigm shift in the understanding of the inheritance of OAG. In 1927 it was stated that "cases of

hereditary glaucoma, though by no means unknown, are yet relatively rare"(James 1927). This example was followed by the 1932 publication of Julia Bell's Treasury of Human Inheritance, which contains a large section on the inheritance of glaucoma (Bell 1932). In it she noted that "… certainly relatively few good pedigrees of the condition have ever been published."

Family history has now been revealed to be one of the most important risk factors for OAG development (Tielsch et al., 1994). The Glaucoma Inheritance Study in Tasmania (GIST) found a positive family history is found in over 50% of cases of glaucoma (Green et al., 2007). Furthermore, the screening of relatives has been proven to be a successful strategy for OAG case detection (Miller and Paterson, 1962; Vernon 1991). Investigators from the Rotterdam Eye Study investigated the familial aggregation of OAG by examining not only first-degree relatives of glaucoma cases identified through their prevalence study but also a matched set of controls (Wolfs et al., 1998). Wolfs and colleagues found that first-degree relatives of OAG patients had a 22% risk of developing glaucoma in comparison to 2.3% in the relatives of controls, implying a 10 fold increased relative risk of the disease in first degree relatives of affected patients compared with the general population (Wolfs et al., 1998). Although this study was rigorously conducted it could, however, underestimate the genetic component of glaucoma, especially if the children of glaucoma cases were too young to manifest the disease. There is often a poor knowledge of glaucoma family history (McNaught et al., 2000). While it is clear that many diseases have a tendency to run in families, it may be difficult to dissect out whether this is due to familial sharing of a similar environment, or to similarity in genetic predisposition. For example, it has been shown that attending medical school

aggregates in families, as probably does a preference for eating vegemite on toast (McGuffin and Huckle, 1990).

Racial differences in prevalence of OAG exist. The prevalence in Africans is estimated to be six times as high, in certain age groups, as that in Caucasians (Buhrmann et al., 2000; Ntim-Amponsah et al., 2004; Racette et al., 2003). The finding of a similar greater prevalence in Africans and African-Americans lessens the likelihood that such differences are primarily due to external societal or environment-specific confounders (Tielsch et al., 1991). The differing genetic composition of African-Americans compared to Caucasian-Americans may account for the difference in OAG prevalence. Interestingly, OAG is thought to be extremely rare in Australian Aboriginals (Hollows 1980; Mann 1966).

Further evidence for a genetic basis of OAG stems from twins studies. The ophthalmic literature is peppered by case descriptions of identical twins concordant for OAG and NTG (Gedda et al., 1970; Ofner and Samples, 1992; Teikari et al., 1987). In a large series by Gottfredsdottir and colleagues, OAG was found to be significantly more concordant in monozygotic twin pairs (98.0%) than their spouses (70.2%)(Gottfredsdottir et al., 1999).

A fundamental genetic paradigm for OAG is also supplemented by the fact that some non-human animal species also develop heritable forms of OAG (Gelatt et al., 1998a). Inherited spontaneous OAG has been identified in rhesus monkeys (*Macaca mulatta*) and both autosomal recessive and dominant OAG is present in dog breeds (in particular the beagle and miniature poodle)(Gelatt et al., 1998a).

In the majority of OAG cases it is likely that more than one genetic predisposition is required to manifest disease, and it is generally well accepted now that OAG is a complex trait. Since the first description of a heritable form of OAG by Benedict in 1842, a number of genetic loci have been reported and a smaller number of genes have been implicated or identified (Table 1-1) (Benedict 1842).

Table 1-1
Identified primary open-angle glaucoma loci.

Loci	OMIM	Gene	Location	Initial linkage / gene identifying study	Typical Phenotype
GLC1A	601652	*myocilin*	1q23-25	(Sheffield et al., 1993; Stone et al., 1997)	JOAG / HTG
GLC1B	606689		2cen-q13	(Stoilova et al., 1996)	NTG / HTG
GLC1C	601682		3q21-24	(Wirtz et al., 1997)	HTG
GLC1D	602429		8q23	(Trifan et al., 1998)	NTG / HTG
GLC1E	602432	*optineurin*	10p15-14	(Rezaie et al., 2002; Sarfarazi et al., 1998)	NTG
GLC1F	603383		7q35-q36	(Wirtz et al., 1999)	HTG
GLC1G	609669	*WDR-36*	5q21-35*	(Monemi et al., 2005; Samples et al., 2004)	NTG / HTG
GLC1H	611276		2p16.3-p15	(Suriyapperuma et al., 2007)	NS
GLC1I	609745		15q11-13	(Allingham et al., 2005b)	NTG / HTG
GLC1J	608695		9q22	(Wiggs et al., 2004)	JOAG
GLC1K	608696		20p12	(Wiggs et al., 2004)	JOAG
GLC1L	137750		3p22-p21	(Baird et al., 2005a)	HTG
GLC1M	610535		5q22.1-q32	(Pang et al., 2006)	JOAG
GLC1N	611274		15q22-q24	(Wang et al., 2006)	JOAG

* Locus may contain more than 1 glaucoma associated gene
Abbreviations: HTG, high-tension glaucoma; NTG, normal tension glaucoma; JOAG, juvenile onset glaucoma; NS, not specified.

***Myocilin* Glaucoma:**

The 1997 discovery of the *myocilin* gene (*MYOC*) has significantly impacted upon many OAG families (Stone et al., 1997). The *MYOC* gene (formerly referred to as the trabecular meshwork-induced glucocorticoid response protein or TIGR) was mapped to 1q where the locus for the juvenile form of OAG had previously been identified (GLC1A)(Sheffield et al., 1993; Stone et al., 1997).

MYOC encodes a predicted 504 amino acid polypeptide and contains two major domains, an N-terminal myosin-like domain and a C-terminal olfactomedin-like domain.(Green and Klein, 2002) The encoding region is divided into three exons, of which the majority of the disease-causing variations are clustered in the olfactomedin homology domain of the third exon. The structure of the MYOC protein has been well conserved through evolution (Mukhopadhyay et al., 2002).

Although MYOC is found ubiquitously in the eye, it is also expressed in many extraocular tissues, suggesting that it may not have an eye-specific function (Fingert et al., 2002; Karali et al., 2000). However, it is in the trabecular meshwork (TM) where the primary consequences of *MYOC* dysfunction are found (Jacobson et al., 2001). In the TM, MYOC has been revealed to principally interact with optimedin, an olfactomedin-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 OAG investigation or therapeutic intervention.

Despite numerous descriptions of nonsense and premature termination mutations, haploinsufficiency of the MYOC protein appears unlikely to be the primary disease-causing mechanism (Wiggs and Vollrath, 2001). Cell expression studies comparing

mutant to normal MYOC secretion levels suggest that OAG develops either because of insufficient or compromised MYOC secretion from TM cells due to congestion of the TM secretory pathway (Jacobson et al., 2001). The work of Liu and Vollrath, which demonstrated that mutant forms of the MYOC protein are misfolded and aggregate in the endoplasmic reticulum, also provided weight to a gain–of–function disease model (Liu and Vollrath, 2004). A model of disease causation principally through reduced Triton solubility of MYOC was further supported by the finding that glaucoma was not induced through genetically increasing or decreasing normal *MYOC* expression (Gould et al., 2004). Interestingly, people who are homozygous for *MYOC* mutations do not seem to manifest severe disease indicating a novel mode of inheritance (Hewitt et al., 2006a; Morissette et al., 1998).

Substantial evidence now exists to suggest that approximately one in 30 unselected OAG patients has a *MYOC* mutation (Fingert et al., 1999). To date more than 40 disease-associated mutations in MYOC have been identified (Fingert et al., 2002), with the Gln368STOP mutation the most common individual glaucoma causing variant worldwide (Fingert et al., 1999). It has been revealed that the majority of patients with this specific mutation have descended from a single ancestor harbouring the *MYOC* Gln368STOP (Baird et al., 2003; Faucher et al., 2002). The second most common *MYOC* mutation identified in Australia, which is also found worldwide, is the Thr377Met mutation (Fingert et al., 1999; Mackey et al., 2003).

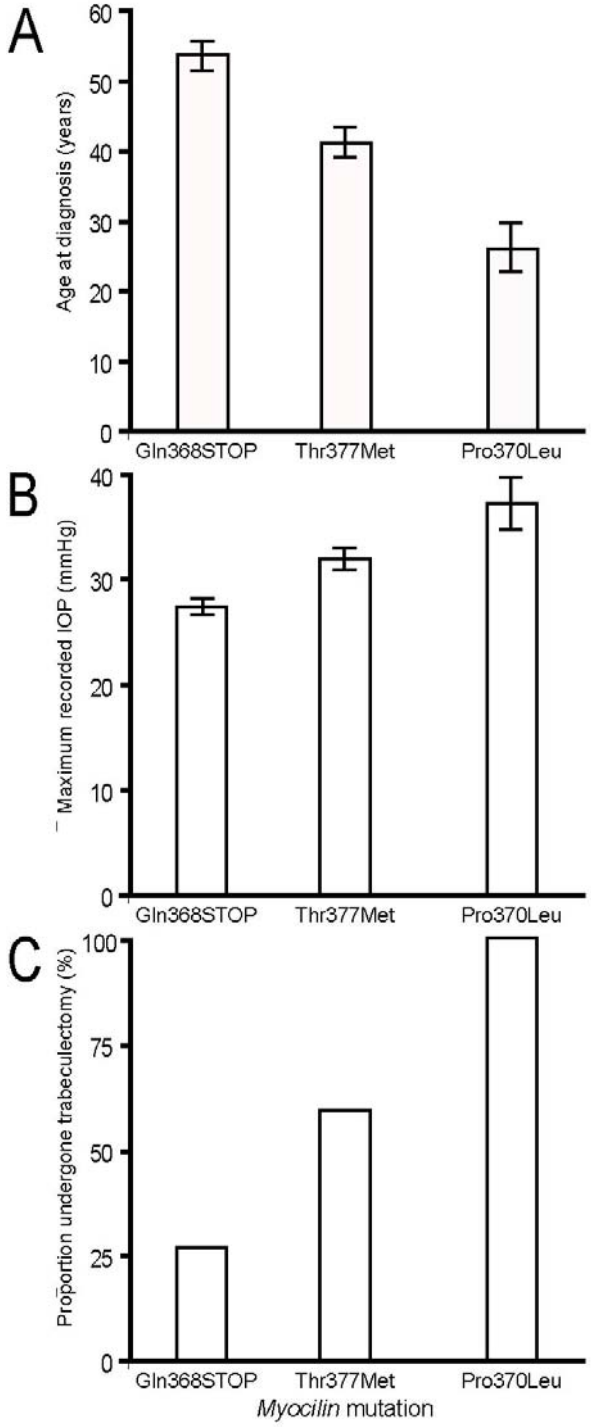
The clinical pattern of *MYOC* glaucoma reflects the specific underlying mutation. *MYOC* is traditionally thought of as having a HTG phenotype, with some mutations (such as Pro370Leu) causing severe juvenile-onset OAG (Alward et al., 1998). The distribution of age and maximum recorded IOP for Australian patients with both the

Gln368STOP and Thr377Met *MYOC* mutations is similar to descriptions of other pedigrees with these mutations (Allingham et al., 1998; Alward et al., 1998; Craig et al., 2001; Graul et al., 2002; Mackey et al., 2003; Puska et al., 2005; Shimizu et al., 2000). A stepwise decrease in the mean age at diagnosis across Australian patients with the Gln368STOP, Thr377Met and Pro370Leu *MYOC* mutations is mirrored by a reciprocal increase in maximum recorded IOP (Figure 1-2)(Hewitt et al. 2006b). It is clear that the specific *MYOC* mutation can be inferred by individual clinical features (genotype-phenotype correlation).

Currently it is not cost-effective to conduct population-based screening for *MYOC* mutations (Aldred et al., 2004). However, the efficacy for genetic screening will increase when conducting comprehensive combined screen of many OAG genes and as the cost of genetic tests decreases markedly due to technological advances. An in-depth investigation of the genotypic and phenotypic association of *MYOC*-related glaucoma is undertaken in Chapter 2.

Figure 1-2

The stepwise decrease in mean age (A) at diagnosis across Australian patients with the Gln368STOP, Thr377Met and Pro370Leu *Myocilin* mutations, with a reciprocal increase in maximum recorded intraocular pressure (B) and proportion requiring filtering surgery (C) (from Hewitt et al. 2006b).



***Optineurin* Glaucoma:**

The second OAG gene identified was the *optineurin* (*OPTN*) gene at the GLC1E locus (Rezaie et al., 2002). The GLC1E locus was initially mapped from a large British pedigree with autosomal dominant NTG (Sarfarazi et al., 1998). Referring to "optic neuropathy-inducing," *OPTN* is located on the short arm of chromosome 10 and encodes a 147 amino acid polypeptide (Rezaie et al., 2002). *OPTN* has a pivotal role in exocytosis as well as Golgi ribbon formation and is potentially involved with the FAS-ligand as well as the tumour necrosis factor-α (TNF-α) apoptotic pathways (Sahlender et al., 2005; Sarfarazi and Rezaie, 2003). Although *OPTN* has been demonstrated to be up-regulated after exposure to TNF-α and dexamethasone (Vittitow and Borras, 2002), its response to elevated IOP remains controversial (Kamphuis and Schneemann, 2003; Vittitow and Borras, 2002).

Mutations in *OPTN* account for approximately 16.7% of familial OAG from an NTG index case, however are only likely to constituted approximately 0.1% of unselected OAG cases (Alward et al., 2003; Aung et al., 2003; Rezaie et al., 2002; Wiggs et al., 2003). The most common *OPTN* disease-causing variant is Glu50Lys (Rezaie et al., 2002). Individuals with this mutation develop aggressive NTG and have a lower age at diagnosis (mean ± SD: 40.8 ± 11.0 years) and greater need for trabeculectomy compared to other non-*OPTN* NTG cases (Aung et al., 2005). The clinical importance of many other *OPTN* variants (in particular Met98Lys) remain controversial (Alward et al., 2003; Aung et al., 2003; Fuse et al., 2004; Jansson et al., 2005; Leung et al., 2003; Rezaie et al., 2002; Tang et al., 2003; Umeda et al., 2004; Weisschuh et al., 2005; Wiggs et al., 2003; Willoughby et al., 2004). In a Japanese cohort Funayama and colleagues found that OAG patients were more likely than control subjects to have both the *TNF-α*/-863A change with the *OPTN* Met98Lys

variant (Funayama et al., 2004). In support of a NTG-modifying variant, Melki et al. reported that the Met98Lys substitution may be associated with a lower IOP at the time of diagnosis and may even modify *MYOC* glaucoma (Melki et al., 2003a).

Additional Glaucoma Genes:

Investigating the genetics of pedigrees with diseases with late age of onset is difficult. The parents of OAG cases are often deceased, whilst the patients' children are frequently too young to manifest disease. Confounding this further is the fact that OAG can be discordant in time, differing in age of onset for some related cases, and there is often also considerable overlap between glaucoma families (Sack et al., 1996). Despite these issues, large pedigrees have been genetically linked and numerous loci have been identified (Table 1-1), although not all have been replicated in later studies.

During the first month of my doctorial candidature, evidence was provided implicating the *WD repeat-containing protein 36* (*WDR36*) gene in causing OAG (Monemi et al., 2005). To date this finding has not been fully replicated in the published literature; however, one preliminary study has not supported this finding (Allingham et al., 2005a). In addition, the original family that provided the initial and only evidence of linkage to the GLC1G locus has not been found to contain coding region mutation in *WDR-36* segregating with the disease phenotype (Kramer et al., 2006). Further discussion and investigation of the *WDR36* gene is performed in the first section of Chapter 5. Researchers must be cautious about heralding novel disease-causing genes until such time as confirmatory replicate studies are reported.

A number of OAG loci have cytogenetic support in the published literature. Cases of congenital glaucoma due to cytogenetic derangement at the GLC1B (Mu et al., 1984), GLC1C (Allderdice et al., 1975; Kondo et al., 1979), GLC1D (Cohn et al., 2005), and GLC1F loci have been described (Kato et al., 2001; Speleman et al., 2000). It is certainly possible that mildly deleterious mutations cause OAG, whilst more significant rearrangement of these underlying genes cause a markedly more severe disease phenotype (such as congenital onset glaucoma). It is also interesting that the GLC1F locus is in close proximity to (but does not seem to overlap) a locus for Pigment Dispersion Syndrome (GPDS1)(Andersen et al., 1997; Anderson et al., 2002).

In 2000, Wiggs and colleagues reported a genome-wide scan for OAG using a sib pair multipoint analysis (Wiggs et al., 2000). This study identified suggestive linkage to a region near the GLC1B locus on chromosome 2 and at loci on chromosomes 14, 17 and 19 (Wiggs et al., 2000). Following this, a genome-wide scan of OAG families of African descent highlighted causative gene regions on chromosomes 2q and 10p (Nemesure et al., 2003). The 10p locus implicated by this study did not include the *OPTN* gene (Nemesure et al., 2003). Recently, a novel OAG locus on the short arm of chromosome 3 was proposed, using a genome-wide scan, as dominantly independently segregating in a large Australian family with some affected members carrying the Gln368STOP *MYOC* mutation (Baird et al., 2005a).

It is noteworthy that many of the implicated OAG loci have been identified from the same clinic base (e.g. GLC1C, GLC1F, GLC1G)(Samples et al., 2004; Wirtz et al., 1997; Wirtz et al., 1999). The upshot of such a finding is that either few research

groups have the facilities for genomic work or that further informative families remain to be identified in other geographic regions.

OAG and Genetic Association studies:

Numerous genetic association studies for OAG have been conducted. Many of these studies have had conflicting results or have not been replicated. When reviewing this ever increasing list of gene alleles studied in OAG (Table 1-2), it is important to note that such tabulation of this data only facilitates crude comparison. These studies often include different racial groups or subtypes of OAG (e.g. HTG versus NTG) and some suffer from inadequate powering or poorly characterized and matched control groups. Frequently, different alleles within the same gene have been studied, making direct comparison of the literature difficult. Should a specific haplotype be revealed to be associated with the disease it is also important to consider that this finding may represent a type one error. Alternatively it may have occurred as the result of linkage disequilibrium (LD) or the influence of neighbouring genes. LD is the tendency of alleles to be inherited together, rather than would be expected given their known frequency in a population and the recombination fraction between the loci. For conciseness, studies investigating the association between various blood groups and OAG have been omitted from Table 1-2. Failure to replicate a genetic association may occur due to locus or allele heterogeneity, as well as under-powering a study to account for LD.

Table 1-2

Conflicting evidence for gene-disease interaction in primary open-angle glaucoma.

Gene / Allele	GenBank Accession No.	Location	Positive / Supporting Studies	Negative / Non-replicating Studies
GSTM1	NM_000561	1p13	(Juronen et al., 2000; Yildirim et al., 2005)	(Jansson et al., 2003)
MTHFR	NM_005957	1p36	(Junemann et al., 2005)	-
MYOC.mt1	NM_000261	1q24	(Colomb et al., 2001; Polansky et al., 2003)	(Alward et al., 2002; Fan et al., 2004; Ozgul et al., 2005; Sjostrand et al., 2002)
REN	NM_000537	1q32	-	(Hashizume et al., 2005)
AGT	NM_000029	1q42	-	(Hashizume et al., 2005)
ACP1	NM_177554	2p25	(Abecia et al., 1996)	-
AGTR1	NM_000685	3q21	-	(Hashizume et al., 2005)
TF	AH010951	3q21	-	(Abecia et al., 1996)
OPA1	NM_015560	3q28	(Aung et al., 2002b; Aung et al., 2002a; Powell et al., 2003)	(Woo et al., 2004)
B2AR	NM_000024	5q32	-	(Gungor et al., 2003)
GLO1	NM_006708	6p21	-	(Abecia et al., 1996)
CDKN1A	NM_000389	6p21	(Tsai et al., 2004)	-
TAP1/2	NM_000593	6p21	(Lin et al., 2004)	-
TNFα	NM_000594	6p21	(Funayama et al., 2004; Lin et al., 2003)	-
EDN1	NM_001955	6p24	-	(Logan et al., 2005)
NOS3	NM_000603	7q36	(Logan et al., 2005)	(Lin et al., 2005)
IGF2	NM_000612	11p15	(Tsai et al., 2003)	-
GSTP1	NM_000852	11q13	-	(Juronen et al., 2000; Yildirim et al., 2005)
CMA1	NM_001836	14q11	-	(Hashizume et al., 2005)
TP53	NM_000546	17p13	(Lin et al., 2002; Ressiniotis et al., 2004a)	(Acharya et al., 2002; Dimasi et al., 2005)
ACE	NM_000789	17q23	-	(Bunce et al., 2005; Hashizume et al., 2005; Ozkur et al., 2004)
MPO	NM_000250	17q23	-	(Lin et al., 2005)
APOE	NM_000041	19q13	(Copin et al., 2002; Fan et al., 2005; Junemann et al., 2004; Mabuchi et al., 2005; Vickers et al., 2002)	(Ressiniotis et al., 2004c; Ressiniotis et al., 2004b)
GSTT1	NM_000853	22q11	-	(Juronen et al., 2000; Yildirim et al., 2005)
AGTR2	NM_000686	Xq22	(Hashizume et al., 2005)	-

If it is challenging enough to replicate causative disease loci in OAG, it seems more difficult to replicate a positive finding for a predisposing genetic risk allele. A part of this problem is that molecular pathways have been used to work backwards to a genetic predisposition. As in the case of *nitric oxide synthase 3 (NOS3)*, different expression patterns were found in the aqueous humour of glaucomatous patients compared to matched patients (Lin et al., 2005; Logan et al., 2005). However, when nucleotide polymorphisms that had been proven to be functionally important in the *NOS3* gene were investigated, conflicting results were obtained (Lin et al., 2005; Logan et al., 2005). Such negative associations may reflect the fact that the initial hypothesis was based on a substance important in the down-stream pathogenetic pathway. The premise that because retinal ganglion cell death in glaucoma occurs through apoptosis, any pro-apoptotic allele in the respective cascade should be found more commonly in OAG cases whilst not unreasonable, may not prove to be the case.

Animal models for OAG have also identified genes involved in glaucoma and susceptibility to optic neurodegeneration. Through a series of back and intercrosses, mutations in the *glycoprotein NMB* (*GPNMB*) gene on the telomeric region of the long arm of chromosome 7 were found to cause pigmentary glaucoma in DBA/2J mice (Anderson et al., 2002). Follow-up studies in this same animal glaucoma model have found that deficiency of the pro-apoptotic *BCL2 associated X protein* gene slow retinal ganglion cell death and that neurodegeneration can be prevented by high-dose radiation with bone marrow transfer (Anderson et al., 2005; Libby et al., 2005). Pathogenetic pathways that may be intrinsically involved in animal models need to

be investigated in human cohorts prior to advocating their adoption in population-based screening platforms or targeted therapy.

The genetics of complex traits: Is glaucoma lagging?

Complex disorders lack a simple Mendelian mode of inheritance, and therefore a single underlying susceptibility gene cannot be assumed. Disease expression in OAG cases most likely involves more than one gene, of which some may display incomplete penetrance or variable expressivity. Nevertheless, the 'holy grail' of genetic research into complex traits, is the identification a single locus of large effect.

The human genome contains approximately three billion nucleotides and close to 30,000 genes (International Human Genome Sequencing Consortium 2004). However, caution must be ascribed when reviewing this figure. Analogous to Matthew Flinders (1774-1814) producing his "General chart of Terra Australis or Australia" in 1804, we now appreciate that this first mapping neglected much of our coastline, including many bays and inlets. Similarly some 200 years later, despite the 'complete genome mapping', many functionally significant regions are still unknown.

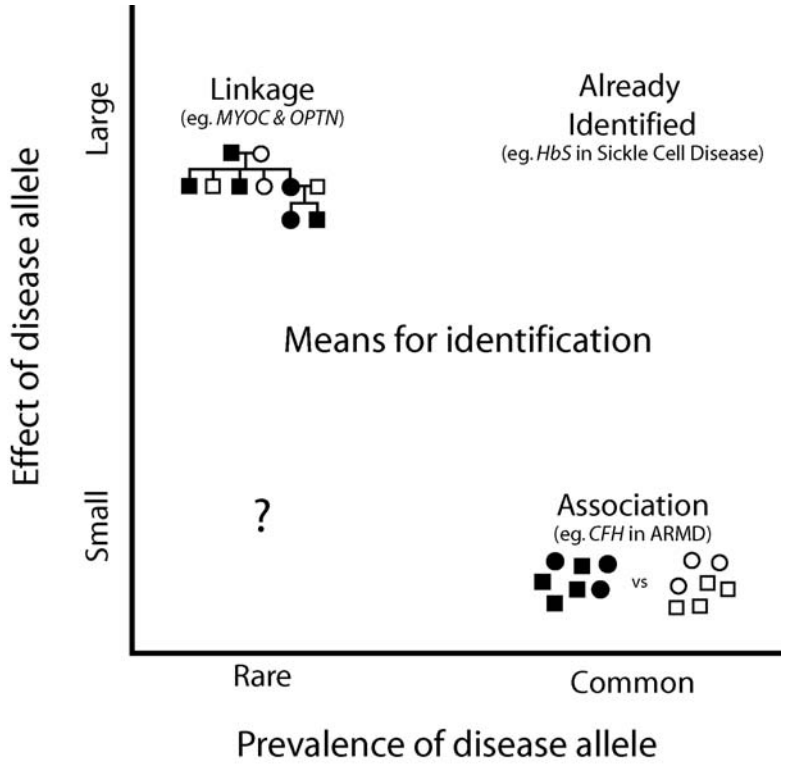
In the human genome, sequence variation include: single nucleotide polymorphisms (SNPs); insertions or deletions of a few nucleotides; and variation in the repeat number of a motif (micro-satellites) (Nowotny et al., 2001). Previous familial and sib-pair linkage studies have relied principally on micro-satellite markers. However, SNPs are more abundant and densely distributed than micro-satellites, occurring approximately every 1,000 basepairs along the human genome, and thus making them more suited to high-resolution genotyping (Nowotny et al., 2001). SNPs can

occur in gene coding regions as well as the intervening regions (introns). To date considerable disease-gene research has focused on the coding (exons) and, to a lesser extent, the promoter regions of genes. However, recent evidence suggests that intronic regions are subject to stronger selective constraint and thus, may be functionally more important than previously presumed (Andolfatto 2005). Obscuring the clarity of understanding in gene function further, is the fact that remarkably few genes are found in the human genome compared to other species (International Human Genome Sequencing Consortium 2004). It is now clear that many genes can produce more than one protein (through alternate splicing) and that different proteins arising from the same gene can have dramatically different functional roles (Zhang et al., 2005).

The allelic architecture for almost all common diseases is still being uncovered. Generally, the disease-causing variants (or mutations) in the population can vary in prevalence (being either rare or common) whilst the effect exerted by the specific allele can also differ in magnitude (small to large effect) (Figure 1-3). Given the paucity of gene identification in complex traits, it is clear that common genes of large effect are extraordinary and do not account for such diseases (Chakravarti 1999). Considering extreme cases, if rare alleles account for the prevalence of common disease it is likely that affected individuals have mutations at only one of many possible disease loci (as may be the case for cardiovascular disorders (Williams et al., 2004)). Conversely if the alleles are common in the population, then people with disease have mutations at multiple loci simultaneously (as may occur in colorectal adenomas (Fearnhead et al., 2004)). Given that rare Mendelian diseases have many rare variants, it is reasonable to postulate that common diseases may also have many rare variants. If multiple common genes are involved in a common complex disease,

it is crucial to determine whether a sole mutation at any particular gene is sufficient and necessary to cause disease (Chakravarti 1999). Given this possibility, dismissal of any gene proposed to be associated with OAG is difficult.

Figure 1-3
Allelic architecture of genetic diseases.



If strong epistasis prevails, a mutation may be necessary for a particular phenotype (Chakravarti 1999). Epistasis classically assumes that genes do not act alone, but rather that particular genotypes or environmental factors formulate gene expression. However, gene-gene interactions are likely to be multifaceted such that despite one beneficial gene-gene allele being present, disease may manifest by a separate gene interaction that is 'endorsed' by a separate detrimental allele. Stochastic environmental factors are also likely to have a marked influence in disease expression. Post-translational modification is the proteolytic cleavage following DNA replication. Many proteins are synthesized as inactive precursors that are activated under physiological conditions by limited proteolysis (such as methylation). Although gene-environment interactions can be modelled, the detection of precise environmental stressors (especially in OAG) has been difficult (Potter 2001). It is clearly a formidable task to cleanly dissect the underlying mechanisms for many complex diseases, in which there are likely to be several genetic and environmental factors involved in the pathophysiology.

The Bottleneck of Glaucoma Genetics:

Many potential avenues exist for untwining the complex genetics of OAG. It is likely that adopting a combination of approaches and pursuing many genetic methods will allow the bottleneck of glaucoma genetics to be broken.

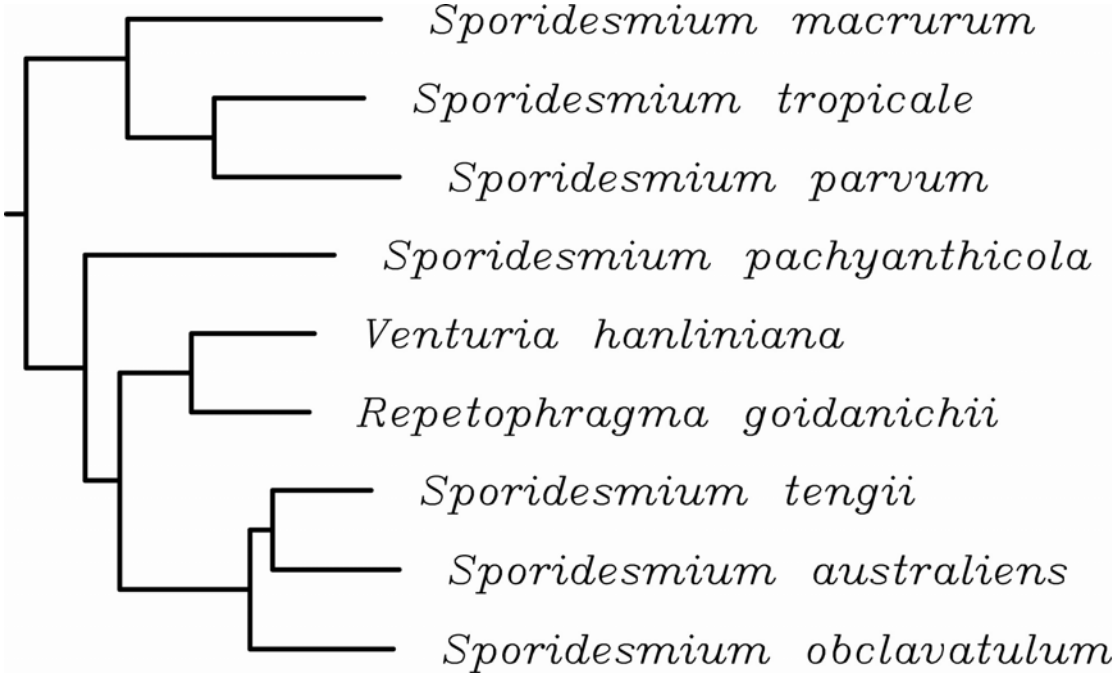
Searching for further pedigrees around the world has had limited success. OAG gene identification by conventional linkage analysis has been greatly complicated by phenocopy or intra-pedigree genetic heterogeneity (Craig et al., 2001; Sack et al., 1996). These issues of phenocopy, where a disease in separate patients seems

clinically identical, yet is found to have a different aetiology; and variable expressivity, where the same gene mutation causes a variety of phenotypic effects, is not unique to glaucoma, let alone the eye. For example prior to recent molecular work, fungi were principally characterised by their morphological appearance. However, an increased understanding of their underlying gene sequences has revealed that despite some fungal species being genetically similar they have remarkably different structures (analogous to phenotypic heterogeneity or variable expressivity). Conversely some taxa which have similar appearance are actually genetically very different (analogous to genotypic heterogeneity) (Figure 1-4).

Compounding the issues of phenocopy and variable expressivity is the initial obscurity of clinical diagnosis in early OAG. The determination of linkage is principally a statistical process and uncertainties introduced about clinical status significantly reduce the power of any such studies. Despite this, pedigree linkage studies have good power for detecting uncommon genes of major effect (as was the case for *MYOC* and *OPTN* glaucoma).

Figure 1-4

Phenocopy and phenotypic heterogeneity (variable expressivity) between asexual fungus species. In this phylogenetic tree, which was generated using the CLUSTAL W program and data on the *28S ribosomal RNA* gene from the NCBI taxonomy database, it is clear that several species of the *Sporidesmium* genus are phylogenetically distant (e.g. *S. australiens* and *S. tropicale*), despite being morphologically similar (phenocopy). Conversely, other taxa (e.g. *Venturia hanliniana* and *Repetophragma goidanichii*) are morphologically different, yet are similar genetically (representing phenotypic heterogeneity or variable expressivity). In this neighbour-joining plot, line distances represent the relative degree of similarity, with the shorter the line being more similar. Based on the work of Shenoy et al. (Shenoy et al., 2006).



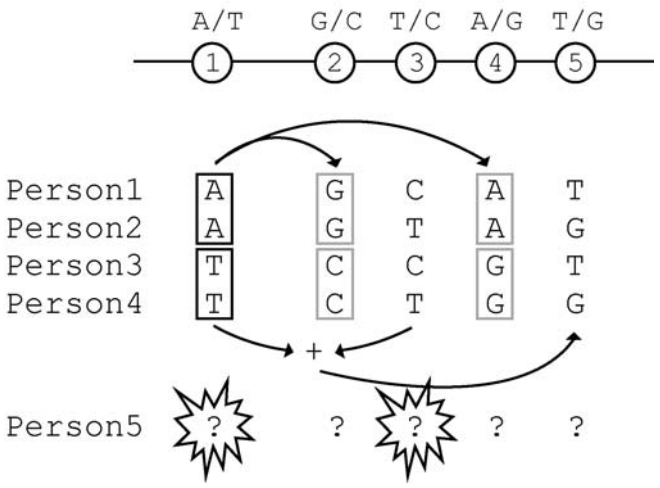
Previous linkage of OAG pedigrees and sibling pairs using micro-satellite markers may have had a high false negative result, being insufficient to detect true loci. With improved knowledge of the SNP variation across the genome, it may be beneficial to reinvestigate these pedigrees using SNP-based platforms. The high SNP density allows loci to be defined more precisely (John et al., 2004). A major technical obstacle for genome-wide SNP investigations, high through-put diploid PCR amplification, has recently been overcome through a highly multiplexed microarray genotyping system (Syvanen 2005). Nonetheless, the difficulty in selection of most advantageous markers and the high cost are drawbacks. Additionally in any SNP selection it is important to know the allele frequencies in the population, something which the HapMap project has addressed (Altshuler et al., 2005). Linkage studies provide a partial scaffold for association studies. Through identifying chromosomal regions of interest, linkage studies substantially reduce the resources required for gene-mapping association.

Whilst the high age at functional impairment or manifestation of OAG increases the difficulty in performing a pedigree linkage analysis, it may improve the power of an association study (through conserved LD regions). From the evolutionary viewpoint, diseases with a late onset of functional impairment may not have conferred a negative selection pressure. Thus, founder haplotypes, as has been found in *MYOC*, may exist (Baird et al., 2003). Recombination hotspots are widespread and account for LD structure across the genome. The efficacy of fine mapping can be improved through adopting a tagged-SNP approach (Figure 1-5). In understanding the majority of common variations in the genome, choosing non-redundant sets of SNPs (i.e. SNPs not in LD) offers considerable efficiency without loss of study power. In this

way genomic regions can be tested for association without requiring the discovery of the exact functional variant (Machini et al., 2005).

Figure 1-5

To identify which variants or SNPs (top of figure) a person carries, one can either genotype all the SNPs (i.e. 1 through to 5), or alternatively tagging SNPs can be selected. Here it can be seen that every person who has an "A" nucleotide at SNP 1, also has a "G" and "A" nucleotide at SNP positions 2 and 4 respectively. Hence, these SNPs are in complete linkage disequilibrium. Further to this, knowledge of SNP 1 and 3 allows the nucleotide at SNP 5 to be determined. Hence, for Person 5 a picture of their genomic sequence at this locus can be identified through only genotyping SNPs 1 and 3.



SNP-based strategies for complex disease have had recent success. Identification of a single risk allele for age-related macular degeneration (AMD) in the *complement factor H* gene was achieved through a focused fine SNP mapping (Edwards et al., 2005; Klein et al., 2005). Given that subjects with severe disease were included in these case-control association studies, thereby increasing the power to detect an

important allele, it is very likely that the prevalence of that allele in unselected AMD has been overestimated.

The integration of expression and other functional data with genetic association studies greatly enhances the power of such investigations. The identification of suitable candidate genes can be performed by gene expression and interaction through gene array experiments. For example *MYOC* was initially implicated through TM dexamethasone induction studies (Stone et al., 1997). As discussed by Stone, focused direct sequencing of the functionally important regions of bioinfomatically determined candidate genes should increase the yield for identifying fundamental disease alleles (Stone 2003).

As discussed above, chromosomal breakpoints at GLC1 (OAG) loci have been implicated in severe glaucoma pathogenesis. Given that there is a proven track record of ocular disease gene identification (e.g. *PAX6*, *PITX2* and *FOXC1*) through chromosomal investigations further review of such methods is warranted in OAG (Cohn et al., 2005). However, it must be acknowledged that chromosomal rearrangement may not account for the haphazard segregation of complex diseases.

Breaking down or "splitting" the OAG phenotype into its constitutional anatomical or pathophysiological components is another means for progress. Much evidence exists for "success of method" in using a SNP-based approached for identifying important quantitative trait loci (QTL) (Hugot et al., 2001). As an aside, maximum value from the genotype data is obtained through ensuring that a QTL approach can also be combined with or performed following an association study. The study of intermediate phenotypes can be more powerful than simply ascertaining whether

disease is present or absent. Such a method for progress has been implemented for IOP and cup-to-disc ratios (Charlesworth et al., 2005).

Risk indicators of OAG correlate highly in families. The Beaver Dam Eye Study (BDES) found that optic nerve parameters (principally vertical cup-to-disc diameter ratios) and IOP are more strongly correlated in siblings than in cousins (Klein et al., 2004). Using a commingling analysis on data from the Blue Mountains Eye Study it was suggested that a major gene accounts for approximately 18% of the variance of IOP (Viswanathan et al., 2004). A genome-wide sib-pair linkage study by the BDES investigators found two potential linkage regions on chromosomes 6 and 13 (Duggal et al., 2005). Using a micro-satellite genome-wide multipoint variance-components linkage analysis in a well investigated Australian *MYOC* pedigree, Charlesworth et al. recently revealed significant linkage of IOP to the long arm of chromosome 10, whilst suggestive linkage for vertical cup-to-disc ratio on the short arm of chromosome 1 (Charlesworth et al., 2005). Naturally however, such results require replication.

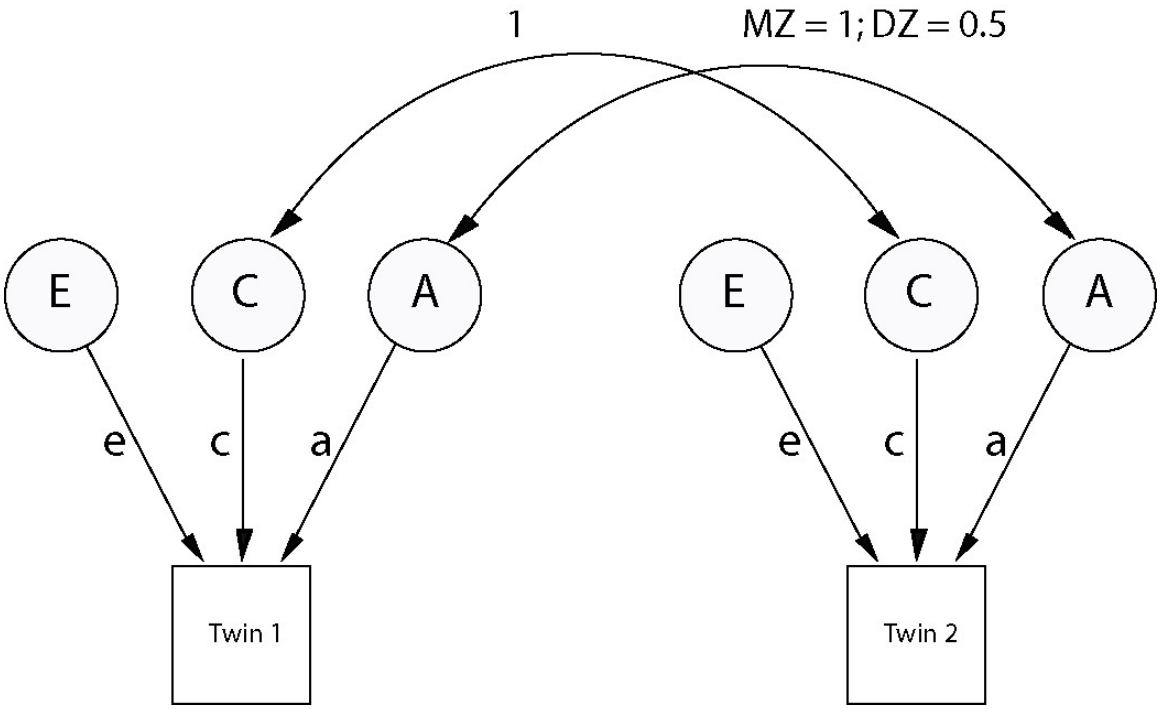
Twin studies are a major tool in determining heritability and identifying disease-causing genes. Twins studies allow for well-controlled association studies, as well as the study of the genetic versus environmental contribution to traits. When referring to twin studies many people assume the case of either two identical twins, one with the disease/trait and one without the disease/trait and conclude that environmental factors are important, alternatively many consider two identical twins reared apart (a rare and unusual situation) where they develop the same disease/trait (often at the same time) and conclude that the cause is likely to be genetic. Although helpful, these cases are rare. A classic twin study ensures a sophisticated analysis of the

variation between a large collection of identical (monozygotic, MZ) twin sets and the variation of a similar number of non-identical (dizygotic, DZ) twin sets. MZ twins have the same genes and a similar early environment, whilst DZ twins share a similar environment but have on average only half of their genes in common. Therefore any greater similarity between MZ twins compared to DZ twins is due to this extra gene 'sharing.' Comparison between the covariance of MZ and DZ twin pairs allows estimation of the genetic and environmental contributions to the trait in question. This can be broken down into dominant versus additive genetic components and shared versus non-shared environmental elements (Figure 1-6).

Once the components of a trait have been modelled, and the importance of genetic effects on human differences has been determined, it is then possible to elucidate the precise location of these genes. Gene identification is performed through using discordant sibling-pair analysis of the DZ twins. There are also several examples of where twin studies have been used to confirm disease causing genes (Nyholt et al., 2005; Zhu et al., 1999; Zhu et al., 2004). One of the most pertinent cases was a recent study investigating the genetics of eye colour, which found that up to 74% of the normal variation in eye colour liability is due to a QTL in the *OCA2* gene (Zhu et al., 2004). The *OCA2* gene has been previously implicated in causing oculocutaneous albinism (Rinchik et al., 1993). Twins Eye Studies have found a high heritability of central corneal thickness (Toh et al., 2005), optic disc cup area (Poulsen JL, et al. IOVS 2005; 46: ARVO E-abstract 1092) and IOP (MacKinnon J, et al. IOVS 2004; 45: ARVO E-abstract 4390).

Figure 1-6

Path model for univariate analysis of a twin study. Observed phenotype on twin 1 and twin 2 are represented as squares, latent factors in circles. A = additive genetic influence, E = unique environmental influence, C = common environmental influence. Regression coefficients are shown in lower case, a = additive genetic, c = common environment, e = unique environment. Both variables A correlate by a factor of 1.0 in MZ twins and by 0.5 in DZ twins (i.e. DZ twins share half their additive genes). Dominant genetic influences can be substituted for common environmental influences and correlate by a factor 1.0 in MZ twin or 0.25 in DZ twins. Given that the common environment is shared, C correlate exactly between the siblings.



Animal models offer another principal means for dissecting complex traits. As discussed previously there has already been some relative success with pigmentary glaucoma. However, whilst animal models are promising they must also be approached vigilantly and their applicability to human disease must be ascertained. Unfortunately, despite mutations in the *GPNMB* gene being found to cause pigmentary glaucoma in mice, no mutations were found in the coding regions in human cases of inherited pigment dispersion glaucoma (Anderson et al., 2002). To date no genes important in spontaneous OAG development in the canine, or rhesus monkey have been identified (Gelatt et al., 1998a).

Translational Research - from the bench to the slitlamp:

Gaps between the translation of current general genetic medical research into the clinical setting are widespread. Whilst the aetiology for many common preventable complex diseases remain to be unravelled, it is less useful to allocate research resources for the genetic detection of untreatable or non-preventable diseases at the demise of treatable ones. Glaucoma is a model genetic disease to investigate. In short, what does OAG genetics mean to the treating ophthalmologist? It will provide the ability to detect and treat a disease with potentially blinding consequences as early as possible.

Prior to the incorporation of genetic tests into the diagnostic algorithm, it is clearly essential to determine the significance of a disease gene variant. Along with differentiating disease mutations from normal genetic variability between individuals, understanding the clinical implications of a specific genotype is elemental. Calculating the pathogenetic probability of sequence variation by reviewing the alteration in gene function or protein structure is only feasible in genes

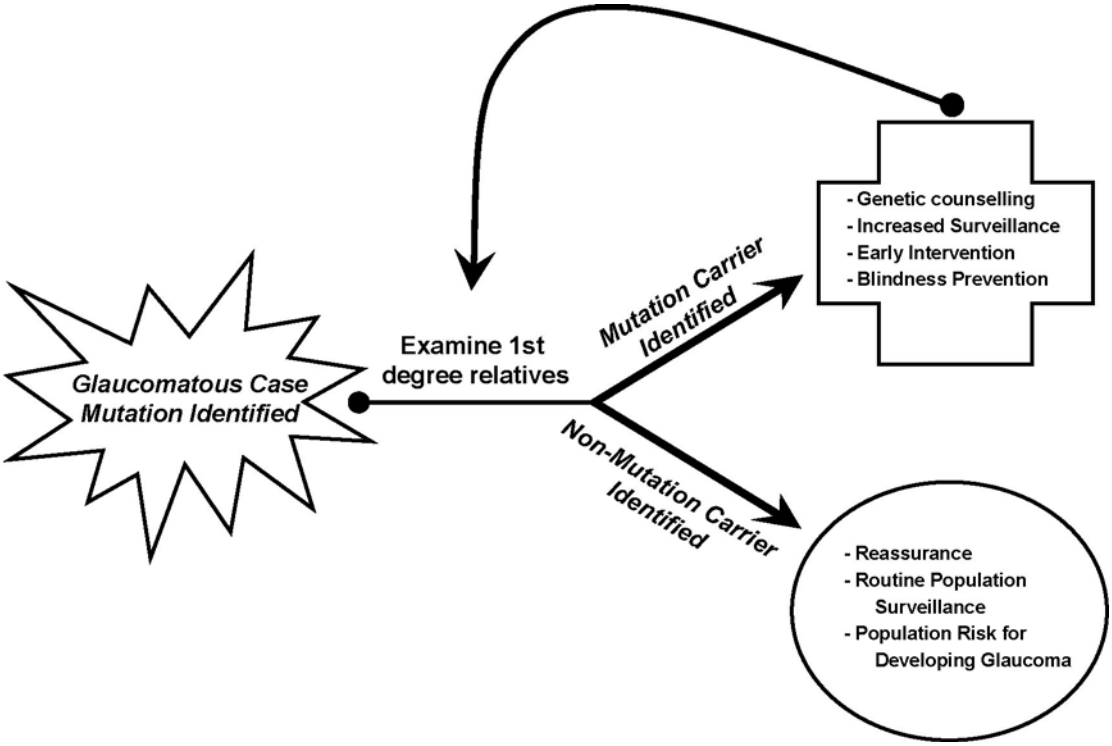
which have been unequivocally statistically implicated in disease causation (Stone 2003). A strong foundation of clinical research is essential prior to amalgamation of genetic counselling and predictive molecular testing. Understanding the sensitivity, spectrum, prevalence and penetrance of gene sequence changes ensures that patients can be adequately informed of the likely implications of carrying such a change. Community-based longitudinal studies, which incorporate both molecular and environmental components, are required.

Once the relative implication of a specific gene mutation has been recognised it is necessary to ensure that any gene-screening platforms are focused and efficient. In Mendelian diseases it has been well demonstrated that disease mutations are unevenly distributed, such that molecular screening can be refined to detect more than half of the clinically important variants with only one tenth the effort (Stone 2003).

In the clinic, detecting those who are at risk, or equally importantly, those who do not require increased clinical surveillance would be cost-beneficial and allow the streamlining of finite resources. Once an OAG patient is identified as having a disease-causing gene mutation(s), all of their first-degree relatives (children, parents and siblings) can be tested for the same mutation(s) (Figure 1-7). If they carry the mutation(s) then they are followed closely for early clinical signs of glaucoma, and their first-degree relatives are also tested. Thus, mutation testing moves out in a stepwise direction from the index case until all the (distant) relatives who harbour the mutation(s) are identified. This process is known as cascade screening, and has been successfully applied in cancer genetics.

Figure 1-7

A schematic of the cascade genetic screening cycle. Note that the first-degree relatives of the non-mutation carriers do not require increased surveillance or molecular examination.



Although the incorporation of cascade screening into clinical practice would dramatically reduce the cost of 'unnecessary clinical screening,' an evidence base for screening regimens is required. We have evaluated the perceptions of family members involved in cascade genetic screening for *MYOC* glaucoma and found them to be generally positive (Healey et al., 2004). Predictive glaucoma testing in appropriate circumstances is acceptable to OAG patients and their family (Healey et al., 2004).

Conclusion and setting of this thesis:

In summary, glaucoma is an ideal disease for genetic investigation. 'Genetic mechanisms' have been unequivocally linked to the disease process. Population-based clinical screening currently misses at least 50% of cases including some with advanced disease. When OAG is detected early and appropriate therapeutic intervention is initiated, blindness from glaucoma is preventable. The primary premise of this thesis is that glaucoma is a complex heterogeneous disease and that an improved understanding of its molecular pathogenesis will have important clinical ramifications.

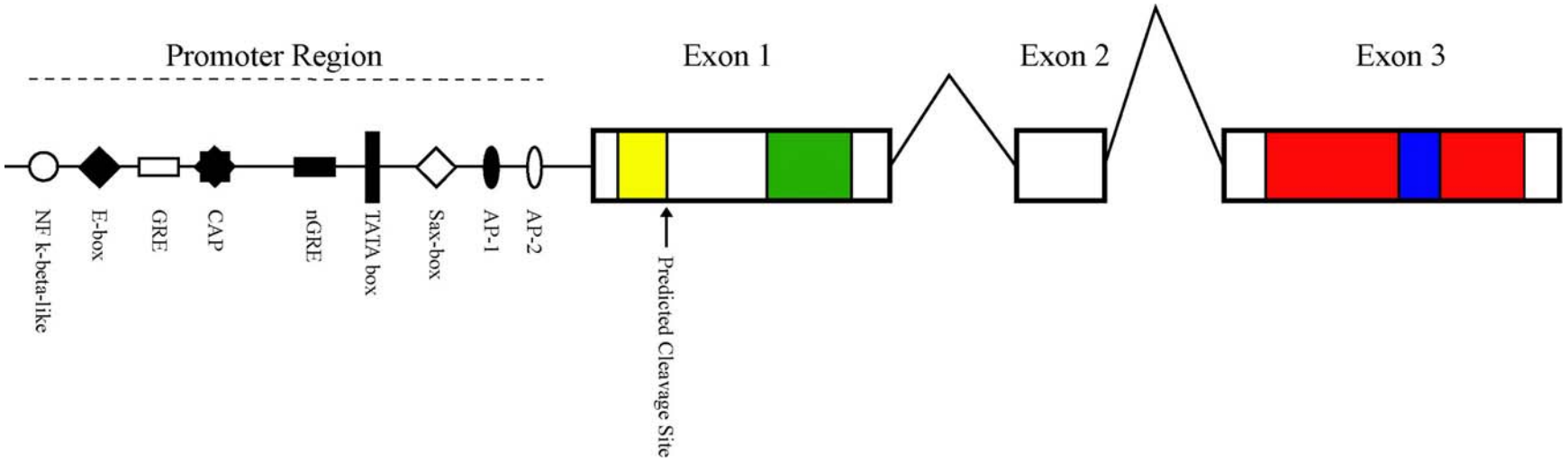
This thesis has been written such that it could be read in its entirety or in discrete portions, such that the subsection of each chapter is preceded by a brief introduction. As a consequence, abbreviations used throughout the thesis appear in full upon first use in each subsection. With the rapid dissemination of knowledge, predominately due to internet peer-review and pre-print online publication, the role of theses as significant learned journals will continue to diminish. This is particularly true in the genetics arena where the lag between novel gene discovery and reporting is shortening. Nevertheless, the role of a major dissertation as a resource for experimental methodology cannot be undervalued and as a consequence a substantive appendix has been included. It is likely that the best academic purpose for this collection of short stories is as a time capsule, whereby the genetic and phenotypic methods, as well as scientific vogue at the time of submission can be securely archived.

Chapter 2 – *MYOCILIN* GLAUCOMA: Dissecting the genotype-phenotype correlations of *myocilin* glaucoma.

As outlined in Chapter 1, the *myocilin* (*MYOC*) gene was the first gene in which mutations were found to cause glaucoma. In 1997 Stone and colleagues identified mutations in the *MYOC* gene (OMIM: 601652; formerly: *trabecular meshwork-induced glucocorticoid response* gene or *TIGR*) in families affected by autosomal dominant JOAG and OAG (Kubota et al., 1997; Polansky et al., 1997; Stone et al., 1997). *Myocilin* maps to the GLC1A locus at 1q24.3-q25.2 (Fingert et al., 2002). The *MYOC* gene has three exons, which contain two major homology domains, an N-terminal myosin-like domain, and a C-terminal olfactomedin-like domain (Figure 2-1), and encodes a 504 amino acid polypeptide. The majority of the identified disease-causing variants are clustered in the evolutionary conserved olfactomedin-domain of exon 3 (Fingert et al., 2002). *MYOC* mutations account for most cases of autosomal dominant JOAG and approximately 1 in 30 unselected cases of OAG (Fingert et al., 1999). *MYOC*-related glaucoma is predominantly associated with an elevated IOP, whereas other forms of OAG are often found in the absence of elevated IOP, and strong phenotype-genotype correlations exist within the spectrum of *MYOC* mutations (Alward et al., 1998). *MYOC* is expressed ubiquitously in the eye and despite some descriptions of nonsense mutations, haploinsufficiency of the MYOC protein has been excluded as the primary disease mechanism (Fingert et al., 2002).

Figure 2-1

Schematic structure of the *Myocilin* gene, displaying its protein homology domains and promoter region organisation. The putative functional coding regions are highlighted in colour, with the yellow, green, red and blue sections representing the secretory signal peptide sequence, protein binding, olfactomedin homology and hydrophobic domains, respectively (Fan et al., 2004; Fingert et al., 2002; Zhou and Vollrath, 1999).



Interestingly OAG is not induced through genetically increasing or decreasing wild-type MYOC expression (Gould et al., 2004), and people homozygous for disease-causing variants do not necessarily manifest disease (Morissette et al., 1998). A gain-of-function disease model was suggested through the observation that mutant forms of the MYOC protein are misfolded and aggregate in the endoplasmic reticulum of trabecular meshwork cells (Jacobson et al., 2001; O'Brien et al., 2000; Tamm 2002; Yam et al., 2007; Zhou and Vollrath, 1999). Trabecular meshwork cells are essential for the homeostatic regulation of aqueous humour outflow from the eye, and dysfunction generally manifests as elevated IOP. Shepard and colleagues have recently demonstrated that there is a mutation-dependent, gain-of-function association between human MYOC and the peroxisomal targeting signal type 1 receptor (PTS1R) (Shepard et al., 2007). This is the first demonstration of a disease resulting from mislocalization of mutant protein to peroxisomes caused by mutation-induced exposure of a cryptic signalling site (Shepard et al., 2007).

Genetic screening offers a comprehensive means for identifying people predisposed to disease development. A corollary of this is the need for detailed understanding of the phenotypic variation associated with specific alleles. Locus-specific phenotypic databases offer a universal means for transferring clinically useful data which can affect the clinical management of the individual's glaucoma and has implications for the screening of family members. This is particularly important in the case of a disease as common as OAG, in which the primary care is provided by ophthalmologists who in turn will increasingly need ready access to updated information regarding the mutation spectrum and associated phenotypes. The first aim of this Chapter is to describe the design and development of a comprehensive

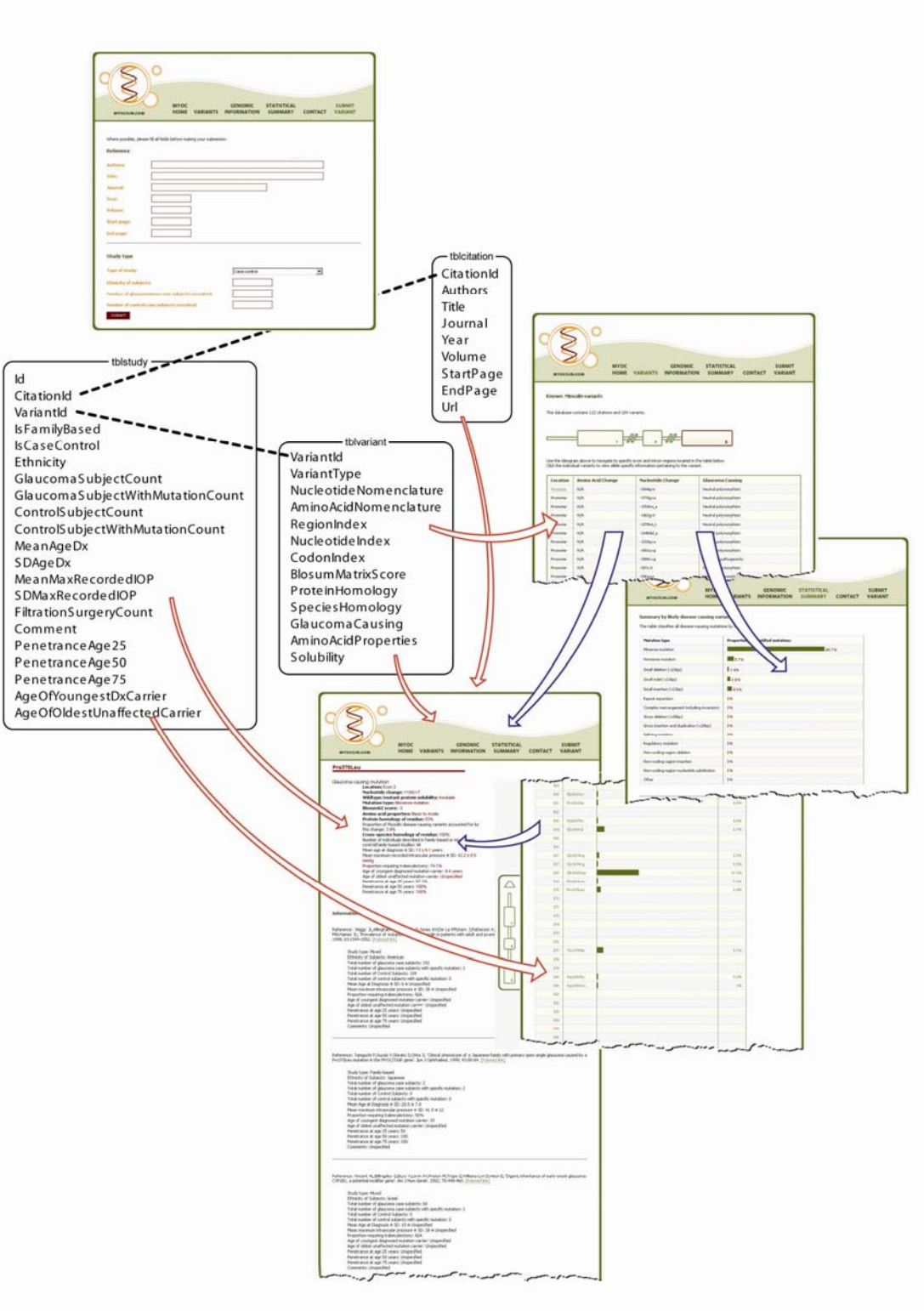
online (www.myocilin.com) database of *MYOC* allele-specific phenotype information.

