Epidemiologic and genetic insights into open-angle glaucoma

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MEINEN ELTERN IN DANKBARKEIT GEWIDMET

(DR. O.W. RAMDAS "DAS CHORIONCARCINOM INTRA GRAVIDITATEM INTAKTAM" 1980)

Contents

Chapter 1

Introduction

Introduction

The eye is a very complex organ with a remarkable architecture (Figure 1). It is responsible for one of the main senses of the human being, in that every living person can observe the world through his/her eyes: “a mirror of life”. Loosing this sense, and thus loss of sight, leads to a significant reduction in quality of life.[1] Therefore it is crucial to prevent or cure the eye from sight-threatening disorders.

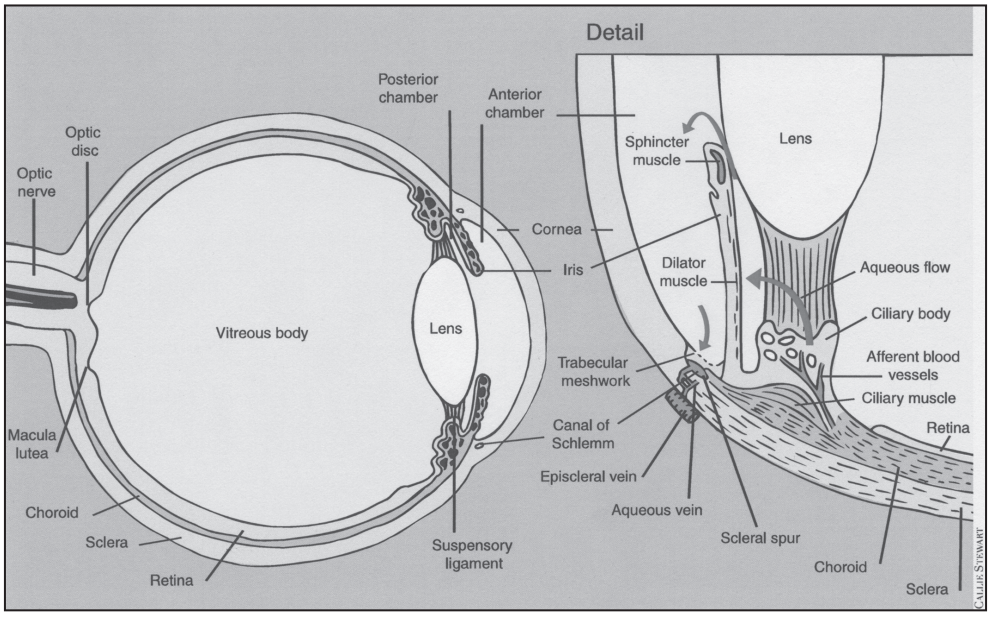
In the past centuries several sight-threatening disorders have been described. One of the major eye disorders affecting the visual performance is glaucoma. A few centuries ago the general thought was that glaucoma was a disease of the lens. The word glaucoma means “opacity of the crystalline lens”. Because a greenish color was observed in eyes with glaucoma, the phenomenon has also been known as green cataract. However, extraction of the deep sea-green colored lens in glaucomatous eyes did not result in restoration of vision, but showed that the lens was often clear rather than opacified. Later on, when the difference between glaucoma and cataract was discovered, the term glaucoma was used for several eye disorders other than cataract. Nowadays we still do not exactly know what glaucoma is, but we know that the optic nerve head is primarily affected instead of the lens.[2] Nevertheless, this does not indicate that the historical findings were all wrong. The greenish color of the pupil has been ascribed to corneal haze and the presence of blood pigments in some forms of glaucoma.[3] Even today, in German the phrase “grüne Star” means glaucoma.

Glaucoma, forms and facts

A multitude of eye disorders have been referred to as forms of glaucoma. All forms have in common morphologic changes at the optic nerve head (glaucomatous optic neuropathy; GON) and corresponding functional changes in the visual field (glaucomatous visual field loss; GVFL). The visual field is defined as all central and peripheral objects located in the field of vision that we observe when fixating on a certain object. In glaucoma the peripheral visual field is generally first affected. Eventually, the central visual field becomes also affected resulting in complete blindness. Glaucoma has been nicknamed the ”silent thief of sight”, because the periphery of the visual field is lost unnoticedly while the visual acuity remains relatively normal until the disease is quite advanced. The damaged visual field cannot be recovered once it is lost.

In this process the pressure within the eye (intraocular pressure; IOP) plays an unequivocal role. The anterior segment of the eye contains aqueous humor, which is produced by the ciliary body. From here aqueous humor flows through the pupil, the anterior chamber, and drains through the uveoscleral route or, more important, through the trabecular meshwork located in the angle formed by the iris and cornea (Figure 1). The IOP-level is determined by the aqueous secretion and the rate of outflow.

Figure 1. The anatomy of eye (left) with a magnification of the area of the ciliary body (right). (from: Fraser S, Manvikar S; Glaucoma - the pathophysiology and diagnosis; Hospital Pharmacist; July/August 2005; Vol. 12; p251-254; with permission)



There are two main forms of glaucoma characterized by the depth of the anterior chamber angle. We call the glaucoma open-angle glaucoma (OAG) if the anterior chamber angle is open. The reduced outflow is presumed to be caused by an increased resistance at the level of the trabecular meshwork. If the anterior chamber angle is closed, we call the glaucoma angle-closure glaucoma (ACG). Glaucoma may also be classified as primary or secondary. In contrast to the latter, the cause of elevation of the IOP in the former form is not known. Because the pathophysiology and treatment of the different forms of glaucoma are so diverse, there is no single definition that adequately encompasses all forms. Primary OAG is more common in Caucasian persons, whereas primary ACG is more common in Asian persons.[4]

The burden of glaucoma is obvious. It is the leading cause of irreversible blindness worldwide. The number of people suffering from glaucoma is expected to increase.[4] It has become a major healthcare issue in ophthalmology. Nevertheless, the etiology of OAG is still obscure. After decades of research only a few risk factors have been identified for OAG. Known risk factors include high age, African descent, elevated IOP, myopia, and a positive family history of OAG – the latter indicating a genetic background. Currently treatment is targeted on regulating IOP.[5,6] Treatment aims to decrease the progression of damage to the optic nerve head and thus the progression of GVFL. Despite the current approach of controlling IOP in OAG, there are also patients with well-controlled IOP, who continue to lose sight.[7] From a pathophysiological point of view, the main factors involved in the OAG course

next to elevated IOP are: bloodflow, ischemia, glial cells activation, and apoptosis, leading to optic nerve head changes (GON). In order to increase our knowledge of this major cause of blindness, we need to find more environmental and genetic risk factors associated with OAG. This should give us a better understanding of the underlying pathophysiologic mechanisms of GON and subsequent GVFL.

