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The genetics of primary open-angle glaucoma in black South Africans

Williams, Susan Eileen Isabella

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THE GENETICS OF PRIMARY OPEN-ANGLE GLAUCOMA IN BLACK SOUTH AFRICANS

Susan Eileen Isabella Williams

A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Doctor of Philosophy

Johannesburg, 2013

DECLARATION

I, Susan Williams, declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other university.



30th day of September 2013

DEDICATION

To my family, in thanks for their encouragement and support:

My husband, Graham

My sisters, Kathy and Julia

And in memory of my parents, Hugh and Elizabeth

ETHICS APPROVAL

The project was approved by the Human Research Ethics Committee (Medical) at the University of the Witwatersrand.

Clearance certificate numbers: M10216 and M110214.

PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS STUDY

1. PUBLICATIONS

Whigham BT, Williams SE, Liu Y, Rautenbach RM, Carmichael TR, Wheeler J, Ziskind A, Qin X, Schmidt S, Ramsay M, Hauser MA, Allingham RR. Myocilin mutations in black South Africans with POAG. *Mol Vis* 2011;17:1064-9.

The first two authors were joint first authors on this paper.

2. PRESENTATIONS

2.1. Congress: OSSA (The Ophthalmological Society of South Africa) Annual Meeting
Venue: Port Elizabeth, South Africa
Date: 24-27 March 2011
Title: Myocilin Glaucoma in Black South Africans
Presenter: S.E.I. Williams (oral presentation)

2.2. Congress: South African Society of Human Genetics Annual Congress
Venue: Cape Town, South Africa
Date: March 2011
Title: The familial extent of primary open-angle glaucoma in black South Africans
Presenter: T.E. Zwane (poster presentation)

2.3. Congress: OSSA (The Ophthalmological Society of South Africa) Annual Meeting

Venue: Sandton, South Africa

Date: 22-25 March 2012

Title: The genetics of Central Corneal Thickness

Presenter: S.E.I. Williams (oral presentation)

2.4. Congress: Wits Faculty of Health Sciences Research Day

Venue: Johannesburg, South Africa

Date: 16 September 2012

Title: Primary Open Angle Glaucoma Genetic Risk Factors in Black South Africans

Presenter: S.E.I. Williams (oral presentation)

This presentation was awarded a prize for the best oral presentation in the "Molecular and Comparative Biosciences" section.

2.5. Congress: ARVO (Association for Research in Vision and Ophthalmology) Annual Meeting

Venue: Seattle, USA

Date: 5-9 May 2013

Title: *MYOC* Variants In POAG In Black South Africans

Presenter: S.E.I. Williams (poster presentation – Abstract accepted)

ABSTRACT

Purpose

Primary Open Angle Glaucoma (POAG) is an important cause of irreversible visual loss in South Africa. POAG is asymptomatic, yet early detection and treatment can prevent visual loss. POAG has a genetic component, and identifying genetic risk factors could facilitate early detection as well as elucidate pathogenic mechanisms of the disease. POAG is more common in populations of African descent, but has been understudied in the context of POAG genetic risk factors in these populations. The purpose of this research was to identify genetic risk factors for POAG in black South Africans.

Methods

Self-identified black South African POAG patients and unaffected control participants were enrolled at St John Eye Hospital in Soweto. The study population was evaluated in case-control association studies for genetic risk factors in the following genomic regions: *CDKN2B/CDKN2BAS-1, MYOC, TMCO1, CAV1/CAV2, CYP1B1, WDR36, COL1A1, COL1A2, COL5A1, COL8A2, ZNF469, SIX1/SIX6, ATOH7* and the chromosome 2p16 locus. The study was powered to detect moderate-sized genetic effects. Family members of participants identified with potentially pathogenic mutations in *MYOC* were counselled, screened for the mutations and clinically screened for glaucoma. Genotyping data from SNPs in *TMCO1, CAV1/CAV2, CDKN2BAS-1* and *SIX1/SIX6* that had also been genotyped in a West African and an African American population were compared with the South African data and the three datasets were combined to create a larger sample of individuals of African descent for association analyses.

Results

There were 247 POAG patients and 255 control participants enrolled in the study that were representative of the black population of South Africa. The POAG participants had advanced disease with more than half having severe visual impairment from the disease. Potentially pathogenic mutations in *MYOC* were identified in 4.2% of the POAG patients (Lys500Arg in 1.2%, Gly374Val in 0.9% and

Tyr453del in 2.3%) and in 20% of the family members screened. The screening successfully identified individuals at high risk for future visual loss and enabled them to receive counselling and follow-up. Lys500Arg is a benign variant, whereas Gly374Val and Tyr453del are pathogenic. Tyr453del is incompletely penetrant. In the association studies, single SNPs in the *COL1A1, ZNF469* and *MYOC* regions showed marginal associations with POAG, but the identified associations did not withstand correction for multiple testing. Ocular quantitative trait association analyses also yielded no significant associations after correction but a significant association was identified with type 2 diabetes mellitus and rs12522383 in *WDR36*. There was also a significant association identified with SNP rs10120688 in *CDKN2BAS-1* and visual impairment in the African American dataset. Combining the three datasets of individuals of African descent increased the power to detect small genetic effects and identified suggestive associations with SNPs in *TMCO1* and *CAV1/CAV2*.

Conclusions

Black South Africans with POAG may have a *MYOC* mutation that either causes or contributes to their risk for developing POAG in approximately 3.3%. *MYOC* mutation screening in affected families successfully demonstrated the value of genetic information in identifying and protecting individuals at risk for visual loss from glaucoma. The genetic risk associated with the candidate genes evaluated in this study and POAG in black South Africans, if such a risk exists, is a small risk. There is therefore a critical need for further genetic association studies in POAG in this significantly affected population to identify other genetic risk factors. This study has important implications for the management and counselling of black South African patients with POAG and their families.

ACKNOWLEDGEMENTS

1. SUPERVISION

I am enormously grateful to my two supportive and encouraging supervisors; Professor Michèle Ramsay from the Division of Human Genetics and Professor Trevor R. Carmichael from the Division of Ophthalmology. I am fortunate to have had two such wonderful supervisors, each of whom provided me with unique support in their field of expertise. Together they have helped me in each step of the research process. They were both always available to meet with me and to advise me. Their recommendations have always been practical and have strengthened this work. Thank you.

2. FINANCIAL ASSISTANCE

2.1 Carnegie Clinical Scientist PhD Fellowship

The Carnegie Foundation is thanked for its support with this fellowship that I held for two years from 1 March 2011 to 28 February 2013.

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The Carnegie Corporation Transformation Project at Wits is thanked for its support with this grant that funded the Illumina BeadXpress Candidate Gene Association Study.

2.3 South African Medical Research Council (MRC) Self-initiated Research Grant (Professor T.R.Carmichael)

The South African MRC is thanked for its support with the grant awarded to Professor Carmichael for the Myocilin Family Study.

3. PRACTICAL ASSISTANCE

Thank you very much to Sister Thandi Zwane from the Division of Human Genetics and the MRC for her tireless research assistance.

Thank you to the staff at St John Eye Hospital for supporting me to do the research.

Thank you to the staff at the Division of Human Genetics and the National Health Laboratory Services (NHLS) Molecular Genetics laboratory for their assistance. I was assisted particularly by:

- Ms Punita Pitamber
- Dr Zané Lombard
- Ms Angela Hobbs
- Ms Tasha Wainstein

I am very grateful to Dr Michael Hauser for his invitation to Durham, North Carolina, for welcoming me to his unit and for allowing me to perform the TaqMan Association Study at the cost of the laboratory. He is also thanked for allowing me access to the data on the African American and Ghanaian databases.

Thank you to the research staff who assisted me at the Duke Center for Human Genetics. I was assisted particularly by:

- Mr Jason Gibson
- Ms Nicole Nelson

Thank you to Professor Scott Hazelhurst for assisting me with the Structure analysis.

Thank you to Ms Hayley Komen for assisting me with formatting the thesis.

4. PARTICIPANTS

The study was only made possible by the participants who kindly and patiently sacrificed their time to enrol in this study. They are thanked for their assistance.

STATEMENT OF CONTRIBUTION

I designed this research study, obtained ethics approval and performed the background research. I was responsible for all the components of the study. My personal contribution for each component of the study is detailed below.

Chapter 2: Phenotypes and Samples

I enrolled and examined each participant.

I was supported by Sister Thandi Zwane with translation, informed consents, the questionnaire and phlebotomy.

I extracted the DNA in 96 of the samples. The remainder were extracted at the NHLS laboratory by Ms Punita Pitamber and students in the department.

I performed all the statistical analyses.

Chapter 3: Myocilin Study

The initial sequencing of the gene in a subset of participants was performed in collaboration with Duke University in Durham, North Carolina. Dr Benjamin Whigham performed the sequencing and was assisted by Dr Yutao Liu.

For the family study I enrolled and examined each family member. I was supported by:

- Sister Thandi Zwane who contacted the probands and their family members and assisted with translation, informed consents and phlebotomy
- Ms Tasha Wainstein, a genetic counsellor, who counselled the probands and their family members

I designed the mutation screening experiment and the primers for the Sanger sequencing. Ms Angela Hobbs assisted me with DNA extractions and she performed

the sequencing.

Chapter 4: Candidate Gene Association Studies

Illumina BeadXpress Association study

I selected the candidate genes and SNPs for inclusion in the BeadXpress study and ordered the custom assay for the experiment.

I observed and assisted Dr Lombard and Ms Pitamber who performed the Illumina GoldenGate Assay.

I analysed the genotyping data with BeadStudio software.

I performed all the statistical analyses.

Professor Hazelhurst assisted me with the Structure analysis.

TaqMan Association study

I travelled to Durham, North Carolina to perform this portion of the study at the Duke Center for Human Genetics in Dr Hauser's laboratory.

The candidate genes and SNPs for inclusion in the TaqMan study were selected by Dr Hauser's team.

I performed the TaqMan genotyping with the support of Mr Gibson.

I performed all the statistical analyses.

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ABBREVIATIONS

CCT	Central corneal thickness
ddNTP	Dideoxynucleotide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
FDR	False discovery rate
FWER	Familywise error rate
GDx NFI	Nerve fibre index measured with the GDxVCC
GDx TSNIT	Average nerve fibre layer thickness measured with the GDxVCC
GWA	Genome-wide association
IOP	Intra-ocular pressure
LD	Linkage disequilibrium
MD	Visual field mean deviation
NTG	Normal tension glaucoma
OR	Odds ratio
PCR	Polymerase chain reaction
POAG	Primary open-angle glaucoma
RNA	Ribonucleic acid
SD	Standard deviation
SNP	Single nucleotide polymorphism
TGF-β	Transforming growth factor β
VCDR	Vertical cup-to-disc ratio

PREFACE

I am a clinical ophthalmologist with an interest in glaucoma that began when I was a registrar at the Wits Division of Ophthalmology attached to St John Eye Hospital in Soweto. I was distressed by the number of patients we were managing with progressive visual impairment as a result of glaucoma that presented late and was difficult to control. My Master of Medicine research project was an attempt to influence glaucoma progression surgically. I compared the gold-standard of trabeculectomy with mitomycin C to viscocanalostomy in a randomised control trial in fellow eyes of patients requiring surgery to both eyes.

My interest in the genetics of glaucoma, however, only came when the prize for the 'Sam and Dora Cohen Junior Lecturer Award' took me to the ARVO (Association for Research in Vision and Ophthalmology) Annual Meeting as part of the 'Host-a-Researcher' programme. This programme partnered me with Dr R. Rand Allingham from Duke University as my mentor. Dr Allingham's passion for and interest in the genetics of glaucoma inspired me, as did the stimulating sessions on the subject at the meeting. By the end of the meeting Dr Allingham and I had agreed to collaborate on a research project evaluating the association of the *LOXL1* gene with exfoliative glaucoma in black South Africans. The productive collaboration (which is ongoing) changed the then current understanding of the association of *LOXL1* with exfoliative glaucoma because of a unique pattern of risk association identified in black South Africans. This convinced me that I should be exploring genetic associations with primary open-angle glaucoma in this significantly affected, yet unique, population.

Professor Michèle Ramsay and Professor Trevor Carmichael encouraged me to register for my doctorate and, with their supervision, to become an independent researcher and clinician scientist.

Chapter 1: Introduction

1. INTRODUCTION

Glaucoma is the commonest cause of irreversible visual loss in South Africa, where the commonest type of glaucoma is primary open-angle glaucoma (POAG).[1] POAG is asymptomatic, but with early diagnosis and treatment, visual loss may be prevented. POAG has a significant genetic component, but the disease is phenotypically and genetically heterogeneous. The genetics of POAG is understudied in populations of African descent. Identifying the genetic factors influencing the disease may lead to a better understanding of the pathophysiology of POAG, may lead to targeted therapies for the condition and may allow an earlier diagnosis.

The purpose of this research was to identify genetic risk factors for primary open-angle glaucoma in black South Africans.

2. LITERATURE REVIEW

2.1 Glaucoma

Glaucoma is a heterogeneous group of conditions that have in common an optic neuropathy characterised by specific structural and functional changes. The structural changes consist of progressive retinal ganglion cell death and consequent loss of the retinal nerve fibres that constitute the optic nerve. This produces excavation, or 'cupping', at the optic nerve head and thinning of the retinal nerve fibre layer. The structural changes have a functional counterpart: visual field changes related to the neuronal loss. The visual field changes typically begin in the mid-periphery and progress to tunnel vision and finally irreversible blindness.

The World Health Organisation, in its 2010 Global Estimates of Visual Impairment, cited glaucoma as the second leading cause of blindness in the world.[2] Cataract is the commonest cause of blindness and glaucoma is the commonest cause of irreversible visual loss worldwide. Using prevalence models constructed by age, sex and ethnicity in 2006, Quigley *et al*.[2] estimated that there would be 60.5 million people worldwide with glaucoma in 2010, with 8.4 million of those blind from glaucoma. The estimate for Africa for 2010 was 6.5 million with glaucoma, with the ratio of glaucoma to population over the age of forty being 4.3% in Africa, the highest in the world.[2] The figures for glaucoma are expected to rise to 79.6 million worldwide and 8.4 million in Africa by 2020. The consequences are growing global costs and societal burdens of glaucoma. Individual and economic costs and the societal burden increase with disease severity; however, early identification of glaucoma and proactive management may prevent glaucoma-related visual loss.[3]

2.2 Primary open-angle glaucoma

The commonest form of glaucoma in South Africa, as it is worldwide, is primary open-angle glaucoma (POAG).[4, 5] The diagnosis of POAG is made when glaucoma is found in the absence of a secondary cause for the glaucoma and when the drainage angle of the eye is judged to be open on gonioscopy. The prevalence of POAG

varies with race. POAG is most common in people of African descent. Population-based surveys in black populations have varying prevalence estimates, but all report higher prevalences than corresponding studies in white populations.[4-14] In two South African prevalence studies (Hlabisa Glaucoma Study[5] undertaken in rural Kwazulu-Natal and Temba Glaucoma Study[4] in the urban North-West Province), the disease (by conservative diagnostic criteria) had an age and gender adjusted prevalence of 2.8% of the population sampled. [4, 5] In both of these prevalence studies more than 33% of the patients with POAG were bilaterally blind. At least one eye was blind in more than 50%. Only 4% of the rural population and 10% of the urban population with POAG had received prior treatment for glaucoma.[4, 5] These findings indicate that POAG is a significant health problem in South Africa. This problem is compounded by the absence of screening programmes which are not currently feasible because they are expensive and impractical.[15]

POAG is phenotypically heterogeneous and may even comprise several disorders that have not yet been systematically defined. POAG typically develops in the fourth to sixth decades, but may occur earlier when it distinguished as being of 'juvenile-onset'. It is asymptomatic and yet, with early detection and appropriate management, irreversible visual loss from POAG may be prevented. The pathophysiology of POAG is not yet fully understood, nor have the factors contributing to its progression been fully characterised. Intraocular pressure (IOP) is the most important risk factor for POAG and the only proven treatable risk factor. The Ocular Hypertension Treatment Study,[16] the Collaborative Normal Tension Glaucoma Trial,[17] the Advanced Glaucoma Intervention Study,[18] the Early Manifest Glaucoma Treatment Trial[19] and the Collaborative Initial Glaucoma Treatment Study[20] were well-conducted, prospective, randomised trials that provided conclusive evidence that IOP reduction slows the development or progression of visual loss caused by glaucoma, irrespective of the initial level of the IOP.

IOP is determined by the equilibrium between aqueous humour production and outflow. Aqueous is secreted by the ciliary body and the conventional outflow is via the trabecular meshwork, into the canal of Schlemm to the episcleral venous circulation. It is thought that the increased IOP that may be observed in POAG is the result of increased outflow resistance through the trabecular pathway.[21] IOP

elevation, or an increase in the pressure gradient across the lamina cribrosa, causes deformation of the lamina cribrosa with corresponding deformation and mechanical stress to retinal ganglion cell axons.[22] The extent of the deformation is determined by the scleral compliance. With the deformation there is remodelling that may result in mechanical failure as well as perfusion changes that contribute to the damage.[23, 24] Other factors, independently or in addition to IOP, that may contribute to the death of retinal ganglion cells in POAG are local ischaemia and hypoxia, excessive stimulation of the glutaminergic system, oxidative stress, inflammatory cytokines and aberrant immunity.[25-28] These other factors may have a more significant role to play in normal tension glaucoma (NTG; POAG occurring in the absence of an elevated IOP).

A family history is present in as many as fifty percent of patients with POAG.[29-31] First-degree relatives of individuals with POAG have a 7-10 times greater risk of developing POAG than the general population.[32, 33] A family history is now considered to be one of the most important risk factors for POAG development.[34] This epidemiological evidence, together with twin studies and familial clustering studies, strongly suggests that genetic factors play a significant role in the pathogenesis of POAG.[32, 35, 36] Investigating and characterising the genetic basis for POAG should help delineate the pathogenesis of the disease. This in turn may lead to the development of new and targeted therapies for POAG. The design of DNA-based tests for POAG could result in presymptomatic genetic testing. Ultimately preventative treatment of the disease may be possible.

2.3 The genetics of POAG

POAG is characterised genetically as a ‘complex’ disorder and is genetically heterogeneous.[37] Familial aggregation studies have tended to suggest that POAG may be subdivided into two groups. In the first group, the disease is transmitted as a Mendelian trait, usually with an autosomal dominant inheritance pattern. This rare form of POAG is usually caused by mutations within single genes: ‘Monogenic POAG’. The frequencies of the mutations are low, but the likelihood of POAG in individuals with those mutations is high. The mutations rarely occur in unaffected

cohorts. This is in contrast to 'Common POAG' that is multifactorial and probably polygenic. The genetic associations detected in this group are risk alleles that do not cause POAG on their own, but may promote the disease in combination with environmental factors and/or other genetic risk factors. These risk alleles occur frequently in POAG and control populations, but are more common in POAG patients.

2.3.1 Monogenic POAG

Between five and ten percent of POAG may be caused by mutations within single genes. Linkage analyses, largely in families demonstrating this form of inheritance of POAG, have documented an association of POAG to several genetic loci.[38-48] Genome-wide scanning in monogenic families has added to this list.[49-51] Table 1.1 documents the sixteen loci that have been designated *GLC1A* to *P* by the Human Genome Organisation (HUGO) Genome Nomenclature Committee (http://www.genenames.org/; 'GLC': glaucoma, '1': POAG, 'A to P': chronological order of genes discovered). Five of the loci (*GLC1A, J, K, M* and *N*) were identified in families with juvenile-onset POAG.[50-52] This uncommon subgroup of POAG occurs when there is onset of POAG before the age of 35 years. Because of the younger age of onset, family studies in juvenile-onset POAG are likely to include more individuals exhibiting the POAG phenotype than those in adult-onset POAG. Within the sixteen POAG associated loci, four genes have been identified – the *MYOC* (myocilin)*, OPTN* (optineurin), *WDR36* (WD repeat domain 36) and *NTF4* (neurotrophin 4) genes.[38, 48, 53, 54]

Table 1.1 POAG associated loci recognised by the HUGO Genome Nomenclature Committee (http://www.genenames.org/)

Symbol	Name	Location	Reference
GLC1A - MYOC	Myocilin, trabecular meshwork inducible glucocorticoid response	1q23-q24	Sheffield *et al.*[49]
GLC1B	glaucoma 1, open angle, B (adult-onset)	2cen-q13	Stoilova *et al.*[39]
GLC1C	glaucoma 1, open angle, C	3q21-q24	Wirtz *et al.*[40]
GLC1D	glaucoma 1, open angle, D (adult-onset)	8q23	Trifan *et al.*[41]
GLC1E - OPTN	Optineurin	10p14	Sarfarazi *et al.*[42]
GLC1F	glaucoma 1, open angle, F (adult-onset)	7q35-q36	Wirtz *et al.*[43]
GLC1G - WDR36	WD repeat domain 36	5q22.2	Monemi *et al.*[44]
GLC1H	glaucoma 1, open angle, H (adult-onset)	2p16.3-p15	Suriyapperuma *et al.*[45]
GLC1I	glaucoma 1, open angle, I	15q11-q13	Allingham *et al.*[46]
GLC1J	glaucoma 1, open angle, J (juvenile-onset)	9q22	Wiggs *et al.*[52]
GLC1K	glaucoma 1, open angle, K (juvenile-onset)	20p12	Wiggs *et al.*[52]
GLC1L	glaucoma 1, open angle, L (adult-onset)	3p22-p21	Baird *et al.*[47]
GLC1M	glaucoma 1, open angle, M (juvenile-onset)	5q	Pang *et al.*[50]
GLC1N	glaucoma 1, open angle, N (juvenile-onset)	15q22-q24	Wang *et al.*[51]
GLC1O - NTF4	neurotrophin 4	19q13.3	Pasutto *et al.*[48]
GLC1P	glaucoma 1, open angle, P	12q14	Fingert *et al.*[55]

References are given as superscript numbers

2.3.1.1 The MYOC gene (myocilin)

The *GLC1A* locus was identified in 1993 using linkage analysis on a large juvenile-onset POAG family in the United States.[49] In 1997, Stone *et al.*[38] identified the *MYOC* gene within this locus. *MYOC,* coding for the 57kD human myocilin protein, was the first gene associated with POAG to be identified. This gene was initially known as the *TIGR* gene for trabecular meshwork inducible glucocorticoid response protein. This was because the protein or gene product was identified while studying the effects of steroids on trabecular meshwork cell cultures; prolonged dexamethasone exposure resulted in increased production of this protein in vitro.[56] The function of the protein is still not fully understood despite both the gene and the protein having been identified 15 years ago. Myocilin is expressed throughout the eye and it is secreted from trabecular meshwork cells to the trabecular meshwork extracellular matrix.[57, 58] It has been postulated that myocilin glaucoma is a disease of protein unfolding; a conformational disorder.[59] There may be defective secretion of mutant myocilin with intracellular accumulation of the misfolded protein within the endoplasmic reticulum.[60] This leads to cell death and a malfunctioning matrix resulting in an increase to outflow resistance of aqueous humour, an increase in

intraocular pressure and glaucoma. The pathophysiology of myocilin glaucoma is therefore thought to be one of a gain-of-toxic-function.[61, 62]

The *MYOC* gene consists of three exons that code for 504 amino acids arranged into a secretion signal sequence, coiled-coil region and an olfactomedin domain.[63] More than 250 sequence variants have been detected within the exons and surrounding non-coding portions of the gene. More than seventy of those are considered to be disease-causing point mutations (http//www.myocilin.com). Most of the mutations occur in exon 3 of the gene which codes for the olfactomedin domain in the protein.[63] Amyloid has been localised to several regions of the olfactomedin domain of myocilin, supporting the theory that myocilin glaucoma caused by mutations within this domain is a protein conformational disorder.[64]

MYOC mutations have been studied in multiple populations.[65-71] Mutations in the *MYOC* gene cause between two and four percent of POAG across the globe.[72] More glaucoma-causing mutations have been found in *MYOC* than any other identified glaucoma risk gene. Although the frequency of disease-associated mutations is similar among African, Caucasian and Asian individuals with POAG, many mutations exist only in a specific racial group.[65, 73] In juvenile-onset POAG the inheritance of myocilin glaucoma is autosomal dominant, but in adult-onset POAG the mode of inheritance is more complicated. Mutations considered to be disease-causing have been identified in normal controls or unaffected family members.[74, 75] Cheng *et al.*[76] in a systematic review and meta-analysis evaluated the association of the five most commonly reported polymorphisms with POAG. They found strong evidence for an association between myocilin polymorphisms and high-tension POAG that was ethnicity dependent.[76] The relationship is, however, not purely Mendelian in that the mutations are often necessary but insufficient.

Despite the wealth of publications relating to myocilin glaucoma, relatively few are in African populations. Given that *MYOC* mutations are often found only in a single population, it is useful to screen *MYOC* in multiple populations. Identifying novel mutations is important for providing targets for functional studies with mutant myocilin. It may also provide the targets for genetic screening within a specific population. Myocilin genotype-phenotype correlations could enhance POAG

management. In addition, it is likely that common sequence variants within *MYOC* may play a role in 'common POAG' as has been suggested in association studies.[77]

2.3.1.2 The OPTN gene (optineurin)

The second gene discovered that is associated with POAG was identified as the *OPTN* gene coding for optineurin (optic neuropathy-inducing protein) and located within the *GLC1E* locus on chromosome 10.[53] The *GLC1E* locus was initially mapped from a large British pedigree with autosomal dominant POAG associated with normal IOP (NTG).[42] Subsequent reports have confirmed the association of the Glu50Lys *OPTN* missense mutation with NTG, but this is likely to account for less than 1% of POAG overall.[78-80] Only the Glu50Lys mutation has shown a strong disease association. The results of association studies with sequence variants in *OPTN* and POAG vary with some showing single nucleotide polymorphisms (SNPs) that confer susceptibility to POAG,[77, 79, 81-84] while others show no association.[83-85] Jia *et al.*,[86] despite finding no common polymorphisms significantly associated with POAG, did show that polymorphisms in this gene, in the *MYOC* gene and in the *APOE* gene might interactively contribute to POAG. This finding is supported by Fan *et al.*[77] and is consistent with the idea that many glaucoma phenotypes are polygenic in origin.

As with myocilin, the exact pathogenetic role of optineurin in glaucoma is uncertain. Optineurin is expressed in many ocular and nonocular tissues, including the trabecular meshwork, nonpigmented ciliary epithelium, retina, brain, heart, liver, skeletal muscle, kidney, and pancreas.[53] It interacts with proteins involved in apoptosis, inflammation and vasoconstriction and may participate in the tumour necrosis factor signalling pathway.[53, 87] It may have a role in optic nerve neuroprotection by reducing retinal ganglion cell susceptibility to tumour necrosis factor-mediated apoptosis.[88] The loss of function of this protein in patients with glaucoma may therefore decrease the threshold for ganglion cell apoptosis.

2.3.1.3 The WDR36 gene (WD repeat domain 36)

WDR36 was identified within the *GLC1G* locus on chromosome 5.[44] It encodes a

protein thought to be involved in T-cell activation and proliferation and in ribosomal RNA processing.[89, 90] The protein is expressed in tissues throughout the eye. Loss of the WDR36 protein activates the P53 stress pathway through disruption of ribosomal RNA maturation.[90] P53 is a key regulator of apoptosis and apoptosis, in turn, is responsible for retinal ganglion cell death in glaucoma.

Monemi *et al.*[44] originally presented *WDR36* as a glaucoma-causing gene but subsequent reports have failed to replicate these findings.[91-94] In addition there are *GLC1G* linked families without mutations in *WDR36*.[50, 54, 95] It is now not certain whether mutations in this gene cause glaucoma or whether they just increase disease susceptibility or modify the severity of the disease in an affected person. The gene does not have an established role in glaucoma pathogenicity at present. Common sequence variants in this gene were, however found to be associated with high tension POAG in Japanese and East Indian studies.[96, 97]

2.3.1.4 The NTF4 gene (neurotrophin 4)

NTF4 codes for neurotrophin 4 that activates tyrosine kinase-B receptor. Tyrosine kinase-B receptor is present in retinal ganglion cells and is thought to protect them from a high IOP, ischaemia and cytotoxins.[98] It is therefore postulated that *NTF4* variants could affect neuronal survival and cause glaucomatous optic neuropathy.[48]

In 2009 Pasutto *et al.*[48] identified eight different sequence variants in the *NTF4* gene in 15 European POAG patients accounting for about 1.7% of the POAG in that population. Only one of the variants occurred in a control subject. A single mutation accounting for less than 1% of POAG patients was subsequently identified in a Chinese population.[99] An association with *NTF4* and Glaucoma was not, however, detected in Indian or Caucasian United States populations.[100, 101] These inconclusive findings make the association of *NTF4* with POAG controversial.

2.3.2 Common POAG

The majority of POAG is thought to be of complex, multifactorial inheritance. It is likely that several genes and environmental factors influence the development of the

phenotype. Identifying genetic risk factors remains very important in this group. Unlike in monogenic POAG, the identification of risk alleles is unlikely to lead to screening or diagnostic tests for the disease. Identifying risk alleles and risk genes will, however, further our understanding of the molecular basis of the disease. This is the first step to new therapies. On the basis of risk genes one may be able to distinguish different subtypes of POAG, each with their own targeted therapy.

Association studies using common genetic variants are a powerful tool for uncovering significant genes in common conditions with multifactorial inheritance.[102, 103] Association studies require large groups of POAG patients and controls. The frequencies of minor alleles at sites of common variation (SNPs) are compared in the POAG and control groups. Where the frequency is significantly higher in the POAG group the alleles are designated 'risk alleles'. Candidate gene association studies, genome-wide association (GWA) studies, and quantitative trait mapping have all been used to identify genetic risk factors for POAG in several diverse populations, but never in black South Africans. The identification of the most significant disease-associated alleles in different populations is important for understanding their contribution to the pathogenesis of POAG.

2.3.2.1 Candidate gene association studies

In this type of association study, the genes are selected for analysis because they are considered to be suitable 'candidates' as POAG risk genes. The selection may include known glaucoma genes or it may be based purely on biological hypotheses. Genes that are thought to influence POAG endophenotypes are also good candidate genes. These are included under 'Quantitative Trait Mapping'.

Table 1.2 summarises some of the candidate gene association studies that have investigated the role of common sequence variants in known glaucoma genes (*MYOC, CYP1B1, OPTN* and *WDR36*) in association with POAG. *CYP1B1,* coding for the cytochrome P450 1B1 enzyme, is known to be associated with primary congenital glaucoma and was, therefore, included in this group.[104] Table 1.2 illustrates that in most of the genes investigated there are both positive and negative replication studies. The controversial findings may be explained by a number of

factors. The studies are in various different population groups with the replication studies often in different groups. Interestingly, none of the studies have been performed in populations of African descent. In addition, the sample sizes and the individual SNPs investigated often vary among the original studies and the replication studies. This makes comparisons of the studies difficult. A meta-analysis of the POAG case-control association studies investigating the *OPTN* gene could only find evidence of an association with one SNP and POAG in Asian populations.[105] Random effects meta-analyses in a total of six published studies on the effects of SNPs in *CYP1B1* on POAG were unable to detect any association.[106]

Table 1.2 POAG candidate gene association studies in known glaucoma genes

Gene	Chr	POAG Phenotype	§ Ancestry	§ Year	§ n	§ OR	Positive Replication	Negative Replication	Meta-analyses
MYOC	1q23-24	NTG	Chinese[77]	2005	681	2.0			
		POAG	Chinese[77]	2005	681			European[107]	
MYOC promoter	1q24	POAG	European[108]	2001				North American,[109] Chinese,[110] Turkish[111]	
CYP1B1	2p22	POAG	Indian[112]	2008	279	6.0	Australian,[113] European[114]	Chinese[115]	Negative[106]
WDR36	5q22	POAG	Japanese[96]	2007	239	2.2	Japanese,[96] Indian,[97] European[116]		
OPTN	10p14	POAG	Chinese[77]	2005	681	2.3		European,[107] South American[117]	Positive[105]
		NTG	Chinese[77]	2005	681	5.0	European[118]	Indian[83]	

References are given as superscipt numbers

Chr, chromosomal location; § Original Study; § n, total sample size (POAG and controls) in the original study; § OR, highest odds ratio reported in the original study; Positive Replication, ancestry in positive replication studies; Negative Replication, ancestry in negative replication studies; Meta-analyses, outcome of meta-analyses

Table 1.3 summarises some of the POAG candidate gene association studies that have investigated genes based on biological hypotheses. Despite the fact that NTG is not considered to be a separate disease entity but merely part of the disease spectrum of POAG, the genetic association studies often separate POAG into NTG and a high-tension group. Some of the associations have only been detected in one or other of these groups. This often reflects the biological hypothesis. For example, *OPA1* was included as a candidate gene because it causes Dominant Optic Atrophy and therefore optic neurodegeneration.[119] Likewise, *APOE* was selected as a candidate gene because alterations in the gene are thought to be associated with Altzheimer's Disease and neurodegeneration.[120] *NOS2* and *NOS3* were selected

because they have gene products (nitric oxide sythases) that regulate ocular blood flow.[121, 122] Neurodegeneration and vascular risk factors may be more important in the pathogenesis of NTG than in high tension POAG, where factors influencing aqueous humour dynamics and IOP may be more relevant. Many of the POAG associations were only identified within single studies (Table 1.3). Where there are repetition studies, there is often conflicting evidence for an association with POAG. In the case of the *MTHFR* gene, there have even been two published meta-analyses of the association with POAG that are contradictory, however the meta-analysis confirming an association only found the association in a population-based model.[123, 124] As with the association studies in glaucoma-associated genes, these discrepancies may relate to the differences in population, phenotypes and SNPs investigated in the studies. The measure of the association in the original studies, as reflected by the odds ratio (OR), varied widely with the genes, from as little as 1.3 in *CYP46A1* to as much as 15.0 in *TAP1*.[125, 126] Total sample size (POAG and control participants) was relatively small (less than 700) in the original studies represented in Table 1.3. Despite several populations being tested for associations, I could only find evidence of one study that included populations of African descent.[127] This particular study failed, in African-Americans and Ghanains with POAG, to confirm the associations with *OPA1* that had been detected in Singaporeans with NTG.[119, 127]

Table 1.3 POAG candidate gene association studies in genes selected on the basis of biological hypotheses

Gene	Chr	POAG Phenotype	§ Ancestry	§ Year	§ n	§OR	Positive Replication	Negative Replication	Meta-analyses
GSTM1	1p13	POAG	European[128]	2000	452	1.8	South American[129]	European,[130] Chinese[115]	
MFN2	1p36	NTG	European[118]	2009	285	1.3			
MTHFR	1p36	POAG	European[131]	2005	147	2.4	Korean[132]	European,[133] Japanese,[134] Chinese,[115] Indian[135]	Positive,[124] Negative[123]
IL1B	2q14	POAG	Chinese[136]	2003	163	4.2		Indian[137]	
IL1A	2q14	POAG	Chinese[136]	2006	323	1.8		Indian[137]	
PARL	3q27	NTG	European[118]	2009	285	1.5			
OPA1	3q28	NTG	Singaporean[119]	2002	363	3.1	Singaporean,[138] Japanese,[134] European[139]	Korean,[140] European-American,[127] African-American,[127] West African,[127] Chinese[115]	Positive[141]
EDNRA	4	NTG	Japanese[142]	2005	650		Korean[143]		
TNFA	6p21	POAG	Chinese[144]	2003	163	2.7	Chinese[115]	European[145]	
TAP1	6p21	POAG	Chinese[126]	2004	171	15.0			
NOS3	7q36	POAG	European[121]	2005	172		European-American,[146] Pakistani,[147] Chinese[148]	Chinese[115]	
TLR4	9q33	NTG, POAG	Japanese[149, 150]	2008	549	1.7	Chinese,[151] Japanese[150]		
LMX1B	9q33	POAG	European[152]	2009	585	0.6		Chinese[151]	
MMP1	11q21-22	POAG	European[153]	2011	449	1.7		European[154]	
CYP46A1	14q32	POAG, NTG	European[125]	2009	268	1.3		European,[155] Chinese[141]	
CDH1	16q22	POAG	Chinese[156]	2006	163	5.5		Chinese[115]	
TP53	17p13	POAG	Chinese[157]	2002	117	2.4	European,[116] Chinese,[115] European-American[158]	Indian,[159] Turkish,[160] Japanese,[161] South American[162]	Positive[163]
APOE	19q13	NTG	Tasmanian[120]	2002	106	2.5	Saudi[164]	European,[165] Turkish[160]	
NOS2	19q13	POAG	European[122]	2005	200				
XRCC1	19q13	POAG	Pakistani[166]	2011	353	2.7			
ERCC2	19q13	POAG	Pakistani[166]	2011	353	3.6			
SLC23A2	20p13	POAG	European[167]	2011	300	1.7			
AGTR2	Xq22-23	NTG	Japanese[168]	2001	698				

References are given as superscipt numbers

Chr, chromosomal location; § Original Study; § n, total sample size (POAG and controls) in the original study; §OR, highest odds ratio reported in the original study; Positive Replication, ancestry in positive replication studies; Negative Replication, ancestry in negative replication studies; Meta-analyses, outcome of meta-analyses

2.3.2.2 Genome-wide association studies

GWA studies for genetic associations have recently been used successfully to identify genetic risk factors for several complex diseases including POAG. The risks identified, however, generally explain a relatively small percentage of the heritability

of the trait in question. GWA studies usually require large cohorts of cases and controls. They explore SNPs across the genome for an association, avoiding the bias of selecting only known protein-coding genes. In order to cover the genome, vast numbers of SNPs need to be investigated, but the premise of linkage disequilibrium can reduce the total number. Linkage disequilibrium is the non-random assortment of alleles on the chromosome because of their proximity to each other and a tendency not to recombine. A 'tag' SNP will be in linkage disequilibrium with several nearby SNPs and may provide proxy information for the genotype at that region. Much of the common variation in the genome can therefore be assayed indirectly. While this is a very useful technique for identifying regions of interest within the genome, defining the causative variant from this technique is more difficult. In addition, testing hundreds of thousands of SNPs for an association increases the threshold for statistical significance of an association. It is usually necessary to correct the acceptable level of significance for one million independent tests (p-value of less than 5×10^{-8}).[169] An additional limitation in GWA studies, specifically in African populations, is that linkage disequilibrium varies with population: African populations have smaller linkage disequilibrium blocks. Most of the modern SNP genotyping arrays are not designed with the African linkage disequilibrium structure taken into account.

The findings of POAG GWA studies are presented in Table 1.4 in the chronological order in which they were published. The first POAG GWA study was published in 2009 at the time this study was conceived.[170] Since then there has been a steady increase in the number of GWA studies and publications, with 2012 seeing the publication of the results of four POAG GWA studies.[171-174] The chromosomal loci identified in POAG GWA studies along with the nearest genes to the loci are documented in Table 1.4.

Table 1.4 The association results of POAG genome-wide association studies presented in chronological order

Reference	Nearest Gene	Chr	POAG Phenotype	Ancestry	Year	n	OR
Nakano *et al.*[170]	*ZP4*	1q43	NTG	Japanese	2009	1575	1.4
	PLXDC2	10p12					1.5
	DKFZp762A217	12q21					1.4
*Jiao *et al.*[175]		2p16	POAG	**Afro-Caribbean**	2009		34.9
Meguro *et al.*[176]	*SRBD1*	2p21	NTG	Japanese	2010	660	2.8
	ELOVL5	6p21-12					1.7
Thorleiffsson *et al.*[177]	*CAV1 / 2*	7q31	POAG	European	2010	36140	1.4
Burdon *et al.*[178]	*TMCO1*	1q24	Advanced POAG	European	2011	4561	1.7
	CDKN2B-AS1	**9p21**					1.5
Osman *et al.*[172]	***CDKN2A / B***	**9p21**		Japanese	2012	7993	0.8
	SIX6	**14q23**					0.8
		2q21					0.9
Nakano *et al.*[171]	***CDKN2B-AS1***	**9p21**	NTG /POAG	Japanese	2012	2219	1.9
		9p21	NTG				2.1
Wiggs *et al.*[173]	***SIX1 / 6***	**14q23**	NTG /POAG	European	2012	6633	1.3
	CDKN2B-AS	**9p21**					0.7
	CDKN2B-AS	**9p21**	NTG				0.6
		8q22					0.6
Takamoto *et al.*[174]	***CDKN2B***	**9p21**	NTG	Japanese	2012	843	2.0

References are given as superscipt numbers

* Locus initially identified using linkage analysis

Chr, chromosomal location; Sample, total sample size (POAG and controls); OR, highest odds ratio

2.3.2.2.1 ZP4, PLXDC2 and DKFZP762A217

ZP4, PLXDC2, and *DKFZP762A217* were the nearest annotated genes to the POAG associated loci reported on chromosomes 1, 10 and 12 respectively by Nakano *et al.*[170] in the first GWA study for POAG. The total sample size of 1575 comprised 827 predominantly NTG cases and 748 controls all from Kyoto, Japan. The three genomic regions identified did not reach genome-wide significance ($p \cong 10^{-5}$) and the findings have yet to be replicated.[151, 170, 179]

2.3.2.2.2 The locus on chromosome 2p16

Jiao *et al.*[175] identified common variants within the chromosome 2p16 locus with a strong association with POAG in the Afro-Caribbean population of Barbados. This population has one of the highest prevalences of POAG worldwide. The 2p16 region was initially identified with microsatellite and SNP markers through linkage analyses in multiplex families and then confirmed with association studies in unrelated POAG

cases and unaffected controls.[175] This was, therefore, not a GWA study, but I include it here because it is one of the few association studies in a population of African descent. It described a novel locus with a major impact on susceptibility to common POAG in an Afro-Caribbean population. Replication studies have had contradictory results. No association was shown with this region and POAG in a Japanese cohort.[180] Kim *et al.*[181] examined SNPs in an extended region around this locus in a Korean population to look for cross-population association, but were unable to detect any associated SNPs. A weak association was found with POAG and one SNP in the 2p16 region in African-Americans (but not in Ghanaians), and in contrast to the original study, the minor allele was associated with less risk.[182] Interestingly, the minor allele frequencies in the African-American and Ghanaian controls were very similar to those in the population from Barbados.[182] A recent association study in Southern Chinese identified an age-specific association between a SNP in this region and late-onset POAG with an odds ratio of 2.2.[151] No study has replicated the level of significance identified by Jiao *et al.*[175]

2.3.2.2.3 SRBD1 and ELOVL5

The Normal Tension Glaucoma Genetic Study Group of the Japan Glaucoma Society identified the 2p21 and 6p21-12 loci as having an association with NTG in a small GWA study of 305 NTG patients and 355 controls.[176] *SRBD1* and *ELOVL5* were the genes identified in the two loci, but only a SNP in *SRBD1* reached genome-wide significance with a p-value of 2.5×10^{-9}.[176] The authors report that *SRBD1* mRNA is higher in the peripheral blood of humans with the risk allele at this SNP.[176] This suggests an involvement, either of the SNP or one in linkage disequilibrium with it, in the regulation of expression of the gene. Mabuchi *et al.*[183] confirmed the association of *SRBD1* with both NTG and high-tension POAG in a Japanese population, however Nakano *et al.*,[171] in another Japanese population, could find no evidence to support the association.

2.3.2.2.4 CAV1 and CAV2 (caveolin1 and 2 and the intergenic region)

CAV1 and *CAV2* are members of the caveolin gene family that are expressed in most human cell types (including the trabecular meshwork in normal and

glaucomatous tissue).[184] The proteins formed by these genes are responsible for the formation of caveolae (invaginations of the plasma membrane) that have a role in transcytosis and signal transduction.[184] They interact with endothelial nitric oxide synthetase to reduce the production of nitric oxide. They regulate transforming growth factor β (TGF-β). *CAV1* is upregulated in the trabecular meshwork one hour after an increase in IOP.[177] A large genome-wide association study in Iceland identified an association with POAG and a SNP on chromosome 7q31.[177] This SNP is within a linkage disequilibrium block that contains *CAV1* and *CAV2*. The authors of the initial discovery went on to replicate the finding in independent cohorts of Europeans from Sweden, the United Kingdom and Australia and Asians from Hong Kong and Shantou.[177] The association was strongest for the NTG subgroup when all the data were combined. The overall odds ratio in the European replication cohort at the associated SNP was 1.2, while that in the Asian cohort was significantly higher at 3.3.[177] In addition, the risk allele frequency was much lower in the Asian controls (<1%) than in the European controls (20-26%) and the linkage disequilibrium block is larger in Asians. This supports the authors' theory that the variant may tag the functional variant with stronger linkage disequilibrium in Asians than Europeans.[177] However, Osman *et al.*[172] in a Japanese GWA study replicated the association in a different marker in 7q31, but with a similar effect size to the European cohort (p = 0.007, OR = 1.3).

Wiggs *et al.*,[185] in an independent association study in a Caucasian population from the United States, confirmed the association of the 7q31 Caveolin region with POAG and reported an odds ratio (consistent with the original report) of 1.3. The association was stronger for NTG and showed a gender effect, with association in women but not men.[185] Kuehn *et al.,*[186] also in a Caucasian population from the United States, failed to replicate a statistically significant association. However the odds ratio from their data was 1.1 (p=0.2) that is similar to the odds ratio in the European replication group of the original study, so the data do not exclude an association but suggest that the study was underpowered to detect so small an effect.[186]

2.3.2.2.5 TMCO1 (Transmembrane and Coiled-coil Domain 1)

The function of the *TMCO1* gene that is located on chromosome 1 upstream of the *MYOC* gene is unknown. The gene has a highly conserved amino acid sequence and is expressed in most human tissues suggesting that it has a critical role.[187] Recessive mutations in the gene cause a rare syndrome known as "*TMCO1* defect syndrome" consisting of mental retardation with skeletal anomalies and craniofacial dysmorphism but no glaucoma.[187] Burdon *et al.*[178] identified *TMCO1* in an Australian GWA study in a Caucasian population. The study in question had a total sample size of 4561 that consisted of 615 patients with advanced glaucoma and severe visual field defects and 3946 controls. The top ranked SNP was identified downstream of the *TMCO1* gene (but within the same linkage disequiibrium block), with an odds ratio of 1.7 ($p = 6 \times 10^{-10}$), but a second SNP of genome-wide significance was identified in the intron of the gene. This finding was replicated in the original study in independent Australian cohorts, one with advanced disease, but in another two with less severe disease.[178] In a follow-up study, the same group evaluated the *TMCO1* gene for association with POAG phenotypes and screened for mutations in the gene in familial POAG.[188] They found no mutations that could account for a monogenic form of POAG. POAG patients carrying the risk allele had a younger age at diagnosis than those homozygous for the major allele at the associated SNP.[188] There is supporting evidence of the association of *TMCO1* with POAG from a recent GWA study evaluating IOP.[189]

2.3.2.2.6 The Cyclin-Dependent Kinase Inhibitors: CDKN2A, CDKN2B and CDKN2B-AS1

CDKN2A and *CDKN2B* are tumour suppressor genes that code for cyclin-dependant kinase inhibitors. These are induced by TGF-β and may regulate cell growth.[190] They play a role in cell cycle arrest, in senescence and in apoptosis in response to stress.[191] *CDKN2A* and *B* are expressed in retinal ganglion cells and are upregulated in animal models of glaucoma in response to elevated IOP.[178] *CDKN2B-AS1* is a long, non-coding, functional RNA thought to be involved in the regulation of several genes including *CDKN2A* and *B*.[192] It is transcribed in the antisense direction of *CDKN2A* and *B.* All three occur within a linkage disequilibrium block in the 9p21

locus. This region is a 'hotspot locus' that has shown an association with various diseases including coronary artery disease and diabetes mellitus.[193, 194] In relation to glaucoma, the locus was initially identified in a vertical cup-to-disc ratio (VCDR) GWA from the population-based Rotterdam Study I and II cohorts.[195] Since then it has been identified in five independent POAG GWA studies in different populations (see above in Table 1.4). There can, therefore, be little doubt that it has an important role to play in glaucoma. It is an authentic variant for POAG that does not appear to be ethnicity specific, although further studies in more varied populations are still required to confirm this.

The first GWA study to document an association with POAG and the 9p21 locus was the Australian Study undertaken by Burdon and co-authors and described above in relation to *TMCO1*.[178] In addition to identifying associations with advanced glaucoma and *TMCO1*, SNPs in *CDKN2B-AS1* also reached genome-wide significance and were replicated in the same three cohorts.[178] The next two GWA studies reporting the association were undertaken in Japanese populations. Osman *et al.*[172] conducted a GWA study using BioBank Japan samples of 1394 POAG cases and 6599 controls. Associated SNPs were selected for replication in an additional set of independent POAG cases (1802) and controls (7212). In a combined analysis of these two datasets, two SNPs in *CDKN2B* reached genome-wide significance.[172] In both SNPs, POAG was associated with the major allele suggesting that the minor alleles are protective from the disease. Nakano *et al.*[171] performed a GWA study in 833 Japanese POAG patients and 686 controls that identified the 9p21 locus. They then combined this dataset with that of their earlier GWA study.[170] A meta-analysis of the combined datasets identified genome-wide significant variants in the *CDKN2B-AS1* locus.[171] A subgroup analysis into NTG and high-pressure POAG suggested that the association was stronger for the NTG group.[171] This finding is supported by the Japanese NTG GWA study of Takamoto *et al*.,[174] as well as the meta-analysis of the NEIGHBOR (National Eye Institute Glaucoma Human Genetics Collaboration) and GLAUGEN (Glaucoma Genes and Environment) GWA studies.[173] The NEIGHBOR and GLAUGEN studies were undertaken in residents of the United States who were of European ancestry. The GLAUGEN study consisted of 976 POAG cases and 1140 controls while the NEIGHBOR study had 2170 cases and 2347 controls. In the meta-analysis of both studies, nineteen SNPs achieved

genome-wide significance. Seventeen of these were in the *CDKN2B-AS* region, while the other two were in the SIX1 / 6 region (see below). In common with the findings of Osman *et al.*,[172] the minor alleles in the *CDKN2B-AS* region were protective in this dataset. In the NTG / high-pressure subgroup analysis, there were only genome-wide significant associations in the NTG group: with SNPs in the *CDKN2B-AS* region and in a newly identified gene desert at 8q22.[173] Takamoto *et al.*,[174] in their Japanese NTG GWA study on a total of 469 NTG cases (286 in the discovery cohort) and 1071 controls (557 in the discovery cohort), identified multiple SNPs in the 9p21 locus that were strongly associated with NTG. The level of association was greater than that in the Caucasian studies, but in common with other studies the minor allele was protective.[174] The predominance of the association of 9p21 with NTG suggests that the involvement of this region with POAG may be on the basis of a neuronal factor in glaucomatous optic neuropathy, rather than high IOP.

Replication association studies have been performed in several different population groups. Notably, the Barbados Family Study of Glaucoma group described a significant association between POAG and a SNP in *CDKN2B-AS* in 272 POAG cases and 165 controls of Afro-Caribbean descent ($p = 0.0029$, odds ratio = 0.4).[196]

2.3.2.2.7 SIX1 and SIX6 (sine oculis-related homeobox 1 and 6 homologs)

SIX1 and *SIX6* are two adjacent genes in the 14q23 region that code for developmentally regulated transcription factors. *SIX6* mRNA is expressed in the retina, optic nerve and brain.[197, 198] Mutations in *SIX6* have been described in patients with anophthalmia and microphthalmia.[199] The locus was first identified as a candidate region for VCDR in a meta-analysis of GWA data from the population-based Rotterdam Study I and II cohorts.[195] The fact that it has subsequently been identified in the GWA studies of Osman *et al*.[172] in a Japanese population, and Wiggs *et al.*[173] in a European population suggests that variants within the gene contribute to the pathogenesis of POAG and are authentic genetic risk factors for the condition. It should, however, be noted that the association of POAG with *SIX1* and *SIX6* that was identified by Wiggs *et al.*[173] did not remain in their subgroup analysis in either the high-pressure group or the NTG group.

2.3.2.3 POAG quantitative trait analysis

Quantitative trait mapping, the analysis of genetic risk through the investigation of individual endophenotypes, has been employed in the search for POAG genetic associations.[200] Endophenotypes are continuous, heritable traits that are associated with the disease but that also manifest in individuals regardless of the disease state. Because they are continuous, individuals may be ranked along the continuum of risk. This can provide more information than a simple dichotomous distribution into case or control status would do. Mapping disease-susceptible genes could therefore be simplified. Central corneal thickness (CCT), IOP, optic disc area and VCDR are all heritable, structural characteristics that may be associated with a POAG phenotype and have therefore been investigated for genetic associations.

2.3.2.3.1 Intraocular pressure

IOP is an obvious candidate for quantitative trait analysis. It is, as mentioned previously, the most important risk factor for POAG. It is an easily measured continuous trait that is highly heritable. Twin studies suggest that the heritability of IOP is in the order of 0.65.[201-203] Segregation analysis has demonstrated a polygenic mode of inheritance of IOP suggesting that association studies are a valid mechanism for establishing genetic risk factors for a raised IOP.[204] IOP exhibits significant genetic correlations with POAG risk.[200]

Five genome-wide linkage studies have identified 15 potential regions of interest for an association with IOP.[95, 204-207] Two of these regions reached genome-wide significance. The first of these regions was the 10q22 locus that was linked to maximum IOP in an Australian study.[205] The second was the 5q22 locus that has been identified in two separate studies.[95, 206] Rotimi *et al.*[95] identified this locus in association with IOP in West African sibling pairs from Nigeria and Ghana affected with type 2 diabetes mellitus. The locus overlaps with the *GLC1G* locus (containing the *WDR36* gene) and the authors believe that their work replicates the *GLC1G* locus.[95] The region is, however, quite large and does contain several other candidate genes for IOP and POAG. The GENDISCAN (Gene Discovery for Complex Traits in Isolated Large Families of Asians of the Northeast) Project replicated the association

of IOP with 5q22 in a Mongolian population.[206]

A GWA study of IOP was undertaken by van Koolwijk *et al.*[189] in 11 972 European participants from the Rotterdam Study cohort and the Erasmus Rucphen Family Study.[189] SNPs in the genes *GAS7* and *TMCO1* reached genome-wide significance. Both of these genes are expressed in ocular cells and tissues associated with glaucoma.[189] They are thought to play a role in aqueous humour dynamics and glaucomatous neuropathy. Both SNPs were also marginally associated with POAG.[189] The results of this study also strongly support an association of *CAV1* and *CAV2* with IOP.[189]

2.3.2.3.2 Vertical cup-to-disc ratio

VCDR is another excellent candidate endophenotype for POAG. The assessment of VCDR forms part of the diagnosis of POAG. VCDR is highly heritable, with heritability estimates from twin studies being in the region of 0.70.[208, 209] The trait has been demonstrated to have significant genetic correlations with POAG risk.[200]

Genome-wide linkage analysis in 2005 first found an association with VCDR and the 1p32 locus in Australians of European descent.[205] In 2010, a meta-analysis of GWA data from the population-based Rotterdam Study I and II cohorts was published that identified associations with VCDR and the *CDKN2B* and *SIX1/SIX6* genes, and optic disc area and the *ATOH7* (atonal homolog 7) gene.[195] *ATOH7* was also associated with VCDR independent of optic disc area and it was later identified in a second GWA study in association with mean disc area.[210] *ATOH7* codes for Math5 that regulates retinal ganglion cell histogenesis and thus may contribute to optic nerve head area. All three regions identified in the Rotterdam study were subsequently found to be associated with POAG in candidate gene association studies, confirming that the trait mapping approach may be used to identify genetic risk factors for POAG.[211, 212] More recently, *CDKN2B* and *SIX1/SIX6* were identified independently in POAG GWA studies (see above in Table 1.4).

2.3.2.3.3 Central corneal thickness

CCT has been acknowledged as important in POAG since the advent of applanation tonometers in the 1950s and the recognition that the measurement of IOP is dependent on the CCT.[213] However, it was the Ocular Hypertension Treatment Study (OHTS) that first showed that CCT was a significant predictor for the development of glaucoma in ocular hypertensives.[214] This resulted in a common misinterpretation that CCT is an independent risk factor for glaucoma, an interpretation not supported by the data because it is not possible to separate out the effect of CCT on IOP measurement error. Attempts to do this by adjusting the measured IOP using CCT correction formulae are flawed because none of the formulae fully capture the corneal-induced error on tonometric measurement.[215, 216] Nevertheless, the suggestion that CCT is a risk factor has been supported by studies citing thinner corneas in POAG patients than in controls of the same populations.[217, 218] In addition, populations of African descent have thinner corneas than other populations and they are disproportionately affected with POAG: a link has been suggested between these two facts.[219, 220] It has been postulated that a thin CCT is a risk factor for POAG because it may be associated with altered biomechanics at the optic nerve head making this structure more susceptible to glaucomatous damage.[221] CCT is one of the most heritable ocular structural features, with heritability estimates from twin studies at approximately 0.90.[222, 223] It is quantitative and easily measured. It is certainly associated with POAG even if it is not known to what extent the association is independent of IOP.[224] It is therefore, potentially, a good candidate for POAG quantitative trait analysis. However, Charlesworth *et al.*,[200] in a bivariate quantitative genetic analysis, were unable to show a significant genetic correlation between CCT and POAG risk. This may have been the result of a lack of power. CCT remains a strong trait for genetic analysis because of its high heritability, and since its association with POAG is controversial, it has been included in POAG quantitative trait mapping approaches.

Candidate gene association studies and GWA studies have successfully identified several CCT genetic associations. The collagen genes are good candidates for an association with CCT because collagen is such an important constituent of the cornea. Mutations in the genes *COL1A1* (on chromosome 17) and *COL1A2* (on

chromosome 7) are known to cause osteogenesis imperfecta, which is associated with thin corneas. Dimasi *et al.*[225] investigated common sequence variants in these genes for an association with CCT in normal subjects and found associations with both genes. Two separate GWA studies for CCT each identified a sequence variant near *COL5A1.*[226, 227] *COL5A1* encodes for type V collagen that is abundant in the corneal stroma. It interacts to form highly ordered and intricate arrays of heterotypic type I and type V collagen fibrils. Mutations in *COL5A1* have been found in patients with Ehlers Danlos syndrome, which is associated with thin corneas.[228] *COL8A2* has been identified in a GWA study from Singapore.[226] *COL8A2* encodes for collagen VIII that is an important component of Descemet's membrane. Studies in mice have shown that inactivation of the genes encoding the collagen type VIII subunits (*COL8A1* and *COL8A2*) results in thinning of the corneal stroma.[229] *COL8A2* sequence variants have been associated with a thinner CCT.[229] *ZNF469* (an uncharacterised zinc-finger protein) is implicated in Brittle Cornea Syndrome that is a very rare disorder characterised by abnormally thin corneas.[230] The locus on chromosome 16q24 where this gene is found has been implicated in CCT in four GWA studies.[226, 227, 231, 232] Other genes implicated in CCT GWA studies in non-glaucomatous populations are *AKAP13, CHSY1, IBTK* and *LRRK1*.[226, 227, 233] The GWA study performed in the NEIGHBOR and GLAUGEN consortia was different in that it evaluated CCT in POAG cases.[234] The authors were only able to replicate the *ZNF469* association with CCT, but found that none of the aforementioned genes were associated with POAG. None of the SNPs in the study reached genome-wide significance, but two POAG candidate genes (*NTM* and *CNTNAP4*) were first identified by association with CCT and then by association analysis with POAG risk.[234] *NTM* was significantly associated with increased POAG risk and decreased CCT in the subset of POAG patients with NTG.[234]

2.3.2.4 Diabetes mellitus and POAG

Diabetes mellitus is not a quantitative trait, but because type 2 diabetes is heritable and considered to have an association with POAG, it could be included in genetic association studies related to POAG. The association of diabetes mellitus with POAG is, however, a controversial one. Four population-based studies (Baltimore Eye Survey[235], Rotterdam Study,[236] Proyecto Vision and Eye Research[237] and Visual

Impairment Project[238]) found no association, but three others reported significant associations (Beaver Dam Eye Study,[239] Blue Mountains Eye Study[240] and Los Angeles Latino Eye Study[241]). Two further, large epidemiological studies in women, the Black Women's Health Study and the Nurses Health Study confirmed this association in women.[242, 243] A meta-analysis in 2004 using data from both men and women found that type 2 diabetes is associated, independently, with an increase in incidence of POAG.[244] The controversial results may reflect different populations studied as well as different definitions of POAG and diabetes mellitus. Those reporting an association suggest that it may be on the basis of diabetic microvascular damage and altered vascular autoregulation of the retina and optic nerve.[241] This, together with compromised neuronal function and metabolism in the retina, may result in glaucomatous optic neuropathy. There is little data available on the association of glaucoma candidate genes with diabetes mellitus. Genetic associations with candidate genes, diabetes and POAG, if identified, would strengthen the evidence for an independent association between these two disease entities.

2.3.3 POAG genetics in populations of African ancestry

Table 1.5 contains a chronological summary of POAG genetic studies that have been undertaken in populations of African descent. Five of the studies evaluated myocilin mutations in populations of African ancestry.[65, 245, 246, 70, 247] Myocilin mutations were uncommon causes of monogenic POAG in Moroccans and African Americans.[70, 247] In Ghana the prevalence of myocilin mutations was estimated to be 4.4% of POAG patients.[245] The mutations observed in the African populations differed from those observed in Europeans. The commonest European mutation (Q368STOP) was not observed in Uganda, Ghana, Morocco or in the African Americans evaluated. [65, 245, 246, 70, 247] A mutational analysis for optineurin in Ghanaians did not identify any pathogenic mutations responsible for monogenic POAG in that population.[84] Linkage analyses performed in African-American and Afro-Caribbean populations to identify new loci for monogenic POAG identified the GLC1I and GLC1P loci as well as loci on 2q and 10p.[248, 46, 55] Rotimi *et al*.[95] identified loci at 5q and 14q in a linkage analysis in West Africans for loci linked to IOP. Association analyses identified the Chromosome 2p16 locus in Afro-Caribbeans, but

this was not replicated in Ghanaians or African-Americans.[175, 182] Association analyses were negative for POAG and *OPA1* in Ghanaians, African-Americans and Afro-Caribbeans.[127, 249] Cao *et al*.[196] performed a candidate gene association study which evaluated *CDC7/TGFBR3, ZP4, SRBD1, ELOVL5, CAV1/CAV2, TLR4, CDKN2B, CDKN2B-AS1, ATOH7, PLXDC2, TMTC2, SIX1*, and *CARD10* as candidate genes for POAG in Afro-Caribbeans. Suggestive associations were identified with SNPs in *CDKN2B* and *ATOH7*. [196]

Table 1.5 A chronological summary of POAG genetic studies in populations of African descent

Reference	Year	Population of African descent	Genes / Loci	POAG Phenotype / Endophenotype	Description
Fingert *et al.*[65]	1999	African-American	*MYOC*	POAG	Mutational analysis
Challa *et al.*[245]	2002	Ghanaian	*MYOC*	POAG	Mutational analysis
Sale *et al.*[246]	2002	Ugandan	*MYOC*	POAG	Q368STOP mutational analysis
Melki *et al.*[70]	2003	Moroccan	*MYOC*	POAG	Mutational analysis
Nemesure *et al.*[248]	2003	Afro-Caribbean	2q / 10p	POAG	Genome-wide linkage analysis
Allingham *et al.*[46]	2005	African-American	*GLC1I*	POAG	Ordered subset analysis using age at diagnosis
Rotimi *et al.*[95]	2006	Ghanaian, Nigerian	5q*(?WDR36)* / 14q	IOP and T2DM	Genome-wide linkage analysis
Yao *et al.*[249]	2006	Afro- Caribbean	*OPA1*	POAG / NTG	Association analysis (no association)
Liu *et al.*[127]	2007	Ghanaian, African-American	*OPA1*	POAG	Association analysis (no association)
Liu *et al.*[84]	2008	Ghanaian	*OPTN*	POAG	Mutational analysis (no mutations)
Jiao *et al.*[175]	2009	Afro- Caribbean	*2p16*	POAG	Linkage and association analysis
Liu *et al.*[182]	2010	Ghanaian, African-American	*2p16*	POAG	Association analysis (no association)
Fingert *et al.*[55]	2011	African-American	*GLC1P*	NTG	Linkage analysis
Liu *et al.*[247]	2012	African-American	*MYOC*	POAG	Mutational analysis
Cao *et al.*[196]	2012	Afro- Caribbean	*ZP4, SRBD1, ELOVL5, CAV1/2, TLR4, CDKN2B/AS1, ATOH7, PLXDC2, TMTC2, SIX1, CARD 10*	POAG	Association analysis (associations with *CDKN2B-AS* and *ATOH7*)

References are given as superscript numbers

T2DM, type 2 diabetes mellitus

It is apparent from Table 1.5 and the above review that the majority of research studies evaluating genetic risk for POAG have been in populations other than those of African descent. It is ironic that this should be the case, since African populations are disproportionately affected with POAG. There are, however, a number of explanations for the paucity of research in these populations. Firstly, most African

nations are developing countries lacking the funding and the infrastructure to perform large-scale genetic studies. Then there are the specific challenges relating to genetic studies in African populations. There is broad consensus that Africa is the origin of modern humans and as a result there is greater genetic diversity in populations of African descent.[250] African populations therefore have more variant SNP sites and greater haplotype diversity with smaller linkage disequilibrium blocks than other populations.[251] This has the disadvantage of increasing the costs of GWA studies in Africans that consequently require denser SNP genotyping. However, the corollary is that fine mapping for causal variants is likely to be more successful than in less diverse populations.[251] The great genetic diversity within Africans also implies that findings in one African population may not necessarily be applied to other African populations. It is therefore important that genetic studies be undertaken in diverse populations of African descent.

3. STUDY OBJECTIVES

3.1 Hypothesis

There are identifiable genetic risk factors associated with primary open-angle glaucoma (POAG) in black South Africans.

3.2 Specific aims

3.2.1 Aim 1: Characterise POAG phenotypically in black South Africans

A group of clinic based black South Africans with POAG was enrolled, characterised phenotypically and compared with a similar group of control participants. DNA samples were banked from each participant for use in primary open-angle genetic studies. The findings are presented in Chapter 2: Phenotypes and samples.

3.2.2 Aim 2: Determine the extent to which POAG in black South Africans is mediated by mutations in the *MYOC* gene

Participants with potentially pathogenic myocilin mutations were identified from within the study population. Their family members were examined and genotyped to understand the role of the mutations in the South African context. The findings are presented in Chapter 3: Myocilin study.

3.2.3 Aim 3: Determine the contribution of genetic variation within candidate genomic regions to POAG in black South Africans

Single nucleotide polymorphisms within POAG candidate genes and regions were genotyped in the study population in two separate case-control association studies to identify common variants associated with the disease in black South Africans.

In the first study, SNPs within candidate genomic regions were simultaneously genotyped in the study population using a medium-throughput genotyping platform. POAG phenotypes, diabetes mellitus and ocular quantitative traits were also

evaluated for an association with the candidate gene variants.

In the second study (undertaken at the Duke Center for Human Genetics in Durham, North Carolina in the United States of America), individual SNPs in candidate genomic regions were genotyped in the study population. The results of genotyping the same SNPs in two further populations of African descent (African Americans from North Carolina and West Africans from Ghana) were available for comparison and combination with the South African study population association results.

The findings are presented in Chapter 4: Candidate gene association studies.

Chapter 2: Phenotypes and samples

1. INTRODUCTION

The purpose of this part of the study was to characterise POAG phenotypically in black South Africans. This was achieved by enrolling a group of self-identified black South Africans with POAG from St John Eye Hospital in Soweto and comparing them with a similar group of unaffected control participants from the same clinic and population.

The phenotyping of the POAG patients was comprehensive. Family histories were included to estimate the extent to which POAG is familial in this population and to identify POAG patients with potentially monogenic forms of the disease. Medical histories were used to evaluate comorbidities, particularly hypertension and diabetes mellitus. Ophthalmological histories in POAG participants were recorded to establish the age at diagnosis of POAG, the POAG features on presentation, treatment prescribed for the condition and the response to that treatment. A comprehensive ophthalmological examination was undertaken to evaluate the extent of visual impairment and to quantify continuous ocular structural traits.

DNA was extracted from the peripheral blood of the participants to create a POAG DNA bank of DNA samples from well phenotyped South African black POAG patients and unaffected control participants from the same population for use in POAG genetic studies in this population.

2. METHODS

2.1 Population and sample

2.1.1 Ethics

This study adhered to the tenets of the Declaration of Helsinki. Written informed consent was obtained from all subjects and the study was approved by the Human Research Ethics Committee of the University of the Witwatersrand (Ethics approval number: M10216; Appendix 1). Appendix 2 contains the participant information forms, the informed consent forms and the DNA sampling informed consents used in the study.

2.1.2 Study population

Participants were enrolled at St John Eye Hospital in Soweto, Johannesburg in the Gauteng Province of South Africa. St John Eye Hospital is the eye department of Chris Hani Baragwanath Hospital, the largest hospital in South Africa. It is a teaching hospital for the University of the Witwatersrand Department of Health Sciences and a public sector hospital managed by the Gauteng Department of Health. The hospital serves the greater Soweto region, but is a tertiary referral centre, so it receives referrals from a larger area of Gauteng and from neighbouring countries. Gauteng is the smallest of South Africa's provinces, but the most populace, with a population of 12.3 million as of the 2011 South African Population Census.[252] Soweto falls under the Johannesburg municipality that has a population of 4.4 million, 3.4 million of whom are self-identified black Africans (http://interactive.statssa.gov.za/superweb). The population of Soweto is an urban population with representatives from all South African ethnic groups.

Self-identified black South African participants with POAG presenting to the St John Eye Hospital outpatients department for diagnosis, treatment and follow-up of their condition were invited to participate in the research. Patients were included regardless of the stage of their condition, what treatment they had received, whether or not their glaucoma was well controlled and when the diagnosis was made.

Unrelated self-identified black South African participants over the age of 60 years (an age at which they were considered less likely to develop POAG later in their lives) with no evidence of exfoliation syndrome or glaucoma and without self-reported familial history of glaucoma were invited to participate as control subjects. Control participants were recruited from people attending the St John Eye Hospital for complaints unrelated to glaucoma like refractive errors, senile cataracts, diabetic eye disease, blepharitis, floaters or itchy eyes.

2.1.2.1 Inclusion Criteria for POAG participants

1. Self-identified black South African participants 18 years of age or older who voluntarily gave informed consent to participate in the study.

2. Participants with a diagnosis of POAG (see 2.1.4) as evidenced by: -

- Characteristic visual field defects and characteristic glaucomatous optic atrophy
- Open angles on gonioscopy

2.1.2.2 Exclusion Criteria for POAG participants

- Participants with any secondary cause for their glaucoma

2.1.2.3 Inclusion Criteria for controls

1. Self-identified black South African participants unrelated to the study subjects who voluntarily gave informed consent to participate in the study as control subjects.

2. Participants older than 60 years old - an age at which they are less likely to develop POAG later in their lives.

2.1.2.4 Exclusion Criteria for controls

1. Participants with a family history of glaucoma.

2. Participants with evidence of: -

- Elevated intraocular pressure
- Glaucomatous disc damage
- Exfoliation syndrome

Patients with POAG and controls in the same population and from the same hospital were previously recruited for an exfoliative glaucoma and *LOXL1* association study.[253] The informed consent for that study specifically included using the DNA extracted for further studies on glaucoma genetics. Those participants that met the inclusion and exclusion criteria for this study, with the exception of the age criterion for controls, were included in the study. The majority of control participants in the *LOXL1* association study were older than sixty, however there were nine control participants who were older than 50 years, but not yet 60 years old who were also included in the study. These nine participants had no evidence of POAG.

2.1.3 Data collection

Appendix 3 contains the data collection sheets used in this study.

2.1.3.1 Questionnaire

The participants answered questions relating to their ethnic ancestry (as determined by their mother tongue and that of their parents and grandparents), their place of birth, their medical history and their ophthalmological history. The ophthalmological history included, in POAG participants, the age at diagnosis of the POAG, the IOP recorded in both eyes when the diagnosis of POAG was made, the treatment they had received for POAG and their response to this treatment. This information, where it was available, was obtained both from the participants and from a record review of outpatient clinic files. The medical history likewise relied on both clinic files and

information from the participants to ascertain the length and type of treatment for common medical conditions such as hypertension and diabetes mellitus. Sister Thandi Zwane, a genetic research assistant from the Division of Human genetics of the University of the Witwatersrand, interviewed the participants to ascertain a family history of glaucoma, eye diseases and blindness.

2.1.3.2 Examination

Each participant had their blood pressure tested and had an estimation of their random blood glucose from a finger-prick haemoglucotest. These tests were not performed on all the participants in the *LOXL1* association study.

I performed a standardized ophthalmological examination on each participant that included the following:

- Best corrected Snellen visual acuity
- Conjunctival assessment and characterisation of drainage bleb where this was appropriate
- Slit lamp corneal assessment
- Ultrasound pachymetry of the central cornea with the IOPac® Handheld Pachymeter (Reichert® Ophthalmic Instruments)
- Goldmann applanation tonometry
- Gonioscopy, Schaffer grading and description of the drainage angle
- Slit lamp anterior chamber, iris and pupil assessment
- Lens assessment
- Indirect stereoscopic fundoscopy
- Clinical assessment of the VCDR with a description of the optic nerve head, the neuroretinal rim and the peripapillary area

2.1.3.3 Investigations

POAG patients had a visual field assessment with the Oculus CenterField® automated perimeter. A translator was available to explain the test to the

participants. When the participants had previously and accurately performed the test and the results were available for review, it was only repeated if this was clinically indicated. The ‘Fixationcheck’ and ‘False positive’ were used to assess reliability. A ‘Fixationcheck’ less than 20% and a ‘False positive’ less than 10% were excluded from analysis as unreliable. The visual field examination was part of the diagnosis for POAG patients. The mean deviation (MD) was recorded for analysis. If the test was only reliable in one eye, then only the reliable test was included in the analyses. Where there were several fields available, the most recent and reliable field was used.

POAG participants had an assessment of their retinal nerve fibre layer using a scanning laser polarimeter, the GDx VCC™ Nerve Fiber Analyzer (Carl Zeiss Meditec). If the participants had previously and accurately performed the test and the results were available for review, it was only repeated if this was clinically indicated. Reliability of the test was assessed using the ‘Q’ value. A ‘Q’ less than 7 was taken as unreliable. The two parameters analysed were the nerve fibre index (NFI) and the average nerve fibre layer thickness (TSNIT). If the test was only reliable in one eye, then only the reliable test was included in the analyses.

For comparative purposes a subgroup of control participants had visual field and RNFL assessments. These assessments were not performed in all control participants.

Each participant had venous blood sampling for DNA extraction.

2.1.4 Glaucoma diagnosis

A diagnosis of POAG was made if the following criteria were fulfilled:

1. Glaucomatous optic neuropathy as evidenced by the following:

- VCDR greater than 0.7 or
- VCDR asymmetry of greater than 0.2 or
- Notching of the neuroretinal rim or

- Inferior and superior neuroretinal rim thinning or
- Retinal nerve fibre layer thinning on an accurate GDx VCC™ assessment (high NFI and low TSNIT values) combined with clinical suspicion

2. Glaucomatous visual field defects (confirmed on two reliable visual field tests) that correlated with the disc appearance.
- If the participant's visual acuity was too poor to perform visual field testing or if the participant was unable to produce a reliable visual field, then a prior reliable diagnosis of POAG recorded in the clinical notes was accepted for the diagnosis of POAG
- Participants with advanced optic disc cupping, severe visual impairment and raised intraocular pressures (> 25mHg) in whom we could not obtain visual fields were also assumed to have POAG provided the other criteria were met

3. Open angles on gonioscopy.

4. No secondary cause for the glaucoma or optic disc appearance.

2.1.5 Diagnosis of diabetes mellitus

A diagnosis of diabetes mellitus was made if the following criteria were met:

- Self-reported history of diabetes and treatment with diet, oral hypoglycaemic medications or insulin
- Diagnosis of diabetes mellitus in clinical records

Participants had random fingerprick haemoglucotests. Where this was greater than 8mmol/l, the participants were appropriately referred and a diagnosis of diabetes was entered if further investigations (haemoglobin A_{1C} and fasting blood glucose levels) proved this to be the case.

A diagnosis of type 1 diabetes mellitus was made if:

- Diagnosis of diabetes was before the age of 30 and
- Participant had insulin dependent diabetes

The duration of the diabetes, for the purposes of this study, was calculated as the difference between the year of diagnosis and the year of the examination.

2.1.6 Diagnosis of hypertension

A diagnosis of hypertension was made if the following criteria were met:

- Self-reported history of hypertension and anti-hypertensive therapy
- Diagnosis of hypertension in clinical records

Participants had random blood pressure measurements with a sphymomanometer. Where the systolic blood pressure (SBP) was greater than 140mmHg or the diastolic blood pressure (DBP) was greater than 90mmHg, the participants were appropriately referred and a diagnosis of hypertension was entered if hypertension was confirmed on repeat measurements.

Mean arterial pressure (MAP) was calculated using the formula:

- MAP = (2DBP + SBP)/2

2.1.7 Glaucoma phenotypes

2.1.7.1 Primary juvenile glaucoma

This is defined by the European Glaucoma Society in their Terminology and Guidelines for Glaucoma as an onset of glaucoma from the tenth to the thirty fifth year of life.[254] Participants in this study were classified as having primary juvenile glaucoma if they were under the age of forty years with advanced glaucoma or if the date of diagnosis of their glaucoma was before their thirty fifth birthday.

2.1.7.2 Normal tension glaucoma

This is part of the continuum of primary open angle glaucoma. POAG participants with an untreated IOP ≤ 21mmHg were considered to have NTG.

2.1.7.3 Family history of blindness

In glaucoma prevalence studies in South Africa, only 10% of the population with POAG had received prior treatment for glaucoma.[4, 5] In this setting a family history of glaucoma is unlikely. A family history of blindness is easier to elicit and may represent a family history of glaucoma in glaucoma sufferers. For this reason participants were classified on the presence or absence of a family history of blindness.

2.1.7.4 Glaucoma stage

Glaucoma is usually staged 1-5 on the status of the Humphrey visual field, where Stage 5 (end-stage/blind) is defined as a visual acuity of less than 6/60 (caused by glaucoma) in the worse eye or an inability to perform the test because of a central scotoma caused by the glaucoma.[255] A Humphrey Automated Perimeter was not available for use in this study. Those participants with a Snellen visual acuity of less than 6/60 in either eye as a result of glaucoma were classified as having 'severe visual impairment' and 'end-stage' glaucoma. For the purposes of this study 'advanced' glaucoma was defined as a VCDR of more than 0.9 in either eye. A VCDR of less than 0.7 in both eyes was classified as 'early' glaucoma.

2.1.7.5 Glaucoma treatment

Glaucoma treatment was subdivided into those patients receiving medical therapy, those that had received laser therapy, those that had undergone a surgical procedure or any combination of these treatments. The patients who had undergone a glaucoma drainage procedure were reviewed separately.

The response to glaucoma therapy was gauged by the IOP at the time of

examination. This was considered ‘controlled’ if it was less than 18mmHg in each eye (or in the treated eye only if one eye was untreated). In some cases the participants were not receiving therapy (if they were seen at diagnosis or if they had defaulted treatment) and some participants were awaiting surgical therapy.

2.1.7.6 Cataracts

Participants were recorded as having lens opacities (cataracts) if they had visually significant lens opacities in either eye or if they were pseudophakic.

2.2 DNA extraction

Genomic DNA was extracted using a salting out procedure from nucleated cells from the venous blood samples at the NHLS Molecular Genetics Laboratory in the Division of Human Genetics. The procedure, modified from Miller *et al.*[256] is described in detail in Appendix 4.

The quality of the DNA was evaluated by running it on a 0.8% agarose gel. The concentration (ng/µl) and the purity (OD260/280) of the DNA was measured using the Nanodrop® ND-1000 Spectrophotometer (Nanodrop Technologies).

The resuspended DNA was stored at 4°C in the DNA sample repository at this laboratory until it was required for the purposes of this study.

2.3 Statistics

Statistical analyses were performed using STATA version 12.

2.3.1 Descriptive statistical analyses

To describe the demographic characteristics, mean ± standard deviation (SD) was used for continuous variables, and categorical variables were summarised by percentages. For comparisons of continuous variables, the t-test was used with p

values that were two-tailed with significance set at $p < 0.05$. For comparisons of categorical variables χ^2 analysis was used unless the values in individual cells were small in which case I used Fisher's exact test. p values < 0.05 were considered to be significant.

2.3.2 Ocular traits of paired eyes

In each participant data were collected from both eyes.

The measurements from only one eye were used where the fellow eye could not be measured or, in POAG participants, if the fellow eye was untreated (in unilateral glaucoma or blindness).

For dichotomous traits (cataracts, diabetic retinopathy, glaucoma drainage surgery etc.), the trait was regarded as being present even when it was only present in one eye. This was appropriate for the disease characterisation and for use in genetic risk factor association studies.[257]

For continuous traits measured in both eyes, Pearson correlation coefficients were determined for measurements from the left and right eyes. p values < 0.05 (two-tailed) were considered to be significant.

For correlated data from both eyes either the data from only one eye may be used or the ocular findings per individual may be summarised.[257, 258] The latter is thought to have more power in genetic studies because averaging ocular measurements between both eyes may give higher heterogeneity estimates than using information from one eye only.[259] Using one eye is statistically simple, but results in a loss of information, as well as the potential for bias arising through the choice of which eye to use.[257] In this study correlated data from both eyes were averaged except for the IOP at diagnosis in the POAG patients. For the IOP at diagnosis I used the higher of the two measurements with the rationale that for this particular trait it was more appropriate to have the maximum measurement for the purpose of characterisation of the disease and for use in genetic risk factor association studies.

2.3.3 Regression models

Logistic regression was used to compute odds ratios.

To adjust for the confounding variables of age and gender, a logistic regression model was used with age and gender as covariates. An adjusted p value was considered significant if $p < 0.05$.

A linear regression model was used to evaluate the effects of age, gender, POAG diagnosis, diabetic diagnosis, hypertensive diagnosis, maximum IOP and IOP at enrolment on CCT. Covariates that were significant in a univariate analysis were included in a multivariate linear regression model. An adjusted p value of $p < 0.05$ was used to validate the model.

Multivariate analyses were used for the glaucoma phenotypes of primary juvenile glaucoma, severe visual impairment and glaucoma surgery. The following covariates were evaluated: gender, age, IOP at diagnosis, VCDR, CCT, advanced glaucoma, severe visual impairment, glaucoma surgery, IOP control and the presence of lens opacities. Covariates that were significant in a univariate analysis were included in a multivariate logistic regression model. An adjusted p value of $p < 0.05$ was used to validate the model. For relationships that tested significant, odds ratios (with 95% confidence intervals) were used to evaluate the clinical usefulness of the models.

3. RESULTS

3.1 Participant demographics (Table 2.1)

Table 2.1 Demographic features of study subjects

		Control	POAG	p	p ⌘
Total = **502** participants		**255**	**247**		
Female (%)		138 (54.1)	124 (50.2)	0.380 ¶	
Age (years)	Range	50 - 91	22 - 87		
	Mean ± SD	70.2 ± 8.3	60.0 ± 13.1	< 0.001 §	
DM (%)		93 (36.5)	57 (23.1)	0.001 ¶	**0.028**
HT (%)		177 (69.4)	132 (53.4)	< 0.001 ¶	0.599

DM, diabetic; HT, hypertensive; p ⌘, p adjusted for age and gender in a logistic regression model
¶ Pearson's χ^2 test
§ T-test

From St John Eye Hospital in Soweto I recruited a total of 502 self-identified black South African participants of whom 247 were patients with POAG and 255 were controls. The demographic characteristics of the study population are summarized in Table 2.1. Females constituted 50% of the patients and 54% of the controls (p = 0.38). The average age of the patients was 60 years (SD = 13.1), but for controls it was significantly higher (70.2 ± 8.3 years; p<0.0001). Significantly more of the controls than the POAG participants were known hypertensives and diabetics (Table 2.1; p ≤ 0.001). The association, when adjusted for age and gender in a logistic regression model, was no longer significant for hypertension (p = 0.599), but remained significant for diabetes (p = 0.028).

The groups were alike in their ethnic identity that was determined by the first language of the participants and that of their parents and grandparents (Fisher's exact p = 0.90; Figure 2.1). The South African Census 2011 demonstrated a similar spread of ethnic identities among black Africans in the whole of South Africa, in Gauteng and in Johannesburg (Figure 2.2); (http://interactive.statssa.gov.za/superweb). The majority languages (IsiZulu, Sesotho and Setswana) were slightly over-represented in the study population compared with South African Census 2011 results while the minority languages (notably English) were slightly under-represented.

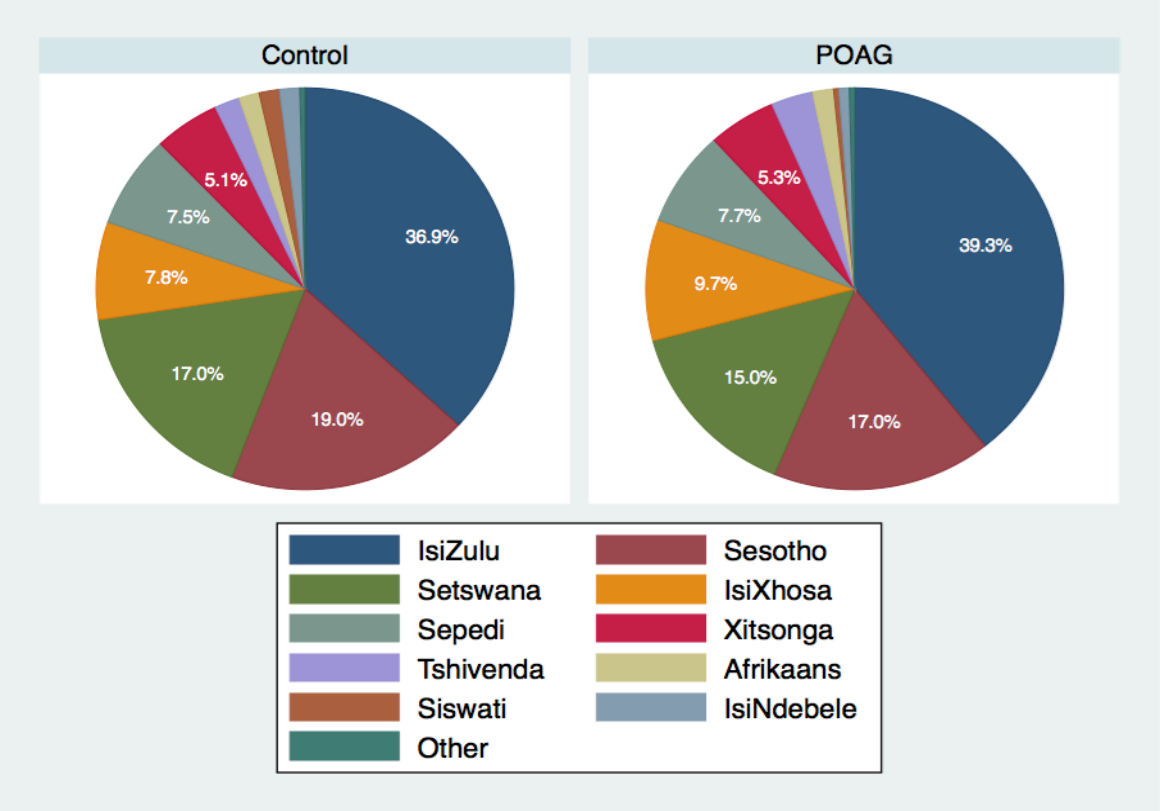

Figure 2.1 First languages spoken within the study population. The proportion of control and POAG participants speaking, as their first language, each of the official languages of South Africa. There were no English first language participants. One participant in each group spoke Chichewa (Other). The groups were alike (Fisher's exact p = 0.904).

