Molecular and Phenotypic Associations in the Open Angle Glaucomas.

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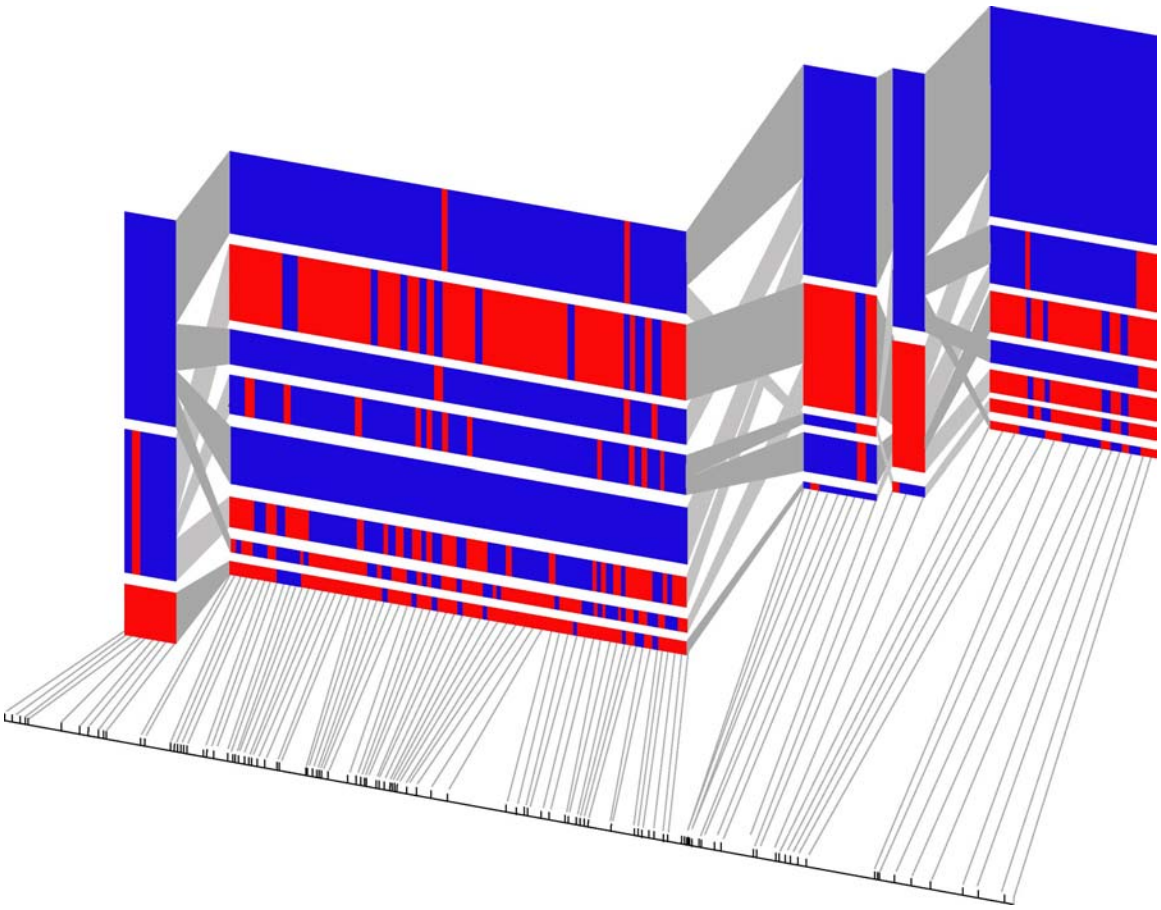

Haplotype Block Structure in the HapMap CEU population of the novel putative glaucoma locus on Xp25. Image based on the work of Dr Ben Fry, colours represent allelic variants and the z-offset emphasises the transition between blocks (see page 197).

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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The Blue Mountains Eye Study (BMES) is cemented in its reputation as one of Australia's greatest contributions to ophthalmic research. I am particularly thankful to Professor Paul Mitchell and Associate Professor Jie Jin Wang who allowed access to BMES samples used in Chapter 5 as well as the protocol sheets which formed the basis for the questionnaire administered in Chapter 3.