Database relationships and website structure

To ensure flexibility and ease of management the *MYOC* database was constructed using the MySQL database package (www.mysql.com). This database contains three significant sheets, with the majority of information being stored in the *tblstudy* sheet. This sheet has one-to-many and many-to-one direct relationships to the *tblvariant* and *tbcitation* sheets, respectively (Figure 2-2). The total number of control and glaucomatous case subjects recruited, as well as the number of case and control subjects identified as carrying a particular *MYOC* variant (as linked to the *tblvariant* sheet) is recorded on the *tblstudy* sheet. For identified variant carriers phenotypic fields in the *tblstudy* sheet include: subject ethnicity, mean and SD age (years) at diagnosis, mean and SD maximum recorded IOP (mmHg), number of patients undergoing trabeculectomy, penetrance at age 25, 50 and 75 years, as well as the age of the youngest diagnosed carrier, and the age of the oldest unaffected carrier. Given that there are no major structural differences between the optic disc in glaucomatous subjects who have *MYOC* mutations compared with individuals with non-*MYOC* related OAG, such morphological data was not recorded in the database (Hewitt et al., 2007a). The primary key for the *tblstudy* sheet (Id) serves to uniquely catalogue each submission and has no direct relationships within the database.

Figure 2-2

Structure of the *myocilin* database. The majority of information stored in this locus-specific dataset is contained in the *tblstudy* sheet. This sheet has one-to-many and many-to-one relationships to the *tblvariant* and *tbcitation* sheets, respectively (dashed lines). Phenotypic and genotypic information can be viewed by selecting a specific variant on the *Variants* webpage or from the *Statistical Summary* page.



The disease-causing status of each sequence variant is coded as a field in the *tblvariant* sheet and as a drop-down menu which allows the selection of the correct variant type. Two fields are used to document the name of each identified variant both at the DNA and protein levels. This information is used to determine the genomic location (nucleotide index), and its corresponding site in *MYOC* (e.g. promoter, exon 1, intron 1 etc). Data relating to the cross-protein and cross-species homology for the particular amino acid is determined by a "lookup" array according to the codon index or number. Additionally within the *tblvariant* sheet, Blosum-62 matrix scores and particular amino acid properties are determined by similar arrays, according to the particular substitution (Henikoff and Henikoff, 1992). The *tbcitation* sheet contains fields allowing for the identification of the submitted source.

A HTML script, which used minimal embedded script so as to eliminate browser-browser variation, was written for the website. Using the heading bar, it is easy to navigate from the homepage to specific information such as identified variants, background genomic data and summary statistics. The specific region of interest can be further investigated by using the ideogram for the *MYOC* gene. For example, when the user selects the third exon of the ideogram they are automatically directed to the variants identified in this corresponding region. The total number of variants and citations recorded in the database is displayed at the top of the *Variants* webpage. On this page, particular phenotypic and genotypic information can be viewed by selecting a specific variant (listed by genomic location). Additionally, one can navigate to the allele specific information from the *Statistical Summary* page.

Nomenclature

Variant designation has been based on the guidelines established by the Human Genome Nomenclature Working Group (Antonarakis 1998; den Dunnen and Antonarakis, 2000). The first nucleotide (A) of the initiator Methionine codon is denoted nucleotide +1 with the nucleotide 5′ to this being numbered –1.

Data integrity

Each study is classified by design as being either a case-control, family-based, or mixed case-control/family-based investigation. The latter subtype designates studies that were initiated on a case-control basis and then extended to examine, in a cascade-screening manner, all of the mutation-carrying proband's available relatives.

Identified variants are classified as being: missense, nonsense, synonymous, or by location: splicing, regulatory, or non-coding. Non-coding region variants are classified as substitutions, insertions or deletions, complex rearrangements or repeat expansions. Deletions, insertions and indels are sub-classified as small (<21bp) or gross (>20bp).

In assigning pathogenic status to a given variant, the following issues are taken into consideration: the predicted disruption of protein translation (e.g. frame-shift mutations and premature stop codons); the frequency of the sequence variant in the control (unaffected) populations; the location of the variant in the *MYOC* gene (i.e. cross-species conservation of coding sequence); evidence for partial segregation with the phenotype within a family; and, when available, results of solubility studies.

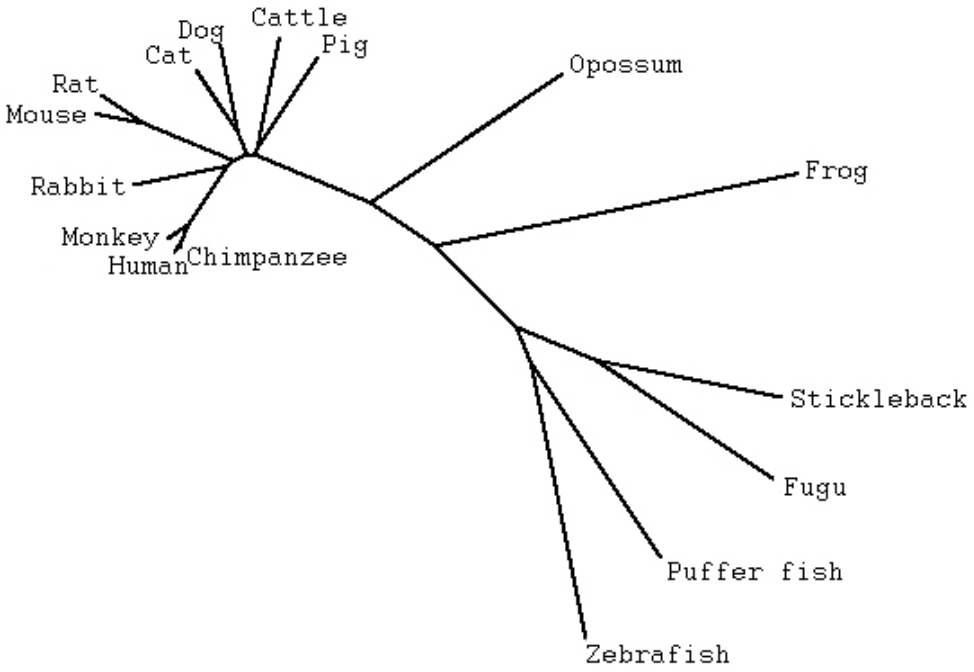
Initial data source:

Phenotype and mutation data have been compiled initially from the published literature. An online search of literature was systematically conducted using PubMed covering all years from 1997-May 2007. Articles cited in reference lists of other manuscripts were also searched. All articles, independent of the language of publication, were included and data were extracted from English-translated abstracts. The database contains web-links to the citation source published by the National Library of Medicine (www.pubmed.com).

Summary of background genomic data

The *Genomic Information* page displays the genomic sequence of *MYOC* and its cross-protein and species homology of the coding regions. The annotated sequence of humans and other animal species (Chimpanzee, Monkey, Rabbit, Mouse, Rat, Cat, Dog, Cattle, Pig, Opossum, Frog, Stickleback, Fugu, Puffer fish, Zebrafish) was obtained from the ensembl database (www.ensembl.org) and the nucleotide sequence renumbered to conform to Nomenclature Working Group guidelines. Protein sequences (MYOC, OLFML2, Q8TEG0, OLFML2B, OLFM1, Q8N8R0, OLFM3, OLFM2, LPHN2, LPHN1, LPHN3, OLFML3, OLFML1, OLFM4, GLDN) were obtained from the protein knowledgebase Swiss-Prot (http://au.expasy.org/sprot/), and the Basic Local Alignment Search Tool of the US National Centre for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST/). Protein and genomic alignments were performed using CLUSTALW, with a BLOSUM-62 protein weighted matrix; a gap open penalty score of 10 and a gap extension penalty score of 0.05 (Chenna et al., 2003). Figure 2-3 displays the phylogenic relationship between the *MYOC* gene sequence differences across species.

Figure 2-3
Unrooted dendrogram of *Myocilin* homology across species.



Database summary statistics:

A large volume of clinically relevant data has been compiled through this locus-specific database. A summary of relevant information generated for each allele is displayed above the list of contributing resources listed on each variant-specific page. Over 180 variants have been identified within the exons and surrounding non-coding regions of the *MYOC* gene (Table 2-1, Table 2-2 and Table 2-3). Approximately 40% of the identified variants have been characterised as disease-causing, with the majority (~85%) of these being missense mutations and clustered in the exon 3 (Figure 2-4 and Figure 2-5). This later information can be viewed on the upper section of the *Statistical Summary* page. The lower section of the *Statistical Summary* page contains the frequency and corresponding genomic location of disease-causing variants identified in case-control designed studies. The relative disease-related prevalence of each variant is displayed in a long format and, to the left of the page, a floating ideogram of the *MYOC* gene is provided to facilitate navigation.

To overcome issues relating to allele-specific penetrance or expressivity, and recruitment bias, phenotypic information is only extracted from investigations which had a family-based or mixed case-control/family-based design. For all phenotypic data the weighted values were calculated according to the number of mutation-carrying subjects phenotyped, thereby providing the most clinically appropriate representation. An example of the data that can be extracted from the database is displayed in Table 2-4, highlighting the strong genotype-phenotype correlations.

Table 2-1

Disease causing variants identified in the *myocilin* gene. Source: www.myocilin.com

Location	Amino Acid Change	Nucleotide Change
Exon 1	Gln19fs	56_72dup
Exon 1	Cys25Arg	73T>C
Exon 1	Arg82Cys	244C>T
Exon 1	Arg91Stop	271C>T
Exon 1	Arg126Trp	376C>T
Exon 1	162ins163	Not Specified
Exon 3	Val251Ala	731G>C
Exon 3	Cys245Tyr	734G>A
Exon 3	Gly246Arg	736G>A
Exon 3	Gly252Arg	754G>A
Exon 3	Glu261Lys	781G>A
Exon 3	Arg272Gly	814C>G
Exon 3	Pro274Arg	821C>G
Exon 3	Thr285Met	854C>T
Exon 3	Trp286Arg	856T>C
Exon 3	Thr293Lys	878C>A
Exon 3	Glu300Lys	898G>A
Exon 3	Ser313Phe	938C>T
Exon 3	Glu323Lys	967G>A
Exon 3	Gln337Glu	1009C>G
Exon 3	Gln337Arg	1010A>G
Exon 3	Arg342Lys	1025G>A
Exon 3	Ile345Met	1035A>G
Exon 3	Tyr347Stop	1041T>G
Exon 3	Ille360Asn	1079T>A
Exon 3	Pro361Ser	1081C>T
Exon 3	Ala363Thr	1087G>A
Exon 3	Gly364Val	1091G>T
Exon 3	Gly367Arg	1099G>A
Exon 3	Gly367Arg	1099G>C
Exon 3	Gln368Stop	1102C>T
Exon 3	Phe369Leu	1105T>C
Exon 3	Pro370Leu	1109C>T
Exon 3	Thr353Lys	1118G>A
Exon 3	Thr377Met	1130C>T

Location	Amino Acid Change	Nucleotide Change
Exon 3	Asp380Asn	1138G>A
Exon 3	Asp380Gly	1138G>C
Exon 3	Asp380Ala	1139A>C
Exon 3	Gly367_Gln368delinsVal	1177GACA>T
Exon 3	Ser393Asn	1178G>A
Exon 3	Ser393Arg	Not Specified
Exon 3	396ins397	Not Specified
Exon 3	Gly399Val	1196G>T
Exon 3	Arg422His	1265G>A
Exon 3	Lys423Glu	1267A>G
Exon 3	Val426Phe	1276G>T
Exon 3	Ala427Thr	1279G>A
Exon 3	Cys433Arg	1297T>C
Exon 3	Gly434Ser	1300G>A
Exon 3	Tyr437His	1309T>C
Exon 3	Ala445Val	1334C>T
Exon 3	Thr448Pro	1342A>C
Exon 3	Asn450Asp	1348A>G
Exon 3	Tyr453del	1357delT
Exon 3	Gly458Asp	1373G>A
Exon 3	Ile465Met	1395C>G
Exon 3	Arg470Cys	1408C>T
Exon 3	Tyr471Cys	1412A>G
Exon 3	Ile477Asn	1430T>A
Exon 3	Ile477Ser	1430T>G
Exon 3	Asn480Lys	1440C>A
Exon 3	Pro481Ser	1441C>T
Exon 3	Pro481Thr	1441C>A
Exon 3	Pro481Arg	1442C>G
Exon 3	Pro481Leu	1442C>T
Exon 3	Glu483Stop	1447G>T
Exon 3	Ile499Phe	1495A>T
Exon 3	Ile499Ser	1496T>G
Exon 3	Ser502Pro	1504T>C
Exon 3	1544ins489Stop	1544insC

Table 2-2
Neutral polymorphisms identified in the *myocilin* gene. Source: www.myocilin.com

Location	Amino Acid Change	Nucleotide Change
Exon 1	Phe4Ser	11T>C
Exon 1	Cys9Ser	26G>C
Exon 1	Gly12Arg	34G>C
Exon 1	Pro13Pro	Not Specified
Exon 1	Pro16Leu	47C>T
Exon 1	Ala17Ser	49G>T
Exon 1	Val18Leu	52G>T
Exon 1	Gln19His	57G>T
Exon 1	Arg46Stop	136C>T
Exon 1	Val53Ala	158T>C
Exon 1	Ser55Thr	164G>C
Exon 1	Asn57Asp	169A>G
Exon 1	Ser69Ser	Not Specified
Exon 1	Val70Val	210C>T
Exon 1	Asn73Ser	218A>G
Exon 1	Arg76Lys	227G>A
Exon 1	Asp77Glu	Not Specified
Exon 1	Arg82His	245G>A
Exon 1	Thr88Thr	Not Specified
Exon 1	Leu95Pro	284T>C
Exon 1	Glu96Glu	288G>A
Exon 1	Gln118Leu	353A>T
Exon 1	Gly122Gly	366C>T
Exon 1	Arg128Arg	384G>C
Exon 1	Thr123Thr	396C>T
Exon 1	Gln134Gln	402A>G
Exon 1	Thr135Ile	404C>T
Exon 1	Leu159Leu	Not Specified
Exon 1	Arg168Arg	504G>A
Exon 1	Leu180Leu	Not Specified
Exon 1	Arg189Gln	566G>A
Intron 1	N/A	IVS1+14g>a
Intron 1	N/A	IVS1+16g>t
Intron 1	N/A	IVS1+19g>c
Exon 2	Ser203Phe	608C>T
Exon 2	Thr204Met	611C>T
Exon 2	Thr204Thr	Not Specified
Exon 2	Asp208Glu	624C>G
Exon 2	Leu215Pro	644T>C
Exon 2	Lys216Lys	648G>A
Exon 2	Thr243Thr	Not Specified
Intron 2	N/A	IVS2+35a>g
Intron 2	N/A	IVS2+73c>t
Intron 2	N/A	IVS2+172c>a
Intron 2	N/A	IVS2+3a>g
Exon 3	Gly244Val	731G>T
Exon 3	Asp247Stop	739G>T
Exon 3	Thr256Met	767C>T
Exon 3	Ala260Ala	780A>G
Exon 3	Lys266Lys	798G>A
Exon 3	Thr285Thr	855G>T
Exon 3	Ile288Ile	864C>T
Exon 3	Thr290Ala	868A>G
Exon 3	Asp302Asp	906G>T
Exon 3	Ile304Ile	Not Specified
Exon 3	Gln309Gln	927G>A
Exon 3	Leu318Leu	Not Specified
Exon 3	Thr325Thr	Not Specified
Exon 3	Val329Met	985G>A
Exon 3	Val329Val	Not Specified
Exon 3	Ser331Ser	Not Specified
Exon 3	Tyr347Tyr	1041T>C
Exon 3	Thr351Thr	Not Specified
Exon 3	Pro370Pro	1110G>A
Exon 3	Thr377Thr	Not Specified
Exon 3	Glu396Glu	1188G>A
Exon 3	Lys398Arg	1193A>G
Exon 3	Val402Ile	1204G>A
Exon 3	Leu403Leu	1209C>T
Exon 3	Glu414Lys	1240G>A
Exon 3	Arg422Cys	1264C>T
Exon 3	Ser425Stop	1274C>G
Exon 3	Ser425Pro	1273T>C
Exon 3	Thr438Ile	1313C>T
Exon 3	Thr438Thr	Not Specified
Exon 3	Val439Val	Not Specified
Exon 3	Arg470His	1409G>A
Exon 3	Tyr473Cys	1418A>G
Exon 3	Ala488Ala	1464C>T
Exon 3	Val495Ile	1483G>A
Exon 3	Lys500Arg	1499A>G
Exon 3	N/A	1515+20a>g
Exon 3	N/A	1515+73g>c
Exon 3	N/A	1515+291a>g
Exon 3	1q23_1q25del	N/A

Table 2-3

Promoter region and variants of uncertain pathogenicity identified in the *myocilin* gene. Source: www.myocilin.com

Location	Amino Acid Change	Nucleotide Change
Promoter	N/A	-2084g>t
Promoter	N/A	-1770g>a
Promoter	N/A	-1760ins_a
Promoter	N/A	-1422g>t
Promoter	N/A	-1378ins_t
Promoter	N/A	-1340del_a
Promoter	N/A	-1333g>a
Promoter	N/A	-1081a>g
Promoter	N/A	-1000c>g
Promoter	N/A	-467gt>ca
Promoter	N/A	-387c>t
Promoter	N/A	-306g>a
Promoter	N/A	-255t>c
Promoter	N/A	-224t>c
Promoter	N/A	-190g>t
Promoter	N/A	-153t>c
Promoter	N/A	-127t>c
Promoter	N/A	-126t>c
Promoter	N/A	-83g>a
Promoter	N/A	-78t>g
Promoter	N/A	-77g>a
Promoter	N/A	-18c>t
Promoter	N/A	-8c>t
Exon 1	Gln48His	144G>T
Exon 1	Arg158Gln	473G>A
Exon 3	Ser331Thr	991T>A
Exon 3	Glu352Lys	1054G>A
Exon 3	Thr353Ile	1058C>T
Exon 3	1q24.3_1q31.2del	N/A

Figure 2-4

Location of disease causing variants (a), and relative cross-species (b) or cross-protein (c) homology. Source: www.myocilin.com

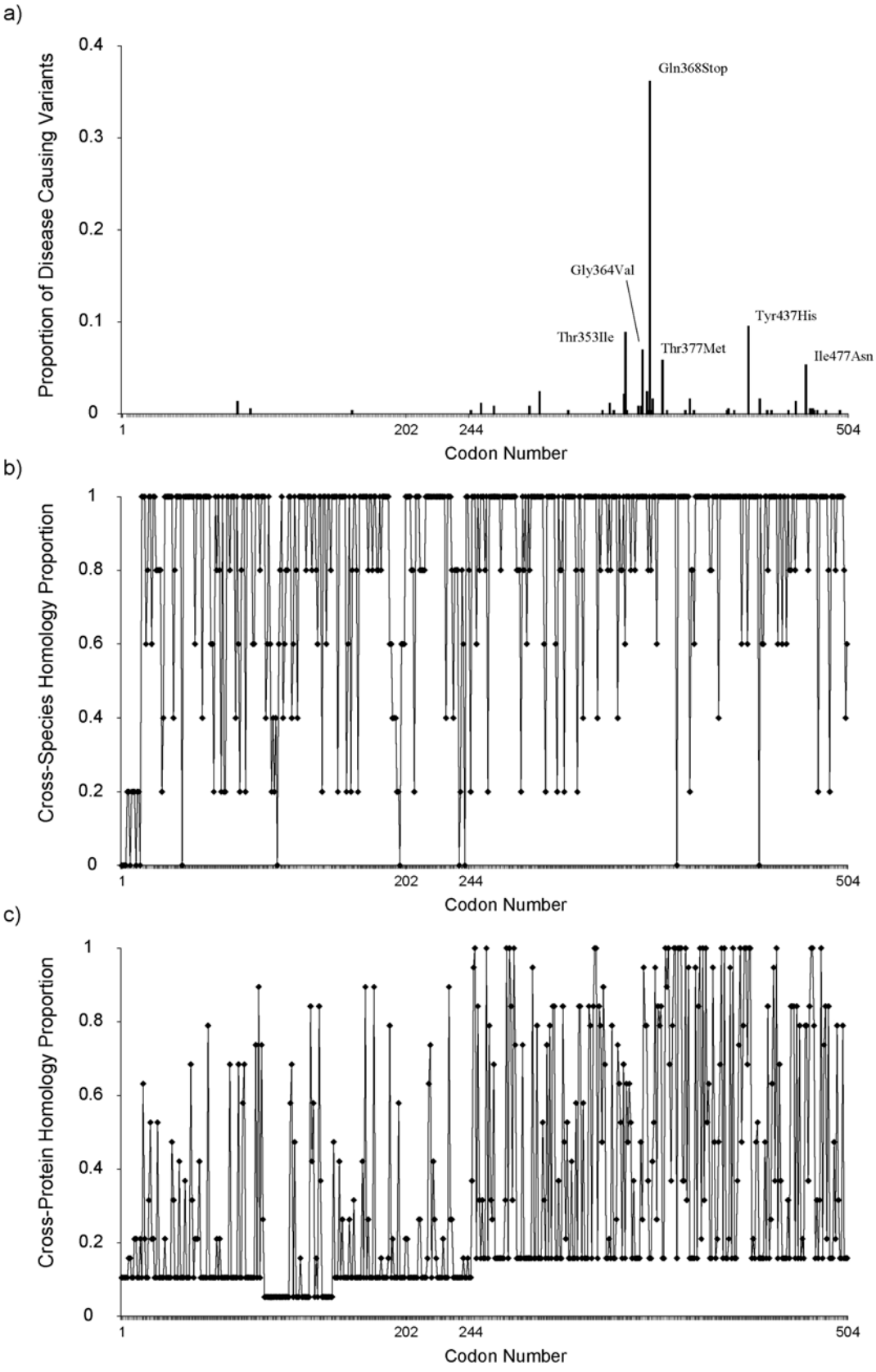


Figure 2-5
Proportion of disease causing variants classified by type of mutation.
Source: www.myocilin.com

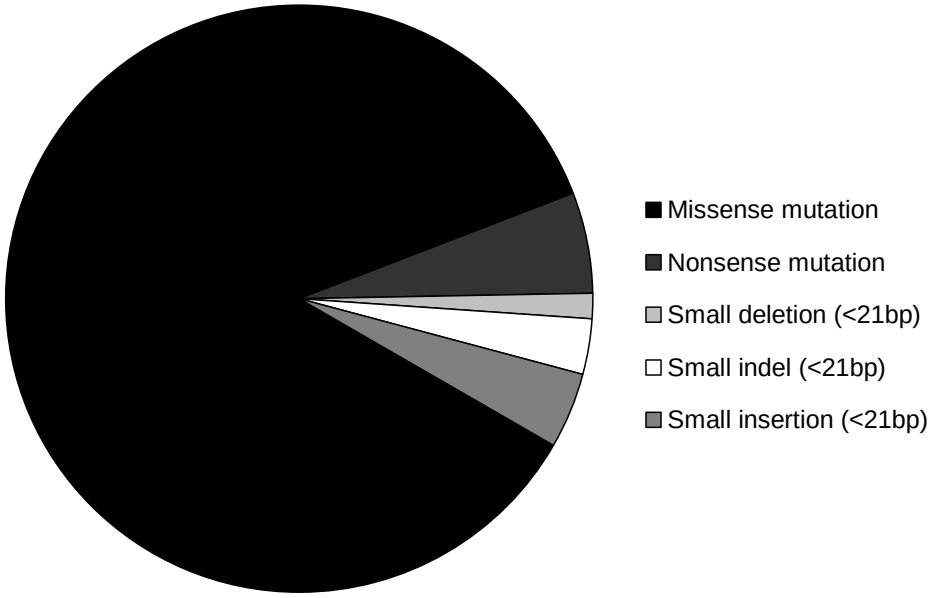


Table 2-4
Mutation-dependent phenotypic variation in the *myocilin* gene. Weighted values calculated from a total of 49 family-based publications extracted through the online database. Source: www.myocilin.com

Myocilin Disease causing Variant	n	Age at diagnosis (years)	(SD)	Maximum recorded IOP (mmHg)	(SD)	Proportion undergoing surgical intervention (%)
Gln368Stop	138	54.4	(10.7)	29.8	(4.4)	34.4
Thr377Met	61	39.9	(13.1)	32.2	(10.1)	53.1
Cys433Arg	8	38.6	(15.1)	33.9	(10.5)	64.7
Gly367Arg	21	34.3	(8.1)	41.1	(10.3)	69.2
Asn480Lys	78	33.2	(4.9)	39.0	(10.6)	65.4
Lys423Glu	156	28.8	(15.4)	31.6	(5.7)	66.7
Gly367_Gln368delinsVal	20	28.2	(12.6)	38.1	(10.0)	80.0
Val426Phe	29	25.0	(13.9)	41.2	(8.1)	50.0
Ile477Asn	73	20.8	(5.8)	41.3	(12.1)	91.3
Pro370Leu	67	13.0	(6.1)	42.2	(8.5)	74.1

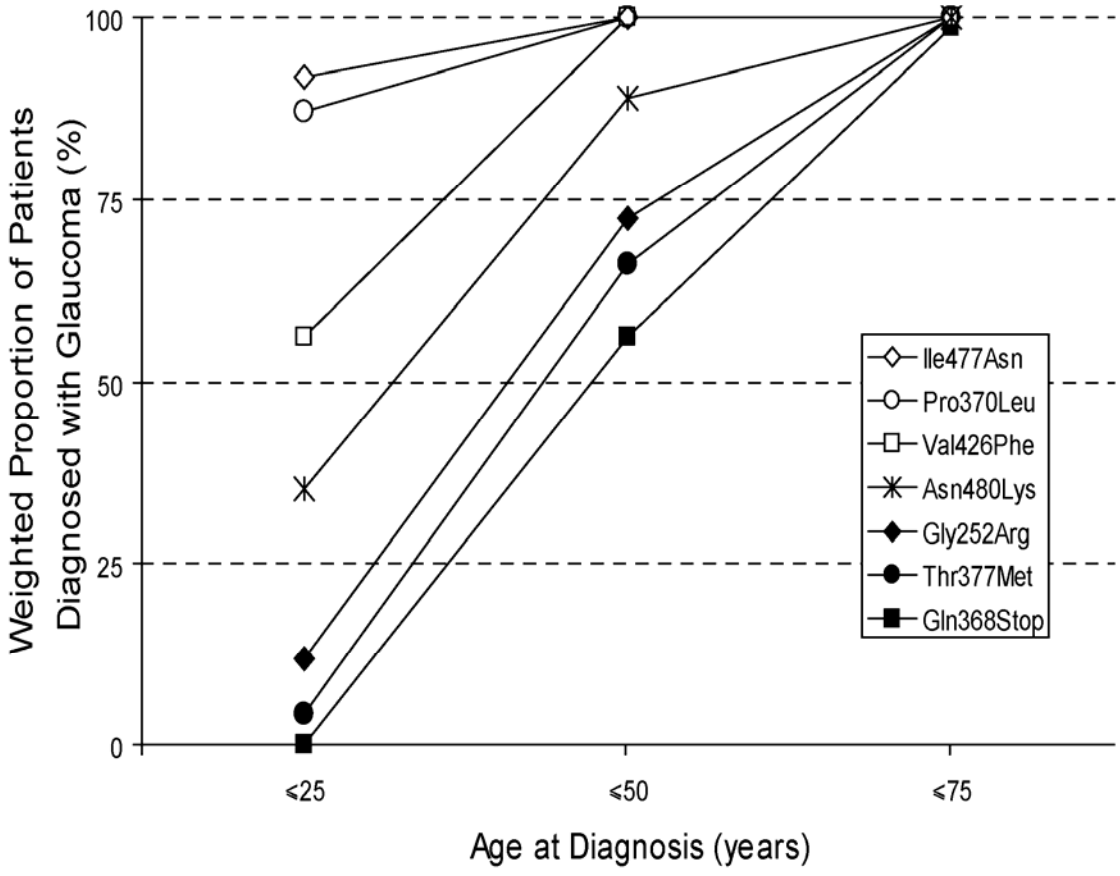
Age-related penetrance was determined for three specific age groups (25, 50 and 75 years). This figure was calculated as the proportion of subjects who were diagnosed less than the specified age, divided by the sum of the number of people diagnosed less than the specified age and any additional people older than this who were not diagnosed with the disease. In reviewing these data, it is acknowledged that this provides a relatively simplistic estimate of penetrance and does not take into account the fact that OAG is a disease spectrum and that separate studies often utilise differing diagnostic criteria to define affected status. To provide a further clinically useful parameter, information relating to the age of the youngest person at diagnosis and the age of the oldest clinically undiagnosed person were also included. Figure 2-6 demonstrates the wide variation in categorised age-dependent penetrance calculated by the weighted means for which more than one published family-based study contributed.

Future data submission and control

Genotypic and phenotypic information can be submitted for upload into this database by using the *Submit Variant* page. Upon entry to this data submission page, the user is asked for his/her name, institution and contact details. Then after entering data such as study design, number of control and glaucomatous case subjects, the user is directed to a form which contains data pertaining to the specific variant identified and its associated phenotypic data. Information for each specific allele is entered separately and additional allelic variants can be incorporated by selecting "Add another variant" once the initial data have been submitted. Submitted data is then manually uploaded using the control panel of the myocilin.com domain host.

Figure 2-6

Genotype-specific differences in disease penetrance. The weighted penetrance for disease-causing variants for which more than one published family-based study contributed. Note the distinct group of mutations causing either juvenile or adult-onset glaucoma and that each variant approximates complete penetrance by 75 years of age.



To maintain patient confidentiality, submitted data will be reviewed to ensure no identifying information (such as subject name or date of birth) has been entered. The source of unpublished submissions can be identified by the listing of contributing individuals or research groups and their respective institutional address.

The ability to directly provide patients, researchers and clinicians with relevant information about particular disease genotype-phenotype correlations advances the possibility for individualised medicine utilising genotype analysis to become a reality. Researchers must be encouraged to collect data from a large number of patients and ensure that its assimilation is in a publicly accessible, user-friendly format. With the increasing availability of genotype-phenotype databases, individual anecdotal evidence for allele-specific disease natural history will be surpassed by much larger datasets providing increased validity to interpretation of genetic tests and enabling clinicians to provide the best possible interpretation of results. We believe integration of this allele-specific phenotypic data relating to *MYOC* will provide a useful resource for clinicians and researchers alike.

The remaining sections of this chapter explore the phenotypic associations with *MYOC*-related glaucoma. Initially the clinical description of a person who is homozygous for the common Gln368STOP *MYOC* mutation is presented. Following this, investigation for a founder effect of the Thr377Met *MYOC* mutation in glaucoma families from differing ethnic backgrounds is undertaken. This work stems from the hypothesis that severe mutations would be detrimental to overall survival thereby conveying a negative selective pressure.

Extending our understanding of mutations which cause disease of intermediate severity the subsequent section investigates the phenotypic effects of the Gly252Arg *MYOC* mutation and investigates its genetic origins. As outlined in Chapter 1 excavation of the optic disc is the final common-endpoint in the primary open-angle glaucomas. The subsequent section of this Chapter investigates whether there are specific features of the optic nerve head which may manifest in *MYOC*–related

glaucoma. Building on this information we then ask whether glaucomatous disc changes are evident prior to diagnosis of glaucoma in *MYOC* pedigrees? This section delineates the place *MYOC*-mutations have in the glaucoma continuum, thereby rounding off this chapter.

A *Myocilin* Gln368STOP homozygote does not exhibit a more severe glaucoma phenotype than heterozygous cases.

Background:

A number of studies have identified individuals homozygous for other *MYOC* mutations. Morissette et al. described four normal individuals aged between 43 and 50 years who were homozygous for the Lys423Glu mutation (Morissette et al., 1998). The Lys423Glu *MYOC* mutation in the heterozygous state typically exhibits a glaucoma phenotype of juvenile onset (i.e. severe), with over two thirds of affected people in the described French-Canadian family diagnosed before the age of 40 (Morissette et al., 1998). However, homozygotes for *MYOC* mutations may not necessarily confer a normal phenotype. Chakrabarti et al. reported primary congenital glaucoma in a patient who was homozygous for the Gln48His *MYOC* mutation (Chakrabarti et al., 2005). Interestingly, a Korean proband who was homozygous for the Arg46STOP *MYOC* mutation displayed a severe glaucoma phenotype, whilst her heterozygous relatives did not exhibit any glaucomatous signs (Yoon et al., 1999). This directly conflicts with the finding of Lam et al. who identified a 77-year-old woman, of Hong Kong-Chinese descent, without glaucoma yet was homozygote for the Arg46STOP (Lam et al., 2000). Further investigation has found the Arg46STOP mutation equally distributed in a glaucomatous (2.0%) and matched control cohort (2.2%) suggesting that this sequence variant could be a neutral variant (Pang et al., 2002).

Methods:

We identified 22 glaucoma pedigrees in which the index case was heterozygous for the Gln368STOP *MYOC* mutation. Cascade genetic screening was performed in these pedigrees. Subjects were seen as part of the Glaucoma Inheritance Study in

Tasmania, which has local ethics committee approval. All recruited subjects underwent a comprehensive clinical examination. Mutation analysis was performed by direct sequencing of amplified DNA. Genomic DNA was isolated and the *MYOC* exon 3 region encompassing the *MYOC* 368 codon was amplified using PCR primers (see page 272) then directly sequenced.

Results:

One individual, from pedigree GQld11, was found to be homozygous for the *MYOC* Gln368STOP mutation (Figure 2-7). At the time of last examination she was 49 years of age and had a Snellen best-corrected visual acuity of 20/15 bilaterally. Initial recorded intraocular pressures were 15mmHg bilaterally. No significant elevation has been recorded upon repeated measurements. Fundus examination revealed small healthy optic discs (Figure 2-8). The proband for that pedigree, (the mother of the homozygous subject), was diagnosed with glaucoma at the age of 61 years. Her highest recorded intraocular pressures were 28 mmHg in each eye. Optic disc examination demonstrated relatively small discs with vertical cup-disc ratios of 0.6 and 0.5 for the right and left eye, respectively. There was prominent loss of the inferior nerve fibre layer in the left eye with a corresponding glaucomatous superior visual field defect.

Figure 2-7

The GQld-11 pedigree illustrating the nuclear family of the Gln368STOP homozygote. The symbol fully filled on the proband (I:1) indicates a primary open angle glaucoma phenotype. Pedigree number and age is displayed. The allelic carrier status of the Gln368STOP mutation for each individual is shown (+ or -) as is the relevant haplotype data.

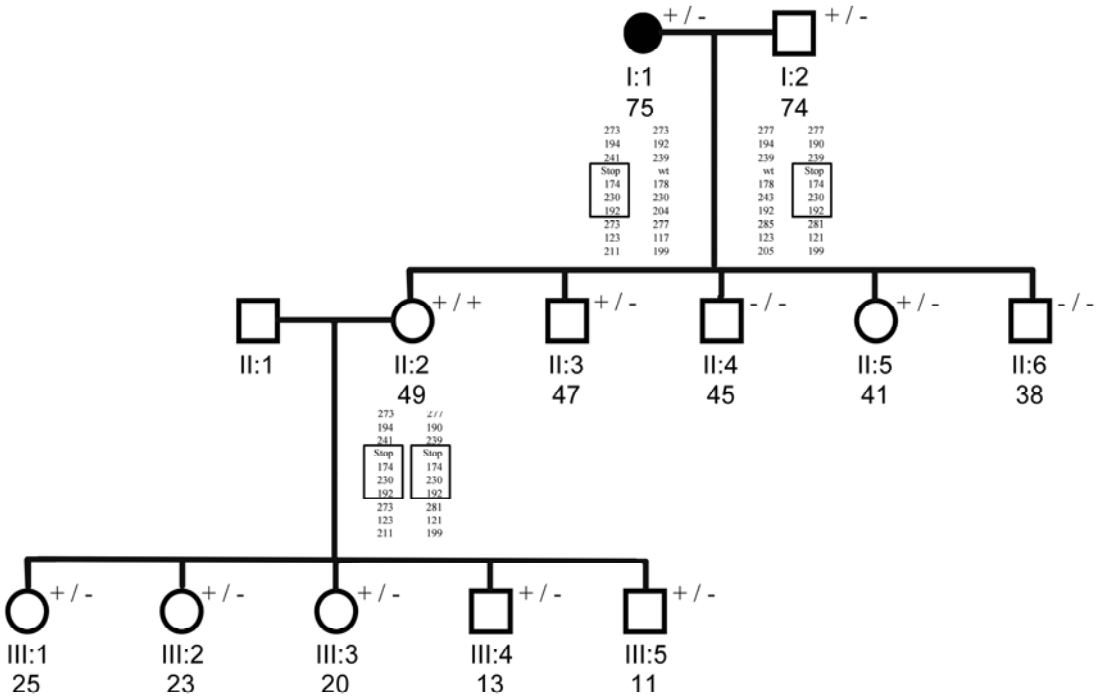
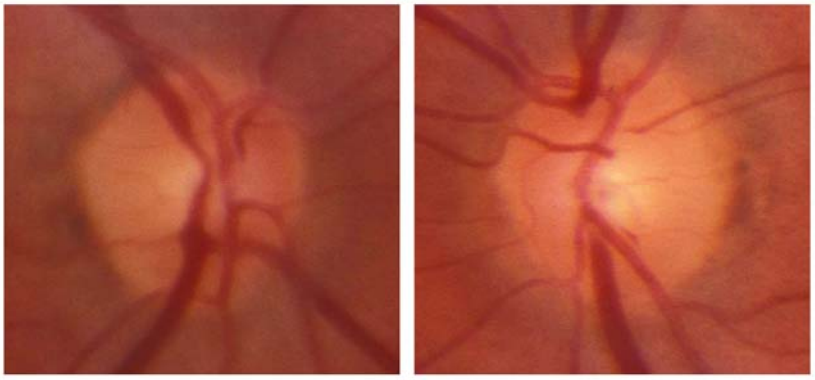


Figure 2-8
Optic disc photographs of individual II:2 who is homozygous for the Gln368STOP *myocilin* mutation.



Examination of the 74-year-old father (found to be heterozygous for the Gln368STOP *MYOC* mutation as expected), as well as all siblings of the homozygous individual did not reveal any signs of glaucoma or ocular hypertension. Penetrance for the Gln368STOP *MYOC* mutation increases with age. In our previously reported Australian cohort the mean age of diagnosis for Gln368STOP carriers was 52.4 ± 12.9 years (Craig et al., 2001). It was interesting to identify a further example of an elderly *MYOC* mutation carrier with no glaucomatous damage. This could convey a co-segregating protective factor which may have been transmitted to his homozygous daughter, although it is noted that she is still just below the mean age of diagnosis for heterozygotes in our population.

Haplotype analysis revealed that a recombinant event had occurred within the 5-prime region 1kb upstream region from the Gln368STOP mutation on the paternal chromosome. This suggests a narrowing of the previously described founded region (Baird et al., 2003).

Discussion:

Given that Gln368STOP is the most common *MYOC* mutation, it is noteworthy that no homozygous individuals have previously been identified in other well investigated pedigrees (Baird et al., 2003; Craig et al., 2001). Recently, the Gln368STOP mutation was screened in an Australian population-based cohort, and was identified to have a carrier frequency of ~1:1,000 (Baird et al., 2005b). Hence, in Australia we would expect five people to be homozygous for Gln368STOP *MYOC*.

In summary, we report the first patient homozygous for the commonest Gln368STOP *MYOC* mutation. She shows no evidence of glaucoma at the age of 49. Although there is the prospect of this individual developing glaucoma in the future, it is clear that she does not manifest a more severe than usual phenotype.

Investigation for founder effects of the Thr377Met *Myocilin* mutation in glaucoma families from differing ethnic backgrounds

Background:

Worldwide the Thr377Met *MYOC* mutation is one of the most commonly identified OAG-causing mutations and it has been previously identified in populations from five different continents. This specific mutation has been identified in four Australian-based families, two families residing in the United States of America and one each from Greece, the Former Yugoslavian Republic of Macedonia (FYROM) India, Finland and Morocco (Allingham et al., 1998; Kanagavalli et al., 2003; Mackey et al., 2003; Melki et al., 2003b; Petersen et al., 2006; Puska et al., 2005; Shimizu et al., 2000; Wiggs et al., 1998). The Thr377Met *MYOC* mutation is located within the C-terminal coding region and renders the protein insoluble (Zhou and Vollrath, 1999). Although this highly penetrant mutation appears to be sufficient to cause disease, evidence supporting gene-gene interaction in adult onset glaucoma has been described in a family harbouring this variant (Petersen et al., 2006). Members of a Greek family who carried both the Thr377Met *MYOC* mutation and a haplotype associated with the GLC1C locus displayed greater disease severity than did those family members with only one disease variant (Petersen et al., 2006).

In general, the Thr377Met mutation confers disease of intermediate severity, with patients typically being diagnosed in their fourth decade. This is younger than the age at which patients with the Gln368Stop mutation are usually diagnosed, yet somewhat older than those carrying other *MYOC* mutations such as Pro370Leu, which is generally diagnosed around age 15 years (Allingham et al., 1998; Kanagavalli et al., 2003; Mackey et al., 2003; Melki et al., 2003b; Petersen et al., 2006; Puska et al., 2005; Shimizu et al., 2000; Wiggs et al., 1998). Following a similar pattern, patients with the Thr377Met mutation tend to have a maximum

recorded intraocular pressure around 30 mmHg, lower than that described for mutations causing juvenile onset glaucoma (such as Pro370Leu) though generally higher than for the Gln368Stop mutation (Shimizu et al., 2000).

Inherited diseases with a relatively late onset may be more likely to have a common disease founder than those which manifest at a younger age. As outlined in the preceding section, evidence for a founding effect has previously been reported for the common Gln368Stop *MYOC* mutation; however, others such as the Gly367Arg mutation have arisen independently several times (Baird et al., 2003; Baird et al., 2005c; Faucher et al., 2002). Small, local founder effects have also been identified for other less common mutations (Angius et al., 1998; Baird et al., 2003; Brezin et al., 1998; Faucher et al., 2002; Melki et al., 2003b). It is unclear whether a OAG variant causing intermediate disease severity would disseminate worldwide. Herein, we identified the haplotypes associated with the Thr377Met MYOC mutation to evaluate a possible founder effect in OAG families from various different ethnic backgrounds. To assess the degree of linkage disequilibrium across *MYOC* in the general population we also investigated data generated from the HapMap consortium.

Methods:

Nine Thr377Met *MYOC* glaucoma pedigrees from the United States of America, Greece, Finland, India and Australia were recruited. The four Australian families had emigrated from Great Britain, Greece and Macedonia. Both families from the United States of America were of Greek ethnicity (Table 2-5). The Indian proband lived in a Sino-Tibetan linguistic region. Ethical approval had previously been obtained by each research group's respective local Institutional Review Board as previously

reported (Allingham et al., 1998; Kanagavalli et al., 2003; Mackey et al., 2003; Melki et al., 2003b; Petersen et al., 2006; Puska et al., 2005; Shimizu et al., 2000; Wiggs et al., 1998).

Table 2-5
Summary of phenotypic features of glaucomatous individuals carrying the Thr377Met *MYOC* mutation.

Family Designation	Country of residence (Ancestral Ethnicity)	n of OAG or OHT individuals described	Mean ± SE age at diagnosis (years)	Mean ± SE maximum recorded IOP (mmHg)	Previous Description of Pedigree
Vic1	Australia (British)	19	39 ± 2.5	29.9 ± 1.8	(Mackey et al., 2003)
Vic118	Australia (Greek)	1	57	60	(Mackey et al., 2003)
Vic119	Australia (Greek)	2	42 ± 10	38 ± 8	(Mackey et al., 2003)
Vic120	Australia (Macedonian)	1	47	30	(Mackey et al., 2003)
Ep1	Greece (Greek)	9	51.3 ± 4.9	32 ± 3.8	(Petersen et al., 2006)
UMJG7	USA (Greek)	3	38 ± 1.7	44 ± 1.7	(Shimizu et al., 2000)
HMS7	USA (Greek)	1	42	24	(Wiggs et al., 1998)
Fin1	Finland (Finnish)	9	34.3 ± 8.8	33.2 ± 5.5	(Puska et al., 2005)
Ind1	India (Sino-Tibetan)	1	52	44	(Kanagavalli et al., 2003)
-	Morocco (Berber)	1	36	70	(Melki et al., 2003b)

Abbreviations: n, number; SE, standard error; OAG, primary open angle glaucoma; OHT, ocular hypertension; IOP, intraocular pressure; USA, United States of America.

Six microsatellite markers (D1S2851, MY3, My5, D1S1619, D1S2790 and D1S242) and four single nucleotide polymorphisms (rs3768570, rs235858, rs171000 and rs235873) located within a four Mb region surrounding the *MYOC* gene were genotyped in available subjects from each pedigree. Haplotypes in each family were constructed by visual inspection of the genotype data with no recombinations

assumed between the markers. Genomic DNA was available from only one individual from the Indian cohort.

To estimate the probability of finding non-coincidental haplotypes segregating with the founded Thr377Met *MYOC* mutation, we determined the frequencies of the associated neighbouring alleles in a sample of OAG patients originating from the same ethnic background. Thirty-three genealogically unrelated OAG patients of Greek ethnicity who on direct sequencing were found not to have the Thr377Met *MYOC* mutation, were recruited. The frequencies of the founded haplotype between the Greek Thr377Met *MYOC* mutation carrying patients and the Greek non-*MYOC* OAG patients was compared using Fisher's exact test (Intercooled Stata 7.0 for Windows; Stata Corporation, College Station, TX, USA).

Allele frequencies and linkage disequilibrium across the chromosome region 1q24 was assessed using HapMap data (www.hapmap.org)(The HapMap Consortium 2003). Phase I and II data were accessed from public release 21. This data release contains all processed data from the project including genotypes from the Affymetrix 500k genotyping array. HapMap comprises data of genotyped individuals from the Centre d'Etude du Polymorphisme Humain collection in Utah, USA; the Yoruba in Ibadan, Nigeria; The Han Chinese in Beijing, China; and the Japanese in Tokyo, Japan (The HapMap Consortium 2003). Haplotype blocks were reviewed using Haploview 3.32 and defined as having an upper confidence interval maximum for strong recombination of 0.9 (Barrett et al., 2005; Gabriel et al., 2002). Markers with a minor allele frequency below 0.05 were excluded (Gabriel et al., 2002).

Results:

Genotyping of the microsatellite markers revealed a shared haplotype in mutation carriers across six families from Greece, the United States of America and Australia (Table 2-6). All of these families were known to be of Greek or Macedonian ethnicity and recombination was found to have occurred in a region telomeric to *MYOC* between D1S1619 and D1S2790.

Table 2-6
Ancestral disease haplotype shared by individuals carrying the Thr377Met *MYOC* mutation from different ethnic backgrounds.

Marker	Chromosome 1 location (Mb)	Greek	Australian (British)	Indian	Finnish
D1S2851	167.048	187	187	177 / 181	187
rs3768570	167.890	-	G	G / A	A
rs235858	168.328	-	G	G / A	A
rs171000	168.330	-	G	G / T	T
MY3	168.336	176	174	174 / 178	178
rs235873	168.344	-	G	G / A	A
My5 (NGA17)	168.454	239	239	239	243
D1S1619	168.469	198	192	198 / 200	192
D1S2790	169.756	-	243	243 / 249	249
D1S242	171.104	-	215	221	217
Number of contributing pedigrees:		6	1	1	1
Number of mutation carrying subjects haplotyped:		26	12	1	4

To investigate whether the haplotype observed in the Greek/ FYROM Thr377Met *MYOC* families was inherited only by chance we genotyped a small cohort of OAG patients of Greek ethnicity who were known not to have the Thr377Met *MYOC* mutation. Although the precise phase could not be fully determined because of the limited availability of other family members, one Greek patient with OAG was found to have markers (between My3 and D1S1619) in common with the founded Greek Thr377Met *MYOC* mutation. However, even with the conservative assumption that

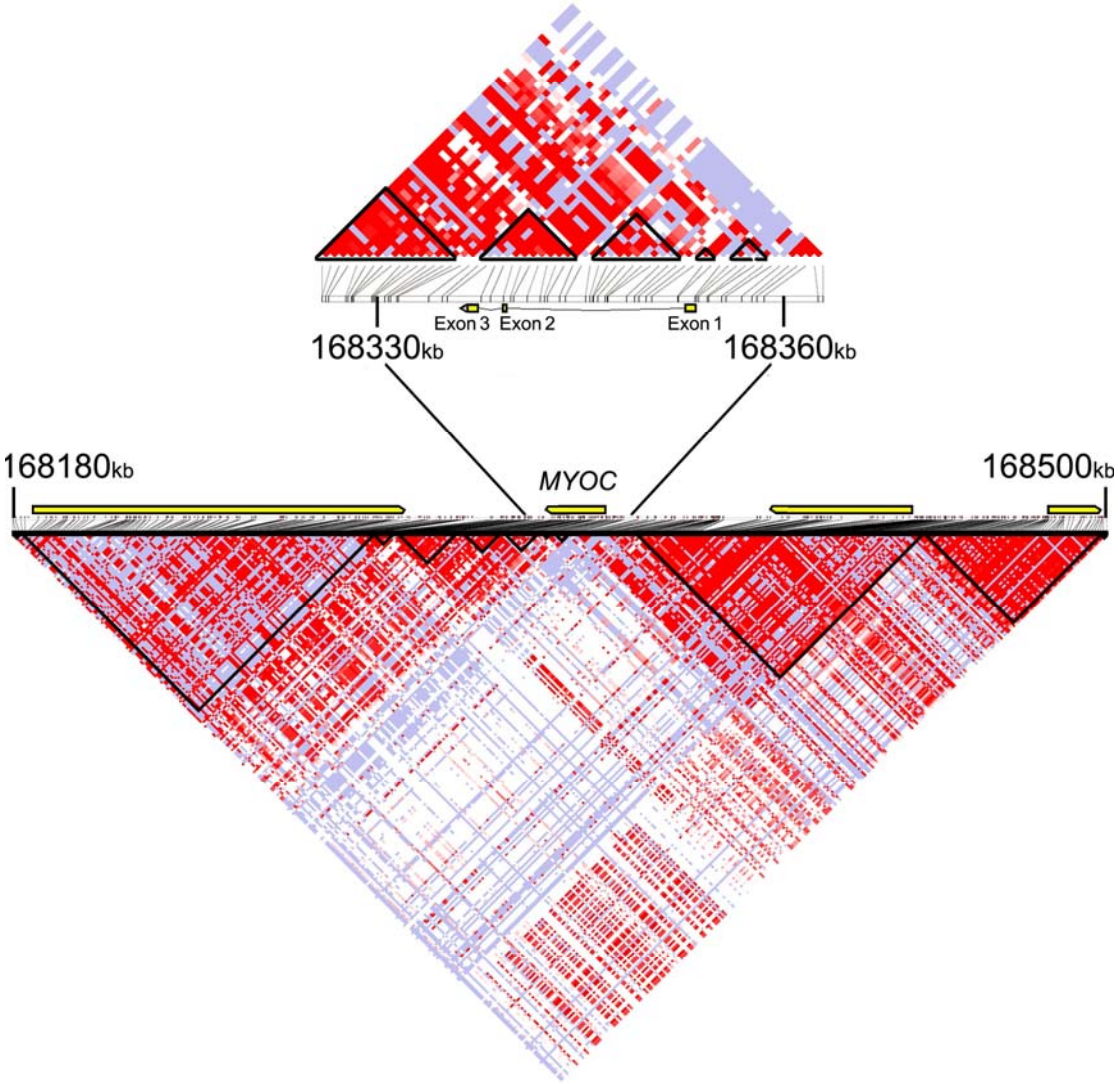
this patient did have the founded MYOC haplotype, our data supports the rejection of the hypothesis that the Greek Thr377Met haplotype merely represents frequent alleles ($p<0.0001$).

The families from Finland and Great Britain were found to have differing haplotypes from each other and from those which originated from Greece or Macedonia. This suggests that the Thr377Met *MYOC* mutation has arisen at least three times worldwide. The Indian proband was found to have some markers in common both with the Finnish pedigree and the family which originated from Great Britain. However, given that only one case from this family could be ascertained, the haplotype segregating with the Thr377Met *MYOC* mutation could not be fully determined.

Reviewing the data generated by the HapMap consortium revealed that linkage disequilibrium across *MYOC*, particularly in the Utah residents, is not strong (Figure 2-9). Larger linkage disequilibrium blocks over the genes neighbouring *MYOC* (*HbxAg transactivated protein 2* and *vesicle-associated membrane protein 4*) were identified. In each population linkage disequilibrium was generally stronger across exons 2 and 3 compared to the 5′ region of the *MYOC* gene.

Figure 2-9

Haplotype blocks of the 320kbp region containing the *myocilin* (*MYOC*) gene, in subjects from the Centre d'Etude du Polymorphisme Humain collection in Utah. The plotted D′ statistic is orientated to the *MYOC* ideogram position and the strength of linkage disequilibrium is displayed in increasing shades of red for higher values. The relative gene locations are indicated by yellow bars.



Discussion:

Pre-symptomatic screening for glaucoma is an attractive prospect and although it may be currently uneconomical to introduce *MYOC* gene screening in a population-wide approach, targeted screening is warranted in selected populations. Our results imply that the Thr377Met *MYOC* mutation should be considered in glaucoma patients of Greek descent in particular.

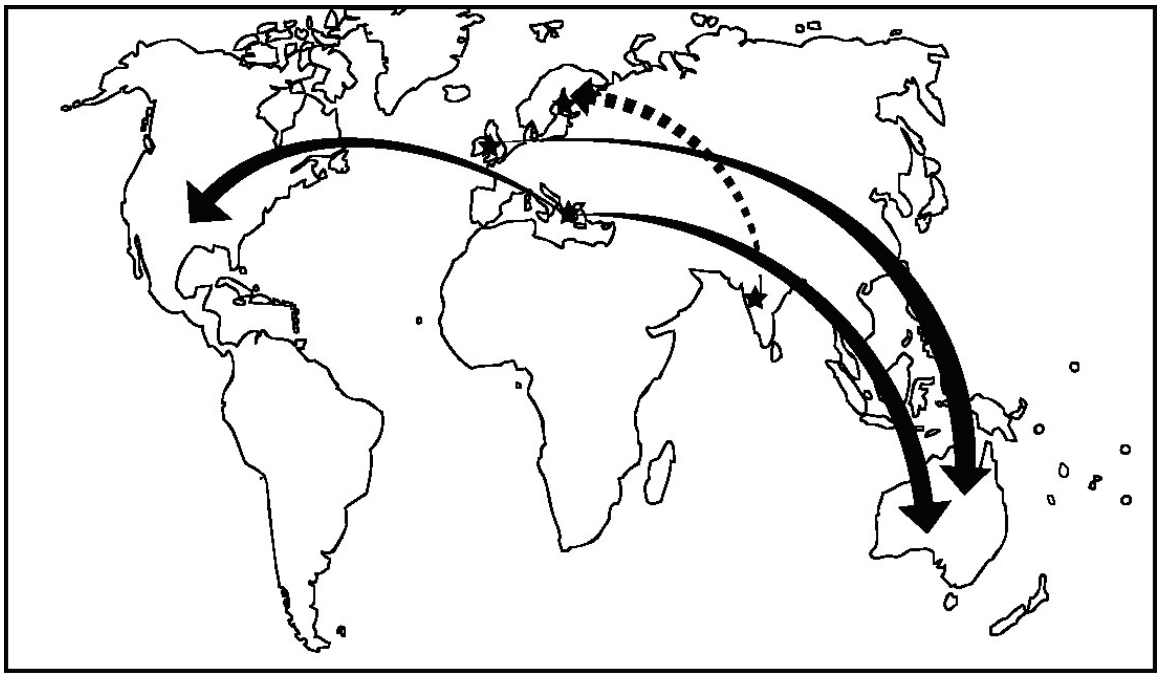
A common haplotype was identified with the Thr377Met *MYOC* in six of the nine genealogically independent pedigrees studied. The northern boundary of Epirus, Greece, where the *Ep1* family in this report lives, forms the southern edge of the FYROM. Given this geographical proximity, we surmised that the Australian families from Greece and the FYROM have a common founder (Figure 2-10). However, the British, Finnish and Indian families have a distinct haplotype from the Greek one, suggesting that the Thr377Met mutation has occurred de novo more than once (Figure 2-11). Unfortunately refinement of the haplotype associated with this mutation in the Indian case was limited by the lack of genetically informative relatives. Nonetheless, we hypothesize that a Finnish-Indian connection would be at least 1500 years old, from when the Finno-Ugric (Huns) people migrated from Central Asia. A limitation of our work is that the location of the centromeric and telomeric recombination in each pedigree was not identified. Such information would allow for the identification of the minimal common genomic distance between mutation carriers and in turn provide an estimation of the age at which the Thr377Met mutation occurred, yet is beyond the scope of this work (Genin et al., 2004; Slatkin 2000).

Figure 2-10
Aghia Paraskevi who is recognised by the Eastern Greek Orthodox Church as the patron saint of sight. This photograph was taken during a field trip at the head of the Vikos Gorge, the world's longest and deepest gorge located in Northern Epirus.

The population spread of human disease is significantly influenced by relative selection pressures and the existence of founder effects raises questions about whether such pressures are playing a role in *MYOC* allele frequencies. Our results coupled with the late age at diagnosis (with the weighted mean across published pedigrees being 41.4 years), suggest that the Thr377Met *MYOC* mutation alone may not be significantly detrimental to reproduction. Although it is possible to arrive at a founder effect through non-selective processes such as genetic drift, it is plausible

that this glaucoma-causing mutation may act as a protective factor against a separate ailment, thereby positively affecting biological fitness. Further insights may come from studies of Thr377Met allele frequencies in the Greek glaucoma populations. Evidence for genetic founder effects in this prevalent age-related yet heterogeneous disease has important implications for future gene identification strategies.

Figure 2-11
The worldwide dissemination of the Thr377Met *MYOC* mutation. Stars indicate location of known origin; solid arrows indicate known migrations; and the dashed arrow indicates an inferred migration.



The haplotype structure across the comparatively small region surrounding *MYOC*, in conjunction with relatively small proportion of disease accounted for by mutations in *MYOC*, suggests that a high density SNP platform would be required for it to have been identified using a genome-wide association case-control approach.

Nevertheless, genes with a different allelic architecture could be identified using such technology and, interestingly, a common disease haplotype has recently been associated with the common Y402H variant of *Complement Factor H* implicated in age-related macular degeneration (Hageman et al., 2005).