In this thesis we focused on primary OAG. We first outlined how to define GON more precisely, as required for adequate risk-factor analyses in an epidemiological setting. Next, we evaluated one of the theories implicated in the pathophysiological pathway of IOP and OAG, and search for other risk factors. Finally, we examined the genetics of OAG with the view to identify new genes and their impact.

GON in epidemiological settings

Glaucomatous damage due to loss of the retinal nerve fiber layer becomes visible as an excavation (cupping) at the optic nerve head (optic disc), and is measured by comparing the diameter or area of cupping with the diameter or area of the whole optic disc. The optic disc cupping occurs more in vertical direction resulting in a vertical ovalisation of the cup. This is quantified as the vertical cup-disc ratio (VCDR), a clinical important parameter in OAG management. Although a single measurement of VCDR is a significant determinant of the risk of developing OAG,[8,9] persons born with a high but stable VCDR are presumably not at risk for OAG. A considerable variation of VCDR and optic disc area in children without OAG has been described.[10] Irrespective of the VCDR at birth, an increase in VCDR over time suggests a loss of retinal ganglion cells in excess of the normal age-related decline, a pathological sign of OAG.

In epidemiological research the presence of GON is often defined as a certain value of VCDR, also called cut-off. Theoretically the development of GON starts together with GVFL. However, if GON is based on a certain cut-off point of VCDR persons may be classified wrongly as having GON. For example, a person born with a high but stable VCDR may be classified as having GON irrespective of glaucomatous changes at the optic nerve head. Furthermore, persons with GVFL might be classified as not having GON. The other way around GVFL might not be detected in persons with GON, due to visual field defects, which are too subtle to be captured with perimetry. Therefore, albeit some misclassification is evitable, it is important to find the optimal cut-off point of VCDR in order to get more precise results in risk-factor analyses.

Evaluating risk factors for OAG

Regarding the most important risk factor for OAG, two main theories exist as to how elevated IOP may initiate glaucomatous damage. An increase in IOP can result in either mechanical pressure or ischemia of the optic nerve head (optic disc). Both mechanisms may independently

contribute to loss of retinal ganglion cells, but the exact mechanism that underlies the structural and functional changes in OAG are still unknown. Especially the vascular role and oxidative stress need further evaluation. Oxidative stress has been suggested to be an important step in both mechanisms leading to OAG.[11] Furthermore, of the mentioned risk factors for OAG, the IOP is currently the only modifiable risk factor. Irrespective of both theories identification of other potential modifiable factors that affect the risk of OAG independent of IOP should provide new insights in the treatment of OAG.

Implicating genes in OAG

Another important risk factor for OAG is a positive family history, suggesting that genes are involved in the genetic etiology of OAG.[12] To date three causative genes (*MYOC*, *OPTN* and *WDR36*) have been established for late-onset OAG. However, the prevalence of these genes in OAG in the general population is low (Table 1).[13] This raises the following question: is the genetic risk underlying OAG caused by a few genes with large effects or by many genes with small effects? Common variants involved in OAG were not identified at the start of this thesis. To detect multiple common genetic variants with small effects large studies are needed. Genome-wide association (GWA) studies are designed to capture such variants. In GWA studies millions of common variants (single nucleotide polymorphisms [SNPs]) across the whole genome are genotyped in a large sample of independent persons, who are preferably from the same origin. This approach has been successful for several diseases (e.g. diabetes mellitus, myocardial infarction, hypercholesterolemia). In ophthalmology the approach has been successful in age-related macular degeneration.

Table 1. Three established genes in OAG

Myocilin (*MYOC*)	Optineurin (*OPTN*)	WD40-repeat 36 (*WDR36*)
Chromosome 1, first reported locus	Chromosome 10, second gene discovered	Chromosome 5
In juvenile or early adult form of OAG	In family members with normal tension glaucoma (NTG)	Reported to be causative, however many studies failed to identify genetic variants in *WDR36* as the causative agent
Associated with IOP	Associated with normal IOP, NTG	
Prevalence 3-5% in OAG-cases	Prevalence rare in OAG and NTG-cases	Prevalence 1.6-17% in OAG-cases
Mechanism in pathogenesis OAG unclear	Mechanism in pathogenesis OAG unclear	Suggestive evidence of the involvement of p53 in OAG

IOP = intraocular pressure; OAG = open-angle glaucoma.

This thesis and its main study population

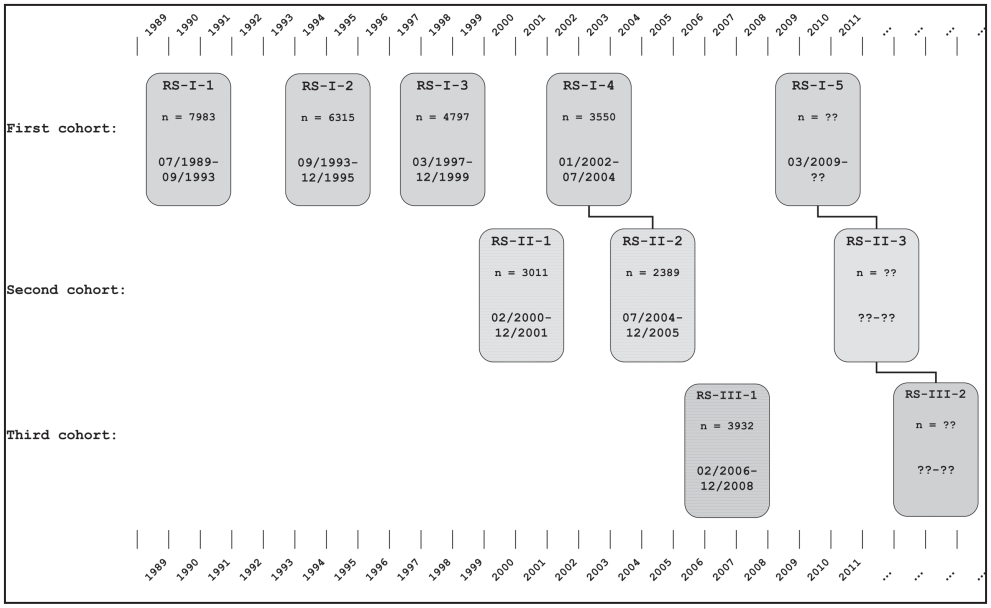
The current thesis uses epidemiological (chapter 2 and 3) and genetic approaches (chapter 4 and 5) on OAG that will discuss most of the issues described above.