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):

1. Dimasi DP, **Hewitt AW**, Green CM, Mackey DA, Craig JE. Lack of association of p53 polymorphisms and haplotypes in high and normal tension open angle glaucoma. Journal of Medical Genetics. 2005; 42:e55.

2. Toh TY, Liew SHM, MacKinnon JR, **Hewitt AW**, Poulsen JL, Spector TD, Gilbert CE, Hammond CJ, Mackey DA. High heritability of Central Corneal Thickness: The Twin Eye Study. Investigative Ophthalmic Visual Sciences. 2005; 46: 3718-3722.

3. **Hewitt AW**, and Burdon KP. The relative contribution of the X chromosome to the eye. Ophthalmic Genetics. 2005; 26: 191-193.

4. **Hewitt AW**, Jeganathan VS, Kidd J, Pesudovs K, Verma N. Is visual function preserved in patients receiving photodynamic therapy for age related macular degeneration? Graefes Archives of Clinical and Experimental Ophthalmology. 2006; 244: 972-977.

5. **Hewitt AW** and Cooper RL. Glaucoma nosology. Clinical & Experimental Ophthalmology. 2006; 34 (1): 94.

6. **Hewitt AW**, Bennett SL, Dimasi DP, Craig JE, Mackey DA. A Myocilin Gln368Stop homozygote does not exhibit a more severe glaucoma phenotype than heterozygous cases. American Journal of Ophthalmology. 2006; 141 (2): 402-403.*

7. **Hewitt AW**, Traill A, Cooper RL, Morgan JE, Mackey DA. Tools for optic cup:disc measurement. Clinical & Experimental Ophthalmology. 2006; 34 (2): 288-289.

8. Wu J, **Hewitt AW**, Green CM, Ring MA, McCartney PJ, Craig JE, Mackey DA. Disease severity of familial glaucoma compared with sporadic glaucoma. Archives of Ophthalmology, 2006; 124: 950-954.

9. **Hewitt AW**, Craig JE, Mackey DA. Complex genetics of complex traits: the case of primary open-angle glaucoma. Clinical and Experimental Ophthalmology. 2006; 34(4): 472-484.*

10. **Hewitt AW**, Dimasi DP, Mackey DA, Craig JE. The WDR-36 D658G mutation is not a glaucoma-causing variant in Australia. American Journal of Ophthalmology. 2006; 142: 324-325.*

11. Craig JE, **Hewitt AW**, Dimasi DP, Toomes C, Howel N, Cohn AC, Mackey DA. The role of the Optineurin Met98Lys variant in hereditary optic neuropathies. British Journal of Ophthalmology. 2006; 90:1420-1424.*

12. **Hewitt AW**, MacKinnon JR, Elder JE, Giubilato A, Craig JE, Mackey DA. Risk of familial transmission of infantile glaucoma in Australia. Ophthalmic Genetics. 2006; 27(3) 93-97.

13. **Hewitt AW**, Bennett SL, Fingert JH, Cooper RL, Stone EM, Craig JE, Mackey DA. The optic nerve head in Myocilin glaucoma. Investigative Ophthalmic Visual Sciences. 2007; 48: 238-243.*

14. **Hewitt AW**, Bennett SL, Richards JE, Booth AP, Inglehearn C, Rashida A, Stone EM, Craig JE, Mackey DA. Myocilin Gly252Arg mutation and glaucoma of intermediate severity in Caucasians. Archives of Ophthalmology. 2007; 125:98-104.*

15. Bennett SL, **Hewitt AW**, Poulsen JL, Kearns LS, Morgan JE, Craig JE, Mackey DA. Screening for glaucomatous disc changes prior to diagnosis of glaucoma in myocilin pedigrees. Archives of Ophthalmology. 2007; 125: 112-116.*

16. Cohn AC, Toomes C, Potter C, Towns KV, **Hewitt AW**, Inglehearn CF, Craig JE, Mackey DA. Autosomal Dominant Optic Atrophy: a review of penetrance and expressivity in patients with OPA1 mutations. American Journal of Ophthalmology. 2007; 143: 656-662.