Confined conclusions based on the HapMap data must be approached with caution and the populations from which the data were generated should not be over-generalised. Nevertheless, it was interesting to find that the linkage disequilibrium across *MYOC* was not as strong as its neighbouring genes. Although we found that linkage disequilibrium was marginally stronger across the regions with greater cross-species homology (particularly exon 3), on a genome-wide level sequence conservation appears not to be an important predictor of linkage disequilibrium (Kato et al., 2006).

In summary, the Thr377Met *MYOC* mutation has arisen at least three times in independent populations. Interestingly however, HapMap data suggest that linkage disequilibrium across *MYOC* is not strong. Evidence for genetic founder effects in this prevalent age-related, yet heterogeneous disease has important implications for future gene identification strategies.

The Gly252Arg *Myocilin* mutation confers glaucoma of intermediate severity and in Caucasians originated from a common founder.

Background:

In 1998 Richards et al. identified the *myocilin* (*MYOC*) Gly252Arg mutation in a Caucasian patient residing in the United States of America (Richards et al., 1998). As described by Shimizu et al., this patient was diagnosed with glaucoma at the age of 26 years and had a maximum recorded intraocular pressure (IOP) of 62 mmHg (Shimizu et al., 2000). In keeping with a juvenile onset glaucoma phenotype, Booth et al. described a large Scottish family harbouring the *MYOC* Gly252Arg mutation (Booth et al., 2000). The mean ± SD age at OAG diagnosis was approximately 30.8 ± 7.3 years, with the mean ± SD maximum recorded IOP 39.3 ± 12.5 mmHg (Booth et al., 2000). Five of the six mutation-carrying individuals who manifested disease had undergone bilateral trabeculectomy (Booth et al., 2000).

Willoughby et al. described a two generation Chinese pedigree who carried the *MYOC* Gly252Arg mutation and had juvenile onset glaucoma (Willoughby et al., 2004). The proband was diagnosed at aged 29, whilst her father had been diagnosed at the age of 38 years and had required bilateral trabeculectomy (Willoughby et al., 2004). Interestingly, these Chinese cases were found to have the Arg545Gln variant in *optineurin* (OPTN), the second gene identified to cause OAG (Willoughby et al., 2004). However, this *OPTN* variant has been found to be distributed equally between Chinese glaucoma cases and ethnically matched normal control subjects (Leung et al., 2003).

The *MYOC* Gly252Arg amino acid substitution is predicted to have a positive charge change and is Triton assay insoluble (Shimizu et al., 2000). Herein, we describe the

phenotype of an Australian pedigree with the *MYOC* Gly252Arg mutation and show that all Caucasian OAG cases with this mutation have a common founder.

Method:

This study was approved by the relevant ethics committees of the Royal Victorian Eye and Ear Hospital and the Royal Hobart Hospital. It was conducted in accordance with the revised declaration of Helsinki. Written informed consent was provided by each subject.

All recruited subjects underwent a comprehensive clinical examination which included: anterior segment examination; gonioscopy; IOP measurement by Goldmann applanation tonometry; pachymetry; refraction; and a mydriatic optic disc assessment. Simultaneous stereoscopic optic disc photographs were digitalized (Nidek fundus camera 3-Dx/F, Nidek, Gamagori, Japan). All subjects over the age of 30, and younger individuals who had optic disc signs suggestive of glaucomatous damage, underwent automated visual field assessment using the Humphrey computerized perimeter (Humphrey Field Analyser II, Zeiss-Humphrey, Dublin, California, USA).

The Mann-Whitney test was used to compare the age at diagnosis and maximum recorded IOP between our OAG cases and those presented previously by Shimizu et al.(Shimizu et al., 2000) and Booth et al (Booth et al., 2000). Fisher's exact test was used to compare the proportion of subjects who had undergone trabeculectomy in our cohort to that described by Booth et al. (Booth et al., 2000). Statistical analysis was performed using Intercooled Stata 7.0 for Windows (Stata Corporation, USA). Power calculations were performed using PS v1.0.17 (Dupont and Plummer, 1997).

Laboratory Techniques:

Genomic DNA was isolated from peripheral blood samples. The *MYOC* Gly252Arg mutation was initially detected with the use of single-strand conformation polymorphism analysis. A template of 12.5 ng of DNA was used in an 8.35 μL polymerase chain reaction (PCR) using primer sequences and conditions previously described (Alward et al., 1998). Amplified products were denatured and underwent electrophoresis.

Subsequent mutation analysis for other members of the family was performed through direct sequencing. The *MYOC* exon 3 amplicon containing the *MYOC* 252 codon was amplified (Alward et al., 1998). The PCR products were purified and directly sequenced (Kit Promega kit Wizard SV Gel PCR clean up system). Sequencing reactions were carried out using the Applied Biosystems Big Dye Terminator kit (Applied Biosystems, Scoresby, Australia), with 25 cycles of 10s at 95^{o}C, 5s at 50^{o}C, followed by 4 min at 60 ^{o}C, as specified by the manufacturer. Sequencing analysis was performed using an Applied Biosystems Prism 310 Genetic Analyzer™ and were reviewed using Sequencher™ (Gene Codes Corporation, Michigan, USA).

The haplotype around the *MYOC* Gly252Arg mutation in affected members of our Australian pedigree was compared to that of OAG affected members of the previously described American and Scottish cases known to have the same mutation (Booth et al., 2000; Richards et al., 1998). Genotyping was performed using nine microsatellite satellite markers (D1S2658, D1S851, MY5, MY3, D1S2815, D1S1619, D1S218, D1S212, D1S2640) using previously described methods (Baird et al., 2003).

Results:

The matriarch and patriarch (born circa 1795) for the six generation Australian Caucasian pedigree (GACT02) are known to have 85 descendants. Key individuals are displayed in Figure 2-12. Eight subjects from this pedigree with the *MYOC* Gly252Arg mutation were found to have glaucoma (Figure 2-12). The mean ± SD age at diagnosis was 46.3 ± 11.4 years (range 31-60 years). Six (75%) of these individuals had undergone filtration surgery (Table 2-7). The highest recorded IOP ranged from 27 to 42 mmHg (mean ± SD: 32.4 ± 5.6). The mean ± SD central corneal thickness was 520 ± 25 μm. Examples of the optic disc and visual field characteristics for these glaucomatous cases are displayed in Figure 2-13.

Table 2-7
Clinical characteristics of individuals with the *MYOC* Gly252Arg mutation.

Subject	Gender	Diagnosis	Age at Most Recent Examination (years)	Age at Diagnosis (years)	Maximum Recorded IOP (mmHg)	Glaucoma Therapy
V:2	F	Normal	39	-	16	nil
V:1	M	Normal	45	-	19	nil
IV:20	M	Normal	58	-	17	nil
V:6	F	OH	42	40	30	Med
V:4	F	OH	49	40	26	Med
V:3	F	OH	51	45	24	nil
IV:18	F	HTG	65	31	32	TRAB OU
IV:13	F	HTG	52	36	30	TRAB OU
IV:2	F	HTG	76	36	42	TRAB OU
IV:9	F	HTG	75	42	32	TRAB OU
IV:16	M	HTG	64	50	40	TRAB OU
IV:14	M	HTG	71	57	27	TRAB OU
IV:11	M	HTG	63	58	28	Med
IV:10	F	HTG	72	60	28	Med

Abbreviations: F, female; M, male; OH, ocular hypertension; HTG, high tension glaucoma; IOP, intraocular pressure; Med, topical glaucoma medication; TRAB OU, trabeculectomy in both eyes.

Figure 2-12

Principal individuals from the GACT02 pedigree. Individual identification code and year of birth is displayed for each symbol. Clear symbols indicate people with no signs of glaucoma, symbols fully shaded depict those with glaucoma, half filled symbols depict subjects with ocular hypertension. Carrier status of the *MYOC* Gly252Arg mutation is displayed (+ or -). Note that individual IV:5 is affected by hearsay.

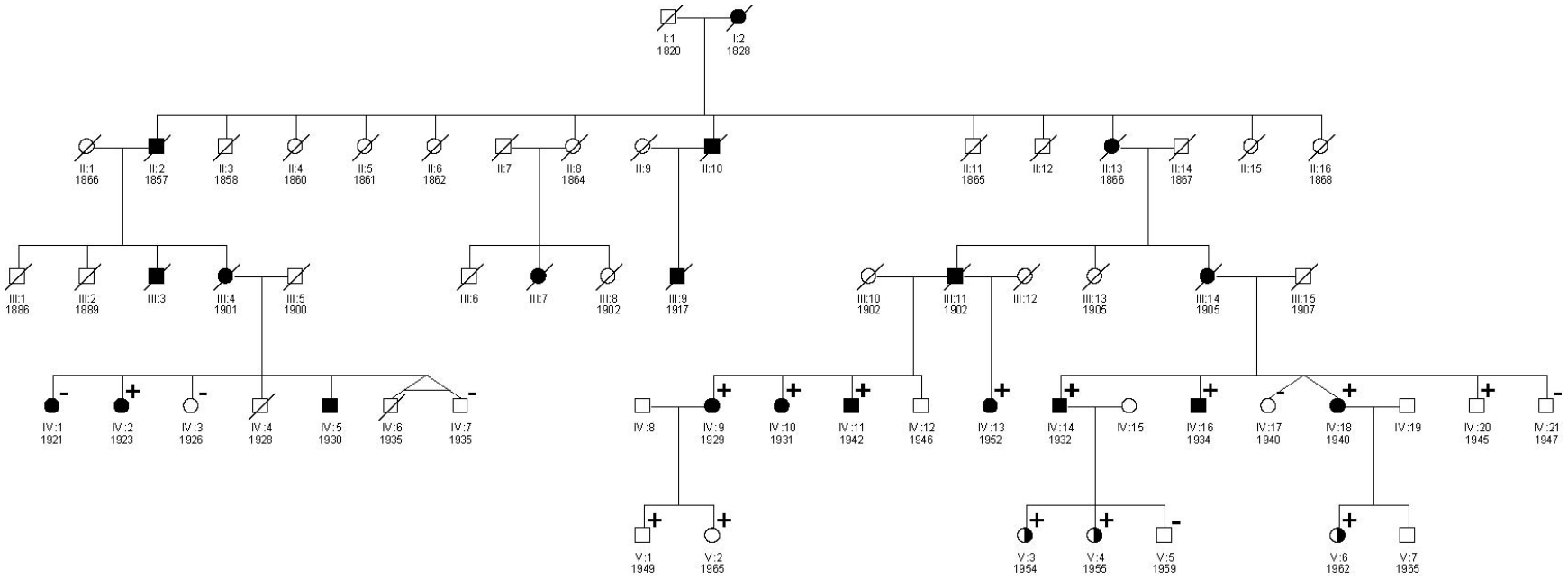


Figure 2-13
Optic disc appearance and Humphrey 24-2 visual field findings of subjects with glaucoma carrying the *MYOC* Gly252Arg mutation.

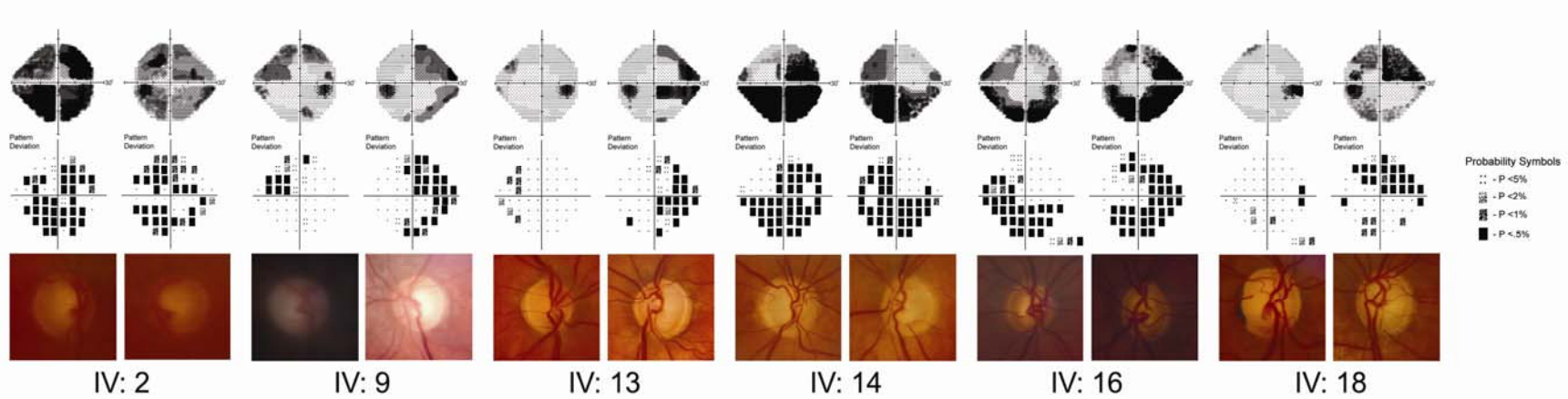
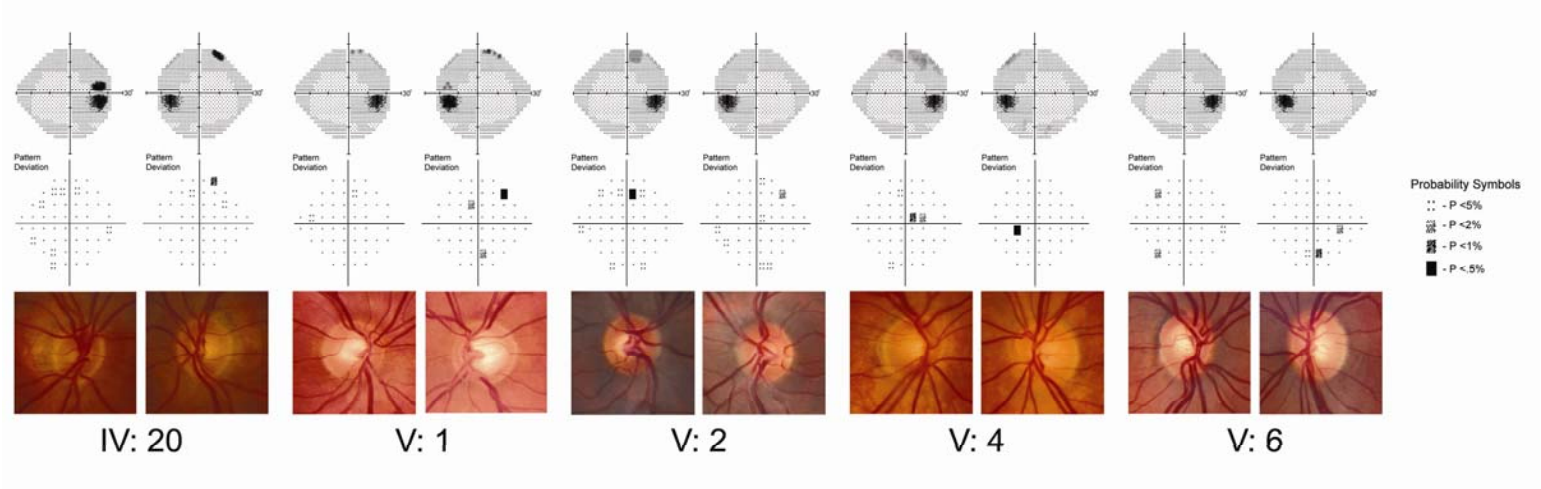


Figure 2-14
Optic disc appearance and Humphrey 24-2 visual field findings of individuals carrying the *MYOC* Gly252Arg mutation yet not currently manifesting glaucoma. Note the large optic discs of subject V:1 who has normal intraocular pressures and that individuals V:4 and V:6 have documented ocular hypertension.



An additional three subjects with the *MYOC* Gly252Arg mutation were diagnosed with ocular hypertension, two (V:4 and V:6) of whom had commenced a prostaglandin receptor agonist at the age of 40 years. Despite being heterozygotic for the Gly252Arg mutation, three subjects (IV:20, V:1, V:2) aged 58,45 and 39 years respectively, did not manifest ocular hypertension or have reproducible visual field loss (Figure 2-14).

A founding haplotype between MY5 and D1S218 was identified across our pedigree and affected mutation-carrying Caucasian subjects living in Scotland and North America (Table 2-8)(Booth et al., 2000; Shimizu et al., 2000). However, the mean age of onset for glaucoma and ocular hypertension in this Australian pedigree was significantly greater than previously presented ($p=0.0026$) (Booth et al., 2000; Shimizu et al., 2000). This finding remained significant when the ocular hypertension cases were excluded ($p=0.0121$). Maximum recorded IOP and the proportion of subjects requiring trabeculectomy did not significantly differ between our cases and that previously described ($p=0.11$ and $p=0.55$ respectively), though we had less than 20% power to detect a difference for these traits (Booth et al., 2000; Shimizu et al., 2000).

Table 2-8
Marker size for common *MYOC* mutation haplotypes.

	Marker								
MYOC Mutation (Ethnicity)	D1S2658	D1S851	MY5	MY3	D1S2815	D1S1619	D1S218	D1S212	Reference:
Gly252Arg (Caucasian)	-	-	241	178	228	192	287	-	Present Study
Gly252Arg (Chinese-Canadian)	279	184	239	178	226	198	283	123	Present Study
Thr377Met (Greek)	-	-	239	176	230	198	-	-	(Hewitt et al., 2007b)
Thr377Met (UK/Australian)	-	-	239	174	232	192	-	-	(Hewitt et al., 2007b)
Thr377Met (Finland)			243	178	187	192			(Hewitt et al., 2007b)
Gln368Stop (Caucasian)	-	-	-	174	230	192	-	-	(Baird et al., 2003; Hewitt et al., 2006a)

Phenocopy was identified in two branches of the pedigree (1: in IV:1 and the son of IV:3 not shown, as well as 2: the granddaughter of II:5). Subject IV:1 was diagnosed with glaucoma at 40 years and had undergone trabeculectomy in both eyes. The matriarch's great grand niece (not shown) was diagnosed with glaucoma aged 59 years; however, she was found not to have the Gly252Arg mutation.

Discussion:

We present evidence of co-segregation of the *MYOC* Gly252Arg myocilin mutation with disease phenotype. The Gly252Arg mutation alters the charge and is predicted to alter neighbouring residues secondary structure, from a β-strand to α-helix across a conserved PCK motif (Rozsa et al., 1998). Further analysis has revealed that the amino acid alteration renders the protein insoluble on Triton solubility assay (Shimizu et al., 2000). The *MYOC* Gly252Arg mutation has not

been identified in any normal control series (Fingert et al., 1999; Shimizu et al., 2000; Stone et al., 1997).

A common founder across Caucasian subjects with the *MYOC* Gly252Arg mutation was identified. Evidence of a common ancestry across other *MYOC* mutations (such as Gln368Stop and Thr377Met) has been described (Baird et al., 2003; Mackey et al., 2003). Such a finding has important implications for future methodology discovering other genes or single-nucleotide polymorphisms predisposing to adult-onset OAG.

Despite a common founder for this specific *MYOC* mutation in Caucasian subjects, the phenotype from this Australian Gly252Arg *MYOC*-mutation-carrying pedigree is less severe than previously described. Although a similar proportion required trabeculectomy, the age of diagnosis for glaucoma in our pedigree is significantly older than that described previously in the literature (Booth et al., 2000; Shimizu et al., 2000). Our data suggest that this mutation should be considered in adult-onset glaucoma cases rather than those solely with a juvenile onset.

From the literature published to date, the Gly252Arg mutation is of comparable severity to the *MYOC* Thr377Met mutation (Mackey et al., 2003; Puska et al., 2005; Shimizu et al., 2000). It is more severe than the *MYOC* Gln368Stop mutation though less severe than other mutations such as Pro370Leu or Lys423Glu (Craig et al., 2001;

Morissette et al., 1998; Shimizu et al., 2000; Suzuki et al., 1997). Using gonioscopy, Booth and colleagues identified abnormal angle blood vessels or mesodermal tissue remnants in the series of Gly252Arg *MYOC*-affected cases they examined (Booth et al., 2000). Interestingly, the drainage angle features described by Booth et al.(Booth et al., 2000) were not noted in our mutation-carrying patients. Such a difference in angle architecture may be the cause or a confounding reason for the differing age of diagnosis between pedigrees.

The optic nerve head in *Myocilin* glaucoma

Background:

The ability to accurately anticipate the likely natural history or clinical progression of a patient would significantly improve therapeutic algorithms and thus result in enhanced visual preservation. The usefulness of molecular diagnosis may be eliminated should discerning features be detectable at the slitlamp. The aim of this study was to describe the morphological features of the optic nerve head (ONH) in *MYOC* glaucoma, compared to severity matched OAG patients known not to have *MYOC* mutations.

Methods:

A case-control design was adopted, whereby case subjects represented patients with a *MYOC* mutation and control subjects were OAG patients who did not have a *MYOC* mutation. A *MYOC* disease-causing mutation was defined as one that altered the predicted amino acid sequence and had previously been found to be consistently more common in glaucomatous cases than in age-matched normal individuals.(Alward et al., 1998) A total of 66 case subjects from 25 genealogically separate pedigrees were identified. These cases were matched by visual field severity to 105 control OAG patients without *MYOC* mutations. This study was approved by the relevant ethics committees of the Royal Victorian Eye and Ear Hospital, and the Royal Hobart Hospital. Written informed consent was obtained from each subject and this study was conducted in accordance with the declaration of Helsinki and subsequent revisions.

Glaucoma was defined by the presence of, in at least one eye, visual field loss with corresponding: optic disc cupping (cup-disc ratio ≥ 0.7); or by a 0.2 inter-eye disparity in cup-disc ratio; or focal rim notching. For inclusion, subjects required an abnormal visual field as graded by the Glaucoma Hemifield Test (using a Humphrey Field Analyser II, Zeiss-Humphrey, Dublin, California, USA). Mean deviation scores in the most recent, reliable visual field test were used to match glaucomatous patients without *MYOC* mutations (control subjects) to mutation-carrying case subjects.

All recruited subjects underwent a comprehensive clinical examination, which included: anterior segment examination; gonioscopy; IOP measurement by Goldmann applanation tonometry; visual field assessment; refraction; and a mydriatic optic disc assessment. Highly myopic eyes, with a refraction exceeding -7 dioptres, were excluded due to confounding myopia-related ONH appearances, including optic disc tilting and tessellation of vessels. Colour 35mm slides of the ONH were taken using a non-telecentric Nidek 3-Dx/F fundus camera (Nidek, Gamagori, Japan). The resultant simultaneous stereoscopic images were digitalized at a high resolution (2102×1435 pixels, 2900 ppi, 36 bit colour) using a Nikon CoolScan IV ED slide scanner (Nikon Corp., Tokyo, Japan).

Preliminary ONH quantification was performed stereoscopically using custom software with a Z-screen (Stereographics Corporation,

California, USA), by a grader masked to subjects' mutation status (Hewitt et al., 2006d; Morgan et al., 2005a; Morgan et al., 2005b; Sheen et al., 2004). This stereoscopic system has been described in detail elsewhere (Morgan et al., 2005a; Morgan et al., 2005b; Sheen et al., 2004). In brief, it consists of a normal CRT monitor and an overlying high speed modulating panel. Flicker-free stereoscopy, achieved by alternatively displaying the component images of the stereo pair on the monitor at 60 Hz, can be viewed using passive polarised glasses. Cursor depth may then be adjusted to coincide with Elschnig's rim, such that the neuroretinal rim as well as the inner margin of the disc can be outlined at the depth of the scleral plane. We corrected for image magnification using: keratometry readings; refraction; and camera specifications, using established methods, to provide scaled estimates of disc parameters (Morgan et al., 2005a; Morgan et al., 2005b; Sheen et al., 2004).

Using this custom software, the optic disc size, neuroretinal rim area, maximal disc and cup diameters, length of the central retinal vessel trunk along the floor of cup, and the size of any disc haemorrhages, as well as parapapillary atrophy were quantified. Parapapillary atrophy was differentiated into: a central beta zone demarcated by visible sclera as well as large choroidal vessels close to the optic disc border; and a peripheral alpha zone with irregular pigmentation (Jonas et al., 1989). A neuroretinal rim notch was defined as a 60^{o} arc of disc, in the centre of which the neuroretinal rim was thinner than 2/3 of the rim width at both peripheral borders of this disc sector. Disc and cup

ovality was determined by the ratios of their respective maximal vertical and horizontal diameters.

Using a Pentax Stereo Viewer-II (Pentax Imaging Company, Golden, Colorado, USA) the colour 35mm slides, with all patient identifying information removed, were subjectively graded. The central retinal artery entry site was categorised as being: “1” far nasal; “2” mid-nasal; “3” central; “4” mid-temporal; or “5” far temporal. Cup depth was graded in a Likert range between “0” for no cupping and “5” for very deep cupping (Jonas and Grundler, 1996). The slope in the majority (>50%) of the neuroretinal rim was scaled as being “1” for very shelved; “3” if vertical and “4” when undermined. When undermined the slope of the remaining neuroretinal rim was graded either “1” to “3”. The presence of nerve fibre layer defects and baring of vessels were also noted. When visible, the lamina cribrosa appearance was recorded. Similar to that described by Miller and Quigley, lamina pore shapes were described as being: “1” round or dot-like; “2” polygonal; “3” oval; and “4” striate or slit-like, in shape (Miller and Quigley, 1987). The configuration of the lamina pores were also characterized as being: “1” circumferential; “2” radial or spiral; “3” hour-glass; and “4” random or disorganized (Miller and Quigley, 1987).

The stereo disc photographs of the ONH of each subject were reviewed by an observer (Sonya L Bennett) masked to mutation status and clinical parameters. To determine the reproducibility and internal

validity of ONH grading, 43 (12.7%) randomly selected optic disc photographs were analysed twice. An identical grade for the central retinal artery entry site, slope of the neuroretinal rim and cup depth were made in over 90% of cases. Identical lamina cribrosa pore shape and orientation grades were made in over 86% of cases. Any ONH features that were rated differently upon re-testing were within one category of the original rank.

Laboratory Techniques:

Mutation screening was conducted using either direct sequencing or single stranded conformation polymorphism (SSCP) analysis. Genomic DNA was isolated from peripheral blood samples, and the coding regions of the *MYOC* were amplified using previously published oligonucleotide primers (Alward et al., 1998). In preparation for SSCP, PCR products were denatured for 3 minutes at 94^{o}C and following electrophoresis were stained with silver nitrate for review. Mutations detected by SSCP were subsequently confirmed by sequencing. Sequencing reactions were carried out using the Applied Biosystems Big Dye Terminator kit (Applied Biosystems, Scoresby, Australia), with 25 cycles of 10s at 95^{o}C, 5s at 50^{o}C, followed by 4 minutes at 60^{o}C, as specified by the manufacturer. Analysis was performed using an Applied Biosystems Prism 310 Genetic Analyzer™ and the resultant outputs were reviewed using Sequencher™ (Gene Codes Corporation, Michigan, USA).

Data Analysis:

The presence of *MYOC*-specific ONH features were investigated through comparing both the 'better' and 'worse' eyes of case and control subjects. The 'worse' eye was determined by mean deviation on the most recent reliable visual field. Case subjects were analysed on a pooled and mutation-specific basis to assess genotype-phenotype correlations.

Intercooled Stata 7.0 for Windows (Stata Corporation, College Station, TX, USA) was used in statistical analysis. The Student t-test was used for parametric data, whilst the Kruskal Wallis and Mann-Whitney tests were applied to determine significant differences in non-parametric data. Differences in categorical proportions were tested using the χ^2 test. The Bonferroni correction was used to account for multiple testing. Power calculations were performed using the PS program version 1.0.17 for Windows (Dupont and Plummer, 1997). Unless otherwise indicated, data is presented as the mean plus or minus the standard deviation (SD).

Results:

A total of 66 patients with a *MYOC* mutation (Gln368Stop, n=38; Thr377Met, n=17; Gly252Arg, n=6; Pro370Leu, n=4; Asp380Gly, n=1) were matched by visual field findings to 105 patients known not to have a *MYOC* mutation. Four eyes from four subjects (three cases and one control) were excluded from analysis due to poor fundus imaging. This cohort had 90% power to identify a ten fold increased

prevalence of disc haemorrhages at the 0.05 significance level. For quantitative traits we had a power of 0.887 to detect a 0.5 SD difference between the *MYOC* mutation group and the non-mutation control group.

Patients with a *MYOC* mutation were diagnosed earlier ($p<0.001$) and had higher maximum recorded IOP ($p<0.001$) than control patients without *MYOC* mutations (Table 2-9). Case subjects with the Gln368Stop mutation were diagnosed later (53.8 ± 12.9 years) than those with any other *MYOC* mutations ($p<0.001$). Case subjects with the Pro370Leu had the lowest age at diagnosis (15.7 ± 9.8 years). There was a stepwise increase in mean maximum recorded IOP between Gln368Stop, Thr377Met, Gly252Arg and Pro370Leu *MYOC* mutation cases, respectively ($p<0.001$). The subject with the Asp380Gly mutation was diagnosed aged 28 years and had a maximum recorded IOP of 44 mmHg.

Table 2-9
Composition of the study groups (mean ± SD; range).

	MYOC Glaucoma	Non-*MYOC* Glaucoma	p
N	66	105	
Gender F / M	36/30	67/38	0.228 (NS)
Age at diagnosis (years)	46.2 ± 15.1; 9 to 83	60.0 ± 11.3; 30 to 82	< 0.0001
Age at review (years)	60.6 ± 16.5; 16 to 96	68.4 ± 10.5; 41 to 93	< 0.0001
Maximum recorded IOP (mmHg)	30.8 ± 10.1; 17 to 60	23.2 ± 6.4; 15 to 44	< 0.0001
Refractive error (D)	-0.12 ± 1.4; -6.19 to +3.5	0.04 ± 1.6; -5.25 to +6.13	0.522 (NS)

Abbreviations: n, number; F, female; M, male; NS, not statistically significant; IOP, intraocular pressure; D, dioptres; MD, mean deviation.

The *MYOC*-mutation-carrying group and the *MYOC*-mutation-free group did not vary significantly in: optic disc area; neuroretinal rim area; steepness and depth of disc cupping; optic cup shape as determined by the ratio of vertical/horizontal cup diameters; and the size of beta as well alpha-zones of parapapillary atrophy (Table 2-10 and Table 2-11). There was no significant difference in visible lamina cribrosa morphology between mutation carriers and non-*MYOC* patients. The proportion of bared vessels did not differ between cases and controls (p=0.952). Although disc haemorrhages were identified more frequently in the control group, this finding was not significant after correction for multiple testing (Table 2-12). In all eyes examined, *MYOC*-mutation carriers had more neuroretinal rim notches than control subjects; once again, however, this was not significant following Bonferroni correction (Table 2-12).

Table 2-10

Optic nerve head characteristics of subject's worse eye as determined by visual field mean deviation (mean ± SD; range).

	MYOC Glaucoma	Non-*MYOC* Glaucoma	p
n	66	105	
Visual Field Severity (MD in dB)	-10.73 ± 10.19; -31.65 to -1.35	-8.90 ± 8.45; -30.80 to -1.10	0.217 (NS)
Optic Disc Size (mm^2)	2.13 ± 0.47; 1.25 to 3.33	2.14 ± 0.45; 1.02 to 3.17	0.892 (NS)
Neuroretinal rim area (mm^2)	1.04 ± 0.42; 0.25 to 2.21	1.14 ± 0.37; 0.28 to 2.59	0.088 (NS)
Neuroretinal rim / Optic Disc Area	0.42 ± 0.21; 0.06 to 0.91	0.46 ± 0.17; 0.10 to 0.86	0.236 (NS)
ONH Shape			
- Disc ovality	1.09 ± 0.07; 0.94 to 1.32	1.10 ± 0.08; 0.89 to 1.31	0.243 (NS)
- Cup ovality	1.16 ± 0.18; 0.81 to 1.71	1.18 ± 0.19; 0.81 to 2.24	0.497 (NS)
Optic disc cupping			
- Steepness	3.01 ± 1.00; 1 to 4	2.97 ± 0.99; 1 to 4	0.779 (NS)
- Depth	4.00 ± 0.97; 1 to 5	3.95 ± 1.14; 1 to 5	0.922 (NS)
Lamina cribrosa architecture			
- n gradable (%)	28 (42.4)	38 (36.2)	0.415 (NS)
- Lamina pore shape	2.18 ± 1.12; 1 to 4	2.14 ± 1.12; 1 to 4	0.904 (NS)
- Lamina pore orientation	3.00 ± 1.32; 1 to 4	3.13 ± 1.24; 1 to 4	0.713 (NS)
Parapapillary atrophy			
- Beta zone (mm^2)	0.20 ± 0.66; 0 to 5.09	0.27 ± 0.61; 0 to 4.74	0.487 (NS)
- Alpha zone (mm^2)	0.88 ± 0.72; 0 to 3.72	1.03 ± 0.71; 0 to 4.15	0.180 (NS)
Site of central retinal artery entry	2.41 ± 0.63; 1 to 4	2.47 ± 0.62; 1 to 4	0.496 (NS)
Length of vessel trunk along floor of cup (mm)	0.26 ± 0.21; 0 to 0.78	0.24 ± 0.20; 0 to 0.96	0.548 (NS)

Abbreviations: n, number; NS, not statistically significant; ONH, optic nerve head.

Table 2-11

Optic nerve head characteristics of subject's better eye as determined by visual field mean deviation (mean ± SD; range).

	MYOC Glaucoma	Non-*MYOC* Glaucoma	p
n	63	104	
Visual Field Severity (MD in dB)	-6.45 ± 8.81; -30.98 to 2.02	-4.70 ± 6.34; -27.82 to 2.62	0.144 (NS)
Optic Disc Size (mm^2)	2.19 ± 0.53; 1.39 to 3.74	2.15 ± 0.53; 1.15 to 3.57	0.640 (NS)
Neuroretinal rim area (mm^2)	1.15 ± 0.42; 0.38to 2.34	1.21 ± 0.39; 0.42 to 2.51	0.413 (NS)
Neuroretinal rim / Optic Disc Area	0.46 ± 0.18; 0.12 to 0.82	0.49 ± 0.18; 0.13 to 0.90	0.339 (NS)
ONH Shape			
- Disc ovality	1.12 ± 0.09; 0.86 to 1.36	1.10 ± 0.07; 0.91 to 1.26	0.301 (NS)
- Cup ovality	1.19 ± 0.21; 0.82 to 1.79	1.19 ± 0.22; 0.71 to 1.85	0.786 (NS)
Optic disc cupping			
- Steepness	2.94 ± 0.92; 1 to 4	2.79 ± 0.98; 1 to 4	0.341 (NS)
- Depth	3.90 ± 0.98; 0 to 5	3.79 ± 1.09; 0 to 5	0.564 (NS)
Lamina cribrosa architecture			
- n gradable (%)	25 (39.7)	30 (28.9)	0.149 (NS)
- Lamina pore shape	1.76 ± 1.20; 1 to 4	1.93 ± 1.01; 1 to 4	0.564 (NS)
- Lamina pore orientation	3.59 ± 0.85; 1 to 4	3.18 ± 1.17; 1 to 4	0.183 (NS)
Parapapillary atrophy			
- Beta zone (mm^2)	0.19 ± 0.35; 0 to 1.68	0.25 ± 0.62; 0 to 3.28	0.439 (NS)
- Alpha zone (mm^2)	0.92 ± 0.72; 0 to 3.29	1.09 ± 0.75; 0 to 4.84	0.161 (NS)
Site of central retinal artery entry	2.40 ± 0.59; 1 to 4	2.34 ± 0.54; 1 to 4	0.522 (NS)
Length of vessel trunk along floor of cup (mm)	0.22 ± 0.17; 0 to 0.73	0.20 ± 0.22; 0 to 1.00	0.581 (NS)

Abbreviations: n, number; NS, not statistically significant; ONH, optic nerve head.

Table 2-12

Disc haemorrhages, neuroretinal rim notching and nerve fibre layer defects in all eyes examined (mean ± SD; 95% confidence interval).

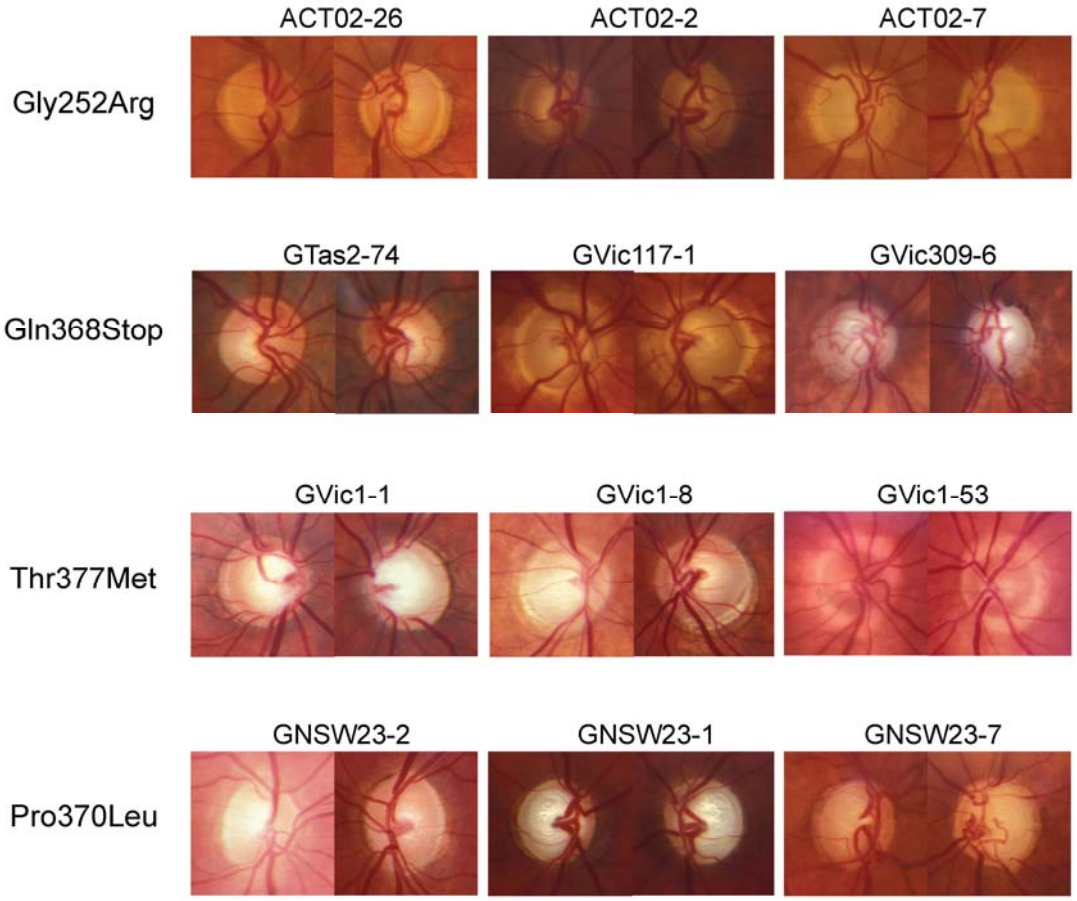
	MYOC Glaucoma	Non-*MYOC* Glaucoma	p
n of eyes	129	209	
Disc haemorrhages			
- Frequency (%)	1 (0.8)	14 (6.7)	0.010
- Size (mm^2)	0.02	0.10 ± 0.07; 0.06 to 0.14	0.165 (NS)
Frequency of neuroretinal rim notching (%)	38 (29.5)	39 (18.7)	0.022
Frequency of nerve fibre layer defects (%)	4 (3.1)	3 (1.4)	0.307 (NS)

Abbreviations: n, number; NS, not statistically significant.

There was a significant stepwise increase in neuroretinal rim area to optic disc area ratios between cases with the Gly252Arg, Thr377Met and Gln368Stop *MYOC* mutations (p=0.003). However, this trait did not statistically differ between Gln368Stop *MYOC*-mutation carriers and glaucomatous patients without *MYOC* mutations (p=0.43). Sub-analysis revealed no other significant *MYOC*-mutation specific morphological characteristics (Figure 2-15).

Figure 2-15

Optic nerve head photographs of individuals with the Gly252Arg (ACT02-26 aged 52; ACT02-2 aged 64; ACT02-7 aged 71), Gln368Stop (GTas2-74 aged 50; GVic117-1 aged 62; GVic309-6 aged 75), Thr377Met (GVic1-1 aged 34, GVic1-8 aged 48; GVic1-53 aged 78) and Pro370Leu (GNSW23-2 aged 18; GNSW23-1 aged 41; GNSW23-7 aged 62) *MYOC* mutations.



Discussion:

No major morphological differences in the ONH of glaucomatous *MYOC* mutation carriers compared to visual field severity matched non-*MYOC* patients were identified. This work supports that of Alward and colleagues, that maximum recorded IOP and age at diagnosis may be the best discerning features of *MYOC* glaucoma (Alward et al., 1998). ONH damage is the final common endpoint for glaucoma and our study underscores the phenomena of phenocopy in this heterogeneous disease.

MYOC protein from patients with the Gly252Arg, Gln368Stop, Pro370Leu and Thr377Met *MYOC* mutations are known to be Triton assay insoluble (Shimizu et al., 2000; Zhou and Vollrath, 1999), and firm phenotype-genotype correlations for age at diagnosis, maximum recorded IOP and neuroretinal rim area to optic disc area ratios were identified. The age of diagnosis and peak IOP in case subjects with the Gln368Stop; Pro370Leu or Thr377Met *MYOC* mutations did not differ from those previously reported (Adam et al., 1997; Allingham et al., 1998; Alward et al., 1998; Puska et al., 2005; Shimizu et al., 2000; Wiggs et al., 1998). The mean age of onset for OAG in our Gly252Arg cases is greater than that previously presented (Booth et al., 2000; Shimizu et al., 2000). The Asp380Gly *MYOC* mutation has previously been identified in a patient with juvenile-onset open-angle glaucoma (JOAG), and the amino acid substitution has a Blosum 62 Matrix score of -1, indicating a low tolerance for this particular exchange during natural selection (Henikoff and Henikoff, 1992; Stone 2003). A non-synonymous change to alanine at this 380 codon renders the MYOC protein insoluble (Liu and Vollrath, 2004).

Although analysis of the ONH slides suggested a lower rate of disc haemorrhages in *MYOC* carriers, after this study was performed two of the *MYOC*-mutation-carrying cases were subsequently documented as having optic disc haemorrhages. Thus, when supplemented with fact that the difference does not remain significant following correction for multiple testing, we are not certain that this difference is actually real. Interestingly, earlier work has suggested that optic disc haemorrhages are less common in patients with JOAG; however the analysis of this work may have been biased through not adjusting for age (Jonas and Budde, 2000). Nevertheless, a similar large review of patients with either high or normal-pressure OAG has also found optic disc haemorrhages to be more frequently associated with the latter group (Tezel et al., 1996).

The finding that neuroretinal rim notches were more common in *MYOC*-mutation carriers than in non-*MYOC* patients was unexpected; once again, however, this finding was not significant following correction for multiple testing. Jonas and Budde observed that neuroretinal rim notching was more common in patients with JOAG than patients with normal-pressure OAG. Given that a subset of *MYOC* mutations are known to cause JOAG we expected to reach a similar conclusion (Jonas and Budde, 2000). Using red-free fundus photographs Jonas and Budde found that, when present, localized retinal nerve fibre layer defects were narrower in subjects with JOAG than patients with normal-pressure OAG (Jonas and Budde, 2000). The number of nerve fibre layer defects identified in our cohort is relatively low and may reflect the inherent difficulty in identifying them from colour slides (Hoyt and Newman, 1972).

Extracellular matrix remodelling at the ONH occurs due to elevated IOP (Agapova et al., 2003). Fibroblast activation and expression of matrix metalloproteinases may alter the lamina pore shape and orientation. Miller and Quigley have previously found that patients with high IOP-related OAG are more likely to have an hour-glass appearance of connective tissue bundles at the lamina cribrosa (Miller and Quigley, 1987). No significant difference in the clinically visible lamina cribrosa architecture between *MYOC* cases and non-*MYOC* glaucomatous controls was noted. We corroborate the finding of Healey et al. that lamina cribrosa pore visibility is greater in ONHs with larger cup sizes (Healey and Mitchell, 2004). Our study does not fully preclude other fundamental differences in the composition of extracellular matrix remodelling between *MYOC* and non-*MYOC* glaucoma patients, and the pattern of pre-glaucomatous IOP spiking in *MYOC* mutation requires further investigation.

Caprioli and Spaeth have previously found that, despite having similar mean total visual field loss, patients with normal-pressure OAG tend to have smaller neuroretinal rim areas, particularly in the inferotemporal regions, than patients with high-pressure OAG (Caprioli and Spaeth, 1985). Given this finding they suggested that the ONH appearance may be useful in differentiating subgroups of OAG. We found that neuroretinal rim tissue was not preferentially lost in any particular region of *MYOC* cases compared to non-*MYOC* patients (data not shown). Additionally, the site of central artery entry and length of the vessel trunk along the floor of the cup, a direct surrogate for vessel bayoneting, did not differ between case and control subjects. After adjusting for disease severity, Tezel et al. concluded that the clinical appearance of the ONH did not differ between patients with normal or high-pressure OAG (Tezel et al., 1996).

Many of the mutation-carrying case subjects were from the same pedigree. A full description of the general clinical features and the overall pedigree structure for some of the Gln368Stop and Thr377Met *MYOC* mutation cases analysed as part of this study's cohort have been presented previously (Craig et al., 2001; Mackey et al., 2003). Randomly selecting only one affected case from each pedigree may prove the most rigorous means for investigation, however the small number available would significantly limit the ability to detect significance in such analysis. The power to detect signature morphological features in specific, uncommon *MYOC* mutations, such as Pro370Leu or Asp380Gly, was limited. Any potential bias introduced through adherence to therapy was minimized through matching the cases to control subjects by disease severity.

In summary, no structural or morphological difference of the ONH could be clearly detected in pooled subjects who had a *MYOC* mutation when compared to non-*MYOC* glaucoma cases. Longitudinal analysis of the ONH in *MYOC* cases may reveal specific, though likely subtle, characteristics important in the natural history of *MYOC*-related optic cup excavation and glaucomatous damage.

Young *Myocilin* mutation carriers and the Glaucoma Continuum: Are glaucomatous disc changes evident prior to diagnosis of glaucoma in *Myocilin* pedigrees?

Background:

The spectrum of OAG disease compounds the difficulty of population-based screening. A continuum exists between clinically undetectable OAG, asymptomatic disease and functional impairment (Weinreb et al., 2004). Within this 'glaucoma continuum', retinal ganglion cell loss and retinal nerve fibre layer damage may precede visual field deficit by at least five years (Katz et al., 1997; Pederson and Anderson, 1980; Quigley et al., 1992). It is known that the prevalence of OAG increases with age; however, there is no sound evidence base delineating the age for the commencement of screening (Crick and Tuck, 1995; Mitchell et al., 1996; Wensor et al., 1998).

Our previous investigations of pedigrees containing common *MYOC* mutations revealed that the mean ± SD age for the diagnosis of glaucoma or ocular hypertension (OHT) was 52.4 ± 12.9 and 40.4 ± 11.0 years for the Gln368STOP and Thr377Met *MYOC* mutation carriers, respectively (Craig et al., 2001; Mackey et al., 2003). The Thr377Met *MYOC* mutation penetrance rate for OHT or glaucoma in Australia is approximately 88% at the age 30 years, whilst the age-related penetrance for OHT or glaucoma is 72% at age 40 years for the Gln368STOP *MYOC* mutation (Craig et al., 2001; Mackey et al., 2003). Maximum IOP readings are typically higher in patients carrying the Thr377Met, who typically have a mean ± SD IOP of 31.7 ± 9.9 mmHg, rather than the Gln368STOP *MYOC* mutation which usually manifests with a mean ± SD IOP of 28.4 ± 4.7 (Craig et al., 2001; Mackey et al., 2003).

We performed cascade genetic screening in Australian pedigrees known to carry either the Gln368STOP or Thr377Met *MYOC* mutation. The aim of this study was to investigate whether structural differences of the optic nerve head are evident in young people who do not have manifest glaucoma but are known to carry common *MYOC* mutations. The main interest of this work was to investigate the early changes of optic nerve in subjects at an established high risk of developing glaucoma compared to subjects who likely have a baseline risk.

Method:

Once a mutation-carrying OAG patient has been identified, each of his/her first-degree relatives can be tested for the same mutation. If these relatives are identified as carrying the at-risk mutation, they are followed closely for early clinical signs of glaucoma, and their first-degree relatives are also tested. Thus, 'cascade genetic screening' moves out in a stepwise direction from the index case until all the relatives who carry the mutation are identified.

Cascade screening was performed for pedigrees with either the Gln368STOP or the Thr377Met *MYOC* mutations identified through the Glaucoma Inheritance Study in Tasmania (GIST). All recruited subjects underwent a comprehensive clinical examination, which included: anterior segment examination; gonioscopy; IOP measurement by Goldmann applanation tonometry; pachymetry; refraction; and a mydriatic optic disc assessment.

Stereoscopic optic disc photographs were taken (Nidek fundus camera 3-Dx/F, Nidek, Gamagori, Japan) and then digitalized at a high resolution (2102×1435 pixels,

2900 ppi, 36 bit colour) using a Nikon CoolScan IV ED slide scanner (Nikon Corp., Tokyo, Japan).

Analysis of optic disc area, optic cup area and neuroretinal rim area was performed stereoscopically using custom software (StereoDx) with a Z-screen (Stereographics Corporation, California, USA)(Morgan et al., 2005a; Morgan et al., 2005b). The technical details of this system have been described elsewhere. In brief, the Z screen comprises a glass plate incorporating a liquid crystal (LC) membrane which is laid over the computer monitor screen. The phase of the LC film is altered at 60 Hz so that the observer, when wearing passive Polaroid glasses, can restrict the view of the component image of the stereopair to the correct eye. The images of the stereopair are each displayed at 60 Hz with the monitor refreshing at 120Hz to provide a flicker free stereoscopic image. The stereoscopic depth of the mouse cursor can be adjusted to coincide with the plane of Elschnig's rim. The inner border of the neuroretinal rim is defined as the point at which a plane, lying at the level of Elschnig's rim intersects with the surface of the retinal nerve fibre layer. Image magnification was corrected on the basis of: keratometry readings; refraction; and camera specifications using established methods to provide scaled estimates of disc parameters (Garway-Heath et al., 1998; Rudnicka et al., 1998). Measurements were performed by a single grader who was masked to the mutation status of each subject.

Glaucoma was defined as the presence of an abnormal visual field with a corresponding optic disc change that was characteristic of glaucoma. Able subjects underwent automated visual field assessment using the Humphrey computerized perimeter (Humphrey Field Analyser II, Zeiss-Humphrey, Dublin, California, USA). Visual field results were deemed appropriate if the rate of false-positive or false-

negative responses were < 20% and there were < 5 fixation losses. Glaucomatous visual field defects were defined as having a total mean defect of less than 2dB, with upon repeated testing, at least two adjacent points in a location typical for glaucoma, having a pattern deviation of < 1%.

Age cut-off criteria were set at approximately one standard deviation below the mean age of glaucoma diagnosis for each mutation. Hence, for the purposes of this study the age limit for recruitment was set at 40 years for the Gln368STOP pedigrees and at 30 years for the Thr377Met pedigrees. Subjects were excluded if they had glaucoma previously diagnosed or a refractive error more than ± 6 D.

This study was approved by the relevant ethics committees of the Royal Victorian Eye and Ear Hospital, and the Royal Hobart Hospital. Written informed consent was obtained from each subject and this study was conducted in accordance with the declaration of Helsinki and its subsequent revisions.

Data Analysis:

A case-control design was adopted. Case subjects were the offspring of index cases identified to also carry their respective pedigree's *MYOC* mutation. Subjects from these same pedigrees found not to have inherited these mutations were defined as control subjects. The optic disc features of *MYOC* mutation carriers and non-carriers were compared. Additionally, disc features were compared on a mutation-by-mutation basis. Vertical and horizontal cup and disc measurements as well as each 60 degree neuroretinal rim segment were analysed separately. The Student's t-test with a Bonferroni correction for multiple-comparison testing was performed using Intercooled Stata 7.0 for Windows (Stata Corporation, USA). Power calculations

were performed using the PS program version 1.0.17 for Windows (Dupont and Plummer, 1997).

Laboratory Techniques:

Mutation analysis was performed through direct sequencing. Genomic DNA was isolated from peripheral blood samples, and the *MYOC* exon 3 amplicon containing the *MYOC* 368 and 377 codons was amplified using previously published intronic primers (Alward et al., 1998). The PCR products were purified and sequenced. The sequencing reactions were carried out using the Applied Biosystems Big Dye Terminator kit (Applied Biosystems, Scoresby, Australia), with 30 cycles of 10s at 95^{o}C, 5s at 50^{o}C, followed by 4 min at 60 ^{o}C, as specified by the manufacturer. Sequencing analysis was performed using an Applied Biosystems Prism 310 Genetic Analyzer™ and were reviewed using Sequencher™ (Gene Codes Corporation, Michigan, USA).

Results:

A total of 30 *MYOC* mutation (case) and 33 mutation-free (control) subjects were recruited. Of the 31 subjects recruited from the nine Gln368STOP *MYOC* pedigrees (GTas2, GTas209, GTas287, GTas309, GTas88, GQld11, GVic122, GVic124, GVic139), 14 (45.2%) were found to carry the *MYOC* mutation. No subject from the Gln368STOP *MYOC* pedigrees was required to be excluded due to new visual field findings suspicious for glaucoma. The Thr377Met *MYOC* mutation was identified in 16 (50.0%) of the 32 subjects who were recruited from two separate pedigrees (GVic1, GVic20). One 26-year-old subject (who was later found to have the Thr377Met mutation) was excluded because of a previous diagnosis of glaucoma (GVic1-IV:1)(Mackey et al., 2003). The fundus image of one eye of another subject

(with a Thr377Met *MYOC* mutation) was of poor quality and was excluded from analysis. Hence, optic discs from a total of 29 *MYOC* mutation carriers were available for analysis. At the 0.05 significance level this cohort had 80% power to detect a 0.18mm^2 difference in neuroretinal rim areas.

Demographic details of the study cohort are displayed in Table 2-13. There was no significant difference in IOP between mutation carriers and non-carriers (P=0.44). Three Thr377Met mutation carriers, aged between 22 and 30 years, were found to have a maximum recorded IOP above 21mmHg though below 25mmHg. The neuroretinal rim area in these subjects' eye with the smallest neuroretinal rim area ranged between 1.214 and 1.297 mm^2.

Vertical cup-to-disc ratios correlated highly with global disc areas (mutation carriers $y=0.168x + 0.125$, $r^2=0.24$, $p=0.006$; non-mutation carriers $y=0.132x + 0.247$, $r^2=0.21$, $p=0.008$). Across the whole cohort the measured optic disc variables approximated a Gaussian distribution. Adjusting for age at the time of examination did not reveal any significance difference in optic disc feature between mutation carriers and non-carriers.

Table 2-13
Demographic and optic disc parameters of young people from Gln368STOP or Thr377Met *myocilin* pedigrees.

	Gln368STOP Non-Carrier		Gln368STOP Carrier		p	Thr377Met Non-carrier		Thr377Met Carrier		p
Number of subjects	17		14			16		15		
Number of females (%)	10	(55.6)	7	(53.9)	0.45*	9	(56.3)	5	(33.3)	0.23*
	mean	(SD)	mean	(SD)		mean	(SD)	mean	(SD)	
Age in years	28.6	(8.0)	21.1	(11.6)	0.17	15.1	(4.7)	18.7	(5.8)	0.07
Maximum recorded IOP in mmHg	14.8	(3.1)	13.8	(3.0)	0.45	15.1	(4.7)	15.2	(3.9)	0.23
Maximum vertical cup:disc ratio	0.57	(0.08)	0.44	(0.15)	0.005**	0.51	(0.12)	0.50	(0.11)	0.93
Maximum horizontal cup:disc ratio	0.53	(0.09)	0.48	(0.16)	0.31	0.52	(0.12)	0.49	(0.11)	0.32
Maximum Global Disc Area in mm^2	1.928	(0.525)	1.836	(0.467)	0.62	1.881	(0.414)	1.697	(0.368)	0.20
Minimum Global Rim Area in mm^2	1.253	(0.276)	1.250	(0.228)	0.97	1.232	(0.183)	1.352	(0.254)	0.14
Minimum Temp. Rim Area in mm^2	0.213	(0.052)	0.202	(0.048)	0.54	0.208	(0.050)	0.252	(0.046)	0.018***
Minimum Temp. Sup. Rim Area in mm^2	0.123	(0.034)	0.134	(0.029)	0.38	0.134	(0.027)	0.142	(0.030)	0.44
Minimum Nasal Sup. Rim Area in mm^2	0.153	(0.041)	0.179	(0.045)	0.10	0.172	(0.039)	0.175	(0.042)	0.84
Minimum Nasal Rim Area in mm^2	0.373	(0.116)	0.353	(0.067)	0.57	0.356	(0.079)	0.408	(0.123)	0.17
Minimum Nasal Inf. Rim Area in mm^2	0.174	(0.065)	0.171	(0.040)	0.91	0.163	(0.040)	0.176	(0.049)	0.41
Minimum Temp Inf. Rim Area in mm^2	0.147	(0.040)	0.151	(0.040)	0.78	0.143	(0.031)	0.154	(0.037)	0.37

Abbreviations: IOP, intraocular pressure; SD, standard deviation; Temp, temporal; Sup, superior; Inf, inferior.
* Calculated using Fisher's exact test
** Not significant following Bonferroni correction for multiple-comparison testing p=0.13
*** Not significant following Bonferroni correction for multiple-comparison testing p=0.46

No notch, nerve fibre layer defect or neuroretinal rim haemorrhage was noted in any eye examined (Figure 2-16 and Figure 2-17). Results from each 60 degree sector of the optic disc area from the worst eye, as judged by the eye with the smallest neuroretinal rim area, are presented in Table 2-13. There was no statistically significant difference in cup-to-disc area ratios (Figure 2-18) or total rim area between Gln368STOP and Thr377Met mutation carriers and non- carriers (OD: P=0.08 and P=0.29; OS: P= 0.51 and P= 0.25, respectively). Combining both mutation groups, there was no statistically significant difference in cup-to-disc area ratios between case and control subjects (OD: P=0.16; OS P=0.34).

Figure 2-16

Right and left optic disc examples of subjects from Gln368STOP *MYOC* pedigrees. a) Mutation carrier GQld11-14 aged 22 years; b) mutation carrier GTas287-11 aged 7 years; c) mutation carrier GVic139-5 aged 32 years; d) mutation carrier GVic124-11 aged 37 years; e) non-carrier GQld11-12 aged 7 years; f) non-carrier GTas287-10 aged 29 years; g) non-carrier GVic139-4 aged 34 years; non-carrier GVic124-7 aged 31 years.

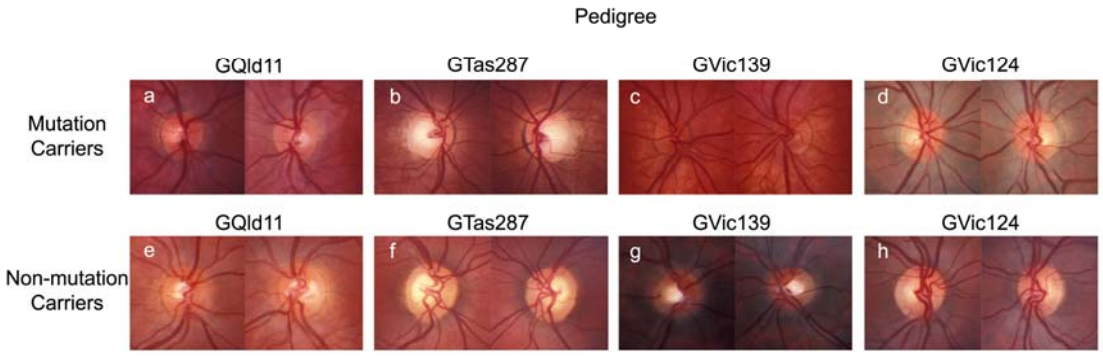


Figure 2-17

Right and left optic disc examples of subjects from Thr377Met *MYOC* pedigrees. a) Mutation carrier GVic1-82 aged 11 years; b) mutation carrier GVic1-84 aged 11 years; c) mutation carrier GVic120-4 aged 13 years; d) mutation carrier GVic1-5 aged 24 years; e) non-carrier GVic1-87 aged 12 years; f) non-carrier GVic1-70 aged 12 years; g) non-carrier GVic1-7 aged 13 years; non-carrier GVic1-68 aged 17 years.