Chapter 2 addresses an epidemiological definition of GON and how to determine the optimal cut-off point in a population-based study. Chapter 3 presents the ten-years incidence of OAG in the Rotterdam Study and addresses the issue whether there is evidence for a vascular component, in terms of perfusion pressure, affecting the risk of developing OAG. Modifiable risk factors such as lifestyle and nutrition-related risk factors are also studied in this section.

Chapter 4 focuses on the genetics of clinical relevant quantitative traits for OAG. These traits are also referred to as endophenotypes. Endophenotypes are defined as (heritable) phenotypes which are related to the disease of interest For example, blood pressure instead of hypertension. The potential endophenotypes described in this chapter comprise the optic disc area, the VCDR, and the IOP. Furthermore, a genetic polygenic model underlying these traits and OAG will be discussed. Chapter 5 assesses the clinical implication of new genes identified for OAG.

All studies described in this thesis were performed as part of the Rotterdam Study (Erasmus Rotterdam Gezondheid Onderzoek; ERGO), comprising of three cohorts. All of which are prospective population-based studies conducted in Ommoord, a district of Rotterdam, the Netherlands.[14,15] Examinations of the first cohort (RS-I) started in 1991, the second cohort (RS-II) in 2000, and the third cohort (RS-III) in 2006 (Figure 2). RS-I and RS-II included participants aged 55 or over at baseline, and RS-III included participants of 45 years or older. Participants of each cohort participated in several follow-up examinations. At each visit at the research center participants were thoroughly examined targeting neurological, cardiovascular, ophthalmological diseases, and more.

Figure 2. Timeline of the Rotterdam Study cohorts (by: Frank J.A. van Rooij).



For OAG, data of RS-I-2 was merged with RS-I-1 (baseline), because of the short time interval between the visits, ophthalmic part started some months later and as a consequence some participants were not examined until RS-I-2, and participants underwent fewer examinations at RS-I-2. Therefore, RS-I-3 and RS-I-4 were considered first and second follow-up, respectively.

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Chapter 2

Normative values of the optic nerve head

2.1 *Heidelberg Retina Tomograph (HRT3) in population-based epidemiology: normative values and criteria for glaucomatous optic neuropathy.*
Ramdas WD, Wolfs RC, Hofman A, de Jong PT, Vingerling JR, Jansonius NM.
Ophthalmic Epidemiol. [accepted]

2.2 *Defining glaucomatous optic neuropathy from a continuous measure of optic nerve damage - the optimal cut-off point for risk-factor analysis in population-based epidemiology.*
Ramdas WD, Rizopoulos D, Wolfs RC, Hofman A, de Jong PT, Vingerling JR, Jansonius NM.
Ophthalmic Epidemiol. [accepted]

Heidelberg Retina Tomograph (HRT3) in population-based epidemiology: normative values and criteria for glaucomatous optic neuropathy

ABSTRACT

Purpose: To establish normative values for Heidelberg Retina Tomograph (HRT3) variables and to develop HRT3-based criteria for glaucomatous optic neuropathy for epidemiological research in a white population.

Methods: Consecutive participants in the Rotterdam Study were examined with HRT and simultaneous stereoscopic fundus photography (ImageNet) in addition to other ophthalmic examinations including intraocular pressure (IOP) measurements and perimetry. Normative values for all HRT3 variables were determined in participants who met all the following criteria: no glaucomatous visual field loss, an IOP of 21 mmHg or less, no IOP lowering treatment, and a negative family history of glaucoma. Sensitivity was determined in participants with glaucomatous visual field loss at a fixed high specificity of 97.5% - a value commonly used in population-based epidemiology.

Results: 2516 participants were included in this study of whom 66 had glaucomatous visual field loss in at least one eye and 1680 fulfilled the criteria for contributing to the normative values. The HRT3 linear cup-disc ratio (LCDR) variable, adjusted for disc area, showed the highest sensitivity, 35%, at the required specificity of 97.5%. The 97.5th percentile of the LCDR was 0.67 for small discs (up to 1.5 mm^2), 0.71 for medium sized discs and 0.76 for large discs (above 2.0 mm^2). The HRT3 Glaucoma Probability Score and previously published linear discriminant functions showed a lower sensitivity than LCDR at this specificity.

Conclusions: At the high specificity of 97.5% as is commonly used in population-based epidemiology, the sensitivity of the HRT3 is low - albeit not lower than that of the vertical cup-disc ratio as assessed with simultaneous stereoscopic fundus photography and analyzed with the ImageNet software. The LCDR variable, stratified for disc area, seems to be the most suitable variable to develop criteria for glaucomatous optic neuropathy for epidemiological purposes.

INTRODUCTION

Glaucoma is one of the main causes of irreversible blindness in the world.[1] Although several risk factors have been identified, its etiology is still largely unknown. Epidemiological research is one of the ways to further increase our understanding of the pathophysiology of this disease. Moreover, epidemiological knowledge is indispensable for health care planning.

Most epidemiological definitions of primary open-angle glaucoma include morphologic changes in the optic nerve head (ONH).[2-4] The ONH can be assessed with fundoscopy or fundus photography. Modern imaging techniques, however, provide better objectivity and standardization.[5] Although these new techniques may have their limitations as well, objectivity and standardization are very important properties for, for example, large-scale epidemiological studies and this makes the performance of these techniques in population-based epidemiology a pivotal issue. The Heidelberg Retina Tomograph (HRT; Heidelberg Engineering, Dossenheim, Germany) uses an imaging technique that has shown long-term stability and backwards compatibility, that is, results remain comparable with those from previous versions.[6-9] The HRT has a glaucoma module, which makes it possible to create a 3-dimensional map of the ONH and the peripapillary retina. From this map, the value of a number of optic disc parameters can be determined.[10] Several studies have shown that these parameters differ significantly between normal eyes and eyes with glaucomatous visual field loss.[3,11-20]

Recently, the Tajimi Study, a population-based study, evaluated ONH characteristics using HRT3 in normal Japanese subjects.[21] They reported low sensitivities for the built-in classification algorithms, in contrast to the high sensitivities found in clinical settings.[22-24] This suggests that clinically used HRT3 variables cannot be used in epidemiological studies as a matter of course.