17. **Hewitt AW**, Gaudette D, Allingham RR, Järvelä I, Kitsos G, Krishnadas SR, Petersen MB, Richards JE, Sundaresan P, Wiggs JL, Mackey DA, Wirtz MK. Investigation for founder effects of the Thr377Met Myocilin mutation in glaucoma families from differing ethnic backgrounds. Molecular Vision. 2007; 13: 487-492.*

18. **Hewitt AW**, Poulsen JP, Alward WL, Bennett SL, Budde WM, Cooper RL, Craig JE, Fingert JH, Foster PJ, Garway-Heath DG, Green CM, Hammond CJ, Hayreh SS, Jonas JB, Kaufman PL, Miller N, Morgan WH, Newman NJ, Quigley HA, Samples JR, Spaeth GL, Pesudovs K, Mackey DA. Heritable features of the optic nerve head: A novel twin method for determining genetic significance. Investigative Ophthalmic Visual Sciences. 2007; 48: 2469-2475.*

19. Dimasi DP, **Hewitt AW**, Straga T, Pater J, MacKinnon JR, Elder JE, Casey T, Mackey DA, Craig JE. The prevalence of CYP1B1 mutations in Australian patients with primary congenital glaucoma. Clinical Genetics. 2007; 72: 255-260.

20. **Hewitt AW**, Kearns LS, Jamieson R, Williamson K, van Heyningen V, Mackey DA. PAX6 mutations may be association with high myopia. Ophthalmic Genetics. 2007; 28: 179-182.

21. **Hewitt AW**. Genetic diseases of the optic nerve head: from embryogenesis to pathogenesis. Expert Review in Ophthalmology. 2007; 2 (5): 769-777.

22. **Hewitt AW**, Mackey DA, Craig JE. Myocilin allele-specific glaucoma phenotype database. Human Mutation. 2008; 29: 207-211.*

23. Zhu G, **Hewitt AW**, Ruddle JB, Kearns LS, Brown SA, MacKinnon JR, Craig JE, Hammond CJ, Martin NG, Mackey DA. Genetic dissection of myopia: Evidence for linkage of ocular axial length to chromosome 5q. Ophthalmology. *2008; 115: 1053-1057.*

24. **Hewitt AW**†, Sharma S†, Burdon KP, Wang JJ, Dimasi DP, Mackey DA, Mitchell P, Craig JE. Ancestral *LOXL1* variants are associated with pseudoexfoliation in Caucasian Australians but with markedly lower penetrance than in Nordic people. Human Molecular Genetics. *2008; 17: 710-716. Online Nov 23 2007,*† Equal contribution by authors.*

25. Green CM, Kearns LS, Wu J, Barbour JM, Wilkinson RM, Ring MA, Craig JE, Wong TL, **Hewitt AW**, Mackey DA. How significant is a family history of glaucoma? Experience from the Glaucoma Inheritance Study in Tasmania (GIST). Clinical and Experimental Ophthalmology. 2007; 35: 739-799.

26. **Hewitt AW**, Sanfilippo P, Ring MA, Craig JE, Mackey DA. Mortality in Primary Open Angle Glaucoma: Two cupped discs and funeral. In Press Eye.*

27. Sherwin J, **Hewitt AW**, Ruddle JB, Mackey DA. The role of genetic isolates in Ophthalmic disease. Ophthalmic Genetics. 2008; 29: 149-161.

