GENETIC EPIDEMIOLOGY OF GLAUCOMA

Leonieke M.E. van Koolwijk

The studies presented in this thesis were conducted at the Departments of Epidemiology and Clinical Genetics, Erasmus University Medical Centre, Rotterdam, The Netherlands; the Rotterdam Eye Hospital and the Rotterdam Ophthalmic Institute, Rotterdam, The Netherlands; and the UCL Institute of Ophthalmology, London, United Kingdom.

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Chapter 4.2

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Part I

INTRODUCTION

Chapter 1.1

Introduction to primary open-angle glaucoma

Glaucoma is a heterogeneous group of optic neuropathies that have in common an accelerated degeneration of retinal ganglion cells and their axons, a subsequent typical excavation of the optic disc and a concomitant pattern of irreversible visual field loss.[1,2] Glaucoma affects approximately 2% of individuals of European descent and up to 10% of individuals of sub-Saharan African descent over 50 years of age.[3] It is a progressive disease, which without adequate treatment can result in severe visual disability and eventually blindness.

Primary open-angle glaucoma (POAG) is the predominant form of glaucoma in Western countries.[4] The disease is distinct from other forms of glaucoma through its age-related, insidious onset and an unobstructed iridocorneal angle with a normal appearance. Traditionally, an elevated intraocular pressure (IOP) was part of the clinical definition. However, an estimated 20 - 50% of all patients with otherwise characteristic POAG have IOPs consistently within the normal range (a condition referred to as "normal tension glaucoma"),[5] whereas most individuals with an elevated IOP do not have any signs of glaucomatous optic neuropathy or visual field loss (a condition called "ocular hypertension").[6] Nevertheless, an elevated IOP is considered an important causative factor and the major risk factor for POAG. The 10-year incidence of glaucomatous visual field loss has been reported to increase by 11% [6-15%] per millimeter of mercury increase in IOP.[7] Moreover, IOP is currently the only modifiable risk factor. Lowering the IOP, either by medication or surgically, has been shown to reduce the risk of conversion from ocular hypertension to glaucoma and to slow down the progression of glaucoma.[8,9]

In addition to an elevated IOP, several other risk factors have been associated with the development and progression of POAG. The most important ones are older age, African descent, a family history of POAG, high myopia, a reduced ocular perfusion pressure (the difference between blood pressure and IOP), and a thinner central cornea.[1,10] Evidence is less consistent for other risk factors, such as migraine, diabetes mellitus, cardiovascular disease, or a larger optic disc.[1,10]

The pathophysiological processes that initiate retinal ganglion cell loss are not completely understood but are generally assumed to occur at the optic nerve head.[11] In the past, these processes have been considered as either being mechanical or vascular. The mechanical theory suggested that elevated levels of IOP directly damaged the retinal nerve fibers as they pass through the lamina cribrosa to form the optic nerve.[11] The vascular theory proposed that insufficient blood flow to the optic nerve head was the main cause of damage.[11] A more contemporary view is that mechanical and vascular factors do not act independently but rather combine to cause the damage to the retinal nerve fibers.

The clinical diagnosis of POAG is based on a matching pattern of structural and functional signs of retinal ganglion cell degeneration, in addition to an open iridocorneal angle.[1,2] Structural signs comprise a characteristic appearance of the optic disc and thin-

ning of the retinal nerve fiber layer (RNFL). At the optic disc, the axons of the retinal ganglion cells come together to form the optic nerve. The optic disc has a central depression or cup, which is pale and does not contain any nerve fibers. The surrounding tissue is called rim and contains the axons of the retinal ganglion cells. Degeneration of retinal ganglion cells by glaucoma results in deepening and widening of the cup (excavation), often expressed as an increased vertical cup-to-disc ratio (VCDR). The RNFL surrounding the optic disc consists of retinal ganglion cell axons and consequently gets thinner as a result of glaucomatous damage. The optic disc and RNFL can be clinically assessed by ophthalmoscopy and by imaging methodologies such as scanning laser polarimetry, confocal scanning laser ophthalmoscopy or optical coherence tomography.[12] Glaucomatous retinal ganglion cell degeneration functionally manifests as typical visual field defects, which can be assessed by measuring light sensitivity at different locations in the central 30° of vision.[1]

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Chapter 1.2

Review of previous work in the genetic epidemiology of glaucoma

Van Koolwijk LME, Bunce C, Viswanathan AC.

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In 1869, Von Graefe described a heritable form of glaucoma and noted that the accurate etiology of this disease remained to be investigated.[1] In 2011, investigation is still ongoing. Although the presence of a genetic component has since been confirmed, unraveling the details of this component has proven difficult. To this end, human geneticists, laboratory scientists, epidemiologists and clinicians have integrated their expertise in the discipline of Genetic Epidemiology.

Genetic epidemiology is the study of how genes produce disease in human populations. It is distinct from two closely allied fields: it differs from classical epidemiology by its explicit consideration of genetic factors and it differs from medical genetics by its emphasis on population-based studies. Genetic epidemiology also studies the joint effects of genes and the environment and includes an incorporation of the underlying biology of the disease into its conceptual models. Genetic epidemiology is increasingly focusing on common diseases. Examples of common diseases in ophthalmology include age-related macular degeneration, myopia, and primary open-angle glaucoma (POAG). In this chapter we consider the methodologies used in genetic epidemiology, and discuss current and future perspectives on POAG genetics.

Genetic studies of POAG are important for two reasons. First, the identification of genes and their biological pathways may elucidate the pathophysiological mechanisms which are poorly understood at present. This knowledge may provide new directions for the development of glaucoma therapy. Second, by knowing the genes and how they predict the onset or progression of POAG, we may be able to create diagnostic and prognostic DNA tests. This risk stratification would be especially valuable in the case of POAG, as many patients are diagnosed only after significant and irreversible visual field damage has occurred. Early treatment may prevent or delay this damage. In addition, many "suspects" who repeatedly attend the glaucoma clinic unnecessarily might be reassured and either dismissed from regular surveillance or followed less frequently based on their genetic risk profile.

The route to a better understanding of POAG etiology starts by investigating whether susceptibility to the disease has a genetic basis and assessing the magnitude and type of this genetic susceptibility. These issues will be discussed in the first section of this chapter. Once a genetic component has been established, the next step is searching for the genes that cause or contribute to POAG. The two main approaches for this are *linkage* and *association*. Gene-finding has been the chief purpose of genetic epidemiologic studies up to now. Linkage and association studies therefore take the lion's share of this chapter. We address the basic principles of these approaches and evaluate how they have contributed to our current knowledge of POAG genetics. Before any results of these gene-finding studies can be usefully translated into ophthalmic practice the significance of the identified genes in the population and in the etiology of POAG needs to be determined. Studies addressing these issues are still in their infancy and will be briefly discussed in the last section of this chapter.

GENETIC SUSCEPTIBILITY TO POAG

Is POAG a genetic disease?

Support for a genetic basis of POAG comes from numerous familial cases, epidemiological studies and twin analyses. Family history has been reported to be an important risk factor for the development of POAG. First degree family members of patients with POAG are estimated to have as much as a tenfold increased risk of the disease compared to the general population.[2] Prevalence studies of POAG have shown significant racial variation.[3-7] Although environmental factors may partly account for this variation, genetic factors are likely to play an important role. This is substantiated by a recent meta-analysis demonstrating no significant differences in POAG prevalence among black populations from America, Europe, West Indies, or Africa.[8] A high concordance of POAG in monozygotic twin pairs further supports a genetic predisposition for POAG.[9]

Except for the rare familial cases with clear Mendelian patterns of inheritance, POAG is generally considered a complex disease: it most likely results from multiple genetic and environmental factors, and from interactions between them. No single factor is necessary or sufficient to cause the disease. An approach to understanding the etiology of complex diseases is provided by the liability threshold theory. Figure 1 shows the distribution of a theoretical variable that represents the liability of developing the disease. Genetic and environmental factors determine an individual's position on this liability distribution. A person with a favorable combination of protective factors in the absence of many risk factors will be at the left side of the distribution. A person with an unfavorable combination of genetic and environmental risk factors will be at the right side. A person whose liability variable exceeds a critical threshold will develop the disease.

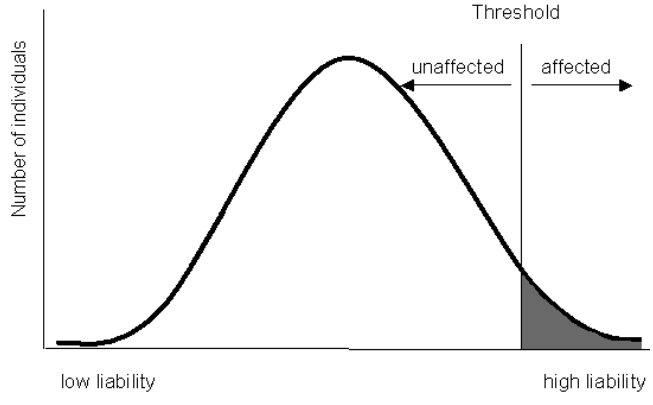


Figure 1. Liability threshold model for complex diseases

Genetic contribution to quantitative POAG traits

The etiological complexity of POAG may be reduced by discretely studying quantitative features of the phenotype: vertical cup-to-disc ratio (VCDR), optic disc neuroretinal rim area, retinal nerve fiber layer (RNFL) thickness, or risk factors such as intraocular pressure (IOP) and central corneal thickness (CCT). These quantitative traits may have simpler genetic origins and may therefore be easier to unravel. Moreover, they do not require an individual to be arbitrarily categorized as "affected" or "unaffected" and are therefore not susceptible to misclassification, which has been a major problem in POAG studies. Another advantage is that quantitative traits can also be studied in individuals without glaucoma, which means that this approach greatly increases the number of individuals available for genetic studies.

The genetic contribution to quantitative traits can be estimated by means of variance components analyses. These analyses separate the total variance of a trait into components attributable to different causes (Figure 2). The first theoretical division entails a component explained by the effects of genotype and a component explained by the effects of environment. The genotypic component represents the proportion of the variance of the trait relating to the particular composition of genes possessed by the individual. The environment refers to all the non-genetic conditions that influence the phenotypic variance. The genotypic component can be subdivided into a segment that includes the cumulative effects of the individual alleles *(additive genetic variance)* and a segment that represents the effects of interactions. The latter may include interactions between the two alleles of one gene *(dominance)*, interactions between the alleles of different genes *(epistasis)* and interactions between genes and environmental factors.

Separating the total phenotypic variance into these components allows an estimation of the relative contribution of the different determinants to the phenotype. The relative

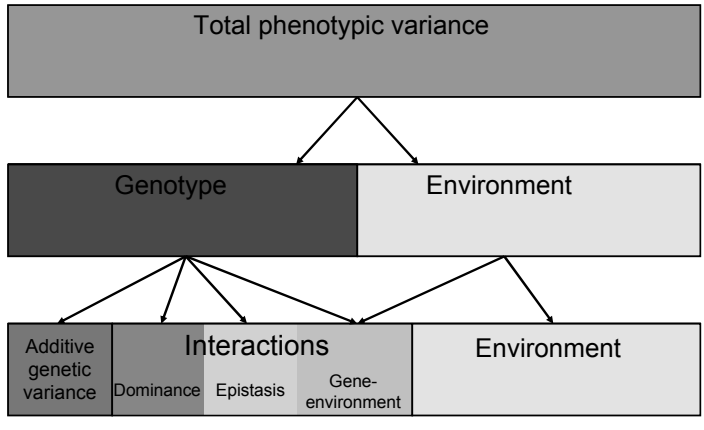


Figure 2. Partitioning of variance in a variance components analysis

contribution of heredity to the phenotype is called *heritability*: It is the proportion of the total variance that is explained by additive genetic effects (i.e., "narrow sense" heritability in contrast to "broad sense" heritability, the latter reflecting all possible genetic contributions to phenotypic variance). The heritability ranges from 0, indicating that additive genetic effects do not contribute to the phenotype, to 1, indicating that the phenotype is completely explained by additive effects of genes. Heritability can be estimated from the resemblance between family members.

Additive genetic effects have been reported to contribute significantly to the variances of quantitative POAG traits. Heritability estimates range from 0.29 to 0.50 for IOP, from 0.48 to 0.80 for VCDR, and from 0.48 to 0.82 for RNFL thickness.[10-15] CCT showed a heritability estimate of 0.95, which suggests that 95% of its variance is explained by the effects of genes.[10,16] These high heritability estimates support quantitative trait strategies to discover new genes for POAG.

However, the analyses described above only provide information on the combined additive effects of all genes. If there is a very large number of contributing genes, each with a very small effect on the phenotype, gene-finding studies would have very little chance of success. To assess gene-finding feasibility, a quantitative trait can be investigated for the presence of a major gene accounting for its variance. A suitable method for this is by the use of commingling analysis. This analysis applies a maximum likelihood method to assess the strength of evidence for the effect of a major gene compared with the null model of no major gene. Furthermore, it estimates the contribution of the major gene to the total variance of the phenotype (locus-specific heritability).

Commingling analysis was performed on IOP data of 3654 persons attending the Blue Mountains Eye Study.[17] The best fitting model for the dataset consisted of a mixture of 3 distributions (Figure 3), which would be consistent with the presence of a major gene in the determination of IOP. This major gene was estimated to account for 18% of the total variance in IOP. The parameters of the best fitting distribution provide some guidance for the planning of future genetic studies. The middle distribution in Figure 3 contains the heterozygotes, i.e. the individuals that carry one copy of the wild-type ("normal") allele and one copy of the rare ("IOP-increasing") allele. It is likely that values of IOP more extreme than 3 residual standard deviations (dotted vertical lines in Figure 3) from the mean of this distribution will be from homozygotes: IOP values less than 18 mmHg will be from persons with two copies of the wild-type allele, and IOP values greater than 33 mmHg will be from persons with two copies of the rare allele. For the purposes of association studies, it would be desirable to compare individuals having at least 1 copy of the rare allele with individuals having no copies. The former group would be those with IOPs higher than 3 residual standard deviations (uninterrupted vertical line) from the mean of the leftmost distribution in Figure 3, which corresponds to IOP values greater

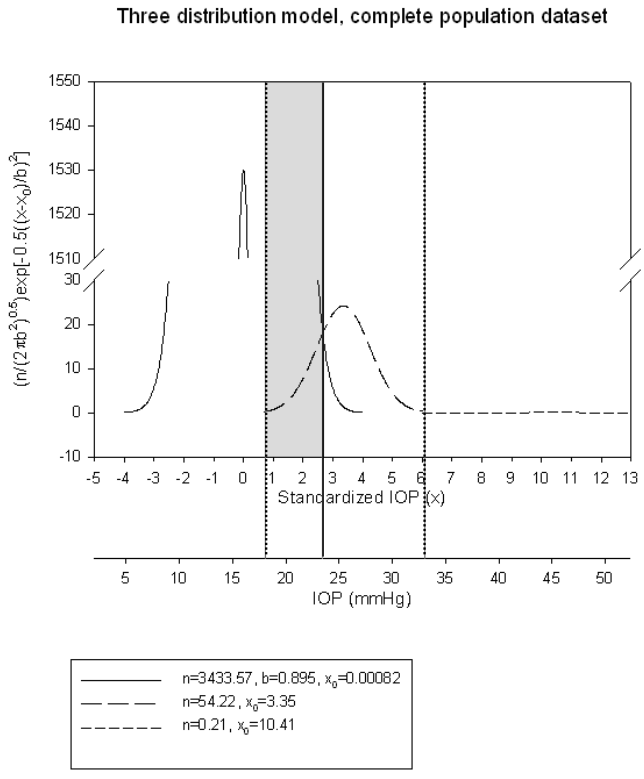


Figure 3. Commingling analysis of IOP in the Blue Mountains Eye Study[17]
This figure shows the model which, after commingling analysis, fitted the population IOP data best. It consists of three normal distributions, each containing *n* individuals, with mean x_0, and with common standard deviation *b*. The dotted vertical lines are placed at 3 standard deviations from the mean of the middle distribution, which contains the heterozygotes. More extreme IOP values (IOP < 18 mmHg or IOP > 33 mmHg) are likely to be from homozygotes. The uninterrupted vertical line is placed at 3 standard deviations from the mean of the leftmost distribution (IOP 23.5 mmHg).
For the purposes of association studies, it would be desirable to compare individuals having at least 1 copy of the risk allele (right from the uninterrupted vertical line, i.e. IOP > 23.5 mmHg) with individuals having no copies (left from the leftmost dotted vertical line, i.e. IOP < 18 mmHg).

than 23.5 mmHg. The latter group would be those with IOPs of less than 18 mmHg, for reasons already discussed.

LINKAGE STUDIES

Having established a likely genetic component in the etiology of a complex disease, the next step is to localize any disease genes. Linkage analysis provides a means to identify the chromosomal location *(locus)* of a disease gene without any prior knowledge about

possible biological mechanisms. Linkage analysis has traditionally been performed to study monogenic diseases in large families with multiple affected members. This approach is called parametric or model-based linkage analysis. It requires an assumption of the genetic model, in which the mode of inheritance, the disease and marker allele numbers and frequencies, and the penetrance of the disease genotype need to be specified. As long as an adequate model can be assumed, parametric linkage provides a powerful method to locate a disease gene. However, it has failed to find the more common genes underlying complex diseases, for which a valid genetic model cannot be specified. The shift towards the genetics of complex diseases has therefore led to the development of new methods of linkage analysis that are nonparametric, or model-free.

Both parametric and nonparametric linkage approaches have been used to identify the chromosomal locations of POAG susceptibility genes. The following sections explain the principles of these methods and consider their roles in POAG genetics.

Linkage analysis of monogenic forms of glaucoma

Parametric linkage analyses investigate the co-segregation of genetic loci in pedigrees. The rationale is that 2 loci lying close together on a chromosome have a high probability of being inherited together. The further apart 2 loci are on a chromosome, the higher the chance of a recombination event occurring between them during meiosis. This would put an end to their co-segregation. Loci on different chromosomes segregate independently. Thus, the probability of 2 loci segregating together is a measure of the genetic distance between them. Similarly, the probability of a genetic marker and the disease segregating together is a measure of the genetic distance between that marker and the disease gene. Designating the whole genome with genetic markers and observing their co-segregation with the disease in a pedigree can subsequently lead to localization of the disease gene. The likelihood of genetic linkage (compared with the null-hypothesis of no linkage) between a genetic marker and the disease is usually expressed as a LOD (logarithm of the odds) score. High, positive LOD scores (traditionally > 3) are evidence for linkage, and low, negative scores (< -2) are evidence against.

Why should we study monogenic forms of POAG while the large majority of POAG cases are considered of complex etiology? One reason is that the loci and the genes that have been identified in these monogenic forms may also determine susceptibility for the more common forms of POAG. Moreover, they may provide a clue to the pathology, disease mechanisms and signalling pathways in POAG. The advantage of dealing with monogenic forms of POAG is that methods of parametric linkage are more straightforward and well-established than the methods used for complex diseases. However, parametric linkage analysis in pedigrees can be complicated by the typically late onset of POAG: the patients' parents are often deceased, while their children may be too young to manifest the disease.

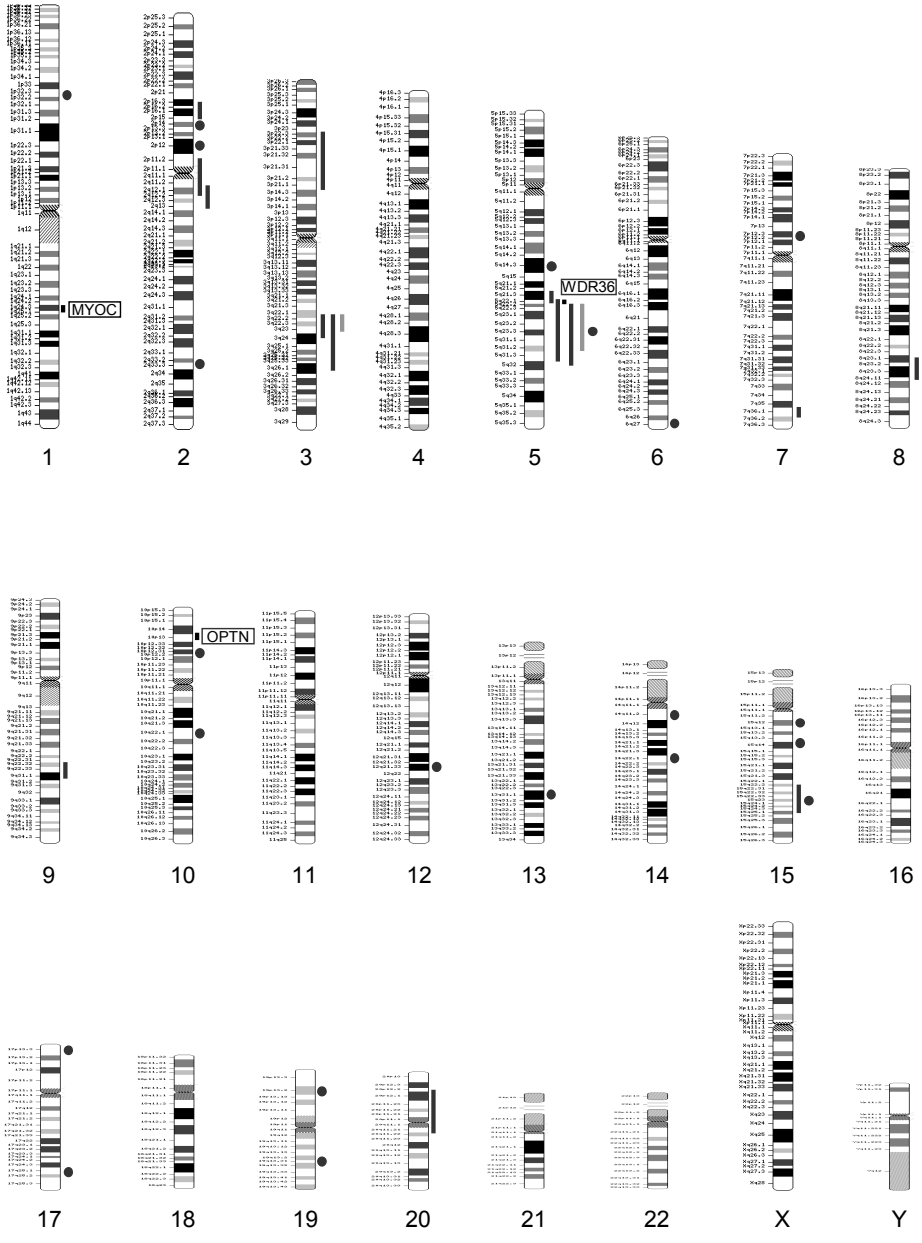


Figure 4. Chromosomal regions linked to POAG

The dark grey lines represent linkage regions that have been identified by analyses in single, large pedigrees. Line boundaries have been determined by haplotype analyses. Light grey lines correspond to replicated or refined loci in the same population. Dark grey dots represent maximum LOD scores from population based (family) studies. The three identified genes are shown in black. References are listed and summarized in Table 1.

Table 1. Linkage Studies of POAG

Locus	Gen	Region	Reference	Study design	Numbers	Phenotype	Markers	Significance
GLC1A		1q24.3	Sheffield VC, Nat Genet 1993;4:47-50	Parametric linkage (AD)	37 (22 affected) members of 1 familiy	JOAG	D1S212	tpLOD 6.5
	MYOC		Stone EM, Science 1997;275:668-670	Candidate gene screening	mutations in 5 out of 8 families	JOAG/POAG	D1S1619-D1S3664	
				& case-control study	330 patients, 471 controls	JOAG/POAG		
GLC1B		2cen-q13	Stoilova D, Genomics 1996;36:142-150	Parametric linkage (AD)	203 (90 affected) members of 17 families,	POAG/NTG	D2S2161-D2S176	tpLOD 6.48
					6 of which linked to locus			
			Charlesworth JC, Ophthalmologica 2006;220:23-30	Non-parametric linkage of candidate loci	15 (7 affected) members of 1 family	POAG	D2S2264-D2S2269	emp $p < 0.01$
GLC1C		3q22.1-q23	Wirtz MK, Am J Hum Genet 1997;60:296-304	Parametric linkage (AD)	44 (9 affected) members of 1 family	POAG	D3S3637-D3S1744	mpLOD 3.20
			Kitsos G, Eur J Hum Genet 2001;9:452-457	Parametric linkage (AD) of candidate loci	34 (10 affected) members of 1 family	POAG	D3S3637-D3S1763	mpLOD 3.88
			Samples JR, Clin Genet 2004;65:40-44	Refining locus in same families	Wirtz & Kitsos families	POAG	D3S3637-D3S3694	
GLC1D		8q23.1-q24.11	Trifan OC, Am J Ophthalmol 1998;126:17-28	Parametric linkage (AD)	20 (8 affected) members of 1 family	POAG	D8S1830-D8S592	tpLOD 3.61
GLC1E		10p13	Sarfarazi M, Am J Hum Genet 1998;62:641-652	Parametric linkage (AD)	39 (16 affected) members of 1 family	NTG	D10S1729-D10S1664	tp&mpLOD 10
	OPTN		Rezaie T, Science 2002;295:1077-1079	Candidate gene screening	mutations in 9 out of 54 families	NTG/POAG		
GLC1F		7q36.1	Wirtz MK, Arch Ophthalmol 1999;117:237-241	Parametric linkage (AD)	25 (10 affected) members of 1 family	POAG	D7S2442-D7S483	mpLOD 4.06

Table 1. Linkage Studies of POAG (continued)

Locus	Gen	Region	Reference	Study design	Numbers	Phenotype	Markers	Significance
GLC1G		5q21.3-q22.1	Samples JR, ARVO 2004		92 (14 affected) members of 1 family		D5S1721-D5S2051	
	WDR36	5q22.1	Monemi S, Hum Mol Genet 2005;14:725-733	Parametric linkage	linkage in 7 out of 148 families	POAG	D5S1466-D5S1480	mpLOD 4.90
				Candidate gene & case-control studies	138 patients, 476 controls	POAG		
GLC1H		2p16.3-p15	Suriyapperuma SP, Arch Ophthalmol 2007;125:86-92	Parametric linkage (AD)	85 (35 affected) members of 7 families	POAG	D2S123-D2S2165	tpLOD 9.30
GLC1I		15q11-13	Allingham RR, IOVS 2005;46:2002-2005	Ordered subset linkage analysis (non-parametric) of candidate locus	370 (227 affected) members of 81 families, 15 of which linked to locus	POAG	GABRB3	mpLOD 3.24
		15q14	Woodroffe A, Exp Eye Res 2006;82:1068-1074	Ordered subset linkage analysis (non-parametric) of candidate locus	167 (107 affected) members of 25 families, 14 of which linked to locus	POAG	D15S1007	mpLOD 2.09 emp p: 0.021
GLC1J		9q22.32-q31.1	Wiggs JL, Am J Hum Genet 2004;74:1314-1320	Parametric linkage (AD)	198 (105 affected) members of 25 families, 10 of which linked to locus	JOAG	D9S1841-D9S271	mpHLOD 4
GLC1K		20p12.2-q11.22	Wiggs JL, Am J Hum Genet 2004;74:1314-1320	Parametric linkage (AD)	198 (105 affected) members of 25 families, 8 of which linked to locus	JOAG	D20S894-D20S878	mpHLOD 4
GLC1L		3p23-p14.3	Baird PN, Hum Genet 2005;117:249-257	Non-parametric linkage	24 affected members of 1 large family, 13 of whom linked to locus	POAG	D3S1768-D3S1289	emp p: 0.003

Table 1. Linkage Studies of POAG (continued)

Locus	Gen	Region	Reference	Study design	Numbers	Phenotype	Markers	Significance
GLC1M		5q22.1-q32	Pang CP, Mol Vis 2006;12:85-92	Parametric linkage (AD)	27 (9 affected) members of 1 family	JOAG	D5S2051-D5S2090	tpLOD 4.82
		5q22.1-q31.2	Fan BJ, Mol Vis 2007;13:779-84	Refining locus in same family	27 (9 affected) members of 1 family	JOAG	D5S2051-NRG2	
		5q23.3	Rotimi CM, IOVS 2006;47:3262-3267	Non-parametric linkage QTL (VCA)	244 sibpairs with DMII	IOP	D5S2120	mpLOD 4.91
GLC1N		15q22.2-q25.1	Wang DY, IOVS 2006;47:5315-5321	Parametric linkage (AD)	25 (8 affected) members of 1 family	JOAG	D15S1036-rs922693	tpLOD 3.31
		2p14 14q11.2 17p13.3 17q25.1 19q13.31	Wiggs JL, Hum Mol Genet 2000;9:1109-1117	Parametric (AD/AR), NPL affected relative pairs, NPL affected sib pairs	41 multiplex families,126 members affected 113 + 69 affected sibpairs	POAG	D2S441 D14S742 D17S926 D17S801 D19S408	NPLOD 2.67 NPLOD 3.68 NPLOD 2.73 NPLOD 2.45 NPLOD 2.17
		2q33.3 10p12.31	Nemesure B, Hum Genet 2003;112:600-609	Parametric (AD / codominant) & non-parametric linkage	662 (256 affected) members of 146 families	POAG	D2S2189 D10S211	tpLOD 2.77 tpLOD 3.26
		10q22.1	Charlesworth JC, IOVS 2005;46:3723-3729	Non-parametric linkage QTL (VCA)	139 (24 affected) members of 1 family	IOP	D10S537	mpLOD 3.3
		1p32				VCDR	D1S197 / D1S220	mpLOD 2.3
		14q22.1	Rotimi CM, IOVS 2006;47:3262-3267	Non-parametric linkage QTL (VCA)	244 sibpairs with DMII	IOP	D14S587	mpLOD 2.95
		6q27 13q31.1	Duggal P, IOVS 2005;46:555-560	Non-parametric linkage QTL (modified Hasemann Elston regr)	218 sibpairs with ARM	IOP	D6S1027 D13S317	p = 0.008 p = 0.00071

Table 1. Linkage Studies of POAG (continued)

Locus	Gen	Region	Reference	Study design	Numbers	Phenotype	Markers	Significance
		2p12	Duggal P, Arch Ophthalmol	Non-parametric linkage	1979 members of 486	IOP	D2S1777	p = 0.007
		5q14.3	2007;125:74-79	QTL (modified Hasemann	families		D5S1725	p = 0.004
		6q27		Elston regr & VCA)			D6S1027	p = 0.003
		7p12.3					D7S1818	p = 0.003
		12q21.33					D12S1064	p = 0.0012
		15q23					D15S131	p = 0.007
		19p13.2					D19S586	p = 0.00006

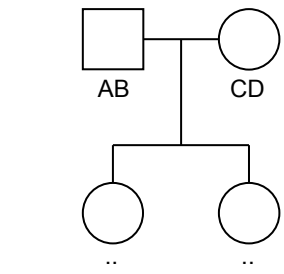
AD = autosomal dominant; QTL = quantitative trait locus; VCA = variance components analysis; AR = autosomal recessive; NPL = non-parametric linkage; regr = regression; DMII = diabetes mellitus type II; ARM = age-related maculopathy; JOAG = juvenile open-angle glaucoma; POAG = primary open-angle glaucoma; NTG = normal tension glaucoma; IOP = intraocular pressure; VCDR = vertical cup-to-disc ratio; tpLOD = two-point logarithm of the odds score; emp = empirical; mpLOD = multipoint logarithm of the odds score; mpHLOD = multipoint heterogeneity logarithm of the odds score; NPLOD = non-parametric logarithm of the odds score.

Fourteen POAG loci have been assigned a GLC1 symbol (GLC1A to GLC1N) by the HUGO Genome Nomenclature Committee (Figure 4 and Table 1). Most studies involved small numbers of large families in which the disease segregated as an autosomal dominant trait. GLC1A, GLC1J, GLC1K, GLC1M and GLC1N were identified in families with the rare, juvenile onset form of POAG (JOAG). JOAG typically manifests before the age of 35 years and exhibits high IOP that often requires surgical therapy. It usually shows autosomal dominant rather than complex inheritance. The JOAG loci may contribute to susceptibility to the common, adult-onset form of POAG. This has already been established for the *MYOC* gene at the GLC1A locus. The role of the other JOAG loci has yet to be fully assessed in adult cases.

Linkage analysis of complex forms of glaucoma

Nonparametric linkage methods investigate the degree of allele sharing by affected family members without assuming a particular mode of inheritance. The rationale is that, in the region of the disease gene, affected relative pairs share copies of the same ancestral alleles more frequently than would be expected based on the degree of their relationship.

The most commonly used method of nonparametric linkage is the **sib pair analysis**, in which pairs of affected siblings from a large number of families are genotyped for



Genotype sib 1

Genotype sib 2	AC	AD	BC	BD
AC	2	1	1	0
AD	1	2	0	1
BC	1	0	2	1
BD	0	1	1	2

Number of alleles shared between sibs

P (sharing 0 alleles) = 4/16 = ¼
P (sharing 1 allele) = 8/16 = ½
P (sharing 2 alleles) = 4/16 = ¼

Figure 5. Allele sharing in sib pairs

markers across the whole genome. If a marker is not linked to the disease, each pair of siblings has a probability of ¼ of sharing no parental alleles, ½ of sharing 1 parental allele, and ¼ of sharing 2 parental alleles (Figure 5). Therefore, according to the null hypothesis of no linkage, a 1: 2: 1 ratio of sib pairs sharing 0, 1, or 2 marker alleles would be expected. For each marker, the observed number of pairs sharing 0, 1 or 2 alleles can be compared with this 1: 2: 1 ratio. Linkage would be suggested if this ratio is significantly shifted towards a higher extent of allele sharing.

A disadvantage of sib pair analysis is that it does not include other affected family members. Potentially important information from, for example, affected aunts, cousins, or grandparents could therefore be lost. To take greater advantage of extended pedigrees, several methods have been developed that do include this additional information. These **affected relative pair analyses** calculate the extent to which affected relatives share copies of the same ancestral marker alleles and compare this with the null hypothesis of random segregation of the marker. Some programs analyze each possible pair of affected individuals whereas others consider the total group of relatives.

Affected sib pair and relative pair analyses usually produce nonparametric LOD (NPL) scores. Interpreting these scores has been challenged by the question of where to put the threshold of statistical significance. In 1995, Lander and Kruglyak, in their guidelines for reporting linkage results, proposed LOD score thresholds of 2.2 for suggestive linkage and 3.6 for significant linkage in affected sib pair analyses.[18] Their significance criteria have been widely applied. Alternatively, some linkage studies perform computer simulations to estimate significance thresholds that are specific to the particular population and circumstances (such as allele frequencies and missing data) under study.

In 2000, Wiggs et al. published the first genome screen of sib pairs with adult onset POAG.[19] They initially studied 113 affected sib pairs originating from 41 mainly Caucasian families. Family sizes ranged from a single affected sib pair to nine affected individuals. Because of this variety in pedigree structures, they used three different analytical methods to assess linkage: a parametric LOD score analysis, a nonparametric affected relative pair method, and a nonparametric sib pair analysis. They revealed 25 chromosomal regions with positive results for at least one analysis. These regions were followed up with extra markers and 69 additional sib pairs. Sib pair analysis using the combined pedigree set of 182 affected sib pairs identified suggestive linkage at chromosomes 2, 14, 17, and 19 (Figure 4, Table 1).

A genome-wide scan as part of the Barbados Family Study of Open-Angle Glaucoma (BFSG) was performed in 146 families of African descent.[20] As in Wiggs' study, a multi-analytical approach was chosen to evaluate the results. Significance levels were estimated by simulation studies. Parametric linkage analysis indicated possible POAG gene regions on chromosomes 2 and 10, with LOD scores > 3.0. Nonparametric affected relative pair

analysis supported linkage on chromosome 2, but did not show any evidence for linkage on chromosome 10. The chromosome 2 locus had not previously been associated with POAG. The locus on chromosome 10 was close to the *OPTN* gene. Sequencing of this gene however did not reveal any pathogenic alterations, suggesting that another gene in this region had caused the linkage result.

One POAG locus (GLC1L) has been identified by means of nonparametric linkage analysis in a single, large pedigree.[21] Previous studies of this six-generation Tasmanian family had detected a mutation in the *MYOC* gene. However, only 9 of the 24 affected family members presented with this mutation. This suggested genetic heterogeneity, indicating that at least one other gene would be expected to determine POAG susceptibility in this family. Parametric linkage methods failed to localize chromosomal POAG regions, probably due to the genetic heterogeneity and incomplete family information. Nonparametric strategies were subsequently employed. These identified a disease locus on the short arm of chromosome 3, for which 11 affected family members shared the same ancestral allele. Interestingly, 7 of them also carried the *MYOC* mutation. The results of this study suggested a possible interaction between the *MYOC* gene and the chromosome 3 locus, although the numbers were too small to substantiate this with significant evidence.

Linkage analysis of quantitative traits

Nonparametric linkage analyses can also be used to study the genetic mechanisms underlying quantitative traits. The most commonly employed methods are based on a strategy developed by Haseman and Elston in 1972.[22] They compared the resemblance of sib pairs for a particular trait with the amount of allele sharing. If a marker is linked to a gene which influences the quantitative trait (i.e., the chromosomal region is a quantitative trait locus, or QTL), sib pairs who carry the same ancestral marker alleles would be expected to be more similar for that trait. Haseman and Elston regressed the squared sib pair difference for the trait on their extent of allele sharing at the marker locus. A significantly negative slope of the regression line would indicate significant linkage. This method has since been modified to increase power, but the basic concept has not changed.

Other methods for analyzing quantitative trait loci (QTLs) are based on variance components partitioning. The total phenotypic variance is separated into components attributable to the effects of a specific chromosomal region (the QTL), residual additive genetic effects, covariate effects, and remaining (environmental, interaction) effects. The parameter of interest is the proportion of the total variance that is explained by a QTL in the region spanned by the tested markers. A maximum likelihood approach can be used to estimate which value of this parameter fits the data best. The likelihood of this model

is then compared with the likelihood of a null model, in which the effect of the QTL is fixed at 0, and a LOD score is produced by the logarithm of the ratio of these likelihoods.

We have shown above that quantitative POAG traits are highly heritable. This suggests that a quantitative trait linkage analysis may be a powerful strategy for identifying new POAG genes. In 2005, Duggal et al. performed a genome-wide linkage analysis for IOP in a subpopulation of the Beaver Dam Eye study.[23] With a modified Haseman-Elston regression method in 218 sibling pairs, they revealed two loci as potential (although not statistically significant) linkage regions for IOP on chromosomes 6 and 13. Neither locus had previously been identified in a genome-wide scan for POAG. When they repeated the genome-wide IOP analysis in a larger sample (n=1979) of the same population, the linkage result on chromosome 13 could not be replicated.[24] The genome-wide linkage analysis in the expanded cohort of the Beaver Dam Eye Study identified seven regions of interest on chromosomes 2, 5, 6, 7, 12, 15, and 19. The region with the strongest evidence for linkage, on the short arm of chromosome 19, had previously been identified in four genome-wide studies on blood pressure. This may suggest a common gene regulating both IOP and blood pressure, two quantitative traits that have already been shown to correlate. Alternatively, this region on chromosome 19 may contain two nearby genes that control IOP and blood pressure independently. The linkage peak on chromosome 2 was very close to the glaucoma locus GLC1B. This locus had been identified in 1996 through classical linkage in six glaucoma families.[25] A recent study confirmed the locus in an extended pedigree from the Glaucoma Inheritance Study in Tasmania.[26] Paradoxically, the glaucoma patients in both studies had normal to slightly elevated IOPs.

Quantitative traits have also been analyzed in the extended Tasmanian POAG pedigree that had previously revealed a mutation in the *MYOC* gene and linkage to the 3p21-22 (GLC1L) region in some, but not all, affected family members.[21] Variance components linkage analyses identified a new locus for IOP on chromosome 10, with a significant LOD score of 3.3.[27] This study was the first to report a locus for VCDR. Suggestive linkage for this trait was found on chromosome 1, with a maximum LOD score of 2.3. The linkage peaks for both IOP and VCDR were substantially reduced after including the *MYOC* mutation status as a covariate in the variance components analysis. This may indicate that the *MYOC* gene interacts with the quantitative trait loci. The different studies in this single family outline the complexity of gene-finding in POAG: they clearly show the heterogeneity, they illustrate that genes that contribute to the phenotype in a different way are likely to be identified by different gene-finding strategies and they touch upon gene-gene interactions, which may have an important role in the etiology of POAG.

We have already considered that quantitative traits may be beneficial in gene-finding studies of POAG for the reason that they may have simpler genetic backgrounds, are not prone to misclassification, and can be studied population-wide. A potential drawback, however, may concern their clinical relevance. Does a gene that has been found to

mediate IOP in the general population really contribute to the development of POAG? Evidently, after a QTL has been identified its role in disease pathogenesis needs to be thoroughly assessed. A weakness of the currently published quantitative trait linkage studies of IOP is that none has been able to adjust for central corneal thickness (CCT). CCT is a potential confounder of IOP measurements[28] as well as a potential risk factor for POAG[29] and has been shown to be highly heritable.[16] The identified loci may therefore, at least to some extent, control CCT rather than IOP.

Linkage studies have so far revealed more than 25 genetic loci for POAG, thereby clearly supporting its complex nature (Figure 4, Table 1). For the vast majority of these loci, however, a gene has not yet been identified. How should we interpret these linkage results? And, how could they guide future gene-finding research? In this respect we may learn from other complex diseases, in which it has been successful to concentrate on gene-finding within loci that had been replicated in independent studies. The *CFH* gene and the *LOC387715* gene for age-related macular degeneration have thus been identified in two repeatedly detected loci on chromosomes 1 and 10, respectively.[30-33]

Many linkage regions for POAG have not been replicated. This may be due to the involvement of multiple genes in the pathogenesis of POAG. It may also be explained by the different study designs: Mendelian linkage approaches are likely to identify loci with rare, highly penetrant mutations whereas nonparametric methods aim for loci with common, low penetrant variants. These loci may or may not be the same. Furthermore, the studies have been performed in different populations, in which different genes may have different contributions to the pathogenesis of POAG. Finally, some loci may have been false positive findings, and other non confirmatory studies may have been false negatives.

GENETIC ASSOCIATION STUDIES

In contrast to the typically genetic concept of linkage, association is an established approach in traditional epidemiology. An association study assesses whether a disease is significantly related to a potential risk factor in a population. A *genetic* association study assesses whether a disease is significantly related to a *genetic variant* in a population. This association exists when individuals with the disease have a significantly higher frequency of a particular risk allele than would be expected from the disease and allele frequencies in the population.

Association between a disease and a genetic variant may occur for two reasons: 1) direct biological action of the genetic variant causes the association; 2) the genetic variant does not have a direct role, but is associated with a causal variant in close proximity due to linkage disequilibrium. Linkage disequilibrium occurs when two loci are so close that

the same alleles will be inherited together over many generations, thereby leading to an association at the population level. This is different from linkage, where the alleles are further apart and – although segregating together within one family – show a variety of combinations across different families.