The aim of this study was to determine normative HRT3 data of a (mainly) white population and to establish an HRT3-based definition of glaucomatous optic neuropathy for epidemiological purposes. For this purpose, we used HRT measurements that were performed in participants in the population-based Rotterdam Study. Data obtained with simultaneous stereoscopic photography (ImageNet; Topcon Corporation, Tokyo, Japan) were available in a subset of this cohort and were compared to the HRT3 data.

MATERIALS AND METHODS

Participants

The present study was performed as part of the Rotterdam Study, a prospective population-based cohort study of residents aged 55 years and older living in Ommoord, a suburb of Rotterdam, the Netherlands. The rationale and study design have been described previously.[25]

All measurements were conducted after the Medical Ethics Committee of the Erasmus University had approved the study protocol and after all participants had provided written informed consent in accordance with the Declaration of Helsinki. The baseline examinations took place between 1991 and 1993; follow-up examinations for glaucoma were performed from 1997 to 1999 and from 2002 to 2006. During the second follow-up examination, both simultaneous stereoscopic photography with the Topcon ImageNet System (Topcon Corporation, Tokyo, Japan) and ONH assessment with the Heidelberg Retina Tomograph (HRT; Heidelberg Engineering, Dossenheim, Germany) were performed during the same visit. All participants from whom HRT data were available were included in this study. For the normative data, participants were required to have no glaucomatous visual field loss (see below), an intraocular pressure (IOP) of 21 mmHg or less at all visits and no IOP-lowering treatment in both eyes, and a negative family history of glaucoma.

Ophthalmic examination

The ophthalmic assessment in the Rotterdam Study, identical at baseline and follow-up examinations, included a medical history, Goldmann applanation tonometry (Haag-Streit, Bern, Switzerland), autorefraction (Topcon RM-A2000; Tokyo Optical Co., Tokyo, Japan), keratometry (Topcon OM-4 Ophthalmometer; Tokyo Optical Co., Tokyo, Japan), best-corrected visual acuity, visual field testing (see below), and ophthalmoscopy and ONH imaging with HRT and ImageNet in both eyes after mydriasis with tropicamide 0.5% and phenylephrine 2.5% eye drops.

Perimetry

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.[2,26] Visual field loss 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 visual field loss 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 visual field loss, 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[26] and the full-threshold HFA 24-2 test results[27] have been described before. In short, visual field loss was considered to be glaucomatous visual field loss only if reproducible and after excluding all other possible causes. Data from ImageNet or HRT were not included in the classification process of the perimetry test results.[27]

Optic nerve head assessment with ImageNet

ImageNet 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 two trained technicians using the ImageNet retinal nerve fiber layer height module. On each stereoscopic pair of optic disc images the technicians marked four points 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. The technicians also marked several points near retinal blood vessels on the stereoscopic pair. These points are used by ImageNet to define a retinal zero-reference plane. All points within the ellipse and at least 150 µm below the zero-reference plane were considered as cup. We used the VCDR as our ImageNet outcome measure.[2] The software is able to calculate the amount of correspondence between points on the two images of the stereoscopic pair. This 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.[28]

Optic nerve head assessment with Heidelberg Retina Tomograph

HRT uses a focused 670-nm diode laser light beam to acquire scans of the ONH region, using the confocal principle. The HRT obtains, during one scan, three series of 16 to 64 confocal frontal slices. A slice covers an angle of 15 x 15 degrees with a resolution of 384 x 384 pixels. From each of these series, a 3-dimensional image of the ONH is reconstructed, from which the software calculates several optic disc parameters. To define the cup, a reference plane is placed 50 µm below the peripapillary retinal surface in the region of the papillomacular bundle. Using the HRT3 software (Heidelberg Eye Explorer [HEyEx] version 1.5.10.0), previously collected data from HRT2 were recalculated and converted to HRT3 (see Discussion). Preceding HRT scanning, autorefraction and keratometry data were obtained. The focusing dial on the camera was adjusted for the corresponding spherical component of the refraction of the eye. No adjustments for astigmatism were applied (see Discussion). All ONH scans were made with a head-to-eye distance between 10 and 20 mm. 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 of more than 50 µm were excluded as recommended in the HRT manual.

Stereometric Heidelberg Retina Tomograph parameters

The HRT provides several stereometric parameters. To enable the calculation of these parameters, manually drawing a contour line to mark the edge of the optic disc is required. This was done by either one or the other of two trained technicians, who placed 4 to 8 points on the disc margin.[29] Identifying the optic disc margin on an HRT scan can sometimes be very

difficult, depending on e.g. the experience of the technician and the image quality of the scan. The agreement between the two technicians was checked in a random subset of scans of 114 right eyes with a topographic standard deviation of 50 µm or less. For this analysis, we used the linear cup-disc ratio (LCDR; the square root of the total cup-disc area ratio) as a measure of the cup-disc ratio, since vertical cup-disc ratio (VCDR) was reported to suffer from HRT software limitations.[30]

Diagnostic algorithms for Heidelberg Retina Tomograph

There are several diagnostic algorithms for HRT published,[15,18,31-33] some of which are built-in in the HRT3 software.[18,31-33] All these algorithms rely on a manually placed contour line, except for the so-called Glaucoma Probability Score (GPS).[33] Most algorithms have a continuous output measure;[15,31,32] the Moorfields Regression Analysis (MRA) has a categorical output (within normal limits, borderline and outside normal limits),[31] whereas GPS has both a continuous and a categorical output.[33] The MRA and GPS classify the ONH not only globally but also separately for six sectors.