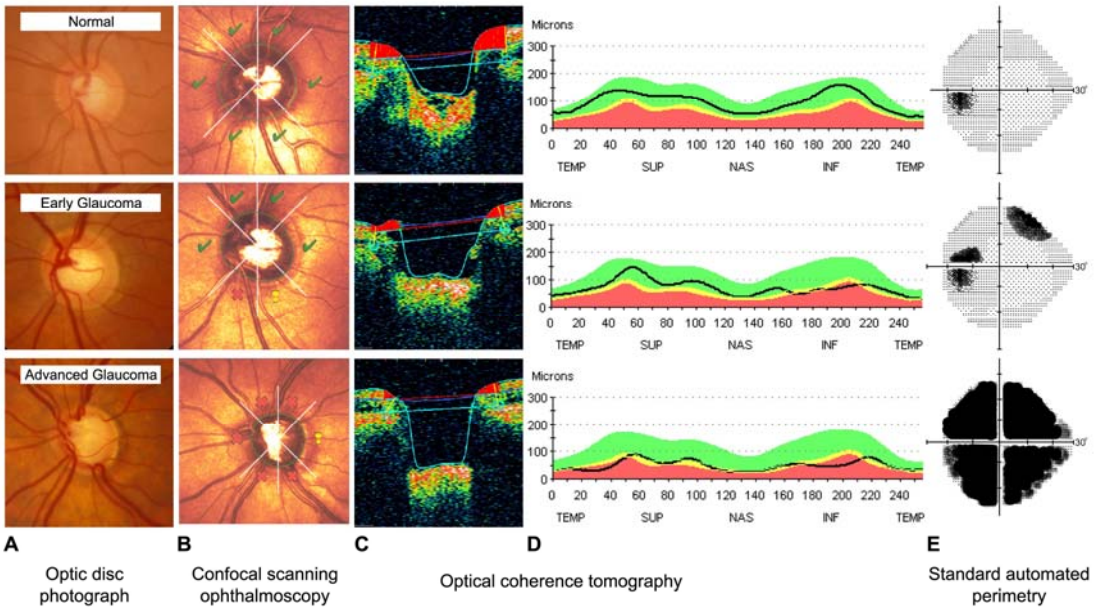
28. Mackey DA, Green CM, Craig JE, **Hewitt AW**. The pathogenesis of the glaucomas: Nature versus nature. Clinical and Experimental Ophthalmology. 2008; 36: 297.

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).

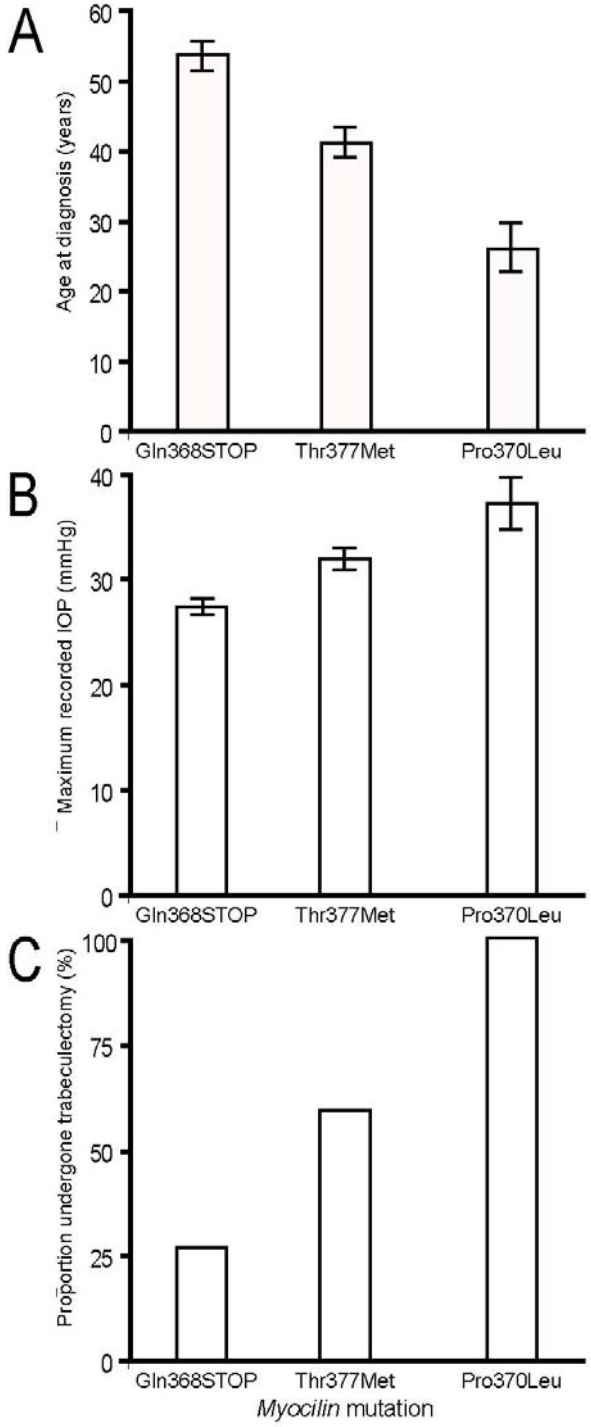
The clinical pattern of *MYOC* glaucoma reflects the specific underlying mutation. *MYOC* is traditionally thought of as having a HTG phenotype, with some mutations (such as Pro370Leu) causing severe juvenile-onset OAG (Alward et al., 1998). The distribution of age and maximum recorded IOP for Australian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