Candidate gene analyses

Many genetic association studies for POAG have been performed. They have either investigated associations with genetic variants in the known, causative genes, or have searched for risk alleles in potentially predisposing candidate genes. These candidate genes have been selected through different strategies. Most commonly, the selection is made through reasoning backwards from the mechanisms that are (assumed to be) involved in the pathogenesis of POAG. This has led to association studies of genes regulating ocular blood flow (nitric oxide synthase and endothelin-1 related genes)[34-36], aqueous humour outflow (renin-angiotensin system genes)[37,38], apoptosis (tumour-protein P53 gene)[39,40], immune system (interleukin 1β and tumour necrosis factor α genes)[41,42], and neurodegeneration (apolipoprotein E gene)[43-46]. Many of these studies have had inconsistent results and the role of these genes in the etiology of POAG is still controversial. A potential disadvantage of this approach is that each (patho-) physiological mechanism probably results from many genes, the effects of which may be influenced by the environment, other genes, or complex gene networks. Working backwards from such a mechanism to one or a set of potential genes may therefore be based on too simplistic a model.

Alternatively, a candidate gene may be selected because of its homology with other disease causing genes. More than 90 % of the mutations in the *MYOC* gene are located in the so-called olfactomedin domain in the third exon. Mukhopadhyay et al. used a bioinformatics approach to search for myocilin-related proteins that had a conserved olfactomedin domain and were expressed in the eye.[47] They thus identified the Noelin 1 and 2 genes as potential candidates for POAG. One association study has since been performed for the Noelin 2 gene (*OLFM2*) in Japanese subjects.[48] A possible disease causing mutation was identified and common genetic variants were suggested to contribute to the glaucoma phenotype by interacting with the optineurin gene. These results still await replication, both in the Japanese and in other populations.

A third group of potential candidates are genes involved in the pathogenesis of related diseases. These diseases could either be complex syndromes of which glaucoma is one of the features, or diseases which show phenotypic similarities to glaucoma. An example is *OPA1*, the gene responsible for autosomal dominant optic atrophy.[49,50] Like glaucoma, optic atrophy is a progressive optic neuropathy caused by degeneration of retinal ganglion cells. The clinical similarities, together with the finding that *OPA1* is expressed in retinal ganglion cells and in the optic nerve, made *OPA1* a promising candidate gene.[51]

Due to the absence of raised IOP, *OPA1* was hypothesized to be most likely associated with normal tension glaucoma. Genetic variants in the *OPA1* gene have indeed been associated with normal tension glaucoma, but not with high tension glaucoma in Caucasian patients.[52,53] A study in Japanese subjects confirmed this, and showed that within the group of high tension glaucoma patients an *OPA1* variant was significantly related to the age at the time of diagnosis.[54] The association between *OPA1* variants and normal tension glaucoma could not be replicated in Korean and African American subjects.[55,56] A second example in this category is *CYP1B1*. Genetic association studies have shown that this gene is not only involved in congenital glaucoma, but also in high tension POAG with juvenile or adult onset.[57,58]

Association analyses following linkage

Association analyses may be used to further investigate previously identified linkage regions. Candidate genes within a linkage region may be selected by means of the methods described above. Association studies for the *ACE* gene[37] (located in a region identified by a genome-wide sib-pair analysis)[19] and the *NOS3* gene[35] (located near the GLC1F locus)[59] have thus been performed. Alternatively, a dense marker set along the total linkage region can be analysed for association. This method has successfully identified variants in the complement factor H and *LOC387715* genes as risk factors for age-related macular degeneration.[30-33]

Genome-wide association analyses

To date, association studies of POAG have focused on candidate genes. With the identification of highly dense and easily genotyped markers called single nucleotide polymorphisms (SNPs), the increasing knowledge of the distribution of linkage disequilibrium throughout the genome, and reducing genotyping costs, a shift is rapidly being made towards genome-wide association studies. Although this approach still has its limitations as discussed below, successes from other complex diseases support the application of genome-wide association studies to gene-finding in glaucoma.* [60]

Confounding in genetic association studies

As in traditional epidemiology, confounding factors have the potential to either generate false-positive or hide true results. One possible source of confounding in genetic association studies is population stratification. Due to recent migration and integration, each apparently homogeneous population may consist of multiple genetically distinct subpopulations. Different disease and allele frequencies between the subpopulations can lead to spurious association because cases and controls may not be properly

* Findings of genome-wide association studies that appeared after the publication of this chapter are discussed in Chapter 5.1

matched (i.e., may derive from different subpopulations). To get round this problem, family-based association methods have been developed. The most widely used is the transmission disequilibrium test (TDT)[61], in which the frequencies of alleles that are transmitted from a parent to an affected child are compared to the frequencies of alleles that are not transmitted. This "internal control" approach is independent of population stratification. Another option is to test the data for possible stratification effects by comparing allele frequencies of several unlinked loci in cases and controls.[62]

A second issue in genetic association studies is that of multiple comparisons. Typically a large number of genetic markers are tested, each with a small a priori probability of association. Even without any true effect, 5% of the markers will show significant association if the usual p-value of 0.05 is respected. The significance level should thus be adjusted but there is no clear consensus on exactly how. The common post-hoc corrections, such as the Bonferroni approach, seem to be too conservative: they assume independence of each test while many markers will actually be correlated owing to linkage disequilibrium. The most reliable current approach is to replicate the findings in a second, independent sample.

Association and linkage

Association and linkage are complementary approaches. The choice to use one or the other depends on the availability of study subjects, expected genetic effects and allele frequencies, technical capacities and funding. Association is assumed to be more powerful than linkage for identifying susceptibility alleles with a small effect, as is often the case in complex diseases. In addition, association studies do not require the recruitment of families with multiple affected cases or special family structures and are therefore easier to conduct. The technical demands and the costs for a genome-wide association study are on the other hand much higher than for a genome-wide linkage study, as more markers need to be genotyped. Furthermore, linkage studies probably have more power to detect susceptibility alleles with low frequencies.

FUTURE PERSPECTIVES

The unambiguous conclusion of the above discussed gene-finding studies is that POAG is a complex disease. Many loci have been identified. The process of identifying genes related to these loci, however, is lagging. Moreover, the currently known genes (*MYOC*[63], *OPTN*[64], *WDR36*[65]) probably contribute to the pathogenesis of POAG in less than 5% of cases in the general population.[66-69] Genes accounting for a more significant proportion of the known heritable component of POAG remain to be identified.

How can we improve and accelerate the gene-finding process of POAG? Can we copy the art from other complex diseases? Recent successes have emphasized the potential value of association studies.[60] This seems a reasonable approach for POAG, as the effect sizes of the susceptibility alleles are expected to be small. In addition, this method has recently become more feasible as a result of emerging high-throughput and affordable genotyping technologies and increasing knowledge of the genome. Association studies may benefit from previous linkage analyses; loci that have repeatedly identified by linkage may be promising association targets.[30-33]

One of the keys to future success in gene-finding is collaboration. Insufficient power has been a common limitation of genetic association studies. Very large cohorts are needed to identify weak susceptibility genes and second cohorts to replicate any findings. Collaboration between research groups is therefore necessary to make progress, as has been realized by the currently increasing numbers of joint initiatives. Inaccurate or incomplete phenotyping has been a second limitation of genetic studies. The complex diagnosis and the insidious onset of POAG have often led to misclassification. Currently improving diagnostic techniques and the opportunity to study quantitative traits may partly solve this problem. A thorough understanding of the phenotype is a prerequisite for a fruitful study design. It is essential to know what clinical features are important and how these can be accurately, efficiently and reliably assessed. Therefore, close collaboration between scientists and clinicians is necessary.

Future studies will not only be directed at finding new genes, but also at elucidating the role of already identified genes. Which gene variants cause disease? How does the gene interact with other genes and with environmental factors? How does the genotype predict the clinical course? And how can we use this information in effectively managing and treating POAG in a particular patient? Before any successful application to clinical practice can be made, these issues need to be resolved.

There is growing support for the role of gene-gene interactions in the pathogenesis of POAG. In a Japanese study of 194 patients with POAG, 217 patients with NTG, and 218 control subjects, Funayama and colleagues showed a possible interaction between the *OPTN* and *TNFα* genes: Genetic variants in *OPTN* were significantly associated with POAG or NTG in individuals carrying particular risk variants in the *TNFα* gene, but not in individuals without these *TNFα* risk variants.[70] Other genes that have been suggested to interact with *OPTN* include *OLFM2*, *APOE*, and *MYOC*.[48,71] For *MYOC*, genetic interactions with *APOE* and *CYP1B1* have been described.[43,58,71] Knowledge of genetic interactions may give insight into the complex pathophysiological mechanisms and into potential targets for glaucoma therapy.

Interactions between genes and environmental factors are also assumed to contribute to the onset and progression of POAG. These interactions have already been demonstrated in other late-onset, complex diseases. In age-related macular degeneration,

for example, risk alleles of the *CFH* and *LOC387715* genes have been shown to significantly interact with cigarette smoking.[72,73] For POAG, in which still little is known about environmental risk factors, such interactions have not yet been revealed. Elucidating these may be of significant importance from a public health perspective for developing blindness-prevention programs.

After a gene has been identified, its role in the etiology of POAG in the population needs to be established. Genetic epidemiologic studies have shown mutations in the *MYOC* gene in 3 to 4 % of sporadic POAG patients.[67,74] More than 50 *MYOC* mutations have been identified in different ethnic groups worldwide. Some (e.g. the Pro370Leu, Tyr437His and Ile477Asn mutations) are particularly associated with severe, early-onset forms of POAG.[75,76] Others are likely to predict a milder clinical course with a later onset. An example is Gln368Stop, which is the most common glaucoma-causing *MYOC* mutation across populations.[67,74,77] For many mutations the correlating phenotype still needs to be fully assessed.

OPTN mutations were initially identified in 16.7% of families with predominantly low-tension glaucoma.[78] Subsequent studies in non-familial cases, however, have reported much lower rates, and *OPTN* mutations are now assumed to be rare causes of low-tension glaucoma.[66,79,80] Associations with high-tension glaucoma have also been suggested, although several studies have not supported this.[66,70,80-82] The prevalence and pathogenicity of genetic variants differ substantially between populations of different ethnicity.[66,70,81,83] Genotype-phenotype correlations for *OPTN* have not been widely studied yet, except for the Glu50Lys mutation, which has been associated with an earlier onset and a more progressive disease course. [79,84] Information on mutation prevalences and genotype-phenotype correlations of all POAG-related genes will be very valuable to create prognostic and diagnostic DNA tests which may assist clinicians in disease management.

WDR36 has been reported to be the third POAG gene, but convincing evidence for its causative role has not yet been produced by population-based studies. The four mutations that had initially been reported by Monemi and colleagues were not found to cause POAG in four independent cohorts.[68,69,85,86] One study of POAG families rather revealed *WDR36* to act as a modifier gene, with gene variants contributing to disease severity.[68] Interestingly, several POAG families have been reported that are linked to this region but do not show any *WDR36* alterations. [86-88] POAG in these families may be caused by *WDR36* variants outside the exons (within the promoter or introns) or by another gene within this region.

CONCLUSION

The complex genetic etiology of POAG is a hard nut for genetic epidemiologists to crack. Although more than 25 chromosomal regions have been linked to the disease, only three genes have currently been identified. These genes contribute to POAG in <5% of the cases in the general population. Hence, genes that explain a more significant fraction remain to be identified.

Genetic linkage and association analyses are the two main methods to search for new genes. These methods may yield more success in the near future due to more accurate and standardized phenotyping together with more sophisticated and cheaper genotyping. Molecular and biological studies will subsequently be needed to resolve the pathophysiological mechanisms, and gene prevalence and genotype-phenotype correlation studies to sensibly translate these findings into ophthalmic practice.

SUMMARY AND KEY POINTS

- It is clear that there is a genetic component to the development of POAG
- There is consensus that the majority of POAG is inherited as a complex disorder
- Linkage and association studies have helped identify loci involved but there is inconsistency between study results and much remains to be elucidated
- Further work requires large scale collaboration involving numerous scientific disciplines and may focus on quantitative traits
- Unraveling the genetic component of POAG might clarify the pathophysiology of the disease and greatly assist in treatment and prevention of visual loss of many thousands of individuals.

ELECTRONIC DATABASE INFORMATION

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/sites/entrez?db=omim, for *MYOC* [MIM 601652], *OPTN* [MIM 602432], *WDR36* [MIM 609669], *OPA1* [MIM 605290], *CYP1B1* [MIM 601771], *ACE* [MIM 106180], *NOS3* [MIM 163729], *APOE* [MIM 107741], *CFH* [MIM 134370], and *LOC387715* [MIM 611313].

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Chapter 1.3

Scope of this thesis

There is an obvious genetic component to the development of glaucoma. However, the details of this component are largely unknown. In Chapter 1.2 we have suggested that current knowledge could be improved by genome-wide association studies (GWASs), accurate phenotyping, quantitative trait approaches, and large-scale collaboration. We have sought to put these suggestions into practice in the studies presented in this thesis.

The main purpose of our research was to gain insight in the genetic etiology of glaucoma by performing GWASs of quantitative glaucoma traits. To substantiate this approach, we first investigated to what extent quantitative glaucoma traits were determined by genetic effects. These studies are described in Chapters 2.1 and 2.2. We subsequently performed GWASs of optic disc parameters and intraocular pressure and thereby had the opportunity to collaborate with various international research groups and investigate our findings in their study populations. Chapters 3.1 and 3.2 describe these GWASs. Our findings in Chapter 3.2 alluded to common genetic pathways underlying glaucoma and Alzheimer's disease. We further explored any associations between these neurodegenerative disorders in the next chapters. We investigated whether cognitive functioning and retinal nerve fiber layer thickness were associated in their physiological spectrum. This study is presented in Chapter 4.1. We also assessed the effects of polymorphisms in *APOE*, a well-established Alzheimer's disease gene, on optic disc characteristics and retinal nerve fiber layer thickness, which is presented in Chapter 4.2. In the general discussion in Chapters 5.1 and 5.2 we view our findings in a wider perspective and speculate on future research directions.

Part 2

STUDIES TO SUPPORT GENE-FINDING FOR QUANTITATIVE GLAUCOMA TRAITS

Chapter 2.1

Genetic contributions to glaucoma: heritability of intraocular pressure, retinal nerve fiber layer thickness and optic disc morphology

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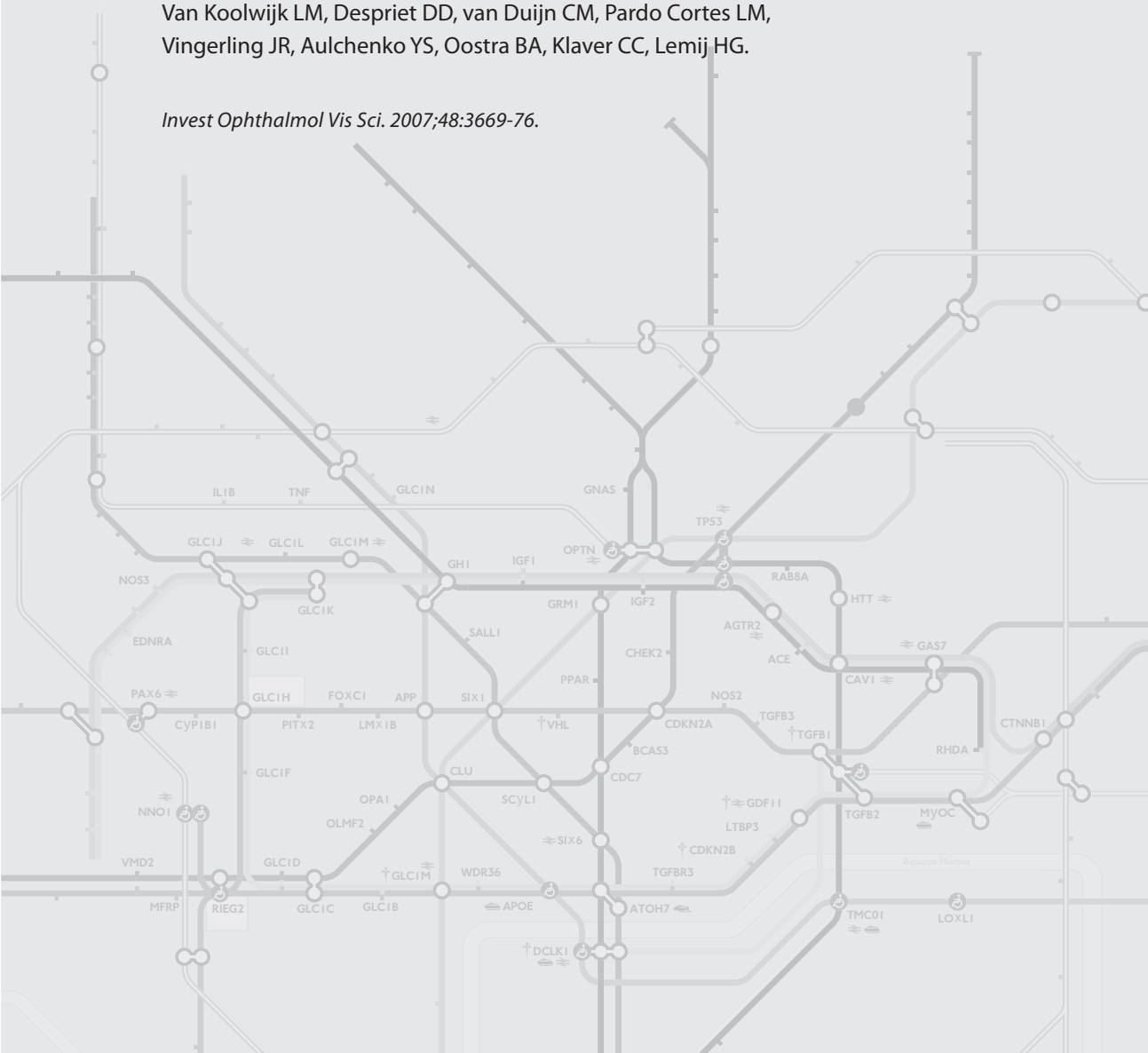

ABSTRACT

Purpose. The genetic etiology of primary open-angle glaucoma (POAG) is still largely unknown, because of its complexity and disparities in its classification. We aimed to determine the genetic contribution to various early, continuous markers of POAG by assessing the heritability of intraocular pressure (IOP), retinal nerve fiber layer (RNFL) thickness, and neuroretinal rim and optic disc parameters in a genetically isolated population.

Methods. A total of 2620 subjects (mean age, 48 yrs; range 18 – 86) from extended pedigrees living in a small town in the Netherlands underwent an extensive ophthalmic examination. Their IOP was measured by Goldmann applanation tonometry, their RNFL thickness by scanning laser polarimetry (GDx VCC), and their optic disc parameters by confocal scanning laser ophthalmoscopy (HRT II). Risk associations were explored by linear regression analyses and heritability estimates by variance component methods.

Results. Inbreeding was present in 2042 (81%) participants, and was significantly associated with a higher IOP ($P < 0.001$). The heritability estimate for IOP was 0.35 (95% confidence interval [CI], 0.27-0.43); for RNFL thickness, 0.48 (95% CI, 0.35-0.60); and for neuroretinal rim area, 0.39 (95% CI, 0.20-0.58). Non-genetic factors accounted for only a small proportion (≤ 0.13) of the variance in all 3 traits.

Conclusions. Early, continuous markers of POAG are strongly determined by additive genetic effects. Our results support a quantitative trait strategy to discover new genes for POAG.

INTRODUCTION

Primary open-angle glaucoma (POAG) is the second leading cause of blindness worldwide.[1] The sharp rise in the ageing population will probably cause a 30% increase in the number of patients with POAG by 2020, with an estimate of 58.6 million affected and 5.9 million bilaterally blind.[2] Established risk factors for POAG are age, race, intraocular pressure (IOP), central corneal thickness (CCT), high myopia, and a positive family history.[3,4] Dissection of the genetic background has resulted in an association with 20 genetic loci and 3 genes (*MYOC, OPTN, WDR36*).[5-7] In addition, genes causing congenital glaucoma or glaucoma-associated developmental syndromes may contribute to adult-onset POAG. Some (e.g. *CYP1B1*) are already recognized to be involved, the role of others (*PITX2, FOXC1, PAX6, LMX1B*) remains to be fully assessed in adult cases.[8-13] The currently identified genes probably contribute to the pathogenesis of POAG in less than 5% of cases in the general population.[14-19] Hence, genes that explain a more significant fraction of POAG remain to be identified.

Gene-finding in POAG has been hampered by etiological and clinical heterogeneity, partly due to non-uniformity of diagnostic criteria. Its insidious onset and slow natural course impede a definite diagnosis at an early stage, whereas including only late-stage, outright POAG will greatly limit genetic studies because relatively few people would be available to participate. These difficulties fuel the question of whether studying heritable, continuous markers of POAG may improve the chance of success in gene-finding.

Quantitative markers of POAG, apart from IOP, are retinal nerve fiber layer (RNFL) thickness and optic disc rim area, both indicators of the number of existing retinal ganglion cells, or indirectly, of any of their loss, typical of glaucoma.[20-22] Previous studies, mostly performed in twins or nuclear families, reported heritability estimates for IOP ranging from 0.29 to 0.50 and for cup-to-disc ratio ranging from 0.48 to 0.80.[23-26] The ranges of these heritability estimates are relatively large, and studies based on more extended pedigrees may provide more precise figures.

We performed a family-based cohort study in a genetically isolated population in the Netherlands and thereby had the opportunity to study large extended pedigrees. As a first investigation into the genetic etiology of early markers of POAG, we explored the heritability estimates for IOP, RNFL thickness, and optic disc rim area. We used the imaging techniques scanning laser polarimetry (SLP) and confocal scanning laser ophthalmoscopy (CSLO) to obtain objective measurements. This study also allowed us to demonstrate the effect of inbreeding and non-genetic factors on these POAG markers.

METHODS

Subjects

Subjects were recruited as part of the Erasmus Rucphen Family (ERF) study. This family-based cohort study was designed to identify susceptibility genes for various complex disorders by studying quantitative traits. The ERF study is being conducted in a genetically isolated population located in the southwest of the Netherlands. This population was founded in the middle of the 18th century by a limited number of individuals (< 400), and was characterized by rapid growth and little immigration until the past few decades. The genealogical database, which contains information on the current inhabitants of this area and their ancestors, includes more than 80,000 records. Genetic characterization of this population has been presented elsewhere.[27-29] The research adhered to the tenets of the Declaration of Helsinki and was approved by the Medical Ethics Committee of Erasmus Medical Centre in Rotterdam. Informed consent was obtained after explanation of the nature and possible consequences of the study.

Eligibility for participation in the study was determined by genealogical background, not by any phenotypes of interest. Twenty-two families were selected who had at least 6 children baptized in the community church between 1880 and 1900. All living descendants of these families aged 18 years and older, as well as their spouses, were invited to attend a series of clinical examinations. A total of 2620 subjects underwent ophthalmic examination.

Based on genotyping half of the sample (N=1430, 437 nuclear families with one or both tested parents and one or more tested offspring) with 5964 single nucleotide polymorphisms, we identified only one nuclear family in which the father was not compatible with any of the three offspring (whereas the siblings were compatible with each other). Exclusion of this family did not alter the heritability estimates. As the percentage of non-paternities is low and is known for only half the sample, the presence of non-paternities was ignored in the data presented.

Clinical examination

All data were collected between June 2002 and February 2005. Non-ophthalmic examinations included anthropometrical measurements, cardiovascular and endocrine assessments, neuropsychological tests, fasting blood samples, and interviews regarding medical history, medication, and putative risk factors.

The ophthalmic examination comprised the assessment of best-corrected visual acuity, refraction, and IOP. Keratometry was determined by an automatic refractometer, and the eyes' axial lengths by an intraocular lens calculator (IOL Master; Carl Zeiss Meditec, Inc., Dublin, CA, USA). Scanning laser polarimetry (SLP) was subsequently performed with the commercially available GDx VCC (Carl Zeiss Meditec, Inc., Dublin, CA, USA). In

mydriasis, participants underwent fundus photography centered on the optic disc (20°, TRC-50XT retinal camera, Topcon Medical Systems, Inc., Paramus, NJ, USA), and confocal scanning laser ophthalmoscopy (CSLO) measurements (Heidelberg Retina Tomograph II [HRT II]; Heidelberg Engineering GmbH, Dossenheim, Germany).

IOP

One of 5 trained examiners performed bilateral IOP measurements with Goldmann applanation tonometry. A drop of fluorescein sodium was instilled in each eye. The tonometer was set at 10, and the prism was carefully applied to the corneal surface of the right eye. Without looking at the scale, the examiner rotated the dial until the inner margins of the two semicircles touched each other. The examiner then moved the slit lamp away from the eye and read the IOP. The tonometer was set at 10, and the measurement was repeated. If the two measurements differed, a third measurement was performed, and the median value was recorded. The procedure was repeated for the left eye.

RNFL thickness

After any refractive error was entered into the GDx VCC software, the birefringence of the anterior segment of each participant was assessed by means of the method described by Zhou and Weinreb.[30] Subsequently, each eye was scanned with adjusted anterior segment compensation to estimate peripapillary RNFL thickness as described by Reus and Lemij.[31] The cut-off for the quality of the image was a GDx VCC scan quality score of 8 or above. Images with lower scores were excluded.

After the boundaries of the optic disc were manually marked, the GDx VCC software positioned two circles centered on the disc: The first had a diameter of ~2.5 mm (54 pixels), the second a diameter of ~3.3 mm (70 pixels). The parameters of RNFL thickness that we used in this study were based on the RNFL thickness measurements between the circles: TSNIT average (temporal-superior-nasal-inferior-temporal), superior average (25° to 144°), inferior average (215° to 334°), and the worst hemifield average (the lowest average value of the RNFL thickness of the hemifields of both eyes).

Neuroretinal rim

Details of the CSLO technique have been described elsewhere.[32] Briefly, imaging was performed after the participant's keratometry data were entered into the software and the settings were adjusted in accordance with the refractive error. Only images with a standard deviation of height measurements below 50 µm were accepted. The optic disc margin was manually marked at the inner edge of Elschnig's ring by one observer (LMEvK). The HRT II software then calculated stereometric parameters of the optic disc and neuroretinal rim. The parameters that we used were disc area, rim area, rim area

superotemporally (45° to 90°), rim area inferotemporally (270° to 315°), rim-to-disc area ratio, vertical cup-to-disc ratio, and cup shape measure, an index of depth variation and steepness of the cup walls.

Statistical analysis

The inbreeding coefficient, which represents the probability that the two alleles at a given locus are identical by descent (i.e. derived from the same ancestral chromosome), was calculated, based on all available genealogical information, by means of PEDIG software.[33] This coefficient was analyzed in quartiles, since its distribution was skewed towards zero.

Other putative covariates of glaucoma pathogenesis that were studied included age, sex, height, body mass index, systolic blood pressure, pulse rate, fasting blood glucose level, blood cholesterol level, IOP, time of IOP measurement, axial length of the eye, spherical equivalent of refractive error, and mean corneal curvature. The glaucoma markers that were studied were IOP, TSNIT average, superior average, inferior average, worst hemifield average, disc area, rim area, superotemporal rim area, inferotemporal rim area, rim-to-disc area ratio, vertical cup-to-disc ratio, and cup shape measure. These markers were based on the eye most representative of glaucoma: the eye with the lower RNFL thickness parameters, rim area parameters, and rim-to-disc area ratio and the eye with the higher IOP, vertical cup-to-disc ratio, and cup shape measure. We calculated the mean of both eyes for the analyses of disc area. If a measurement could be obtained in only 1 eye, the parameters of this eye were included in the analyses.

Associations were explored by univariate and multivariate linear regression analyses (SPSS version 11.0 for Windows). All determinants below the 0.10 significance level in the multivariate analyses were retained in the final model for heritability estimation. The distribution of the multivariate regression residuals in the final model was tested for normality with the non-parametric, one-sample Kolmogorov-Smirnov test. To reduce kurtosis of the distribution in parameters describing RNFL thickness or rim area, we excluded the upper and lower 0.5 percentile values of these traits. We further transformed traits that were skewed using natural logarithm (disc area, rim area), or exponential function (vertical cup-to-disc ratio).

We estimated the heritability by means of a variance component maximum likelihood analysis, as implemented in the SOLAR 2.1.2. software package.[34, 35] A variance component analysis separates the observed phenotypic variance into components that are attributable to different causes. Heritability describes the relative importance of the component that is attributable to heredity. This component is called the additive genetic variance and represents the cumulative effects of alleles. Heritability can be estimated from the resemblance between family members. We first examined the proportion of the phenotypic variance associated with the covariates. Subsequently, we

estimated the proportion of the remaining phenotypic variance explained by additive genetic effects. Finally, the heritability of each glaucoma marker was calculated as the proportion of the total phenotypic variance explained by additive genetic effects. In addition, we investigated the genetic correlation between rim area and disc area with a linear bivariate analysis.

RESULTS

Demographic and clinical characteristics of the study population have been presented in Table 1. The population was almost all Caucasian, and ages ranged from 18 to 86 years.

A total of 2042 (81%) participants had an inbreeding coefficient greater than zero, indicating at least some degree of inbreeding. The median inbreeding coefficient was 0.00187, and 186 (7.4%) participants had an inbreeding coefficient of at least 0.016, indicating that their parents were second cousins or closer relatives.

Table 1. Characteristics of the Study Population (N=2518)

Characteristic	
Age, mean (yrs) ± SD	47.98 ± 14.26
< 55 yrs, %	65.4
55 + yrs, %	34.6
Male gender, %	44.1
Inbreeding > 0, %	81.1
Inbreeding coefficient, median (range)	0.00187 (0.00000 – 0.06286)
Height men, mean (cm) ± SD	174.72 ± 7.21
Height women, mean (cm) ± SD	161.82 ± 6.51
Body mass index men, mean (kg/m^2) ± SD	27.27 ± 4.18
Body mass index women, mean (kg/m^2) ± SD	26.51± 4.96
Systolic blood pressure, mean (mm Hg) ± SD	139.54 ± 20.16
Pulse rate, mean (bpm) ± SD	70.49 ± 12.21
Fasting blood glucose, mean (mmol / L) ± SD	4.58 ± 0.99
Length of eye axis, mean (mm) ± SD	23.22 ± 1.05
Spherical equivalent, mean (D) ± SD	0.04 ± 1.93
High myopia (≤ –6 D), %	1.4
Corneal curvature, mean (mm) ± SD	7.71 ± 0.26

SD = standard deviation

Table 2. Distribution of IOP, RNFL thickness and optic disc morphology

	Age category < 55	Age category 55+
	N = 1596	*N = 838*
IOP, mean (mmHg) ± SD	15.27 ± 3.05	15.98 ± 2.79
IOP ≥ 22 mm Hg, %	0.9	1.6
RNFL Thickness	*N = 1085*	*N = 446*
TSNIT average, mean (µm) ± SD	57.18 ± 5.92	55.00 ± 5.97
Superior average, mean (µm) ± SD	68.82 ± 7.71	64.13 ± 8.59
Inferior average, mean (µm) ± SD	64.12 ± 8.62	61.19 ± 7.82
Worst hemifield average, mean (µm) ± SD	63.08 ± 7.96	59.68 ± 7.69
Optic Disc Morphology	*N = 458*	*N = 284*
Disc area, mean (mm^2) ± SD	1.86 ± 0.34	1.89 ± 0.36
Rim area, mean (mm^2) ± SD	1.36 ± 0.23	1.35 ± 0.27
Rim area sup-temp, mean (mm^2) ± SD	0.16 ± 0.04	0.16 ± 0.04
Rim area inf-temp, mean (mm^2) ± SD	0.18 ± 0.04	0.17 ± 0.05
Rim-to-disc area ratio, mean ± SD	0.76 ± 0.12	0.73 ± 0.15
Vertical cup-to-disc ratio, mean ± SD	0.37 ± 0.20	0.40 ± 0.24
Cup shape measure, mean ± SD	- 0.16 ± 0.06	- 0.14 ± 0.06

IOP = intraocular pressure; RNFL = retinal nerve fiber layer; TSNIT = temporal-superior-nasal-inferior-temporal; SD = standard deviation.

IOP

Tonometry was successfully performed in 2457 (93.8 %) subjects. Twenty-three (0.9%) subjects received IOP lowering therapy or had a history of these medications and were excluded from the IOP analyses.

IOP values of the right and the left eye were statistically significantly correlated (Pearson correlation coefficient 0.855, p-value <0.001). IOP was statistically significantly higher in the right eye than in the left eye (paired samples t-test; mean difference 0.10 mm Hg; 95% CI, 0.03-0.16).

Data have been presented for the eye with the higher IOP. The distribution of the IOP in the study population has been given in Table 2, and the results of the linear regression and variance component analyses in Table 3. Age, inbreeding, and fasting glucose were significantly associated with IOP, and corneal curvature was inversely related. These covariates accounted for 0.05 of the total phenotypic variance of IOP. Of the remaining variance, the proportion explained by additive genetic effects was 0.37 (95% CI, 0.29-0.45). The heritability estimate, calculated as the contribution of genetic factors to the total phenotypic variance, was 0.35 (95% CI, 0.27-0.43)

Table 3. Linear regression and variance component analyses of intraocular pressure

Determinants	Regression coefficient (SE); p-value	Proportion explained by determinants	Proportion explained by additive genetic effects	
			of remaining variance	of total variance
age	0.025 (0.004); p < 0.001	0.050	0.368 (0.042); p < 0.001	0.350 (0.040); p < 0.001
male gender	0.147 (0.128); p = 0.246			
inbreeding	0.201 (0.061); p < 0.001			
pulse rate	0.009 (0.005); p = 0.069			
fasting glucose	0.262 (0.065); p < 0.001			
corneal curvature	-0.869 (0.245); p < 0.001			

SE = standard error; IOP = intraocular pressure

RNFL thickness

Only 1552 (59 % of total) subjects underwent RNFL thickness measurements, because this procedure was introduced after the study had commenced. Twenty-one (1.4 %) subjects were excluded due to poor quality of the images.

The distributions of the RNFL thickness parameters have been presented in Table 2. The nerve fiber indicator (NFI) was ≥40 (i.e., suggestive of glaucoma) in 20 (1.8%) of 1085 subjects younger than 55 years, and 41 (9.2%) of 446 subjects 55 years of age or older.

The results of the linear regression and variance component analyses have been reported in Table 4. Inbreeding and IOP were not statistically significantly related to any RNFL thickness parameter. Age and systolic blood pressure were inversely associated with all parameters, although the relation between systolic blood pressure and inferior average did not reach statistical significance (*P*=0.144). Axial length, spherical equivalent and corneal curvature, all of which are covariates of refractive error, were significantly associated with most RNFL thickness parameters.

The proportion of the total phenotypic variance of RNFL thickness explained by the determinants in Table 4 ranged from 0.03 (inferior average) to 0.07 (superior average). Of the remaining variance, the proportion explained by additive genetic effects was 0.50 (95% CI, 0.36-0.63) for TSNIT average, 0.43 (95% CI, 0.31-0.56) for superior average, 0.50 (95% CI, 0.37-0.63) for inferior average, and 0.49 (95% CI, 0.37-0.60) for worst hemifield average. The heritability estimates for all parameters of RNFL thickness were highly significant, and ranged from 0.40 (95% CI, 0.29-0.52) for superior average to 0.49 (95% CI, 0.36-0.61) for inferior average.

Neuroretinal rim and optic disc morphology

Data of the first 750 participants were included in this analysis. Of these, 8 (1.1%) subjects were excluded because of poor quality of the topographic images.

Table 4. Linear regression and variance component analyses of retinal nerve fiber layer thickness

	Determinants	Regression coefficient (SE); p-value	Proportion explained by determinants	Proportion explained by additive genetic effects	
				of remaining variance	of total variance
TSNIT average	age	- 0.055 (0.013); p < 0.001	0.038	0.495 (0.067); p < 0.001	0.476 (0.064); p < 0.001
	male gender	- 0.337 (0.321); p = 0.294			
	inbreeding	- 0.116 (0.149); p = 0.435			
	bmi	0.084 (0.036); p = 0.021			
	sbp	- 0.020 (0.009); p = 0.033			
	axial length	0.664 (0.212); p = 0.002			
	spherical equiv	0.241 (0.118); p = 0.040			
Superior average	age	- 0.108 (0.016); p < 0.001	0.070	0.432 (0.063); p < 0.001	0.402 (0.058); p < 0.001
	male gender	- 0.257 (0.412); p = 0.529			
	bmi	0.103 (0.048); p = 0.034			
	sbp	- 0.028 (0.013); p = 0.030			
	fasting glucose	- 0.364 (0.239); p = 0.129			
	corneal curvature	3.702 (0.796); p < 0.001			
Inferior average	age	- 0.074 (0.018); p < 0.001	0.033	0.503 (0.067); p < 0.001	0.487 (0.064); p < 0.001
	male gender	- 0.729 (0.458); p = 0.112			
	sbp	- 0.019 (0.013); p = 0.144			
	axial length	0.987 (0.300); p < 0.001			
	spherical equiv	0.194 (0.166); p = 0.242			
Worst hemifield average	age	- 0.088 (0.016); p < 0.001	0.060	0.485 (0.061); p < 0.001	0.456 (0.057); p < 0.001
	male gender	- 0.165 (0.400); p = 0.682			
	bmi	0.083 (0.047); p = 0.077			
	sbp	- 0.031 (0.012); p = 0.011			
	fasting glucose	- 0.294 (0.234); p = 0.208			
	corneal curvature	3.131 (0.777); p < 0.001			

SE = standard error; TSNIT = temporal-superior-nasal-inferior-temporal; bmi = body mass index; sbp = systolic blood pressure; spherical equiv = spherical equivalent

The distributions of all optic disc parameters that were measured have been presented in Table 2. The Moorfields regression classification was outside normal limits in 13 (2.8%) of 458 subjects less than 55 years of age, and 29 (10.2%) of 284 subjects more than 55 years of age.

Table 5 shows the results of the linear regression and variance component analyses. Inbreeding and IOP were not associated with any optic disc parameter. Age showed a statistically significant association with disc area, rim-to-disc area ratio, and vertical cup-to-disc ratio. Determinants of refractive error (axial length, spherical equivalent, and corneal curvature) were significantly related to most parameters.

These covariates explained a fraction of 0.13 of the variance in disc area, 0.02 to 0.06 of the variance in rim area parameters, and 0.06 of the variance in cup shape measure. Of the remaining variance, additive genetic effects accounted for 0.59 (95% CI, 0.42-0.77) of disc area, 0.41 (95% CI, 0.21-0.61) to 0.84 (95% CI, 0.69-0.98) of rim area parameters, and 0.42 (95% CI, 0.21-0.63) of cup shape measure. This resulted in heritability estimates of 0.52 (95% CI, 0.36-0.67) for disc area, 0.39 (95% CI, 0.20-0.58) to 0.79 (95% CI, 0.65-0.93) for rim area parameters, and 0.40 (95% CI, 0.20-0.59) for cup shape measure.

We analyzed co-aggregation of rim and disc area and found a low genetic correlation (r genetic =0.16; SE = 0.13, P-value = 0.26).

DISCUSSION

This study was performed to assess the heritability of early, continuous POAG markers in a large family-based cohort study by using objective imaging techniques. IOP, RNFL thickness, and neuroretinal rim area were strongly genetically determined, with heritability estimates of 0.35, 0.48, and 0.39, respectively. Non-genetic factors, although significantly associated with glaucoma phenotypes, were responsible for only a small proportion of the variance of these traits.

The design of our study had several limitations. First, genetically isolated populations may exhibit genetic drift, and their genetic composition may therefore deviate from the general population. We performed simulation studies in the ERF population, which showed that the effects of genetic drift on the frequency of common alleles were negligable.[28] Thus, we believe it is valid to generalize our results to an outbred population. A second limitation is that we studied a relatively young and healthy cohort, which may reduce the clinical relevance of our findings. This problem appeared to be small, since the proportion of subjects aged 60+ years was considerable (20%), and the range of outcome variables was representative of the clinical spectrum. Third, measurements of RNFL thickness and neuroretinal rim area were for logistic reasons not performed on the total study population. We do not think that this affected the outcome of our results,

Table 5. Linear regression and variance component analyses of optic disc morphology

	Determinants	Regression coefficient (SE); p-value	Proportion explained by determinants	Proportion explained by additive genetic effects	
				of remaining variance	of total variance
Disc area	age	0.016 (0.006); p = 0.008	0.130	0.593 (0.089); p < 0.001	0.516 (0.078); p < 0.001
	male gender	0.480 (0.196); p = 0.014			
	inbreeding	0.106 (0.074); p = 0.153			
	height	- 0.011 (0.011); p = 0.317			
	sbp	0.006 (0.003); p = 0.097			
	axial length	- 0.405 (0.119); p < 0.001			
	spherical equiv	- 0.317 (0.059); p < 0.001			
	corneal curvature	2.819 (0.373); p < 0.001			
Rim area	age	0.039 (0.028); p = 0.162	0.037	0.407 (0.102); p < 0.001	0.392 (0.098); p < 0.001
	male gender	1.886 (0.665); p = 0.005			
	inbreeding	0.088 (0.357); p = 0.801			
	sbp	- 0.019 (0.018); p = 0.276			
	axial length	- 1.487 (0.565); p = 0.009			
	spherical equiv	- 0.986 (0.281); p < 0.001			
	corneal curvature	5.541 (1.793); p = 0.002			
Rim area sup-temp	age	0.003 (0.006); p = 0.617	0.025	0.426 (0.095); p < 0.001	0.415 (0.093); p < 0.001
	male gender	- 0.059 (0.218); p = 0.787			
	height	0.023 (0.013); p = 0.066			
	axial length	- 0.198 (0.093); p = 0.034			
	spherical equiv	- 0.184 (0.051); p < 0.001			

Table 5. Linear regression and variance component analyses of optic disc morphology (continued)

	Determinants	Regression coefficient (SE); p-value	Proportion explained by determinants	Proportion explained by additive genetic effects	
				of remaining variance	of total variance
Rim area inf-temp	age	- 0.003 (0.007); p = 0.667	0.020	0.483 (0.097); p < 0.001	0.473 (0.095); p < 0.001
	male gender	0.155 (0.161); p = 0.332			
	inbreeding	0.066 (0.088); p = 0.453			
	sbp	- 0.004 (0.004); p = 0.363			
	axial length	- 0.244 (0.138); p = 0.077			
	spherical equiv	- 0.179 (0.068); p = 0.009			
	corneal curvature	0.770 (0.436); p = 0.077			
Rim-to-disc area ratio	age	- 0.010 (0.004); p = 0.024	0.055	0.835 (0.075); p < 0.001	0.789 (0.071); p < 0.001
	male gender	0.006 (0.145); p = 0.968			
	height	0.010 (0.009); p = 0.250			
	sbp	- 0.004 (0.003); p = 0.089			
	spherical equiv	0.042 (0.026); p = 0.110			
	corneal curvature	- 1.001 (0.194); p < 0.001			
Vertical cup-to-disc ratio	age	0.017 (0.004); p < 0.001	0.058	0.684 (0.089); p < 0.001	0.645 (0.084); p < 0.001
	male gender	- 0.054 (0.088); p = 0.535			
	sbp	0.004 (0.002); p = 0.085			
	spherical equiv	- 0.065 (0.024); p = 0.007			
	corneal curvature	0.883 (0.181); p < 0.001			
Cup shape measure	age	0.018 (0.011); p = 0.089	0.060	0.420 (0.105); p < 0.001	0.395 (0.099); p < 0.001
	male gender	0.568 (0.360); p = 0.114			
	height	- 0.067 (0.020); p < 0.001			
	bmi	- 0.056 (0.028); p = 0.043			
	sbp	0.012 (0.006); p = 0.074			
	fasting glucose	0.213 (0.146); p = 0.144			
	axial length	0.390 (0.117); p < 0.001			

SE = standard error; sup-temp = superotemporal; inf-temp = inferotemporal; bmi = body mass index; sbp = systolic blood pressure; spherical equiv = spherical equivalent

because the subsets were chosen randomly and had sufficient statistical power. Fourth, we did not have the opportunity to study central corneal thickness (CCT), a potential confounder of IOP measurements as well as an important risk factor for POAG.[36, 37] CCT has previously been reported to account for between 1% and 6% of the total variance in IOP measured with Goldmann applanation tonometry.[38-41] The heritability of CCT has been estimated to be 0.95.[42] Because we did not include CCT into the variance component analysis of IOP, CCT-determining genes may have been incorrectly considered as IOP-determining genes, thus inflating our heritability estimate of IOP.