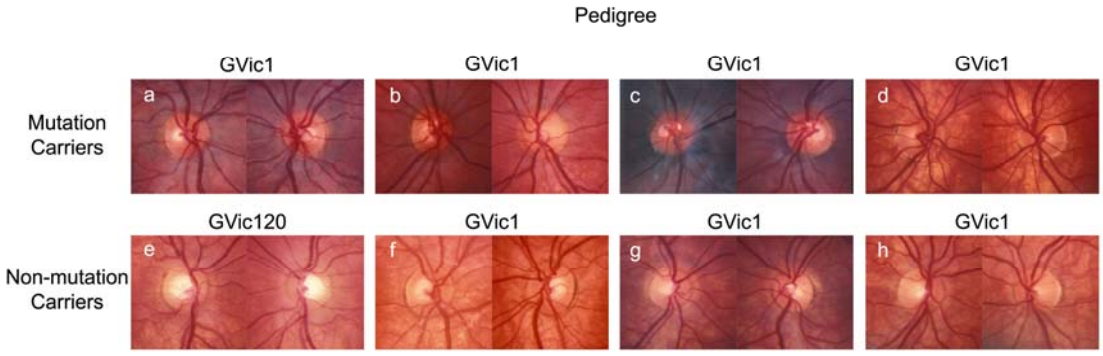
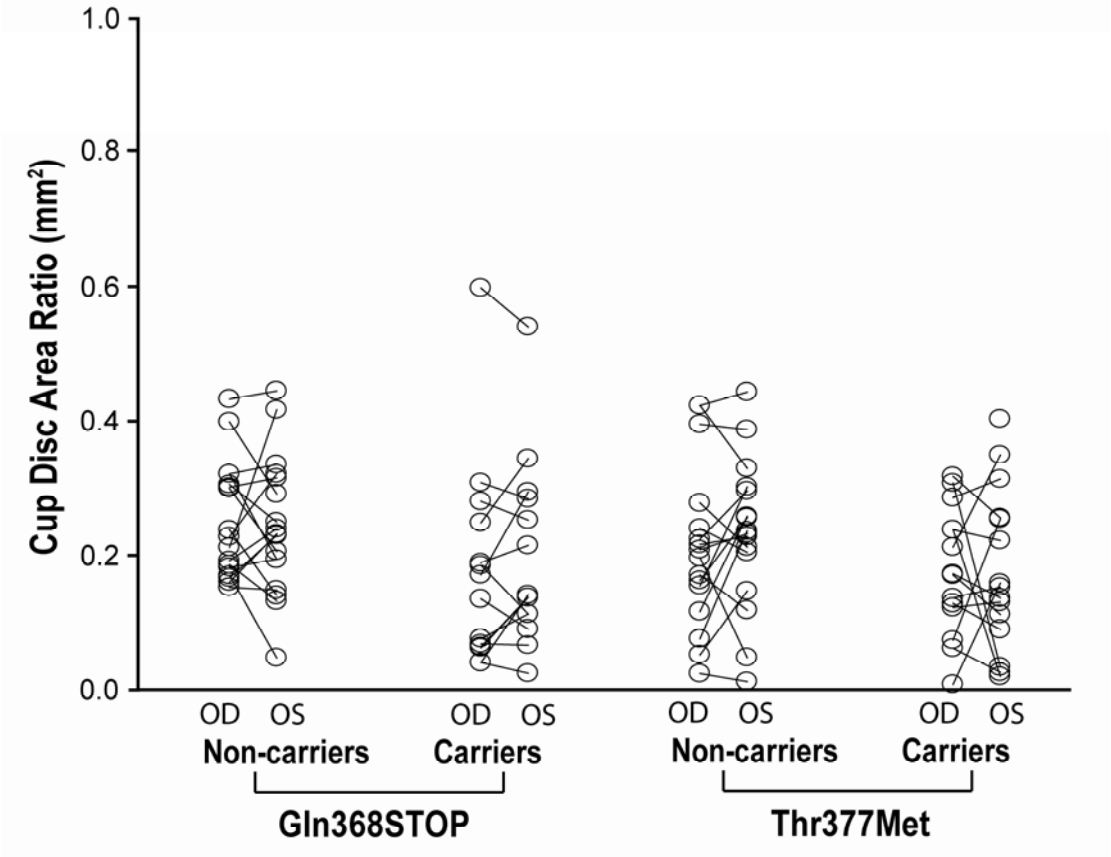


Figure 2-18

The distribution of cup-to-disc area ratios for the Gln368STOP and Thr377Met mutation carriers and non-carriers. The right (OD) and left (OS) eyes of each subject are joined by a line.



One individual (GTas287-11) with the Gln368STOP mutation was found to have outlying cup-to-disc area ratios of 0.6 (Figure 2-16b). It is important to note that these measurements were not corrected for disc size, which in this individual were larger than normal. Because the individual was seven years old at the time of examination, reliable visual fields were not available; however, repeated IOP readings have all been consistently <19 mmHg bilaterally. No significant difference in cup-to-disc area ratios between pedigrees was identified ($p>0.05$).

Discussion:

We found no quantifiable difference in the structure of the optic nerve head, either in cup-to-disc area ratio or neuroretinal rim area, between young individuals who are carriers and those who are non-carriers of common *MYOC* mutations. Although confounded by penetrance and heterogeneity, the results from our study suggest that young patients with either the Gln368STOP or Thr377Met mutation cannot be clinically differentiated from family members who do not have these mutations. It is clear that these results are applicable only to *MYOC* glaucoma. It is certainly possible that other OAG-related genes, particularly those influencing normal tension glaucoma, may not follow this natural history.

This study underscores the importance of baseline optic disc evaluation in predisposed cases. Observing and analysing serial optic disc photographs and IOP measurements of these young individuals will enhance our understanding of the disease mechanisms underlying *MYOC* glaucoma. Airaksinen et al. and Zeyen et al. have demonstrated that in eyes with initially normal visual fields, from glaucoma patients diagnosed by contralateral ocular findings, the rate of neuroretinal rim area loss is approximately 0.47% to 1.7% per year (Airaksinen et al., 1992; Zeyen and Caprioli, 1993). It is possible that the trajectory of neuroretinal rim loss in *MYOC* glaucoma differs significantly to that of OAG caused by other mechanisms.

The finding of Gln368STOP mutation carriers having smaller vertical cup-to-disc ratios than their non-mutation carrying counterparts was unexpected and may either represent a statistical anomaly, given that the finding was no longer significant following correction for multiple testing, or it may be due to ascertainment bias.

Considerable lengths were taken to ascertain the most optimal internal control set, that is a non-mutation carrier from the same family, thereby reducing any confounding bias introduced by co-associated traits. Ideally to avoid confounding traits only one randomly selected member per pedigree affected would be included, however the resultant loss of power in this study would negate any such benefit.

Along with being one of the most sensitive features in differentiating patients with OAG from normal (Airaksinen et al., 1985; Balazsi et al., 1984), a small neuroretinal rim area is a principal morphological predictive factor for disease development and progression (Jonas et al., 2004). Further to this, the superior and inferior poles of the optic disc lose nerve fibres at a selectively greater rate (Quigley et al., 1982). Mutation carriers were not found to have a smaller total neuroretinal rim area than non-carriers and, importantly, no preferential loss of neuroretinal rim tissue was noted in the superior or inferior regions.

These results lend support to the hypothesis that *MYOC* glaucomatous optic nerve changes occur following a sustained elevation of IOP (Alward et al., 1998). In contrast *optineurin* mutations are thought to induce retinal ganglion cell apoptosis (Sarfarazi and Rezaie, 2003). Large diurnal variations in IOP do occur in individuals with OAG and may dramatically influence the degree of optic disc damage (Asrani et al., 2000). It is also possible that either intermittent spiking or a stepwise increase in IOP may occur prior to a sustained elevation in IOP. The optic disc features of the seven-year-old subject carrying the Gln368STOP *MYOC* mutation are suspicious of glaucomatous damage. It is interesting that he has never been found to have an elevated IOP and other causes for optic neuropathy have been excluded. Along with *MYOC* glaucoma being principally associated with a high IOP, the prevalence of

glaucomatous disc damage has also been shown to dramatically increase above IOP of 21 mmHg (Mitchell et al., 1996). Investigation of the diurnal IOP variation in *MYOC* cases is warranted.

In this study we actively conducted cascade genetic screening for *MYOC* glaucoma. Genetic screening and counselling has been shown to be appreciated by families known to carry *MYOC* mutations (Healey et al., 2004). Whilst population-based molecular screening for *MYOC* changes alone are not currently cost-effective, with the advance of genetic studies more genes implicated in OAG will be identified (Aldred et al., 2004). Increasing our understanding of the genetic mechanisms of OAG will have a significant public health impact. Genetic screening will principally highlight those people who do not require vigilant pre-disease clinical screening.

Chapter 3 – GLAUCOMA AS A SYSTEMIC DISEASE.

This chapter investigates the role of systemic factors in primary open angle glaucoma. Primary open angle glaucoma is a common neurodegenerative disease. While many systemic diseases are associated with or predispose to neuronal death, the converse that there could be systemic manifestations of glaucoma is also possible. All cause mortality data for a large population were reviewed and after adjustment for age and male gender, a significant association between open angle glaucoma and death due to ischaemic heart disease was identified. Then in the final section of this chapter we investigated the association of systemic disease in 1700 individuals with OAG. Significant independent associations for a past history of migraine and atherosclerosis were identified for familial glaucoma. The identification of potential risk factors for different forms of this disease may assist in the identification of phenotypic subtypes, each with potentially different pathogenetic mechanisms that may be modified by intervention and disease-prevention strategies.

Aetiology of mortality in the Open Angle Glaucomas: "two cupped discs and a funeral".

Background:

Antagonistic pleiotropy, whereby the molecular pathoaetiology for an age-related disease may harbour or confer an overall survival benefit before the reproductive age, is one of the peculiar aspects of diseases that typically manifest relatively late in life, such as primary open angle glaucoma (OAG) (Capri et al., 2006). Unfortunately such relationships are generally difficult to study. The investigation of all-cause mortality data can provide insight into the phenotypic pleiotropy or significant associations between a systemic disease and another condition.

Previous work has suggested an association between cardiac disease and glaucoma (Klein et al., 1999; Lee et al., 2006a; Lee et al., 2003). Analysis of data from the United States National Health Interview Survey revealed an increased risk of mortality due to cardiovascular disease in participants with glaucoma (Lee et al., 2003). They also identified a modest association between glaucoma, in people without reported visual impairment, and cancer (Lee et al., 2003). However, a limitation of this cross-sectional study was that glaucoma status was determined by self report (Lee et al., 2003). Data from the Blue Mountains Eye Study in Australia, have suggested that glaucoma patients younger than 75 years of age have a greater risk of cardiovascular mortality, particularly following exposure to topical timolol (Lee et al., 2006a). However, the conclusion from this work has been criticised for its relatively small sample size following age-stratification (Sharkawi and Franks, 2008).

In this study we investigated the cause for mortality in people with OAG. We specifically sought to replicate the association between cardiovascular disease and glaucoma using data from a relatively homogenous, Caucasian population. In our large cohort, which comprised mortality information from over 27,000 people of whom 741 were known to have OAG, we identified a modest, yet statistically significant association between death due to ischaemic heart disease and OAG.

Methods:

Specific approval for this study was obtained from the Southern Tasmania Health and Medical Human Research ethics committee. Raw data were obtained from the Tasmanian Registrar of Birth Deaths and Marriages, for the years December 1996 to December 2005. This database contained re-identifiable demographic details, as well as all available data on the cause of death as listed on the medical death certificate for each person registered as deceased in the Australian state of Tasmania. For the past decade Tasmania has had a relatively stable population of approximately half a million people, who are predominantly of Anglo-Celtic ancestry (Australian Bureau of Statistics. 2001). All-cause mortality data, for all people who were at least 40 years of age at their time of death, were classified using the ICD-10 guidelines, by one trained grader (Paul Sanfilippo). This grader was masked to subjects' glaucoma status. The ICD-10 coded data were then re-identified and merged with the Glaucoma Inheritance Study in Tasmania (GIST) database.

The GIST has been described in depth previously (Coote et al., 1996; Green et al., 2007; Wu et al., 2006). In brief, separate audits of all glaucoma patients attending all ophthalmic practices in Tasmania, between 1994 and 1996, were performed. Subsequently, surveys inviting patients to participate in the study were directly

mailed to over 3,800 Tasmanian patients who had been investigated or treated for glaucoma. Additional surveys were distributed to all optometric and general practitioner clinics. Over 500 index OAG cases were found to have died or relocated from Tasmania prior to 1996. Initially a total of 2062 participants were examined, with definitive OAG being diagnosed in 1700 (82.4%) of these participants (Green et al., 2007). Since this original enrolment, an additional 709 people have been reviewed by GIST and diagnosed with OAG.

For the purpose of this study, a diagnosis of OAG by a treating ophthalmologist was deemed to be appropriate for inclusion into the GIST database. Confirmation of disease status was performed when examined by the GIST team, where patients with OAG were required to have, in at least one eye: optic disc cupping (cup-disc ratio≥0.7); or an 0.2 inter-eye disparity in cup-disc ratio; or focal rim notching with corresponding visual field loss. Visual field assessments were conducted using the Humphrey Visual Field Analyzer threshold 24-2 algorithm and graded as abnormal if three contiguous regions on pattern standard deviation had a probability of normality of <5%, or if the Glaucoma Hemifield Test was abnormal. Subjects were required to have an open iridocorneal angle on gonioscopic examination. Clinical details, such as age at diagnosis, cup-disc ratio and maximum recorded intraocular pressure (IOP) were recorded in the GIST database.

To assess the internal validity in ICD-10 classification, the assigned mortality code was graded twice for 6,000 people. An identical all-cause mortality code was assigned on both occasions for 5,944 (99.1%) people. Contingency tables were used for crude analysis and then models adjusting for age at death as well as gender were constructed. Binary logistic regression analysis was performed using SPSS v14.0.0

(SPSS Inc, Chicago, IL, USA). The odds ratio was computed as e^{β}, where β is the regression coefficient of the covariate and the 95% confidence interval was calculated as $e^{\beta +/- 1.96 \times SE\beta}$, where SEβ is the standard error of the regression coefficient. Nonparametric continuous variables were compared using the Mann-Whitney U test. Data are presented as the mean ± SD.

Results:

During the defined nine-year study period, a total of 33,879 deaths were recorded. Data were unavailable for 4,868 (14.4 %) people and 1725 (5.1%) were younger at the time of death than the designated age (40 years) for inclusion into this study. Hence, information from 27,286 people (80.5% of the full Tasmanian Registry) was reviewed. The mean age at death for the study sample was 78.4 ± 11.5 (range:41-109) years and this cohort comprised 13,919 (51.0%) females.

Eight hundred and five patients with OAG were identified by cross tabulation with the GIST database. Full mortality information was available for 741 (92.0%) people known to have OAG, of whom 375 (50.6%) were female (Table 3-1).

Table 3-1
Glaucoma patients' clinical characteristics and availability of mortality data.

	Mortality Data Available (n = 741)			Mortality Data Unavailable (n = 61)			p*
n female (%)	375 (50.6%)			32 (52.5%)			0.78
	Mean ± SD	Range		Mean ± SD	Range		
Age at Death (years)	83.8 ± 7.8	52 - 101		80.2 ± 6.4	65 - 93		<0.001
Age Enrolled in the GIST (years)	79.3 ± 7.6	50 - 97		77.4 ± 7.2	60 - 92		0.036
Age at Diagnosis (years)	69.0 ± 11.1	30 - 93	(n=333)	65.6 ± 9.6	40 - 78	(n=47)	0.11
Maximum CDR	0.77 ± 0.17	0.1 - 1	(n=518)	0.78 ± 0.20	0.2 - 1	(n=56)	0.22
Inter-eye difference in CDR	0.10 ± 0.12	0 - 0.6	(n=518)	0.09 ± 0.13	0 - 0.6	(n=56)	0.38
Maximum recorded IOP (mmHg)	25.4 ± 8.6	15 - 75	(n=435)	26.6 ± 9.0	15 - 48	(n=48)	0.53

* test of difference performed using the χ^2 test for gender and the Mann-Whitney U test for continuous variables.
Abbreviations: n, number; SD, standard deviation; GIST, Glaucoma Inheritance Study in Tasmania; CDR, cup-disc ratio; IOP, intraocular pressure.

The mean age at death for OAG subjects included in this study was 83.8 ± 7.8 years. These subjects died at a significantly older age than people not known to have OAG ($p<0.0001$). Additionally, there were significantly fewer people with OAG who died before the age of 80 years (n with OAG=199, $p<0.0001$). The 61 patients for whom mortality data were not available were significantly younger at the age of death and enrolment in the GIST compared to those patients with complete mortality data (Table 3-1).

Pooled analysis of mortality due to all forms of circulatory diseases revealed a significant association with OAG (Table 3-2). Following adjustment for the age at death and male gender, the odds ratio for death due to ischaemic heart disease in people with OAG was significant (OR=1.30, 95%CI: 1.08 - 1.56; $p=0.006$). This association increased when analysis was restricted to people who were less than 80 years of age at their time of death. This finding was not reflected in the pooled analysis of mortality due to all forms of circulatory disease. Although there was a trend towards an association between ischaemic heart disease mortality and OAG in people who were at least 80 years at their time of death this was not statistically significant. Analysis of other sub-types of circulatory disease mortality revealed no other significant association with OAG. Crude analysis revealed that there were significantly fewer people with OAG who died due to metastatic cancer ($p<0.0001$); however, this did not remain significant following adjustment for age and gender (Table 3-2). No association between mortality cause and signs of OAG disease severity, such as cup-disc ratio, age at diagnosis or maximum recorded IOP, was identified (data not shown).

Table 3-2

Odds ratio and 95% confidence interval, for different causes of mortality in people with glaucoma, stratified by age at death. All values are adjusted for age at death and male gender. Significant results are highlighted in a bold type font.

	Age Group		
	All Ages	< 80 years	≥ 80 years
All circulatory diseases	**1.21 (1.04 - 1.40)**	1.20 (0.90 - 1.61)	**1.22 (1.02 - 1.45)**
Ischaemic Heart Disease	**1.30 (1.08 - 1.56)**	**1.72 (1.13 - 2.61)**	1.21 (0.98 - 1.49)
Other heart diseases	1.17 (0.99 - 1.39)	1.27 (0.89 - 1.82)	1.14 (0.94 - 1.37)
Diseases of the arteries, arterioles and capillaries	1.00 (0.79 - 1.25)	1.05 (0.63 - 1.73)	0.99 (0.77 - 1.29)
Cerebrovascular Disease	1.05 (0.86 - 1.28)	0.87 (0.65 - 1.15)	1.04 (0.83 - 1.30)
Cancer (all)	0.87 (0.73 - 1.03)	0.87 (0.65 - 1.15)	0.87 (0.71 - 1.07)
Cancer (metastatic)	0.90 (0.70 - 1.15)	0.79 (0.52 - 1.16)	1.02 (0.74 - 1.42)
Female breast cancer	0.78 (0.41 - 1.48)	1.07 (0.43 - 2.67)	0.64 (0.26 - 1.57)
Lung cancer	1.05 (0.75 - 1.48)	0.87 (0.56 - 1.34)	1.35 (0.77 - 2.36)
Colorectal cancer	0.90 (0.64 - 1.26)	1.48 (0.75 - 2.90)	0.70 (0.47 - 1.05)
Prostate cancer	0.72 (0.47 - 1.12)	0.32 (0.10 - 1.01)	0.89 (0.55 - 1.43)
Chronic obstructive pulmonary disease	0.97 (0.76 - 1.24)	0.88 (0.58 - 1.33)	0.96 (0.71 - 1.30)
Influenza and pneumonia	1.06 (0.88 - 1.28)	1.00 (0.66 - 1.50)	1.09 (0.88 - 1.34)
Renal Failure (Acute and Chronic)	1.12 (0.88 - 1.42)	0.95 (0.56 - 1.58)	1.15 (0.88 - 1.51)
Liver Failure	1.02 (0.58 - 1.79)	1.17 (0.54 - 2.52)	1.05 (0.46-2.39)

Discussion:

In this study we found that OAG increased the likelihood for death due to ischaemic heart disease. This effect was strongest when analysis was restricted to data from people who died before the age of 80 years. In this age group the odds ratio for death due ischaemic heart disease in OAG patients was 1.72 (95% CI: 1.13-2.61). This supports previous work, which also identified an association between cardiovascular mortality and glaucoma (Klein et al., 1999; Lee et al., 2006a; Lee et al., 2003). In a study investigating the cause of mortality in a diabetic population, from Wisconsin in the United States of America, ischaemic heart disease was found to be associated with a history of glaucoma (Klein et al., 1999). This finding was subsequently supported in two population-based studies where participants were not selected for diabetes status (Lee et al., 2006a; Lee et al., 2003).

The pathoaetiological relationship between OAG and ischaemic heart disease is unclear. However, it has been postulated that an association could possibly arise as an adverse complication of OAG treatment or due to a common overlapping pathogenic pathway (Leske 2003). Acetazolamide treatment for OAG is known to be associated with an increased risk of mortality (Egge and Zahl, 1999), and the systemic effects of other IOP-modulating medications is well established (Leske 2003). Lee and colleagues identified a significant relationship between topical β-blocker use and cardiovascular mortality (Lee et al., 2006a). Unfortunately in our study, data pertaining to OAG treatment were not available.

Altered haemodynamics at the optic nerve head is known to be associated with retinal ganglion cell apoptosis (Hayreh 1969), and a vascular pathogenic origin for the glaucomas has long been debated (Flammer et al., 2002). It is certainly plausible

that some people with glaucoma may have altered haemodynamics of vessels both at the optic nerve head and the heart (Broadway and Drance, 1998; Weinreb 1992). Interestingly the *Myocilin* gene, which has been unequivocally implicated in glaucoma and is not known to serve any physiological role, has been found to be highly expressed in the heart (Fingert et al., 2002).

Using a clinic-based population, Tattersall and colleagues found that despite better IOP control, OAG patients with a poor life expectancy lost more functional vision than severity-matched patients (Tattersall et al., 2005). In our study no association between disease severity and mortality cause was identified. Additionally, we did not replicate the association between cancer mortality and OAG, which was suggested by Lee and colleagues (Lee et al., 2003). Following adjustment for age at death and gender, there was no significant difference in cancer mortality rates in OAG patients and the general population.

This retrospective study has a number of important limitations. Given that data from medical notification certificates, as submitted to the regional registry for Births Deaths and Marriages, were used, substantial bias could have been introduced due to incorrect or misinformed documentation being provided by the primary care physician. Nonetheless, data integrity on the ICD-10 coding for this study was optimised by use of one trained grader. Misclassification bias could have also been introduced due to the fact that approximately half of all people with OAG in our community are undiagnosed (Mitchell et al., 1996; Wensor et al., 1998). Nonetheless, should the association with ischaemic heart disease and OAG be genuine and not related to treatment, we would expect that that the conferred risk ratio would be strengthened by the inclusion of all actual cases of OAG from the

study population. Finally, the age restriction (<80 years) was performed post hoc and as such is also subject to classification bias.

Our study supports the findings of others, and suggests that a review for signs of cardiovascular disease may be warranted in people diagnosed with OAG. In time, as further work investigates the underlying molecular mechanisms of OAG, the precise pathoaetiology link between ischaemic heart disease and glaucoma should be uncovered.

Association of systemic disease with familial and sporadic primary open angle glaucoma: the Glaucoma Inheritance Study in Tasmania.

Background:

Several epidemiological studies have specifically aimed to identify important factors predisposing to primary open angle glaucoma (OAG) (Gordon et al., 2002; Leske et al., 2003; Miglior et al., 2007). Whilst any identified risk factor can be useful for diagnostic screening or susceptibility profiling, traits that are amenable to intervention allow for the immediate establishment of active disease-prevention strategies. For example, non-modifiable factors such as increasing age, family history, and ethnicity can passively allow for the identification of those at highest risk of glaucoma blindness. Conversely, although only a few systemic conditions are known to have an aetiological association with OAG, some putative risk factors such as hypertension and diabetes mellitus are modifiable (Boland and Quigley, 2007; Leske 2007; Pache and Flammer, 2006). In addition, the identification of associated comorbidities ensures refined case and phenotypic definition - essential for a complete understanding of the pathogenesis of any disease.

Up to two-thirds of all OAG is familial (Green et al., 2007; Wolfs et al., 1998), and despite the relatively large number of cross-sectional epidemiologic studies investigating the associations between glaucoma and medical systemic risk factors, there has been a paucity of work examining the potential links of these traits with familial and sporadic OAG. This study examined a large cohort of people diagnosed with OAG and their available relatives. OAG cases were categorised into familial and sporadic subgroups based on genealogical data, with the aim of comparing the

prevalence of various putative disease-associated and potentially modifiable risk factors between these groups.

Methods:

This study was conducted in accordance with the Declaration of Helsinki and subsequent revisions. Written informed consent was obtained from patients participating in the Glaucoma Inheritance Study in Tasmania (GIST), which was approved by the relevant ethics committees of the University of Tasmania, the Royal Hobart Hospital, and the Royal Victorian Eye and Ear Hospital.

Study Design:

A cross-sectional, retrospective study design was utilised. People living in the Australian state of Tasmania, who had been investigated or treated for OAG since 1980, were identified and invited to participate in this study (Green et al., 2007; Sack et al., 1996). The Tasmanian population is relatively homogenous, with the majority (91%) of people born in Australia and descended from Anglo-Celtic stock (Australian Bureau of Statistics. 2001). The remaining residents are born outside Australia, with 5% originating from the United Kingdom (Australian Bureau of Statistics. 2001).

Between 1994 and 1996, approximately 4,000 surveys were directly mailed or distributed via optometrists, general practitioners, glaucoma support groups and local pharmacies to people thought to have OAG (Green et al., 2007; Sack et al., 1996). The diagnosis of respondents was then confirmed by the GIST clinicians. Subsequently, all living relatives over the age of 40 years, of index cases were invited to undergo a comprehensive ocular examination. Both maternal and paternal

relatives from each family were invited to participate (Sack et al., 1996). Over 1,042 unaffected family members were examined by GIST (Green et al., 2007). Other first-degree relatives had been examined by local ophthalmologists or optometrists, and were reported to be normal.

Examination protocol and case definition:

Each person reviewed in the GIST underwent comprehensive ocular examination and a detailed medical history was obtained. Masked examiners assessed various glaucoma parameters. Best-corrected Snellen visual acuity and Humphrey visual field testing (Zeiss-Humphrey, Dublin, California, USA) was performed. Measurement of intraocular pressure (IOP) was undertaken using Goldmann applanation tonometry prior to gonioscopy. Two independent observers clinically graded the optic disc and stereo-photographs of the optic nerve head were taken using a Nidek 3-Dx/F fundus camera (Nidek, Gamagori, Japan). If there was a disagreement in OAG status of a patient, a consensus between the ophthalmologists was reached through open discussion.

In this study, OAG was defined as an optic neuropathy with optic nerve head excavation and signs of thinning of the neuroretinal rim or other characteristic optic disc features such as Drance type nerve fibre layer haemorrhage, notching, as well as significant focal or diffuse retinal nerve fibre layer tissue loss (Coote et al., 1996). An abnormal Glaucoma Hemi-field test or a visual field defect consistent with the neuroretinal rim changes was also required (Coote et al., 1996). Patients were excluded if there was evidence of secondary forms of glaucoma such as trauma, inflammation, pigment dispersion, anterior segment dysgenesis or other significant anterior segment pathology. Patients with pseudoexfoliative-associated glaucoma

were not specifically excluded. In total 362 subjects were excluded: 241 were found to have secondary or congenital forms of glaucoma; 27 had a visual field defect or optic disc pathology not related to OAG; 26 had anterior segment dysgenesis; and 68 subjects were unable to complete the study protocol.

A detailed questionnaire enquiring about knowledge of family history, demographic data, current medications, and medical history of systemic disorders including: hypertension, angina, thyroid disease and diabetes mellitus, was administered. Patients were also questioned about a past history of migraine headaches, whether they regularly experienced cold hands or feet similar to that of Raynaud's phenomenon, and if they had ever had a severe hypovolaemic episode requiring a blood transfusion. Problems with vision, past eye disease or eye treatment, and ocular symptoms were also documented. When available, the past medical history was cross-referenced with their general practitioner medical summaries.

Information provided from the family history surveys was collated and merged with local genealogical databases, such as Tasmanian Family Link which contains all reported births, deaths, burials, and marriages that have occurred in Tasmania (www.archives.tas.gov.au/nameindexes/colonial). A research genealogist (Maree Ring) then reconstructed 309 pedigrees by connecting index cases and all known relatives. Each OAG case was categorised by the closest relative known to have OAG. Familial glaucoma was defined as having a fourth-degree or closer relative affected with OAG. First-degree relatives include one's parents, offspring and siblings. The relationship of each successive branch away from an index case increases by a degree, such that fourth-degree relatives comprise second cousins' children and great-great-grandparents.

Statistical Analysis:

Logistic regression analysis was employed in the comparison of the prevalence of atherosclerosis, hypertension, migraine, Raynaud's phenomenon, diabetes, thyroid disease, corticosteroid use, previous transfusion, and current cigarette use, between patients with familial or sporadic OAG. Two models were constructed. The first model adjusted for male gender and age at review, with the second model also adjusting for disease severity. The second model specifically included: gender, age at review, age at diagnosis, maximum cup:disc ratio and maximum recorded IOP. Stratification by the number of OAG-affected relatives was also undertaken. Familial clustering was examined for reviewing individual pedigrees. All odds ratio (OR) estimates presented are accompanied by 95% confidence limits, and analysis was undertaken using SPSS v14.0 (SPSS Inc., Chicago, USA). Power calculations were performed using the PS Power and Sample Size Calculations program v2.1.30 (Dupont and Plummer, 1997).

Results:

A total of 3800 potential OAG cases were identified, of whom 2062 were examined. Many potential OAG cases had died over the 15-year period or could not be located, whilst others declined or were too elderly or infirm to participate. As reported previously, 1012 (59.5%) subjects were found to have familial OAG, and 688 (40.5%) subjects had no known or identified relative affected with OAG (sporadic glaucoma) (Green et al., 2007).

Patients in the familial OAG group were found to be younger both at the age of review as well as diagnosis (Table 3-3). The familial OAG group also had worse

cup:disc ratios, yet lower maximum recorded IOP measurements, compared to sporadic OAG patients (Wu et al., 2006).

Table 3-3
Demographic and clinical features of the study sample. Data presented as mean ± SD.

	Familial OAG (n=1012)	Sporadic OAG (n=688)	p
n female	580 (57.3%)	388 (56.4%)	0.71
Age at Review (years)	70.6 ± 12.6	72.6 ± 10.3	0.001
Age at Diagnosis (years)	61.4 ± 13.0 (n=780)	64.0 ± 12.6 (n=575)	<0.001
Maximum CDR	0.74 ± 0.18	0.73 ± 0.18	0.016
Inter-eye difference in CDR	0.12 ± 0.18	0.11 ± 0.16	0.11
Maximum recorded IOP (mmHg)	25.4 ± 8.0	26.0 ± 8.2	0.001

Abbreviations: n, number; OAG, primary open angle glaucoma; CDR, cup:disc ratio; IOP, intraocular pressure.

Analysis revealed that there was no significant difference in the prevalence of hypertension, Raynaud's phenomenon, diabetes mellitus, or thyroid disease between familial and sporadic OAG cases (Table 3-4). Additionally, exposure to corticosteroids, cigarette smoking and hypovolaemic events requiring blood transfusion did not differ between these groups.

Table 3-4

Odds ratios and 95% confidence intervals for systemic disease and risk factor exposure in patients with familial glaucoma compared to those with sporadic glaucoma. Significant associations are displayed in bold type font.

	Crude		Model 1*		Model 2†	
	OR (95% CI)	p	OR (95% CI)	p	OR (95% CI)	p
Atherosclerosis	1.28 (0.95 - 1.72)	0.11	**1.42 (1.05 - 1.92)**	**0.024**	**1.40 (1.03 - 1.91)**	**0.030**
Hypertension	1.09 (0.86 - 1.39)	0.47	1.12 (0.88 - 1.44)	0.35	1.23 (0.84 - 1.78)	0.29
Migraine	**1.75 (1.22 - 2.52)**	**0.002**	**1.63 (1.13 - 2.36)**	**0.009**	**1.67 (1.15 - 2.42)**	**0.007**
Raynaud's Phenomenon	1.06 (0.82 - 1.36)	0.66	1.10 (0.85 - 1.41)	0.47	1.09 (0.84 - 1.41)	0.51
Diabetes	0.76 (0.53 - 1.10)	0.15	0.78 (0.54 - 1.12)	0.18	0.76 (0.52 - 1.10)	0.14
Thyroid Disease	1.18 (0.81 - 1.72)	0.39	1.19 (0.81 - 1.75)	0.38	1.17 (0.79 - 1.73)	0.45
Corticosteroid Use	0.84 (0.66 - 1.08)	0.19	0.84 (0.66 - 1.08)	0.18	0.87 (0.67 - 1.12)	0.28
Previous Transfusion	0.83 (0.63 - 1.10)	0.20	0.86 (0.65 - 1.14)	0.30	0.85 (0.64 - 1.13)	0.26
Current Cigarette Use	1.23 (0.84 - 1.78)	0.29	1.11 (0.76 - 1.63)	0.59	1.23 (0.83 - 1.81)	0.30

* adjusted for male gender and age at review

† adjusted for male gender, age at review, age at diagnosis, maximum cup:disc ratio and maximum recorded intraocular pressure

Table 3-5

Odds ratios and 95% confidence intervals for migraine and atherosclerosis stratified by number of OAG affected relatives. The crude power to detect a difference at the $\alpha = 0.05$ level is displayed.

		n (%)	Crude		Model 1*		Model 2†		Power
			OR (95% CI)	p	OR (95% CI)	p	OR (95% CI)	p	
Migraine	1st degree affected relative (Familial OAG)	108 (16.5%)	1.93 (1.32 - 2.83)	<0.001	1.80 (1.22 - 2.65)	0.003	1.79 (1.21 - 2.64)	0.004	0.83
	≥ 2nd degree affected relative (Familial OAG)	51 (14.3%)	1.38 (0.86 - 2.23)	0.18	1.33 (0.81 - 2.16)	0.26	1.33 (0.81 - 2.16)	0.26	0.35
	No Affected Relative (Sporadic OAG)	76 (11.0%)	-		-		-		
Atherosclerosis	1st degree affected relative (Familial OAG)	134 (20.4%)	1.16 (0.84 - 1.60)	0.38	1.31 (0.94 - 1.83)	0.11	1.28 (0.92 - 1.80)	0.15	0.11
	≥ 2nd degree affected relative (Familial OAG)	84 (23.6%)	1.55 (1.06 - 2.25)	0.023	1.67 (1.14 - 2.45)	0.009	1.69 (1.15 - 2.51)	0.008	0.45
	No Affected Relative (Sporadic OAG)	129 (18.8%)	-		-		-		

* adjusted for male gender and age at review

† adjusted for male gender, age at review, age at diagnosis, maximum cup:disc ratio and maximum recorded intraocular pressure

A past history of migraine was found to be significantly associated with familial OAG (Table 3-4). After adjustment for age at review, sex and disease severity, the OR for familial disease was 1.67 (95%CI: 1.15 - 2.42). This effect was primarily driven by cases who had a first-degree relative also affected by OAG (Table 3-5); however, this study was underpowered to detect an association between migraine and OAG cases with a second-degree or more distant relative similarly affected. There were 42 pedigrees that contributed at least five cases to this study, and the median proportion of cases affected with migraine in these pedigrees was 0.17 (mean ± SD: 0.22 ± 0.23). Twelve of these 42 pedigrees were found to have no members with a past history of migraine, whilst all cases (n = 5) in one pedigree (GTas25) reported migraine symptoms.

After adjustment for male gender and the age at review, the presence of atherosclerosis was found to be more common in cases with familial glaucoma than in people with sporadic disease. This remained significant when the full model, which also adjusted for disease severity, was used (Table 3-4). When analysis was restricted to people who had a first-degree relative affected, no association between atherosclerosis and familial OAG was identified (Table 3-5). The proportion of people with atherosclerosis from pedigrees with at least five case members participating in this study ranged from 0 to 0.55 (median: 0.20, mean ± SD: 0.23 ± 0.16), and there were seven pedigrees that contributed at least five cases to this study, yet reportedly had no members with manifest atherosclerosis.

Discussion:

In this study, which investigated systemic associations in 1700 people with OAG, we identified a significant association for a past history of migraine and atherosclerosis between familial and sporadic disease. We have previously reported that familial glaucoma is generally more severe than sporadic forms of the disease (Wu et al., 2006). Our present data highlight some important systemic associations that may contribute to this discordance in OAG phenotypic severity.

Ocular vasospasm is established as a putative risk factor for OAG, and may be associated with generalised vasospastic tendency manifested in migraine, Raynaud's phenomenon (cold extremities) and Prinzmetal angina (due to coronary artery spasm rather than atherosclerosis) (Carter et al., 1990; Flammer et al., 2001; Grieshaber and Flammer, 2005). In a Japanese-based case-control study, Usui and colleagues failed to find a significant correlation in migraine prevalence between OAG and normal subjects (Usui et al., 1991). Moreover, a separate study revealed that there were no differences between low-tension and high-tension glaucoma groups with respect to organic vascular pathologic findings (Carter et al., 1990). In the population-based Beaver Dam Eye Study, no association between OAG and migraine headache was identified (Klein et al., 1993). However, following age-stratification, a significant association with prevalent disease was identified in the Blue Mountains Eye Study population (Wang et al., 1997). After stratifying participants into ten-year age groups, Wang and colleagues found an increased OR for OAG among people aged 70-79 years with a history of typical migraine (OR 2.5; CI 1.2-5.2) (Wang et al., 1997). Our data suggest that central vasospastic features of OAG may be confined to familial forms of the disease. It must be noted that it is certainly possible that the sporadic forms of OAG may also have a similar but weaker association compared to

people from the general population; however, such investigation is beyond the scope of this study.

Despite the tendency for atherosclerosis in familial OAG, no association was identified for systemic hypertension, diabetes or cigarette smoking. Each of these factors is known to contribute to atherosclerotic plaque formation (Aboyans et al., 2007). Similarly, we found no significant association between cold extremities, which reflect vasospasm of peripheral arteries (Raynaud's phenomenon), and familial OAG. This lack of association conflicts with the finding of migraine and may reflect differences between central and peripheral vasospastic tendencies.

It is certainly acknowledged that the identified association of migraine and atherosclerosis with familial glaucoma may be spurious and clearly requires replication. Both migraine and atherosclerosis have been shown to have major heritable components (Duffy et al., 1993; Nyholt et al., 2005). Hence, the difference in disease prevalence between patients delineated as having familial or sporadic OAG may merely reflect co-segregation of each disease or similar environmental exposures rather than a pleiotropic effect of heritable forms of OAG. However, no major effect of familial clustering was identified for either migraine or atherosclerosis. Given the likelihood of allele sharing, we expected firm familial associations to be strongest in the first-degree relatives, compared to that in more distantly related patients, and although such a relationship was identified in migraine no such pattern existed for atherosclerosis.

In this study, the proportion of people who had a history of ever using corticosteroids was not significantly different between familial and sporadic OAG. As this is a

cross-sectional study, we were unable to examine the temporal relationship with corticosteroid use and, unfortunately, no dose-effect data were available.
A strong association between previous use of inhaled corticosteroids and findings of glaucoma or ocular hypertension in persons with a family history of glaucoma has previously been reported. Moreover, this risk increased with higher doses (OR 6.3; CI 1.0-38.6) for persons who used more than four puffs per day (Mitchell et al., 1999a). Some evidence for a genetic basis for steroid-induced ocular hypertension exists (Armaly 1967b; Reuling and Schwartz, 1970; Schwartz et al., 1972). Clinic-based studies have demonstrated that inheritance of steroid induced ocular hypertension may have an autosomal recessive pattern and may be associated with familial glaucoma (Bartlett et al., 1993; Davies 1968).

The major limitations of this study include recall and misclassification bias. Despite being cross-referenced with medical summaries provided by local physicians when available, a history for each systemic outcome used in this study was primarily elicited through structured questioning. Further to this, the degree of atherosclerosis is not likely to correlate closely with self-reported end-organ symptoms, such as angina, and consequently more formal documentation of systemic disease is warranted. Although great efforts were made to ensure full pedigree ascertainment, it is also certainly possible that some sporadic cases with an actual family history of the disease were misclassified.

In summary, this study identified a significant difference for a past history of migraine or presence of atherosclerosis between patients with familial forms of OAG compared to people with sporadic disease. No association for hypertension, diabetes, Raynaud's phenomenon, thyroid disease, corticosteroids use or cigarette smoking

was identified. These results imply that there are important systemic differences between familial and sporadic disease. An understanding of such differences and systemic comorbidities will be useful for further work investigating the underlying molecular mechanisms of this disease.

Chapter 4 – BLINDING GLAUCOMA: Biometric associations of severe glaucoma.

In this chapter the role of central corneal thickness as a risk factor for disease severity in a cohort of patients with advanced glaucoma was investigated. Then in a subset of this cohort, we compared different automated optic nerve head imaging modalities. It was found that the Stratus optical coherence tomography (OCT) retinal nerve fibre layer clock hour analysis accurately detected all cases of advanced glaucoma. However, the OCT retinal nerve fibre layer quadrant average scan, as well as the Moorfields Regression Analysis on Heidelberg Retinal Topography II and Heidelberg Retinal Topography 3 systems were surprisingly not completely sensitive despite the advanced nature of the glaucoma cases.

The clinical significance of central corneal thinness in glaucoma: the conundrum of pathogenesis thickens.

Background:

Intraocular pressure (IOP) has long been identified as a major risk factor for Primary Open Angle Glaucoma (OAG) and prospective randomised trials have shown that lowering IOP slows glaucomatous visual field loss (van der Valk et al., 2005). The relative thickness of the cornea centrally is known to confound the clinical measurement of IOP, with a lower central corneal thickness (CCT) generally leading to an underestimation of IOP (Ehlers et al., 1975; Goldmann and Schmidtq, 1957). A failure to recognise the confounding effects of CCT could lead to the misclassification of patients based on IOP in regards to glaucoma risk and diagnosis (Herndon et al., 1997). Patients may suffer a delay in diagnosis or be under-treated if they have thin CCT. Conversely, individuals with thick CCT may have falsely high IOP readings and be treated unnecessarily due to overestimation of their true IOP. Recently however, several lines of evidence have indicated a role for CCT in the development and progression of glaucoma above and beyond that associated with IOP correction (Brandt et al., 2001).

The large prospective Ocular Hypertension Treatment Study (OHTS) reported that lower CCT measurements were a significant predictor for the development of OAG, independent of IOP (Brandt et al., 2001; Gordon et al., 2002). This finding was subsequently confirmed by the European Glaucoma Prevention Study (EGPS) (Miglior et al., 2007), and pooled analysis of data from these studies validated the use of CCT in OAG risk-prediction models (Gordon et al., 2007). The role of CCT as a risk factor for prevalent disease or for the progression of OAG is less well established.

A recently published systematic review found that there was no strong evidence for an association between CCT and deterioration in visual field loss (Dueker et al., 2007). Interestingly, initial results from the Early Manifest Glaucoma Trial (EMGT) found no relationship between OAG and CCT (Leske et al., 2003). However, after a longer follow-up period (median of eight years) a thinner CCT was revealed to be a significant factor for disease progression, particularly in patients with higher baseline IOP (Leske et al., 2007). The aim of this study was to document the role of CCT as a risk factor for disease progression in a cohort of patients with advanced OAG. This work was based on the premise that by virtue of severe visual field loss, patients must have had significantly progressive disease.

Methods:

A retrospective case-control design was adopted. Subjects with advanced OAG and disease-free controls were recruited between October 2005 and September 2007. Case subjects were referred from local Ophthalmologists, the Royal Society for the Blind, and Glaucoma Australia, as the initial part of the Australian and New Zealand Registry of Advanced Glaucoma. The tenets of the declaration of Helsinki were adhered to and informed consent was obtained from each patient. The joint ethics committee of Flinders University and Flinders Medical Centre approved this study.

Patients with OAG were included in this study if they had severe visual field loss and corresponding signs of advanced glaucomatous optic neuropathy in at least one eye. Severe visual field loss due to OAG was defined as a minimum of 10/16 points in the central 10^{o} of visual field in one eye having a pattern deviation less than 0.5% due to OAG, as measured on a reliable field from the Humphrey Field Analyser II 24-2

(Zeiss-Humphrey, Dublin, California, USA). IOP was not used as an inclusion criterion for case subjects.

Control subjects were recruited from the same geographical region, through the local media and residential villages. For inclusion each control subject was required to have a normal retinal nerve fibre layer analysis as determined by scanning laser polarimetry (GDx VCC, Carl Zeiss Meditec, Inc., Sydney, Australia), an IOP less than or equal to 21 mmHg measured using a Tonopen (Mentor, Norwell, Massachusetts, USA) and a normal visual field on Humphrey FDT (Carl Zeiss Meditec, Inc., Sydney, Australia) testing.

After instillation of topical anaesthetic (0.5% amethocaine HCl), CCT was measured in all subjects using an ultrasound Pachmate DGH 55 (DGH Technology Inc., Exton PA, USA). Eyes were excluded for any of the following: signs suggestive of corneal dystrophy, corneal scars, previous refractive surgery or recent (within six months) ocular surgery. Analysis was performed using results for both the right and left eye as well as for the mean of the two eyes.

Results:

The demographic features of the study participants are presented in Table 4-1. Control subjects were statistically significantly older at the time of examination than the case subjects were at diagnosis ($p<0.0001$). The mean ± SD maximum recorded IOP for case subjects was 28.7 ± 8.7 mmHg. CCT was well correlated between the right and left eyes of participants ($r^2=0.94$, $p<0.0001$). Patients with advanced OAG

in at least one eye due to OAG were found to have significantly lower CCT measurements compared to the normal control cohort ($p<0.0001$).

CCT measurements below 500μm conferred an age and gender adjusted odds ratio (OR) of 6.36 (95%CI: 3.93-10.30; Wald $\chi^2=64.71$) for glaucoma blindness. Conversely, there were significantly fewer patients with thick CCT (greater than 570μm) who were blind from glaucoma compared to the normal control subjects (OR for developing disease with a CCT > 570 = 0.39, 95%CI: 0.23–0.65; $\chi^2=19.89$). The three thinnest quintiles for CCT measurements were significantly associated with an increased likelihood of blindness from glaucoma compared to the thickest CCT quintile (Table 4-2).

Table 4-1
Demographic features of the study cohorts.

	Controls	Cases	p
No.	271	213	
No. Female (%)	147 (54.2%)	99 (46.5%)	0.09
Age at Examination (SD)	75.7 (8.3)	77.4 (10.3)	0.04
Age at Diagnosis (SD)	-	62.2 (13.9)	-
Worst Eye MD (SD)	-	-21.9 (6.7)	-
Trabeculectomy in at least one eye (%)	-	114 (53.5%)	-
CCT OD (SD)	545.5 (35.7)	512.0 (37.2)	<0.0001
CCT OS (SD)	543.4 (34.2)	511.0 (36.6)	<0.0001
Individual mean CCT (SD)	544.5 (34.2)	511.4 (35.9)	<0.0001

Abbreviations: No., number; MD, mean deviation; SD, standard deviation.

Table 4-2
Number of participants and corresponding odds ratio for developing blinding glaucoma, by central corneal thickness quintiles.

	Central Corneal Thickness Quintile				
	1	2	3	4	5
Range (μm)	≤ 496	497 - 518	519 - 540	541 – 563	≥ 564
No. with OAG	77	52	38	27	19
No. of controls	21	44	59	70	77
Crude OR	3.97 (2.62 - 6.01)	2.74 (1.76 - 4.26)	1.98 (1.23 - 3.18)	1.41 (0.84 - 2.35)	
χ^2	67.03	24.34	8.71	1.72	Reference
p	2.67×10^{-16}	8.07×10^{-7}	0.0032	0.19	

Abbreviations: No., number; OAG, primary open angle glaucoma; OR, odds ratio.

Discussion:

In this study we found that CCT was strongly associated with advanced OAG. These retrospective data support other recent studies, which have also concluded that a lower CCT is related to increased glaucoma severity (Herndon et al., 2004; Hewitt and Cooper, 2005) and with the progression of disease in preperimetric glaucomatous optic neuropathy (Medeiros et al., 2003). Our strategy of concentrating analysis on the most severe aspect of OAG is novel and was chosen to maximise the probability of identifying clinically relevant associations. An overriding hypothesis of this retrospective 'hyper-disease' case-control design was that each patient had at some point undergone 'progressive visual field loss.' A further conjecture is that clinicians would step patients up the therapeutic ladder when they had been identified as having worsening optic disc damage or visual field loss, regardless of their recorded target IOP level. Nonetheless, our study design and specific control inclusion criteria restricted the inclusion of IOP for multivariate modelling.

The work performed by the OHTS and EGPS has established the importance of a low CCT measurement in the conversion of ocular hypertension to OAG (Gordon et al., 2002; Miglior et al., 2007). Multivariate modelling of baseline features, including various applanation tonometry correction algorithms, failed to reduce the clinical significance of CCT recordings in the screening of patients with ocular hypertension (Brandt 2004; Brandt 2007). When supplemented with the most recent data from the EMGT (Leske et al., 2007), it appears that, in addition to elevated IOP, the biomechanical properties of the eye (albeit being relatively poorly reflected by the CCT) are an important feature in OAG pathogenesis. In a clinic-based study Congdon and colleagues found that a thinner CCT was associated with greater optic disc damage, and that corneal hysteresis was independently associated with glaucoma risk (Congdon et al., 2006). In addition to altered corneal-biomechanical properties, a thin CCT may reflect distorted optic nerve head biomechanics.

It has been shown that the biomechanical properties of the peripapillary sclera are dramatically altered by exposure to chronic elevations in IOP (Downs et al., 2005), and that the lamina cribrosa is thinner, as well as having altered anatomic relationships, in eyes with elevated IOP due to secondary glaucoma (Jonas et al., 2003). The correlation between lamina cribrosa thickness and CCT is difficult to study *in situ*. Although the histopathological work of Jonas and Holbach found no biometric relationship between the thickness of the lamina cribrosa and that of the cornea, such a study was considerably impeded by the use of processed specimens, in which many CCT readings were artificially beyond the normal population range (Jonas and Holbach, 2005). Tengroth, who was one of the first to investigate the global protein profiles throughout the eye of OAG patients, hypothesised that the molecular properties and composition of the lamina cribrosa would be more similar to the trabecular meshwork than the sclera (Rehnberg et al., 1987; Tengroth et al.,

1985; Tengroth and Ammitzboll, 1984a; Tengroth and Ammitzboll, 1984b). More recent work by Steely and colleagues has found a high degree of overlap in protein expression profiles of the lamina cribrosa and corneal stroma (Steely, Jr. et al., 2000).

Interestingly Mabuchi and colleagues have reported that transgenic mice with targeted mutations in the gene disrupting the α-1 subunit of the collagen type I (*Col1a1*), develop sustained IOP elevation and progressive optic nerve axon loss (Mabuchi et al., 2004). The *COL1A1* gene is known to be expressed in the developing cornea (von der et al., 1977), and mutations in this gene have been associated with osteogenesis imperfecta, which is one of the commonest forms of connective tissue disorder (Gajko-Galicka 2002). People with osteogenesis imperfecta are known to have remarkably thin corneas (Everekliogluet al., 2002). This finding is intriguing, given that mice do not have a formed lamina cribrosa at the optic nerve head (Gelatt et al., 1998b). Thus, this animal model is likely to reflect altered biomechanical properties throughout the eye.

In summary, the data from this study suggest that CCT measurement should be included as a routine part of the examination in all patients with OAG, not solely in the screening of patients with ocular hypertension. A thin CCT appears to be a significant risk factor for glaucoma progression, with a CCT below 500μm conferring an OR of approximately 6.4 (95%CI: 3.9-10.3) for advanced visual field loss.

The sensitivity of confocal laser tomography versus optical coherence tomography in detecting advanced glaucoma.

Background:

Automated imaging offers a less subjective means for diagnosing and staging glaucomatous optic neuropathy (GON). Whilst the ability of different imaging modalities to detect early glaucoma has been validated, their efficacy in identifying advanced disease has not been well established (Hougaard et al., 2007; Iliev et al., 2006; Medeiros et al., 2004; Zelefsky et al., 2006).

The incorporation of built-in normative databases is one of the inherent strengths in using automated imaging technologies in the setting of diagnostic or therapeutic monitoring (Moreno-Montanes et al., 2007). This allows for the direct comparison or regression of optic disc features measured in clinic-based patients to that of people from the general population (Moreno-Montanes et al., 2007). The output from these algorithms inherently aids in the minimisation of subjective biases and can guide clinicians in optic disc assessment.

The aim of this study was to determine whether the normative databases used in automated imaging devices accurately detect all cases of end-stage GON. The underlying supposition was that the ability of automated imaging devices to detect all cases of definitive disease should be validated prior to their widespread utilisation in disease screening. Of the various screening devices, the role of optical

coherence tomography (OCT) and confocal scanning laser ophthalmoscopy (CSLO) in clinical practice has received the most attention (Harasymowycz et al., 2005). We specifically sought to determine whether the manufacturer-supported normative algorithm in the Heidelberg Retinal Topography (HRT; Heidelberg Engineering, Heidelberg, Germany) CSLO was more sensitive than that incorporated into the Stratus OCT (Carl Zeiss Meditec, Inc.).

Methods:

Informed consent was obtained from each patient and this study was conducted in accordance with the Declaration of Helsinki and its subsequent revisions. Ethical approval was obtained from the relevant joint committee of Flinders University and Flinders Medical Centre.

Patients with advanced glaucoma were recruited between October 2005 and September 2007, through the cooperation of local ophthalmologists, the Royal Society for the Blind and Glaucoma Australia. Advanced POAG was defined as glaucomatous optic neuropathy with visual field loss due to primary open angle glaucoma. Subjects were required to have 10 of the 16 points in their central 10 degrees of visual field in one eye with a pattern deviation less than 0.5%, as measured on a Humphrey Field Analyser II 24-2 (Zeiss-Humphrey, Dublin, California, USA), Maximum recorded intraocular pressure and optic disc parameters were not used alone as inclusion criteria. Subjects with severe refractive error (spherical equivalent

exceeding ±6 dioptres) or potential visual field impairing retinal comorbidity, were excluded.

Following full pupillary dilation, CSLO and OCT was performed using the HRT II (Heidelberg Engineering, Heidelberg, Germany) or the Zeiss Stratus OCT (software v.4.0.4, Carl Zeiss Meditec, Inc.) machines, respectively. Only those scans that were free of artefact and had a topography standard deviation less than 30 on the HRT or a signal strength of at least five on the OCT were accepted. Cylindrical correction with the manufacturer-provided lenses was used during CSLO imaging. With the HRT II software, the optic disc margin was defined at the inner margin of Elschnig's rim by one trained observer (AWH), who was masked to the OCT findings. After preliminary data extraction, the images obtained using the HRT II software were directly imported into the HRT 3 program. The fastRNFL scan of the OCT was used to image the peripapillary retinal nerve fibre layer (RNFL). Scan placement was continuously monitored and adjusted as appropriate.

Results from the worse eye of each subject, as judged by mean deviation (MD) on visual field, were used for analysis. Images from left eyes were flipped to appear as right eyes for the purpose of direct comparison. The severity of visual field deficit was also determined using the Advanced Glaucoma Intervention Study (AGIS) defect score (The AGIS Investigators 1994). Briefly, this was determined by the number and depth of depressed adjacent test sites, in the nasal as well

as upper and lower hemifields, on the total deviation plot (The AGIS Investigators 1994). Scores range from 0 (no defect) to 20 (all test sites depressed) (The AGIS Investigators 1994).

The Moorfields Regression Analysis (MRA) from the HRT II and HRT 3 programs was used as the primary outcomes for the CSLO machine (Wollstein et al., 1998). The MRA discriminates between normal and glaucomatous optic discs using neuroretinal rim area following adjustment for age and optic disc size (Wollstein et al., 1998). The normative database used for the MRA in the HRT 3 program has been extensively expanded compared to the HRT II software (Heidelberg Engineering 2005). Optic disc segment data, generated from the Glaucoma Probability Score (GPS) of the HRT 3 software package, were also reviewed (Swindale et al., 2000). The GPS provides an assessment of the three-dimensional optic disc and RNFL shape, independent of the operator defined disc boundary (Swindale et al., 2000). Output from both the clock hour/sector averages and the quadrant averages of the RNFL scans from the OCT were also analysed. All automatically generated data points from both the CSLO and OCT were classified as being within normal limits (1), borderline (2), outside normal limits (3) or not determinable (0).

The visual field confirmation of disease status was used as the reference standard for analysis. To be classified as abnormal on automated imaging, the optic disc was required to have at least one region highlighted as being outside normal limits. To determine the

variability in optic disc margin demarcation using the HRT II software, fifty optic discs from different subjects were graded twice. Across all sectors there was excellent agreement in MRA outcomes (median $\kappa = 0.85$, range: 0.70–0.94).

McNemar's test was used in the analysis of disease status proportions between imaging modalities. The total number of sectors classified as being outside normal limits on HRT II and HRT 3 was compared using the Wilcoxon signed ranks test. The Spearman rank coefficient was used to correlate the outcomes from MRA between the HRT II and HRT 3 normative databases. Statistical analysis was performed using SPSS v15 (SPSS Inc, Chicago, IL, USA) and R (R Foundation for Statistical Computing, Vienna, Austria). Data are presented as mean ± SD.

Results:

A total of 157 Caucasian subjects, with a mean age of 76.2 ± 10.6 years, were recruited. Sixty-seven (42.7%) subjects were female. The average MD on visual field testing was -21.79 ± 6.58 dB and AGIS disability scores ranged from 6 to 20 (mean 17.01±3.80). Following analysis on the HRT II software, the mean optic disc and optic cup areas were 1.981 ± 0.401 mm^2 and 1.354 ± 0.465 mm^2, respectively.

At least one sector in each study eye was identified as being outside normal limits by the OCT sector RNFL analysis (100% sensitivity). Five eyes were classified as being within normal limits on the OCT

quadrant average analysis (96.8% sensitivity). Each of these five eyes was found, by HRT II and HRT 3, to have at least one sector outside normal limits. Four eyes were found to be either completely within normal limits or borderline on the HRT II MRA (sensitivity of 97.5%) (Figure 4-1). One of these eyes was subsequently identified as being outside normal limits by the HRT 3 MRA (Figure 4-1). No systematic feature, such as optic disc size, cup depth or image quality, was identified to account for the eyes inappropriately labelled as normal (data not shown). No eye was found to be within normal limits by the GPS (Figure 4-2). However, a substantial number (22/157, 14.0%) eyes had segments that were not classifiable.