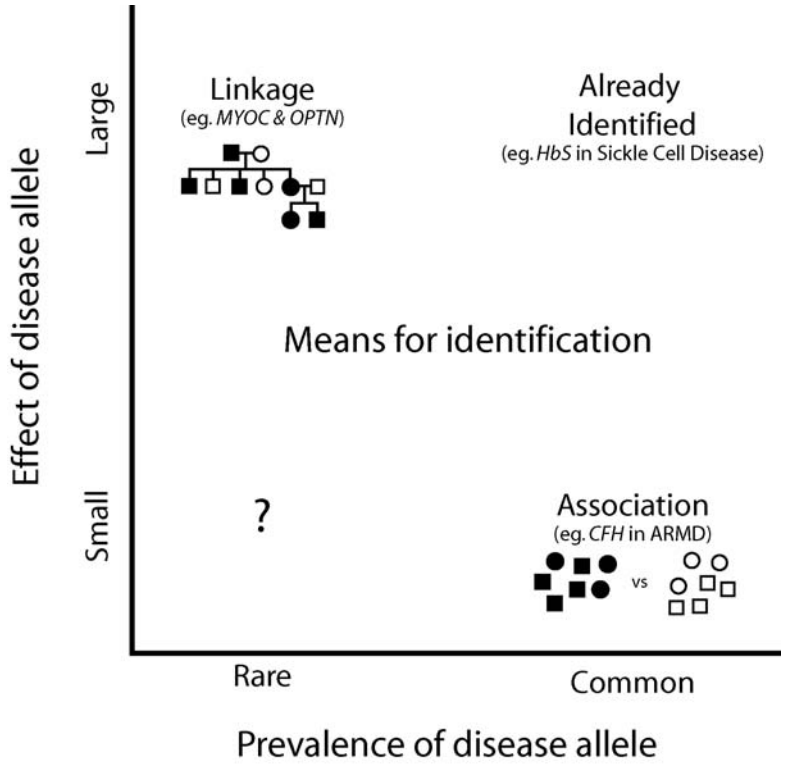
In the human genome, sequence variation include: single nucleotide polymorphisms (SNPs); insertions or deletions of a few nucleotides; and variation in the repeat number of a motif (micro-satellites) (Nowotny et al., 2001). Previous familial and sib-pair linkage studies have relied principally on micro-satellite 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.

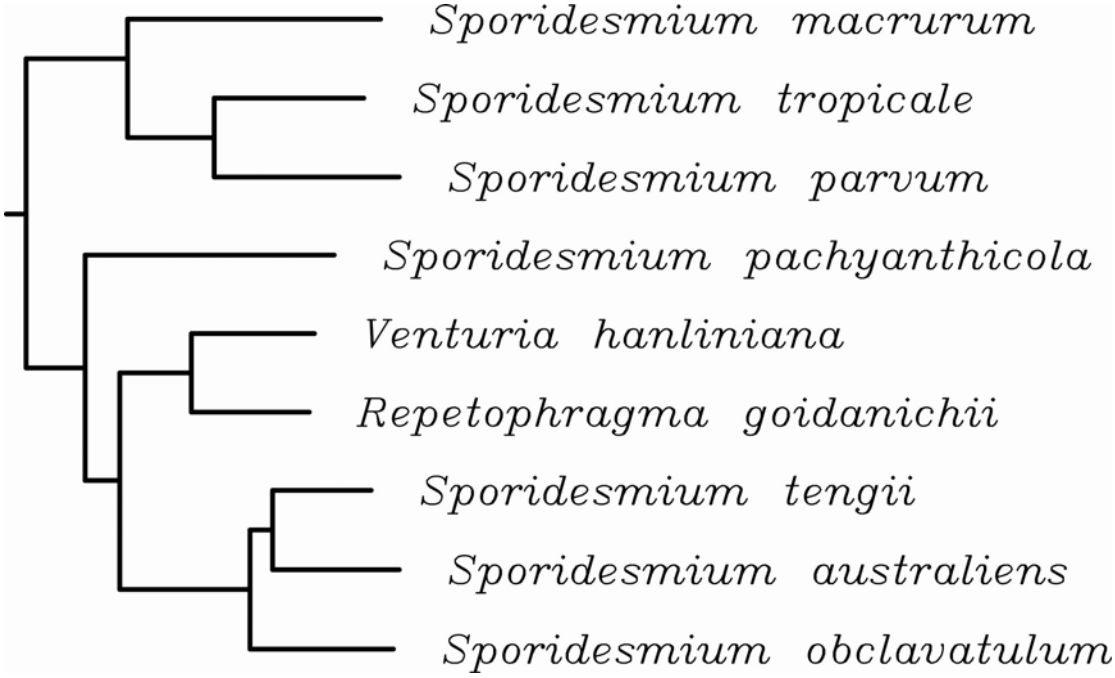
Searching for further pedigrees around the world has had limited success. OAG gene identification by conventional linkage analysis has been greatly complicated by phenocopy or intra-pedigree genetic heterogeneity (Craig et al., 2001; Sack et al., 1996). These issues of phenocopy, where a disease in separate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