Statistical analysis

For assessing the agreement between both technicians for HRT, we used Bland-Altman analysis[34] applied to the HRT LCDR values of the 114 eyes of 114 participants analyzed by both technicians, independently of each other. For comparison, the VCDR of the ImageNet slides of these 114 eyes were also subjectively assessed by two glaucoma specialists (RCWW and NMJ). For the normative database, we calculated mean values, standard deviations and 97.5th percentiles of the various HRT3 parameters in participants without glaucomatous visual field loss. Differences between right and left eyes were analyzed with paired t-tests; differences between males and females and between participants with and without glaucomatous visual field loss with independent sample t-tests and chi-square statistics. The effects of disc area and refraction were analyzed using ANOVA, after stratification. For this purpose, the square root of the disc area was used, since the area itself showed a skewed distribution. The disc area was classified as small (square root of disk area up to mean –2/3 standard deviation [s.d.]), medium sized, or large (above mean +2/3 s.d.). Refraction was stratified into five categories: up to -4.00 D, -3.99 to -1.01 D, -1.00 to +1.00 D, +1.01 to +3.99 D, and +4.00 D and above. Those eyes with a previous cataract extraction were excluded from the determination of the normative data stratified for refractive error but were included in all other analyses. A p-value of 0.05 or less was considered statistically significant. In case of multiple comparisons, Bonferroni correction was applied. Receiver Operator Characteristic (ROC) curves were made for all continuous variables, using participants with glaucomatous visual field loss to establish sensitivity. Areas under the ROC curve and sensitivities at a specificity of 97.5% were determined. In an accompanying article, we showed that this specificity is close

to optimal from the point of view of risk-factor analysis in population-based epidemiology. [Ramdas, et al.; Chapter 2.2] Positive predictive values and sensitivities were determined for a range of cut-off points for the HRT3 variable with the highest sensitivity at a specificity of 97.5%. Classification by this HRT3 variable was compared to classification by ImageNet VCDR at a specificity of 97.5%, using McNemar's test. As we did not use HRT or ImageNet data for the original classification process (but only perimetry data), we also applied the ISGEO criteria[35] by including ImageNet data to the classification process, and subsequently recalculated the sensitivity of the proposed criteria for glaucomatous optic neuropathy according to HRT. All statistical analyses were performed using SPSS version 15.0.0 for Windows (SPSS inc., Chicago, IL, USA; 2006).