RNFL thickness and optic disc morphology were not associated with IOP in these relatively young individuals not suspected of having glaucoma. An elevated IOP has been recognized as an important risk factor for glaucoma.[37, 43] IOP levels have been shown to correlate with optic disc characteristics in eyes of subjects with ocular hypertension.[44] This might suggest that IOP affects the RNFL and optic disc pathophysiologically rather than physiologically. The results of Chang et al. support this hypothesis.[25] Other studies, however, reported a significant effect of IOP on optic disc morphology in normal subjects.[45, 46]

How do our findings relate to other studies into POAG heritability? Our estimate of 0.35 for IOP is remarkably similar to the 2 most recent studies of the heritability of IOP. Klein et al. estimated a heritability of 0.35 from the parent-child correlation in the Beaver Dam Eye Study.[24] Chang et al. reported a heritability estimate of 0.36 in a population-based cohort of Caucasian sibships 65 to 84 years of age.[25] The heritability estimate of RNFL thickness in our study (0.48) was much lower than the estimate reported by Hougaard et al. (0.78-0.82), who studied monozygotic and dizygotic twins.[47] Heritability may be population specific. Even populations with similar genetic backgrounds may show different heritability estimates due to different environmental variances and different study designs. Heritability studies based on twins assume that the environmental correlations among monozygotic and dizygotic twins are equal. However, if this "equal environment assumption" does not hold, the heritability estimated from twin data alone may be higher than heritability derived from extended families, as in our study.[48] Different measurement methods (OCT in Hougaard et al. versus GDx VCC in our study) and different adjustments for covariates may also contribute to the heritability differences. There are no former studies that assessed the heritability of optic disc rim area, but 3 studies estimated the heritability of cup-to-disc ratio as a proxy. Our estimate for this parameter of 0.64 was in their reported range of 0.48-0.80.[24-26]

We were able to study optic disc morphology objectively with the HRT II, a technique that had not been used before in heritability studies. We found heritabilities of 0.52 for disc area, 0.39 for total rim area, and 0.47 for inferotemporal rim area. We found a markedly higher estimate (0.79) for the rim-to-disc ratio, indicating a larger genetic contribution for the combination than for any of the parameters separately. Speculating

that different sets of genes may determine rim and disc area, we performed a bivariate analysis of these parameters and found no evidence of any genetic correlation.

As a general rule, inbreeding increases the probability that the gene profile comprises two identical alleles. In our study, inbreeding was significantly associated with a higher IOP, suggesting the presence of causative recessive alleles in the genetic background of this trait. Evidence from previous studies supports this notion. Recessive mutations in the *CYP1B1* gene not only link to congenital glaucoma and anterior segment dysgenesis, but also play a role in high pressure POAG with juvenile or adult onset.[8,9] Inbreeding was not associated with RNFL thickness or neuroretinal rim area. Therefore, our study does not support a recessive inheritance of these POAG markers.

In conclusion, we have demonstrated that IOP, RNFL thickness, and neuroretinal rim area are continuous POAG markers that are strongly determined by genetic effects. Genome-wide association methods have been successfully applied to map genes for other complex disorders. A quantitative trait analysis greatly enhances the statistical power of this technology. The high heritabilities that we found in the current study encourage us to use this approach for identifying new POAG genes.

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Chapter 2.2

Major genetic effects in glaucoma: commingling analysis of optic disc parameters in an older Australian population

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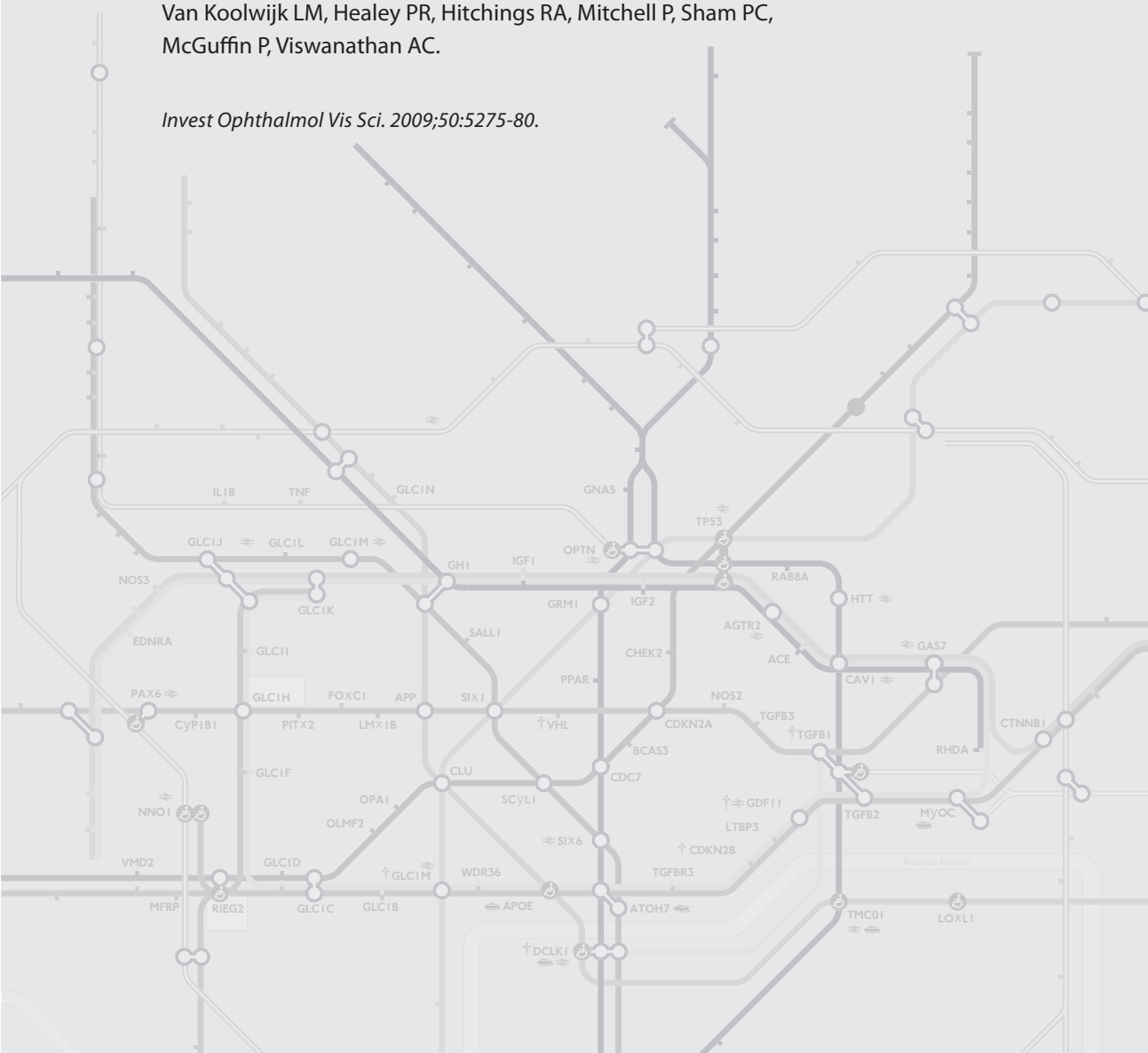

ABSTRACT

Purpose. To test the hypothesis that there is a major genetic determinant of vertical disc diameter (VDD) and vertical cup-to-disc ratio (VCDR) in a large, population-based sample.

Methods. Data were collected from 3654 individuals, 49 years of age or older, participating in the Blue Mountains Eye Study. VDD and VCDR were determined from stereo optic disc photographs. Commingling analyses in SKUDRIVER/SKUMIX were performed in non-glaucomatous eyes to investigate whether the observed VDD and VCDR data were best described by a 1-, 2-, or 3-distribution model.

Results. VDD data did not show evidence of commingling. After adjustment for the effects of age, VDD and intraocular pressure, the best model for VCDR consisted of a mixture of three distributions in Hardy-Weinberg equilibrium. The proportion of the variance in VCDR explained by this mixing component was 0.58.

Conclusions. Findings from this study are consistent with the presence of a major gene that accounts for 58% of the variance in VCDR. These results strongly support further efforts to identify the genetic variants responsible for this quantitative trait, which is a key constituent of the phenotype of primary open-angle glaucoma.

INTRODUCTION

Primary open-angle glaucoma (POAG) is a progressive optic neuropathy with an established genetic component to its origin.[1-3] In the majority of cases, POAG is inherited as a complex disease: it is assumed to result from many interactive genetic and environmental factors, none of which individually is necessary or sufficient to cause the disease. Although high-throughput genotyping technologies are becoming increasingly feasible and affordable, and underlying methodologies to unravel complex diseases are developing rapidly, the multifactorial etiology of POAG is still proving a hard nut to crack. More than 25 chromosomal regions have been linked to the disease, but only 3 genes (*MYOC*[4], *OPTN*[5], *WDR36*[6]) have been identified. These genes most likely contribute to the pathogenesis of POAG in less than 5% of cases in the general population.[7-10] Genes accounting for a greater proportion of the known heritable component of POAG thus remain to be identified.

The etiologic complexity of POAG can be reduced by separately studying quantitative features of the phenotype, such as the vertical optic disc diameter (VDD) or the vertical cup-to-disc ratio (VCDR). Elucidating the genetic determinants of these quantitative features in healthy eyes may improve our understanding of any damage to the optic disc in glaucomatous eyes. Quantitative traits are likely to be more powerful in detecting new genes than the dichotomous POAG trait. They also may have simpler genetic backgrounds, can be studied in entire populations, and are less prone to misclassification.

Obviously, a prerequisite of considering quantitative POAG traits for gene-finding studies is that these traits have a genetic basis. Previous estimates of the heritability of VDD or disc area ranged from 0.52 to 0.73.[11-13] Heritability estimates of VCDR ranged from 0.48 to 0.65.[12-14] These estimates were based on family studies and provided information on the additive effects of all involved genes. Although a high heritability itself may already be promising for gene-finding studies, it would be interesting to know whether the heritable component solely involves genes of small effect or also includes one or more genes that have a relatively large effect. The latter would be easier to detect in gene-finding studies, and their presence would therefore even more resolutely support quantitative trait based strategies.

To date, no investigation into possible major genetic effects on VDD and VCDR has been made. A suitable method of examining the population distribution of a quantitative trait for major genetic effects is the use of commingling analysis.[15] This is a form of model fitting that employs the method of maximum likelihood.[16] Commingling analysis investigates the strength of evidence for a single gene of major effect and provides an estimate of the locus-specific heritability, which is the proportion of the total phenotypic variance explained by the effect of the major gene.

To explore the feasibility of applying VDD and VCDR to population-based gene-finding strategies, we performed a commingling study on optic disc data from the Australian Blue Mountains Eye Study cohort. We investigated the population distribution of VDD and VCDR for major genetic determinants and estimated their locus-specific heritability.

METHODS

Study population

The Blue Mountains Eye Study (BMES) is a population-based survey of vision and common eye diseases in the Blue Mountains region west of Sydney, Australia. The study adhered to the tenets of the Declaration of Helsinki and was approved by the Western Sydney Area Health Service Human Ethics Committee. Written, informed consent was obtained from all participants. The population has been described in detail elsewhere.[17] In brief, all permanent non-institutionalized residents 49 years of age or older were invited to participate. Of the 4433 eligible individuals, 3654 (82.4%) attended baseline eye examinations between 1992 and 1994. Of the 779 nonparticipants, 501 (11.3%) refused, 68 (1.5%) had died, and 210 (4.8%) had moved away from the area. The response rate compares well with the best population-based research in glaucoma.[18-20]

Clinical examination and optic disc grading

All subjects underwent comprehensive eye examinations, including assessment of subjective refraction with a logMAR chart,[21] and measurement of intraocular pressure (IOP) with Goldmann applanation tonometry. Visual fields were initially assessed with a 30° suprathreshold screening test (Humphrey 76-point test). Full-threshold Humphrey 30-2 visual field tests of each eye were subsequently performed in subjects with suspected glaucoma.

After pupil dilation, 30° colour stereoscopic optic disc photographs were taken with a Zeiss FF3 fundus camera (Carl Zeiss Meditec, Dublin, CA). Slide transparencies (35 mm) were mounted in clear plastic sheets. Optic disc parameters were assessed by means of a Donaldson stereo viewer with a template of small circles (Pickett circles number 1203) placed under one of the stereo pair, as described and validated previously.[22] The vertical disc diameter (VDD) was measured to the nearest 0.01 mm as the longest diameter between the inner limits of the scleral ring in a range between clock hours 11 to 1 and 5 to 7. The optic cup was determined by its contour, with the outer margin taken to be the point where the wall met the plane of the disc surface at the level of the scleral ring. The vertical cup-to-disc ratio (VCDR) was calculated from the disc and cup measurements. Optic disc measurements were corrected for the magnification effect of the eye-camera system according to spherical equivalent refraction, as described by

Bengtsson and Krakau.[23] All photographs were graded by one or both of two trained graders. The chief investigator (PM) adjudicated discrepancies. Inter-observer variability was assessed in a masked fashion in a random sample of 100 optic discs and was in the excellent agreement range.[24]

Selection criteria

Because the magnification correction for optic disc measurements used in this study is inaccurate after cataract surgery, subjects who were aphakic or pseudophakic in both eyes were excluded from analyses (n = 108). If only one eye of a subject was phakic, this eye rather than its non-phakic fellow was considered for analysis. If both eyes were phakic, one eye was chosen at random for inclusion in the analysis. Eyes with tilted optic discs (n = 78) or with other disc anomalies, such as colobomata (n = 1), disc drusen (n = 1), or optic atrophy (n = 1), were excluded from the dataset. A further 16 eyes were excluded because of high myopia (spherical equivalent greater than -8 D).[25] The main analyses in this study were performed on a "normal" population, which excluded patients with glaucoma in either eye (n = 90). The diagnosis of glaucoma was made on the basis of typical glaucomatous visual fields loss on the Humphrey 30-2 test, combined with matching optic disc rim thinning, as described previously.[24] For the analyses of VDD, 86 eyes were excluded because no gradable optic disc photographs were available, and for the analyses of VCDR, an additional 5 eyes were excluded because covariate data were incomplete. This left valid data from 3273 and 3268 subjects for the analyses of VDD and VCDR, respectively.

Statistical analysis

Before the commingling study, univariate and multivariate linear regression analyses were performed (SPSS version 11.5 for Windows; SPSS, Chicago, IL) to detect whether any adjustments were needed for the effects of explanatory covariates. Putative covariates of VDD and VCDR that were studied included age, sex, height, history of migraine, intraocular pressure (IOP), and (for VCDR analysis only) VDD. Adjusted VDD and VCDR data were standardized to have a mean of 0 and a variance of 1.

Commingling analysis investigates whether the observed distribution of a quantitative trait is best modeled by a single distribution or by an admixture of multiple distributions. The latter could indicate that a gene of major effect underlies the trait. If this major gene has an allele frequency of q; genotypic means of m_1, m_2 and m_3; and within-genotype variance s^2, a likelihood function L for an individual observation is defined under Hardy-Weinberg equilibrium as:[26]

$$L(q, m_1, m_2, m_3, s^2; x) = q^2 f(x; m_1, s^2) + 2q(1-q) f(x; m_2, s^2) + (1-q)^2 f(x; m_3, s^2)$$

where *x* is the observed trait value of a randomly ascertained member of the population and $f(x; m, s^2)$ is a normal density function with mean *m* and variance s^2. The overall likelihood of this mixture model is computed as the product of the likelihoods of the individual observations. The maximum likelihood of this model may be compared with that of the null model, which consists of a single normal distribution, for a test of the major gene effect.

The software used to implement the commingling analysis was the C++ program SKUDRIVER, written by one of the authors (ACV), and the program SKUMIX.[15] Both programs are available at http://statgen.iop.kcl.ac.uk/skudriver/. The commingling analysis was performed on the adjusted and standardized VDD and VCDR data, and comprised maximum likelihood estimation for each of three models: single distribution (the null model), 2-distribution (i.e., fully dominant or recessive) and 3-distribution model.

SKUDRIVER takes as input a user-specified range of starting values for each of the following variables (see also Figure 1): within-genotype variance (V), homozygote mean (U), dominance (D), displacement (T), allele frequency (Q), power transform variables (P and R), and inbreeding coefficient (F). Displacement (T) is defined as the difference between the mean values of the two homozygote distributions. Dominance (D) represents the mean value of the heterozygote distribution relative to the two homozygotes.

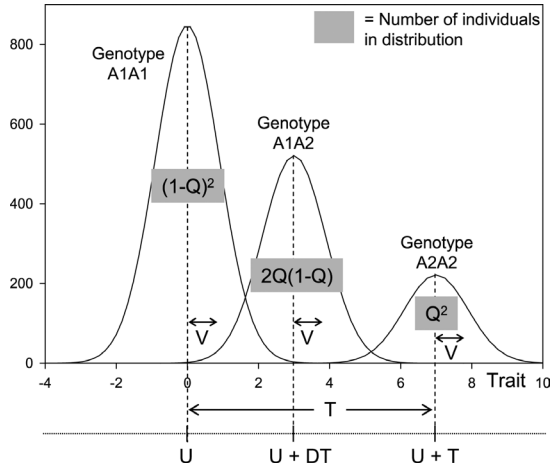

Figure 1. Principles of commingling analysis
Schematic presentation of the input variables of a commingling analysis.
V = within-genotype variance, which is the same for all three distributions
U = mean trait value of people carrying the A1A1 genotype
T = displacement, which is difference between the mean trait values of people carrying the A1A1 genotype and people carrying the A2A2 genotype
D = dominance, which represents the mean trait value of people carrying the A1A2 genotype relative to the two homozygous (A1A1 and A2A2) distributions;
Q = Frequency of the A2 allele

Thus, the three genotypic means are at U, U + DT and U + T. Since the input parameters in SKUDRIVER can be specified as either "fixed" or "estimated", the user may constrain the model to a single distribution by fixing the value of T as 0, or may specify a two-distribution model by fixing the value of D as 0 or 1. Q is assigned to be the frequency of the allele associated with the displaced distribution so that in the three-distribution model, under Hardy-Weinberg equilibrium, the proportions of the population within each of the distributions are $(1-Q)^2$, $2Q(1-Q)$, and Q^2. However, the program also allows deviation from Hardy-Weinberg equilibrium by introducing an inbreeding coefficient F, so that the proportions within the distributions become $(1-Q)^2 + FQ(1-Q)$, $2Q(1-Q)(1-F)$, and $Q^2 + FQ(1-Q)$.[27]

One of the important features of the software is the facility to specify starting values P and R of a power transformation to reduce skewness of the form $y = R / P [(x/R + 1)^P - 1]$, where R is chosen such that every $x/R + 1$ is positive in the sample and P is optimized as part of the maximum likelihood estimation. This method allows the fit of multiple distributions to be assessed after skewness has been removed, which is important, since skewness in itself may lead to the mistaken conclusion that more than one distribution is present.[15] Significant skewness may be tested for by a likelihood ratio test comparing a model in which P is fixed to a value of 1 (untransformed model) with a corresponding model in which P is not constrained (transformed model).

Each of the possible starting values for the parameters in SKUDRIVER was used to perform a maximum likelihood estimation with the program SKUMIX. In this way a grid search of the likelihood surface is conducted, minimizing problems of singularities or local maxima.[28] The SKUMIX program provided a measure of the goodness of fit for the 1-, 2- and 3-distribution models, both with and without a power transformation, expressed as minus twice the logarithm of the likelihood (-2 log *L*). Hypothesis testing was achieved by referring the difference in this quantity between two models to a chi-squared distribution with degrees of freedom equal to the difference in the number of free parameters. Since multiple comparisons were made, each p-value was corrected with a Bonferroni correction to avoid spuriously significant results.[29] The best fitting model was chosen according to the Akaike Information Criterion (AIC), defined as -2 log *L* + twice the number of free parameters.[30] The AIC penalizes for adding free parameters and thus selects the most parsimonious model that fits the data well. The Akaike weight (*w*) was used to assess model selection uncertainty.[31] It represents the probability that the model is the best among the whole set of models.

RESULTS

Demographic and ophthalmic characteristics of the study population are presented in Table 1. Ages ranged from 49 to 96 years, with a mean of 65.5. The population was mainly Caucasian with a minority (0.7%) of Aboriginal, Negroid, Oceanian, Asian, and Indian ethnicity. The mean VDD was 1.51 mm and the mean VCDR was 0.43. The population distributions of VDD and VCDR are shown in Figure 2. No statistically significant association was found between VDD and any of the studied covariates. Therefore, no additional correction other than standardization was made to the VDD data prior to commingling analysis. Skewness and kurtosis of the standardized VDD distribution were 0.191 and 0.152, respectively. VCDR was statistically significantly associated with age (multivariate

Table 1. Characteristics of the study population

Characteristic	
Total number	3273
Age (y), mean ± SD	65.5 ± 9.4
Age 49 – 59 y, N (%)	964 (29.5)
Age 60 – 69 y, N (%)	227 (37.5)
Age 70 – 79 y, N (%)	832 (25.4)
Age 80+ y, N (%)	250 (7.6)
Male gender, N (%)	1438 (43.9)
White race, N (%)	3248 (99.3)
Vertical Disc Diameter (mm), mean ± SD	1.51 ± 0.17
Vertical Cup-to-Disc Ratio, mean ± SD	0.43 ± 0.14
Intraocular pressure (mm Hg), mean ± SD	16.0 ± 2.7

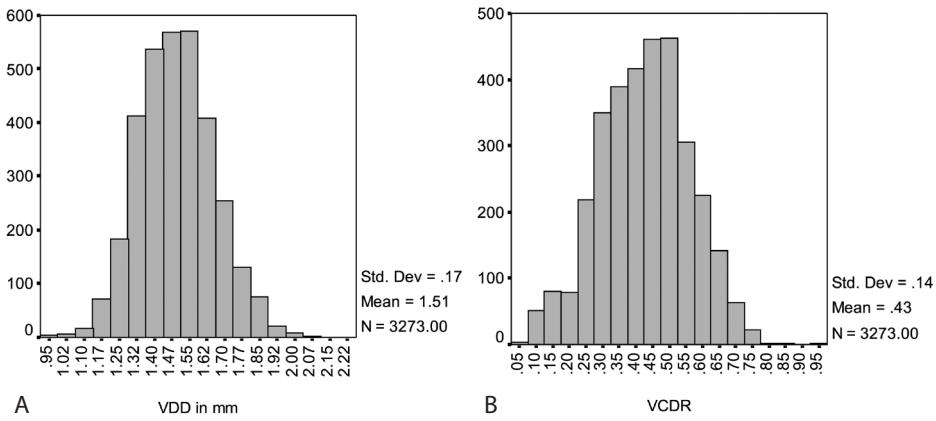

Figure 2. (A) Distribution of vertical disc diameter of random eye **(B)** Distribution of vertical cup-to-disc ratio of random eye

regression coefficient [B] = 0.001, p < 0.001), VDD (B = 0.003, p < 0.001) and IOP (B = 0.004, p < 0.001). The standardized residuals of this multivariate regression model were used for further analysis. The distribution of the standardized and adjusted VCDR data had a skewness of -0.068 and a kurtosis of -0.097.

The results of the commingling analysis of VDD are presented in Table 2. Under the hypothesis of one distribution, significant skewness was removed by the power transformation (χ^2_1 = 9.16, p = 0.027 after Bonferroni correction). This is reflected by the findings that the skewness of the untransformed data was 0.191, whereas after the power transformation (with R fixed as 11.0 and P optimized by SKUMIX/SKUDRIVER as 0.35) the skewness was 0.002. The power transform did not significantly improve the fit of the data when two or three distributions were specified. For the untransformed data, the 2-distribution model fitted the data significantly better than the 1-distribution model (χ^2_2 = 10.92, p = 0.047 after Bonferroni correction), but the 3-distribution model did not fit better than the 2-distribution model. Considering the transformed models only, neither dataset provided evidence of commingling. Allowing the inbreeding coefficient (F) to vary (i.e., allowing departure from Hardy-Weinberg equilibrium) did not significantly improve the fit of any of the commingled models. According to the AIC, the most conservative and parsimonious model that fitted the data well was the 1-distribution transformed model. The Akaike weights show that this model was only 1.12 (0.29 / 0.26) times more likely than the 2-distribution untransformed model to be the best, indicating a considerable degree of model-selection uncertainty.

Table 2. Commingling analysis of vertical disc diameter

MODEL	-2 x log likelihood + constant	X^2 (df) compared with untransformed[a]	X^2 (df) compared with 1 distribution[b]	X^2 (df) compared with 2 distributions[c]	Akaike Information Criterion[d]	Akaike weight[e]
1 distribution, untrans	4643.69				4647.69	0.01
1 distribution, trans	4634.52	9.16 (1) *			4640.52	0.29
2 distribution, untrans	4632.77		10.92 (2) *		4640.77	0.26
2 distribution, trans	4631.11	1.66 (1)	3.42 (2)		4641.11	0.22
3 distribution, untrans	4631.87		11.82 (3)	0.90 (1)	4641.87	0.15
3 distribution, trans	4631.08	0.79 (1)	3.44 (3)	0.02 (1)	4643.08	0.08

[a] Difference between *-2 log likelihood* of the given model and the untransformed model for the same number of distributions

[b] Difference between *-2 log likelihood* of the given model and the corresponding (i.e. transformed or untransformed) model for 1 distribution

[c] Difference between *-2 log likelihood* of the given model and the corresponding (i.e. transformed or untransformed) model for 2 distributions

[d] *-2 log likelihood* + twice the number of free parameters

[e] Represents the relative likelihood of the model

*p < 0.05 after Bonferroni correction for multiple comparisons

untrans = untransformed; trans = transformed

The results of the commingling analysis of VCDR, after adjustment for the effects of age, VDD, and IOP, are presented in Table 3. When the models were compared by means of maximum likelihood ratio tests and the p-values corrected for multiple comparisons by using the Bonferroni method, the data provided no significant evidence of skewness or commingling. However, when considering the AIC, the best fitting model was the 3-distribution transformed model. This model had a 0.25 probability of fitting best, but was closely followed by the 3-distribution untransformed model with a 0.22 probability, resulting in an evidence ratio of 1.14 for the relative likelihood of the transformed versus the untransformed 3-distribution model. Neither model was improved by allowing the inbreeding coefficient to vary. The parameters of the 3-distribution transformed model were: residual variance 0.44, homozygote mean -0.07, dominance 0.46, displacement -2.73, allele frequency 0.23, power transform variable P -0.57, power transform variable R (fixed) 11.0, inbreeding coefficient (fixed) 0. These parameters gave rise to the distributions shown in Figure 3. When the back-transformed, unstandardized regression residuals were considered, the middle distribution (which represents individuals carrying 1 copy of the rare allele) had a mean of -0.15, and the leftmost distribution (which represents individuals carrying 2 copies of the rare allele) had a mean of -0.29. As the total variance was 1.05, the residual variance of 0.44 implied that the variance due to the commingling was 0.61 and the locus-specific heritability was 0.58.

Table 3. Commingling analysis of vertical cup-to-disc ratio

MODEL	-2 x log likelihood + constant	X^2 (df) compared with untransformed[a]	X^2 (df) compared with 1 distribution[b]	X^2 (df) compared with 2 distributions[c]	Akaike Information Criterion[d]	Akaike weight[e]
1 distribution, untrans	4635.09				4639.09	0.10
1 distribution, trans	4633.91	1.18 (1)			4639.91	0.07
2 distribution, untrans	4630.03		5.06 (2)		4638.03	0.17
2 distribution, trans	4627.84	2.19 (1)	6.07 (2)		4637.84	0.19
3 distribution, untrans	4627.55		7.54 (3)	2.48 (1)	4637.55	0.22
3 distribution, trans	4625.33	2.23 (1)	8.58 (3)	2.51 (1)	4637.33	0.25

Commingling analysis was performed after adjustment for the effects of age, vertical disc diameter, and intraocular pressure

[a] Difference between *-2 log likelihood* of the given model and the untransformed model for the same number of distributions

[b] Difference between *-2 log likelihood* of the given model and the corresponding (i.e. transformed or untransformed) model for 1 distribution

[c] Difference between *-2 log likelihood* of the given model and the corresponding (i.e. transformed or untransformed) model for 2 distributions

[d] *-2 log likelihood* + twice the number of free parameters

[e] Represents the relative likelihood of the model

untrans = untransformed; trans = transformed

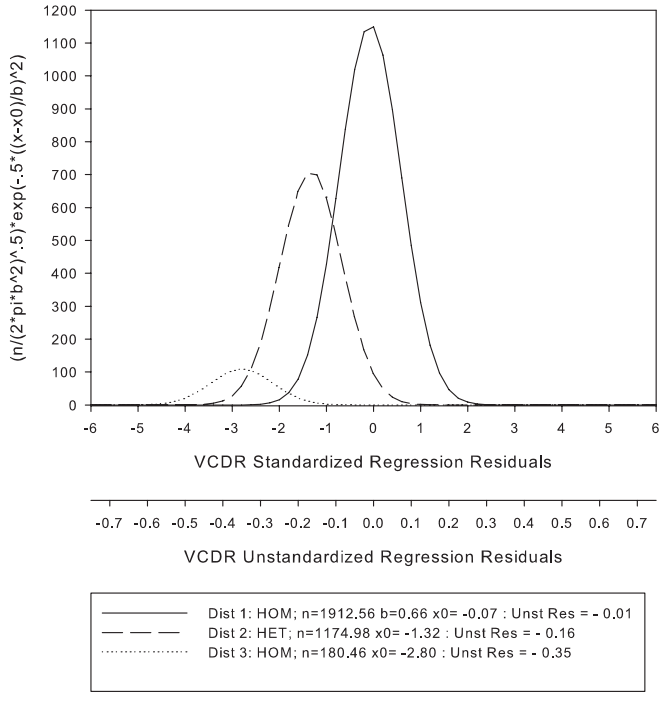

Figure 3. Vertical cup-to-disc ratio: transformed three distributions model
This figure shows the model which, after commingling analysis, fitted the population VCDR data best. It consists of three normal distributions, each containing *n* subjects and having mean *x0*, with common standard deviation *b*. The unstandardized values corresponding to *x0* have been given in the lower X-axis.

DISCUSSION

We sought to test the hypothesis of a major genetic determinant of VDD and VCDR by analyzing the distribution of these traits in a large Australian population. Commingling analysis of VDD did not provide statistically significant evidence of a major gene effect. The most satisfactory model for VCDR, after adjustment for the effects of age, VDD and IOP, consisted of a mixture of three distributions and would be compatible with the presence of a major gene with minor allele frequency 0.23 accounting for 58% of the variance in VCDR. The rare allele of this gene would reduce VCDR by 0.15 in heterozygotes (36% of the population) and by 0.29 in homozygotes (6% of the population).

The design of our study had two important limitations. First, commingling analysis can provide evidence of a mixture of distributions but cannot reveal the origin of the mixing component. Evidence of commingling therefore does not necessarily imply evidence of a major genetic effect, as environmental sources of commingling cannot be ruled out. However, as a genetic origin of VCDR is biologically plausible and compatible with previous literature,[12-14] Hardy-Weinberg proportions are respected, and environmental

factors of major effect are unknown, it is likely that a gene of major effect explains this commingling. Second, the exclusion of patients with glaucoma is a possible source of selection bias. The exclusion was necessary because we do not know whether the same processes are responsible for VCDR in healthy and glaucomatous eyes. When patients with glaucoma were included, commingling analysis of VCDR resulted in a three-distribution model with dominance –0.59, displacement 2.05, and allele frequency 0.17. The rightmost distribution in this model (n=93; SD=0.75; mean=2.05, corresponding to 0.27 unstandardized residuals) was very similar to the distribution of the included glaucoma population (n=83; SD=1.05; mean=2.04, corresponding to 0.27 unstandardized residuals). This result may indicate that patients with glaucoma form a separate distribution and that the SKUMIX program is able to correctly disentangle this admixture. However, a major genetic origin of the commingling in this heterogeneous population could be disputed. The results for VDD did not change after the glaucoma cases were included.

The lack of distributional effects in VDD in our study appears to disagree with previous work, in which heritability estimates of VDD or disc area ranged from 0.52 to 0.73.[11-13] Our result may be explained by the conservative design of the SKUMIX program, which implements the commingling analysis. This design, which 'had to guard against claiming separate distributions where none exist'[15] has been tested experimentally.[32] When both commingling and segregation analyses were applied to simulated pedigree data in which a major locus was segregating, more than 20% of the samples provided evidence of segregation of a single locus but not of commingling. Our results for VDD therefore do not preclude a genetic determinant of major effect. This finding is also suggested by the Akaike weights, which show a considerable model selection uncertainty and provide some support for a 2-distribution model (fully dominant or recessive gene) as well. Another possible explanation of the discrepancy with previous heritability studies is that the latter assessed the additive effects of all involved genes. A collection of several loci with small effects rather than a single major gene determining VDD may lead to high heritability estimates without evidence of commingling.[27;33]

Additive genetic effects have been reported to account for 48% to 65% of the total variance in VCDR.[12-14] Our estimate of 58% for its locus-specific heritability suggested that an important part of this additive genetic variance might be attributable to the effect of a single locus. Moreover, our study provided a model elucidating the allele frequencies, dominance and displacement associated with this locus.

The parameters of the best fitting model indicated that the rare allele of the major locus would cause a significant and clinically detectable reduction in VCDR in a substantial proportion of the population. This finding warrants some speculation on the clinical relevance of this potential locus. VCDR has been reported to predict the development of POAG in individuals with ocular hypertension and in healthy individuals.[34-37] However, one might question whether a large VCDR in these studies was an actual risk factor or

rather an early sign of POAG, and consequently, whether the smaller VCDR associated with the major locus in our study would actually reduce POAG risk. Identifying the gene and exploring its function could shed light on this issue.

Our results provide some guidance for the planning of future gene-finding studies in this population. The commingled model with the high locus-specific heritability of VCDR strongly supports a quantitative trait based approach. A gene that accounts for 58% of the trait variance would require sample sizes of approximately 450 and 150 sib pairs to have an 80% power to be detected by genome-wide linkage and association methods, respectively.[38;39] If a dichotomous trait based association analysis were to be considered, it would be desirable to compare individuals having at least one copy of the rare allele with individuals having no copies. The former group would consist of individuals with VCDRs smaller than the lower extreme of the rightmost distribution, say those with VCDR values less than three residual standard deviations from the mean of this distribution; that is, standardized VCDR regression residuals of less than –0.07 – 3*0.66 = -2.05. This translates to VCDRs that are at least 0.26 smaller than would be expected based on age, VDD and IOP. The second group would be those with VCDRs greater than the upper extreme of the middle distribution; that is, those with standardized VCDR regression residuals greater than –1.32 + 3*0.66 = 0.66, corresponding to VCDRs of at least 0.08 greater than predicted from the covariates.

In conclusion, commingling analysis in this large, Australian population provided evidence of a mixture of distributions in VCDR. The result was consistent with the presence of a major gene accounting for 58% of the total variance in VCDR. Although a high heritability of VCDR has been reported, our study is the first to suggest that a major gene may be responsible for this trait. This finding strongly supports further efforts to identify the genetic variants responsible for VCDR, which is an important feature of the POAG phenotype.

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Part 3

GENOME-WIDE ASSOCIATION STUDIES

Chapter 3.1

A genome-wide association study of optic disc parameters

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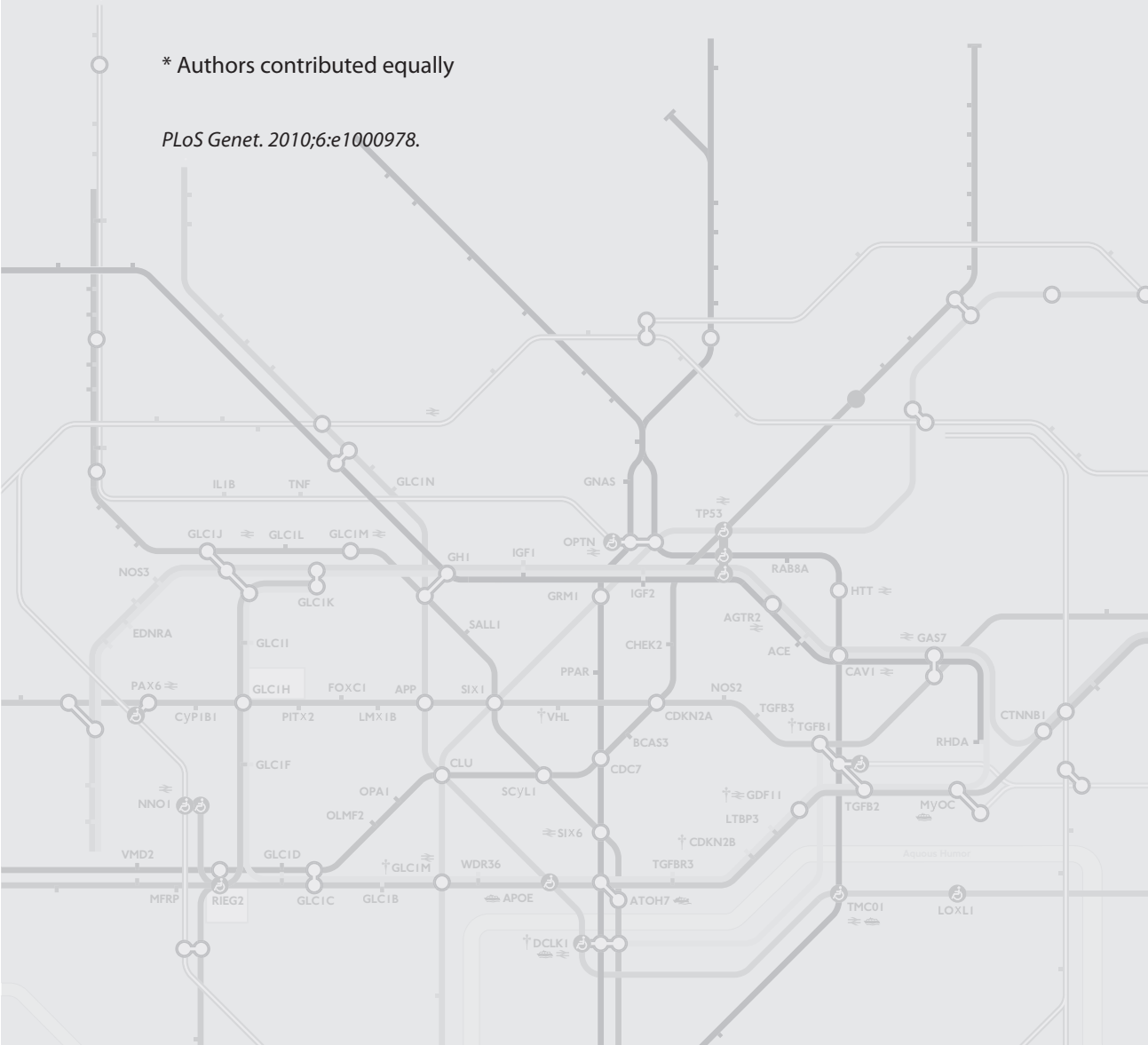

ABSTRACT

The optic nerve head is involved in many ophthalmic disorders, including common diseases such as myopia and open-angle glaucoma. Two of the most important parameters are the size of the optic disc area and the vertical cup-to-disc ratio (VCDR). Both are highly heritable but genetically largely undetermined. We performed a meta-analysis of genome-wide association (GWA) data to identify genetic variants associated with optic disc area and VCDR. The gene discovery included 7,360 unrelated individuals from the population-based Rotterdam Study I and Rotterdam Study II cohorts. These cohorts revealed two genome-wide significant loci for optic disc area, rs1192415 on chromosome 1p22 ($p=6.72x10^{-19}$) within 117kb of the *CDC7* gene and rs1900004 on chromosome 10q21.3-q22.1 ($p=2.67x10^{-33}$) within 10kb of the *ATOH7* gene. They revealed two genome-wide significant loci for VCDR, rs1063192 on chromosome 9p21 ($p=6.15x10^{-11}$) in the *CDKN2B* gene and rs10483727 on chromosome 14q22.3-q23 ($p=2.93x10^{-10}$) within 40 kbp of the *SIX1* gene. Findings were replicated in two independent Dutch cohorts (Rotterdam Study III and Erasmus Rucphen Family study; N=3,612), and the TwinsUK cohort (N=843). Meta-analysis with the replication cohorts confirmed the four loci and revealed a third locus at 16q12.1 associated with optic disc area, and four other loci at 11q13, 13q13, 17q23 (borderline significant), and 22q12.1 associated with VCDR. *ATOH7* was also associated with VCDR independent of optic disc area. Four of the loci were marginally associated with open-angle glaucoma. The protein pathways in which the loci of optic disc area are involved overlap with those identified for VCDR, suggesting a common genetic origin.