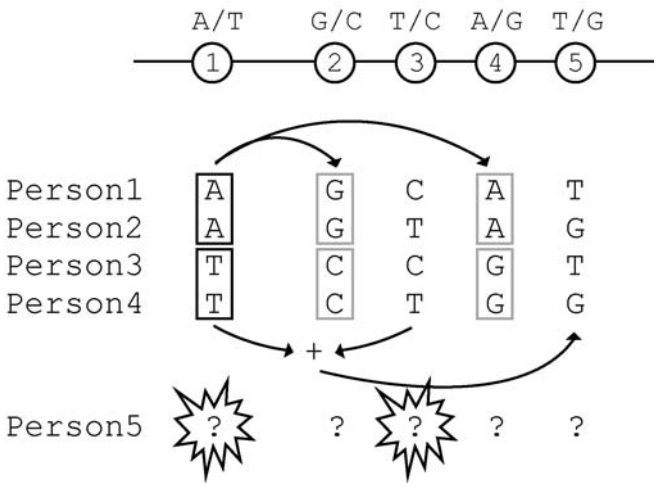
Previous linkage of OAG pedigrees and sibling pairs using micro-satellite markers may have had a high false negative result, being insufficient to detect true loci. With improved knowledge of the SNP variation across the genome, it may be beneficial to reinvestigate these pedigrees using SNP-based platforms. 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.

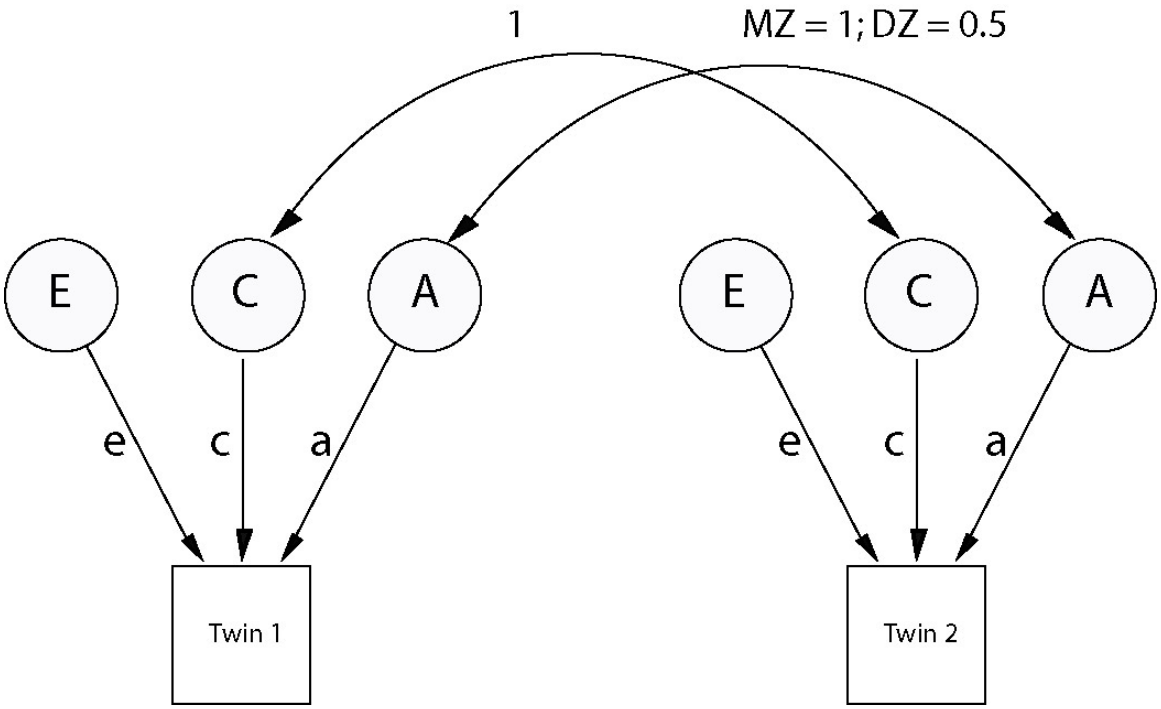
Twin studies are a major tool in determining heritability and identifying disease-causing genes. Twins studies allow for well-controlled association studies, as well as the study of the genetic versus environmental contribution to traits. When referring to twin studies many people assume the case of 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