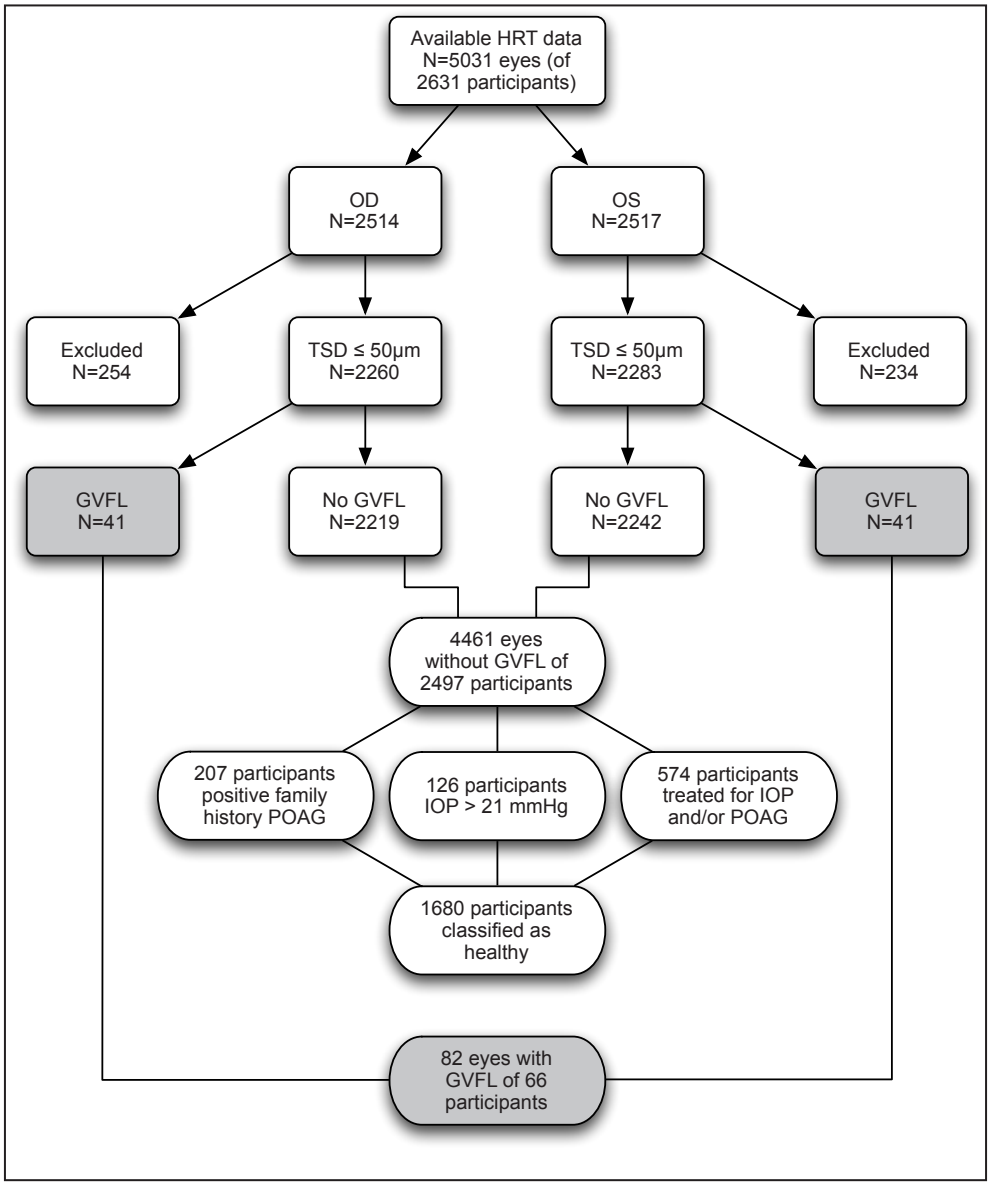
RESULTS

The baseline examination of the ophthalmic part of the Rotterdam Study (1991-1993) included 6806 participants of whom 3088 attended the second follow-up between October 2002 and January 2006. Of these 3088 participants including 116 with glaucomatous visual field loss, 2631 (including 81 with glaucomatous visual field loss) underwent an HRT examination on one or both eyes (457 participants were not scanned due technical reasons not related to the participants) resulting in a total of 5031 scanned eyes We excluded 488 of these 5031 eyes because of a topographical standard deviation > 50 µm; this resulted in the exclusion of 115 of 2631 participants with unreliable scans in both eyes. Of the remaining 2516 participants (95.6%), 66 (2.6%) had glaucomatous visual field loss in at least one eye (Figure 1). The global index mean deviation on full-threshold perimetry of the cases with glaucomatous visual field loss was on average -10.4 dB (range: -28.2 to -1.1 dB, median: -9.3 dB) in the included eye. Following the Hodapp classification system,[36] 18.2% of the included eyes had early glaucomatous loss, 27.3% moderate loss, and 54.5% severe glaucomatous loss. Of the remaining participants without glaucomatous visual field loss, 1680 had an IOP of 21 mmHg or less and no IOP-lowering treatment in both eyes, and a negative family history of glaucoma. These 1680 participants (healthy subjects) were used to establish the normative data.

Table 1 summarizes the general characteristics of the study population. Participants without glaucomatous visual field loss were significantly younger and had a slightly better image quality in terms of the topographical standard deviation.

As mentioned in the Methods section, all HRT parameters except GPS require manual outlining of the optic disc margin. The influence of this manual outlining was studied by comparing HRT3 LCDR values based on the outlining of identical HRT scans by two different technicians in a random subset of 114 right eyes. Bland-Altman analysis showed good agreement between the two technicians (mean difference 0.00; 95% CI of difference -0.02 to 0.01; limits of agreements -0.15 to 0.14). For comparison, we also determined the agreement

Figure 1. Flowchart showing the inclusion of 1680 healthy subjects and 66 participants with glaucomatous visual field loss out of 2631 participants from the population-based study cohort. Healthy subjects were participants without glaucomatous visual field loss, with an intraocular pressure (IOP) of 21 mmHg or less, without IOP-lowering treatment and with a negative family history of glaucoma.



HRT = Heidelberg Retina Tomograph; TSD = topography standard deviation; GVFL = glaucomatous visual field loss; POAG = primary open-angle glaucoma.

between two glaucoma specialists assessing subjectively the VCDR using the ImageNet slides (mean difference -0.02; 95% CI of difference -0.05 to 0.00; limits of agreements -0.25 to 0.20), between HRT and ImageNet (mean difference -0.05; 95% CI of difference -0.07 to -0.03; limits of agreements -0.25 to 0.16), between HRT and the glaucoma specialists (mean difference 0.05; 95% CI of difference 0.02 to 0.08; limits of agreements -0.23 to 0.32), and between ImageNet and the glaucoma specialists (mean difference 0.10; 95% CI of difference 0.08 to 0.13; limits of agreements -0.14 to 0.34).

Table 1. Characteristics of the participants used for the normative data (healthy subjects) and the participants with glaucomatous visual field loss (mean ± standard deviation with range between brackets unless stated otherwise)

	Healthy Subjects (N=1680)	Participants with GVFL (N=66)*	p-value
Age (year)	74.1 ± 5.6 (65 to 95)	78.0 ± 5.5 (66 to 92)	<0.001
Gender, % female	56.3	48.5	0.213
Spherical equivalent (D)**	1.2 ± 2.4 (-12.0 to 10.5)	0.4 ± 2.6 (-7.0 to 6.3)	0.037
TSD (µm)	20.7 ± 10.0 (0 to 50)	25.4 ± 11.1 (8 to 50)	<0.001

* = 35% based on Goldmann perimetry[27]; ** 220 eyes excluded because of missing refractive data or history of cataract surgery; GVFL = glaucomatous visual field loss; TSD = topography standard deviation.

After adjusting for multiple comparisons, there were no significant differences between right and left eyes, except for the mean RNFL thickness (0.209 versus 0.216; p=0.001), contour line modulation temporal-superior (0.158 versus 0.168; p<0.001) and the RB discriminant function[32] (0.929 versus 1.032; p<0.001). There were also no significant differences between men and women, except for contour line modulation temporal-inferior (0.132 versus 0.147; p<0.001). In the remainder of this study, we will only present the results for a randomly selected eye of each participant.

Table 2 shows normative values for the HRT3 parameters and corresponding values in participants with glaucomatous visual field loss. Most HRT3 parameters differed significantly between both groups except for disc area and maximum cup depth. Table 3 presents the normative values after stratification for refractive error (spherical equivalent). After adjusting for multiple comparisons, differences between the subgroups were found for rim area, rim volume (higher in myopic eyes), height variation contour, contour line modulation temporal-superior and topographic standard deviation.

Table 4 shows area under the ROC curve values and sensitivities at 97.5% specificity, using the cut-off points as displayed in Table 2 (97.5th percentile). Sensitivities of MRA are lacking in Table 4 because MRA has a categorical output. Only MRA segment temporal – with borderline classified as within normal limits – had a specificity close to 97.5%; all other MRA parameters had specificities below 95%. The corresponding sensitivity of MRA segment temporal was 18.8%. Variables with the highest sensitivities at 97.5% specificity were LCDR, VCDR and the sectorial GPS variables temporal/inferior and nasal/inferior. As mentioned

in the Methods section, VCDR was reported to suffer from HRT software limitations.[30] Furthermore, the HRT3 software often failed to calculate the sectorial GPS values. This failure was not related to the presence of glaucomatous visual field loss (that is, to the presence of glaucomatous damage; p=0.769) and happened in 872 of 4543 scans with a topographical standard deviation of 50 µm or less; in 330 of these 872 scans the HRT3 was not able to calculate a global GPS value either. Therefore, we will mainly concentrate on LCDR. Figure 2 shows ROC curves for HRT3 LCDR and for the three linear discriminant functions.