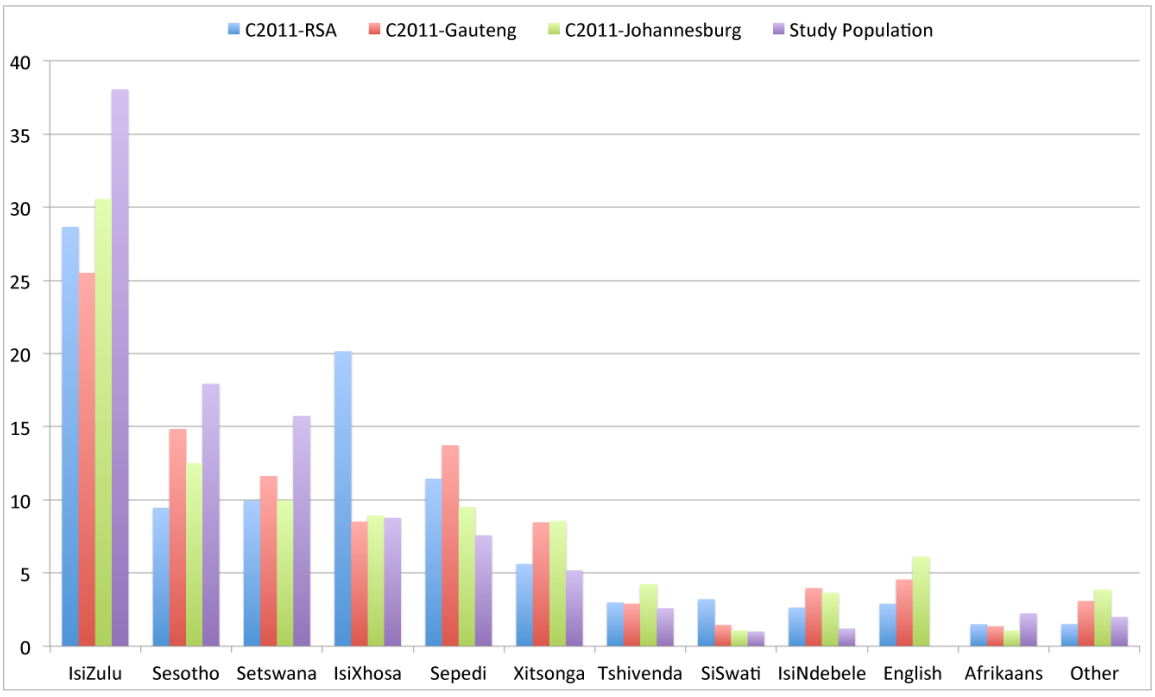

Figure 2.2 First language spoken: Study population compared with South African Census 2011 results in self-identified black Africans. The languages are presented along the x-axis. The y-axis represents the frequency in percent.

C2011-RSA, census 2011 in black Africans in the whole country

C2011-Gauteng, census 2011 in black Africans in Gauteng

C2011-Johannesburg, census 2011 in black Africans in Johannesburg

3.2 Ophthalmic family history (Table 2.2)

Table 2.2 Ophthalmic family history of study subjects

	Control	POAG	p
Total = **502** participants	**255**	**247**	
Family history of glaucoma (%)	N/A	18 (7.3)	
Family history of eye problems (%)	36 (14.1)	79 (32.0)	< 0.001
Family history of blindness (%)	18 (7.1)	60 (24.3)	< 0.001

p, Pearson's χ^2 test
N/A, not applicable: a family history of glaucoma was an exclusion criterion for controls

The ophthalmic family history is summarized in Table 2.2. There were 18 (7%) POAG participants with a family history of glaucoma. Significantly more POAG patients than control participants had a family history of non-specific eye 'problems' (32% POAG versus 14% in controls; P < 0.001) and a family history of blindness (24% POAG versus 7% in controls; P < 0.001).

3.3 Paired eye data

Pearson pairwise correlation coefficients for continuous traits measured in both eyes and the two-tailed significance of the relationship are presented in Table 2.3 along with the mean in each eye for each measurement. Individuals in whom measurements were only made in one eye or, in POAG participants, if one eye was untreated (in unilateral glaucoma or blindness), were excluded from this analysis.

Table 2.3 Correlation of paired eye data

	POAG				**Control**			
	RE	LE	r	p	RE	LE	r	p
CCT (µm)	505.1	507.0	0.88	<0.0001	513.5	515.4	0.87	<0.0001
VCDR	0.89	0.89	0.52	<0.0001	0.38	0.38	0.92	<0.0001
IOP1 (mmHg)	16.6	15.7	0.50	<0.0001	13.4	13.4	0.95	<0.0001
IOP2 (mmHg)	31.4	30.7	0.72	<0.0001	N/A	N/A	N/A	N/A
VF MD (dB)	-10.9	-10.0	0.47	<0.0001	-4.8	-4.2	0.82	<0.0001
GDx NFI (µm)	64.7	56.7	0.32	0.0041	24.9	21.7	0.28	0.0441
GDx TSNIT	37.4	39.9	0.41	0.0006	53.5	54.9	0.31	0.0370

RE, mean in right eyes; LE, mean in left eyes; r, Pearson pairwise correlation coefficient for measurements from the left and right eyes, p, p-value for two-tailed significance of r
IOP1, IOP at enrolment; IOP2, IOP at diagnosis; VF MD, Oculus visual field MD

There was a correlation between right and left eye measurements for all the continuous traits evaluated (CCT, VCDR, IOP at diagnosis and at enrolment, Oculus

MD and GDx NFI and TSNIT). The correlation in the POAG patients was strongest for CCT where it was very similar to the correlation for CCT in the control participants ($r = 0.88$ in POAG; $r = 0.87$ in controls). The correlation between left and right eyes was very strong for VCDR ($r = 0.92$) and IOP ($r = 0.95$) in the controls, whereas it was less so (but still significant; $p<0.0001$) for the POAG patients reflecting the asymmetrical nature of POAG. Similarly the correlation between left and right eye Oculus MD was stronger for the controls. The correlations for the GDx measurements were not as strong in both POAG and control participants suggesting more inter-eye variability, but the correlations were still significant ($p < 0.05$).

Left and right eyes correlated, therefore the mean of measurements from both eyes for each individual in whom there were appropriate measurements in both eyes was used in further analyses of CCT, VCDR, IOP at enrolment, Oculus MD and GDx NFI and TSNIT.

The maximum IOP recorded from either of the eyes in each individual was used in analyses of the IOP at diagnosis. The use of the ‘worse’ eye in this instance strengthens the disease characterisation.

3.4 Clinical findings (Table 2.4)

Table 2.4 Clinical features of study subjects

		Control	POAG	p	OR (95% CI)	p ⌘	OR ⌘ (95% CI)
Total = **502** participants		**255**	**247**				
MAP	n	203	217				
(mmHg)	Range	61.3 - 189.7	73.3 - 158.7				
	Mean ± SD	100.1 ± 16.2	100.8 ± 13.6	0.640 §	1.00 (0.99-1.01)		
IOP	n	255	247				
(mmHg)	Range	6 - 18	4 - 37				
	Mean ± SD	13.2 ± 2.6	16.2 ± 5.6	< 0.001 §	1.20 (1.14 - 1.26)	**< 0.001**	1.20 (1.13 - 1.27)
CCT	n	213	219				
(µm)	Range	420 - 609	379 - 587.5				
	Mean ± SD	513.5 ± 36.1	505.1 ± 37.9	0.020 §	0.99 (0.99 - 1.00)	**0.001**	0.99 (0.98 – 1.00)
VCDR	n	255	247				
	Range	0.1 - 0.7	0.3 - 1				
	Mean ± SD	0.37 ± 0.13	0.89 ± 0.13	< 0.001 §	5.32×10^{9} (1.6×10^{7} – 1.8×10^{12})	**< 0.001**	4.73×10^{9} (1.1×10^{7} – 2.0×10^{12})
Cataracts (%)		244 (95.7)	122 (49.4)	< 0.001 ¶	0.04 (0.02 - 0.09)	**< 0.001**	0.08 (0.04 - 0.16)

MAP, Mean arterial pressure; IOP, intraocular pressure at the time of enrolment; n, number of participants in whom the investigation was performed

OR (95%CI), odds ratio (95% confidence intervals)

⌘ adjusted for age and gender in a logistic regression model

¶ Pearson's χ^2 test

§ T-test

Most of the controls (96%) had lenticular opacities or had had cataract surgery. This was in contrast to the 49% of glaucoma patients with cataracts (p<0.001). Adjusting for age and gender did not alter this significant difference. Every participant had an IOP measurement at enrolment and an estimation of VCDR. The participants that were included from the *LOXL1* association study did not all have CCT estimations or sphygmomanometry. The mean arterial pressure (MAP) was similar in the two groups (p = 0.64), but the IOP at the time of enrolment (which was frequently a treated IOP), the VCDR and the CCT were all significantly different in control and POAG participants (p < 0.05). The significance of a thinner CCT in association with POAG remained when adjusted for age and gender in a logistic regression model (P = 0.001).

3.4.1 Central corneal thickness (Figure 2.3)

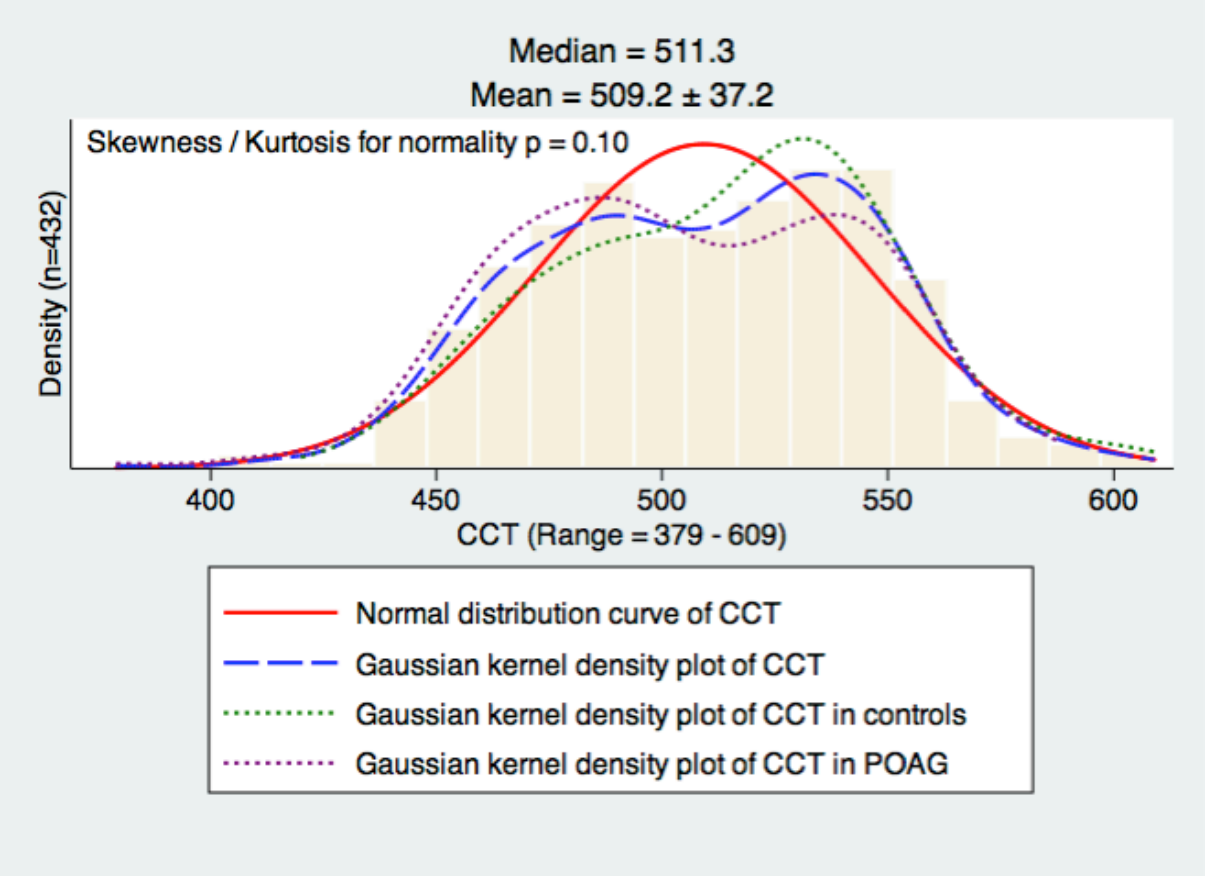

Figure 2.3 Central corneal thickness. Histogram of central corneal thickness in µm along the x-axis with the density in the study population on the y-axis. A normal distribution curve and Gaussian kernel density plots are superimposed on the histogram.

A CCT measurement was performed in 432 of the 502 study participants. The mean CCT for the group was 509.2µm (SD = 37.2µm). The histogram (Figure 2.3) illustrates an approximately bimodal distribution of CCT in this population. This is not caused by the thicker CCTs in controls, because the distribution is approximately bimodal in both cases and controls as illustrated by the Gaussian kernel density plots for cases and controls that overlie the histogram in Figure 2.3. Table 2.5 evaluates the effect that age, gender, IOP and diagnoses of POAG, diabetes mellitus and hypertension have on CCT. The mean CCT was thinner in older participants, in males, in hypertensives, in non-diabetics and in patients with POAG. The association was only significant for POAG ($p = 0.020$). IOP, either at diagnosis or at enrolment, did not affect CCT ($p > 0.05$). When age, gender, POAG, hypertensive and diabetic diagnoses were included in a multivariate linear regression model, age, POAG diagnosis and diabetic diagnosis were all significant components ($p < 0.05$).

Table 2.5 Central corneal thickness

	Mean CCT ± SD		p	Coefficient (95% CI) ❖	p ⌘	Coefficient (95% CI) ⌘
Age (M)	**≤ 66 (n = 224)**	**> 66 (n = 208)**				
	512. 0 ± 35.7	506.2 ± 38.7	0.106 §			
Age (n=432)			0.093 ❖	-0.25 (-0.54 - 0.04)	**0.014**	-0.41 (-0.74 - -0.08)
Male	**Female (n=200)**	**Male (n=232)**				
	512.3 ± 37.9	506.6 ± 36.5	0.109 §	-5.77 (-12.81 - 1.28)	0.191	-4.74 (-11.86 - 2.38)
POAG	**POAG (n=219)**	**Control (n=213)**				
	505.1 ± 37.9	513.5 ± 36.1	0.020 §	-8.36 (-15.37 - -1.35)	**0.002**	-12.26 (-19.87 - -4.66)
DM	**DM (n=132)**	**Non DM (n=300)**				
	514.2 ± 37.3	507.1 ± 37.1	0.067 §	7.12 (-0.5 - 14.74)	**0.035**	8.54 (0.59 - 16.48)
HT	**HT (n=269)**	**Non HT (n=163)**				
	507.5 ± 37.6	512.1 ± 36.5	0.210 §	4.6 (-11.86 - 2.66)	0.266	-4.58 (-12.64 - 3.49)
IOP Dx (n=390)			0.602 ❖	-0.08 (-0.38 - 0.22)		
IOP (n=432)			0.677 ❖	0.17 (-0.62 - 0.94)		

Age (M), age divided into 2 groups based on the median of 66 years; DM, diabetic; HT, diagnosed and treated for hypertension; Non HT, no history of hypertension; IOP Dx, IOP at diagnosis in POAG and at enrolment for controls; IOP, IOP at enrolment

Coefficient (95%CI), linear regression coefficient (95% confidence intervals)

❖ Univariate linear regression analysis

§ T-test

⌘ Adjusted for age, gender, POAG, hypertensive and diabetic diagnoses in a linear regression model

3.5 Structural and functional assessments

Visual fields were documented in 195 of the POAG patients. The remaining 52 patients were unable to complete the test, the most common reason being a visual acuity insufficient to perform the test. The visual field was reliable in at least one eye in 112 of the patients. The remaining 83 patients did not produce reliable fields on repeated testing. A small subgroup of 86 of the control participants also underwent a visual field assessment. The visual field was reliable in at least one eye in 44 of the patients. Figure 2.4 compares the mean deviation in the accurate visual field tests for the groups of POAG patients and control participants. The mean deviation was significantly different in the two groups ($p < 0.001$). The mean deviation was surprisingly low in the control group. In all instances this was a diffuse loss and often attributed to lenticular opacities.

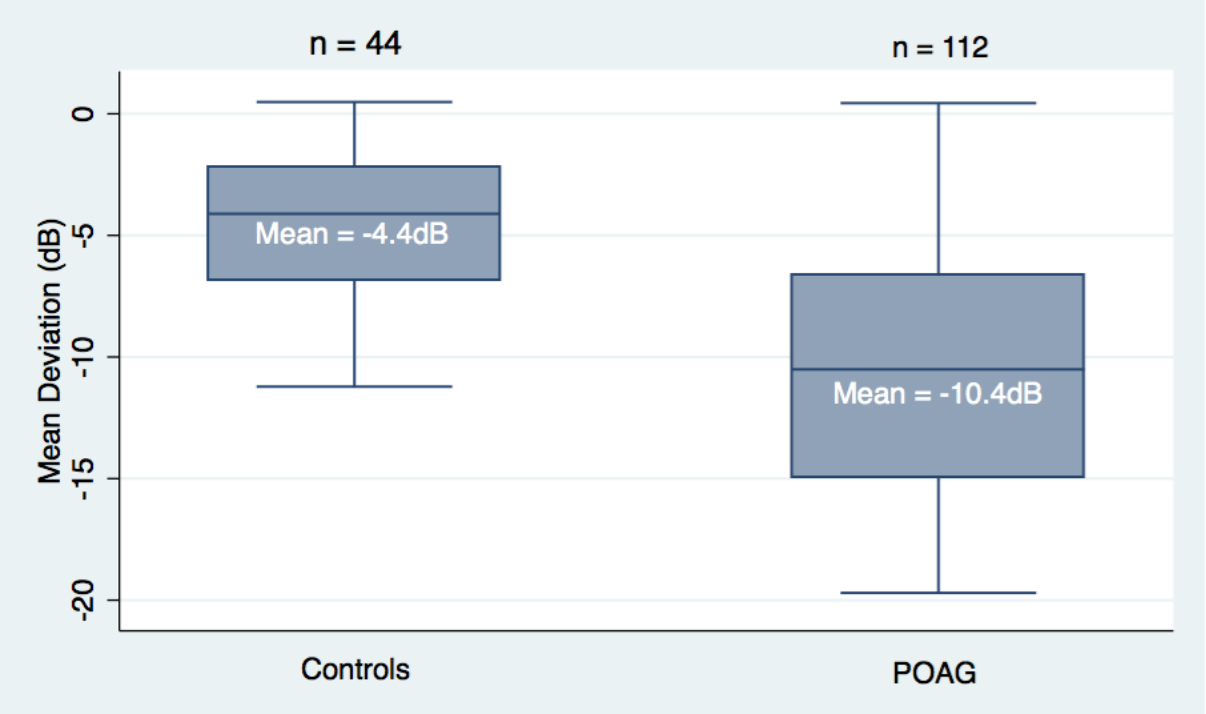

Figure 2.4 Oculus visual field mean deviation. Box-and-whisker plots in the POAG and control participants of Oculus visual field mean deviation. The bottoms and tops of the boxes are the lower and upper quartiles respectively, and the bands near the middle of the boxes is the median. The ends of the whiskers represent the upper and lower adjacent values. The means are recorded within the boxes. There was a significant difference in mean deviation in the two groups ($p < 0.001$).

A GDx VCC™ assessment of retinal nerve fibre layer thickness was performed in 175 of the POAG patients. The remaining 82 patients were unable to complete the test, the most common reason being a visual acuity insufficient to perform the test. The GDx was reliable in at least one eye in 141 of the patients. A small subgroup of 66 of the control participants also underwent a GDx VCC™ retinal nerve fibre layer assessment. The GDx was reliable in at least one eye in 58 of the patients. Figure 2.5 compares the GDx NFI and GDx TSNIT in the accurate GDX assessments for the groups of POAG patients and control participants. The two groups were significantly different in both GDx NFI and the average retinal nerve fibre layer thickness ($p < 0.001$).

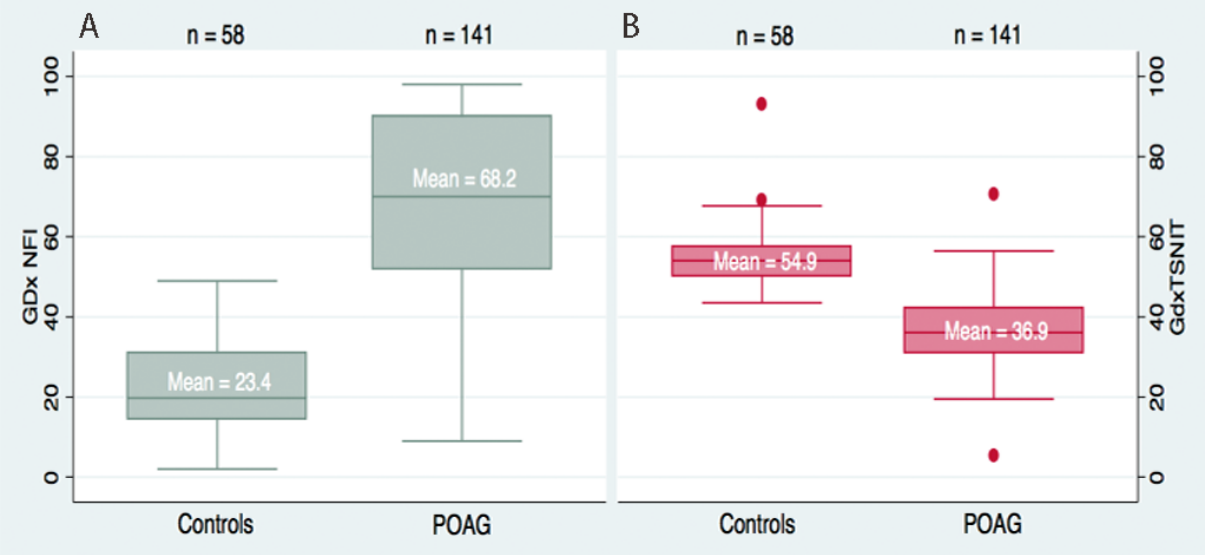

Figure 2.5 GDx VCC™ retinal nerve fibre layer assessments. Box-and-whisker plots in the POAG and control participants of:
A. GDx NFI - Nerve fibre index ($p < 0.001$)
B. GDx TSNIT - GDx average retinal nerve fibre layer thickness measured in µm ($p < 0.001$)
The bottoms and tops of the boxes are the lower and upper quartiles respectively, and the bands near the middles of the boxes are the median. The ends of the whiskers represent the upper and lower adjacent values. Any data not included between the whiskers is plotted as an outlier with a dot. The mean is given within the boxes.

3.6 Diabetes mellitus (Table 2.6)

Table 2.6 Comparison of diabetic and non-diabetic participants

		Diabetic	**Non-diabetic**	p	OR (95% CI)	p ⌘	OR (95% CI) ⌘
Total = **502** participants		**150** (29.9%)	**352** (70.1%)				
Female (%)		89 (59.3)	173 (49.2)	0.037 ¶	1.51 (1.03 - 2.22)	0.074	1.43 (0.97 - 2.12)
Age (years)	Mean ± SD	67.8 ± 8.5	64.1 ± 13.1	0.001 §	1.02 (1.01 - 1.04)	0.058	1.02 (0.99 - 1.04)
POAG (%)		57 (38.0)	190 (54.0)	0.001 ¶	0.52 (0.35 - 0.77)	**0.031**	0.63 (0.41 - 0.96)
HT (%)		126 (84.0)	183 (52.0)	< 0.001 ¶	4.85 (2.99 - 7.87)	**< 0.001**	4.33 (2.60 - 7.21)
HGT	n	123	297				
(mmol/l)	Mean ± SD	8.8 ± 4.1	5.9 ± 1.6	< 0.001 §	1.59 (1.41 - 1.79)	**< 0.001**	1.56 (1.38 - 1.76)
CCT	n	132	300				
(µm)	Mean ± SD	514.2 ± 37.3	507.1 ± 37.1	0.067 §	1.01 (0.99 - 1.01)	0.066	1.01 (0.99 - 1.01)

HT, hypertensive; HGT, random blood glucose from a haemoglucotest; n, number of participants in whom the investigation was performed
OR (95%CI), odds ratio (95% confidence intervals)
⌘ adjusted for age, gender and POAG diagnosis in a logistic regression model
¶ Pearson's χ^2 test
§ T-test

Within the complete group of 502 participants, 150 (30%) were known diabetics. Table 2.6 compares the diabetics to the non-diabetics. The proportion of females was greater for diabetics (p=0.037), however this association was no longer significant when corrected for age and POAG diagnosis in a logistic regression model (p=0.074). The diabetics were older (68 ± 9 years versus 64 ± 13 years; p = 0.031). Significantly more of the diabetics were also hypertensive even with adjustment for gender, age and a diagnosis of POAG (p > 0.001). The participants that were included from the *LOXL1* association study did not all have finger-prick haemoglucotests performed on them. The mean random blood glucose as determined by the haemoglucotest was higher in diabetics (9 ± 4 mmol/l versus 6 ± 2 mmol/l; p < 0.0001). A means comparison of CCT in the diabetics and non-diabetics with a t-test found that the mean CCT in diabetics was marginally thicker (p = 0.067). With adjustment for age, gender and POAG diagnosis in a logistic regression model, this association remained unchanged with a small effect size with the odds ratio close to 1.0.

The majority (95%) of the diabetics had a diagnosis of type 2 diabetes mellitus (Table 2.7). The remainder had type 1 diabetes mellitus. The diabetics requiring insulin, with or without the addition of oral hypoglycaemics, comprised 30% of the group. The mean length of time since diabetic diagnosis was 12 years. Diabetic retinopathy was present in 25% and cystoid macular oedema in 17% of diabetics. Table 2.7 documents these diabetic phenotypes as well as a comparison of the phenotypes in POAG patients and in controls. The POAG diabetics were younger (p = 0.001). CCT was thinner in the POAG diabetics, but this did not reach significance (p = 0.117). There were similar proportions of POAG and control participants with Type 1 diabetes (p = 0.260) and requiring treatment with insulin (p = 0.971). The mean time in years since the diagnosis of diabetes had been made was the same in both groups. However, significantly more of the control group had diabetic retinopathy (p<0.001) and cystoid macular oedema (0.003) than the POAG participants.

Table 2.7 Comparison of POAG and control diabetics

		Total	POAG	Control	p	p ⌘	OR ⌘ (95% CI)
n		**150**	**57**	**93**			
Age (years)	**Mean ± SD**	67.8 ± 8.5	65.0 ± 8.8	69.6 ± 7.9	0.001 §	**0.002**	0.93 (0.89 - 0.97)
Female (%)		89 (59.3)	32 (56.1)	57 (61.3)	0.533 ¶		
CCT (µm)	**Mean ± SD**	514.2 ± 37.3	507.8 ± 37.8	518.2 ± 36.6	0.117 §		
T1DM (%)		8 (5.33)	5 (8.77)	3 (3.23)	0.260 ⋆		
Insulin requiring (%)		45 (30.00)	17 (29.82)	28 (30.11)	0.971 ¶		
Diabetic retinopathy (%)		38 (25.33)	5 (8.77)	33 (35.48)	< 0.001 ⋆	**< 0.001**	0.15 (0.05 - 0.43)
Cystoid macular oedema (%)		25 (16.67)	3 (5.26)	22 (23.66)	0.003 ⋆	**0.004**	0.15 (0.04 - 0.55)
Time since	**Range**	1 - 40	1 - 40	1 - 40			
diagnosis (years)	**Mean ± SD**	11.56 ± 9.49	11.56 ± 9.03	11.56 ± 9.80	0.99 §		

T1DM, type 1 diabetes mellitus
OR (95%CI), odds ratio (95% confidence intervals)
¶ Pearson's χ^2 test
⋆ Fisher's exact test
§ T-test
⌘ adjusted for age and gender in a logistic regression model

3.7 Hypertension (Table 2.8)

Table 2.8 Comparison of hypertensive and normotensive subjects

		HT	**Non HT**	p	OR (95% CI)	p ⌘	OR ⌘ (95% CI)
Total = **502** participants		**309** (61.6%)	**193** (38.4%)				
Female (%)		187 (60.5)	75 (38.9)	< 0.001 ¶	2.41 (1.67 - 3.49)	**< 0.001**	2.23 (1.51 - 3.29)
Age (years)	**Mean ± SD**	68.5 ± 9.5	60.0 ± 13.8	< 0.001 §	1.07 (1.05 - 1.09)	**< 0.001**	1.07 (1.05 - 1.09)
POAG (%)		132 (42.7)	115 (59.6)	< 0.001 ¶	0.51 (0.35 - 0.73)	0.683	0.92 (0.60 - 1.4)
DM (%)		126 (40.8)	24 (12.4)	< 0.001 ¶	4.85 (2.99 - 7.87)	**< 0.001**	4.35 (2.61 - 7.24)
MAP (mmHg)	**n**	263	157				
	Mean ± SD	102.0 ± 16.2	98.0 ± 12.1	0.008 §	1.02 (1.00 - 1.03)	**0.040**	1.02 (1.00 - 1.03)
CCT (µm)	**n**	269	163				
	Mean ± SD	507.5 ± 37.6	512.1 ± 36.5	0.210 §	1.00 (0.99 - 1.00)	0.668	1.00 (0.99 - 1.00)

HT, diagnosed and treated for hypertension; Non HT, no history of hypertension; OR (95%CI), odds ratio (95% confidence intervals)
DM, diabetic; MAP, Mean arterial pressure; n, number of participants in whom the investigation was performed
¶ Pearson's χ^2 test
§ T-test
⌘ adjusted for age and gender in a logistic regression model

Known hypertensives receiving treatment for this condition comprised 62% of the study group (309 participants). There was a significantly greater proportion of females ($p < 0.001$) in the hypertensive subgroup. The difference remained significant when corrected for age in a logistic regression model. The hypertensives had a mean age of 68 years compared to the non-hypertensives whose mean age was 60 years ($p < 0.0001$). Correcting for gender did not alter the significance ($p < 0.001$). Diabetic comprised 41% of the hypertensive group versus 12% of the non-hypertensives ($p < 0.001$). The mean arterial pressure was higher in hypertensives (102 ± 16 mm/Hg versus 98 ± 12 mm/Hg; $p = 0.0077$). The thicker mean CCT in non-hypertensives did not reach statistical significance.

3.8 Glaucoma phenotypes (Table 2.9)

Table 2.9 Characteristics of POAG participants

		POAG
Total number of POAG participants		247
Age at diagnosis (years)	n	205
	Range	17 - 84
	Mean ± SD	55.0 ± 13.6
	Primary juvenile glaucoma (%)	**26 (12.7)**
IOP at diagnosis (mmHg)	n	202
	Range	18 - 68
	Mean ± SD	34.9 ± 9.1
	Normal tension glaucoma (%)	6 (3.0)
Glaucoma stage	n	247
	Advanced (%)	176 (71.3)
	Early (%)	14 (5.7)
	Severe visual impairment (%)	**130 (52.6)**
	One eye (%)	103 (41.7)
	Both eyes (%)	27 (10.9)
Glaucoma Treatment	n	247
	Drainage surgery (%)	**133 (53.9)**
	Medical therapy alone (%)	95 (38.5)
	Laser treatment (%)	37 (15.0)
	IOP control (%)	169 (68.4)

n, number of participants in whom the variable was recorded

The glaucoma diagnostic and treatment history of the POAG participants is described in Table 2.9. The mean age at diagnosis was 55 years and 26 (13%) of the POAG participants had juvenile onset open angle glaucoma. A subgroup of the patients did not know when the diagnosis of POAG was made and had no clinic records. In this same group the IOP at diagnosis was not known. The mean IOP at

diagnosis was 35mmHg (SD = 9mmHg) and 6 of the patients had normal tension glaucoma (IOP less than or equal to 21mmHg). A diagnosis of advanced glaucoma (VCDR greater than 0.9 in either eye) was present in 71% of participants and early glaucoma (VCDR less than 0.7 in both eyes) in 6%. Over half (53%) of the patients had severe visual impairment (visual acuity less than 6/60) in at least one eye. The patients were treated surgically in 54% of cases, only with medical therapy in 38% and in 15% the treatment consisted of laser therapy either alone or in combination with medical or surgical therapy. The treatment had resulted in an IOP at enrolment of less than 18mmHg in treated eyes in 68% of the patients, who were judged to have achieved 'IOP control'.

3.8.1 Primary juvenile glaucoma (Table 2.10)

Table 2.10 Primary juvenile glaucoma

		Juvenile	**Adult-onset POAG**	p	OR (95% CI)	p ⌘	OR ⌘ (95% CI)
Total = **247** POAG		**26** (10.5%)	**221** (89.5%)				
Female (%)		6 (23.1)	118 (53.4)	0.003 ⋆	0.26 (0.10 - 0.68)	0.801	0.74 (0.07 - 7.59)
Age	**Mean ± SD**	35 ± 10.2	63.0 ± 7.6	< 0.001 §	0.65 (0.55 - 0.78)	**< 0.001**	0.61 (0.48 - 0.78)
IOP (mmHg)	**n**	24	179				
	Mean ± SD	38.4 ± 9.6	34.6 ± 9.4	0.063 §	1.04 (1.00 - 1.08)		
VCDR	**n**	26	221				
	Mean ± SD	0.94 ± 0.10	0.89 ± 0.13	0.079 §	56.2 (0.6 - 5456.2)		
CCT (µm)	**n**	25	194				
	Mean ± SD	519.0 ± 32.5	503.3 ± 38.3	0.052 §	1.01 (1.00 - 1.02)		
Advanced glaucoma (%)		24 (92.3)	152 (68.8)	0.011 ⋆	5.45 (1.25 - 23.70)	0.831	1.63 (0.02 - 142.70)
SVI (%)		20 (76.9)	110 (49.8)	0.012 ⋆	3.33 (1.30 - 8.62)	0.372	5.18 (0.14 - 192.29)
Glaucoma Surgery (%)		18 (69.2)	115 (52.0)	0.144 ⋆	2.07 (0.87 - 4.97)		
IOP control (%)		21 (80.8)	148 (67.0)	0.186 ⋆	2.04 (0.74 - 5.63)		
Family history of blindness (%)		4 (15.4)	56 (25.3)	0.338 ⋆	0.53 (0.18 - 1.62)		
Cataracts (%)		6 (23.1)	116 (52.5)	0.006 ⋆	0.27 (0.10 - 0.70)	**0.046**	36.0 (1.1 - 1207.8)

Juvenile, primary juvenile glaucoma; OR (95%CI), odds ratio (95% confidence intervals)
IOP, IOP at diagnosis; n, number of participants in whom the variable was recorded; SVI, severe visual impairment
⋆ Fisher's exact test
§ T-test
⌘ Adjusted for age, gender, advanced glaucoma, severe visual impairment and cataracts in a multivariate logistic regression model

The 26 glaucoma patients known to have primary juvenile glaucoma (onset from 10 to 35 years of age) were predominantly male (77% versus 46% in the non-juvenile

POAG group; p = 0.003). By definition they were considerably younger than the presumed 'Adult-onset' group. The IOP at diagnosis was higher and the VCDR was greater in this group, although neither difference reached statistical significance (p > 0.05). The CCT was thicker (marginally significant; p = 0.052) in the juvenile group. The proportion of primary juvenile glaucoma patients with advanced glaucoma and severe visual impairment was significantly greater than in the 'Adult-onset' POAG (p < 0.05), but the adult-onset group had more participants with lenticular opacities (p = 0.006). The variables gender, age, advanced glaucoma, severe visual impairment and cataracts reached statistical significance in a univariate model and were included in a multivariate logistic regression model. Gender, advanced glaucoma and severe visual impairment were no longer significant factors associated with primary juvenile glaucoma in this model. Cataracts were significantly associated with 'adult-onset' POAG in a univariate model, but once an age correction was factored in they were associated with primary juvenile glaucoma.

3.8.2 Severe visual impairment (Table 2.11)

Table 2.11 Severe visual impairment (SVI)

		SVI	**No SVI**	p	OR (95% CI)	p ⌘	OR ⌘ (95% CI)
Total = **247** POAG		**130** (52.6%)	**117** (47.4%)				
Female (%)		57 (43.9)	67 (57.8)	0.029 ¶	0.57 (0.34 - 0.95)	0.901	0.96 (0.51 - 1.82)
Age (years)	**Mean ± SD**	58.8 ± 13.8	61.5 ± 12.4	0.107 §	0.98 (0.97 - 1.00)	0.769	1.00 (0.97 - 1.02)
Age Dx (years)	**Mean ± SD**	53.9 ± 14.5	56.1 ± 12.4	0.262 §	0.99 (0.97 - 1.00)		
IOP (mmHg)	**n**	109	93				
	Mean ± SD	36.7 ± 9.2	32.9 ± 9.4	0.004 §	1.05 (1.01 - 1.08)	0.139	1.03 (0.99 - 1.06)
VCDR	**n**	130	117				
	Mean ± SD	0.94 ± 0.11	0.85 ± 0.13	<0.001 §	475 (36 - 6203)	**<0.001**	1937 (73 - 51529)
CCT (µm)	**n**	115	103				
	Mean ± SD	505.2 ± 36.7	505.4 ± 39.4	0.973 §	1.00 (0.99 - 1.01)		
Glaucoma Surgery (%)		79 (60.8)	54 (46.6)	0.026 ¶	1.78 (1.07 - 2.95)	0.612	1.18 (0.61 - 2.28)
IOP control (%)		85 (65.4)	83 (71.6)	0.341 ¶	0.76 (0.45 - 1.32)		
Cataracts (%)		67 (51.5)	55 (47.4)	0.479 ¶	1.20 (0.73 - 1.98)		

SVI, severe visual impairment; OR (95%CI), odds ratio (95% confidence intervals)
Age Dx, age at diagnosis of POAG; IOP, IOP at diagnosis; n, number of participants in whom the variable was recorded
¶ Pearson's χ^2 test
§ T-test
⌘ Adjusted for age, gender, IOP, VCDR and glaucoma surgery in a multivariate logistic regression model

Of the 130 patients with a Snellen visual acuity of worse than 6/60 in at least one eye, 44% were female (versus 58% in the group without severe visual impairment; p

= 0.029). The mean age in the severely visually impaired group was 59 years. This did not differ significantly from the group without severe visual impairment. The two groups did have a significantly different mean IOP at diagnosis (p = 0.004) and VCDR (p < 0.001). More of the severely visually impaired participants had undergone glaucoma drainage surgical procedures (p = 0.026). The variables gender, IOP, VCDR and glaucoma surgery reached statistical significance as predictors of severe visual loss in POAG in a univariate model and were included, together with age, in a multivariate logistic regression model. The only feature that still remained a significant predictor of severe visual loss in this model was a larger VCDR (adjusted p < 0.001).

3.8.3 Glaucoma drainage surgery (Table 2.12)

Table 2.12 Glaucoma drainage surgery

		Surgery	No surgery	p	OR (95% CI)	p ⌘	OR (95% CI) ⌘
Total = **247** POAG		**133**	**114**				
Female		68 (51.1)	56 (49.1)	0.753 ¶	1.08 (0.66 - 1.79)		
Age (years)	**Mean ± SD**	59.4 ± 14.0	60.8 ± 12.0	0.425 §	0.99 (0.97 - 1.01)		
Age Dx (years)	**Mean ± SD**	52.2 ± 14.4	58.1 ± 11.8	0.002	0.97 (0.95 - 0.99)	0.161	0.98 (0.96 - 1.01)
IOP (mmHg)	**n**	109	94				
	Mean ± SD	37.7 ± 9.6	31.8 ± 8.2	<0.001 §	1.08 (1.04 - 1.12)	**0.001**	1.07 (1.03 - 1.12)
VCDR	**n**	132	114				
	Mean ± SD	0.94 ± 0.09	0.85 ± 0.15	<0.001 §	450 (35 - 5756)	**0.010**	57 (3 - 1250)
CCT (µm)	**n**	121	98				
	Mean ± SD	504.1 ± 40.0	506.4 ± 35.4	0.662 §	1.00 (0.99 - 1.01)		
SVI (%)		79 (59.4)	51 (44.7)	0.026 ¶	1.78 (1.07 - 2.95)	0.439	1.31 (0.66 - 2.62)
IOP control (%)		99 (74.4)	70 (61.4)	0.035 ¶	1.78 (1.04 - 3.08)	0.12	1.70 (0.87 - 3.33)
Cataracts (%)		71 (53.4)	51 (44.7)	0.157 ¶	1.44 (0.87 - 2.38)		

Surgery, glaucoma drainage surgery; OR (95%CI), odds ratio (95% confidence intervals)

IOP, IOP at diagnosis; Age Dx, age at diagnosis of POAG; n, number of participants in whom the variable was recorded; SVI, severe visual impairment

¶ Pearson's χ^2 test

§ T-test

⌘ Adjusted for age at diagnosis, IOP, VCDR, severe visual impairment and IOP control in a multivariate logistic regression model

Patients that had undergone glaucoma surgical procedures were similar to those that had not in terms of age and gender and both groups had similar CCTs. The age at diagnosis, however, was younger in the surgery group (p = 0.002). The surgery group had a higher mean IOP at diagnosis (p < 0.001) and a larger VCDR (p < 0.001). A higher proportion of these individuals had advanced glaucoma and severe visual impairment. IOP control was achieved in 74% of the surgical patients that had

an IOP less than 18mmHg in the treated eye/eyes on enrolment. This is in contrast to 61% achieving IOP control in the medical and laser therapy group of patients (p = 0.035). The surgery group did not have a significantly higher proportion of lenticular opacities (p = 0.157). The variables age at diagnosis, IOP, VCDR, severe visual impairment and IOP control reached statistical significance for an association with glaucoma surgery in a univariate model and were included in a multivariate logistic regression model. The significant associations that remained in this model were the higher IOP (adjusted p = 0.001) and the larger VCDR (adjusted p = 0.010) in the surgery group.

3.8.4 Family history of blindness (Table 2.13)

Table 2.13 Family history of blindness

		Family history	No family history	p	OR (95% CI)
Total = **247** POAG		**60** (24.3%)	**187** (75.7%)		
Female (%)		30 (50.0)	94 (50.3)	0.971 ¶	0.99 (0.55 - 1.77)
Age (years)	**Mean ± SD**	59.3 ± 11.8	60.3 ± 13.6	0.605 §	0.99 (0.97 - 1.02)
Age Dx (years)	**Mean ± SD**	55.3 ± 12.3	54.8 ± 13.9	0.841 §	1.00 (0.98 - 1.03)
IOP (mmHg)	**n**	49	154		
	Mean ± SD	36.5 ± 10.0	34.5 ± 9.3	0.209 §	1.02 (0.99 - 1.06)
VCDR	**n**	60	186		
	Mean ± SD	0.93 ± 0.10	0.89 ± 0.13	**0.042 §**	17.8 (1.1 - 297.6)
CCT (µm)	**n**	53	166		
	Mean ± SD	509.3 ± 34.8	503.8 ± 38.9	0.363 §	1.00 (0.99 - 1.01)
SVI (%)		31 (51.7)	99 (52.9)	0.833 ¶	0.94 (0.52 - 1.68)
Glaucoma Surgery (%)		36 (60.0)	97 (51.9)	0.272 ¶	1.39 (0.77 - 2.50)
IOP control (%)		40 (66.7)	129 (69.0)	0.864 ¶	0.95 (0.51 - 1.77)
Cataracts (%)		25 (41.7)	97 (51.9)	0.158 ¶	0.66 (0.36 – 1.18)

OR (95%CI), odds ratio (95% confidence intervals)

IOP, IOP at diagnosis; Age Dx, age at diagnosis of POAG; n, number of participants in whom the variable was recorded; SVI, severe visual impairment

¶ Pearson's χ^2 test

§ T-test

The 60 patients with a family history of blindness did not differ significantly from the 187 patients with no family history of blindness in terms of gender, age, age at diagnosis, IOP at diagnosis or CCT (p > 0.05). The proportion in either group with severe visual impairment, glaucoma drainage surgery, IOP control or cataracts was also similar (p > 0.05). The only difference in the groups was a larger VCDR in the group with a family history (p = 0.042).

4. DISCUSSION

4.1 Ethnicity

The study population of POAG and control participants, all of whom were self-identified black African participants who were enrolled at St John Eye Hospital in Soweto, Johannesburg, was representative of the black population of Johannesburg, of Gauteng and of South Africa. Within the study population were participants who spoke, as their first language, each of the eleven official languages of South Africa (IsiZulu, Sesotho, Setswana, IsiXhosa, Sepedi, Xitsonga, Tshivenda, Siswati, IsiNdebele, English and Afrikaans) with the exception of English. That the majority languages were slightly over-represented in the study population may reflect the specific demographics of Diepkloof (where the hospital is situated) or Soweto relative to Johannesburg as a whole.

4.2 Comorbidities

A significant proportion of the study population (62%) had been diagnosed with hypertension. Age is a recognised risk factor for hypertension in South Africa as it is worldwide, and the high prevalence of hypertension in the study population reflects both the mean age of the study population (65 years) and the prevalence of hypertension in this population.[260] Population surveys in South Africa have reported a prevalence of hypertension of about 50% in the over 55 year age group.[261, 262] Diabetes mellitus and hypertension were both significant comorbidities in the study population. The proportion of diabetics in the study population was 30%. Sub-Saharan Africa traditionally has a lower prevalence of diabetes than developed nations.[263] Within Sub-Saharan Africa, the prevalence is highest in South Africa with diabetes affecting approximately 9% of the population over the age of 55 years.[264] In this context the 30% prevalence we found is high and may reflect the selection bias introduced because this was a clinic population. In keeping with the findings in this study population, a Sub-Saharan meta-analysis has revealed that more than 90% of Sub-Saharan diabetics have type 2 diabetes mellitus.[263] A quarter of the diabetics in this study had diabetic retinopathy, consistent with reports from South Africa and

worldwide.[265-267] The diabetics in this study had thicker CCTs (after adjusting for age, gender, POAG and hypertensive diagnoses) and this finding is also supported by the literature.[268, 269]

4.3 Central corneal thickness

CCT is recognised to be thinner in populations of African descent.[219, 220] This study population is no exception with a mean CCT of 509μm that is considerably lower than the mean of 555μm that is widely quoted as the 'normal' CCT for populations of European descent. CCT has been reported to become thinner with age, although not consistently.[270] Our findings suggest that this is the case in the study population.

4.4 Comparison of POAG and control groups

The POAG and control groups of the study population were of similar sizes and were similar in gender and ethnic identity.

4.4.1 Age

The POAG and control groups had significantly different ages. This was because older controls than POAG participants were deliberately enrolled to strengthen the diagnostic certainty of the 'control' label. This selection bias resulted in more hypertensive controls than hypertensive POAG patients.

4.4.2 Clinic population selection bias

The controls were a clinic population enrolled from St John Eye Hospital. In that age group (over 60 years old) the commonest reasons for management at the clinic are cataracts, diabetes and glaucoma. Consequently more of the control participants had lenticular opacities (or were pseudophakic) and more were diabetic than the POAG participants even after adjusting for the older age of the controls. Diabetes is thought to be an independent risk factor for POAG, so one might otherwise have expected more POAG patients to be diabetic than the controls. The selection bias may also be

a reason for the significantly greater proportion of control diabetics with diabetic retinopathy and cystoid macular oedema than the POAG diabetics. This difference could also be explained by better ophthalmological follow-up of diabetic patients that also have POAG.

4.4.3 Vertical cup-to-disc ratio

VCDR was an important diagnostic criterion in this study and was consequently significantly different in POAG patients and controls.

4.4.4 Automated perimetry

Automated perimetry formed part of the diagnosis of POAG. The mean deviation on perimetry was significantly different in the POAG and control participants despite the relatively small group of controls that had the investigation. The control participants had a lower mean deviation than might have been anticipated because so many had lenticular opacities. The field loss in all the controls was diffuse.

POAG and control participants performed equally poorly on the Oculus CenterField® test with reliable fields recorded in 57% and 51% respectively. One might have expected a worse performance in the POAG patients because of their worse visual acuity, but perhaps the controls performed worse because frequently they did not repeat the test, so they did not have the benefit of a 'learning effect'. Another factor in the poor performance may be the Oculus CenterField® machine. Newer generation automated perimeters have faster protocols and are more ergonomically designed. Greater comfort and speed might result in more reliable tests.

4.4.5 Nerve fibre analysis

A nerve fibre analysis using scanning laser polarimetry (GDx VCC™) was completed in 71% of the POAG patients and in 26% of the controls. This investigation confirmed that the nerve fibre layer was significantly thinner in POAG patients. The nerve fibre index was below 50 in all controls and above 50 in most of the POAG patients suggesting that it is a reasonable indicator of glaucoma.

4.4.6 Intra-ocular pressure

A raised IOP was not a diagnostic criterion for POAG in this study but IOP is the most important risk factor for POAG. Consequently the mean IOP, both at enrolment, when it was frequently a treated IOP, and at diagnosis, was significantly higher in the POAG participants.

4.4.7 Central corneal thickness

The association of CCT with POAG is controversial.[216] In this study population the POAG patients had a thinner mean CCT even with adjustment for age, gender, diabetes and hypertension. This association has previously been reported in other population groups.[217, 218] However, it is not possible to separate out the effect of CCT on IOP measurement error with Goldmann applanation tonometry, so this is not evidence that CCT is an independent risk factor for POAG in this population.

4.4.8 Family history of blindness

A family history of glaucoma is another important risk factor for POAG. We excluded controls with family histories of glaucoma. However, significantly more of the POAG patients than controls had family histories of eye problems and blindness, despite there being more control participants with cataracts and with diabetes. Both cataracts and diabetes are important causes of blindness that are heritable. It is reasonable, therefore, to assume that the difference in family history of blindness is attributable to undiagnosed glaucoma within the POAG families.

4.5 POAG phenotypes

Phenotypically, the POAG patients were young, had advanced disease and high IOPs. This picture, although different from that in developed nations, is fairly typical of the reports from Sub-Saharan Africa.[11, 271-273]

4.5.1 Primary juvenile glaucoma

The mean age at diagnosis in the study population POAG patients was 55 years. The age at diagnosis is not the same as the age at onset. In the context of patients presenting with advanced disease, the age of onset could be many years before the age at diagnosis. It is likely, therefore, that the proportion of patients with juvenile onset glaucoma was underestimated. Finding significant and specific phenotypic features in the juvenile onset group was limited by its small size. There were more males in the juvenile group and there were more patients with advanced cupping and severe visual impairment, but these features did not remain significant in a multivariate model. Primary juvenile glaucoma is thought to be monogenic (and autosomal dominant) more frequently than adult-onset POAG and this would lead one to expect more significant family histories of glaucoma or blindness. This was not the case in this group. A surprising association was identified with primary juvenile glaucoma and cataracts after adjusting for age, gender, advanced glaucoma and severe visual impairment. I think this may be because a greater proportion of the juvenile-onset group had glaucoma surgical procedures even though this difference did not reach statistical significance. Cataracts are a recognised complication of glaucoma drainage surgery.

4.5.2 Glaucoma management

The mean maximum IOP at diagnosis in the POAG patients was 35mmHg. This is very high. Considering that medical therapy can be expected to lower the IOP by a maximum of about 40%, this implies that a significant proportion of this group of patients would only achieve an IOP less than 18mmHg with more invasive therapy. It is not therefore surprising that 54% of the patients had undergone a glaucoma drainage surgical procedure. Glaucoma drainage surgery was correlated with higher IOPs and more advanced cupping. Although more of the patients who had surgery developed cataracts, this difference was not significant. Probably as a result of the high surgical rate in this POAG group, IOP control had been achieved at the time of enrolment in 69% of the patients. A higher proportion of surgically treated patients achieved IOP control than non-surgically treated, but again the difference was not significant.

4.5.3 Normal tension glaucoma

The high mean IOP also reflects how few patients there were in this group with NTG. The Hlabisa[5] and Temba[4] Glaucoma Prevalence studies that were undertaken in rural and urban South Africa respectively, both found that almost half of the POAG cases detected had untreated IOPs in the normal tension range. It is surprising then that there were so few patients with NTG in this cohort. This may be the result of under-diagnosis.

There is no formal screening programme for glaucoma in South Africa, but St John Eye Hospital is a referral centre. Many of the glaucoma referrals are from optometrists who routinely perform tonometry on patients. Perhaps, as a result, the referrals to St John Eye Hospital are preferentially ocular hypertensive patients.

The gold standard for glaucoma diagnosis is disc changes on fundoscopy matched to visual field defects. More than 50% of the study patients could not perform visual field testing, either at all or reliably. While the inability to produce reliable fields may relate to individual patient difficulty with the test or the instrument, in many instances this reflects the advanced disease state. In the absence of reliable fields, and in patients with glaucomatous discs, a diagnosis of POAG was accepted if the IOP was greater than 25mmHg. This almost certainly introduced a bias away from patients with NTG.

4.5.4 Glaucoma stage

The fact that so many of the study patients had advanced disease (71%) and severe visual impairment (53%) suggests that not only NTG, but POAG itself is under-diagnosed in this population. The South African glaucoma prevalence studies confirm this.[4, 5] The low prevalence of a family history of glaucoma in the POAG group, taken together with the high prevalence of a family history of eye problems and blindness, provides more supportive evidence for an under-diagnosed condition. The only predictor of severe visual loss in the POAG participants in this study in the regression model including age, gender, IOP, VCDR and glaucoma surgery was a larger VCDR.

4.5.5 Family history of blindness

A larger VCDR was also the only factor associated with a family history of blindness. This suggests that the monogenic, autosomal dominant form of POAG in this population is phenotypically very similar to the entire group.

4.6 Limitations

There was a selection bias introduced by enrolling control subjects from St John Eye Hospital that were over the age of 60 years (see 4.4.2). The commonest reasons for management at the clinic in this age group are cataracts, diabetes and glaucoma, so in the control population, cataracts and diabetes were over-represented. Selecting controls of an older age was, however, important because it enhanced the diagnostic certainty of the control label. It is still possible, although unlikely, that there were pre-symptomatic and pre-perimetric POAG patients classified as controls.

The study population was adequately sized to phenotype POAG in this group of clinic patients. Some of the POAG subtypes, like juvenile-onset glaucoma and NTG, were, however, uncommon and therefore the subgroups were too small to ascertain if there were significant phenotypic differences.

There were missing clinic records from some of the participants, so it was not always possible to determine the ocular status at the time of diagnosis. In addition not all of the participants included from the *LOXL1* study had investigations like CCT, blood pressure and haemoglucotest. However, these investigations were available for the majority of the participants.

A relatively small group of the controls had retinal nerve fibre layer and visual field assessments. The performance, particularly on visual field testing, was poor in all participants. This is a theoretical concern because the diagnosis of POAG rests on the combination of visual field defects with glaucomatous optic nerve head changes. Practically, however, the patients seen at St John Eye Hospital are often in so advanced a disease state as to make the diagnosis straightforward in the absence of

fields. Including IOP in the diagnosis of these advanced cases may have biased the dataset away from participants with advanced NTG.

4.7 Summary

In summary the study population consisted of representative South African black POAG patients and control participants from the same population. A significant proportion of both groups were diabetic and hypertensive. The POAG group frequently had a family history of blindness. The POAG patients were relatively young. They had high IOPs and many had had glaucoma surgical procedures. The POAG in this group was mostly advanced disease and more than half of the patients had severe visual impairment from POAG.

DNA samples from the group were banked in the sample repository at the NHLS Molecular Genetics Laboratory.

Chapter 3: Myocilin Study

1. INTRODUCTION

The purpose of this part of the study was to explore myocilin mutations in black South Africans.

This was initially achieved by identifying sequence variants in the *MYOC* gene in this population. This was done by sequencing the exons of the gene in 100 POAG patients and 97 control participants from the study population. This was undertaken at Duke University in Durham, North Carolina, United States of America.

In the second part of the study additional study participants were evaluated for the mutations that were discovered from sequencing. This was achieved by including the mutations in an assay designed for use with the Illumina® BeadXpress genotyping platform.

Finally, relatives of participants identified as carrying potentially pathogenic myocilin mutations were screened. The relatives were screened both for glaucoma and for the mutations. The purpose of this was to determine the pathogenicity of the mutations, and the penetrance and phenotypic characteristics of POAG associated with the mutations.

2. MYOCILIN MUTATIONS IN BLACK SOUTH AFRICANS WITH POAG

This portion of the research was undertaken in Michael Hauser's laboratory in the Duke Center for Human Genetics. The results were published in the following article:[274]

> **Whigham BT, Williams SE,** Liu Y, Rautenbach RM, Carmichael TR, Wheeler J, Ziskind A, Qin X, Schmidt S, Ramsay M, Hauser MA, Allingham RR. **Myocilin mutations in black South Africans with POAG.** *Mol Vis* 2011; 17:1064-9.

Benjamin Whigham (from Duke University) and I were joint first authors on this paper.

Aliquots of the DNA samples from the first 97 control and first 100 POAG participants that were enrolled in the study population (Chapter 2) were sent to Michael Hauser's laboratory in Durham. Whigham designed primers flanking the entire coding sequence of *MYOC* and at least 80 base pairs into each intron of *MYOC*. Polymerase chain reaction (PCR) amplifications of the region were purified and sequenced and the sequences analysed. Whigham also sequenced the *MYOC* exons in a further South African group that consisted of 13 POAG cases and 34 controls that had been included in a previous study on exfoliation glaucoma by Rautenbach *et al.*[275] All of the additional cases were IsiXhosa speaking participants enrolled from the East London Hospital Complex. There were no potentially pathogenic mutations identified in the additional cases and no variants different from those in the 197 participants from this study population. Although the East London participants were included in the paper, I have not included them further in this discussion.

A total of 19 *MYOC* variants, 12 in coding regions and 7 in non-coding regions of the gene, were identified in the sampled population (Table 3.1). Seven of the variants were novel variants.[274] Of the 12 coding variants, 7 were silent variants that were not predicted to change the amino acid sequence of the protein, 4 were missense

changes and one was a deletion causing a frameshift. The frameshift mutation (Tyr453del) was thought to be glaucoma-causing and in this study was identified in one control and 3 POAG participants. Only two of the variants, Gly374Val and Lys500Arg, were not identified in control participants. These were both missense changes occurring, as with the frameshift mutation, in exon 3 of the gene. It is in this part of the gene, the part that codes for the olfactomedin-like domain of the protein, that most pathogenic myocilin mutations are situated. A further silent variant in exon 3 (Thr325Thr) was more common in POAG than in controls and two intronic variants (rs79255460 and rs2032555) were commoner in control participants.

Table 3.1 List of *MYOC* variants identified from sequencing 100 POAG and 97 control participants of the study population

Nucleotide Sequence Change *	Location	Amino acid change	dbSNP ID	Observed in controls (n=97)	Observed in POAG (n=100)
c.1-91 -92delCT §	Promoter	Non-coding	-	2	1
c.1-83G>A	Promoter	Non-coding	rs2075648	1	0
c.39T>G	Exon 1	Pro13Pro	rs12082573	6	6
c.227G>A	Exon 1	Arg76Lys	rs2234926	1	0
c.477A>G	Exon 1	Leu159Leu	rs61730977	6	6
c.604+13A>C §	Intron 1	Non-coding	-	5	4
c.604+50G>A §	Intron 1	Non-coding	-	6	6
c.612G>T	Exon 2	Thr204Thr	rs57824969	2	3
c.654G>A §	Exon 2	Glu218Glu	-	2	2
c.730+35G>A	Intron 2	Non-coding	rs2032555	15	8
c.731-73C>T §	Intron 2	Non-coding	rs79255460	9	1
c.731-23G>A §	Intron 2	Non-coding	-	1	2
c.975G>A	Exon 3	Thr325Thr	rs61730976	3	13
c.1041T>C	Exon 3	Tyr347Tyr	rs61730974	1	0
c.1054G>A	Exon 3	Glu352Lys	rs61745146	4	4
c.1121G>T §	Exon 3	Gly374Val	-	0	2
c.1188G>A	Exon 3	Glu396Glu	rs61730975	6	9
c.1357delT	Exon 3	Tyr453del	-	1	3
c.1499A>G	Exon 3	Lys500Arg	rs145977437	0	2

*Nucleotides numbered as in Ensembl accession number ENSG00000034971 (transcript ENST00000037502)
§ Novel variants

2.1 Tyr453del

The Tyr453del mutation is a frameshift mutation in exon 3 of the *MYOC* gene that results in the addition of 10 ectopic amino acids and truncates the MYOC protein. Nonsense mediated RNA decay and consequently haploinsufficiency of the myocilin protein are not thought to be causes of POAG: myocilin-mediated POAG has a gain-

of-function disease model.[276] The mechanism of POAG in association with this mutation is not therefore known, however other frameshift mutations in exon 3 of the gene have been reported to be strongly associated with glaucoma (http//www.myocilin.com).[277] The mutation was first identified by Fingert *et al.*[65] in an African American POAG patient. Liu *et al.*[247] subsequently identified the mutation in one of 529 African American POAG patients. It has also been identified in a glaucoma patient of admixed African and Andean descent.[278] It was not found in Ghanaian cases from West Africa and it has not been reported in non-African populations.[247] Ours has been the only report of this mutation in a control participant with no evidence of glaucoma.[274] The mutation was observed at the high rate of 3% in this study population compared with less than 0.5% in the other reports of this mutation. The mutation has been classified a 'Glaucoma causing mutation' (http//www.myocilin.com).[277] Given that we identified an unaffected carrier, the mutation may be pathogenic but incompletely penetrant.

2.2 Gly374Val

The Gly374Val mutation is a missense mutation that was a novel mutation that we identified in two POAG patients heterozygous for the mutation, but in no control participants. The SIFT algorithm returns a score of 0 with a prediction that Gly374Val is a damaging mutation.[279] The same result is achieved with PROVEAN (v1.1) that returns a score of -8.436 against the prediction cutoff of less than -2.5 for a deleterious mutation.[280] The glycine amino acid at this position in the protein is highly conserved. The mutation is therefore unsurprisingly also predicted to be 'Probably Damaging' using Polyphen 2 which returned a score of 1.00 (sensitivity: 0.00; specificity: 1.00).[281] This suggests that Gly374Val is a cause of POAG in this population.

2.3 Lys500Arg

The Lys500Arg missense mutation in exon 3 of *MYOC* is considered to be of 'uncertain pathogenicity' (http//www.myocilin.com).[277] It was not found in any controls in the study population, but was identified in two POAG patients who were

heterozygous for both this mutation and a silent mutation (Glu396Glu). The Glu396Glu mutation was identified in another 7 POAG patients and in 6 unaffected controls. Glu396Glu has been reported in several populations to occur in both POAG patients and controls.[65, 70, 109, 251] It is considered a 'neutral polymorphism' (http//www.myocilin.com).[277] The Lys500Arg mutation returns a score of 0.91 using the SIFT algorithm with a prediction that it is a tolerated variant.[279] PROVEAN (v1.1) predicts that the mutation is neutral with a score of 0.374 (against the prediction cutoff of less than -2.5 for a deleterious mutation).[280] The mutation is also predicted to be 'Benign' using Polyphen 2 which returned a score of 0.001 (sensitivity: 0.99; specificity: 0.15).[281] When we published the results of this study, the mutation had never been reported to occur in an unaffected control, although it had been described in one African American individual from the general population.[65] It has, however, subsequently been described in two controls and four glaucoma patients by Liu *et al.*[251] in their large African American dataset. The contribution of Lys500Arg to glaucoma is therefore uncertain.

3. IDENTIFYING ADDITIONAL PARTICIPANTS WITH POTENTIAL MUTATIONS

3.1 Methods

The study population, selection criteria, examination and investigations have been described in detail in Chapter 2.

SNPs were designed to look for the mutations identified in Table 3.1 (above) in the study population using the Illumina® BeadXpress genotyping platform. The *MYOC* gene sequence was downloaded from the UCSC (University of California Santa Cruz) Genome Browser. The start of the coding sequence of the gene was identified in the GRCh37/hg19 assembly of the human reference sequence, and then the exons were identified. The positions of the sequence variants were identified by counting the base pairs. The dbSNP recognised SNPs were submitted to Illumina along with the novel variants and potentially pathogenic mutations that were submitted as genome sequences. In the case of the one base pair deletion the sequence in which it occurs is as follows: GCT**T**ATGA, so the following SNP was designed: GCT[**T/**A]TGA with reverse sequence orientation. The SNPs designed to look for the mutations were included in the Illumina assay design for the BeadXpress experiment described in Chapter 4.2. The SNP details, sequence and final Illumina® Score (a value on the 0.000 to 1.000 scale to reflect the ability to perform a successful assay) for the three potentially pathogenic mutations using the Assay Design Tool are in Table 3.2. Five of the other mutations (four novel intronic mutations and one neutral synonymous polymorphism in exon 3) that occurred in controls and POAG participants with similar frequencies, failed the Assay Design Tool and were excluded from the design. The final design therefore included the three potentially pathogenic myocilin mutations and eleven other variants that had been identified in this population.

Table 3.2 Potential disease causing variants in the *MYOC* gene included in the Illumina Assay Design

Amino acid change	Nucleotide change	Sequence	Chr	Coordinate	Sequence Orientation	Illumina® Score
Gly374Val	c.1121G>T	CTCATCAGCCAGTTTATGCAGGGCTACCCTTCTAAGGTT CACATACTGCCTAGGCCACTGGAAAGCACGGGTGCTGT GGTGTACTCGGGGAGCCTCTATTTCCAGGGCGCTGAGT CCAGAACTGTCATAAGATATGAGCTGAATACCGAGACAG TGAAGGCTGAGAAGGAAATCCCTGGAGCTGGCTACCAC GGACAGTTCCCGTATTCTTGGG**[T/G]**TGGCTACACGGAC ATTGACTTGGCTGTGGATGAAGCAGGCCTCTGGGTCATT TACAGCACCGATGAGGCCAAAGGTGCCATTGTCCTCTCC AAACTGAACCCAGAGAATCTGGAACTCGAACAAACCTGG GAGACAAACATCCGTAAGCAGTCAGTCGCCAATGCCTTC ATCATCTGTGGCACCT	1	171605459	REVERSE	0.885
Lys500Arg	c.1499A>G	TGTACACCGTCAGCAGCTACACCTCAGCAGATGCTACCG TCAACTTTGCTTATGACACAGGCACAGGTATCAGCAAGA CCCTGACCATCCCATTCAAGAACCGCTATAAGTACAGCA GCATGATTGACTACAACCCCCTGGAGAAGAAGCTCTTTG CCTGGGACAACTTGAACATGGTCACTTATGACATCA**[A/G]**GCTCTCCAAGATGTGAAAAGCCTCCAAGCTGTACAGGC AATGGCAGAAGGAGATGCTCAGGGCTCCTGGGGGGAGC AGGCTGAAGGGAGAGCCAGCCAGCCAGGGCCCAGGCA GCTTTGACTGCTTTCCAAGTTTTCATTAATCCAGAAGGAT GAAC	1	171605081	REVERSE	0.965
Tyr453del	c.1357delT	CTCTCCAAACTGAACCCAGAGAATCTGGAACTCGAACAA ACCTGGGAGACAAACATCCGTAAGCAGTCAGTCGCCAAT GCCTTCATCATCTGTGGCACCTTGTACACCGTCAGCAGC TACACCTCAGCAGATGCTACCGTCAACTTTGCT**[A/T]**TGA CACAGGCACAGGTATCAGCAAGACCCTGACCATCCCATT CAAGAACCGCTATAAGTACAGCAGCATGATTGACTACAA CCCCCTGGAGAAGAAGCTCTTTGCCTGGGACAACTTGAA CATGGTCACTTATGACATCAAGCTCTCCAAGATGTGAAAA GCCTCCAAGCTGTACAGGCAATGGCAGAAGGAGATGCT	1	171605223	REVERSE	0.889

Chr, Chromosome
Genome Build = GRCh37/hg19

The DNA samples from the study participants were genotyped with the Illumina® GoldenGate Assay and the BeadXpress Reader as described in Chapter 4.2. The genotyping was repeated on a subset of participants in whom *MYOC* had been sequenced in order to confirm the validity of the designed SNPs. Additional participants with potential mutations that were identified among the remainder of the group genotyped were included in the family study.

3.2 Results

BeadXpress genotyping and BeadStudio analysis was successful in DNA samples from 429 participants of whom 215 had a diagnosis of POAG and 214 were control participants (see Chapter 4.3). These 429 samples included 161 samples that had been sequenced for the exons in the *MYOC* gene. A comparison of the BeadXpress genotyping results for SNPs in *MYOC* with the results obtained by sequencing the *MYOC* exons in these 161 samples confirmed the validity of the BeadXpress genotyping. All of the SNP genotyping results correlated and the potential mutations were all correctly identified. Table 3.3 contains the 14 *MYOC* variants that were first

identified by sequencing in 197 participants and then genotyped in 429 DNA samples. The number of POAG and control individuals carrying each mutation is recorded in both of these groups.

Table 3.3 List of *MYOC* variants identified from sequencing 100 POAG and 97 controls of the study population and subsequently genotyped using the Illumina® BeadXpress

			Identified by sequencing		**Identified by Illumina® BeadXpress genotyping**	
Nucleotide Sequence Change *	Amino acid change	dbSNP ID	Controls (97)	POAG (100)	Controls (214)	POAG (215)
c.1-83G>A	Non-coding	rs2075648	1	0	6	4
c.39T>G	Pro13Pro	rs12082573	6	6	18	17
c.227G>A	Arg76Lys	rs2234926	1	0	5	1
c.477A>G	Leu159Leu	rs61730977	6	6	19	18
c.604+13A>C	Non-coding	-	5	4	11	9
c.612G>T	Thr204Thr	rs57824969	2	3	3	2
c.730+35G>A	Non-coding	rs2032555	15	8	25	28
c.731-73C>T	Non-coding	rs79255460	9	1	15	14
c.975G>A	Thr325Thr	rs61730976	3	13	12	23
c.1054G>A	Glu352Lys	rs61745146	4	4	10	12
c.1121G>T	Gly374Val	-	0	2	0	2
c.1188G>A	Glu396Glu	rs61730975	6	9	12	14
c.1357delT	Tyr453del	-	1	3	2	5
c.1499A>G	Lys500Arg	rs145977437	0	2	0	2

*Nucleotides numbered as in Ensembl accession number ENSG00000034971 (transcript ENST00000037502)

SNPs rs2032555 and rs79255460 were commoner in controls in the group sequenced, however this difference was not maintained in the larger group. SNP rs2234926 did, however occur in more controls and SNP rs61730976 was more prevalent in POAG patients. When comparing the minor allele frequencies in POAG and controls in these two SNPs, neither association was statistically significant (Table 3.4).

Table 3.4 POAG association analyses of SNPs rs2234926 and rs61730976

SNP	MAF in POAG cases	MAF in controls	p	OR (95% CI)
rs2234926	0.002	0.012	0.123 ⋆	0.20 (0.02 - 1.70)
rs61730976	0.054	0.028	0.058 ¶	1.97 (0.97 - 4.01)

MAF, minor allele frequency; OR (95%CI), odds ratio (95% confidence intervals)
⋆ Fisher's exact test
¶ χ^2 test

3.2.1 Gly374Val

In the initial sequencing of 100 POAG patients and 97 controls, we identified two POAG participants who had this probably damaging mutation in the *MYOC* gene. With the BeadXpress genotyping platform both of these participants were correctly identified as having the mutation. No additional participants were found to carry the variant. The demographic and clinical characteristics of participants with the Gly374Val mutation compared with the entire group of POAG participants (see Chapter 2) are documented in Table 3.5. The participants with the mutations were both Sesotho speaking and they had thinner corneas than the whole group of POAG participants, but in other respects were approximately representative of this group. Neither of the participants with the mutation had other variants in the *MYOC* gene.

Table 3.5 Demographic and clinical characteristics of participants with the Gly374Val mutation compared with all the POAG participants

Participant code	PP002TN	PP061MT	**All POAG participants (247)**
Identified by sequencing	Yes	Yes	**2/100**
Identified by BeadXpress genotyping	Yes	Yes	**2/215**
Age (years)	68	52	**60.0 ± 13.1**
Gender	Female	Female	**50.2% Female**
Language	Sesotho	Sesotho	**17% Sesotho**
Family history of glaucoma	No	No	**7.3%**
Family history of blindness	No	No	**24.3%**
Diabetic	Yes	No	**23.1%**
Hypertensive	Yes	Yes	**53.4%**
Age at diagnosis (years)	52	52	**55.0 ± 13.6**
IOP (mmHg)	30	50	**34.9 ± 9.1**
CCT (µm)	450	472	**505.1 ± 37.9**
VCDR	0.90	1.00	**0.89 ± 0.13**
Cataracts	Yes	No	**49.0%**
IOP control	Yes	No	**68.0%**
SVI	No	Both eyes	**53.0%**
Glaucoma Surgery	Yes	No	**54.0%**
Other *MYOC* variants	No	No	

IOP, IOP at diagnosis; SVI, severe visual impairment

3.2.2 Lys500Arg

This non-synonymous coding SNP was identified in two POAG participants in the initial sequencing of 197 participants. The BeadXpress genotyping that was repeated

on 161 of these samples (including one of the samples with the variant) and 268 other samples, correctly detected the previously sequenced sample and identified one other POAG participant carrying the variant. The demographic and clinical characteristics of participants with the Lys500Arg variant compared with the entire group of POAG participants are documented in Table 3.6. One of the POAG participants with the variant had primary juvenile glaucoma with an age at diagnosis of 28 years. All three of the participants identified as carrying the Lys500Arg variant also carried a second variant, the Glu396Glu variant.

Table 3.6 Demographic and clinical characteristics of participants with the Lys500Arg mutation compared with the entire group of POAG participants

Participant code	P16JL	P38WM	PP171GN	**All POAG participants (247)**
Identified by sequencing	Yes	Yes	No	**2/100**
Identified by BeadXpress genotyping	Yes	No	Yes	**2/215**
Age (years)	56	63	**34**	**60.0 ± 13.1**
Gender	Male	Male	Male	**50.2% Female**
Language	Sepedi	Sesotho	IsiZulu	**39% IsiZulu; 17% Sesotho; 7.7% Sepedi**
Family history of glaucoma	No	No	No	**7.3%**
Family history of blindness	No	No	No	**24.3%**
Diabetic	Yes	No	No	**23.1%**
Hypertensive	Yes	No	No	**53.4%**
Age at diagnosis (years)	54	61	**28**	**55.0 ± 13.6**
IOP (mmHg)	32	26	Unknown	**34.9 ± 9.1**
CCT (µm)	493.5	487.5	567.5	**505.1 ± 37.9**
VCDR	0.90	0.93	0.95	**0.89 ± 0.13**
Cataracts	No	No	No	**49.0%**
IOP control	Yes	Yes	Yes	**68.0%**
SVI	No	No	One eye	**53.0%**
Glaucoma Surgery	No	No	No	**54.0%**
Other *MYOC* variants	**Glu396Glu**	**Glu396Glu**	**Glu396Glu**; Glu352Lys	**6.5% Glu396Glu**

IOP, IOP at diagnosis; SVI, severe visual impairment

3.2.3 Glu396Glu (rs61730975)

The Glu396Glu variant, that corresponds to a nucleotide change (c.1188G>A) in *MYOC* (SNP rs61730975), is considered a neutral polymorphism. The results of BeadXpress genotyping and POAG association analysis (using PLINK v1.07) of this SNP are in Table 3.7. The minor allele of the SNP occurred in 14 POAG participants

and 12 control individuals and was not associated with POAG when evaluated using logistic regression with an additive model after adjusting for age and gender.

Table 3.7 The association of SNP rs61730975 with POAG

SNP	MAF in POAG cases	MAF in controls	p	OR (95% CI)*	p*
rs61730975	0.033	0.028	0.699	0.84 (0.33 – 2.10)	0.706

MAF, minor allele frequency; OR (95%CI), odds ratio (95% confidence intervals)
*Adjusted for age and gender

3.2.4 Tyr453del

The frameshift deletion (c.1357delT) was detected in four participants in the initial sequencing of 197 participants. Three of these participants had POAG and one was a control participant. With the BeadXpress genotyping platform all four of these participants were correctly identified as having the mutation and an additional three individuals (two POAG and one control) were found to carry the mutation. The demographic and clinical characteristics of POAG participants with the Tyr453del variant compared with the entire group of POAG participants are documented in Table 3.8. There was no particular phenotypic pattern associated with those POAG participants with the mutation.

Table 3.8 Demographic and clinical characteristics of POAG participants with the Tyr453del mutation compared with all the POAG participants

Participant code	P36JN	P43TM	PP003RM	PP177JZ	PP085LN	**All POAG participants (247)**
Identified by sequencing	Yes	Yes	Yes	No	No	**3/100**
Identified by BeadXpress	Yes	Yes	Yes	Yes	Yes	**5/215**
Age (years)	70	66	78	50	73	**60.0 ± 13.1**
Gender	Female	Male	Female	Male	Female	**50.2% Female**
Language	IsiZulu	Sesotho	Setswana	Setswana	IsiXhosa	**39% IsiZulu; 17% Sesotho; 15% Setswana; 10% IsiXhosa**
Family history of glaucoma	No	No	Yes	No	No	**7.3%**
Family history of blindness	No	Yes	Yes	No	No	**24.3%**
Diabetic	No	No	Yes	No	Yes	**23.1%**
Hypertensive	Yes	No	Yes	No	Yes	**53.4%**
Age at diagnosis	70	60	72	50	70	**55.0 ± 13.6**
IOP (mmHg)	32	32	Unknown	36	36	**34.9 ± 9.1**
CCT (µm)	438	502	528	510	498	**505.1 ± 37.9**
VCDR	0.98	1.00	0.85	1.00	1.00	**0.89 ± 0.13**
Cataracts	Yes	No	Yes	No	Yes	**49.0%**
IOP control	Yes	Yes	Yes	No	No	**68.0%**
SVI	One eye	Both eyes	No	No	One eye	**53.0%**
Glaucoma Surgery	No	Yes	Yes	No	No	**54.0%**
Other *MYOC* variants	No	Glu352Lys	c.731-73 C>T	Thr325Thr	No	

IOP, IOP at diagnosis for POAG subjects or at enrolment for controls; SVI, severe visual impairment

Table 3.9 contains the demographic and clinical characteristics of control participants with the Tyr453del variant compared with the entire group of control participants. The controls were demographically similar to the POAG patients carrying the mutation. They were both over the age of 70 years and had no evidence of glaucoma.

Table 3.9 Demographic and clinical characteristics of control participants with the Tyr453del mutation compared with all the control participants

Participant code	CC012LN	CC141FM	All control participants (255)
Identified by sequencing	Yes	No	1/100
Identified by BeadXpress	Yes	Yes	2/214
Age (years)	80	71	70.2 ± 8.3
Gender	Female	Female	54.1% Female
Language	Tshivenda	Sesotho	19% Sesotho; 2% Tshivenda
Family history of glaucoma	No	No	0%
Family history of blindness	Yes	No	7.1%
Diabetic	Yes	Yes	36.5%
Hypertensive	Yes	Yes	69.4%
IOP (mmHg)	14	14	13.2 ± 2.6
CCT (µm)	542	507	513.5 ± 36.1
VCDR	0.3	0.4	0.37 ± 0.13
Cataracts	Yes	Yes	95.7%

IOP, IOP at enrolment

The participants identified with the Tyr453del, Gly374Val or Lys500Arg mutations were invited to participate in the Myocilin Family Study.

4. MYOCILIN FAMILY STUDY

4.1 Methods

4.1.1 Ethics

The study adhered to the tenets of the declaration of Helsinki.

Ethics clearance was obtained from the University of the Witwatersrand Human Research Ethics Committee (Ethics Clearance Certificate M110214 – Appendix 5).

Appendix 6 contains the participant information sheets that were used in the study. The informed consent forms may be found in Appendix 2 (Sections 3 and 4).

4.1.2 Study Participants

Participants (probands) that had been identified as having one of the three potential disease-causing variants in *MYOC* were contacted.

The probands received detailed genetic counselling on the significance of the genetic finding. Family pedigrees were constructed. The pedigrees were drawn with the Progeny Online Pedigree Drawing App (http://www.progenygenetics.com/online-pedigree/).

Relatives of the probands were invited to participate in the study.

4.1.2.1 Inclusion Criteria for participants

- Relatives of the probands that the probands chose to contact and inform of their diagnosis and genetic findings
- Relatives of the probands 18 years of age or older who voluntarily gave informed consent to participate in the study

4.1.3 Genetic Counselling

Each participant received genetic counselling.

4.1.4 Data Collection

Appendix 3 contains the questionnaire and data collection sheets used in the study.

4.1.4.1 Questionnaire

The participants answered questions relating to their ethnic ancestry (as determined by their mother tongue and that of their parents and grandparents), their place of birth, their medical history and their ophthalmological history.

4.1.4.2 Examination

Each participant had their blood pressure tested and had an estimation of their random blood glucose from a finger-prick haemoglucotest.

I performed a standardized ophthalmological examination on each participant that included the following:

- Best corrected Snellen visual acuity
- Conjunctival assessment
- Slit lamp corneal assessment
- Goldmann applanation tonometry
- Gonioscopy, Schaffer grading and description of the drainage angle
- Slit lamp anterior chamber, iris, pupil and lens assessment
- Indirect stereoscopic fundoscopy
- Clinical assessment of the VCDR as well as description of the optic nerve head, the neuroretinal rim and the peripapillary area

4.1.4.3 Investigations

If it was clinically warranted (glaucoma suspects) the study participants had an

assessment of their retinal nerve fibre layer using the GDx VCC™. Reliability of the test was assessed using the 'Q' value. A 'Q' less than 7 was taken as unreliable. The two parameters analysed were the NFI and the average nerve fibre layer thickness (TSNIT).

Likewise, a visual field assessment using the Oculus CenterField® automated perimeter was performed on participants who were glaucoma suspects. The 'Fixationcheck' and 'False positive' were used to assess reliability. A 'Fixationcheck' less than 20% and a 'False positive' less than 10% were excluded from analysis as unreliable.

4.1.4.4 POAG Diagnosis

A diagnosis of POAG was made if the following criteria were fulfilled:

1. Glaucomatous optic neuropathy as evidenced by the following:
- VCDR greater than 0.7 or
- VCDR asymmetry of greater than 0.2 or
- Notching of the neuroretinal rim or
- Inferior and superior neuroretinal rim thinning or
- Retinal nerve fibre layer thinning on an accurate GDx VCC™ assessment (high NFI and low TSNIT values) combined with clinical suspicion

2. Glaucomatous visual field defects that correlated with the disc appearance.

3. Open angles on gonioscopy.

4. No secondary cause for the glaucoma or optic disc appearance.

Where there was glaucomatous optic neuropathy without visual field changes, then the participant was classified as a POAG suspect.

4.1.5 DNA extraction

Each participant had venous blood sampling for DNA extraction.

Genomic DNA was extracted using a salting out procedure from nucleated cells from the venous blood samples at the NHLS Molecular Genetics Laboratory in the Division of Human Genetics. The procedure, modified from Miller *et al.*[256] is described in detail in Appendix 4.

The quality of the DNA was evaluated by running it on a 0.8% agarose gel. The concentration (ng/µl) and the purity (OD260/280) of the DNA were measured using the Nanodrop® ND-1000 Spectrophotometer (Nanodrop Technologies). DNA samples were diluted to a final concentration of 100ng/µl using 1X TRIS-EDTA (TE) buffer and stored at -20°C.

4.1.6 Genotyping

All three of the potential disease-causing variants occur within a 500 base pair region in exon 3 of the *MYOC* gene. The nucleotide sequence for exon 3 of *MYOC* along with the variants is illustrated in Figure 3.1. The region was amenable to amplification with the polymerase chain reaction (PCR) and the variants were then detected with cycle sequencing. The probands were sequenced as positive controls for the sequence variants and negative controls were included from within the study population.

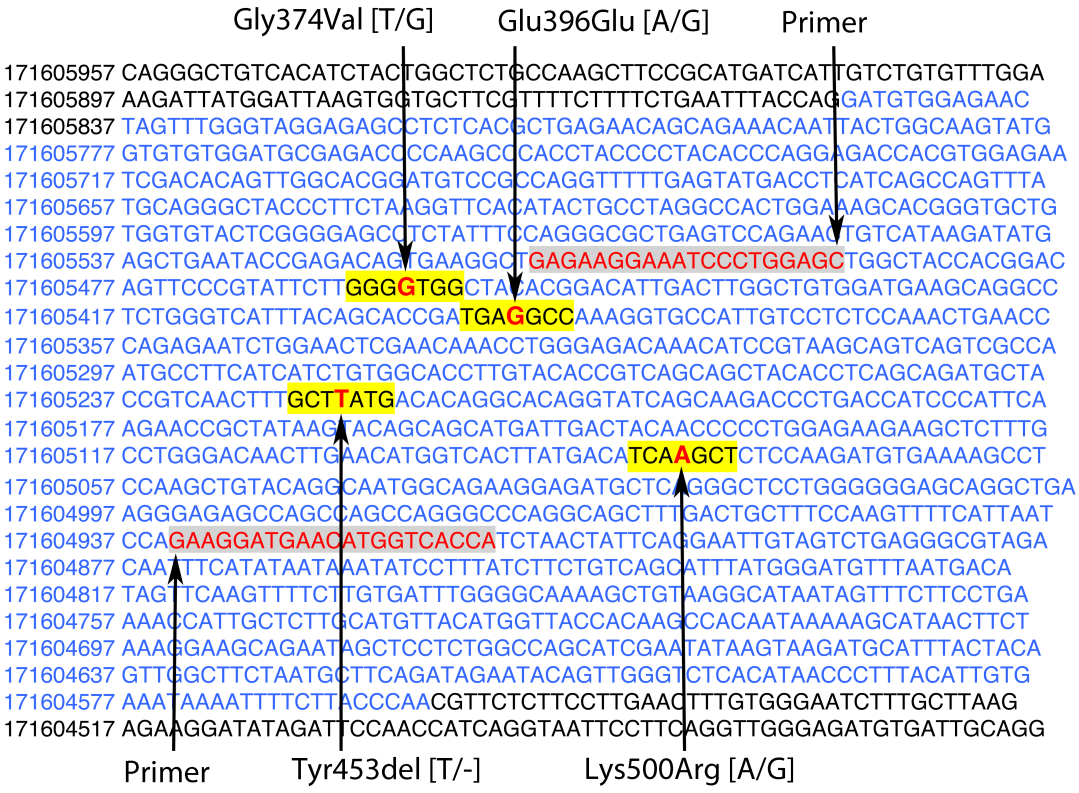

Figure 3.1 The nucleotide sequence for exon 3 of the myocilin gene (GRCH37). The coding sequence is in blue. The primers are in red. The nucleotides that are altered in the potential mutations are bold red and highlighted in yellow.

4.1.6.1 The polymerase chain reaction

This technique, first described by Mullis *et al.,*[282] involves the amplification of a specific region of genomic DNA. Amplification is achieved by successive cycles of heating and cooling to temperatures specific for denaturation of double stranded DNA, annealing of the primers and the extension of the new product.

Conventional PCR was used to amplify the portion of interest within exon 3 of the *MYOC* gene.

4.1.6.1.1 Materials

- DNA samples from each participating relative, probands (positive controls) and negative controls
- Primers
- KAPA *Taq* ReadyMix (KAPA Biosystems)

The primers used to amplify the region of interest within exon 3 of *MYOC* are highlighted in Figure 3.1 (above) and the primer details are in Table 3.10.