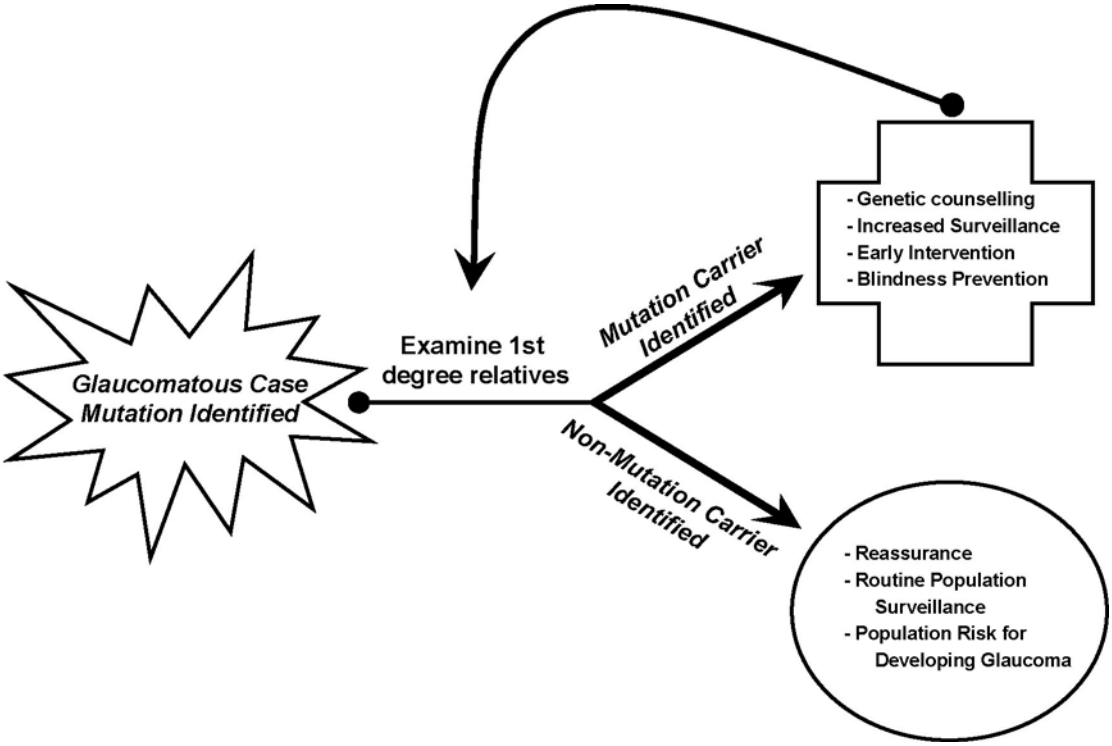
which have been unequivocally statistically implicated in disease causation (Stone 2003). A strong foundation of clinical research is essential prior to amalgamation of genetic counselling and predictive molecular testing. Understanding the sensitivity, spectrum, prevalence and penetrance of gene sequence 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.