AUTHOR SUMMARY

Morphologic characteristics of the optic nerve head are involved in many ophthalmic diseases. Its size, called the optic disc area, is an important measure and has been associated with e.g. myopia and open-angle glaucoma (OAG). Another important and clinical parameter of the optic disc is the vertical cup-to-disc ratio (VCDR). Although studies have shown a high heritability of optic disc area and VCDR, the specific genetic factors involved are still undetermined. We therefore conducted a genome-wide association (GWA) study on these quantitative traits, using data of over 11,000 Caucasian participants, and we related the findings to myopia and OAG. We found evidence for association of three loci with optic disc area: *CDC7/TGFBR3* region, *ATOH7,* and *SALL1*; and seven loci with VCDR: *CDKN2B*, *SIX1*, *SCYL1*, *CHEK2*, *ATOH7, DCLK1*, and *BCAS3* (borderline significant). None of the loci could be related to myopia. There was marginal evidence for association of *ATOH7, CDKN2B, SIX1,* and *SCYL1* with OAG, which remains to be confirmed. The present study reveals new insights into the physiological development of the optic nerve and may shed light on the pathophysiological protein pathways leading to (neuro-) ophthalmic diseases such as OAG.

INTRODUCTION

The optic nerve head, or optic disc, is the place where the axons of the retinal ganglion cells leave the eye and form the optic nerve. Its morphology, visible by ophthalmoscopy, is important in the diagnosis and follow-up of patients with (neuro-) ophthalmic diseases, such as ischemic and hereditary optic neuropathies, optic neuritis, papilledema and primary open-angle glaucoma (OAG). Optic disc parameters of interest are the surface of the optic nerve head referred to as the optic disc area (measured in units of mm^2), and the vertical cup-to-disc ratio (VCDR). The optic disc area is associated with general characteristics (such as body height) as well as ocular ones (such as axial length).[1,2] The relation to axial length makes the optic disc size directly relevant for nearsightedness (myopia), one of the most common ophthalmic disorders. Furthermore, it has been suggested that larger optic discs may suffer more from intraocular pressure-related stress, a strong risk factor for OAG.[3] However, the association of the size of the optic disc to OAG is not clear since it has been argued that larger optic discs may have a larger anatomical reserve for various optic neuropathies such as OAG due to a higher number of nerve fibers[4]. Effects may even partially counteract each other.[4]

The VCDR is a parameter commonly used in the clinical glaucoma management.[5] The VCDR is determined by comparing (in a vertical direction) the size of the cup, a region without axons, to the size of the optic disc. An increase in VCDR may indicate the occur-

rence of glaucomatous changes of the optic nerve head, referred to as glaucomatous optic neuropathy.[6] In addition, an unusual large VCDR at a single observation is a significant determinant of glaucoma.[7,8] The heritability of the optic disc area and VCDR are estimated to be around 52-59% and 48-80%, respectively, suggesting a major role for genetic factors.[9-12] This prompted us to study the genes determining the optic disc area and VCDR as endophenotypes for myopia and OAG.

To identify genetic determinants of optic disc area and VCDR, we performed a genome-wide association study (GWAS) of optic disc area and VCDR using data from Caucasian participants of the Rotterdam Study [RS] (cohort I and II, in which participants have an identical age distribution and eye assessment) and replicated our findings in three independent cohorts of Caucasian ethnicity: the Rotterdam Study III [RS-III, a younger cohort], the Erasmus Rucphen Family [ERF] study and the TwinsUK cohort (see Materials and Methods for details of all cohorts). Next, we examined whether the genome-wide significant single nucleotide polymorphisms (SNPs) were related to myopia and OAG using data from patients with (one of) these diseases from the Rotterdam Study I.

RESULTS

Study samples

The discovery cohorts included 5,312 (RS-I) and 2,048 (RS-II) participants who were genotyped and had reliable optic disc data, resulting in a total of 7,360 participants included in the primary GWAS discovery set. A small fraction (205 from RS-I and 90 from RS-II) had missing or unreliable baseline data; for these we used the data available at follow-up. From RS-III, 1,966 participants were included, and from ERF 1,646, resulting in a total of 10,972 participants when the discovery and replication cohorts from the Netherlands were combined, and 11,815 when the 843 participants of TwinsUK were also included. Table 1 summarizes the general characteristics of the discovery and replication cohorts. There are significant differences between the cohorts in terms of age (discovery cohort is older), gender (TwinsUK includes mainly women) and optic disc parameters (due to different disc-assessment techniques [see Materials and Methods]; the analyses were adjusted for this difference).

Figure S1 and S2 show the Q-Q plots for the observed versus expected p-values for each individual study and for the meta-analysis of the discovery and replication cohorts for optic disc area and VCDR, respectively. Genomic control for all four cohorts showed low dispersion for optic disc area as well as for VCDR with inflation factors in the range of 1.024 - 1.061.

Table 1. Characteristics of the five study populations presented as mean ± standard deviation (range) unless stated otherwise

	RS-I/RS-II	RS-III	ERF	TwinsUK
Total sample size (N)	7,360	1,966	1,646	843
Age (years)	67.0 ± 8.4 (55 – 99)	55.6 ± 5.5 (45 – 89)	46.8 ± 14.1 (18 – 84)	56.1 ± 12.7 (16 – 83)
Gender, N(%) female	4,208 (57.2)	1,102 (56.1)	942 (57.2)	818 (97.0)
Disc area (mm^2)*	2.40 ± 0.48 (0.58 – 6.20)	1.92 ± 0.45 (0.70 – 7.20)	1.92 ± 0.37 (1.07 – 4.33)	2.59 ± 0.65 (0.75 – 6.96)
Vertical cup-to-disc ratio*	0.50 ± 0.14 (0.00 – 0.89)	0.42 ± 0.17 (0.00 – 1.00)	0.46 ± 0.15 (0.00 – 0.84)	0.32 ± 0.10 (0.07 – 0.70)

* = In RS-I, RS-II and TwinsUK measured with stereoscopic images, in RS-III and ERF with confocal scanning laser ophthalmoscopy

Optic disc area

Figure 1A presents the $^{-10}$log p-plot for the primary discovery cohort for optic disc area and shows two loci on chromosomes 1 and 10, including 192 SNPs that are beyond the genome-wide significance threshold of $5x10^{-8}$. Exclusion of OAG (N=188) and myopia

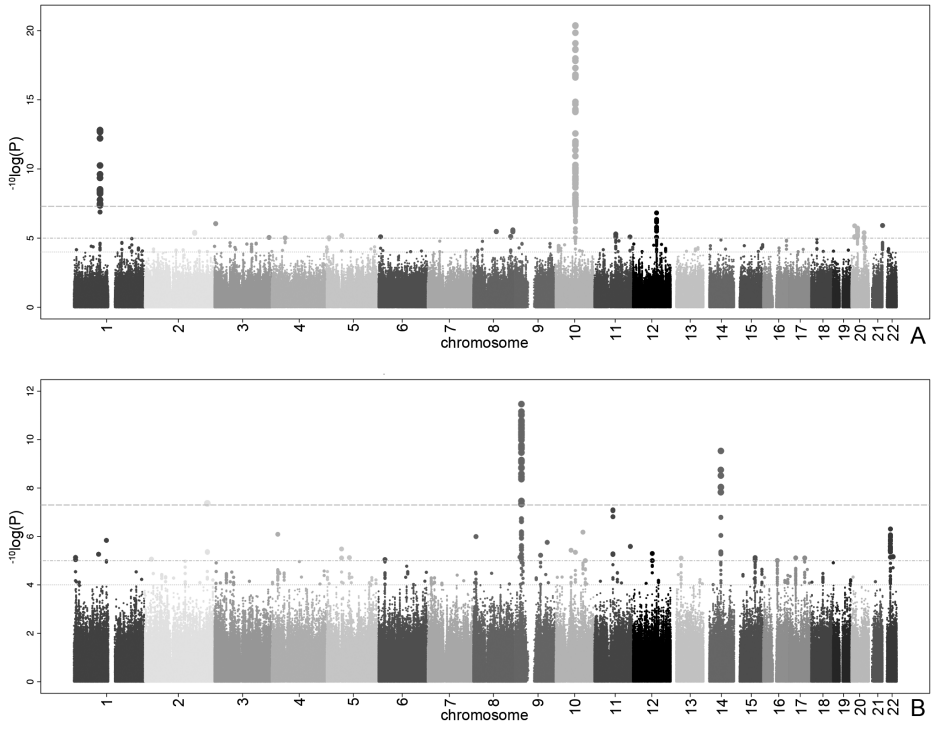

Figure 1. The -10log p-plots for the meta-analyzed RS-I/RS-II genome-wide association study
Plot (A) of disc area and plot (B) of vertical cup-to-disc ratio. The upper line represents the genome-wide significance threshold: $p=5x10^{-8}$. The middle and bottom lines represent the 10^{-5} and 10^{-4}, respectively.

Table 2. Top SNPs of all loci associated (p-value < 10^{-6}) with disc area in the meta-analysis: Results for each individual cohort and for the meta-analysis itself (presented as the effects per minor allele)

SNP	Chromosome location	Position	MA	RS-I/RS-II				RS-III			
				MAF	Beta	SE	P-value	MAF	Beta	SE	P-value
rs1900004	10q21.3-q22.1	69670887	T	0.22	-0.114	0.009	$2.67x10^{-33}$*	0.23	-0.082	0.017	$1.85x10^{-6}$
rs1192415	1p22	91849685	G	0.18	0.091	0.010	$6.72x10^{-19}$*	0.18	0.059	0.019	$1.69x10^{-3}$
rs1362756	16q12.1	50015791	C	0.29	0.036	0.009	$4.85x10^{-5}$	0.28	0.032	0.016	$4.92x10^{-2}$

* = significant at a p-value of $5x10^{-8}$; SNP = single nucleotide polymorphism; b = base pairs; MA(F) = minor allele (frequency); SE = standard error

Table 3. Results of replication in the TwinsUK cohort of the three revealed loci for disc area with their meta-analyzed results of all five cohorts

Most significant SNP	Minor allele	Minor allele frequency	Chromosome location	Position
Disc area				
rs1900004	T	0.24	10q21.3-q22.1	69670887
rs1192415	G	0.18	1p22	91849685
rs1362756	C	0.30	16q12.1	50015791

* = significant at a p-value of 0.05; ** = significant at a p-value of $5x10^{-8}$;
SNP = single nucleotide polymorphism; SE = standard error

(N=115) cases did not alter the results. Replication analyses in two independent cohorts of Dutch origin (RS-III and ERF study) showed that the findings from all cohorts were consistent in the direction of the effect with p-values ranging from $1.69x10^{-3}$ to $2.39x10^{-10}$ (Table 2). The combined analysis of the discovery and Dutch replication cohorts yielded an overall p-value $1.82x10^{-27}$ for rs1192415 (optic disc area increased by 0.064 ± 0.006 mm^2 [beta ± standard error] when persons heterozygous for the reference allele were compared with those homozygous), and p-value $2.05x10^{-32}$ for rs1900004 (optic disc area decreased by 0.068 ± 0.006 mm^2). Table 2 shows the results for the top SNPs of all loci with p-values <10^{-6} observed in the meta-analysis. The meta-analysis of the four Dutch cohorts revealed a cluster of 10 SNPs on chromosome 16q12.1 showing borderline genome-wide significant evidence for association with the optic disc area ($p=6.48x10^{-8}$). When the Dutch data were combined with the TwinsUK series (Table 3), this region became genome-wide significant ($p=5.07x10^{-9}$). Table 3 shows that the chromosome 1 and 10 regions were also replicated consistently in the TwinsUK cohort.

The regions of interest for optic disc area are shown in Figure 2. The first region on chromosome 1p22 is located between the cell division cycle 7 (*CDC7*) and the transforming growth factor beta receptor 3 (*TGFBR3*) genes, but the SNPs in the intergenic region were most significant. The genome-wide significant region on chromosome 10q21.3-

Table 2. (continued)

ERF				Meta-analysis				Name	Distance (b)	Number of SNPs on loci with p-value <10-6
MAF	Beta	SE	P-value	MAF	Beta	SE	P-value			
0.21	-0.033	0.008	$5.28x10^{-5}$	0.22	-0.068	0.006	$2.05x10^{-32}$*	ATOH7/PBLD	9021	175
0.25	0.049	0.008	$2.39x10^{-10}$*	0.22	0.064	0.006	$1.82x10^{-27}$*	CDC7/TGFBR3	116719	61
0.27	0.023	0.007	$1.56x10^{-3}$	0.28	0.028	0.005	$6.48x10^{-8}$	SALL1	1154095	10

Table 3. (continued)

delta disc area per allele (mm^2)		P-value	delta disc area per allele in meta-analysis of all five cohorts (mm^2)		P-value in meta-analysis of all five cohorts
Beta	SE		Beta	SE	
-0.133	0.038	$4.64x10^{-4}$*	-0.070	0.006	$2.71x10^{-35}$**
0.091	0.041	$2.60x10^{-2}$*	0.065	0.006	$2.77x10^{-28}$**
0.097	0.037	$8.29x10^{-3}$*	0.030	0.005	$5.07x10^{-9}$**

q22.1 was quite large and included several genes. The region includes the Myopalladin (*MYPN*) gene, the heterogeneous nuclear ribonucleoprotein H3 (2H9) (*HNRNPH3*) gene, RUN and FYVE domain containing (*RUFY2*) gene, DNA replication helicase 2 homolog (yeast) (*DNA2*) gene, and the solute carrier family 25 (mitochondrial carrier; Graves disease autoantigen), member 16 (*SLC25A16*) gene. However, the most significant evidence was found in the region between the atonal homolog 7 (*ATOH7*) gene and the phenazine biosynthesis-like protein domain containing *(PBLD)* gene. The nearest gene in the third region on chromosome 16q12.1 was the sal-like 1 (*SALL1*) gene. Together, the three SNPs associated with optic disc area explained up to 2.7% of the variation in optic disc area.

Next, we evaluated the association of these loci with clinically relevant ophthalmic outcomes (myopia and OAG; Table S1). None of the optic disc area loci were associated with myopia-related outcomes (p-values ranging from 0.09 to 0.80). Of the three loci associated with optic disc area we found only the 10q21.3-q22.1 locus to be marginally associated with OAG (p=0.04 for rs1900004).

Vertical cup-to-disc ratio

All analyses of VCDR were adjusted for optic disc area. Figure 1B presents the $^{-10}$log p-plot for the discovery cohorts (meta-analyzed RS-I/RS-II GWAS) for VCDR and shows two loci

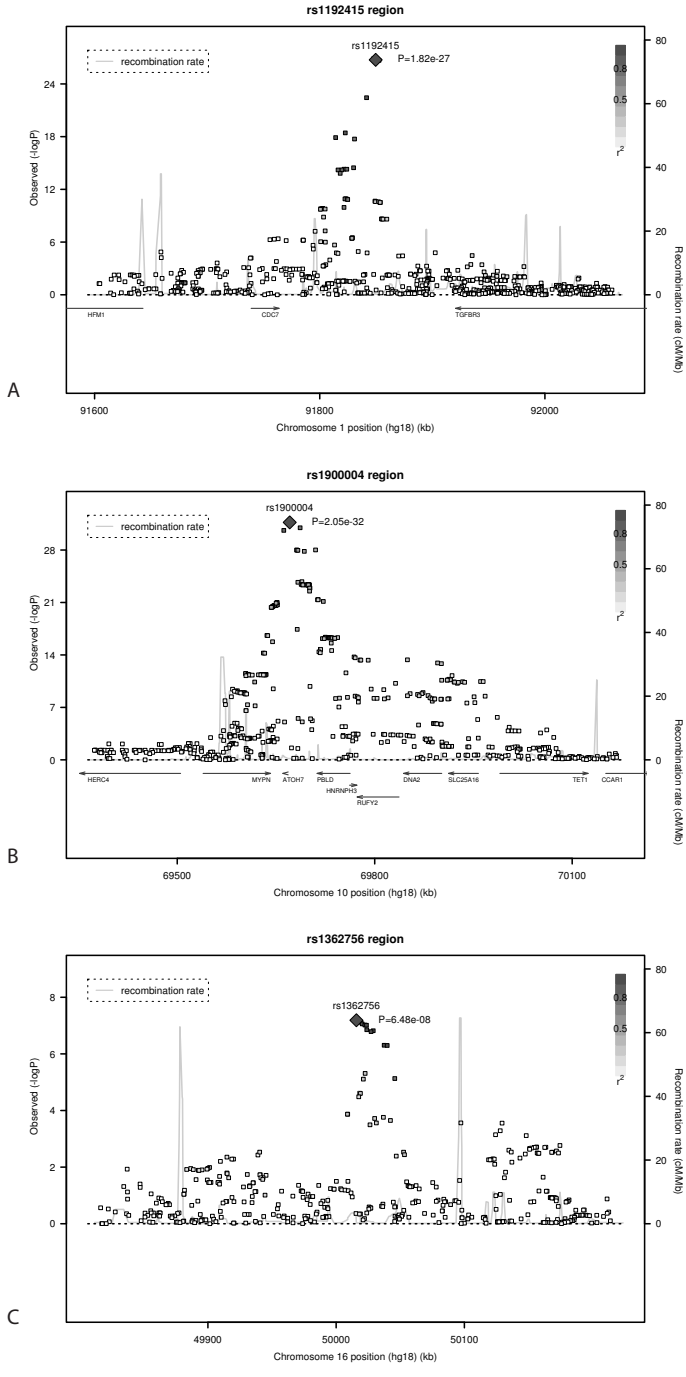

Figure 2. Regional plots of the three loci associated with optic disc area
Plots (A-C) show the loci on chromosomes 1, 10, and 16, respectively.

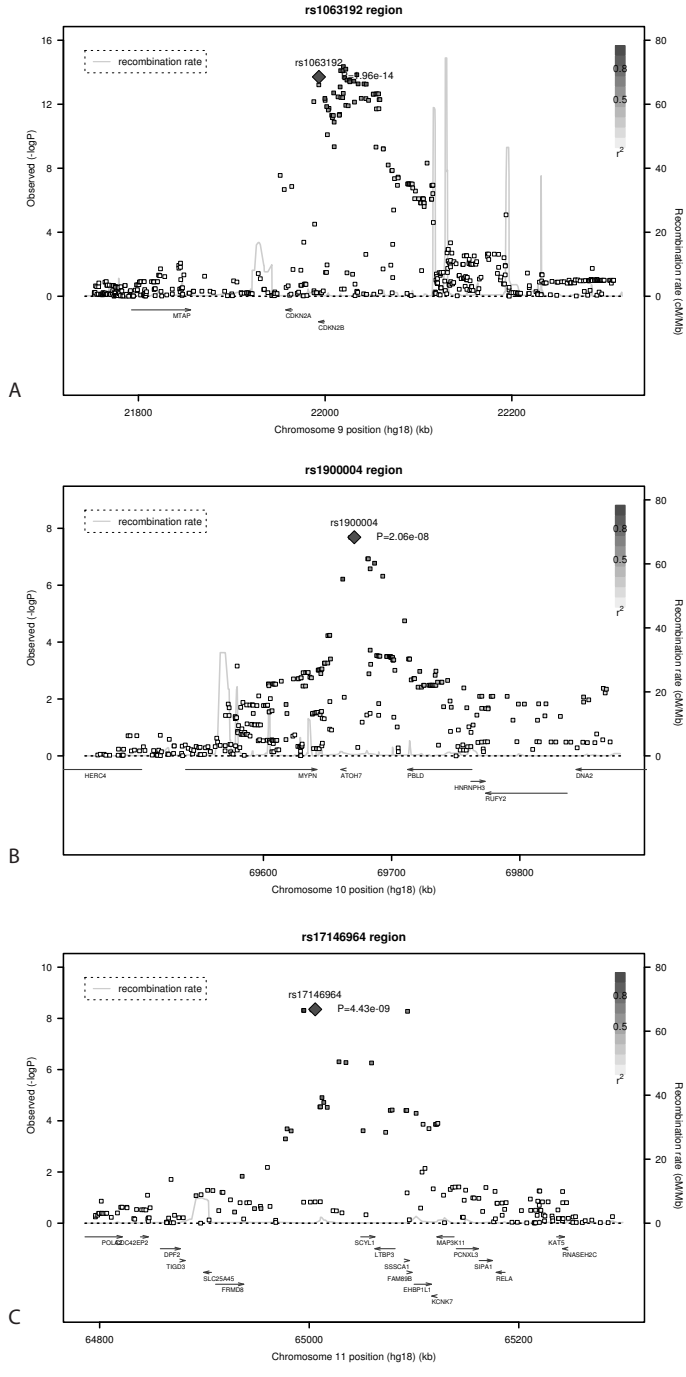

Figure 3. Regional plots of the six loci associated with vertical cup-to-disc ratio
Plots (A-F) show the loci on chromosomes 9, 10, 11, 13, 14, and 22, respectively.

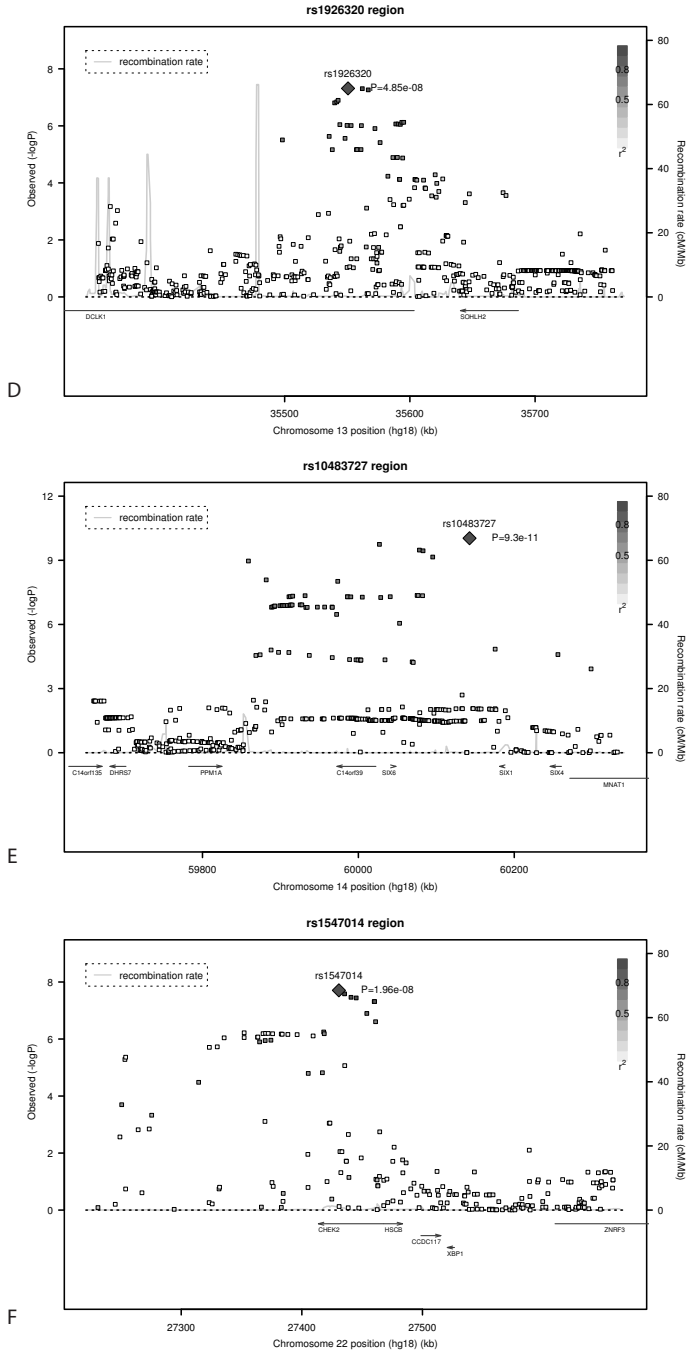

Figure 3. (continued)

reaching genome-wide significance at a threshold of $5x10^{-8}$. Adjustment for the intraocular pressure did not alter the results nor did exclusion of the OAG cases. The combined analysis of the discovery and two Dutch replication cohorts yielded an overall p-value of $1.96x10^{-14}$ for rs1063192 and $9.30x10^{-11}$ for rs10483727 (Table 4). The regions of interest for VCDR are shown in Figure 3. The genome-wide significant region on chromosome 9 included two genes from the same gene family (cyclin-dependent kinase inhibitor 2A [*CDKN2A*] and *CDKN2B*). For chromosome 14, several genes were included in the region of interest. The strongest association was found for rs10483727 close to the sin oculis homeobox homolog 1 (*SIX1*) gene, but also several SNPs flanking *SIX6* were genome-wide significant as well as one SNP between RNA-binding motif 8B (*RBM8B*) and the protein phosphatase 1A (*PPM1A*) gene. Furthermore, there were four other loci that showed consistent evidence of association and reached genome-wide significance in the combined analysis of all Dutch cohorts (Table 4). These included the chromosome 10q21.3-q22.1 region identified for the optic disc area (Table 2). For chromosome 11q13, the most significant SNPs were found in between the FERM domain containing 8 (*FRMD8*) and the SCY1-like (*SCYL1*) gene. The region of interest also harboured latent transforming growth factor beta binding protein 3 (*LTBP3*). The genome-wide significant SNPs within this locus were all in the same linkage disequilibrium block, hampering determination of the most important variant (Figure 3). Of the other two genome-wide significant loci, the SNPs point to the doublecortin–like kinase 1 (*DCLK1*) for chromosome 13q13, and CHK2 checkpoint homolog (*CHEK2*) for chromosome 22q12.1 (Figure 3).

Finally, when we combined all top SNPs from the joint analysis of the four Dutch cohorts with the TwinsUK, one additional borderline genome-wide significant region emerged as genome-wide significant. The region comprises 2 SNPs on chromosome 17q23 ($p=2.81x10^{-8}$; Table 5). The combined effect of the seven loci associated with VCDR explained 2.2% of the variation in the VCDR. Also for the VCDR none of the loci were associated with myopia at $p<0.05$. When we evaluated the association with OAG, four of the loci associated with VCDR were also found to be marginally associated with OAG: 9q21 (p=0.017), 14q22-23 (p=0.021), 11q13 (p=0.049), and the overlapping gene *ATOH7* discussed earlier.

DISCUSSION

In the present study we identified three genetic loci (10q21.3-q22.1, 1p22 and 16q12.1) associated with optic disc area, and seven genetic loci (9q21, 14q22-23, 10q21.3-q22.1, 11q13, 13q13, 17q23, and 22q12.1) associated with VCDR. Of these, one (10q21.3-q22.1) was associated with both quantitative traits. For these regions, the evidence for the association was genome-wide significant and our findings were consistently replicated in

Table 4. Top SNPs of all loci associated (p-value < 10^{-6}) with vertical cup-to-disc ratio in the meta-analysis: Results for each individual cohort and for the meta-analysis itself (presented as the effects per minor allele)

SNP	Chromosome location	Position	MA	RS-I/RS-II				RS-III			
				MAF	Beta	SE	P-value	MAF	Beta	SE	P-value
rs1063192	9q21	21993367	G	0.45	-0.014	0.002	$6.15x10^{-11}$*	0.46	-0.013	0.005	$1.38x10^{-2}$
rs10483727	14q22-23	60142628	T	0.40	0.014	0.002	$2.93x10^{-10}$*	0.39	0.001	0.005	$7.81x10^{-1}$
rs17146964	11q13	65005721	G	0.21	-0.014	0.003	$7.94x10^{-8}$	0.21	-0.013	0.007	$5.65x10^{-2}$
rs1547014	22q12.1	27430711	T	0.29	-0.011	0.002	$7.20x10^{-6}$	0.30	-0.019	0.006	$1.02x10^{-3}$
rs1900004	10q21.3-q22.1	69670887	T	0.22	-0.012	0.003	$4.49x10^{-6}$	0.23	-0.021	0.006	$8.90x10^{-4}$
rs1926320	13q13	35550617	C	0.24	0.011	0.003	$1.45x10^{-5}$	0.25	0.020	0.006	$1.29x10^{-3}$
rs8068952	17q23	56641426	G	0.24	-0.012	0.003	$7.85x10^{-6}$	0.24	-0.014	0.006	$2.54x10^{-2}$
rs12025126	1p36.2-p36.1	8682141	C	0.28	-0.009	0.003	$3.93x10^{-4}$	0.27	-0.011	0.006	$6.62x10^{-2}$
rs2159128	19p13.3	901380	G	0.13	-0.016	0.005	$3.16x10^{-4}$	0.14	-0.021	0.010	$3.67x10^{-2}$

* = significant at a p-value of $5x10^{-8}$; SNP = single nucleotide polymorphism; b = base pairs;
MA(F) = minor allele (frequency); SE = standard error

Table 5. Results of replication in the TwinsUK cohort of the revealed loci for vertical cup-to-disc ratio with their meta-analyzed results of all five cohorts

Most significant SNP	Minor allele	Minor allele frequency	Chromosome location	Position
VCDR				
rs1063192	G	0.44	9p21	21993367
rs10483727	T	0.44	14q22-23	60142628
rs17146964	G	0.20	11q13	65005721
rs1547014	T	0.26	22q12.1	27430711
rs1900004	T	0.24	10q21.3-q22.1	69670887
rs1926320	C	0.25	13q13	35550617
rs8068952	G	0.19	17q23	56641426
rs12025126	C	0.28	1p36.2-p36.1	8682141
rs2159128	G	0.08	19p13.3	901380

* = significant at a p-value of 0.05; ** = significant at a p-value of $5x10^{-8}$; SNP = single nucleotide polymorphism;
VCDR = vertical cup-to-disc ratio; SE = standard error

the independent replication cohorts. The SNPs in these loci were common variants with minor allele frequencies ranging from 0.21 to 0.46. The genome-wide significant SNPs of the present study were not in linkage disequilibrium with known missense mutations. The combined effect of the three SNPs involved in the optic disc area explained 2.7%, while the seven loci associated with VCDR explained 2.2% of the variation.

The region with the strongest statistical evidence for association was a locus on chromosome 10q21.3-q22.1, which was associated with both optic disc area and VCDR, and

Table 4. (continued)

ERF				Meta-analysis				Name	Distance (b)	Number of SNPs on loci with p-value <10-6
MAF	Beta	SE	P-value	MAF	Beta	SE	P-value			
0.47	-0.015	0.005	$2.54x10^{-3}$	0.46	-0.014	0.002	$1.96x10^{-14}$*	CDKN2B	0	88
0.45	0.014	0.005	$4.95x10^{-3}$	0.41	0.012	0.002	$9.30x10^{-11}$*	SIX1	39878	10
0.21	-0.010	0.006	$1.05x10^{-1}$	0.21	-0.014	0.002	$4.43x10^{-9}$*	SCYL1	43403	8
0.32	-0.010	0.005	$7.34x10^{-2}$	0.29	-0.011	0.002	$1.96x10^{-8}$*	CHEK2	0	29
0.21	-0.007	0.006	$2.98x10^{-1}$	0.22	-0.013	0.002	$2.06x10^{-8}$*	ATOH7/PBLD	9021	10
0.27	0.008	0.006	$1.41x10^{-1}$	0.24	0.012	0.002	$4.85x10^{-8}$*	DCLK1	0	15
0.20	-0.007	0.006	$2.47x10^{-1}$	0.23	-0.012	0.002	$3.11x10^{-7}$	BCAS3	0	2
0.32	-0.019	0.005	$3.82x10^{-4}$	0.29	-0.011	0.002	$4.14x10^{-7}$	RERE	0	5
0.11	-0.032	0.011	$2.45x10^{-3}$	0.13	-0.019	0.004	$7.05x10^{-7}$	ARID3A	0	1

Table 5. (continued)

delta disc area per allele (mm^2)		P-value	delta disc area per allele in meta-analysis of all five cohorts (mm^2)		P-value in meta-analysis of all five cohorts
Beta	SE		Beta	SE	
-0.007	0.005	$1.33x10^{-1}$	-0.013	0.002	$4.35x10^{-15}$**
0.012	0.005	$1.36x10^{-2}$*	0.012	0.002	$1.01x10^{-11}$**
-0.004	0.006	$5.25x10^{-1}$	-0.012	0.002	$3.72x10^{-9}$**
-0.005	0.005	$3.15x10^{-1}$	-0.011	0.002	$1.48x10^{-8}$**
-0.005	0.006	$3.83x10^{-1}$	-0.012	0.002	$1.72x10^{-8}$**
0.010	0.006	$5.92x10^{-2}$	0.012	0.002	$1.23x10^{-8}$**
-0.018	0.007	$6.69x10^{-3}$*	-0.012	0.002	$2.81x10^{-8}$**
-0.010	0.005	$6.64x10^{-2}$	-0.011	0.002	$5.69x10^{-8}$
-0.012	0.010	$2.41x10^{-1}$	-0.018	0.004	$2.98x10^{-7}$

included multiple genes. Although the genome-wide significant region is very large for the optic disc area analysis, the *ATOH7* gene showed the most significant evidence of association with VCDR. *ATOH7* is the human ortholog of *Atoh7*.[13] *Atoh7* is highly expressed in retinal progenitor cells during the early stages of retinal neurogenesis in zebrafish, chick, frog, and mouse.[14-17] Targeted disruption of *Atoh7* in mice causes a specific loss of retinal ganglion cells and optic nerves, and a concomitant increase in cone photoreceptors.[18] Overexpression of *Atoh7* and interaction with the *neuroD* gene in chickens

increases the amount of retinal ganglion cells and photoreceptors.[19] The duration of expression of *ATOH7* is regulated by several proteins, including Growth and Differentiation Factor 11 (*GDF11*).[20] Another factor involved in this genetic pathway is Sonic hedgehog (*SHH*), which mediates the direction of growth as the eye develops from the central part towards the periphery (including the optic nerve).[21] Thus, the *SHH* and *GDF11* regulate *ATOH7*, which in turn regulates *Brn3b*. This gene may play a role in further differentiation of the retinal ganglion cells and is expressed in post-mitotic retinal ganglion cell precursors. Retinal ganglion cells differentiate into the lower retinal epithelium (later becoming the retinal ganglion cell layer). At the same time, the dendrites reach the bipolar, horizontal, and amacrine cells in the inner retinal plexiform layer, while their axons form the optic nerve, optic chiasm, superior colliculus and lateral geniculate nucleus.[21] Although *ATOH7* has been implicated in retinal development in animals, this gene has not been linked to the development of any optic nerve pathology in humans. The analyses of VCDR showed that *ATOH7* (rs1900004) was also significantly associated with VCDR, independent of optic disc area. This suggests that this gene is involved in both the optic disc area and VCDR.

The 1p22 region is second in terms of strength of association based on the p-values. This region includes the genes *CDC7* and *TGFBR3* associated with optic disc area. *CDC7* encodes a cell division cycle protein with kinase activity. Overexpression of this gene has been found in neoplastic transformations in some tumors. Although this region is associated with the optic disc area, the protein that *CDC7* encodes for interacts with the *CDKN2A* protein associated with VCDR. However, also *TGFBR3* is of interest because of the interaction of *ATOH7* with *GDF11*, a member of the bone morphogenetic protein (BMP) and the TGFbeta superfamily. *GDF11* interacts with the latent transforming growth factor beta binding protein 3 (*LTBP3*). In our analyses targeting VCDR, we found genome-wide significant evidence for an association of *LTBP3* with VCDR (see below). While *CDKN2A* is not known to be involved in TGFbeta signalling, *CDKN2B* has been implicated in this pathway. In the VCDR analysis, the most significant SNPs on chromosome 9p21 were located within the *CDKN2B* gene. This gene (also known as *p15Ink4b*) lies adjacent to the tumor suppressor gene *CDKN2A* and encodes a cyclin-dependent kinase. The protein encoded by *CDKN2B* is thought to play a role in cell growth regulation and is induced by transforming growth factor beta (*TGFB*).[22] The *p15ink4b* protein phosphorylates and inactivates the retinoblastoma tumor suppressor (*pRb*) protein.[23] Deletions of this gene and of the retinoblastoma 1 gene are often found in malignant gliomas and melanomas.[24] A recent study in mice found that *p15Ink4b* was ectopically expressed in both zinc finger E-box binding homeobox 1(*Zeb1)* mutant cells and neuroectodermally derived cells, including the developing retina, optic nerve, and muscles surrounding the eye.[25] Taken together, our findings point to a central role of TGFbeta in the development of the optic disc and VCDR. TGFbeta is a multifunctional cytokine that

modulates developmental and repair processes in several tissues. TGFbeta signalling has been implicated in a wide variety of diseases including inflammation, autoimmune disorders, fibrosis, cancer and cataracts. The *CDKN2B/CDKN2A* region has recently also been associated with myocardial infarction and type 2 diabetes mellitus.[26]

Regarding the optic disc area, we found one additional region genome-wide significantly associated when pooling the data of the Dutch and TwinsUK studies. Although the chromosome 16q12.1 region concerns a gene desert, the closest gene in the third locus associated with optic disc area is *SALL1*. Defects in this gene are a cause of Townes-Brocks syndrome, an autosomal dominant disorder characterized by the triad of imperforate anus, dysplastic ears, and thumb malformations.[27] Ocular symptoms in this syndrome may include microphthalmia, iris and chorioretinal colobomata, and lamellar cataract. Only rare variants have been implicated in Townes-Brocks syndrome, while the association we report here is with common variants. *SALL1* encodes a zinc finger transcriptional repressor. When considering the protein pathway, *SALL1* interacts with *SIX1*.[28] Rare variants in *SIX1* are involved in branchio-oto-renal syndrome.[29] We found that common variants in *SIX1* were genome-wide significantly associated with VCDR.

Regarding VCDR, chromosome 14q22-23 was genome-wide significant in the discovery cohorts and was replicated consistently in the other cohorts. The region includes two genes which are obvious candidates: *SIX1* and *SIX6* (the latter also known as *Optx2* and about 94kb distance from rs10483727). *SIX6* is involved in eye development. Defects in this gene have been associated with anophthalmia in mice and in humans.[30-32] Embryological studies have shown expression in the ventral optic stalk, which later becomes the optic nerve.[33] In the adult mouse retina, *Optx2* mRNA has been found in cells within the ganglion cell layer and inner nuclear layer.[34] In human, *SIX6* is expressed in the developing retina, optic nerve and other brain structures.[31]

There were three more genome-wide significant loci on chromosomes 11q13, 13q13 and 22q12.1 associated with VCDR (Table 2). On 11q13 most SNPs were found close to *SCYL1*, which has been associated with optic nerve atrophy in mice.[35] However, also the presence of *LTBP3* in this region is of interest, as this protein binds to *TGFB1*, *TGFB2*, and *TGFB3*, and is thus involved in the same signalling pathway as *CDKN2B. LTBP3* is further of interest because of its homology to *LTBP2*, which has been implicated in primary congenital glaucoma.[36,37] The *DCLK1* gene on 13q13 is expressed in the optic tectum.[38] This is a probable kinase that may be involved in a calcium signaling pathway controlling neuronal migration in the developing and mature brain. Finally, the *CHEK2* gene on chromosome 22q12.1 has been associated with several types of cancer, including breast cancer.[39] A literature search did not show a direct link between *CHEK2* and the eye, however one study reported mapping of a locus on chromosome 22q12.1–q13.1 (*OPA5*) to autosomal dominant optic atrophy and one case-report described an association of chromosome 22q11.2 deletion syndrome with optic disc swelling, which is

probably caused by the resulting hypocalcaemia.[40,41] Regarding the association of *CHEK2* with breast cancer, it is of interest that also one borderline significant SNP is located in a gene breast carcinoma amplified sequence 3 (*BCAS3*) involved in this pathway.

Although our study has convincingly identified SNPs involved in optic disc area and VCDR, there are also a number of limitations. At this point, we cannot relate the identified quantitative trait loci to a single clinical outcome. There was some marginal evidence suggesting that four of the genes involved in the development of the optic disc area and VCDR are relevant to OAG. However, the findings were far from genome-wide significant and remain to be confirmed. Another limitation concerns the differences in methodology. Two of the four replication cohorts, RS-III and ERF, used confocal scanning laser ophthalmoscopy to determine the optic disc area, while the other studies, RS-I, RS-II and TwinsUK, used digitized stereoscopic images. Although this may be considered a drawback, we do not think this distorted our results, since several studies compared both methods and found high correlations for all stereometric parameters.[42-44] Moreover, since our findings replicated in all cohorts, differences across measurements are probably small and unlikely to influence our results, beyond that the estimation of the effects (beta-coefficients) may differ across studies. Finally, the TwinsUK study served as a replication cohort in this study, but is also involved as a replication cohort for a GWAS based on a discovery cohort from Australia.[45] Both Dutch and Australian cohorts independently implicated *ATOH7* as playing a role in optic disc phenotypes and both utilize the TwinsUK data to replicate their findings. Although the association of *ATOH7* was genome-wide significant in the Dutch validation cohorts, this overlap in replication samples should be taken into account.

In conclusion, by conducting GWA analyses, we found genome-wide significant evidence for the association of three genetic loci with optic disc area, and another six with VCDR. Although multiple genes were included in the regions of interest, the most interesting ones for optic disc area were *CDC7* and *TGFBR3* on chromosome 1p22, *ATOH7* on chromosome 10q21.3-22.1 (also for VCDR), and *SALL1* on chromosome 16q12. Regions of interest for VCDR were *CDKN2A* and *CDKN2B* on chromosome 9p21, *SIX1* and *SIX6* on chromosome 14q22-23, *SCYL1* and *LTBP3* on chromosome 11q13, *CHEK2* on chromosome 22q12.1, *DCLK1* on chromosome 13q13, and *BCAS3* on chromosome 17q23. There are several pathways implicated but the most interesting is the TGFbeta signalling pathway that appears to play a key role. Further research is needed to implicate these findings in the pathophysiology of the eye.

MATERIALS AND METHODS

Study populations

The Rotterdam Study I (RS-I) is a prospective population-based cohort study of 7,983 residents aged 55 years and older living in Ommoord, a suburb of Rotterdam, the Netherlands.[46] Baseline examinations for the ophthalmic part took place between 1991 and 1993; follow-up examinations were performed from 1997 to 1999 and from 2002 to 2006.

The RS-II and RS-III are two other prospective population-based cohort studies of 3,011 residents aged 55 years and older, and 3,392 residents aged 45 years and older, respectively. The rationale and study design are similar to those of the RS-I.[46] The baseline examinations of RS-II took place between 2000 and 2002; follow-up examinations were performed from 2004 to 2005. Baseline examinations of RS-III took place between 2006 and 2009.

The Erasmus Rucphen Family (ERF) Study is a family-based cohort study in a genetically isolated population in the southwest of the Netherlands with over 3,000 participants between 18 and 86 years of age. Cross-sectional examinations took place between 2002 and 2005. The rationale and study design of this study have been described elsewhere.[47,48] All measurements in these studies were conducted after the Medical Ethics Committee of the Erasmus University had approved the study protocols and all participants had given a written informed consent in accordance with the Declaration of Helsinki.

Finally, the TwinsUK adult twin registry is a volunteer cohort of over 10,000 healthy twins based at St Thomas' Hospital in London. Participants were recruited and examined between 1998 and 2008. A total of 843 had complete data, all of whom were Caucasian. This cohort is predominantly female, as only 3% of included participants were male.