Cup-disc ratios are reported to depend on disc area[37,38] and a possible way to improve

Table 2. Normative values of Heidelberg Retina Tomograph parameters based on a randomly chosen eye of the healthy subjects and the corresponding values in the participants with glaucomatous visual field loss

	Healthy Subjects (N=1680)			Participants with GVFL (N=66)		
	Mean	s.d.	p97.5	Mean	s.d.	p-value
Disc area (mm^2)	1.77	0.42	2.65	1.85	0.45	0.114
Cup area (mm^2)	0.40	0.32	1.14	0.73	0.50	<0.001*
Rim area (mm^2)	1.37	0.36	2.23	1.11	0.43	<0.001*
Cup-disc area ratio	0.22	0.15	0.53	0.38	0.22	<0.001*
Rim-disc area ratio	0.78	0.15	1.00	0.62	0.22	<0.001*
Cup volume (mm^3)	0.08	0.09	0.33	0.16	0.17	<0.001*
Rim volume (mm^3)	0.34	0.17	0.78	0.26	0.15	<0.001*
Mean cup depth (mm)	0.17	0.09	0.38	0.22	0.11	<0.001*
Maximum cup depth (mm)	0.49	0.24	1.00	0.56	0.25	0.019
Height variation contour (mm)	0.36	0.16	0.62	0.40	0.17	0.023
Cup shape measure	-0.18	0.08	-0.04	-0.11	0.09	<0.001*
Mean RNFL thickness (mm)	0.21	0.08	0.35	0.17	0.08	<0.001*
RNFL cross sectional area (mm^2)	0.98	0.39	1.76	0.81	0.37	0.001*
Horizontal cup-disk ratio	0.40	0.23	0.85	0.53	0.26	<0.001*
Vertical cup-disk ratio	0.33	0.25	0.79	0.57	0.30	<0.001*
Maximum contour elevation (mm)	-0.12	0.12	0.06	-0.06	0.11	<0.001*
Maximum contour depression (mm)	0.24	0.15	0.52	0.34	0.18	<0.001*
CLM temporal-superior (mm)	0.16	0.09	0.32	0.14	0.10	0.243
CLM temporal-inferior (mm)	0.14	0.09	0.31	0.06	0.13	<0.001*
Average variability (s.d.; mm)	0.03	0.02	0.09	0.04	0.02	0.008*
Reference height (mm)	0.26	0.13	0.52	0.31	0.14	0.002*
Linear cup-disc ratio	0.42	0.19	0.73	0.58	0.22	<0.001*
GPS (global)	0.35	0.28	0.91	0.60	0.32	<0.001*
FSM discriminant function value[31]	1.48	1.99	5.97	-0.57	2.43	<0.001*
RB discriminant function value[32]	0.94	0.83	2.52	0.13	1.06	<0.001*

* significant at a Bonferroni adjusted p-value of 0.002; GVFL = glaucomatous visual field loss; s.d. = standard deviation; p97.5 = 97.5th percentile; RNFL = retinal nerve fiber layer; CLM = contour line modulation; GPS = glaucoma probability score.

the sensitivity could be by taking disc area into account. Figure 3 shows mean values and 97.5th percentiles for HRT3 LCDR and ImageNet VCDR, stratified for the square root of the disc area. The means of HRT3 LCDR ($p<0.001$) and ImageNet VCDR ($p<0.001$) increased significantly with disc area. The HRT3 LCDR cut-off values for small (disc area up to 1.5 mm^2), medium sized and large (disc area above 2.0 mm^2) optic discs were 0.67, 0.71 and 0.76. The corresponding 99.5th percentiles were 0.82, 0.76, and 0.84, respectively. Figure 4 presents the distributions of the square root of the disc area for both systems.

Table 3. Normative values of Heidelberg Retina Tomograph Parameters stratified for refractive error

	Refractive Error*					p-value
Refraction range (D)	Up to -4.00	-3.99 to -1.01	-1.00 to 1.00	1.01 to 3.99	4.00 and up	
	Mean ± s.d.	Mean ± s.d.	Mean ± s.d.	Mean ± s.d.	Mean ± s.d.	
Disc area (mm^2)	1.96 ± 0.52	1.80 ± 0.46	1.76 ± 0.43	1.77 ± 0.39	1.69 ± 0.43	0.005
Cup area (mm^2)	0.40 ± 0.39	0.35 ± 0.37	0.41 ± 0.33	0.41 ± 0.30	0.35 ± 0.27	0.135
Rim area (mm^2)	1.56 ± 0.45	1.45 ± 0.44	1.35 ± 0.36	1.36 ± 0.33	1.35 ± 0.36	<0.001**
Cup-disc area ratio	0.20 ± 0.16	0.18 ± 0.16	0.22 ± 0.15	0.22 ± 0.14	0.19 ± 0.14	0.032
Rim-disc area ratio	0.80 ± 0.16	0.82 ± 0.16	0.78 ± 0.15	0.78 ± 0.14	0.81 ± 0.14	0.032
Cup volume (mm^3)	0.06 ± 0.08	0.06 ± 0.09	0.08 ± 0.10	0.08 ± 0.10	0.06 ± 0.07	0.103
Rim volume (mm^3)	0.47 ± 0.26	0.40 ± 0.22	0.34 ± 0.16	0.33 ± 0.15	0.35 ± 0.19	<0.001**
Mean cup depth (mm)	0.16 ± 0.08	0.16 ± 0.09	0.18 ± 0.10	0.18 ± 0.09	0.17 ± 0.08	0.095
Maximum cup depth (mm)	0.47 ± 0.24	0.45 ± 0.24	0.49 ± 0.25	0.49 ± 0.24	0.47 ± 0.22	0.320
Height variation contour (mm)	0.45 ± 0.37	0.39 ± 0.26	0.35 ± 0.15	0.35 ± 0.11	0.36 ± 0.13	<0.001**
Cup shape measure	-0.18 ± 0.07	-0.18 ± 0.07	-0.17 ± 0.07	-0.18 ± 0.08	-0.18 ± 0.06	0.727
Mean RNFL thickness (mm)	0.21 ± 0.09	0.21 ± 0.09	0.21 ± 0.07	0.21 ± 0.07	0.22 ± 0.09	0.401
RNFL cross sectional area (mm^2)	1.03 ± 0.47	1.02 ± 0.46	0.98 ± 0.36	0.98 ± 0.36	1.03 ± 0.44	0.532
Horizontal cup-disk ratio	0.40 ± 0.27	0.37 ± 0.26	0.40 ± 0.23	0.41 ± 0.22	0.37 ± 0.20	0.131
Vertical cup-disk ratio	0.33 ± 0.27	0.29 ± 0.26	0.34 ± 0.25	0.34 ± 0.24	0.29 ± 0.23	0.060
Maximum contour elevation (mm)	-0.14 ± 0.11	-0.14 ± 0.23	-0.11 ± 0.10	-0.11 ± 0.09	-0.12 ± 0.10	0.030
Maximum contour depression (mm)	0.32 ± 0.37	0.25 ± 0.14	0.24 ± 0.16	0.24 ± 0.13	0.25 ± 0.14	0.012
CLM temporal-superior (mm)	0.19 ± 0.11	0.17 ± 0.10	0.15 ± 0.09	0.15 ± 0.08	0.17 ± 0.09	0.001**
CLM temporal-inferior (mm)	0.12 ± 0.12	0.13 ± 0.11	0.14 ± 0.09	0.14 ± 0.09	0.15 ± 0.10	0.361
Average variability (s.d.; mm)	0.03 ± 0.02	0.03 ± 0.02	0.03 ± 0.02	0.03 ± 0.02	0.03 ± 0.02	0.184
Reference height (mm)	0.28 ± 0.16	0.26 ± 0.13	0.26 ± 0.14	0.26 ± 0.13	0.27 ± 0.14	0.708
Linear cup-disc ratio	0.39 ± 0.21	0.37 ± 0.21	0.43 ± 0.19	0.43 ± 0.18	0.41 ± 0.18	0.004
TSD (µm)	24.00 ± 9.89	22.97 ± 10.09	20.08 ± 9.95	19.44 ± 9.38	19.36 ± 8.55	<0.001**
GPS (global)	0.30 ± 0.29	0.30 ± 0.26	0.38 ± 0.29	0.36 ± 0.28	0.36 ± 0.29	0.082
FSM discriminant function value[31]	2.17 ± 3.10	1.93 ± 2.98	1.39 ± 1.88	1.39 ± 1.78	1.54 ± 1.74	0.005
RB discriminant function value[32]	1.04 ± 1.01	1.00 ± 0.86	0.90 ± 0.79	0.94 ± 0.79	1.09 ± 0.88	0.165