Table 3.10 Primer sequences used

***MYOC* exon**	**Forward primer sequence (5' – 3')**	**Reverse primer sequence (5' – 3')**	**PCR product size (bp)**	**Covered genomic region (GRCH37)**
Exon 3	GAGAAGGAAATCCCTGGAGC	TGGTGACCATGTTCATCCTTC	598	chr1:171,604,914-171,605,511

KAPA *Taq* ReadyMix (KAPA Biosystems) contains wild-type *Taq* polymerase, reaction buffer and deoxynucleoside triphosphates (dNTPs). *Taq* polymerase is a heat-stable DNA polymerase originally isolated from the bacterium Thermus aquaticus. It synthesizes a new DNA strand from the dNTPs using single-stranded DNA (produced by thermal denaturation) as a template and the annealed primers for initiation of synthesis.

Table 3.11 contains the concentrations and volumes of the PCR components.

Table 3.11 PCR components

PCR Components	**Final concentration**	**Volume (ul)**
DNA	100ng/ul	1
Primer forward	10mM	2
Primer reverse	10mM	2
KAPA Taq ReadyMix	1U/µl	25
ddH20	N/A	20
TOTAL		**50**

Table 3.12 documents the PCR protocol that consists of denaturation of the double-stranded DNA sample, annealing of primers to the single-stranded DNA and extension of the synthesized PCR product.

Table 3.12 PCR protocol

Cycle step	**Temperature (°C)**	**Time**	**Number of cycles**
Initial Denaturation	95	2 minutes	1
Denaturation	95	30 seconds	
Annealing	55	30 seconds	35
Extension	72	2 minutes	
Final extension	72	5 minutes	1
Hold	4		

The PCR fragments were run on a 2% agarose gel to check that the correct DNA fragment was amplified. A 100 base pair ladder was included in the gel in order to see the size of band of PCR product. Figure 3.2 shows an image from one of the gels demonstrating the 598 base pair fragment produced by the PCR.

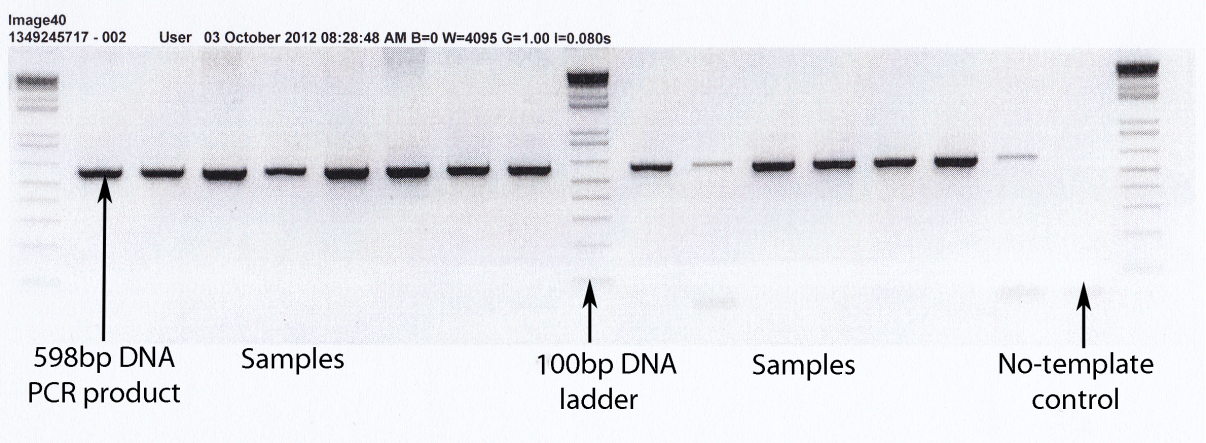

Figure 3.2 Agarose gel picture. Agarose gel (2%) picture illustrating the 598bp PCR fragments that were amplified from the DNA samples compared with a 100bp DNA ladder and a no-template control.

4.1.6.2 PCR fragment purification

The method employed to obtain a pure PCR product in preparation for DNA sequencing was the enzyme-based method involving Exonuclease I (New England Biolabs) and Shrimp Alkaline Phosphatase (Roche). These enzymes digested the remaining genomic DNA, unused primers and dNTPs that may otherwise interfere with cycle sequencing.

4.1.6.2.1 Materials

- Shrimp Alkaline Phosphatase (1U/µl) (Roche Cat. No. 1 758 250)
- Exonuclease I (20U/µl) (New England Biolabs Cat. No. M0293S)
- PCR products

The proportions of these components used in the Exonuclease – Shrimp Alkaline Phosphatase cleanup of the PCR products are contained in Table 3.13.

Table 3.13 Components used in the Exonuclease – Shrimp Alkaline Phosphatase cleanup of the PCR products

Component (concentration)	Volume (µl)
Exonuclease I (20U/ µl)	0.1
Shrimp Alkaline Phosphatase (1U/µl)	0.7
Milli Q water	1.2
PCR product	5
Total	**7**

4.1.6.3 Cycle sequencing

The sequencing method developed by Sanger[283] forms the basis of the cycle sequencing reaction. This follows the same basic procedure as PCR and is also performed in a thermocycler, but only one primer is used in each sequencing reaction so that the amplification of the product is linear and not exponential. Dideoxynucleotides (ddNTPs) labelled with fluorescent dyes are used to interrupt the extension of the DNA strand. Each ddNTP (ddCTP, ddATP, ddTTP and ddGTP) has a different fluorescent dye attached to it. The concentration of dNTPs (allowing extension) in the reaction is much greater than that of ddNTPs (interrupting extension). Therefore the thermocycling product consists of fragments of DNA ranging in length from the length of the primer plus one nucleotide to the length of the primer plus all nucleotides in the PCR product. The fragments are separated on a gel. A laser within an automated DNA sequencing machine energises the dyes attached to each ddNTP so that the 3' nucleotide base can be identified.

4.1.6.3.1 Materials

- Purified PCR product
- Primer
- BigDye® v3.1 (Applied Biosystems)

The BigDye® Terminator v3.1 Cycle Sequencing Kit provides the required reagent components for the sequencing reaction in a ready reaction, pre-mixed format. It contains *Taq* DNA polymerase, dNTPs in large concentration and ddNTPs with fluorescent dyes in low concentration.

The components for the cycle sequencing are contained in Table 3.14. A forward reaction was set up for each PCR fragment with the exception of one sample, where a reverse reaction was used to confirm the findings of the forward reaction. Cycle sequencing was performed in the GeneAmp® PCR system 9700 (Applied Biosystems). The thermal cycling conditions are documented in Table 3.15. Once cycle sequencing was complete, the cycle sequencing products were cleaned using the Microsep plate. Capillary electrophoresis was done using the ABI 3130*xl* Genetic analyzer (Applied Biosystems). Sequence analysis was achieved with BioEdit. Electropherograms were visualised with 4Peaks software (http://4peaks.en.softonic.com).

Table 3.14 Components for the cycle sequencing

PCR components	**Stock concentration**	**Final concentration**	**Volume (µl)**
Purified PCR product	N/A	N/A	6
Primer	10µM	0.5µM	1
BigDye® v3.1 (Applied Biosystems)	N/A	N/A	4
ddH20	N/A	N/A	8
TOTAL			**20**

Table 3.15 Thermal cycling conditions for the cycle sequencing

Temperature	**Time**	**Number of cycles**
96 °C	30 seconds	30
50 °C	15 seconds	
60 °C	4 minutes	1
4 °C	10 minutes	1
24 °C	Hold	

4.2 Results

4.2.1 Gly374Val

We located and provided genetic counselling to one of the two participants that we identified with the Gly374Val mutation (PP002TN). Four relatives were included in the study and were sequenced to look for the mutation. None of the individuals tested carried the mutation (Figure 3.3).

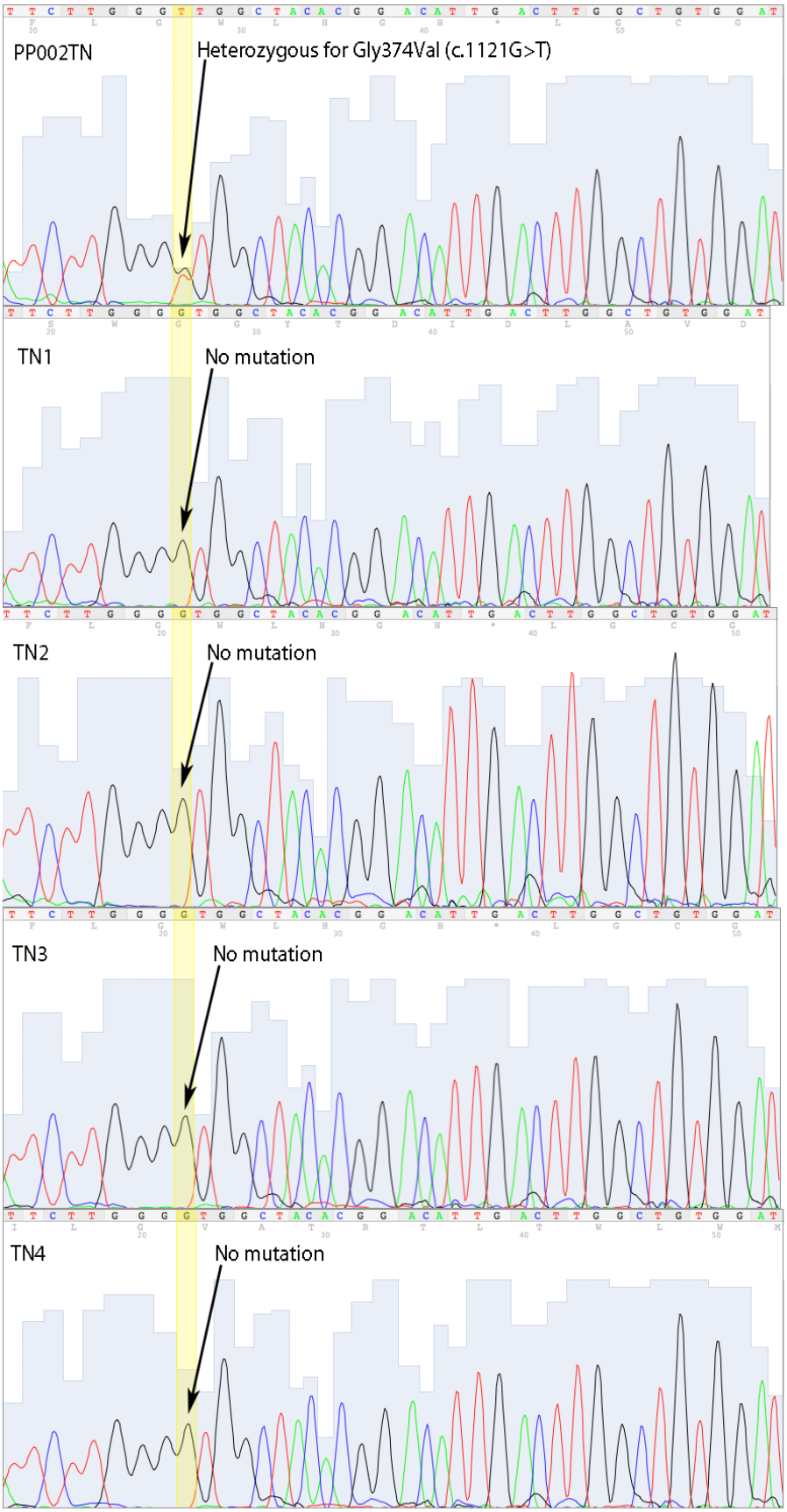

Figure 3.3 Electropherograms for Gly374Val. Electropherograms of the BigDye cycle sequences from PP002TN and the family members TN1-4 in the region of the Gly374Val mutation on exon 3 of the *MYOC* gene. PP002TN is heterozygous at the c.1121 nucleotide and carries the Gly374Val mutation. TN1-4 are all homozygous for the G allele at this position and therefore do not carry the mutation.

PP002TN Family

Figure 3.4 is the family pedigree for PP002TN. Four of her relatives (labelled TN1 - 4 in Figure 3.4) agreed to participate in the study. The relatives were a daughter (TN1), two cousins (TN2 and TN3) and the daughter of TN3 (TN4).

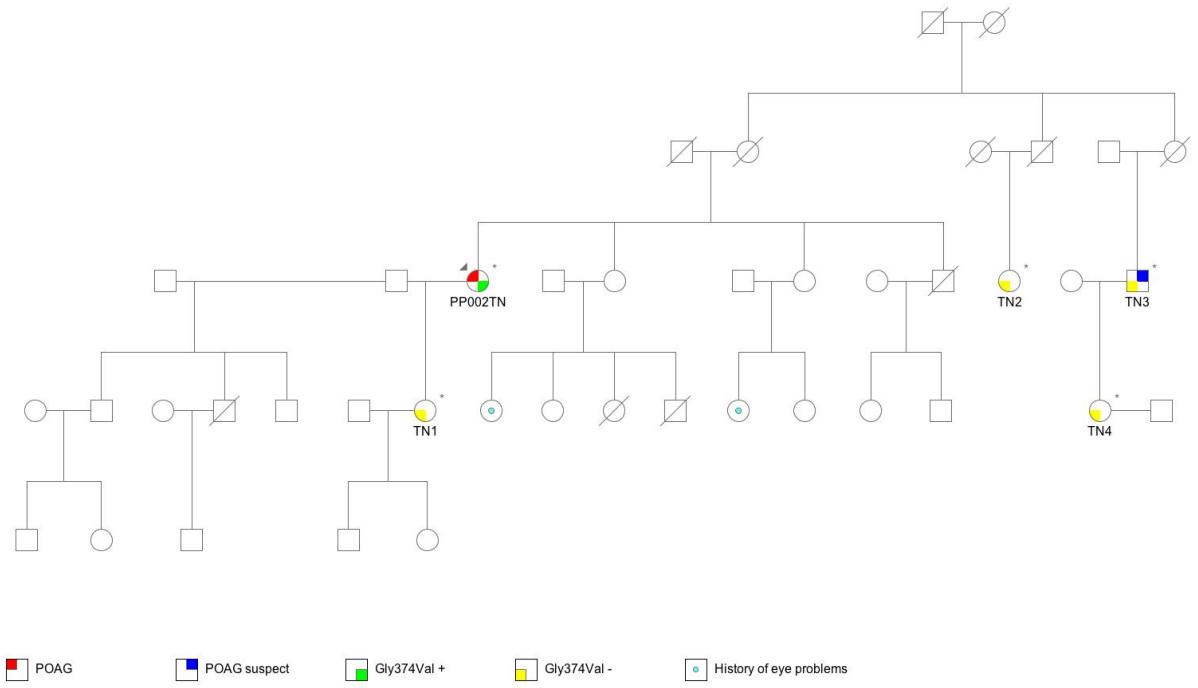

Figure 3.4 Family pedigree for PP002TN

The demographic and clinical findings in each of these relatives compared with the proband are documented in Table 3.16. Three of the relatives did not have POAG and did not have the Gly374Val mutation. TN3 (a first cousin to the proband) was classified as a glaucoma suspect. He had disc asymmetry with an unusual appearing optic disc on the right that was shallowly cupped with a peripapillary capillary loop. He also had IOP asymmetry between the eyes with an IOP of 22mmHg in the right eye. Visual field and GDx examinations were, however normal. TN3 did not carry the Gly374Val mutation.

Table 3.16 Demographic and clinical characteristics and mutation screening of relatives of PP002TN compared with the proband (PP002TN)

Participant code	TN1	TN2	TN3	TN4	**Proband PP002TN**
Relationship to proband	Daughter	Cousin	Cousin	Daughter of TN3	
POAG	**No**	**No**	**Suspect**	**No**	**Yes**
Ophthalmic history	High myopia, pseudophakia	Untreated refractive error	Right pseudophakia	Nil	
Age (years)	53	72	82	60	**68**
Gender	Female	Female	Male	Female	**Female**
Language	Sesotho	Sepedi	Sesotho	Sesotho	**Sesotho**
Diabetic	No	No	No	No	**Yes**
Hypertensive	No	Yes	Yes	Yes	**Yes**
IOP (mmHg)	16	16	*20	18	**30**
VCDR	0.2	0.4	*0.6	0.3	**0.9**
Cataracts	Yes	No	Yes	No	**Yes**
Gly374Val	**No**	**No**	**No**	**No**	**Yes**
Other *MYOC* variants	No	No	No	No	**No**

IOP, IOP at diagnosis for the proband or on examination for the relatives; * Asymmetry between the two eyes

4.2.2 Lys500Arg

We located and provided genetic counselling to one of the three participants that we identified with the Lys500Arg mutation (P16JL). Six relatives were included in the study and were sequenced to look for the variant. One relative (JL3) was found to be heterozygous for the Lys500Arg variant as well as for the Glu396Glu variant (Figure 3.5). The finding was confirmed on reverse sequencing.

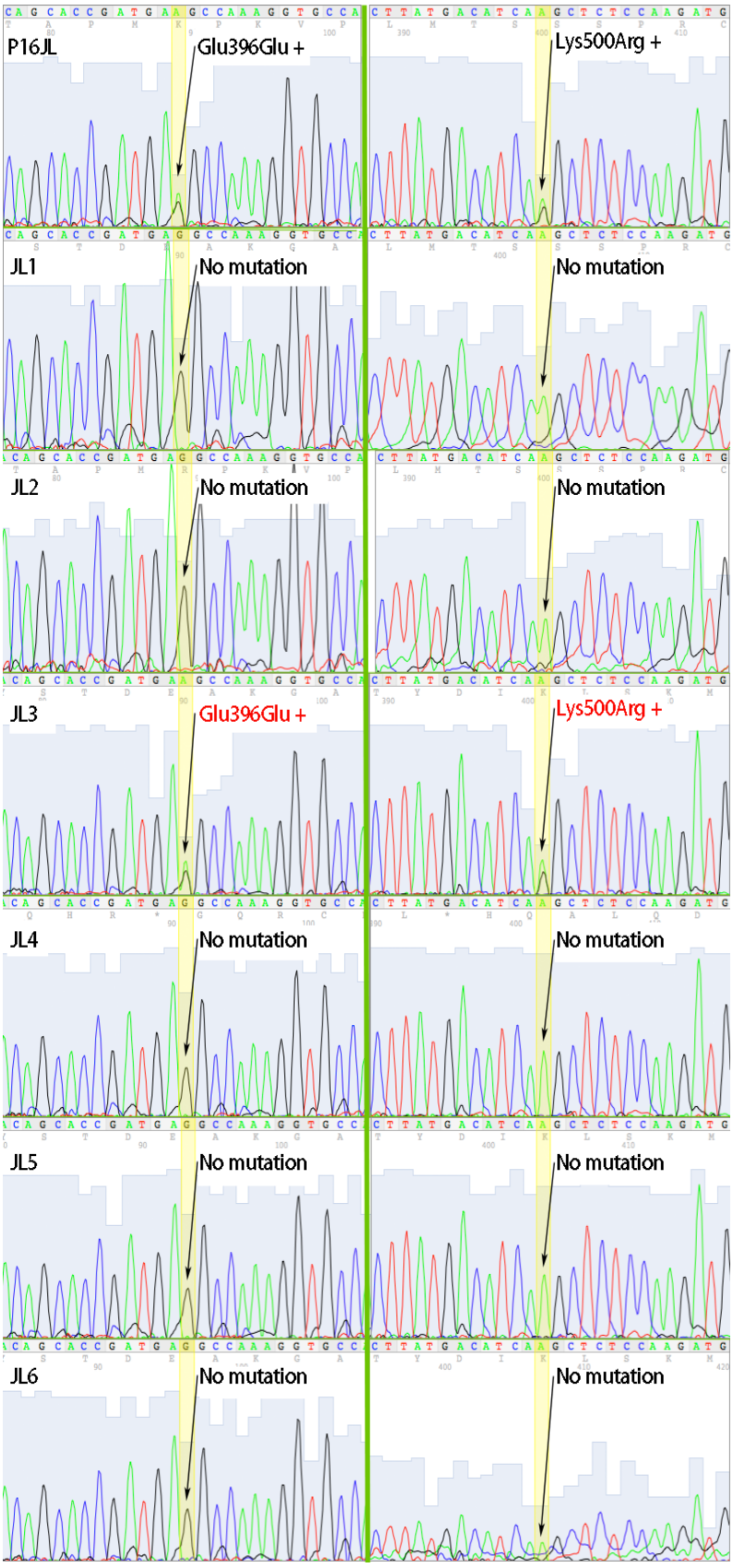

Figure 3.5 Electropherograms for Lys500Arg. Electropherograms of the BigDye cycle sequences from P16JL and the family members JL1-6 in the regions of the Glu396Glu (c.1188G>A; rs61730975) and Lys500Arg (c.1499A>G) variants on exon 3 of the *MYOC* gene. P16JL is heterozygous for both of these variants, as is JL3. JL1,2,4,5 and 6 are all homozygous for the G allele at position c.1188 and the A allele at position c.1499 and therefore do not carry the variants.

P16JL Family

Figure 3.6 is the family pedigree for P16JL. Six of his relatives (labelled JL1 - 6 in Figure 3.6) agreed to participate in the study. The relatives were four siblings (JL1 - 4), a daughter (JL5), and a granddaughter (JL6).

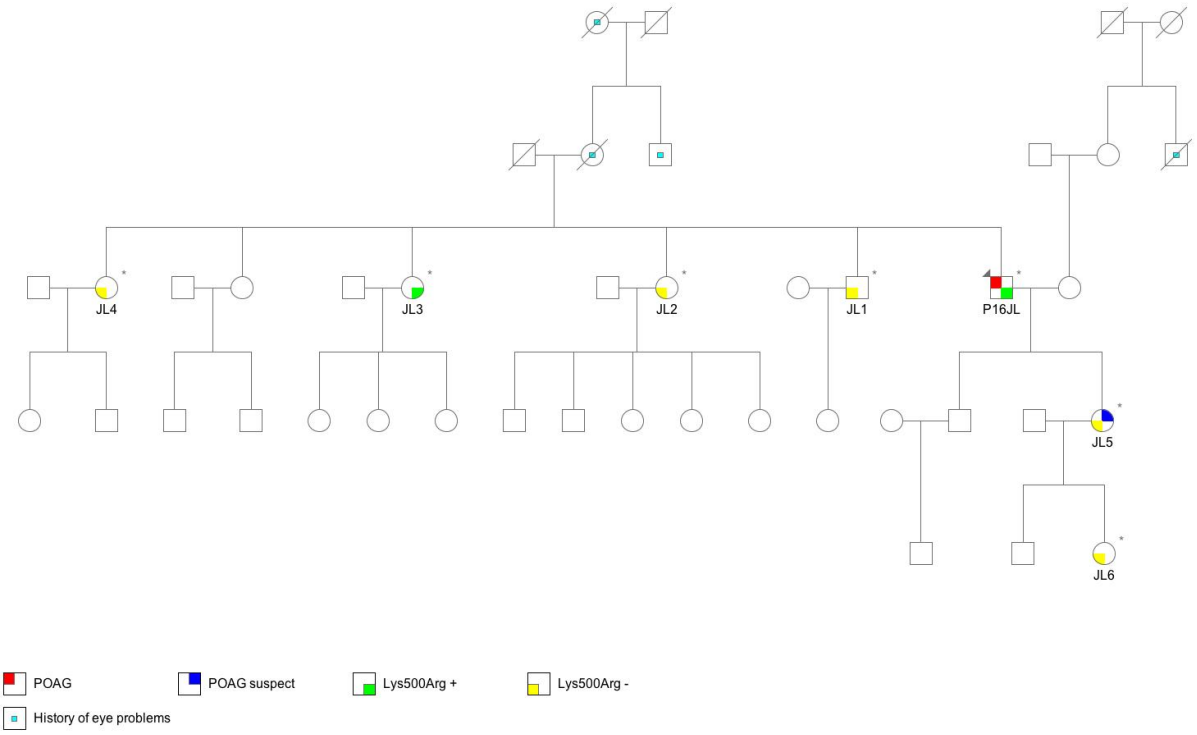

Figure 3.6 Family pedigree for P16JL

The demographic and clinical findings in each of these relatives compared with the proband are documented in Table 3.17. Five of the relatives did not have POAG. One of these was the 52 year-old sibling (JL3) who was found to harbour the Lys500Arg and Glu396Glu variants. The proband's daughter (JL5) was a glaucoma suspect with large VCDRs but a normal visual field and GDx. She did not carry either of the variants.

Table 3.17 Demographic and clinical characteristics and mutation screening of relatives of P16JL compared with the proband (P16JL)

Participant code	JL1	JL2	JL3	JL4	JL5	JL6	**Proband P16JL**
Relationship to proband	Sibling	Sibling	Sibling	Sibling	Daughter	Grand-daughter	
POAG	**No**	**No**	**No**	**No**	**Suspect**	**No**	**Yes**
Ophthalmic history	Diabetic retinopathy	Nil	Nil	Nil	Refractive error	Refractive error	
Age (years)	58	54	52	46	40	18	**56**
Gender	Male	Female	Female	Female	Female	Female	**Male**
Language	Sepedi	Sepedi	Sepedi	Sepedi	Sepedi	Setswana	**Sepedi**
Diabetic	Yes	No	No	No	No	No	**Yes**
Hypertensive	Yes	Yes	No	No	Yes	No	**Yes**
IOP (mmHg)	18	16	14	15	14	14	**32**
VCDR	0.3	0.6	0.3	0.5	0.9	0.2	**0.9**
CCT	516	510	501	519	486	454	**493.5**
Cataracts	Yes	Yes	No	No	No	No	**No**
Lys500Arg	**No**	**No**	**Yes**	**No**	**No**	**No**	**Yes**
Other *MYOC* variants	No	No	**Glu396Glu**	No	No	No	**Glu396Glu**

IOP, IOP at diagnosis for the proband or on examination for the relatives

4.2.3 Tyr453del

We located and provided genetic counselling to six of the seven participants that we identified with the Tyr453del mutation. Fifteen relatives were included in the study and were sequenced to look for the mutation. Four of the individuals tested carried the mutation (Figure 3.7).

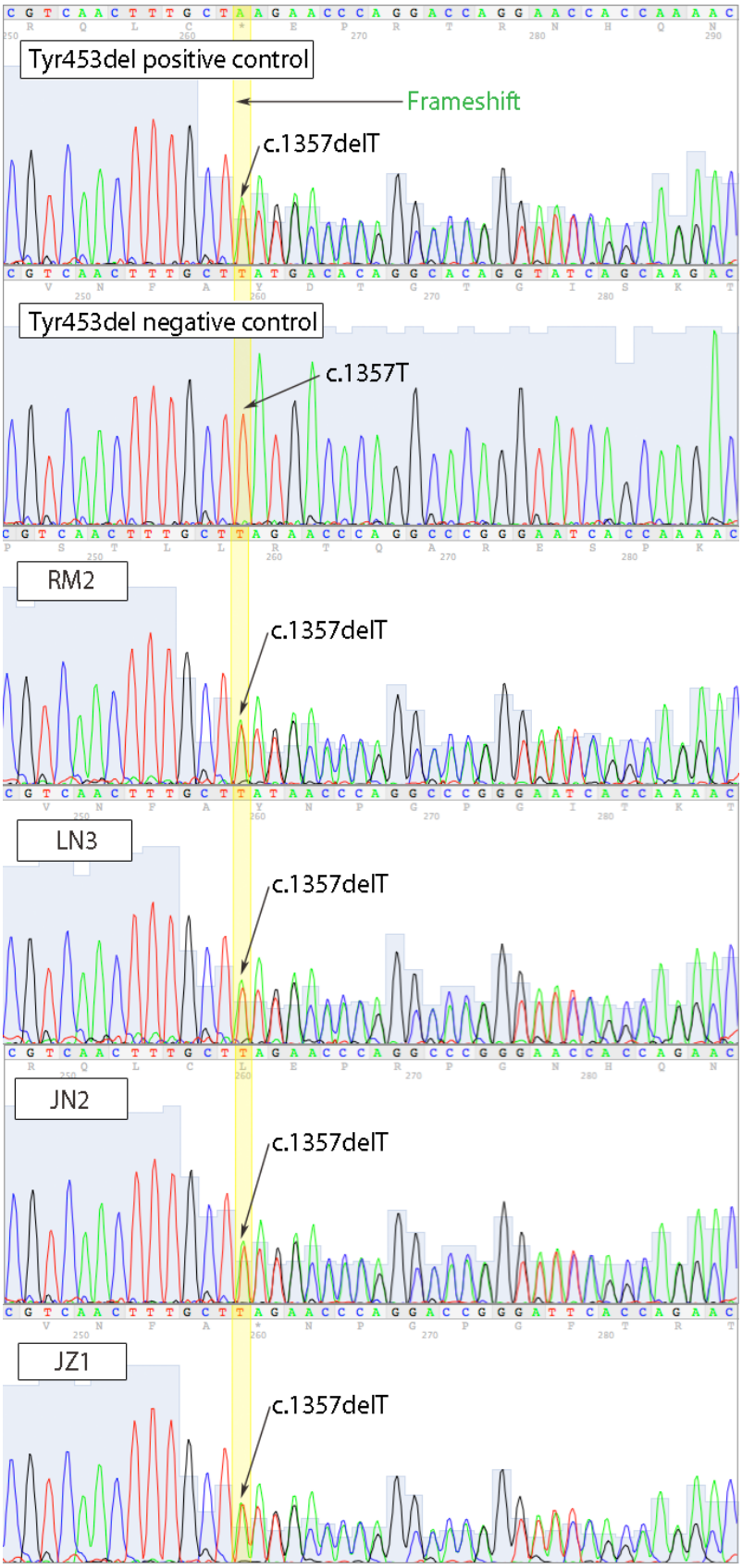

Figure 3.7 Electropherograms for Tyr453del. Electropherograms of the BigDye cycle sequences in the region of the Tyr453del (c.1357delT) mutation on exon 3 of the *MYOC* gene. The Tyr453del negative control is homozygous for the T allele at position c.1357. The Tyr453del positive control is heterozygous for a T deletion and consequent frameshift at this position. Family members RM2, LN3, JN2 and JZ1 all carry the mutation. The other family members tested (not illustrated) were all homozygous for the T allele at position c.1357 and therefore do not carry the mutation.

4.2.3.1 P36JN Family

Figure 3.8 is the family pedigree for P36JN. Four of her children (labelled JN1 - 4 in Figure 3.8), from three different fathers, agreed to participate in the study.

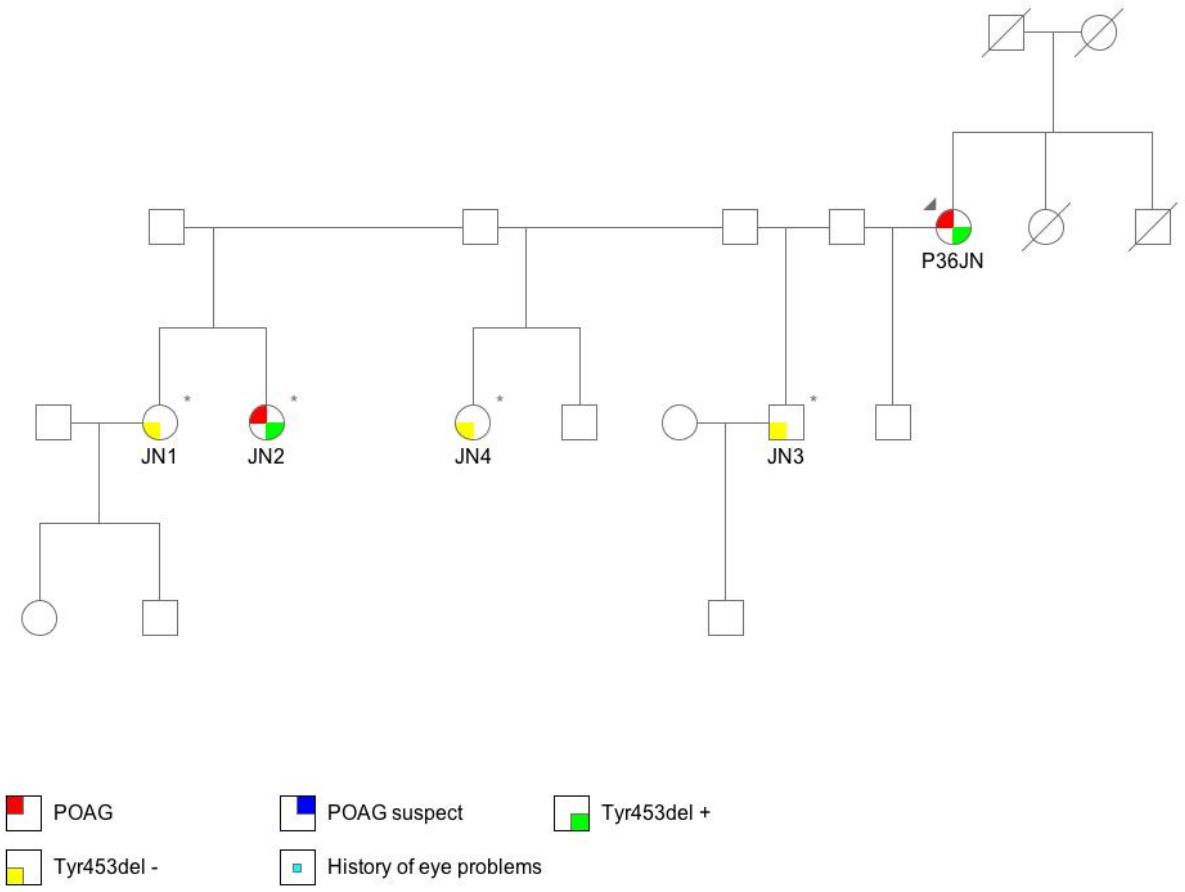

Figure 3.8 Family pedigree for P36JN

The demographic and clinical findings in each of these relatives compared with the proband are documented in Table 3.18. Two of the daughters (JN1 and JN4) did not have POAG and did not carry the mutation. The son, JN3, had vertical cup-to-disc ratios of 0.7 in each eye. The cups were symmetrical, deep and concentric. There was no corresponding visual field loss and no thinning of the retinal nerve fibre layer on GDx. JN3 did not carry the Tyr453del mutation. The third daughter, JN2, was diagnosed with POAG on the basis of glaucomatous optic neuropathy with corresponding visual field defects and thinning of her retinal nerve fibre layer on GDx. She had raised intraocular pressures. She was started on glaucoma treatment. She was found to carry the Tyr453del mutation.

Table 3.18 Demographic and clinical characteristics and mutation screening of relatives of P36JN compared with the proband (P36JN)

Participant code	JN1	JN2	JN3	JN4	**Proband P36JN**
Relationship to proband	Daughter	Daughter	Son	Daughter	
POAG	**No**	**Yes**	**No**	**No**	**Yes**
Ophthalmic history	Nil	Nil	Refractive error	Nil	
Age (years)	59	51	35	31	**70**
Gender	Female	Female	Male	Female	**Female**
Language	IsiZulu	IsiZulu	IsiZulu	IsiZulu	**IsiZulu**
Diabetic	No	No	No	No	**No**
Hypertensive	No	Yes	No	No	**Yes**
IOP (mmHg)	18	32	16	14	**32**
VCDR	0.30	0.90	0.70	0.30	**0.98**
Cataracts	No	No	No	No	**Yes**
Tyr453del	**No**	**Yes**	**No**	**No**	**Yes**

IOP, IOP at diagnosis for the proband or on examination for the relatives

4.2.3.2 PP003RM Family

Figure 3.9 is the family pedigree for PP003RM. Two of her children (labelled RM1 and RM2 in Figure 3.9) agreed to participate in the study.

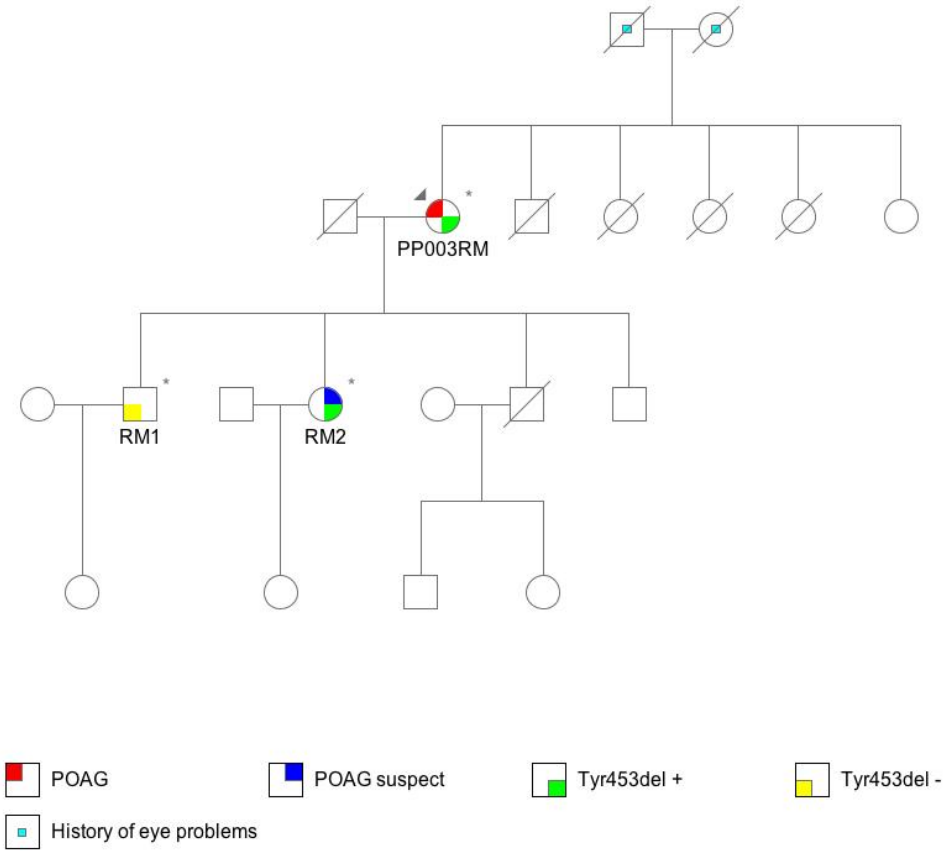

Figure 3.9 Family pedigree for PP003RM

The demographic and clinical findings in the proband's son and daughter compared with the proband are documented in Table 3.19. The son, RM1, did not have POAG and did not carry the mutation. The 43 year-old daughter, RM2, had vertical cup-to-disc asymmetry of 0.2. The left optic disc had temporal and superior thinning of the neuroretinal rim. There was no thinning of the retinal nerve fibre layer on GDx (NFIs of 20 and 17 and TSNITs of 52.0µm and 56.0µm in the right and left eyes respectively). Her first visual field examination was grossly unreliable. The second was more reliable. Both eyes in that test had a Glaucoma Asymmetric Test that was outside normal limits with an MD of -3.86dB in the right eye and -5.62dB in the left eye. The visual field examination will be repeated to confirm the findings. The pattern of loss did not correspond completely with the optic disc appearance. In addition there was IOP asymmetry with an IOP of 18mmHg recorded on two separate occasions in the right eye, while the left eye on the same two occasions had an IOP of 12mmHg. RM2 was therefore classified as a POAG suspect. RM2 was found to carry the Tyr453del mutation.

Table 3.19 Demographic and clinical characteristics and mutation screening of relatives of PP003RM compared with the proband (PP003RM)

Participant code	RM1	RM2	**Proband PP003RM**
Relationship to proband	Son	Daughter	
POAG	**No**	**Suspect**	**Yes**
Ophthalmic history	Nil	Nil	
Age (years)	58	43	**78**
Gender	Male	Female	**Female**
Language	Sesotho	Sesotho	**Setswana**
Diabetic	No	No	**Yes**
Hypertensive	Yes	No	**Yes**
IOP (mmHg)	18	*18	**Unknown**
VCDR	0.30	*0.50	**0.85**
Cataracts	No	No	**Yes**
Tyr453del	**No**	**Yes**	**Yes**

IOP, IOP at diagnosis for the proband or on examination for the relatives
* Asymmetry between the two eyes

4.2.3.3 PP085LN Family

Figure 3.10 is the family pedigree for PP085LN. Two of her children (labelled LKN1 and LKN2 in Figure 3.10) agreed to participate in the study.

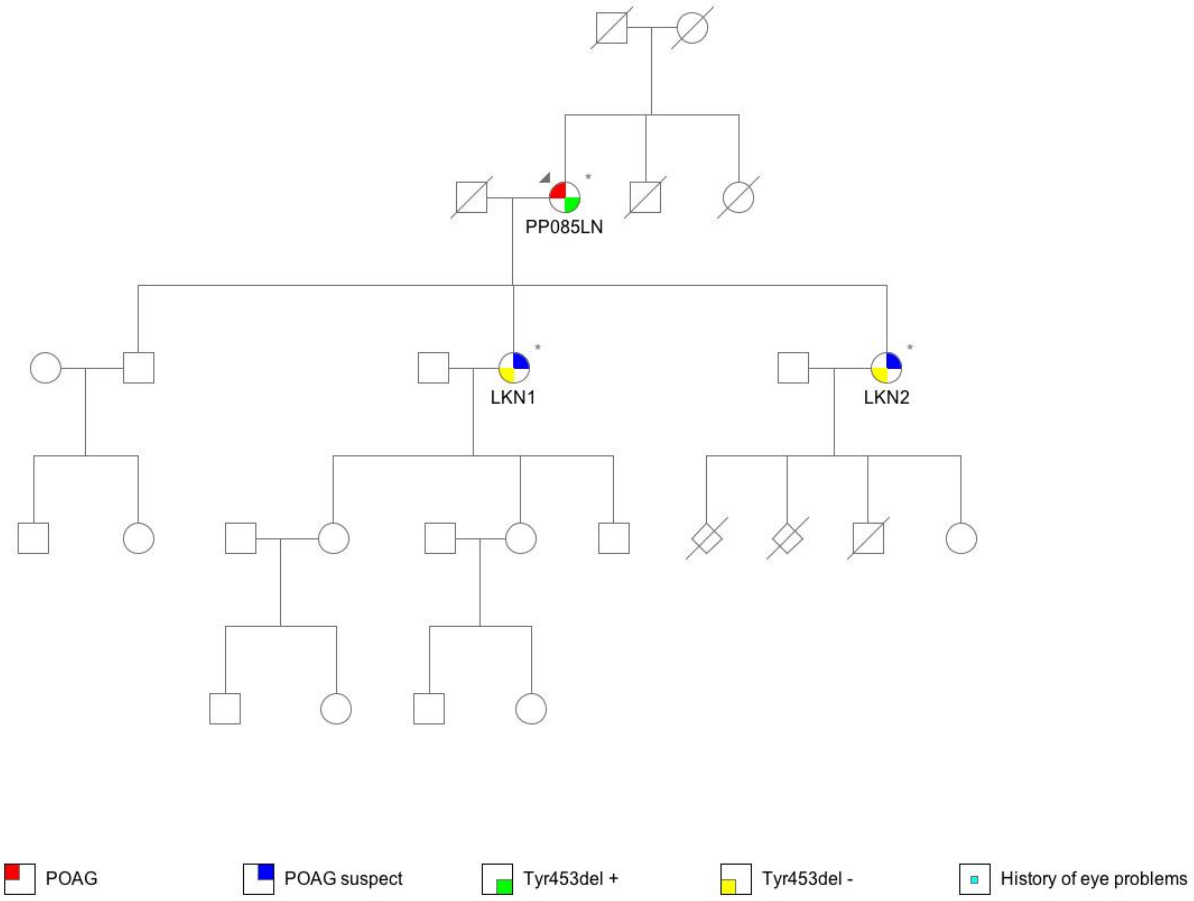

Figure 3.10 Family pedigree for PP085LN

The demographic and clinical findings in the proband's two daughters compared with the proband are documented in Table 3.20. The older daughter, LKN1, had vertical cup-to-disc asymmetry. The right disc had temporal cupping of 0.7 with superior and inferior neuroretinal rim thinning. The left optic disc had a thick neuroretinal rim and was 0.3 cupped. There was thinning of the retinal nerve fibre layer in the right eye on GDx (NFI of 39 compared with 31 on the left; TSNIT of 40.4μm compared with 43.9μm on the left). The visual field examination revealed full fields. The younger daughter (LKN2) also had disc asymmetry. The VCDR on the right was 0.7 with inferior thinning. On the left the VCDR was 0.5 with an inferior notch. LKN2 had higher NFIs on GDx (41 right and 53 left) and thinning of the retinal nerve fibre layer inferiorly on the right (inferior average thickness of 42.9μm). Her visual field examination was normal. Both LKN1 and LKN2 were therefore classified as POAG suspects. Neither was found to carry the Tyr453del mutation.

Table 3.20 Demographic and clinical characteristics and mutation screening of relatives of PP085LN compared with the proband (PP085LN)

Participant code	LKN1	LKN2	**Proband PP085LN**
Relationship to proband	Daughter	Daughter	
POAG	**Suspect**	**Suspect**	**Yes**
Ophthalmic history	Nil	Nil	
Age (years)	56	42	**73**
Gender	Female	Female	**Female**
Language	IsiXhosa	IsiXhosa	**IsiXhosa**
Diabetic	No	No	**Yes**
Hypertensive	Yes	Yes	**Yes**
IOP (mmHg)	16	19	**36**
VCDR	*0.5	*0.6	**1.0**
MD (dB)	-1.7	-1.9	**-14.4**
GDx NFI	35	47	**88**
GDx TSNIT (µm)	42.2	43.2	**29.4**
Cataracts	No	No	**Yes**
Tyr453del	**No**	**No**	**Yes**

IOP, IOP at diagnosis for the proband or on examination for the relatives
*Asymmetry between two eyes

4.2.3.4 P43TM Family

Figure 3.11 is the family pedigree for P43TM. His two surviving children (labelled TM1 and TM2 in Figure 3.11) agreed to participate in the study.

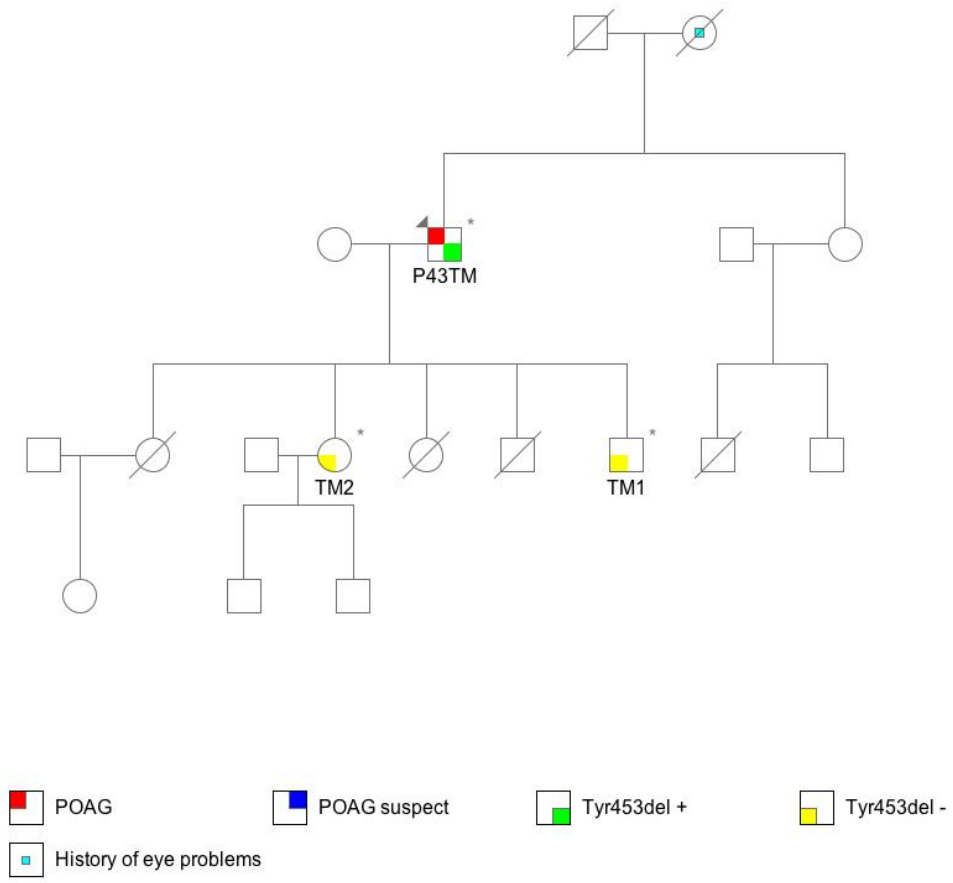

Figure 3.11 Family pedigree for P43TM

The demographic and clinical findings in the proband's son and daughter compared with the proband are documented in Table 3.21. Neither child had POAG and neither carried the Tyr453del mutation.

Table 3.21 Demographic and clinical characteristics and mutation screening of relatives of P43TM compared with the proband (P43TM)

Participant code	TM1	TM2	**Proband P43TM**
Relationship to proband	Son	Daughter	
POAG	**No**	**No**	**Yes**
Ophthalmic history	Nil	Nil	
Age (years)	33	28	**66**
Gender	Male	Female	**Male**
Language	Sesotho	Sesotho	**Sesotho**
Diabetic	No	No	**No**
Hypertensive	No	Yes	**No**
IOP (mmHg)	14	10	**32**
VCDR	0.2	0.3	**1.0**
Cataracts	No	No	**No**
Tyr453del	**No**	**No**	**Yes**

IOP, IOP at diagnosis for the proband or on examination for the relatives

4.2.3.5 PP177JZ Family

Figure 3.12 is the family pedigree for PP177JZ. One of his children (labelled JZ1 in Figure 3.12) agreed to participate in the study.

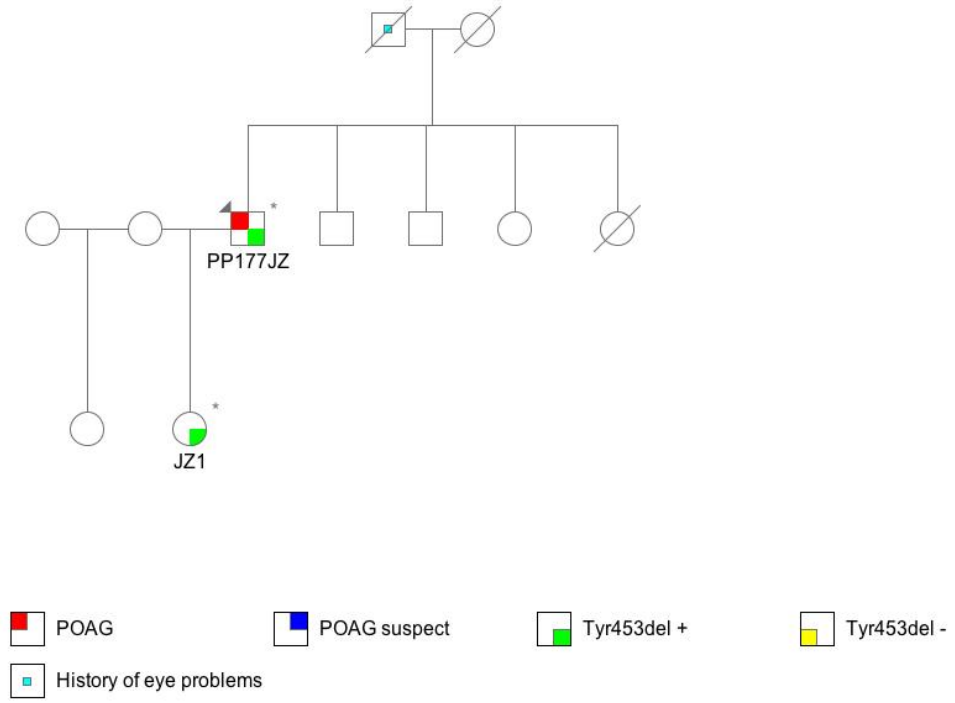

Figure 3.12 Family pedigree for PP177JZ

The demographic and clinical findings in the proband's daughter (JZ1) compared with the proband are documented in Table 3.22. JZ1 is 19 years old and has no evidence of POAG yet, but she was found to carry the Tyr453del mutation.

Table 3.22 Demographic and clinical characteristics and mutation screening of relative to PP177JZ compared with the proband (PP177JZ)

Participant code	JZ1	**Proband PP177JZ**
Relationship to proband	Daughter	
POAG	**No**	**Yes**
Ophthalmic history	Nil	
Age (years)	19	**50**
Gender	Female	**Male**
Language	Sepedi	**Tswana**
Diabetic	No	**No**
Hypertensive	No	**No**
IOP (mmHg)	12	**36**
VCDR	0.4	**1.0**
Cataracts	No	**No**
Tyr453del	**Yes**	**Yes**

IOP, IOP at diagnosis for the proband or on examination for JZ1
*Asymmetry between two eyes

4.2.3.6 CC012LN Family

CC012LN was a control participant. She is 80 years old and does not have POAG, but was found to carry the Tyr453del mutation. Figure 3.13 is the family pedigree for CC012LN. Four of her relatives (labelled LN1 - 4 in Figure 3.13) agreed to participate in the study. The relatives were a son (LN3), two daughters (LN1 and LN2) and a granddaughter (LN4, the daughter of LN1).

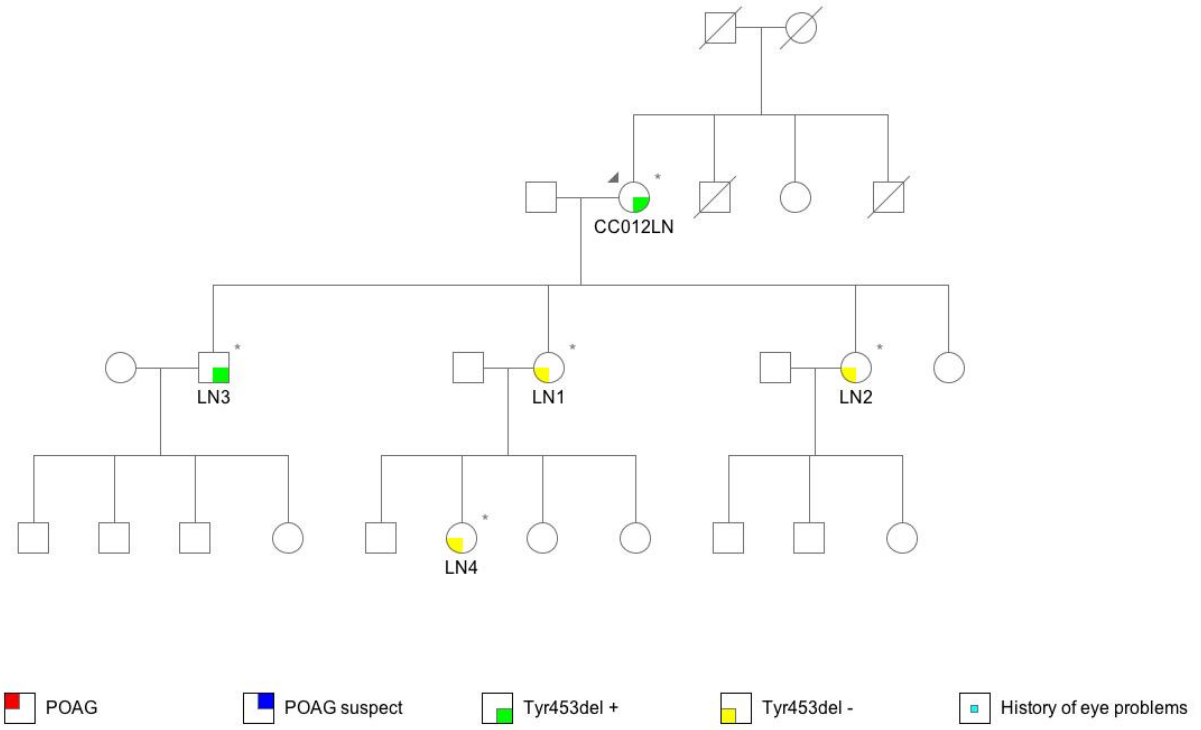

Figure 3.13 Family pedigree for CC012LN

The demographic and clinical findings in each of these relatives compared with the proband are documented in Table 3.23. None of the relatives were diagnosed with POAG. The 38 year-old son (LN3) was found to carry the Tyr453del mutation.

Table 3.23 Demographic and clinical characteristics and mutation screening of relatives of CC012LN compared with the proband (CC012LN)

Participant code	LN1	LN2	LN3	LN4	**Proband CC012LN**
Relationship to proband	Daughter	Daughter	Son	Granddaughter	
POAG	**No**	**No**	**No**	**No**	**No**
Ophthalmic history	Refractive error	Nil	Nil	Nil	**Cataracts**
Age (years)	59	44	38	25	**80**
Gender	Female	Female	Male	Female	**Female**
Language	Tshivenda	Tshivenda	Tshivenda	Tshivenda	**Tshivenda**
Diabetic	No	No	No	No	**Yes**
Hypertensive	Yes	No	No	No	**Yes**
IOP (mmHg)	16	14	18	16	**14**
VCDR	0.6	0.3	0.4	0.4	**0.3**
Cataracts	No	No	No	No	**Yes**
Tyr453del	**No**	**No**	**Yes**	**No**	**Yes**

IOP, IOP on examination

4.2.3.7 Tyr453del mutations

Including the seven index cases, we identified a total of eleven individuals who carry the Tyr453del mutation. Of these, six had a definite diagnosis of POAG. Table 3.24 provides an estimation of the penetrance of POAG caused by this mutation in this small sample at the ages of 40, 50, 60, 70 and 80 years. Penetrance increases from 25% at the age of 50 years, to 86% at the age of 80 years.

Table 3.24 An estimation of POAG penetrance at different ages in study participants carrying the Tyr453del mutation

Code	Relative	Age	Diagnosis	POAG at 40	POAG at 50	POAG at 60	POAG at 70	POAG at 80
P36JN		70	POAG	X	X	X	✓	✓
P43TM		60	POAG	X	X	✓	✓	✓
PP003RM		72	POAG	X	X	X	✓	✓
PP177JZ		50	POAG	X	✓	✓	✓	✓
PP085LN		70	POAG	X	X	X	✓	✓
CC012LN		80	Control	X	X	X	X	X
CC141FM		71	Control	X	X	X	X	N/A
JN2	P36JN	51	POAG	X	✓	✓	✓	✓
RM2	PP003RM	43	POAG suspect	X	N/A	N/A	N/A	N/A
JZ1	PP177JZ	19	No POAG	N/A	N/A	N/A	N/A	N/A
LN3	CC012LN	38	No POAG	X	N/A	N/A	N/A	N/A
Penetrance				**0/10 (0%)**	**2/8 (25%)**	**3/8 (38%)**	**6/8 (75%)**	**6/7 (86%)**

Age, age at diagnosis for POAG patients or on enrolment for other participants

The clinical, demographic and POAG characteristics of those Tyr453del mutation positive individuals who also have a diagnosis of POAG compared with all the POAG study participants are recorded in Table 3.25. The Tyr453del POAG patients had high IOPs and advanced cupping. The average age at diagnosis was 62 years. The POAG participants with the mutation had a similar phenotype to the whole group. The only statistically significant difference was in the VCDR that was larger in the Tyr453del positive POAG patients ($p = 0.043$).

Table 3.25 Demographic and clinical characteristics of POAG participants with the Tyr453del mutation compared with all the POAG participants

	Tyr453del+ (n=6)	POAG (n=247)	p
Age (years ± SD)	64.7 ± 10.6	60.0 ± 13.1	0.340 §
Gender	66.7% Female	50.2% Female	0.684 ⋆
Language	33% IsiZulu; 17% Sesotho; 33% Setswana; 17% IsiXhosa	39% IsiZulu; 17% Sesotho; 15% Setswana; 10% IsiXhosa	
Family history of glaucoma	33.3%	7.3%	0.074 ⋆
Family history of blindness	33.3%	24.3%	0.637 ⋆
Diabetic	33.3%	23.1%	0.626 ⋆
Hypertensive	66.7%	53.4%	0.689 ⋆
Age at diagnosis (years ± SD)	62.2 ± 9.1	55.0 ± 13.6	0.119 §
IOP (mmHg ± SD)	33.6 ± 2.0	34.9 ± 9.1	0.184 §
CCT (µm ± SD)	490.7 ± 29.5	505.1 ± 37.9	0.294 §
VCDR ± SD	0.96 ± 0.06	0.89 ± 0.13	**0.043 §**
MD (dB ± SD)	-9.9 ± 5.1	-10.4 ± 5.4	0.860 §
GDx NFI ± SD	59.3 ± 17.5	68.2 ± 22.9	0.394 §
GDx TSNIT (µm ± SD)	38.4 ± 5.8	36.9 ± 8.7	0.651 §
Cataracts	50.0%	49.4%	1.000 ⋆
IOP control	60.0%	68.4%	1.000 ⋆
SVI	50.0%	52.6%	1.000 ⋆
Glaucoma Surgery	40.0%	53.9%	0.258 ⋆

IOP, Intraocular presssure at diagnosis; SVI, severe visual impairment
⋆ Fisher's exact test
§ T-test

5. DISCUSSION

Sequencing of the *MYOC* exons and the flanking non-coding regions identified 19 sequence variants. Fourteen of these were screened in the study population. Nine variants (four non-coding, four synonymous SNPs and a single non-synonymous SNP) occurred with similar frequencies in POAG and control participants suggesting no association with POAG. Two SNPs (Arg76Lys = rs2234926 and Thr325Thr = rs61730976) varied in minor allele frequency between POAG patients and control participants, but not significantly. There were three potentially pathogenic mutations identified: Gly374Val, Lys500Arg and Tyr453del.

5.1 Gly374Val

It is probable that the Gly374Val mutation is a novel glaucoma-causing mutation. It was only observed in POAG patients and not in control participants. It is predicted to be damaging to the MYOC protein. However, this mutation only occurred with a prevalence of 0.9% in the POAG participants, suggesting that it is an uncommon cause of glaucoma in this population. Determining the typical phenotype of Gly374Val POAG is not possible with only two individuals. It is however interesting that the patients had neither a family history of glaucoma nor of blindness. They both had thin corneas, high IOPs and advanced cupping. The family study did not identify any additional individuals with the mutation. A first cousin of the index case who did not carry the mutation, was identified as a POAG suspect. If he goes on to develop glaucoma it may be the result of other genetic influences on this heterogeneous disease. This has implications for glaucoma genetic counselling and screening in South Africa. Screening for the mutation in POAG patients would only be practical if there was simultaneous and inexpensive testing for other mutations, for example Tyr453del. Cascade screening in relatives could potentially identify individuals for closer follow-up and early treatment of the condition. Further studies are required before any estimations may be made on the penetrance of the mutation. From a counselling perspective, not carrying the mutation does not imply protection from POAG, but carrying the mutation would imply a much higher risk for developing the disease.

5.2 Lys500Arg

The evidence suggests that Lys500Arg is a benign polymorphism that is probably not associated with an increased risk of POAG. The mutation is predicted to be tolerated and has been described in control subjects in other African populations.[251] Initially the mutation was identified only in POAG patients within the study population, but the family study identified an unaffected participant carrying the mutation. This participant was a sibling to the index case and a similar age to the severely affected proband who had advanced cupping and high IOPs. In this context it seems unlikely that the unaffected carrier will go on to develop POAG although this cannot be excluded. The unaffected Lys500Arg carrier also carried the Glu396Glu variant that was common to the three other POAG participants with Lys500Arg. Glu396Glu is considered a benign polymorphism that, in this population, is in linkage disequilibrium with Lys500Arg. Glu396Glu, without Lys500Arg, occurred in similar frequencies in POAG and control participants. In addition to being identified in an unaffected individual, the Lys500Arg mutation was not present in the daughter of the index case who had features suggestive of POAG. If she goes on to develop glaucoma, this will add further weight to the suggestion that Lys500Arg is benign and a poor predictor for disease development. It is probably of no value as a screening test in this population.

5.3 Tyr453del

The Tyr453del frameshift mutation is thought to be a glaucoma-causing mutation. It is likely that the mutation causes gain-of-function due to a truncated protein. It is the most important glaucoma-causing mutation identified in this population with a prevalence of 2.3%. This is much more common than reports of the mutation in other studies. For example it has been reported in African Americans with a prevalence of between 0.2% and 0.3%.[65, 251] It has only been reported in POAG individuals with African ancestry. Interestingly the mutation was identified in two unaffected controls in our study population, both over the age of 70 years. They are unlikely to develop glaucoma at this stage. This suggests incomplete penetrance associated with the mutation. The mutation may be necessary but not sufficient to cause the disease in

carriers of the mutation.

The penetrance of Tyr453del in this population increases with age. Penetrance is estimated at 25% at the age of 50 years and increases to an estimated 86% at the age of 80 years. The estimates are limited by the small sample size and by an inability to determine the age of onset of POAG. Because the POAG participants all had advanced disease, it is likely that the age of onset of POAG was substantially lower than the age at diagnosis and actual prevalence is therefore higher in the younger age groups.

The phenotype of POAG associated with the Tyr453del mutation is one of adult-onset with high IOPs and large VCDRs. The phenotype is not distinguishable from the average black South African POAG patient seen at St John Eye Hospital, so selective screening for the mutation would not be possible.

The family study successfully identified a previously undiagnosed individual with POAG. She is now receiving appropriate therapy for the disease. A high-risk POAG suspect and a 19 year-old at high risk of developing POAG were also identified. Both will be closely monitored for POAG development. Two POAG suspects who did not carry the mutation will also be monitored. They may go on to develop the disease and if they do this would suggest another, probably genetic, aetiological mechanism in this particular family.

Based on these findings, screening for Tyr453del has a place in the management of POAG in black South Africans. As the costs of genetic screening come down, so this will become more readily available. Cascade screening in the relatives of POAG patients identified with the mutation is valuable. Participant counselling and interpreting the results of the testing is not, however, straightforward. The presence of the mutation implies a high risk for disease development, but the converse does not apply and relatives without the mutation should still receive routine follow-up.

5.4 Limitations

Myocilin mutations are not a common contribution to POAG in this population, so the groups that were identified with the mutations were not large enough to determine accurately if there were specific phenotypic features of the glaucoma mediated by the mutations.

Tracing family members of individuals with the mutations was a further challenge in investigating penetrance.

Identifying further individuals with mutations and following this up with the screening of further family members would strengthen the conclusions of this study.

5.5 Summary

Black South Africans with POAG may have a *MYOC* mutation that either causes or contributes to their risk for developing POAG in approximately 3.3%. The commonest mutation is a frameshift mutation (Tyr453del) that is incompletely penetrant. That the mutation is necessary but insufficient introduces a counselling dilemma. Mutation screening can, however, identify high-risk individuals who can be monitored to detect early signs of the disease. In this small study, this approach led to the diagnosis of POAG in one family member who is now receiving treatment for the condition and the identification of two other high-risk individuals who will be closely monitored for the development of POAG. The Gly374Val mutation is predicted to be damaging to MYOC. It is an uncommon cause of POAG in this population. The Lys500Arg mutation is predicted to be benign and tolerated. In the family with the mutation, it did not segregate with the disease suggesting that it is a neutral polymorphism. These findings have important implications for the management and counselling of black South African patients with POAG and their families.

Chapter 4: Candidate Gene Association Studies

1. INTRODUCTION

The purpose of this part of the study was to explore the contribution of genetic variation at candidate genes or chromosomal regions to the pathogenesis of POAG in black South Africans using a case-control association study.

Secondary aims were to evaluate the association in this population with ocular quantitative traits (IOP, CCT and VCDR) and genetic variation in candidate regions; and diabetes mellitus with genetic variation in POAG candidate genes.

The study consisted of two distinct parts:

In the first experiment the genotyping was performed on the study population using the medium-throughput Illumina® BeadXpress platform. This genotyping system is geared to genotype 48 to 384 SNPs simultaneously in one sample. The system allows for genotyping of custom designed assays. In this way SNPs from thirteen candidate regions were simultaneously evaluated in the study population.

In the second experiment the genotyping on the study population was performed using TaqMan® assays for individual SNPs from four candidate regions. I undertook this second experiment in Dr Michael Hauser's laboratory in the Duke Center for Human Genetics in Durham, North Carolina, United States of America. The candidate regions and SNPs had been selected by Dr Michael Hauser's team and had already been evaluated for association with POAG in two large groups of individuals of African descent (from Dr R. Rand Allingham): a group of African Americans and a group of West Africans from Ghana. I was given access to this raw data and could therefore both compare and combine the findings from these three distinct populations of African descent.

2. ILLUMINA BEADXPRESS ASSOCIATION STUDY

2.1 Methods

2.1.1 Population and sample

The study population, selection criteria, examination and investigations have been described in detail in Chapter 2.

2.1.2 Candidate region selection

POAG candidate region selection was based on location within known POAG loci or genes identified in association studies as having variants associated with POAG. The purpose of including these regions was not to replicate studies in other populations, but rather to identify appropriate candidates for a targeted search for gene associations with POAG in this population. Candidate genes for association with CCT, VCDR and IOP were also included as POAG candidate genes. The candidate region selection took place from January to June 2011. The selection was based on a literature review at that time. A current literature review of the subject with a detailed description of each region can be found in Chapter 1.

The following thirteen genes or chromosomal loci were included in the association study:

- **Known POAG genes**
    - *MYOC*
    - *CYP1B1*
    - *WDR36*

WDR36 was also included because of the reported association of the locus with IOP.[95, 206]

- **Regions identified in POAG association studies**
    - Chromosome 2p16 locus
    - *CAV1* and *CAV2* and the intergenic region

CAV1 and *CAV2* were also included because of the evidence suggesting an association of the region with IOP.[188]

- **Regions identified in CCT association studies**
  - *COL1A1*
  - *COL1A2*
  - *COL5A1*
  - *COL8A2*
  - *ZNF469* region

- **Regions identified in optic disc parameter association studies**
  - *CDKN2B*
  - *SIX1* and *SIX6* and the intergenic region
  - *ATOH7*

At the time of candidate gene selection the genome-wide association of SNPs within the *CDKN2B, SIX1* and *SIX6* regions and POAG had not yet been published.

2.1.3 Single nucleotide polymorphism selection

SNP selection took place from January to June 2011. The approach to selecting representative SNPs within each of the selected genes or loci was as follows:

I began with a literature review and included SNPs ('literature SNPs') that had shown an association in other studies and in other population groups.

The next step was to determine the size of each region and to determine the linkage disequilibrium patterns within each region. Small regions or regions with significant linkage disequilibrium could be adequately covered with tag SNPs. The regions were explored both in HapMap (using HapMap data release #27) and in Haploview.[284] In exploring linkage disequilibrium the HapMap data from the Yoruba in Ibadan, Nigeria (YRI) was used. This population was used as a proxy for the black South African population because at the time of SNP selection there were no data on black South

Africans in HapMap and the Yoruban dataset was the largest for a group of African descent. There is tremendous diversity in populations of African descent, so it is likely that the study population differed from the Yoruban population. This is a weakness in the SNP selection process and one that I attempted to mitigate by comparing the YRI HapMap data with that for the Luhya in Webuye, Kenya (LWK) and the Maasai in Kinyawa, Kenya (MKK).

For regions too large to be covered with tag SNPs, only the previously associated SNPs ('literature SNPs') were used. Where the regions could be covered with tag SNPs, tag SNP data were downloaded from HapMap using the 'Tagger Multimarker' protocol which aggressively searches for multi-marker predictors to capture all alleles of interest (SNPs and/or haplotypes).[285] In running 'Tagger', the HapMap YRI data were used. The 'literature SNPs' were 'force included'. A minimal coefficient of determination (r^2) at which all alleles were to be captured was selected to be 0.8. Setting the threshold to 1.0 would have resulted in a non-redundant set of tag SNPs where all untyped SNPs would have a perfect proxy. SNPs were selected with a minor allele frequency of more than 0.05 in the Yoruba. In this manner a list of SNPs was created for each gene or region.

The list of SNPs was sent to Illumina for processing and 'scoring' using the Illumina® Assay Design Tool. SNP information was obtained from the most current sources at the time, Human Genome Build 37.1 and dbSNP version 131 (accessed in June 2011). Minor allele frequency was added to each SNP, as was a 'SNP Score' and 'Design Rank'. The 'SNP score' is a value on the 0.000 –1.000 scale to reflect the ability to design a successful assay. GoldenGate® validated SNPs that have original assay oligonucleotides on file have 'SNP scores' of 1.1. The 'Design Rank' results can be 0, 0.5 or 1. A 'SNP Score' of less than 0.400 has a rank of 0 and implies a low success rate and a high risk to produce an unsuccessful pooled query oligonucleotide assay. A 'SNP Score' of 0.400 to less than 0.600 has a rank of 0.5 and implies a moderate success rate and a moderate risk to produce an unsuccessful pooled query oligonucleotide assay. A 'SNP Score' of 0.600 to 1.1 has a rank of 1 and implies a high success rate and a low risk to produce an unsuccessful pooled query oligonucleotide assay.

SNPs with a rank of 0 or 0.5 were removed. Where 2 SNPs were less than 61 base pairs away from each other (this generates the 'Failure Code'=340), the literature was reviewed and the more relevant 'literature SNP' chosen. If the SNPs too close to each other were tag SNPs, then first one SNP was 'force excluded' and then the other and 'Tagger' was re-run to see which of the two it was better to exclude. Alternatively only the higher scoring SNP was included. In this way new files of successful SNPs to 'force include' and failed SNPs to 'force exclude' were created in a re-run of 'Tagger' to identify any new SNPs. These files were also used to run the 'Tagger' application in Haploview in the two populations from Kenya to find SNPs in these populations that differed from the YRI SNPs. Where new SNPs were identified the complete files were sent to Illumina for repeat scoring. This process was repeated until SNPs had been selected that all had high scores and therefore predicted the successful production of a pooled query oligonucleotide assay. Figure 4.1 is a flow chart illustrating this process.

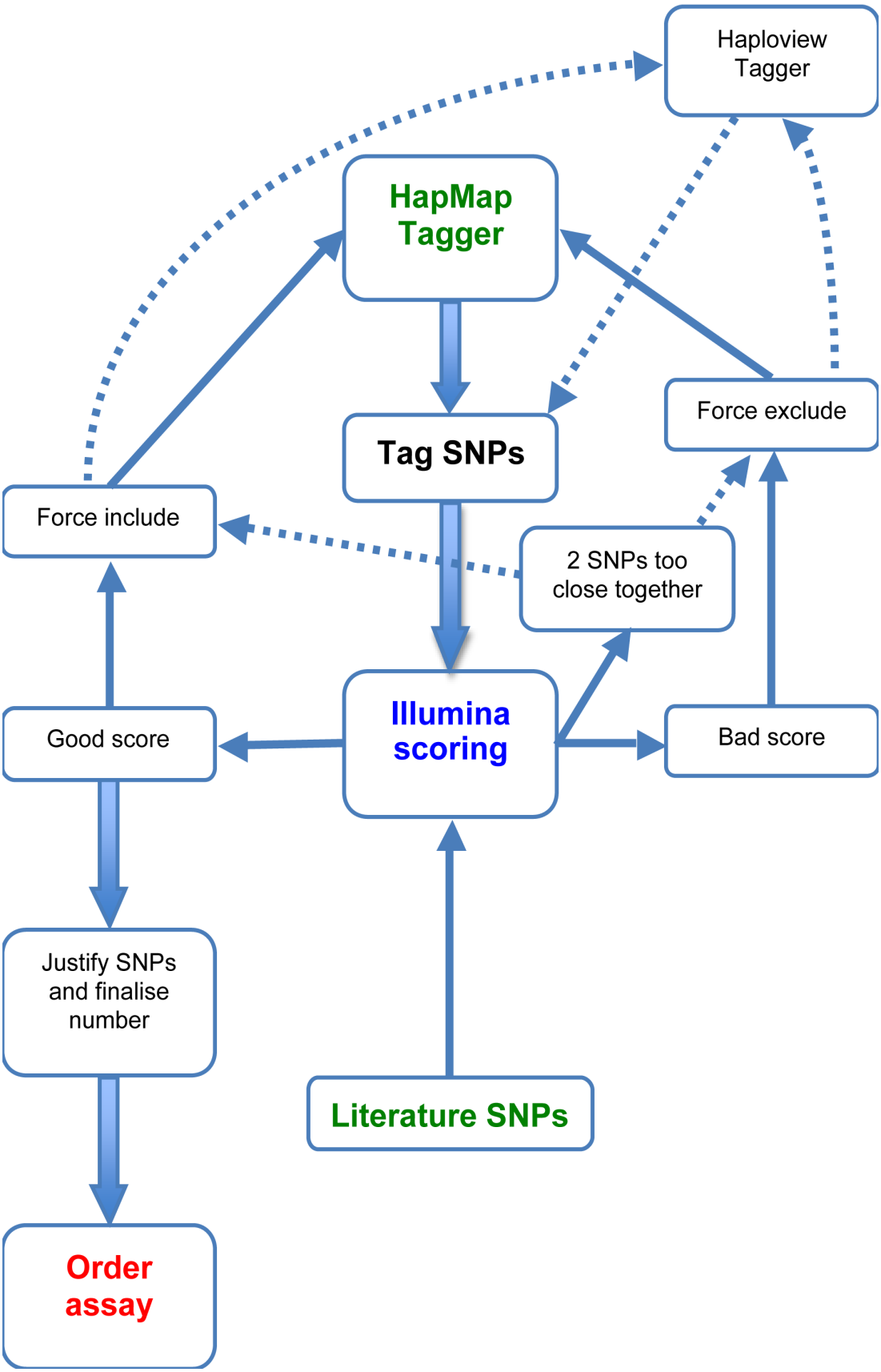

Figure 4.1 SNP selection. The process used to select SNPs for candidate genes incorporating HapMap Tagger and SNPs included from the literature review (Literature SNPs) and culminating in Illumina scoring and a final list of SNPs.

The specific SNP selection process for each gene or region is described below. Linkage disequilibrium (LD) plots for each region in the Yoruba were generated in Haploview (version 4.2)[286] using the SNPs I selected in that region that had HapMap genotyping information on the Yoruba that could be downloaded into Haploview.

2.1.3.1 The locus on chromosome 2p16 (Figure 4.2)

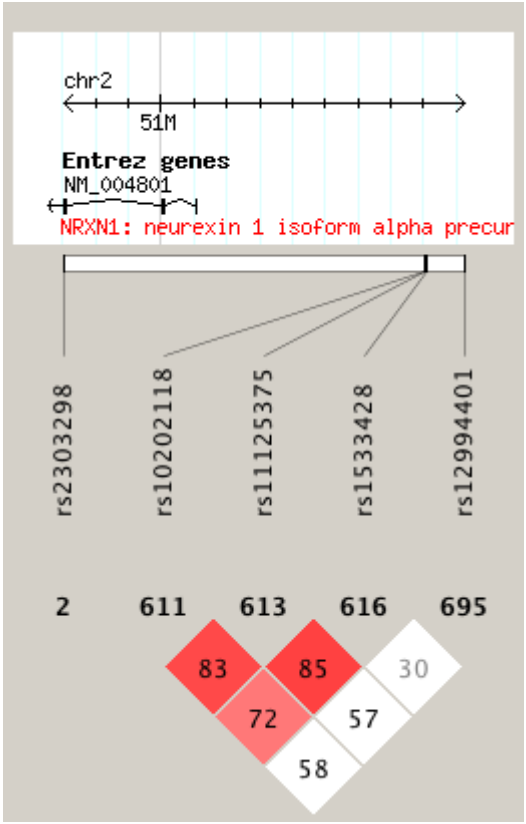

Figure 4.2 Haploview linkage disequilibrium plot of SNPs selected for genotyping from the locus on chromosome 2p16 showing the LD patterns of the SNPs in the Yoruba from HapMap genotyping data (HapMap Version 3 Release 27). Above is the location of the region on Chromosome 2 along with the location of a gene (neurexin 1 isoform alpha precursor) within the region. Below is an indication of the position of the SNPs along with a diagram of the block structure of the region. LD plots were identified by strong LD. Depth of red/pink colour indicates the computed pairwise D' value; deeper pink indicates a higher D' value.

The chromosome 2p16 region identified in the study from Barbados[175] is a 2Mbp (mega base pair) region around the chromosomal marker D2S2156. This marker had the highest LOD ($\log_{10}$ of odds) score for linkage with POAG in that study.[175] The authors of that study selected 30 SNPs within this 2Mbp region. They found a significant association with two SNPs: rs1533428 had the highest LOD score and rs12994401 was also significantly associated although it was in linkage disequilibrium with the former SNP (D'=0.72).[175] All the other SNPs that showed an

association were clustered in a 100kbp region (600kbp away from D2S2156) that yielded 89 tag SNPs when tag SNP data on it were downloaded from HapMap. The region was too large to be covered adequately with tag SNPs in this study, so only the 8 SNPs that had shown an association in the Barbados study were added to the assay design as 'literature' SNPs. One of these SNPs received a low Illumina® Assay Design Tool score and was excluded. A total of seven 'literature' SNPs were therefore included in the final design for this region.

2.1.3.2 MYOC (myocilin) (Figure 4.3)

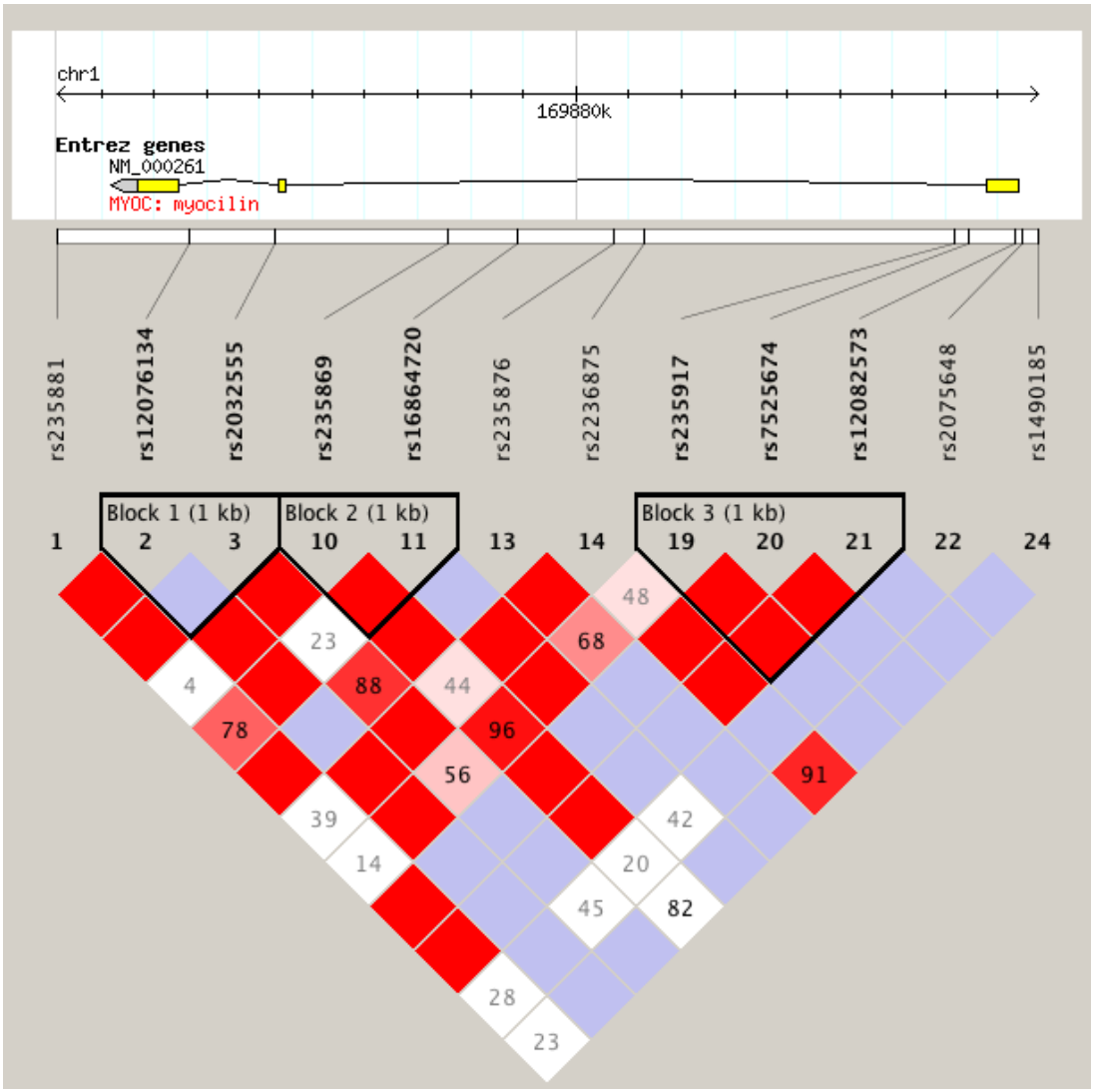

Figure 4.3 Haploview linkage disequilibrium plot of SNPs selected for genotyping from the *MYOC* gene showing the LD patterns of the SNPs in the Yoruba from HapMap genotyping data (HapMap Version 3 Release 27). Above is the location of the region on Chromosome 1 along with the location of the gene within the region. Exons are illustrated with yellow bolcks. Below is an indication of the position of the SNPs along with a diagram of the block structure of the region. LD plots were identified by strong LD. Depth of red/pink colour indicates the computed pairwise D' value; deeper pink indicates a higher D' value. The blue blocks represent high D' but low LOD values. SNPs 1 (rs235881) and 24 (rs1490185) were included in the plot to demarcate the region, but were not selected for genotyping.

The nineteen sequence variants (including the three potentially pathogenic mutations) that we identified through sequencing *MYOC* (see Chapter 3.2) were submitted to Illumina as genome sequences unless they were known SNPs and could be included in the dbSNP list. Five of the variants (none of which had differed in frequency in the controls when compared to the cases in the original subset) failed the Illumina® Assay Design Tool and were excluded from the assay. In this way a total of eleven 'literature' SNPs and a further three potentially disease-causing mutations were included in the assay.

There were eight tag SNPs (one of which was also a 'literature SNP') in the *MYOC* gene from tag SNP data downloading from HapMap. Two of these failed the Illumina® Assay Design Tool and were replaced by two others in the final assay. Only one allele remained uncaptured by the final selection of SNPs that captured 18 of 19 alleles with a mean r^2 of 0.974.

A total of 18 SNPs and a further three potentially disease-causing mutations (for the purposes of the Myocilin Study – Chapter 3) within the *MYOC* gene were included in the final assay.