Ophthalmic examination

The ophthalmic assessment in RS-I and RS-II, both for baseline and follow-up, included a medical history, autorefraction, keratometry, visual field testing and optic nerve head imaging with Topcon ImageNet System of both eyes after mydriasis with topical tropicamide 0.5% and phenylephrine 2.5%. RS-III was similar to RS-I except for optic nerve head imaging with confocal scanning laser ophthalmoscopy (Heidelberg Retina Tomograph 2 [HRT]). The ophthalmic assessment in ERF included a medical history, autorefraction, keratometry and optic nerve head imaging with the HRT of both eyes after pharmacologic mydriasis. In the TwinsUK optic disc parameters were measured from stereo disc photographs using the Nidek-3DX stereo camera, with digitized images scanned from Polaroid images and StereoDx stereoscopic planimetric software (StereoDx) using a Z-screen (StereoGraphics Corp) and software obtained from James Morgan from Cardiff University software, Wales, UK.[49]

Optic nerve head assessment

ImageNet, which was used in RS-I and RS-II, takes simultaneous stereoscopic images of the optic disc at a fixed angle of 20°, using a simultaneous stereoscopic fundus camera (Topcon TRC-SS2; Tokyo Optical Co., Tokyo, Japan). Images were analyzed by using the ImageNet retinal nerve fiber layer height module. On each stereoscopic pair of optic disc images four points were marked on the disc margin, defined as the inner border of the peripapillary ring or the outer border of the neural rim, if a scleral ring was visible. Next, the software drew an ellipse using these points to outline the disc margin and to determine the cup. The amount of correspondence between the marked points on the two images of the stereoscopic pair is expressed as a "bad points" percentage, which indicates the percentage of points lacking correspondence. This percentage can be used as an indicator of image quality. Images with 25% or more bad points were excluded.[50]

HRT 2, used in RS-III and ERF, acquires scans of the optic nerve head region with a focused 670-nm diode laser light beam. The HRT obtains, during one scan, three series of 16 to 64 confocal frontal slices. From each of these series, a 3-dimensional image of the optic nerve head is reconstructed, from which the software calculates several optic disc parameters. To define the cup, the HRT places a reference plane 50 mm below the peripapillary retinal surface in the region of the papillomacular bundle.

Imaging was performed after the participant's keratometry data were entered into the software and the settings were adjusted in accordance with the refractive error. In RS-III all HRT 2 data were converted to HRT 3. As an indicator of image quality we used the topographic standard deviation of the scan, which is a measure of the variability among the three series of a single HRT scan. Scans with a topographic standard deviation exceeding 50 mm were excluded. The inter-observer variability and agreement for both systems have been described elsewhere.[42] Details of the optic disc measurements in TwinsUK have been described elsewhere.[51]

Myopia and open-angle glaucoma assessment

Myopia was defined as a spherical equivalent of -6.00D or lower. For each eye the spherical equivalent was calculated by means of the standard formula: spherical equivalent = spherical component + (cylindrical value/2). The mean spherical equivalent of both eyes was included. Those eyes with a history of cataract surgery were excluded from this analysis.

OAG diagnosis was primarily based on glaucomatous visual field loss (VFL). The visual field of each eye was screened with a Humphrey Field Analyzer (HFA II 740; Zeiss, Oberkochen, Germany) using a 52-point threshold-related supra-threshold test that covered the central field with a radius of 24°. This test was modified from a standard 76-point screening test.[52,53] VFL was defined as non-response in at least three contiguous test points (or four including the blind spot). If the first test was unreliable (>33% false-

positive or false-negative catch trials) or a reliable test showed VFL in at least one eye, a second supra-threshold test was performed on that eye. If the second supra-threshold test was reliable and showed VFL, a full-threshold HFA 24-2 test (second follow-up) or Goldmann perimetry (Haag Streit, Bern, Switzerland; baseline and first follow-up) was performed on both eyes. The classification process of the Goldmann perimetry test results[52] and the full-threshold HFA 24-2 test results [Czudowska, et al. unpublished data] have been described previously. In short, VFL was considered to be glaucomatous VFL only if reproducible and after excluding all other possible causes. For the present study, participants were considered as having glaucomatous VFL if they had glaucomatous VFL in at least one eye during either follow-up round. Cases had to have an open anterior chamber angle and no history or signs of angle closure or secondary glaucoma were allowed.[53] Criteria for glaucomatous optic neuropathy, such as VCDR, were not included in the criteria for OAG.

Genotyping

In the RS-I, RS-II and RS-III cohorts, DNA was genotyped by using the Illumina Infinium II HumanHap550chip v3.0 array according to the manufacturer's protocols. Details have been described elsewhere.[54] After exclusion of participants for reasons of low-quality DNA, a total of 5,974 participants were available with genotyping data from RS-I, 2,157 participants from RS-II, and 2,082 from RS-III. In ERF, DNA was genotyped on four different platforms (Illumina 6k, Illumina 318K, Illumina 370K and Affymetrix 250K), which were then merged. After exclusion of participants for whom genotyping data were unavailable, 2,385 had genotyping data. As we did not use the same microarray for the various study populations we imputed our genotype data using HapMap CEU as reference population, resulting in over 2.5 million SNPs. Extensive quality control analyses have been performed in each cohort. Finally, the genotyping of the TwinsUK cohort took place in stages; in the first stage participants were genotyped by using Illumina's HumanHap 300K duo chip, whereas in the second stage participants were genotyped with Illumina's HumanHap610 Quad.

Statistical analysis

Statistical analysis within studies

If we had data on both eyes then we chose a random eye. In cases of missing or unreliable baseline data on both eyes, we used follow-up data where available. Results from the RS-I and RS-II cohorts were combined, because both studies were identical in population structure. Within each study, linear regression models were used to examine the associations between SNPs and optic disc area adjusted for age and gender. The analyses of VCDR were further adjusted for optic disc area. Using these linear regres-

sion models, we calculated regression coefficients with corresponding 95% confidence intervals (CI). To adjust for multiple testing a p-value of $5x10^{-8}$ or less was considered statistically significant. As a secondary analysis we performed the analyses of VCDR with the same additive models but with further adjustment for intraocular pressure and its treatment.

All statistical analyses were performed with SPSS version 15.0.0 for Windows (SPSS inc., Chicago, IL, USA; 2006), MACH2 QTL as implemented in GRIMP[55] and R statistical package version 2.8.1 for Linux (www.r-project.org). For the analysis of the family based data we used the GenABEL package to adjust for relationships.[56]

Meta-analysis

First, we replicated the top SNPs of the discovery cohorts in the two Dutch replication cohorts (RS-III and ERF). To adjust for familial relationships of participants in ERF we used the score test described by Chen and Abecasis which is implemented in the GenABEL package.[57] Meta-analyses were performed with Metal for Linux (www.sph.umich.edu/csg/abecasis/metal) to summarize the global effect through the four cohorts. To obtain optimal and unbiased results we used genomic control and the inverse variance method of each effect size estimate.[58] This was only done for the SNPs that were genotyped or imputed in all four cohorts. SNPs which deviated significantly from Hardy-Weinberg equilibrium ($p<0.0001$) or had a minor allele frequency <0.05 were excluded in the present study. Next, we replicated all top SNPs from the joint analysis of the four Dutch cohorts in a combined analysis with the TwinsUK.

Finally, we tested in RS-I whether the identified loci were associated with other ophthalmic traits such as myopia by using the spherical equivalent of the refractive error, and OAG based on glaucomatous visual field loss. This was done by using logistic regression analyses adjusted for age and gender.

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Supporting information for

A genome-wide association study of optic disc parameters

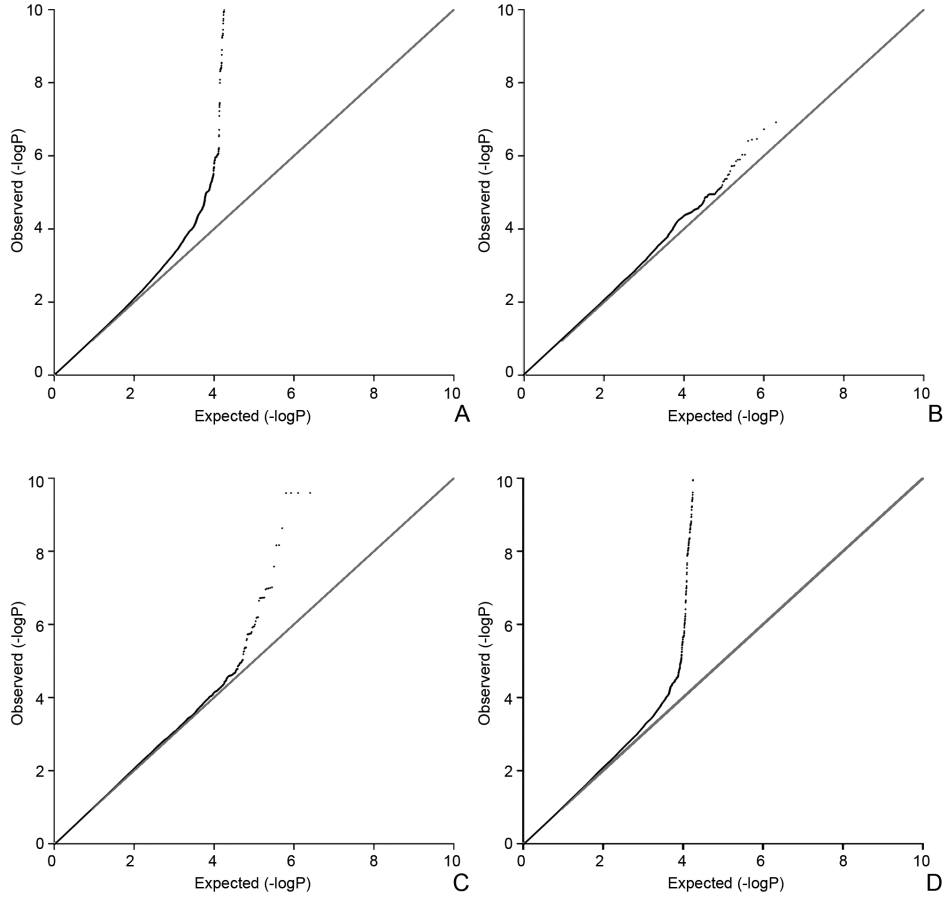

Figure S1. Optic disc area Q-Q plots for the observed versus expected p-values for the discovery cohorts (A), the individual replication cohorts (B,C), and for the meta-analysis (D).

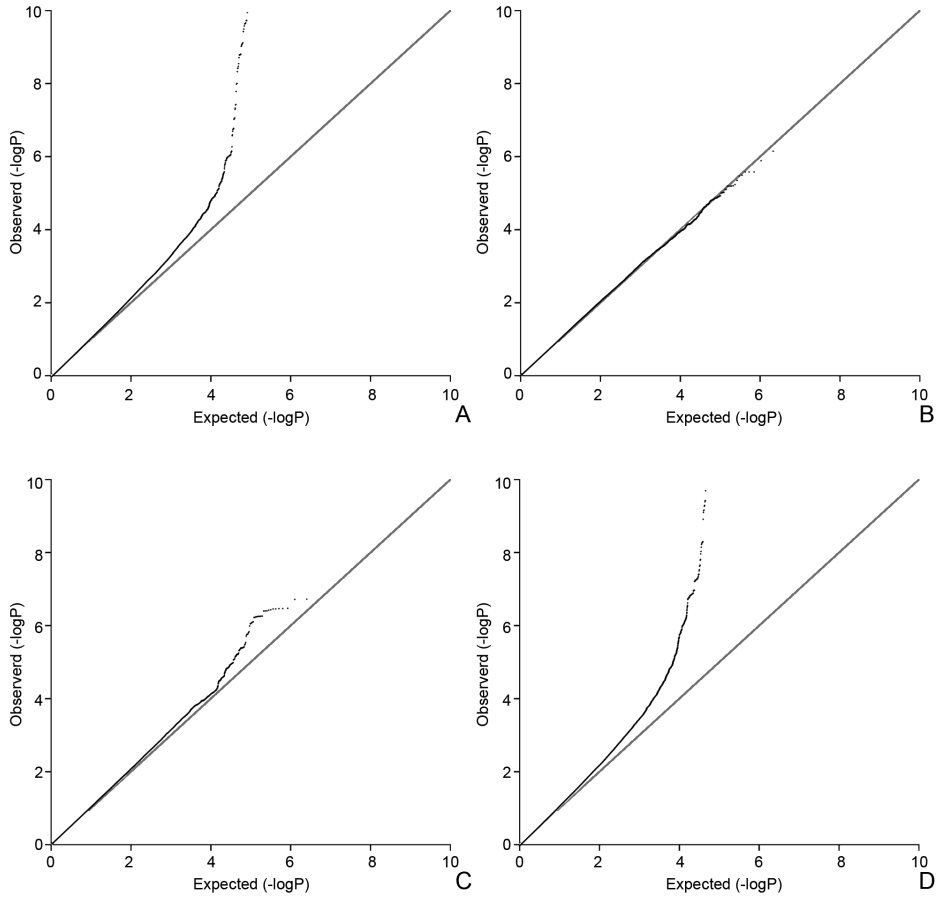

Figure S2. Vertical cup-to-disc ratio Q-Q plots for the observed versus expected p-values for the discovery cohorts (A), the individual replication cohorts (B,C), and for the meta-analysis (D).

Table S1. Characteristics of the patients with open-angle glaucoma presented as mean ± standard deviation (range) unless stated otherwise.

	RS-I	
	Cases (N=188)	Controls (N=5,548)
Age (years)	75.5 ± 7.4 (56 - 94)	74.5 ± 7.8 (55 - 105)
Gender, N (%) female	85 (45.2)	3289 (59.3)
Intraocular pressure (mmHg)	18.2 ± 6.2 (6.0 – 54.6)	15.2 ± 3.5 (5.0 – 58.5)
Intraocular pressure treatment, N (%)	37 (19.7)	93 (1.7)

RS = Rotterdam Study

Chapter 3.2

Common genetic determinants of intraocular pressure and primary open-angle glaucoma

Van Koolwijk LME*, Ramdas WD*, Ikram MK, Jansonius NM, Pasutto F, Hysi PG, Macgregor S, Janssen SF, Hewitt AW, Viswanathan AC, ten Brink J, Hosseini SM, Amin N, Despriet DDG, Willemse-Assink JJM, Kramer R, Rivadeneira F, Struchalin M, Aulchenko YS, Weisschuh N, Zenkel M, Mardin CY, Gramer E, Welge-Lüssen U, Montgomery GW, Carbonaro F, Young TL, the DCCT/EDIC research group, Bellenguez C, McGuffin P, Foster PJ, Topouzis F, Mitchell P, Wang JJ, Wong TY, Czudowska MA, Hofman A, Uitterlinden AG, Wolfs RCW, de Jong PTVM, Oostra BA, Paterson AD, Wellcome Trust Case Control Consortium 2, Mackey DA, Bergen AB, Reis A, Hammond CJ, Vingerling JR, Lemij HG, Klaver CCW, van Duijn CM.

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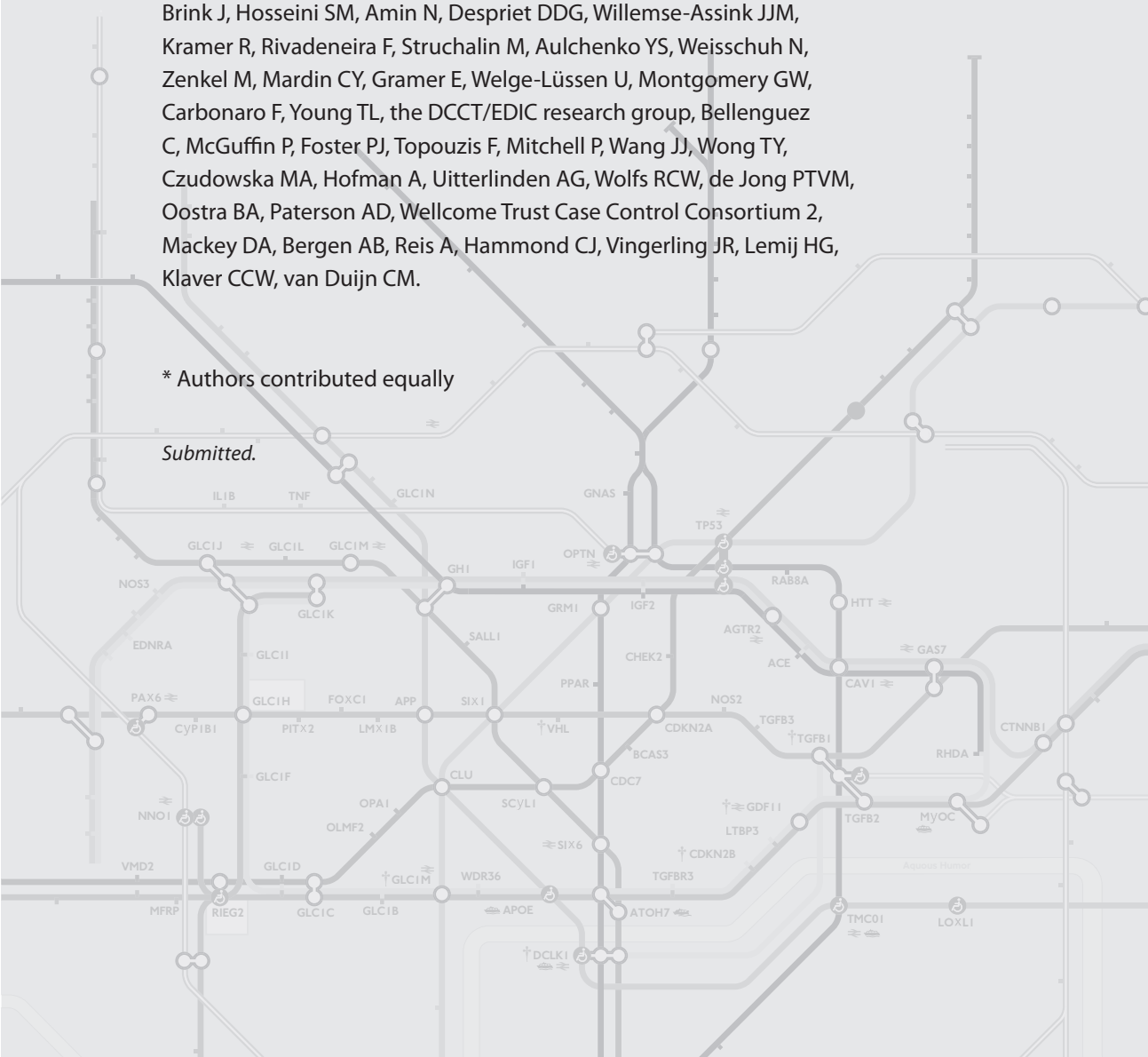
Submitted.



ABSTRACT

Intraocular pressure (IOP) is a highly heritable risk factor for primary open-angle glaucoma and is the only target for current glaucoma therapy. The genetic factors which determine IOP are largely unknown. We performed a genome-wide association study for IOP in 11,972 participants from 4 independent population-based studies in The Netherlands. We replicated our findings in 7,482 participants from 4 additional cohorts from the UK, Australia, Canada, and the Wellcome Trust Case-Control Consortium 2 / Blue Mountains Eye Study. IOP was significantly associated with rs11656696, located in *GAS7* at 17p13.1 ($p=1.4x10^{-8}$), and with rs7555523, located in *TMCO1* at 1q24.1 ($p=1.6x10^{-8}$). In a meta-analysis of 4 case-control studies (total N=1,432 glaucoma cases), both variants also showed evidence for association with glaucoma ($p=2.4x10^{-2}$ for rs11656696 and $p=9.1x10^{-4}$ for rs7555523). *GAS7* and *TMCO1* are highly expressed in the ciliary body and trabecular meshwork as well as in the retina, and functionally interact with known glaucoma disease genes. These data suggest that we have identified two clinically relevant genes involved in IOP regulation.

AUTHOR SUMMARY

Glaucoma is a major eye disease in the elderly and is the second leading cause of blindness worldwide. The numerous familial glaucoma cases, as well as evidence from epidemiological and twin studies, strongly support a genetic component in developing glaucoma. However, it has proven difficult to identify the specific genes involved. Intraocular pressure (IOP) is the major risk factor for glaucoma and the only target for the current glaucoma therapy. IOP has been shown to be highly heritable. We investigated the role of common genetic variants in IOP by performing a genome-wide association study. Discovery analyses in 11,972 participants and subsequent replication analyses in a further 7,482 participants yielded two common genetic variants that were associated with IOP. The first (rs11656696) is located in *GAS7* at chromosome 17, the second (rs7555523) in *TMCO1* at chromosome 1. Both variants were associated with glaucoma in a meta-analysis of 4 case-control studies. *GAS7* and *TMCO1* are expressed in the ocular tissues that are involved in glaucoma. Both genes functionally interact with the known glaucoma disease genes. These data suggest that we have identified two genes involved in IOP regulation and glaucomatous neuropathy.

INTRODUCTION

Primary open-angle glaucoma (hereafter referred to as glaucoma) is a progressive optic neuropathy responsible for 12.3% of global blindness.[1] The evidence for a genetic etiology of glaucoma is well-established.[2] However, genes consistently implicated so far (*MYOC*, *OPTN*, *WDR36*)[3-5] are relevant only in a limited number of families and explain a small proportion of the glaucoma cases in the general population.[6-8] So far, 2 genome-wide association studies (GWASs) for glaucoma have been published. A study from Iceland identified a common variant near *CAV1* and *CAV2*.[9] Both genes are expressed in the trabecular meshwork as well as in retinal ganglion cells. A Japanese study identified 3 putative loci, although none of these reached genome-wide significance.[10] Such significance for association with glaucoma was achieved in an Afro-Caribbean population for a locus on chromosome 2p by focused genotyping in a previously identified linkage region.[11]

Intraocular pressure (IOP) is the major risk factor of glaucoma and existing glaucoma therapies are exclusively aimed at lowering IOP. An elevated IOP (> 21 mmHg) influences both the onset and progression of glaucoma.[12] Genetic effects have been shown to account for a significant proportion of the variance in IOP, with heritability estimates ranging from 0.29 to 0.67.[13-17] Five genome-wide linkage studies of IOP have been performed.[18-22] These resulted in 15 potential regions of interest, 2 of which were genome-wide significantly linked to IOP. The first was identified in an Australian glaucoma pedigree and was located on 10q22.[18] The second was identified in individuals without glaucoma in West Africa and Mongolia and was located in the 5q22-23 region, which had already been implicated in glaucoma (*WDR36* gene and GLC1M locus).[3, 21-23] Taken together, these findings suggest that extensive heterogeneity underlies the genetics of IOP and that the same genetic factors may possibly affect both the variance in normal IOP and the risk of getting glaucoma. Thus, unraveling the genetic background of IOP may shed light upon the pathophysiology of glaucoma. To date, no GWAS has been reported for IOP.

To identify genetic determinants of IOP, we performed a GWAS in 11,972 participants from 4 independent population-based studies in The Netherlands, and we replicated our findings in 7,482 participants from 4 additional independent cohorts of Caucasian ancestry. We investigated whether the IOP associated SNPs were also related to glaucoma in 1,432 glaucoma cases. Lastly, we examined expression levels of the identified candidate genes in human ocular tissues. We identified common variants in *GAS7* and *TMCO1* that altered the susceptibility to both IOP and glaucoma.

RESULTS

Discovery studies

Genotypic and IOP data were available for 11,972 participants from the Rotterdam Study cohort I (RS-I), RS-II, RS-III, and the Erasmus Rucphen Family (ERF) Study (Table 1). Genomic inflation factors of the individual cohorts' analyses ranged between 1.006 and 1.037. Four SNPs on chromosome 17p13.1 were significantly associated with IOP in the discovery meta-analysis ($p<5x10^{-8}$; Figure 1, Table 2). These SNPs are located in the growth arrest-specific 7 (*GAS7*) gene (Figure 2).[24] The SNP that showed strongest association with IOP was rs11656696. The effect of the rs11656696 alleles was consistent across all 4 discovery cohorts (Table S1). A further 6 chromosomal loci showed more moderate but nevertheless suggestive associations with IOP ($p<1x10^{-5}$; Table 2, Figure S1) and were also taken to the replication phase. Of these, rs7555523 is located in the trans-membrane and coiled-coil domains 1 (*TMCO1*) gene on chromosome 1q24.1 (Figure 2),[24] which is located 7.6 MB from *MYOC*.

Table 1. Characteristics of the discovery cohorts

Characteristic	RS-I	RS-II	RS-III	ERF
Participants with valid data (N)	5,794	2,102	2,041	2,035
Age (y), mean ± SD (range)	68.8 ± 8.9 (55 – 100)	64.4 ± 8.0 (55 – 95)	55.7 ± 5.8 (45 – 97)	48.8 ± 14.4 (18 – 86)
Male gender (%)	41.2	45.7	43.9	43.3
IOP (mmHg), mean ± SD (range)	14.7 ± 3.4 (5 – 59)	14.4 ± 3.4 (7 – 32)	13.6 ± 3.0 (5 – 30)	15.3 ± 3.1 (6 – 33)
IOP ≥ 22 mmHg (%)	3.3	3.3	1.9	1.2
Participants with IOP lowering treatment (%)	2.4	3.9	1.5	0.9
Vertical cup-to-disc ratio, mean ± SD (range)	0.50 ± 0.14 (0.00 – 0.89)	0.50 ± 0.14 (0.05 – 0.87)	0.42 ± 0.17 (0.00 – 1.00)	0.43 ± 0.16 (0.00 – 0.83)
Disc area (mm2), mean ± SD (range)	2.42 ± 0.48 (0.58 – 5.44)	2.32 ± 0.48 (1.06 – 6.20)	1.92 ± 0.45 (0.70 – 7.20)	1.90 ± 0.35 (1.07 – 3.95)

IOP = intraocular pressure; SD = standard deviation; RS= Rotterdam Study; ERF = Erasmus Rucphen Family study

We examined at least 416 KB of the chromosomal regions spanning the known disease genes *MYOC*, *OPTN*, and *WDR36* in more detail in the discovery meta-analysis. None of the 1507 SNPs assessed in total showed significant association with IOP (Figure S2).[24] We also evaluated 10 SNPs which had approached genome-wide significance in earlier association studies (Table S2).[9-11] Of these, rs4236601 in the *CAV1-CAV2* region, previously identified in Caucasians, was consistently associated with increased IOP in our discovery meta-analysis (beta=0.19, 95%CI=0.09-0.29, $p=1.1x10^{-4}$).[9] Of the three regions identified in Japan, only rs7081455 on chromosome 10 showed nominal evidence for association

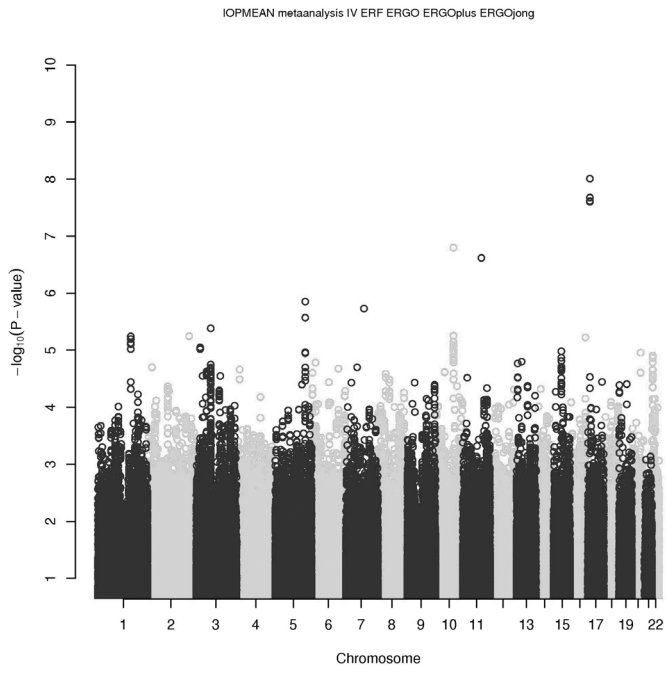


Figure 1. Results of the meta-analysis of the gene discovery cohorts

Table 2. Results of the meta-analysis of the gene discovery cohorts: loci associated with IOP ($p < 10^{-5}$)

SNP	Chrom	Position	MA	MAF	Gene region	#SNPs*	Beta	SE	P-value
rs11656696	17p13.1	9974404	A	0.43	*GAS7*	4	-0.26	0.05	9.8E-09
rs7894966	10q23.2	88608604	G	0.04	*BMPR1A*	8	0.67	0.13	1.6E-07
rs216146	5q32	149426114	T	0.39	*CSF1R*	2	0.22	0.05	1.4E-06
rs2117760	3p13	70933151	A	0.32	*FOXP1*	1	0.22	0.05	4.1E-06
rs7555523	1q24.1	163985603	C	0.13	*TMCO1*	11	0.30	0.07	5.7E-06
rs1826598	16q23.1	76130456	A	0.11	*ADAMTS18, NUDT7*	1	0.32	0.07	6.0E-06
rs9841621	3p24.3	18384081	G	0.01	*SATB1*	5	-0.81	0.18	8.9E-06

SNP = single nucleotide polymorphism; Chrom = chromosome; MA(F) = minor allele (frequency); SE = standard error

* number of SNPs with $p<10^{-5}$ in the region

According NCBI build 37.1, rs11656696 is located at position 10033679 in the growth-arrest-specific gene *GAS7* while an earlier build allocated the SNP at 9974404 (http://www.ncbi.nlm.nih.gov).

with IOP (beta=0.12, 95%CI=0.08-0.16, $p=4.6x10^{-3}$). Our data did not replicate the association in the 2p16 locus which was previously identified in Afro-Caribbeans. Finally, we examined the two chromosomal regions that had previously been identified in genome-wide linkage studies of IOP.[18, 22] Both regions showed suggestive evidence of association with IOP in our discovery meta-analysis: Rs7894966, located in the bone morphogenetic

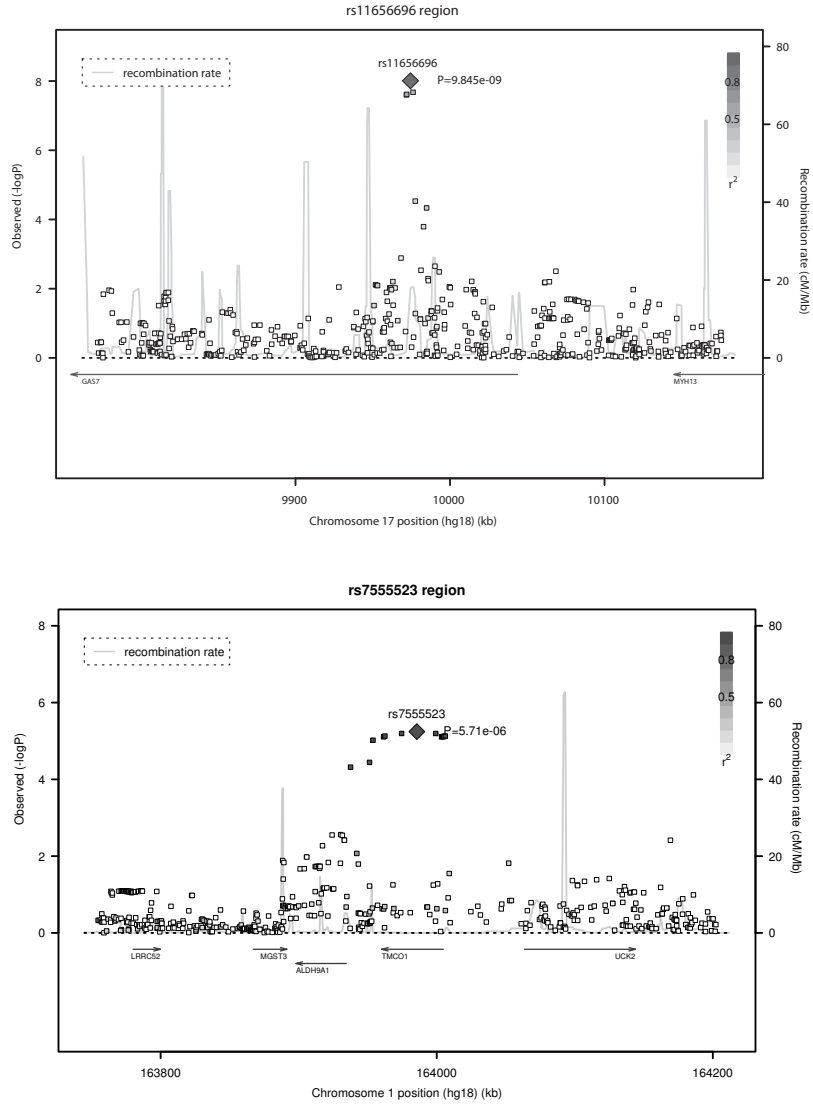


Figure 2. Regional association plots of the 17p13.1 and 1q24.1 regions in the discovery meta-analysis

protein receptor 1A (*BMPR1A*) gene on chromosome 10q23.2, is in the region previously identified in an Australian linkage study of IOP (16.2 MB from the peak LOD score);[18] Rs216146, in the colony stimulating factor 1 receptor (*CSF1R*) gene on chromosome 5q32, is close to the region that previously showed genome-wide significant linkage to IOP in West Africans.[22] This SNP is located at a distance of 21.0 MB to the peak LOD score, 10.0 MB to the glaucoma locus *GLC1M*, and 39.0 MB to *WDR36*.

Replication studies

Replication of the IOP association was done in 4 additional cohorts from the TwinsUK study (N=2,235), the Australian Twin study (N=1,807), the Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications study (DCCT/EDIC; N=1,304), and the Wellcome Trust Case-Control Consortium 2 / Blue Mountains Eye Study (WTCCC2/BMES; N=2,136) (Supporting Information). The results of the replication analyses are presented in Table 3. Although in most studies the association did not reach nominal significance ($p<0.05$), most likely explained by the low statistical power of these relatively small studies, the directionality of the effects was consistent across the 4 replication cohorts for most SNPs. The exceptions were rs7894966 and rs216146 for which the effects were in opposite direction compared to the discovery cohorts. When the gene discovery and replication cohorts were combined, two intronic SNPs reached genome-wide significance. Each copy of the rs11656696 minor allele (A), located in *GAS7*, was associated with a 0.19 mmHg IOP reduction (95% confidence interval [CI]=0.12-0.26 mmHg; $p=1.4x10^{-8}$), and each copy of the rs7555523 minor allele (C), located in *TMCO1*, with a 0.28 mmHg IOP increase (95%CI=0.18-0.37 mmHg; $p=1.6x10^{-8}$).

Glaucoma case-control studies

We investigated the associations of the *GAS7* rs11656696 minor allele (A) and the *TMCO1* rs7555523 minor allele (C) with glaucoma in 4 case-control studies from the Netherlands and Germany (Supporting Information). The results are presented in Figure 3. For rs11656696 A we found a decreased glaucoma risk in the Amsterdam Glaucoma Study (AGS; OR=0.71, 95%CI=0.51-0.99) and the Erlangen and Tübingen study (OR=0.82, 95%CI=0.69-0.97), but not in RS-I and the Genetic Research in Isolated Populations (GRIP) program. When combining the 4 case-control studies, rs11656696 A showed a decreased glaucoma risk (OR=0.88, 95%CI=0.78-0.98, $p=2.4x10^{-2}$). For rs7555523 C, we found an increased glaucoma risk in all 4 case-control studies. Combined, these studies showed an increased glaucoma risk with an OR of 1.31 (95%CI=1.12-1.53, $p=9.1x10^{-4}$).

Expression studies

We examined gene expression levels in human ocular tissues and observed moderate to high expression of *GAS7,* and high expression of *TMCO1* in the ciliary body (CB), the secretory neuroepithelium that produces the aqueous humor (Table 4). Both genes were moderately to highly expressed in the choroid, the retinal pigment epithelium and photoreceptors.

Table 3. Results of the replication analyses and the joint analysis of discovery and replication cohorts

		Replication analyses					
		TwinsUK			Australian Twins		
SNP	Chrom	Beta	SE	P-value	Beta	SE	P-value
rs11656696	17p13.1	-0.32	0.11	3.9E-03	-0.11	0.10	2.9E-01
rs7894966	10q23.2	-1.15	0.37	1.7E-03	-0.32	0.29	2.6E-01
rs216146	5q32	0.00	0.11	9.8E-01	-0.08	0.11	4.8E-01
rs2117760	3p13	0.12	0.11	2.9E-01	-0.03	0.11	7.7E-01
rs7555523	1q24.1	0.24	0.15	9.6E-02	0.23	0.16	1.4E-01
rs1826598	16q23.1	0.15	0.15	3.4E-01	0.10	0.17	5.6E-01
rs9841621	3p24.3	-0.42	0.36	2.4E-01	-0.34	0.33	3.0E-01

SNP = single nucleotide polymorphism; Chrom = Chromosome; SE = standard error;
DCCT/EDIC = Diabetes Control and Complications Trial / Epidemiology of Diabetes Interventions and Complications study; WTCCC2/BMES = Wellcome Trust Case-Control Consortium 2 / Blue Mountains Eye Study

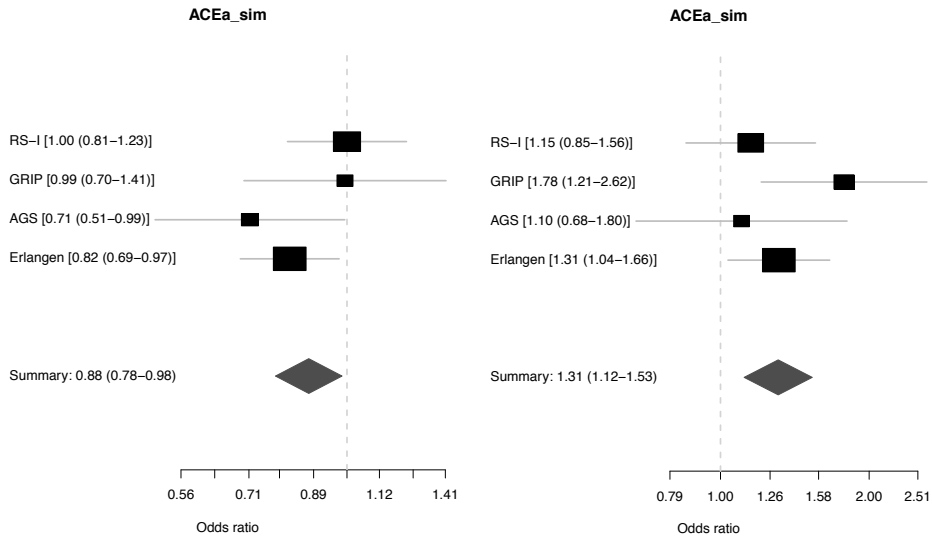


Figure 3. Association of rs11656696 and rs7555523 with glaucoma

DISCUSSION

We identified rs11656696 in *GAS7* and rs7555523 in *TMCO1* as common variants associated with IOP. In a joint analysis of the discovery and replication cohorts each copy of the rs11656696 minor allele (A; allele frequency 0.43) was associated with a 0.19 mmHg decrease in IOP (95%CI=0.12-0.26 mmHg), whereas each copy of the rs7555523 minor allele (C; allele frequency 0.13) was associated with a 0.28 mmHg increase in IOP

Table 3. (continued)

						Joint analysis of discovery and replication cohorts		
DCCT/EDIC			WTCCC2/BMES					
Beta	SE	P-value	Beta	SE	P-value	**Beta**	**SE**	**P-value**
0.04	0.11	6.8E-01	-0.06	0.09	4.9E-01	**-0.19**	**0.03**	**1.4E-08**
-0.11	0.29	6.9E-01				**0.30**	**0.10**	**3.6E-03**
-0.08	0.11	4.7E-01	-0.08	0.09	3.4E-01	**0.09**	**0.03**	**4.9E-03**
0.05	0.11	6.5E-01				**0.16**	**0.04**	**2.8E-05**
0.18	0.17	2.9E-01	0.30	0.13	1.8E-02	**0.28**	**0.05**	**1.6E-08**
0.20	0.18	2.7E-01	0.01	0.13	9.3E-01	**0.22**	**0.05**	**2.0E-05**
-0.31	0.40	4.3E-01	-0.15	0.31	6.3E-01	**-0.54**	**0.12**	**1.4E-05**

Table 4. Gene expression levels in human ocular tissues

Gene	CB-PE	CB-NPE	Choroid	RPE	Photoreceptors	TM *
GAS7	55 (1.3)	57 (2.0)	73 (5.1)	76 (1.7)	78 (8.6)	78 (3.1)
TMCO1	93 (1.5)	93 (1.0)	86 (2.5)	88 (1.9)	88 (2.4)	88 (1.5)

The two genes are ranked by increasing expression, calculated by the mean percentiles (SD) of the expression levels. Gene expression of CB-PE and CB-NPE (n=4), choroid (n=3), photoreceptors (n=3) and RPE (n=6) were performed on Agilent Human 44k microarray of post-mortem donor eyes without glaucoma or any other ocular diseases.

* Data from Liton et al., performed on Affymetrix Human U133 microarray, showing mean percentiles (SD) of human gene expression levels in TM tissue from 3 healthy eyes.[26]

CB-PE = ciliary body, pigmented epithelium; CB-NPE = ciliary body, non-pigmented epithelium; RPE = retinal pigment epithelium; TM = trabecular meshwork.

(95%CI=0.18-0.37 mmHg). Both variants showed marginal evidence for association with glaucoma when combining data from 4 case-control studies, although for rs11656696 significance was only obtained in 2 studies.