Figure 4-1

Four subjects (a – d) found on HRT II Moorfields Regression Analysis, to have a normal or borderline optic disc. The images from HRT II, HRT 3, Stratus OCT retinal nerve fibre layer assessment, as well as the Pattern Standard Deviation on 24-2 Humphrey visual field are displayed (left to right).

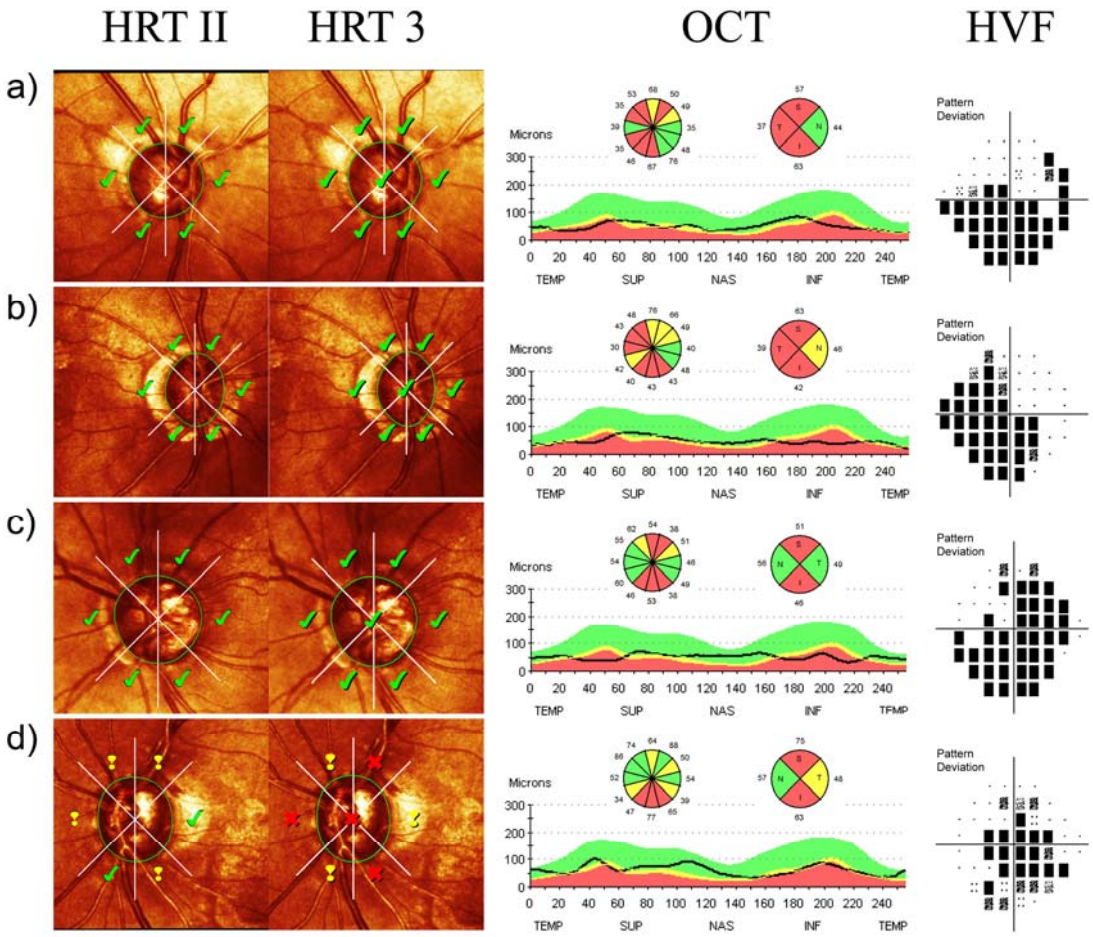
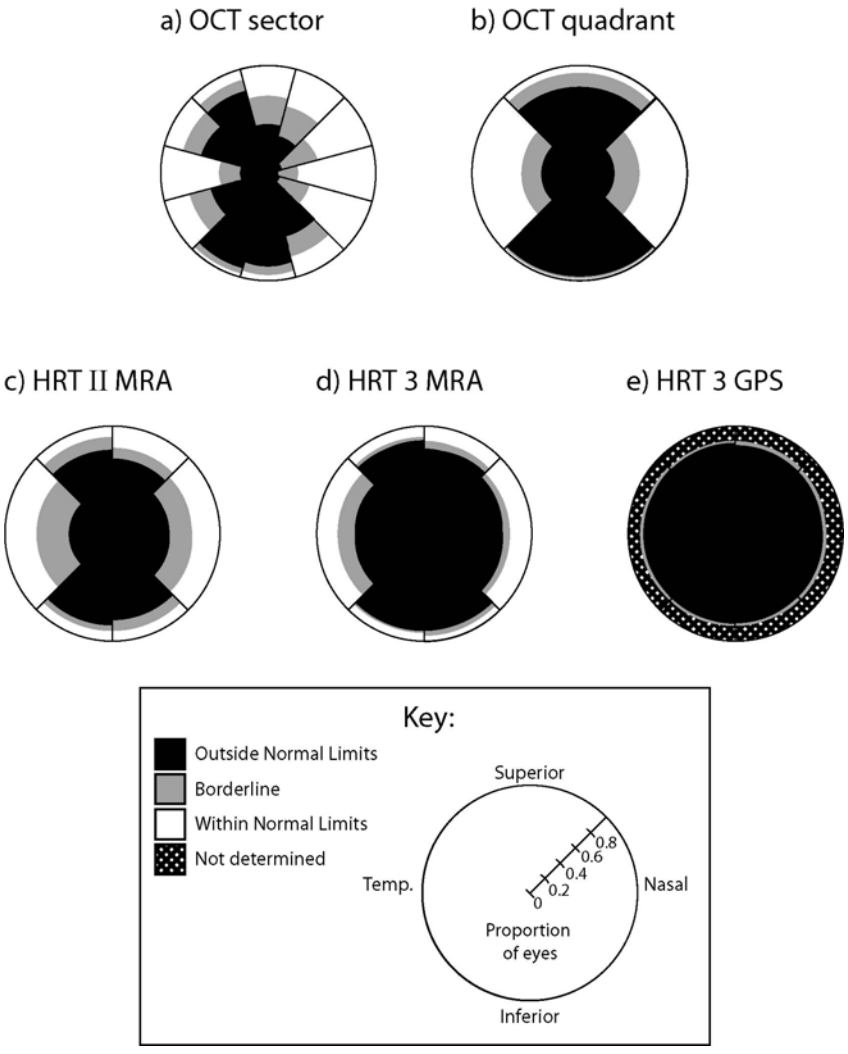


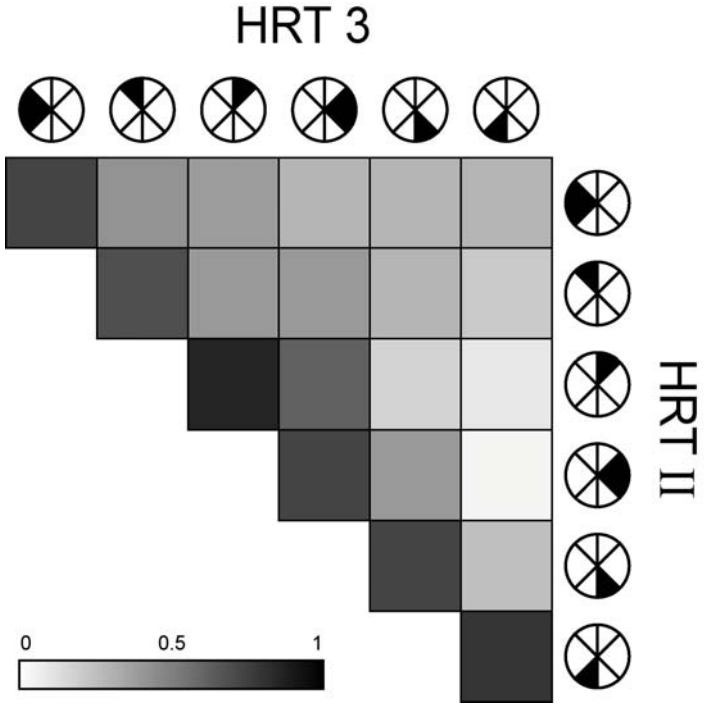
Figure 4-2

Segmental plots showing proportion of glaucomatous eyes graded as being outside, borderline or within normal limits by the normative algorithms. a) and b) Stratus OCT; b) – e) Heidelberg Retinal Topography. Abbreviations: Temp, temporal; OCT, optical coherence tomography; HRT, Heidelberg Retinal Topography; MRA, Moorfields Regression Analysis; GPS, Glaucoma Probability Score.



The location and relative proportion of sectors outside and within normal limits for each imaging modality is displayed in Figure 4-2. Although there were more eyes found to have an abnormal nasal quadrant compared to the temporal quadrant on OCT, this did not reach statistical significance (p=0.47). There were significantly more eyes identified as having at least half of all optic disc segments outside normal limits on the OCT clock hour analysis compared to the OCT quadrant analysis (p<0.001). A greater proportion of eyes were found to have at least half of all optic disc segments outside normal limits on HRT 3 MRA compared to HRT II MRA (p=0.008). Similarly, the total number of segments classified as being outside normal limits was greater on HRT 3 MRA than HRT II MRA (p<0.001). The superonasal segment was found to have the greatest correlation between HRT II and HRT 3 MRA (Figure 4-3).

Figure 4-3
Correlation (r^2) in Moorfields Regression Analysis outcomes between the HRT II and HRT 3 normative databases. Sectors are displayed as a right eye.



Discussion:

In this study, we found that all cases were observed to have at least one abnormal optic disc segment when the OCT RNFL clock hour/sector average analysis was used. However, the OCT RNFL quadrant average analysis, as well as the MRA on HRT II and HRT 3 programs, were not completely sensitive in detecting patients with well established GON. The diagnostic sensitivity of HRT 3 was identified as being only marginally better than that of the HRT II (98.7% vs 97.5%). Nonetheless, significantly more optic disc segments in the whole cohort were identified as being outside normal limits on the HRT 3 MRA than on HRT II MRA. The power to investigate the underlying scan or optic disc features for the eyes identified as being normal on MRA and OCT RNFL quadrant average analysis was limited. Medeiros et al. have shown that the sensitivity for glaucoma detection in advanced disease (i.e. high AGIS scores) using the Stratus OCT is poorer in people with large optic discs, whilst the HRT II MRA has a poorer sensitivity in people with small optic discs (Medeiros et al., 2006).

Analysis of the regions with weak correlation between MRA on the HRT II and HRT 3 systems provides some indication of the optic disc segments where the HRT 3 normative database has been improved (Figure 4-3). From our data it is possible to infer that the regression database has been enhanced in the assessment of optic discs with advanced inferotemporal and nasal damage.

The inferior disc segment was the most common site identified for glaucomatous damage, as assessed by all imaging modalities. Interestingly, the proportion of eyes with abnormal segments were in direct opposition to the normal “ISNT” rule for the

MRA of the HRT scans, thus supporting its use as a diagnostic aid for glaucoma (Jonas et al., 1988). The rim area of healthy optic discs has been previously observed to be thickest in the inferior, followed by the superior, nasal and temporal sectors (Jonas et al., 1988). No optic disc segment was preferentially highlighted as being more damaged on GPS analysis.

It was also noteworthy that no eye was classified as being within normal limits on the GPS analysis using the HRT 3 software. A major caveat of this finding was that there were a relatively high proportion of optic discs (14.0%) for which no segmental GPS classification could be made. Ferreras and colleagues have recently reported that the diagnostic performance of the GPS was similar to that of the MRA (Ferreras et al., 2007), whilst our findings suggest that the GPS may outperform the MRA in the assessment of advanced disease.

A weakness of our study is that each patient was only imaged once with each device. DeLeón Ortega and colleagues have revealed that the test–retest variability on HRT II testing is greater in eyes with moderate or severe damage compared to early GON or normal people (DeLeon Ortega et al., 2007). Interestingly, their work also found that the reproducibility in averaged RNFL thickness measured using the OCT was greater in the severe GON group compared to those with less severe disease (DeLeon Ortega et al., 2007). An additional limitation of the present study is that stereoscopic optic disc photographs were not utilised in the demarcation of the optic disc margin for the MRA. Nonetheless, we had good intra-observer reproducibility.

In conclusion, the Stratus OCT RNFL clock hour normative database performs better in the detection of advanced GON than other modalities studied. Status OCT quadrant average, as well as the MRA on HRT II and HRT 3 systems, were

comparable but not completely sensitive in detecting severe glaucoma. The findings from this study may have important ramifications for future applications in community-based screening programs for glaucoma. It is quite clear that one should have an expectation of close to 100% sensitivity in a screening test for severe cases such as those used in this study. It is certain that sensitivity could only fall if less severe cases were included in the analysis. As objective means for diagnosing and monitoring glaucomatous progression are becoming more readily available, clinicians must remain vigilant in their clinical assessment of the optic disc.

Chapter 5 – GLAUCOMA GENETIC ASSOCIATIONS: Probing for genetic predispositions in Open Angle Glaucoma.

Individual risk profiling could prevent glaucoma blindness by pre-symptomatic screening followed by tailored therapy after disease is detected. Analogous to fire fighting, just as total fire bans and smoke alarms prevent catastrophic damage, knowledge of the genomic profile of at-risk individuals could ensure that patients receive adequate pre-symptomatic clinical screening and early intervention. Additionally, just as all fires (i.e. oil-based versus electrical versus wood) can not be extinguished with just with any form of hydrant, it is likely that understanding the molecular mechanisms underpinning a particular patient's disease would allow for the use of individualised medicine. This chapter principally explores the genetic underpinnings of the glaucomas.

Work investigating the relative importance of the Asp658Gly variant in the *Winged Domain 40- repeat 36* gene and the Met98Lys variant in the *optineurin* gene was performed. The role of *lysyl oxidase–like 1* gene sequence variants in the pathogenesis of pseudoexfoliative glaucoma was also investigated. The final section of this chapter describes the use of equimolar DNA pools in a case-control genome-wide association study. Novel putative genetic risk variants for OAG were identified on chromosomes 3q21, 6p25, 14q13 and Xq25.

Case-control study of the *WDR-36* Asp658Gly mutation indicates it is a neutral variant.

Background:

Despite many positive linkage studies, relatively few OAG-causing genes have been identified (Hewitt et al., 2006b). In 2004, Samples et al. reported a large American family of Dutch ancestry with linkage to chromosome 5q-*GLC1G* (Samples et al., 2004). Additional evidence for a glaucoma-causing gene in this region was provided by Monemi and colleagues (Monemi et al., 2003). More recently, Monemi et al. presented data suggesting that mutations in the *WD40-Repeat36* (*WDR36*) gene at the GLC1G locus cause OAG (Monemi et al., 2005). One amino acid variation in particular (Asp658Gly), was present in a large *GLC1G*-linked family (Monemi et al., 2005). Although a further six potential or predicted disease susceptibility mutations were reported, the Asp658Gly variant was the sole mutation that was statistically significantly more common in glaucoma cases compared to controls. Furthermore, the large family with the Asp658Gly variant had been used to refine the critical region for the *GLC1G* gene. This missense mutation was found in 13 of the 670 (1.94%) unrelated cases whilst not in any the 238 normal controls (Monemi et al., 2005). The aim of this study was to investigate the prevalence and associated phenotype of the *WDR36* Asp658Gly mutation in Australian patients with glaucoma, and to establish its frequency in normal population controls.

Methods:

In total 249 Caucasian individuals with well-characterised OAG were recruited through the Glaucoma Inheritance Study in Tasmania (GIST). 217 age-matched control subjects were recruited from the same population as the OAG cases. Our control cohort comprised 151 people who resided in local retirement homes, and 66

unrelated people who were recruited through an adjuvant genetic study. Control subjects were included if examination showed they were free of ocular hypertension or OAG. Informed consent was provided by each participant and ethical approval was obtained from local committees.

Molecular techniques and primer sequence were kindly provided by M.Sarfarazi (University of Connecticut Health Centre). PCR amplification of exon 17 was performed using intronic primers (forward: 5′-AAAACATTTTCTGCATCTCTTATCC-3′; reverse: 5′-CAAGCAGATGTTGAATCACTCAG-3′). Conditions are listed in the Appendix. The 389bp amplimer obtained was digested with the *BglI* restriction enzyme (Genesearch, Australia). Digested fragment products of 272bp and 117bp were produced in the presence of the Asp658Gly allele and were detected by gel electrophoresis.

Results:

The Asp658Gly sequence variant was found in equal prevalence between our cases and matched control subjects. Four of the 249 (1.6%) OAG cases and four of the 217 (1.8%) control subjects carried this variant (χ^2=0.04, p=0.84). Each control subject had no known family history of glaucoma. The phenotype and family history status of case subjects is displayed in Table 5-1. Two of the Gly658-carrying case subjects had a OAG-affected third-degree relative (both had one affected first cousin), the mother of one Asp658Gly-carrying case subject had OAG, whilst the remaining case had a sibling who was said to be affected with OAG (not available for examination). None of the offspring of these probands had been diagnosed with OAG. There were no significant differences in the phenotype or family history status between

glaucoma cases with or without the *WDR36* Asp658Gly variant. This is in marked distinction to the situation we have previously reported for the commonest disease-causing variant in the glaucoma-causing gene *Myocilin* (Gln368STOP), which causes disease of greater severity and with a stronger family history than unselected cases (Craig et al., 2001).

Table 5-1
Phenotypic composition of glaucoma cases with the *WDR36* Asp658Gly variant compared with non-carriers.

	OAG Asp658Gly non-carriers	OAG Asp658Gly carriers	p
N	245	4	
Gender F/M	156 / 89	1 / 3	0.11[a]
Age at Review (SD)	72.9 (11.5)	73.0 (8.3)	0.91[b]
Age at Diagnosis (SD)	64.0 (12.4)	60.0 (9.7)	0.91[b]
Worse eye CDR (SD)	0.80 (0.14)	0.80 (0.18)	0.97[b]
Maximum recorded IOP in mmHg (SD)	25.2 (8.7)	28.0 (8.2)	0.53[b]
Degree of Closest OAG Affected Relative:			
Sporadic OAG	64 (26.1%)	0	0.19[a]
1st Degree Relative Affected	124 (50.1%)	2* (50.0%)	
2nd Degree Relative Affected	22 (9.0%)	0	
≥ 3rd Degree Relative Affected	35 (14.3%)	2 (50.0%)	

Abbreviations: n, number; F, female; M, male; SD, standard deviation for mean difference; CDR, vertical cup:disc ratio; IOP, intraocular pressure; OAG, primary open angle glaucoma.
* - relative affected by hearsay, refer to text
a - χ^2 test
b - Mann-Whitney test

Discussion:

We set out to identify families and determine the associated phenotype with the previously reported common dominant *WDR36* Asp658Gly mutation. We found the variation to be equally prevalent in unaffected controls. In the cases with this mutation, the examined pedigrees from two of the probands were found not to belong to large autosomal dominant families. Taken together, these findings lead us to conclude that this variant is likely to be a neutral variant in Australia. Given that the Asp658Gly variant was used as the principal evidence supporting *WDR36* as a disease causing gene, our results indicate that additional populations should be carefully assessed for this variant before concluding that *WDR36* is a glaucoma gene.

The role of the Met98Lys *Optineurin* variant in Glaucoma:

Background:

As outlined in Chapter 1, *Optineurin* (*OPTN*), located at chromosome 10p13, was the second gene identified as causing OAG. *OPTN* has been shown to be mutated in some families with normal tension glaucoma (NTG)(Rezaie et al., 2002), whilst investigations into other polymorphisms (in particular *OPTN* Met98Lys) have yielded conflicting results(Alward et al., 2003; Aung et al., 2003; Leung et al., 2003; Rezaie et al., 2002; Tang et al., 2003). In the original paper describing *OPTN*, it was proposed that the Met98Lys variant was very strongly associated with glaucoma(Rezaie et al., 2002). The Met98Lys allele alters the binding affinity (Iwata T, et al. IOVS 2003; 44: ARVO E-abstract 1114) of *OPTN* to RAB8, a protein which interacts with Huntington protein. Furthermore, it has recently been reported that *OPTN* is involved in neural degeneration by metabotropic glutamate receptor signalling via interaction with Huntington protein (Anborgh et al., 2005). OPTN is involved in both the apoptotic FAS-ligand as well as the tumour necrosis factor-α (TNF-α) pathway (Sarfarazi and Rezaie, 2003) and dysfunction of *OPTN* could be deleterious to cells under mitochondrial stress. We investigated the role of the *OPTN* Met98Lys polymorphism as a risk factor in a large cohort of OAG patients. Although other *OPTN* sequence variations have been reported, they have not been evaluated in this study.

Methods:

Patient Selection:

Written informed consent was obtained from each subject and this study was conducted in accordance with the Declaration of Helsinki and its subsequent revisions. Approval was obtained from the relevant Institutional Review Boards of the Royal Victorian Eye and Ear Hospital and the Royal Hobart Hospital.

Subjects with OAG were recruited through the Glaucoma Inheritance Study in Tasmania (GIST). GIST subjects are derived from a predominantly Caucasian population. OAG was defined by the presence of, in at least one eye: optic disc cupping (cup-disc ratio≥0.7); or by a 0.2 inter-eye disparity in cup-disc ratio; or focal rim notching with corresponding visual field loss. Visual field assessments were conducted using the Humphrey Visual Field Analyzer threshold 24-2 algorithm and graded as abnormal if the mean deviation or pattern standard deviation had a probability of normality of <5%, or if the Glaucoma Hemifield Test was abnormal. Subjects were required to have an open iridocorneal angle on gonioscopic examination. HTG was diagnosed in subjects who had glaucoma and an untreated IOP>21 mmHg. Subjects diagnosed with NTG had never been found to have an applanation IOP measurement >21 mmHg. A diagnosis of NTG or HTG was made by the treating ophthalmologist and this sub-classification then confirmed in the GIST follow-up. The severity of the disease phenotype was determined in the OAG group by the age at diagnosis, severity of optic disc cupping and GIST severity score. In brief, the GIST score is a combined assessment of visual field severity, optic disc cupping and degree of IOP elevation(Coote et al., 1996). A GIST score of 0.7, 0.8, 0.9 and 1.0 infer mild, moderate, severe or very severe disease, respectively. For a

more detailed description of the derivation of the OAG diagnostic criteria or the GIST score the reader is referred to Coote et al. (Coote et al., 1996).

A subset of the OAG group had previously been identified as having a disease-causing mutation in the *MYOC* gene (Craig et al., 2001; Mackey et al., 2003). Given the possibility for phenotypic modification effects between *MYOC* and *OPTN* and the fact that a proportion of the *MYOC* subjects were recruited from the same pedigree, the *MYOC* cohort was analysed separately from the NTG and HTG OAG groups.

Control subjects were recruited from the same geographical region and were matched by age to the OAG cohort. The control cohort comprised 152 people who resided in local retirement homes and 66 unrelated people who were recruited through an adjuvant genetic study. Control subjects were included if they were found on examination to be free of ocular hypertension, OAG and optic atrophy.

Laboratory Techniques:

For the *OPTN* gene screening, a 621 bp fragment spanning the Met98Lys mutation site was amplified using a Hybaid PCR Express Thermal Cycler (Hybaid Ltd, UK). Primer sequences were as follows: F: 5'-GACCAGGCAAAACACCAATCC-3' and R: 5'-CCTTAGCTCCTAGTAACCATAG-3' (Geneworks, Adelaide, Australia). Following PCR restriction digest was then performed on the products using the *Stu1* enzyme. The 603T>A nucleotide change present in the Met98Lys polymorphism carriers creates a Stu1 restriction site, thus resulting in digested fragments of 416 bp and 205 bp, respectively.

Statistical Analysis:

Unless otherwise indicated data is presented as mean ± SD. Allele frequencies were analysed using the Chi-square test through Intercooled Stata 7.0 (Stata Corporation, USA). The nonparametric Kruskal-Wallis test was used to examine disease severity in OAG subjects. Power calculations were performed using the PS program version 1.0.17 for Windows (Dupont and Plummer, 1997). This study had 80% power to detect a 5 year difference in age at diagnosis, a 5 mmHg difference in IOP and a 0.09 difference in cup:disc ratio between the *OPTN* Met98Lys heterozygotes and non-variant carriers, at the 0.05 significance level.

Results:

OPTN Met98Lys in the control cohort:

218 (151 female) control subjects who had no clinical sign of optic atrophy on examination were recruited. The age at recruitment was 70.9±16.6 years. 17 (7.8%) control subjects were found to have the *OPTN* Met98Lys variant in the heterozygous state.

OPTN Met98Lys in OAG:

498 OAG subjects were studied, of whom 128 (25.7%) were classified as having NTG. There were 222 (60.2%) and 82 (64.1%) females in the HTG and NTG groups, respectively. The age at diagnosis for the overall OAG cohort was 62.9±12.0 years (Table 5-2). Compared to the HTG group, patients with NTG were diagnosed at an older age (p=0.0046) and had larger cup-disc ratios in their worst eye (p=0.0002).

Table 5-2
Phenotypic breakdown of OAG subjects with and without the *OPTN* Met98Lys variant.

	n	Mean ± SD age at Examination	p	Mean ± SD age at Diagnosis	p	Mean ± SD GIST severity score	p	Mean ± SD Maximum IOP	p	Mean ± SD Maximum CDR	p
OAG without Met98Lys	470	72.5 ± 10.9	0.49	62.8 ± 12.1	0.24	0.85 ± 0.11	0.27	24.0 ± 8.3	0.07	0.78 ± 0.16	0.76
OAG with Met98Lys	28	73.9 ± 8.9		65.7 ± 10.1		0.83 ± 0.12		20.9 ± 4.4		0.77 ± 0.14	
HTG without Met98Lys	354	72.0 ± 11.06	0.55	61.9 ± 12.2	0.74	0.86 ± 0.11	0.28	26.1 ± 8.3	0.71	0.77 ± 0.17	0.66
HTG with Met98Lys	16	71.6 ± 7.1		63.7 ± 7.7		0.83 ± 0.13		24.4 ± 3.4		0.76 ± 0.16	
NTG without Met98Lys	116	74.2 ± 10.5	0.22	65.4 ± 11.3	0.30	0.83 ± 0.10	0.91	16.9 ± 2.7	0.78	0.83 ± 0.12	0.14
NTG with Met98Lys	12	77.1 ± 10.5		68.1 ± 12.3		0.83 ± 0.10		17.3 ± 1.4		0.79 ± 0.10	
Overall (OAG)*	498	72.6 ± 10.8		62.9 ± 12.0		0.85 ± 0.11		23.8 ± 8.2		0.78 ± 0.16	

* Excluding *myocilin* mutation carriers

Abbreviations: HTG, high tension glaucoma; NTG, normal tension glaucoma; OAG, primary open-angle glaucoma; n, number; SD, standard deviation; GIST, Glaucoma Inheritance Study in Tasmania; IOP, intraocular pressure; CDR, worst eye cup-disc ratio.

28 OAG subjects were identified as having the *OPTN* Met98Lys variant. Analysing the OAG group by the presence of the *OPTN* Met98Lys variant revealed that there was a trend towards a lower maximum IOP (24.0±8.3 versus 20.9±4.4; p=0.072). The presence of the Met98Lys variant in the OAG group did not influence age at diagnosis, GIST severity score, or maximum cup-disc ratio in the worst eye (Table 5-2).

There were significantly more *OPTN* Met98Lys carriers in the NTG group (12/128; 9.4%) compared to the HTG group (16/370; 4.3%; p=0.033). This association was however not significant after Bonferroni correction. In addition, there was no significant difference between the NTG and control cohorts (p=0.609). The difference in frequency between the HTG group and control group approached significance (p=0.077). The presence of the *OPTN* Met98Lys variant did not appear to modify the OAG phenotype in either the HTG or NTG subgroups (Table 5-2).

<u>*OPTN* Met98Lys in *MYOC* OAG:</u>

29 subjects (15 female) who had the *MYOC* Gln368Stop mutation and OAG were recruited from 14 pedigrees, and 16 subjects (10 female) who had OAG with the *MYOC* Thr377Met mutation were recruited from three pedigrees.

Two of the *MYOC* Gln368Stop mutation carriers were found to also carry the *OPTN* Met98Lys variant and had maximum recorded IOPs of 22 and 24 mmHg with corresponding maximal cup-disc ratios of 0.7 and 0.8, respectively. The maximum recorded IOP and cup-disc ratio in the *MYOC* Gln368Stop mutation carriers who did not have the *OPTN* Met98Lys variant were 33.8±10.5 mmHg and 0.81±0.17,

respectively. One subject who had both the Thr377Met *MYOC* mutation and the *OPTN* Met98Lys variant was found to have a maximum recorded IOP of 24 mmHg and a maximum cup-disc ratio of 0.8 in the worst eye. The maximum recorded IOP and cup-disc ratio in the *MYOC* Thr377Met mutation carriers who did not have the *OPTN* Met98Lys variant were 28.5±7.6 mmHg and 0.82±0.17, respectively. However, analysis of pooled *MYOC* mutation carriers revealed no statistical difference in maximal recorded IOP (p=0.066) or cup-disc ratios (p=0.283) in the Met98Lys carriers.

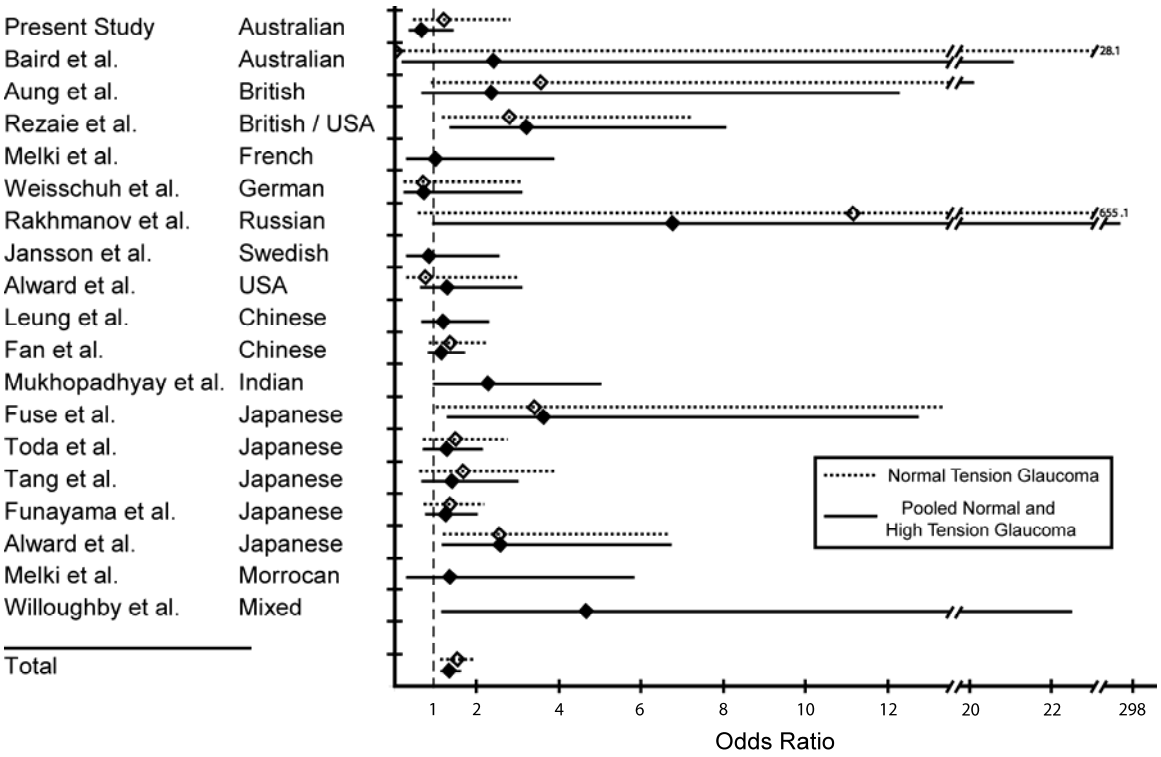
Discussion:

Investigation of the *OPTN* Met98Lys polymorphism in OAG has yielded conflicting findings. Although in our study more *OPTN* Met98Lys carriers were found in the NTG group compared to the HTG group (p=0.033), this finding did not remain statistically significant following correction for multiple testing. Importantly there was no significant difference in carrier frequency between the NTG and control cohorts (p=0.609). We performed a meta-analysis of all published work to date on the *OPTN* Met98Lys variant in glaucoma. A weak but highly significant association with OAG was found (OR: 1.35; 95% Confidence Interval: 1.16-1.58; p=0.0001) (Figure 5-1) (Alward et al., 2003; Aung et al., 2003; Baird et al., 2004; Fan et al., 2005; Funayama et al., 2004; Fuse et al., 2004; Jansson et al., 2005; Leung et al., 2003; Melki et al., 2003a; Mukhopadhyay et al., 2005; Rakhmanov et al., 2005; Rezaie et al., 2002; Tang et al., 2003; Toda et al., 2004; Weisschuh et al., 2005; Willoughby et al., 2004). This finding remains significant after exclusion of the original study (Rezaie et al., 2002) describing the positive association (OR: 1.29; 95% Confidence Interval: 1.10-1.51; p=0.0011).

With the numbers available in this study, a statistically significant difference was not reached between maximal recorded IOP and presence of the *OPTN* Met98Lys variant (24.0±8.3 for non-carriers *vs* 20.9±4.4 for carriers; p=0.07). The response of OPTN expression to elevated IOP remains controversial (Vittitow and Borras, 2002). Melki et al. found that subjects positive for Met98Lys have a significantly lower IOP than *MYOC* mutation carriers who do not have the Met98Lys variant (Melki et al., 2003a). It should, however, be noted that *MYOC* mutations are almost invariably associated with substantially elevated IOP (Alward et al., 1998). We found that our double mutant subjects tended to have lower IOP than their single *MYOC* mutation-carrying counterparts. Such a finding may explain the previous case report describing an NTG *MYOC* mutation patient and adds to the emerging evidence for gene-gene interaction in OAG (Mardin et al., 1999; Petersen et al., 2006). Incomplete disease-gene segregation can occur in complex heterogeneous diseases due multiple additive effects of minor risk alleles (Hewitt et al., 2006b).

Figure 5-1

Meta-analysis of all published case:control studies of the *OPTN* Met98Lys variant in open-angle glaucoma. Diamonds represent odds ratio point estimates, with the dashed and solid bars indicating the respective 95% confidence interval. Dashed bars indicate subjects with normal tension glaucoma, solid bars indicate pooled case subjects with normal or high tension glaucoma. Note that the overall analysis has an odds ratio greater than 1.



In conclusion, this large study of the *OPTN* Met98Lys variant in optic neuropathies found no major gene effect. However, a trend towards association with NTG was again found, prompting a meta-analysis of all published work examining this variant in OAG. This revealed a weak but highly significant association with OAG. It is anticipated that multiple genetic modifiers may act collectively to influence optic nerve diseases. Very large well-characterized cohorts of cases and matched controls are required to dissect these potentially additive complex interactions which are unlikely to segregate in typical Mendelian patterns.

Ancestral *LOXL1* variants are associated with pseudoexfoliation in Caucasian Australians but with markedly lower penetrance than in Nordic people

Background:

The allelic architecture for many human diseases is being rapidly uncovered. Improved access to array-based technology, in conjunction with the Human Genome and International HapMap projects, have permitted a comprehensive examination of the genetic underpinnings of many traits important to human health (Daiger 2005; International Human Genome Sequencing Consortium 2004; The HapMap Consortium 2003; The Wellcome Trust Case Control Consortium 2007). Results from the opening barrage of genome-wide investigations have begun to answer the question of the contribution provided by common genetic variants in the aetiology of common disease. Proponents of the common disease-common variants hypothesis, which predicts that genes responsible for the majority of common diseases have relatively simple allelic spectra, have argued that if the loci contributing to common disease have a moderately sized set of disease alleles, the allelic spectra should be simple (Lander 1996; Reich and Lander, 2001). Conversely, given that variants predisposing to common complex diseases generally appear to have lower penetrance and weaker selection pressures than rarer Mendelian disease-associated alleles, some have argued that they are likely to be underpinned by considerably greater degrees of allelic heterogeneity (Pritchard 2001; Pritchard and Cox, 2002). In this section, we describe a locus-specific contribution made to a disease that is relatively uncommon in our study population, but find a remarkably similar allelic architecture to that of the Nordic population in which the disease is extremely common.

Pseudoexfoliation syndrome (OMIM:177650) is a generalised disorder of the extracellular matrix characterised clinically by the pathological accumulation of abnormal fibrillar material in the anterior segment of the eye (Figure 5-2) (Schlotzer-Schrehardt and Naumann, 2006). In addition to being a significant risk factor for glaucomatous optic neuropathy, pseudoexfoliation syndrome has also been associated with lens zonule weakness, cataract formation, and systemic vascular complications (Mitchell et al., 1997; Naumann et al., 1998; Ringvold 2001; Schlotzer-Schrehardt and Naumann, 2006). The prevalence of pseudoexfoliation syndrome varies markedly between populations, with the highest rates (up to 40%) being documented in people residing in Nordic countries, whilst Anglo-Celtic Caucasians have a remarkably lower prevalence (Forsius 1979; Mitchell et al., 1999b; Ringvold 1996).

Figure 5-2
Pseudoexfoliative material on the anterior lens capsule (courtesy of A. Chappell).

Recently Thorleifsson and colleagues performed a genome-wide scan of Icelandic patients with glaucoma and pseudoexfoliation syndrome (Thorleifsson et al., 2007). They identified two nonsynonymous SNPs in exon 1 of the *lysyl oxidase–like 1* (*LOXL1*) gene which together conferred a population attributable risk in Icelandic and Swedish individuals of 99% for developing pseudoexfoliation (Thorleifsson et al., 2007). Interestingly, the highest risk diplotype was identified in approximately a quarter of the general population who had not been clinically examined, a not unexpected finding given the high prevalence of pseudoexfoliation in their population (Thorleifsson et al., 2007).

We investigated the role of *LOXL1* sequence variation in a large Caucasian population recruited through the Blue Mountains Eye Study (BMES), a population-based study of 4,838 individuals aged 49 years or older, who had a comprehensive baseline eye examination, including a specific examination for pseudoexfoliation syndrome. The two previously identified coding variants in *LOXL1* were found to be strongly associated with pseudoexfoliation syndrome. However, despite our study population's remarkably lower lifetime incidence of the disease, the underlying allelic architecture at *LOXL1* was almost identical to that described by Thorleifsson et al. in Iceland and Sweden (Thorleifsson et al., 2007).

Methods:

Subject recruitment:

Subjects participated in the BMES, as previously described (Attebo et al., 1996; Mitchell et al., 1996). In brief, the BMES is a population-based cohort study investigating the aetiology of common ocular diseases among suburban residents aged 49 years or older, living in the Blue Mountains region, west of Sydney,

Australia. The population in this area is stable and ethnically homogeneous of predominantly Anglo-Celtic descent. Subjects were recruited during one of four surveys between 1992 and 2004. The baseline BMES survey was conducted between 1992 and 1993, recruiting a total of 3654 participants (82.4% of 4433 eligible persons identified in a private census). Of these people, 2564 (70.2%) were re-examined during the five- and ten-year follow-up studies. An ancillary study conducted between 1998 and 2000 examined an additional 1174 people who had either reached the eligible age (49+ years) for participation or had relocated into the study area (85.2% of 1378 newly eligible persons identified in a second private census). DNA samples were obtained during the five-year follow-up and ancillary surveys. Approval for this study was obtained from the relevant Human Research Ethics committees of the Westmead Millennium Institute at the University of Sydney, as well as from Flinders Medical Centre and Flinders University.

Pseudoexfoliation syndrome was diagnosed at slitlamp examination by ophthalmologists as part of a comprehensive ocular examination, including pupil dilation. Given the inherent difficulties in detecting pseudoexfoliation after cataract surgery, participants who had undergone bilateral cataract surgery were considered unclassifiable. Analysis was performed comparing the diagnosed pseudoexfoliation cases against both the sub-population in whom pseudoexfoliation had been clinically excluded and the total unselected population. Glaucoma diagnosis was strictly based on concordant findings of typical glaucomatous visual field defects on the Humphrey 30-2 test together with corresponding optic disc rim thinning, including an enlarged cup-disc ratio (≥ 0.7) or cup-disc ratio asymmetry (≥ 0.3) between the two eyes (Mitchell et al., 1996).

Genotyping and Data Analysis:

Using the software program Tagger, SNPs across *LOXL1* were selected on the basis of linkage disequilibrium patterns within European people from the Centre d'Étude du Polymorphisme Humain from Utah (CEPH), as part of the International HapMap Project (de Bakker et al., 2005; The HapMap Consortium 2003). 12 tagging SNPs, which captured all alleles with an r^2 of 0.8, were selected and genotyped (Figure 1). This included the forced selection of SNP rs3825942 which had been implicated as being associated with pseudoexfoliation by the work of Thorleifsson et al (Thorleifsson et al., 2007). The additional previously identified disease-associated SNP (rs1048661), which was not included as part of the International HapMap Project, was also specifically genotyped in our population (Thorleifsson et al., 2007).

Genotyping was performed with the use of Sequenom iPLEX GOLD chemistry on an Autoflex Mass Spectrometer at the Australian Genome Research Facility (Queensland, Australia). Map coordinates provided are those from National Centre for Biotechnology Information Build 36 (August 2007). The SNP name designations were obtained from dbSNP and HapMap. Individuals with >5% missing genotypes were excluded from analysis. SNP genotyping in control samples was checked for compliance with the Hardy–Weinberg equilibrium by using Haploview 4.0 (Barrett et al., 2005).

Haplotypes and diplotypes for each individual were estimated using the expectation maximization algorithm in HAPLO.STATS. We performed association analysis using contingency tables of individual SNPs, haplotypes, and diplotypes using Haploview 4.0, SNPStats and SPSS (v14.0 SPSS Inc, Chicago, IL) (Barrett et al., 2005; Sole et al., 2006). Given that the results did not change considerably when

only the pseudoexfoliation-free controls were used, data are presented for the total unselected control cohort. An online genetics power calculator was used to estimate the power of this study, after considering a variety of effect sizes and allele frequencies (Purcell et al., 2003). Assuming a multiplicative genetic model and a heterozygous odds ratio of 1.5, our study of unselected cases had a power of 90% to detect a disease-associated allele with a population frequency of at least 0.50.

Homology Modelling:

The LOXL1 protein sequence of humans and other animal species was obtained from the Ensembl database. Protein alignments were performed using CLUSTALW, with a BLOSUM-62 protein-weighted matrix; a gap open penalty score of 10 and a gap extension penalty score of 0.05 (Thompson et al., 1994).

Results:

Complete genotyping data of the *LOXL1* SNPs were available for 2508 participants, of whom 86 (3.4%) had been diagnosed with pseudoexfoliation syndrome. The disease status of 335 (13.4%) participants was unclassifiable. The demographic features of our population-based study cohort are presented in Table 5-3. All genotyped SNPs were in Hardy–Weinberg equilibrium.

Table 5-3

Demographic features of the study population. Pseudoexfoliation status was not classifiable if participants had undergone cataract surgery. The unselected control group comprises both the subjects who had no clinical signs of pseudoexfoliation and those whose clinical status was unclassifiable.

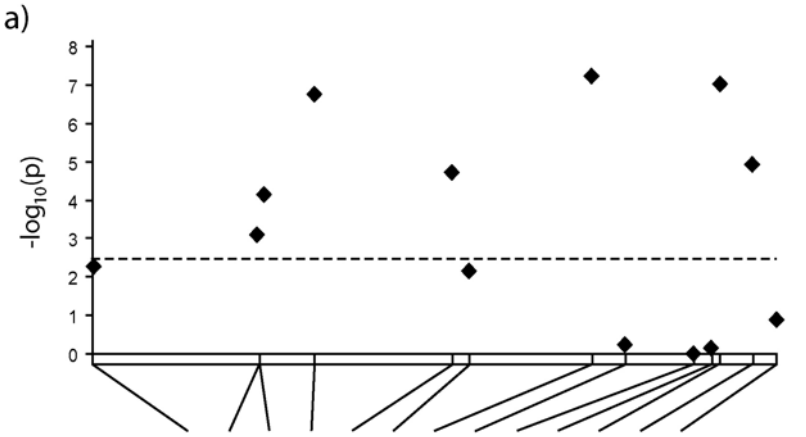
	Cases (Pseudoexfoliation)	Controls without pseudoexfoliation	Pseudoexfoliation not classifiable	Total unselected controls
Total number	86	2087	335	2422
Female (%)	54 (62.8)	1152 (55.2)	221 (66)	1373 (56.7)
Mean age (SD) yrs	76.4 (8.1)	68.6 (10.0)*	77.3 (8.7)	69.9 (10.3)*
Number glaucoma (%)	11 (12.8)	79 (3.8)*	30 (9.0)	109 (4.5)*

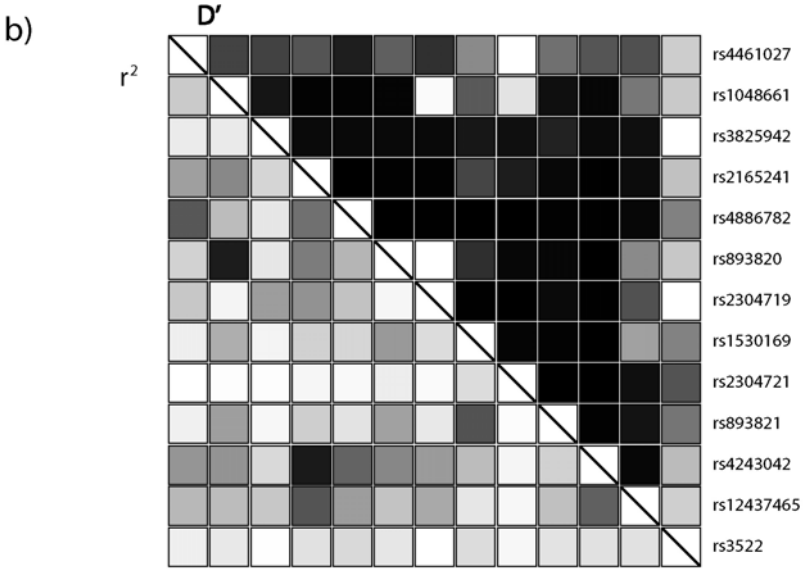
* $p<0.001$ compared to case subjects.

Seven SNPs were found to be associated with pseudoexfoliation, after age-adjustment and Bonferroni correction (Figure 5-3). However, no tagging SNP remained significant after additional adjustment for the two nonsynonymous SNPs located in exon 1 of *LOXL1* (rs1048661: Arg141Leu; and rs3825942: Gly153Asp). The age-adjusted odds ratios for developing pseudoexfoliation and having the G allele at SNP rs1048661 or the G allele at rs3825942 were 1.86 (95%CI: 1.27-2.76) and 3.81 (95%CI: 1.88-9.02), respectively. The high risk haplotype for these SNPs (G,G) conferred an odds ratio of 4.74 (95%CI: 2.32-11.26) compared to the lower risk haplotype (G,A). The (T,A) haplotype expected to have the lowest risk at these SNPs was not observed in our population, consistent with the findings of Thorleifsson et al (Thorleifsson et al., 2007). Two copies of the high risk (G,G) haplotype (the high risk diplotype) were identified in 54.7% of cases compared with 24.6% of our control population. The high risk diplotype conferred an odds ratio of 7.20 (95%CI: 3.04-20.75) compared with no copies of the high risk haplotype (Figure 5-3).

Figure 5-3

Association analysis of the *LOXL1* gene. (a) displays the χ^2 allelic association between cases and all unselected controls at each SNP and their relative location. The dashed line represents the Bonferroni adjusted level for significance. Pairwise D′ and r^2 values are displayed in panel (b) and the odds ratios (OR) as well as p values for important haplotypes as well as diplotypes, are revealed in panel (c). The estimated frequency (*f*) in cases and controls is also shown. * Individuals with the high risk diplotype were compared to those with no copies of the high risk haplotype.





c)

		OR (95% CI)	p	*f* case	*f* control
Haplotypes	C G G T A T C C C T T T C	1.56 (1.12 - 2.16)	0.005	0.38	0.28
	T G A C G T T C C T A C C	0.15 (0.02 - 0.55)	0.002	0.01	0.07
	- G G - - - - - - - - - -	2.71 (1.91 - 3.92)	3.8×10^{-9}	0.74	0.51
	- T G - - - - - - - - - -	0.54 (0.36 - 0.78)	7.8×10^{-4}	0.22	0.34
	- G A - - - - - - - - - -	0.27 (0.11 - 0.55)	1.3×10^{-4}	0.05	0.15
Diplotypes	C G G T A T C C C T T T C T G G T G T C C C T T T C	4.12 (1.53 - 9.50)	0.003	0.08	0.02
	C G G T A T C C C T T T C C G G T A T C C C T T T C	1.44 (0.59 - 3.05)	0.39	0.09	0.06
	- G G - - - - - - - - - - - G G - - - - - - - - - -	* 7.20 (3.04 - 20.75)	1.8×10^{-7}	0.55	0.25

We investigated the distribution of *LOXL1* risk diplotypes in glaucoma cases without pseudoexfoliation syndrome, versus controls without glaucoma and found no significant difference (p=0.66). Pseudoexfoliative glaucoma was strongly associated with the high risk *LOXL1* diplotype. However, the magnitude of this association was not greater than that with pseudoexfoliation syndrome alone, possibly reflecting the relatively small number of pseudoexfoliative glaucoma cases in this population-based study (data not shown).

The two significant disease-associated nonsynonymous coding SNPs in *LOXL1* were found to be at a very high frequency in our control population, similar to the findings of Thorleifsson et al., despite the substantially lower prevalence of pseudoexfoliation in our population (Forsius 1979; Mitchell et al., 1999b; Ringvold 1996; Thorleifsson et al., 2007). The allele frequency of these SNPs in our control population did not significantly differ from the allele frequency reported in the Nordic populations (Table 5-4). Both disease-associated SNPs were identified at a slightly higher allele frequency in Nordic cases compared to the Australian cases. However, this difference was statistically significant only for rs3825942 (Table 5-4).

To investigate the origins of these disease-associated coding variants we examined their cross-species homology. Both disease-associated variants are well conserved across mammalian species (Figure 5-4). It is noteworthy that the common ancestral wildtype allele at each SNP is the disease-associated allele.

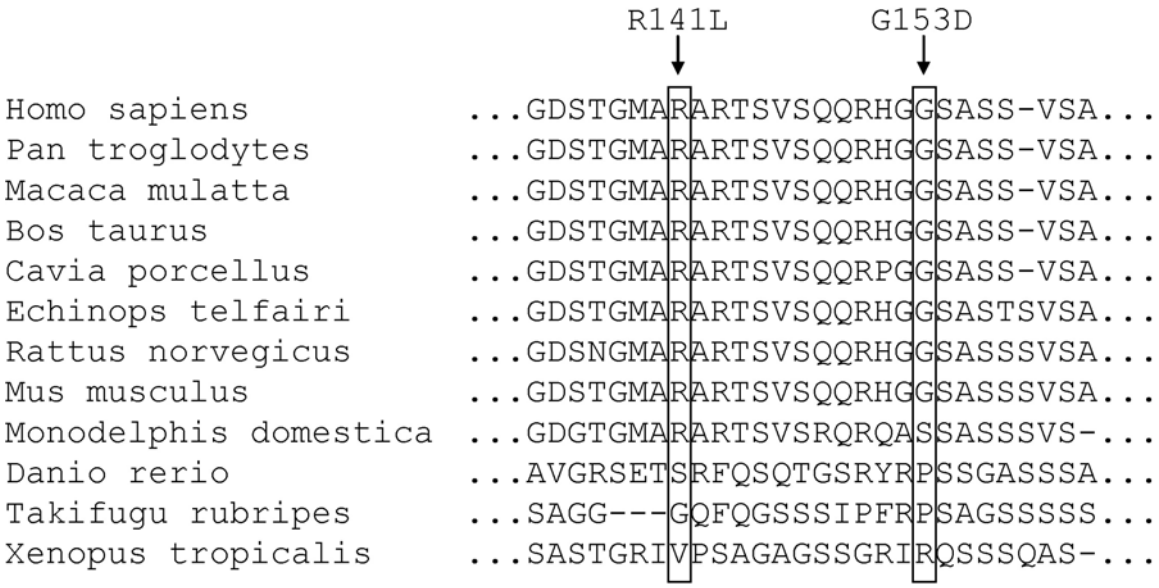
Table 5-4

Prevalence of pseudoexfoliation syndrome and allele frequencies of the disease-associated nonsynonymous *LOXL1* coding SNPs in the Nordic population compared to our Anglo-Celtic population. Data from combined pseudoexfoliative glaucoma and glaucoma free pseudoexfoliation syndrome subjects extrapolated from Thorleifsson and colleagues (16). OR, odds ratios; CI, confidence interval; *f*, frequency. χ^2 comparison between Nordic and Anglo-Celtic allele frequencies: † p = 0.22, ‡ p = 0.13, § p = 0.0007, $^{¥}$ p = 0.45.

	Nordic				Anglo-Celtic			
Disease Prevalence over age 80 years	40.0%				4.6%			
	f cases	*f* controls	OR (95% CI)	p	*f* cases	*f* controls	OR (95% CI)	p
rs1048661 (G)	0.83	0.64	2.53 (2.06-3.12)	2.04×10^{-19}	$0.78^{\dagger}$	$0.66^{\ddagger}$	1.86 (1.27-2.76)	8.49×10^{-4}
rs3825942 (G)	0.99	0.86	19.56 (8.93-53.53)	2.36×10^{-23}	$0.95^{\S}$	$0.84^{¥}$	3.81 (1.88-9.02)	7.83×10^{-5}

Figure 5-4

Cross species homology of the LOXL1 protein region containing the disease associated nonsynomous variants. The variants R141L and G153D correspond to SNPs rs1048661 and rs3825942 respectively. Note that the wildtype is the disease associated allele.



Discussion:

Pseudoexfoliation syndrome is a relatively uncommon age-related disease characterised by a generalized fibrillar degeneration of elastin containing tissues. Pseudoexfoliation causes severe chronic open angle glaucoma with blindness, is associated with cataract formation, increased complications at cataract surgery, and also may be associated with systemic vascular disease (Mitchell et al., 1997; Naumann et al., 1998; Ringvold 2001; Schlotzer-Schrehardt and Naumann, 2006). In this study we confirmed, using a non-Nordic population, the findings of Thorleifsson and colleagues, that two coding variants in the *LOXL1* gene are strongly associated with pseudoexfoliation (Thorleifsson et al., 2007). The *LOXL1* association is of such a magnitude that it is clearly identifiable in our population, despite the fact that a small proportion of the control samples in this study are likely to have

pseudoexfoliation undiagnosed due to previous cataract surgery, or will develop it at a later age.

We found that the high risk diplotype across the two coding SNPs conferred a high odds ratio (7.20, 95%CI: 3.04-20.75) for developing pseudoexfoliation. Despite our general population having an approximate nine-fold lower lifetime incidence of pseudoexfoliation than the Nordic population, a similar proportion of people in our unaffected control group were found to carry the high risk diplotype (25%), as compared to those studied by Thorleifsson et al. (Forsius 1979; Mitchell et al., 1999b; Ringvold 1996; Thorleifsson et al., 2007). This is even more remarkable considering that the control population in the Thorleifsson study was not specifically examined for the condition and hence would be expected to contain affected but undiagnosed individuals with pseudoexfoliation.

This example of relatively uncommon diseases being caused by common variants is not likely to be unique. It is well appreciated that the frequency of disease-associated alleles at each genetic locus is subject to the joint effects of selection, mutation and random genetic drift (Pritchard 2001). We found that the disease-associated variants in *LOXL1* were well conserved across species and that, peculiarly, the less common "mutations" that have arisen are protective against pseudoexfoliation. Given the interesting phenotypic observation of uterine prolapse in a *LOXL1* knockout mouse (Liu et al., 2004), we speculate that pseudoexfoliation associated ancestral variants in *LOXL1* could result in lower miscarriage rates.

Pseudoexfoliation material is comprised of a cross-linked, highly glycosylated and enzymatically resistant glycoprotein–proteoglycan complex (Schlotzer-Schrehardt

and Naumann, 2006). This structure generally bears epitopes of the basement membrane and the elastic fibre systems (Schlotzer-Schrehardt and Naumann, 2006). *LOXL1* belongs to a group of proteins responsible for catalysing the oxidative deamination of lysine residues of tropoelastin (Borel et al., 2001; Noblesse et al., 2004; Thomassin et al., 2005). In turn, this deamination causes spontaneous cross-linking and formation of elastin polymer fibres (Liu et al., 2004).

Pseudoexfoliation syndrome represents a complex, late-onset disease, and our finding of a similar allelic architecture to that described by Thorleifsson and colleagues, in the face of their study population's higher prevalence for this disease, suggests that other genetic or environmental factors are important in its genesis. Despite the high population attributable risk of *LOXL1* alleles in the Nordic study, which is in large part due to the remarkably high population frequency of the "risk alleles", our findings suggest that further research into the molecular underpinnings of pseudoexfoliation is clearly warranted.

A genome-wide association screen for Open Angle Glaucoma identifies susceptibility variants on chromosomes 3q21, 6p25, 14q13 and Xq25.

Background:

The candidate gene approach has been the basis for hundreds of genetic studies (see Chapter 1). A specific-hypothesis driven approach relies on understanding the biology of the disease and precise gene function. For many complex diseases, such as primary open angle glaucoma (OAG), the underlying biology is poorly understood and the number of potential candidate genes is legion (Hewitt et al., 2006b). In addition, the roles of the majority of human genes are not understood, thereby foregoing their potential for inclusion in a focused, candidate gene study design. Genome-wide association (GWA) is a relatively new means of investigation and has been successful in identifying single nucleotide polymorphisms (SNPs) associated with age-related macular degeneration (AMD), pseudoexfoliation, type I diabetes, type 2 diabetes, breast cancer and obesity (amongst others) (Easton et al., 2007; Frayling et al., 2007; Klein et al., 2005; The Wellcome Trust Case Control Consortium 2007; Thorleifsson et al., 2007).

GWA holds great promise, particularly in the case of common complex diseases, because no prior information about underlying molecular mechanisms is required. Additionally, putative regions of association typically extend over relatively short genomic distances (compared with loci identified from pedigree-based linkage which can identify dauntingly large regions), thus the number of genes to be thoroughly assessed may be small enough that all genes at a locus can be investigated, regardless of known function (The Wellcome Trust Case Control Consortium 2007). Furthermore, unlike linkage studies in rare (though important) pedigrees, it is

possible that positive results may be more readily applicable to unselected cases, such those attending general outpatient clinics.

Unfortunately, the high cost of genome-wide arrays has been the major barrier inhibiting the widespread utilisation of such study design (Macgregor et al., 2006). Well-powered GWA investigations generally cost more than AUS$1.5 million (Michael Sandery personal communication). Equimolar pooling of DNA heralds as the most promising approach for cost reduction. In equimolar pooling, rather than individually genotyping each subject in each of the case and controls cohorts, the equimolar DNA pools of all "cases" and all "controls" are constructed separately and then genotyped (Pearson et al., 2007). Pooling has recently been shown to be possible using large scale microarrays (Hanson et al., 2007; Macgregor et al., 2006; Melquist et al., 2007; Pearson et al., 2007). Compared with individual genotyping, pooling can cost 100-fold less (Michael Sandery personal communication), thereby making GWA studies substantially more affordable.