2.1.3.3 CYP1B1 (cytochrome P450 1B1) (Figure 4.4)

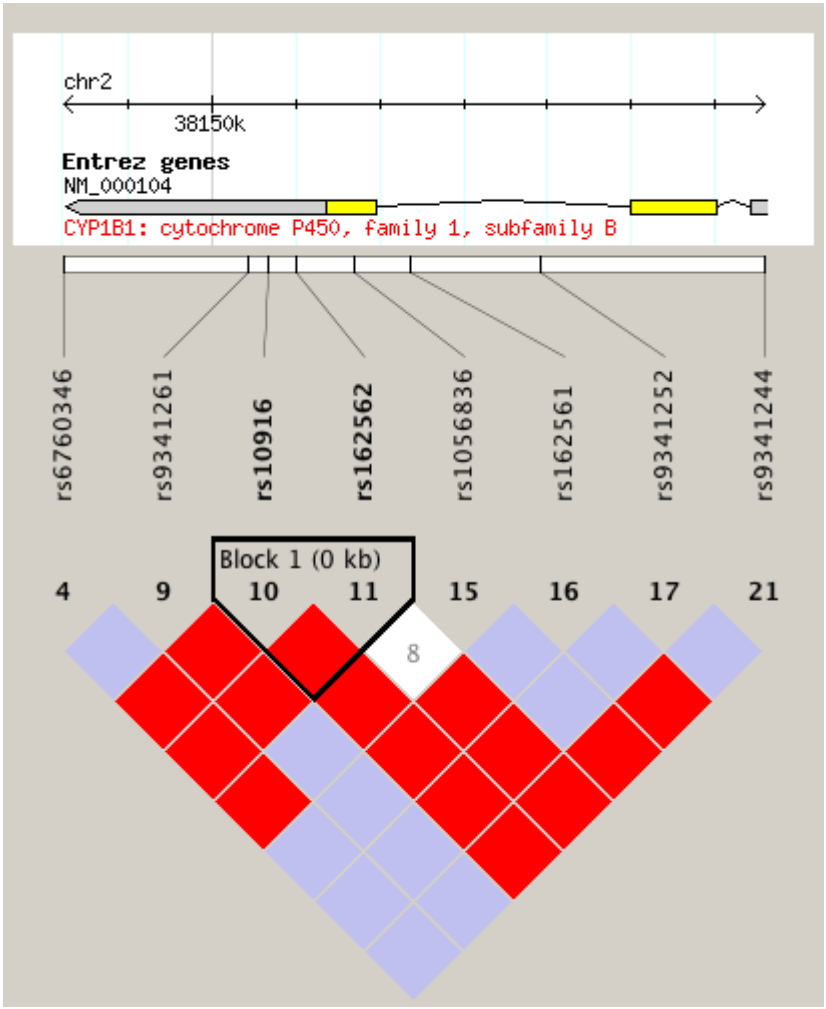

Figure 4.4 Haploview linkage disequilibrium plot of SNPs selected for genotyping from the *CYP1B1* gene showing the LD patterns of the SNPs in the Yoruba from HapMap genotyping data (HapMap Version 3 Release 27). Above is the location of the region on Chromosome 2 along with the location of the gene within the region. Exons are illustrated with yellow bolcks. Below is an indication of the position of the SNPs along with a diagram of the block structure of the region. LD plots were identified by strong LD. Depth of red/pink colour indicates the computed pairwise D' value; deeper pink indicates a higher D' value. The blue blocks represent high D' but low LOD values. SNPs 1 and 21 were included in the plot to demarcate the region, but were not selected for genotyping.

Burdon *et al.*[113] evaluated seven tag SNPs in the *CYP1B1* gene in a case-control association study and found a nominal association with POAG and SNPs tagged by rs162562 and rs10916 and represented in a common haplotype. These two SNPs were included as 'literature' SNPs. There were ten tag SNPs in *CYP1B1* from tag SNP data downloading on the Yoruban from HapMap. The literature SNPs were included in this group. There were two additional SNPs in the Luhya when Haploview tagger was run, but both scored poorly and were excluded. After the Illumina® Assay Design Tool process and the discarding of SNPs that failed, were low-scoring or were too close together, seven tag SNPs (including the two 'literature' SNPs)

remained which captured nine of twelve alleles with a mean r^2 of 0.981. These were included in the final assay.

2.1.3.4 CAV1 and CAV2 (caveolin) and the intergenic region (Figure 4.5)

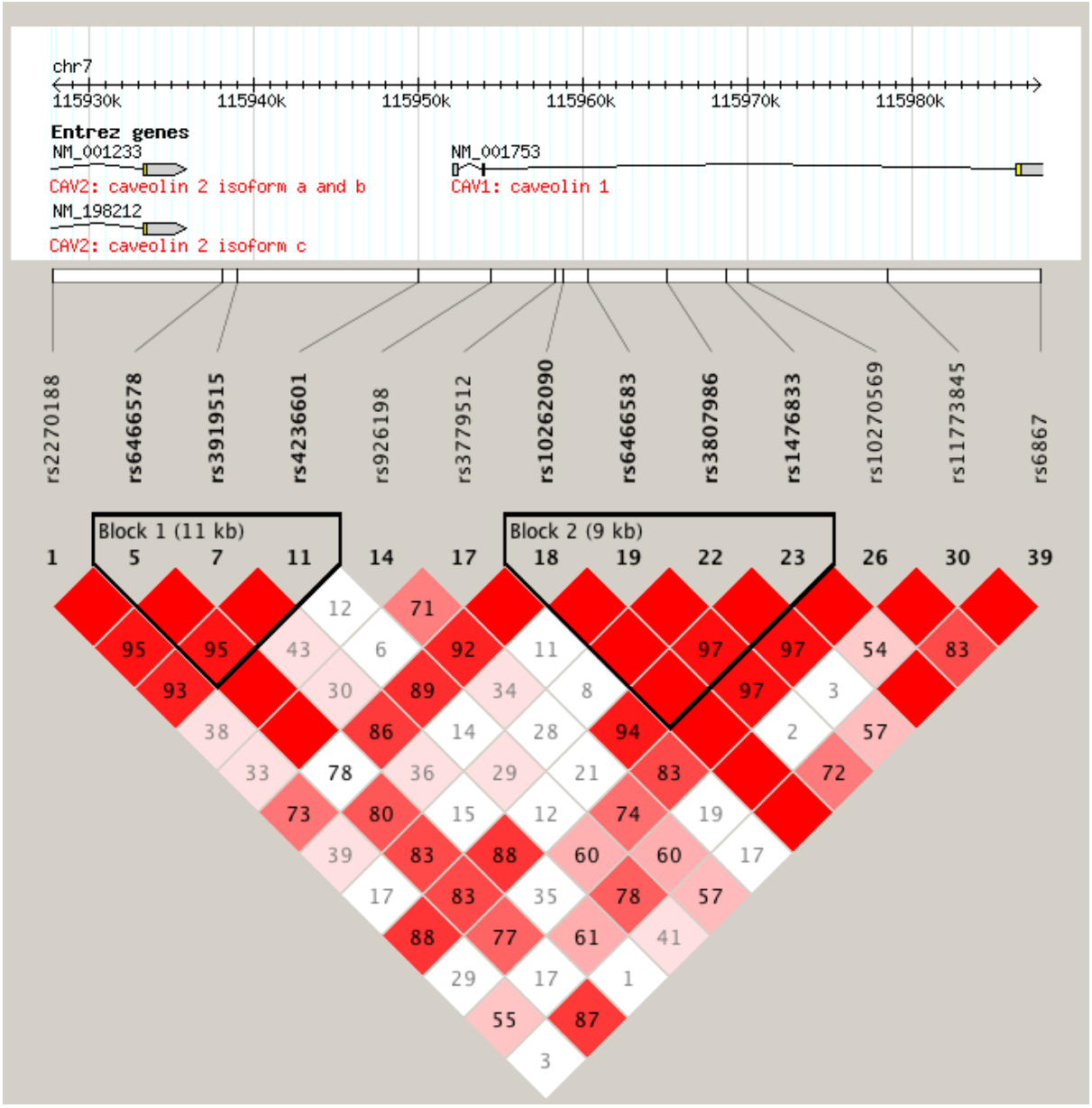

Figure 4.5 Haploview linkage disequilibrium plot of SNPs selected for genotyping from the *CAV1* and *CAV2* gene region showing the LD patterns of the SNPs in the Yoruba from HapMap genotyping data (HapMap Version 3 Release 27). Above is the location of the region on Chromosome 7 along with the location of the gene within the region. Exons are illustrated with yellow bolcks. Below is an indication of the position of the SNPs along with a diagram of the block structure of the region. LD plots were identified by strong LD. Depth of red/pink colour indicates the computed pairwise D' value; deeper pink indicates a higher D' value. SNPs 1(rs2270188) and 39(rs6867) were included in the plot to demarcate the region, but were not selected for genotyping.

The genome-wide association study in Iceland identified the association with POAG and SNP rs4236601 that lies within a linkage disequilibrium block that contains

CAV1 and *CAV2*.[177] This significant SNP (rs4236601) was included in the assay design as a 'literature' SNP. Because the SNP is in the intergenic region between *CAV1* and *CAV2*, both genes and the intergenic region (from chromosome 7:115926680 to 115988466) were included when tag SNP data were downloaded from HapMap (YRI). There were sixteen tag SNPs in this region. Three failed the Illumina® Assay Design Tool process, but one of these was replaced by a surrogate SNP. A total of fourteen tag SNPs were therefore included in the assay. These SNPs captured 66 of 68 alleles with a mean r^2 of 0.952.

2.1.3.5 COL8A2 (collagen type VIII alpha 2) (Figure 4.6)

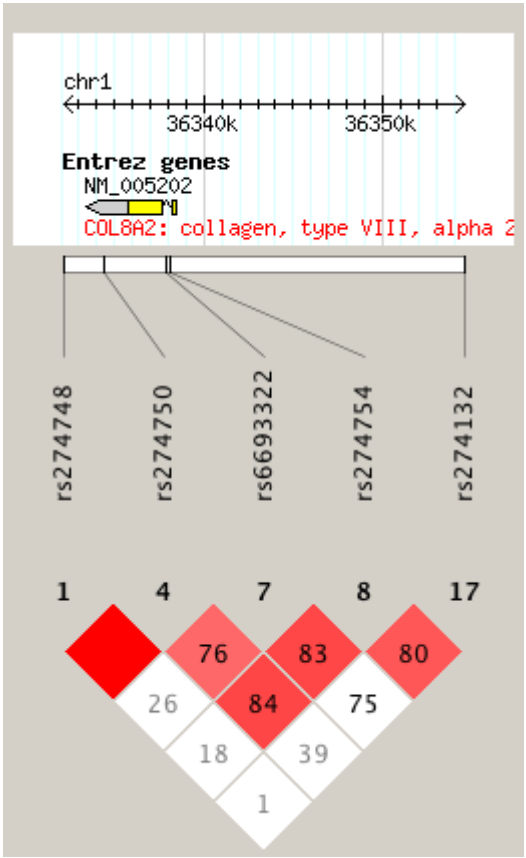

Figure 4.6 Haploview linkage disequilibrium plot of SNPs selected for genotyping from the *COL8A2* gene showing the LD patterns of the SNPs in the Yoruba from HapMap genotyping data (HapMap Version 3 Release 27). Above is the location of the region on Chromosome 1 along with the location of the gene within the region. Exons are illustrated with yellow bolcks. Below is an indication of the position of the SNPs along with a diagram of the block structure of the region. LD plots were identified by strong LD. Depth of red/pink colour indicates the computed pairwise D' value; deeper pink indicates a higher D' value. The blue blocks represent high D' but low LOD values. SNPs 1 (rs274748) and 17 (rs274132) were included in the plot to demarcate the region, but were not selected for genotyping.

The two SNPs evaluated by Desronvil *et al.*[229] for an association with CCT were included as 'literature' SNPs. The SNP that in that study had shown no association

with CCT failed the Illumina® Assay Design Tool process and was excluded. There were three tag SNPs that covered the gene when HapMap tagger was run for the Yoruba. One of these was the significant 'literature SNP'. All three SNPs were included in the final assay design. There were no additional SNPs in the Luhya or in the Maasai when Haploview Tagger was run using these populations.

2.1.3.6 COL1A1 (collagen type I alpha 1) (Figure 4.7)

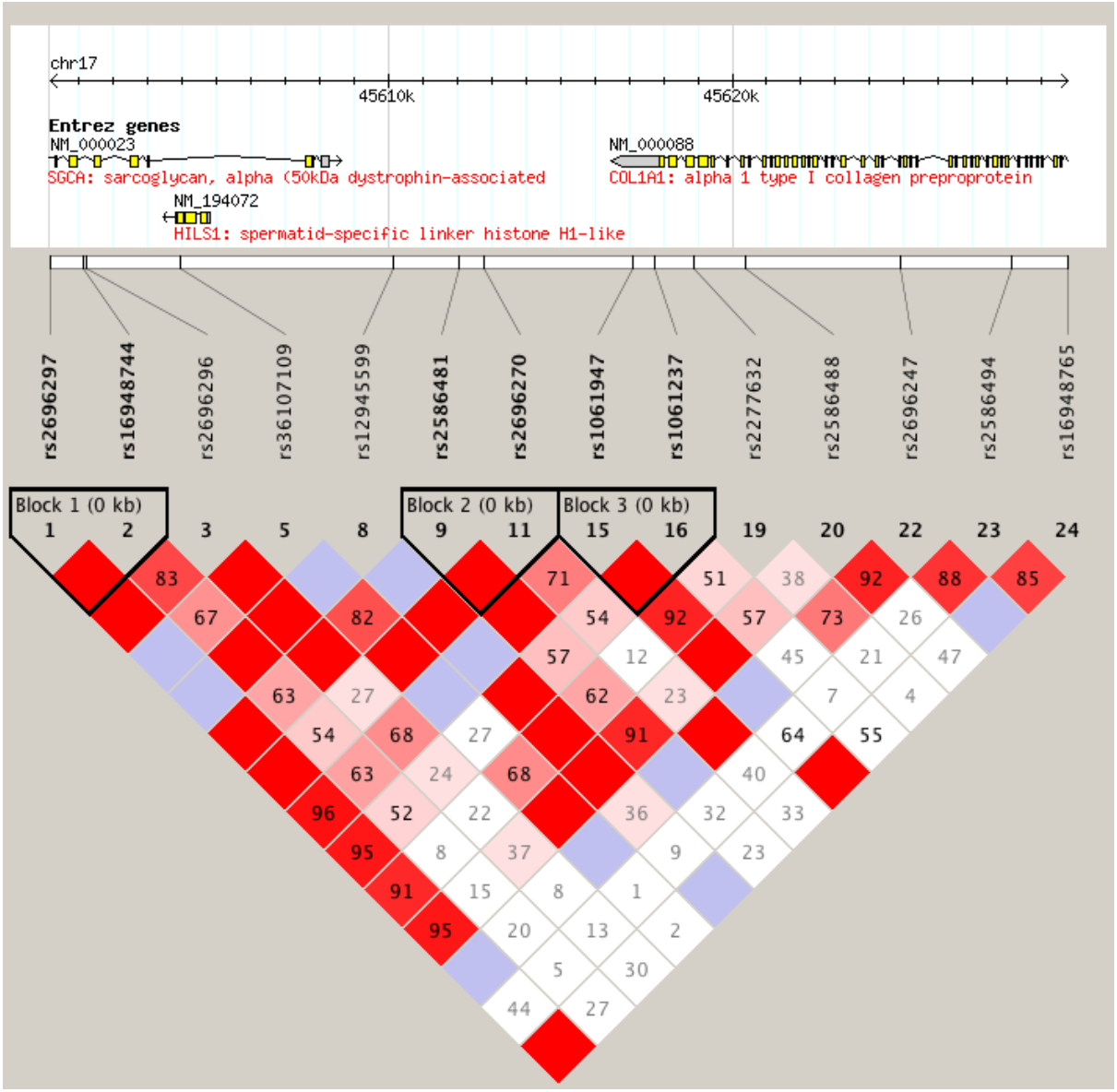

Figure 4.7 Haploview linkage disequilibrium plot of SNPs selected for genotyping from the *COL1A1* gene region showing the LD patterns of the SNPs in the Yoruba from HapMap genotyping data (HapMap Version 3 Release 27). Above is the location of the region on Chromosome 17 along with the location of the gene and two other Entrez genes within the region. Exons are illustrated with yellow bolcks. Below is an indication of the position of the SNPs along with a diagram of the block structure of the region. LD plots were identified by strong LD. Depth of red/pink colour indicates the computed pairwise D' value; deeper pink indicates a higher D' value. The blue blocks represent high D' but low LOD values.

Dimasi *et al.*[225] reported a significant association of the SNP rs2696297 on chromosome 17 (near *COL1A1*) with CCT. Downloading tag SNP data from the Yoruba in HapMap for the region including both the associated SNP (rs2696297) and the gene (chr17:45600000..45633999) yielded 25 tag SNPs. Seven of these SNPs received a low score in the Illumina® Assay Design Tool process and were substituted with two new SNPs. The 'literature' SNP was included in the final design that consisted of 21 tag SNPs that capture 34 of 40 alleles with a mean r^2 of 0.97.

2.1.3.7 COL1A2 (collagen type I alpha 2) (Figure 4.8)

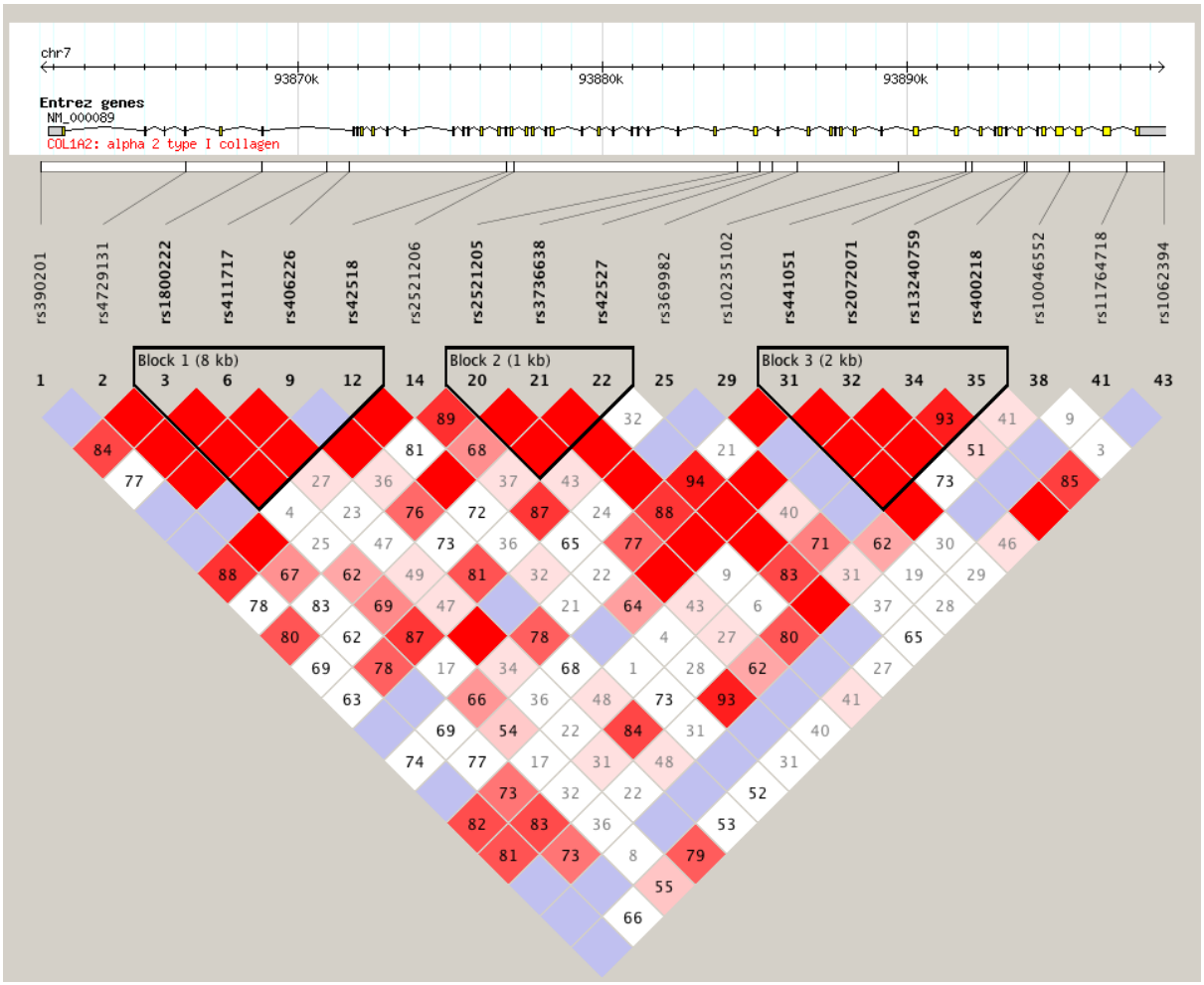

Figure 4.8 Haploview linkage disequilibrium plot of SNPs selected for genotyping from the *COL1A2* gene showing the LD patterns of the SNPs in the Yoruba from HapMap genotyping data (HapMap Version 3 Release 27). Above is the location of the region on Chromosome 7 along with the location of the gene within the region. Exons are illustrated with yellow bolcks. Below is an indication of the position of the SNPs along with a diagram of the block structure of the region. LD plots were identified by strong LD. Depth of red/pink colour indicates the computed pairwise D' value; deeper pink indicates a higher D' value. The blue blocks represent high D' but low LOD values. SNP 1(rs390201) was included in the plot to demarcate the region, but was not selected for genotyping.

COL1A2 is on chromosome 7. Downloading tag SNP data from the Yoruba in

HapMap for the region including the three SNPs forming the significant haplotype discovered by Dimasi *et al.*[225] and the gene (chr7:93861809..93898480) yielded 28 tag SNPs (which included one of the 'literature' SNPs). Two SNPs received a low score in the Illumina® Assay Design Tool process and two SNPs were too close together. Three of these SNPs were excluded and were substituted with two other SNPs so that 27 tag SNPs that capture 47 of 48 alleles with a mean r^2 of 0.982 were included in the final design. The other two 'literature' SNPs that make up the associated haplotype were also included in the final assay design.

2.1.3.8 COL5A1 (collagen type V alpha 1) (Figure 4.9)

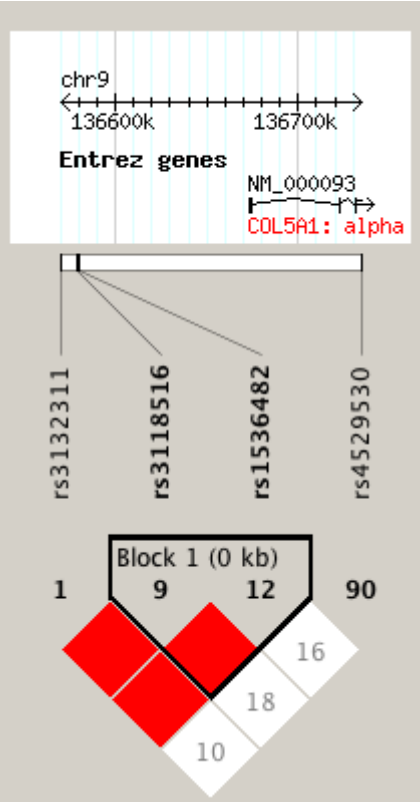

Figure 4.9 Haploview linkage disequilibrium plot of SNPs selected for genotyping from the *COL5A1* gene region showing the LD patterns of the SNPs in the Yoruba from HapMap genotyping data (HapMap Version 3 Release 27). Above is the location of the region on Chromosome 9 along with the location of the gene within the region. Below is an indication of the position of the SNPs along with a diagram of the block structure of the region. LD plots were identified by strong LD. Depth of red/pink colour indicates the computed pairwise D' value; deeper pink indicates a higher D' value. The blue blocks represent high D' but low LOD values. SNPs 1 (rs3132311) and 90 (rs4529530) were included in the plot to demarcate the region, but were not selected for genotyping.

COL5A1 is a large gene (2Mbp in size) that yielded 90 SNPs when tag SNP data were downloaded from HapMap (YRI). For this reason only SNPs that had been found to have an association with CCT were included from this gene in the assay

design.[226, 231] Five 'literature SNPs' were evaluated, one was excluded for a low score, so a total of four 'literature SNPs' were included in the final assay design.

2.1.3.9 CDKN2B (cyclin-dependent kinase inhibitor 2B) (Figure 4.10)

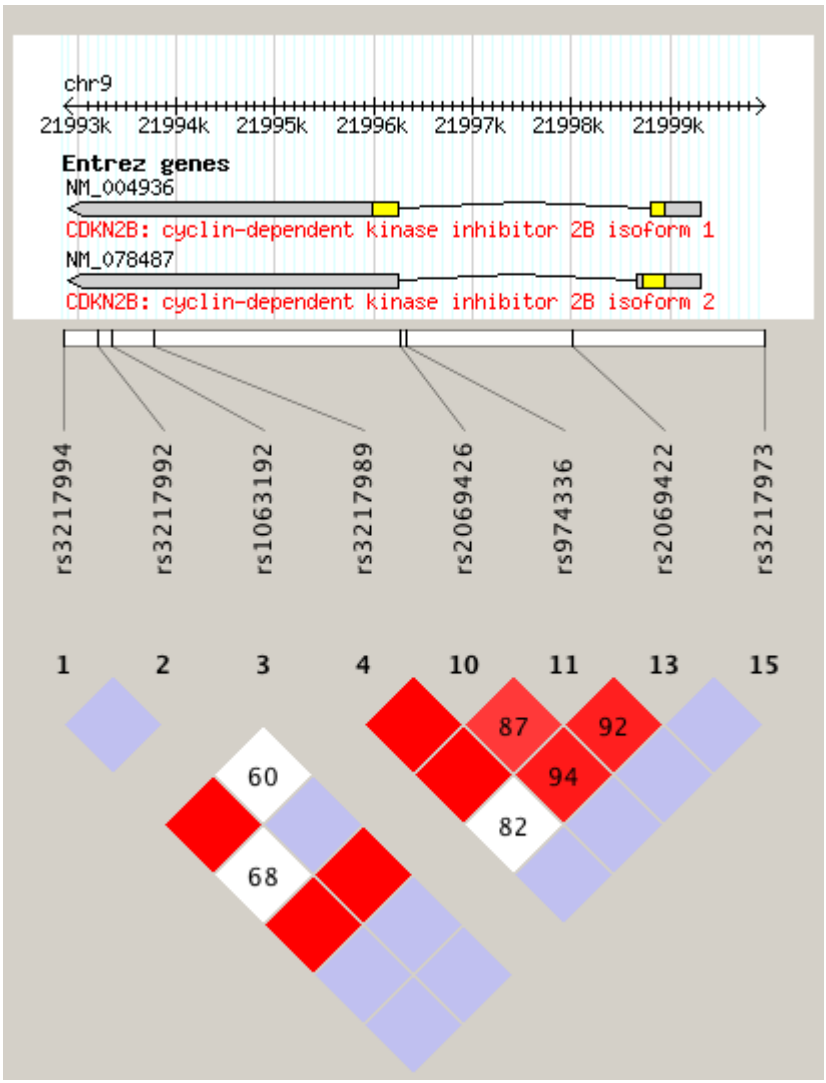

Figure 4.10 Haploview linkage disequilibrium plot of SNPs selected for genotyping from the *CDKN2B* gene showing the LD patterns of the SNPs in the Yoruba from HapMap genotyping data (HapMap Version 3 Release 27). Above is the location of the region on Chromosome 9 along with the location of the gene within the region. Exons are illustrated with yellow bolcks. Below is an indication of the position of the SNPs along with a diagram of the block structure of the region. LD plots were identified by strong LD. Depth of red/pink colour indicates the computed pairwise D' value; deeper pink indicates a higher D' value. The blue blocks represent high D' but low LOD values. SNPs 1 (rs3217994) and 15 (rs3217973) were included in the plot to demarcate the region, but were not selected for genotyping.

CDKN2B was tagged in the Yoruba in HapMap by five SNPs. The single SNP (rs1063192) that was found to be associated with VCDR in the Rotterdam Study[195] had a minor allele frequency of 0 in the Yoruba, but it was also included in the design. Running Tagger in Haploview in the Luhya produced an additional two

SNPs, one of which had a low score and was excluded. The final assay design thus included seven SNPs that, in the Yoruba, captured all alleles with an r^2 of 1.0.

2.1.3.10 ATOH7 (atonal homolog 7) (Figure 4.11)

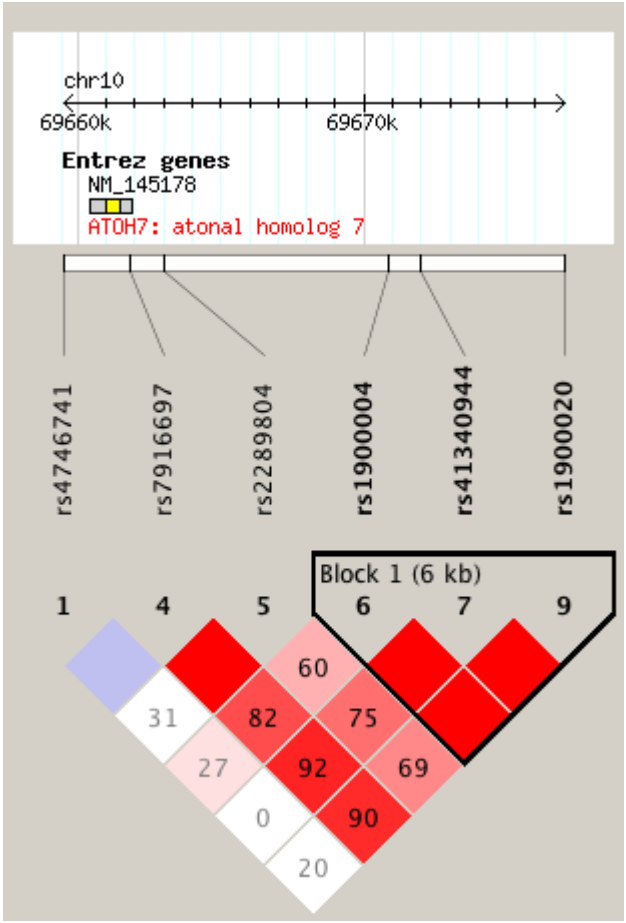

Figure 4.11 Haploview linkage disequilibrium plot of SNPs selected for genotyping from the *ATOH7* gene showing the LD patterns of the SNPs in the Yoruba from HapMap genotyping data (HapMap Version 3 Release 27). Above is the location of the region on Chromosome 10 along with the location of the gene within the region. Exons are illustrated with yellow bolcks. Below is an indication of the position of the SNPs along with a diagram of the block structure of the region. LD plots were identified by strong LD. Depth of red/pink colour indicates the computed pairwise D' value; deeper pink indicates a higher D' value. The blue blocks represent high D' but low LOD values. SNP 1 (rs4746741) was included in the plot to demarcate the region, but was not selected for genotyping.

The SNP (rs1900004) near *ATOH7* that was found to be associated with optic nerve head area in the genome-wide association data meta-analysis on the Rotterdam Study cohorts was included as a 'literature' SNP.[195] Downloading tag SNP data from the Yoruba in HapMap for the region including the 'literature SNP' (rs1900004) and *ATOH7* (chr10:69660000..69680000) yielded six tag SNPs (which included rs1900004). These SNPs captured all alleles with an r^2 of 1.0 and were all included

in the final design. There were no additional SNPs in Haploview Tagger for the Luhya or the Maasai.

2.1.3.11 SIX1 and SIX6 (sine oculis-related homeobox 1 and 6 homologs) (Figure 4.12)

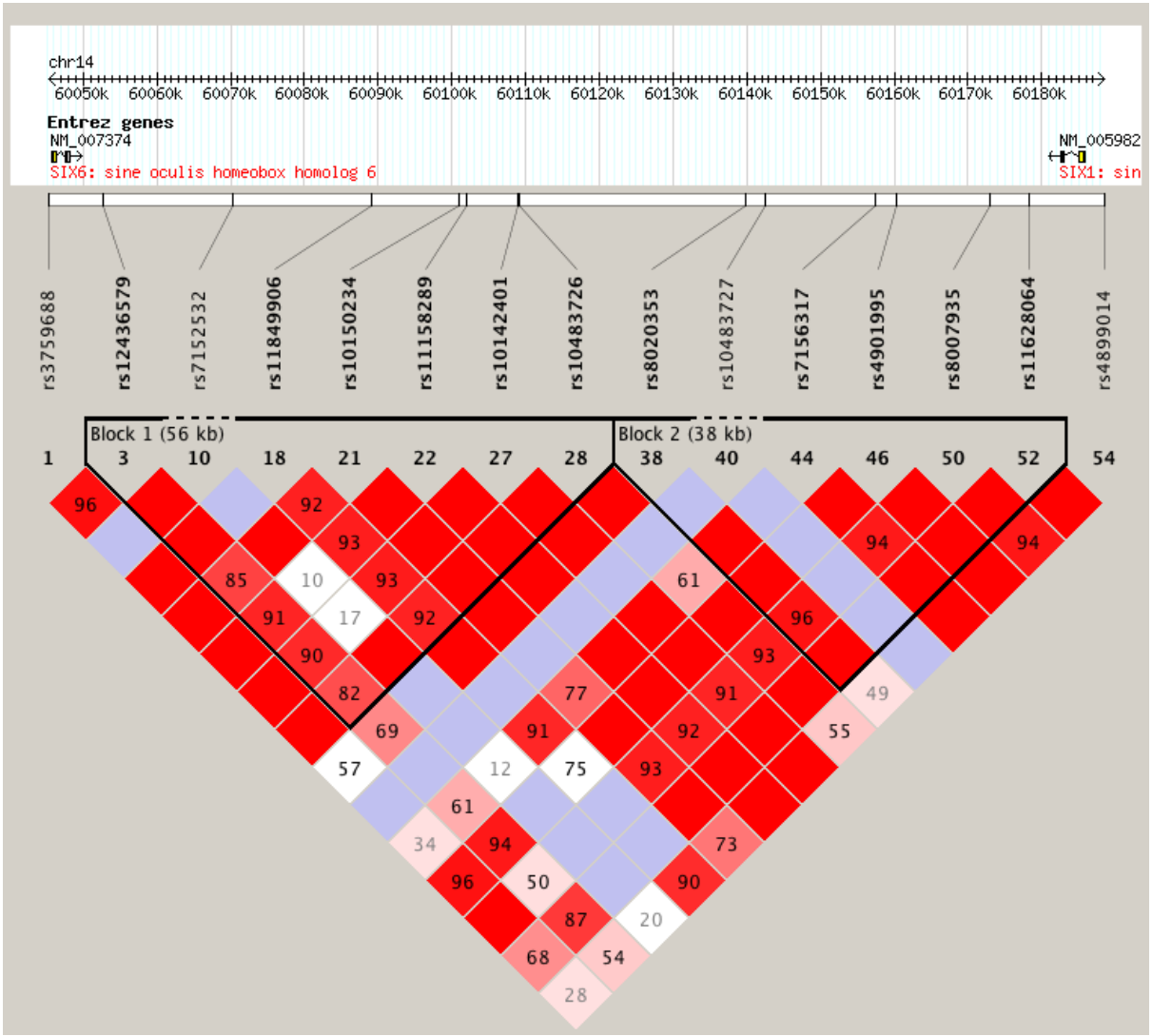

Figure 4.12 Haploview linkage disequilibrium plot of SNPs selected for genotyping from the *SIX1* and *SIX6* gene region showing the LD patterns of the SNPs in the Yoruba from HapMap genotyping data (HapMap Version 3 Release 27). Above is the location of the region on Chromosome 14 along with the location of the two genes within the region. Exons are illustrated with yellow bolcks. Below is an indication of the position of the SNPs along with a diagram of the block structure of the region. LD plots were identified by strong LD. Depth of red/pink colour indicates the computed pairwise D' value; deeper pink indicates a higher D' value. The blue blocks represent high D' but low LOD values. SNPs 1 (rs3759688) and 54 (rs4899014) were included in the plot to demarcate the region, but were not selected for genotyping.

SIX1 and *SIX6* are both small genes and the intervening intergenic region is large, but with large linkage disequilibrium blocks. The region (chr14:60045775.. 60185934) was therefore covered by fourteen tag SNPs in the Yoruba. Two of the

SNPs had low scores and were substituted for two others. The 'literature' SNP (rs10483727: associated with a bigger VCDR and a higher risk of POAG[212]) in the intergenic region of *SIX1* and *SIX6* was included in the design so that fifteen tag SNPs in fifteen tests captured 127 of 128 alleles in the Yoruba with a mean r^2 of 0.933. Also included in the final design assay were the four highest scoring additional SNPs from Haploview Tagger in the Luhya and the highest scoring additional SNP from Haploview Tagger in the Maasai.

2.1.3.12 WDR36 (WD repeat domain 36) (Figure 4.13)

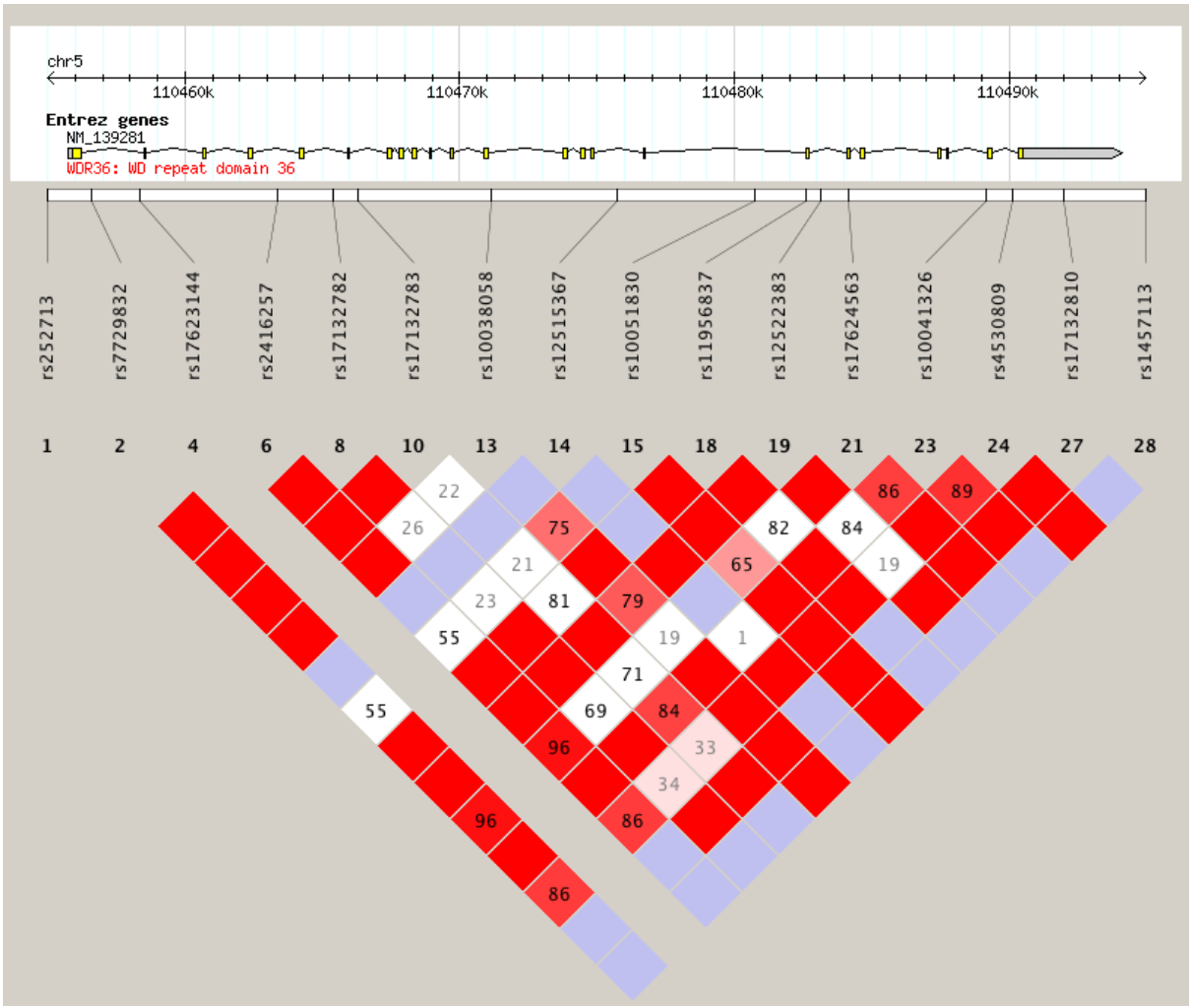

Figure 4.13 Haploview linkage disequilibrium plot of SNPs selected for genotyping from the *WDR36* gene showing the LD patterns of the SNPs in the Yoruba from HapMap genotyping data (HapMap Version 3 Release 27). Above is the location of the region on Chromosome 5 along with the location of the gene within the region. Exons are illustrated with yellow bolcks. Below is an indication of the position of the SNPs along with a diagram of the block structure of the region. LD plots were identified by strong LD. Depth of red/pink colour indicates the computed pairwise D' value; deeper pink indicates a higher D' value. The blue blocks represent high D' but low LOD values. SNP 1 (rs252713) was included in the plot to demarcate the region, but was not selected for genotyping.

WDR36 was tagged in the Yoruba in HapMap by eighteen SNPs. Three SNPs received a low score in the Illumina® Assay Design Tool process and two SNPs were too close together. Four of these SNPs were excluded and were substituted with two other SNPs so that sixteen tag SNPs that capture 45 of 49 alleles with a mean r^2 of 0.977 were included in the final design. Running Tagger in Haploview in the Luhya produced an additional nine SNPs, four of which had low scores and were excluded. Running Tagger in Haploview in the Maasai produced an additional two SNPs. The final assay design thus included 23 SNPs.

2.1.3.13 The locus near ZNF469 (zinc finger protein 469) (Figure 4.14)

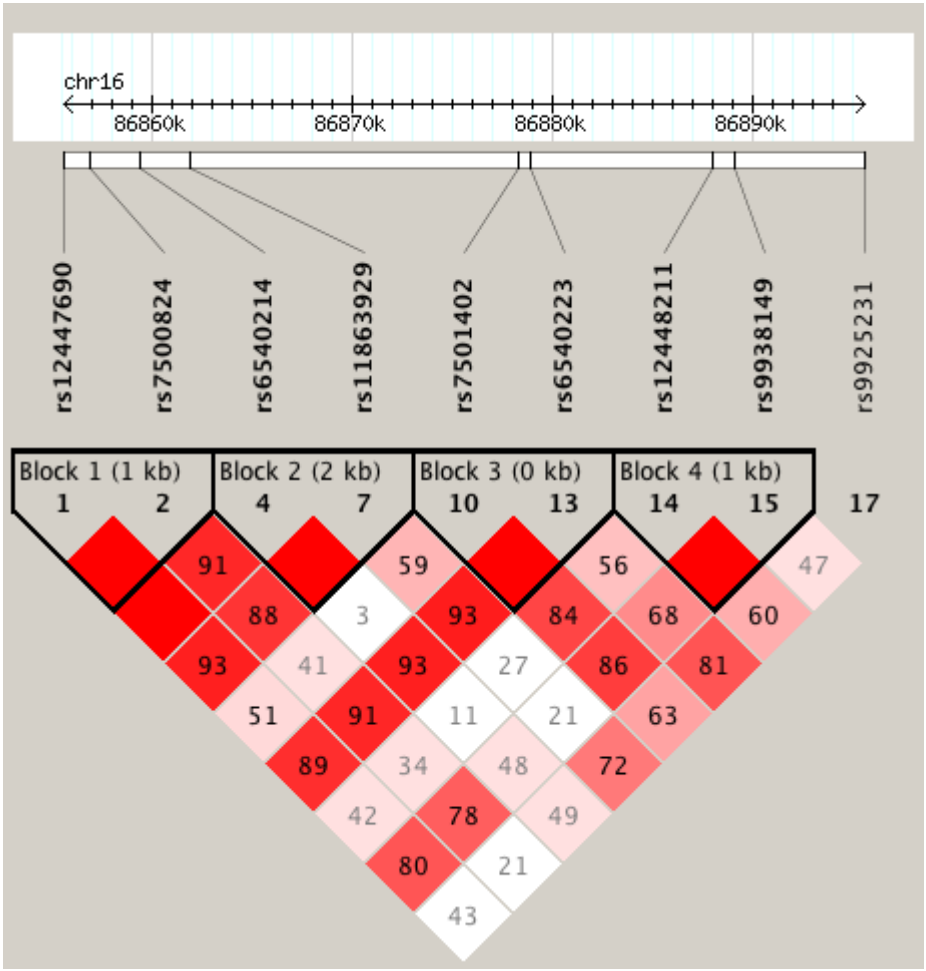

Figure 4.14 Haploview linkage disequilibrium plot of SNPs selected for genotyping from the *ZNF469* gene region showing the LD patterns of the SNPs in the Yoruba from HapMap genotyping data (HapMap Version 3 Release 27). Above is the location of the region on Chromosome 16. Below is an indication of the position of the SNPs along with a diagram of the block structure of the region. LD plots were identified by strong LD. Depth of red/pink colour indicates the computed pairwise D' value; deeper pink indicates a higher D' value.

Ten 'literature' SNPs were selected from the three genome-wide association studies implicating this region for an association with CCT.[226, 227, 231] Downloading tag SNP data in the Yoruba from the gene alone (chr16:88493879.. 88507165) from HapMap tagged four SNPs. Three of these scored poorly in the Illumina® Assay Design Tool process, so only the remaining SNP (rs11644804) was included in the assay and only 1 of 5 alleles in the gene was captured. Downloading tag SNP data in the Yoruba from the 50kbp region around the 'literature SNPs' (chr16:86855000.. 86900000) from HapMap tagged ten SNPs, which included six of the 'literature' SNPs. These were sent for scoring along with the remaining four 'literature' SNPs. Six of these fourteen SNPs (four of which were 'literature' SNPs) scored poorly in the Illumina® Assay Design Tool process and were substituted with one other. One additional SNP was added when the region was interrogated with data from the Luhya using Haploview Tagger. The assay design for the region of the 'literature' SNPs therefore consisted of six 'literature' SNPs, an additional three tag SNPs in the Yoruba and one in the Luhya. These ten SNPs capture 14 of 18 alleles with a mean r^2 of 0.979.

The final assay design for this region consisted of eleven SNPs: the single tag SNP from the gene as well as the ten SNPs comprising of 'literature SNPs' and tag SNPs from the region surrounding the 'literature SNPs.

2.1.3.14 Ancestry informative markers

Lao *et al*.[287] identified ten SNPs that were informative of geographic population structure and genetic ancestry. The ten SNPs were included in the assay design together with eight SNPs that discriminate among southern African populations and that were identified locally (unpublished data).[288] These eighteen SNP markers were therefore considered informative of ancestry and were included in the assay in addition to the candidate region SNPs for the population substructure analysis.

The candidate genes or regions, their chromosomal location and the number of SNPs genotyped within the regions that were included in the final Illumina order for the 192 SNP VeraCode GoldenGate Genotyping Assay Kit are summarised in Table 1. Appendix 7 (Table A7.1) contains the complete list of SNPs that was included in the assay together with the alleles at each SNP and their chromosomal location.

Table 4.1 Candidate genes or regions, their chromosomal location and the number of SNPs genotyped within the regions in this study

Genes / regions	Location	Literature SNPs	Tag SNPs	**Assay SNPs**
MYOC	chr1:169871181..169888396	11	8	**18**
	Potentially pathogenic mutations	3	N/A	**3**
Chr2p16	chr2:51000000..52000000	7	0	**7**
CYP1B1	chr2:38148250..38156796	2	7	**7**
CAV1/CAV 2	chr7:115926680..115988466	1	14	**15**
COL8A2	chr1:36333433..36338437	1	3	**3**
COL1A2	chr7:93861809..93902000	3	27	**29**
COL1A1	chr17:45600000..45633999	1	21	**21**
COL5A1	chr9:136673473..136876507	4	0	**4**
CDKN2B	chr9:21992906..21999312	1	6	**7**
ATOH7	chr10:69660000..69680000	1	6	**6**
SIX1/SIX6	chr14:60045775..60185934	1	19	**20**
WDR36	chr5:110455769..110494099	0	23	**23**
ZNF469	chr16:86855000..86900000	6	8	**11**
AIMS				**18**
TOTAL				**192**

AIMS, SNPs informative of ancestry

Literature SNPs, SNPs derived from the literature review; Tag SNPs, SNPs derived from the HapMap Multimarker Tagger protocol and HapMap data in the Yoruba, the Luhya and the Webuye; Assay SNPs, SNPs included in the genotyping assay

2.1.4 Genotyping with the Illumina® GoldenGate Assay and the BeadXpress Reader

2.1.4.1 Illumina® GoldenGate Assay

This assay targets specific SNPs in genomic DNA samples. The genotyping application is based on sequence-specific extension and ligation of correctly hybridized query oligonucleotides, which are distinguished by their shared primer landing sites.[289] The approach is illustrated in Figure 4.15.[289]

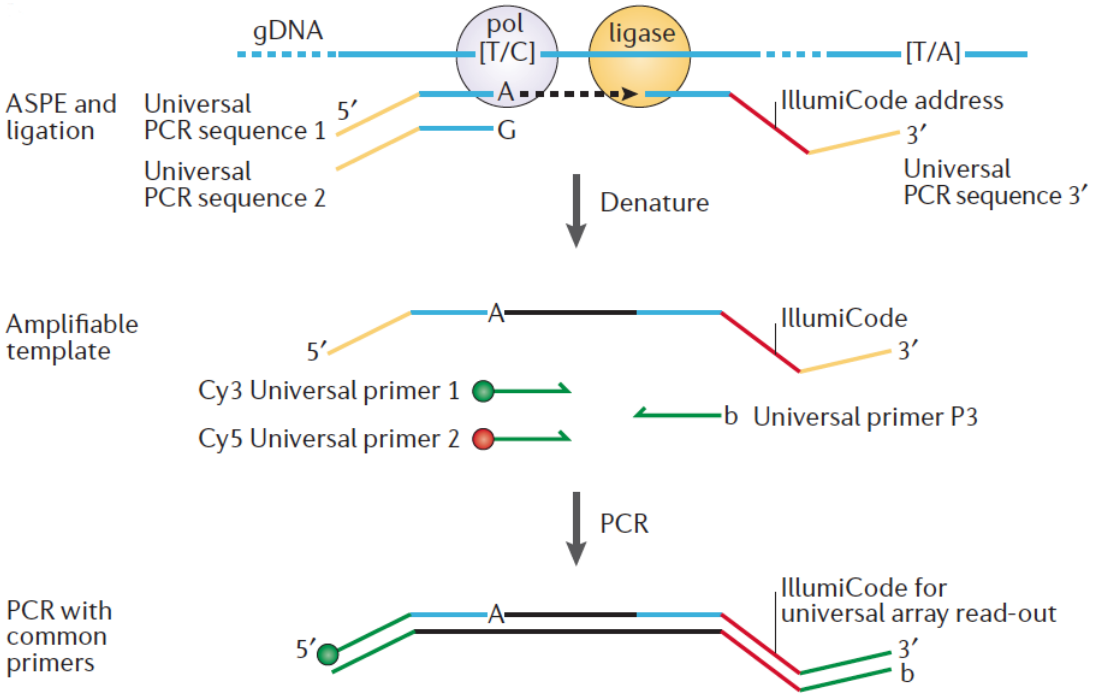

Figure 4.15 Goldengate assay genotyping.This uses extension ligation between annealed locus-specific oligonucleotides (LSOs) and allele-specific oligonucleotides (ASOs). An allele-specific primer-extension (ASPE) step is used to preferentially extend the correcty matched ASO (at the 3' end) up to the 5' end of the LSO primer. Ligation then closes the nick. A subsequent PCR amplification step is used to amplify the appropriate product using common primers to 'built-in' universal PCR sites in the ASO and LSO sequences. The resultant products are hybridised and read out on an array of universal-capture probes (complimentary to IllumiCodes).[Fan JB, Chee MS, Gunderson KL. Highly parallel genomic assays. Nature reviews Genetics 2006;7:632-644.][287]

In the GoldenGate Assay, the DNA samples from each participant were first activated through a chemical reaction with biotin. The biotinylated DNA was then purified from excess biotin. Assay oligonucleotides (two allele-specific oligonucleotides and one locus-specific oligonucleotide for each of the 192 selected SNPs) were added and hybridized to the DNA, and the mixture was bound to streptavidin-conjugated paramagnetic particles. This step immobilised the genomic DNA on a solid support and enabled stringent washing that removed excess and incorrectly hybridised probes. Thereafter allele-specific extension and ligation of the hybridized oligonucleotides was performed. The extended and ligated products formed a synthetic template that was transferred to a universal polymerase chain reaction (PCR) with fluorescently labelled primers, and amplified. The strand containing the fluorescent signal in the PCR products was isolated and hybridized to VeraCode beads. VeraCode beads are rod-shaped glass microbeads (measuring ~240 x 28 μm) that are digitally inscribed with novel barcodes, with their surface

available for the attachment of an oligonucleotide complementary to the specified address sequence. After the hybridization, the VeraCode beads were washed and scanned on the BeadXpress Reader.

Appendix 8 contains the detailed procedure for the Illumina® GoldenGate Assay.

2.1.4.2 BeadXpress Reader laser scanning

VeraCode beads were scanned by the high-throughput, two-colour BeadXpress Reader and the genotype data was captured electronically.

The BeadXpress Reader uses lasers to excite the Cy3 and Cy5 fluors of the single-stranded PCR products bound to the VeraCode beads. Light emissions from these fluors are then recorded in a data file. Fluorescence data are analysed to derive genotyping results using Illumina® BeadStudio software.

2.1.4.3 BeadStudio analysis

The BeadStudio software package was used to cluster the fluorescence data and generate genotypes for each sample and each SNP. The software was used for quality control on both the samples and the SNPs.
Samples were excluded if there was evidence of:

- Contamination
- Call rates less than 0.95
- Problems with allele specific extension
- Problems with PCR uniformity
- Problems with hybridisation
- Inconsistency of gender estimation (from the genotyping) and known gender

SNPs were excluded if they were not in Hardy-Weinberg equilibrium in the control group ($p < 0.01$) or if there was:

- Low call frequency
- Poor cluster separation

- Low 50% GC rate (GC is an estimation of the confidence of the genotype call for any given sample with a value between 0 and 1 and the 50% GC rate is the median GC for all samples at one SNP)
- Questionable clustering

2.1.5 Statistics

2.1.5.1 Sample size

The study was designed to detect moderate effects.

A sample size of 472 individuals was initially selected for investigation because that corresponds to five plates of 96 DNA samples each with two control samples repeated on each plate. An assumption was made that up to 10% of the samples would fail genotyping, so a total sample size of 425 was assumed with approximately equal proportions of POAG and control participants.

2.1.5.2 Power calculations for POAG association studies

Power calculations were performed using Quanto Version 1.2.[290, 291] A 'log-additive' mode of inheritance was assumed. The odds ratio that could be detected 80% of the time with 95% confidence in an unmatched case-control study of genetic association in POAG in South Africa (assuming a disease prevalence of 2.8%) in 213 cases and 213 controls was calculated for population disease allele frequencies ranging from 0.01 to 0.99. Figure 4.16 is a graphical representation of the calculated values. The shaded regions in Figure 4.16 are the odds ratios that this study is powered to detect. This study was adequately powered to detect odds ratios of 0.6 or less and 1.5 or more when the frequencies of the disease alleles in the population were between 0.2 and 0.7. Where the population disease allele frequencies were greater or less than this, this sample size was only powered to detect more significant odds ratios. The study was not powered to detect small effects with odds ratios between 0.6 and 1.5.

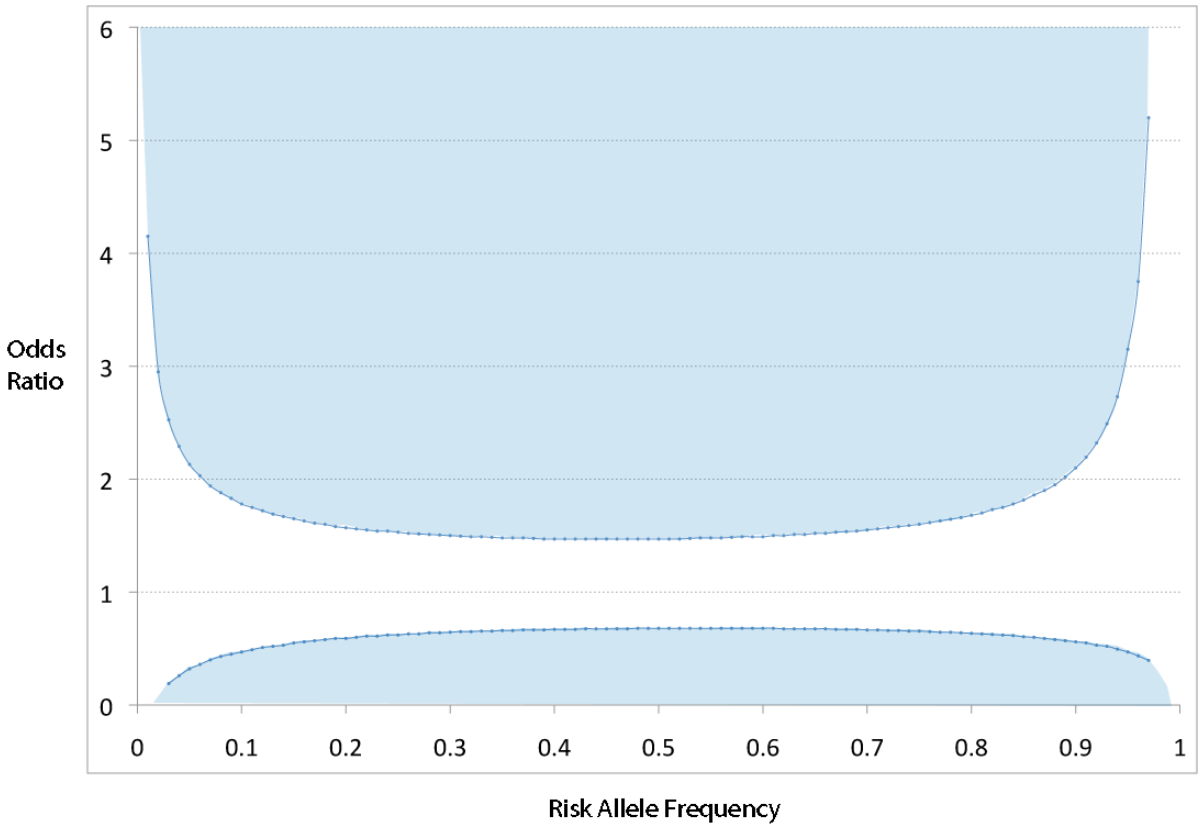

Figure 4.16 Power calculations for POAG association. The odds ratio (y-axis) that can be detected 80% of the time with 95% confidence in an unmatched case-control study of genetic association in POAG in South Africa (assuming a disease prevalence of 2.8%) in 213 cases and 213 controls for population disease allele frequencies (x-axis) ranging from 0.01 to 0.99 calculated using Quanto Version 1.2. A 'log-additive' mode of inheritance was assumed. The shaded area represents the odds ratios that this study is adequately powered to detect for a given risk allele frequency.

The study was not designed to replicate published reports. However it was possible to estimate the power of this study to replicate literature reports in the few SNPs included from the literature that had the relevant information. The effect size of POAG associations with individual SNPs reported in the literature (at the time of SNP selection) is documented in Table 2 along with an estimation of the power of the sample size in this study to detect the association. The data mirror the calculations represented in Figure 4.16. For example the effect of association with SNPs in the chromosome 2p16 region is large and this sample size is adequately powered to detect the association. In contrast, the effect of SNP association with *CYP1B1* reported by Burdon et al.[113] is very small and this sample size is underpowered to detect the association. The minor allele frequencies of the SNPs in the Yoruba from HapMap data have been included in the table. Where the minor allele frequency in the Yoruba differs significantly from that in the original population investigated, the power of a given sample size to detect the association is affected, either positively as in SNP rs4236601 in the *CAV1/CAV2* region or negatively for the

SNPs in *CDKN2B* and *SIX1/SIX6*.

Table 4.2 The strength of POAG associations reported in the literature and the power of this study to detect those associations

Gene	SNP	Reference	MAF (POAG)	MAF (Control)	OR	Power	MAF (YRI)	Power (YRI)
C2p16	rs1533428	Jiao *et al.*[175]	0.58	0.32	4.2	100	0.35	100
	rs12994401	Jiao *et al.*[175]	0.41	0.08	6.2	100	0.02	100
CYP1B1	rs162562	Burdon *et al.*[113]	0.20	0.23	1.1	8	0.25	8
	rs10916	Burdon *et al.*[113]	0.21	0.23	1.2	13	0.37	17
CAV1&2	rs4236601	Thorleifsson *et al.*[177]	0.23	0.29	1.4	57	0.45	72
CDKN2B	rs1063192	Fan *et al.*[212]	0.34	0.42	0.7	55	0.01	8
SIX1/6	rs10483727	Fan *et al.*[212]	0.47	0.40	1.3	55	0.02	9

MAF, minor allele frequency; Power, the power (in percent) to detect the association with 95% confidence in 213 cases and 213 controls; MAF (YRI), HapMap data on minor allele frequency in the Yoruba; Power (YRI), the power (in percent) to detect the association with 95% confidence in 213 cases and 213 controls assuming the minor allele frequency found in the Yoruba

2.1.5.3 Power calculations for CCT association studies

The genetic effect (change in mean CCT per allele) that could be detected 80% of the time with 95% confidence in 425 individuals was calculated for population risk allele frequencies ranging from 0.01 to 0.99 using Quanto Version 1.2.[291, 292] A 'log-additive' mode of inheritance was assumed. A population mean of 510μm with a standard deviation of 37μm was used in the calculations. Figure 4.17 represents the calculated values. The graph demonstrates that this sample size was adequately powered to detect genetic effects of greater than 8μm in CCT when the frequencies of the risk alleles in the population were between 0.2 and 0.8. Where the population risk allele frequencies were greater or less than this, this sample size was only powered to detect more significant genetic effects.

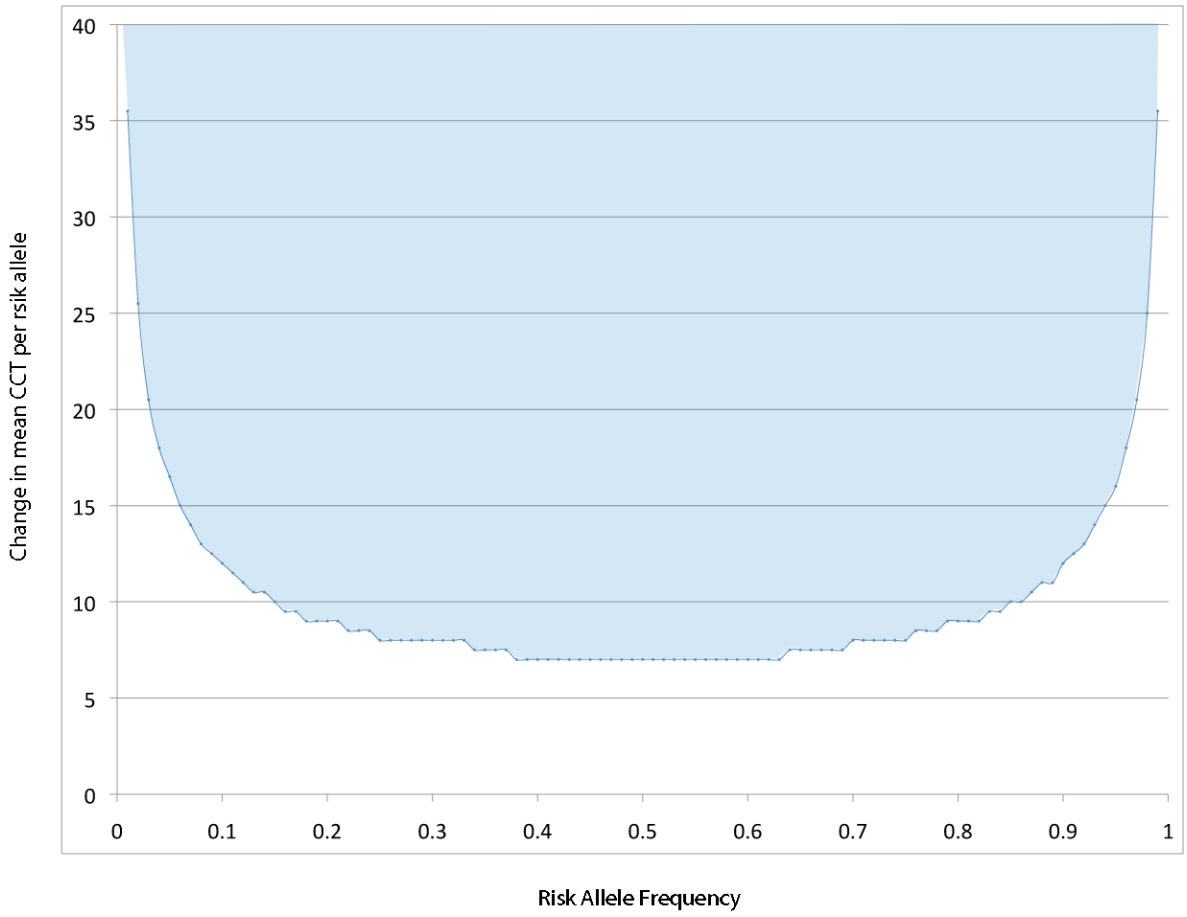

Figure 4.17 Power calculations for CCT association. The change in mean CCT in µm (y-axis) per allele that can be detected 80% of the time with 95% confidence in 425 individuals in South Africa for population risk allele frequencies (x-axis) ranging from 0.01 to 0.99 calculated using Quanto Version 1.2. A 'log-additive' mode of inheritance and a population mean for CCT of 510µm with a standard deviation of 37µm was assumed. The shaded area represents the changes in mean CCT per risk allele that this study is adequately powered to detect for a given risk allele frequency.

2.1.5.4 Power calculations for VCDR association studies

The genetic effect (change in mean VCDR per allele) that could be detected 80% of the time with 95% confidence in 425 individuals was calculated for population risk allele frequencies ranging from 0.01 to 0.99 using Quanto Version 1.2.[291, 292]A 'log-additive' mode of inheritance was assumed. A population mean of 0.4 with a standard deviation of 0.2 was used in the calculations. Figure 4.18 represents the calculated values. The graph demonstrates that this sample size was adequately powered to detect genetic effects of greater than 0.05 in VCDR when the frequencies of the risk alleles in the population were between 0.2 and 0.8. Where the population risk allele frequencies were greater or less than this, this sample size was only powered to detect more significant genetic effects.

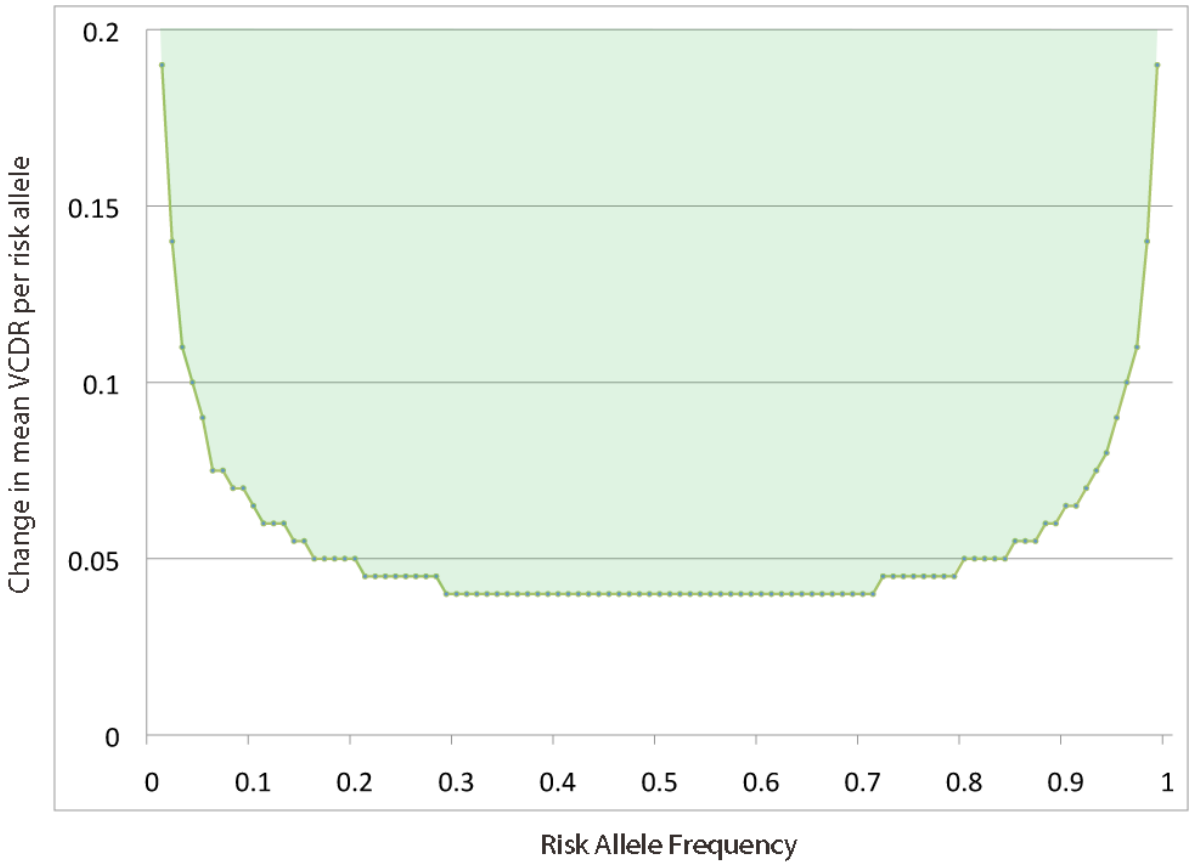

Figure 4.18 Power calculations for VCDR association. The change in mean VCDR (y-axis) that can be detected 80% of the time with 95% confidence in 425 individuals in South Africa for population risk allele frequencies (x-axis) ranging from 0.01 to 0.99 calculated using Quanto Version 1.2. A 'log-additive' mode of inheritance and a population mean for VCDR of 0.4 with a standard deviation of 0.2 was assumed. The shaded area represents the changes in mean VCDR per risk allele that this study is adequately powered to detect for a given risk allele frequency.

Genetic effects of greater than 0.05 in VCDR are moderate effects. Published VCDR association studies (at the time of SNP selection) suggest that this level would be unlikely to replicate previously identified associations that account for only 2.2% of the variation in VCDR (Table 4.3).[195] However the regions are good candidate regions for an association with POAG and were included in the study for that reason.

Table 4.3 The genetic effect of VCDR associations reported in the literature

Gene	SNP	Reference	MAF	Genetic effect
CDKN2B	rs1063192	Ramdas *et al.*[195]	0.46	0.013
SIX1	rs10483727	Ramdas *et al.*[195]	0.41	0.012

MAF, minor allele frequency; Genetic effect, change in mean VCDR per allele

2.1.5.5 Power calculations for IOP association studies

The genetic effect (change in mean IOP per allele) that could be detected 80% of the

time with 95% confidence in 425 individuals was calculated for population risk allele frequencies ranging from 0.01 to 0.99 using Quanto Version 1.2.[291, 292]A 'log-additive' mode of inheritance was assumed. A population mean of 13mmHg with a standard deviation of 5mmHg was used in the calculations. Figure 4.19 represents the calculated values. The graph demonstrates that this sample size is adequately powered to detect genetic effects of greater than 1mmHg in IOP when the frequencies of the risk alleles in the population are between 0.2 and 0.8. Where the population risk allele frequencies are greater or less than this, this sample size is only powered to detect more significant genetic effects. *WDR36* was the only gene that was evaluated for an association with IOP. There were only tag SNPs and no 'literature' SNPs evaluated in this gene.

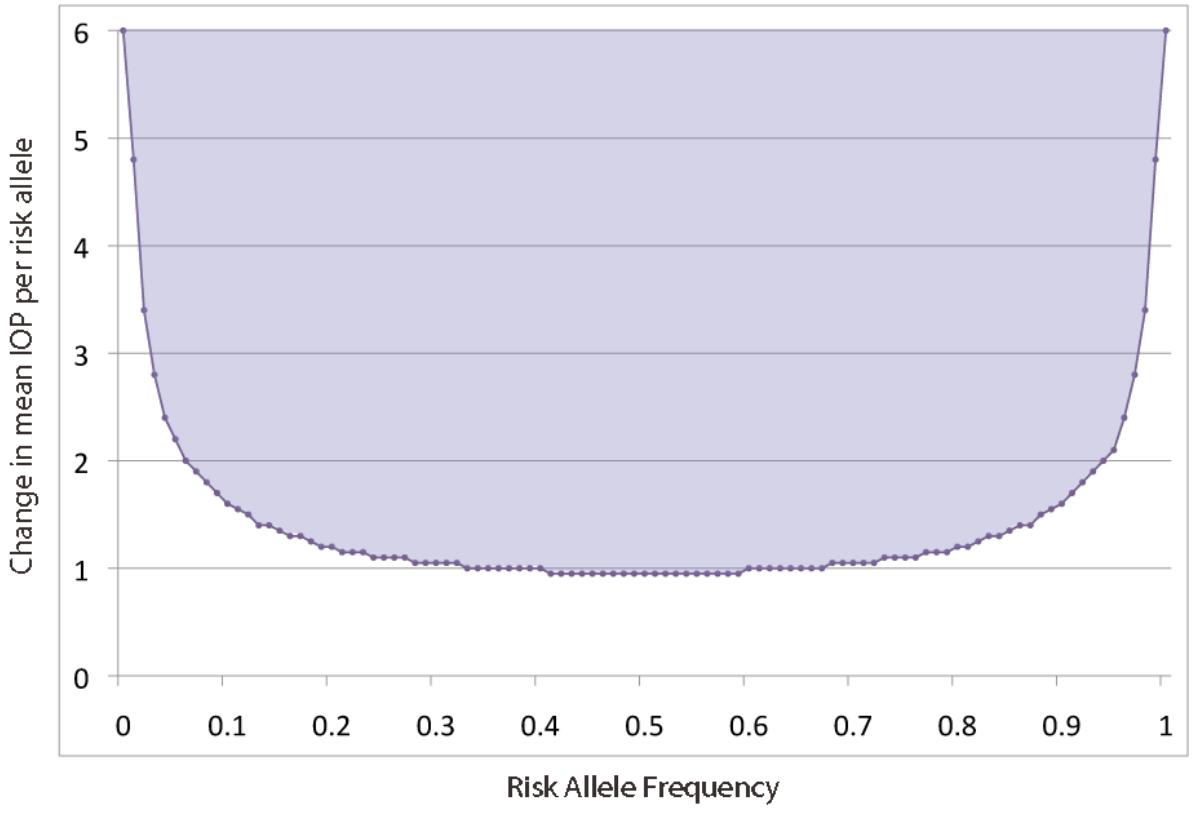

Figure 4.19 Power calculations for IOP association.The change in mean IOP in mmHg (y-axis) that can be detected 80% of the time with 95% confidence in 425 individuals in South Africa for population risk allele frequencies (x-axis) ranging from 0.01 to 0.99 calculated using Quanto Version 1.2. A 'log-additive' mode of inheritance and a population mean for IOP of 13mmHg with a standard deviation of 5mmHg was assumed. The shaded area represents the changes in mean IOP per risk allele that this study is adequately powered to detect for a given risk allele frequency.

2.1.5.6 Descriptive statistical analyses

Descriptive analyses were performed using STATA version 12 as described in Chapter 2.2.3.

2.1.5.7 Association analyses

SNP and haplotype association analyses were performed using PLINK (v1.07) and Haploview (v4.2).[284, 293]The minimum genotyping rate for SNPs and individuals to be included in the statistical analyses was set to be 90%.

Hardy-Weinberg equilibrium was assessed by using the χ^2 test. SNPs that were not in Hardy-Weinberg equilibrium ($p < 0.01$) in the group of controls were excluded from further analysis.

The minor allele frequencies of each SNP between patients with POAG and control participants were compared by using the χ^2 test or Fisher's exact test. Odds ratios and confidence intervals were calculated using logistic regression.

The same approach was used in the association analyses for diabetes. The minor allele frequencies of each SNP between diabetic and non-diabetic participants were compared by using the χ^2 test or Fisher's exact test. Odds ratios and confidence intervals were calculated using logistic regression.

Permuted associations were tested with random label swapping of cases and controls and repeating the association analysis 100 000 times to see if the observed test statistic was similar to the permuted test statistic. The permuted association generated a p value that was a pointwise estimate of an individual SNP's significance ((R+1)/(N+1) where R was the number of times the permuted test was greater than the observed test and N was the number of permutations (100 000)).[293]

Where associations were discovered with individual SNPs, the linkage disequilibrium patterns of those regions were explored and haplotype blocks were defined from the algorithm from Gabriel *et al.*[294] Haplotype associations with POAG were tested using

these haplotype block definitions and the χ^2 test.

Associations of the SNPs in *COL1A1, COL1A2, COL5A1, COL8A2* and *ZNF469* with CCT; the SNPs in *CDKN2B, SIX1* and *SIX6*, and *ATOH7* with VCDR; and the SNPs in *WDR36* and *CAV1/CAV2* with IOP were evaluated by using linear regression.

Regional association results were plotted using LocusZoom v1.1.[295]

2.1.5.8 Adjusting associations for covariates

Covariates that were potential confounders were included in statistical models.

Gender: Gender could potentially be correlated with the variants, but not mediate their effects. This was therefore included as a covariate in regression modelling in all of the analyses.

Age: Age is an unlikely confounder assumed to be independent of the variants. However, it was included in the quantitative trait analyses because it is likely to be beneficial in reducing noise and increasing the power to detect associations by explaining some of the variability in the outcome.[296] Age was similarly included in the diabetic association analysis because in this population diabetes is associated with age (Chapter 2), but the sample ascertainment (pertaining to diabetes) was random, so age and the variants remained independent. The same is not true for the POAG association analyses. Age is correlated with POAG in the study population (Chapter 2), but the correlation was induced by a selection bias. Pirinen *et al.*[297] have shown that in the presence of induced correlation omitting the covariate from a logistic regression model can increase the power particularly when the covariate is a strong risk factor and the disease prevalence is low as is the case with age and POAG in this study. Regression models both including and omitting age were employed for the POAG association analyses.

POAG diagnosis (POAG or control): Diabetes mellitus, CCT, IOP and VCDR are all risk factors for POAG, making POAG diagnosis a confounder in association analyses of these traits. POAG diagnosis (POAG or control) was therefore included as a

covariate in the regression models for diabetes, CCT, VCDR and IOP association analyses.

2.1.5.9 Correcting for multiple comparisons

In the association analyses, multiple comparisons of SNPs covering all investigated genes were made. Corrections for multiple testing were performed using several methods.

The Bonferroni, Holm and Šidák procedures were used to control for the familywise error rate (FWER).

The procedures by Benjamini and Hochberg, and Benjamini and Yekutieli controlled for the false discovery rate (FDR).

Maximum permuted association testing generated a p-value that controlled for the fact that SNPs were tested simultaneously during permuted association. It compared the observed test statistic with the maximum of all permuted statistics for each replicate.

Some of the SNPs are in linkage disequilibrium with each other, so these standard methods of correction for multiple testing are overly conservative, because the tests are not completely independent. Correction of significant p values for multiple testing was therefore also undertaken using the online SNPSpD (SNP spectral decomposition) interface (http://genepi.qimr.edu.au/general/daleN/SNPSpD/).[298, 299] The correction method on this interface relies on SNP and genotyping data from the user to determine linkage disequilibrium between SNPs. The linkage disequilibrium is used to generate an effective number of independent tests that can then be used to adjust the correlated tests as if they were independent.[298]

2.1.5.10 Structure analysis

This was performed using EIGENSOFT (v 4.2).[300] A principal components analysis on the data was used to identify structure and correlation between cases/controls

and eigenvectors of the structure. Comparisons of this dataset with other African and European datasets were performed to detect admixture and were plotted using R (v 2.15.2).[301]

2.2 Results

2.2.1 Study population

The original study population consisted of 502 individuals (see Chapter 2.3). Five plates of 96 DNA samples each were genotyped using the BeadXpress genotyping platform. There were two 'control' samples repeated on each plate therefore genotyping was performed on 472 unique DNA samples. The samples in each of the POAG (247 individuals) and control (255 individuals) groups with the best quality and quantity of DNA were selected for the genotyping.

Quality control on the genotyping revealed that in 35 samples there were call rates of less than 0.95 and these samples were excluded from the analysis. One additional sample was excluded for failing first hybridisation and seven samples were excluded where there was inconsistency of gender estimation (from the genotyping) and known gender. SNP genotyping was successful in 429 participants (215 POAG patients and 214 controls).

The demographic features and clinical findings in this subgroup of the study subjects are detailed in Appendix 9 in Tables A9.1 and A9.2. Appendix 9 also contains Figures A9.1 to A9.3 that compare the demographics and clinical findings of the POAG and control participants in the entire study population and in the POAG and control participants in whom genotyping was successful. The subgroup of 429 participants in whom genotyping was successful was representative of the entire study population of 502 individuals that was analysed in Chapter 2.3 in demographics, in tribal identity and in clinical findings. The conclusions in Chapter 2.4 on the whole study population may therefore be extrapolated to this subgroup.

2.2.2 Structure Analysis

A principal component analysis of 137 of the genotyped SNPs (including the 18 SNPs informative of ancestry) that were common to this and HapMap African (YRI, LWK) and European (CEU) and other African (Khomani Bushmen, Hadza and

Sandawe)[302] datasets showed little structure in the study group and no difference between POAG or control status and eigenvectors of the structure. Combining the data with these other African and European data sets revealed a homogeneous population distinct from the other populations with no evidence of admixture (Figure 4.20).

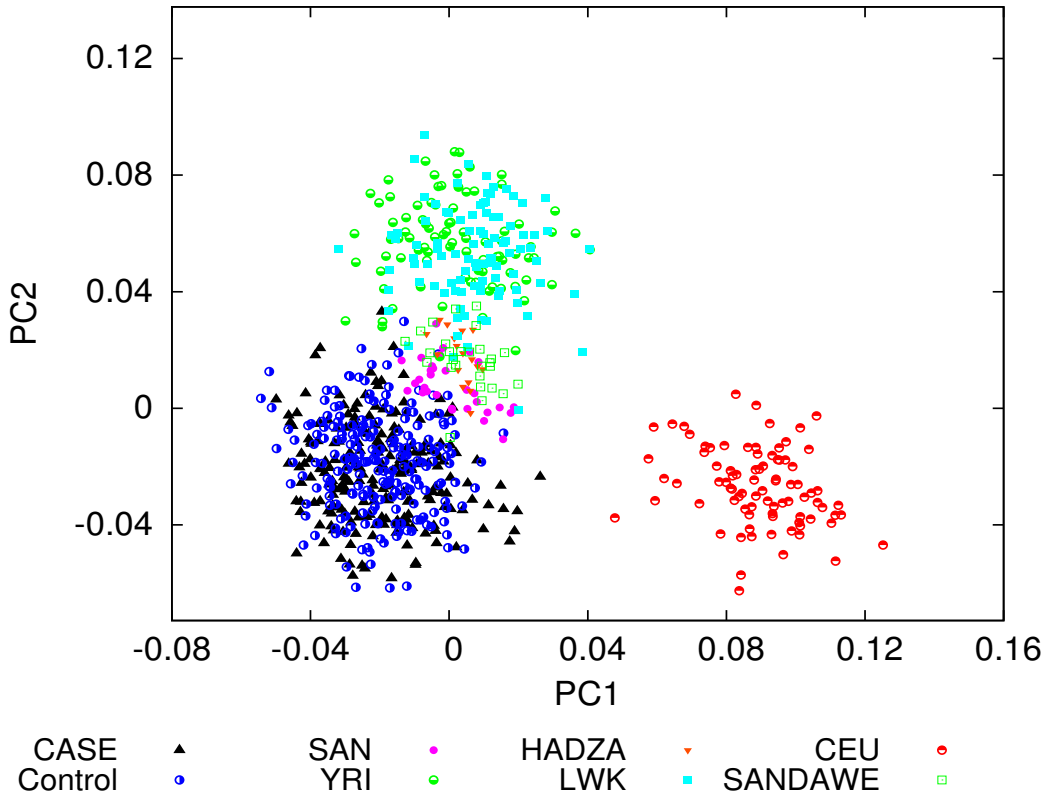

Figure 4.20 Structure analysis. Structure analysis of 137 SNPs in common in this data (CASE, POAG; Control, Controls) and in other African populations (SAN, San from Southern Africa; HADZA, Hadzabe from Tanzania; YRI, Yoruba from Nigeria; LWK, Luhya from Kenya; SANDAWE, Sandawe from Tanzania) and a European population (CEU, Utah residents with European ancestry).

2.2.3 SNP quality control

One hundred and seventy-one SNPs were selected to tag the common genetic variation in *MYOC, CDKN2B, CAV1/2, CYP1B1, WDR36, COL1A1, COL1A2, COL5A1, COL8A2, ZNF469, SIX1/6, ATOH7* and the chromosome 2p16 locus.

Twelve SNPs either failed quality control testing using BeadStudio software or had genotype distributions in the control group that were not consistent with Hardy-Weinberg equilibrium ($p < 0.01$). These twelve SNPs were excluded from further

analysis. Table 4.4 contains the details of the SNPs, their gene or region and their reason for exclusion. The remaining panel of 159 SNPs was genotyped in this case-control study of 215 patients with POAG and 214 healthy controls.

Table 4.4 SNPs failing quality control and excluded from analysis

SNP	Gene /Region	Low call frequency	Poor cluster separation	HWE (controls)	Low 50% GC	Questionable clustering
rs4236601	*CAV1/2*	x		x		x
rs2069426	*CDKN2B*	x				x
rs162549	*CYP1B1*		x		x	x
rs8010936	*SIX1/6*	x	x	x	x	x
rs11863929	*ZNF7*					x
rs6540214	*ZNF9*		x		x	x
rs17132782	*WDR36*		x	x	x	x
rs2075554	*COL1A1*	x		x		x
rs16948767	*COL1A1*		x	x	x	
rs6466583	*CAV1/2*			x		
rs2586481	*COL1A1*			x		
rs10916	*CYP1B1*			x		

HWE (controls), SNP genotype distribution not consistent with Hardy-Weinberg equilibrium in the control group; GC, an estimation of the confidence of the genotype call for any given sample with a value between 0 and 1; 50% GC, the median GC for all samples at one SNP

2.2.4 POAG association analysis

2.2.4.1 Minor allele frequencies

Allele frequencies in the POAG and control groups for each SNP are provided in Appendix 10 (Table A10.1). For the SNPs that were included because they were ‘literature SNPs’, the minor allele frequency in the control group was compared to the minor allele frequency reported in the literature for each SNP. The results of this comparison are summarised in Figure 4.21.

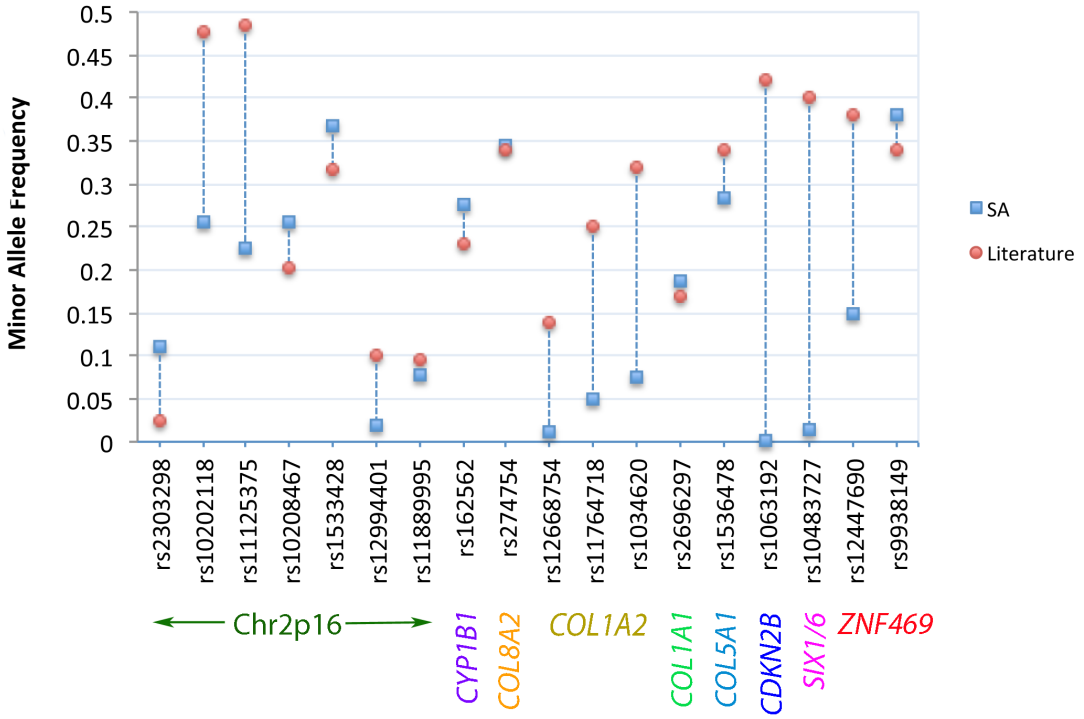

Figure 4.21 Minor allele frequency comparison. Minor allele frequency comparison in the study population controls (SA) and as cited in the literature (Literature). The genomic regions are listed below the SNPs. Chr2p16, the chromosome 2p16 genomic region (Jiao *et al.*[175]); *CYP1B1* (Burdon *et al.*[113]); *COL8A2* (Desronvil *et al.*[229]); *COL1A1* and *COL1A2* (Dimasi *et al.*[225]); *COL5A1* (Vitart *et al.*[227]); *CDKN2B* (Fan *et al.*[212]); *SIX1/6* (Fan *et al.*[212]); *ZNF469* rs12447690 (Vitart *et al.*[227]); *ZNF469* rs9938149 (Vithana *et al.*[226]).