GAS7 is located in a chromosomal region previously identified by a linkage study of glaucoma.[25] We observed moderate to high expression of *GAS7* in the ciliary body (CB), the secretory neuroepithelium that produces the aqueous humor. Previously, Liton and colleagues already reported expression of *GAS7* in human trabecular meshwork (TM), which is the main tissue involved in aqueous humor outflow.[26] Together, the CB and TM largely control IOP. Significant downregulation of *GAS7* expression was observed in TM of glaucomatous eyes.[26] In absence of the (in vivo) typical mechanical forces on the TM, a similar effect was also observed in cultured TM cells.[26] High *GAS7* expression was found in amacrine cells in the mouse retina, while lower expression was found in retinal cell types which are usually not affected by glaucoma.[27] Protein pathway analyses and evidence from previous literature allude to functional effects of *GAS7* in both the TM and retina. *GAS7* has been implicated in cell remodelling, possibly facilitated through its capacity to

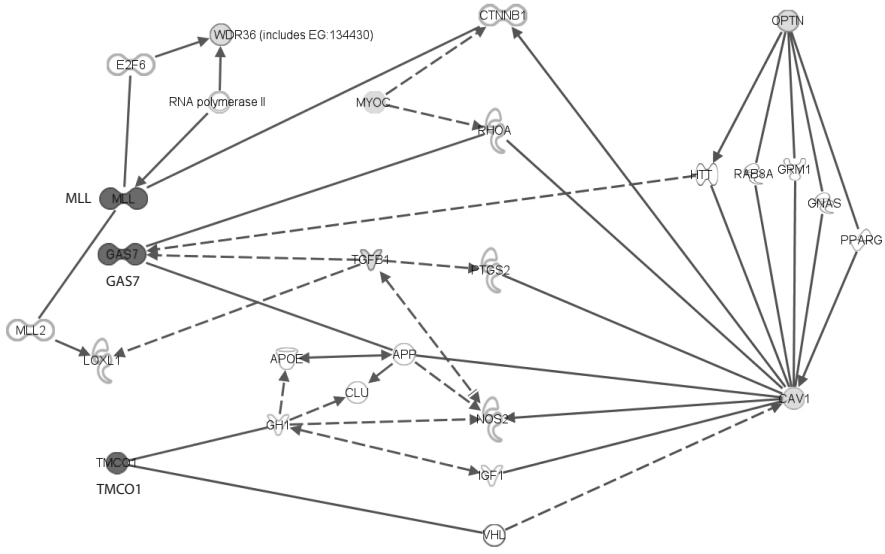


Figure 4. Biochemical and functional interactions between (putative) glaucoma disease genes
Ingenuity diagram of biochemical and functional interactions between the newly identified GAS7 and TMCO1 disease genes implicated in elevated IOP and glaucoma, and previously known glaucoma disease genes (WDR36, MYOC, OPTN, CAV1). Functional relationships in the knowledge database Ingenuity (www.ingenuity.com) are a compilation of all known gene-relevant biochemical and functional data of in vivo and in vitro experiments involving (molecules, cells and tissues of) rats and mice and man, as well as data from zebrafish and Drosophila and ongoing clinical trials in man. The query genes/proteins GAS7 (including it's drosophila homologue MLL) and TMCO1 are presented in dark grey. Known glaucoma disease genes are given in light grey. Blank genes/molecules are generated by the knowledge database to construct a functional network under the criteria specified by the investigator. The diagram was generated using the function "Path Explorer".
In general, solid lines indicate a direct, experimentally verified, physical relationship between two molecules, for example a physical protein-protein interaction, or an enzym-DNA interaction, etc. Dotted lines refer to the existence of an indirect functional relationship, such as co-upregulation in cell cultures under specific experimental conditions. WDR36 = WD Repeat-containing protein 36; OPTN = optineurin; MYOC = myocilin; GAS7 = growth arrest-specific 7; MLL = myeloid/lymphoid or mixed-lineage leukemia; TMCO1 = transmembrane and coiled-coil domains 1; CAV1 = caveolin 1; TGFB1 = transforming growth factor beta 1; CTNNB1 = catenin (cadherin-associated protein) beta 1; RHOA = ras homolog gene family, member A; E2F6 = E2F transcription factor 6; VHL = von Hippel-Lindau; HTT = huntingtin; NOS2 = nitric oxide synthase 2; LOXL1 = lysyl oxidase-like 1; APOE = apolipoprotein E; APP = amyloid beta (A4) precursor protein; CLU = clusterin.
As shown, GAS7 (MLL) and TMCO1 interact multiple times and in several ways with previously known glaucoma disease genes. For a specific description of these interactions, see text.

associate with actin and mediate the reorganization of microfilaments.[28, 29] In neuronal cells, *GAS7* expression is critical for neurite formation.[28, 30] *MYOC*, the major glaucoma gene previously associated with elevated IOP cases, also affects the actin cytoskeletal structure

and neurite outgrowth.[31] Whereas *MYOC* has an inhibitory effect on neurite outgrowth, *GAS7* is involved in the formation of neurites. Interestingly, experimental ischemic retinal damage in rats, resembling retinal damage due to glaucoma, leads to extensive remodelling of inner retinal neurons.[32] *GAS7* may also contribute to remodelling of the TM, as is the case for the myocilin protein which has been shown to alter the actin structure and modulate TM cell morphogenesis.[33] *GAS7* interacts with *MYOC*, as well as with other genes implicated in glaucoma, such as *OPTN*, *WDR36*, *CAV1*, *NOS2*, *FOXC1*, *APOE*, *APP* and *CLU* (Figure 4; www.ingenuity.com). The latter three genes are primarily known for their association with Alzheimer's Disease, a neurodegenerative disease previously linked to glaucoma.[34] *GAS7* interacts with both *MYOC* and *CAV1* through β-catenin (*CTNNB1*) and RhoA (*RHOA*). B-catenin anchors the actin cytoskeleton and is part of the Wnt signalling pathway, which has previously been implicated in trabecular outflow regulation.[35, 36] RhoA signalling regulates the intracellular levels of phosphorylated myosin light chain, which directly influence trabecular meshwork cellular contraction and thus aqueous humor outflow.[37] Finally, *GAS7* is regulated by transforming growth factor (TGF) beta, which has previously been implicated in trabecular outflow as well as in the development of the optic disc (the primary site of neuronal damage by glaucoma).[38-40] The frequency of the *GAS7* rs11656696 A-allele is 0.44 in the HapMap CEU population of European ancestry whereas it is 0.12 in the HapMap Yorubian population of African ancestry. The lower frequency of the A-allele in the African population is consistent with the higher prevalence of glaucoma with elevated IOP in this population and warrants further research into the association of rs11656696 with IOP and glaucoma in African populations.

The second variant that we found to be associated with IOP and glaucoma was rs7555523 in *TMCO1*, a highly evolutionary conserved gene of largely unknown function.[41, 42] Rs7555523 is located in a region which previously showed suggestive evidence for linkage with blood pressure.[43] IOP and blood pressure have already been shown to correlate.[44] *TMCO1* is highly expressed in the human TM and CB, and to a lesser extent in the retina (Table 4).[26] *TMCO1* interacts with *CAV1* via VHL (Figure 4). A homozygous frameshift mutation in *TMCO1* has been associated with a genetic syndrome involving multiple organ systems, including renal agenesis and hydronephrosis.[41] Extensive ophthalmologic examination was not reported, however a high incidence of strabismus was noticed.

No previous GWASs of IOP have been conducted to date. When comparing our findings to those of association studies of glaucoma, we found an overlap with 2 regions. First, rs4236601 in the *CAV1-CAV2* region, previously identified in Caucasians, was consistently associated with an increased IOP in our discovery meta-analysis.[9] Our findings in this region did not reach genome-wide significance. However, multiple testing adjustment by using a Bonferroni correction for the 10 SNPs evaluated (Table S2) yields a criterion for significance of $p<5x10^{-3}$. Thus, our findings strongly support an association between the *CAV1-CAV2* region and IOP, despite the fact that the original report that identified

CAV1-CAV2 did not find any evidence for a stronger relation to high pressure glaucoma. Second, a locus on chromosome 10p, which had previously been identified in Japan, also passed this Bonferroni threshold.[10] Similar to Nakano and coworkers, we could not assign a specific glaucoma disease gene to this region. The replication of this locus in our study is remarkable as most patients with glaucoma in Japan present with normal tension glaucoma (i.e., glaucoma with IOP ≤ 21 mmHg).

A potential limitation of our study design is that we did not measure central corneal thickness (CCT) in the discovery cohorts. CCT is a potential confounder of IOP measurements and may be an IOP-independent risk factor for glaucoma.[45, 46] CCT has previously been reported to account for 1-6% of the variance in IOP measured with Goldmann applanation tonometry.[47-50] To examine the distortion of IOP by CCT, we assessed the associations of rs11656696 and rs75555623 with IOP in the TwinsUK cohort after including CCT as a covariate in the multivariate model. The association changed from -0.316 (95%CI= -0.536 – -0.096) to -0.400 (95%CI= -0.620 – -0.180) for rs11656696 and from 0.242 (95%CI= -0.048 – 0.532) to 0.220 (95%CI= -0.080 – 0.520) for rs7555523 after correction for CCT, suggesting that the controlling for CCT only produces relatively minor changes with respect to the effect size and significance of association.

In conclusion, this genome-wide association study in 8 independent Caucasian cohorts identified rs11656696 in *GAS7* at chromosome 17p13.1 and rs7555523 in *TMCO1* at chromosome 1q24.1 as common genetic variants associated with IOP. The variants were also marginally associated with glaucoma. *GAS7* and *TMCO1* are expressed in ocular cells and tissues implicated in glaucoma. Biochemical protein interactions with known glaucoma disease genes, as well as functional data support the involvement of these genes in aqueous humor dynamics and glaucomatous neuropathy.

MATERIALS AND METHODS

Ethics statement

All participating studies adhered to the tenets of the Declaration of Helsinki and were approved by their Medical Ethics Committees. Written, informed consent was obtained from all participants.

Outline of the study

For the gene discovery phase, we combined data of 11,972 participants derived from 4 large, independent population-based cohort studies in The Netherlands: the Rotterdam Study cohort I (RS-I), RS-II, RS-III, and the Erasmus Rucphen Family (ERF) Study. Replication of the findings was sought in 4 independent populations: the TwinsUK Adult Twin study, the Australian Twin Study, the Diabetes Control and Complications Trial /

Epidemiology of Diabetes Interventions and Complications study (DCCT/EDIC),[51] and the Wellcome Trust Case-Control Consortium 2 / Blue Mountains Eye Study (WTCCC2/BMES). Clinical relevance of the identified loci was assessed by evaluating associations between the variants and glaucoma. To this end, we performed case-control analyses using 4 different glaucoma cohorts from The Netherlands and Germany. Finally, we examined the expression levels of the identified candidate genes in ocular tissues.

Discovery studies

Participants

The RS-I is a prospective population-based cohort study of 7,983 residents 55 years of age and older living in Ommoord, a suburb of Rotterdam, The Netherlands.[52] Baseline ophthalmic examinations took place from 1991 to 1993, follow-up examinations from 1997 to 1999 and from 2002 to 2006. The RS-II is an independent cohort of another 3,011 new respondents in the same age range as RS-I.[52] Baseline examinations were performed from 2000 to 2002 and follow-up examinations from 2004 to 2005. The RS-III was based on the same protocol as RS-I and RS-II, and included 3,932 residents with a different age range, being 45 years and older. Baseline examinations took place from 2006 to 2009. Finally, ERF is a family-based cohort study in a genetically isolated population in the southwest of The Netherlands with over 3,000 participants 18 years of age and older.[16, 53] Examinations took place from 2002 to 2005.

Clinical examination

In all discovery cohorts, the IOP was measured with Goldmann applanation tonometry (Haag-Streit, Bern, Switzerland), which is the international standard for IOP assessment in ophthalmic research and clinical practice. IOP was measured twice per eye. If the two measurements in one eye differed, a third measurement was performed, and the median value was recorded.[16, 54] The IOP measurement was part of a comprehensive ophthalmic examination, including the assessment of visual acuity, refraction, keratometry, fundus photography, and imaging of the optic disc.

Genotyping

In the RS-I, RS-II and RS-III cohorts, DNA was genotyped with the Illumina Infinium II HumanHap550 chip v3.0 array. In the ERF study, DNA was genotyped on 4 different platforms (Illumina 6k, Illumina 318K, Illumina 370K and Affymetrix 250K), which were then merged. Genotype data were imputed by using HapMap CEU build 35 as the reference population, resulting in over 2.5 million SNPs. For details please see the Supporting Information.

Replication studies

SNPs showing the strongest associations in the discovery phase were carried forward and assessed for association with IOP in 2,235 participants from the TwinsUK Study, 1,807 from the Australian Twin Study, 1,304 from the DCCT/EDIC Study, and 2,136 from the WTCCC2/BMES Study. The TwinsUK, Australian Twin and WTCCC2/BMES were also population-based studies, and participants were ascertained regardless of their phenotypes or clinical status. The DCCT/EDIC study comprised only patients with type 1 diabetes included in a preventive trial. Descriptions of the study populations, clinical examinations, and genotyping methods of the replication cohorts are provided in the Supporting Information and Table S3.

Glaucoma case-control studies

SNPs showing the strongest associations in the discovery and replication phase were also evaluated in 4 series of glaucoma patients. The first series included 188 participants from RS-I in whom the technician measuring IOP was completely ignorant of the presence of glaucoma. Controls were healthy participants of RS-I. The second case-control study was an independent series of 104 glaucoma cases from an isolated population (Genetic Research in an Isolated Population [GRIP] study), with the ERF population as a control group. The third study included 152 cases and 141 controls recruited from all over The Netherlands as part of the Amsterdam Glaucoma Study (AGS). The last case-control study comprised a series of 988 glaucoma cases and 378 controls ascertained in Erlangen and Tübingen, Germany. Details of the clinical evaluation and glaucoma diagnosis in these studies are described in the Supporting Information and Table S4.

Statistical analyses

Discovery analysis

Analyses were performed for the mean IOP of both eyes or for one eye if data on the other eye were missing. In the gene discovery analyses, IOP levels were imputed for those who received IOP-lowering medication or had a history of IOP lowering surgery, because the initial IOP levels were unknown. Based on a reported average of a 30% IOP reduction caused by IOP lowering medication, estimated in a meta-analysis, IOP values of those receiving this medication were divided by 0.7 to estimate pre-treatment IOP.[55] In participants with a history of IOP-lowering surgery, pre-treatment IOP was assumed to be at least 30 mmHg.

Associations between IOP and genome-wide loci were assessed with linear regression models under the assumption of an additive model for the effect of the risk allele. Analyses were adjusted for age and sex. Genomic inflation factors (λ) were calculated to evaluate any population stratification. Analyses were performed with the ProbABEL

package from the ABEL set of programs (http://mga.bionet.nsc.ru/yurii/ABEL/).[56] To adjust for familial relationships of participants in ERF, the score test for relatives was applied by using the genomic kinship matrix as implemented in the GenABEL package of R statistical software (http://cran.r-project.org).[56-58]

The results from the 4 cohorts were subjected to an inverse variance meta-analysis. Genomic control was used to correct the standard errors of the effect estimates before pooling.[59] The genome-wide threshold for statistical significance was set at a p-value of $5x10^{-8}$ to adjust for multiple testing.[60] Meta-analyses were performed with METAL software (http://www.sph.umich.edu/csg/abecasis/metal/index.html).

Results of the discovery meta-analysis were also used to explore regions in the immediate vicinity of the known glaucoma genes (*MYOC*, *OPTN*, *WDR36*) as well as the regions which had approached genome-wide significance in previous GWASs of glaucoma and previous linkage studies of IOP.[9-11, 18, 22]

Replication analysis

Loci which were suggestive ($p<1x10^{-5}$) of association with IOP in the discovery meta-analysis were taken forward to the replication phase. If two or more significantly associated SNPs within a locus were in linkage disequilibrium (LD), only the SNP with the best probability of association (lowest p-value) was selected. Linear regression analyses adjusted for age and sex were performed under the assumption of an additive effect of the risk allele. The results from the discovery and replication cohorts were combined by using an inverse variance meta-analysis (METAL software).

Glaucoma case-control analysis

SNPs that were genome-wide significantly associated with IOP in the meta-analysis of the discovery and replication cohorts were assessed in the 4 glaucoma case-control studies. Logistic regression analyses adjusted for age and sex were performed (SPSS version 15.0 for Windows; SPSS, Chicago, IL) and a pooled effect estimate was calculated (Rmeta software [http://cran.r-project.org/web/packages/rmeta/index.html]).

Gene expression and pathway analyses

Retinal expression data were obtained essentially as described by Booij and colleagues.[61] Human healthy donor eyes (n=4) were collected in collaboration with the Dutch Cornea Bank and snap frozen. History of the donor eyes revealed no glaucoma or other eye diseases. Cryosections (20µm) of the CB were cut and mounted on PEN membrane slides (Carl Zeiss MicroImaging). With the use of laser dissection microscopy, the CB epithelium was cut out. RNA isolation (RNeasy Micro Kit, Qiagen) and amplification (Amino Allyl MessageAmp II aRNA Amplification, Ambion Applied Biosystems) were conducted according to the manufacturers' protocols. After labelling of experimental aRNA with Cy5

and reference aRNA (composed of RPE and choroid) with Cy3, we performed hybridization on catalogue human 4x44k microarrays (Agilent Technologies). Mean expression intensity data were normalized with R software (R Development Core Team, 2009). The mean expression data were further subdivided based on percentiles in Windows Excel. We used the 90th, 50th and 10th percentile of the mean expression intensity to categorize our data into groups with high (>90th), moderate (50th-90th), low (10th-50th) and very low (<10th) expression. Pathway analysis was conducted in Ingenuity Knowledge Base (Ingenuity Systems, www.ingenuity.com). We looked for functional links between *GAS7* (*MLL* in rodents) and *TMCO1* and molecules known to play a role in glaucoma.

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Supporting information for

Common genetic determinants of intraocular pressure and primary open-angle glaucoma

ADDITIONAL METHODOLOGY

Genotyping and imputation methods for the discovery cohorts

In the three cohorts from the Rotterdam Study, DNA was genotyped with the Illumina Infinium II HumanHap550 chip v3.0 array. In the ERF study, DNA was genotyped on four different platforms (Illumina 6k, Illumina 318K, Illumina 370K and Affymetrix 250K), which were then merged. Participants with low call rate (<97.5%), excess heterozygosity (>0.336), or mismatch between reported and genetically determined sex were excluded from the analysis. For each study, single-nucleotide polymorphisms (SNPs) were filtered to satisfy a call rate >98% and a Hardy-Weinberg equilibrium test p-value >$1x10^{-5}$. Genotype data were imputed by using HapMap CEU build 35 as the reference population, resulting in over 2.5 million SNPs (Markov Chain Haplotyping (MaCH) package; http://www.sph.umich.edu/csg/abecasis/MACH). After quality control, a total of 5,974 participants from RS-I, 2,157 (RS-II), 2,082 (RS-III), and 2,385 (ERF) had valid genotype data.

Replication Cohorts

Descriptive characteristics of the replication cohorts are presented in Table S3.

TwinsUK Adult Twin study

Participants were recruited from the TwinsUK Adult Twin Registry, based at St. Thomas' Hospital, London. They were twin volunteers from the general population, and were part of a twin study on glaucoma heritability. Intraocular pressure (IOP) was measured by using the Ocular Response Analyser (ORA-Reichert®, Buffalo, NY), a non-contact air-puff tonometer which ejects an air impulse lasting 20 milliseconds and monitors the time course changes of the cornea by an electro-optical collimation detector system. Genotyping was carried out by using Illumina (San Diego, CA) genotyping platforms; the Human Hap 300k Duo and Human Hap610 Quad array. All SNPs passed quality control criteria (Hardy-Weinberg equilibrium p>0.001, minor allele frequency of at least 0.04, genotyping success rate for the SNP at least 95%). Imputation was calculated with reference to HapMap release 22 CEU by using IMPUTE version 2. Data from 2,235 participants, from 1,417 sibships/families (of which 209 monozygotic), were included in the analyses. For a subset of 2,093 participants from 1,331 sibships, also CCT data were available. The Goldmann-correlated IOP, which the manufacturers have calibrated with Goldmann applanation tonometry, was used as the outcome measure most comparable with the discovery cohort in this study. The mean IOP was calculated from 4 readings (2 from each eye) for each participant. Every association analysis was performed by using Merlin, given the family data. Zigocity was included in the model and was useful in modelling environmental variance.

Australian Twin study

The Australian Twin Eye Study comprises participants examined as part of the Twins Eye Study in Tasmania or the Brisbane Adolescent Twins Study. In most participants, the IOP was measured with the TONO-PEN XL (Reichert, Inc. New York, USA) as outlined in Mackey et al.[1] The Australian cohorts were genotyped on the Illumina Human Hap610W Quad array, with part of the sample typed alongside the TwinsUK cohort and the remainder typed as a separate contract with DeCODE genetics. The inclusion criteria for the SNPs were a minor allele frequency >0.01, Hardy-Weinberg equilibrium $p \geq 10^{-6}$, and a SNP call rate >95% or Illumina Beadstudio Gencall Score ≥0.7, resulting in 543,862 SNPs. Imputation was done with reference to HapMap release 22 CEU by using MACH (http://www.sph.umich.edu/csg/abecasis/MACH/).[2] Data from 1,807 people, from 863 families, were included in the analyses. The mean IOP of both eyes was used as outcome variable. Association analyses were performed in Merlin (http://www.sph.umich.edu/csg/abecasis/merlin/) by using the –fastassoc option. Age, sex and measurement technique (tonopen or Goldmann applanation tonometry) were fitted as covariates. Ancestry, initially determined through self-reporting, was verified through Principal Component decomposition.

Diabetes Control and Complications Trial / Epidemiology of Diabetes Interventions and Complications study (DCCT/EDIC)

The Diabetes Control and Complications Trial (DCCT, 1982-1993) is a multicenter, randomized clinical trial to compare conventional and intensive diabetic treatments with regard to their effects on the development and progression of long-term diabetic complications. The goal of intensive therapy was to normalize plasma glucose level. A total of 1,441 patients with type 1 diabetes were separated into two cohorts (primary prevention cohort and secondary intervention cohort) based on diabetes duration and presence of complications at baseline. The DCCT was prematurely stopped in 1993; after it was conclusively shown that intensive treatment delays the development and progression of long-term diabetic complications. Most of the DCCT subjects were further followed in the Epidemiology of Diabetes Intervention and Complications study (EDIC, 1994-present), an observational study to look at long-term effects of glycemic exposure. During the DCCT, participants went through annual ophthalmic exams. In each visit IOP was measured in both eyes by Goldmann applanation tonometry (on average 6 measurements). Genotyping was performed by using the Illumina 1M chip (San Diego, CA). After quality control measures, data of 841,342 SNPs with a minor allele frequency >0.01 were available. Genotypes for a total of 2.5M SNPs were imputed based on HapMap II CEU. After exclusion of any participants with a history of glaucoma, any prior eye surgery or ophthalmic medications, presence of angle neovascularization, as well as exclusion of any individuals who were likely to be admixed between white Europeans and other

ethnic groups, data from 1,304 participants were included in the analyses.[3] The mean IOP of both eyes was used as the outcome phenotype. To correct potential outliers, the top and bottom 0.5 percentiles of data were winsorized.

Wellcome Trust Case-Control Consortium 2 (WTCCC2) / Blue Mountains Eye Study (BMES)

Participants of the Wellcome Trust Case Control Consortium 2 (WTCCC2) are part of the Blue Mountains Eye Study (BMES), a population-based eye disease survey in individuals living in the Blue Mountains region, west of Sydney, Australia. The BMES protocol has been described in detail previously.[4] Intraocular pressure (IOP) was measured by applanation tonometry with a Goldmann tonometer (Haag-Streit, Bern, Switzerland). Samples were genotyped on the Human660W-Quad. Imputation was performed with IMPUTE2[5] which adopts a two-stage approach using both haploid and diploid reference panels. For the haploid reference panel, we used HapMap2 and HapMap3 SNP data for the 120 non-related CEU trios (see www.hapmap.org), and for the diploid reference we used the 1958 Birth Cohort (58C) and the United Kingdom Blood Service (UKBS) control data, merging genotypes from the Illumina 1.2M Duo chip and Affymetrix Genome Wide Human SNP array 6.0. Outlying individuals on the basis of call rate, heterozygosity, relatedness, ancestry, and signal intensity, or where there was discordance of reported gender and findings of gender specific markers, were excluded. The SNPs considered in this study passed the following quality control criteria in the WTCCC2/BMES data: minor allele frequency higher than 0.01, missing rate lower than 2%, Hardy Weinberg $p > 1x10^{-3}$, Fisher information higher than 0.98 and no plate effect. Imputed SNPs had imputation information higher than 0.90. After exclusion of any participants who had undergone eye surgery, who were on medication designed to lower IOP or who had outlying values of IOP, data from 2,136 individuals were considered in the analysis. The mean IOP of both eyes was considered as the response variable and the analysis was adjusted on age and sex. We performed single SNP analysis under an additive model using missing data likelihood score tests as implemented in SNPTEST.

Glaucoma case-control studies

Demographic and clinical characteristics of the glaucoma cases and controls of the 4 studies are presented in Table S4.

Glaucoma case-control study in RS-I

A total of 188 prevalent and incident glaucoma cases were recruited as part of RS-I. Glaucoma diagnosis was based on glaucomatous visual field loss. Cases were classified as glaucoma if the participant was classified as having glaucomatous visual field loss during at least one of the examination rounds. The visual field of each eye was screened by using a 52-point supra-threshold test that covered the central visual field with a ra-

dius of 24° (Humphrey Field Analyzer [HFA] II 740; Carl Zeiss, Oberkochen, Germany). The test was modified from a standard 76-point screening test and tested the same locations as used in the Glaucoma Hemifield Test.[6, 7] If the first visual field test was unreliable, or a reliable test showed visual field loss in at least one eye, a second supra-threshold test was performed on that eye. In participants in which visual field loss remained present on the second supra-threshold test or the test was unreliable again, Goldmann kinetic perimetry (baseline and first follow-up; Haag-Streit, Bern, Switzerland) or full-threshold HFA testing with 24-2 grid (second follow-up visit) was performed on both eyes by a skilled perimetrist. The classification process of both Goldmann perimetry and full-threshold HFA test results has been described before.[6, 8]

Genetic Research in Isolated Populations (GRIP) program.

A total of 104 patients with glaucoma were recruited in three local hospitals in the region of the ERF population. These patients did not participate in the ERF study, which was used as control population. The diagnosis of glaucoma was made by the ophthalmologist in attendance and verified by a glaucoma specialist (HGL). The diagnosis was based on a glaucomatous appearance of the optic disc (notching or thinning of the neuroretinal rim), combined with a matching glaucomatous visual field defect and open angles on gonioscopy. Visual fields were tested with standard automated perimetry by means of the HFA 24-2 SITA Standard test program or the Octopus 101 (Haag Streit, Bern, Switzerland) G2 program with TOP strategy. Visual field test results had to be reliable and reproducible. Patients with any other known disease that could cause visual field defects were excluded. Genotyping was performed with the 318K array of the Illumina Infinium II whole-genome genotyping assay (HumanHap300-2). Genotyping quality control criteria and methods of imputations were identical to those in the ERF study.

Amsterdam Glaucoma Study

A total of 152 patients with glaucoma and 141 control persons were recruited from eye clinics, meetings of the glaucoma patients' association, nursing homes, and fairs for the elderly. Preferably spouses of cases were used as control persons. If no spouse was available any non-related acquaintance was considered as suitable. In all persons, ophthalmoscopy and biomicroscopy with a 90 diopter lens were performed and digital stereo images of the optic nerve head were taken in mydriasis. Criteria for glaucoma included a glaucomatous optic neuropathy (vertical cup-disc ratio (VCDR) > 0.7) with corresponding glaucomatous visual field loss in at least one eye or a VCDR ≥ 0.8 when no visual field was available. Criteria for a control were age older than 60 years, and a VCDR ≤ 0.6 on fundus photography. Genotyping was performed by means of Taqman®.

Glaucoma case-control studies Erlangen and Tübingen

A total of 988 glaucoma cases and 378 healthy controls were recruited as part of case-control studies in Erlangen and Tübingen, Germany. Controls were age and gender matched to the patients. All participants underwent standardized clinical examinations for glaucoma at the Ophthalmology Department of the University of Erlangen-Nuremberg and at the University Eye Hospital in Würzburg and Tübingen, respectively. The examinations included optic nerve head imaging (Heidelberg Retina Tomograph [HRT] 1 and 2; or biomicroscopy with a Goldmann lens and a Haag-Streit slit lamp), visual field testing, and 24-hour Goldmann applanation tonometry profile with five measurements.[9, 10] Glaucoma was defined as the presence of glaucomatous optic disc damage (as classified according to Jonas)[11, 12] in at least one eye, with a corresponding visual field defect. A pathologic visual field was defined by a pathologic Bebie curve, three adjacent test points with more than 5 dB sensitivity loss, or at least one point with more than 15 dB sensitivity loss. Genotyping was preformed by means of selected pre-developed TaqMan® Genotyping Assays (Applied Biosystems, Foster City, CA, USA), following the manufacturer's instructions.

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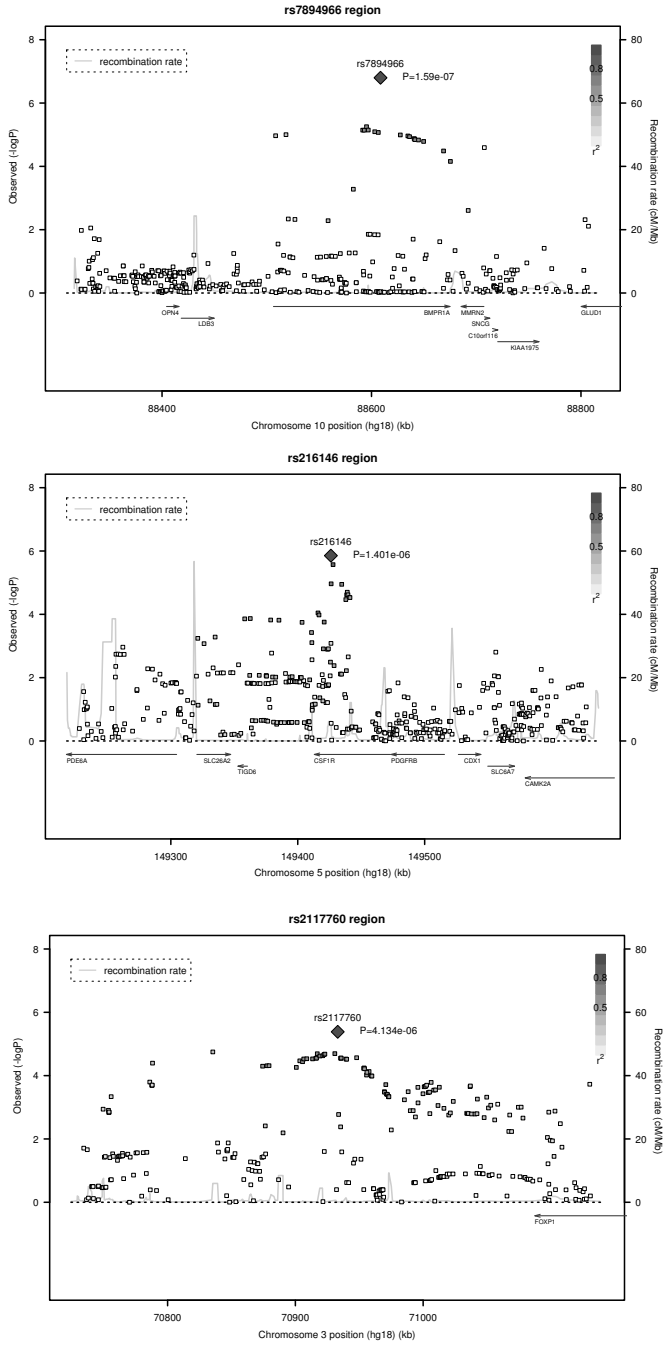

Figure S1. Regional association plots of loci associated with IOP (5x10-8 < p-value < 1x10-5) in the discovery meta-analysis

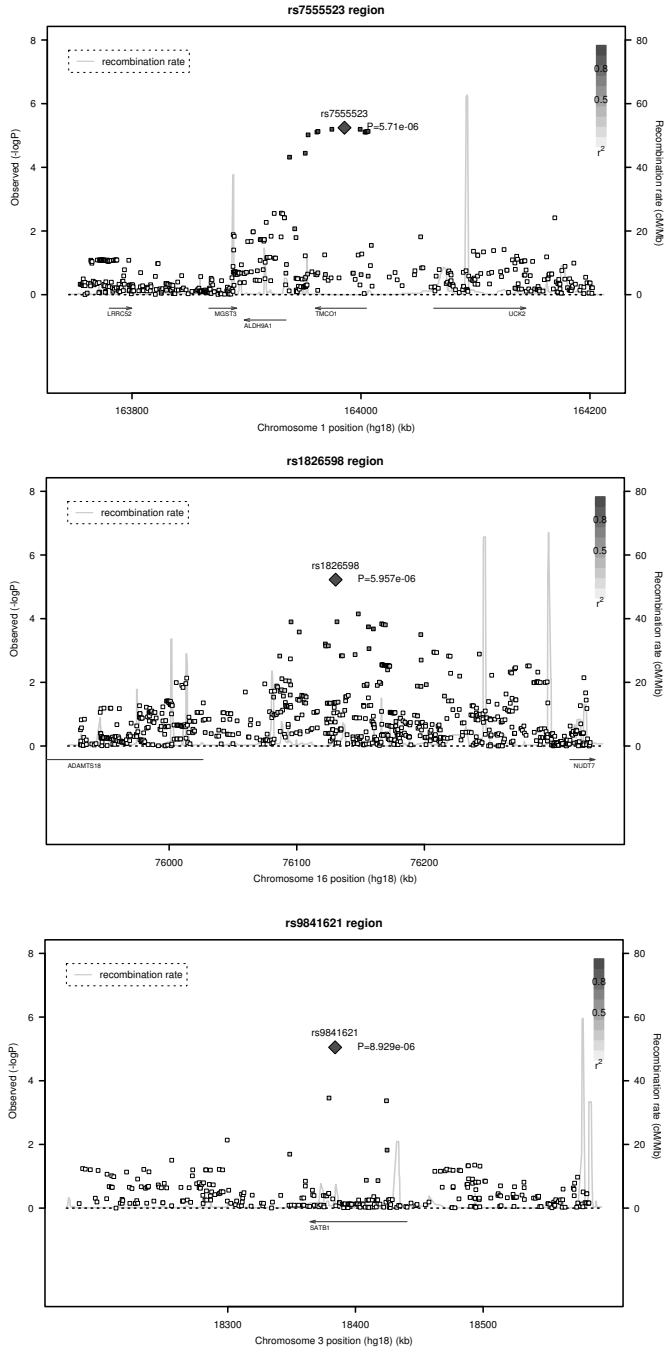

Figure S1. (continued)

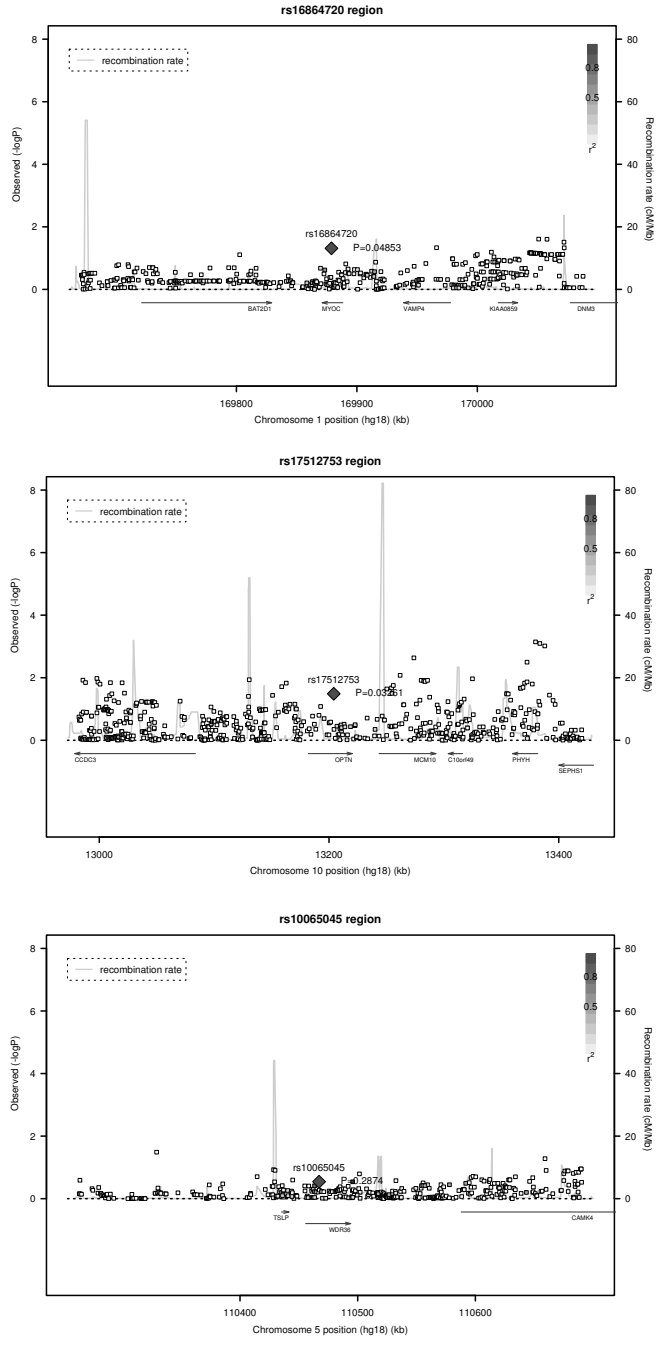

Figure S2. Regional association plots of MYOC, OPTN, and WDR36 regions in the discovery meta-analysis

Table S1. Loci associated with IOP with p-values $<10^{-5}$ after meta-analyses: results of individual cohorts

SNP	Chrom	RS-I			RS-II		
		Beta	SE	P-value	Beta	SE	P-value
rs11656696	17p13.1	-0.351	0.064	$3.4x10^{-8}$	-0.107	0.107	$3.1x10^{-1}$
rs7894966	10q23.2	0.909	0.195	$3.0x10^{-6}$	0.226	0.308	$4.6x10^{-1}$
rs216146	5q32	0.238	0.067	$3.7x10^{-4}$	0.115	0.110	$3.0x10^{-1}$
rs2117760	3p13	0.242	0.070	$5.5x10^{-4}$	0.336	0.114	$3.2x10^{-3}$
rs7555523	1q24.1	0.331	0.097	$6.2x10^{-4}$	0.515	0.162	$1.5x10^{-3}$
rs1826598	16q23.1	0.517	0.103	$5.4x10^{-7}$	0.185	0.171	$2.8x10^{-1}$
rs9841621	3p24.3	-1.079	0.277	$1.0x10^{-4}$	-0.434	0.466	$3.5x10^{-1}$

SNP = single nucleotide polymorphism; Chrom = Chromosome; SE = standard error; RS = Rotterdam Study; ERF = Erasmus Rucphen Family study

Table S2. Association results for SNPs identified in previous association studies

SNP [allele]	Chrom	Freq	Discovery meta-analysis			RS-I		
			Beta	SE	P-value	Beta	SE	P-value
Nakano et al								
rs547984[C]	1q43	0.55	0.03	0.04	4.9E-01	0.03	0.06	6.8E-01
rs540782[G]	1q43	0.57	0.04	0.04	3.6E-01	0.04	0.06	5.0E-01
rs693421[G]	1q43	0.57	0.04	0.04	3.5E-01	0.04	0.06	4.9E-01
rs2499601[T]	1q43	0.46	0.06	0.04	1.9E-01	0.08	0.06	2.3E-01
rs7081455[G]	10p12.31	0.45	0.12	0.04	4.6E-03	0.16	0.06	1.4E-02
rs7961953[G]	12q21.31	0.88	0.01	0.07	8.5E-01	0.07	0.10	4.7E-01
Jiao et al								
rs1533428[T]	2p16	0.30	0.02	0.05	7.0E-01	0.02	0.07	7.6E-01
rs12994401[T]	2p16	0.19	-0.04	0.05	4.8E-01	0.04	0.08	6.2E-01
Thorleifsson et al								
rs4236601[A]	7q31	0.29	0.19	0.05	1.1E-04	0.24	0.07	7.6E-04
rs1052990[G]	7q31	0.36	0.17	0.04	1.6E-04	0.22	0.07	8.7E-04

SNP = single nucleotide polymorphism; Chrom = Chromosome; Freq = Frequency; SE = standard error; RS = Rotterdam Study; ERF = Erasmus Rucphen Family study

Table S1. (continued)

RS-III			ERF		
Beta	SE	P-value	Beta	SE	P-value
-0.241	0.101	$1.7x10^{-2}$	-0.156	0.120	$1.9x10^{-1}$
0.561	0.267	$3.6x10^{-2}$	0.697	0.300	$2.0x10^{-2}$
0.247	0.098	$1.2x10^{-2}$	0.252	0.113	$2.6x10^{-2}$
0.130	0.104	$2.1x10^{-1}$	0.150	0.112	$1.8x10^{-1}$
0.223	0.142	$1.2x10^{-1}$	0.100	0.166	$5.5x10^{-1}$
0.127	0.157	$4.2x10^{-1}$	0.164	0.177	$3.5x10^{-1}$
-0.777	0.422	$6.5x10^{-2}$	-0.620	0.368	$9.2x10^{-2}$

Table S2. (continued)

RS-II			RS-III			ERF		
Beta	SE	P-value	Beta	SE	P-value	Beta	SE	P-value
-0.17	0.11	9.8E-02	0.06	0.10	5.1E-01	0.21	0.11	4.8E-02
-0.17	0.11	1.1E-01	0.08	0.10	3.8E-01	0.19	0.11	7.2E-02
-0.17	0.11	1.2E-01	0.09	0.10	3.6E-01	0.19	0.11	7.3E-02
-0.13	0.10	2.0E-01	0.17	0.09	7.5E-02	0.06	0.11	5.5E-01
0.02	0.11	8.4E-01	0.15	0.10	1.3E-01	0.11	0.11	2.9E-01
-0.25	0.16	1.2E-01	-0.06	0.15	6.9E-01	0.18	0.15	2.2E-01
0.09	0.12	4.5E-01	0.10	0.10	3.4E-01	-0.16	0.12	1.6E-01
-0.14	0.13	2.8E-01	-0.02	0.12	8.8E-01	-0.16	0.13	2.1E-01
0.08	0.11	4.7E-01	0.27	0.11	1.1E-02	0.05	0.12	6.9E-01
0.00	0.11	9.7E-01	0.26	0.10	8.9E-03	0.11	0.11	3.2E-01

Table S3. Characteristics of the replication cohorts

Characteristic	TwinsUK	Australian Twins	DCCT/EDIC	WTCCC2/ BMES
Participants with valid data (N)	2,235	1,807	1,304	2,136
Age (y), mean ± SD (range)	56.8 ± 11.7 (16 – 83)	22.2 ± 12.7 (5 – 90)	26.8 ± 7.1 (13 – 39)	62.8 ± 8.2 (49 – 91)
Male gender (%)	2.5	44	53	43
IOP (mmHg), mean ± SD (range)	15.6 ± 3.1 (7 – 30)	15.8 ± 3.0 (6 – 30)	15.7 ± 2.7 (9 – 22)	15.5 ± 2.8 (8 – 27)
IOP ≥ 22 mmHg (%)	3.3	1.3	1.0	1.9
Participants with IOP lowering treatment (%)	1	0	0	0
Disc area (mm^2), mean ± SD (range)	2.6 ± 0.7 (0.7-7.0)**	2.1 ± 0.4 (1.1-3.6)	*	*
Vertical cup-disc ratio, mean ± SD (range)	0.32 ± 0.10 (0.07-0.70)**	0.45 ± 0.13 (0.09-0.88)	*	0.41 ± 0.14 (0.07-0.95)

* not measured

**available for subset of 843 TwinsUK participants only, mean age 56 years.

IOP = intraocular pressure; SD = standard deviation; DCCT/EDIC = Diabetes Control and Complications Trial / Epidemiology of Diabetes Interventions and Complications study; WTCCC/BMES = Wellcome Trust Case-Control Consortium / Blue Mountains Eye Study

Table S4. Characteristics of the glaucoma case-control studies

	RS-I		GRIP		AGS		Erlangen and Tübingen	
	Cases	Controls	Cases	Controls	Cases	Controls	Cases	Controls
Number of participants	188	5,548	104	2,035	152	141	988	378
Age (y), mean ± SD (range)	75.5 ± 7.4 (56 – 94)	74.5 ± 7.8 (55 – 105)	73.3 ± 9.2 (51 – 91)	48.8 ± 14.4 (18 – 86)	73.1 ± 11.1 (27 – 98)	72.2 ± 8.2 (55 – 92)	66.5 ± 14.1 (12 – 104)	73.9 ± 6.4 (34 – 97)
Male gender (%)	54.8	40.7	47.1	43.3	54.4	43.7	39.0	40.2
IOP (mmHg), mean ± SD (range)	18.2 ± 6.2 (6 – 55)	15.2 ± 3.5 (5 – 59)	25.9 ± 8.6 (12 – 62)	15.3 ± 3.1 (6 – 33)	27.0 ± 8.3 (13 – 54)	*	27.8 ± 9.3 (11 – 65)	<21.0
IOP ≥ 22 mm Hg (%)	20.7	3.3	60.6	1.2	73.3	*	50.8	0
Participants with IOP lowering treatment (%)	19.7	1.7	100.0	0.9	100.0	0.0	99.0	0.0

* not measured

IOP = intraocular pressure; SD = standard deviation; RS = Rotterdam Study; GRIP = Genetic Research in Isolated Populations; AGS = Amsterdam Glaucoma Study

Part 4

STUDIES TO INVESTIGATE ANY ASSOCIATIONS BETWEEN THE EYE AND THE BRAIN

Chapter 4.1

Association of cognitive functioning with retinal nerve fiber layer thickness

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ABSTRACT

Purpose. The brain areas that are responsible for cognitive functioning have the same embryonic origin as the retina. We assessed the association between cognitive functioning and retinal nerve fiber layer (RNFL) thickness in a large, population-based sample.