In this study we sought first to validate the use of equimolar DNA pools for GWA. Klein and colleagues individually genotyped case subjects with AMD and age-matched normal controls. They identified a SNP in the *complement factor H* (*CFH*) gene that was strongly associated with AMD (Klein et al., 2005). This work has been well validated and the *CFH* gene has been clearly implicated in the pathogenesis of AMD (Edwards et al., 2005; Hageman et al., 2005; Haines et al., 2005). We constructed equimolar DNA pools on a relatively small cohort of patients with AMD as well as age-matched controls and found that SNPs at the *CFH* locus reached genome-wide significance ($p < 10^{-7}$) for association with AMD. We then utilised this

novel pooling methodology to investigate the genomic architecture of OAG and identified a number of putative disease-associated regions.

Methods:

Subject Recruitment:

All participants provided written informed consent. Ethical approval was obtained from the joint committees of the Flinders Medical Centre and Flinders University (Adelaide, South Australia), the Royal Hobart Hospital and the University of Tasmania (Hobart, Tasmania), as well as from the Westmead Millennium Institute and the University of Sydney.

Age-Related Macular Degeneration Cohort:

Consecutive patients diagnosed with exudative AMD at the Flinders Medical Centre, South Australia, were recruited. Peripheral blood from 131 patients was collected in EDTA tubes. Disease-free age-matched controls from the same geographical region were recruited as outlined below.

Open Angle Glaucoma Case-Control Cohorts:

Three case-control cohorts were utilised in the OAG arm of this study (Table 5-5). Equimolar DNA pools were constructed using two independent OAG cohorts - of case subjects with severe glaucoma blindness and age-matched normal controls - from South Australia (SA) and Tasmania.

Table 5-5

Study composition and demographic features. All subjects were individually genotyped; however, only those marked with an * were used in the genome-wide association.

Cohort	Disease Status	n	Gender (F/M)	Age at Examination (SD)	Age at Diagnosis (SD)	Known Family History of Glaucoma (%)
South Australia	Case*	205	97 / 108	77.4 (9.9)	61.8 (13.7)	107 (52.2)
	Control*	216	113 / 103	75.6 (8.4)	-	0
Tasmania	Case*	183	113 / 70	75.8 (9.4)	63.5 (13.6)	154 (84.2)
	Control*	153	96 / 57	74.6 (12.2)	-	0
	Case	301	179 / 122	72.3 (11.7)	62.5 (12.7)	176 (58.5)
Blue Mountains	Control	488	251 / 237	81.8 (4.0)	-	0
	Case	154	99 / 55	77.6 (9.6)	66.3 (15.0)	42 (27.3)

Abbreviations: n, number; F, female; M, male, SD, standard deviation.

The SA case cohort, which represented the first spoke of the Australian and New Zealand Registry of Advanced Glaucoma, was recruited through the cooperation of local ophthalmologists, the Royal Society for the Blind, and Glaucoma Australia. Control subjects were ascertained through local media advertisement and from independent-living retirement villages.

The Tasmanian cohort was recruited through the Glaucoma Inheritance Study in Tasmania (GIST) (Sack et al., 1996). This cohort has been ascertained over the previous decade and approximately 2,000 unselected Tasmanian OAG cases, representing close to full population ascertainment of diagnosed glaucoma cases, have been reviewed (Green et al., 2007). The subset of subjects at the severe end of the OAG spectrum (n=183) were included for the pooling experiment whilst individual genotyping was performed on an additional 301 GIST cases who did not meet the severity criteria for inclusion into the pooling experiment (see below). GIST case subjects were unselected in terms of known family history. One hundred and fifty-three people were specifically recruited, from local Tasmanian aged care facilities or through an adjuvant genetic study, to act as controls subjects for the GIST OAG pool. Control subjects for the less severe GIST OAG cohort were selected through the Blue Mountains Eye Study (BMES).

The BMES is a population-based cohort study investigating the aetiology of common ocular diseases among suburban residents aged 49 years or older, living in the Blue Mountains region, west of Sydney, Australia. Subjects were recruited during one of four surveys between 1992 and 2004. A total of 488 control subjects were utilised. In addition 154 OAG cases identified during this population-based survey were also genotyped individually.

All control subjects were required to have no known family history of OAG, as well as a normal intraocular pressure, optic disc and visual field. The SA-based control series were additionally required to have no signs of AMD, thereby ensuring their utilisation for the validating DNA pooling experiment. Additionally those control subjects in the SA and GIST cohort were age and gender matched to the cases. The population-based BMES control cohort comprised the eldest subgroup of people meeting control inclusion criteria.

Case subjects for the OAG SA and GIST pools were required to have in their worse eye: a vertical cup:disc ratio ≥ 0.95, a best-corrected visual acuity worse than 6/60 due to OAG, or on a reliable Humphrey Visual Field (Carl Zeiss Pty. Ltd., Sydney, Australia) a mean deviation of $\leq$ -22db and at least 10 out of 16 central squares involved with a Pattern Standard Deviation of $< 0.5\%$. The field loss had to be due to OAG, and the less severely affected eye was required to have signs of glaucomatous disc damage and a glaucomatous field defect. Case subjects not meeting this degree of severity had concordant findings of typical glaucomatous visual field defects on the Humphrey 24-2 (for GIST) or 30-2 (for BMES) test together with corresponding optic disc rim thinning, including an enlarged cup-disc ratio (≥ 0.7) or cup-disc ratio asymmetry (≥ 0.2) between the two eyes. Intraocular pressure (IOP) was not considered in the diagnostic criteria. Clinical exclusion criteria included: i) pseudoexfoliative glaucoma, ii) pigmentary glaucoma, iii) angle closure or mixed mechanism glaucoma; iv) secondary glaucoma due to aphakia, rubella, rubeosis or inflammation; v) congenital or infantile glaucoma, juvenile glaucoma with age of onset less than 20 years; or vi) glaucoma in the presence of a known syndrome. Subjects were excluded from this study if they had previously been identified as

having a disease-causing mutation in *myocilin* gene (accounting for 3-4% of OAG in our combined data sets) (Fingert et al., 1999).

Pool construction and genotyping:

Genomic DNA was extracted using Qiagen (Qiagen Pty Ltd, Victoria, Australia) column-based kits and quantified using the Fluoroskan Ascent system (Thermo Labsystems, Massachusetts, USA) together with Picogreen reagents (Molecular Probes, P-7589, Invitrogen Pty Ltd, Victoria, Australia). The samples selected for pool construction were confirmed to have a concentration between 50 and 80ng/μL. Once standardised the Eppendorf epMotion 5075 robot (Eppendorf AG, Hamburg, Germany) was used to pipette an equal amount of DNA from each sample into the final pool, similar to that described by Pearson and colleagues, such that the minimum pipetting volume was 5μL (Pearson et al., 2007). Each pool was then mixed gently and allowed to homogenise.

Array-based genotyping was performed at the Queensland Institute of Medical Research on the Illumina Beadstation platform using the high density Infinium HumanHap 550 beadchip (Illumina Inc. California, USA). The Illumina platform was chosen for its recently released maximal genome coverage with HapMap tag SNPs and high level of bead/SNP redundancy. The Infinium HumanHap 550 array is estimated in Caucasians to capture 88% of variants, with an $r^2 \geq 0.8$ (The HapMap Consortium 2007). The Illumina Beadstation software was toggled to output bead level intensity data (i.e. raw green/red beadscores). A Tecan Freedom EVO robot (Tecan Group Ltd., Mannedorf, Switzerland) was used in the post-amplification processing of arrays to improve standardisation. The SA AMD and OAG cohorts

were assayed with six technical repeats; the case-control pools from the GIST cohort were processed in triplicate.

Individual genotyping:

Age-related Macular Degeneration Case-Control Cohort:

Custom primers were designed to flank the Tyr402His (rs1061170) *CFH* variant (F: 5′-tcattgttatggtccttaggaaa-3′ and R: 5′-ggatgcatctgggagtagga-3′) and obtained from Geneworks, Adelaide, Australia. PCR was performed using 50 ng of genomic DNA, 0.5U HotStarTaq Plus DNA polymerase (QIAGEN, Valencia, CA, USA), 10x QIAGEN PCR buffer, 2mM dNTPs, 5pM of each oligonucleotide primer and water to a total volume of 20µl. The amplification conditions involved an initial activation step of 95ºC for 15 minutes, followed by 30 cycles consisting of 30 seconds of denaturation at 94ºC, 30 seconds of annealing at 51ºC (Set 3); 54°C (Set 2); 58°C (Set 1) and 30 seconds of extension at 72ºC. Final extension was 72ºC for 1 minute. A SNaPshot^{TM} Multiplex assay (Applied Biosystems, Foster City, California USA) was used to identify the Y402H *CFH* variant. Using the following probe sequence: 5′-attttccttatttggaaaatggatataatcaaaat-3′; reactions were performed according to manufacturer's instructions.

Primary Open Angle Glaucoma Case-Control Cohort:

Replication investigation of the most promising candidate SNPs from the OAG GWA was performed at the Australian Genome Research Facility (Queensland, Australia). Three multiplexes, containing 93 of the top 138 SNPs identified from the GWA as well as three additional SNPs, were constructed and processed using the Sequenom iPLEX GOLD chemistry on an Autoflex Mass Spectrometer. One additional SNP from each of the three loci where SNPs had reached genome-wide

significance from the pooling data were included. To ensure genotyping integrity, two of these SNPs were selected because of a high-linkage disequilibrium within European people from the Centre d'Étude du Polymorphisme Humain from Utah (CEPH) of HapMap (rs3011384 tagging rs2919009, with a r^2=0.887; and rs473219 tagging rs1789792 with a r^2=1.0) (The HapMap Consortium 2003). A nonsynonymous coding variant (rs10126452; His147Gln) in the *Winged-Domain Repeat-40 C* (*WDR40C*) gene, near rs5933350, which was not genotyped by the HapMap consortium, was also included.

Analysis and Power Calculations:

The microarray data were analysed as described previously (Macgregor et al., 2006; Macgregor et al., 2008). In brief, SNPs that had negative bead intensity scores or less than 20 control bead-level allele contributing data points were discarded. Bead intensity scores were calibrated on a strand-by-strand basis. Allele frequency estimates that were highly variable across arrays were also removed (Macgregor et al., 2008). Sources of pooling error were then estimated and assumed to follow a binomial distribution (Macgregor 2007). The χ^2 test for allelic association between cases and controls was applied all the SNPs that passed quality control (QC) (Macgregor et al., 2006).

Analysis of the individual genotyping was performed at the allelic level using Haploview 4.0 and at the genotypic level using SNPStats (Barrett et al., 2005; Sole et al., 2006). Autosomal SNPs were checked for compliance with the Hardy–Weinberg equilibrium in the full cohort (Barrett et al., 2005). SNPs on the X chromosome were analysed similar to that previously described (The Wellcome Trust Case Control Consortium 2007). Males were considered as if they were homozygous females and

were not assumed to be in Hardy–Weinberg equilibrium (The Wellcome Trust Case Control Consortium 2007). Bonferroni correction for multiple-testing was adopted both at the level for genome-wide and follow-up levels. Permutation testing using Haploview 4.0 was also performed (Barrett et al., 2005) and the web-based Genetic Association Interaction Analysis (GAIA) application was used to test for interaction between loci (Macgregor and Khan, 2006). Map coordinates were obtained from NCBI Build 36 (August 2007) and SNP details from dbSNP (b126) (Wheeler et al., 2007). Cross-species homology data in the UCSC Genome Bioinformatics Site (hg18, released March 2006) and population allele frequency data from HapMap (release 22, March 2007) were also reviewed (Hinrichs et al., 2006; The HapMap Consortium 2003).

Power for the OAG study was calculated post-hoc, using the algorithms developed by Skol et al. for two stage studies (Skol et al., 2006). Assuming a multiplicative relative risk, a risk allele frequency of 0.3, a type-I error rate of 0.0001 and a SNP:causal-variant r^2 of 0.8 (a conservative estimate for the Illumina 550K chip), the power to detect association with joint analysis for genotype relative risks of 1.5, 1.6, 1.7 and 1.8 was 0.23, 0.46, 0.68 and 0.83, respectively.

Results:

Validation of equimolar DNA pooling for GWA:

Individual genotyping of the AMD cases and SA controls revealed a highly significant ($p=2.2x10^{-12}$) association for the *CFH* risk allele (rs1061170). The odds ratio (OR) for developing AMD was 1.96 (95% CI:1.07-3.58) for heterozygotes and 9.23 (95% CI:4.67-18.25) in people homozygous for the disease-associated allele.

A total of 316,867 SNPs passed all QC parameters across all arrays used in this initial experiment. There was an array error of approximately 0.0011 per array. A heavy tail was noted on the quantile-quantile plot, consistent with previous pooling experiments (Figure 5-5). The known association of exudative AMD with the *CFH* gene was easily detected, being the most strongly associated region of all SNPs on the HumHap550 array (Figure 5-6). The SNP rs1061170, which was genotyped on an individual basis, did not pass QC (Table 5-6). Nonetheless, four SNPs at the *CFH* locus were found to reach genome-wide significance (Figure 5-6). SNP rs1329428 in *CFH* had an uncorrected p value of 2.3×10^{-11}.

Table 5-6

SNPs at the *CFH* locus on chromosome 1 that were identified as being associated with age-related macular degeneration, using equimolar DNA pooling. Linkage disequilibrium (r^2) in the HapMap CEPH population and genomic distance (bp) from the disease associated SNP (rs1061170) is displayed.

Rank in Pool	rs name	bp from rs1061170	r^2
1	rs1329428	43573	0.309
2	rs6428357	16334	0.586
3	rs1332666	325442	0.221
4	rs2019724	15680	0.591
7	rs379489	-34214	No CEPH data
8	rs6667243	282256	0.275
9	rs12137359	470744	0.064
18	rs800292	-17004	0.101

Figure 5-5

Quantile-quantile plots of test statistics obtained from the genome-wide association of exudative age-related macular degeneration and blind primary open angle case-control DNA pools.

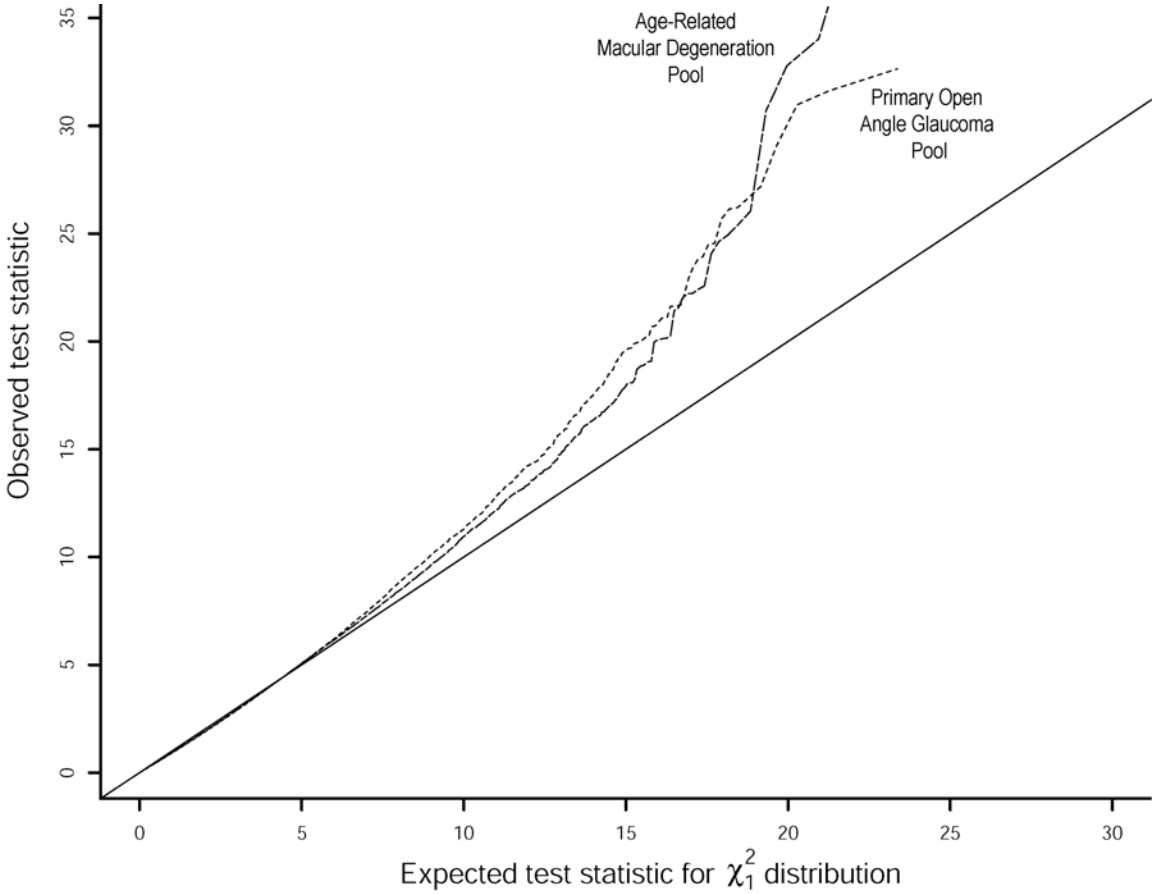
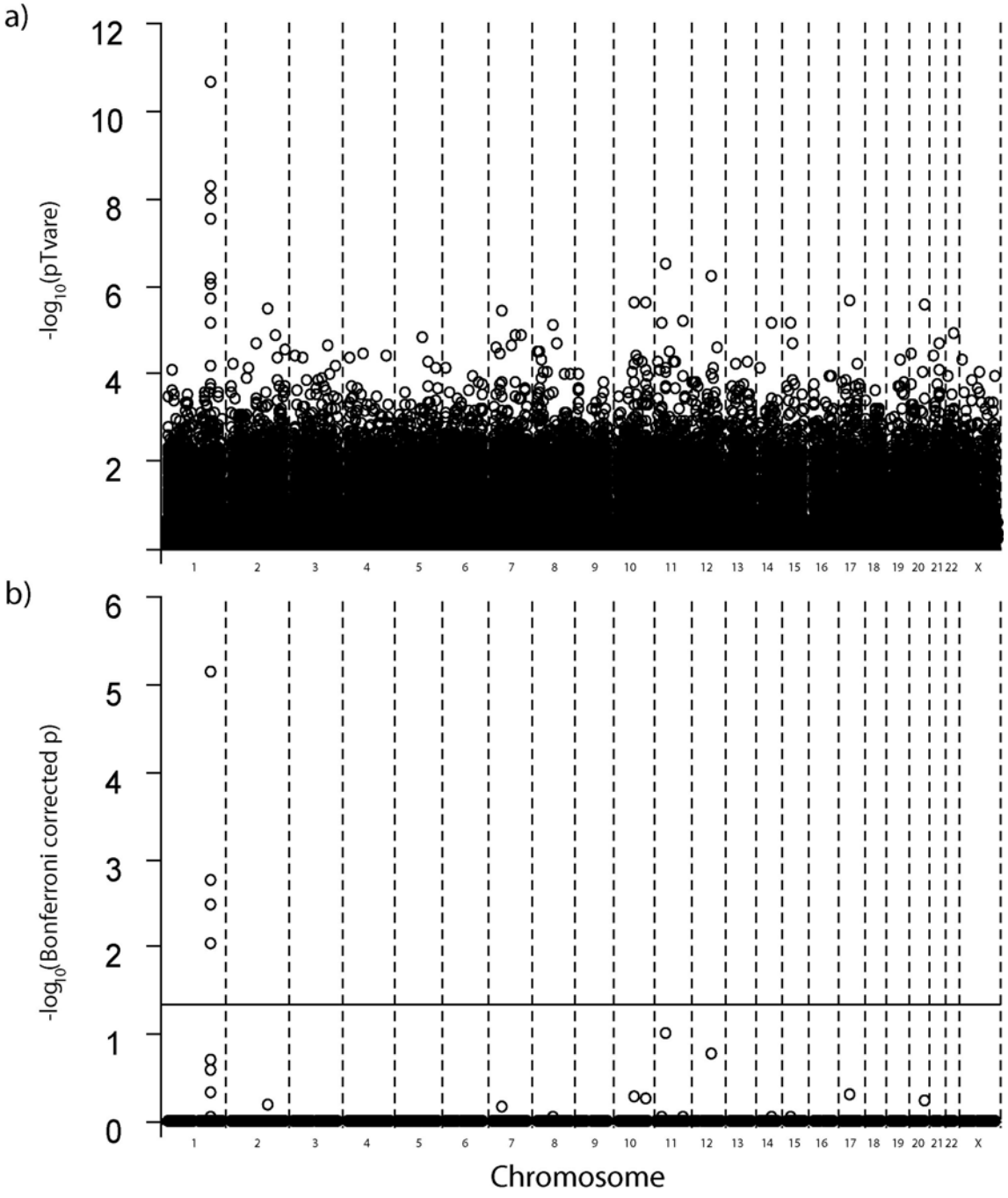


Figure 5-6

Validation of the DNA pooling strategy. SNPs genotyped from the pooled case-control series of age-related macular degeneration cases and age-matched controls, plotted according to chromosomal location. Panel a) displays the $-\log_{10}$ raw p values; panel b) reveals the $-\log_{10}$ Bonferroni corrected p values with the horizontal line indicating SNPs which are significant at a genome-wide level ($<1.25 \times 10^{-7}$). Note the cluster of highly associated SNPs at the *complement factor H* locus on chromosome 1.



Genome-wide Association in Open-Angle Glaucoma:

Across all OAG pool arrays, a total of 374,128 SNPs passed QC. The overall pooling error was approximately $9x10^{-5}$, which is approximately eight times smaller than the expected binomial sampling variance. This implies that approximately 80% of the available information has been extracted in this pooling experiment, as compared to individual genotyping (i.e. the effective case sample size was n~290). For the SA samples, the array error for a single array is ~0.0005. The pool construction error estimate was approximately $-3x10^{-5}$. It was not possible to separate array error and pool construction error on the GIST arrays. However, the combined estimate of approximately 0.0006 suggests that the pooling performance was similar to the SA samples and that the pool construction error was small. Joint analysis of the OAG GWA datasets revealed that there were 669 SNPs which reached a nominal significance level of $<10^{-3}$ (Figure 5-7). Three SNPs (rs2919009, rs1789792 and rs5933350) reached genome wide significance (Figure 5-8). Two of these SNPs (rs2919009, rs1789792) were known to have poor quantile QC parameters, and one (rs5933350) was located on the X chromosome (Figure 5-8).

Figure 5-7
Overlap in SNPs with a nominal p value of <0.001 when the South Australian and Tasmanian glaucoma case-control cohorts were analysed separately or as one.

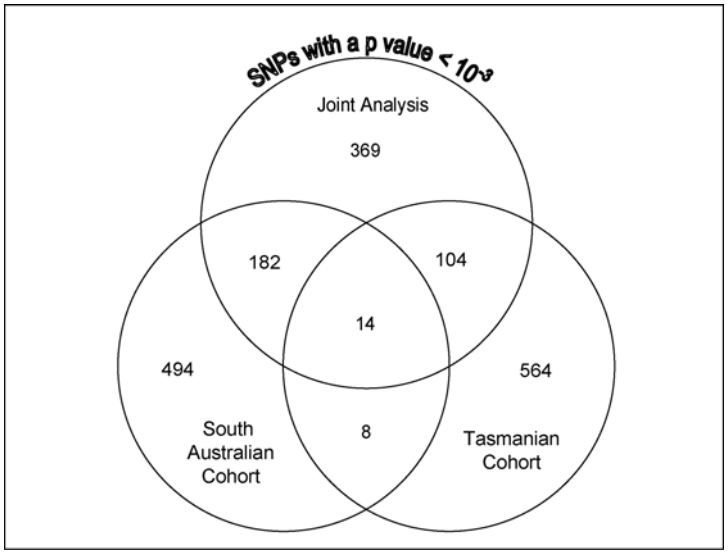
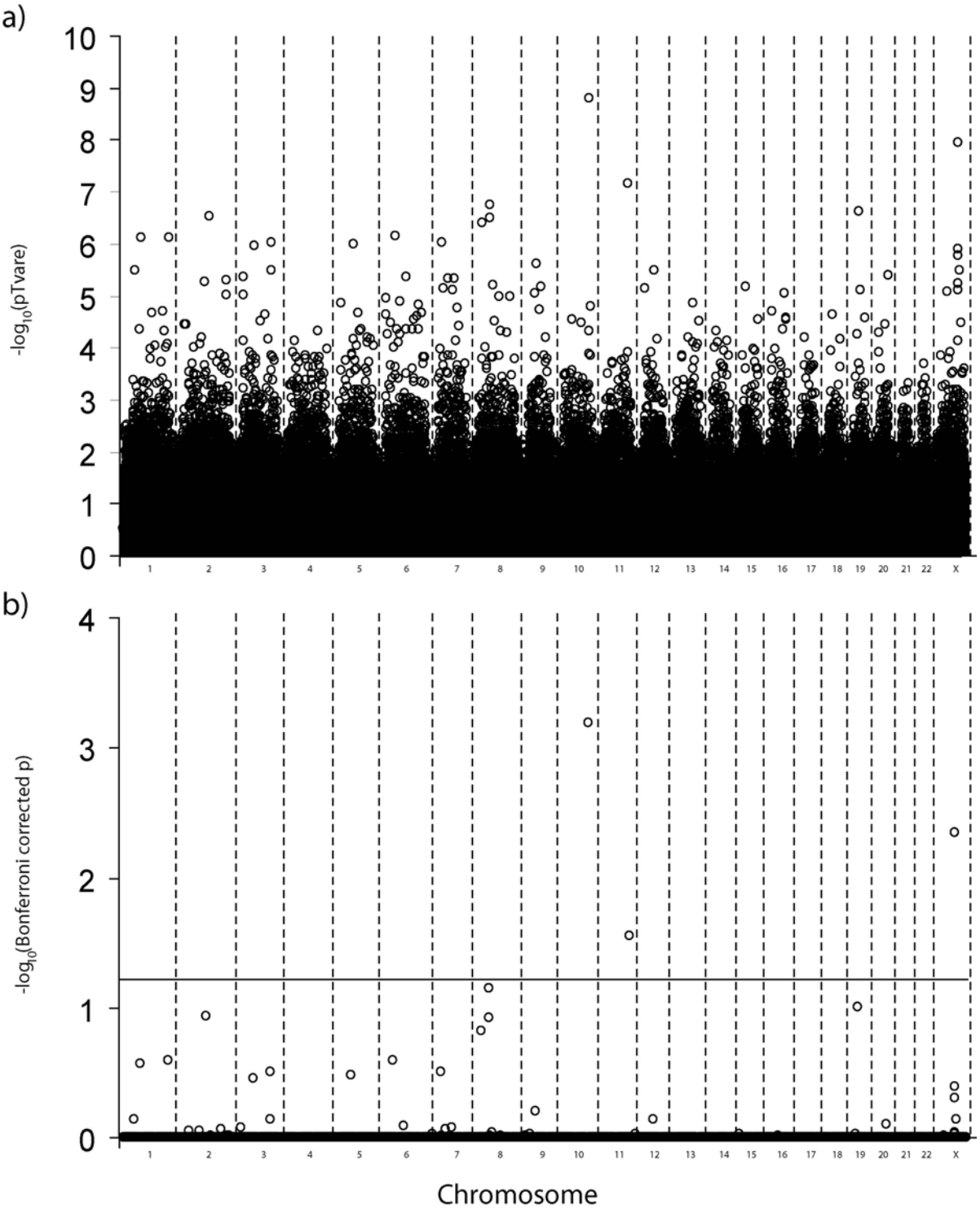


Figure 5-8

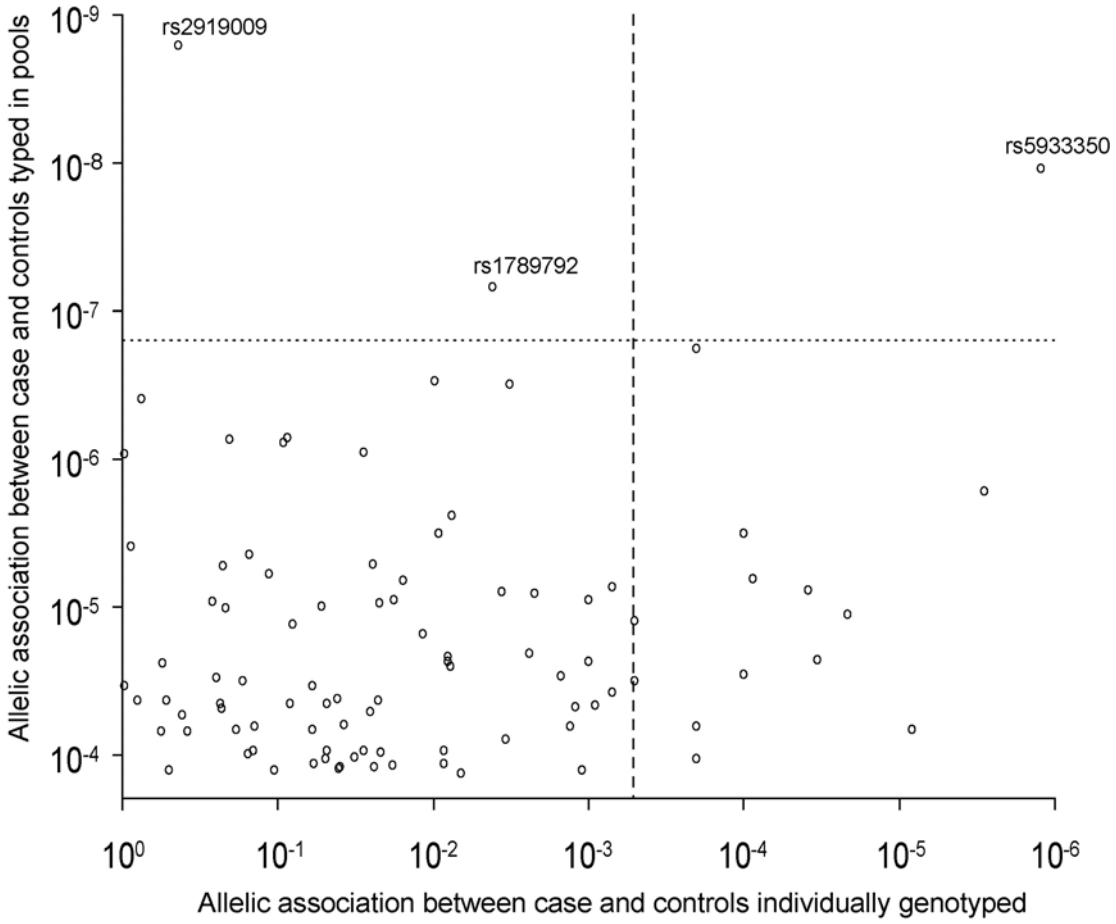
Joint analysis of the pooled severe case-examined normal control genome-wide association series of advanced glaucoma cases and age-matched control subjects. SNPs are plotted according to chromosomal location. Panel a) displays the $-\log_{10}$ raw p values; panel b) reveals the $-\log_{10}$ Bonferroni corrected p values.



Follow-up individual genotyping in Open-Angle Glaucoma:

The correlation between allelic associations for SNPs derived from pooling array-based GWA and individual genotyping is displayed in Figure 5-9. One SNP on the X chromosome (rs5933350) remained significant at the Bonferroni-correction level set for genome-wide and individual follow-up significance levels.

Figure 5-9
Correlation between allelic associations for SNPs derived from pooling array-based and individual genotyping. Horizontal and vertical dashed line indicates the Bonferroni-correction level set for genome-wide and individual follow-up significance, respectively. Note one SNP (rs5933350) remained significant in both analyses.



Five SNPs (rs5933350, rs10126452, rs1937250, rs1937245, rs1290579) at Xq25 were individually genotyped and found to be strongly associated with OAG (χ^2 range: 14.4-22.9). The mean r^2 between the SNPs at this locus was 0.921 and SNP:rs5933350 had the lowest permutated p value for allelic association (0.0005). The effect at this locus was greater in the subjects who met the criteria for advanced glaucoma (Figure 5-10). This genomic region is relatively poorly conserved across species (Figure 5-11). The coding SNP in WDR40C (rs10126452) was found to be in complete linkage disequilibrium with its neighbouring SNPs (rs5933350, rs1937250).

Figure 5-10
Genotype frequencies in cases and controls for the X-linked SNP: rs5933350. Data is presented for the whole cohort as well just the severe cases-control subset, who were used in the initial genome-wide association.

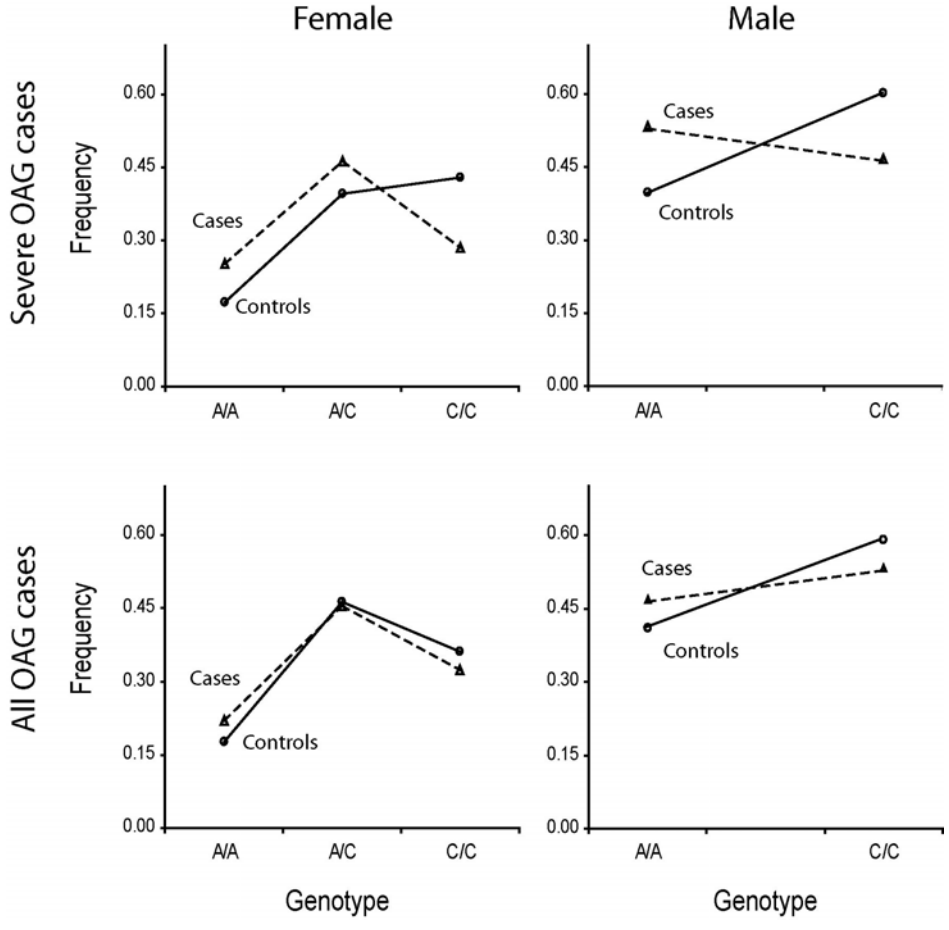
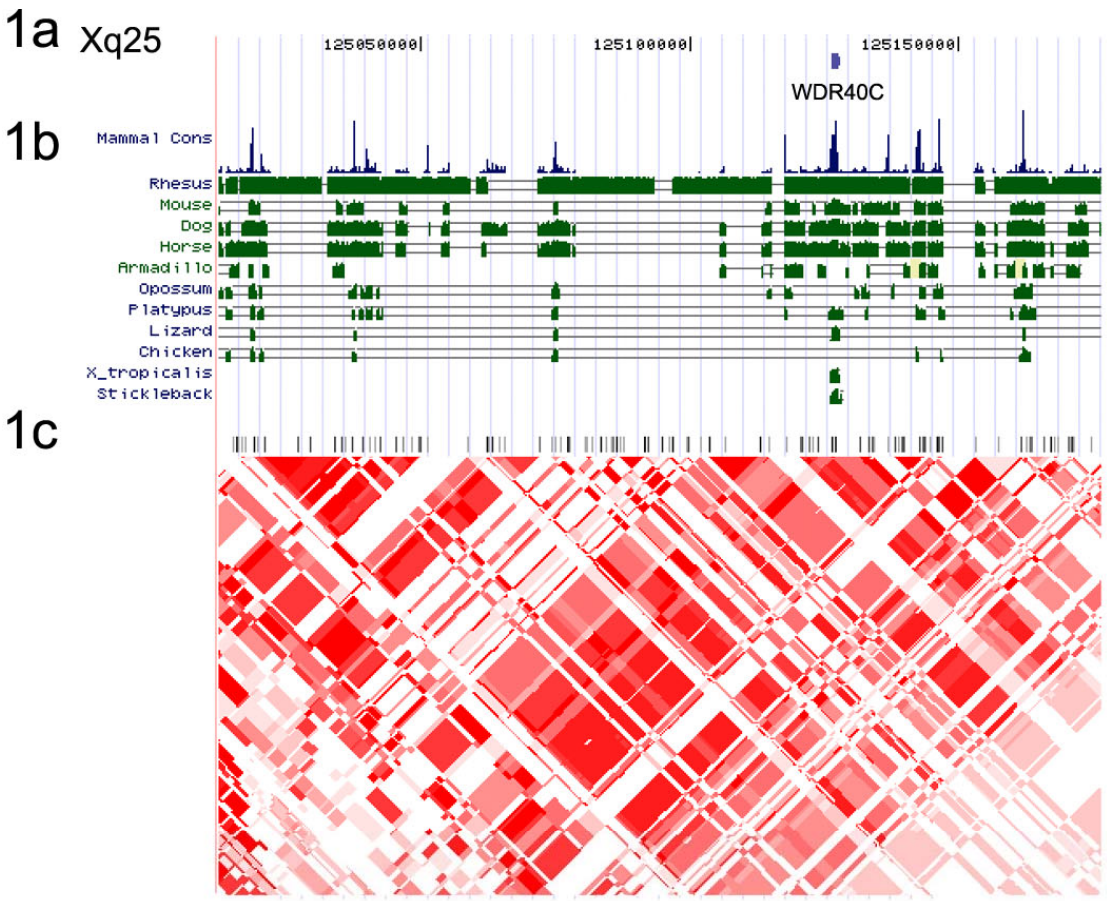
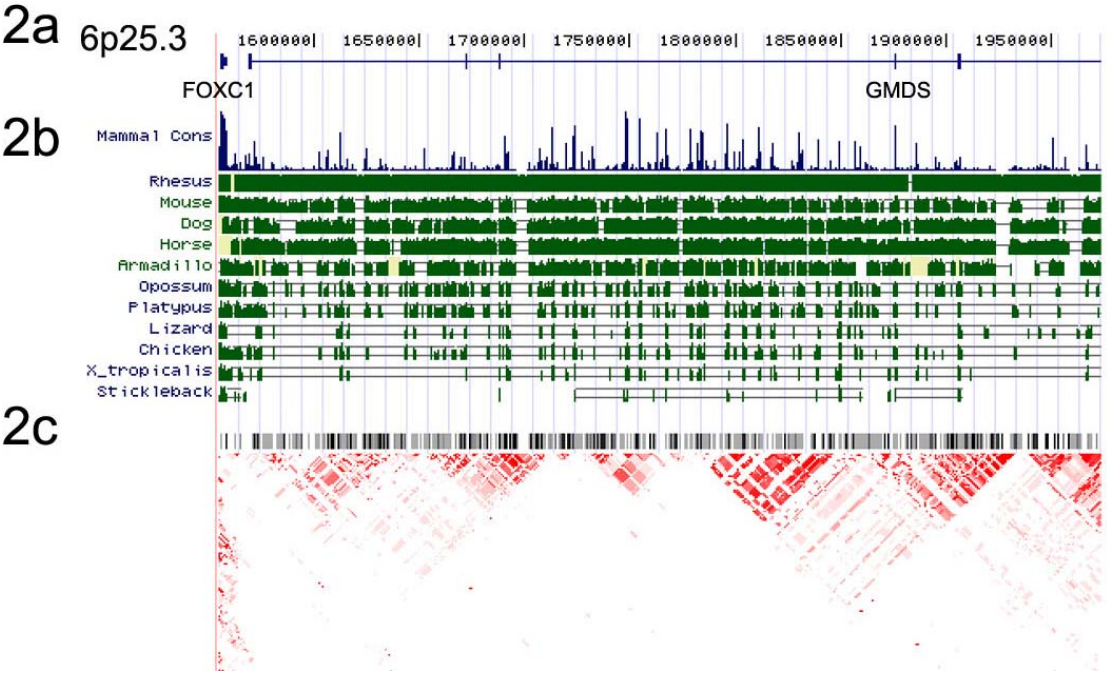


Figure 5-11

Putative novel primary open angle glaucoma loci. A 164kb region on the long arm of the X chromosome is displayed in panel 1a and a 418kb region at 6p25.3 is displayed in panel 2a. The pattern of cross species homology at these loci is presented in panels 1b and 2b. Panels 1c and 2c reveal the SNPs genotyped by the HapMap Consortium and the corresponding pattern of LD (r^2) in the CEPH population.





Following individual genotyping, 18 autosomal SNPs were found to have a significant ($p \leq 0.001$) allelic or genotypic association with OAG (Table 5-7). Three of these SNPs (rs10934884; rs9392354; rs10132688) remained highly significant on joint analysis of the full cohort. Being homozygous for the high risk allele in at least two of these three loci, conferred an OR for developing OAG equal to 2.13 (95%CI: 1.57-2.88; $\chi^2=26.5$, $p=2.64 \times 10^{-7}$), when compared to people who had no high risk allele in the homozygous state. This effect increased to an OR of 3.43 (95%CI: 2.11-5.60; $\chi^2=28.9$, $p=7.62 \times 10^{-8}$) when analysis was refined to solely those cases at the severe end of the OAG disease spectrum. No evidence for direct epistasis at these loci was found (data not shown). The linkage disequilibrium between two significant SNPs on chromosome 6 (rs11969985, rs9392354) was relatively strong ($r^2 = 0.71$).

Discussion:

Using the emerging technique of equimolar DNA pooling we were easily able to identify a strong association between markers at the *CFH* locus and AMD. It was interesting to note that the AMD pooling array error was larger than that observed in the SA or GIST OAG pools. Nevertheless, this problem was partly ameliorated when the AMD cases were compared with the SA controls, because the pooling error was easily small enough to still be able to identify significant association. The estimates of pooling error in the SA and GIST cohorts agreed well and the pooling construction error in these cohorts was remarkably small relative to the array error, similar to that observed previously (Macgregor 2007).

Table 5-7

Summary of individually genotyped autosomal SNPs with a significant ($p \leq 0.001$) allelic or genotypic association with open angle glaucoma. Data is presented for the whole cohort as well just the severe cases-control cohort who were used in the initial GWA. SNPs in bold indicate those in which analysis of the full cohort remained highly significant.

dbSNP name	chr	Risk Allele	Group	HWE p	Case-Cont f	P	Codominant Model			Dominant Model		Recessive Model		Log-Additive Model	
							Hetero OR (95% CI)	Homo OR (95%CI)	p	OR (95%CI)	p	OR (95%CI)	p	OR (95%CI)	p
rs10934884	**3**	**G**	**Severe**	**0.36**	**0.755, 0.648**	**8.1×10^{-6}**	**1.45 (0.85-2.47)**	**2.51 (1.48-4.25)**	**0.0001**	**1.96 (1.18-3.26)**	**0.0082**	**1.86 (1.39-2.49)**	**<0.0001**	**1.64 (1.31-2.06)**	**<0.0001**
			All	**0.09**	**0.739, 0.672**	**2.8×10^{-5}**	**1.09 (0.77-1.54)**	**1.62 (1.16-2.28)**	**0.0001**	**1.36 (0.98-1.89)**	**0.064**	**1.52 (1.25-1.84)**	**<0.0001**	**1.35 (1.17-1.57)**	**0.0001**
rs7638638	3	G	Severe	0.82	0.330, 0.253	0.001	1.63 (1.20-2.20)	1.82 (1.05-3.14)	0.0025	1.66 (1.24-2.21)	0.0006	1.45 (0.86-2.47)	0.16	1.46 (1.17-1.84)	0.001
			All	0.87	0.298, 0.275	0.1355	1.17 (0.96-1.43)	1.17 (0.81-1.68)	0.28	1.17 (0.97-1.42)	0.11	1.09 (0.77-1.55)	0.63	1.12 (0.96-1.30)	0.14
rs495703	5	T	Severe	0.22	0.756, 0.668	2.0×10^{-4}	2.42 (1.28-4.59)	3.33 (1.77-6.29)	0.0003	2.87 (1.55-5.33)	0.0004	1.55 (1.16-2.08)	0.0033	1.58 (1.25-2.01)	0.0001
			All	0.31	0.720, 0.671	0.0024	1.40 (0.97-2.02)	1.71 (1.19-2.45)	0.0073	1.55 (1.09-2.20)	0.013	1.29 (1.06-1.56)	0.01	1.27 (1.09-1.48)	0.0021
rs10461474	5	T	Severe	0.93	0.806, 0.736	0.0011	1.60 (0.80-3.19)	2.33 (1.19-4.58)	0.0053	2.03 (1.04-3.94)	0.034	1.55 (1.16-2.08)	0.0032	1.49 (1.17-1.90)	0.0012
			All	1.00	0.810, 0.763	0.001	1.22 (0.75-1.98)	1.64 (1.02-2.63)	0.0048	1.48 (0.93-2.36)	0.097	1.37 (1.13-1.67)	0.0015	1.32 (1.11-1.55)	0.0011
rs11969985	6	G	Severe	0.06	0.919, 0.848	2.1×10^{-5}	1.92 (0.59-6.25)	3.70 (1.18-11.60)	0.0002	3.26 (1.04-10.21)	0.029	2.05 (1.43-2.94)	0.0001	1.93 (1.40-2.66)	<0.0001
			All	0.10	0.885, 0.854	0.0083	2.08 (1.00-4.34)	2.49 (1.22-5.08)	0.014	2.39 (1.17-4.87)	0.012	1.27 (1.02-1.59)	0.036	1.30 (1.06-1.58)	0.0096
rs9392354	**6**	**A**	**Severe**	**0.26**	**0.814, 0.723**	**3.3×10^{-5}**	**1.46 (0.76-2.82)**	**2.50 (1.32-4.73)**	**0.0002**	**2.06 (1.10-3.85)**	**0.021**	**1.81 (1.34-2.43)**	**0.0001**	**1.65 (1.29-2.10)**	**<0.0001**
			All	**0.66**	**0.790, 0.734**	**1.0×10^{-4}**	**1.51 (0.97-2.34)**	**1.99 (1.30-3.06)**	**0.0006**	**1.79 (1.17-2.73)**	**0.0059**	**1.40 (1.15-1.70)**	**0.0007**	**1.36 (1.16-1.60)**	**0.0001**
rs2947941	6	G	Severe	0.20	0.879, 0.807	1.0×10^{-4}	4.10 (0.89-18.97)	6.85 (1.50-31.19)	0.0002	5.92 (1.30-26.91)	0.0064	1.78 (1.30-2.46)	0.0004	1.80 (1.34-2.43)	0.0001
			All	0.41	0.861, 0.826	0.0056	1.12 (0.56-2.22)	1.50 (0.77-2.94)	0.017	1.38 (0.71-2.71)	0.34	1.36 (1.10-1.68)	0.0047	1.31 (1.08-1.59)	0.005
rs584189	6	G	Severe	0.84	0.776, 0.698	5.0×10^{-5}	1.38 (0.74-2.55)	2.15 (1.17-3.93)	0.0022	1.78 (0.99-3.21)	0.051	1.63 (1.22-2.18)	0.0008	1.51 (1.20-1.91)	0.0005
			All	0.34	0.767, 0.731	0.0162	1.54 (0.99-2.38)	1.74 (1.13-2.67)	0.028	1.65 (1.09-2.52)	0.017	1.20 (0.99-1.45)	0.068	1.22 (1.04-1.42)	0.016
rs17500237	7	A	Severe	0.41	0.854, 0.779	2.0×10^{-4}	1.13 (0.50-2.52)	2.02 (0.93-4.41)	0.0008	1.70 (0.78-3.68)	0.17	1.82 (1.33-2.49)	0.0002	1.64 (1.25-2.14)	0.0002
			All	0.43	0.836, 0.798	0.0039	1.30 (0.75-2.23)	1.67 (0.99-2.83)	0.017	1.55 (0.92-2.61)	0.098	1.33 (1.08-1.63)	0.007	1.29 (1.08-1.54)	0.0043
rs757931	7	C	Severe	1.00	0.735, 0.654	7.0×10^{-4}	1.28 (0.75-2.16)	1.98 (1.18-3.35)	0.0028	1.61 (0.97-2.67)	0.06	1.62 (1.22-2.16)	0.0009	1.47 (1.17-1.83)	0.0007
			All	0.69	0.721, 0.686	0.0272	1.03 (0.73-1.47)	1.28 (0.91-1.81)	0.072	1.16 (0.83-1.62)	0.38	1.25 (1.03-1.51)	0.022	1.18 (1.01-1.36)	0.031
rs17055703	8	A	Severe	0.96	0.349, 0.260	2.0×10^{-6}	1.41 (1.04-1.90)	2.64 (1.53-4.55)	0.0006	1.57 (1.18-2.09)	0.0021	2.25 (1.33-3.81)	0.0017	1.53 (1.22-1.91)	0.0002
			All	0.95	0.340, 0.300	0.0121	1.21 (0.98-1.48)	1.45 (1.04-2.02)	0.044	1.25 (1.03-1.51)	0.024	1.32 (0.96-1.82)	0.084	1.20 (1.04-1.39)	0.012
rs10509391	10	G	Severe	0.31	0.847, 0.776	5.0×10^{-4}	1.00 (0.47-2.15)	1.76 (0.85-3.67)	0.0014	1.48 (0.72-3.07)	0.28	1.76 (1.29-2.39)	0.0003	1.57 (1.21-2.03)	0.0006
			All	0.58	0.843, 0.816	0.0358	0.81 (0.45-1.43)	1.06 (0.61-1.86)	0.041	0.98 (0.57-1.71)	0.95	1.29 (1.05-1.59)	0.016	1.21 (1.01-1.45)	0.038

continued over

dbSNP name	chr	Risk Allele	Group	HWE p	Case-Cont f	P	Codominant Model			Dominant Model		Recessive Model		Log-Additive Model	
							Hetero OR (95% CI)	Homo OR (95%CI)	p	OR (95%CI)	p	OR (95%CI)	p	OR (95%CI)	p
rs7913531	10	C	Severe	0.97	0.662, 0.579	9.0×10^{-4}	1.46 (0.94-2.26)	2.08 (1.33-3.27)	0.0032	1.71 (1.13-2.59)	0.01	1.55 (1.16-2.09)	0.0034	1.44 (1.16-1.78)	0.0007
			All	1.00	0.641, 0.601	0.0174	1.09 (0.82-1.46)	1.36 (1.01-1.83)	0.045	1.21 (0.92-1.58)	0.18	1.27 (1.05-1.55)	0.016	1.19 (1.03-1.36)	0.016
rs3741974	12	A	Severe	1.00	0.278, 0.203	7.0×10^{-4}	1.57 (1.15-2.12)	2.20 (1.14-4.23)	0.0025	1.64 (1.22-2.19)	0.0009	1.85 (0.97-3.52)	0.057	1.53 (1.20-1.95)	0.0006
			All	0.71	0.257, 0.220	0.012	1.31 (1.07-1.61)	1.30 (0.85-2.00)	0.023	1.31 (1.08-1.59)	0.0061	1.17 (0.77-1.79)	0.45	1.23 (1.05-1.44)	0.011
rs10132688	**14**	**C**	**Severe**	**0.55**	**0.504, 0.420**	**0.0012**	**1.45 (1.04-2.03)**	**1.92 (1.28-2.89)**	**0.0055**	**1.58 (1.15-2.17)**	**0.0042**	**1.52 (1.07-2.16)**	**0.018**	**1.39 (1.14-1.71)**	**0.0013**
			All	**0.89**	**0.489, 0.431**	**8.0×10^{-4}**	**1.30 (1.04-1.63)**	**1.58 (1.20-2.08)**	**0.0034**	**1.38 (1.12-1.70)**	**0.0028**	**1.34 (1.06-1.70)**	**0.014**	**1.26 (1.10-1.45)**	**0.0008**
rs2182035	14	C	Severe	0.66	0.495, 0.427	0.0085	1.76 (1.26-2.47)	1.61 (1.07-2.42)	0.0033	1.71 (1.25-2.35)	0.0008	1.13 (0.80-1.60)	0.48	1.30 (1.06-1.59)	0.011
			All	0.27	0.486, 0.442	0.0114	1.24 (0.99-1.55)	1.38 (1.06-1.81)	0.045	1.28 (1.04-1.58)	0.019	1.21 (0.96-1.52)	0.11	1.18 (1.03-1.35)	0.015
rs1912637	15	T	Severe	0.46	0.137, 0.074	8.7×10^{-5}	1.84 (1.27-2.66)	NA (0.00-NA)	0.0001	1.96 (1.36-2.82)	0.0003	NA (0.00-NA)	0.0047	2.00 (1.41-2.84)	0.0001
			All	0.34	0.116, 0.085	0.0028	1.32 (1.03-1.68)	6.48 (1.44-29.03)	0.0016	1.39 (1.09-1.77)	0.008	6.15 (1.37-27.56)	0.0044	1.43 (1.14-1.80)	0.0021
rs9940998	16	A	Severe	0.76	0.370, 0.289	0.001	1.49 (1.09-2.03)	2.05 (1.24-3.40)	0.004	1.59 (1.18-2.13)	0.0021	1.69 (1.05-2.73)	0.029	1.45 (1.16-1.81)	0.0009
			All	0.98	0.359, 0.330	0.0799	1.24 (1.01-1.53)	1.21 (0.88-1.67)	0.11	1.23 (1.01-1.50)	0.036	1.09 (0.80-1.47)	0.59	1.14 (0.99-1.32)	0.071

Abbreviations: chr, chromosome; HWE, Hardy–Weinberg equilibrium; Case-Cont f, case-control frequency; Hetero, heterozygotic for the risk allele; Homo, homozygotic for the risk allele; OR, odds ratio; CI, confidence interval; NA, not calculable.

The subsequent aim of this work sought to specifically identify important, common genetic variants associated with OAG, irrespective of known family history for this disease. Validation and replication was performed on 93 highly associated SNPs identified from the pooling study. Individual genotyping across the three case-control cohorts eliminated all potential false positive results associated with pooling artefact. Our design of using multiple cohorts and joint analysis of the full case-control series also aimed at reducing type 1 errors.

From the pooled GWA analysis there was poor overlap in the top SNPs identified from each OAG case-control cohort or on joint analysis (Figure 5-7). The predominant driver of this poor overlap between the top hits is random sampling from the population. The pooling error in this study was approximately 10 times smaller than the random sampling error. Thus, the expected overlap for the top SNPs from each cohort individually is relatively small. This finding of stratification bias is clearly independent of DNA pooling. Simulation demonstrated that only approximately four (range two to six commonly observed) SNPs were expected to overlap between the top 100 SNPs from each cohort when the best 100 of 300,000 SNPs are typed. A poor overlap between SNPs reflects the modest sample sizes involved in this GWA.

To minimise population stratification, strict inclusion criteria of cases and uniformity of phenotyping across our OAG case-control cohorts ensured that they were equivalent from a diagnostic perspective. Additionally, within each of the three geographic regions, all of the case and control individuals were drawn from the same representative population. All subjects in this study identified themselves as being of non-Hispanic European ancestry. It was clear that our study had modest power to

detect loci with weaker effects. Nevertheless, true associations showing strong evidence for association, such as that observed in AMD, should not have been missed. We successfully identified a number of genomic regions associated with OAG.

Interestingly one of these putative loci was located on the X chromosome, a chromosome not previously implicated in OAG, but one which has been implicated with Leber's Hereditary Optic Neuropathy, another form of inherited optic neuropathy (Hudson et al., 2005). Association analysis of SNPs on the sex chromosomes requires a different approach to the autosomes (The Wellcome Trust Case Control Consortium 2007). Given that males are hemizygous for X, and that in females many genes on the X chromosome undergo Lyonisation, the relative genotypic weighting compared to autosomal alleles differed in our single locus analysis (The Wellcome Trust Case Control Consortium 2007). Such analysis demands that there is no difference in gender composition between case-control cohorts. The haplotype block in the HapMap CEPH population across the X chromosomal region identified as being associated with OAG spanned approximately 200kb and there is one gene known to map to this region (*WDR40C*; NM_001013628). Given the high linkage disequilibrium at this locus, it was not surprising to find that the nonsynonymous (rs10126452; His147Gln) variant in *WDR40C,* which had not been typed by the HapMap Consortium, was completely tagged by neighbouring SNPs.

One autosomal SNP (rs10934884) that was identified as being strongly associated with OAG severity, is located approximately 5Mb from the microsatellite marker (D3S3637) bordering the *GLC1C* locus (OMIM: 601682) on chromosome 3q21. This

SNP is located in an intronic region of the *Winged-Domain Repeat-10* (*WDR10*; NM_052985) gene (Gross et al., 2001). The identification of associated SNPs located in or near genes from the *Winged-Domain Repeat* family is particularly noteworthy. As discussed previously, in 2005 Monemi and colleagues implicated the *Winged Domain 40- Repeat 36* (*WDR36*; NM_139281) gene (Monemi et al., 2005). However, there has been conflicting support for this finding (Hauser et al., 2006; Hewitt et al., 2007a; Pasutto et al., 2008; Weisschuh et al., 2007). *Winged-Domain Repeats* are minimally conserved regions of approximately 40 amino acids, which facilitate the formation of heterotrimeric complexes (Neer et al., 1994). Members of this gene family are involved in a variety of cellular processes, including signal transduction and apoptosis (Smith et al., 1999).

Two significant autosomal SNPs (rs11969985, rs9392354), located in an intronic region of the *GDP-mannose 4,6-dehydratase* (GMDS, NM_001500) gene, are approximately 308kb centromeric to a gene associated with ocular anterior segment dysgenesis and glaucoma (*forkhead box C1*, [*FOXC1*] gene), in a region with relatively poor linkage disequilibrium (Figure 5-11). *FOXC1* belongs to a family of transcription factors and although the specific function of this gene has not yet been fully elucidated, it has been shown to play a role in the regulation of embryonic and ocular development (Murphy et al., 2004). Mutations in this gene cause various forms of severe glaucoma phenotypes such as primary congenital glaucoma, autosomal dominant iridotrabecular-dysgenesis, and Axenfeld-Rieger anomaly (Table 5-8). However, this gene has not previous been implicated in OAG (Nishimura et al., 1998; Strungaru et al., 2007). It is certainly plausible that *cis* acting non-coding variants (tagged by rs11969985 or rs9392354) could modulate *FOXC1* expression, thereby facilitating a less severe glaucoma phenotype or a very subtle

form of iridotrabecular-dysgenesis (Scheetz et al., 2006). Interestingly, we have previously reported that people with anterior segment dysgenesis are at a lifetime risk for developing glaucoma (Hewitt et al., 2006c).

Table 5-8
Previously reported variants and associated phenotype in the *forkhead box C1* (*FOXC1*) gene located on chromosome 6p25.