2.2.4.2 Association analysis

Appendix 10 contains the results of the single SNP association testing with a diagnosis of POAG in the study population. Allele frequencies, results of the χ^2 test and Fisher's exact test for each SNP may be found in Table A10.1. The results of logistic regression (unadjusted, adjusted for gender only and adjusted for both gender and age in an additive model) are provided for each SNP in Table A10.2. The associations are summarised in Figure 4.22.

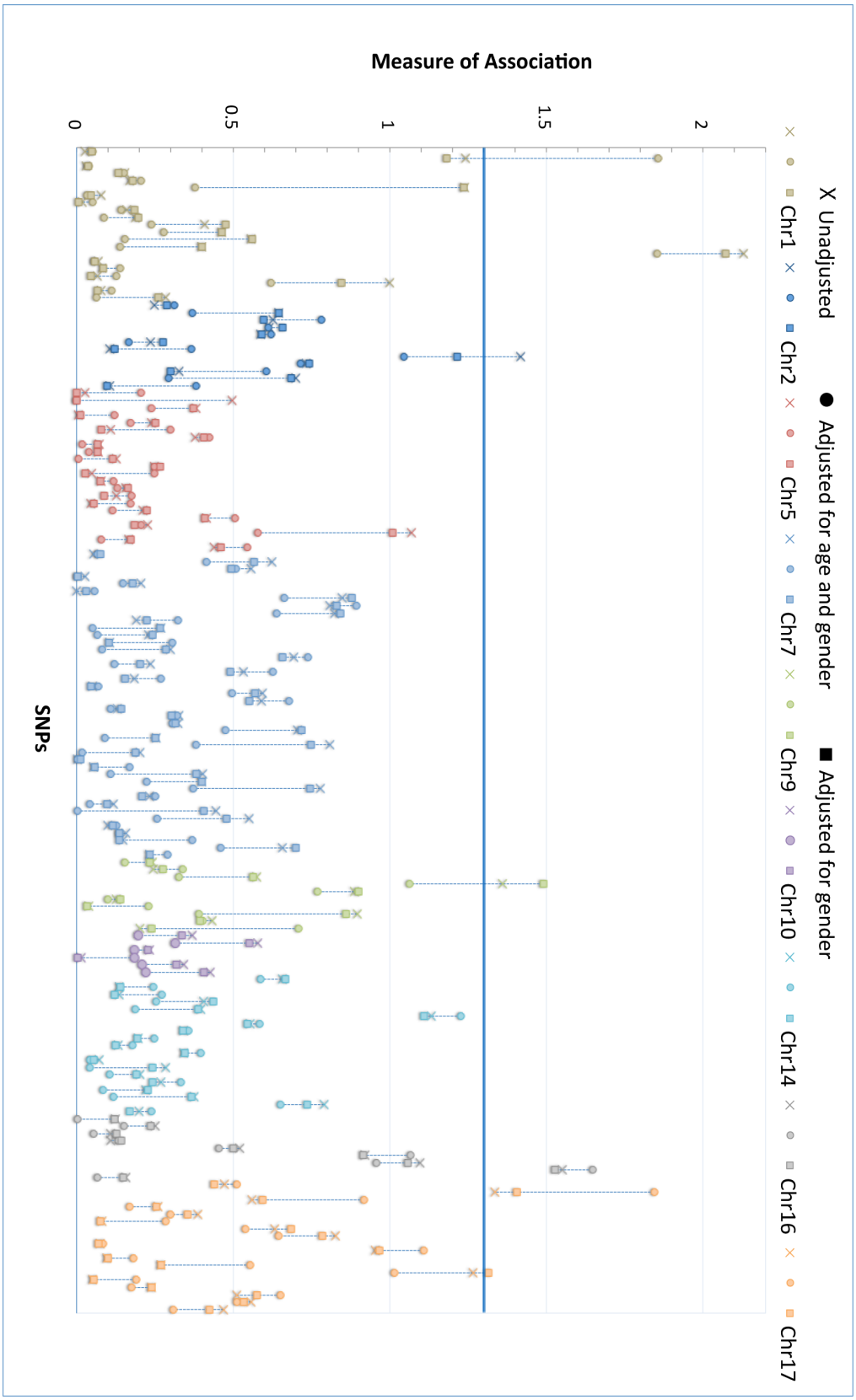

Figure 4.22 Summary of association of SNPs with POAG by logistic regression. The measure of association is represented on the y-axis by -$\log_{10}$p. For each SNP a comparison is made of the unadjusted association with the association adjusted for age and gender or adjusted for gender only. The SNPs represented are in *COL8A2* (chromosome 1), *MYOC* (chromosome 1), *CYP1B1* (chromosome 2), Chromosome 2p16 region, *WDR36* (chromosome 5), *COL1A2* (chromosome 7), *CAV1/CAV2* (chromosome 7), *CDKN2B* (chromosome 9), *COL5A1* (chromosome 9), *ATOH7* (chromosome 10), *SIX1/SIX6* (chromosome 14), *ZNF469* (chromosome 16) and *COL1A1* (chromosome 17). The blue line represents p=0.05. Bonferroni corrected p=0.0003 (0.05/159) which is 3.5 on the logarithmic scale (not illustrated).

There was no evidence of association between POAG in black South Africans and the *CAV1/2* region, *CYP1B1, WDR36, COL1A2, COL5A1,* the *SIX1/SIX6* region and *ATOH7* ($p > 0.05$). There were five SNPs with a nominally significant association with POAG in this population without adjustment for age and gender ($p < 0.05$). These were individual SNPs in *MYOC* (rs235917), the chromosome 2p16 region (rs11125375), *CDKN2B* (rs974336), *COL1A1* (rs16948744) and the *ZNF469* region (rs9925231). The strongest association was with SNP rs235917 in *MYOC* (p=0.0074). The association with SNP rs11125375 in the chromosome 2p16 region did not remain significant in either of the regression models that included covariates. The other four SNPs remained associated with POAG after adjusting for gender alone with little loss in power. Adjusting for both gender and age resulted in a small loss of power in the associations with SNP rs235917 in *MYOC* and SNP rs974336 in *CDKN2B,* but introduced a new nominally significant SNP in *COL8A2* (rs6693322). Table 4.5 contains the association analyses of these SNPs.

Table 4.5 SNPs associated with POAG

Genomic region	SNP : MA	MAF POAG : Controls	p	OR* (95%CI)	p*	OR** (95%CI)	p**
COL8A2	rs6693322 : G	0.11 : 0.07	0.0573	1.56 (0.97 – 2.51)	0.0659	1.92 (1.14 - 3.24)	0.0139
MYOC	rs235917 : A	0.08 : 0.04	**0.0074**	2.23 (1.23 – 4.05)	0.0084	2.32 (1.19 - 4.52)	0.0140
Chr 2p16	rs11125375 : T	0.29 : 0.22	0.0381	1.32 (0.15 – 1.77)	0.0610	1.33 (0.96 – 1.84)	0.0898
CDKN2B	rs974336 : A	0.24 : 0.30	0.0436	0.70 (0.51 – 0.97)	0.0322	0.74 (0.52 – 1.05)	0.0862
COL1A1	rs16948744 : G	0.37 : 0.44	0.0462	0.75 (0.57 – 0.99)	0.0391	0.68 (0.50 - 0.93)	0.0143
ZNF469	rs9925231 : A	0.50 : 0.43	0.0282	1.36 (1.03 – 1.78)	0.0298	1.43 (1.05 -1.93)	0.0225

*Adjusted for gender; ** Adjusted for age and gender
MA, minor allele; MAF, minor allele frequency; p, unadjusted p-value; OR (95%CI), odds ratio (95% confidence interval)

The SNPs in *MYOC, COL1A1* and *ZNF469* that were consistently associated with POAG in all three logistic regression models were selected for further analysis. A measure of the effect of the association for the three SNPs is illustrated in Figure 4.23.

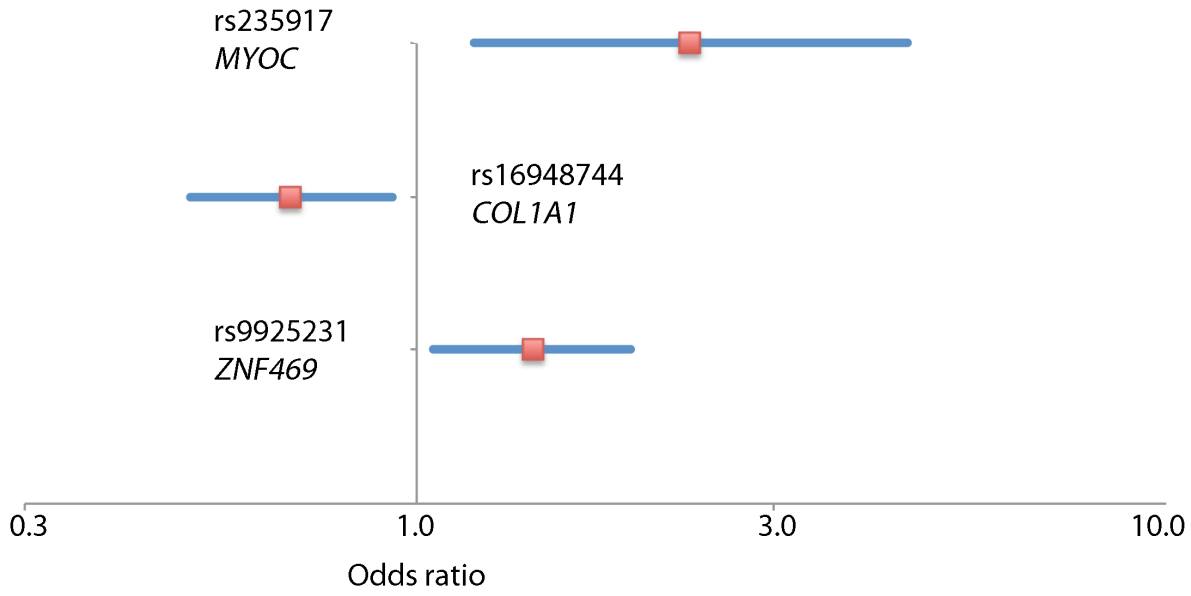

Figure 4.23. Forest plot of the odds ratios for each SNP associated with POAG. The red squares indicate the odds ratios with the 95% confidence intervals denoted by the blue lines.

2.2.4.3 Correction for multiple testing on SNP panel

Bonferroni corrected p-value for significance = 0.05/159 SNPs
= 0.0003

SNPSpD[295] corrected p-value for significance = 0.05/117 effective independent loci
= 0.0004

The adjusted associations did not withstand Bonferroni or SNPSpD correction for multiple comparisons. Permutation testing confirmed the associations of the significant SNPs with POAG, but the associations did not withstand correction for multiple testing using the maximum permuted statistic ($p > 0.05$, Table 4.6). In addition, no other form of correction for multiple testing (Holm and Šidák FWER methods or the FDR procedures by Benjamini and Hochberg, and Benjamini and Yekutieli) yielded a significant association ($p > 0.05$; Table 4.6).

Table 4.6 Permutation testing and correcting for multiple comparisons in SNPs associated with POAG

Genomic region	SNP	p	EMP1	EMP2	BONF	HOLM	SIDAK SS	SIDAK SD	FDR BH	FDR BY
MYOC	rs235917	0.01	0.01	0.600	1.00	1.00	0.69	0.69	0.95	1.0
COL1A1	rs16948744	0.05	0.05	1.000	1.00	1.00	1.00	1.00	0.95	1.0
ZNF469	rs9925231	0.03	0.03	0.968	1.00	1.00	0.99	0.99	0.95	1.0

p, unadjusted p-value; EMP1, p value that is a pointwise estimate of an individual SNPs significance after permuted testing with 100 000 permutations; EMP2, p value that controls for the fact that 158 other SNPs were tested in the permuted association; BONF, corrected p value for multiple comparisons using the Bonferroni method; HOLM, Holm step-down adjusted p values; SIDAK SS, Sidak single-step adjusted p values; SIDAK SD, Sidak step-down adjusted p values; FDR BH, Benjamini and Hochberg step-up FDR control; FDR BY, Benjamini and Yekutieli step-up FDR control

In a glaucoma subgroup analysis, there was no association with a family history of blindness, the presence of severe visual impairment or juvenile onset POAG and the POAG associated SNPs (Table 4.7). However, participants that had undergone glaucoma drainage surgery were more likely to have the risk allele of rs9925231 (*ZNF469* region) than those who had not had surgery (unadjusted p = 0.006; Table 4.7).

Table 4.7 Glaucoma subgroup analysis with POAG associated SNPs

SNP		Family history of blindness	Severe visual impairment	Glaucoma drainage surgery	Juvenile onset glaucoma
	n - cases	67	114	119	25
rs235917 (*MYOC*)	MAF cases	0.037	0.066	0.071	0.100
	MAF controls	0.066	0.060	0.058	0.059
	p	0.201	0.769	0.467	0.247
	p*	0.161	0.914	0.775	0.936
rs16948744 (*COL1A1*)	MAF cases	0.381	0.364	0.399	0.440
	MAF controls	0.407	0.417	0.405	0.401
	p	0.564	0.161	0.886	0.583
	p*	0.486	0.072	0.779	0.881
rs9925231 (*ZNF469*)	MAF cases	0.493	0.513	0.542	0.580
	MAF controls	0.460	0.448	0.436	0.458
	p	0.487	0.089	**0.005**	0.093
	p*	0.557	0.126	**0.006**	0.123

MAF, minor allele frequency; p, unadjusted p value; p*, p value adjusted for age and gender

2.2.4.4 MYOC

SNP rs235917 in the *MYOC* gene had the most significant association with POAG (p=0.0074). The linkage disequilibrium patterns in the SNPs analysed in this gene are illustrated in Figure 4.24. The algorithm taken from Gabriel *et al*.[294] defined a single haplotype block, with three haplotypes. The associated SNP (rs235917) was not within the block.

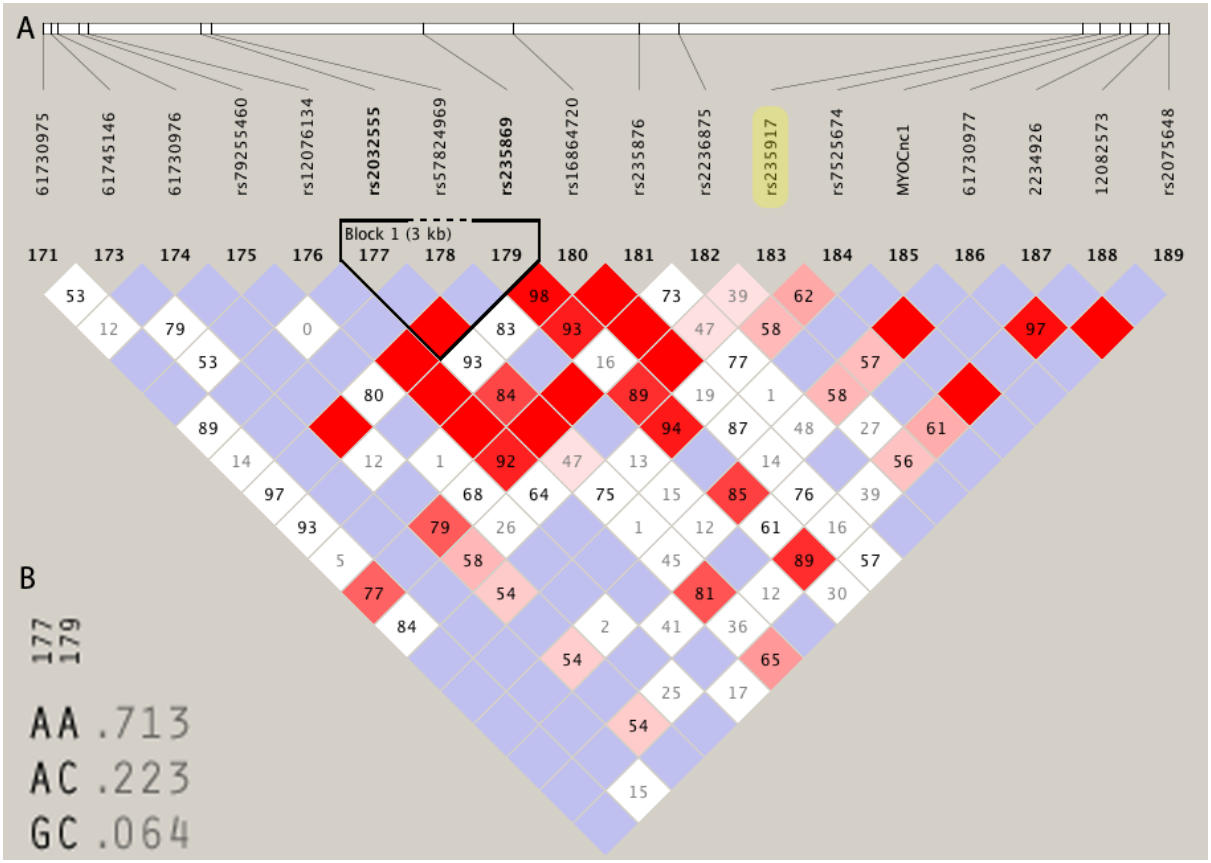

Figure 4.24 Haploview linkage disequilibrium plot of SNPs genotyped in the *MYOC* gene showing the LD patterns of the SNPs in this population.

A: A diagram of the LD block structure of the region. LD plots were identified by strong LD. Depth of red/pink colour indicates the computed pairwise D' value; deeper pink indicates a higher D' value. The blue blocks represent high D' but low LOD values. SNP 183 (rs235917 - highlighted in yelllow) was associated with POAG. Block 1 represents a haplotype block.

B: The haplotype frequencies in the haplotype block. Marker numbers above the haplotypes indicate the SNPs. The frequency of each haplotype within a block is given to the right of the haplotype.

Table 4.8 contains the haplotype association analysis of the *MYOC* haplotypes making up the block. None had a significant association with POAG (Table 4.8).

Table 4.8 *MYOC* haplotype association analysis with POAG

Block	Haplotype	HF POAG	HF Controls	χ^2	p
Block 1 (rs2032555 and rs235869)	AA	0.700	0.727	0.744	0.39
	AC	0.233	0.213	0.493	0.48
	GC	0.067	0.061	0.160	0.16

HF, haplotype frequency

2.2.4.5 COL1A1

SNP rs16948744 in the *COL1A1* gene had an association with POAG (p=0.0143). The linkage disequilibrium patterns in the SNPs analysed in this gene are illustrated in Figure 4.25. The algorithm taken from Gabriel *et al*.[294] defined a single haplotype

block, with three haplotypes. The associated SNP (rs16948744) was not within the block. Table 4.9 contains the haplotype association analysis of the *COL1A1* haplotypes making up the block. None had a significant association with POAG (Table 4.9).

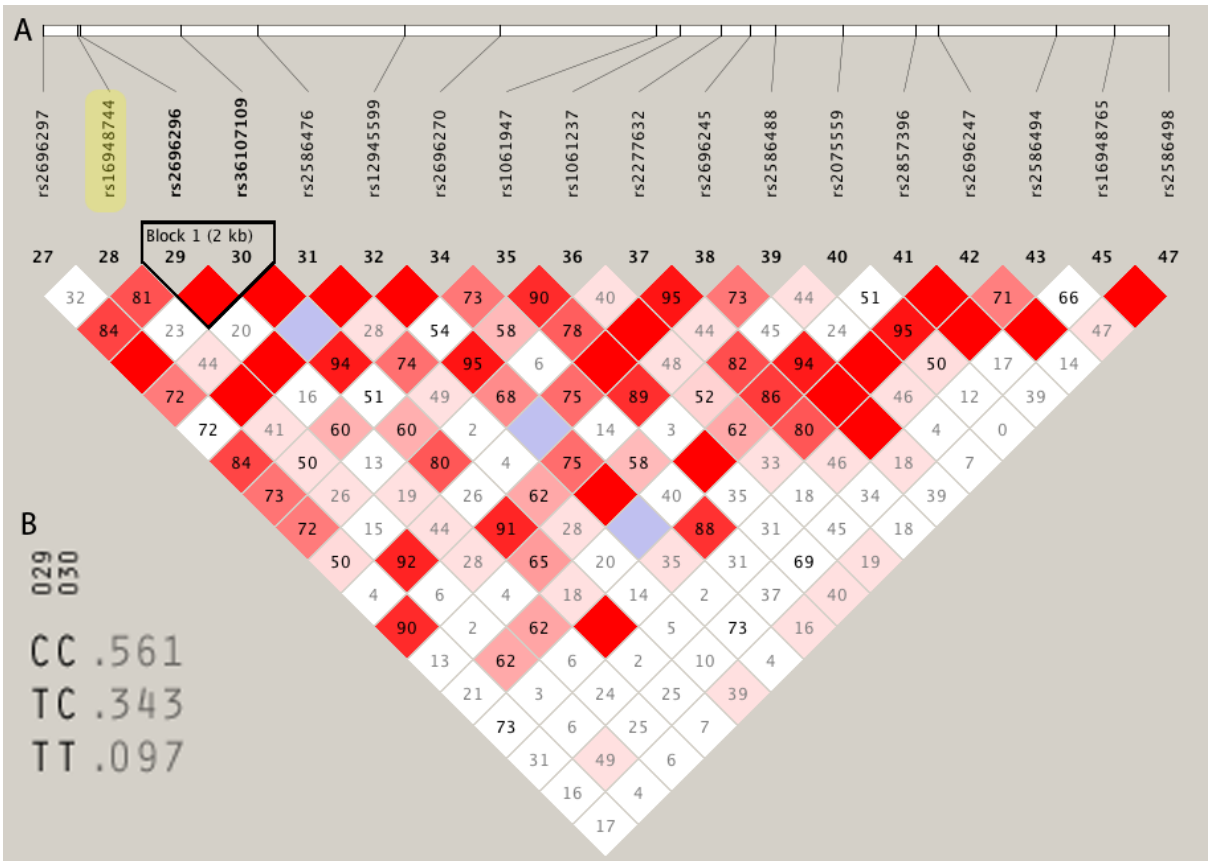

Figure 4.25 Haploview linkage disequilibrium plot of SNPs genotyped in the *COL1A1* gene showing the LD patterns of the SNPs in this population.

A: A diagram of the LD block structure of the region. LD plots were identified by strong LD. Depth of red/pink colour indicates the computed pairwise D' value; deeper pink indicates a higher D' value. The blue blocks represent high D' but low LOD values. SNP 28 (rs16948744 - highlighted in yellow) was associated with POAG. Block 1 represents a haplotype block.

B: The haplotype frequencies in the haplotype block. Marker numbers above the haplotypes indicate the SNPs. The frequency of each haplotype within a block is given to the right of the haplotype.

Table 4.9 *COL1A1* haplotype association analysis with POAG

Block	Haplotype	HF POAG	HF Controls	χ^2	p
Block 1 (rs2696296 and rs36107109)	CC	0.579	0.542	1.193	0.27
	TC	0.330	0.355	0.591	0.44
	TT	0.091	0.103	0.360	0.55

HF, haplotype frequency

2.2.4.6 ZNF469 gene region

SNP rs9925231 in the *ZNF469* gene region had an association with POAG (p = 0.0282) and with glaucoma drainage surgery (p = 0.006). The linkage disequilibrium patterns in the SNPs analysed in this gene are illustrated in Figure 4.26. The algorithm taken from Gabriel *et al*.[294] defined three haplotype blocks, with three haplotypes each. The associated SNP (rs9925231) was not within the blocks, but a fourth haplotype block was analysed that contained the associated SNP with the two SNPs in haplotype block 3. Table 4.10 contains the haplotype association analysis with both POAG and glaucoma drainage surgery of the *ZNF469* gene region haplotypes making up the blocks. The GC haplotype of Block 3 (rs12448211 and rs9938149) was associated with glaucoma drainage surgery (p = 0.005) as was the GCA haplotype of the block containing the associated SNP (rs12448211, rs9938149 and rs9925231) (p = 0.004).

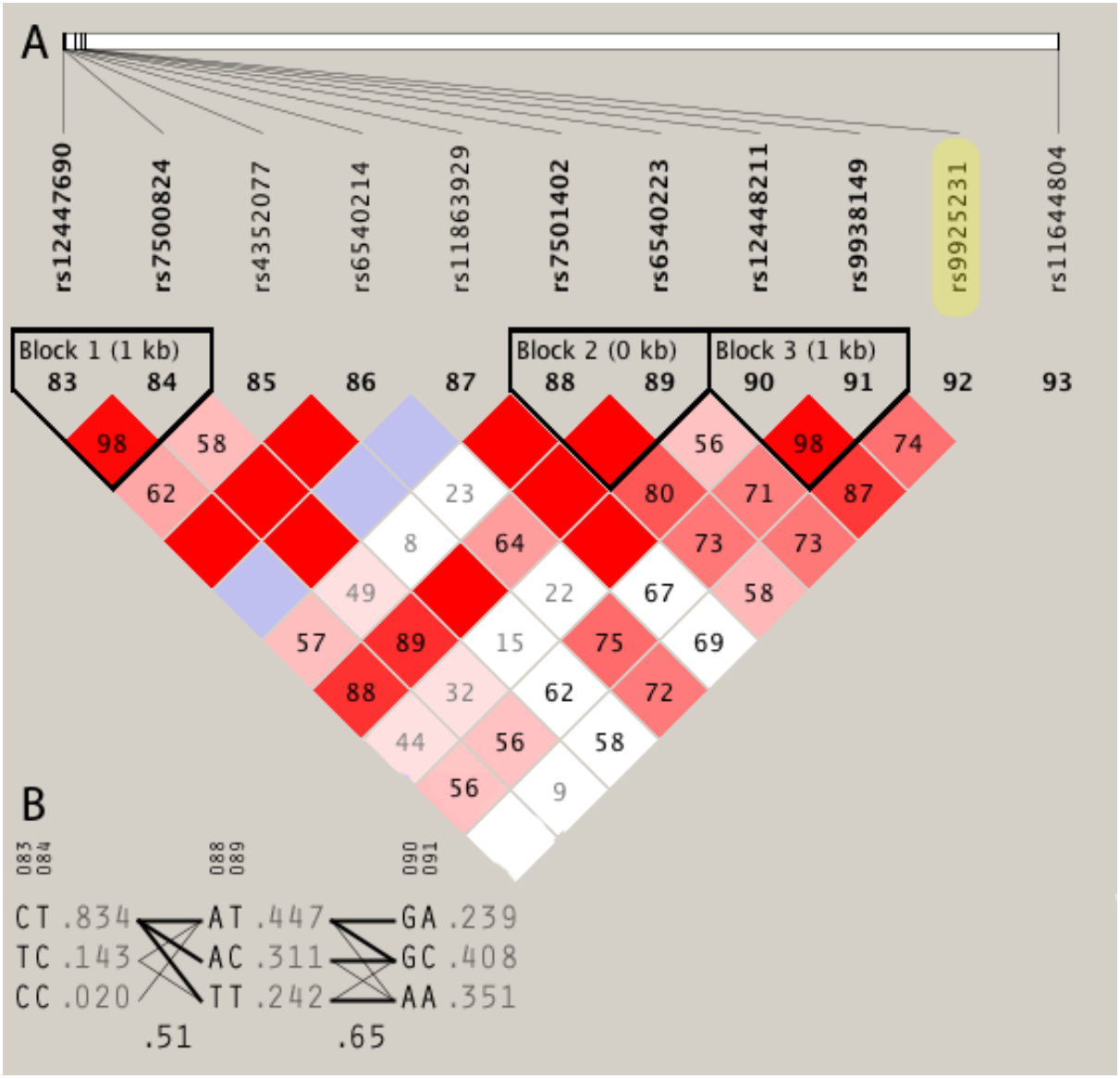

Figure 4.26 Haploview linkage disequilibrium plot of SNPs genotyped in the *ZNF469* gene region showing the LD patterns of the SNPs in this population.

A: A diagram of the LD block structure of the region. LD plots were identified by strong LD. Depth of red/pink colour indicates the computed pairwise D' value; deeper pink indicates a higher D' value. Blocks 1,2 and 3 represent haplotype blocks. SNP rs9925231 (highlighted in yellow) was associated with POAG.

B: The haplotype frequencies in the haplotype blocks. Marker numbers above the haplotypes indicate the SNPs. The frequency of each haplotype within a block is given to the right of the haplotype. The thickness of the lines connecting the haplotypes across blocks represents the relative frequency (i.e., high [thick] versus low [thin]) with which a given haplotype is associated with the haplotype in the neighbouring block.

Table 4.10 *ZNF469* gene region haplotype association analysis with POAG and glaucoma drainage surgery

Block	Haplotype	HF POAG	HF Controls	χ^2	p	HF Surgery	HF Controls	χ^2	p
Block 1 (rs12447690 and rs7500824)	CT	0.842	0.827	0.338	0.560	0.827	0.840	0.214	0.644
	TC	0.139	0.147	0.103	0.750	0.148	0.142	0.042	0.837
	CC	0.016	0.023	0.553	0.460	0.025	0.018	0.501	0.479
Block 2 (rs7501402 and rs6540223)	AT	0.426	0.467	1.480	0.220	0.407	0.461	2.055	0.152
	AC	0.327	0.294	1.097	0.300	0.360	0.292	3.716	0.054
	TT	0.247	0.238	0.078	0.780	0.233	0.247	0.175	0.676
Block 3 (rs12448211 and rs9938149)	GC	0.439	0.377	3.362	0.070	**0.487**	**0.381**	8.041	**0.005**
	AA	0.327	0.376	2.301	0.130	0.284	0.377	6.520	**0.011**
	GA	0.234	0.244	0.112	0.740	0.229	0.242	0.170	0.680
Block with associated SNP (rs12448211, rs9938149 and rs9925231)	GCA	0.389	0.389	4.370	0.037	**0.433**	**0.328**	8.110	**0.004**
	AAG	0.311	0.311	1.644	0.200	0.273	0.356	5.401	**0.020**
	GAG	0.137	0.137	0.982	0.322	0.131	0.157	0.910	0.340
	GAA	0.097	0.097	0.598	0.439	0.099	0.086	0.336	0.562
	GCG	0.049	0.049	0.211	0.646	0.055	0.051	0.047	0.829
	AAA	0.016	0.016	0.807	0.369	0.011	0.022	1.082	0.298

HF, haplotype frequency; HF Surgery, haplotype frequency in POAG patients that had glaucoma drainage surgery

2.2.5 IOP association analysis

Among the 22 SNPs in *WDR36* and the 13 SNPs in *CAV1/CAV2* that were tested for an association with IOP using linear regression, a single SNP in *WDR36* (rs1457113) had a minor allele that was marginally associated with a higher IOP after adjusting for diagnosis (POAG or control), age and gender (p = 0.04). The mean IOP associated with each genotype for this SNP is documented in Table 4.11 along with the measure of association.

Table 4.11 Association of SNP rs1457113 in *WDR36* with IOP

SNP	Genotype	n (%)	IOP (mean ± SD in mmHg)
rs1457113	CC	1 (0.2)	42 ± 0
	TC	58 (14.9)	26.4 ± 14.3
	TT	331 (84.9)	22.7 ± 12.3
	Total	390 (100)	**p = 0.0173**
			Adjusted **p = 0.0404**

p was obtained from linear regression; Adjusted p, p obtained from linear regression with adjustment for diagnosis (POAG or control), age and gender

2.2.5.1 Correction for multiple testing on SNP panel

Bonferroni corrected p-value for significance = 0.05/35 SNPs
= 0.001

SNPSpD[299] corrected p-value for significance = 0.05/24 effective independent loci
= 0.002

The associations did not remain significant after adjustment for multiple testing.

2.2.6 CCT association analysis

There were 71 SNPs in the genes/regions thought to be associated with CCT, namely *COL1A1, COL1A2, COL5A1, COL8A2*, and the *ZNF469* region. In the linear regression association analysis between these SNPs and CCT, the T allele of SNP rs7500824 in the *ZNF469* region and the C allele of SNP rs2521206 in *COL1A2* were both weakly associated with a thinner central cornea after adjusting for diagnosis (POAG or control), age and gender ($p < 0.05$; Table 4.12).

Table 4.12 Association of SNPs with CCT

SNP (region)	Genotype	n (%)	CCT (mean ± SD in μm)
rs7500824	CC	12 (3.2)	531.2 ± 31.7
(*ZNF469*)	TC	97 (26.3)	512.4 ± 38.7
	TT	260 (70.5)	505.7 ± 49.1
	Total	369 (100)	**p=0.0446**
			Adjusted **p=0.0472**
rs2521206	CC	37 (10.0)	499.0 ± 36.2
(*COL1A2*)	TC	154 (41.7)	506.7 ± 55.4
	TT	178 (48.3)	511.5 ± 38.8
	Total	369 (100)	**p=0.1173**
			Adjusted **p=0.0449**

p was obtained from linear regression; Adjusted p, p obtained from linear regression with adjustment for diagnosis (POAG or control), age and gender

2.2.6.1 Correction for multiple testing on SNP panel

Bonferroni corrected p-value for significance = 0.05/71 SNPs
= 0.0007

SNPSpD[299] corrected p-value for significance = 0.05/47 effective independent loci
= 0.001

The association did not remain significant after adjustment for multiple testing.

2.2.7 VCDR association analysis

We found no evidence of association between the 31 SNPs in the regions of *CDKN2B, SIX1/6*, and *ATOH7* and VCDR in this population.

2.2.8 Diabetic association analysis

Appendix 11 contains the results of the single SNP association testing with a diagnosis of diabetes mellitus in the study population. Allele frequencies, results of the χ^2 test and Fisher's exact test for each SNP may be found in Table A11.1. The results of logistic regression including the adjustment for age, gender and glaucoma diagnosis (POAG or control) of single SNP association testing with each of the 159 SNPs and diabetes mellitus are provided in Table A11.2. The associations are summarised in Figure 4.27.

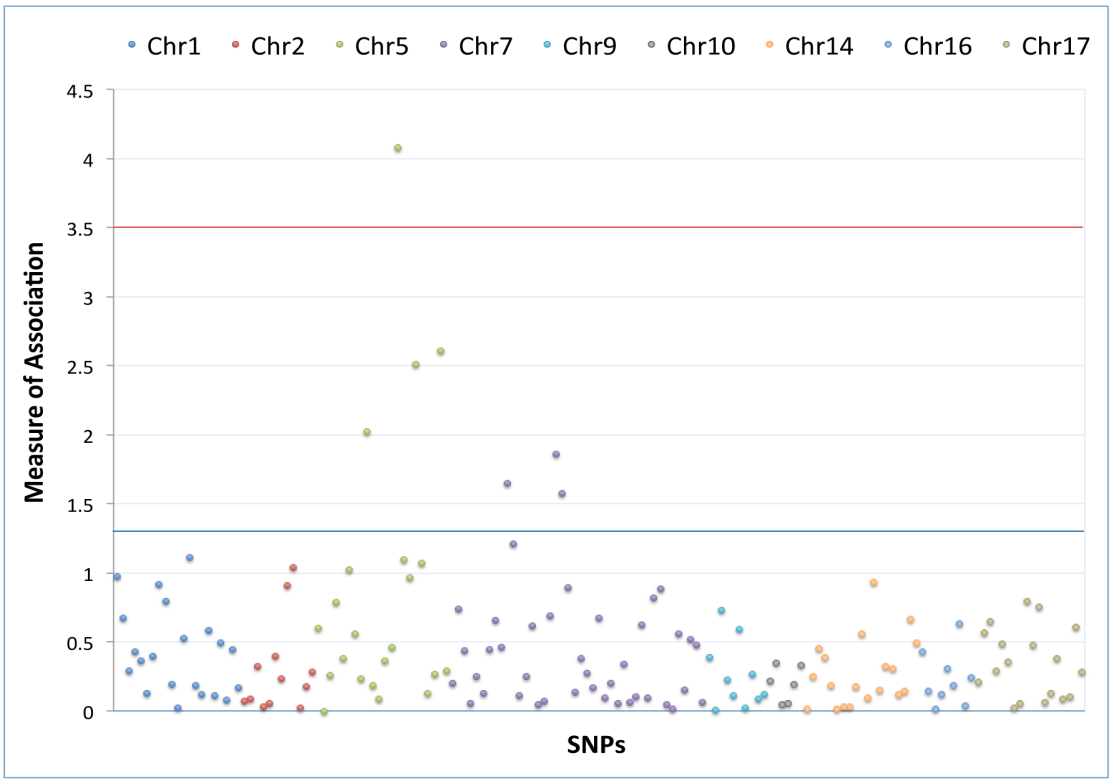

Figure 4.27 Summary of association of SNPs with diabetes mellitus by logistic regression. The measure of association is represented on the y-axis by $-\log_{10}p$. The association was calculated by logistic regression adjusted for diagnosis (POAG or control), age and gender. The SNPs represented are in *COL8A2* (chromosome 1), *MYOC* (chromosome 1), *CYP1B1* (chromosome 2), Chromosome 2p16 region, *WDR36* (chromosome 5), *COL1A2* (chromosome 7), *CAV1/CAV2* (chromosome 7), *CDKN2B* (chromosome 9), *COL5A1* (chromosome 9), *ATOH7* (chromosome 10), *SIX1/SIX6* (chromosome 14), *ZNF469* (chromosome 16) and *COL1A1* (chromosome 17). The blue line represents p=0.05. The red line represents the Bonferroni corrected P=0.0003 (0.05/159).

The two regions with significant SNP associations ($p < 0.05$) after adjustment for age, gender and diagnosis (POAG or control) were within the *WDR36* and *COL1A2* genes. The association analysis in the significant SNPs in *WDR36* and *COL1A2* is detailed in Table 4.13. A measure of the effect of the association for these seven SNPs (using odds ratios) is illustrated in Figure 4.28.

Table 4.13 SNPs with an association to diabetes mellitus

Genomic region	SNP : MA	MAF DM : nonDM	p	OR (95% CI)*	p*	BONF*
WDR36	**rs12522383 : T**	**0.256 : 0.143**	**0.000076**	**2.06 (1.43 – 2.95)**	**0.000083**	**0.013**
WDR36	rs17132810 : T	0.205 : 0.125	**0.002428**	1.81 (1.23 – 2.67)	**0.002464**	0.392
WDR36	rs11241098 : T	0.205 : 0.127	**0.003078**	1.78 (1.21 – 2.62)	**0.003107**	0.494
WDR36	rs12515367 : G	0.050 : 0.020	**0.015230**	2.60 (1.17 – 5.78)	**0.009476**	1.000
COL1A2	rs4266 : G	0.271 : 0.192	**0.009281**	1.57 (1.12 – 2.21)	**0.013750**	1.000
COL1A2	rs42521 : C	0.430 : 0.346	**0.019890**	1.43 (1.06 – 1.93)	**0.022650**	1.000
COL1A2	rs10235102 : T	0.112 : 0.067	**0.023880**	1.77 (1.07 – 2.93)	**0.026560**	1.000

*Adjusted for diagnosis (POAG or control), age and gender

MA, minor allele; MAF DM, minor allele frequency in diabetic patients; MAF nonDM, minor allele frequency in non-diabetic participants; p, unadjusted p-value; OR (95% CI), odds ratio (95% confidence intervals); BONF, corrected p value for multiple comparisons using the Bonferroni method

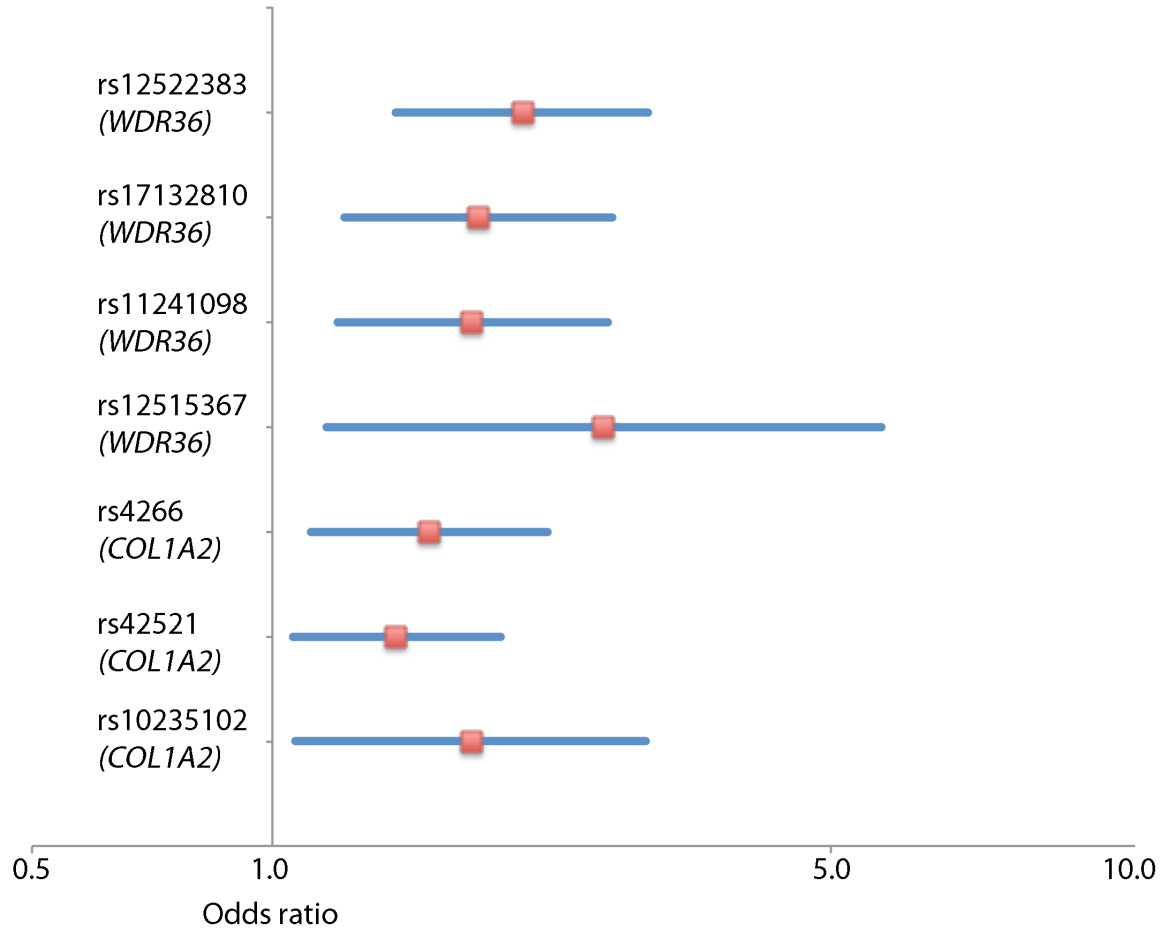

Figure 4.28. Forest plot of the odds ratios (x-axis) for each SNP associated with diabetes mellitus. The red squares indicate the odds ratios with the 95% confidence intervals denoted by the blue lines.

2.2.8.1 Correction for multiple testing on SNP panel

Bonferroni corrected p-value for significance = 0.05/159 SNPs
= 0.0003

SNPSpD[295] corrected p-value for significance = 0.05/117 effective independent loci
= 0.0004

The association of diabetes mellitus with SNP rs12522383 in *WDR36* remained significant after correction for multiple testing using the SNPSpD and Bonferroni methods. Permutation testing confirmed the associations of the significant SNPs with

diabetes mellitus. The most significant SNP, rs12522383 in *WDR36*, remained significantly associated after correction for multiple testing with the maximum permuted statistic (p = 0.012; Table 4.14). In addition, other forms of correction for multiple testing (Holm and Šidák FWER methods and the FDR procedure by Benjamini and Hochberg) yielded a significant association for this SNP (p < 0.05; Table 4.14).

Table 4.14 Permutation testing and multiple comparison corrections for SNPs associated with diabetes mellitus

Genomic region	SNP	p	EMP1	EMP2	BONF	HOLM	SIDAK SS	SIDAK SD	FDR BH	FDR BY
WDR36	**rs12522383**	**0.000076**	**0.00014**	**0.012**	**0.012**	**0.012**	**0.012**	**0.012**	**0.012**	0.068
WDR36	rs17132810	**0.002428**	**0.00211**	0.278	0.386	0.384	0.321	0.319	0.163	0.922
WDR36	rs11241098	**0.003078**	**0.00288**	0.334	0.489	0.483	0.387	0.384	0.163	0.922
WDR36	rs12515367	**0.015230**	**0.02234**	0.859	1.000	1.000	0.913	0.907	0.484	1.000
COL1A2	rs4266	**0.009281**	**0.00980**	0.701	1.000	1.000	0.773	0.767	0.369	1.000
COL1A2	rs42521	**0.019890**	**0.01687**	0.915	1.000	1.000	0.959	0.955	0.527	1.000
COL1A2	rs10235102	**0.023880**	**0.02598**	0.944	1.000	1.000	0.979	0.975	0.542	1.000

p, unadjusted p-value; EMP1, p value that is a pointwise estimate of an individual SNPs significance after permuted testing with 100000 permutations; EMP2, p value that controls for the fact that 158 other SNPs were tested in the permuted association; BONF, corrected p value for multiple comparisons using the Bonferroni method; HOLM, Holm step-down adjusted p values; SIDAK SS, Sidak single-step adjusted p values; SIDAK SD, Sidak step-down adjusted p values; FDR BH, Benjamini and Hochberg step-up false discovery rate control; FDR BY, Benjamini and Yekutieli step-up false discovery rate control

2.2.8.2 WDR36 and diabetes mellitus

SNP rs12522383 in the *WDR36* gene had a significant association with a diabetic phenotype after adjustment for age, gender and glaucoma diagnosis (Bonferroni corrected p<0.05). Three other SNPs in the gene were also associated with diabetes mellitus, but not after Bonferroni correction. The linkage disequilibrium patterns in this group in the SNPs analysed in this gene are illustrated in Figure 4.29. The algorithm taken from Gabriel *et al.*[294] defined two haplotype blocks. The most significantly associated SNP (rs12522383) was not within either block. The regional plot of the SNPs in *WDR36* and their association with diabetes mellitus is illustrated in Figure 4.30. Two of the other associated SNPs (rs11241098 and rs12515367) are in linkage disequilibrium with rs12522383 (Figure 4.30). In the haploype analysis the two haplotype blocks from Figure 4.29 as well as a haplotype block consisting of the three associated SNPs in linkage disequilibrium (rs12522383, rs11241098 and rs12515367) were therefore analysed. The results of this association analysis are documented in Table 4.15. None of the haplotypes in Block 1 was associated with

diabetes. The TA haplotype in Block 2 was associated with diabetes, but not as significantly as SNP rs11241098 which is part of the block. The CCA haplotype (that does not contain the risk allele of rs12522383) of the block formed by the associated SNPs in linkage disequilibrium is significantly protective against a diabetic phenotype, however the association is only a little stronger than that for the T-allele of SNP rs12522383 alone ($p = 6.9 \times 10^{-5}$ versus $p = 7.6 \times 10^{-5}$).

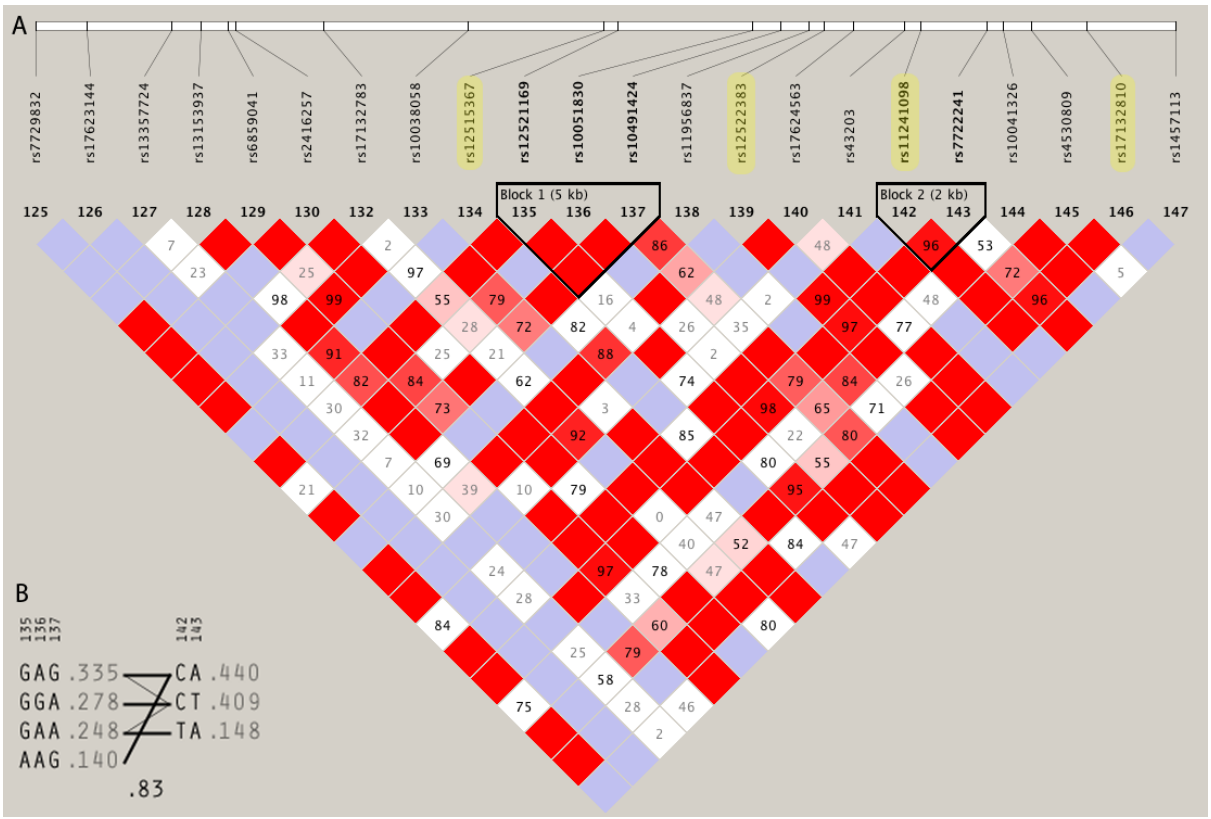

Figure 4.29 Haploview linkage disequilibrium plot of SNPs genotyped in the *WDR36* gene region showing the LD patterns of the SNPs in this population.

A: A diagram of the LD block structure of the region. LD plots were identified by strong LD. Depth of red/pink colour indicates the computed pairwise D' value; deeper pink indicates a higher D' value. The blue blocks represent high D' but low LOD values. The SNPs associated with diabetes are highlighted in yellow. Blocks 1 and 2 represents haplotype blocks.

B: The haplotype frequencies in the haplotype blocks. Marker numbers above the haplotypes indicate the SNPs. The frequency of each haplotype within a block is given to the right of the haplotype. The thickness of the lines connecting the haplotypes across blocks represents the relative frequency (i.e., high [thick] versus low [thin]) with which a given haplotype is associated with the haplotype in the neighbouring block.

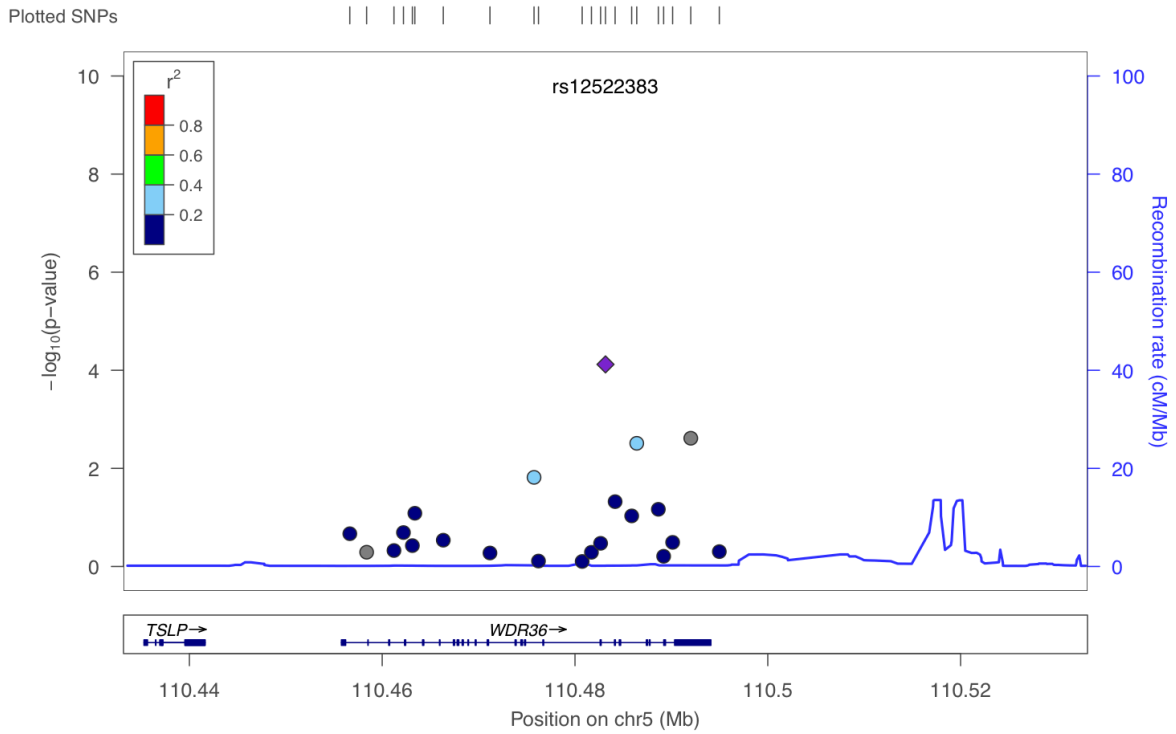

Figure 4.30 Regional plot of the association of SNPs in WDR36 with diabetes mellitus. On the left y-axis is the -$\log_{10}$p for each SNP genotyped. On the right y-axis and the line is the recombination rate estimated from this data. The annotated genes in the region (*WDR36* and *TSLP*) are indicated along the bottom of the figure. SNPs are coloured based on their linkage disequilibrium with the most significant SNP (rs12522383), where purple indicates the index SNP, light blue indicates 0.2 < r2 < 0.4, and dark blue indicates r2 ≤ 0.2.

Table 4.15 *WDR36* gene haplotype association analysis with diabetes mellitus

Block	Haplotype	HF diabetics	HF non-diabetics	χ^2	p
Block 1 (rs12521169, rs10051830 and rs10491424)	AAG	0.146	0.137	0.109	0.7408
	GAG	0.312	0.345	0.874	0.3499
	GGA	0.271	0.281	0.084	0.7721
	GAA	0.271	0.237	1.117	0.2905
Block 2 (rs11241098 and rs7722241)	CT	0.361	0.431	3.592	0.0581
	TA	0.201	0.127	7.874	**0.0051**
	CA	0.438	0.443	0.019	0.8914
Associated SNP block (**rs12522383**, rs11241098 and rs12515367)	TCG	0.050	0.017	7.761	**0.0053**
	TTA	0.205	0.126	8.951	**0.0028**
	CCA	**0.744**	**0.857**	15.850	**0.00007**

HF, haplotype frequency

A diabetic subgroup analysis of association with SNP rs12522383 confirmed the association for type 2 diabetes mellitus but the SNP is not associated with diabetic retinopathy (p = 0.237; Table 4.16).

Table 4.16 Diabetic subgroup analysis for association with rs12522383 in *WDR36*

Diabetic phenotype	n cases	MAF cases	MAF controls	p	p*
Type 1	7	0.357	0.174	0.07533	0.07713
Type 2	122	0.250	0.148	**0.00043**	**0.00043**
Diabetic retinopathy	30	0.233	0.173	0.23730	0.26460

*Adjusted for diagnosis (POAG or control), age and gender
MAF, minor allele frequency; p, unadjusted p-value

3. TAQMAN ASSOCIATION STUDY

3.1 Methods

3.1.1 Population and sample

The study population, selection criteria, examination and investigations have been described in detail in Chapter 2.

3.1.2 Candidate region selection

The team from Michael Hauser's laboratory at the Duke Center for Human Genetics selected the candidate regions and SNPs. POAG candidate region selection was based on location within genomic regions identified in POAG GWA studies using populations of European ancestry. The regions included in the study were:

- *TMCO1*
- *CAV1*
- *CAV2*
- *CDKN2BAS*
- *SIX1* and *SIX 6* and the intergenic region

A detailed description of the genes and regions and a discussion of the current literature on the subject can be found in Chapter 1.

3.1.3 Single nucleotide polymorphism selection

Tagging SNPs were selected, using the Yoruba from Nigeria as the reference population, to cover the genomic regions using HaploView with r^2 greater than 0.6 and a minor allele frequency of greater than 0.05.

The candidate genes or regions, their chromosomal location and the number of SNPs selected within the regions for genotyping are summarised in Table 4.17.

Appendix 12 contains the complete list of SNPs that was genotyped in this study together with the chromosomal location and the alleles at each SNP. There was an overlap of nine SNPs that were genotyped in both the BeadXpress study and the TaqMan® study. This overlap was used to validate the genotyping results from each study.

Table 4.17 Candidate genes or regions, their chromosomal location and the number of SNPs genotyped within the regions in this study

Genomic region	Chromosome	Location	Tag SNPs
TMCO1	1	163951690..164006222	8
CAV2	7	115933310..115949965	6
CAV1	7	115954680..115987033	7
CDKN2BAS-1	9	21992906..22122076	19
SIX1/SIX6	14	59855477..60185934	9
TOTAL			**49**

Tag SNPs, SNPs derived from the Haploview Tagger protocol

3.1.4 Sample preparation and export

An export permit for aliquots of DNA from 500 individuals was obtained from the Department of Health (Reference J1/2/4/2). Aliquots of the DNA from each individual in the study population were normalised to a concentration of 50ng/µl and a volume of 200µl. The normalised samples were couriered (on ice) to the Duke Center for Human Genetics.

3.1.5 Genotyping with TaqMan-based allelic discrimination assays

Genotyping of blood DNA was performed with TaqMan® allelic discrimination assays (Applied Biosystems).

TaqMan® allelic discrimination for SNP genotyping is a 5' nuclease assay that uses the 5' to 3' exonuclease activity of Taq DNA polymerase.[303] There is a separate assay for each SNP under investigation. PCR is performed using flanking primers to amplify the SNP polymorphic site in an assay with fluorescent oligonucleotide probes designed to hybridise to that SNP. There are two allele-specific probes that each consist of a different 5' reporter dye (fluorophore) and a 3' quencher dye. The close proximity of the dyes enables the quencher dye to eliminate the fluorophore's signal

using FRET (Förster fluorescence resonance energy transfer). An allele-specific probe that is complementary to the SNP allele will bind to the target DNA strand during PCR amplification and then get degraded by the 5' nuclease activity of Taq DNA polymerase as it extends the DNA from the primers. This cleavage of the probe releases the fluorophore from the quencher resulting in fluorescence of that reporter dye. If the probe and target DNA sequences do not match, there is reduced hybridisation. This prevents the nuclease from acting on the probe and the fluorophore is not released from the quencher. The amount of fluorescent signal from each of the two fluorophores determines the allele or alleles present in the target region.[304] There are no post-PCR processing steps.

3.1.5.1 Materials

3.1.5.1.1 Genomic DNA

Aliquots of 20ng of genomic DNA from each sample were delivered to the bottom surface of each well of 384-well optical reaction plates. In addition to the samples being analysed, the following controls were included in each plate:

- No template control – this contained no DNA template so fluorescence levels were due to the uncleaved probe
- Known genotype – two controls with known genotype

The DNA was dried down completely by evaporation at room temperature in a dark, amplicon-free location covered with lint-free tissue. There were two plates of samples prepared for each of the 49 SNPs.

3.1.5.1.2 SNP genotyping assay

For each of the 49 SNPs there was a SNP genotyping assay that contained:

- Sequence-specific forward and reverse primers to amplify the polymorphic sequence of interest
- A TaqMan® probe labelled with a 5' VIC® dye and a 3' quencher (tetramethylrhodamine) to detect the allele 1 sequence

- A TaqMan® probe labelled with a 5' FAM (6-carboxy-fluorescein) dye and a 3' quencher (tetramethylrhodamine) to detect the allele 2 sequence

3.1.5.1.3 Universal PCR Master Mix

This single reaction buffer contains a premix of the necessary components (except primers and probes) for the 5' nuclease assay. It contains AmpliTaq Gold™ DNA polymerase, reaction buffer and dNTPs. AmpliTaq Gold™ DNA polymerase is a DNA polymerase that is active only after incubation at elevated temperatures ('hot start' PCR). It synthesises a new DNA strand from the dNTPs using single-stranded DNA (produced by thermal denaturation) as a template and the annealed primers for initiation of synthesis. The 'hot start' reduces the requirements on primer design.

3.1.5.2 Protocol

3.1.5.2.1 Prepare reaction mix

The reaction mix for each assay was prepared in a sterile centrifuge tube, inverted and centrifuged. The volumes of each component required per well of the 384-well optical reaction plates are in Table 4.18.

Table 4.18 TaqMan® reaction mix components

PCR Components	**Volume per well (ul)**
TaqMan® Universal PCR Mastermix	2.5
SNP genotyping assay	0.25
DNAse free H_2O	2.25
TOTAL	**5**

3.1.5.2.2 Prepare reaction plate

For each SNP assay, 5 ul of the reaction mix was pipetted into each well of the two 384-well plates prepared with dried down DNA. The plate was sealed with an adhesive cover, vortexed and centrifuged.

3.1.5.2.3 PCR

Table 4.19 documents the PCR protocol for the thermal cycler that consisted of AmpliTaq Gold™ enzyme activation, denaturation of the double-stranded DNA sample, annealing of primers to the single-stranded DNA and extension of the synthesized PCR product.

Table 4.19 PCR protocol

AmpliTaq Gold™ enzyme activation	**PCR (40 cycles)**	
HOLD	Denature	Anneal / Extend
10 minutes at 95°C	15 seconds at 92°C	1 minute at 60°C

3.1.5.2.4 Allelic discrimination plate read and analysis

The fluorescent signal intensity was measured directly in the reaction well by the ABI ViiA7 Sequence Detector (Applied Biosystems). Algorithms within the software extract the contribution of each component dye to the observed spectrum and produce a scatter diagram plotting the allele-specific components of each reaction. An increase in signal of either FAM or VIC® indicates homozygosity for the FAM-specific or VIC®-specific allele while an increase in both signals indicates heterozygosity.

3.1.6 SNP quality control

Genotyping efficiency of 95% and matching genotypes of quality-control samples within and across all plates was required to include samples in the statistical analysis.

3.1.7 Statistics

3.1.7.1 Descriptive statistical analyses

Descriptive analyses were performed using STATA version 12 as described in Chapter 2.2.3.

3.1.7.2 Association analyses

Association analyses were performed on the following datasets:

- The study population

The study population comprised the South African study population as described in Chapter 2.

- African American dataset

The investigators from the Duke Center for Human Genetics provided me with the raw genotyping data on two populations of African American POAG patients and unaffected control participants. The first consisted of self-identified African Americans enrolled at the Duke Medical Center in Durham, North Carolina. The second consisted of African Americans enrolled by external collaborators elsewhere in the United States of America. The second dataset had incomplete genotyping results on several of the SNPs. For this reason I only analysed the first population.

- Ghanaian dataset

The investigators from the Duke Center for Human Genetics provided me with the raw genotyping data on a population of West African POAG patients and unaffected controls from Ghana. The participants had been enrolled in Ghana for collaborative studies with the Duke Center for Human Genetics.

- Combined dataset

The combined dataset consisted of the study population together with the African American and Ghanaian datasets.

SNP association analyses were performed using PLINK (v1.07) and HaploView (v4.2).[284, 293] The minimum genotyping rate for SNPs and individuals to be included

in the statistical analyses was set to be 90%.

Hardy-Weinberg equilibrium was assessed by using the χ^2 test. SNPs that were not in Hardy-Weinberg equilibrium ($p < 0.01$) in the group of controls were excluded from further analysis.

The minor allele frequency of each SNP in patients with POAG and in control participants was compared in each dataset by using the χ^2 test or Fisher's exact test. Odds ratios and confidence intervals were calculated using logistic regression. The same approach was used in the association analyses for severe visual loss from glaucoma. The allele frequency in each SNP in the subset of POAG patients with a visual acuity of less than 6/60 in either eye and in control participants was compared in each dataset by using the χ^2 test or Fisher's exact test. Odds ratios and confidence intervals were calculated using logistic regression.

The association analyses for diabetes were performed in the South African study population only. The minor allele frequency of each SNP in South African diabetic and in South African non-diabetic participants was compared by using the χ^2 test or Fisher's exact test. Odds ratios and confidence intervals were calculated using logistic regression.

Associations of the SNPs in *CDKN2B, SIX1* and *SIX6* with VCDR and the SNPs in *TMCO1* and *CAV1/CAV2* with IOP were evaluated by using linear regression in each dataset.

3.1.7.2.1 Adjusting associations for covariates

Covariates that were potential confounders were included in statistical models.

Gender: Gender could potentially be correlated with the variants, but not mediate their effects. This was therefore included as a covariate in regression modelling in all of the analyses.

Population: In the analyses of the combined dataset, population (South African,

African American or Ghanaian) was included as a potentially confounding covariate.

Age: Age is an unlikely confounder assumed to be independent of the variants. However, it was included in the quantitative trait analyses because it is likely to be beneficial in reducing noise and increasing the power to detect associations by explaining some of the variability in the outcome.[296] Age was similarly included in the diabetic association analysis because in the South African study population diabetes is associated with age (Chapter 2), but the sample ascertainment (pertaining to diabetes) was random, so age and the variants remained independent. The same was not true for the POAG association analyses. Age was correlated with POAG in the study population (Chapter 2) and in the other populations analysed, but the correlation was induced by a selection bias (approximately equal numbers of POAG and controls selected). Pirinen *et al.*[297] have shown that in the presence of induced correlation omitting the covariate from a logistic regression model can increase the power particularly when the covariate is a strong risk factor and the disease prevalence is low as is the case with age and POAG in this study. Regression models both including and omitting age were employed for the POAG association analyses.

POAG diagnosis (POAG or control): Diabetes mellitus, IOP and VCDR are all risk factors for POAG, making POAG diagnosis a confounder in association analyses of these traits. POAG diagnosis (POAG or control) was therefore included as a covariate in the regression models for diabetes, VCDR and IOP association analyses.

3.1.7.2.2 Correcting for multiple comparisons

In the association analyses, multiple comparisons of SNPs covering the genomic regions of interest were made. Corrections for multiple testing were performed using the Bonferroni method as well as the online SNPSpD (SNP spectral decomposition) interface (http://genepi.qimr.edu.au/general/daleN/SNPSpD/).[298,299]

3.1.7.3 Power calculations

Power calculations were performed using Quanto Version 1.2.[290, 291] A 'log-additive' mode of inheritance and a disease risk for POAG of 3% were assumed. The odds ratios used in the calculations were those reported (for specific SNPs represented in this panel) in the GWA studies that identified the genomic associations. Minor allele frequencies for each SNP and each population were calculated from the genotyping dataset for that population. The power to detect these odds ratios with 95% confidence 80% of the time was considered adequate.

3.2 Results

3.2.1 Study populations

3.2.1.1 South African dataset

The original study population consisted of 502 individuals (see Chapter 2.3). TaqMan® allelic discrimination for each of the 49 selected SNPs was successful in 366 of these individuals, of whom 179 had POAG and 187 were unaffected controls.

The demographic features and clinical findings in this subgroup of the study subjects are detailed in Appendix 13 in Tables A13.1 and A13.2. A summary of the data compared with the other African datasets may be found in Table 4.20 overpage. Appendix 13 also contains Figures A13.1 to A13.3 that compare the demographics and clinical findings of the POAG and control participants in the entire study population and in the 179 POAG and 187 control participants in whom genotyping was successful. The subgroup of 366 participants in whom genotyping was successful was representative of the entire study population of 502 individuals that was analysed in Chapter 2.3 in demographics, in tribal identity and in clinical findings. The conclusions in Chapter 2.4 on the whole study population may therefore be extrapolated to this subgroup.

3.2.1.2 African American dataset

Genotyping results in this population were available for 1419 individuals (686 POAG cases and 733 unaffected controls). There were 659 females in the dataset (46%). The mean age of the group was 61 years with the POAG patients older than the controls. Phenotypic data were incomplete, however in the participants in whom the data were available, the mean IOP and mean VCDR were significantly greater in the POAG than in the control participants. The IOP may have been a treated IOP in some instances. Within the POAG group, 23% had severe visual impairment (visual acuity less than 6/60 in either eye) from glaucoma. A summary of the data compared with the other African datasets may be found in Table 4.20 overpage.

3.2.1.3 Ghanaian dataset

Genotyping results in this population were available for 1069 individuals (484 POAG cases and 585 unaffected controls). There were 548 females in the dataset (49%). The mean age of the group was 63 years with the POAG patients older than the controls. Phenotypic data were incomplete, however in the participants in whom the data were available, the mean IOP and mean VCDR were significantly greater in the POAG than in the control participants. The IOP recorded was that on examination, which may have been a treated IOP in some instances. Within the POAG group, 52% had severe visual impairment (visual acuity less than 6/60 in either eye) from glaucoma. A summary of the data compared with the other African datasets may be found in Table 4.20 overpage.

3.2.1.4 Combined dataset

The combined dataset consisted of 2854 individuals of African descent of whom 1348 had glaucoma and 1506 were unaffected control subjects. There were 1388 females in the dataset (51%). The mean age of the group was 62 years with the POAG patients older than the controls. Phenotypic data were incomplete, however in the participants in whom the data were available, the mean IOP and mean VCDR were significantly greater in the POAG than in the control participants. Since the IOP was an untreated IOP at diagnosis in the South African dataset and it was sometimes a treated IOP in the other datasets, comparing and combining the IOP data is problematic. Within the POAG group, 36% had severe visual impairment (visual acuity less than 6/60 in either eye) from glaucoma.

Table 4.20 summarises the demographics of the individuals comprising each of these four datasets. The datasets differ in that the South African POAG participants were younger than the African American or Ghanaian patients, whereas the South African controls were older than the controls in the other two groups. The IOP comparison is not useful because the IOP recorded was not always the IOP at diagnosis. There were fewer African American POAG patients with severe visual impairment from glaucoma (23%) than in the South African or Ghanaian datasets (50% and 52% respectively).

Table 4.20 Demographics of the populations included in the POAG association analyses

Group		Total (POAG : Control)				
		South Africans	African Americans	Ghanaians	p	Combined
n		**366 (179 : 187)**	**1419 (686 : 733)**	**1069 (483 : 586)**		**2854 (1348 : 1506)**
Female	n	181 (84 : 97)	659 (316 : 343)	548 (212 : 336)	0.06 (0.67 ; **<0.05**) ¶	1388 (612 : 776)
	%	49.5 (46.9 : 51.9)	46.4 (46.1 : 46.8)	51.3 (43.9 : 57.3)		48.6 (45.4 : 51.5)
Age (years)	n	366 (179 : 187)	1260 (682 : 578)	563 (425 : 138)		2189 (1286 : 903)
	Mean	64.8 (59.4 : 69.9)	61.0 (67.0 : 54.0)	62.5 (63.4 : 59.7)	**<0.05 (<0.05: <0.05)** §	62.1 (64.8 : 58.2)
	SD	12.4 (13.6 : 8.4)	13 (12.4 : 9.6)	12.7 (12.3 : 13.5)		12.9 (12.8 : 11.9)
IOP (mmHg)	n	334 (147 : 187)	150 (130 : 20)	892 (389 : 503)		1376 (666 : 710)
	Mean	22.8 (35.2 : 13.2)	24.5 (26.0 : 14.7)	22.4 (31.3 : 15.6)	N/A	22.7 (31.0 : 15.0)
	SD	12.8 (9.5 : 2.7)	9.9 (9.8 : 3.1)	10.3 (9.7 : 3.4)		10.9 (10.1 : 3.6)
VCDR	n	366 (179 : 187)	1220 (674 : 546)	561 (427 : 134)		2147 (1280 : 867)
	Mean	0.63 (0.89 : 0.38)	0.63 (0.87 : 0.33)	0.75 (0.86 : 0.39)	<0.05 (0.45 : <0.05) §	0.67 (0.87 : 0.35)
	SD	0.29 (0.13 : 0.13)	0.41 (0.41 : 0.13)	0.23 (0.10 : 0.11)		0.36 (0.30 : 0.13)
VA	n	366 (179 : 187)	1241 (676 : 565)	549 (412 : 137)		2156 (1267 : 889)
	SVI	89 (89 : N/A)	155 (155 : N/A)	213 (213 : N/A)	<0.05 (**<0.05** : N/A) ¶	457 (457 : N/A)
	% SVI	24.3 (49.7 : N/A)	12.5 (22.9 : N/A)	38.8 (**51.7** : N/A)		21.2 (36.1 : N/A)

¶ Pearson's χ^2 test

§ One-way analysis of variance

IOP, IOP at diagnosis in the South African dataset; VA, visual acuity measurement available; SVI, severe visual impairment caused by glaucoma

3.2.2 SNP quality control

None of the 49 SNPs selected to cover the genomic regions of interest failed quality controls.

In the South African analysis a single SNP in the *CAV1* gene (rs3779512) had a genotype distribution in the control group that was not consistent with Hardy-Weinberg equilibrium ($p < 0.01$). All 49 SNPs had genotype distributions consistent with Hardy-Weinberg equilibrium ($p > 0.01$) in the other datasets. The rs3779512 SNP was excluded from further analysis in analyses of the South African data alone.

This panel of 49 SNPs included 9 SNPs that were genotyped in the study population as part of the BeadXpress Candidate Gene Study (Appendix 12; Table A12.1). A comparison of the genotyping results from the TaqMan® assays and the BeadXpress genotyping for each individual for each of these SNPs confirmed the validity of each method. The genotyping results correlated exactly except for SNP rs3779512 that had already been excluded from the analysis on the basis of Hardy-Weinberg estimation.

3.2.3 Correcting for multiple testing on SNP panel

Bonferroni corrected p-value for significance = 0.05/49 SNPs
= 0.0010

SNPSpD[295] corrected p-value for significance = 0.05/38 effective independent loci
= 0.0013

3.2.4 Power calculations

Table 4.21 summarises the power calculations for each of the populations analysed. The African American and combined datasets were well powered to detect all the reported associations with 95% confidence. The Ghanaian dataset was underpowered to detect the association of SNPs rs7518099 in *TMCO1* (78%), rs4236601 in *CAV1* (76%) and rs4901977 in *SIX1/6* (73%) with POAG but was otherwise adequately powered. The South African study population dataset was underpowered to detect any of the reported associations.

Table 4.21 Power calculations for the replication of glaucoma SNP associations from GWA studies for each dataset

Gene	SNP	Reference	OR		Ref.	WA	AA	SA	ALL
TMCO1	rs4656461	Burdon *et al.*[178]	1.51	**MAF**	0.12	0.25	0.24	0.31	0.27
				Power (%)		99	100	75	100
	rs7518099	Burdon *et al.*[178]	1.49	**MAF**	0.12	0.09	0.11	0.08	0.10
				Power (%)		78	95	35	100
CAV1/2	rs4236601	Thorleifsson *et al.*[177]	1.27	**MAF**	0.29	0.42	0.40	0.41	0.41
				Power (%)		76	90	35	100
CDKN2b	rs7049105	Wiggs *et al.*[173]	0.71	**MAF**	0.50	0.26	0.26	0.18	0.25
				Power (%)		90	98	39	100
	rs2151280	Wiggs *et al.*[173]	0.71	**MAF**	0.42	0.27	0.33	0.22	0.29
				Power (%)		90	99	44	100
	rs10120688	Burdon *et al.*[178]	1.32	**MAF**	0.48	0.36	0.46	0.37	0.41
				Power (%)		85	97	44	100
	rs10120688	Wiggs *et al.*[173]	0.71	**MAF**	0.40	0.36	0.46	0.37	0.41
				Power (%)		95	100	58	100
SIX1/6	rs4901977	Wiggs *et al.*[173]	1.27	**MAF**	0.44	0.34	0.42	0.47	0.34
				Power (%)		73	90	36	99

OR, odds ratio reported in the reference; MAF, minor allele frequency; Ref., minor allele frequency reported in the reference; WA, dataset of West Africans from Ghana; AA, African American dataset from Duke; SA, South African study population; ALL, combined dataset

3.2.5 South African study population association analyses

3.2.5.1 POAG association analysis

Appendix 14 contains the results of single SNP association testing with a diagnosis of POAG in the South African study population dataset for each SNP in Appendix 12. Allele frequencies, results of the χ^2 test and Fisher's exact test are documented in Table A14.1. The results of the logistic regression including the adjustment for gender and the adjustment for both age and gender are provided in Table A14.2. The gender-adjusted associations are summarised in Figure 4.31. There was no evidence of an association between POAG in black South Africans and this panel of SNPs in *TMCO1*, *CDKN2BAS-1,* the *CAV1/2* region*,* and the *SIX1/6* region.

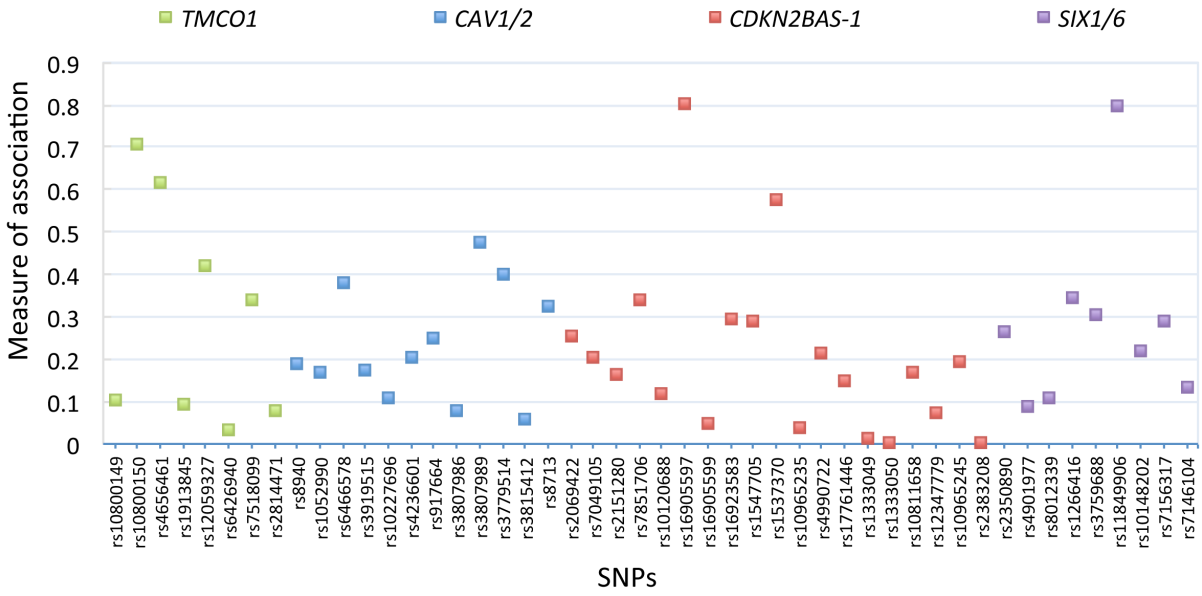

Figure 4.31. Association of SNPs with POAG in the South African study population. The y-axis is the measure of association (represented by $-\log_{10}p$) by logistic regression adjusted for gender in *TMCO1, CAV1/2, CDKN2BAS-1* and *SIX1/6*. On the logarithmic scale p = 0.05 is equivalent to 1.3 (not illustrated).

3.2.5.2 Diabetic association analysis

An association analysis of each of the 48 SNPs with a diagnosis of diabetes mellitus revealed no significant associations after adjusting for a diagnosis of POAG, gender and age in a logistic regression model.

3.2.5.3 Severe visual impairment from glaucoma

In a glaucoma subset analysis, each of the 48 SNPs was evaluated for an association with severe visual impairment (visual acuity of less than 6/60 in either eye) from glaucoma. There were no significant associations after adjusting for gender in a logistic regression model.

3.2.5.4 IOP association

A recent IOP GWA study by van Koolwijk *et al.*[189] implicated both *TMCO1* and the *CAV1/CAV2* genomic region, so the SNPs in these regions were further evaluated by linear regression with adjustment for age, gender and diagnosis (POAG or control) for an association with IOP in this population. There were no significant

associations identified.

3.2.5.5 VCDR association

Both the *CDKN2B* and *SIX1/SIX6* genomic regions were identified in a VCDR GWA study,[195] so the SNPs in the gene were further evaluated by linear regression with adjustment for age, gender and diagnosis (POAG or control) for an association with VCDR in this population. The adjusted associations are summarised in Figure 4.32.

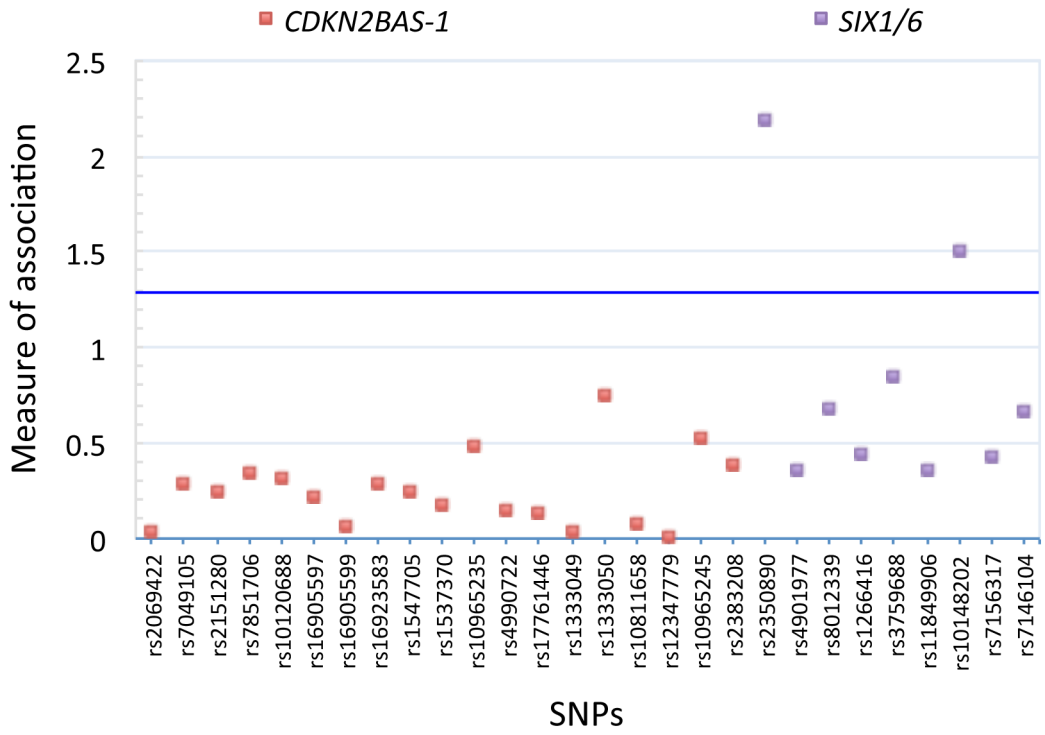

Figure 4.32 Association of SNPs with VCDR in the South African study population. The y-axis is the measure of association (represented by $-\log_{10}p$) by linear regression adjusted for age, gender and diagnosis (POAG or control) in *CDKN2BAS-1* and *SIX1/6*. On the logarithmic scale p = 0.05 is equivalent to 1.3 (blue line).

Two SNPs in the *SIX1/SIX6* genomic region (rs2350890 and rs10148202) were associated with VCDR in the adjusted model ($p<0.05$). Neither SNP was evaluated in this region in the BeadXpress study. Table 4.22 contains details of the associations. Neither association withstood correction for multiple testing using the Bonferroni method ($p>0.0018$).

Table 4.22 Association of SNPs in *SIX1/SIX6* with VCDR

SNP	Genotype	Frequency	VCDR (mean ± SD)	p	Adjusted p
rs2350890	CC	0.22	0.64 ± 0.30	0.4350	**0.0065**
	TC	0.54	0.60 ± 0.28		
	TT	0.24	0.68 ± 0.29		
rs10148202	AA	0.03	0.66 ± 0.23	0.1949	**0.0317**
	GA	0.21	0.67 ± 0.28		
	GG	0.76	0.62 ± 0.29		

p was obtained from linear regression; Adjusted p, p obtained from linear regression with adjustment for diagnosis (POAG or control), age and gender

3.2.6 Comparing association analyses in the populations of African descent

3.2.6.1 TMCO1

3.2.6.1.1 Allele frequencies

The allele frequencies for each SNP included in the *TMCO1* region in the South African study population and in the Ghanaian and African American datasets were similar to each other. The allele frequencies are compared with each other and with two HapMap populations (YRI, Yoruba from Nigeria and CEU, Utah residents with European ancestry) in Figure 4.33. The figure illustrates that the populations of African descent all have quite similar allele frequencies, whereas in many of the SNPs in this region the population of European descent has markedly different allele frequencies. In SNPs rs4656461, rs7518099 and rs2814471 the allele frequencies were similar across all the populations illustrated.

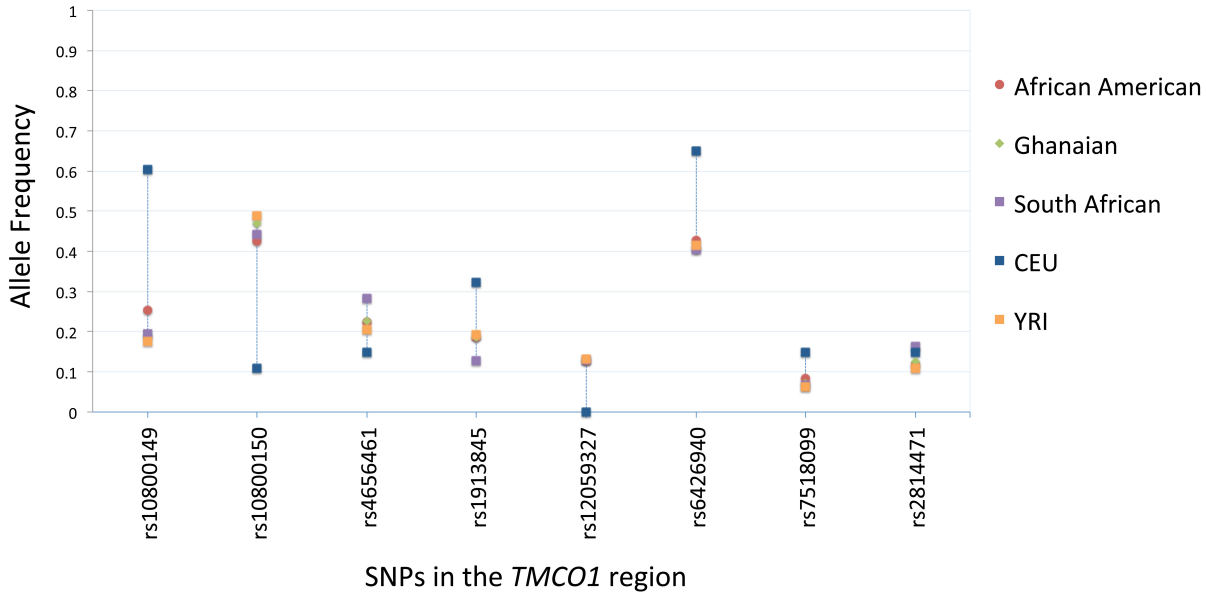

Figure 4.33 Allele frequencies of the SNPs in the *TMCO1* region. Allele frequencies are represented on the y-axis in the South African, Ghanaian and African American datasets compared with those from two HapMap populations (YRI, Yoruba from Nigeria; CEU, Utah residents with European ancestry).