Methods. Neuropsychological and ophthalmic examinations were performed in 1485 healthy individuals (mean age, 46 years; range, 18 – 85) from the Erasmus Rucphen Family (ERF) study, a study in a genetic isolate from the Netherlands. Different domains of cognitive functioning were assessed with the Dutch Adult Reading Test, the Rey Auditory Verbal Learning Test, semantic fluency, the Trail Making Test, the Stroop Color-Word Test and Block Design. RNFL thickness was measured with scanning laser polarimetry (GDx VCC). The association between cognitive test scores and peripapillary RNFL thickness was studied with linear regression analyses, adjusting for age, sex, level of inbreeding, and refractive error.

Results. After adjustment for confounders, a better cognitive performance was statistically significantly associated with a thicker RNFL in all tests ($P < 0.03$) except for the Stroop Color-Word Test ($P = 0.15$). RNFL thickness explained up to 2.8% ($R^2 = 0.028$) of the total variance in cognitive test scores. The association diminished in age groups beyond 40 years.

Conclusions. The present study shows that cognitive functioning is associated with RNFL thickness in healthy young individuals. The lack of association in older individuals suggests that loss of neurons in the cerebrum and retina is not concomitant and may have different origins.

INTRODUCTION

Cognitive functioning refers to the ability to attend to complex external or internal stimuli, to identify the relevant features of these stimuli, and to make appropriate responses (including storing aspects of this information).[1] In accordance with their complexity and importance in daily life, cognitive functions take up the major part of the central nervous system (CNS).

The eye is the only part of the body that provides a direct view of the CNS. The retina can be examined by means of direct or indirect ophthalmoscopy. Moreover, the thickness of the retinal nerve fiber layer (RNFL), which contains the axons of the retinal ganglion cells, can be objectively measured with imaging techniques such as scanning laser polarimetry (SLP). To what extent these easily quantifiable properties of the retina could provide insight into the concealed parts of the CNS, is largely unknown.

Both the retina and the brain areas that are responsible for cognitive functioning originate from the embryonic prosencephalon. The premise of retinal involvement in cognitive functioning has been supported by studies describing an increased prevalence of glaucoma in patients with Alzheimer's Disease (AD).[2;3] Other supportive evidence comes from histopathologic postmortem studies demonstrating retinal ganglion cell loss in patients with AD,[4;5] and from in vivo studies reporting a reduced RNFL thickness in patients with AD.[6;7] However, these few studies were limited to a small number of selected patients and focused on the extreme disease ends of cognitive and retinal functioning. Whether associations exist in their physiological spectrum is currently unknown. We had the opportunity to study the association between cognitive functioning and RNFL thickness in a large, population-based sample of healthy subjects. We assessed a broad range of cognitive functions by means of an extensive neuropsychological examination and we measured RNFL thickness with SLP. We also assessed whether cognitive functioning was associated with other ophthalmic and non-ophthalmic factors.

METHODS

Study population

Subjects were recruited as part of the Erasmus Rucphen Family (ERF) study, a family-based cohort study in a genetically isolated population in the Netherlands. This population was founded in the middle of the 18th century by fewer than 400 individuals. Eligibility for participation in the study was determined by genealogical background, not by any phenotypes of interest. Twenty-two families were selected who had at least 6 children baptized in the community church between 1880 and 1900. All living descendants of these families aged 18 years and older, as well as their spouses, were invited to attend

a series of clinical examinations. Genetic characterization of the study population has been presented elsewhere.[8-10]

The research adhered to the tenets of the Declaration of Helsinki, and was approved by the Medical Ethics Committee of the Erasmus Medical Centre in Rotterdam. Informed consent was obtained after explanation of the nature and possible consequences of the study.

A total of 1641 subjects underwent neuropsychological and RNFL thickness assessments. Participants with visual acuity of less than 20/40 in the best eye (n = 3), poor quality RNFL thickness measurements in both eyes (n = 21), or missing genealogical information (n = 131) or refractive error data (n = 1) were excluded from the dataset, leaving 1485 subjects for the analysis. For the Trail Making Test, the Stroop Color-Word Test and the Dutch Adult Reading Test, 3 subjects were excluded because of illiteracy, and for the Stroop test, a further 20 subjects were excluded because of color blindness.

Neuropsychological assessment

All neuropsychological tests were performed during a 50-minutes examination in a standardized environment. The Dutch Adult Reading Test (a validated Dutch version of the National Adult Reading Test[11]), consisting of a series of words with irregular pronunciation, was used as a measure of general cognitive ability.[12]

Memory function was assessed by means of the Dutch version of the Rey Auditory Verbal Learning Test.[13;14] Short-term memory was defined as the number of correctly recalled words in the first trial, learning as the sum of correctly recalled words in trials 2 to 5, and delayed recall as the number of correctly recalled words after 20 minutes. Executive functioning was established with three tests: semantic fluency (animals and professions),[15] part B of the Trail Making Test as a measure of concept shifting, [16] and card III of the Stroop Color-Word Test as a measure of susceptibility to interference.[17] Visuospatial and visuoconstructive abilities were assessed with the subtest Block Design of the Wechsler Adult Intelligence Scale and were scored by the number of correctly replicated geometric designs adjusted for time of completion.[18]

RNFL thickness measurements

RNFL thickness was evaluated with scanning laser polarimetry (SLP). This technique, featured in the GDx VCC (Carl Zeiss Meditec, Inc., Dublin, CA, USA), measures the retardation of a polarized scanning laser beam induced by the birefringence of retinal ganglion cell axons. It subsequently converts this retardation into RNFL thickness.

Two GDx VCC scans were performed per eye. The first scan assessed anterior segment birefringence with the method described by Zhou and Weinreb.[19] The GDx VCC software then automatically adjusted the anterior segment compensator to each individual eye. Subsequently, the second scan was performed to measure RNFL thickness as described

by Reus and Lemij.[20] The cut-off for image quality was a GDx VCC scan quality score of 8 or above. Images with lower scores were excluded.

After the boundaries of the optic disc were manually marked, the software positioned two circles centered on the disc: the first had a diameter of ~2.5 mm (54 pixels), the second a diameter of ~3.3 mm (70 pixels). The average RNFL thickness between these circles was used in our analyses.

Statistical analysis

If high-quality GDx VCC images could be obtained in both eyes, one eye was chosen randomly for inclusion in the analysis. If a high-quality image could be obtained in only one eye of a subject, this eye, rather than its fellow, was considered for analysis. The inbreeding coefficient, which represents the degree of consanguinity between a subject's parents, was calculated by means of PEDIG software.[21] Statistical analyses were performed with commercial software (SPSS version 11.0 for Windows; SPSS, Chicago, IL).

Characteristics of the study population were evaluated for men and women separately, and differences were tested with an independent samples t-test (normally distributed continuous variables), a Mann Whitney U test (not normally distributed continuous variables), or a Pearson Chi-square test (dichotomous variables). Multiple regression analyses were performed with cognitive test scores as outcome variables and RNFL thickness, age, sex, inbreeding coefficient and the spherical equivalent of the refractive error as predictor variables. The distribution of the multiple regression residuals was tested for normality with the non-parametric, one-sample Kolmogorov-Smirnov test. Outcome variables that were skewed were transformed by means of natural logarithm (Trail Making Test part B, Stroop Color-Word Test card III) or square root (Rey Auditory Verbal Learning Test trial 1, Block Design). Subsequently, systolic and diastolic blood pressure, blood cholesterol level, and fasting blood glucose level were added as predictor variables. The analyses were also stratified by age tertiles.

RESULTS

Demographic and clinical characteristics of the study population are presented in Table 1. Ages ranged from 18 to 85 years, with a mean of 45.9 years. The majority of subjects (78%) had consanguineous parents, and the median inbreeding coefficient indicated that these parents shared a common ancestor 4 to 5 generations back. Men and women differed statistically significantly in educational level and in cognitive performance on some of the tests. Women performed significantly better than men on the Rey Auditory Verbal Learning Test ($P < 0.001$). Men performed significantly better on Block Design ($P < 0.001$).

Table 1. Characteristics of the Study Population

	Men (N =679)	Women (N=806)
Age, mean (yrs) ± SD	46.5 ± 14.0	45.4 ± 13.8
Inbreeding > 0, %	78.6	77.5
Inbr coeff, median (2.5th– 97.5th percentiles)	0.00123 (0.00000 – 0.02237)	0.00062 (0.00000 – 0.02131)
Highest level of education†		
Elementary school, %	29.8	28.0
Junior vocational training, %	60.6	67.5
Senior vocational or academic training, %	9.6	4.6
Syst blood pressure†, mean (mm Hg) ± SD	141.6 ± 16.6	134.7 ± 19.9
Diast blood pressure†, mean (mm Hg) ± SD	81.3 ± 9.4	78.9 ± 9.9
Blood cholesterol, mean (mmol /L) ± SD	5.5 ± 1.1	5.6 ± 1.0
Blood glucose†, mean (mmol/L) ± SD	4.7 ± 1.0	4.3 ± 0.8
Ophthalmic		
Randomized eye right, %	46.1	50.9
RNFL thickness, mean (µm) ± SD	57.6 ± 6.2	57.7 ± 6.2
Spherical equivalent of refraction, mean (D) ± SD	- 0.2 ± 1.9	0.0 ± 1.8
Cognitive functioning *median test scores* (2.5th– 97.5th percentiles)*		
15 word test I †	4 (1 – 8)	5 (2 – 8)
15 word test II-V †	33 (14 – 49)	36 (18 – 52)
15 word test VI †	7 (2 – 13)	8 (2 – 14)
Semantic fluency	38 (21 – 58)	37 (20 – 56)
Trail Making Test B	75 (33 – 208)	73 (34 – 230)
Stroop Color-Word Test part III †	96 (66 – 180)	93 (61 – 171)
Block Design †	29 (11 – 65)	24 (10 – 63)
Dutch Adult Reading Test	66 (20 – 92)	64 (20 – 90)

SD = standard deviation; Inbr coeff = inbreeding coefficient; syst = systolic; diast = diastolic; RNFL = retinal nerve fiber layer;

Differences between the groups were tested for statistical significance with an independent samples t-test for normally distributed continuous variables, with the Mann Whitney U test for not normally distributed continuous variables, and with the Pearson Chi-square test for dichotomous variables.

† $p < 0.05$

* The 15 word test I was scored by the number of correctly recalled words in the 1st trial; 15 word test II-V by the sum of correctly recalled words in the 2nd to 5th trials; 15 word test VI by the number of correctly recalled words after 20 minutes; semantic fluency by the sum of correctly named items in two categories (animals and professions); Trail Making Test B by the time in seconds to completion of the task; Stroop Colour-Word test part III by the time in seconds to completion of the task; Block Design by the number of correctly replicated geometric designs; the Dutch Adult Reading Test by the number of correctly pronounced words.

Table 2. Multiple linear regression analyses of RNFL thickness and other covariates on cognitive functioning

	RNFL thickness	Age	Male gender	Inbreeding	Spherical equivalent
15 word test I ‡	0.004 (0.002); p = 0.012	- 0.012 (0.001); p < 0.001	- 0.090 (0.021); p < 0.001	- 0.016 (0.009); p = 0.079	- 0.012 (0.006); p = 0.040
15 word test II-V	0.098 (0.031); p = 0.002	- 0.312 (0.015); p < 0.001	- 3.243 (0.383); p < 0.001	- 0.342 (0.171); p = 0.046	- 0.324 (0.110); p = 0.003
15 word test VI	0.024 (0.011); p = 0.026	- 0.084 (0.005); p < 0.001	- 0.909 (0.131); p < 0.001	- 0.136 (0.058); p = 0.020	- 0.110 (0.038); p = 0.003
Semantic fluency	0.099 (0.038); p = 0.009	- 0.237 (0.018); p < 0.001	1.005 (0.467); p = 0.032	- 0.890 (0.209); p < 0.001	- 0.360 (0.134); p = 0.007
Trail Making Test B * ‡	- 0.006 (0.002); p = 0.001	0.019 (0.001); p < 0.001	- 0.005 (0.022); p = 0.829	0.031 (0.010); p = 0.001	0.017 (0.006); p = 0.007
Stroop Color-Word Test part III * ‡	- 0.002 (0.001); p = 0.150	0.010 (0.001); p < 0.001	0.016 (0.014); p = 0.239	0.001 (0.006); p = 0.915	0.004 (0.004); p = 0.295
Block Design ‡	0.015 (0.005); p = 0.001	- 0.054 (0.002); p < 0.001	0.333 (0.059); p < 0.001	- 0.087 (0.026); p = 0.001	- 0.066 (0.017); p < 0.001
Dutch Adult Reading Test	0.305 (0.071); p < 0.001	- 0.414 (0.034); p < 0.001	1.819 (0.880); p = 0.039	- 2.669 (0.395); p < 0.001	- 1.246 (0.251); p < 0.001

Presented data are regression coefficients (SE) and p-values in a multiple regression model including age, gender, inbreeding and refractive error.

SE = standard error

* Higher test scores represent lower cognitive performance

‡ transformed variables

The results of the multiple linear regression analyses are provided in Table 2. Better cognitive performance was statistically significantly associated with a higher RNFL thickness in all tests ($P < 0.03$) except for the Stroop Color-Word Test card III ($P = 0.15$). These results were independent of age, sex, level of inbreeding, refractive error and also of additional possible confounders including systolic and diastolic blood pressure, serum cholesterol, and fasting glucose levels. Older age was statistically significantly associated with a lower cognitive performance on all tests ($P < 0.001$). Inbreeding and spherical equivalent of any refractive error were inversely related to all cognitive tests, except for the Stroop Color-Word Test card III, although the association between inbreeding and the first trial of the 15-word test did not attain statistical significance ($\beta = -0.016$; $P = 0.079$).

Univariate linear regression of RNFL thickness on cognitive functioning showed R^2 (coefficient of determination) values ranging from 0.012 for the Stroop Color-Word Test card III to 0.028 for the Dutch Adult Reading Test. The multiple regression model with RNFL thickness, age, sex, level of inbreeding, and spherical equivalent of refractive error as predictor variables had R^2 values in the range from 0.164 (semantic fluency) to 0.365 (Block Design).

When stratified by age tertiles (Table 3) the association between cognitive functioning and RNFL thickness remained significant for 6, 2, and 1 of the 8 cognitive tests in the categories 18-39 years of age, 39-53 years of age, and 53 years of age or older, respectively.

Table 3. Multiple linear regression analyses of RNFL thickness on cognitive functioning stratified by age tertiles

	Age 18.0 – 38.9 y	Age 38.9 – 52.5 y	Age 52.6 – 85.3 y
15 word test I ‡	0.006 (0.003); p = 0.047	0.004 (0.003); p = 0.196	0.001 (0.003); p = 0.676
15 word test II-V	0.120 (0.050); p = 0.016	0.047 (0.058); p = 0.423	0.101 (0.054); p = 0.061
15 word test VI	0.036 (0.017); p = 0.039	- 0.002 (0.020); p = 0.912	0.026 (0.018); p = 0.155
Semantic fluency	0.109 (0.061); p = 0.075	0.094 (0.070); p = 0.178	0.082 (0.063); p = 0.192
Trail Making Test B * ‡	- 0.005 (0.002); p = 0.031	- 0.007 (0.003); p = 0.044	- 0.007 (0.004); p = 0.060
Stroop Color-Word Test part III * ‡	0.000 (0.002); p = 0.810	- 0.002 (0.002); p = 0.184	- 0.002 (0.002); p = 0.327
Block Design ‡	0.025 (0.009); p = 0.003	0.017 (0.009); p = 0.071	0.004 (0.007); p = 0.593
Dutch Adult Reading Test ‡	0.245 (0.085); p = 0.004	0.278 (0.139); p = 0.046	0.400 (0.146); p = 0.006

Presented data are regression coefficients (SE) and p-values for RNFL thickness in a multiple regression model including age, gender, inbreeding, and refractive error.
SE = standard error
* Higher test scores represent lower cognitive performance
‡ transformed variables

DISCUSSION

The present population-based study demonstrated an association between cognitive functioning and RNFL thickness in their physiological range. This association particularly manifested in young to middle-aged adults and diminished in individuals 40 years of age or older. RNFL thickness explained only a small proportion of the variance in cognitive functioning.

The design of our study had three limitations. First, an isolated population may differ from the general population in its genetic and environmental composition. Our results may therefore not apply to other populations. However, simulation studies have shown that common allele frequencies in the ERF population do not deviate from the general population.[9] Including the inbreeding coefficient as a potential confounding factor in our analyses did not change the results. We therefore believe that the association between the level of cognitive functioning and RNFL thickness may be generalized to the outbred population. Second, a cross-sectional study design does not allow for inferences on causal relationships. To address these, longitudinal studies would be needed. Third, we did not determine the reproducibility of the RNFL thickness measurements in this population. Previous studies on intersession and interoperator variability in other populations have shown that the GDx VCC provides highly reproducible measurements.[22-25] We have no reason to suspect a lower reproducibility in our healthy and relatively young population, in which cataract and other ocular conditions that could interfere with measurement variability are presumably less prevalent. Strengths of our study are the large sample size, the extensive assessment of different cognitive domains, the objective RNFL thickness measurements and the adjustments for a variety of confounders.

Previous studies have concentrated on the pathophysiological spectrum of cognitive functioning and retinal properties. In addition to an increased prevalence of glaucoma in patients with AD,[2;3] common pathogenetic mechanisms for these neurodegenerative diseases have been suggested. These include the role of apolipoprotein E and amyloid-β.[26-28] Histopathologic and in vivo studies have reported retinal ganglion cell loss in patients with AD.[4-7] A study by Iseri et al. hinted at a possible correlation between the amount of retinal ganglion cell loss and the severity of cognitive impairment in a group of 14 patients with AD, but should be interpreted cautiously, as no adjustments for age (a potentially important confounder) had been made, and inconsistencies between the different retinal measurements were reported.[6]

Our finding of a reduced association between cognitive functioning and RNFL thickness at older age is inconsistent with results in previous studies. Considering the particular strong effects in the young age category, we think that the relationship between a higher level of cognitive functioning and a thicker RNFL may reflect a better development of tissues that originate from the prosencephalon as a whole. We also speculate that any

damage to any of these tissues, including the RNFL, would be unlikely to run an equal course. Generalised loss might, in principle, differentially affect the various neuronal tissues of prosencephalic origin. Selective loss to the RNFL, for instance, could be caused by increased intraocular pressure (glaucoma), which would be unlikely to affect cognitive functioning. Glaucoma and other ocular diseases particularly manifest in the elderly population. One might therefore expect the relationship between RNFL thickness and cognitive functioning to weaken with age, which is what we have indeed found.

Most previous studies into neurologic diseases have used optical coherence tomography (OCT) to measure the RNFL thickness in vivo.[6;7;29;30] The principle of OCT is analogous to the ultrasound B-scan, but instead of sound OCT uses interference patterns of backscattered near-infrared light.[31] Although this technique is fundamentally different from SLP, RNFL measurements of both techniques have been shown to correlate well and to have a comparable diagnostic accuracy for glaucoma.[32;33] SLP measurements are based on the birefringence of the RNFL, which is assumed to be induced by the ganglion cell axons without any contribution from the supporting astrocytes and Müller cells.[34;35] This technique may therefore provide a more direct measure of pathology in for example AD, in which an increased astrocyte/neuron ratio has been reported.[36] However, this may not apply to our study, in which we only consider the physiological spectrum of cognitive functioning.

Our study showed that higher scores on cognitive tests were associated with a more negative refractive error. This finding is consistent with those in previous studies, which have reported that intelligence and educational attainment, both correlates of cognitive functioning,[37-39] are significantly related to myopia.[40-43]

The coefficient of determination (R^2) of the multiple regression model ranged from 0.16 for semantic fluency to 0.37 for Block Design. This indicates that the predictor variables in the model together account for 16% to 37% of the total variance in cognitive test scores. RNFL thickness alone explained up to 2.8% of the variance in cognitive test scores. Hence, despite the statistically significant slope of the regression line, RNFL thickness is by no means a precise predictor of cognitive ability. The tempting idea of making inferences about cognitive functioning or decline by easily and safely measuring RNFL thickness is therefore not realistic at the present time. Technologies for in vivo imaging of the retina have recently advanced quickly. The latest devices offer an axial imaging resolution of 2-3 µm, thus approaching the level of detail achieved in histopathology.[44] It would be of interest to investigate the association between cognitive functioning and these more accurate RNFL thickness measurements and to explore the applicability of the new imaging devices to neurologic practice.

In conclusion, our study is the first to our knowledge to show a significant association between the level of cognitive functioning and RNFL thickness in a healthy population. Although any clinical implications would currently be limited, our results may warrant further investigations into the causality and future applicability of this association.

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Chapter 4.2

The effect of the Apolipoprotein E gene on the optic disc and retinal nerve fiber layer

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ABSTRACT

Purpose. The Apolipoprotein E (*APOE*) ε2/3/4 polymorphism is known to affect the risk of neurodegenerative diseases such as Alzheimer's disease. The role of *APOE* in the pathogenesis of glaucoma is controversial. We investigated the association between the *APOE* ε2/3/4 polymorphism and quantitative endophenotypes of glaucoma in a large Caucasian population.

Methods. The study population comprised 1878 participants of the Erasmus Rucphen Family (ERF) study, a family-based cohort study in an isolated population in the Netherlands. We assessed optic disc parameters with confocal scanning laser ophthalmoscopy and retinal nerve fiber layer (RNFL) thickness with scanning laser polarimetry. We investigated whether these endophenotypes of glaucoma were associated with the number of *APOE**4 alleles as well as with *APOE* genotypes.

Results. The *APOE**4 allele was not associated with any glaucoma endophenotype. *APOE**2 was borderline significantly associated with more favourable optic disc parameters but not with a thicker RNFL.

Conclusions. The borderline significant association between *APOE**2 and optic disc parameters calls for more extensive follow-up studies. This variant may have a role in a common etiological pathway of glaucoma and Alzheimer's disease.

INTRODUCTION

The Apolipoprotein E (*APOE*) ε2/3/4 polymorphism is known to affect the risk of neurodegenerative diseases such as Alzheimer's disease (AD). The allele encoding the ε4 variant (*APOE**4) has been associated with a 3.68 [3.30-4.11] fold increased risk of AD, based on a meta-analysis of all studies published to date (www.alzgene.org). *APOE**2 has been associated with a 0.62 [0.46-0.85] reduction in the risk of AD (www.alzgene.org). The relationship between *APOE* and AD may be attributed to the neurodegeneration which underlies AD but there is also evidence that *APOE* determines neuronal capacity already early in life. *APOE* has also been implicated in ophthalmic disorders including age-related macular degeneration (AMD), for which the *APOE**4 allele was associated with a reduced risk.[1] We have previously found an association between cognitive functioning and retinal nerve fiber layer (RNFL) thickness, an important parameter in glaucoma (see Chapter 4.1).[2] The role of *APOE* in the pathogenesis of glaucoma is controversial.[3,4] We investigated the association of the *APOE* ε2/3/4 polymorphism with quantitative endophenotypes of glaucoma. We assessed the optic disc parameters disc area (the size of the optic disc area), neuroretinal rim area and vertical cup-to-disc ratio (both measures of neuronal capacity, or indirectly, of any optic nerve degeneration), and cup shape measurement (an index of depth variation and steepness of the cup walls). We further studied the RNFL thickness, which was related to cognitive functioning in our earlier study.

METHODS

The study population comprised 1878 participants of the Erasmus Rucphen Family (ERF) study, a family-based cohort study in an isolated population in the Netherlands. Participants of the ERF study are not selected on any disease phenotype. Optic disc area, neuroretinal rim area, vertical cup-to-disc ratio (VCDR) and cup shape measurement were determined by confocal scanning laser ophthalmoscopy (HRT II; Heidelberg Engineering GmbH, Dossenheim, Germany), and RNFL thickness by scanning laser polarimetry (GDx VCC; Carl Zeiss Meditec, Inc., Dublin, CA, USA). Details of these measurements have been provided elsewhere.[5] *APOE* genotypes were determined on 5 ng/µl genomic DNA samples by means of Taqman allelic discrimination technology on an ABI Prism 7900 HT Sequence Detection System with SDS v 2.1 (Applied Biosystems, Foster City, CA).

One eye was randomly selected for inclusion in the analysis. Associations between optic disc parameters, RNFL thickness and the number of *APOE**4 alleles were assessed by means of multiple linear regression analyses adjusted for age and sex. Associations between optic disc parameters, RNFL thickness and *APOE* genotypes were assessed

by means of one-way ANOVA and independent-samples t tests. Since we analyzed a specific candidate gene and its functionally relevant alleles, no adjustments were made for multiple testing. The six possible genotypes for *APOE* were analyzed separately, with those homozygous for the wildtype allele (*APOE*33) as reference. Further, the data on the strongest risk allele were grouped into *APOE**4 carriers (heterozygotes: *APOE*24, *APOE*34; and homozygotes: *APOE*44). Those carrying the protective allele (*APOE**2) were grouped into *APOE**2 carriers (heterozygotes: *APOE*23; and homozygotes; *APOE*22). Following the findings in AD research, those with an *APOE*24 genotype were classified as *APOE**4 carriers. *APOE**2 and *APOE**4 carriers were compared to those homozygous for the *APOE**3 variant. To maximize the contrast, we also compared the *APOE**2 and *APOE**4 carriers to each other.

Table 1. Characteristics of the Study Population (N=1878)

Characteristic	
Age (y)	47.2 ± 13.9
Male gender, %	44.6
Optic disc parameters (N = 1878)	
Disc area (mm^2)	1.90 ± 0.36
Rim area (mm^2)	1.48 ± 0.26
VCDR	0.31 ± 0.22
CSM	- 0.19 ± 0.07
RNFL thickness parameters (N=1280)	
RNFL thickness (µm)	57.66 ± 6.02
RNFL thickness superior (µm)	69.56 ± 8.42
RNFL thickness inferior (µm)	65.36 ± 8.64
APOE characteristics	
Genotype distribution, %	
ε2 / ε2	0.1
ε2 / ε3	7.2
ε2 / ε4	1.9
ε3 / ε3	54.1
ε3 / ε4	32.3
ε4 / ε4	4.3
Allele distribution, %	
ε2	4.7
ε3	73.9
ε4	21.4

Data shown for age and ophthalmic characteristics are means ± standard deviation; Data shown for male gender and APOE characteristics are percentages.
VCDR = vertical cup-to-disc ratio; CSM = cup shape measurement; RNFL = retinal nerve fiber layer; APOE = Apolipoprotein E.

Table 2. Association between optic disc parameters, RNFL thickness and the APOE ε4 allele

	Optic disc parameters					**RNFL thickness parameters**			
ε4 alleles	*N (%)*	Disc area (mm2)	Rim area (mm2)	VCDR	CSM	*N (%)*	RNFLt (μm)	RNFLt sup (μm)	RNFLt inf (μm)
0	*1154 (61.4)*	1.90 ± 0.35	1.48 ± 0.27	0.31 ± 0.22	-0.18 ± 0.07	*792 (61.9)*	57.66 ± 6.12	69.45 ± 8.50	65.32 ± 8.81
1	*643 (34.2)*	1.89 ± 0.36	1.45 ± 0.24	0.32 ± 0.22	-0.19 ± 0.07	*443 (34.6)*	57.63 ± 5.97	69.66 ± 8.30	65.35 ± 8.41
2	*81 (4.3)*	1.88 ± 0.38	1.47 ± 0.30	0.31 ± 0.20	-0.19 ± 0.07	*45 (3.5)*	59.03 ± 5.31	71.15 ± 7.53	67.29 ± 8.75
P-value *		0.29	0.13	0.69	0.26		0.39	0.23	0.35

Data shown are means ± standard deviation

* P-value of linear regression analyses

VCDR = vertical cup-to-disc ratio; CSM = cup shape measurement; RNFLt = retinal nerve fiber layer thickness; sup =superior; inf = inferior

Table 3. Association between optic disc parameters, RNFL thickness and the APOE genotype

	Optic disc parameters					**RNFL thickness parameters**			
Genotype	*N (%)*	Disc area (mm2)	Rim area (mm2)	VCDR	CSM	*N (%)*	RNFLt (μm)	RNFLt sup (μm)	RNFLt inf (μm)
ε2 / ε2	*2 (0.1)*	1.66 ± 0.24	1.50 ± 0.01	0.13 ± 0.18	-0.22 ± 0.001	*1 (0.1)*	62.45	78.59	70.75
ε2 / ε3	*136 (7.2)*	1.91 ± 0.32	1.52 ± 0.24	0.28 ± 0.21	-0.20 ± 0.06	*96 (7.5)*	57.79 ± 6.17	69.09 ± 8.12	65.16 ± 8.64
ε2 / ε4	*36 (1.9)*	1.80 ± 0.31	1.40 ± 0.23	0.33 ± 0.21	-0.18 ± 0.06	*23 (1.8)*	56.89 ± 5.14	68.71 ± 6.77	66.11 ± 6.70
ε3 / ε3	*1016 (54.1)*	1.90 ± 0.36	1.47 ± 0.27	0.31 ± 0.22	-0.18 ± 0.07	*695 (54.3)*	57.64 ± 6.12	69.48 ± 8.55	65.34 ± 8.84
ε3 / ε4	*607 (32.3)*	1.90 ± 0.37	1.46 ± 0.24	0.31 ± 0.22	-0.19 ± 0.07	*420 (32.8)*	57.67 ± 6.02	69.71 ± 8.38	65.31 ± 8.50
ε4 / ε4	*81 (4.3)*	1.88 ± 0.38	1.47 ± 0.30	0.31 ± 0.20	-0.19 ± 0.07	*45 (3.5)*	59.03 ± 5.31	71.15 ± 7.53	67.29 ± 8.75
P-value *		0.49	0.12	0.49	0.23		0.65	0.63	0.73

Data shown are means ± standard deviation

* P-value of one-way ANOVA

VCDR = vertical cup-to-disc ratio; CSM = cup shape measurement; RNFLt = retinal nerve fiber layer thickness; sup =superior; inf = inferior

RESULTS

Characteristics of the study population are presented in Table 1. The average age of the participants was 47.2 years (standard deviation 13.9). One percent of the participants received IOP-lowering therapy or had a history of these medications. Results of the association analyses are presented in Tables 2 and 3. There was no statistically significant association between optic disc parameters, RNFL thickness, and the number of *APOE**4 alleles (Table 2). Table 3 shows the results of the genotype-specific analyses, which also did not show any association between optic disc parameters, RNFL thickness, and *APOE**4. However, participants with the *APOE*22 and *APOE*23 genotypes tended to have larger rim areas ($p = 0.07$) when compared with *APOE*33. A significant difference emerged ($p = 0.01$) when *APOE**2 carriers were compared with *APOE**4 carriers. This translates into a lower VCDR in *APOE**2 carriers when compared with *APOE*33 ($p = 0.10$) and with *APOE**4 carriers ($p = 0.08$), although these findings were not statistically significant. Consistently, *APOE**2 carriers had a lower cup shape measurement ($p = 0.02$) when compared with *APOE*33. The same trend (although not significant; $p = 0.11$) was seen when *APOE**2 carriers were compared with *APOE**4 carriers. *APOE**2 was not associated with any RNFL thickness parameters.

DISCUSSION

The findings from this study did not provide evidence of any association between glaucoma endophenotypes and the *APOE**4 allele in a healthy population. In AD, this allele is primarily associated with the underlying neurodegenerative process. We found some evidence that *APOE**2 was associated with more favourable optic disc parameters in this healthy population. *APOE**2 is related to a reduced risk of AD. In addition, *APOE**2 has been associated with larger regional cortical thicknesses and volumes in patients with mild cognitive impairment and AD.[6] An association of *APOE**2 with a larger rim area would be in line with these findings and may suggest that this variant is related to the amount of nerve fibers. However, our findings were borderline significant and the association did not consistently emerge from the RNFL thickness analyses. Although our results are inconclusive, they do warrant further investigation of any associations between *APOE**2 and ophthalmic parameters representing the amount of nerve fibers in a more extensive study. Moreover, it will be of interest to further explore whether *APOE**2 has any protective effect against glaucoma.

The high prevalence of glaucoma in patients with Alzheimer's disease may suggest a common pathogenetic mechanism.[7] Our results do not support that the most common genetic risk factor for AD (*APOE**4) is implicated in the major endophenotypes of

glaucoma. Protein pathway analyses have suggested that *APOE* interacts with several genes implicated in glaucoma and intraocular pressure (its major risk factor), including *CAV1*, *GAS7* and *TMCO1*. The findings of our study may indicate that the *APOE**2 variant, by determining nerve capacity, has a role in a common pathogenetic mechanism of AD and glaucoma.

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Part 5

GENERAL DISCUSSION

Chapter 5.1

Gene-finding in glaucoma

Primary open-angle glaucoma (hereafter referred to as glaucoma) is a major eye disease in the elderly and is the leading cause of irreversible blindness worldwide. There is ample evidence supporting a genetic etiology of glaucoma. However, identifying the specific genes involved has proven difficult. The genes that have been consistently implicated so far (*MYOC*, *OPTN*, and *WDR36*)[1-3] are relevant only in a limited number of families and contribute to the pathogenesis of glaucoma in less than 5% of the cases in the general population. Genes explaining a greater proportion of the known heritable component of glaucoma thus remain to be identified.

The studies presented in this thesis were performed to gain insight into the genetic etiology of glaucoma by identifying genes for quantitative traits of this disease. These quantitative traits were either clinical markers of glaucoma (such as vertical cup-to-disc ratio [VCDR]) or a major risk factor (intraocular pressure [IOP]). We initially investigated the genetic contributions to these traits in a heritability study and found strong evidence that these traits are highly genetically determined. In a subsequent commingling study our findings further suggested the presence of a major gene accounting for the variance in VCDR. The results from these explorative studies strongly supported further efforts to identify the genes responsible for quantitative glaucoma traits. We consequently performed genome-wide association studies (GWASs) for optic disc parameters and IOP. With regard to the first, we found genome-wide significant evidence for the association of 3 chromosomal regions with the size of the optic disc area, and 7 chromosomal regions with VCDR. Although multiple genes could be implicated in these regions, the most interesting ones were *ATOH7* at chromosome 10q21.3-22.1 (associated with both traits), *CDC7 / TGFBR3* at 1p22 and *SALL1* at 16q12 (both associated with optic disc area), and *CDKN2A / CDKN2B* at 9p21, *SIX1 / SIX6* at 14q22-23, *SCYL1 / LTBP3* at 11q13, *CHEK2* at 22q12.1, *DCLK1* at 13q13 and *BCAS3* at 17q23 (all associated with VCDR). For IOP, we found genome-wide significant evidence for associations with common variants in *GAS7* at 17p13.1 and in *TMCO1* at 1q24.1. The chromosomal regions identified by our GWASs, in addition to the regions previously implicated in glaucoma, have been presented in Figure 1 and summarized in Table 1.

Expression studies, protein pathway analyses, and previously reported functional data allude to several pathogenetic mechanisms through which the identified genes may contribute to the development of glaucoma (Figure 2). For each quantitative trait we identified genes that are involved in the transforming growth factor (TGF) beta signalling pathway. The TGF-β family of cytokines regulates proliferation, differentiation, adhesion, migration, and other functions in many cell types. TGF-β1 and TGF-β2 have already been implicated in glaucoma, by both regulating trabecular outflow and remodelling the lamina cribrosa.[4,5] Other cell cycle regulation pathways also emerged from our study. *CHEK2*, *CDKN2A*, and *CDC7* are involved in the p53 signal transduction pathway. P53 is an important regulator of apoptosis, which is thought to be the main mecha-

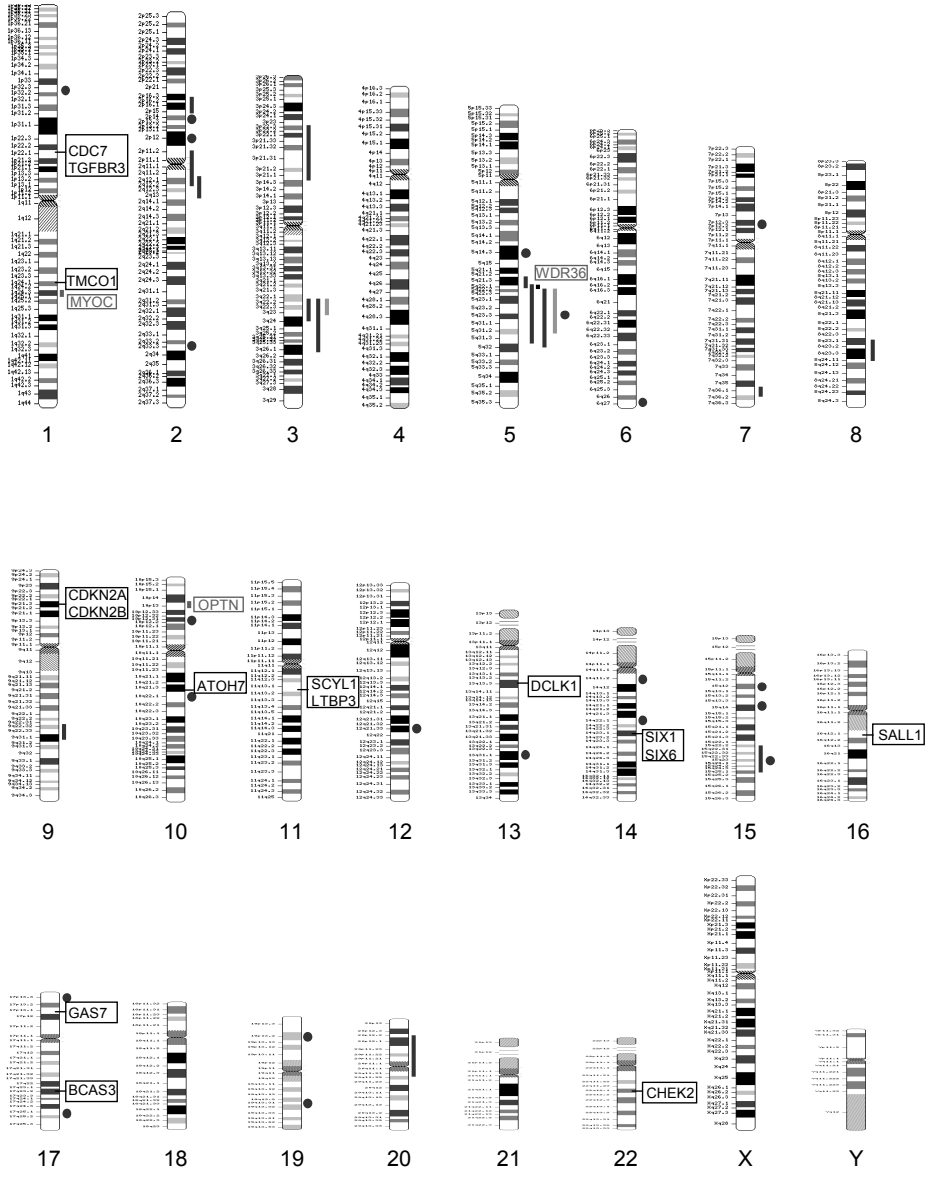

Figure 1. Chromosomal regions identified by our genome-wide association studies together with previously identified loci

Potential glaucoma genes within the chromosomal regions identified by our genome-wide association studies have been written in black. Previously identified genes have been written in grey. The dark grey lines represent linkage regions that have previously been identified by analyses in single, large pedigrees. Line boundaries have been determined by haplotype analyses. Light grey lines correspond to replicated or refined loci in the same population. Dark grey dots represent previously reported maximum LOD scores from population based (family) studies.