Variant	BLOSSUM62 score	Associated Phenotype	Source
Gln23Ter	-1	Axenfeld-Rieger syndrome	(Mirzayans et al., 2000)
Thr68Thr	5	-	dbSNP
Pro69Pro	7	-	dbSNP
Ser82Thr	-1	Iridogoniodysgenesis type 1	(Mears et al., 1998)
Ile87Met	1	Axenfeld-Rieger anomaly and glaucoma	(Mears et al., 1998)
Phe112Ser	-1	Rieger syndrome/Axenfeld anomaly/Peters anomaly	(Nishimura et al., 1998)
Ile126Met	1	Severe Axenfeld anomaly and glaucoma	(Nishimura et al., 1998)
Ser131Leu	-2	Rieger anomaly and glaucoma	(Nishimura et al., 1998)
Cys135Cys	9	-	dbSNP
Leu137Val	1	-	dbSNP
Asn426Asp	1	-	dbSNP
Ala486Ala	4	-	dbSNP
11-bp DEL	-	Rieger anomaly and iris hypoplasia	(Nishimura et al., 1998)
Duplication	-	Iris hypoplasia and glaucoma	(Lehmann et al., 2002)
22-BP INS	-	Axenfeld anomaly	(Nishimura et al., 1998)

The SNP rs10132688 has been mapped to 14q13 and is located between two genes *Cofilin 2* (*CFL2*), and *Bromodomain adjacent to zinc finger domain 1A* (*BAZ1A*). The linkage disequilibrium between these two genes is relatively strong (Gillett et al., 1996; Jones et al., 2000). Mutations in *CFL2* cause a rare form of myopathy (Agrawal et al., 2007), whilst BAZ1A forms a DNA-binding heterodimer and is involved in chromatin remodelling (Poot et al., 2000).

Clearly further work is required before the association of variants at these novel loci with OAG is definitively proven. Replication in additional disease cohorts, in addition to functional molecular studies, will remain essential facets of such work. A caveat on such investigations is that should the true causal variant(s) at any of these loci be identified, then they are likely to have a greater effect size than that observed here. A list of statistically similar SNPs at the major autosomal loci is provided in Table 5-9 (Nyholt 2006b).

In summary, well-characterised ethnically homogeneous cohorts of OAG cases, coupled with the power of high density genetic markers in a GWA study, were used to identify novel putative genetic risk factors for OAG. Our strategy of concentrating analysis on the cases with definitive end-stage glaucomatous visual field loss was chosen to maximize the probability of finding strong novel genetic associations. Additionally, our design of undertaking GWA on a subset of the full cohort is well established (Easton et al., 2007). Despite being labour-intensive, there is a very high chance that novel genes accounting for the major common genetic contribution to many inherited human diseases could be uncovered relatively cheaply using equimolar DNA pooling.

Table 5-9

Statistically similar SNPs (ssSNP) in the novel autosomal OAG loci. The corresponding pattern of linkage disequilibrium (r^2) in the CEPH population of the HapMap Consortium and distance (bp) from the SNP implicated in this study (testSNP) is presented.

Chr	testSNP	ssSNP	bp from testSNP	r^2
3	rs10934884	rs140695	-34126	1
3	rs10934884	rs2811469	95188	0.606
6	rs9392354	rs9378670	3144	1
6	rs9392354	rs9378671	12934	1
6	rs9392354	rs9378672	13855	1
6	rs9392354	rs6935616	16453	1
6	rs9392354	rs6920883	16841	1
6	rs9392354	rs12530335	20093	1
6	rs9392354	rs11242735	27440	1
6	rs9392354	rs9378676	36027	1
6	rs9392354	rs11242738	49102	1
6	rs9392354	rs7745086	51803	1
6	rs9392354	rs7753375	55843	1
6	rs9392354	rs11242730	2426	0.967
6	rs9392354	rs11242734	19660	0.928
6	rs9392354	rs11242739	49954	0.875
6	rs9392354	rs12164011	494	0.825
6	rs9392354	rs9405540	-2679	0.824
6	rs9392354	rs4580917	5603	0.823
6	rs9392354	rs9392353	-6529	0.822
6	rs9392354	rs7749136	-10959	0.821
6	rs9392354	rs9392356	4022	0.772
6	rs9392354	rs9378319	25440	0.734
6	rs9392354	rs9392358	25073	0.715
6	rs9392354	rs12055694	43920	0.715
6	rs9392354	rs9378684	82704	0.715
6	rs9392354	rs9405541	24574	0.714
6	rs9392354	rs17134651	44341	0.714
6	rs9392354	rs9405546	63639	0.702
6	rs9392354	rs9405157	-39313	0.63
6	rs9392354	rs11242740	101542	0.552
6	rs9392354	rs11965306	120709	0.552
6	rs9392354	rs17134732	132955	0.552
6	rs9392354	rs9392371	162609	0.552
6	rs9392354	rs12528102	171397	0.552
6	rs9392354	rs9378685	118483	0.542
6	rs9392354	rs3778557	115891	0.522
14	rs10132688	None		

Chapter 6– USING TWINS TO DISSECT THE GLAUCOMA PHENOTYPE.

As introduced in Chapter 1, twins provide the perfect biological experiment (Martin et al., 1997). Twin studies have become a major tool in determining heritability and identifying disease-causing genes for many common conditions (Martin et al., 1997).

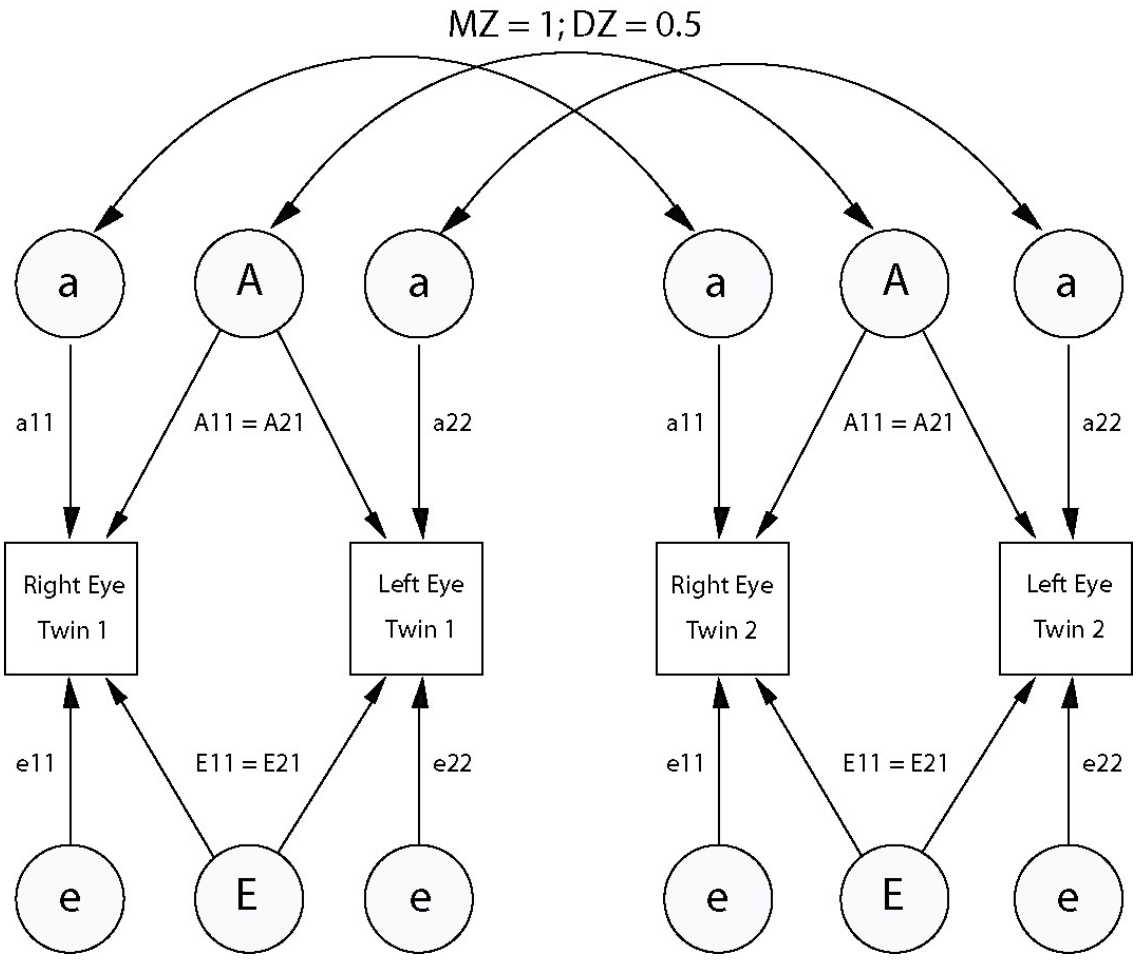
In a classical twin study, comparison of the covariance between monozygotic and dizygotic twin pairs allows for the estimation of the genetic and environmental contributions to the trait in question. This can be broken down into dominant versus additive genetic components and shared versus non-shared environmental elements. Although a simplistic path diagram for this partitioning was displayed in Chapter 1 (Figure 1-6), clearly a bivariate extension is required for the analysis of ocular traits (Figure 6-1).

Extensions of the classical twin study can allow for the localisation of quantitative trait loci. Gene identification is accomplished through performing a discordant sibling-pair analysis of the dizygotic twins. Essentially by statistically identifying the shared genomic regions for all dizygotic twins who are concordant for a trait compared to the gene regions that differ between dizygotic twins who are discordant for the trait, the trait-associated gene loci can be uncovered.

This chapter proposes a novel twin design and is aimed at the identification of the principal heritable components of visible optic nerve head structures. Such a novel twin design could be readily applied to other unique applications such as in the

endophenotypic breakdown of brainwave activity or in discerning discrete patterns of male baldness.

Figure 6-1
The extended path diagram displaying the general bivariate AE model used to partition additive (A and a), and unique (E and e) environmental components on a trait for both eyes (right and left).



Heritable features of the optic nerve head: A novel twin method for determining genetic significance.

Background:

Understanding the principal factors that contribute to variation in human traits is important because genetic or environmental determinants of these traits may also be related to disease susceptibility. Quantitative traits are inherently more powerful for disease loci identification than attempting to map dichotomous (present/absent) diseases such as glaucoma, given their relative rarity in the population (Williams and Blangero, 2004).

The optic nerve head (ONH), which is bounded by Elschnig's ring as demarcated by the termination of Bruch's membrane, is comprised of numerous quantifiable anatomical structures (Jonas et al., 1999). Ganglion cell axons converge at the ONH, to exit the eye in a crude retino-topographic pattern (Fitzgibbon and Taylor, 1996). Similarly, retinal arteries and veins either enter or leave the eye, respectively, through this scleral foramen. Various diseases with a genetic basis manifest clinically in the form of altered architecture of both the intra- and parapapillary regions of the ONH (Traboulsi 1998). It would be helpful to understand which ONH characteristics should be prioritized for quantitative trait loci (QTL) analysis.

In the prioritisation of ONH traits for QTL investigation, it should be remembered that different disease processes may result in the same phenotypic appearance, but also that there is phenotypic heterogeneity in that a single disease may cause a variable ONH phenotype. For example, in addition to the classic glaucomatous optic cup excavation (which can be focal or diffuse) one may also see retinal artery narrowing and expansion of the zone of beta-parapapillary atrophy in primary open-

angle glaucoma (Airaksinen et al., 1984; Jonas et al., 1999). Additionally, other genetic diseases cause other abnormalities of the ONH, such as the presence of telangiectatic vessels in some patients with Leber's hereditary optic neuropathy (Yen et al., 2006). Nonetheless, the genes underlying the many diseases that affect the ONH may be identified through investigating its chief heritable components.

The aim of this study was to determine which features of the ONH are primarily genetically determined. ONH photographs from randomly selected twin pairs were viewed by practitioners who have a sub-specialist interest in the optic nerve. These experts, who were masked to zygosity, attempted to identify which pairs they thought were monozygotic (MZ) and which they thought were dizygotic (DZ). The underlying premise was that the ONH traits that are most highly heritable would be those on which the experts who were the most proficient at correctly identifying twin-pair zygosity based their decision. In a second experiment, a random series of an additional set of MZ twins were selected and the right ONH photograph was displayed with the expert then randomly viewing either the same person's left ONH, flipped horizontally to appear as a right ONH, or the right ONH from their MZ pair. Each expert was asked to nominate which of the latter two most resembled the ONH first viewed. It was reasoned that major epigenetic factors would account for variation in ONH morphology should each expert be able to consistently nominate the ONH photographs from the same person. Conversely, mirroring or laterality would be important in ONH embryogenesis if the experts were consistently nominating the right ONH photographs from the MZ pair as being most similar. Overall, the results from this study allow for the prioritization of quantifiable ONH traits for further genetic investigation.

Materials and Methods:

Subjects Recruitment and Study Protocol:

Twin pairs were identified as part of the Twins Eye Study in Tasmania (TEST), and were recruited from the general population through local media campaigns as well as through a national registry. The Australian twin registry includes more than 30,000 sets of twins, and invitations then were directly sent to all registered Tasmanian twins (>1,000 eligible sets). The relevant ethical committees of the University of Tasmania as well as the Royal Victorian Eye and Ear Hospital approved this study, and the tenets of the Declaration of Helsinki were followed. Each subject or his/her respective legal guardian provided written informed consent prior to participation.

All recruited twins underwent a comprehensive clinical examination that included: anterior segment examination; intraocular pressure measurement; corneal pachymetry; refraction; and a mydriatic optic disc assessment. Simultaneous stereoscopic optic disc photographs were obtained using a Nidek 3-Dx/F fundus camera (Nidek, Gamagori, Japan) on 35mm ektachrome slide film (Kodak, Melbourne, Australia). For all twin pairs, zygosity was determined by DNA analysis using the following polymorphic microsatellite markers: D2S2211 (7 alleles); D3S1267 (13 alleles); D6S257 (11 alleles); D8S284 (8 alleles); D11S4151 (6 alleles); D12S345 (10 alleles); D14S283 (9 alleles) and D17S1852 (12 alleles). Using the models developed by Nyholt, our genotyping protocol will falsely classify a DZ pair as MZ in 1 of 4907 cases.(Nyholt 2006a)

Seventy-seven twin pairs were selected at random from the complete TEST set (n>400). The mean ± SD age of the selected twins was 30.6 ± 11.8 years (range: 7-63 years). Colour 35mm slides of each subject's ONH were viewed using a Pentax

Stereo Viewer-II (Pentax Imaging Company, Colorado, USA). All identifying information was removed from each stereoscopic slide prior to grading.

Experiment Design:

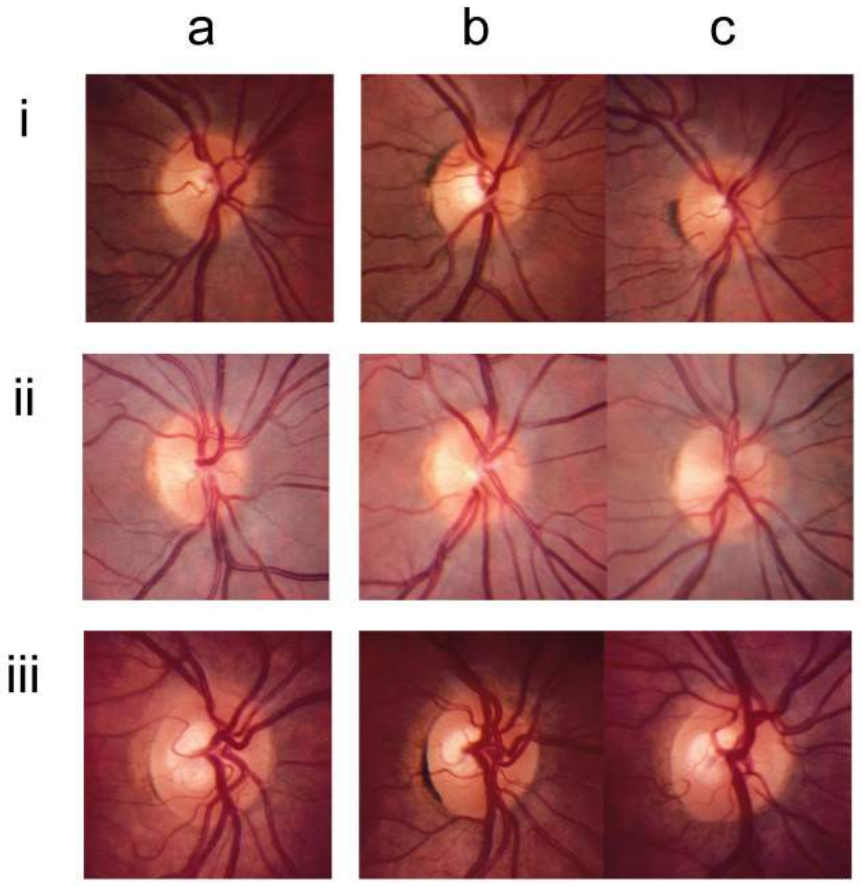
Following an initial pilot (viewed by: Richard L Cooper; Paul L Kaufman; Christopher J Hammond; Sohan S Hayreh), 15 optic disc experts (Wallace LM Alward, Sonya L Bennett, Wido M Budde, Jamie E Craig, John H Fingert, Paul J Foster, David F Garway-Heath, Catherine M Green, Jost B Jonas, Neil R Miller, William H Morgan, Nancy J Newman, Harry A Quigley, John R Samples, George L Spaeth), masked to zygosity, viewed the selected slides. Prior to viewing, these experts were questioned in an unstructured manner about ONH traits that they believed were inherited. Then after viewing a standardised teaching set of five pairs of MZ and five sets of DZ twins, the experts were asked to indicate, in a forced-choice manner, the zygosity of 50 twin pairs. Upon completion of the full set, experts were questioned qualitatively about how their decisions were reached and then asked to quantitatively weight (between 0 and 10) the relative importance assigned to specific ONH traits. These specific ONH traits included: CDR; optic disc size; optic cup size; optic cup depth; optic disc shape; optic cup shape; overall neuroretinal rim appearance; retinal vessel diameters; location of the vascular trunk; vascular pattern within the optic disc margin; vascular pattern beyond the optic disc margin; the presence of cilioretinal vessels; and the presence of parapapillary atrophy.

In the second experiment, each expert was shown ONH photographs from 17 sets of MZ twin pairs. The experts then viewed a right ONH photograph from one of the MZ twin pairs and attempted to determine if the same individual's left ONH, flipped

horizontally to appear as a right ONH, or the right ONH from their MZ twin most resembled the first photograph (Figure 6-2).

Figure 6-2

Optic nerve head photographs of monozygotic (MZ) twin pairs where each expert was asked, after viewing the right ONH from one of the MZ twin pairs (Panel a), whether the same individual's left ONH, flipped horizontally to appear as a right ONH, or the right ONH from their MZ twin most resembled the first. In examples i and iii ONH photographs (a) and (c) are from the same individual, whilst in example ii (a) and (b) are from the same individual.



Data Analysis:

As a measure of reproducibility and to test for fatigability, the proportion of correctly identified twin pairs in the first set of 25 were compared using Fischer's exact test with that in the last set of 25. Statistical analysis was performed using SPSS 12.0.1 (SPSS Inc, Chicago, IL, USA).

The responses to the post-experiment structured questionnaire were analysed using a Rasch approach with the Winsteps 3.61.1 programme (www.winsteps.com).(Linacre 2004) Rasch analysis allowed the usefulness of specific ONH traits, as weighted by different graders, to be measured on a common logit scale, thereby allowing direct comparison. The Rasch model does not assume values for response categories (e.g. 0,1,2…) rather it assumes that all categories are on the same underlying latent variable.(Rasch 1966) Categories that were disordered or underutilized were collapsed into adjacent categories. Category probability curves were reviewed to ensure goodness of fit in the probability of observing the relative weighting of each collapsed category at each point on the latent ONH variable (Pesudovs et al., 2004). A rank-ordered analysis was used, whereby each grader's ability was rated by the percent of twin pairs for whom they had nominated the correct zygosity (Linacre 2006). This percentage then was empirically adjusted from proportions to logits to allow sensible fit statistics. The t-standardized, mean square statistics, which compare the predicted responses with the observed, were reviewed to monitor the compatibility of the data with the Rasch model. Outlier sensitive fit (outfit) mean square is the conventional sum of squared standardized residuals and is sensitive to occasional responses that are different to the expected response, whilst for the information-weighted fit (infit) mean square each squared standardized residual value is first weighted by its variance and then summed, so as to tolerate extreme

responses (Pesudovs et al., 2004). Values predictive of measurement are deemed to generally fall between 0.5 and 1.5 (Pesudovs et al., 2004).

Results:

The proportions of correctly nominated twin pair zygosities ranged from 74% to 90% (median 82%) across the 15 graders. Two of the ophthalmic experts correctly determined the zygosity of twin pairs 45 out of 50 times. The zygosity of 20 (40%) twin pairs were correctly nominated by all of the 15 experts, whilst the zygosity of three (6%) sets of twins (Figure 6-3) was correctly identified by fewer than five of the graders.

For each individual expert and across the panel as a whole, the proportion of incorrect zygosity calls did not differ ($p>0.1$) between the first and second halves of the viewed set. MZ twins were more frequently erroneously identified as DZ than vice versa (Table 6-1). The three graders who specified more DZ twins than MZ generally performed poorly on the grading, each with scores less than 84%.

Figure 6-3

Optic nerve head photographs of twin pairs. Panel 1 contains examples of twin pairs for whom the correct zygosity was identified correctly by each expert whilst Panel 2 contains examples of twin pairs whose zygosity was most frequently incorrectly nominated. In both panels twin pair (i) are dizygotic, whilst twin pairs (ii) and (iii) are monozygotic. Right (OD) and left (OS) optic nerve heads are displayed.

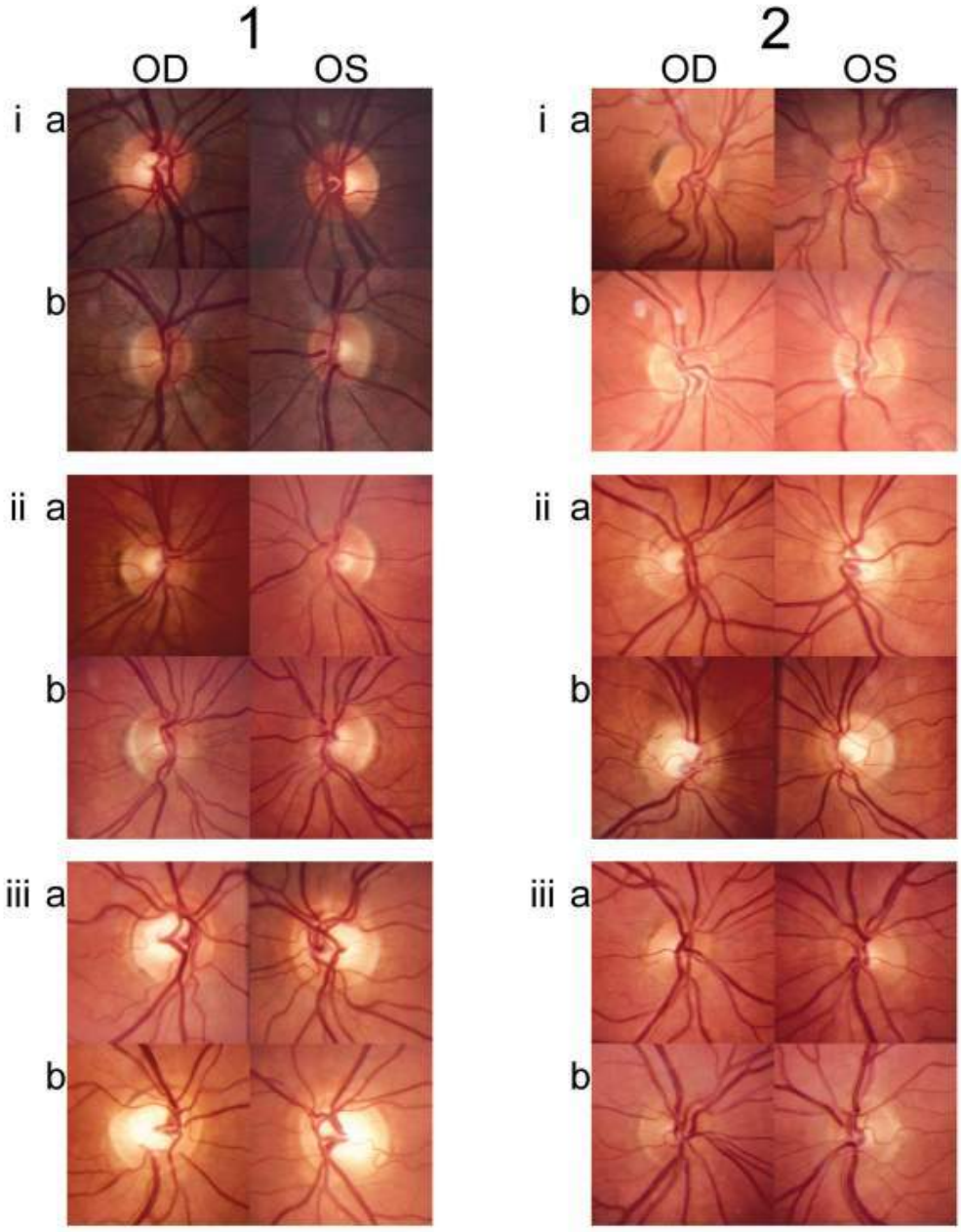


Table 6-1

Relative score and responses from each ophthalmic expert when qualitatively questioned prior to the experiment regarding the most heritable features of the optic nerve head and following the experiment about how their made judgment was reached.

Expert	Percent of correct zygosity nominations	No. of MZ twins nominated as DZ	No. of DZ twins nominated as MZ	Pre-Experiment Response	Post-Experiment Response	Percent of correct MZ matching
A	90	3	2	Size and shape of disc and cup; tilting of disc; depth of cup; slope/profile of neuroretinal rim; vessel pattern; myopic crescent may be useful	Shape and depth of cup; shape and tilting of disc; angulation of vessels through the laminar; vessel pattern not useful; cilioretinal vessels not useful	47.1
B	90	3	2	Size of disc; size and shape of cup; vessel arrangement; scleral crescent may be useful; clarity of neuroretinal rim; neuroretinal rim shape	Orientation of disc; size of disc; size of cup; shape and site of prominence of neuroretinal rim; vessel pattern not useful	70.6
C	86	4	3	Size and shape of disc; vessel trunk course; depth and shape of cup; parapapillary atrophy may be useful	Shape and size of disc; depth, shape and size of cup; location of highest region of nerve fibre layer; parapapillary atrophy	76.5
D	86	5	2	Size of disc; tilting and shape of disc; depth of cup / nerve head; CDR; vasculature features likely not to be useful	Shape, size and orientation of disc; elevation of neuroretinal rim; trunk position in nerve	58.8
E	86	5	2	CDR; location and branching of vessels; titling and shape of disc; colour of optic nerve (relative temporal pallor)	Shape of disc; depth of cup; branching pattern of vessels not useful	70.6
F	84	5	3	Size and shape of disc; size of cup; cilioretinal vessels may be useful; arrangement and direction of vessels may be useful	Size of disc; Orientation of disc; shape and depth of the cup; orientation of the vessel trunk	58.8
G	84	4	4	Size of disc; size and shape of cup; tilting of disc; parapapillary atrophy; vascular branching pattern likely not to be useful	Size and shape of disc; pattern of vessels outside the disc margin; nerve fibre layer volume not useful	64.7
H	82	6	3	Size and shape of cup; size and shape of disc; temporal vessel branching pattern, parapapillary pigmentation; depth of trunk branching	Shape and size of cup, shape and size of disc; presence of cilioretinal vessels; pattern of vasculature	70.6
I	82	4	5	Vascular pattern; position of vascular branching; size of disc; site of maximal neuroretinal rim thickness	Size and orientation of the disc; vascular pattern; parapapillary abnormalities; contour of neuroretinal rim	52.9
J	82	3	6	VCDR; parapapillary atrophy; site of maximal neuroretinal rim thickness (ISNT); size of disc; displacement of vessels likely not to be useful	Arrangement or sectorial location of cupping; vascular pattern;	64.7
K	80	5	5	Size of disc; CDR; shape of disc and cup	Size of disc; CDR; shape of cup; contour of neuroretinal rim	64.7
L	80	6	4	Size of disc; CDR; shape of disc; degree of disc tilting; vascular pattern	Diameter of disc; choroidal pattern; vascular pattern; shape of disc	47.1
M	78	8	3	Size and shape of disc; size of cup; vasculature pattern; colour of parapapillary region; depth of cup	Size of disc; size of cup; pattern of vessels; depth of cup; parapapillary pigmentation	82.4
N	76	2	10	Size and shape of disc; shape of cup; individual characteristics likely to be less important than overall gestalt of ONH pattern	Pattern of choroid and retinal pigment epithelium; blood vessel pattern; neuroretinal rim width not useful	76.5
O	74	7	6	Size of disc and cup; vessel pattern; colour of the optic nerve	Height of the optic nerve relative to retinal plane; vessel pattern; optic disc size more useful than cup size; tilt of disc; peripapillary atrophy	64.7

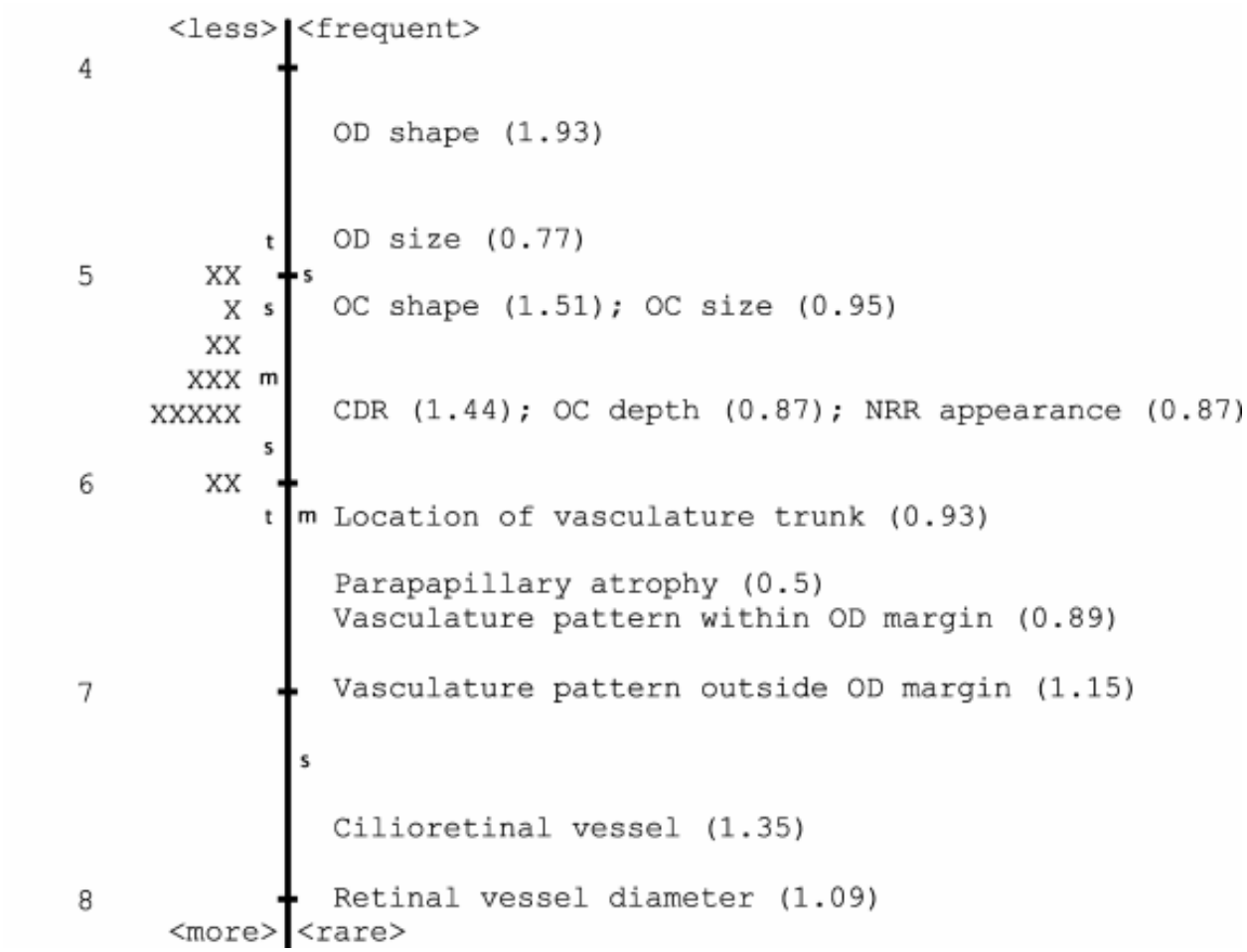
Abbreviations: No., number; MZ, monozygotic; DZ, dizygotic; CDR, cup to disc ratio; VCDR, vertical cup to disc ratio; ISNT, inferior>superior>nasal>temporal neuroretinal rim sector thickness.

Upon completion of the experiment, each expert had an altered opinion regarding the traits that he or she believed were heritable, relative to the specific responses in the qualitative initial interview (Table 6-1). Rasch analysis of the quantitative weighting revealed that experts who correctly identified the zygosity in more than 85% of cases placed most weighting on the size and shape of the optic disc and optic cup, whilst experts with the lowest scores placed greater weighting on the ONH vasculature (Figure 6-4). The empirically adjusted ability of the experts, each represented by an 'X' is displayed, with better performing experts and less useful ONH characteristics for determining zygosity appearing near the bottom of the figure. The item optic disc shape had noticeable off-variable noise (infit mean square = 1.93).

During the second experiment, the ONH photographs from the same individual were matched correctly on average 64.7% of the time (range: 47.1% to 82.4%). Across the experts, there was no correlation between the results of the zygosity nominating experiment and the second ONH matching experiment (Spearman's rho: -0.16, p=0.27). Two of the three best performing experts in this second experiment also had the greatest disparity in the proportion of twin zygosities incorrectly nominated as MZ or DZ (Table 6-1).

Figure 6-4

Performance of ophthalmic experts and the relative usefulness of specific optic nerve head features in determining zygosity. To the *left* of the solid line are the graders, represented by *X*, and on the *right* are the ONH items. More able graders and less useful items, for determining zygosity by ONH appearance are near the bottom. The t-standardized information-weighted mean square statistic is displayed in parentheses, with the column of figures on the left representing the logit values of the rank-adjusted proportions. Abbreviations: s, one standard deviation; t, two standard deviations; m, mean; OD, optic disc; OC, optic cup; CDR, cup-disc ratio; NRR, neuroretinal rim.



Discussion:

The degree to which genes and environmental factors determine ONH morphology is of fundamental importance for the full understanding of the aetiology of common blinding diseases such as glaucoma. We used a novel approach to dissect the heritable features of the ONH. In determining the heritable components, the results from this study suggest that quantification of the shape and size of the optic disc and cup should receive a greater priority than quantification of ONH vascular features. Factors such as vascular pattern or tortuosity are difficult to quantify; hence, our findings are strengthened by the fact that this is a qualitative study rather than a quantitative one. The relative partitioning of genetic and environmental components of particular traits allows for informed investigation of underlying pathogenesis. Given the vast array of potentially quantifiable ONH structures, prioritising those that should be investigated over others allows for efficient use of finite resources.

The tight coupling of ONH traits and disease underscores the relevance of the genetic liability that is associated with those specific features (Traboulsi 1998). Mutations in both nuclear and mitochondrial genes alter ONH architecture in the diseased state (Alexander et al., 2000; Delettre et al., 2000; Wallace et al., 1988). Glaucoma, a disease of progressive excavation of the optic disc, for example, has been demonstrated to have a genetic basis (Rezaie et al., 2002; Stoilov et al., 1997; Stone et al., 1997). It is noteworthy that each expert grader differed in their responses prior to and after completing the experiment. The initial unstructured questioning allowed exploration of the pre-experiment biases regarding heritable traits of the ONH. Interestingly, in this initial questioning, optic disc size was regarded as the most important trait by 11 of the 15 experts. The post-experiment questioning gave insight into each grader's intuition on these heritable features. Three of the top five

performing experts commented that vascular pattern was not useful in determining twin zygosity, whilst the four poorest performing graders principally utilised these features in determining the zygosity.

The second experiment performed in this study investigated the relative phenomenon of mirroring, laterality and environment effects on the ONH. Unique environmental factors and variable expression of genetic factors (e.g., differences in methylation) may account for phenotypic differences in MZ twins (Singh et al., 2002). In classical twin studies, the equal-environment assumption is widely accepted (Martin et al., 1997), and within individuals there is a degree of ONH asymmetry (Jonas et al., 1999). However, the ONH features of some MZ twins are surprisingly dissimilar, thereby suggesting that stochastic epigenetic events significantly influence ONH architecture. This phenomenon is the likely reason for the two sets of MZ twins pairs that most experts incorrectly determined as being DZ pairs in the initial experiment. Although the phenomenon of mirror-imaging has been described in MZ twins (Satoh et al., 1995; Townsend et al., 1986), it does not appear to be of marked significance in ONH development. The principal limitation of this second experiment was the relatively small number of MZ twins used. Increasing both the number of expert graders as well as the number of twins used would allow for a clearer demarcation of traits commonly discordant between MZ twins and the possible identification of novel factors influencing the ONH appearance (Oates et al., 2006).

Populations of twins provide a powerful opportunity for disentangling complex genetic and environmental interactions (Martin et al., 1997). A classical twin study allows analysis of the variance and covariance between MZ and DZ twin pairs. Comparison between the covariance of MZ and DZ twin pairs allows partitioning

into dominant versus additive genetic components and shared versus non-shared environmental elements (Martin et al., 1997). There has been a small number of low-powered twin studies investigating the ONH and these generally support the results of our findings. Twins were included in the cohort used by Armaly in his landmark paper that concluded that the CDR of the ONH was genetically determined (Armaly 1967a). Subsequently, Schwartz and colleagues used ONH photographs from twins to estimate that the heritability of the CDR ranges between 70% and 80% (Schwartz et al., 1975; Schwartz et al., 1976). Teikari and Airaksinen also identified greater CDR correlations between MZ twins than DZ pairs (Teikari and Airaksinen, 1992). The parapapillary retinal nerve fibre layer thickness has been found to have a relatively high heritability (82%), whilst the presence of cilioretinal arteries are influenced by additive genetic factors with an estimated heritability of 71% (Hougaard et al., 2003; Taarnhoj et al., 2005). Huntzinger and Christian concluded that vascular tortuosity was likely to be more genetically determined than other features such as vessel length, branching points and number of vessels crossing the optic disc margin (Huntzinger and Christian, 1978). Although family-based studies investigating ONH hereditability have focused on few phenotypic features, their findings do also generally support the overall results of our study. The size of optic cup and disc (Bengtsson 1980; Chang et al., 2005; Klein et al., 2004), have been found to be more hereditable than retinal vessel thickness (Xing et al., 2006).

In summarising this study, Rasch analysis demonstrated that both the shape and size of the optic disc and cup were more useful in determining twin zygosity than vascular parameters. Thus, these traits are particularly likely to be highly heritable. Nonetheless, epigenetic variation does cause minor asymmetry of the ONH. Determining the genetic and environmental variants which influence ONH

morphology will allow for the elucidation of the molecular pathogenesis of diseases that alter optic nerve architecture.

Chapter 7 – CONCLUSION : Dimensional complexities in a molecularly and phenotypically heterogeneous disease.

The eye is part of one of the most remarkable systems in vertebrates. Indeed, Darwin himself marvelled at how an organ of such extreme perfection could have formed through natural selection (Darwin 1859). Across different species, the eye exemplifies the phenomena of convergent, divergent as well as parallel evolution (Weiss and Buchanan, 2004). Stemming from the observation of repeated apparently independent evolution for such a complex organ, it has been postulated that the development of primitive visual apparatuses were the principal driving force behind the Cambrian explosion (Parker 2003).

From cellular organisation in the visual cortex, to the high degree of pre-retinal processing or interesting characteristic of avascularity in the metabolically active translucent cornea, many inroads have been made into understanding the human visual system (Ambati et al., 2006; Hubel and Wiesel, 1962; Kolb 2003; Weiland et al., 2005). Nonetheless, the underlying biological mechanism(s) leading to many important blinding diseases remain Delphian. Recent advances in molecular technology, computational methods and a greater appreciation of the complexities in both homeostatic as well as diseased states will allow for rejuvenated and novel avenues of investigation. It is foreseen that in the immediate future a “systems biology” approach to understanding complex diseases will need to be adopted.

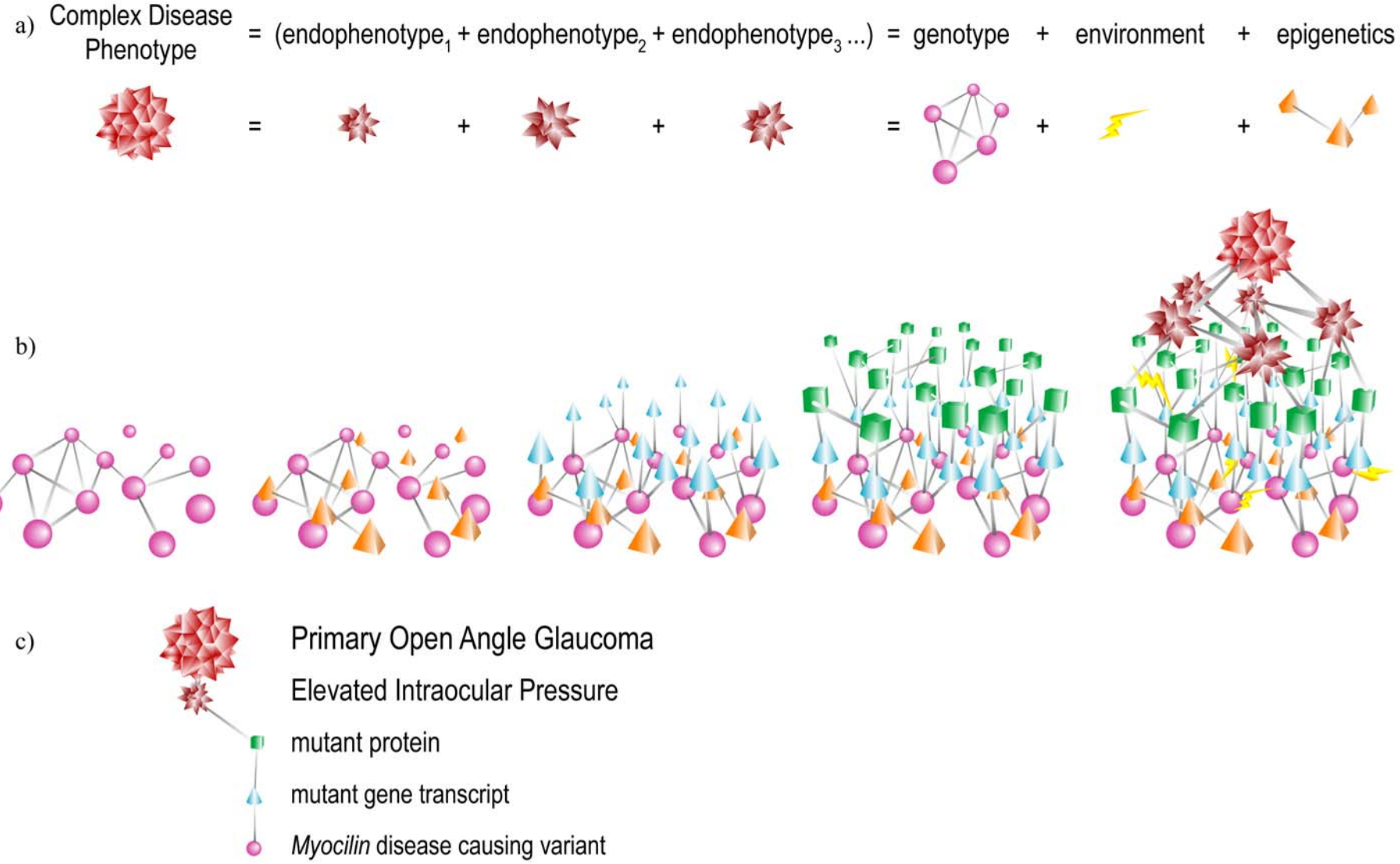
Complex diseases and integrational biology:

The central biological dogma, that the expression of most biological traits is influenced by many genetic and environmental influences, has shifted with the emerging recognition that stochastic epigenetic stressors also have a fundamental role in phenotypic determination. As well discussed by Petronis, there needs to be a paradigm shifting incorporation of epigenetic factors in the investigation of complex diseases (Petronis 2006). The collision of germ-line, somatic, environmental and stochastic factors conspire in the dynamic web of a complex phenotype (Figure 7-1). Most complex phenotypes themselves can be broken into fundamental components, whereby each sub-trait can combine or constitute a diagnostic pillar of a disease. For example, endophenotypes for the Primary Open Angle Glaucomas (OAG) include elevated intraocular pressure, trabecular meshwork dysfunction, compromised optic nerve head haemodynamics and susceptibility to retinal ganglion cell apoptosis.

A systems biology approach allows for the integration of information at all biological levels (Kitano 2002a). The interactions between each contributing factor can be displayed as a vector, whereby, the importance of each complex association between elements can be defined for a particular disease. When unravelling the fundamental pathogenic associations for a complex disease, many independent lines of enquiry, including genomic sequence, gene expression, protein expression, bioinformatics, epidemiology and cellular interaction, are studied simultaneously (Kitano 2002b). The following sections will discuss briefly these principal mechanisms contributing to different lines of investigation for a systems-based approach in the study of a complex disease.

Figure 7-1

The molecular milieu of a complex disease: a) the constitutional breakdown of a complex disease; b) the hierarchical structure from the genomic level (far left) to the transcriptomic and proteomic levels (middle panels) which culminate in the overall complex disease phenotype (far right), note that hypothetical interactions are displayed by lines; c) the schema of the complex disease phenotype reduced to reveal only the *Myocilin* genotype-to-phenotype spine.



Genetic variants can be depicted as representing the foundation of a complex disease (Figure 7-1). However, it must be appreciated that in accepting this notion, as well as the central molecular principal that DNA is transcribed into RNA which is in turn translated into a protein, does not negate the fact the fact that other DNA-independent influences make significant contributions. Nonetheless, investigation at the genomic level offers a convenient framework by which to construct the molecular web of a complex trait. Adding to this, it the fact that substantial advances have been made in both understanding the degree of genetic variation in man as well as the means to rapidly survey individuals' genomic profile.

Genome-wide sequencing will clearly obviate the need for HapMap and a tagging sequence approach, but will dramatically increase the difficulties in data management (The HapMap Consortium 2003; The HapMap Consortium 2007). The corollary of increasing the number of markers (or variants identified by full sequencing) is that the number of subjects (or samples) required to be screened must also increase to allow statistical significance to be reached and spurious associations avoided. Comprehensive catalogues of human variations (such as dbSNP) will continue to be of major importance.

In the wake of data from the present wave of genome-wide association studies, it will be interesting to uncover the proportion of causative genomic variants identified within known protein coding genes. A reductionalistic approach to the attribution of causative variants should be avoided. For example, in the initial work investigating the pathogenic variant in a second major locus for age-related macular degeneration it was relatively more convenient to consider an amino-acid changing variant (Ala69Ser) in a hypothetical protein (*LOC387715*) than it was to investigate for and

uncover a promoter region variant (-152G>A) in high linkage disequilibrium (*HTRA1*) (DeWan et al., 2006; Jakobsdottir et al., 2005; Yang et al., 2006). It is hypothesised that the relative proportion of Mendelian diseases compared to complex diseases, which develop due to mutations in protein coding regions or sequences highly cross-species conserved regions, will be markedly different. Recently, Bustamante and colleagues observed strong evidence of natural selection for rapid amino acid evolution and divergence between humans and chimpanzees (Bustamante et al., 2005).

The effect size for many common genetic risk variants is likely to be relatively small. The majority of variants identified in the initial, large Wellcome Trust Case Control Consortium study were found to generally confer a risk between 1.2 and 1.5 (The Wellcome Trust Case Control Consortium 2007). Although considerably more data mining for epistatic interaction of these specific genome wide studies is required, it has been hypothesized that the identified genetic polymorphisms alone are unlikely to fully account for the familial clustering of these common heterogeneous diseases. Despite the fact that there are likely to be many genes (which may have rare variants of small effect) which remain to be identified, it is just as likely that non-genetic influences may have just an important role in disease development. Similar to Feynman asking the gentleman who had lost his cars keys in a dark gutter why he was searching for them beneath the street lamp only to receive the response "because that is where the light is" (Feynman 1985), as anatomists of complex traits we should ask ourselves why are we dissecting complex traits only at the genomic level?

As discussed previously, a systems biology approach which incorporates a strong emphasis on epigenetics will be fundamental in the analysis of complex phenotypic

variation (Petronis 2006). Epigenetics is the process by which patterns of gene expression are modified in a mitotically heritable manner (Whitelaw and Martin, 2001). The heritability of such phenomena involve DNA-independent mechanisms (Whitelaw and Martin, 2001). Epigenetic modifications include DNA and chromatin modification, such as methylation state (van Vliet et al., 2007). Interestingly, although the epigenetic state is initially established in early development, recent evidence has suggested that that the epigenetic state for some parts of the genome are not cleared. Thus, these regions can manifest with transgenerational inheritance (Whitelaw and Whitelaw, 2006).

The development of a Human Methylation-Map, analogous to the HapMap project, should be developed. Such a resource would be invaluable for the identification of genomic regions that are easily targeted and commonly susceptible to differences in epigenetic state and alteration in transcript expression. The major confounder of such work will be the relatively high degree of tissue-specificity; for example, the patterns of leukocyte methylation may not be transferable to the eye (or vice versa). Additionally, ageing and cellular senescence will dramatically alter methylation state. Nonetheless, such a resource may prove extremely useful in diverse diseases.

It is well accepted that only a tiny proportion of the human genome encodes protein, and that most complex genetic phenomena in the higher organisms are RNA-directed (Birney et al., 2007; Pheasant and Mattick, 2007). The interesting antithesis of such observations is the increasing realisation that the majority of the human genome is transcribed (much on both strands) (Mattick 2007). RNA editing alters the protein-coding transcribed genomic products, involved in diverse roles such as cell maturation, function and DNA repair (Mattick 2007). Thousands of non-protein-

coding transcripts are dynamically expressed during cellular differentiation and development (Mattick 2007).

Interestingly, precise patterns of RNA expression have been identified and many RNA transcripts show well-defined subcellular localisation (Mercer et al., 2008). Alu elements in the human genome are the result of positive selection as a modular substrate for RNA editing, in turn driven by selection for higher order function (Taft et al., 2007). It has recently been postulated that the re-writing of productive changes back to the genome may constitute the molecular basis of long term cognitive adaptations and memory. Memory may reflect RNA-mediated retro-transcription in specific, individual neurons (Professor John Mattick personal communication).

Just as the profile of genomic variation between human populations (as well other species) has been developed (The HapMap Consortium 2003; The HapMap Consortium 2007), the gene expression profile for a number of different bodily regions or systems has also been compiled. Much work has focussed specifically on the brain (Lein et al., 2007). Standardised expression atlases provide a primary resource for a wide variety of investigations relating to both the homeostatic and diseased function of different tissues (Wheeler et al., 2007).

Proteomics allows for the study of structure, function and interactions of differently transcribed proteins. Protein characterisation ideally includes direct amino acid sequence analysis (via mass-spectrometry), investigation of post-translational modification and splice variants, as well as the identification of binding partners and sub-cellular localisation (Guo et al., 2007). The density and versatility of protein-arrays needs to be expanded, thereby ensuring that global protein expression patterns

can be studied during specific disease states or pathological processes (Guo et al., 2007).

Molecular events coalesce culminating in the overall phenotype. Most disease phenotypes themselves can be deconstructed into major constitutional endophenotypes. Clinicians need to stand up to the mark and continue to breakdown disease definitions or sub-characterise diseased states better. This is true not only in the ophthalmic field, but also for other medical specialties. For example, it is clear that a diagnosis of schizophrenia most likely represents the conglomeration of a number of varied subtypes, which in a DSM-IV reductionist sense can be conveniently partitioned so as to streamline treatment. Indeed, separating the dichotomous diagnosis of "asthma" into quantitative traits, such as the volume of air forcibly expired in one second, is essential for adherence to therapeutic management plans. A better taxonomy for OAG must be developed. One of the principal pitfalls in understanding the molecular basis of the glaucomas is the challenging issue of not being able to define the early stages of disease well enough. A potential means to avert this issue is to reduce phenotypic complexity by selecting those people who have end-stage optic neuropathy. Nonetheless, it is appreciated that the molecular paths by which different people develop advanced OAG may differ.

Although some molecular cascades may be particular to one endophenotype, for some diseases there is likely to be substantial overlap in factors that contributed to different facets of a disease. Nevertheless, an understanding of how the heritable components of a pleiotropic disease combine together can be used to increase the power for identifying significant biological association (Klei et al., 2008).

At the epidemiological level it is important to investigate both the phenotypic features of a disease as well the likely environmental stressors that may contribute to it. It is well established that environmental factors can have direct effects at the genomic, transcriptomic and proteomic level. The establishment of the epigenetic state at almost any locus can be modified by the environment, and RNA editing appears to be a major mechanism by which environmental signals can overwrite hard-wired genetic information to modify both gene function and regulation (Mattick 2007).

As we understand the nature of complex diseases better, the defined role for separate genetic and epidemiological studies is lessening (Palmer 2004). Genetic-epidemiological studies are emerging as particularly powerful means for investigation. In the absence of immediately obvious genetic or environmental risk factors, combining data from these fields, which were once vehemently opposed, will become increasingly important in the description of spatial variation in disease risk.

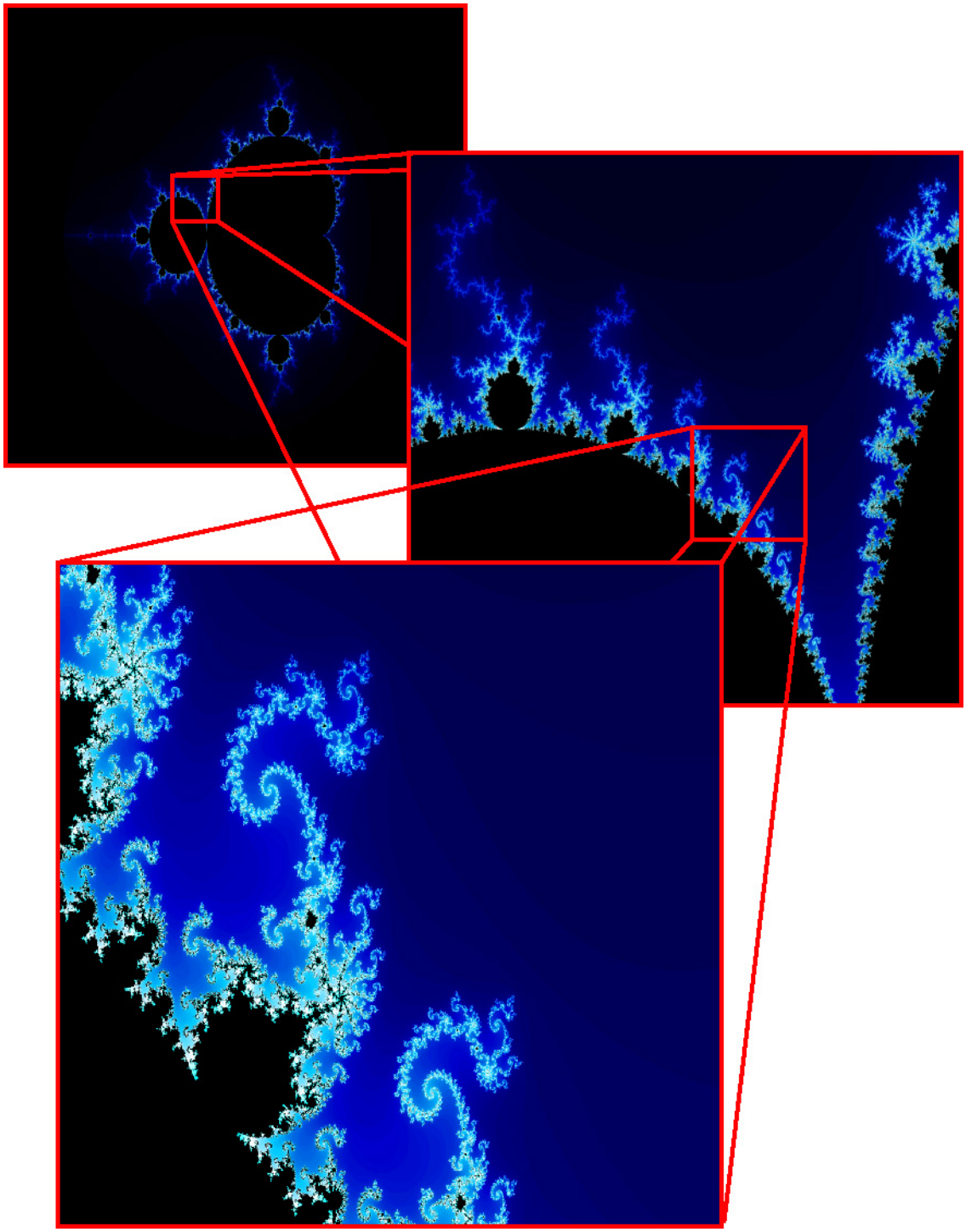
Individual risk profile can be likened to pieces of a pie; whereby, each molecular event contributes a slice, and a particular disease will manifest when a significant proportion of the pie has been digested. Just as some slices of this pie may be large, so too will the relative effect size of different underlying pathogenic mechanisms. Appreciating such a model allows for the acceptance of incomplete segregation of a complex disease and one particular causative mutation within a pedigree. As more case-based and family-based cohorts are investigated, the need to visualise each pedigree's associated molecular and phenotypic variation will become more important. Such a model of pedigree construction needs to extend beyond the traditional static two-dimensional programs (see www.genepi.org.au/celestial3d.html).

The final dimension of this schema for a complex molecular web is that of time. It is generally well established that different genes are up- or down-regulated as an animal ages (McCarroll et al., 2004). These expression profiles result in dramatic physiological and pathological changes. Time-space relationships need to be factored into a complete systems-biology disease model.

The de-construction of the dynamic and interacting milieu of complex traits has begun in earnest and the dynamics of RNA and protein expression in gene circuits is beginning to be understood. In 2005 Cheung et al. reported the use of genomic and transcriptomic data to identify *cis* and *trans* acting regulatory regions (Cheung et al., 2005). More recently this approach has also been used for the retina (Scheetz et al., 2006). In vivo protein-DNA interactions have also been studied and used to connect transcription factors with their direct targets, thereby improving our understanding of gene regulatory networks and scaffolds (Johnson et al., 2007; Kim and Ren, 2006).