3.2.6.1.2 POAG association

The POAG association analyses for each SNP in the *TMCO1* region after adjustment for gender are illustrated in Figure 4.34. The combined dataset was also adjusted for population (South African, Ghanaian or African American).

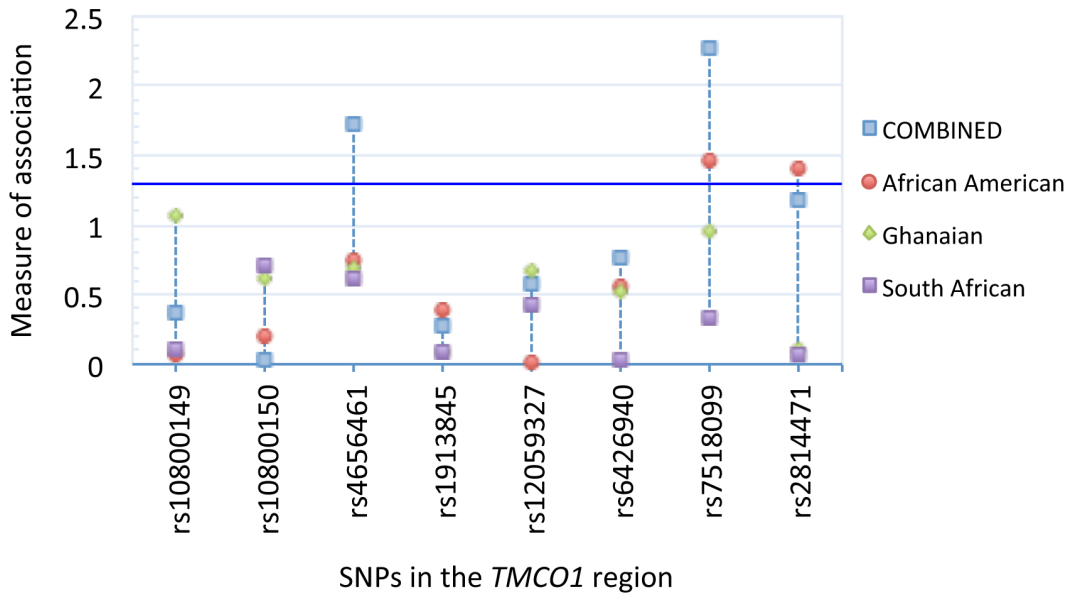

Figure 4.34 POAG association of SNPs in *TMCO1* in populations of African descent. The y-axis is the measure of association with POAG (represented by $-\log_{10}p$) by logistic regression adjusted for gender in the South African, African American and Ghanaian datasets and adjusted for gender and population in the combined dataset. On the logarithmic scale p = 0.05 is equivalent to 1.3 (blue line).

Two SNPs (rs4656461 and rs7518099) were associated with glaucoma in the combined dataset. SNP rs7518099 also showed an association in the African American dataset, as did another SNP (rs2814471) that was not associated in the combined group. Details of these associations are tabulated in Table 4.23. None of the adjusted associations withstood correction for multiple testing using either the Bonferroni method ($p > 0.0010$) or the p corrected using the SNPSpD interface ($p > 0.0013$).

Table 4.23 SNPs in *TMCO1* associated with POAG

SNP : MA	Population	MAF POAG : Controls	p	OR* (95% CI)	p*	OR** (95% CI)	p**
rs4656461 : G	South African	0.32 : 0.28	0.2539	1.21 (0.88 - 1.65)	0.2416	1.29 (0.91 - 1.83)	0.1501
	Ghanaian	0.26 : 0.23	0.1160	1.14 (0.93 - 1.39)	0.2006	1.34 (0.96 - 1.88)	0.0866
	African American	0.24 : 0.22	0.1694	1.13 (0.95 - 1.34)	0.1759	1.20 (0.97 - 1.49)	0.1000
	Combined	**0.26 : 0.23**	**0.0171**	**1.15 (1.02 - 1.30)**	**0.0192**	**1.15 (1.00 - 1.33)**	**0.0526**
rs7518099 : C	South African	0.08 : 0.07	0.4781	1.23 (0.71 - 2.13)	0.4563	1.32 (0.73 - 2.36)	0.3560
	Ghanaian	0.09 : 0.07	0.0738	1.29 (0.94 - 1.76)	0.1108	1.38 (0.82 - 2.31)	0.2247
	African American	0.11 : 0.08	**0.0301**	1.31 (1.02 - 1.68)	**0.0345**	1.31 (0.96 - 1.79)	0.0833
	Combined	**0.10 : 0.08**	**0.0035**	**1.3 (1.08 - 1.56)**	**0.0054**	**1.27 (1.02 - 1.58)**	**0.0346**
rs2814471 : C	South African	0.17 : 0.16	0.8698	1.04 (0.70 - 1.56)	0.8338	1.08 (0.70 - 1.67)	0.7302
	Ghanaian	0.13 : 0.13	0.5705	1.04 (0.80 - 1.34)	0.7851	1.07 (0.71 - 1.62)	0.7534
	African American	0.14 : 0.12	**0.0363**	1.26 (1.01 - 1.56)	**0.0400**	1.25 (0.96 - 1.63)	0.1030
	Combined	**0.14 : 0.13**	**0.0574**	**1.15 (0.99 - 1.34)**	**0.0662**	**1.11 (0.92 - 1.33)**	**0.2740**

*Adjusted for population (South African, Ghanaian or African American) and gender; **Adjusted for population (South African, Ghanaian or African American), age and gender

MA, minor allele; MAF, minor allele frequency; p, unadjusted p-value; OR (95% CI), odds ratio (95% confidence intervals)

The two SNPs associated in the combined dataset (rs4656461 and rs7518099) were identified by Burdon *et al.*[178] in the GWA study that first described the association of *TMCO1* with POAG. The study was in an Australian Caucasian population with a total sample size of 4561.[178] Figure 4.35 represents a comparison of the odds ratios for the associations as reported by the GWA study in the combined discovery and replication sets and for each of the datasets explored in this study.

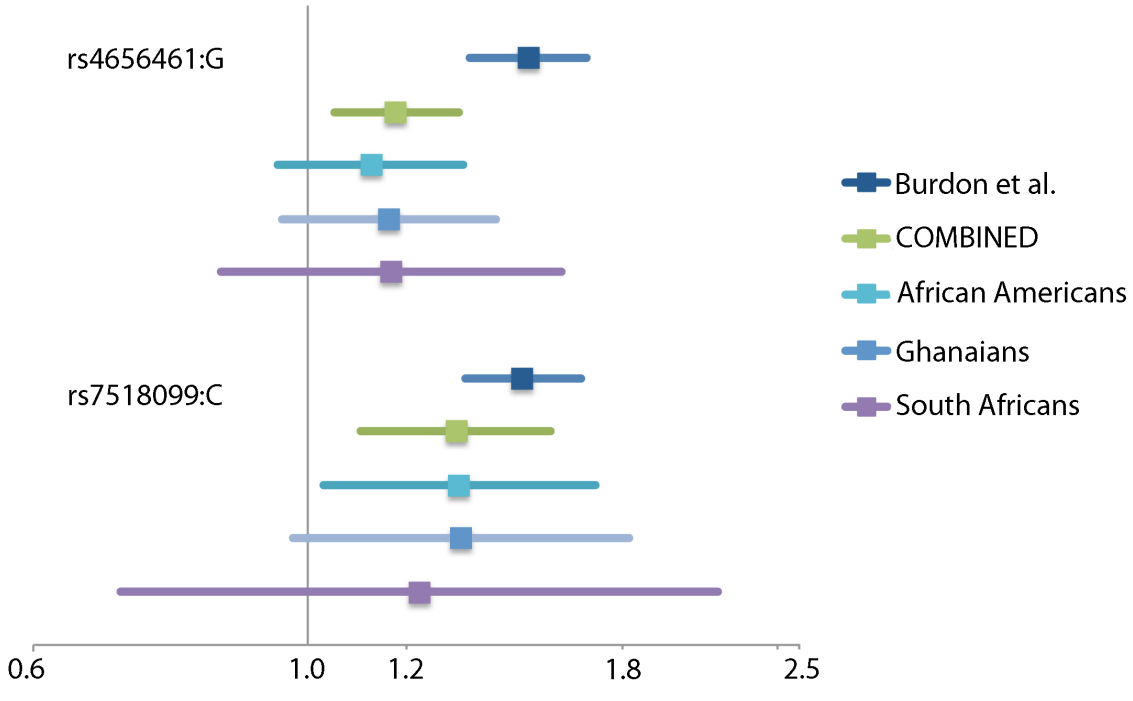

Figure 4.35 Forest plot of the odds ratios for SNPs rs4656461:G and rs7518099:C in *TMCO1*. Odds ratios (x-axis) are represented from the publication by Burdon *et al.*,[178] the South African, Ghanaian, African American and combined datasets. The squares indicate the odds ratios with the 95% confidence intervals denoted by the lines.

From the figure it is apparent that the odds ratios for association with the risk alleles of these SNPs and POAG are very similar for the populations of African descent. The association, however, only becomes significant in the larger datasets when the confidence intervals are smaller. In the combined dataset this is the case, whereas the South African dataset is the smallest with wide confidence intervals crossing the axis (OR = 1.0). The risk conferred is smaller than that reported by Burdon *et al.*[178] and therefore requires a larger sample size to confirm that risk.

3.2.6.1.3 Severe visual impairment from glaucoma

In a glaucoma subset analysis, SNPs in *TMCO1* were evaluated for an association with severe visual impairment (visual acuity of less than 6/60 in either eye) from glaucoma in each population. None of the SNPs genotyped in the region was found to be associated with severe visual impairment caused by glaucoma in any of the individual populations or in the combined African dataset.

3.2.6.1.4 IOP association

A recent IOP GWA study by van Koolwijk *et al.*[189] implicated *TMCO1*, so the SNPs in the gene were further evaluated by linear regression with adjustment for age, gender, population and diagnosis (POAG or control) for an association with IOP. SNP rs1913845 was marginally associated with IOP (p=0.0476) in only the African American dataset. This result should be interpreted with caution because IOP data were only available for 150 individuals in that dataset. None of the other SNPs genotyped in the region was found to be associated with IOP in any of the individual populations or in the combined African dataset.

3.2.6.2 CAV1/CAV2

3.2.6.2.1 Allele frequencies

The allele frequencies for each SNP included in the *CAV1/CAV2* region in the South African study population and in the Ghanaian and African American datasets were similar to each other. The allele frequencies are compared with each other and with two HapMap populations (YRI, Yoruba from Nigeria and CEU, Utah residents with European ancestry) in Figure 4.36. The figure illustrates a marked difference in the African study populations and the European population for most of the SNPs in this genomic region. In addition the allele frequencies from the Yoruba differ from the three African study populations in SNPs rs3919515, rs4236601, rs3779512 and rs3779514.

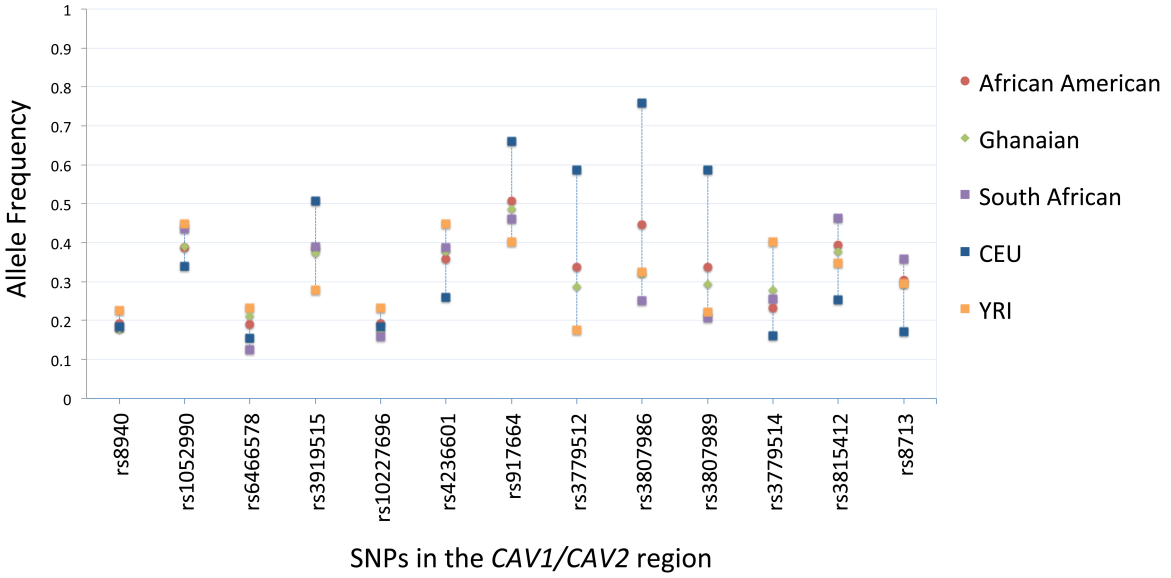

Figure 4.36 Allele frequencies of the SNPs in the *CAV1/CAV2* region. Allele frequencies are represented on the y-axis in the South African, Ghanaian and African American datasets compared with those from two HapMap populations (YRI, Yoruba from Nigeria; CEU, Utah residents with European ancestry).

3.2.6.2.2 POAG association

Figure 4.37 summarises the POAG association analyses for each SNP in the *CAV1* and *CAV2* regions after adjustment for gender. The combined dataset was also adjusted for population (South African, Ghanaian or African American).

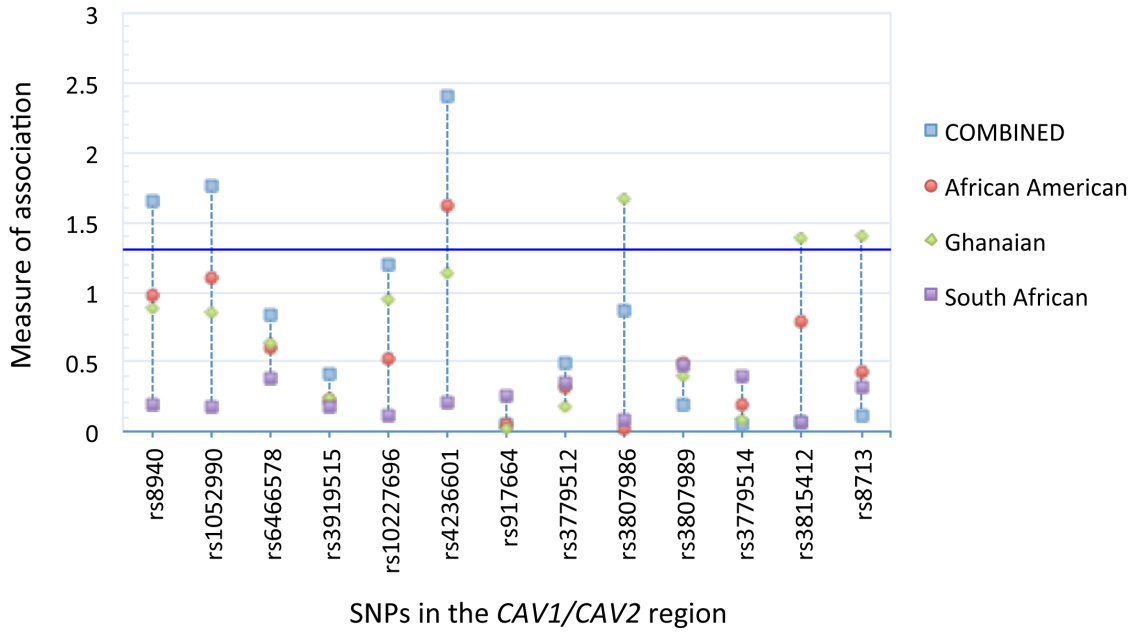

Figure 4.37 POAG association of SNPs in *CAV1/CAV2* in populations of African descent. The y-axis is the measure of association with POAG (represented by $-\log_{10}p$) by logistic regression adjusted for gender in the South African, African American and Ghanaian datasets and adjusted for gender and population in the combined dataset. On the logarithmic scale p = 0.05 is equivalent to 1.3 (blue line).

Three SNPs (rs8940, rs1052990 and rs4236601) were associated with glaucoma in the combined dataset ($p<0.05$). SNP rs4236601 also showed an association in the African American dataset. Details of the associations with POAG for each of these SNPs in each of the four African datasets are tabulated in Table 4.24. Only the association with rs4236601 remained after including age in the logistic regression analysis. None of the associations withstood correction for multiple testing using either the Bonferroni method ($p > 0.0010$) or the p corrected using the SNPSpD interface ($p > 0.0013$).

Table 4.24 SNPs in *CAV1/CAV2* associated with POAG in the combined dataset

SNP : MA	Population	MAF POAG : Controls	p	OR* (95% CI)	p*	OR** (95% CI)	p**
rs8940 : G	South African	0.20 : 0.18	0.6608	1.09 (0.75 - 1.58)	0.6495	1.09 (0.72 - 1.65)	0.6762
	Ghanaian	0.21 : 0.18	0.1132	1.18 (0.95 - 1.47)	0.1309	1.36 (0.93 - 1.98)	0.1099
	African American	0.22 : 0.19	0.1131	1.17 (0.97 - 1.41)	0.1058	1.17 (0.93 - 1.48)	0.1919
	Combined	**0.21 : 0.19**	**0.0233**	**1.17 (1.02 - 1.33)**	**0.0223**	**1.16 (0.99 - 1.36)**	**0.0691**
rs1052990 : C	South African	0.45 : 0.44	0.6560	1.06 (0.80 - 1.42)	0.6757	1.00 (0.73 - 1.37)	0.9953
	Ghanaian	0.42 : 0.39	0.1248	1.14 (0.96 - 1.36)	0.1419	1.14 (0.86 - 1.51)	0.3524
	African American	0.42 : 0.39	0.0793	1.15 (0.98 - 1.33)	0.0780	1.15 (0.95 - 1.39)	0.1458
	Combined	**0.43 : 0.39**	**0.0185**	**1.14 (1.02 - 1.27)**	**0.0172**	**1.10 (0.97 - 1.25)**	**0.1356**
rs4236601 : A	South African	0.41 : 0.39	0.6000	1.08 (0.80 - 1.45)	0.6252	1.06 (0.77 - 1.46)	0.7364
	Ghanaian	0.42 : 0.38	0.0658	1.18 (0.98 - 1.4)	0.0735	1.12 (0.84 - 1.49)	0.4242
	African American	0.40 : 0.36	0.0249	1.19 (1.02 - 1.39)	0.0240	1.20 (0.99 - 1.46)	0.0644
	Combined	**0.41 : 0.37**	**0.0042**	**1.17 (1.05 - 1.31)**	**0.0039**	**1.15 (1.01 - 1.31)**	**0.0374**

*Adjusted for population (South African, Ghanaian or African American) and gender; **Adjusted for population (South African, Ghanaian or African American), age and gender

MA, minor allele; MAF, minor allele frequency; p, unadjusted p-value; OR (95% CI), odds ratio (95% confidence intervals)

The most significantly associated SNP (rs4236601) was identified in association with POAG in the GWA study reported by Thorleifsson *et al.*[177] The GWA study in question was in a discovery cohort of 1263 POAG patients and 34 877 controls from Iceland, but the replication cohorts included two Asian populations.[177] Figure 4.38 represents a comparison of the odds ratios for the associations as reported by that

GWA study in the combined discovery and replication sets and for each of the datasets explored in this study.

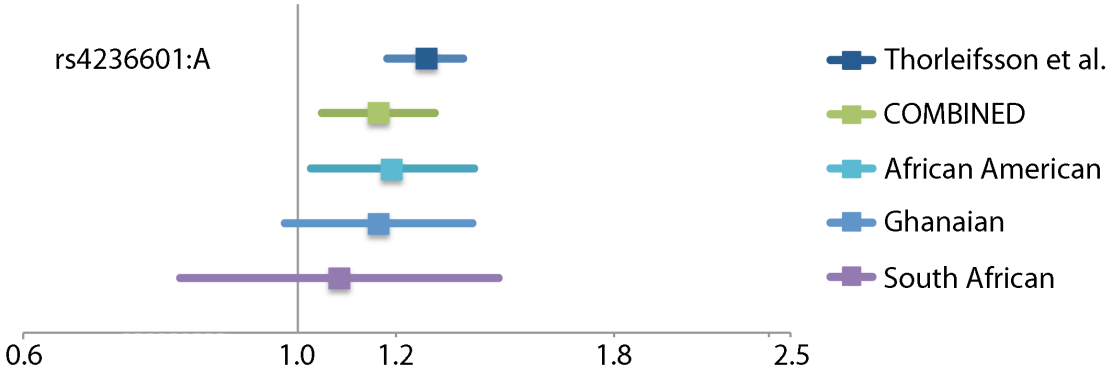

Figure 4.38 Forest plot of the odds ratios for SNP rs4236601:A in *CAV1*. Odds ratios (x-axis) are represented from the publication by Thorleifsson *et al.*,[177] the South African, Ghanaian, African American and combined datasets. The squares indicate the odds ratios with the 95% confidence intervals denoted by the lines.

From the figure it is apparent that the odds ratios for association with the A allele of SNP rs4236601 and POAG are similar for the populations of African descent. The association, however, only becomes significant in the larger datasets when the confidence intervals are smaller. In the combined and African American datasets this is the case, whereas the South African dataset is the smallest with wide confidence intervals crossing the axis (OR = 1.0). The risk conferred by the allele is smaller than that reported by Thorleifsson *et al.*[177] in all the African populations and is smallest for the South African dataset. A larger sample size would be required to confirm this risk.

A further three SNPs in *CAV1/CAV2* (rs3807986, rs3815412 and rs8713) showed an association only in the Ghanaian dataset. Table 4.25 contains the association results for these SNPs in the Ghanaian dataset only. None of the associations remained significant when age was included as a covariate in the logistic regression model and no SNP withstood correction for multiple testing using either the Bonferroni method ($p > 0.0010$) or the p corrected using the SNPSpD interface ($p > 0.0013$).

Table 4.25 SNPs in *CAV1/CAV2* associated with POAG in the Ghanaian dataset

SNP : MA	MAF POAG : Controls	p	OR* (95% CI)	p*	OR** (95% CI)	p**
rs3807986 : T	0.36 : 0.32	**0.0332**	1.24 (1.03 - 1.49)	**0.0212**	1.25 (0.92 - 1.69)	0.1491
rs3815412 : G	0.42 : 0.38	0.0632	1.21 (1.01 - 1.45)	**0.0408**	1.12 (0.84 - 1.5)	0.4428
rs8713 : C	0.33 : 0.29	0.0601	1.22 (1.01 - 1.47)	**0.0398**	1.1 (0.81 - 1.49)	0.5466

*Adjusted for gender; **Adjusted for age and gender

MA, minor allele; MAF, minor allele frequency; p, unadjusted p-value; OR (95% CI), odds ratio (95% confidence intervals)

3.2.6.2.3 Severe visual impairment from glaucoma

In a glaucoma subset analysis, SNPs in *CAV1/CAV2* were evaluated for an association with severe visual impairment (visual acuity of less than 6/60 in either eye) from glaucoma in each population. The results of the logistic regression analysis for each SNP in the region including population and gender as covariates are represented in each population in Figure 4.39.

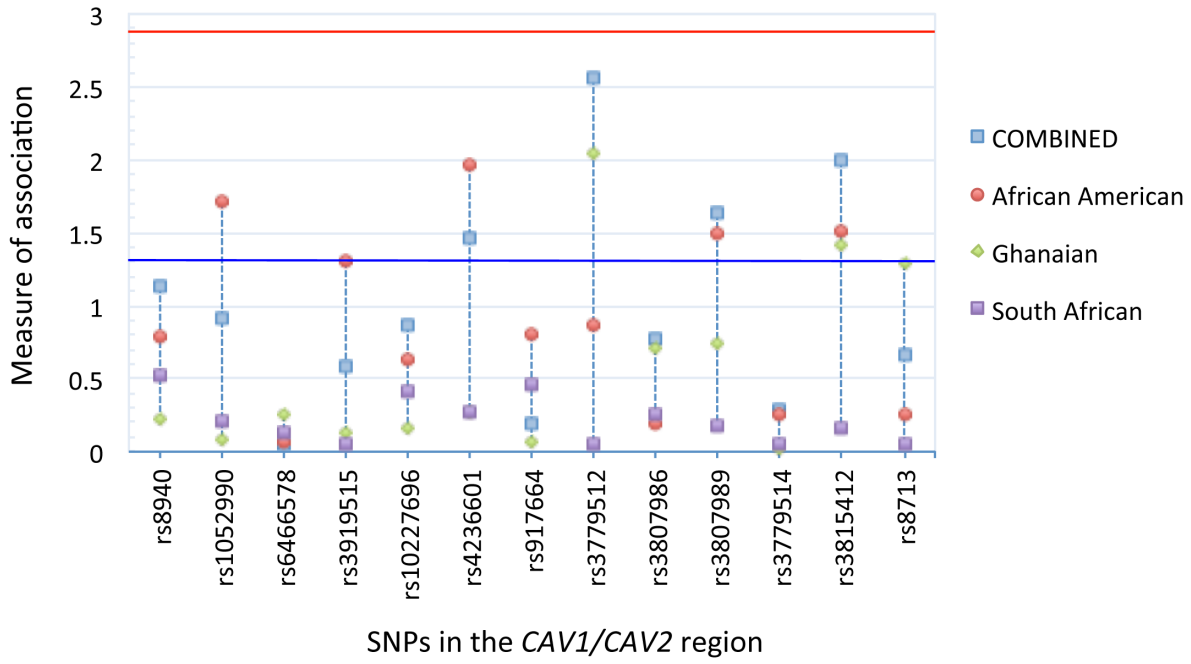

Figure 4.39 Association of SNPs in *CAV1/CAV2* with severe visual impairment caused by glaucoma in populations of African descent. The y-axis is the measure of association with severe visual impairment (represented by $-\log_{10}p$) by logistic regression adjusted for gender in the South African, African American and Ghanaian datasets and adjusted for gender and population in the combined dataset. On the logarithmic scale p = 0.05 is equivalent to 1.3 (blue line). The red line represents the p corrected for multiple testing using the SNPSpD interface.

In the combined dataset there was an association with severe visual impairment and four SNPs (rs8940, rs4236601, rs3779512 and rs3815412) in the region ($p<0.05$). SNP rs4236601 was the SNP most strongly associated with POAG in the POAG

association analysis and rs8940 was also associated with POAG, however rs3779512 was not associated with POAG in any of the populations and rs3815412 was only marginally associated with POAG in the Ghanaian dataset. SNP rs4236601 was also associated with severe visual loss in the African American dataset, as was one additional SNP (rs1052990). SNPs rs3779512 and rs3815412 were also associated with severe visual impairment from glaucoma in the Ghanaian dataset, as was one additional SNP (rs8713). There were no associations with SNPs in this region and visual loss in the South African dataset. None of the associations withstood correction for multiple testing using either the Bonferroni method ($p > 0.0010$) or the p corrected using the SNPSpD interface ($p > 0.0013$).

3.2.6.2.4 IOP association

The results of the IOP GWA study by van Koolwijk *et al.*[189] supported an association of *CAV1/CAV2* with IOP, so the SNPs in the gene were further evaluated by linear regression with adjustment for age, gender, population and diagnosis (POAG or control) for an association with IOP. None of the SNPs genotyped in the region was found to be associated with IOP in any of the individual populations or in the combined African dataset.

3.2.6.3 CDKN2BAS-1

3.2.6.3.1 Allele frequencies

The allele frequencies for each SNP included in the *CDKN2BAS-1* region in the South African study population and in the Ghanaian and African American datasets were similar to each other. The allele frequencies are compared with each other and with two HapMap populations (YRI, Yoruba from Nigeria and CEU, Utah residents with European ancestry) in Figure 4.40. The figure illustrates that the populations of African descent all have quite similar allele frequencies, whereas in many of the SNPs in this region the population of European descent has markedly different allele frequencies. In SNPs rs2069422, rs10120688, rs17761446, rs12347779 and rs2383208 the allele frequencies were similar across all the populations illustrated.

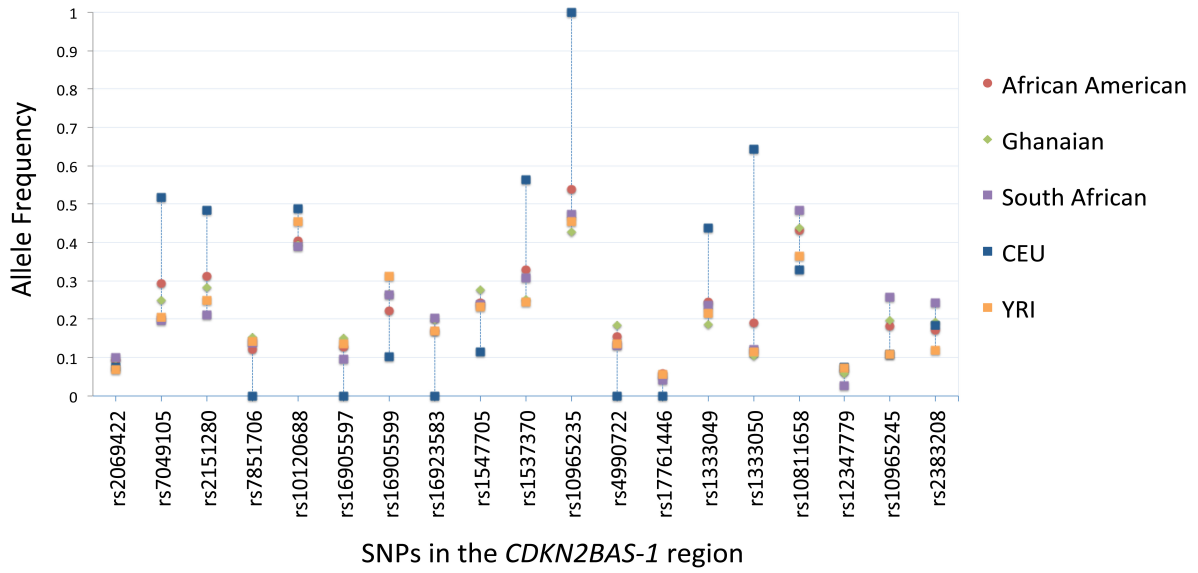

Figure 4.40 Allele frequencies of the SNPs in the *CDKN2BAS-1* region. Allele frequencies are represented on the y-axis in the South African, Ghanaian and African American datasets compared with those from two HapMap populations (YRI, Yoruba from Nigeria; CEU, Utah residents with European ancestry).

3.2.6.3.2 POAG association

Figure 4.41 summarises the POAG association analyses for each SNP in the *CDKN2BAS-1* region after adjustment for gender. The combined dataset was also adjusted for population (South African, Ghanaian or African American).

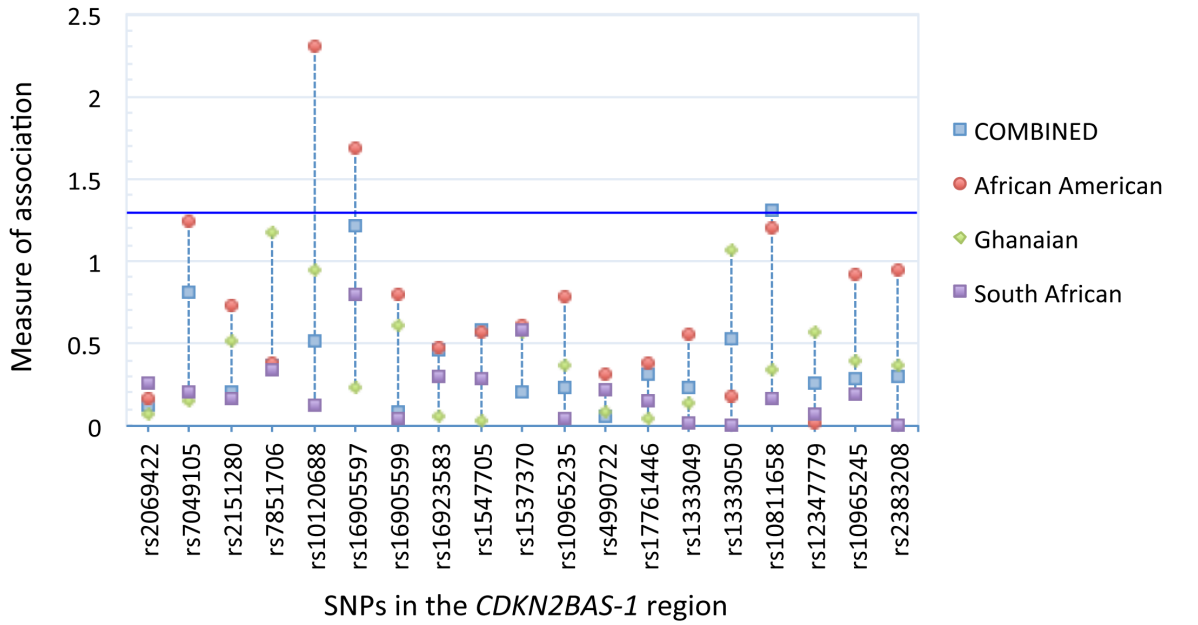

Figure 4.41 POAG association of SNPs in *CDKN2BAS-1* in populations of African descent. The y-axis is the measure of association with POAG (represented by $-\log_{10}p$) by logistic regression adjusted for gender in the South African, African American and Ghanaian datasets and adjusted for gender and population in the combined dataset. On the logarithmic scale p = 0.05 is equivalent to 1.3 (blue line).

Two SNPs (rs10120688 and rs16905597) were associated with glaucoma in the African American dataset (p<0.05). A single SNP, rs10811658, showed a weak association in the combined dataset (p=0.048). Details of these associations are tabulated in Table 4.26.

Table 4.26 SNPs in *CDKN2BAS-1* associated with POAG

SNP	Population	MA	MAF POAG : Controls	p	OR* (95% CI)	p*	OR** (95% CI)	p**
rs10120688	South African	A	0.38 : 0.39	0.7717	0.95 (0.71 - 1.29)	0.7579	1.01 (0.72 - 1.39)	0.9766
	Ghanaian	A	0.36 : 0.39	0.1265	0.87 (0.73 - 1.04)	0.1147	0.72 (0.54 - 0.96)	**0.0262**
	African American	A	0.46 : 0.40	**0.0049**	1.24 (1.07 - 1.44)	**0.0050**	1.19 (0.99 - 1.43)	0.0679
	Combined	**A**	**0.41 : 0.40**	**0.2859**	**1.06 (0.95 - 1.18)**	**0.3038**	**0.99 (0.87 - 1.12)**	**0.8733**
rs16905597	South African	A	0.07 : 0.10	0.1623	0.67 (0.39 - 1.17)	0.1579	0.67 (0.37 - 1.21)	0.1849
	Ghanaian	A	0.16 : 0.15	0.5284	1.07 (0.84 - 1.35)	0.5825	1.29 (0.86 - 1.94)	0.2177
	African American	A	0.10 : 0.13	**0.0190**	0.76 (0.60 - 0.96)	**0.0207**	0.74 (0.56 - 0.98)	**0.0335**
	Combined	**A**	**0.12 : 0.13**	**0.0818**	**0.86 (0.74 - 1.01)**	**0.0617**	**0.95 (0.78 - 1.15)**	**0.5815**
rs10811658	South African	G	0.47 : 0.48	0.6909	0.94 (0.70 - 1.26)	0.6776	0.85 (0.61 - 1.17)	0.3099
	Ghanaian	A	0.45 : 0.44	0.4193	1.07 (0.90 - 1.27)	0.4613	1.08 (0.82 - 1.43)	0.5905
	African American	A	0.47 : 0.43	0.0635	1.15 (0.99 - 1.34)	0.0626	1.12 (0.93 - 1.35)	0.2422
	Combined	**A**	**0.47 : 0.44**	**0.0452**	**1.11 (1.00 - 1.24)**	**0.0484**	**1.08 (0.95 - 1.23)**	**0.2165**

*Adjusted for population (South African, Ghanaian or African American) and gender; **Adjusted for population (South African, Ghanaian or African American), age and gender

MA, minor allele; MAF, minor allele frequency; p, unadjusted p-value; OR (95% CI), odds ratio (95% confidence intervals)

The association with rs16905597 in African Americans remained when age was included as a covariate in the logistic regression analysis, however the other associations were no longer significant. SNP rs10120688, that was not associated with POAG in the unadjusted or gender adjusted Ghanaian dataset, was associated with POAG in this population when age was included as a covariate. The risk allele (rs10120688 : A) in the African American dataset was, however, protective in the Ghanaian dataset. Combining the datasets therefore removed any association. None of the associations withstood correction for multiple testing using either the Bonferroni method (p > 0.0010) or the p corrected using the SNPSpD interface (p >

0.0013).

Three of the SNPs genotyped in the region (including SNP rs10120688) had been identified as having an association with POAG from GWA studies.[173, 178] Figure 4.42 represents a comparison of the odds ratios for the associations as reported by the GWA studies and from the populations of African descent. From the report by Burdon *et al.*,[178] the data from the combined discovery (total sample size of 4561 individuals of European descent) and replication sets were used and in the report by Wiggs *et al.*,[173] the data from the meta-analysis of the results of the NEIGHBOR (2170 POAG and 2347 controls of European descent) and GLAUGEN (976 POAG and 1140 controls of European descent) studies were used.

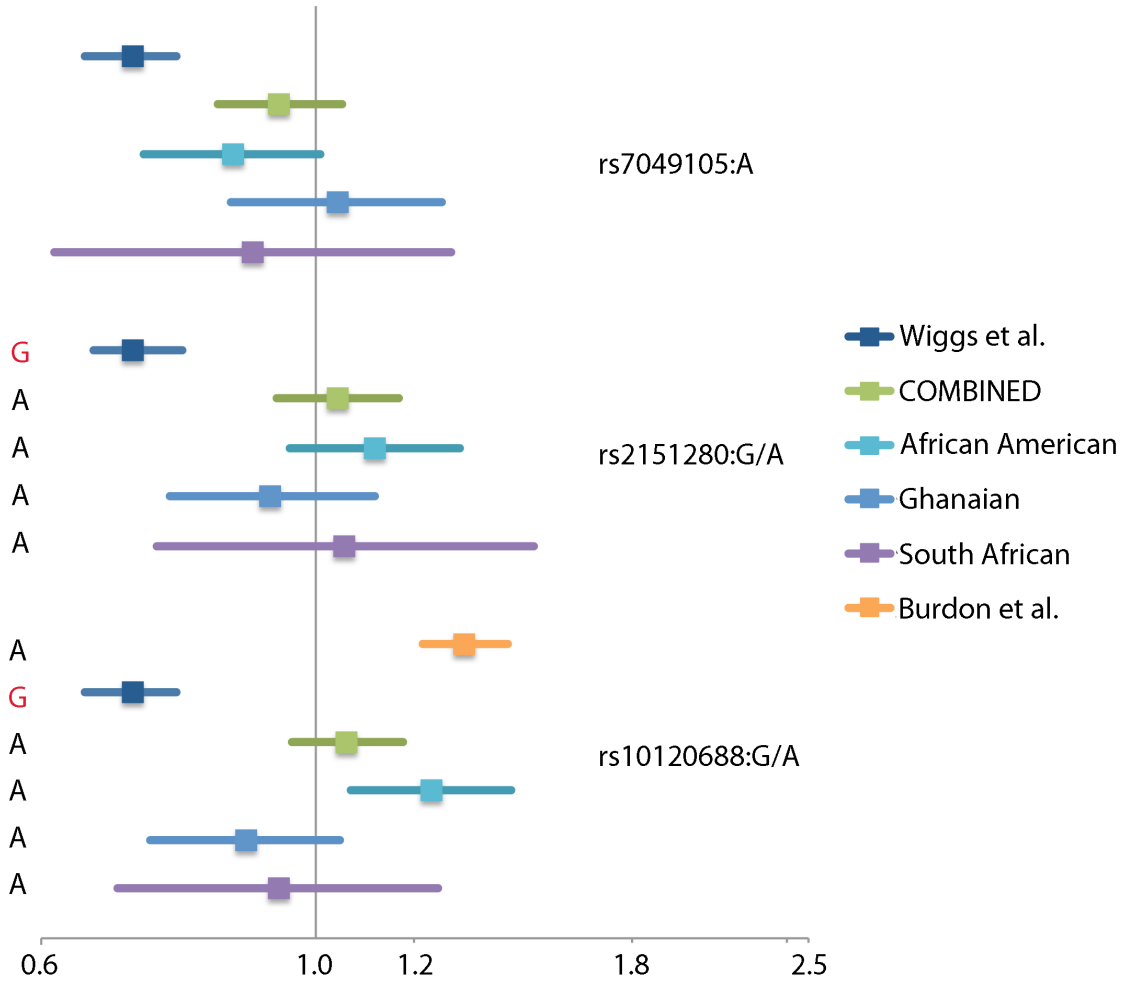

Figure 4.42 Forest plot of the odds ratios for SNP rs7049105:A, rs2151280 and rs10120688 in *CDKN2BAS-1*. Odds ratios (x-axis) are represented from the publications by Wiggs *et al.*,[173] Burdon *et al.*,[178] the South African, Ghanaian, African American and combined datasets. The squares indicate the odds ratios with the 95% confidence intervals denoted by the lines. The alleles for the odds ratios for SNPs rs2151280 and rs10120688 are on the left of the odds ratios.

From the figure it is apparent that the odds ratios for association with the risk alleles of these SNPs and POAG differ with the different populations. The odds ratios for rs10120688 reported by Burdon *et al.*[178] and Wiggs *et al.*[173] are deceptively dissimilar because they report on alternate alleles. This association with POAG is only replicated in the African American dataset. In the Ghanaian and South African datasets the A allele is marginally protective.

3.2.6.3.3 Severe visual impairment from glaucoma

In a glaucoma subset analysis, SNPs in *CDKN2BAS-1* were evaluated for an association with severe visual impairment (visual acuity of less than 6/60 in either eye) from glaucoma in each population. The results of the logistic regression analysis for each SNP in the region including population and gender as covariates are represented in each population in Figure 4.43.

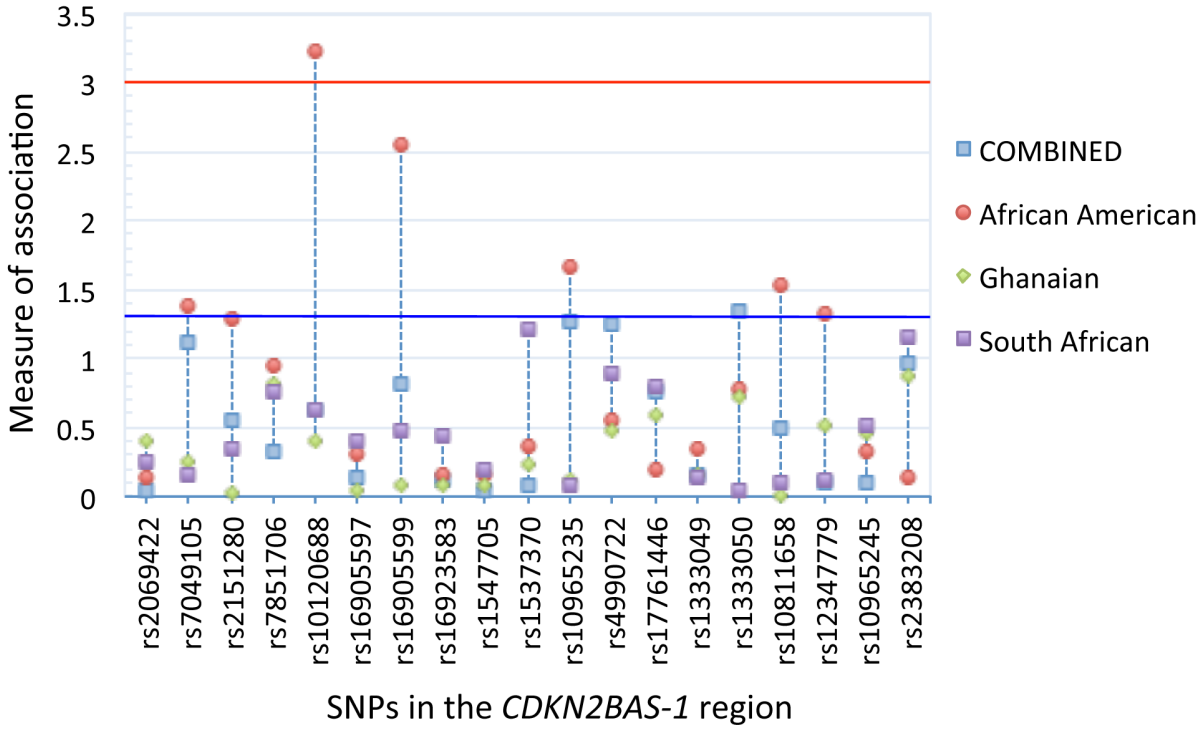

Figure 4.43 Association of SNPs in *CDKN2BAS-1* with severe visual impairment caused by glaucoma in populations of African descent. The y-axis is the measure of association with severe visual impairment (represented by $-\log_{10}p$) by logistic regression adjusted for gender in the South African, African American and Ghanaian datasets and adjusted for gender and population in the combined dataset. On the logarithmic scale p = 0.05 is equivalent to 1.3 (blue line). The red line represents the Bonferroni corrected level of significance (p=0.0010).

There were six SNPs (rs7049105, rs10120688, rs16905599, rs10965235, rs10811658 and rs12347779) in the region associated with severe visual loss from glaucoma in the African American dataset ($p<0.05$) and one SNP (rs1333050) marginally associated with visual loss in the combined group ($p=0.046$). The most significantly associated SNP in the African American group (rs10120688) was also the SNP most significantly associated with POAG in that population and the SNP identified in two POAG GWA studies in European populations.[173, 178] None of the other SNPs associated with visual loss was associated with POAG. The association with rs10120688 and visual loss from glaucoma in the African American dataset withstood correction for multiple testing using the Bonferroni method ($p=0.0006$; Bonferroni $p<0.0010$). The details of the association testing for this SNP are in Table 4.27.

Table 4.27 Association analysis of SNP rs10120688 (A allele) and severe visual impairment from glaucoma

Population	A allele frequency SVI : Controls	p	OR* (95% CI)	p*	OR** (95% CI)	p**
South African	0.34 : 0.39	0.2329	0.80 (0.55 - 1.16)	0.2346	0.84 (0.55 - 1.29)	0.4270
Ghanaian	0.37 : 0.39	0.4391	0.91 (0.72 - 1.14)	0.4009	0.77 (0.55 - 1.07)	0.1166
African American	**0.51 : 0.40**	**0.0004**	**1.55 (1.21 - 1.99)**	**0.0006**	**1.51 (1.12 - 2.03)**	**0.0061**
Combined	0.42 : 0.40	0.3057	1.10 (0.94 - 1.28)	0.2356	1.04 (0.88 - 1.23)	0.6453

*Adjusted for population (South African, Ghanaian or African American) and gender; **Adjusted for population (South African, Ghanaian or African American), age and gender

SVI, severe visual impairment from POAG; p, unadjusted p-value; OR (95% CI), odds ratio (95% confidence intervals)

The odds ratios for association of SNP rs10120688 with severe visual loss in POAG in the African datasets are represented graphically in the Forest plot in Figure 4.44.

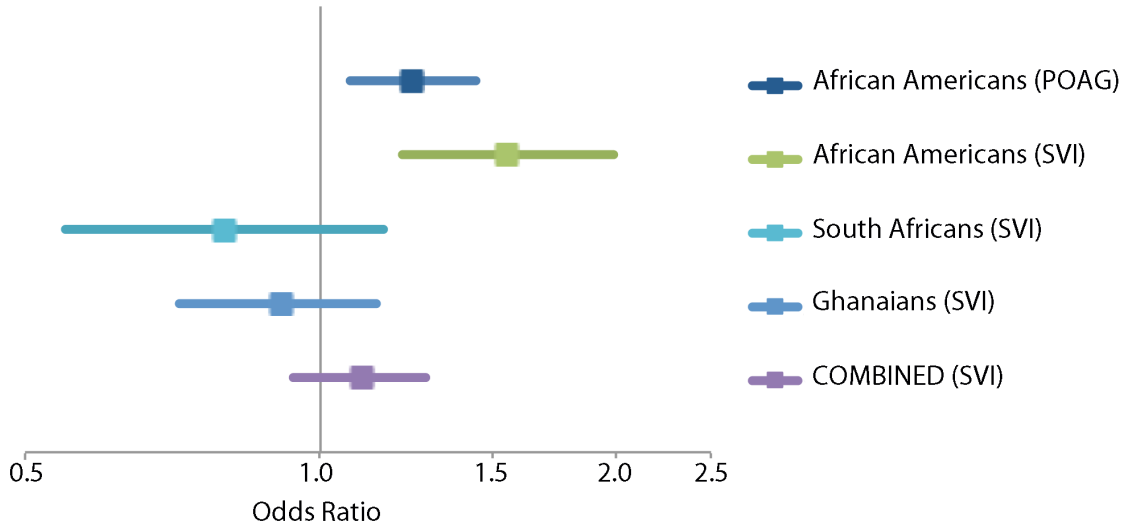

Figure 4.44 Forest plot of the odds ratios for the association of SNP rs10120688:A with severe visual impairment. Odds ratios (x-axis) are represented from the South African, Ghanaian, African American and combined datasets in the association with severe visual impairment (SVI). For comparison the odds ratio for association with POAG is represented in the African American dataset alone (POAG). The squares indicate the odds ratios with the 95% confidence intervals denoted by the lines.

From the figure it is apparent that the risk allele for severe visual impairment in African Americans is, if anything, protective in the other two African populations.

None of the other associations with SNPs in the region and visual loss from glaucoma withstood correction for multiple testing using either the Bonferroni method ($p > 0.0010$) or the p corrected using the SNPSpD interface ($p > 0.0013$).

3.2.6.3.4 VCDR association

The *CDKN2B* region was identified in a VCDR GWA study,[195] so the SNPs in the gene were further evaluated by linear regression with adjustment for age, gender, population and diagnosis (POAG or control) for an association with VCDR. None of the SNPs genotyped in the region was found to be associated with VCDR in the South African, Ghanaian or combined datasets. SNP rs1537370 was nominally associated with VCDR in the African American dataset only ($p=0.033$). The SNP was not associated with POAG or severe visual loss from POAG in this or any of the other populations tested. The association did not withstand correction for multiple testing.

3.2.6.4 SIX1/6

3.2.6.4.1 Allele frequencies

The allele frequencies for each SNP included in the *SIX1/SIX6* region in the South African study population and in the Ghanaian and African American datasets were similar to each other. The allele frequencies are compared with each other and with two HapMap populations (YRI, Yoruba from Nigeria and CEU, Utah residents with European ancestry) in Figure 4.45. The figure illustrates that the populations of African descent all have quite similar allele frequencies, whereas in many of the SNPs in this region the population of European descent has markedly different allele frequencies. In SNPs rs2350890, rs11849906, rs10148202 and rs7146104 the allele frequencies were more similar across all the populations illustrated.

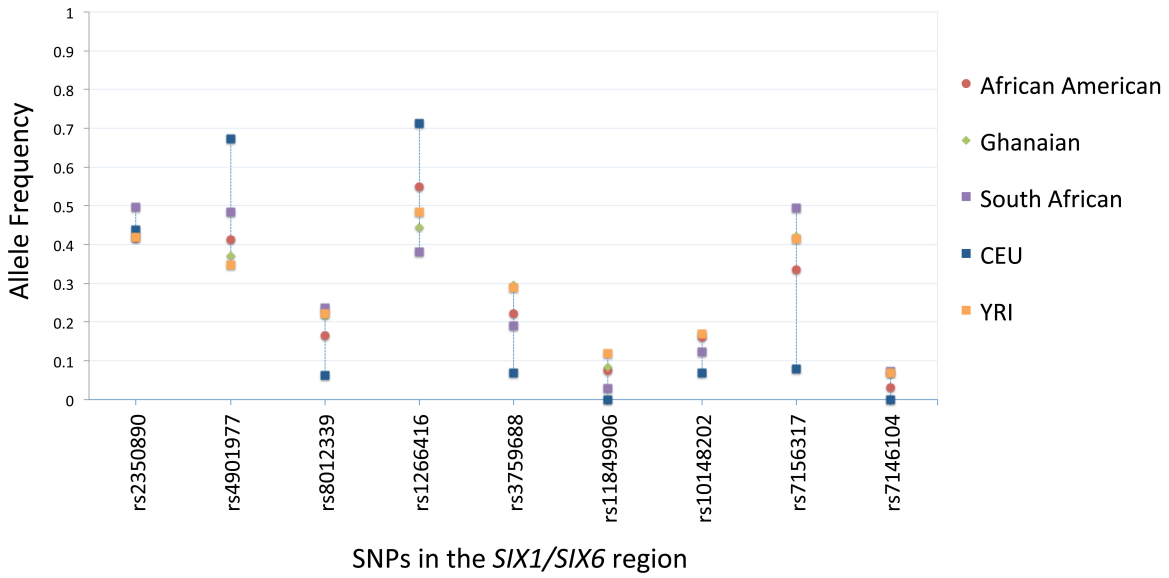

Figure 4.45 Allele frequencies of the SNPs in the *SIX1/SIX6* region. Allele frequencies are represented on the y-axis in the South African, Ghanaian and African American datasets compared with those from two HapMap populations (YRI, Yoruba from Nigeria; CEU, Utah residents with European ancestry).

3.2.6.4.2 POAG association

Figure 4.46 summarises the POAG association analyses for each SNP in the *SIX1/SIX6* region after adjustment for gender. The combined dataset was also adjusted for population (South African, Ghanaian or African American). There were no SNPs in this region associated with POAG in any of the populations.

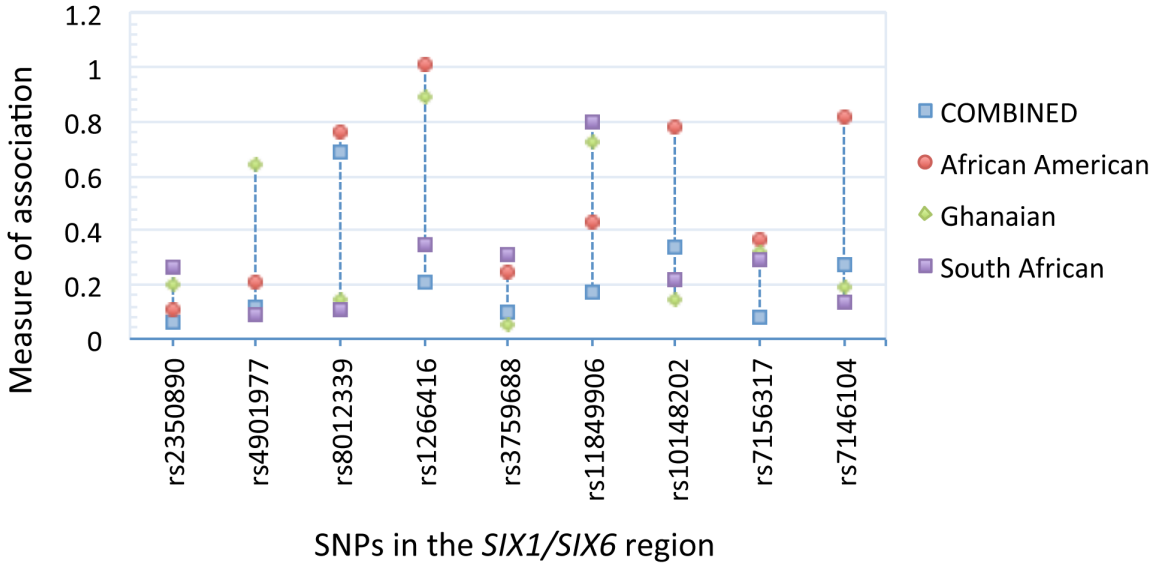

Figure 4.46 POAG association of SNPs in *SIX1/SIX6* in populations of African descent. The y-axis is the measure of association with POAG (represented by $-\log_{10}p$) by logistic regression adjusted for gender in the South African, African American and Ghanaian datasets and adjusted for gender and population in the combined dataset. On the logarithmic scale p = 0.05 (not illustrated).

One of the SNPs genotyped in the region (rs4901977) had been identified as having an association with POAG in the GWA study from the NEIGHBOR and GLAUGEN consortia.[173] Figure 4.47 represents a comparison of the odds ratios for the associations from the meta-analysis of the results of the NEIGHBOR and GLAUGEN studies (Wiggs *et al.*[173]) and from the populations of African descent.

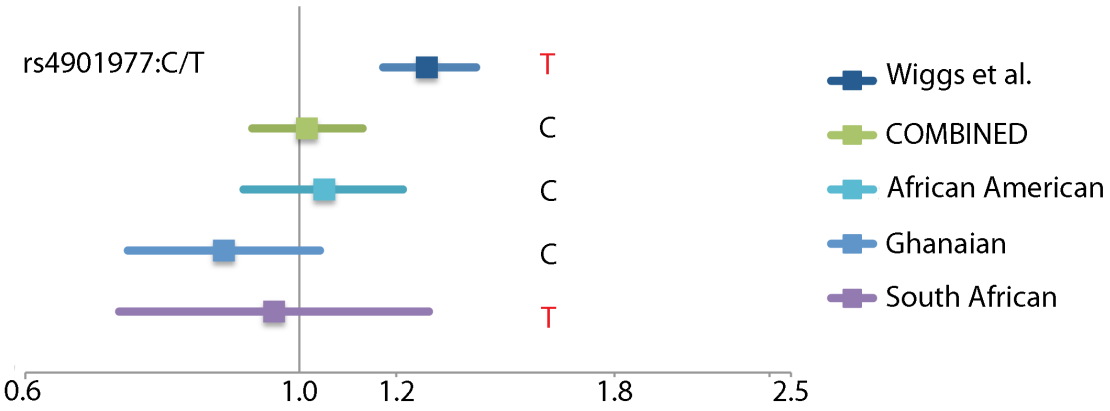

Figure 4.47 Forest plot of the odds ratios for SNP rs4901977 in *SIX1/SIX6*. Odds ratios (x-axis) are represented from the publication by Wiggs *et al.*,[173] the South African, Ghanaian, African American and combined datasets. The squares indicate the odds ratios with the 95% confidence intervals denoted by the lines. The alleles for the odds ratios are on the right of the odds ratios.

From the figure it is apparent that the risk associated with SNP rs4901977 identified

by Wiggs *et al.*[173] is not replicated in the populations of African descent either individually or together even when the alternate alleles used to report the odds ratios are taken into account.

3.2.6.4.3 Severe visual impairment from glaucoma

In a glaucoma subset analysis, SNPs in the *SIX1/SIX6* genomic region were evaluated for an association with severe visual impairment (visual acuity of less than 6/60 in either eye) from glaucoma in each population. None of the SNPs genotyped in the region was found to be associated with visual loss from glaucoma in the South African, Ghanaian or combined datasets. SNP rs7146104 was nominally associated with severe visual loss (after adjustment for gender) in the African American dataset only (p=0.025). The SNP was not associated with POAG in this or any of the other populations tested. The association did not withstand correction for multiple testing.

3.2.6.4.4 VCDR association

The *SIX1/SIX6* region was identified in a VCDR GWA study,[195] so the SNPs in the gene were further evaluated by linear regression with adjustment for age, gender, population and diagnosis (POAG or control) for an association with VCDR. None of the SNPs genotyped in the region was found to be associated with VCDR in the African American, Ghanaian or combined datasets. The SNPs associated with VCDR in the South African dataset (rs2350890 and rs10148202) were not associated with POAG or severe visual loss from POAG in this or any of the other populations tested.

4. DISCUSSION

4.1 Study population

The study population of black South Africans was representative of the region in which the study was conducted. It consisted of individuals with different ethnic affiliations evidenced by their different home languages. However the languages are all Southern Bantu languages (part of the Niger-Kordofanian linguistic macrofamily) and a structure analysis on the SNPs genotyped in the BeadXpress Study confirmed that the population is homogeneous but distinct from other African populations. This is not surprising in the context of the great genetic diversity in populations of African descent because of their ancestral age.[302]

The comparison with other populations of African descent was extended in the TaqMan® Genotyping Study performed at Duke University where there was a unique opportunity to compare and combine the genotyping results from the South African study population and Ghanaian and African American groups. The Ghanaian and South African populations fall into the same genetic cluster (one of six in Africa) identified by Tishkoff *et al.*[305] from a principal components analysis of 121 African populations. This may reflect the spread of Bantu-speaking populations from West Africa across Eastern and Southern Africa within the past 3000 to 5000 years. African Americans, while an admixed population, are also estimated to be of predominantly Niger-Kordofanian ancestry with approximately 13% European admixture and 8% admixture with other African populations.[305] African Americans have been shown to cluster genetically closely with West Africans, consistent with the historic slave trade origin of African Americans who are largely descended from ancestors in West Africa.[305] The allele frequency comparison suggests that the African Americans in this analysis were closer to the other two African populations and to the Yoruba from Nigeria than to the HapMap European population. In fact, the three African groups compared in the TaqMan® genotyping analysis have sufficient similarities (based on this small analysis of only 49 SNPs) to justify grouping them together to increase the power to detect small genetic effects in populations of African descent.

4.2 Association analyses

The primary objective of the study was to evaluate SNPs in candidate genomic regions for an association with POAG. In each instance this was performed with a χ^2 test or Fisher's exact test (unadjusted) or in a logistic regression model adjusted for gender or for both age and gender. The inclusion of gender as a covariate is justified because it is a potential confounder. The inclusion of age as a covariate tended to reduce the power to detect associations, without having a marked effect on the odds ratio for the effect size, however in some cases the inclusion of age introduced associations that were not otherwise identified as being significant. This may represent an artefact because age was strongly correlated with POAG in the study population as a result of selection bias. Adjustments for gender alone were therefore given more weight in the analyses.

4.3 Candidate gene associations

The candidate genomic regions that were evaluated in this study were selected for inclusion because they were located within known POAG loci, they had been identified in POAG association studies or they had been identified in ocular quantitative trait association studies. A detailed overview of the findings for each genomic region follows, but a summary of the candidate gene associations identified in this study is presented in Table 4.28.

Table 4.28 Summary of associations identified in the candidate gene association studies

Population			**SA**			**WA**	**AA**	**COMB**	**EUR**
Association	POAG	IOP	CCT	VCDR	DM	POAG	POAG	POAG	POAG
Known POAG Genes									
MYOC	**x**								
CYP1B1									
WDR36		**x**			**x**				
Regions identified in POAG association studies									
C2p16									
TMCO1							**x**	**x**	**x**
CAV1/CAV2						**x**	**x**	**x**	**x**
CDKN2B/BAS-1							**x**		**x**
SIX1/SIX6				**x**					**x**
Regions identified through ocular quantitative trait associations									
COL1A1	**x**								
COL1A2			**x**		**x**				
COL5A1									
COL8A2									
ZNF469	**x**		**x**						
ATOH7									

Associations are marked with an 'x', a red 'x' represents an association that withstands correction for multiple testing
SA; South African study population; DM, diabetes mellitus; WA, Ghanaian dataset; AA; African American dataset; COMB; combined African dataset; EUR, identified in European populations

4.3.1 Known POAG genes

4.3.1.1 MYOC

Pathogenic mutations in the *MYOC* gene have been demonstrated to be important in a percentage of black South Africans with POAG who presumably have a monogenic form of the disease (Chapter 3). In this candidate gene association study, 18 common variants in the gene were evaluated for an association with POAG in black South Africans. One variant had a weak association with POAG, indicating that this gene may be important in both the monogenic and genetically complex forms of the disease.

4.3.1.2 CYP1B1

This study failed to confirm the associations with POAG that have been reported with polymorphisms in the *CYP1B1* gene. This lack of association is corroborated by a recent meta-analysis of genetic polymorphisms in *CYP1B1* with POAG, which concluded that conflicting results reported in the literature may be the result of

different genetic structures among different populations.[106]

4.3.1.3 WDR36

There were no associations with POAG in black South Africans and SNPs in *WDR36*, but a weak association (that did not withstand correction for multiple testing) was identified with IOP and one SNP (rs1457113) in the gene. The 5q22 locus that contains the gene has been identified in association with IOP in two genome-wide linkage studies.[95, 206] The genetic effect of the risk allele of SNP rs1457113 on IOP was small in this study, but replicating the association in a larger group could suggest that *WDR36* is the associated gene within the locus.

A much more significant association with a SNP in *WDR36* that did withstand Bonferroni correction was identified with a self-reported history of diabetes mellitus in this population, and more specifically, type 2 diabetes mellitus. This SNP and three others that showed more marginal associations with diabetes mellitus in this study had no overlap with the SNP associated with IOP. This genetic association is not among the 3659 genes reported with diabetes mellitus and recorded in the Phenopedia section of the Human Genome Epidemiology Network (HuGENet™) as of July 2012.[306] A weakness of this finding is that body mass index was not recorded in this study, so the association results were not corrected for body mass index. The association with diabetes mellitus we have detected needs to be replicated in order for its significance to be confirmed.

Confirmation of an association with diabetes and a recognised POAG locus would strengthen the evidence for an independent association between these two disease entities. Although it is thought that the two diseases are associated on the basis of diabetic microvascular damage and altered vascular autoregulation of the retina and optic nerve, an alternate hypothesis may be related to elevated IOP associated with diabetes.[241, 243] Associations of *WDR36* with both diabetes mellitus and IOP could substantiate this hypothesis.

4.3.2 Regions identified in POAG association studies

4.3.2.1 Chromosome 2p16 region

This study did not identify a significant association with POAG in black South Africans and the seven SNPs genotyped in the chromosome 2p16 region that had all shown significant associations in the Afro-Caribbean population of Barbados.[175] The study was well powered to detect the magnitude of risk reported in that study. One SNP, rs11125375, showed a marginal association with the T allele, but the association did not withstand adjustment for gender or for age and gender. The odds ratio for the association was 1.4, which this study was underpowered to detect. This allele was the alternate allele to the risk allele identified by Jiao *et al.*[175] The minor allele frequencies of the SNPs in this study were different to the original study, suggesting that despite the commonality of African descent, these two populations are not similar. As with our study, no association was shown with this region and POAG in Japanese and Korean cohorts.[181, 307] Only a weak association has been reported with POAG and one SNP in this region in African-Americans (but not in Ghanaians).[182] This suggests that the reported association may be specific to the original population in which it was identified. It could be a local founder effect or it may represent genetic drift or region specific selection pressures. Alternately there may be an unidentified causal variant in this region with different levels of linkage disequilibrium with the genotyped SNPs in different populations.

4.3.2.2 TMCO1

Genotyping results were available from eight SNPs in the *TMCO1* genomic region in the South African, Ghanaian and African American datasets. The South African dataset alone did not have the statistical power to detect the odds ratio (OR = 1.5) reported in the GWA study by Burdon *et al.*[178] However, combining all three of these datasets increased the power. In the combined dataset, an association approaching significance after adjusting for gender and population and with correction for multiple testing was identified in a SNP (rs7518099) that the above-mentioned GWA study identified, but with smaller odds ratios. That this SNP was included in an attempt to replicate a previously identified association suggests that applying correction for

multiple testing may be too stringent. This provides evidence for an association with glaucoma and this region in different populations. It is therefore likely that the *TMCO1* gene locus represents a common pathway for glaucoma susceptibility across all populations. Further investigations into the function of the gene and its role in glaucoma are warranted.

The region was also evaluated for an association with IOP because it had been identified by van Koolwijk *et al.*[189] in an IOP GWA study. No convincing association with IOP was detected in any of the datasets of African origin suggesting that in these populations the association with POAG may be independent of IOP. However, the phenotypic data on IOP was incomplete, so the findings should be interpreted with caution.

4.3.2.3 CAV1 / CAV2

SNPs in the *CAV1* and *CAV2* genomic region were evaluated in both the BeadXpress genotyping project and in the TaqMan® study. Where the SNPs overlapped, the genotyping results concurred. None of the SNPs evaluated in this region was found to be associated with POAG in the South African study population, however the study was not powered to detect small genetic effects. In both the Ghanaian and African American datasets there were marginal associations with POAG and SNPs in *CAV1/CAV2*. Combining the South African dataset with the Ghanaian and African American datasets produced an association in SNP rs4236601 nearing significance after correction for multiple testing. This SNP was the most significantly associated SNP that had been detected by Thorleifsson *et al.*[177] in their GWA study and was therefore included in the study for replication purposes, suggesting that correction for multiple testing may not be appropriate. As was the case with the SNPs in *TMCO1*, the odds ratio was smaller for this African dataset than it was for the European dataset in which the association was first detected. An association with the *CAV1/CAV2* genomic region and glaucoma has been demonstrated in Europeans, in Asians and now in Africans suggesting that it plays a role in glaucoma susceptibilty across all populations, but the different odds ratios suggest different degrees of risk association.

A biological hypothesis for a role for the caveolin gene products in glaucoma exists with their regulatory capacity for TGF-β. In addition, the results of the IOP GWA study by van Koolwijk *et al.*[189] support an association of this region with IOP. No association with IOP was identified for SNPs in this region (bearing in mind the limitation of incomplete phenotypic information) and the study populations investigated. Nonetheless, the region, and how it affects a glaucoma phenotype, are deserving of further study in African populations.

4.3.2.4 CDKN2B / CDKN2BAS-1

Tagging SNPs and 'literature SNPs' were evaluated in the 9p21 locus using both BeadXpress and TaqMan® genotyping. Where the SNPs overlapped, the genotyping results concurred. The region was first identified in a VCDR GWA study, so each SNP was evaluated for an association with both POAG and with VCDR. This study did not show an association with any of the SNPs and VCDR in the South African study population. This result was anticipated because the genetic effect on VCDR of the 'literature SNP' identified by Ramdas *et al.*[195] was too small to be detected with this sample size. Expanding the association with VCDR to include the Ghanaian and African American datasets revealed a nominal association with one SNP in the region and VCDR in the African American dataset, but the association did not withstand correction for multiple testing and was not present in the combined dataset.

The POAG association analysis also revealed no significant associations with any of the SNPs in the South African study population. This may be because the association with POAG has been demonstrated to be stronger with the NTG phenotype:[171, 173, 174] NTG was very uncommon in the South African study population. Alternately there may be a small genetic risk associated with POAG in this population that this sample size was insufficient to detect.

Combining the datasets of African descent and thus increasing the sample size did not yield any effects that withstood correction for multiple testing. An association with POAG and this region has however been demonstrated in a population of African descent by Cao *et al.*[196] in the Afro-Caribbean population of Barbados. Furthermore,

the African American dataset alone had a SNP (rs10120688) with an association to POAG that approached significance after correction for multiple testing. This same SNP, that was also identified by Wiggs *et al.*[173] and Burdon *et al.*[178] in populations of European descent, was significantly associated with severe visual loss from glaucoma in the African American group. Comparing the association analyses for this SNP in the three African datasets revealed similar minor allele frequencies in the control groups, but the minor allele was the risk allele for both POAG and severe visual loss from POAG in the African Americans whereas the minor allele was, if anything, protective in the other two groups. Combining the three African datasets in this study therefore, not surprisingly, removed the significance seen in the African American population alone. This suggests that there are population differences at this locus within these three different populations of African descent. Combining the datasets in this instance, while increasing the power, in fact masks genuine population specific signals.

The *CDKN2B / CDKN2BAS-1* region has been convincingly demonstrated to be associated with POAG and NTG in several populations, but the findings of this study suggest that the genetic effect varies with different populations and within populations originating from Africa.

4.3.2.5 SIX1 / SIX6

SNPs in *SIX1, SIX6* and the intergenic region were evaluated in both the BeadXpress genotyping project and in the TaqMan® study. Where the SNPs overlapped, the genotyping results concurred. The region was first identified, along with the 9p21 region, by Ramdas *et al.*[195] in association with VCDR. The BeadXpress study did not show an association with any of the SNPs and VCDR in the South African study population. Two SNPs (rs2350890 and rs10148202) within the region, that were only evaluated with TaqMan® genotyping, did however demonstrate an association with VCDR after adjusting for POAG diagnosis, age and gender. The associations did not withstand correction for multiple testing and were not found in either of the other African populations or the combined dataset.

There were no SNPs that were associated with POAG in the South African study

population or in either of the other two African populations evaluated or in the combined group of individuals of African descent. The combined sample size was sufficient to replicate the effect size reported in the literature suggesting that the POAG susceptibility alleles in *SIX1/SIX6* discovered in European and Japanese populations play a greatly reduced or negligible role in populations of African descent.[172, 173]

4.3.3 Regions identified in ocular quantitative trait association studies

4.3.3.1 Collagen genes (COL1A1, COL1A2, COL5A1 and COL8A2)

The collagen genes *COL1A1, COL1A2, COL5A1* and *COL8A*2 were selected as candidate genes for POAG because of their association with CCT. Despite the study being powered to detect genetic effects on CCT of more than 8μm per risk allele, no significant associations were detected with SNPs in these regions and CCT in this population. There was a marginal association with a SNP in *COL1A2* and CCT and, in addition, individual SNPs in *COL1A1*and *COL8A2* showed a nominal association with POAG in the study population. These associations did not withstand correction for multiple testing. This lack of association confirms the findings of Ulmer *at al*.[234] who evaluated CCT in a POAG GWA study. As with this South African study, they found no associations with these genes for either CCT or POAG in the European population they evaluated.

The association analysis with diabetes mellitus revealed a nominal association with three SNPs in *COL1A2* and diabetes mellitus. The associations did not withstand correction for multiple testing.

4.3.3.2 ZNF469

The 16q24 genomic region was also included as a POAG candidate gene for its association with CCT. In this study a single SNP in the region was weakly associated with CCT. A different SNP was associated with POAG and in a glaucoma subset analysis was associated with glaucoma drainage surgery. None of the associations remained significant after correction for multiple testing.

4.3.3.3 ATOH7

ATOH7 was included as a POAG candidate gene in the BeadXpress study because the gene was identified in a GWA study in association with optic disc area and VCDR.[195] A SNP in the region was identified in association with POAG in the Afro-Caribbean population of Barbados in a study of similar size to this one.[196] The BeadXpress study was unable to replicate the association of SNPs in the gene with POAG. This provides further evidence of population differences between these two populations of African origin. This study did not identify an association with SNPs in the region and VCDR indicating that in this population there is a reduced role of this region in the susceptibility to optic disc parameters.

4.4 Limitations

The candidate genes selected were mostly selected out of work on European and Asian populations. European, Asian and African populations differ and may have different risk factors.

The tagging SNPs were selected using the Yoruba from Nigeria as the reference population, but also comparing the SNPs with tagging SNPs for the Luhya and Maasai from Kenya. The diversity within African populations suggests that these populations are imperfect proxies for black South Africans.

The candidate gene association studies were designed to detect moderate genetic risk effects in the South African dataset. This study did not detect any such effects. This does not exclude effects accounting for a small percentage of the risk for glaucoma. GWA studies have only found small risk effects to date. A larger sample size would be required to detect such effects. Combining datasets of different populations of African descent to achieve this increased the power to detect associations common to all the groups, but did not increase the power to detect population-specific effects.

4.5 Summary

This first candidate gene association study in a black South African population did not detect any significant genetic effects within the regions included in the study. Combining the South African data with data from a West African population and an African American population significantly increased the power to detect associations and identified associations of POAG with the *CAV1, CAV2* and *TMCO1* genomic regions. These are likely, therefore, to be common pathways for glaucoma susceptibility across all populations. The risk effect of the associated alleles was, however, less than that recorded in other populations.

The African American population alone had a significant association with a SNP in *CDKN2BAS-1* and severe visual impairment from glaucoma that was not strengthened by combining the African populations. This suggests that the African populations have different susceptibilities in this and potentially other genomic regions and that evaluating different African populations individually is still important.

A quantitative trait analysis did not find associations withstanding multiple correction with candidate genes and IOP, VCDR or CCT, but a significant association was identified with a SNP in *WDR36* and diabetes mellitus. This finding needs replication.

The results of the study suggest that the POAG genetic susceptibility alleles found in other populations play a reduced role in populations of African ancestry underscoring the need for large genome-wide association studies in African populations. Combining populations of African descent may be used in future studies of this type to increase the power to detect small genetic effects. Populations of African descent are, however, genetically different and should also be evaluated independently to identify population specific effects.

Chapter 5: Conclusion

1. PROBLEM DEFINITION

Glaucoma, an optic neuropathy characterised by specific functional and structural alterations to the optic nerve, is the most important cause of irreversible visual loss in South Africa. Primary open-angle glaucoma (POAG), the commonest type of glaucoma in South Africa, is consequently an important cause of morbidity in this country. The visual loss caused by POAG, while irreversible, may be prevented with early detection and treatment of the condition.

The pathogenesis of POAG is incompletely understood, however it is widely accepted that genetic factors plays a role in the development of the disease. POAG is genetically heterogeneous.

A subgroup of individuals with POAG has an apparently 'monogenic' form of the disease. The most important gene associated with this type of POAG is the *MYOC* gene that codes for myocilin. *MYOC* mutations have been documented to cause POAG in approximately 3% to 5% of individuals in several different population groups from around the world. Exploring the gene for mutations in different populations has identified numerous pathogenic mutations that are thought to mediate glaucoma through a gain-of-function model. The pathogenesis of myocilin glaucoma is, however, still unknown and the identification of new mutations remains important in understanding the disease. In addition, mutations specific to individual populations may be amenable to genetic screening in that population and may prevent visual loss.

The majority of POAG patients have a complex, multifactorial pattern of inheritance. Numerous genes have been identified as risk factors in this group. Many of the associations have only been reported in single studies, in one population group or in association with specific POAG phenotypes. More recently there have been successful genome wide association (GWA) studies that have identified common variants as genetic risk factors. These common variants are, however, only responsible for a small percentage of the risk for POAG. Identifying risk alleles

remains important for understanding the molecular basis of the disease, distinguishing subtypes and developing targeted therapies for the disease.

The most important risk factor for POAG is IOP that is an ocular quantitative trait. Evaluating common variants for associations with this, and other ocular quantitative traits implicated in POAG (CCT and VCDR) is therefore another approach to unravel the genetics of glaucoma.

Diabetes mellitus is thought to be an independent risk factor for POAG. Common genetic risk factors for both POAG and diabetes mellitus may help to define the association of these two common conditions.

Populations of African descent are significantly affected with POAG. It is commoner in African populations, occurs at a younger age and is more severe. Despite this, African populations are understudied with respect to POAG and more specifically with respect to the genetics of POAG. There have been no prior studies on the genetics of POAG in black South Africans. African populations are the most diverse in the world because of their ancestral age. Genetic studies in African populations could therefore identify new associations.

The purpose of this research was, therefore, to identify genetic risk factors for POAG in black South Africans. Ultimately it is hoped that this will translate into a better understanding of the pathogenesis of the disease, new treatments, earlier diagnosis and the prevention of irreversible visual loss from glaucoma.

2. STUDY OBJECTIVES

2.1 Hypothesis

The hypothesis was that there are identifiable genetic risk factors associated with POAG in black South Africans. The findings in this study support the hypothesis.

2.2 Specific aims

2.2.1 Aim 1: Characterise POAG phenotypically in black South Africans

The study population consisted of self-identified black South African adult POAG and control participants from the St John Eye Hospital in Soweto. The population was representative of the black population of South Africa with ten of the country's eleven official languages reflected as first languages in the sample. A significant proportion of the study population had hypertension and/or diabetes mellitus appropriate for the mean age (65 years) of the sample and the prevalence of these conditions in South Africa.

The POAG patients were deliberately younger than the controls to strengthen the diagnostic certainty in the control group. Hypertension, diabetes mellitus and lenticular opacities were over-represented in the control group because of the age bias and the fact that the controls were non-glaucomatous eye-clinic patients. Despite this there were significantly more POAG patients than control subjects with a family history of blindness. This provides evidence for the importance of the genetic component to the disease in this population.

The POAG participants had a mean age of diagnosis younger than that reported in non-African populations (despite also presenting late in the disease), they had higher IOPs at diagnosis and the disease was more advanced. More than half of the POAG patients in the study population were severely visually impaired as a result of the glaucoma. The only predictor for severe visual loss in this population was a larger VCDR.

The POAG patients had thinner corneas than the controls, but the mean CCT for the entire study population was significantly lower than that recorded in non-African populations. CCT was thicker in the diabetics and became thinner with age.

There were relatively few POAG patients known to have a juvenile onset for the condition, although it is likely that there were more patients in this category, but who presented at an older age with advanced disease. There were very few POAG patients in this study population with NTG.

POAG in this group of patients was treated medically, surgically or with laser either alone or in combination. More than half of the POAG participants were treated with glaucoma drainage surgery. This occurred more frequently in patients with higher IOPs and more advanced cupping. IOP control was achieved in more than two-thirds of the entire group of patients.

2.2.2 Aim 2: Determine the extent to which POAG in black South Africans is mediated by mutations in the *MYOC* gene

Sequence variants were identified within the exons and flanking non-coding regions of the *MYOC* gene in the study population. Most of the variant alleles occurred with similar frequencies in POAG and control participants suggesting that they were not associated with POAG. Two SNPs had allele frequencies that varied in the two groups, but not significantly. There were three potentially pathogenic mutations identified and subsequently explored in family members.

The first of these was the Lys500Arg mutation that was only identified in POAG patients and not in controls in the study population. However, the mutation is predicted to be tolerated and has been described in control subjects in other African populations. In addition this study identified one unaffected family member with the mutation and another family member who was a POAG suspect without the mutation. The evidence suggests that the mutation is a benign polymorphism.

The Gly374Val mutation was identified as a novel glaucoma-causing mutation. The mutation is predicted to be damaging to the protein. It occurred only in POAG

patients and not in controls with a prevalence of 0.9% in the POAG population. It was not identified in any family members.

The Tyr453del frameshift mutation was a known glaucoma-causing mutation thought to cause gain-of-function from a mutated protein that has only been described in African populations. It occurred in the South African POAG group with a prevalence of 2.3%: more commonly than in any other African population in which it has been observed. The mutation is incompletely penetrant because it was identified in two unaffected controls from the study population. Penetrance of the mutation, as estimated from this very small sample, increases from 25% at the age of 50 years to 80% at the age of 80 years. Despite the challenges of incomplete penetrance, family screening successfully identified a family member with undiagnosed POAG who is now receiving treatment for the condition. A further two family members considered to be at high risk for developing the disease were also identified and will be monitored for disease development. Visual loss has therefore been prevented through the screening of this mutation.

POAG in black South Africans is therefore mediated by mutations in the *MYOC* gene in approximately 3.3%. Screening of the mutations in black South African POAG patients would identify families suitable for cascade screening that would in turn allow for early diagnosis and the prevention of visual loss from glaucoma.

2.2.3 Aim 3: Determine the contribution of genetic variation within candidate genomic regions to POAG in black South Africans

Association studies using common genetic variants are a useful tool for uncovering genes associated with common conditions. The candidate genomic regions evaluated in this study were selected for inclusion because they were located within known POAG loci, they had been identified in POAG association studies in other populations or they had been identified in association with ocular quantitative traits.

The study was powered to detect moderate genetic effects that would consequently be most valuable clinically. There were no significant associations that withstood correction for multiple testing detected with POAG in black South Africans and

common variants in any of the genomic regions included in the study (*MYOC, CYP1B1, WDR36*, chromosome 2p16, *TMCO1, CAV1/CAV2, CDKN2B/CDKN2BAS-1, SIX1/SIX6, COL1A1, COL1A2, COL5A1, COL8A2, ZNF469* AND *ATOH7*). Notably the study did not corroborate a very significant association and strong genetic effect with a region on chromosome 2p16 and POAG that had been detected in an Afro-Caribbean population suggesting that that effect may be population-specific.

The absence of moderate effects does not preclude small genetic effects in the genes evaluated. In fact, weak associations with small genetic effects were identified with POAG in this population and common variants in *MYOC, ZNF469, COL1A1* and *COL8A2.* These are therefore target regions for further research in this population. The *ZNF469* genomic region was also marginally associated with CCT in the study population. VCDR association testing revealed a weak association with SNPs in *SIX1/SIX6*. IOP association testing identified a nominal association with a SNP in *WDR36*. Interestingly, a much more significant association was identified with SNPs in the *WDR36* gene and the POAG risk factor, diabetes mellitus. This finding needs replication.

Comparing and combining the association testing in this population with a West African population and an African American population for the *TMCO1, CAV1/CAV2, CDKN2BAS-1* and *SIX1/SIX6* regions yielded further insights. All three of these populations are of predominantly Niger-Kordofanian ancestry (although the African American population is an admixed population), so combining the three populations of African descent had the effect of creating a larger dataset that was adequately powered to detect small genetic effects. This resulted in the replication of associations with SNPs in the *TMCO1* and *CAV1/CAV2* regions and POAG. These regions, although associated with a smaller risk effect in POAG in African populations, are likely common pathways for glaucoma susceptibility across all populations. They are therefore important regions for further study.

Since significant population structure is evident among African populations, pooling of data across the populations does, however, need to be approached with care. As evidence of this a SNP in *CDKN2BAS-1* that had been identified in separate GWA

studies in European and Asian populations was significantly associated with severe visual impairment from glaucoma in African Americans and nominally associated with POAG in the same population. There was no association with either the West African or South African populations and combining all three populations with African Ancestry led to a loss of association. It is possible that the effect is specific to NTG as is suggested by the European and Asian studies, however NTG was an uncommon diagnosis in all three of the populations of African descent. The effect may be specific to only a subset of African populations or may be associated with European population admixture. Regardless of the reasons, this suggests that African populations should be evaluated independently as well as together.

The study results suggest that genetic variation at common variants within the specific candidate genomic regions evaluated in this study only contributes, if anything, a small percentage to the risk for POAG in black South Africans. A larger sample size would provide more certainty by being powered to detect smaller effects, however a search for associations with other genomic regions in African populations would also be valuable.

3. FURTHER WORK

The myocilin study has been extended, in an ongoing project, to pilot screening (for the two mutations) on all willing POAG patients attending the eye clinics affiliated to the University of the Witwatersrand. Cascade screening will then be offered to the relatives of patients with mutations. This will provide more information on the phenotype associated with the mutations, on penetrance and on the value of this approach for screening in this country.

Marginal associations that were identified with variants in candidate genes can be explored in larger black South African study populations to confirm or reject their involvement in the disease. Country-wide collaborative studies could achieve the numbers required.

While a candidate gene approach is practical, it is apparent that populations of African descent have different genetic contributions to POAG risk. A GWA study in these populations could provide new insights into POAG pathogenesis. This would require large numbers of participants that could be achieved collaboratively among African populations. Ultimately, however, further genetic studies into this South African population are warranted.