Table 1. Genes identified for quantitative glaucoma traits by the genome-wide association studies presented in this thesis

Gene	Location	Associated trait	Association with glaucoma *
CDC7 / TGFBR3	1p22.1	optic disc area	n.s.
SALL1	16q12.1	optic disc area	n.s.
ATOH7	10q21.3-q22.1	optic disc area and VCDR	1.28 [1.12 - 1.47]
CDKN2A / CDKN2B	9p21.3	VCDR	0.76 [0.70 - 0.84]
SIX1 / SIX6	14q23.1	VCDR	1.20 [1.10 - 1.31]
SCYL1 / LTBP3	11q13.1	VCDR	n.s.
DCLK1	13q13.3	VCDR	n.s.
CHEK2	22q12.1	VCDR	n.s.
BCAS3	17q23.2	VCDR	n.s.
GAS7	17p13.1	IOP	0.88 [0.78 - 0.98]
TMCO1	1q24.1	IOP	1.31 [1.12 - 1.53]

* presented as odds ratio [95% confidence interval]
n.s. = not significant; VCDR = vertical cup-to-disc ratio; IOP = intraocular pressure

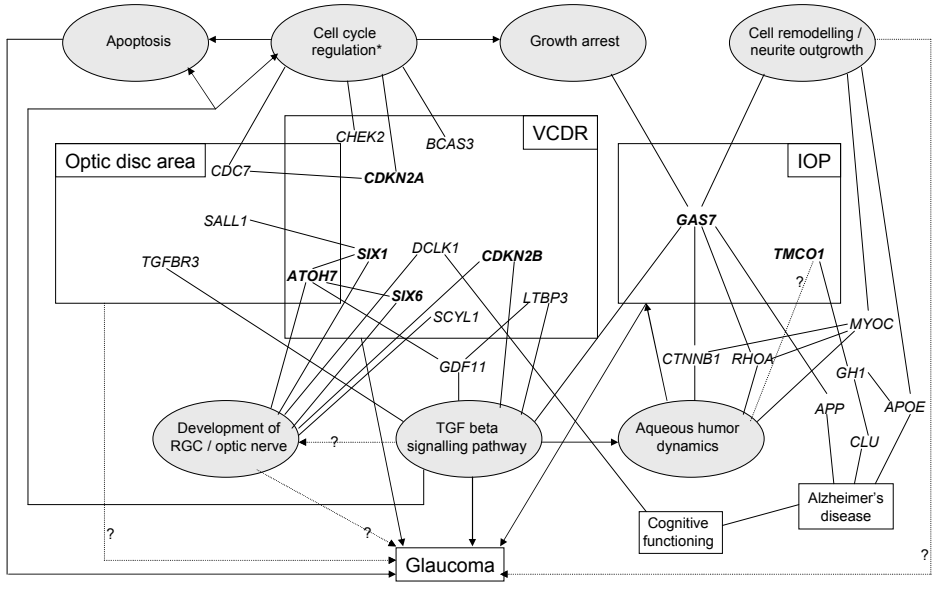

Figure 2. Diagram showing potential pathogenetic mechanisms through which the identified genes may contribute to the development of glaucoma
The genes that have been associated with glaucoma are in bold.
* Including the p53 signal transduction pathway

nism behind the retinal ganglion cell death in glaucoma.[6] Association studies between polymorphisms in the p53 gene and glaucoma have yielded conflicting results.[7,8] Other genes from the p53 signal transduction pathway have been shown to be upregulated in experimental glaucoma.[9] It will be of interest to further explore this pathway in relation to glaucoma. Our studies identified several genes that are involved in the early development of retinal ganglion cells and the optic nerve. This finding calls for further studies on how these genes may contribute to the typically late onset of glaucoma, which may initially feel counterintuitive.

The various methodologies used in our studies had in common that they, where possible, aimed to facilitate gene-finding for glaucoma by reducing its etiological complexity. For example, some of our studies have been conducted in a genetically isolated population. Gene-finding studies in isolated populations may benefit from both a reduced genetic variability and a lower environmental heterogeneity, resulting in an enhanced power to detect new genes.[10] A second methodology by which we sought to reduce complexity was by studying quantitative traits. Intuitively, the pathogenetic mechanisms underlying any disturbance in IOP regulation at the front of the eye may be distinct from those causing an increased susceptibility to neuronal damage at the back of the eye. By investigating to what extent genetic risk scores of IOP could explain the variance in VCDR, we have indeed confirmed that the genes involved in IOP and VCDR hardly overlap.[11] Genetic studies may therefore benefit from discretely studying these traits. As concomitant benefits, quantitative traits are not susceptible to misclassification and can be studied population-wide, thereby also contributing to an increased study power.

A potential drawback of studying quantitative traits may relate to their clinical relevance. Are the genetic variants that we have found to mediate the optic disc area, VCDR and IOP in a healthy population also relevant to the onset of glaucoma? In a meta-analysis of 6 case-control studies (total N = 3,161 glaucoma cases and 42,837 controls), we investigated whether the genetic variants that we had identified for optic disc parameters were also associated with glaucoma.[12] Of the 8 variants evaluated, we found statistically significant associations with glaucoma for *CDKN2A / CDKN2B* (identified for VCDR), *SIX1 / SIX6* (identified for VCDR), and *ATOH7* (identified for both optic disc area and VCDR). A subsequent study in a US Caucasian sample of 539 glaucoma cases and 336 controls confirmed these associations for *CDKN2A/B* and *SIX1/6*.[13] In this study, *ATOH7* was not independently associated with glaucoma. However, a significant interaction between *ATOH7* and *SIX1/6* was identified such that persons who carried the *SIX1/6* risk genotype were more likely to develop glaucoma if they also carried the *ATOH7* genotype associated with a larger optic disc area. This may indicate that a large optic disc area alone is not necessarily a risk factor for glaucoma, as has been suggested previously,[14] but that it can significantly increase the risk of getting glaucoma when coupled with risk factors

mediating the VCDR. Although this particular interaction needs to be reconfirmed, this study nicely illustrates, in addition to replication, one of the other essential next steps after gene-finding: studying the interactions with other genes and with environmental factors. Interactions are assumed to play a fundamental role in developing glaucoma. Elucidating any interactions may therefore greatly contribute to our understanding of its complex etiology.

We also investigated the clinical relevance of the genetic variants in *GAS7* and *TMCO1* that we had identified for IOP. In a meta-analysis of 4 case-control studies (total N = 1,432 glaucoma cases and 8,102 controls), both variants showed evidence for association with glaucoma, although for *GAS7* this association was only observed in 2 studies. Additional studies are needed to shed light on the role of these genetic variants in glaucoma. Finally, we examined to what extent genetic risk scores based on our GWAS results of quantitative traits could predict the risk of getting glaucoma.[11] A benefit of this risk scores method is that it also takes into account the truly associated genetic variants that did not reach genome-wide significance. Our VCDR risk scores explained up to 4.7% of the variance in glaucoma, which exceeded the variance explained by age and gender. This suggests a substantial overlap in the genetic etiologies of VCDR and glaucoma. For IOP such a genetic overlap with glaucoma was not supported by our risk scores analyses in this particular case-control study (derived from the Rotterdam Study, RS-I). It would be of interest to also evaluate the effects of the IOP risk scores in other studies.

An apparent discrepancy exists between the results of our commingling analyses, which suggested a major gene determining VCDR, and the findings of our GWAS, which – despite sufficient power – could not detect such a major gene. Even with the conservative design of the SKUMIX program,[15] which implements the commingling analysis, the major gene effect suggested in chapter 2.2 may be a case of simulation of Mendelism, as has been described by Edwards in 1960.[16,17] To investigate how Mendelism may be simulated, McGuffin and Huckle in 1990 collected data on the frequency of attending medical school in adult relatives of medical students.[18] A complex segregation analysis provided stronger evidence for transmission of a major genetic effect (and even suggested a recessive mode of inheritance!) than for the much more intuitively plausible hypothesis of multifactorial transmission. Likewise, we may have been misled by the circumstantial evidence provided by our commingling analysis for a major gene determining VCDR.

We have not been the only ones seeking to unravel the genetic etiology of glaucoma. Especially in the last 5 years - the rising GWAS era – discreet successes have been achieved slowly but surely. In 2007, the first GWAS of glaucoma yielded 2 common exonic variants in *LOXL1* that explained a significant proportion of the cases of exfoliation glaucoma in a European population.[19] Exfoliation glaucoma is a form of secondary glaucoma in which aqueous humor outflow is obstructed by fibrillar extracellular (exfoliative) material. The

data suggested that *LOXL1* was particularly associated with the accumulation of microfibrillar deposits in the anterior segment of the eye (exfoliation syndrome) rather than with the consequent onset of secondary glaucoma. Subsequent studies in populations of Caucasian, African and Asian ancestry confirmed the association of *LOXL1* with exfoliation glaucoma and did not support any association with other subtypes of glaucoma, such as primary open-angle glaucoma.[20-23] For the latter, 2 GWASs have been published. A study in 1,263 affected cases and 34,877 controls from Iceland identified a common variant near *CAV1* and *CAV2*.[24] Both genes are expressed in the trabecular meshwork as well as in retinal ganglion cells. In our GWAS we identified an association between the *CAV1-CAV2* region and IOP. A study of 545 patients with glaucoma and 297 controls from Iowa could not replicate the association of this region with glaucoma, suggesting that this region is not a strong risk factor in all populations.[25] The second GWAS on glaucoma has been performed in 827 Japanese cases and 748 controls and has identified 3 putative loci, although none of these reached genome-wide significance.[26] One of these loci showed evidence for association with IOP in our GWAS, which is surprising as most glaucoma patients in Japan present with normal tension glaucoma (i.e., glaucoma with IOP ≤ 21 mmHg). A case-control study in an Indian population could not replicate the association with glaucoma for any of the three Japanese loci.[27] Further studies across different populations should shed further light on the role of these variants in glaucoma. In addition to the identification of common genetic variants by the GWASs described above, Pasutto *et al*. performed a candidate gene study of *NTF4* and reported that rare mutations in this gene accounted for about 1.7% of the patients with glaucoma in a European population.[28] The Rotterdam Study (RS) I and the Genetic Research in an Isolated Population (GRIP) study functioned as independent replication cohorts for the German study. Among the 211 patients with glaucoma from RS I, two carried a missense mutation (A88V) in *NTF4*. *NTF4* mutations were not detected in the 104 patients with glaucoma from GRIP. Subsequent studies in a US population of European ancestry as well as in an Indian population could not demonstrate an association between any coding variants in *NTF4* and an increased risk of glaucoma.[29,30] Results from a case-control study in a Chinese population suggested that *NTF4* mutations may be a rare cause of glaucoma in individuals of Chinese ancestry.[31]

Despite so many efforts, the complex etiology of glaucoma is still far from understood and much more remains to be elucidated. Future directions following from the studies described in this thesis encompass studies to further explore our findings as well as studies to identify additional genes. To start with the first, we will have to validate the role of the genetic variants that we have identified across populations of different ethnicities. The associations of *ATOH7* and *TGFBR3* with optic disc area have been confirmed in a Singaporean study comprising participants of Indian and Malay ancestry.[32] This may indicate that - although glaucoma in different populations may manifest in a different way

- there are at least some shared genetic pathways. The relevance of the other identified loci to quantitative traits as well as glaucoma in populations of other ethnicities should be one focus of future research. Of particular interest would be a study of the association of *GAS7* with IOP and glaucoma in populations of African ancestry, given the significant difference in risk allele frequencies of the identified genetic variant between European and African populations. A next step should also be to discover the underlying causes behind the identified associations. These could for example be any regulatory elements of gene expression, which can be revealed by assessing whether the genetic variants are associated with the expression of nearby genes. The identified associations may also be explained by one or more rare causal variants in the surrounding regions (synthetic association).[33] As these rare variants have been shown to be possibly megabases away from the identified associations, follow-up sequencing should include extensive chromosomal regions. In the chapters 3.1 and 3.2 we have already discussed the presumed functions of the identified genes and we have speculated on the potential mechanisms through which these genes could contribute to the onset of glaucoma. Our hypotheses were based on previous functional studies which did not specifically investigate any ophthalmic pathology. Further functional research aiming to particularly investigate the role of these genes in glaucoma is therefore critical to understand any underlying pathways.

What should be future strategies to discover additional genes? To answer this question we should first make up our minds on what type of genes we think we should be looking for. With the achieved though limited successes of recent GWASs, should we still adhere to the "common disease – common variant" hypothesis? Or may the missing heritability better explained by yet unknown rare variants of relatively large effect? By evaluating how genetic risk scores based on our GWAS data could predict VCDR, IOP and glaucoma in an independent population, we found strong evidence for a polygenic model underlying both VCDR and glaucoma, but not IOP.[11] Our data suggested that a multitude of common variants, each with a very small effect, may collectively account for a substantial proportion of the variance in VCDR and glaucoma. To identify these genetic variants with small effects larger scale GWASs are needed. The logical first step will be to perform a meta-analysis of the currently available GWAS data of various research groups. However, limitations of this approach concern the differences in ethnicity, phenotypic heterogeneity due to different measurement methods across different studies, and non-uniformity of diagnostic criteria for glaucoma. More accurate and standardized phenotyping may open up new perspectives for future research. A potential resource for future studies on glaucoma may be the European Glaucoma Society GlaucoGENE project, which is a pan-European glaucoma-specific genetic epidemiology research network.[34] This initiative aims at creating a central database comprising genetic and phenotypic information from people throughout Europe. It specifically focuses on comprehensive

and standardized phenotyping, which will allow accurate classification and separate investigation of glaucoma subtypes as well as detailed studies of genotype-phenotype correlations. An attractive new strategy may be to classify glaucoma subtypes based on whether or not patients respond to a specific treatment, and to separately analyze, for example, beta-blocker responders and non-responders, or prostaglandin responders and non-responders. Under the hypothesis of different pathogenetic mechanisms underlying glaucoma in these different subtypes, this might again be a methodology that increases gene-finding power by reducing the etiological complexity of glaucoma.

Our genetic risk scores analysis did not support a polygenic model underlying the variance in IOP. A commingling analysis in an Australian population provided evidence of a rare variant with a large effect on IOP.[35] To identify rare variants, sequencing of entire genomes will be necessary. Although whole-genome sequencing or whole-exome sequencing of large populations are currently expensive and still facing various methodological issues, these may be promising methods for gene-finding in the near future. Before we have reached that point, various more feasible interim designs have been proposed.[36] First, families with multiple affected individuals may be sequenced. Since (very) large glaucoma families have been available for previous research, this may be a suitable method for glaucoma. Second, individuals at the extreme ends of a trait may be sequenced. In our case it would be very interesting not only to look at the extreme ends of quantitative glaucoma traits but also to consider ocular hypertension and normal tension glaucoma as the extreme ends of optic nerve vulnerability. Third, we may start to sequence candidate regions. Apart from the loci identified by our GWASs, any replicated previous linkage findings may also be promising targets (Figure 1). The results of the studies described in this thesis further point to 2 additional approaches to select candidate regions. First, the loci that we have identified in both our GWASs are connected to the TGF beta signalling pathway. Sequencing any (other) genes involved in this pathway may help elucidating how this pathway is involved in glaucoma. Second, the association between cognitive functioning and retinal nerve fiber layer thickness warrants sequencing of loci known to be involved in other neurodegenerative diseases, such as Alzheimer's disease.

The established glaucoma genes *MYOC* and *OPTN* have been identified by linkage analyses in families in which glaucoma segregated as an autosomal dominant trait. Although these genes only have little impact on the glaucoma cases in the general population, they do have clinical relevance in glaucoma families carrying a mutation. In these particular families, genetic testing may identify the individuals who are at an increased risk of getting glaucoma. These individuals can be offered intensive screening programs allowing early diagnosis and treatment in order to as much as possible prevent irreversible neuronal damage. Despite the high heritability, it may be questioned whether this "personalized medicine" will be feasible for the sporadic form of glaucoma,

in which multiple interacting genes and environmental factors are involved. Even if we could uncover all these genes and understand the causal pathways they are involved in, DNA testing will reveal an enormous amount of unique genetic profiles which will probably not allow any sensible risk prediction.[37] We certainly do not want to infer that we should desist from any further efforts to unravel the genetic etiology of glaucoma. The pathogenesis of glaucoma is very poorly understood and the only current target for glaucoma therapy is lowering of the IOP. Genetic studies are and will be critical to elucidate the pathogenetic mechanisms underlying glaucoma and to provide clues for developing new therapeutic strategies that will eventually prevent blindness in many thousands of individuals.

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Chapter 5.2

Common pathways in glaucoma and Alzheimer's disease

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Glaucoma and Alzheimer's disease (AD) are progressive, neurodegenerative disorders that particularly affect the elderly population. Both disorders are characterized by the loss of neuronal cells through apoptotic mechanisms. For glaucoma, this involves apoptosis of retinal ganglion cells, resulting in deficits in the visual field and ultimately in blindness. For AD, it concerns the loss of cholinergic basal forebrain cells, resulting in deficits in cognitive functioning and ultimately in dementia. For long, a relationship between glaucoma and AD has been speculated on. It has been questioned whether glaucoma is an ocular manifestation of AD[1] or even whether AD is a cerebral form of glaucoma.[2]

Support for an association between these neurodegenerative disorders initially emerged from studies noting a high prevalence of glaucoma among patients with AD.[3;4] However, these clinical studies had several methodological limitations from an epidemiological perspective (including their retrospective nature, potential selection bias, and inaccurate diagnostic criteria for glaucoma) and results of subsequent studies were inconsistent. Despite the lack of convincing epidemiological evidence supporting the clinical observations, glaucoma and AD have ample biological similarities. Particularly, the loss of retinal ganglion cells and thinning of the retinal nerve fiber layer, both typical features of glaucoma, have also been demonstrated in mild cognitive impairment and AD.[5;6] In a study of 1485 healthy individuals with an age range from 18 to 85 years, we have previously shown that retinal nerve fiber layer thickness and cognitive functioning are significantly associated even in their physiological spectrum.[7] Other similarities between glaucoma and AD include the particular involvement of the magnocellular visual pathway, the damage to the lateral geniculate nucleus, and disruptions in the circadian rhythm.[8]

A diversity of hypotheses has been proposed to explain the poorly understood association between glaucoma and AD. The association has been suggested to be mediated by brain atrophy and decreased cerebrospinal fluid pressures in patients with AD, leading to abnormal pressure gradients across the lamina cribrosa at the back of the eye.[9] This would cause glaucomatous damage to the optic nerve in a similar fashion as if there were an elevated intraocular pressure (the major risk factor of glaucoma). Another hypothesis involves infection with *Helicobacter pylori* as a possible shared risk factor for both glaucoma and AD.[10] It has also been argued that there may be an earlier manifestation of glaucomatous visual field loss in patients with AD as a result of reduced neuronal reserves in these patients.[8] Finally, there is increasing evidence supporting various hypotheses of common pathogenetic mechanisms explaining the association between glaucoma and AD. Osawa *et al*., for example, have recently demonstrated an aggregation of optineurin (encoded by the established glaucoma gene *OPTN*) in neurofibrillary tangles and dystrophic neurites in AD.[11] Optineurin was also detected in the typical pathologic lesions of other neurodegenerative diseases, such as skein-like

and round hyalin inclusions in amyotrophic lateral sclerosis, and Lewy bodies and Lewy neurites in Parkinson's disease, suggesting that optineurin may be involved in a common neurodegenerative process.

We have recently found new support for common genetic pathways underlying glaucoma and AD. By performing a genome-wide association study, we identified 2 common genetic variants associated with intraocular pressure and glaucoma. The first variant was located in *GAS7* (growth arrest-specific 7), a gene previously implicated in cell remodeling and in the formation of neurites in neuronal cells. The second variant was located in *TMCO1* (trans-membrane and coiled-coil domains 1), a highly evolutionary conserved gene of largely unknown function. *GAS7* and *TMCO1* are expressed in the ocular tissues involved in glaucoma. Interestingly, a protein pathway analysis (*www.ingenuity.com*) of these genes revealed several interactions with genes known to be involved in AD (Figure). *GAS7* directly interacts with amyloid beta precursor protein (*APP*). *TMCO1* interacts with apolipoprotein E (*APOE*) and clusterin (*CLU*) via growth hormone 1 (*GH1*). In addition, caveolin1 (*CAV1*), a gene recently implicated in glaucoma, directly interacts with *APP*.

The role of APP in the pathogenesis of AD is well recognized. It is the key pathway in AD. Abnormal processing of APP leads to an increased production of amyloid-β (Aβ), which is the major component of senile plaques in AD. Mutations in *APP* have been associated with inherited, early-onset forms of AD.[12] Cumulating evidence suggests a role for APP and Aβ in retinal ganglion cell apoptosis in glaucoma. Abnormal processing of APP and increased levels of Aβ in retinal ganglion cells have been demonstrated in rats with experimental glaucoma.[13] Moreover, targeting the Aβ formation pathway has been shown to effectively reduce glaucomatous retinal ganglion cell apoptosis in vivo.[14] APP directly interacts with *GAS7*, associated with intraocular pressure, as well as *CAV1*, associated with glaucoma. While there is no direct link, two other AD genes emerged in the glaucoma pathway: *APOE* and *CLU*. *APOE* is a susceptibility gene for both sporadic and familial late-onset forms of AD.[15] Apolipoprotein E has an important role in lipid transport and cholesterol homeostasis within the central nervous system. Similar to *GAS7*, Apolipoprotein E has been shown to be essential for neurite outgrowth. Several studies have investigated the associations between common *APOE* polymorphisms and glaucoma. The reported results are inconsistent. *CLU* is an AD gene recently identified by two genome-wide association studies.[16;17] *CLU* encodes clusterin, which is, like APOE, a major brain apolipoprotein acting as a molecular chaperone for Aβ. CLU may participate in Aβ clearance from the brain by forming complexes that cross the blood-brain barrier. *CLU* has been examined in pseudoexfoliation syndrome, a risk factor for the pseudoexfoliation subtype of glaucoma, but its role in the pathogenesis of this syndrome is controversial. It is of interest that *TMCO1* through growth hormone 1 gene (*GH1*) interacts with both *APOE* and *CLU*.

Although the role of *GAS7* and *TMCO1* in the pathogenesis of glaucoma needs to be further biologically substantiated, the particular interactions of these genes with the

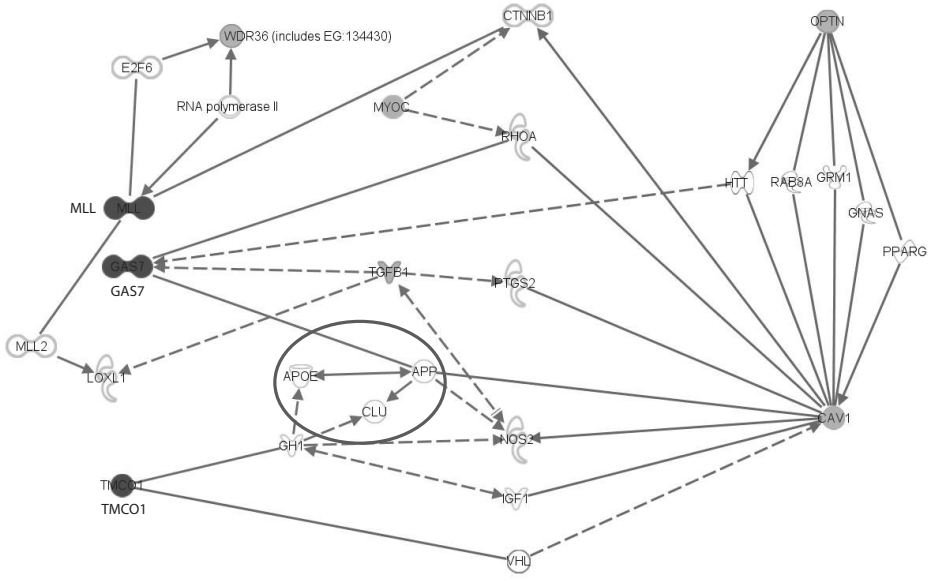

Figure. Biochemical and functional interactions between (putative) glaucoma and AD genes
Ingenuity analyses of biochemical and functional interactions between the newly identified *GAS7* and *TMCO1* disease genes implicated in intraocular pressure and glaucoma, and previously known glaucoma disease genes (*WDR36, MYOC, OPTN, CAV1*) revealed interactions with Alzheimer's disease genes (*APP, APOE, CLU*). Functional relationships in the knowledge database Ingenuity (www.ingenuity.com) are a compilation of all known gene-relevant biochemical and functional data of in vivo and in vitro experiments involving (molecules, cells and tissues of) rats and mice and man, as well as data from zebrafish and Drosophila and ongoing clinical trials in man. The query genes/proteins GAS7 (including it's drosophila homologue MLL) and TMCO1 are presented in dark grey. Known glaucoma disease genes are given in light grey. The circle surrounds the Alzheimer's disease genes APOE, APP, and CLU. Blank genes/molecules are generated by the knowledge database to construct a functional network under the criteria specified by the investigator. The diagram was generated using the function "Path Explorer".
In general, solid lines indicate a direct, experimentally verified, physical relationship between two molecules, for example a physical protein-protein interaction, or an enzym-DNA interaction, etc. Dotted lines refer to the existence of an indirect functional relationship, such as co-upregulation in cell cultures under specific experimental conditions. WDR36 = WD Repeat-containing protein 36; OPTN = optineurin; MYOC = myocilin; GAS7 = growth arrest-specific 7; MLL = myeloid/lymphoid or mixed-lineage leukemia; TMCO1 = transmembrane and coiled-coil domains 1; CAV1 = caveolin 1; TGFB1 = transforming growth factor beta 1; CTNNB1 = catenin (cadherin-associated protein) beta 1; RHOA = ras homolog gene family, member A; E2F6 = E2F transcription factor 6; VHL = von Hippel-Lindau; HTT = huntingtin; NOS2 = nitric oxide synthase 2; LOXL1 = lysyl oxidase-like 1; APOE = apolipoprotein E; APP = amyloid beta (A4) precursor protein; CLU = clusterin.

known AD genes support a joint pathogenesis of glaucoma and AD. Moreover, they provide attractive clues to future research. A pathogenetic link between glaucoma and AD may facilitate the interchange of knowledge and so help in elucidating the currently so poorly understood etiologies of these disorders. To reveal the unknown mechanisms

in glaucoma, we may benefit from the known pathways in AD, and vice versa. Moreover, new insights in the origin of neurodegeneration may also be extended to other disorders, such as Parkinson's disease. Not only the common pathways in glaucoma and AD, but also their specific differences at the molecular level will contribute to our understanding of neurodegeneration.

Elucidating the common pathogenesis of glaucoma and AD may provide common opportunities for new diagnostic targets. These could, for example, include the genotyping of (a combination of) specific polymorphisms in susceptibility genes or the assessment of particular protein levels. Another focus of overlapping diagnostic opportunities may emanate from a clinical resemblance between the two disorders: thinning of the retinal nerve fiber layer. By using an old generation ophthalmic imaging technology (time-domain optical coherence tomography [OCT]), specific patterns of retinal nerve fiber loss have already been suggested in AD.[5] The currently available next generation OCT (Fourier-domain OCT) allows much faster imaging with a higher resolution. This new technology greatly assists the ophthalmologist in diagnosing glaucoma. Although it may be too early to imagine these ophthalmic imaging devices also being an indispensable tool in the neurological practice, the opportunity to assess the retina and optic nerve head easily and in so much detail certainly warrants further research into the neurological applicability of these devices.

Insight in the common genetic pathways of glaucoma and AD may also create openings for the development of new therapeutic strategies. Guo *et al*. already reported Aβ being a promising target in glaucoma treatment, which is one of several attractive opportunities to further explore.[14] The glutamate excitotoxic cascade may be another pathway of interest. Memantine, an N-methyl-D-aspartate (NMDA) glutamate receptor antagonist, is used in the treatment of moderate to severe AD.[18] Preclinical studies support a neuroprotective effect of memantine on retinal ganglion cells in animal models of glaucoma.[19;20] A phase III randomized controlled clinical trial did not show any significant difference in glaucoma progression between patients receiving memantine and patients receiving a placebo.[21] However, not all the results of that study have to date been reported on. A difficulty in clinical studies is how to define and measure the clinical outcome, glaucoma progression. Future studies may in this respect benefit from the next generation imaging methodologies.

In conclusion, by revealing interactions between recently identified glaucoma candidate genes and genes involved in AD, we have found further support for a common pathogenesis of glaucoma and AD. Elucidating the shared pathways between these and other neurodegenerative diseases may lead to a better understanding of the etiology of neurodegeneration and moreover provide a point of departure for developing diagnostic and therapeutic strategies.

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Chapter 6

SUMMARY / SAMENVATTING

SUMMARY

Glaucoma is a progressive optic neuropathy responsible for 12.3% of global blindness. There is ample evidence supporting a genetic etiology of glaucoma. However, identifying the specific genes and pathogenetic mechanisms involved has proven difficult. The studies presented in this thesis were performed to gain insight in the genetic etiology of glaucoma by focusing on quantitative traits of this disease.

The thesis starts with an introduction to glaucoma and its quantitative traits in **Chapter 1.1.** A review of preceding work in the genetic epidemiology of glaucoma has been provided in **Chapter 1.2**. Previous studies have linked more than 25 chromosomal regions to glaucoma but only identified three genes (*MYOC, OPTN,* and *WDR36*) with little impact on sporadic glaucoma in the general population. We concluded that further gene-finding research could benefit from more accurate and detailed phenotyping, large-scale interdisciplinary collaborations, genome-wide association approaches and quantitative trait – based strategies. We have sought to apply our recommendations to the gene-finding studies presented in this thesis.

As a first investigation into the genetic etiology of quantitative glaucoma traits, we performed a heritability study (**Chapter 2.1**). We explored the genetic contributions to clinical markers of glaucoma (retinal nerve fiber layer [RNFL] thickness and optic disc parameters) as well as a major risk factor (intraocular pressure; IOP). We used the imaging techniques scanning laser polarimetry and confocal scanning laser ophthalmoscopy to ensure objective phenotyping. The heritability estimates of the studied traits ranged from 0.35 to 0.79, indicating an extensive genetic component. Our results supported further efforts to identify the genes responsible for these quantitative glaucoma traits.

A different approach of exploring the genetic etiology of quantitative glaucoma traits has been applied in **Chapter 2.2**, in which we sought to test the hypothesis that there is a major genetic determinant of vertical disc diameter (VDD) and vertical cup-to-disc ratio (VCDR). We examined the population distributions of these traits by the use of a commingling analysis. VDD data were best fitted by a 1-distribution model, which would not support a major genetic effect. VCDR data were best modelled by a mixture of three distributions. These results would be consistent with a major genetic determinant of VCDR and support gene-finding studies for this trait.

We subsequently performed genome-wide association studies (GWASs) for the size of the optic disc area and VCDR (**Chapter 3.1**). We found genome-wide significant evidence for the association of 3 chromosomal regions with the size of the optic disc area, and 7 chromosomal regions with VCDR. Although multiple genes could be implicated in these regions, the most interesting ones were *ATOH7* at chromosome 10q21.3-22.1 (associated with both traits), *CDC7/TGFBR3* at 1p22 and *SALL1* at 16q12 (both associated with optic disc area), and *CDKN2A/CDKN2B* at 9p21, *SIX1/SIX6* at 14q22-23, *SCYL1/LTBP3*

at 11q13, *CHEK2* at 22q12.1, *DCLK1* at 13q13 and *BCAS3* at 17q23 (all associated with VCDR). Previously reported functional studies of these genes may suggest a critical role of the transforming growth factor (TGF) beta signalling pathway in the development of the optic disc and VCDR.

In **Chapter 3.2** we have presented our GWAS for IOP. We found genome-wide significant evidence for associations of common variants in *GAS7* at 17p13.1 and in *TMCO1* at 1q24.1 with IOP. Both variants were also marginally associated with glaucoma. *GAS7* and *TMCO1* are highly expressed in the ciliary body and trabecular meshwork as well as in the retina. Biochemical protein interactions with the known glaucoma genes as well as previously reported functional data support the involvement of these genes in aqueous humor dynamics and glaucomatous neuropathy.

A protein pathway analysis of *GAS7* and *TMCO1* revealed several interactions with genes known to be involved in Alzheimer's disease (AD). We further explored any associations between (quantitative) glaucoma and AD traits in the next chapters. We first investigated whether cognitive functioning and RNFL thickness were associated in their physiological spectrum. To this end, we assessed a broad range of cognitive functions and measured RNFL thickness in a large, population-based sample of healthy subjects. This study, which has been presented in **Chapter 4.1**, showed that a better cognitive performance was significantly associated with a thicker RNFL. The association particularly manifested in young to middle-aged adults and diminished in age groups beyond 40 years, which may suggest that loss of neurons in the cerebrum and retina is not concomitant and may have different origins.

In **Chapter 4.2**, we investigated whether quantitative glaucoma traits were associated with common variants in the Apolipoprotein E (*APOE*) gene, a genetic risk factor for AD. Our findings did not provide evidence of any association between optic disc parameters, RNFL thickness and the *APOE**4 allele, which is the allele associated with an increased risk of AD. We found borderline significant evidence that *APOE**2 (the allele with a protective effect against AD) was associated with a larger neuroretinal rim area and a lower VCDR. However, we did not observe any association between *APOE**2 and a thicker RNFL. Our findings may hint at a role of *APOE**2 in a common etiological pathway of glaucoma and AD, but more extensive studies are needed to support this hypothesis.

In **Chapter 5.1**, we have viewed the results of our gene-finding studies in a wider perspective, and we have provided suggestions for future gene-finding strategies. We have elaborated on any common pathways in glaucoma and AD in **Chapter 5.2**. Elucidating these pathways may lead to a better understanding of the etiology of neurodegeneration and may create openings for the development of new diagnostic and therapeutic strategies.

SAMENVATTING

Glaucoom is een aandoening waarbij de zenuwcellen van de oogzenuw langzaam afsterven. Hierdoor treedt er verlies van het gezichtsveld op en kan, in een laat stadium, blindheid ontstaan. Van alle blindheid op aarde, wordt ruim 12% veroorzaakt door glaucoom. Er is veel onderzoek gedaan naar het ontstaan van glaucoom. Uit al dat onderzoek is naar voren gekomen dat erfelijke factoren een belangrijke rol spelen. Echter, het ontdekken van de specifieke genen en mechanismen die verantwoordelijk zijn voor het ontstaan van glaucoom is lastig gebleken. Met het onderzoek beschreven in dit proefschrift hebben wij geprobeerd meer inzicht te verkrijgen in de erfelijke achtergrond van glaucoom. Daartoe hebben we ons vooral geconcentreerd op zogenaamde "kwantitatieve eigenschappen" van deze aandoening. Deze kwantitatieve eigenschappen beschrijven bijvoorbeeld de dikte van de zenuwvezellaag in het netvlies, bepaalde kenmerken van de oogzenuw of een belangrijke risicofactor voor glaucoom: de oogdruk.

Dit proefschrift begint met een korte inleiding over glaucoom (**Hoofdstuk 1.1**). Vervolgens geven we in **Hoofdstuk 1.2** een overzicht van de voorafgaande studies naar de erfelijkheid van glaucoom. In deze studies werden meer dan 25 gebiedjes op de chromosomen ontdekt waarin genen voor glaucoom zouden kunnen liggen. Verdere zoektochten in deze gebiedjes leverden tot nu toe echter maar drie genen op (*MYOC*, *OPTN* en *WDR36*) die slechts een beperkte rol blijken te hebben bij het ontstaan van glaucoom in de algemene bevolking. Wij concludeerden dat verder onderzoek naar de erfelijkheid van glaucoom gebaat zou kunnen zijn bij nauwkeurigere meetmethodes, grootschalige samenwerkingsverbanden, genoomwijde associatiestudies (een techniek waarmee je variaties in het hele erfelijk materiaal onderzoekt), en op kwantitatieve eigenschappen gebaseerde analyses. Dit zijn de speerpunten van onze eigen studie geworden.

In een eerste, verkennende studie onderzochten we de bijdrage van erfelijke factoren aan de dikte van de zenuwvezellaag in het netvlies, verschillende kenmerken van de oogzenuw en de oogdruk (**Hoofdstuk 2.1**). We gebruikten specifieke oogcameras om de zenuwvezellaag en oogzenuwkenmerken nauwkeurig en objectief te kunnen meten. Uit onze resultaten bleek dat de onderzochte kwantitatieve glaucoomeigenschappen voor een belangrijk deel (35 – 79%) door erfelijke factoren bepaald worden. Een zodanig onmiskenbare erfelijke component rechtvaardigde nader onderzoek naar de onderliggende genen.

Een andere methode om de erfelijke achtergrond van kwantitatieve glaucoomeigenschappen te verkennen, hebben we toegepast in **Hoofdstuk 2.2**. In dit hoofdstuk keken we niet naar het totale effect van alle betrokken genen, maar onderzochten we de hypothese dat één gen met een groot effect verantwoordelijk zou zijn voor kenmerken van de oogzenuw. De kenmerken die wij onderzochten waren de verticale papil

diameter (VPD; de diameter van de oogzenuw op de plek waar deze het oog verlaat) en de verticale cup-disc ratio (VCDR; deze maat zegt iets over de hoeveelheid zenuwvezels op de plek waar de oogzenuw het oog verlaat – hoe hoger de waarde, hoe minder zenuwvezels). De resultaten voor VPD ondersteunden de hypothese niet. De resultaten voor VCDR zouden wel kunnen wijzen op de aanwezigheid van een gen met een groot effect, wat weer zou pleiten voor verder genetisch onderzoek.

Vervolgens hebben wij van 2,5 miljoen plekjes op het erfelijk materiaal (DNA) onderzocht of zij gerelateerd waren aan de papiloppervlakte (de oppervlakte van de oogzenuw op de plek waar deze het oog verlaat) en de VCDR (**Hoofdstuk 3.1**). Hiertoe hebben we een genoomwijde associatie studie uitgevoerd. We vonden drie regio's die geassocieerd waren met de papiloppervlakte en zeven regio's met de VCDR. Hoewel veel verschillende genen in deze regio's verantwoordelijk zouden kunnen zijn voor de gevonden associatie, lijken de meest interessante: *ATOH7* (geassocieerd met zowel papiloppervlakte als VCDR), *CDC7/TGFBR3* en *SALL1* (beide geassocieerd met papiloppervlakte), en *CDKN2A/CDKN2B*, *SIX1/SIX6*, *SCYL1/LTBP3*, *CHEK2*, *DCLK1* en *BCAS3* (alle geassocieerd met VCDR). Eerder gepubliceerde functionele studies van deze genen wijzen op een mogelijke rol van het zogenaamde "transforming growth factor (TGF) beta" signaleringspad in de ontwikkeling van de oogzenuw.

Hoofdstuk 3.2 beschrijft onze genoomwijde associatie studie voor oogdruk. We toonden aan dat genetische varianten in het *GAS7* gen op chromosoom 17 en het *TMCO1* gen op chromosoom 1 geassocieerd waren met deze risicofactor voor glaucoom. In een vervolgstudie in hetzelfde hoofdstuk lieten we bovendien zien dat deze varianten ook geassocieerd waren met glaucoom zelf. *GAS7* en *TMCO1* zijn genen die tot uitdrukking komen in de oogweefsels die de oogdruk reguleren en ook in het netvlies. Zowel eerder beschreven interacties tussen *GAS7*, *TMCO1* en de reeds bekende glaucoomgenen als de resultaten van gepubliceerde functionele studies ondersteunen de hypothese dat deze genen betrokken zijn bij het reguleren van de oogdruk en het ontstaan van glaucoom.

Een analyse van de eiwitnetwerken van *GAS7* en *TMCO1* liet verschillende interacties zien met genen die een rol spelen bij het ontstaan van de ziekte van Alzheimer. We besloten mogelijke relaties tussen (kwantitatieve) eigenschappen van glaucoom en Alzheimer nader te onderzoeken. Eerst bestudeerden we of cognitief functioneren en zenuwvezellaagdikte geassocieerd waren in hun fysiologische spectrum. Hiertoe testten we een breed palet aan cognitieve functies en bepaalden we de zenuwvezellaagdikte in een grote groep gezonde personen. Deze studie, beschreven in **Hoofdstuk 4.1**, toonde aan dat betere cognitieve prestaties significant geassocieerd waren met een dikkere zenuwvezellaag in het netvlies. De associatie viel vooral op bij jong volwassenen en was minder duidelijk aanwezig bij personen boven de 40 jaar. Dit zou erop kunnen wijzen dat het verlies van zenuwcellen in de hersenen en in het netvlies gedurende het leven niet gelijk op gaat en verschillende oorzaken heeft.

In **Hoofdstuk 4.2** onderzochten we of kwantitatieve glaucoomeigenschappen geassocieerd zijn met varianten in het Apolipoproteine E (*APOE*) gen, een erfelijke risicofactor voor de ziekte van Alzheimer. Onze resultaten lieten geen verband zien tussen enerzijds oogzenuwkenmerken en zenuwvezellaagdikte en anderzijds *APOE*4*, de variant die een verhoogd risico geeft op de ziekte van Alzheimer. Wel waren er aanwijzingen dat *APOE**2 (de variant die beschermt tegen de ziekte van Alzheimer) geassocieerd was met meer zenuwvezels in de oogzenuw. Echter, een dikkere zenuwvezellaag in het netvlies werd bij deze variant niet gezien. Onze bevindingen zouden erop kunnen wijzen dat *APOE**2 een rol speelt in een gemeenschappelijk ontstaansmechanisme van glaucoom en de ziekte van Alzheimer. Uitgebreidere studies zijn nodig om deze hypothese nader te onderzoeken.

In **Hoofdstuk 5.1** hebben we de resultaten van onze genetische studies in een breder perspectief geplaatst en suggesties gedaan voor toekomstig onderzoek. We zijn dieper ingegaan op gemeenschappelijke ontstaansmechanismen van glaucoom en de ziekte van Alzheimer in **Hoofdstuk 5.2**. Het ophelderen van deze mechanismen zou meer inzicht kunnen geven in het verlies van zenuwcellen en zou aanknopingspunten kunnen bieden voor de ontwikkeling van nieuwe diagnostische tests en behandelmethoden.

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Uit: Erik of het klein insectenboek, Godfried Bomans

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Leonieke

ABOUT THE AUTHOR

Leonieke Maria Elisabeth van Koolwijk was born on March 24th 1977 in Nijmegen, the Netherlands. After graduating (cum laude) from secondary school (Stedelijk Gymnasium Nijmegen) in 1995, she entered medical school at the Medical Faculty of the University of Utrecht. During her studies, she participated in research projects at the Department of Pulmonology, Wilhelmina Children's Hospital, Utrecht, under supervision of Prof. dr. C.K. van der Ent. She did a clinical internship in orthopedics at the University Hospital of Salamanca, Spain (1998), she worked as a volunteer in Urubamba, Peru (2000), and did her rotations in paediatrics and otorhinolaryngology at the Tygerberg Hospital, University of Stellenbosch, South Africa (2002). In 2003, she obtained her medical degree en started working as a resident at the Department of Clinical Genetics, Erasmus University Medical Centre, Rotterdam. In 2004, she started the research described in this thesis at the Departments of Epidemiology and Clinical Genetics, Erasmus University Medical Centre, Rotterdam, in close collaboration with the Rotterdam Eye Hospital and the Rotterdam Ophthalmic Institute, under supervision of Prof. dr. B.A. Oostra, Prof. dr. H.G. Lemij, Prof. dr. ir. C.M. van Duijn, and Dr. C.C.W. Klaver. She spent a year in London, United Kingdom, to work as a research fellow at Moorfields Eye Hospital and the UCL Institute of Ophthalmology, under supervision of A.C. Viswanathan, MD, PhD (2007), after which she continued her research in Rotterdam. In 2009-2010 she worked as a resident at the department of Clinical Genetics, VU Medical Centre, Amsterdam, the Netherlands. In 2006 she married Zeeger de Jongh. They have two children, Quirine (2008) and Reimert (2010).

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