It is clear that the merging of quasi-parallel or independent avenues of molecular investigation will have a significant contribution in understanding complex diseases. Approaches using genome-wide association (or even full genome sequencing) must

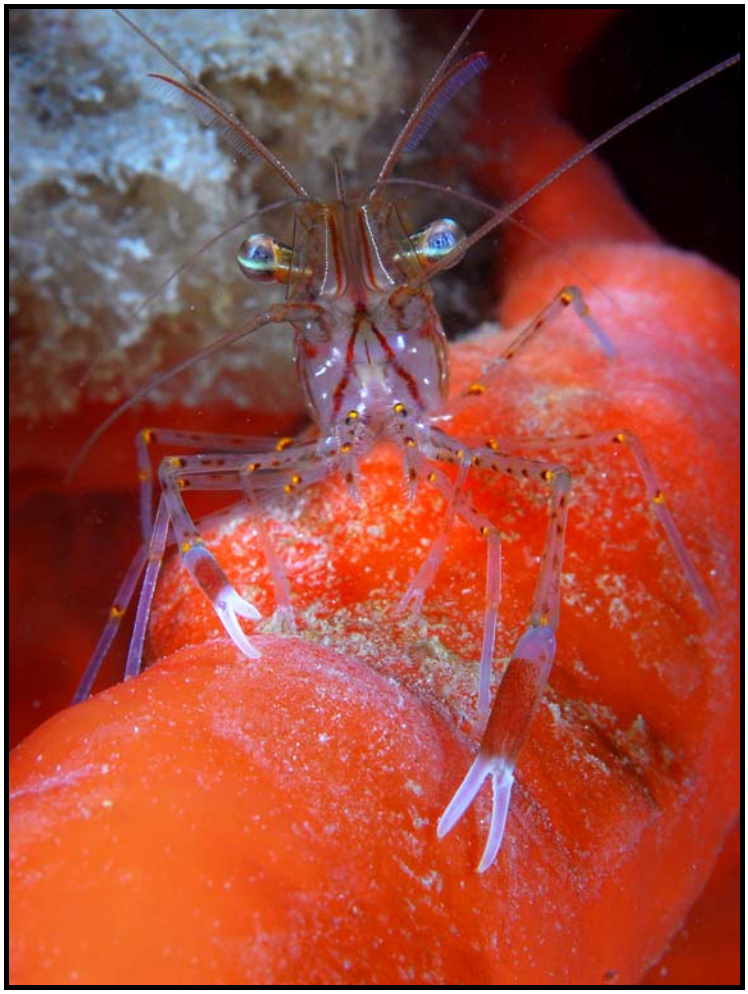
Figure 7-2
Increasing complexity of a dimensionally finite system. This Mandelbrot image was generated using Winfract version 18.21 (shareware of The Stone Soup Group).



eventually incorporate other molecular sectors, such as gene expression and protein profiling. As such whilst our knowledge of one particular molecular level advances, the number of refined questions also increases (Polkinghorne 1996). Our understanding of a complex system can be likened to that of a fractal image, whereby focussing in upon a region merely leads to another level of complexity (Figure 7-2). Whilst the dimensions of a biological system or disease pathogenesis may be finite, their complexity may not be.

The analysis of high dimensional data is paramount when pursuing a systems biology path of investigation. Data could be potentially merged from genomic, transcriptomic, epigenetic, proteomic, environmental and endophenotypic studies. Each of these lines of investigation will contribute incomprehensible dimensions to the analysis, simultaneously. Raw multidimensional data cannot be graphically represented and is probably currently beyond our capabilities of visualization. We generally have difficulty understanding dimensions beyond that which we can experience. For example, regardless of how wonderfully colourful a coral reef may seem to us (with a visual sensitivity for three distinct light wavelengths), we cannot comprehend the kaleidoscopic perception of shrimps which have photoreceptors for 12 different wavelengths of light (Figure 7-3) (Marshall and Oberwinkler, 1999; Marshall 1988).

Figure 7-3
A shrimp (*Palaemon serenus*) with eyes capable of perceiving a kaleidoscopic spectrum of light – easily exceeding our comparatively simple degree of trichromatic perception and possibly beyond the comprehension of our primary visual cortex. Photographed beneath Blairgowrie marina, courtesy of A. Newton.



Principal component analysis (PCA) is one common means for reducing the dimensionality of data. PCA can be summarized as a method of transforming the original variables into new uncorrelated variables (principal components) which are weighted averages of the original variables (Peterson 2003; Scholz and Selbig, 2007). A measure of the amount of information conveyed by each variable is reflected in its variance. Such a technique allows the visualisation of data in two or

three dimensions. Additionally, Bayesian models have been expanded to permit the joint spatial analysis of numerous interacting variables (Molitor et al., 2004). However, these methodological extensions are accompanied by an increase in the complexity of the model structures and parameterization choice.

Analytical tools unique to the fields of statistical mechanics and information theory should be merged and their usage must become more diverse. An integrational biological approach demands the development of numerically efficient analytical methods. Fortunately in time mathematical breakthroughs will be transferred to other disciplines (such as complex genetics). For example, the recent proof of the Poincaré conjecture may prove useful in understanding topographical aspects of pharmacogenomics, molecular evolution, signal transduction pathways, or stress responses in cancer biology (Mackenzie 2006).

On a final note of optimism, exciting prospects for computational methodology and analytical tools continue to be developed (Purcell et al., 2007). It is certainly a very exciting time for molecular medicine. We are at a stage where major technological techniques are at our disposal (albeit somewhat restricted by cost) such that the biological scientific community is reaching a dramatic point of marked increasing returns whereby we have more publicly available data then ever before. The fissure between theoretical and experimental biology is narrowing.

Scientists need to continually prod at the boundaries of our understanding of nature. Indeed Darwin's notion of natural selection – or gradual change – though being a marvellous example of paradigm shifting thought, is now considered a remarkably simple idea (which is currently easily taught at high school); however, the intricacies

and understanding of epistasis or trajectories of beneficial epigenetic states are currently less so. However, no doubt in time, these concepts too will become minor paragraphs in Messel's high school science texts. The common pool of knowledge will become deeper the further we swim in it.

As molecular scientists and phenotyping clinicians we must strive to appreciate the wonderful complexities of dynamic and inter-related biological systems. Dobzhansky's insight that "almost everything makes sense in biology in the light of evolution" cannot be understated (Dobzhansky 1973). Nonetheless, a primitive awe for nature should not be lost. Such scientific misplacement is probably best portrayed in William Blake's (1757–1827) painting "Sir Isaac Newton" where the fellow of the Royal Society, whose hair is dramatically curled appearing like cerebral gyri protruding beyond his scalp, is hunched over, focusing on a geometric problem (possibly representing scientific reductionalism) and thereby ignoring the wonderfully textured natural world behind him.

Context of findings from this dissertation:

Glaucoma, which is a complex heterogeneous disease, presents an ideal case for genetic investigation. OAG is the commonest subtype in Caucasians and was the focus of this thesis. When detected early, OAG is amenable to therapeutic intervention. Prospective randomized trials have clearly shown that IOP lowering with drugs, laser, or surgical intervention slows glaucoma visual loss (van der Valk et al., 2005). Hence, glaucoma blindness should be largely preventable if cases at high risk could be identified at an early stage of their disease. However, all forms of treatment carry cost and morbidity. The annual cost of glaucoma treatment in the USA has recently been estimated at AUS$3.5 billion per year (Lee et al., 2006b). Approximately 10% of glaucoma cases reach the level of legal blindness in their lifetime, with many less severe cases remaining asymptomatic (Grant and Burke, Jr., 1982).

Unfortunately, current population-based clinical screening lacks efficacy (Nduaguba and Lee, 2006). However, if individuals with a genetic predisposition for developing OAG can be identified, then efficient and cost-effective population-based screening programs could be designed. The impact of glaucoma treatment in minimizing visual disability is hampered by the inability to distinguish between early stage cases (or "glaucoma suspects") at high and low risk of progression to severe disease. This can lead to delayed diagnosis and under-treatment of high-risk cases with poor outcomes for those individuals, and over-treatment of low-risk cases with unnecessary cost and morbidity. Identifying important genetic or molecular markers for glaucoma will improve screening programs, and enable optimal use of limited resources.

Although the heritability of OAG as a complex disease is widely acknowledged, the underlying genes for the majority of cases are still unknown. Mutations in the *Myocilin* (*MYOC*) gene cause juvenile OAG, and a subset (~4%) of adult onset disease (Fingert et al., 1999; Stone et al., 1997). Mutations in *Optineurin* (*OPTN*) account for a small number of pedigrees with dominant OAG with generally low intraocular pressure (Alward et al., 2003; Aung et al., 2005; Sarfarazi and Rezaie, 2003). Together, established glaucoma-causing genes currently account for less than 5% of unselected OAG cases in our population. Other putative linked loci remain to be replicated (Hewitt et al., 2006b). Previous work indicates that the cause of at least 95% of OAG cases remains to be identified. This thesis explored the genotypic mechanisms and phenotypic features intimately involved in the glaucomatous process. There were two predominant sections of this thesis which constituted the bulk of the original work undertaken.

The first of these two major chapters, investigated the genotype-phenotype correlations in *MYOC*, a gene that has been unequivocally associated with OAG. Chapter 2 commenced with a comprehensive catalogue of *MYOC* variants, highlighting the strong genotype–phenotype correlations associated with specific myocilin mutations. This theme was then extended with the phenotypic description of an individual homozygous for the common Gln368STOP *MYOC* mutation.

A secondary theme of Chapter 2 explored the evidence for common founder effects for *MYOC* mutations. It was shown that the second most commonly identified *MYOC* variant in Australia, Thr377Met, has arisen at least three separate times worldwide. The final sections of this chapter directly explored the genotype-phenotype correlations in *MYOC*-related glaucoma. The phenotype of a pedigree with the

Gly252Arg *MYOC* mutation was described, and then the optic nerve head characteristics in people with *MYOC* mutations was investigated. *MYOC* mutation carriers were found to have a lower prevalence of optic disc haemorrhages; however, no other major structural or morphologic difference of the anterior optic nerve head was detected, when compared to individuals with non-*MYOC* glaucoma. This novel finding may generally reflect the association between *MYOC*-related glaucoma and elevated IOP. Armed with this information, we then showed that no structural differences of the optic nerve head are evident in young people known to carry myocilin mutations, but who do not have manifest glaucoma. Our work on *MYOC* generally supports that of others, and shows 1) that it is possible to accurately dissect out or refine genotypic as well as phenotypic sub-types of glaucoma; and 2) that genetic founder effects are likely to exist for a large proportion of monogenic OAG (Fingert et al., 2002).

The second major section of this thesis presented work investigating other genetic mechanisms in OAG. The Asp658Gly variant in the *Winged Domain 40- repeat 36* (*WDR36*) gene was found in a case-control study to be a neutral variant in the Australian population. Meta-analysis of the common *optineurin* (*OPTN*) Met98Lys variant, confirmed that its association with OAG, although weak, is highly statistically significant. Work is presented investigating the role of sequence variants in exon 1 of the *lysyl oxidase–like 1* (*LOXL1*) gene in a Caucasian population-based cohort. Replicating previous work (Thorleifsson et al., 2007), two nonsynonymous variants in exon 1 of *LOXL1* (Arg141Leu;Gly153Asp) were found to be strongly associated with pseudoexfoliative glaucoma.

The final section of Chapter 5 described the use of equimolar DNA pools in a case-control genome-wide association study which identified novel putative genetic risk variants for OAG on chromosomes 3q21, 6p25, 14q13 and Xq25. To an extent, our technique of using equimolar DNA pooling was well validated in a separate study, where we were easily able to identify a strong association between markers at the *complement factor H* locus and age-related macular degeneration. Clearly further work is required before the association of variants at these novel OAG loci are definitively proven. Replication in additional disease cohorts, in addition to functional molecular studies, will remain essential facets of such work. Despite being labour-intensive, there is a very high chance that novel genes accounting for the major common genetic contribution to many inherited human diseases could be uncovered relatively cheaply using equimolar DNA pooling.

Nestled between these major sections, were two chapters investigating the role of glaucoma as a systemic disease and the biometric associations of advanced OAG. In a large cohort, comprising mortality data from over 27,000 people of whom 741 were known to have OAG, we identified a statistically significant association between death due to ischaemic heart disease and OAG. Following adjustment for the age at death and male gender, the odds ratio for death due to ischaemic heart disease in people with OAG was significant (OR=1.30, 95%CI: 1.08 - 1.56; p=0.006). Also in Chapter 3, work investigating the association between systemic disease and familial and sporadic forms of OAG was presented. OAG cases from the Glaucoma Inheritance Study in Tasmania were categorised into familial (n=1012, 59.5%) and sporadic (n=688, 40.5%) subgroups based on comprehensive genealogical screening. We identified a significant difference for a past history of migraine or presence of atherosclerosis between patients with familial forms of OAG compared to people

with sporadic disease. After adjustment for age at review, sex and disease severity, the OR for familial OAG and migraine was 1.67 (95%CI: 1.15 - 2.42). No association for hypertension, diabetes, Raynaud's phenomenon, thyroid disease, corticosteroids use or cigarette smoking was identified. Taken together these results imply that there are important systemic differences between familial and sporadic disease.

In Chapter 4 the role of central corneal thickness as a risk factor for disease progression in a cohort of patients with advanced OAG was documented. This work was based on the premise that by virtue of severe visual field loss, patients must have had significantly progressive disease. A thin central cornea was found to be a significant risk factor for glaucoma progression, with a central corneal thickness below 500μm conferring an OR of approximately 6.4 (95%CI: 3.9-10.3) for advanced visual field loss. These results suggest that central corneal measurement should be included as a routine part of the examination in all patients with OAG, not solely in the screening of patients with ocular hypertension. Our strategy of concentrating analysis on the cases with definitive end-stage glaucomatous visual field loss was chosen to maximize the probability of identifying strong clinically relevant associations. A separate study included in this chapter investigated the role of automatic optic disc imaging devices in the phenotypic documentation of OAG. It was found that the Stratus optical coherence tomography (OCT) retinal nerve fibre layer quadrant average scan, as well as the Moorfields Regression Analysis on Heidelberg Retinal Topography II and Heidelberg Retinal Topography 3 systems were equivalent, but not entirely sensitive, compared to the OCT clock hour analysis, in detecting advanced glaucoma. These novel findings may have important ramifications for phenotype-based disease screening programs.

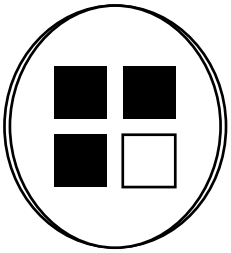
Chapter 6 of this thesis sought to identify the principal heritable components of visible optic nerve head structures in a population-based sample of twins. In determining the genetic components of the optic nerve head, the results of this study suggest that the shape and size of the optic disc and cup are more heritable and should receive a greater priority for quantification than vascular features. Further to this, epigenetic variation is likely to cause minor asymmetry of the anterior optic nerve head. Determining the genetic and environmental variants that influence optic disc morphology will allow for the elucidation of the molecular pathogenesis of diseases that alter optic nerve architecture, such as OAG.

The next step for glaucoma genetics:

As genetic testing for glaucoma becomes more readily available, the differentiation between non-impairing polymorphisms and disease-causing variants becomes more clinically relevant (Stone 2003). Clinical outcome studies are required to correlate specific disease-causing variants with the phenotype, thereby bridging the clinician to the laboratory (Stone 2003).

Accurate phenotypic descriptions, when compiled with relevant genetic information should enhance clinicians' understanding of the specific natural history of an individual patient's disease. Additionally, the ability to identify individuals with a genetic predisposition for developing OAG would allow efficient, cost-effective population-based screening programs to be designed. The large-scale assimilation of relevant data is essential for accurate comprehensive genetic counselling and the translation of genomic information back into the clinic. Glaucoma is a complex heterogeneous disease and, in time, the principal pathological mechanisms will be fully dissected. The establishment of a prospective series of people referred for tertiary review for signs suggestive of glaucoma would be extremely worthwhile. In order to fully close the research loop of the molecular studies investigating the Primary open angle glaucomas, it is of paramount importance to optimize screening of "glaucoma suspects".

Appendices



THE GENETIC STUDY OF ADVANCED GLAUCOMA

Demographic Information

X1	*(IDnum)*	*_____ ID number*
X2	*(surname)*	*Surname _____*
X3	*(firstname)*	*First Name (s) _____*
X4	*(DOB)*	*What is your date of birth? _____ / _____ / _____*
X5	*(address)*	*Your address? _____*
X6	*(post)*	*Postcode _____*
X7	*(phone)*	*Your phone number? _____*
X8	*(rels)*	*Do you have any relatives living in Tasmania?* *yes …1 no … 2* *If yes please list their names and address:* *1. Name _____* *Address _____* *2. Name _____* *Address _____* *3. Name _____* *Address _____*

Spouse's name …….

Your father's name.....date of birth...../.../.....Place.....

Your father's father's name.....date of birth..../..../.....Place.....

Your father's mother's full name.....date of birth...../..../.....Place.....
(and maiden name)

Your mother's name.....date of birth...../..../.....Place.....
(and maiden name)

Your mother's father's name.....date of birth...../..../.....Place.....

Your mother's mother's full name.....date of birth...../..../.....Place.....
(and maiden name).

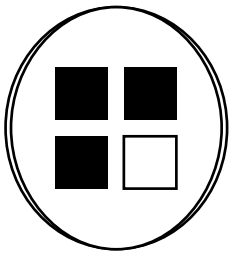
Other Relatives affected with glaucoma (please note if deceased)

Name.....Relationship.....Address.....

Name.....Relationship.....Address.....

Name.....Relationship.....Address.....

Please Draw the family tree:



THE GENETIC STUDY OF ADVANCED GLAUCOMA
Interview based Questionnaire

A1	*(IDnum)*	_____ *ID number*
A2	*(date)*	*Exam date* _____ / _____ / _____ *Time* _____
A3	*(gender)*	*Gender: Female ….. 1 Male ….. 2*
A4	*(age)*	*Age* _____ *yrs*
A5	*(eyedr)*	*Who is your eye doctor?* _____
A6	*(GP)*	*Who is your GP?* _____
A7		*How did you hear about this study?* _____
B1	*(birthpl)*	*Where were you born?* _____ *(write state or territory if in Australia, country if born overseas)*
B2	*(ageAust)*	*If not born in Australia, how old were you when you came to Australia?* ___*yrs*
B3	*(spoken)*	*What language(s) do you speak?* _____

Family History: Have your parents or your brothers or sisters or children had:

		glaucoma *(tick or list number if relevant)*	*cataract*	*macular degeneration*	*blind*	*other (turned eye)*
G1	*Mother*					
G2	*Father*					
G3	*Brothers*					
G4	*Sisters*					
G5	*Natural Children*					

G6		*Is your father still alive? yes …… 1 no ……. 2 don't know …… 8*
G7		*How old was he when he died? _____ yrs*
G8		*Is your mother still alive? yes …… 1 no ……. 2 don't know …… 8*
G9		*If no, how old was she when she died? _____ yrs*

Employment History

C1	*(qualif)*	*After leaving school did you get any other qualifications or learn a trade?* *yes …….. 1* *no …….. 2 goto C3* *don't know ..... 8 goto C3*
C2	*(qualnm)*	*Qualification name _____*
C3	*(jobstat)*	*Are you still employed?* *houseduties …… 1 medical disability …….. 2* *retired ……..…….. 3 unemployed …..…..…… 4* *employed ……..……..… 5 other_____*
C4	*(retired)*	*If retired, how old were you when you retired? _____ yrs don't know …-99*
C5	*(presjob)*	*If employed, what is your present occupation? _____*
C6	*(mainjob)*	*In your working life what has been your main job?* *_____*
C7	*(othjobs)*	*Could you list other jobs you have had? (for ≥ 5yrs) what age?* *_____ _____* *_____ _____* *_____ _____*
D1	*(marital)*	*What is your marital status?* *never married ……..…. 1 divorced ……..…….. 4* *now married ….……..….. 2 widowed ….…..…..…… 5* *separated but not divorced … 3*

If now married or widowed:

D2 *(spoujob)* *What kind of work does / did your spouse do for most of his / her life?*

_____

D3 *(pension)* *Do you receive a blind pension?*

yes …….. 1 *no …….. 2*

don't know 8

D4 *(abode)* *What sort of a place do you live in?*

own house …… 1 *boarding house …….. 6*

own flat …….. 2 *nursing home ……. 7*

rent house…….. 3 *with relatives …….. 8*

rent flat …….. 4 *caravan ……..…….. 9*

hostel ……..…. 5 *other* _____

D5 *(wholive1)* *Does anybody lives with you?*

nobody …….. 1 *daughter …….. 4*

spouse …….. 2 *son ……..…….. 5*

friend ……... 3 *other* _____

D6 *Do you get regular help at home from the following?*

(MOW) *Meals on wheels* *yes ….. 1 no …. 2*

(nurse) *community nurse* *yes ….. 1 no …. 2*

(hmhelp) *home help* *yes ….. 1 no …. 2*

(othhelp) *other* _____

D7 *(dinner)* *Who usually cooks your main meal?*

you …….. 1 *other relative …….. 5*

spouse …….. 2 *friend ……..…….. 6*

daughter …….. 3 *Meals on wheels …… 7*

son …….. 4 *other* _____

D8 *(cleanhs)* *Who usually cleans your house?*

you …….. 1 *other relative …….. 5*

spouse …….. 2 *friend ……..…….. 6*

daughter …….. 3 *home help …..…….. 7*

son …….. 4 *other* _____

D9 *(whoshop)* *Who usually does your shopping?*

you …….. 1 *other relative …….. 5*

spouse …….. 2 *friend ……..…….. 6*

daughter …….. 3

son …….. 4 *other* _____

D10	*(drive)*	*Have you ever driven a car regularly?* *yes, still driving ….. 1 goto D23* *yes, stopped …….. 2 goto D21* *never driven …..….. 3 goto D27*
D11	*(carlast)*	*How long ago did you stop driving? _____ yrs*
D12	*(carvis)*	*Did you stop because of problems with your vision?* *yes ……..….. 1* *no ……...….. 2 don't know ….. 8*
D13	*(accident)*	*Have you had any car accidents in the last year?* *yes ……..….. visual contribution yes … 1 no … 2 don't know … 3* *no ……...….. 4* *don't know ….. 8*
D14	*(accidNo)*	*If yes, how many accidents? _____*
D15	*(drability)*	*Do you think your driving ability is as good now as it used to be?* *yes ……..….. 1* *no ……...….. 2 don't know ….. 8*
D16	*(drabilityvis)*	*If no, do you think this might be related to your vision?* *yes ……..….. 1* *no ……...….. 2 don't know ….. 8*
		Are you able to:
D17	*(seefar)*	*recognise a friend across the street?* *yes ……. 1 no ……. 2 don't know ….. 8*
D18	*(seenear)*	*recognise a friend in close proximity to you?* *yes ……. 1 no ……. 2 don't know ….. 8*
D19	*(seeTV)*	*recognise detail on TV?* *yes ……. 1 no ……. 2 don't know ….. 8*
D20	*(readnewsp)*	*Can you read the ordinary print in the newspaper reasonably well, with or without reading glasses?* *yes ……. 1 no ……. 2 don't know ….. 8*
D21	*(lastrd)*	*If no, when were you last able to do this? _____*
D22	*(magnif)*	*Do you need to use a magnifier to read a newspaper?* *yes ……. 1 no ……. 2 don't know ….. 8*
D23	*(hearing)*	*Have you ever had a problem with your hearing?* *yes ……..….. 1* *no ……...….. 2 don't know ….. 8*
D24	*(hearsev)*	*If yes, is it?* *mild ….…….. 1 moderate ….. 2 severe ……. 3*
D25		*If yes, which ear? left …. 1 right …. 2 both …. 3*

Risk Factor Questions

Many environmental factors have been linked to eye disease I would like to ask you about them …

E1	*(smoking)*	*Have you ever smoked cigarettes, cigars, or a pipe regularly?* *yes …….. 1* *no ……..….. 2 goto E6* *don't know ….. 8 goto E6*
E2	*(smokyrs)*	*How long have / did you smoke for? _____ yrs*
E3	*(smokecurr)*	*Have you given up smoking? yes ….. 1 no …. 2*
E4	*(smokecease)*	*When did you give up smoking? _____ yrs ago*
E5	*(smokeamount)*	*How much do you / did you smoke each day?* *_____ manufactured cigs/day* *_____ grams "hand-rolled"* *_____ cigars / week* *_____ grams pipe tobacco / week*
E6	*(cigspou)*	*Does or did your partner / husband / wife smoke?* *yes ……. 1* *no ….….. 2 don't know ….….. 8*
E7	*(tea)*	*How many cups of tea would you usually drink each day?* *_____ cups per day*
E8	*(Ttype)*	*How do you usually make your tea?* *tea bags ……..….. 1* *tea leaves ……...….. 2 don't know ……... 8*
E9	*(coffee)*	*How many cups of coffee would you usually drink each day?* *_____ cups per day*
E10	*(Ctype)*	*How do you usually make your coffee?* *instant ……..….. 1* *percolated …...….. 2 don't know ……..... 8*
E11	*(pastcaf)*	*About how many cups of coffee or tea did you drink per day 10 years ago? Tea - number of cups _____* *Coffee - number of cups _____*
E12		*Do you mostly eat butter or margarine?* *only butter……..…….……..... 1* *mostly butter, but some margarine …. 2* *equal amounts of both .....…… 3* *mostly margarine, but some butter..... 4* *only margarine…….…..... 5*

E13		*How often each week would you eat leafy green vegetables (like spinach, silver beet)?* *_____ per week (on average)*
E14		*What is your favourite food?* *_____*
E15	*(alcohol)*	*How many days a week would you usually have an alcoholic drink now?* *never ……..…….. 1* *3-4 days a week …. 4* *< once a week ..…. 2* *5-6 days a week …. 5* *1-2 days a week …. 3* *every day …….…..… 6*
E16	*(alctype)*	*What do you mostly drink?* *light beer ……. 1* *spirit …..……..….. 4* *beer …..……..….. 2* *other _____* *wine …..……..….. 3*
E17	*(alcnum)*	*On the days when you have a drink, how many do you usually have? _____*
E18	*(alcmore)*	*Has there been a period in your life when you drank quite a bit more than you do now?* *yes …….….. 1* *no ……...….. 2* *don't know ….. 8*
E19	*(alcstop)*	*If yes, how many years ago was that? _____ yrs*
F1	*(colour)*	*As a teenager what was the natural colour of your hair? Was it:* *blonde ……. 1* *black ……...….. 4* *red ….....…….. 2* *other _____* *brown ……... 3* *don't know ….. 8*
F2	*(burn)*	*In the past, when your skin was exposed to the summer sun, did it?* *always burn, never tan ……..….. 1* *usually burn, tan with difficulty ……. 2* *burn and tan about average ….…….. 3* *rarely burn, tan above average ….... 4* *don't know ……..…..... 8*
F3	*(burnno)*	*How many bad sunburns, that is with soreness lasting more than a day, would you estimate you have had during your life, including childhood?* *none …….. 1* *> 10 ……...….. 4* *one ….…….. 2* *don't know ….. 8* *2 - 10 …….. 3*
F4	*(skincol)*	*What is your natural, non-exposed skin colour?* *very fair ….....…….. 1* *dark olive …….. 4* *fair ……....…….. 2* *brown …….…….. 5* *light olive ….....….... 3* *black ….…….. 6*

General Medical History

Now I would like to ask you a few questions about your medical

M1	*(hospadm)*	*Have you had any admissions to a hospital in the last 12 months?*
		_____ times don't know …. -88
M2		*If so, what for ... which hospital …*
		1._____
		2._____
		Has a doctor ever said that you have any of the following conditions?
M3		*high blood pressure yes / no / don't know age diagnosed _____*
M4		*angina yes / no / don't know age diagnosed _____ (chest pain from your heart)*
M5		*heart attack yes / no / don't know age diagnosed _____ (a coronary, myocardial infarct)*
M6		*stroke yes / no / don't know age diagnosed _____*
M7		*high cholesterol yes / no / don't know age diagnosed_____*
M8		*diabetes yes / no / don't know age diagnosed_____ (sugar in your urine or high blood sugar)*
M9		*treated with 1) diet alone? _____yrs 2) tablets? _____yrs*
		3) insulin? _____yrs
M10		*cancer yes / no / don't know age diagnosed _____*
M11		*What type of cancer?_____*
M12		*Treated with: surgery ……..... 1 no treatment ….. 4*
		radiation ……..... 2 don't know ……... 5
		Chemotherapy .. 3
M13		*sunspots or skin cancer yes / no / don't know age diagnosed _____ (treated by freezing or surgery)*
M14		*thyroid condition yes / no / don't know age diagnosed _____*
M15		*Treated with: radioactive iodine ……..... 1*
		surgery ……...……..... 2
		thyroxine tablets …….... 3
M16		*asthma yes / no / don't know age diagnosed _____*
M17		*arthritis yes / no / don't know age diagnosed _____*
M18		*What type: osteoarthritis ……..... 1 Other_____*
		rheumatoid …….... 2
M19		*gout yes / no / don't know age diagnosed_____*

M20	*Have you ever needed a blood transfusion?* *yes … 1 no … 2 possible …. 3 don't know …. 8*
M21	*Details* _____
M22	*Do you regularly get cold hands or feet? yes / no / don't know*
M23	*Have you ever had migraine headaches?* *(severe headaches, usually on one side, may have changes in vision like zigzag lines or blurring, often nausea or vomiting and usually need to lie down with the lights off)* *yes, typical …..…. 1 if yes, age started _____ yrs* *yes, but atypical .. 2 age stopped _____ yrs* *no …….. 3 don't know ……... 8*
	Have you had any other serious or major illnesses or operations?
M24	*1.* _____
M25	*2.* _____
M26	*3.* _____
M27	*4.* _____
	Would you please list the medication / drugs you are taking? *Name of drug approximate period*
M28	*1.* _____|_____
M29	*2.* _____|_____
M30	*3.* _____|_____
M31	*4.* _____|_____
M32	*5.* _____|_____
M33	*6.* _____|_____
M34	*7.* _____|_____
M35	*8.* _____|_____
M36	*9.* _____|_____
M37	*10.* _____|_____
	Are you allergic to anything?
M38	*1.* _____|_____
M39	*2.* _____|_____

M40	*Over the past year about how often have you taken an aspirin tablet?* *(Solprin, Disprin, Ecotrin or Cardiprin, but not Panadol, Dymadon or Digesic)* *Was it: less than once a month …… 1 goto M43* *more than once a month but less than once a week … … 2 goto M43* *once a week or more ….…… 3 goto M42*
M41	*How many aspirin tablets do you usually take each week? ____*
M42	*Is that…...: more than …… 1 less than …...… 3* *the same …...… 2 don't know …… 8* *….. the number of aspirins you were taking 10 years ago?*
M43	*Have you ever taken steroid tablets such as Prednisone, Cortisone or Decadron for asthma, arthritis, or another condition for longer than one month altogether?* *yes … 1 no … 2 don't know …. 8*
M44	*If yes, about how long altogether were you taking these tablets?* *_____ weeks _____ months _____ years*
M45	*What dose were you taking? _____*
M46	*What condition did you take them for? _____*
M47	*Have you ever used a Becotide puffer, a brown coloured puffer in asthma and other chest problems?* *yes … 1 no … 2 don't know …. 8*
M48	*If yes, how often do / did you need to take it? puffs _____ (per week/month)*
M49	*During the past 12 months, have you had any falls where you have landed on the ground or floor? Number of falls* *_____*
M50	*If fallen, how many of these falls do you think were due to problems with you vision? Number of falls* *_____*

Female Medical History (females only)

Now I would like to ask you a few questions about your menstrual history

W1	*How old were you when you started having periods? _____ yrs*
W2	*Have you stopped having periods?* *yes … 1 no … 2 don't know …. 8*
W3	*If yes, how old were you when you stopped? _____ yrs*
W4	*Have you had a hysterectomy, that is, an operation to remove the uterus?* *yes … 1 no … 2 don't know …. 8*
W5	*if yes at what age? _____ yrs*
W6	*Where both ovaries removed?* *yes … 1 no … 2 don't know …. 8*
W7	*Did your periods stop naturally or because of a hysterectomy?* *naturally …… 1 hysterectomy …….. 2* *other …..…… 3 don't know ……..... 8*
W8	*Have you ever been on hormone replacement therapy such as oestrogens and or progesterones for menopausal symptoms or after the menopause?* *yes … 1 no … 2 don't know …. 8*
W9	*Have you ever taken the oral contraceptive pills for birth control or other medical reasons?* *yes … 1 no … 2 don't know …. 8*
W10	*Have you ever been pregnant?* *yes ….. 1 _____ times* *no ……... 2 don't know …….. 8*
W11	*Of these pregnancies, how many children have you had?* _____

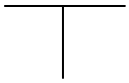
Past Ocular History

And finally I would like to ask you a few questions about your eyes

O1	*Are you using any eye drops at present? yes … 1 no … 2 don't know …. 8 Drug name how long have you been taking these?*
O2	*1. _____|_____*
O3	*2. _____|_____*
O4	*3. _____|_____*
O5	*4. _____|_____*
O6	*Any steroid eye drops? yes … 1 no … 2 don't know …. 8*
O7	*Have you been told that you have glaucoma?* *yes … 1 no … 2 don't know …. 8*
O8	*How many years ago were you first told? _____ yrs _____ age*
O9	*How was your glaucoma diagnosed?* _____ _____
O10	*Have you ever used eye drops or medications for glaucoma?* *yes … 1 no … 2 don't know …. 8*
O11	*Have you ever had an operation or laser treatment for glaucoma?* *yes … 1 no … 2 don't know …. 8*
O12	*Details* _____ _____
O13	*What is the highest pressure in your right eye _____ left eye _____ ?*
O14	*Have you ever been told that you have macular degeneration, sometimes called 'hardening of the arteries at the back of the eye' or 'degeneration of the retina'?* *yes … 1 no … 2 don't know …. 8*
O15	*How many years ago were you first told? _____ yrs _____ age*
O16	*If yes, have you had laser treatment for macular degeneration?* *yes … 1 no … 2 don't know …. 8*
O17	*Details* _____ _____

O18	*Have you ever been told that you have cataracts?* *yes … 1 no … 2 don't know …. 8*
O19	*How many years ago were you first told? _____ yrs _____ age*
O20	*If yes have you ever had a cataract operation?* *yes … 1 no … 2 don't know …. 8*
O21	*Details* _____ _____
O22	*Have you ever had YAG laser to improve your vision after cataract surgery?* *yes … 1 no … 2 don't know …. 8*
O23	*Details* _____ _____
O24	*Have you ever been told you have a problem in the retina or the back of the eye?* *like retinal detachment or vessel blockage or bleeding?* *yes … 1 no … 2 don't know …. 8*
O25	*How many years ago were you first told? _____ yrs _____ age*
O26	*Details* _____ _____
O27	*Have you ever had any serious eye injury requiring doctor's care?* *yes … 1 no … 2 don't know …. 8*
O28	*Details* _____ _____
O29	*Do you have any other eye problems or eye surgery that I haven't asked you about?* *1. Details* _____ _____
O30	*2. Details* _____ _____

Participant Examination Data Sheet

Predilation Exam:	***Right***	***Left***	**Tick when done:**
Acuity:	*s*		**Hx**
	⊂		**VA**
Refraction: *Distance*			**CCT**
and/or Readers			**IOP**
Keratometry *H*			**Blood**
V			**Photo**
Pachymetry			**HVF** (1st Ever)
Axial Length			**HVF** (Most recent)
IOP Time:			**Letter**
Anterior Segment			

Dilated Exam:	***Right***	***Left***
Cup/Disc ratio		
Fundus		
Disc Size (S,M,L)		
Other Disease		

1st Ever Reliable HVF	***Right***	***Left***
Date:		
MD		
5o PSD <0.5%	*/4*	*/4*

Most Recent HVF	***Right***	***Left***
Date:		
MD		
5o PSD <0.5%	*/4*	*/4*

GLAUCOMA SUBGROUP:

- ☐ *High Tension*
- ☐ *Normal Tension*
- ☐ *PDS*
- ☐ *PXF*
- ☐ *JOAG*
- ☐ *COAG / PIG*
- ☐ *Other* _____

Exclusion Criteria:
Signs of:

- ☐ Primary Angle Closure
- ☐ Secondary Glaucoma due to:
  - ☐ Aphakia
  - ☐ Rubella
  - ☐ Neovascularisation
  - ☐ Inflammation

☐ **Progressor**

- ☐ **Lost to follow-up**
- ☐ **Progressed despite Max Rx**

☐ **Presented with Severe Glc**

Participant Information Sheet

THE GENETIC STUDY OF ADVANCED GLAUCOMA

Department of Ophthalmology, Flinders University (08) 8404 2035

Eye.Genetics@fmc.sa.gov.au

This is a research project, and you do not have to be involved. If you do not wish to participate, your medical care will not be affected in any way. It does not involve alteration to treatment which would be continued by your own ophthalmologist.

This study is looking for people with severe glaucoma to find genes that cause glaucoma. We are inviting individuals who are affected with severe glaucoma to be involved in the study. Additionally, we are recruiting people who are of a similar age, but ***do not*** *have glaucoma. This is at no cost to you. We would only need to see you once for a full glaucoma examination, and the taking of a blood sample. The blood sample will be used to extract DNA which will be stored for analysis. Comparing the DNA from people with severe glaucoma to those who do not have glaucoma will help us in finding genes that may cause severe glaucoma. You are under no obligation to provide this and it may not carry any direct benefit to you.*

If you choose to participate, you will be given:

- *An explanation of the eye examination procedures.*
- *An explanation of any technical terms used.*
- *A written record of the results of your testing.*

Whilst there may be no direct benefit to you from this study, our hope is that it will provide information about risk factors for glaucoma. This will help us to better treat patients in the future*.*

During this study many potential glaucoma genes (some of which may currently be unknown) will be looked at. This will be achieved in the following way: a blood sample will be taken from your arm. DNA (the genetic material) will be extracted from the blood and stored for analysis. Genes will be examined in the DNA. Genes from patients with glaucoma will be compared to those without glaucoma looking for differences. At this stage we don't know what we will find. If we find differences in some genes, then this could lead to new or improved methods of detecting and treating glaucoma. You will be informed and counselled about any important results which directly affect you. Your views on this feedback will also be sought.

Apart from the blood test for DNA extraction, the other tests will be those that form part of a routine eye examination for glaucoma. This will include:

1) Administration of a Topical Anaesthetic Eye-drop and Pupil Dilating Eye-drop.
This is so we can measure the eye pressure and look at the optic nerve.

2) Measurement of Central Corneal Thickness by Contact Ultrasound Pachymetry
This is a very rapid test involving brief touching of a probe to the cornea after the anaesthetic eye drop so you won't feel it. This measurement is now thought to be important in glaucoma.

3) Measurement of Intraocular Pressure.
High Intraocular pressure is a major risk factor for glaucoma. You will have had this measured on many occasions.

4) Photographic images of the of the Optic Nerve will be taken.
This is to create a permanent record of the optic nerve appearance so we can do measurements without having to see you again.

The blood test is just like any other standard blood test from the arm that you will have had in the past. There is a small risk of bruising and discomfort when having a blood sample taken. The eye drops used are exactly the same as those used in standard full eye examinations. These may briefly feel uncomfortable and a very small proportion of people are allergic to these drops. If you know of such an allergy, then no drops would be given. If you, as a participant of this research, suffer injury, compensation may, at the discretion of Flinders Eye Centre, be paid without litigation. However, compensation is not automatic and you may have to take legal action in order to receive payment.

The DNA will be stored at Flinders University. You should be aware that the research team intends to publish the results of this study in medical journals, but that no participant would be identified. Our research team has been successful in securing funding for research at the highest Government funded levels, and prefers to publish all our results in medical and scientific journals. The information from the study will belong solely to the investigators and their collaborators. It may become necessary in the future to involve commercial partners to fund our work, and this may in turn lead to some information being used for commercial development and the generation of profit.

Flinders University and their collaborators will own the results of any scientific development. You may withdraw from the study at any time, without prejudice, and have your sample destroyed.

Should you require further details about the project before, during or after the study, please contact Dr Jamie Craig at the Flinders Eye Centre: Tel (08) 8404 2035. This study has been reviewed by the Flinders Clinical Research Ethics Committee. Should you wish to discuss the project with someone not directly involved, in particular in relation to matters concerning policies, your rights as a participant, or should you wish to make a confidential complaint, you may contact the Administrative Officer - Research, Ms. Carol Hakof: Tel (08) 8204 4507.

Department of Ophthalmology, Flinders University (08) 8404 2035

Eye.Genetics@fmc.sa.gov.au

WHAT IS GLAUCOMA?

Glaucoma is an eye disorder where the pressure in the eye is too high for the safety of the optic nerve. If left untreated, this pressure can cause damage to the nerve at the back of the eye and result in loss of peripheral (side) vision. If this loss of peripheral vision progresses, people may no longer be able to hold a driver's licence. Late in the progression of glaucoma, central vision may be lost as well. Once vision is lost through glaucoma, it can never be restored.

Glaucoma can be treated by lowering the pressure in the eyes. If the pressure is lowered adequately further visual loss is slowed. This is usually done with eye drops, but sometimes laser or surgery is required.

Early diagnosis and treatment is therefore essential in preventing visual loss from glaucoma. Large studies show that half the people in Australia who have glaucoma are unaware of the condition, because there are usually no symptoms. This is why glaucoma is often called "the sneak thief of sight". Many of these people have relatives with glaucoma.

Ophthalmologists (eye specialists) diagnose glaucoma with three tests: a test of eye pressure to see if it is raised, a test of the visual field to measure the amount (if any) of peripheral vision that has been lost, and signs of "cupping" or damage to the optic nerve.

In the later stages, glaucoma is relatively easily diagnosed, however it is far more difficult in the early stages. Thus, many people in the early stages are described as "glaucoma suspects." Given glaucoma is only slowly progressive and that its treatment with drops and surgery carries some risks of side effects, ophthalmologists do not usually commence treatment until the diagnosis is certain.

RESEARCH INTO GLAUCOMA

Recent research has shown that in many circumstances glaucoma is a familial disease. However, this is often not clear-cut and we are still learning more about the genetics of glaucoma. Only a small number of genes have been identified. The first glaucoma gene, called GLCIA or Myocilin (it has also been called TIGR), was identified in 1997. When affected people have a faulty copy of this gene there is a 50% chance that they pass onto their children.

This study will compare gene changes between people who have very severe glaucoma and those who do not. This will hopefully allow the identification of new genes which cause glaucoma. Results from this study will be published and we will provide you with a copy of these publications in the future if you wish. You would not be identified in these scientific publications.

REACTIONS TO KNOWING YOUR TEST RESULTS

Before giving you the relevant results of your personal DNA testing, we need to inform you of some of the issues involved in knowing the results.

Medical research has now been able to identify the gene responsible in a number of diseases, and has gained some experience in passing on the findings to the family members who have been tested for the gene. Previously, most genetic testing involved serious diseases where

there has been no effective treatment, and some people did not want to know the results of their tests. Many guidelines have been developed using this experience.

In this study, we will look at genes already known to cause glaucoma (myocilin, optineurin and WDR36). If we find a change in any of these genes, this may be useful information, and you may choose to know the results. We will also be looking for new genes for glaucoma and it may take some years before all the results of this study are known. We may never find any changes in some participants.

It is important to remember that ***glaucoma is not life-threatening,*** *and can be treated to prevent or delay visual loss. Early diagnosis is a good thing.*

CHOOSING TO KNOW YOUR TEST RESULTS - IF YOU CARRY A CHANGE IN A KNOWN GLAUCOMA GENE

People who already have glaucoma and are told they carry the abnormal gene will have confirmation of something they already suspected. People who were "glaucoma suspects" or were "normal" and are told they carry this gene will know they are at high risk of progressing to glaucoma. We do not yet know if all people with the abnormal gene will get glaucoma, but we currently recommend that they are screened regularly, at least once a year if they are over 18 years of age.

Treatment is likely to be recommended at the first signs of glaucoma, and the treatment given will depend on the ophthalmologist who is treating you.

We will be happy to discuss the research and the results of your tests with your doctors.

CHOOSING TO KNOW YOUR TEST RESULTS - IF YOU DO NOT CARRY A CHANGE IN A KNOWN GLAUCOMA GENE

We already know that the causative genes for the majority of glaucoma cases are not yet identified. One of the reasons for doing this study is to try to identify new genes that can cause severe glaucoma. This may be of benefit in the future to help prevent severe glaucoma.

CHOOSING NOT TO KNOW YOUR TEST RESULTS

If you decide not to be told the results of your testing, it will not change your treatment in any way.

BENEFITS OF KNOWING YOUR TEST RESULTS

If there is a change identified in a specific glaucoma gene, there may be benefits in the future in determining the best kinds of treatment for specific cases. There may also be benefits which arise in terms of possible testing for other family members in the future. This could help to determine who is at highest risk.

At present, there are usually no obvious disadvantages in terms of lifestyle or in terms of insurances (Life, Disability or Health), but in future this situation may change and individuals known to carry the abnormal gene may be subject to policy restrictions.

Please feel free to ask any questions. We will endeavour to provide any further information you would like. Please contact A/Prof Jamie Craig for further information on (08) 8404 2035.

FLINDERS MEDICAL CENTRE
FLINDERS UNIVERSITY OF SOUTH AUSTRALIA

Consent for Participation in Research

I, _____ *request and give consent to my involvement in the research project:*

first or given names *surname*

The Genetic Study of Advanced Glaucoma

I acknowledge that the nature, purpose and contemplated effects of the research project, especially as far as they affect me and have been explained to my satisfactio

_____ *and my consent is given voluntarily.*

first or given names *surname*

I acknowledge that the details of the following procedures:

1) Administration of a Topical Anaesthetic Eye-drop and Pupil Dilating Eye-drop
2) Measurement of Central Corneal Thickness by Contact Ultrasound Pachymetry
3) Measurement of Intraocular Pressure
4) Photography of the Optic Nerve
5) Collection of a Blood Sample for DNA Testing

have been explained to me, including indications of risks; any discomfort involved; anticipation of length of time and the frequency with which the procedure will be performed.

I have understood and am satisfied with the explanations that I have been given.
I have been provided with a written information sheet.
I understand that my involvement in this research project and the procedure may not be of any direct benefit to me and that I may withdraw my consent at any stage without affecting my rights or the responsibilities of the researchers in any respect.
I give consent for my medical records to be reviewed by this research team.
I acknowledge that I have been informed that should I receive an injury as a result of taking part in this study, I may need to start legal action in order to receive compensation.
I declare that I am over the age of 18 years.

I ***would / would not*** *like to be contacted if my results from this study would be useful for myself or my family.*

Signature of research participant: _____ *Date:* _____

Signature of Witness: _____

Printed Name of Witness: _____

I, _____ *have described to* _____

the research project and the nature and effects of the procedures involved. In my opinion he/she understands the explanation and has freely given his/her consent.

Signature: _____ *Date:* _____

Status in project: _____

Case Examination Feedback Sheet

THE GENETIC STUDY OF ADVANCED GLAUCOMA

University of Flinders
Department of Ophthalmology
Flinders Medical Centre
Bedford Dve
Bedford Park, 5042
South Australia
Phone: (08) 8404 2035
Email: Eye.Genetics@fmc.sa.gov.au

Dear Doctor.....

Your patient.....

Date ……./……../……..

was involved in the Genetic Study of Advanced Glaucoma.

On examination today the findings were:

	Right	Left
Visual Acuity	.....	.....
Visual Field	.....	.....
Intraocular Pressure	.....	.....
Central Corneal Thickness	.....	.....
Optic Disc	.....	.....

This study is **NOT** involved in the ongoing management of the patients, but we are happy to provide any details to treating doctors should this be required.

Yours Sincerely,

A/Prof Jamie Craig

Control Examination Feedback Sheet

THE GENETIC STUDY OF ADVANCED GLAUCOMA

University of Flinders
Department of Ophthalmology
Flinders Medical Centre
Bedford Dve
Bedford Park, 5042
South Australia
Phone: (08) 8404 2035
Email: Eye.Genetics@fmc.sa.gov.au

Dear Doctor.....

Your patient.....

Date ……./……../……..

was involved in the Genetic Study of Advanced Glaucoma, as a **NORMAL CONTROL** subject in this study. We are hoping to determine risk factors for developing severe glaucoma. On examination today the findings were:

	Right	Left
Visual Acuity	.....	.....
Visual Field	.....	.....
Intraocular Pressure	.....	.....
Central Corneal Thickness	.....	.....
Optic Disc	.....	.....

In the opinion of the team your patient falls into the following category:

☐ 1. Appears to have glaucoma

☐ 2. Appears not to have glaucoma

☐ 3. Shows no signs of glaucoma at present, but is too young to say positively that they will not develop the disease later.

☐ 4. Shows some equivocal features, which may develop into glaucoma.

☐ 5. Has another eye disease that requires management

(detail: ……)

Patients who are too young (generally under 65 years) or have equivocal findings should be checked again by an ophthalmologist within 2 years. The opinion of the team does not necessarily indicate that treatment should be initiated, nor what form treatment should take. This study is not involved in the ongoing management of the patients, but will be happy to provide any details to treating doctors should this be required.

Yours Sincerely,

A/Prof Jamie Craig

DNA extraction from whole blood

Standard Protocol: Purification of DNA from Whole Blood Using the QIAamp Blood Maxi Kit (Spin Protocol)

QIAamp DNA Blood Maxi Kit	**(10)**	**(50)**
Catalog no.	**51192**	**51194**
Number of preps	**10**	**50**
QIAamp Maxi Spin Columns	10	50
Collection Tubes (50 ml)	10	50
Buffer AL*	126 ml	2 x 330 ml
Buffer AW1* (concentrate)	27 ml	121 ml
Buffer AW2 (concentrate)	17 ml	81 ml
Buffer AE	22 ml	110 ml
QIAGEN Protease	1 vial‡	5 vials‡

* Not compatible with disinfecting agents containing bleach. Contains a chaotropic salt..
† Resuspension volume 4.4 ml
‡ Resuspension volume 5.5 ml

This protocol is adapted for the purification of genomic DNA from up to 10 ml of whole blood.

Important points before starting

■ Do not use more than 1 x 10^8 white blood cells.

■ All centrifugation steps are carried out at room temperature (15–25°C). Do not use a fixed-angle rotor.

Things to do before starting

■ Equilibrate samples to room temperature (15–25°C) before starting.
■ Prepare a 70°C water bath for use in step 4 of the protocol.
■ Ensure that Buffer AW1, Buffer AW2, and QIAGEN Protease have been prepared.
■ If a precipitate has formed in Buffer AL, redissolve by incubating at 56°C.

Procedure

1. Pipet 500 µl QIAGEN Protease into the bottom of a 50 ml centrifuge tube.
2. Add 10 ml blood and mix briefly (most of our tubes contain 8ml).
Bring the volume of the sample up to 10 ml with PBS, if necessary, before adding to the centrifuge tube.
Note: QIAGEN Protease (or proteinase K) can be added to samples that have already been dispensed into centrifuge tubes. In this case, it is important to ensure proper mixing after adding the enzyme.
3. Add 10 ml Buffer AL, and mix thoroughly by inverting the tube 15 times, or vortexing for 3-4 seconds.
To ensure adequate lysis, the sample must be mixed thoroughly with Buffer AL to yield a homogenous solution.
Note: Do not add QIAGEN Protease directly to Buffer AL.

4. Incubate at 70°C for 10 min.
DNA yield reaches a maximum after lysis for 10 min at 70°C, but longer incubation times will not adversely affect yield.
5. Add 8 ml ethanol (96–100%) to the sample, and mix by inverting the tube 10 times, or vortexing for 3-4 seconds.
In order to ensure efficient binding, it is essential that the sample is mixed thoroughly after addition of ethanol to yield a homogeneous solution.
Note: Only use 96–100% ethanol. Other alcohols may result in reduced yield and purity. Do not use denatured alcohol, which contains other substances such as methanol or methylethylketone.
6. Carefully transfer one half of the solution from step 5 onto the QIAamp Maxi column placed in a 50 ml centrifuge tube (provided), taking care not to moisten the rim. Close the cap and centrifuge at 1850 x *g* for 3 min.
If more convenient, lysate can be loaded onto the QIAamp Maxi column up to the level of the top screw thread on the 50 ml centrifuge tube.
If the solution has not completely passed through the membrane, centrifuge again at a slightly higher speed.
Do not overtighten caps. If caps are tightened until they snap they may loosen during centrifugation and damage the centrifuge.
Note: Always hold the closed QIAamp Maxi columns in an upright position as liquid may pass through the ventilation slots on the rims of the columns even if caps are closed.
7. ● Remove the QIAamp Maxi column, discard the filtrate, and place the QIAamp Maxi column back into the 50 ml centrifuge tube. Load the remainder of the solution from step 5 onto the QIAamp Maxi column. Close the cap and centrifuge again at 1850 x *g* for 3 min.
Note: Wipe off any spillage from the thread of the 50 ml centrifuge tube before reinserting the QIAamp Maxi column.
Do not wet the rim of the QIAamp Maxi column. Close each column in order to avoid cross-contamination during centrifugation. If the solution has not completely passed through the membrane, centrifuge again at a slightly higher speed.
8. Remove the QIAamp Maxi column, discard the filtrate, and place the QIAamp Maxi column back into the 50 ml centrifuge tube.
Note: Wipe off any spillage from the thread of the 50 ml centrifuge tube before reinserting the QIAamp Maxi column.
If the filtrate is not removed, the nozzle of the QIAamp Maxi column will be submerged in the filtrate, and washing efficacy will be reduced.
9. Carefully, without moistening the rim, add 5 ml Buffer AW1 to the QIAamp Maxi column. Close the cap and centrifuge at 4000 x *g* for 2 min.
Note: Do not discard the flow-through at this stage. Continue directly with step 10.
10. Carefully, without moistening the rim, add 5 ml Buffer AW2 to the QIAamp Maxi column. Close the cap and centrifuge at 4000 x *g* for 20 min.
Note: The increased centrifugation time should remove all traces of Buffer AW2 from the QIAamp Maxi column before elution. If the centrifugal force is below 4000 x *g*, incubating the QIAamp Maxi column for 10 min at 70°C in an incubator to evaporate residual ethanol is recommended. Residual ethanol in the eluate may cause inhibition of PCR leading to false-negative results.
11. Place the QIAamp Maxi column in a clean 50 ml centrifuge tube (provided), and discard the collection tube containing the filtrate.
Note: Use a wet tissue paper to wipe any spillage off the QIAamp Maxi column before insertion into the 50 ml centrifuge tube.

12. Pipet 1 ml Buffer AE or distilled water, equilibrated to room temperature (15–25°C), directly onto the membrane of the QIAamp Maxi column and close the cap. Incubate at room temperature for 5 min, and centrifuge at 4000 x *g* for 4 min.

For long-term storage of DNA, eluting in Buffer AE and storing in aliquots at –20°C is recommended since DNA is subject to acid hydrolysis if dissolved in water.

13. Reload the eluate (1 ml) containing the DNA onto the membrane of the QIAamp Maxi column. Close the cap and incubate at room temperature for 5 min. Centrifuge at 4000 x *g* for 10 min.

Note: Less than 1 ml will be eluted from the column, but this has no effect on DNA yield.

Oragene Saliva DNA purification protocol.

Purification of the total 4 mL sample

1. Incubate the Oragene/saliva sample in the Oragene vial at 50°C in a water bath or air incubator for a minimum of 1 hour.

The sample may be incubated overnight if this is more convenient.

This incubation step only needs to be done once.

2. Divide the total 4 mL Oragene/saliva sample into four 1.5 mL microcentrifuge tubes, each
containing approximately 1 mL of sample.

3. Add 40 µL (1/25th volume) of Oragene Purifi er (supplied with kit) to each tube and mix gently by inversion.

The sample will become turbid as impurities are precipitated.

4. Incubate the four tubes on ice for 10 minutes.

5. Centrifuge the four tubes for 3 minutes at 15,000 × g at room temperature.

6. Carefully pipette the clear supernatant from each tube and combine them all into one 15 mL centrifuge tube without disturbing the pellets. Discard the pellets.

6. Add 4 mL (equal volume) of room-temperature 95% ethanol to the supernatant and mix gently by inversion. Invert at least 5 times.

A clot of DNA may be visible.

7. Let the solution stand for 10 minutes at room temperature so that the DNA is fully precipitated. Do not incubate at -20°C because impurities may co-precipitate with the DNA.

8. Centrifuge for 10 minutes at 1,100 × g (3,500 rpm) at room temperature.

9. Discard the supernatant without disturbing the DNA pellet (may or may not be visible). Remove ethanol as thoroughly as possible.

10. Once all of the ethanol has been removed, dissolve the DNA pellet in 500 µL of TE or other
standard buffer.

The expected concentration of the rehydrated DNA is 20 to 200 ng/µL.

11. To fully dissolve the DNA, vigorous vortex followed by incubation for a minimum of 1 hour at room temperature, preferably overnight. Alternatively, incubation for 10 minutes at 50°C is also effective.

***Myocilin* Sequencing:**

Fragment ID	Primer Sequence	Fragment Size	Nucleotides covered
Myoc_e1.1F	CACCTCTCAGCACAGCAGAG	478 bp	1 - 404
Myoc_e1.1R	GTAGGCAGTCTCCAACTCTCTG		
Myoc_e1.2F	CCATGTCAGTCATCCATAACTTAC	505 bp	224 - 604
Myoc_e1.2R	TAGGAGAAAGGGCAGGCAG		
Myoc_e2F	CAACATAGTCAATCCTTGGGC	269 bp	605 - 730
Myoc_e2R	ATACTGATTCTCTGAACACAGCAC		
Myoc_e3.1F	GGGCTGTCACATCTACTGGC	555 bp	731 - 1159
Myoc_e3.1R	GCTGTAAATGACCCAGAGGC		
Myoc_e3.2F	GCTGAATACCGAGACAGTGAAG	590 bp	1066 - 1515
Myoc_e3.2R	AACTTGGAAAGCAGTCAAAGC		

PCR Reagents:

	Volume & [conc] in PCR for 25 µl PCR	Stock solution	
10x PCR Buffer	2.5 µL (1X)	10 X	
H_2O	7.55 µL		
$MgCl_2$ [50 mM]	0.75 µL (1.5 mM)	50 mM	
dNTP	5.0 µL (200 µM of each dNTP)	1 mM of each 4 mM (total)	
Primers	0.5 µM (of each, F and R)	6.25 µM (of each) 2.0 µL (each primer)	
Platinum *Taq*	1.0 units	5 U/µL	0.2 µL
DNA	100 ng	20 ng/µL	5.0 µL
	25.0 µl		

PCR Conditions:

Cycles	temp	minutes:seconds	
1X	94°C	5:00	
15X	94°C	0:30	
	61°C*	0:50	
*Decrease after 5 cycles by 1°C every 5 cycles.			
	72°C	1:00	
35X	94°C	0:30	
	58°C	0:50	
	72°C	1:00	
1X	72°C	10:00	
	20°C	5:00	end

The web resource Primer3 was used in the design of all primers used for single SNP screening. (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi).

Reagents for 20 µl PCR

10x PCR Buffer	2.5 µL (1X)
$MgCl_2$ [50 mM]	0.75 µL (1.5 mM)
dNTP	2mM of each dNTP)
Primers	0.5 µM (of each, F and R)
HotStar *Taq*	0.5 units
DNA	50 ng
H_2O	to a total volume of 20µl.

PCR Conditions:

Cycles	temp	minutes:seconds	
1X	95°C	15:00	
30X	94°C	0:30	
	*°C	0:30	
	72°C	0:30	
1X	72°C	1:00	
	4°C	5:00	end

* Optimal annealing temperature (°C) as specified.

***Optineurin* Met98Lys variant:**

Fragment size: 621 bp

Fragment ID	Forward	Reverse	Temp
OPTN_M98K	GACCAGGCAAAACACCAATCC	CCTTAGCTCCTAGTAACCATAG	52°C

***WD40-Repeat 36* Asp658Gly variant:**

Fragment size: 389 bp

Fragment ID	Forward	Reverse	Temp
WDR_D658G	AAAACATTTTCTGCATCTCTTATCC	CAAGCAGATGTTGAATCACTCAG	51°C

Complement Factor H *Tyr402His variant*

Fragment size: 190 bp
Annealing temperatures of 51ºC (Set 3); 54°C (Set 2); 58°C (Set 1).

Fragment ID	Forward	Reverse
CFH_Y402H	TCATTGTTATGGTCCTTAGGAAA	GGATGCATCTGGGAGTAGGA
CFH_Y402HSNAP	ATTTTCCTTATTTGGAAAATGGATATAATCAAAAT	

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