The association of diabetes mellitus and SNPs in WDR36 requires confirmation in an independent dataset of black South Africans in whom body mass index has also been measured.

4. CONCLUSION

From this first study into the genetics of POAG in black South Africans one can conclude that genetic risk factors are important in the pathogenesis of POAG in black South Africans.

POAG in black South Africans is under-diagnosed. Individuals with the disease present with higher IOPs and more advanced disease at a younger age than their European counterparts. Irreversible severe visual impairment from the disease occurs in over half of the disease sufferers indicating that this is a significant health problem in South Africa.

Mutations in the *MYOC* gene contribute to POAG in approximately 3.3% of individuals with the disease. Screening for these mutations can identify families in which cascade screening would be appropriate. This can identify, as it did in this study, undiagnosed or high-risk individuals and ultimately prevent irreversible visual loss.

While no moderate risk associations were identified that withstood correction for multiple testing with common variants in candidate genes and POAG or ocular quantitative traits, there were trends suggesting directions for further investigation.

This study has identified genetic risk factors for POAG in black South Africans that may prevent visual loss. This study can further target research that may ultimately lead to a reduction in irreversible visual loss from this common and devastating condition.

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APPENDICES

APPENDIX 1 - ETHICS CLEARANCE CERTIFICATE M10216

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Dr Susan Williams

CLEARANCE CERTIFICATE	**M10216**
PROJECT	The Genetics of Primary Open-Angle Glaucoma in Black South Africans: Candidate Gene Association Study
INVESTIGATORS	Dr Susan Williams.
DEPARTMENT	Division of Opthalmology
DATE CONSIDERED	26/02/2010
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 26/02/2010 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable
cc: Supervisor : Prof TR Carmichael

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...

APPENDIX 2 - PARTICIPANT INFORMATION AND CONSENT FORMS

1. Control participant information

1.1 Introduction and summary

Hello

My name is Dr Sue Williams. I am a consultant eye specialist at St John and I will be conducting the study we are speaking about. This form is a summary of the fuller information sheet, and does not replace it.

We are studying a condition called primary open angle glaucoma (POAG) that may cause blindness. It seems that this may be inherited in the genes. The purpose of this study is to take a blood sample from patients in this country with POAG. We will then take the DNA (which contains our genes) out of that blood without doing any other tests on the blood. We want to find the genes that are associated with POAG. We will also be doing eye examinations, eye tests and blood tests on people, like you, who don't have POAG – to compare the genes of people with POAG to those of people without POAG.

This study will not help you in any way but it will help us to understand the disease better and may help us to develop new ways to treat the disease in the future.

Once we have done the gene tests on your DNA we will store a sample of your DNA, but we will only use it for other tests for glaucoma. It will not be used for any other purpose.

You are free to join the study or not to join the study. It is your choice. If you don't want to join the study we don't mind and your treatment won't change.

There is much more information in the main patient information sheet; please read it carefully, and discuss the details with me if necessary.

Thank you for your interest.

1.2 Control participant information

I (Dr Susan Williams) am undertaking research into the underlying genetic cause for primary open angle glaucoma (POAG). I will be doing the research at the St John Eye Hospital where I am a consultant.

I would like to invite you to enter this study as a control participant. I am studying patients with POAG, but I need to compare them to healthy individuals. You do not have POAG so I would like your participation as a healthy control. This will mean answering some questions

about your background, your health and your eyes. Your eyes will be examined. You will then have a blood sample taken.

You don't have to join this study. You can also decide to leave the study at any time.
If you do not want to be part of this study or decide to leave the study it will not affect your regular treatments or medical care in any way.

INFORMATION ABOUT PRIMARY OPEN ANGLE GLAUCOMA (POAG)

POAG is a type of glaucoma. Glaucoma is a condition where the optic nerve (that connects the eye to the brain and carries information from the eye to the brain) is gradually damaged. This damage first reduces side vision then central vision. It eventually causes blindness. A high pressure in the eyes is one of the causes for the damage. POAG can be treated with eye drops or surgery to lower the pressure inside the eyes if it is discovered early. If there has already been some loss of vision, this loss is permanent and treatment does not reverse the damage but can prevent further damage.

We don't know what causes POAG. It seems likely that POAG is an inherited disease because it occurs more commonly in some parts of the world and within families. Researchers in other parts of the world have found genes (part of the DNA that we inherit from our parents that is found in all the cells of our bodies) that are associated with POAG. There have not been any studies looking for gene associations in Southern Africans.

The purpose of this research is to study a few genes that may be associated with glaucoma in a group of Southern African patients with glaucoma and to compare these gene findings with a similar group that do not have glaucoma. We hope that the results will help us to understand more about glaucoma. This might lead to new treatments for the condition as well as new ways to make the diagnosis of glaucoma early before people lose vision from the disease.

INFORMATION ABOUT DNA SAMPLING

In order to study your genes we take a sample of blood from your arm. We then extract your DNA from this blood sample. DNA is the chemical inside the nucleus of all our cells that we inherit from our parents. It carries the genetic instructions for making us living organisms. Extracting the DNA allows us to study your genes. Once we have extracted your DNA, we will study the possible glaucoma genes for this research. The remainder of your DNA will be stored at the NHLS Molecular Genetics Laboratory sample repository here in South Africa. In the future if further tests become available for genes involved in glaucoma we may use your DNA to look for these genes. We will not use your DNA for any other purpose.

EXPECTED BENEFITS

This study will not help you, but it will help us to understand POAG better and may help us to develop new ways to treat the disease in the future.

WHAT TO EXPECT FROM THE STUDY

Once you have signed the consent form we will ask you some questions about your background, your health and your eyes. I shall then perform a complete eye examination on you. This examination will include dilating your eyes. This is part of a routine eye examination. It may make your vision a bit blurred and sensitive to light for up to six hours. It is not advisable to drive if your pupils are dilated. You will also undergo a visual field test

and an analysis of the nerve layer around your optic nerve. These tests will confirm that you do not suffer from glaucoma. All of this information will be used in the research study, but your personal details will be withheld. Then a nursing sister will take four teaspoons (20ml or 2 tubes) of blood from a vein in your arm. Venipunctures (drawing blood) are normally done as part of routine medical care and present a slight risk of discomfort. Drawing blood may result in faintness, inflammation of the vein, pain, bruising or bleeding at the puncture site. There is also a slight possibility of infection. Your protection is that experienced personnel perform the procedures under sterile conditions. This blood will be sent to the laboratory where your DNA will be extracted from it. Most of your DNA will then be stored under the code we have assigned to you (not your name). Some of the DNA will be used to assess the glaucoma genes we are researching.

If we would like you to come on a separate day for any of these tests then we will pay you R50 to cover your transport costs.

CONFIDENTIALITY

Only the investigators of the study will know your name and personal details. You will be given a code and number for the study so that no one knows who you are. The blood samples and DNA will not be attached to your name – they will only have your code and number on them.

CONTACT DETAILS

If you have any problems you can contact me at any time on this telephone number: -
Dr Susan Williams – 082 467 6999.
If you have any complaints or problems with this research you may report them to the Chair of the Human Research Ethics Committee at the University of the Witwatersrand on this telephone number – (011) 717 1234.

2. POAG participant information

2.1 Introduction and summary

Hello

My name is Dr Sue Williams. I am a consultant eye specialist at St John and I will be conducting the study we are speaking about. This form is a summary of the fuller information sheet, and does not replace it.

We are studying a condition that you have called primary open angle glaucoma (POAG) and that may cause blindness. It seems that this may be inherited in the genes. The purpose of this study is to do eye examinations, eye tests and to take a blood sample from patients in this country with POAG. We will then take the DNA (which contains our genes) out of that blood without doing any other tests on the blood. We want to find the genes that are associated with POAG. We will also be using the records from all you eye related tests to help us find patterns between specific results from these tests or examinations and the DNA changes. In addition, we will be doing blood tests on other people who don't have POAG – to compare the genes of people with POAG to those of people without POAG.

This study will not help you in any way but it will help us to understand the disease better and may help us to develop new ways to treat the disease in the future.

Once we have done the gene tests on your DNA we will store a sample of your DNA, but we will only use it for other tests for glaucoma. It will not be used for any other purpose.

You are free to join the study or not to join the study. It is your choice. If you don't want to join the study we don't mind and your treatment won't change.

There is much more information in the main patient information sheet; please read it carefully, and discuss the details with me if necessary.

Thank you for your interest.

2.2 POAG participant information

I (Dr Susan Williams) am undertaking research into the underlying genetic cause for primary open angle glaucoma (POAG). I will be doing the research at the St John Eye Hospital where I am a consultant.

I would like to invite you to enter this study as a participant. This will mean answering some questions about your background, your health and your eyes. Your eyes will be examined. You will then have a blood sample taken. We will ask you to return to the hospital on another occasion to talk to a genetic counsellor so that we can find out if this is a condition that runs in your family. We will compensate you for the cost of returning to the hospital for our research.

You don't have to join this study. You can also decide to leave the study at any time.
If you do not want to be part of this study or decide to leave the study it will not affect your regular treatments or medical care in any way.

INFORMATION ABOUT PRIMARY OPEN ANGLE GLAUCOMA (POAG)

POAG is a type of glaucoma. Glaucoma is a condition where the optic nerve (that connects the eye to the brain and carries information from the eye to the brain) is gradually damaged. This damage first reduces side vision then central vision. It eventually causes blindness. A high pressure in the eyes is one of the causes for the damage. POAG can be treated with eye drops or surgery to lower the pressure inside the eyes if it is discovered early. If there has already been some loss of vision, this loss is permanent and treatment does not reverse the damage but can prevent further damage.

We don't know what causes POAG. It seems likely that POAG is an inherited disease because it occurs more commonly in some parts of the world and within families. Researchers in other parts of the world have found genes (part of the DNA that we inherit from our parents that is found in all the cells of our bodies) that are associated with POAG. There have not been any studies looking for gene associations in Southern Africans.

The purpose of this research is to study a few genes that may be associated with glaucoma in a group of Southern African patients with glaucoma and to compare these gene findings with a similar group that do not have glaucoma. We hope that the results will help us to understand more about glaucoma. This might lead to new treatments for the condition as well as new ways to make the diagnosis of glaucoma early before people lose vision from the disease.

INFORMATION ABOUT DNA SAMPLING

In order to study your genes we take a sample of blood from your arm. We then extract your DNA from this blood sample. DNA is the chemical inside the nucleus of all our cells that we inherit from our parents. It carries the genetic instructions for making us living organisms. Extracting the DNA allows us to study your genes. Once we have extracted your DNA, we will study the possible glaucoma genes for this research. The remainder of your DNA will be stored at the NHLS Molecular Genetics Laboratory sample repository here in South Africa. In the future if further tests become available for genes involved in glaucoma we may use your DNA to look for these genes. We will not use your DNA for any other purpose.

EXPECTED BENEFITS

This study will not help you, but it will help us to understand POAG better and may help us to develop new ways to treat the disease in the future.

WHAT TO EXPECT FROM THE STUDY

Once you have signed the consent form we will ask you some questions about your background, your health and your eyes. I shall then perform a complete eye examination on you. This examination will include dilating your eyes. This is part of a routine eye examination. It may make your vision a bit blurred and sensitive to light for up to six hours. It is not advisable to drive if your pupils are dilated. You will also undergo a visual field test and an analysis of the nerve layer around your optic nerve. These tests are routine in the diagnosis and management of glaucoma. All of this information will be used in the research study, but your personal details will be withheld. Then a nursing sister will take four teaspoons (20ml or 2 tubes) of blood from a vein in your arm. Venipunctures (drawing blood) are normally done as part of routine medical care and present a slight risk of discomfort. Drawing blood may result in faintness, inflammation of the vein, pain, bruising or bleeding at the puncture site. There is also a slight possibility of infection. Your protection is that experienced personnel perform the procedures under sterile conditions. This blood will be sent to the laboratory where your DNA will be extracted from it. Most of your DNA will then be stored under the code we have assigned to you (not your name). Some of the DNA will be used to assess the glaucoma genes we are researching.

On a separate occasion a genetic counsellor, who will determine whether this disease is likely to run in your family, will interview you and ask you questions about the health of your family. If you have family members with glaucoma we may ask you to bring them to the hospital for us to include them in the study.

If we would like you to come on a separate day for any of these tests then we will pay you R50 to cover your transport costs.

CONFIDENTIALITY

Only the investigators of the study will know your name and personal details. You will be given a code and number for the study so that no one knows who you are. The blood samples

and DNA will not be attached to your name – they will only have your code and number on them.

CONTACT DETAILS

If you have any problems you can contact me at any time on this telephone number: -
Dr Susan Williams – 082 467 6999.

If you have any complaints or problems with this research you may report them to the Chair of the Human Research Ethics Committee at the University of the Witwatersrand on this telephone number – (011) 717 1234.

3. Consent form

I, the undersigned, have been fully informed as to the procedure to be followed and have been given a description of the discomforts, risks and benefits of joining this study.

In signing this form, I agree to participate in this study and for my medical records to be used as part of the research.

I understand that I am free to refuse to be part of this study or to withdraw my consent and stop participating in this study at any time. If I do withdraw I understand that this will not affect my regular treatment or medical care in any way. If I choose not to be part of this study I will not be treated any differently.

I understand that if I have any questions at any time, they will be answered.

I understand that my confidentiality will be maintained at all times through the use of a code and numbering system.

A signed copy of this consent form will be made available to me if I want one.

Date:…… Participant:..…… ….……..
Name Signature

Participant date of birth……

Date:…… Witness:…….. ……..
Name Signature

PARTICIPANT CODE:……

4. Consent form for DNA storage for possible future use

I, the undersigned, have been fully informed as to the procedure to be followed and have been given a description of the discomforts, risks and benefits of having my DNA sampled and stored for possible further research.

In signing this form, I agree to have my DNA sampled and stored at the NHLS Molecular Genetics Laboratory sample repository.

I understand that most of the DNA that is extracted from my blood will be stored in Johannesburg and may be used for further genetic testing into glaucoma only. This may include sending the samples overseas for further tests. This process will be monitored by the Wits Ethics Committee.

I understand that if I have any questions at any time, they will be answered.

I understand that I can choose to have my DNA discarded at any time.

I understand that my confidentiality will be maintained at all times through the use of a code and numbering system.

A signed copy of this consent form will be made available to me if I want one.

Date:…… Participant:…… ……..
Name Signature

Participant date of birth……

Date:…… Witness:…..…… ……..
Name Signature

PARTICIPANT CODE……

APPENDIX 3 - DATA COLLECTION SHEETS

1. Questionnaire

Date:	BP=	HGT=	**Participant Code:**
Date of birth:		Age:	
Place of birth:		Home language:	
Mother's language:		Maternal grandmother's language:	
		Maternal grandfather's language:	
Father's language:		Paternal grandmother's language:	
		Paternal grandfather's language:	
History of eye problems:			
Any eye surgery?			
Any eye drops?			
Family history of eye problems: (specific questions:- anyone with glaucoma? anyone blind? anyone use eyedrops every day from a hospital? anyone have eye surgery? if so, did the eye surgery improve the vision?			
Draw a pedigree of family eye problems:			
Medical history:			
Diabetic? (if yes do they use tablets or insulin. When was the diagnosis made?)			
Hypertensive? (if yes what medication are they using. When was the diagnosis made?)			
Other illnesses?			
Other information:			

2. Ophthalmic history and examination

Participant code:……

Date of examination:..……..…….

Date of birth:……..……..

Ophthalmic history:

Date of diagnosis of POAG:…….

IOP at diagnosis: RE:……. LE:…….

Ophthalmic treatment:……

……

Previous ophthalmic surgery:…….

……

Examination:

Right Eye		**Left Eye**
	GDx NFI	
	GDx TSNIT	
	Oculus MD	
	Visual Acuity	
	Conjunctiva	
	Cornea	
	GAT	
	Gonioscopy	
	CCT	
	Anterior chamber	
	Pupil	
	Lens	
	Vitreous	
	Fundus	
	Vertical disc diameter	
	Cupping	
	Diagram if required	

APPENDIX 4 - DNA EXTRACTION USING SALTING-OUT

Day One:

1. 5 to 10ml of whole blood was decanted into a 50 ml polypropylene tube (a NUNC). The extraction procedure was usually done in a batch of 16 samples.
2. Each NUNC tube containing the whole blood was filled to the 40ml mark with cold sucrose-Triton-X lysing buffer. This detergent lyses the red blood cells.
3. The NUNC tubes were inverted several times to mix and centrifuged for 10 minutes.
4. The supernatant fluid, containing lysed red blood cells, was poured off as "Blood Waste". A reddish-white pellet containing the leucocytes was left in the tube.
5. The pellets were washed with 20 - 25ml of the cold sucrose-Triton-X lysing buffer.
6. The NUNC tubes were placed on ice for 5 minutes.
7. The NUNC tubes were centrifuged for 5 minutes. The wash was repeated to ensure that most of the red cell debris was removed in the supernatant.
8. 3ml T20E5, 0.2ml 10% SDS (sodium dodecyl sulphate) and 0.5 ml Proteinase-K mix was added to each sample. This step lyses the white blood cells and degrades the protein. The detergent SDS acts by breaking up the lipid bilayer of the cell membrane.
9. The components of the NUNC tube were mixed by inversion and incubated overnight in the 42°C incubator.

Day Two:

1. 1ml saturated NaCl (clear liquid) was added to the lysate and the components were vigorously agitated for 15 seconds by inversion.
2. The tubes with the salt were placed on ice for 10 minutes and then centrifuged for 30 minutes such that a white pellet, containing proteins precipitated by salt, was visible.
3. The supernatant fluid containing the DNA was transferred to new NUNC tubes labelled with the participant codes. Two volumes of absolute ethanol kept at room temperature were added to the tubes. In the presence of a high salt

concentration, DNA is not soluble in ethanol, therefore DNA was precipitated out of solution with the addition of the absolute ethanol.

4. After gentle agitation, the DNA was spooled or fished out of the solution, then washed in 70% ice-cold ethanol to remove excess salt that could interfere with enzymatic reactions. The DNA was placed in safety-lock Eppendorf tubes labelled with the participant codes. All traces of liquid were removed from the Eppendorf tubes. The DNA was air dried and resuspended in appropriate amount of 1x TRIS-EDTA (TE) buffer (usually 200µl to 1000µl).
5. If the DNA was not visible or was fragmented, the DNA was precipitated at minus 70°C for 30 minutes. The NUNC tube was then centrifuged for 20 minutes to pellet the DNA. The supernatant fluid was discarded and the DNA pellet was washed with 70% ethanol and centrifuged for 10 min. The ethanol was discarded and the NUNC tube inverted on a paper towel to dry the pellet before resuspending the DNA pellet in an appropriate amount of 1x TE buffer (usually 20-100µl). This was then placed in a safety-lock Eppendorf tube labelled with the participant code.

APPENDIX 5 - ETHICS CLEARANCE CERTIFICATE M110214

M110214

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Dr Susan EI Williams

CLEARANCE CERTIFICATE	**M110214**
PROJECT	Genetics counselling, cascade screening and eye examination of first-second-degree relatives of
primary-	open angle glaucoma patients identified as having
	MYOC mutations.
INVESTIGATORS	Dr Susan EI Williams.
DEPARTMENT	Division of Human Genetics/NHLS
DATE CONSIDERED	25/02/2011
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 06/05/2011 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable
cc: Supervisor : Prof M Ramsay

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**
PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...

APPENDIX 6 - PARTICIPANT INFORMATION FORMS

1. Introduction and summary

Hello

My name is Dr Sue Williams. I am a consultant eye specialist at St John and I will be conducting the study we are speaking about. This form is a summary of the fuller information sheet, and does not replace it.

We are studying a condition called primary open angle glaucoma (POAG) that may cause blindness particularly if it is only picked up late in the disease process. It seems that this is a family disease. Family diseases are passed on in our genes which we inherit from our parents. The genes are contained in the DNA which is found in every cell of our bodies. We have discovered that your relative has a gene that may cause glaucoma. This may mean that you have POAG or may be at risk of developing it.

We would like to invite you to participate in this study. If you would like to participate, we will examine your eyes, perform eye tests and take a blood sample from your arm. We will then take the DNA out of that blood without doing any other tests on the blood. If we find that you have POAG we will treat you for the condition and we may be able to prevent visual loss. We will test your DNA for the gene that your relative has. If we find that you have the gene for the condition but do not show any signs of the condition we will contact you with the information and urge you to visit us every year for examination for signs of the disease so that treatment may be started early in the disease and visual loss may be prevented.

We would further like to invite you to allow us to store a sample of your DNA for other tests related to glaucoma. It will not be used for any other purpose. You are free to have the sample discarded at any time.

You are free to join the study or not to join the study. It is your choice. If you don't want to join the study we don't mind and if we find you have the condition, your treatment won't change.

There is much more information in the main patient information sheet; please read it carefully, and discuss the details with me if necessary.

Thank you for your interest.

2. Participant information

I (Dr Susan Williams) am undertaking research into the underlying genetic cause for primary open angle glaucoma (POAG). I will be doing the research at the St John Eye Hospital where I am a consultant.

I would like to invite you to enter this study as a participant. This will mean answering some questions about your background, your health and your eyes. Your eyes will be examined. You will then have a blood sample taken. If we find that you have glaucoma or are at risk of developing glaucoma we will treat you appropriately to prevent visual loss from the condition.

You don't have to join this study. You can also decide to leave the study at any time.
If you do not want to be part of this study or decide to leave the study it will not affect your treatments or medical care in any way.

INFORMATION ABOUT PRIMARY OPEN ANGLE GLAUCOMA (POAG)
POAG is a type of glaucoma. Glaucoma is a condition where the optic nerve (that connects the eye to the brain and carries information from the eye to the brain) is gradually damaged. This damage first reduces side vision then central vision. It eventually causes blindness. A high pressure in the eyes is one of the causes for the damage. POAG can be treated with eye drops or surgery to lower the pressure inside the eyes if it is discovered early. If there has already been some loss of vision, this loss is permanent and treatment does not reverse the damage but can prevent further damage.

We don't know what causes POAG. It seems likely that POAG is an inherited disease because it occurs more commonly in some parts of the world and within families. Family diseases are inherited through our genes. The genes are contained in the DNA which is found in all the cells of our bodies. Researchers in other parts of the world have found genes that are associated with POAG. We have found a gene that may cause glaucoma in your relative.

The purpose of this research is to study the relatives of people who may have the gene for the disease to see whether the relatives have the gene or the disease.

INFORMATION ABOUT DNA SAMPLING

If you would like to be a part of this study, then in order to study your genes we will take a sample of blood from your arm. Your blood contains cells. All the cells in our bodies contain a chemical called DNA that we inherit from our parents. It carries the instructions for making us living organisms. It contains all our genes. We can take the DNA out of the cells that are in the sample of blood that we take from your arm. Once we have removed the DNA from the blood we can study your genes. In this study we will look for the gene that your relative has that we think is a glaucoma gene. If you are interested in helping us with further research into glaucoma then the remainder of your DNA will be stored at the NHLS Molecular Genetics Laboratory sample repository here in South Africa. You may sign a separate consent form for this. In the future if further tests become available for genes involved in glaucoma we may then use your DNA to look for these genes. We will not use your DNA for any other purpose.

EXPECTED BENEFITS

If you have glaucoma, we will pick it up during our examination. We can then start treating the condition and we may be able to prevent you from losing vision as a result of the disease.

If you have the gene for the condition, but do not have the disease, we will contact you to give you this information and we can then schedule follow-up consultations to look for the disease which will enable us to start treatment early on in the course of the disease if this becomes necessary.

WHAT TO EXPECT FROM THE STUDY

Once you have signed the consent form we will ask you some questions about your background, your health and your eyes. I shall then perform a complete eye examination on you. This examination will include dilating your eyes. This is part of a routine eye examination. It may make your vision a bit blurred and sensitive to light for up to six hours. It is not advisable to drive if your pupils are dilated. You may also undergo a visual field test and an analysis of the nerve layer around your optic nerve. These tests are routine in the diagnosis and management of glaucoma. All of this information will be used in the research study, but your personal details will be withheld. Then a nursing sister will take two teaspoons (10ml or 2 tubes) of blood from a vein in your arm. Venipunctures (drawing blood) are normally done as part of routine medical care and present a slight risk of discomfort. Drawing blood may result in faintness, inflammation of the vein, pain, bruising

or bleeding at the puncture site. There is also a slight possibility of infection. Your protection is that experienced personnel perform the procedures under sterile conditions. This blood will be sent to the laboratory where your DNA will be taken out of the cells in the blood. Some of the DNA will be used to assess the glaucoma gene that your relative has.

If you sign the separate 'DNA Sampling' consent form, then the remainder of your DNA will be stored under the code we have assigned to you (not your name).

We will pay you R150 to cover your transport costs on attending the hospital.

CONFIDENTIALITY

Only the investigators of the study will know your name and personal details. You will be given a code and number for the study so that no one knows who you are. The blood samples and DNA will not be attached to your name – they will only have your code and number on them.

CONTACT DETAILS

If you have any problems you can contact me at any time on this telephone number: -
Dr Susan Williams – 082 467 6999.

If you have any complaints or problems with this research you may report them to the Chair of the Human Research Ethics Committee at the University of the Witwatersrand on this telephone number – (011) 717 1234.

APPENDIX 7 - BEADXPRESS ASSOCIATION STUDY SNP TABLE

Table A7.1 List of SNPs within each candidate region that were included in the Illumina® GoldenGate Assay

Genomic region	SNP	Chr	Position	Alleles	Comment
COL8A2	rs274750	1	36561956	[T/G]	
	rs6693322	1	36565362	[A/G]	
	rs274754	1	36565617	[A/G]	
MYOC	MYOCc3	1	171605081	[T/C]	Potential Mutation
	MYOCcdel	1	171605223	[T/A]	Potential Mutation
	rs61730975	1	171605392	[T/C]	
	MYOCc2	1	171605459	[A/C]	Potential Mutation
	rs61745146	1	171605526	[T/C]	
	rs61730976	1	171605605	[A/G]	
	rs79255460	1	171605922	[A/G]	
	rs12076134	1	171606054	[T/G]	
	rs2032555	1	171607702	[A/G]	
	rs57824969	1	171607855	[A/C]	
	rs235869	1	171610959	[A/C]	
	rs16864720	1	171612267	[T/C]	
	rs235876	1	171614114	[T/C]	
	rs2236875	1	171614683	[T/G]	
	rs235917	1	171620579	[A/G]	
	rs7525674	1	171620834	[T/C]	
	MYOCnc1	1	171621135	[T/G]	
	rs61730977	1	171621275	[A/G]	
	rs2234926	1	171621525	[T/C]	
	rs12082573	1	171621713	[T/G]	
	rs2075648	1	171621834	[T/C]	
AIM	rs723854	1	192511012	[G/C]	AIM
AIM	rs1876482	2	17362568	[T/C]	AIM
CYP1B1	rs162549	2	38295456	[T/A]	
	rs9341261	2	38296939	[T/G]	
	rs10916	2	38297170	[T/G]	
	rs162562	2	38297515	[T/G]	
	rs1056836	2	38298203	[C/G]	
	rs162561	2	38298877	[T/G]	
	rs9341252	2	38300431	[A/G]	
Chromosome 2p16	rs2303298	2	50850686	[A/G]	
	rs10202118	2	51950324	[A/G]	
	rs11125375	2	51952149	[T/C]	
	rs10208467	2	51957943	[G/C]	
	rs1533428	2	51959258	[A/G]	
	rs12994401	2	52072246	[T/C]	
	rs11889995	2	52163410	[A/G]	
AIM	rs952718	2	215888624	[T/G]	AIM
AIM	rs1344870	3	21307401	[A/C]	AIM
AIM	rs720096	4	179551071	[C/G]	AIM
WDR36	rs7729832	5	110428744	[A/G]	
	rs17623144	5	110430488	[C/G]	

Chr, chromosome; AIM, SNP informative of ancestry

(Table A7.1 continues overpage)

Genomic region	SNP	Chr	Position	Alleles	Comment
	rs13357724	5	110433323	[T/C]	
	rs13153937	5	110434307	[A/G]	
	rs6859041	5	110435231	[A/G]	
	rs2416257	5	110435490	[A/G]	
WDR36	rs17132782	5	110437494	[A/G]	
	rs17132783	5	110438439	[A/G]	
	rs10038058	5	110443281	[A/G]	
	rs12515367	5	110447853	[A/G]	
	rs12521169	5	110448313	[A/G]	
	rs10051830	5	110452845	[A/G]	
	rs10491424	5	110453806	[A/G]	
	rs11956837	5	110454757	[A/G]	
	rs12522383	5	110455266	[T/C]	
	rs17624563	5	110456263	[C/G]	
	rs43203	5	110457983	[T/C]	
	rs11241098	5	110458508	[T/C]	
	rs7722241	5	110460747	[A/T]	
	rs10041326	5	110461298	[A/C]	
	rs4530809	5	110462230	[A/G]	
	rs17132810	5	110464114	[T/C]	
	rs1457113	5	110467074	[T/C]	
AIM	rs1363448	5	140783596	[T/C]	AIM
AIM	rs217538	6	108483470	[G/C]	AIM
AIM	rs65264	7	28545611	[A/G]	AIM
COL1A2	rs4729131	7	94028427	[A/G]	
	rs3814967	7	94029982	[A/G]	
	rs1800222	7	94030899	[A/G]	
	rs411717	7	94033031	[T/C]	
	rs406226	7	94033800	[T/C]	
	rs3763466	7	94035892	[A/G]	
	rs17166249	7	94036547	[A/G]	
	rs42518	7	94038934	[T/C]	
	rs2521206	7	94039187	[T/C]	
	rs42521	7	94040816	[G/C]	
	rs42522	7	94040963	[A/G]	
	rs42524	7	94043239	[C/G]	
	rs42526	7	94045576	[A/G]	
	rs2521205	7	94046541	[A/C]	
	rs3736638	7	94047266	[T/G]	
	rs42527	7	94047677	[A/G]	
	rs369982	7	94048482	[T/C]	
	rs4266	7	94050608	[T/G]	
	rs10235102	7	94051795	[T/G]	
	rs441051	7	94054000	[T/C]	
	rs2072071	7	94054223	[A/G]	
	rs13240759	7	94055933	[T/C]	
	rs400218	7	94056006	[T/C]	
	rs10046552	7	94057418	[T/G]	
	rs12668754	7	94059236	[A/G]	

Chr, chromosome; AIM, SNP informative of ancestry

(Table A7.1 continues overpage)

Genomic region	SNP	Chr	Position	Alleles	Comment
	rs11764718	7	94059317	[T/C]	
	rs1062394	7	94060509	[A/C]	
	rs1034620	7	94063415	[T/C]	
	rs11982782	7	94063700	[A/T]	
CAV1/2	rs8940	7	116146074	[C/G]	
	rs10258482	7	116150095	[T/G]	
	rs6466578	7	116150869	[T/C]	
	rs3919515	7	116151784	[G/C]	
	rs4236601	7	116162729	[A/G]	
CAV1/2	rs926198	7	116167208	[A/G]	
	rs3779512	7	116171063	[T/G]	
	rs10262090	7	116171568	[T/C]	
	rs6466583	7	116173064	[T/C]	
	rs3807986	7	116177825	[T/C]	
	rs1476833	7	116181475	[A/C]	
	rs10270569	7	116182782	[T/C]	
	rs11773845	7	116191301	[T/G]	
	rs9886215	7	116191651	[T/C]	
	rs3807992	7	116197245	[A/G]	
AIM	rs679047	9	12883664	[T/A]	AIM
CDKN2B	rs3217992	9	22003223	[A/G]	
	rs1063192	9	22003367	[T/C]	
	rs3217989	9	22003790	[T/C]	
	rs2069426	9	22006273	[A/C]	
	rs974336	9	22006348	[A/G]	
	rs3217980	9	22006607	[A/G]	
	rs2069422	9	22008026	[T/G]	
AIM	rs2077559	9	36014850	[T/C]	AIM
COL5A1	rs1536478	9	137432248	[A/G]	
	rs3118516	9	137439792	[A/G]	
	rs1536482	9	137440528	[T/C]	
	rs7044529	9	137568051	[T/C]	
ATOH7	rs6480320	10	69991668	[A/G]	
	rs7916697	10	69991853	[A/G]	
	rs2289804	10	69993032	[T/C]	
	rs1900004	10	70000881	[T/C]	
	rs41340944	10	70002020	[A/G]	
	rs1900020	10	70007008	[A/G]	
AIM	rs714857	11	15974389	[T/C]	AIM
AIM	rs953386	13	110943692	[A/G]	AIM
SIX1/6	rs12436579	14	60983087	[T/G]	
	rs17097585	14	60987630	[T/C]	
	rs8006274	14	60988311	[T/C]	
	rs1007152	14	60999493	[A/G]	
	rs7152532	14	61000665	[A/G]	
	rs11849906	14	61019559	[A/G]	
	rs1955698	14	61029936	[A/G]	
	rs10150234	14	61031284	[G/C]	
	rs11158289	14	61032390	[T/C]	

Chr, chromosome; AIM, SNP informative of ancestry

(Table A7.1 continues overpage)

Genomic region	SNP	Chr	Position	Alleles	Comment
	rs10142401	14	61039403	[A/G]	
	rs10483726	14	61039476	[T/C]	
	rs8020353	14	61070296	[T/C]	
	rs10483727	14	61072875	[A/G]	
	rs2057135	14	61077022	[T/C]	
	rs8010936	14	61085974	[A/T]	
	rs7156317	14	61087740	[T/C]	
	rs7146104	14	61089336	[A/G]	
	rs4901995	14	61090629	[C/G]	
	rs8007935	14	61103375	[A/C]	
	rs11628064	14	61108569	[T/C]	
AIM	rs722869	14	97277005	[G/C]	AIM
AIM	rs735612	15	34076642	[A/C]	AIM
AIM	rs2089740	15	36310531	[A/C]	AIM
AIM	rs1823718	15	74147244	[A/G]	AIM
ZNF469	rs12447690	16	88298124	[T/C]	
	rs7500824	16	88299491	[T/C]	
	rs4352077	16	88300429	[A/G]	
	rs6540214	16	88301976	[T/C]	
	rs11863929	16	88304433	[C/G]	
	rs7501402	16	88320911	[T/A]	
	rs6540223	16	88321436	[T/C]	
	rs12448211	16	88330513	[A/G]	
	rs9938149	16	88331640	[A/C]	
	rs9925231	16	88338107	[A/G]	
	rs11644804	16	89976224	[T/C]	
COL1A1	rs2696297	17	48245180	[T/C]	
	rs16948744	17	48246162	[A/G]	
	rs2696296	17	48246231	[T/C]	
	rs36107109	17	48248998	[T/C]	
	rs2586476	17	48251111	[T/C]	
	rs12945599	17	48255183	[T/C]	
	rs2586481	17	48257099	[T/C]	
	rs2696270	17	48257812	[T/C]	
	rs1061947	17	48262119	[T/C]	
	rs1061237	17	48262775	[T/C]	
	rs2277632	17	48263903	[T/C]	
	rs2696245	17	48264717	[A/G]	
	rs2586488	17	48265426	[T/C]	
	rs2075559	17	48267291	[G/C]	
	rs2857396	17	48269302	[T/C]	
	rs2696247	17	48269903	[T/C]	
	rs2586494	17	48273155	[T/G]	
	rs2075554	17	48274309	[A/G]	
	rs16948765	17	48274760	[A/C]	
	rs16948767	17	48275292	[A/C]	
	rs2586498	17	48276252	[A/G]	
AIM	rs1858465	17	51142920	[T/A]	AIM
AIM	rs2112527	19	9603751	[A/G]	AIM

Chr, chromosome; AIM, SNP informative of ancestry

APPENDIX 8 - ILLUMINA GOLDENGATE ASSAY: DETAILED PROCEDURE

Illumina manufactured the unique SNP kit that consisted of:

- Query assay oligonucleotides (oligos) targeting the specific 192 SNPs selected
- Five 96-plex VeraCode bead plates – each well containing the beads providing unique identifiers for each SNP
- Reagents that activate DNA, that elute extended and ligated products, that extend and ligate, that wash away non-specifically hybridised and excess oligonucleotides from the genomic DNA, that bind double-stranded PCR products to magnetic particles, that make the hybridisation plate and that wash the VeraCode beads
- Precipitation and resuspension solutions
- Buffers – oligonucleotide hybridisation and complementary DNA and genomic DNA binding buffer and universal buffers for washing paramagnetic beads and magnetic particles
- Uracil DNA Glycosylase used to help prevent PCR product contamination

1. Pre-PCR protocol

1.1 Make single-use DNA plates

In this process sufficient DNA of each individual sample to be used once in the assay was activated by adding 5µl of the appropriate DNA activation reagent (containing Biotin) to 5µl of each DNA sample (normalised to 50ng/µl) in 96-well microplates. The plates were then pulse centrifuged, vortexed and incubated at 95°C for 30 minutes. DNA is denatured at 95°C, leaving biotinylated single stranded DNA in the plates.

1.2 Precipitate the DNA in the single-use DNA plate

The single stranded biotinylated DNA in the plates was precipitated by adding 5µl of a precipitation solution to each well, then vortexing the plate before adding 15µl 2-propanol to each sample. DNA is insoluble in propanol. The plates were centrifuged for 20 minutes at 3000xg. The supernatant was decanted onto an absorbent pad and

the precipitated DNA was left to dry for 15 minutes.

1.3 Resuspend the DNA in the single-use DNA plate

The precipitated DNA in each well was resuspended in 10µl of the resuspension reagent.

1.4 Make an allele-specific extension plate

10µl of the GoldenGate Assay custom oligo pool (containing the query oligonucleotides - two allele-specific oligonucleotides and one locus-specific oligonucleotide - for each of the 192 selected SNPs) was added to each well of five new 96-well microplates. To each of these wells 30µl of oligo hybridisation and DNA binding buffer (containing streptavidin-conjugated paramagnetic particles) was added. The biotinylated samples from the single-use DNA plates were transferred to the appropriate wells of the allele-specific extension plates. These plates were heat sealed and placed in a preheated heat block and allowed to drop in temperature from 70°C to 30°C over 2 hours such that the query oligonucleotides for each sequence target of interest were allowed to anneal to the biotinylated genomic DNA samples. The genomic DNA was simultaneously captured by paramagnetic particles through the binding of biotin to streptavidin.

1.5 Add master mix for extension/ligation

Mis-hybridised and excess oligonucleotides were washed away in repeated steps using a raised bar magnetic capture plate to allow the paramagnetic beads to remain in the wells while all liquid was discarded and replaced. In this process a washing reagent was used to resuspend the beads (off the magnetic plate) twice before repeating the process twice with a universal buffer. To each washed genomic DNA sample, 37µl of an extension and ligation master mix was added. The extension and ligation reaction occurred in 15 minutes at 45°C.

1.6 Make polymerase chain reaction (PCR) plate

64µl of Titanium Taq DNA polymerase and 50µl Uracil DNA Glycosylase were added to the ‘master mix for PCR’ reagent. This reagent contains three universal primers: two are labeled with fluorescent dyes and the third is biotinylated. The biotinylated primer allows capture of the PCR product and elution of the strand containing the

fluorescent signal. 30µl of this mixture was placed in each well of five new 96-sample plates.

1.7 Inoculate the PCR plate

The allele specific extension plates were placed on the raised bar magnetic plate and, leaving the beads in the wells, the supernatant was discarded. The beads were washed with universal buffer as described above.

35µl of the reagent for inoculating PCR was added to each column of the plates. The plates were placed on a heat block preheated to 95°C for 1 minute. The temperature denatures the DNA and frees the templates formed in the extension and ligation process from the genomic DNA.

The plates were then placed on the raised bar magnet to capture all the beads, but this time the supernatant containing the complementary templates was transferred to the corresponding wells of the PCR plates while the beads (still bound to the genomic DNA) were discarded.

The PCR plates were transferred to the thermal cycler.

2. Post-PCR protocol

2.1 Thermal cycle the PCR plate

The sealed plates were placed in the thermal cycler and run at 37°C for 10 minutes, 95°C for 3 minutes, then 34 cycles of 95°C for 35 seconds, 56°C for 35 seconds and 72°C for 2 minutes and finally 72°C for 10 minutes and 4°C for 5 minutes. The thermal cycling fluorescently labels and amplifies the templates generated in the pre-PCR process.

2.2 Bind PCR products

Magnetic particle reagent used to bind biotinylated double-stranded PCR products was added to each of the wells of the PCR plate. The solution was then transferred to the corresponding columns of filter plates. The filter plates were stored at ambient

temperature, protected from light, for 60 minutes so that the PCR product could bind to the paramagnetic particles.

2.3 Make intermediate plate for VeraCode Bead Plate

The filter plates containing the bound PCR products were placed onto a filter plate adapter and centrifuged at 1000xg for 5 minutes at 25°C to displace the waste fluid into waste plates while the bound PCR products were captured by the filter. Universal buffer was added to each well of the filter plates and the process was repeated.

The reagent for hybridisation was added to each well of new empty 96-well plates and the waste plates were replaced with these intermediate plates.
Sodium hydroxide was dispensed into each well of the filter plates to lower the pH and break the bonds between the biotinylated PCR products and the streptavidin-conjugated paramagnetic particles. The plates were centrifuged at 1000xg for 5 minutes at 25°C to displace the fluid containing the PCR products into the intermediate plates. The filter plates holding the paramagnetic particles were discarded.

2.4 Hybridise VeraCode Bead Plate

A second reagent for hybridisation was neutralised with sodium hydroxide. 50µl of this solution was dispensed into each well of the intermediate plates. 100µl of each assay product was then transferred into the corresponding well of the VeraCode Bead Plate containing the digitally inscribed glass microbeads.

The VeraCode Bead Plates with the samples were placed into the VeraCode Vortex Incubator (an incubating microplate shaker) and incubated at 45°C and at a speed of 850rpm for 3 hours during which time the beads were hybridized to the PCR products.

2.5 Wash VeraCode Bead Plate

The VeraCode Bead pellets were washed with 200µl of buffer that was then aspirated with the vacuum manifold, leaving the beads at the bottom of the wells. The wash was repeated.

APPENDIX 9 - INDIVIDUALS IN WHOM BEADXPRESS GENOTYPING WAS SUCCESSFUL

Table A9.1 Demographic features of study subjects in whom BeadXpress genotyping was successful

Group		Total	POAG	Control	p	Adjusted p
n		429	215	214		
Female (%)		222 (51.75)	107 (49.77)	115 (53.74)	0.411 ¶	
Age (years)	Range		22-87	50-91		
	Mean ± SD		59.8 ± 13.4	70.2 ± 8.3	**<0.001 §**	
FAMILY HISTORY						
Family history of glaucoma (%)			15 (6.98)	N/A		
Family history of blindness (%)			52 (24.19)	15 (7.01)	**< 0.001 ¶**	
HOME LANGUAGE					0.492 ⋆	
	Afrikaans (%)	8 (1.86)	4 (1.86)	4 (1.87)		
	IsiNdebele (%)	5 (1.17)	1 (0.47)	4 (1.87)		
	Sepedi (%)	33 (7.69)	16 (7.44)	17 (7.94)		
	Sesotho (%)	70 (16.32)	32 (14.88)	38 (17.76)		
	Siswati (%)	5 (1.17)	1 (0.47)	4 (1.87)		
	Xitsonga (%)	23 (5.36)	12 (5.58)	11 (5.14)		
	Setswana (%)	69 (16.08)	30 (13.95)	39 (18.22)		
	Tshivenda (%)	11 (2.56)	8 (3.72)	3 (1.40)		
	IsiXhosa (%)	36 (8.39)	21 (9.77)	15 (7.01)		
	IsiZulu (%)	167 (38.93)	89 (41.40)	78 (36.45)		
	Other (%)	2 (0.47)	1 (0.47)	1 (0.47)		
MEDICAL HISTORY						
DM (%)		129 (30.07)	49 (22.79)	80 (37.38)	**0.001 ¶**	**0.037**
	Type I	7 (5.43)	5 (10.20)	2 (2.50)	0.104 ⋆	0.211
	Diabetic retinopathy	30 (23.26)	3 (6.12)	27 (33.75)	**<0.001 ⋆**	**<0.001**
HT (%)		266 (62.00)	118 (54.9)	148 (69.17)	**0.002 ¶**	0.810

DM, diabetic; HT, hypertensive; Adjusted p, p adjusted for age and gender in a logistic regression model
¶ Pearson's χ^2 test
⋆ Fisher's exact test
§ T-test

Table A9.2 Clinical features of study subjects in whom BeadXpress genotyping was successful

Group		POAG	Control	p (t-test)	Adjusted p
n		215	214		
SVI (%)		114 (53.02)			
Glaucoma drainage surgery (%)		119 (55.35)			
Age at diagnosis (years)	n	179			
	Range	17 - 84			
	Mean ± SD	54.48 ± 13.83			
	Juvenile onset (%)	25 (11.63)			
MAP	n	190	166		
	Range	73.33 - 158.67	61.33 - 189.67		
	Mean ± SD	101.55 ± 13.80	99.81 ± 16.56	0.282	
IOP	n	176	214		
	Range	18-68	6-19		
	Mean ± SD	35.2 ± 9.5	13.4 ± 2.7	**<0.001**	
	NTG (%)	6 (3.41%)			
CCT	n	194	175		
	Range	379-586	420-609		
	Mean ± SD	506.0 ± 38.4	513.8 ± 37.2	**0.049**	**0.004**
VCDR	n	214	213		
	Range	0.3-1.0	0.1-0.7		
	Mean ± SD	0.90 ± 0.13	0.37 ± 0.13	**<0.001**	

SVI, Snellen visual acuity < 6/60 in at least one eye secondary to glaucoma; MAP, Mean arterial pressure; IOP, IOP at diagnosis for POAG subjects or at enrolment for control subjects

Adjusted p, p adjusted for age and gender in a logistic regression model

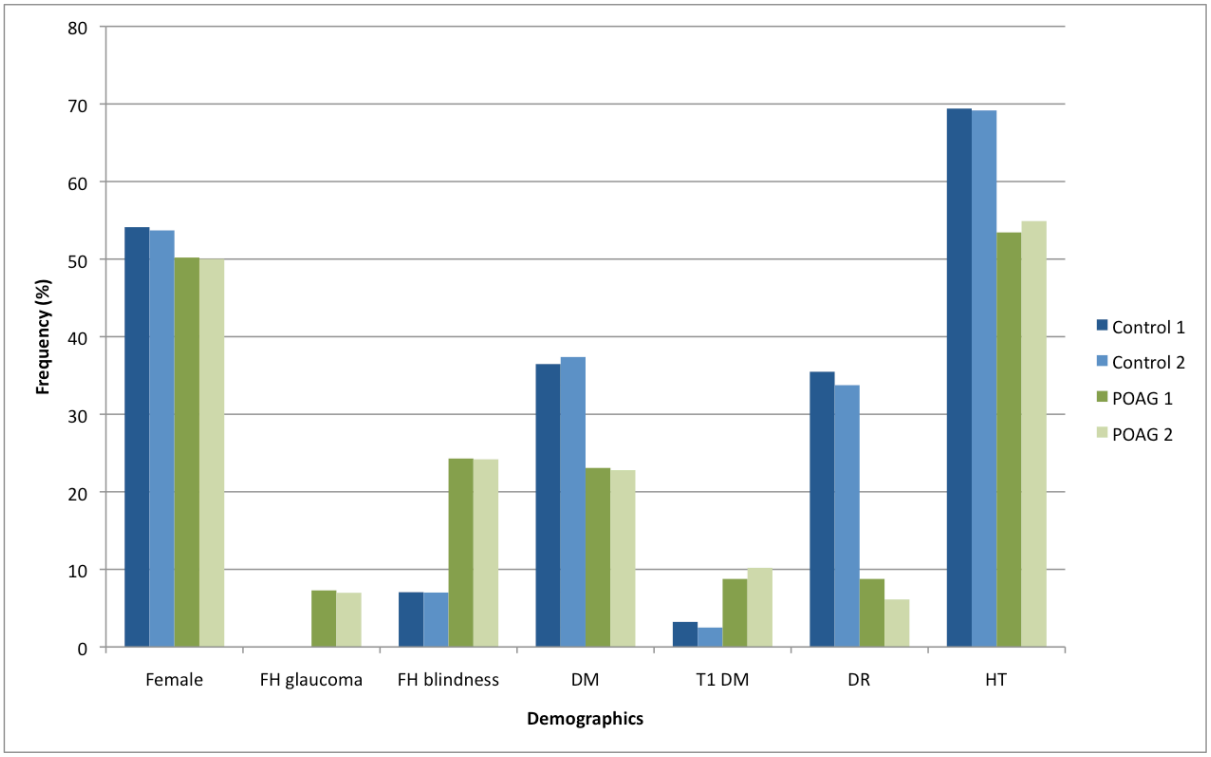

Figure A9.1 Comparison of the demographics of the POAG and control participants in the entire study population (POAG 1, Control 1) and in the POAG and control participants in whom BeadXpress genotyping was successful (POAG 2, Control 2). The y-axis represents frequency in percent and each demographic criterion is represented along the x-axis.

FH Glaucoma, family history of glaucoma; FH blindness, family history of blindness; DM, diabetes mellitus; T1 DM, type 1 diabetes mellitus; DR, diabetic retinopathy; HT, hypertensive

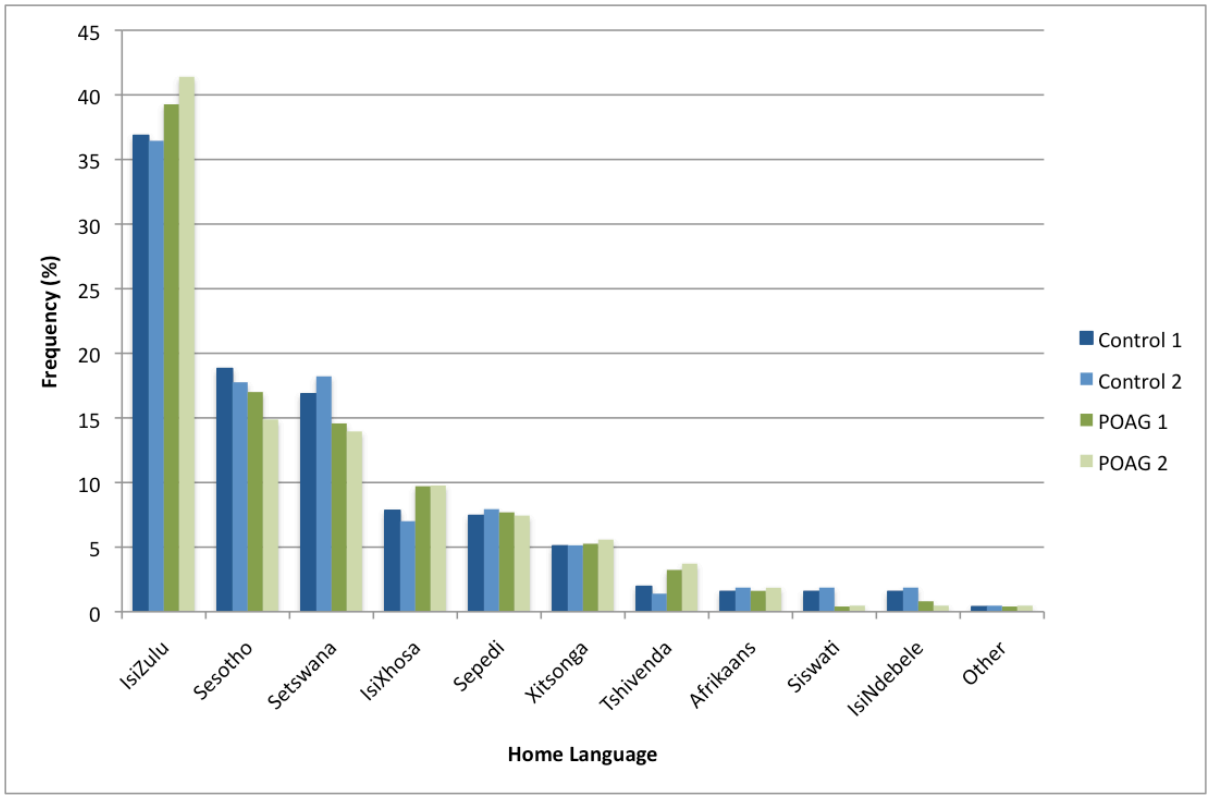

Figure A9.2 First language spoken: comparison of the proportions speaking each South African language of POAG and control participants in the entire study population (POAG 1, Control 1) and in the POAG and control participants in whom BeadXpress genotyping was successful (POAG 2, Control 2). The y-axis represents frequency in percent and the languages are represented along the x-axis.

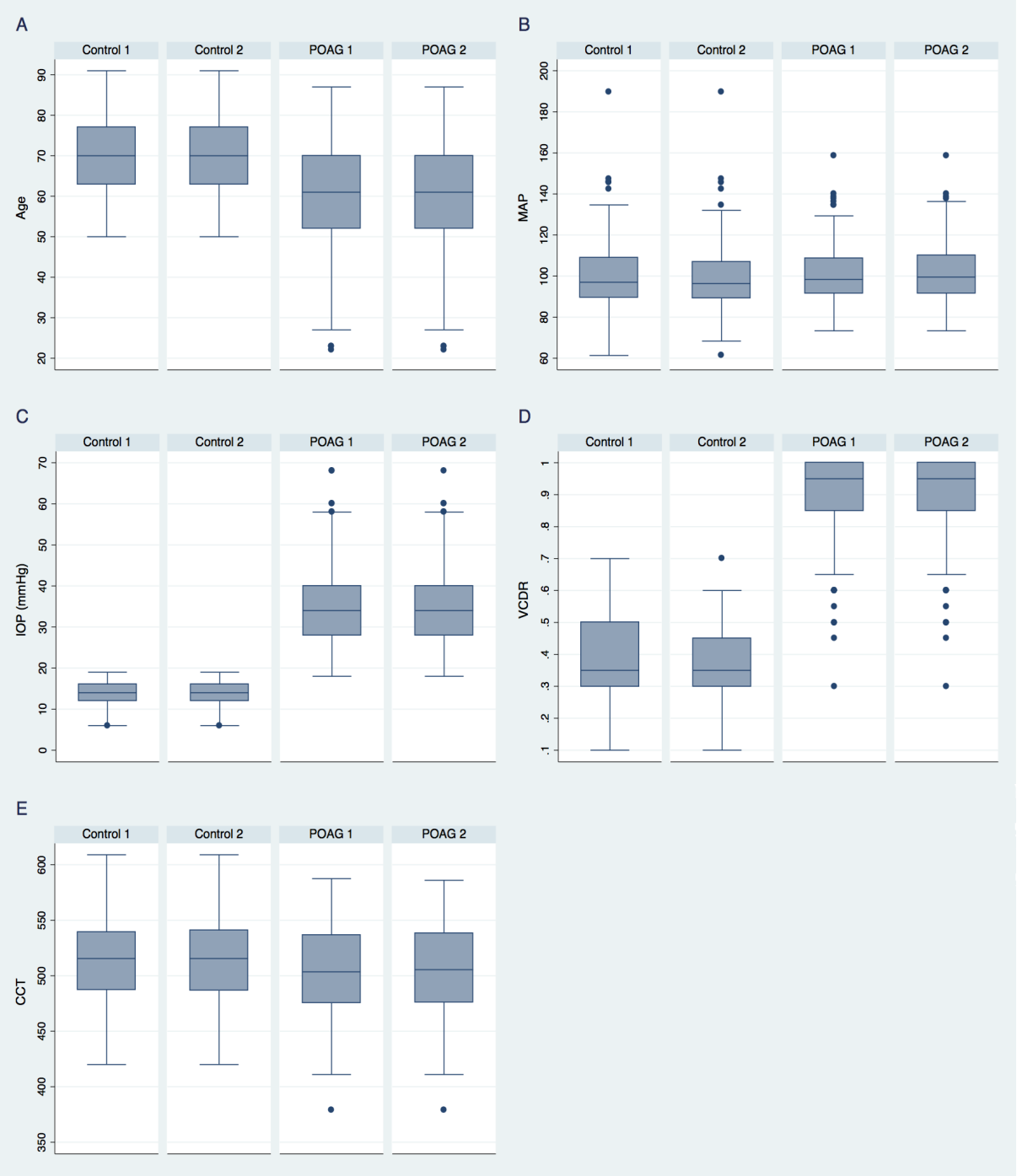

Figure A9.3 Box-and-whisker plots in the POAG and control participants in the entire study population (POAG 1 and Control 1) and in the POAG and control participants in whom BeadXpress genotyping was successful (POAG 2 and Control 2) of:

A - Age

B - Mean arterial pressure (MAP)

C - Intraocular pressure (IOP) in mmHg

D - Vertical cup-to-disc ratio (VCDR)

E - Central corneal thickness (CCT) in μm

The bottom and top of the box are the lower and upper quartiles respectively, and the band near the middle of the box is the median. The ends of the whiskers represent the upper and lower adjacent values. Any data not included between the whiskers is plotted as an outlier with a dot.

APPENDIX 10 - POAG ASSOCIATION ANALYSIS IN THE BEADXPRESS ASSOCIATION STUDY

Table A10.1 Single SNP association testing with a diagnosis of POAG in the study population

Genomic region	Ch	SNP	MA	MAF POAG	MAF Controls	χ^2	p	p*
COL8A2	1	rs274750	T	0.45	0.44	0.0058	0.9392	0.9453
COL8A2	1	rs6693322	G	0.11	0.07	3.6140	0.0573	0.0710
COL8A2	1	rs274754	A	0.35	0.35	0.0071	0.9330	0.9411
MYOC	1	rs61730975	T	0.03	0.03	0.1492	0.6993	0.8426
MYOC	1	rs61745146	T	0.03	0.02	0.1772	0.6738	0.8296
MYOC	1	rs61730976	A	0.05	0.03	3.6050	0.0576	0.0830
MYOC	1	rs79255460	A	0.03	0.04	0.0439	0.8341	0.8569
MYOC	1	rs12076134	G	0.20	0.20	0.0027	0.9585	1.0000
MYOC	1	rs2032555	G	0.07	0.06	0.1602	0.6890	0.7807
MYOC	1	rs57824969	A	0.00	0.01	0.2059	0.6500	0.6860
MYOC	1	rs235869	C	0.30	0.27	0.7441	0.3883	0.4068
MYOC	1	rs16864720	T	0.14	0.16	0.8961	0.3438	0.3898
MYOC	1	rs235876	C	0.07	0.06	1.1830	0.2767	0.3334
MYOC	1	rs2236875	T	0.20	0.18	0.7120	0.3988	0.4305
MYOC	**1**	**rs235917**	**A**	**0.08**	**0.04**	**7.1660**	**0.0074**	**0.0101**
MYOC	1	rs7525674	C	0.04	0.04	0.0349	0.8519	0.8649
MYOC	1	MYOCnc1	G	0.02	0.03	0.0488	0.8251	1.0000
MYOC	1	rs61730977	G	0.04	0.05	0.0320	0.8581	0.8715
MYOC	1	rs2234926	T	0.00	0.01	2.7040	0.1001	0.1230
MYOC	1	rs12082573	G	0.04	0.04	0.0449	0.8323	0.8678
MYOC	1	rs2075648	T	0.01	0.01	0.4142	0.5198	0.5457
CYP1B1	2	rs9341261	G	0.03	0.04	0.3358	0.5622	0.5774
CYP1B1	2	rs162562	T	0.32	0.28	1.4690	0.2254	0.2437
CYP1B1	2	rs1056836	G	0.17	0.20	1.4060	0.2358	0.2494
CYP1B1	2	rs162561	T	0.20	0.17	1.3650	0.2427	0.2530
CYP1B1	2	rs9341252	G	0.05	0.04	1.2820	0.2575	0.3255
C2p16	2	rs2303298	A	0.10	0.11	0.3074	0.5793	0.6576
C2p16	2	rs10202118	A	0.25	0.26	0.0763	0.7824	0.8132
C2p16	**2**	**rs11125375**	**T**	**0.29**	**0.22**	**4.3020**	**0.0381**	**0.0418**
C2p16	2	rs10208467	G	0.22	0.25	1.7570	0.1851	0.1983
C2p16	2	rs1533428	A	0.39	0.37	0.5196	0.4710	0.4822
C2p16	2	rs12994401	T	0.03	0.02	1.6510	0.1988	0.2800
C2p16	2	rs11889995	A	0.07	0.08	0.0780	0.7800	0.7964
WDR36	5	rs7729832	A	0.12	0.12	0.0066	0.9354	1.0000
WDR36	5	rs17623144	C	0.00	0.00	0.9965	0.3182	1.0000
WDR36	5	rs13357724	T	0.01	0.00	0.6620	0.4159	0.6864
WDR36	5	rs13153937	A	0.05	0.05	0.0003	0.9873	1.0000
WDR36	5	rs6859041	G	0.29	0.28	0.3163	0.5739	0.5967
WDR36	5	rs2416257	A	0.13	0.12	0.0792	0.7783	0.8377
WDR36	5	rs17132783	G	0.17	0.15	0.6545	0.4185	0.4562
WDR36	5	rs10038058	A	0.25	0.25	0.0388	0.8439	0.8749
WDR36	5	rs12515367	G	0.03	0.03	0.0365	0.8484	1.0000
WDR36	5	rs12521169	A	0.14	0.14	0.1050	0.7460	0.7676

Ch, Chromosome; MA, minor allele; MAF, minor allele frequency; p, p-value obtained from the χ^2 test; p*, p-value from Fisher's exact test

(Table A 10.1 continues overpage)

Genomic region	Ch	SNP	MA	MAF POAG	MAF Controls	χ^2	p	p*
WDR36	5	rs10051830	G	0.29	0.27	0.3333	0.5637	0.5930
WDR36	5	rs10491424	G	0.47	0.48	0.0178	0.8940	0.9455
WDR36	5	rs11956837	G	0.03	0.04	0.0460	0.8301	0.8525
WDR36	5	rs12522383	T	0.17	0.18	0.1516	0.6970	0.7211
WDR36	5	rs17624563	C	0.18	0.17	0.1077	0.7428	0.7876
WDR36	5	rs43203	T	0.06	0.06	0.0162	0.8988	1.0000
WDR36	5	rs11241098	T	0.14	0.16	0.2564	0.6126	0.6338
WDR36	5	rs7722241	T	0.43	0.40	0.7599	0.3833	0.4042
WDR36	5	rs10041326	C	0.07	0.08	0.2907	0.5898	0.6057
WDR36	5	rs4530809	G	0.23	0.19	2.9650	0.0851	0.0943
WDR36	5	rs17132810	T	0.14	0.15	0.1697	0.6804	0.7023
WDR36	5	rs1457113	C	0.08	0.06	0.8300	0.3623	0.4257
COL1A2	7	rs4729131	A	0.08	0.08	0.0216	0.8830	0.8994
COL1A2	7	rs3814967	A	0.36	0.39	1.3950	0.2376	0.2593
COL1A2	7	rs1800222	A	0.37	0.34	1.1870	0.2759	0.2857
COL1A2	7	rs411717	C	0.27	0.26	0.0060	0.9383	1.0000
COL1A2	7	rs406226	C	0.13	0.12	0.2454	0.6203	0.6772
COL1A2	7	rs3763466	A	0.14	0.14	0.0000	1.0000	1.0000
COL1A2	7	rs17166249	G	0.29	0.24	2.1640	0.1413	0.1633
COL1A2	7	rs42518	C	0.10	0.07	2.0180	0.1554	0.1861
COL1A2	7	rs2521206	C	0.30	0.34	2.0720	0.1501	0.1638
COL1A2	7	rs42521	C	0.38	0.36	0.2136	0.6440	0.6707
COL1A2	7	rs42522	G	0.23	0.22	0.3827	0.5361	0.5667
COL1A2	7	rs42524	G	0.07	0.06	0.2966	0.5860	0.6736
COL1A2	7	rs42526	A	0.44	0.43	0.0763	0.7823	0.8358
COL1A2	7	rs2521205	A	0.34	0.36	0.4532	0.5008	0.5201
COL1A2	7	rs3736638	T	0.25	0.29	1.6240	0.2025	0.2178
COL1A2	7	rs42527	A	0.15	0.14	0.3075	0.5792	0.6276
COL1A2	7	rs369982	T	0.19	0.16	1.1090	0.2923	0.3205
COL1A2	7	rs4266	G	0.21	0.22	0.2033	0.6521	0.6785
COL1A2	7	rs10235102	T	0.08	0.08	0.0212	0.8841	0.9007
COL1A2	7	rs441051	T	0.27	0.24	1.2960	0.2550	0.2719
COL1A2	7	rs2072071	A	0.21	0.24	1.2930	0.2555	0.2877
COL1A2	7	rs13240759	C	0.12	0.13	0.1114	0.7385	0.7591
COL1A2	7	rs400218	C	0.20	0.18	0.5204	0.4707	0.4851
COL1A2	7	rs10046552	G	0.19	0.21	0.5132	0.4738	0.4957
COL1A2	7	rs12668754	A	0.02	0.01	1.6730	0.1959	0.2975
COL1A2	7	rs11764718	T	0.06	0.05	0.3481	0.5552	0.6498
COL1A2	7	rs1062394	A	0.11	0.14	2.0250	0.1547	0.1786
COL1A2	7	rs1034620	C	0.08	0.07	0.2357	0.6273	0.7049
COL1A2	7	rs11982782	T	0.47	0.46	0.0001	0.9923	1.0000
CAV1&2	7	rs8940	C	0.19	0.19	0.0235	0.8781	0.9307
CAV1&2	7	rs10258482	T	0.44	0.41	0.7252	0.3944	0.4070
CAV1&2	7	rs6466578	C	0.16	0.14	0.7117	0.3989	0.4432
CAV1&2	7	rs3919515	C	0.37	0.41	1.9160	0.1663	0.1837
CAV1&2	7	rs926198	A	0.49	0.47	0.3025	0.5823	0.5853
CAV1&2	7	rs3779512	G	0.37	0.36	0.0908	0.7631	0.7776
CAV1&2	7	rs10262090	C	0.11	0.13	0.8460	0.3577	0.3989

Ch, Chromosome; MA, minor allele; MAF, minor allele frequency; p, p-value obtained from the χ^2 test; p*, p-value from Fisher's exact test

(Table A 10.1 continues overpage)

Genomic region	Ch	SNP	MA	MAF POAG	MAF Controls	χ^2	p	p*
CAV1&2	7	rs3807986	T	0.28	0.25	1.1650	0.2805	0.3141
CAV1&2	7	rs1476833	A	0.29	0.28	0.0680	0.7943	0.8202
CAV1&2	7	rs10270569	T	0.18	0.17	0.1527	0.6960	0.7175
CAV1&2	7	rs11773845	T	0.23	0.22	0.1397	0.7086	0.7429
CAV1&2	7	rs9886215	C	0.32	0.36	1.5050	0.2200	0.2483
CAV1&2	7	rs3807992	A	0.48	0.47	0.3030	0.5820	0.5852
CDKN2B	9	rs3217992	A	0.06	0.05	0.3203	0.5714	0.6624
CDKN2B	9	rs1063192	C	0.00	0.00	0.3298	0.5658	1.0000
CDKN2B	9	rs3217989	C	0.32	0.29	1.2400	0.2654	0.2986
CDKN2B	**9**	**rs974336**	**A**	**0.23**	**0.30**	**4.0720**	**0.0436**	**0.0445**
CDKN2B	9	rs3217980	A	0.08	0.11	2.3060	0.1289	0.1607
CDKN2B	9	rs2069422	G	0.11	0.12	0.1066	0.7440	0.8278
COL5A1	9	rs1536478	A	0.29	0.29	0.0116	0.9142	0.9399
COL5A1	9	rs3118516	A	0.19	0.15	2.3310	0.1268	0.1427
COL5A1	9	rs1536482	T	0.46	0.43	0.8139	0.3670	0.3722
COL5A1	9	rs7044529	T	0.39	0.41	0.2379	0.6257	0.6759
ATOH7	10	rs6480320	A	0.29	0.27	0.6287	0.4278	0.4479
ATOH7	10	rs7916697	G	0.20	0.23	1.2540	0.2628	0.2788
ATOH7	10	rs2289804	C	0.50	0.49	0.2991	0.5845	0.6323
ATOH7	10	rs1900004	C	0.24	0.24	0.0017	0.9669	1.0000
ATOH7	10	rs41340944	G	0.43	0.46	0.5598	0.4544	0.4919
ATOH7	10	rs1900020	G	0.49	0.46	0.7957	0.3724	0.3752
SIX1&6	14	rs12436579	T	0.47	0.51	1.5070	0.2195	0.2456
SIX1&6	14	rs17097585	T	0.09	0.08	0.1199	0.7291	0.8069
SIX1&6	14	rs8006274	T	0.27	0.25	0.1216	0.7273	0.7558
SIX1&6	14	rs1007152	G	0.27	0.25	0.7338	0.3917	0.4362
SIX1&6	14	rs7152532	A	0.03	0.02	0.7086	0.3999	0.5181
SIX1&6	14	rs11849906	G	0.05	0.03	3.1980	0.0737	0.1036
SIX1&6	14	rs1955698	A	0.43	0.47	1.1780	0.2777	0.3029
SIX1&6	14	rs10150234	G	0.43	0.46	0.5596	0.4544	0.4922
SIX1&6	14	rs11158289	T	0.40	0.42	0.2242	0.6359	0.6769
SIX1&6	14	rs10142401	G	0.32	0.33	0.1148	0.7348	0.7710
SIX1&6	14	rs10483726	C	0.48	0.50	0.5622	0.4534	0.4947
SIX1&6	14	rs8020353	T	0.26	0.26	0.0373	0.8469	0.8763
SIX1&6	14	rs10483727	G	0.01	0.01	0.4142	0.5198	0.5457
SIX1&6	14	rs2057135	C	0.44	0.43	0.2408	0.6236	0.6305
SIX1&6	14	rs7156317	C	0.50	0.48	0.3796	0.5378	0.5397
SIX1&6	14	rs7146104	A	0.07	0.06	0.2762	0.5992	0.6837
SIX1&6	14	rs4901995	C	0.15	0.14	0.6484	0.4207	0.4383
SIX1&6	14	rs8007935	A	0.23	0.19	1.9590	0.1617	0.1772
SIX1&6	14	rs11628064	T	0.09	0.10	0.2323	0.6298	0.6475
ZNF469	16	rs12447690	T	0.14	0.15	0.1014	0.7501	0.7722
ZNF469	16	rs7500824	C	0.16	0.17	0.3416	0.5589	0.5802
ZNF469	16	rs4352077	A	0.06	0.05	0.0823	0.7742	0.8797
ZNF469	16	rs7501402	T	0.25	0.24	0.0784	0.7795	0.8113
ZNF469	16	rs6540223	C	0.33	0.29	1.0690	0.3012	0.3370
ZNF469	16	rs12448211	A	0.33	0.38	2.4170	0.1200	0.1326
ZNF469	16	rs9938149	C	0.44	0.38	3.0700	0.0798	0.0824

Ch, Chromosome; MA, minor allele; MAF, minor allele frequency; p, p-value obtained from the χ^2 test; p*, p-value from Fisher's exact test

(Table A 10.1 continues overpage)

Genomic region	Ch	SNP	MA	MAF POAG	MAF Controls	χ^2	p	p*
ZNF469	**16**	**rs9925231**	**A**	**0.50**	**0.43**	**4.8180**	**0.0282**	**0.0288**
ZNF469	16	rs11644804	T	0.25	0.26	0.1547	0.6941	0.6966
COL1A1	17	rs2696297	C	0.16	0.19	0.9256	0.3360	0.3688
COL1A1	**17**	**rs16948744**	**G**	**0.37**	**0.44**	**3.9750**	**0.0462**	**0.0511**
COL1A1	17	rs2696296	T	0.42	0.46	1.1930	0.2747	0.3022
COL1A1	17	rs36107109	T	0.09	0.10	0.3598	0.5486	0.5655
COL1A1	17	rs2586476	T	0.31	0.33	0.6831	0.4085	0.4210
COL1A1	17	rs12945599	T	0.08	0.07	0.0489	0.8251	0.8968
COL1A1	17	rs2696270	C	0.43	0.39	1.4220	0.2331	0.2388
COL1A1	17	rs1061947	T	0.15	0.18	2.0820	0.1490	0.1670
COL1A1	17	rs1061237	C	0.48	0.47	0.0414	0.8388	0.8905
COL1A1	17	rs2277632	C	0.46	0.40	2.5440	0.1107	0.1134
COL1A1	17	rs2696245	G	0.07	0.07	0.0643	0.7998	0.8920
COL1A1	17	rs2586488	T	0.50	0.48	0.3787	0.5383	0.5398
COL1A1	17	rs2075559	G	0.39	0.45	3.7110	0.0540	0.0616
COL1A1	17	rs2857396	C	0.09	0.09	0.0196	0.8887	0.9065
COL1A1	17	rs2696247	C	0.15	0.14	0.3122	0.5764	0.6253
COL1A1	17	rs2586494	T	0.20	0.17	1.0390	0.3081	0.3352
COL1A1	17	rs16948765	C	0.06	0.08	1.1870	0.2759	0.2873
COL1A1	17	rs2586498	G	0.36	0.33	0.9137	0.3391	0.3511

Ch, Chromosome; MA, minor allele; MAF, minor allele frequency; p, p-value obtained from the χ^2 test; p*, p-value from Fisher's exact test

Table A10.2 Logistic regression modelling of single SNP association with POAG in the study population

Genomic region	SNP	p1	OR1 (95% CI)	p2	OR2 (95% CI)	p3	OR3 (95% CI)
COL8A2	rs274750	0.9392	1.01 (0.77 - 1.32)	0.8932	1.02 (0.78 - 1.34)	0.8861	0.98 (0.72 - 1.32)
COL8A2	**rs6693322**	**0.0573**	**1.59 (0.98 - 2.57)**	**0.0659**	**1.56 (0.97 - 2.51)**	**0.0139**	**1.92 (1.14 - 3.24)**
COL8A2	rs274754	0.933	1.01 (0.76 - 1.35)	0.9203	1.02 (0.76 - 1.36)	0.9099	0.98 (0.72 - 1.35)
MYOC	61730975	0.6993	1.17 (0.53 - 2.55)	0.7356	1.15 (0.52 - 2.55)	0.7059	0.84 (0.33 - 2.1)
MYOC	61745146	0.6738	1.2 (0.51 - 2.81)	0.6621	1.21 (0.51 - 2.87)	0.6215	1.27 (0.49 - 3.26)
MYOC	61730976	0.0576	1.97 (0.97 - 4.01)	0.058	2.02 (0.98 - 4.17)	0.4166	1.43 (0.6 - 3.39)
MYOC	rs79255460	0.8341	0.93 (0.45 - 1.9)	0.8983	0.96 (0.48 - 1.9)	0.9238	0.96 (0.45 - 2.05)
MYOC	rs12076134	0.9585	1.01 (0.72 - 1.41)	0.985	1 (0.72 - 1.38)	0.8841	0.97 (0.68 - 1.4)
MYOC	rs2032555	0.689	1.12 (0.65 - 1.93)	0.6532	1.13 (0.66 - 1.96)	0.7202	1.12 (0.61 - 2.03)
MYOC	rs57824969	0.65	0.66 (0.11 - 3.98)	0.6362	0.65 (0.11 - 3.92)	0.8166	0.79 (0.11 - 5.69)
MYOC	rs235869	0.3883	1.14 (0.85 - 1.53)	0.3346	1.16 (0.85 - 1.59)	0.5757	1.1 (0.78 - 1.56)
MYOC	rs16864720	0.3438	0.83 (0.57 - 1.21)	0.3443	0.83 (0.56 - 1.22)	0.5256	0.87 (0.57 - 1.33)
MYOC	rs235876	0.2767	1.35 (0.78 - 2.34)	0.2742	1.35 (0.79 - 2.31)	0.6987	1.12 (0.63 - 2.02)
MYOC	rs2236875	0.3988	1.16 (0.82 - 1.64)	0.3977	1.16 (0.82 - 1.63)	0.7228	1.07 (0.73 - 1.56)
MYOC	**rs235917**	**0.0074**	**2.21 (1.22 - 4)**	**0.0084**	**2.23 (1.23 - 4.05)**	**0.014**	**2.32 (1.19 - 4.52)**
MYOC	rs7525674	0.8519	0.94 (0.48 - 1.85)	0.8767	0.95 (0.49 - 1.83)	0.8723	0.94 (0.43 - 2.03)
MYOC	MYOCnc1	0.8251	0.91 (0.38 - 2.16)	0.8198	0.91 (0.39 - 2.1)	0.7262	1.18 (0.46 - 3.05)
MYOC	61730977	0.8581	0.94 (0.5 - 1.79)	0.9	0.96 (0.51 - 1.8)	0.7431	0.88 (0.43 - 1.84)
MYOC	2234926	0.1001	0.2 (0.02 - 1.7)	0.1429	0.2 (0.02 - 1.72)	0.2387	0.26 (0.03 - 2.42)
MYOC	12082573	0.8323	0.93 (0.48 - 1.8)	0.8576	0.94 (0.5 - 1.79)	0.771	0.89 (0.42 - 1.9)
MYOC	rs2075648	0.5198	0.66 (0.19 - 2.36)	0.5493	0.68 (0.19 - 2.44)	0.8599	1.13 (0.28 - 4.5)
CYP1B1	rs9341261	0.5622	0.8 (0.38 - 1.69)	0.5143	0.78 (0.36 - 1.66)	0.4858	0.74 (0.33 - 1.71)
CYP1B1	rs162562	0.2254	1.21 (0.89 - 1.64)	0.2259	1.21 (0.89 - 1.65)	0.4268	1.15 (0.82 - 1.62)
CYP1B1	rs1056836	0.2358	0.81 (0.57 - 1.15)	0.2521	0.82 (0.58 - 1.15)	0.1656	0.76 (0.52 - 1.12)
CYP1B1	rs162561	0.2427	1.23 (0.87 - 1.74)	0.2202	1.24 (0.88 - 1.76)	0.2433	1.25 (0.86 - 1.83)
CYP1B1	rs9341252	0.2575	1.46 (0.76 - 2.8)	0.2569	1.46 (0.76 - 2.8)	0.2384	1.54 (0.75 - 3.15)
C2p16	rs2303298	0.5793	0.88 (0.57 - 1.37)	0.53	0.87 (0.56 - 1.35)	0.6792	0.9 (0.56 - 1.46)
C2p16	rs10202118	0.7824	0.96 (0.7 - 1.3)	0.7564	0.95 (0.71 - 1.29)	0.4288	0.87 (0.62 - 1.23)
C2p16	**rs11125375**	**0.0381**	**1.39 (1.02 - 1.89)**	**0.0607**	**1.32 (0.99 - 1.77)**	**0.0898**	**1.33 (0.96 - 1.84)**
C2p16	rs10208467	0.1851	0.81 (0.59 - 1.11)	0.1808	0.81 (0.59 - 1.11)	0.1922	0.79 (0.56 - 1.13)
C2p16	rs1533428	0.471	1.11 (0.84 - 1.46)	0.4992	1.1 (0.84 - 1.43)	0.2472	1.19 (0.89 - 1.59)
C2p16	rs12994401	0.1988	1.77 (0.73 - 4.26)	0.206	1.78 (0.73 - 4.33)	0.507	1.39 (0.53 - 3.67)
C2p16	rs11889995	0.78	0.93 (0.56 - 1.55)	0.8015	0.94 (0.56 - 1.57)	0.4147	0.79 (0.45 - 1.39)
WDR36	rs7729832	0.9354	1.02 (0.67 - 1.54)	0.9956	1 (0.65 - 1.52)	0.6203	1.13 (0.7 - 1.82)
WDR36	rs17623144	0.3182	N/A	N/A	N/A	0.9993	N/A
WDR36	rs13357724	0.4159	2 (0.36 - 10.98)	0.4247	2.01 (0.36 - 11.08)	0.5743	1.68 (0.27 - 10.38)
WDR36	rs13153937	0.9873	1 (0.54 - 1.83)	0.9705	0.99 (0.55 - 1.79)	0.7557	0.9 (0.47 - 1.74)
WDR36	rs6859041	0.5739	1.09 (0.81 - 1.47)	0.5586	1.1 (0.8 - 1.5)	0.6694	1.08 (0.76 - 1.53)
WDR36	rs2416257	0.7783	1.06 (0.71 - 1.58)	0.8335	1.05 (0.69 - 1.58)	0.5016	1.17 (0.74 - 1.87)
WDR36	rs17132783	0.4185	1.16 (0.81 - 1.68)	0.3928	1.18 (0.81 - 1.7)	0.3759	1.2 (0.8 - 1.81)
WDR36	rs10038058	0.8439	1.03 (0.76 - 1.41)	0.8576	1.03 (0.74 - 1.43)	0.9562	1.01 (0.7 - 1.45)
WDR36	rs12515367	0.8484	1.08 (0.49 - 2.4)	0.858	1.08 (0.48 - 2.42)	0.9144	0.95 (0.4 - 2.29)
WDR36	rs12521169	0.746	1.07 (0.72 - 1.57)	0.7647	1.06 (0.72 - 1.55)	0.9849	1 (0.66 - 1.54)
WDR36	rs10051830	0.5637	1.09 (0.81 - 1.47)	0.5396	1.1 (0.81 - 1.5)	0.563	1.11 (0.79 - 1.56)
WDR36	rs10491424	0.894	0.98 (0.75 - 1.28)	0.936	0.99 (0.75 - 1.31)	0.5645	0.91 (0.67 - 1.25)
WDR36	rs11956837	0.8301	0.92 (0.44 - 1.94)	0.8386	0.92 (0.43 - 1.97)	0.7587	0.88 (0.39 - 2)

OR (95% CI), Odds ratio (95% confidence intervals); p1/OR1, unadjusted; p2/OR2, adjusted for gender; p3/OR3, adjusted for age and gender

(Table A10.2 continues overpage)

Genomic region	SNP	p1	OR1 (95% CI)	p2	OR2 (95% CI)	p3	OR3 (95% CI)
WDR36	rs12522383	0.697	0.93 (0.66 - 1.32)	0.687	0.93 (0.65 - 1.32)	0.7403	0.94 (0.64 - 1.38)
WDR36	rs17624563	0.7428	1.06 (0.75 - 1.51)	0.8174	1.04 (0.73 - 1.48)	0.6659	1.09 (0.73 - 1.62)
WDR36	rs43203	0.8988	1.04 (0.59 - 1.83)	0.8828	1.04 (0.59 - 1.85)	0.6692	0.87 (0.45 - 1.66)
WDR36	rs11241098	0.6126	0.91 (0.62 - 1.32)	0.5974	0.9 (0.62 - 1.32)	0.7674	0.94 (0.62 - 1.43)
WDR36	rs7722241	0.3833	1.13 (0.86 - 1.48)	0.3901	1.13 (0.85 - 1.51)	0.3115	1.18 (0.86 - 1.62)
WDR36	rs10041326	0.5898	0.87 (0.52 - 1.45)	0.6538	0.9 (0.58 - 1.42)	0.6207	0.88 (0.53 - 1.46)
WDR36	rs4530809	0.0851	1.34 (0.96 - 1.86)	0.0978	1.31 (0.95 - 1.82)	0.2633	1.23 (0.86 - 1.77)
WDR36	rs17132810	0.6804	0.92 (0.63 - 1.35)	0.6689	0.92 (0.63 - 1.35)	0.8352	0.96 (0.63 - 1.46)
WDR36	rs1457113	0.3623	1.28 (0.76 - 2.15)	0.3448	1.3 (0.76 - 2.21)	0.2842	1.39 (0.76 - 2.51)
COL1A2	rs4729131	0.883	0.96 (0.58 - 1.59)	0.841	0.95 (0.58 - 1.55)	0.8553	0.95 (0.55 - 1.64)
COL1A2	rs3814967	0.2376	0.85 (0.64 - 1.12)	0.27	0.85 (0.65 - 1.13)	0.3851	0.87 (0.64 - 1.19)
COL1A2	rs1800222	0.2759	1.17 (0.88 - 1.55)	0.3192	1.15 (0.87 - 1.53)	0.309	1.18 (0.86 - 1.61)
COL1A2	rs411717	0.9383	1.01 (0.75 - 1.37)	0.9885	1 (0.73 - 1.37)	0.9903	1 (0.71 - 1.42)
COL1A2	rs406226	0.6203	1.11 (0.74 - 1.67)	0.6614	1.1 (0.73 - 1.66)	0.7073	1.09 (0.69 - 1.73)
COL1A2	rs3763466	1	1 (0.68 - 1.48)	0.9338	0.98 (0.67 - 1.45)	0.8771	0.97 (0.63 - 1.48)
COL1A2	rs17166249	0.1413	1.26 (0.93 - 1.7)	0.1325	1.27 (0.93 - 1.72)	0.2165	1.24 (0.88 - 1.75)
COL1A2	rs42518	0.1554	1.41 (0.88 - 2.27)	0.1475	1.43 (0.88 - 2.31)	0.1273	1.52 (0.89 - 2.61)
COL1A2	rs2521206	0.1501	0.81 (0.61 - 1.08)	0.1438	0.81 (0.61 - 1.08)	0.2284	0.83 (0.6 - 1.13)
COL1A2	rs42521	0.644	1.07 (0.81 - 1.41)	0.5941	1.08 (0.81 - 1.44)	0.4723	1.12 (0.82 - 1.54)
COL1A2	rs42522	0.5361	1.11 (0.8 - 1.53)	0.542	1.11 (0.8 - 1.53)	0.8877	0.97 (0.68 - 1.4)
COL1A2	rs42524	0.586	1.17 (0.67 - 2.03)	0.5726	1.18 (0.67 - 2.06)	0.8556	1.06 (0.56 - 2.01)
COL1A2	rs42526	0.7823	1.04 (0.79 - 1.36)	0.7883	1.04 (0.79 - 1.36)	0.4935	1.11 (0.83 - 1.49)
COL1A2	rs2521205	0.5008	0.91 (0.69 - 1.2)	0.5167	0.91 (0.69 - 1.21)	0.8254	0.97 (0.71 - 1.32)
COL1A2	rs3736638	0.2025	0.82 (0.61 - 1.11)	0.2194	0.83 (0.61 - 1.12)	0.1824	0.8 (0.57 - 1.11)
COL1A2	rs42527	0.5792	1.11 (0.76 - 1.63)	0.6242	1.11 (0.74 - 1.65)	0.7551	1.07 (0.69 - 1.67)
COL1A2	rs369982	0.2923	1.21 (0.85 - 1.73)	0.3227	1.2 (0.84 - 1.73)	0.236	1.28 (0.85 - 1.93)
COL1A2	rs4266	0.6521	0.93 (0.67 - 1.29)	0.7013	0.94 (0.67 - 1.31)	0.5361	0.89 (0.62 - 1.29)
COL1A2	rs10235102	0.8841	0.96 (0.59 - 1.58)	0.9009	0.97 (0.59 - 1.6)	0.8507	0.95 (0.55 - 1.64)
COL1A2	rs441051	0.255	1.2 (0.88 - 1.63)	0.2686	1.2 (0.87 - 1.64)	0.3187	1.2 (0.84 - 1.69)
COL1A2	rs2072071	0.2555	0.83 (0.6 - 1.15)	0.2796	0.84 (0.61 - 1.15)	0.2097	0.8 (0.56 - 1.13)
COL1A2	rs13240759	0.7385	0.93 (0.62 - 1.4)	0.721	0.93 (0.63 - 1.38)	0.7772	0.94 (0.61 - 1.45)
COL1A2	rs400218	0.4707	1.14 (0.8 - 1.6)	0.4959	1.13 (0.8 - 1.6)	0.4779	1.15 (0.78 - 1.71)
COL1A2	rs10046552	0.4738	0.88 (0.63 - 1.24)	0.4842	0.89 (0.64 - 1.23)	0.4924	0.88 (0.62 - 1.26)
COL1A2	rs12668754	0.1959	2.01 (0.68 - 5.94)	0.1914	2.07 (0.69 - 6.17)	0.3337	1.8 (0.55 - 5.92)
COL1A2	rs11764718	0.5552	1.2 (0.66 - 2.17)	0.5598	1.2 (0.66 - 2.18)	0.8118	1.08 (0.56 - 2.09)
COL1A2	rs1062394	0.1547	0.74 (0.49 - 1.12)	0.178	0.75 (0.5 - 1.14)	0.4147	0.83 (0.53 - 1.3)
COL1A2	rs1034620	0.6273	1.13 (0.69 - 1.86)	0.6479	1.13 (0.68 - 1.88)	0.9565	1.02 (0.58 - 1.78)
COL1A2	rs11982782	0.9923	1 (0.77 - 1.31)	0.9683	0.99 (0.76 - 1.3)	0.9948	1 (0.74 - 1.35)
CAV1&2	rs8940	0.8781	0.97 (0.69 - 1.37)	0.8776	0.97 (0.69 - 1.37)	0.676	0.92 (0.63 - 1.35)
CAV1&2	rs10258482	0.3944	1.13 (0.86 - 1.48)	0.4154	1.12 (0.86 - 1.46)	0.7793	1.04 (0.78 - 1.4)
CAV1&2	rs6466578	0.3989	1.18 (0.81 - 1.71)	0.3974	1.17 (0.81 - 1.7)	0.595	1.12 (0.74 - 1.69)
CAV1&2	rs3919515	0.1663	0.82 (0.63 - 1.08)	0.1798	0.83 (0.64 - 1.09)	0.4245	0.89 (0.66 - 1.19)
CAV1&2	rs926198	0.5823	1.08 (0.82 - 1.41)	0.6183	1.07 (0.82 - 1.4)	0.5587	1.09 (0.81 - 1.47)
CAV1&2	rs3779512	0.7631	1.04 (0.79 - 1.38)	0.7966	1.04 (0.8 - 1.35)	0.9033	0.98 (0.74 - 1.31)
CAV1&2	rs10262090	0.3577	0.82 (0.54 - 1.25)	0.3925	0.84 (0.56 - 1.25)	0.995	1 (0.65 - 1.55)
CAV1&2	rs3807986	0.2805	1.18 (0.87 - 1.6)	0.3327	1.16 (0.86 - 1.56)	0.5506	1.11 (0.79 - 1.54)
CAV1&2	rs1476833	0.7943	1.04 (0.77 - 1.4)	0.7671	1.05 (0.78 - 1.41)	0.7442	0.95 (0.68 - 1.32)

OR (95% CI), Odds ratio (95% confidence intervals); p1/OR1, unadjusted; p2/OR2, adjusted for gender; p3/OR3, adjusted for age and gender

(Table A10.2 continues overpage)

Genomic region	SNP	p1	OR1 (95% CI)	p2	OR2 (95% CI)	p3	OR3 (95% CI)
CAV1&2	rs10270569	0.696	1.07 (0.75 - 1.53)	0.7319	1.07 (0.74 - 1.52)	0.7278	1.07 (0.72 - 1.6)
CAV1&2	rs11773845	0.7086	1.06 (0.77 - 1.47)	0.73	1.06 (0.77 - 1.45)	0.4273	1.15 (0.81 - 1.64)
CAV1&2	rs9886215	0.22	0.84 (0.63 - 1.11)	0.1997	0.83 (0.62 - 1.11)	0.3465	0.86 (0.62 - 1.18)
CAV1&2	rs3807992	0.582	1.08 (0.82 - 1.41)	0.5832	1.08 (0.82 - 1.41)	0.5124	1.11 (0.82 - 1.49)
CDKN2B	rs3217992	0.5714	1.18 (0.67 - 2.09)	0.5825	1.18 (0.66 - 2.1)	0.6974	1.14 (0.59 - 2.18)
CDKN2B	rs1063192	0.5658	2 (0.18 - 22.09)	0.5292	2.17 (0.19 - 24.3)	0.4585	2.58 (0.21 - 31.41)
CDKN2B	rs3217989	0.2654	1.18 (0.88 - 1.58)	0.2721	1.18 (0.88 - 1.57)	0.4686	1.13 (0.82 - 1.55)
CDKN2B	**rs974336**	**0.0436**	**0.73 (0.54 - 0.99)**	**0.0322**	**0.7 (0.51 - 0.97)**	**0.0862**	**0.74 (0.52 - 1.05)**
CDKN2B	rs3217980	0.1289	0.7 (0.44 - 1.11)	0.1261	0.69 (0.43 - 1.11)	0.1702	0.7 (0.42 - 1.17)
CDKN2B	rs2069422	0.744	0.93 (0.61 - 1.43)	0.7242	0.92 (0.6 - 1.43)	0.7951	0.94 (0.58 - 1.52)
COL5A1	rs1536478	0.9142	1.02 (0.76 - 1.37)	0.9275	1.01 (0.76 - 1.35)	0.5892	0.92 (0.67 - 1.26)
COL5A1	rs3118516	0.1268	1.32 (0.92 - 1.9)	0.1381	1.31 (0.92 - 1.87)	0.4068	1.18 (0.8 - 1.75)
COL5A1	rs1536482	0.367	1.13 (0.86 - 1.48)	0.4046	1.12 (0.86 - 1.47)	0.3961	1.14 (0.84 - 1.54)
COL5A1	rs7044529	0.6257	0.93 (0.71 - 1.23)	0.5741	0.92 (0.7 - 1.22)	0.1946	0.82 (0.61 - 1.11)
ATOH7	rs6480320	0.4278	1.13 (0.84 - 1.52)	0.4592	1.12 (0.83 - 1.5)	0.6329	1.08 (0.78 - 1.5)
ATOH7	rs7916697	0.2628	0.83 (0.6 - 1.15)	0.2813	0.84 (0.6 - 1.16)	0.484	0.88 (0.61 - 1.27)
ATOH7	rs2289804	0.5845	1.08 (0.82 - 1.41)	0.5909	1.08 (0.83 - 1.4)	0.6546	1.07 (0.8 - 1.43)
ATOH7	rs1900004	0.9669	0.99 (0.73 - 1.36)	0.9934	1 (0.73 - 1.37)	0.6538	1.09 (0.76 - 1.55)
ATOH7	rs41340944	0.4544	0.9 (0.69 - 1.18)	0.4796	0.91 (0.7 - 1.19)	0.6165	0.93 (0.69 - 1.25)
ATOH7	rs1900020	0.3724	1.13 (0.86 - 1.48)	0.3922	1.12 (0.86 - 1.47)	0.5999	1.08 (0.81 - 1.45)
SIX1&6	rs12436579	0.2195	0.85 (0.65 - 1.11)	0.2152	0.84 (0.64 - 1.11)	0.2588	0.84 (0.63 - 1.13)
SIX1&6	rs17097585	0.7291	1.09 (0.67 - 1.76)	0.7236	1.09 (0.68 - 1.75)	0.5682	1.17 (0.69 - 1.98)
SIX1&6	rs8006274	0.7273	1.06 (0.78 - 1.43)	0.7578	1.05 (0.78 - 1.41)	0.5323	1.11 (0.8 - 1.55)
SIX1&6	rs1007152	0.3917	1.14 (0.84 - 1.55)	0.3665	1.15 (0.85 - 1.57)	0.5546	1.11 (0.79 - 1.55)
SIX1&6	rs7152532	0.3999	1.44 (0.61 - 3.42)	0.4078	1.45 (0.6 - 3.46)	0.6471	1.25 (0.48 - 3.22)
SIX1&6	rs11849906	0.0737	1.95 (0.93 - 4.09)	0.078	1.98 (0.93 - 4.21)	0.059	2.22 (0.97 - 5.08)
SIX1&6	rs1955698	0.2777	0.86 (0.66 - 1.13)	0.2838	0.86 (0.66 - 1.13)	0.2605	0.84 (0.63 - 1.14)
SIX1&6	rs10150234	0.4544	0.9 (0.69 - 1.18)	0.4561	0.9 (0.69 - 1.18)	0.4369	0.89 (0.66 - 1.2)
SIX1&6	rs11158289	0.6359	0.94 (0.71 - 1.23)	0.6375	0.94 (0.71 - 1.23)	0.5633	0.91 (0.67 - 1.24)
SIX1&6	rs10142401	0.7348	0.95 (0.72 - 1.27)	0.7503	0.95 (0.72 - 1.27)	0.66	0.93 (0.68 - 1.28)
SIX1&6	rs10483726	0.4534	0.9 (0.69 - 1.18)	0.4522	0.9 (0.69 - 1.18)	0.3998	0.88 (0.66 - 1.18)
SIX1&6	rs8020353	0.8469	1.03 (0.76 - 1.4)	0.8817	1.02 (0.76 - 1.39)	0.9032	1.02 (0.73 - 1.43)
SIX1&6	rs10483727	0.5198	0.66 (0.19 - 2.36)	0.5713	0.71 (0.22 - 2.3)	0.9034	0.92 (0.25 - 3.43)
SIX1&6	rs2057135	0.6236	1.07 (0.82 - 1.4)	0.6425	1.07 (0.82 - 1.39)	0.7842	1.04 (0.77 - 1.4)
SIX1&6	rs7156317	0.5378	1.09 (0.83 - 1.42)	0.5695	1.08 (0.83 - 1.4)	0.4651	1.12 (0.83 - 1.49)
SIX1&6	rs7146104	0.5992	1.15 (0.68 - 1.97)	0.5921	1.16 (0.68 - 1.98)	0.8204	1.07 (0.59 - 1.95)
SIX1&6	rs4901995	0.4207	1.17 (0.8 - 1.71)	0.4292	1.16 (0.8 - 1.67)	0.7598	1.07 (0.71 - 1.6)
SIX1&6	rs8007935	0.1617	1.27 (0.91 - 1.77)	0.1837	1.25 (0.9 - 1.73)	0.2231	1.25 (0.87 - 1.8)
SIX1&6	rs11628064	0.6298	0.9 (0.57 - 1.41)	0.6754	0.91 (0.58 - 1.42)	0.5763	0.87 (0.53 - 1.42)
ZNF469	rs12447690	0.7501	0.94 (0.64 - 1.37)	0.7529	0.94 (0.64 - 1.38)	0.9925	1 (0.65 - 1.54)
ZNF469	rs7500824	0.5589	0.9 (0.62 - 1.29)	0.5813	0.9 (0.63 - 1.29)	0.7033	0.92 (0.62 - 1.38)
ZNF469	rs4352077	0.7742	1.09 (0.6 - 1.98)	0.7432	1.11 (0.61 - 2.01)	0.8781	1.05 (0.54 - 2.04)
ZNF469	rs7501402	0.7795	1.05 (0.77 - 1.43)	0.7176	1.06 (0.77 - 1.47)	0.7324	1.07 (0.74 - 1.53)
ZNF469	rs6540223	0.3012	1.17 (0.87 - 1.56)	0.3158	1.16 (0.87 - 1.55)	0.3505	1.17 (0.85 - 1.61)
ZNF469	rs12448211	0.12	0.8 (0.6 - 1.06)	0.1212	0.79 (0.59 - 1.06)	0.0856	0.75 (0.55 - 1.04)
ZNF469	rs9938149	0.0798	1.28 (0.97 - 1.68)	0.0874	1.27 (0.97 - 1.68)	0.1099	1.29 (0.94 - 1.75)
ZNF469	**rs9925231**	**0.0282**	**1.35 (1.03 - 1.77)**	**0.0298**	**1.36 (1.03 - 1.78)**	**0.0225**	**1.43 (1.05 - 1.93)**

OR (95% CI), Odds ratio (95% confidence intervals); p1/OR1, unadjusted; p2/OR2, adjusted for gender; p3/OR3, adjusted for age and gender

(Table A10.2 continues overpage)

Genomic region	SNP	p1	OR1 (95% CI)	p2	OR2 (95% CI)	p3	OR3 (95% CI)
ZNF469	rs11644804	0.6941	0.94 (0.69 - 1.28)	0.7118	0.94 (0.68 - 1.3)	0.8545	0.97 (0.68 - 1.37)
COL1A1	rs2696297	0.336	0.84 (0.59 - 1.2)	0.3629	0.85 (0.6 - 1.21)	0.3061	0.82 (0.55 - 1.2)
COL1A1	**rs16948744**	**0.0462**	**0.76 (0.58 - 1)**	**0.0391**	**0.75 (0.57 - 0.99)**	**0.0143**	**0.68 (0.5 - 0.93)**
COL1A1	rs2696296	0.2747	0.86 (0.66 - 1.13)	0.2551	0.86 (0.65 - 1.12)	0.1203	0.79 (0.59 - 1.06)
COL1A1	rs36107109	0.5486	0.87 (0.55 - 1.37)	0.555	0.87 (0.55 - 1.37)	0.6764	0.9 (0.55 - 1.48)
COL1A1	rs2586476	0.4085	0.89 (0.66 - 1.18)	0.4407	0.89 (0.67 - 1.19)	0.5	0.9 (0.65 - 1.23)
COL1A1	rs12945599	0.8251	1.06 (0.64 - 1.76)	0.8372	1.05 (0.64 - 1.73)	0.5178	1.2 (0.69 - 2.07)
COL1A1	rs2696270	0.2331	1.18 (0.9 - 1.55)	0.2068	1.19 (0.91 - 1.57)	0.2893	1.18 (0.87 - 1.6)
COL1A1	rs1061947	0.149	0.77 (0.53 - 1.1)	0.1644	0.78 (0.54 - 1.11)	0.226	0.79 (0.53 - 1.16)
COL1A1	rs1061237	0.8388	1.03 (0.79 - 1.35)	0.8525	1.03 (0.78 - 1.35)	0.8192	1.04 (0.77 - 1.4)
COL1A1	rs2277632	0.1107	1.25 (0.95 - 1.63)	0.1082	1.25 (0.95 - 1.65)	0.0779	1.31 (0.97 - 1.78)
COL1A1	rs2696245	0.7998	1.07 (0.63 - 1.83)	0.7953	1.07 (0.63 - 1.81)	0.6582	1.14 (0.64 - 2.02)
COL1A1	rs2586488	0.5383	1.09 (0.83 - 1.42)	0.5381	1.09 (0.83 - 1.42)	0.2793	1.18 (0.87 - 1.59)
COL1A1	**rs2075559**	**0.054**	**0.77 (0.58 - 1.01)**	**0.0483**	**0.75 (0.57 - 1)**	**0.0963**	**0.77 (0.56 - 1.05)**
COL1A1	rs2857396	0.8887	0.97 (0.61 - 1.54)	0.8782	0.97 (0.61 - 1.52)	0.6419	1.13 (0.69 - 1.85)
COL1A1	rs2696247	0.5764	1.12 (0.76 - 1.64)	0.5745	1.12 (0.76 - 1.64)	0.6651	1.1 (0.72 - 1.67)
COL1A1	rs2586494	0.3081	1.2 (0.85 - 1.69)	0.266	1.23 (0.86 - 1.75)	0.2234	1.28 (0.86 - 1.89)
COL1A1	rs16948765	0.2759	0.75 (0.44 - 1.27)	0.2926	0.77 (0.47 - 1.26)	0.3067	0.75 (0.43 - 1.31)
COL1A1	rs2586498	0.3391	1.15 (0.87 - 1.52)	0.3752	1.13 (0.86 - 1.5)	0.4894	1.12 (0.82 - 1.52)

OR (95% CI), Odds ratio (95% confidence intervals); p1/OR1, unadjusted; p2/OR2, adjusted for gender; p3/OR3, adjusted for age and gender

APPENDIX 11 - DIABETES MELLITUS ASSOCIATION ANALYSIS IN THE BEADXPRESS ASSOCIATION STUDY

Table A11.1 Single SNP association testing with a diagnosis of diabetes mellitus in the study population

Genomic region	Ch	SNP	MA	MAF DM	MAF non DM	χ^2	p	p*
COL8A2	1	rs274750	T	0.40	0.46	2.5860	0.1078	0.1167
COL8A2	1	rs6693322	G	0.10	0.08	1.0770	0.2993	0.2983
COL8A2	1	rs274754	A	0.33	0.35	0.3561	0.5507	0.5711
MYOC	1	61730975	T	0.03	0.03	0.2361	0.6270	0.6661
MYOC	1	61745146	T	0.02	0.03	0.6136	0.4335	0.4921
MYOC	1	61730976	A	0.04	0.04	0.0561	0.8128	1.0000
MYOC	1	rs79255460	A	0.05	0.03	1.1220	0.2895	0.3197
MYOC	1	rs12076134	G	0.22	0.19	1.6070	0.2048	0.2247
MYOC	1	rs2032555	G	0.05	0.07	2.0030	0.1570	0.1745
MYOC	1	rs57824969	A	0.01	0.01	0.2239	0.6361	0.6419
MYOC	1	rs235869	C	0.29	0.28	0.0571	0.8112	0.8060
MYOC	1	rs16864720	T	0.18	0.14	2.5740	0.1086	0.1194
MYOC	**1**	**rs235876**	**C**	**0.04**	**0.08**	**4.3940**	**0.0361**	**0.0358**
MYOC	1	rs2236875	T	0.17	0.19	0.4418	0.5063	0.5674
MYOC	1	rs235917	A	0.05	0.07	0.4043	0.5249	0.6437
MYOC	1	rs7525674	C	0.05	0.04	1.6250	0.2025	0.2587
MYOC	1	MYOCnc1	G	0.02	0.03	0.0384	0.8446	1.0000
MYOC	1	61730977	G	0.06	0.04	1.2880	0.2565	0.2851
MYOC	1	2234926	T	0.01	0.01	0.0263	0.8712	1.0000
MYOC	1	12082573	G	0.05	0.04	1.0670	0.3016	0.3596
MYOC	1	rs2075648	T	0.01	0.01	0.0004	0.9833	1.0000
CYP1B1	2	rs9341261	G	0.03	0.04	0.1049	0.7460	0.8394
CYP1B1	2	rs162562	T	0.28	0.30	0.3748	0.5404	0.5556
CYP1B1	2	rs1056836	G	0.20	0.17	0.6056	0.4365	0.4411
CYP1B1	2	rs162561	T	0.18	0.18	0.0000	0.9986	1.0000
CYP1B1	2	rs9341252	G	0.05	0.05	0.0042	0.9483	1.0000
C2p16	2	rs2303298	A	0.09	0.11	0.7705	0.3801	0.4017
C2p16	2	rs10202118	A	0.23	0.26	0.5830	0.4451	0.4933
C2p16	**2**	**rs11125375**	**T**	**0.21**	**0.28**	**4.4370**	**0.0352**	**0.0407**
C2p16	2	rs10208467	G	0.27	0.22	2.9370	0.0866	0.0962
C2p16	2	rs1533428	A	0.38	0.38	0.0055	0.9408	1.0000
C2p16	2	rs12994401	T	0.02	0.03	0.6136	0.4335	0.4921
C2p16	2	rs11889995	A	0.07	0.08	0.4582	0.4985	0.5728
WDR36	5	rs7729832	A	0.10	0.13	1.1270	0.2884	0.3024
WDR36	5	rs17623144	C	0.00	0.00	0.4353	0.5094	1.0000
WDR36	5	rs13357724	T	0.00	0.01	0.5320	0.4658	0.6738
WDR36	5	rs13153937	A	0.07	0.05	1.5250	0.2169	0.2390
WDR36	5	rs6859041	G	0.26	0.29	0.9566	0.3281	0.3652
WDR36	5	rs2416257	A	0.10	0.14	2.4590	0.1168	0.1202
WDR36	5	rs17132783	G	0.14	0.17	1.2510	0.2634	0.3105
WDR36	5	rs10038058	A	0.23	0.26	0.5065	0.4767	0.4941
WDR36	**5**	**rs12515367**	**G**	**0.05**	**0.02**	**5.7400**	**0.0166**	**0.0252**

Ch, Chromosome; MA, minor allele; MAF DM, minor allele frequency in diabetics; MAF nonDM, MAF in non diabetics; p, p-value obtained from the χ^2 test; p*, p-value from Fisher's exact test

(Table A11.1 continues overpage)

Genomic region	Ch	SNP	MA	MAF DM	MAF non DM	χ^2	p	p*
WDR36	5	rs12521169	A	0.14	0.14	0.0510	0.8214	0.8300
WDR36	5	rs10051830	G	0.27	0.28	0.1280	0.7205	0.7405
WDR36	5	rs10491424	G	0.46	0.48	0.4156	0.5191	0.5520
WDR36	5	rs11956837	G	0.04	0.03	0.8653	0.3523	0.4101
WDR36	**5**	**rs12522383**	**T**	**0.25**	**0.14**	**15.0500**	**0.0001**	**0.0002**
WDR36	5	rs17624563	C	0.14	0.19	3.4190	0.0644	0.0780
WDR36	5	rs43203	T	0.04	0.07	2.9370	0.0866	0.1150
WDR36	**5**	**rs11241098**	**T**	**0.20**	**0.13**	**8.3570**	**0.0038**	**0.0049**
WDR36	5	rs7722241	T	0.37	0.43	3.2140	0.0730	0.0822
WDR36	5	rs10041326	C	0.08	0.07	0.2062	0.6498	0.6722
WDR36	5	rs4530809	G	0.19	0.22	1.1340	0.2869	0.3170
WDR36	**5**	**rs17132810**	**T**	**0.20**	**0.13**	**8.7820**	**0.0030**	**0.0047**
WDR36	5	rs1457113	C	0.06	0.08	0.5159	0.4726	0.5636
COL1A2	7	rs4729131	A	0.07	0.08	0.4066	0.5237	0.5815
COL1A2	7	rs3814967	A	0.42	0.36	3.0720	0.0797	0.0914
COL1A2	7	rs1800222	A	0.32	0.37	1.8320	0.1759	0.1878
COL1A2	7	rs411717	C	0.25	0.27	0.2465	0.6196	0.6739
COL1A2	7	rs406226	C	0.13	0.12	0.0718	0.7888	0.8209
COL1A2	7	rs3763466	A	0.13	0.14	0.3752	0.5402	0.5907
COL1A2	7	rs17166249	G	0.24	0.28	1.0030	0.3166	0.3545
COL1A2	7	rs42518	C	0.07	0.10	1.7300	0.1885	0.2389
COL1A2	7	rs2521206	C	0.30	0.33	0.5684	0.4509	0.4736
COL1A2	**7**	**rs42521**	**C**	**0.43**	**0.35**	**4.8790**	**0.0272**	**0.0307**
COL1A2	**7**	**rs42522**	**G**	**0.18**	**0.24**	**3.9720**	**0.0463**	**0.0500**
COL1A2	7	rs42524	G	0.06	0.07	0.1740	0.6766	0.7608
COL1A2	7	rs42526	A	0.45	0.43	0.2723	0.6018	0.6006
COL1A2	7	rs2521205	A	0.38	0.34	1.6550	0.1983	0.2135
COL1A2	7	rs3736638	T	0.28	0.26	0.1492	0.6993	0.7374
COL1A2	7	rs42527	A	0.13	0.15	0.2961	0.5863	0.6727
COL1A2	7	rs369982	T	0.14	0.19	2.3810	0.1228	0.1402
COL1A2	**7**	**rs4266**	**G**	**0.27**	**0.19**	**6.3400**	**0.0118**	**0.0146**
COL1A2	**7**	**rs10235102**	**T**	**0.11**	**0.07**	**4.8850**	**0.0271**	**0.0395**
COL1A2	7	rs441051	T	0.21	0.27	3.3800	0.0660	0.0728
COL1A2	7	rs2072071	A	0.24	0.22	0.3611	0.5479	0.5937
COL1A2	7	rs13240759	C	0.11	0.13	0.7703	0.3801	0.4347
COL1A2	7	rs400218	C	0.17	0.20	0.7442	0.3883	0.4458
COL1A2	7	rs10046552	G	0.19	0.20	0.1549	0.6939	0.7118
COL1A2	7	rs12668754	A	0.01	0.02	2.0820	0.1491	0.2545
COL1A2	7	rs11764718	T	0.05	0.05	0.0004	0.9841	1.0000
COL1A2	7	rs1062394	A	0.15	0.12	1.3150	0.2514	0.2631
COL1A2	7	rs1034620	C	0.07	0.08	0.1951	0.6587	0.7834
COL1A2	7	rs11982782	T	0.48	0.46	0.3755	0.5400	0.5518
CAV1&2	7	rs8940	C	0.19	0.19	0.0086	0.9260	0.9248
CAV1&2	7	rs10258482	T	0.42	0.42	0.0206	0.8860	0.9401
CAV1&2	7	rs6466578	C	0.13	0.16	0.8701	0.3509	0.4030
CAV1&2	7	rs3919515	C	0.40	0.39	0.1432	0.7052	0.7042
CAV1&2	7	rs926198	A	0.50	0.46	1.1030	0.2936	0.2988

Ch, Chromosome; MA, minor allele; MAF DM, minor allele frequency in diabetics; MAF nonDM, MAF in non diabetics; p, p-value obtained from the χ^2 test; p*, p-value from Fisher's exact test

(Table A11.1 continues overpage)

Genomic region	Ch	SNP	MA	MAF DM	MAF non DM	χ^2	p	p*
CAV1&2	7	rs3779512	G	0.40	0.36	1.4930	0.2217	0.2483
CAV1&2	7	rs10262090	C	0.13	0.12	0.1991	0.6554	0.6481
CAV1&2	7	rs3807986	T	0.26	0.27	0.1076	0.7428	0.8007
CAV1&2	7	rs1476833	A	0.26	0.29	1.0930	0.2958	0.3222
CAV1&2	7	rs10270569	T	0.17	0.18	0.1340	0.7143	0.7681
CAV1&2	7	rs11773845	T	0.24	0.21	0.8363	0.3605	0.3726
CAV1&2	7	rs9886215	C	0.32	0.35	0.4740	0.4912	0.5304
CAV1&2	7	rs3807992	A	0.48	0.47	0.0615	0.8042	0.8236
CDKN2B	9	rs3217992	A	0.07	0.06	0.3436	0.5578	0.5310
CDKN2B	9	rs1063192	C	0.00	0.00	0.0131	0.9089	1.0000
CDKN2B	9	rs3217989	C	0.33	0.30	0.8541	0.3554	0.3749
CDKN2B	9	rs974336	A	0.26	0.27	0.1076	0.7428	0.8007
CDKN2B	9	rs3217980	A	0.09	0.10	0.0180	0.8932	1.0000
CDKN2B	9	rs2069422	G	0.09	0.12	1.2760	0.2587	0.2876
COL5A1	9	rs1536478	A	0.28	0.29	0.0080	0.9286	1.0000
COL5A1	9	rs3118516	A	0.15	0.18	1.1300	0.2877	0.3194
COL5A1	9	rs1536482	T	0.43	0.44	0.1451	0.7033	0.7091
COL5A1	9	rs7044529	T	0.38	0.41	0.5267	0.4680	0.4957
ATOH7	10	rs6480320	A	0.29	0.28	0.1060	0.7448	0.7417
ATOH7	10	rs7916697	G	0.21	0.22	0.0965	0.7560	0.7863
ATOH7	10	rs2289804	C	0.50	0.50	0.0010	0.9745	1.0000
ATOH7	10	rs1900004	C	0.24	0.24	0.0015	0.9689	1.0000
ATOH7	10	rs41340944	G	0.46	0.44	0.3312	0.5649	0.6008
ATOH7	10	rs1900020	G	0.45	0.48	0.7029	0.4018	0.4140
SIX1&6	14	rs12436579	T	0.49	0.49	0.0234	0.8783	0.8822
SIX1&6	14	rs17097585	T	0.08	0.09	0.3190	0.5722	0.6897
SIX1&6	14	rs8006274	T	0.28	0.25	0.8441	0.3582	0.3971
SIX1&6	14	rs1007152	G	0.24	0.27	0.9427	0.3316	0.3524
SIX1&6	14	rs7152532	A	0.03	0.03	0.0223	0.8814	0.8193
SIX1&6	14	rs11849906	G	0.03	0.04	0.0747	0.7847	0.8474
SIX1&6	14	rs1955698	A	0.46	0.45	0.0689	0.7930	0.8228
SIX1&6	14	rs10150234	G	0.45	0.44	0.0345	0.8527	0.8813
SIX1&6	14	rs11158289	T	0.40	0.41	0.0970	0.7554	0.7630
SIX1&6	14	rs10142401	G	0.30	0.34	0.8586	0.3541	0.3838
SIX1&6	14	rs10483726	C	0.48	0.49	0.0358	0.8500	0.8820
SIX1&6	14	rs8020353	T	0.29	0.25	2.1440	0.1431	0.1498
SIX1&6	14	rs10483727	G	0.02	0.01	0.4505	0.5021	0.5012
SIX1&6	14	rs2057135	C	0.45	0.43	0.3017	0.5828	0.6005
SIX1&6	14	rs7156317	C	0.50	0.48	0.3068	0.5797	0.6033
SIX1&6	14	rs7146104	A	0.07	0.07	0.1776	0.6734	0.6596
SIX1&6	14	rs4901995	C	0.13	0.15	0.3374	0.5614	0.5989
SIX1&6	14	rs8007935	A	0.23	0.20	0.9696	0.3248	0.3586
SIX1&6	14	rs11628064	T	0.08	0.10	0.7457	0.3878	0.4537
ZNF469	16	rs12447690	T	0.13	0.15	0.6671	0.4141	0.4617
ZNF469	16	rs7500824	C	0.16	0.17	0.0820	0.7746	0.8408
ZNF469	16	rs4352077	A	0.05	0.05	0.0004	0.9841	1.0000
ZNF469	16	rs7501402	T	0.25	0.24	0.1166	0.7328	0.7296

Ch, Chromosome; MA, minor allele; MAF DM, minor allele frequency in diabetics; MAF nonDM, MAF in non diabetics; p, p-value obtained from the χ^2 test; p*, p-value from Fisher's exact test

(Table A11.1 continues overpage)

Genomic region	Ch	SNP	MA	MAF DM	MAF non DM	χ^2	p	p*
ZNF469	16	rs6540223	C	0.29	0.32	0.9871	0.3205	0.3351
ZNF469	16	rs12448211	A	0.37	0.35	0.4022	0.5259	0.5334
ZNF469	16	rs9938149	C	0.37	0.43	2.7490	0.0973	0.1107
ZNF469	16	rs9925231	A	0.45	0.47	0.3390	0.5604	0.6023
ZNF469	16	rs11644804	T	0.28	0.25	0.6395	0.4239	0.4434
COL1A1	17	rs2696297	C	0.17	0.18	0.0931	0.7603	0.8450
COL1A1	17	rs16948744	G	0.38	0.41	1.1250	0.2889	0.3236
COL1A1	17	rs2696296	T	0.41	0.45	1.5220	0.2173	0.2315
COL1A1	17	rs36107109	T	0.11	0.09	0.5124	0.4741	0.5298
COL1A1	17	rs2586476	T	0.35	0.31	1.5350	0.2154	0.2323
COL1A1	17	rs12945599	T	0.08	0.07	0.5236	0.4693	0.4814
COL1A1	17	rs2696270	C	0.41	0.41	0.0101	0.9198	0.9399
COL1A1	17	rs1061947	T	0.17	0.16	0.0102	0.9196	0.9203
COL1A1	17	rs1061237	C	0.51	0.46	1.3580	0.2439	0.2624
COL1A1	17	rs2277632	C	0.45	0.42	0.3385	0.5607	0.5997
COL1A1	17	rs2696245	G	0.05	0.08	1.8330	0.1758	0.1868
COL1A1	17	rs2586488	T	0.50	0.49	0.0507	0.8219	0.8241
COL1A1	17	rs2075559	G	0.43	0.41	0.4023	0.5259	0.5473
COL1A1	17	rs2857396	C	0.08	0.10	0.5436	0.4609	0.5212
COL1A1	17	rs2696247	C	0.13	0.15	0.1755	0.6752	0.7499
COL1A1	17	rs2586494	T	0.18	0.19	0.0802	0.7770	0.8488
COL1A1	17	rs16948765	C	0.05	0.08	1.4840	0.2232	0.2467
COL1A1	17	rs2586498	G	0.35	0.34	0.0415	0.8386	0.8759

Ch, Chromosome; MA, minor allele; MAF DM, minor allele frequency in diabetics; MAF nonDM, MAF in non diabetics; p, p-value obtained from the χ^2 test; p*, p-value from Fisher's exact test

Table A11.2 Logistic regression modelling of single SNP association with diabetes mellitus in the study population

Genomic region	SNP	p1	OR1 (95% CI)	p2	OR2 (95% CI)
COL8A2	rs274750	0.1078	0.79 (0.58 - 1.06)	0.0795	0.76 (0.56 - 1.03)
COL8A2	rs6693322	0.2993	1.3 (0.79 - 2.13)	0.2285	1.36 (0.83 - 2.24)
COL8A2	rs274754	0.5507	0.91 (0.66 - 1.25)	0.5471	0.9 (0.65 - 1.25)
MYOC	61730975	0.627	1.23 (0.54 - 2.79)	0.382	1.47 (0.62 - 3.51)
MYOC	61745146	0.4335	0.67 (0.24 - 1.84)	0.4162	0.65 (0.23 - 1.84)
MYOC	61730976	0.8128	0.91 (0.43 - 1.93)	0.7544	1.14 (0.51 - 2.53)
MYOC	rs79255460	0.2895	1.49 (0.71 - 3.11)	0.4223	1.35 (0.65 - 2.77)
MYOC	rs12076134	0.2048	1.26 (0.88 - 1.8)	0.1378	1.31 (0.92 - 1.88)
MYOC	rs2032555	0.157	0.62 (0.32 - 1.21)	0.1486	0.61 (0.31 - 1.19)
MYOC	rs57824969	0.6361	1.54 (0.26 - 9.26)	0.6541	1.52 (0.25 - 9.32)
MYOC	rs235869	0.8112	1.04 (0.75 - 1.43)	0.7519	1.06 (0.75 - 1.49)
MYOC	rs16864720	0.1086	1.38 (0.93 - 2.04)	0.1645	1.34 (0.89 - 2.04)
MYOC	rs235876	0.0361	0.48 (0.24 - 0.97)	0.0732	0.53 (0.26 - 1.06)
MYOC	rs2236875	0.5063	0.88 (0.6 - 1.29)	0.6072	0.9 (0.62 - 1.33)
MYOC	rs235917	0.5249	0.82 (0.44 - 1.53)	0.7394	0.9 (0.47 - 1.71)
MYOC	rs7525674	0.2025	1.56 (0.78 - 3.13)	0.2756	1.48 (0.73 - 2.97)
MYOC	MYOCnc1	0.8446	0.91 (0.35 - 2.37)	0.7483	0.85 (0.33 - 2.23)
MYOC	61730977	0.2565	1.46 (0.76 - 2.84)	0.341	1.39 (0.71 - 2.71)
MYOC	2234926	0.8712	1.15 (0.21 - 6.32)	0.8138	0.81 (0.14 - 4.64)
MYOC	12082573	0.3016	1.43 (0.72 - 2.83)	0.3775	1.36 (0.68 - 2.72)
MYOC	rs2075648	0.9833	0.99 (0.25 - 3.84)	0.6618	0.73 (0.18 - 2.96)
CYP1B1	rs9341261	0.746	0.87 (0.38 - 2)	0.8364	0.91 (0.39 - 2.16)
CYP1B1	rs162562	0.5404	0.9 (0.65 - 1.26)	0.7508	0.95 (0.67 - 1.34)
CYP1B1	rs1056836	0.4365	1.16 (0.8 - 1.68)	0.5186	1.13 (0.78 - 1.65)
CYP1B1	rs162561	0.9986	1 (0.69 - 1.46)	0.9688	1.01 (0.69 - 1.48)
CYP1B1	rs9341252	0.9483	1.02 (0.51 - 2.05)	0.8554	1.07 (0.52 - 2.18)
C2p16	rs2303298	0.3801	0.8 (0.49 - 1.31)	0.3776	0.79 (0.48 - 1.32)
C2p16	rs10202118	0.4451	0.88 (0.62 - 1.23)	0.5341	0.9 (0.63 - 1.27)
C2p16	rs11125375	0.0352	0.69 (0.49 - 0.98)	0.1084	0.76 (0.54 - 1.06)
C2p16	rs10208467	0.0866	1.34 (0.96 - 1.87)	0.1096	1.32 (0.94 - 1.87)
C2p16	rs1533428	0.9408	0.99 (0.73 - 1.34)	0.9787	1 (0.74 - 1.34)
C2p16	rs12994401	0.4335	0.67 (0.24 - 1.84)	0.6547	0.79 (0.28 - 2.24)
C2p16	rs11889995	0.4985	0.82 (0.46 - 1.46)	0.4992	0.81 (0.45 - 1.48)
WDR36	rs7729832	0.2884	0.77 (0.48 - 1.24)	0.342	0.79 (0.48 - 1.29)
WDR36	rs17623144	0.5094	N/A	0.9993	N/A
WDR36	rs13357724	0.4658	0.46 (0.05 - 3.94)	0.5529	0.51 (0.06 - 4.65)
WDR36	rs13153937	0.2169	1.48 (0.79 - 2.76)	0.1731	1.55 (0.82 - 2.93)
WDR36	rs6859041	0.3281	0.85 (0.61 - 1.18)	0.3638	0.85 (0.6 - 1.21)
WDR36	rs2416257	0.1168	0.69 (0.43 - 1.1)	0.1376	0.69 (0.42 - 1.13)
WDR36	rs17132783	0.2634	0.79 (0.52 - 1.19)	0.2487	0.78 (0.51 - 1.19)
WDR36	rs10038058	0.4767	0.88 (0.63 - 1.24)	0.5266	0.89 (0.62 - 1.28)
WDR36	**rs12515367**	**0.0166**	**2.57 (1.16 - 5.71)**	**0.01**	**3.01 (1.3 - 6.95)**
WDR36	rs12521169	0.8214	1.05 (0.69 - 1.6)	0.6956	1.09 (0.71 - 1.67)
WDR36	rs10051830	0.7205	0.94 (0.68 - 1.31)	0.7435	0.94 (0.67 - 1.34)
WDR36	rs10491424	0.5191	0.91 (0.68 - 1.22)	0.4357	0.88 (0.65 - 1.21)
WDR36	rs11956837	0.3523	1.44 (0.67 - 3.08)	0.3607	1.45 (0.65 - 3.23)
WDR36	**rs12522383**	**0.0001**	**2.03 (1.41 - 2.91)**	**0.0001**	**2.14 (1.46 - 3.15)**

OR (95% CI), Odds ratio (95% confidence intervals); p1/OR1, unadjusted; p2/OR2, adjusted for age, gender and POAG diagnosis (POAG or control)

(Table A11.2 continues overpage)

Genomic region	SNP	p1	OR1 (95% CI)	p2	OR2 (95% CI)
WDR36	rs17624563	0.0644	0.68 (0.45 - 1.03)	0.1079	0.71 (0.47 - 1.08)
WDR36	rs43203	0.0866	0.54 (0.27 - 1.1)	0.1017	0.55 (0.27 - 1.13)
WDR36	**rs11241098**	**0.0038**	**1.76 (1.2 - 2.59)**	**0.0038**	**1.84 (1.22 - 2.77)**
WDR36	rs7722241	0.073	0.76 (0.56 - 1.03)	0.0932	0.76 (0.55 - 1.05)
WDR36	rs10041326	0.6498	1.13 (0.66 - 1.95)	0.7746	1.07 (0.66 - 1.76)
WDR36	rs4530809	0.2869	0.82 (0.57 - 1.18)	0.4964	0.88 (0.61 - 1.27)
WDR36	**rs17132810**	**0.003**	**1.79 (1.21 - 2.63)**	**0.003**	**1.87 (1.24 - 2.82)**
WDR36	rs1457113	0.4726	0.81 (0.45 - 1.45)	0.4902	0.81 (0.44 - 1.49)
COL1A2	rs4729131	0.5237	0.83 (0.48 - 1.46)	0.6052	0.86 (0.49 - 1.52)
COL1A2	rs3814967	0.0797	1.31 (0.97 - 1.76)	0.1782	1.24 (0.91 - 1.68)
COL1A2	rs1800222	0.1759	0.81 (0.59 - 1.1)	0.3168	0.85 (0.62 - 1.17)
COL1A2	rs411717	0.6196	0.92 (0.66 - 1.28)	0.8149	0.96 (0.67 - 1.37)
COL1A2	rs406226	0.7888	1.06 (0.68 - 1.65)	0.5937	1.13 (0.72 - 1.79)
COL1A2	rs3763466	0.5402	0.87 (0.57 - 1.35)	0.709	0.92 (0.59 - 1.43)
COL1A2	rs17166249	0.3166	0.84 (0.6 - 1.18)	0.409	0.86 (0.61 - 1.22)
COL1A2	rs42518	0.1885	0.69 (0.4 - 1.2)	0.2059	0.69 (0.39 - 1.22)
COL1A2	rs2521206	0.4509	0.89 (0.65 - 1.21)	0.3753	0.86 (0.63 - 1.19)
COL1A2	**rs42521**	**0.0272**	**1.4 (1.04 - 1.89)**	**0.0315**	**1.42 (1.03 - 1.95)**
COL1A2	**rs42522**	**0.0463**	**0.69 (0.48 - 1)**	**0.0799**	**0.71 (0.49 - 1.04)**
COL1A2	rs42524	0.6766	0.88 (0.47 - 1.62)	0.7502	0.9 (0.47 - 1.72)
COL1A2	rs42526	0.6018	1.08 (0.81 - 1.45)	0.6594	1.07 (0.79 - 1.44)
COL1A2	rs2521205	0.1983	1.22 (0.9 - 1.65)	0.2932	1.18 (0.87 - 1.61)
COL1A2	rs3736638	0.6993	1.07 (0.77 - 1.48)	0.8463	1.03 (0.74 - 1.44)
COL1A2	rs42527	0.5863	0.89 (0.58 - 1.36)	0.811	0.95 (0.6 - 1.49)
COL1A2	rs369982	0.1228	0.73 (0.49 - 1.09)	0.183	0.75 (0.49 - 1.15)
COL1A2	**rs4266**	**0.0118**	**1.55 (1.1 - 2.18)**	**0.0176**	**1.55 (1.08 - 2.22)**
COL1A2	**rs10235102**	**0.0271**	**1.75 (1.06 - 2.89)**	**0.0301**	**1.8 (1.06 - 3.07)**
COL1A2	rs441051	0.066	0.72 (0.51 - 1.02)	0.1107	0.74 (0.52 - 1.07)
COL1A2	rs2072071	0.5479	1.11 (0.79 - 1.57)	0.659	1.08 (0.76 - 1.53)
COL1A2	rs13240759	0.3801	0.82 (0.52 - 1.28)	0.3833	0.82 (0.52 - 1.29)
COL1A2	rs400218	0.3883	0.85 (0.58 - 1.24)	0.4871	0.87 (0.58 - 1.3)
COL1A2	rs10046552	0.6939	0.93 (0.64 - 1.34)	0.628	0.91 (0.64 - 1.32)
COL1A2	rs12668754	0.1491	0.35 (0.08 - 1.56)	0.207	0.37 (0.08 - 1.72)
COL1A2	rs11764718	0.9841	1.01 (0.53 - 1.92)	0.831	1.08 (0.55 - 2.09)
COL1A2	rs1062394	0.2514	1.28 (0.84 - 1.96)	0.5296	1.15 (0.74 - 1.79)
COL1A2	rs1034620	0.6587	0.88 (0.51 - 1.53)	0.8625	0.95 (0.53 - 1.69)
COL1A2	rs11982782	0.54	1.1 (0.82 - 1.47)	0.4476	1.12 (0.83 - 1.52)
CAV1&2	rs8940	0.926	1.02 (0.7 - 1.47)	0.924	1.02 (0.7 - 1.49)
CAV1&2	rs10258482	0.886	0.98 (0.73 - 1.32)	0.8854	1.02 (0.76 - 1.38)
CAV1&2	rs6466578	0.3509	0.82 (0.54 - 1.25)	0.4134	0.84 (0.55 - 1.28)
CAV1&2	rs3919515	0.7052	1.06 (0.79 - 1.43)	0.9014	1.02 (0.76 - 1.37)
CAV1&2	rs926198	0.2936	1.17 (0.87 - 1.56)	0.1946	1.22 (0.9 - 1.64)
CAV1&2	rs3779512	0.2217	1.21 (0.89 - 1.63)	0.1595	1.23 (0.92 - 1.65)
CAV1&2	rs10262090	0.6554	1.11 (0.71 - 1.72)	0.949	1.01 (0.66 - 1.57)
CAV1&2	rs3807986	0.7428	0.95 (0.68 - 1.32)	0.8915	1.02 (0.73 - 1.43)
CAV1&2	rs1476833	0.2958	0.84 (0.6 - 1.17)	0.3114	0.84 (0.6 - 1.18)
CAV1&2	rs10270569	0.7143	0.93 (0.63 - 1.37)	0.8162	0.95 (0.64 - 1.43)
CAV1&2	rs11773845	0.3605	1.17 (0.83 - 1.66)	0.3417	1.19 (0.83 - 1.68)

OR (95% CI), Odds ratio (95% confidence intervals); p1/OR1, unadjusted; p2/OR2, adjusted for age, gender and POAG diagnosis (POAG or control)

(Table A11.2 continues overpage)

Genomic region	SNP	p1	OR1 (95% CI)	p2	OR2 (95% CI)
CAV1&2	rs9886215	0.4912	0.9 (0.66 - 1.22)	0.3562	0.86 (0.62 - 1.19)
CAV1&2	rs3807992	0.8042	1.04 (0.78 - 1.39)	0.7479	1.05 (0.78 - 1.42)
CDKN2B	rs3217992	0.5578	1.2 (0.65 - 2.19)	0.4286	1.29 (0.69 - 2.43)
CDKN2B	rs1063192	0.9089	1.15 (0.1 - 12.75)	0.9915	0.99 (0.08 - 11.51)
CDKN2B	rs3217989	0.3554	1.16 (0.85 - 1.59)	0.2215	1.22 (0.89 - 1.69)
CDKN2B	rs974336	0.7428	0.95 (0.68 - 1.32)	0.5282	0.89 (0.62 - 1.27)
CDKN2B	rs3217980	0.8932	0.97 (0.59 - 1.6)	0.7277	0.91 (0.54 - 1.53)
CDKN2B	rs2069422	0.2587	0.76 (0.46 - 1.23)	0.2353	0.73 (0.44 - 1.22)
COL5A1	rs1536478	0.9286	0.99 (0.71 - 1.36)	0.8977	1.02 (0.74 - 1.41)
COL5A1	rs3118516	0.2877	0.8 (0.54 - 1.2)	0.5132	0.87 (0.58 - 1.31)
COL5A1	rs1536482	0.7033	0.94 (0.7 - 1.27)	0.9588	0.99 (0.73 - 1.34)
COL5A1	rs7044529	0.468	0.9 (0.66 - 1.21)	0.6772	0.94 (0.69 - 1.27)
ATOH7	rs6480320	0.7448	1.06 (0.76 - 1.46)	0.5482	1.11 (0.8 - 1.53)
ATOH7	rs7916697	0.756	0.94 (0.66 - 1.35)	0.5073	0.88 (0.61 - 1.27)
ATOH7	rs2289804	0.9745	1.01 (0.75 - 1.35)	0.8973	1.02 (0.76 - 1.37)
ATOH7	rs1900004	0.9689	0.99 (0.71 - 1.4)	0.8041	0.96 (0.67 - 1.36)
ATOH7	rs41340944	0.5649	1.09 (0.81 - 1.46)	0.7431	1.05 (0.78 - 1.41)
ATOH7	rs1900020	0.4018	0.88 (0.66 - 1.18)	0.5788	0.92 (0.68 - 1.24)
SIX1&6	rs12436579	0.8783	1.02 (0.76 - 1.37)	0.968	0.99 (0.73 - 1.35)
SIX1&6	rs17097585	0.5722	0.86 (0.5 - 1.47)	0.5358	0.84 (0.49 - 1.45)
SIX1&6	rs8006274	0.3582	1.17 (0.84 - 1.62)	0.3127	1.19 (0.85 - 1.66)
SIX1&6	rs1007152	0.3316	0.85 (0.6 - 1.19)	0.3585	0.85 (0.6 - 1.2)
SIX1&6	rs7152532	0.8814	1.07 (0.43 - 2.66)	0.6695	1.23 (0.48 - 3.19)
SIX1&6	rs11849906	0.7847	0.9 (0.41 - 1.97)	0.9843	1.01 (0.44 - 2.3)
SIX1&6	rs1955698	0.793	1.04 (0.78 - 1.39)	0.9303	1.01 (0.75 - 1.37)
SIX1&6	rs10150234	0.8527	1.03 (0.77 - 1.38)	0.9292	1.01 (0.75 - 1.37)
SIX1&6	rs11158289	0.7554	0.95 (0.71 - 1.28)	0.6891	0.94 (0.69 - 1.28)
SIX1&6	rs10142401	0.3541	0.86 (0.63 - 1.18)	0.2997	0.84 (0.61 - 1.17)
SIX1&6	rs10483726	0.85	0.97 (0.73 - 1.3)	0.7988	0.96 (0.72 - 1.29)
SIX1&6	rs8020353	0.1431	1.28 (0.92 - 1.77)	0.0978	1.33 (0.95 - 1.85)
SIX1&6	rs10483727	0.5021	1.54 (0.43 - 5.51)	0.7182	1.25 (0.38 - 4.1)
SIX1&6	rs2057135	0.5828	1.09 (0.81 - 1.46)	0.4633	1.12 (0.83 - 1.51)
SIX1&6	rs7156317	0.5797	1.09 (0.81 - 1.45)	0.4855	1.11 (0.83 - 1.49)
SIX1&6	rs7146104	0.6734	1.13 (0.64 - 2)	0.5735	1.18 (0.66 - 2.12)
SIX1&6	rs4901995	0.5614	0.88 (0.58 - 1.35)	0.6852	0.92 (0.61 - 1.39)
SIX1&6	rs8007935	0.3248	1.19 (0.84 - 1.7)	0.1743	1.28 (0.9 - 1.84)
SIX1&6	rs11628064	0.3878	0.8 (0.48 - 1.33)	0.2938	0.76 (0.45 - 1.27)
ZNF469	rs12447690	0.4141	0.84 (0.55 - 1.28)	0.3375	0.81 (0.52 - 1.25)
ZNF469	rs7500824	0.7746	0.94 (0.63 - 1.4)	0.6674	0.92 (0.62 - 1.37)
ZNF469	rs4352077	0.9841	1.01 (0.53 - 1.92)	0.9967	1 (0.52 - 1.94)
ZNF469	rs7501402	0.7328	1.06 (0.76 - 1.49)	0.8339	1.04 (0.72 - 1.49)
ZNF469	rs6540223	0.3205	0.85 (0.62 - 1.17)	0.435	0.88 (0.63 - 1.22)
ZNF469	rs12448211	0.5259	1.1 (0.81 - 1.5)	0.7485	1.05 (0.77 - 1.45)
ZNF469	rs9938149	0.0973	0.78 (0.57 - 1.05)	0.1928	0.81 (0.59 - 1.11)
ZNF469	rs9925231	0.5604	0.92 (0.68 - 1.23)	0.8146	0.96 (0.71 - 1.31)
ZNF469	rs11644804	0.4239	1.14 (0.82 - 1.59)	0.5172	1.12 (0.79 - 1.6)
COL1A1	rs2696297	0.7603	0.94 (0.64 - 1.39)	0.5678	0.89 (0.6 - 1.33)

OR (95% CI), Odds ratio (95% confidence intervals); p1/OR1, unadjusted; p2/OR2, adjusted for age, gender and POAG diagnosis (POAG or control)

(Table A11.2 continues overpage)

Genomic region	SNP	p1	OR1 (95% CI)	p2	OR2 (95% CI)
COL1A1	rs16948744	0.2889	0.85 (0.63 - 1.15)	0.2884	0.85 (0.62 - 1.15)
COL1A1	rs2696296	0.2173	0.83 (0.62 - 1.12)	0.2347	0.83 (0.62 - 1.13)
COL1A1	rs36107109	0.4741	1.19 (0.74 - 1.93)	0.5476	1.16 (0.71 - 1.9)
COL1A1	rs2586476	0.2154	1.22 (0.89 - 1.66)	0.3022	1.18 (0.86 - 1.63)
COL1A1	rs12945599	0.4693	1.22 (0.71 - 2.09)	0.4676	1.22 (0.71 - 2.08)
COL1A1	rs2696270	0.9198	0.98 (0.73 - 1.32)	0.9402	0.99 (0.73 - 1.34)
COL1A1	rs1061947	0.9196	1.02 (0.69 - 1.51)	0.8195	0.95 (0.64 - 1.42)
COL1A1	rs1061237	0.2439	1.19 (0.89 - 1.6)	0.2084	1.22 (0.9 - 1.65)
COL1A1	rs2277632	0.5607	1.09 (0.81 - 1.46)	0.4038	1.14 (0.84 - 1.55)
COL1A1	rs2696245	0.1758	0.65 (0.34 - 1.22)	0.1629	0.63 (0.33 - 1.2)
COL1A1	rs2586488	0.8219	1.03 (0.77 - 1.38)	0.8605	1.03 (0.76 - 1.39)
COL1A1	rs2075559	0.5259	1.1 (0.82 - 1.48)	0.733	1.06 (0.77 - 1.44)
COL1A1	rs2857396	0.4609	0.82 (0.49 - 1.39)	0.3865	0.79 (0.47 - 1.34)
COL1A1	rs2696247	0.6752	0.91 (0.6 - 1.39)	0.7795	0.94 (0.61 - 1.45)
COL1A1	rs2586494	0.777	0.95 (0.65 - 1.38)	0.7323	0.93 (0.63 - 1.39)
COL1A1	rs16948765	0.2232	0.68 (0.37 - 1.27)	0.2339	0.7 (0.39 - 1.26)
COL1A1	rs2586498	0.8386	1.03 (0.76 - 1.4)	0.5904	1.09 (0.8 - 1.48)

OR (95% CI), Odds ratio (95% confidence intervals); p1/OR1, unadjusted; p2/OR2, adjusted for age, gender and POAG diagnosis (POAG or control)

APPENDIX 12 – TAQMAN ASSOCIATION STUDY SNP TABLE

Table A12.1 List of SNPs within each candidate region that were included in the TaqMan® association study

Genomic region	SNP	Ch	Position	Alleles	Comment
TMCO1	rs10800149	1	163951691	[A/C]	
	rs10800150	1	163951706	[C/T]	
	rs4656461	1	163953829	[C/T]	
	rs1913845	1	163962203	[T/C]	
	rs12059327	1	163965807	[C/T]	
	rs6426940	1	164000107	[C/T]	
	rs7518099	1	164003504	[C/T]	
	rs2814471	1	164006222	[C/T]	
CAV2	rs8940	7	115933310	[G/C]	BeadXpress
	rs1052990	7	115935606	[C/A]	
	rs6466578	7	115938105	[C/T]	BeadXpress
	rs3919515	7	115939020	[C/G]	BeadXpress
	rs10227696	7	115949183	[A/G]	
	rs4236601	7	115949965	[A/G]	BeadXpress
CAV1	rs917664	7	115954680	[A/T]	
	rs3779512	7	115958299	[C/A]	BeadXpress
	rs3807986	7	115965061	[T/C]	BeadXpress
	rs3807989	7	115973477	[C/T]	
	rs3779514	7	115976419	[A/G]	
	rs3815412	7	115977929	[G/A]	
	rs8713	7	115987033	[C/A]	
CDKN2B	rs2069422	9	21998026	[G/T]	
	rs7049105	9	22018801	[A/G]	
	rs2151280	9	22024719	[A/G]	
	rs7851706	9	22037437	[T/C]	
	rs10120688	9	22046499	[A/G]	
	rs16905597	9	22058074	[A/G]	
	rs16905599	9	22059144	[T/C]	
	rs16923583	9	22063334	[A/T]	
	rs1547705	9	22072375	[C/A]	
	rs1537370	9	22074310	[C/T]	
	rs10965235	9	22105105	[A/C]	
	rs4990722	9	22105217	[T/G]	
	rs17761446	9	22108102	[G/T]	
	rs1333049	9	22115503	[C/G]	
	rs1333050	9	22115913	[T/C]	
	rs10811658	9	22118600	[A/G]	
	rs12347779	9	22118709	[G/C]	
	rs10965245	9	22120515	[A/G]	
	rs2383208	9	22122076	[G/A]	
SIX1/6	rs2350890	14	59855477	[T/C]	
	rs4901977	14	59858929	[G/A]	
	rs8012339	14	59867298	[C/A]	
	rs1266416	14	59892415	[T/C]	
	rs3759688	14	60045332	[A/C]	
	rs11849906	14	60089312	[G/A]	BeadXpress
	rs10148202	14	60125693	[A/G]	
	rs7156317	14	60157493	[G/A]	BeadXpress
	rs7146104	14	60159089	[A/G]	BeadXpress

Ch, chromosome; BeadXpress, SNPs genotyped in both the TaqMan® and the BeadXpress study

APPENDIX 13 - INDIVIDUALS IN WHOM TAQMAN GENOTYPING WAS SUCCESSFUL

Table A13.1 Demographic features of study subjects in whom TaqMan® genotyping was successful

Group		Total	POAG	Control	p	Adjusted p
n		366	179	187		
Female (%)		181 (49.5)	84 (46.9)	97 (51.9)	0.344 ¶	
Age (years)	Range		22 - 87	50 - 91		
	Mean ± SD		59.4 ± 13.6	69.9 ± 8.4	**<0.001** ⋆	
FAMILY HISTORY						
Family history of glaucoma (%)			11 (6.1)	N/A		
Family history of blindness (%)			41 (22.9)	13 (7.0)	**< 0.001** ¶	
HOME LANGUAGE					0.740 ⋆	
	Afrikaans (%)	6 (1.6)	3 (1.7)	3 (1.6)		
	IsiNdebele (%)	4 (1.1)	1 (0.6)	3 (1.6)		
	Sepedi (%)	30 (8.2)	15 (8.4)	15 (8.0)		
	Sesotho (%)	58 (15.9)	25 (14.0)	33 (17.6)		
	Siswati (%)	5 (1.4)	1 (0.6)	4 (2.1)		
	Xitsonga (%)	18 (5.36)	10 (5.6)	8 (4.3)		
	Setswana (%)	61 (16.7)	26 (14.5)	35 (18.7)		
	Tshivenda (%)	11 (3.0)	7 (3.9)	4 (2.1)		
	IsiXhosa (%)	29 (7.9)	16 (8.9)	13 (7.0)		
	IsiZulu (%)	142 (38.8)	74 (41.3)	68 (36.4)		
	Other (%)	2 (0.6)	1 (0.6)	1 (0.5)		
MEDICAL HISTORY						
DM (%)		110 (30.1)	41 (22.9)	69 (36.9)	**0.004** ¶	0.101
	Type I	7 (6.4)	5 (12.2)	2 (2.9)	0.100 ⋆	0.165
	Diabetic retinopathy	24 (21.8)	4 (9.8)	20 (29.0)	**0.019** ⋆	**0.011**
HT (%)		224 (61.2)	95 (53.1)	129 (69.0)	**0.002** ¶	0.828

DM, diabetic; HT, hypertensive; Adjusted p, p adjusted for age and gender in a logistic regression model
¶ Pearson's χ^2 test
⋆ Fisher's exact test
§ T-test

Table A13.2 Clinical features of study subjects in whom TaqMan® genotyping was successful

Group		POAG	Control	p (t-test)	Adjusted p
n		179	187		
SVI (%)		89 (49.7)			
Glaucoma drainage surgery (%)		91 (50.8)			
Age at diagnosis (years)	n	146			
	Range	17 - 84			
	Mean ± SD	54.1 ± 13.7			
	Juvenile onset (%)	22 (12.3)			
MAP (mmHg)	n	157	135		
	Range	73.3 - 140.0	61.3 - 146.0		
	Mean ± SD	100.6 ± 13.3	98.9 ± 14.4	0.295	
IOP (mmHg)	n	147	187		
	Range	18 - 68	6 - 19		
	Mean ± SD	35.2 ± 9.5	13.2 ± 2.7	**<0.001**	
	NTG (%)	5 (2.8%)			
CCT (µm)	n	162	148		
	Range	379 - 586	420 - 609		
	Mean ± SD	505.6 ± 37.9	515.6 ± 37.6	**0.021**	**0.007**
VCDR	n	179	187		
	Range	0.3 - 1.0	0.1 - 0.6		
	Mean ± SD	0.89 ± 0.13	0.38 ± 0.13	**<0.001**	

SVI, Snellen visual acuity < 6/60 in at least one eye secondary to glaucoma; MAP, Mean arterial pressure; IOP, IOP at diagnosis for POAG subjects or at enrolment for control subjects

Adjusted p, p adjusted for age and gender in a logistic regression model

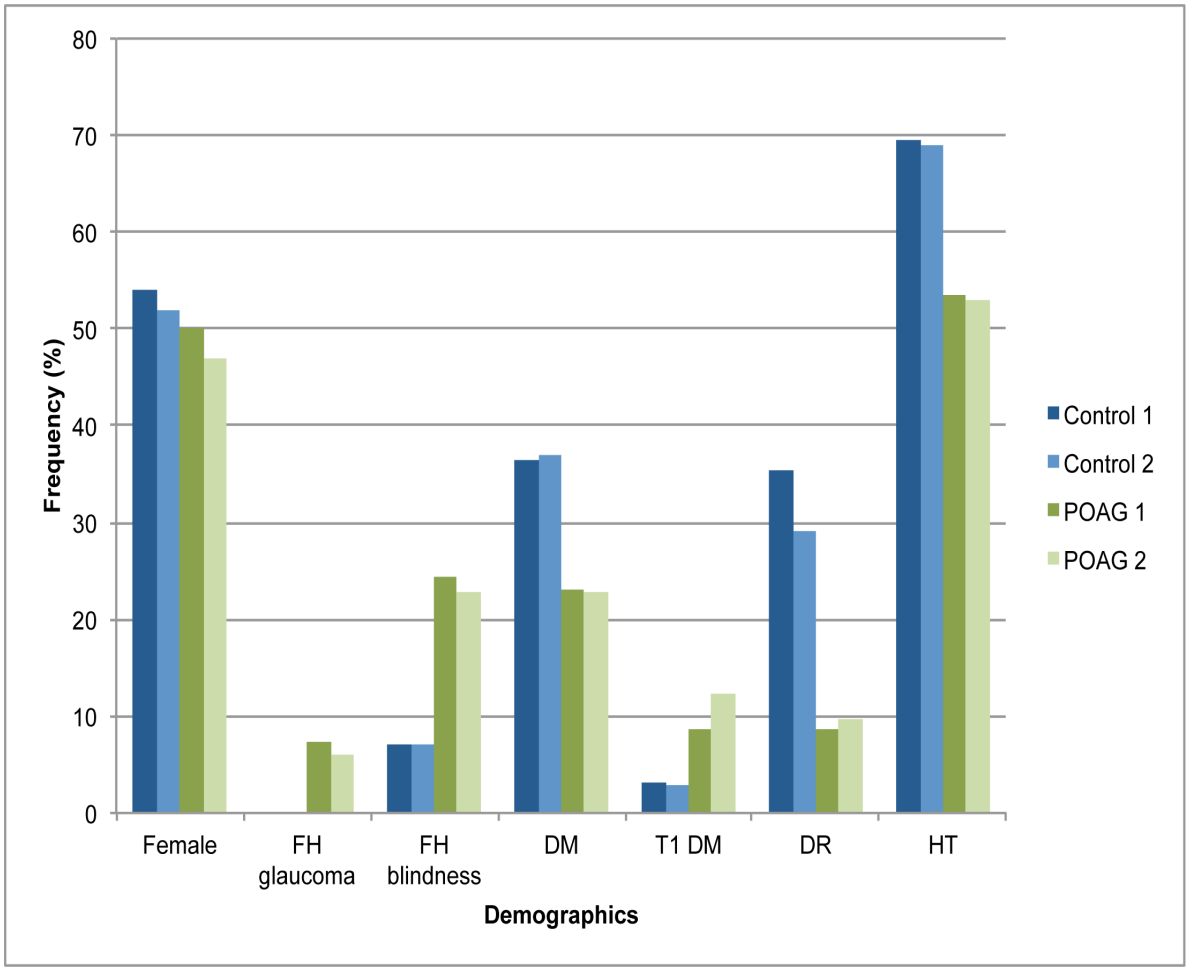

Figure A13.1 Comparison of the demographics of the POAG and control participants in the entire study population (POAG 1, Control 1) and in the POAG and control participants in whom TaqMan® genotyping was successful (POAG 2, Control 2). The y-axis represents frequency in percent and each demographic criterion is represented along the x-axis.

FH Glaucoma, family history of glaucoma; FH blindness, family history of blindness; DM, diabetes mellitus; T1 DM, type 1 diabetes mellitus; DR, diabetic retinopathy; HT, hypertensive

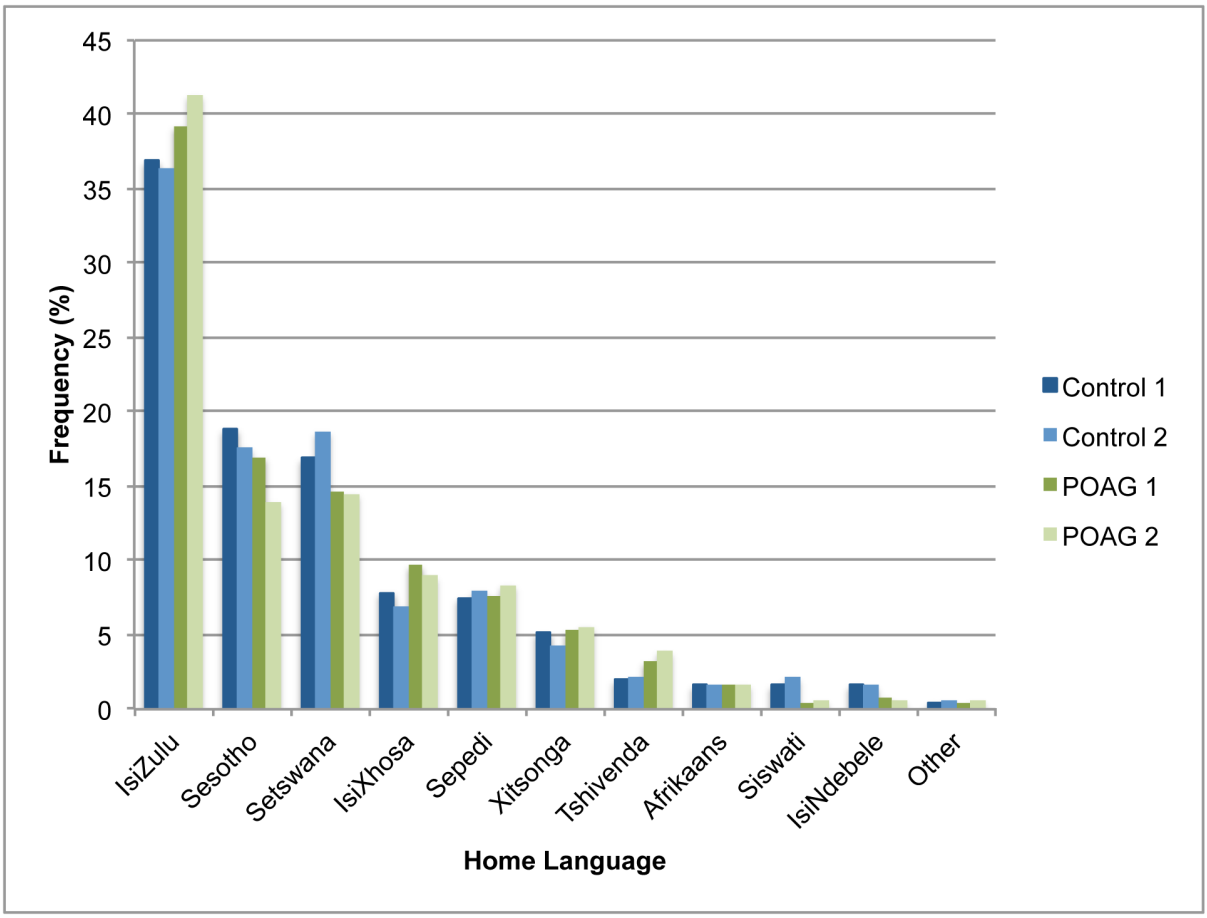

Figure A13.2 First language spoken: comparison of the proportions of POAG and control participants in the entire study population (POAG 1, Control 1) and in the POAG and control participants in whom TaqMan® genotyping was successful (POAG 2, Control 2). The y-axis represents frequency in percent and the languages are represented along the x-axis.

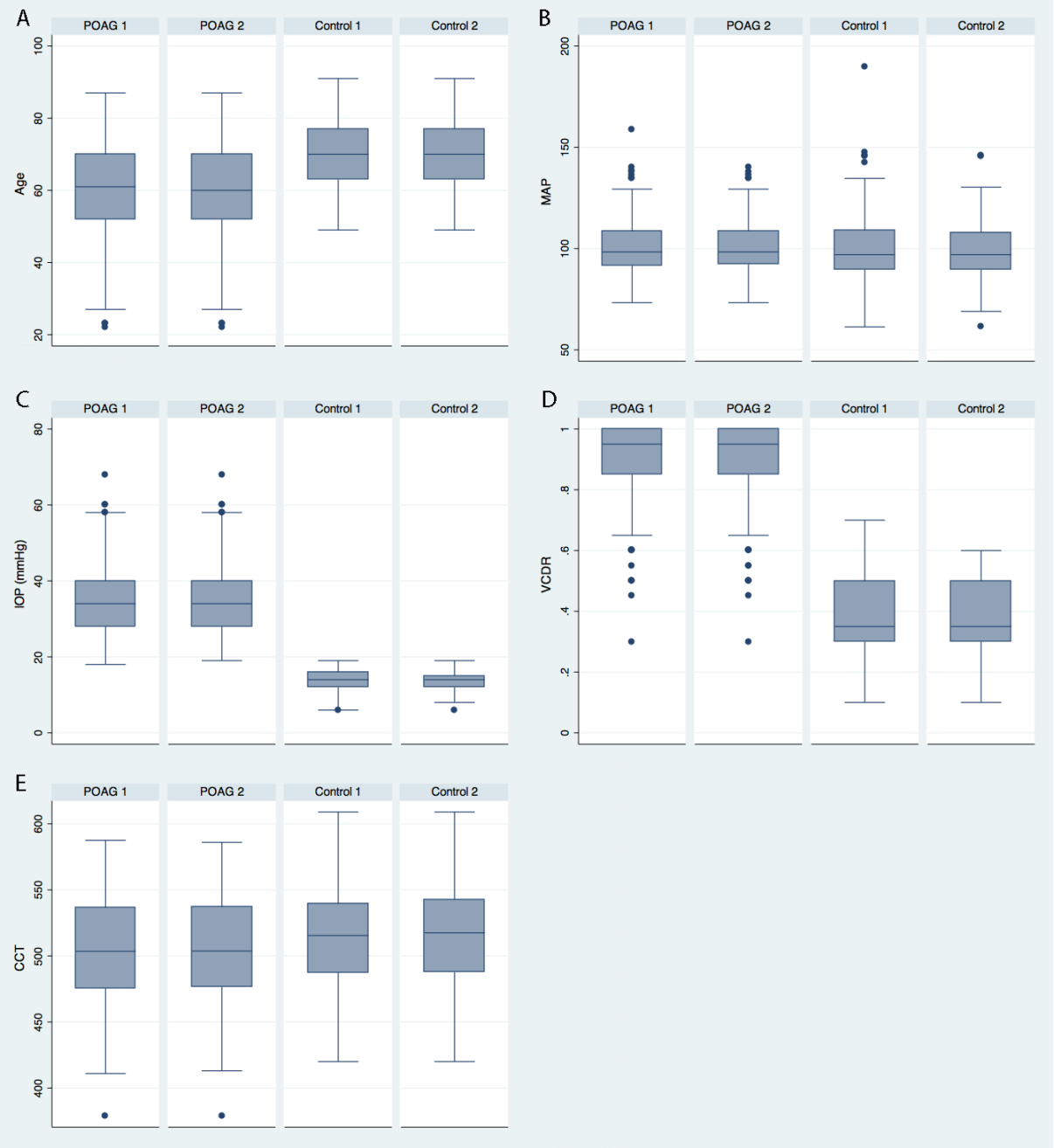

Figure A13.3 Box-and-whisker plots in the POAG and control participants in the entire study population (POAG 1 and Control 1) and in the POAG and control participants in whom TaqMan® genotyping was successful (POAG 2 and Control 2) of:

A - Age

B - Mean arterial pressure (MAP)

C - Intraocular pressure (IOP) in mmHg

D - Vertical cup-to-disc ratio (VCDR)

E - Central corneal thickness (CCT) in μm

The bottom and top of the box are the lower and upper quartiles respectively, and the band near the middle of the box is the median. The ends of the whiskers represent the upper and lower adjacent values. Any data not included between the whiskers is plotted as an outlier with a dot.

APPENDIX 14 - POAG ASSOCIATION ANALYSIS IN THE TAQMAN STUDY

Table A14.1 Single SNP association testing with a diagnosis of POAG in the South African study population

Genomic region	Ch	SNP	MA	MAF POAG	MAF Controls	χ^2	p	p*
TMCO1	1	rs10800149	A	0.19	0.19	0.0756	0.7834	0.8520
TMCO1	1	rs10800150	C	0.49	0.44	1.6610	0.1974	0.2110
TMCO1	1	rs4656461	C	0.32	0.28	1.3020	0.2539	0.2620
TMCO1	1	rs1913845	T	0.12	0.13	0.0373	0.8470	0.9118
TMCO1	1	rs12059327	C	0.15	0.13	0.7616	0.3828	0.3989
TMCO1	1	rs6426940	C	0.40	0.40	0.0006	0.9797	1.0000
TMCO1	1	rs7518099	C	0.08	0.07	0.5033	0.4781	0.4841
TMCO1	1	rs2814471	C	0.17	0.16	0.0269	0.8698	0.9214
CAV1/2	7	rs8940	G	0.20	0.18	0.1926	0.6608	0.7072
CAV1/2	7	rs1052990	C	0.45	0.44	0.1984	0.6560	0.7103
CAV1/2	7	rs6466578	C	0.15	0.13	0.6718	0.4124	0.4521
CAV1/2	7	rs3919515	C	0.37	0.39	0.2051	0.6506	0.7036
CAV1/2	7	rs10227696	A	0.17	0.16	0.0670	0.7958	0.8421
CAV1/2	7	rs4236601	A	0.41	0.39	0.2750	0.6000	0.6517
CAV1/2	7	rs917664	T	0.48	0.46	0.3901	0.5322	0.5565
CAV1/2	7	rs3807986	T	0.26	0.25	0.0705	0.7906	0.7998
CAV1/2	7	rs3807989	C	0.24	0.21	0.9682	0.3251	0.3315
CAV1/2	7	rs3779514	A	0.23	0.26	0.7522	0.3858	0.3932
CAV1/2	7	rs3815412	G	0.46	0.46	0.0158	0.9001	0.9413
CAV1/2	7	rs8713	C	0.33	0.36	0.5337	0.4651	0.4880
CDKN2BAS-1	9	rs2069422	G	0.11	0.10	0.3740	0.5409	0.5538
CDKN2BAS-1	9	rs7049105	A	0.18	0.20	0.2130	0.6444	0.7091
CDKN2BAS-1	9	rs2151280	A	0.22	0.21	0.1910	0.6621	0.7217
CDKN2BAS-1	9	rs7851706	T	0.16	0.14	0.5826	0.4453	0.4688
CDKN2BAS-1	9	rs10120688	A	0.38	0.39	0.0842	0.7717	0.8211
CDKN2BAS-1	9	rs16905597	A	0.07	0.10	1.9530	0.1623	0.1790
CDKN2BAS-1	9	rs16905599	T	0.26	0.26	0.0217	0.8830	0.9332
CDKN2BAS-1	9	rs16923583	A	0.18	0.20	0.4572	0.4989	0.5168
CDKN2BAS-1	9	rs1547705	C	0.22	0.24	0.4247	0.5146	0.5358
CDKN2BAS-1	9	rs1537370	C	0.35	0.31	1.1380	0.2860	0.3057
CDKN2BAS-1	9	rs10965235	C	0.47	0.47	0.0106	0.9182	0.9414
CDKN2BAS-1	9	rs4990722	T	0.14	0.13	0.2575	0.6119	0.6698
CDKN2BAS-1	9	rs17761446	G	0.04	0.04	0.2027	0.6525	0.7074
CDKN2BAS-1	9	rs1333049	C	0.24	0.24	0.0048	0.9446	1.0000
CDKN2BAS-1	9	rs1333050	T	0.12	0.12	0.0000	0.9961	1.0000
CDKN2BAS-1	9	rs10811658	G	0.47	0.48	0.1581	0.6909	0.7116
CDKN2BAS-1	9	rs12347779	G	0.02	0.03	0.0188	0.8911	1.0000
CDKN2BAS-1	9	rs10965245	A	0.24	0.26	0.2642	0.6072	0.6092
CDKN2BAS-1	9	rs2383208	G	0.24	0.24	0.0030	0.9566	1.0000
SIX1/6	14	rs2350890	C	0.48	0.50	0.2715	0.6024	0.6080
SIX1/6	14	rs4901977	G	0.48	0.48	0.0595	0.8073	0.8259
SIX1/6	14	rs8012339	C	0.24	0.24	0.0598	0.8069	0.8638
SIX1/6	14	rs1266416	C	0.36	0.38	0.5065	0.4767	0.4940
SIX1/6	14	rs3759688	A	0.21	0.19	0.4380	0.5081	0.5211
SIX1/6	14	rs11849906	G	0.05	0.03	2.0860	0.1486	0.1850
SIX1/6	14	rs10148202	A	0.14	0.12	0.3084	0.5787	0.5878
SIX1/6	14	rs7156317	A	0.48	0.51	0.4461	0.5042	0.5096
SIX1/6	14	rs7146104	A	0.08	0.07	0.0942	0.7589	0.7841

Ch, Chromosome; MA, minor allele; MAF, minor allele frequency; p, p-value obtained from the χ^2 test; p*, p-value from Fisher's exact test

Table A14.2 Logistic regression modelling of single SNP association with POAG in the South African study population

Genomic region	SNP	p1	OR1 (95% CI)	p2	OR2 (95% CI)	p3	OR3 (95% CI)
TMCO1	rs10800149	0.7834	0.95 (0.66 - 1.37)	0.7895	0.95 (0.67 - 1.36)	0.5628	0.89 (0.6 - 1.32)
TMCO1	rs10800150	0.1974	1.21 (0.91 - 1.61)	0.1961	1.21 (0.91 - 1.61)	0.2212	1.22 (0.89 - 1.68)
TMCO1	rs4656461	0.2539	1.2 (0.88 - 1.65)	0.2416	1.21 (0.88 - 1.65)	0.1501	1.29 (0.91 - 1.83)
TMCO1	rs1913845	0.847	0.96 (0.62 - 1.48)	0.8078	0.95 (0.61 - 1.48)	0.5084	0.85 (0.51 - 1.39)
TMCO1	rs12059327	0.3828	1.2 (0.79 - 1.82)	0.379	1.21 (0.79 - 1.83)	0.4069	1.22 (0.76 - 1.95)
TMCO1	rs6426940	0.9797	1 (0.74 - 1.34)	0.9259	0.99 (0.73 - 1.33)	0.6474	0.93 (0.67 - 1.29)
TMCO1	rs7518099	0.4781	1.22 (0.7 - 2.13)	0.4563	1.23 (0.71 - 2.13)	0.356	1.32 (0.73 - 2.36)
TMCO1	rs2814471	0.8698	1.03 (0.7 - 1.52)	0.8338	1.04 (0.7 - 1.56)	0.7302	1.08 (0.7 - 1.67)
CAV1/2	rs8940	0.6608	1.09 (0.75 - 1.57)	0.6495	1.09 (0.75 - 1.58)	0.6762	1.09 (0.72 - 1.65)
CAV1/2	rs1052990	0.656	1.07 (0.8 - 1.43)	0.6757	1.06 (0.8 - 1.42)	0.9953	1 (0.73 - 1.37)
CAV1/2	rs6466578	0.4124	1.19 (0.78 - 1.82)	0.416	1.19 (0.79 - 1.79)	0.3496	1.24 (0.79 - 1.95)
CAV1/2	rs3919515	0.6506	0.93 (0.69 - 1.26)	0.6692	0.94 (0.7 - 1.25)	0.6988	0.94 (0.68 - 1.29)
CAV1/2	rs10227696	0.7958	1.05 (0.71 - 1.56)	0.7763	1.06 (0.71 - 1.58)	0.637	1.11 (0.72 - 1.73)
CAV1/2	rs4236601	0.6	1.08 (0.81 - 1.45)	0.6252	1.08 (0.8 - 1.45)	0.7364	1.06 (0.77 - 1.46)
CAV1/2	rs917664	0.5322	1.1 (0.82 - 1.46)	0.5643	1.09 (0.81 - 1.46)	0.7561	1.05 (0.76 - 1.45)
CAV1/2	rs3807986	0.7906	1.05 (0.75 - 1.46)	0.8378	1.03 (0.75 - 1.43)	0.8932	0.98 (0.68 - 1.4)
CAV1/2	rs3807989	0.3251	1.19 (0.84 - 1.69)	0.3359	1.19 (0.84 - 1.69)	0.4339	1.17 (0.79 - 1.72)
CAV1/2	rs3779514	0.3858	0.86 (0.62 - 1.21)	0.3955	0.86 (0.61 - 1.21)	0.3364	0.83 (0.57 - 1.21)
CAV1/2	rs3815412	0.9001	0.98 (0.74 - 1.31)	0.8711	0.98 (0.72 - 1.32)	0.805	0.96 (0.68 - 1.34)
CAV1/2	rs8713	0.4651	0.89 (0.66 - 1.21)	0.4737	0.9 (0.67 - 1.21)	0.7504	0.95 (0.68 - 1.32)
CDKN2BAS-1	rs2069422	0.5409	1.16 (0.73 - 1.85)	0.5559	1.15 (0.72 - 1.86)	0.4143	1.24 (0.74 - 2.1)
CDKN2BAS-1	rs7049105	0.6444	0.92 (0.64 - 1.32)	0.6261	0.91 (0.62 - 1.33)	0.7862	0.94 (0.63 - 1.42)
CDKN2BAS-1	rs2151280	0.6621	1.08 (0.76 - 1.53)	0.6869	1.08 (0.75 - 1.55)	0.6001	1.11 (0.75 - 1.66)
CDKN2BAS-1	rs7851706	0.4453	1.17 (0.78 - 1.76)	0.4567	1.17 (0.78 - 1.76)	0.4332	1.2 (0.76 - 1.89)
CDKN2BAS-1	rs10120688	0.7717	0.96 (0.71 - 1.29)	0.7579	0.95 (0.71 - 1.29)	0.9766	1.01 (0.72 - 1.39)
CDKN2BAS-1	rs16905597	0.1623	0.68 (0.4 - 1.17)	0.1579	0.67 (0.39 - 1.17)	0.1849	0.67 (0.37 - 1.21)
CDKN2BAS-1	rs16905599	0.883	0.98 (0.7 - 1.36)	0.8897	0.98 (0.7 - 1.36)	0.9161	0.98 (0.69 - 1.4)
CDKN2BAS-1	rs16923583	0.4989	0.88 (0.61 - 1.27)	0.5061	0.89 (0.62 - 1.27)	0.6743	0.92 (0.62 - 1.36)
CDKN2BAS-1	rs1547705	0.5146	0.89 (0.63 - 1.26)	0.5102	0.89 (0.63 - 1.26)	0.5286	0.88 (0.6 - 1.3)
CDKN2BAS-1	rs1537370	0.286	1.18 (0.87 - 1.61)	0.2642	1.21 (0.87 - 1.68)	0.3542	1.19 (0.82 - 1.72)
CDKN2BAS-1	rs10965235	0.9182	0.99 (0.74 - 1.31)	0.9109	0.98 (0.73 - 1.33)	0.9136	0.98 (0.7 - 1.37)
CDKN2BAS-1	rs4990722	0.6119	1.11 (0.73 - 1.69)	0.6089	1.12 (0.73 - 1.73)	0.9064	1.03 (0.63 - 1.67)
CDKN2BAS-1	rs17761446	0.6525	0.84 (0.4 - 1.78)	0.7115	0.87 (0.42 - 1.82)	0.7201	1.16 (0.52 - 2.56)
CDKN2BAS-1	rs1333049	0.9446	1.01 (0.72 - 1.42)	0.9651	1.01 (0.71 - 1.42)	0.4936	0.88 (0.6 - 1.28)
CDKN2BAS-1	rs1333050	0.9961	1 (0.64 - 1.55)	0.9937	1 (0.64 - 1.56)	0.7991	1.07 (0.66 - 1.73)
CDKN2BAS-1	rs10811658	0.6909	0.94 (0.71 - 1.26)	0.6776	0.94 (0.7 - 1.26)	0.3099	0.85 (0.61 - 1.17)
CDKN2BAS-1	rs12347779	0.8911	0.94 (0.38 - 2.34)	0.8434	0.92 (0.38 - 2.21)	0.5713	0.76 (0.29 - 1.97)
CDKN2BAS-1	rs10965245	0.6072	0.92 (0.65 - 1.28)	0.6363	0.92 (0.66 - 1.29)	0.8392	0.96 (0.67 - 1.39)
CDKN2BAS-1	rs2383208	0.9566	0.99 (0.71 - 1.39)	0.9945	1 (0.72 - 1.4)	0.2564	1.24 (0.85 - 1.8)
SIX1/6	rs2350890	0.6024	0.93 (0.69 - 1.24)	0.5413	0.91 (0.67 - 1.23)	0.3904	0.86 (0.62 - 1.21)
SIX1/6	rs4901977	0.8073	0.96 (0.72 - 1.29)	0.8115	0.97 (0.73 - 1.28)	0.8347	0.97 (0.71 - 1.33)
SIX1/6	rs8012339	0.8069	1.04 (0.75 - 1.46)	0.7806	1.05 (0.75 - 1.46)	0.9856	1 (0.69 - 1.44)
SIX1/6	rs1266416	0.4767	0.9 (0.67 - 1.21)	0.4522	0.89 (0.66 - 1.2)	0.3657	0.86 (0.62 - 1.2)
SIX1/6	rs3759688	0.5081	1.13 (0.79 - 1.62)	0.4926	1.13 (0.8 - 1.61)	0.4002	1.18 (0.8 - 1.73)
SIX1/6	rs11849906	0.1486	1.75 (0.81 - 3.75)	0.159	1.75 (0.8 - 3.83)	0.0814	2.15 (0.91 - 5.06)
SIX1/6	rs10148202	0.5787	1.13 (0.74 - 1.73)	0.6018	1.12 (0.74 - 1.68)	0.6664	1.1 (0.7 - 1.73)
SIX1/6	rs7156317	0.5042	0.91 (0.68 - 1.21)	0.5128	0.91 (0.68 - 1.21)	0.4774	0.89 (0.65 - 1.23)
SIX1/6	rs7146104	0.7589	1.09 (0.63 - 1.87)	0.7323	1.1 (0.64 - 1.89)	0.4797	1.24 (0.68 - 2.27)

OR (95% CI), Odds ratio (95% confidence intervals); p1/OR1, unadjusted; p2/OR2, adjusted for gender; p3/OR3, adjusted for age and gender