* 192 of 1680 participants (11.4%) excluded because of missing data or history of cataract surgery; ** significant at a Bonferroni adjusted p-value of 0.002; s.d. = standard deviation; RNFL = retinal nerve fiber layer; CLM = contour line modulation; TSD = topography standard deviation; GPS = glaucoma probability score.

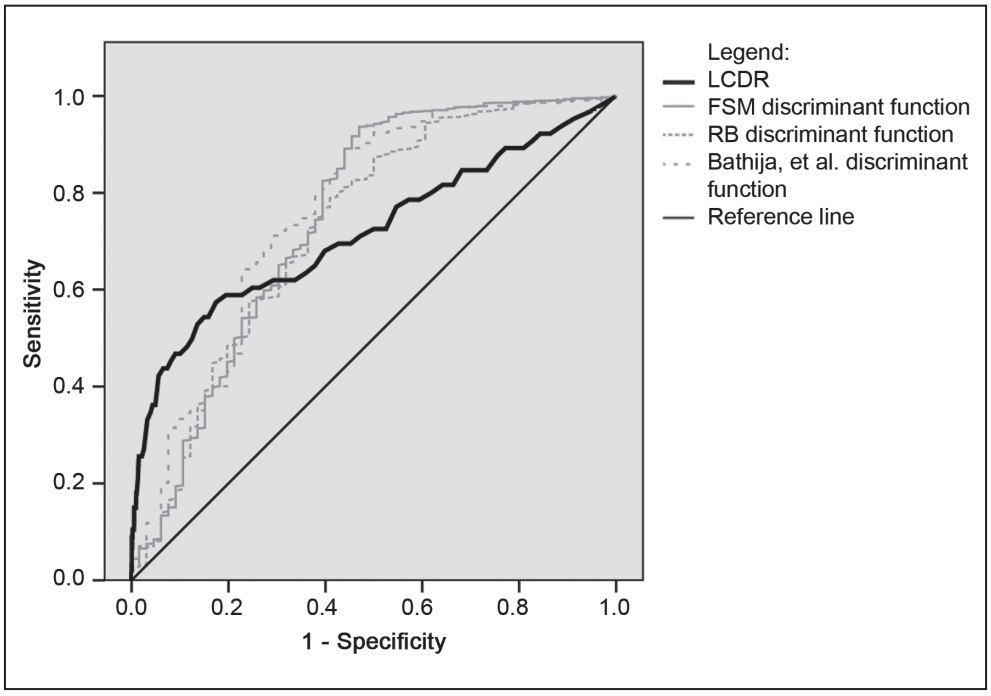
Table 4. Area under the receiver operator characteristic curve and sensitivity at 97.5% specificity for Heidelberg Retina Tomograph parameters

	AUC	Sensitivity (%)
Disc area (mm^2)	0.553	6.1
Cup area (mm^2)	0.696	24.2
Rim area (mm^2)*	0.685	3.5
Cup-disc area ratio	0.718	27.3
Rim-disc area ratio*	0.718	5.2
Cup volume (mm^3)	0.670	12.1
Rim volume (mm^3)*	0.668	5.8
Mean cup depth (mm)	0.636	9.1
Maximum cup depth (mm)	0.589	6.1
Height variation contour (mm)	0.591	10.6
Cup shape measure	0.712	22.7
Mean RNFL thickness (mm)*	0.644	9.9
RNFL cross sectional area (mm^2)*	0.627	4.0
Horizontal cup-disk ratio	0.645	16.7
Vertical cup-disk ratio	0.745	28.8
Maximum contour elevation (mm)	0.651	19.7
Maximum contour depression (mm)	0.679	10.6
CLM temporal-superior (mm)*	0.564	1.3
CLM temporal-inferior (mm)*	0.688	6.0
Average variability (s.d.; mm)	0.654	3.0
Reference height (mm)	0.611	6.1
Linear cup-disc ratio	0.718	27.3
MRA result	0.717	**
MRA global	0.724	**
MRA temporal	0.641	**
MRA temporal/superior	0.692	**
MRA temporal/inferior	0.740	**
MRA nasal	0.620	**
MRA nasal/superior	0.655	**
MRA nasal/inferior	0.704	**
GPS global	0.749	22.6
GPS temporal	0.747	22.6
GPS temporal/superior	0.760	24.5
GPS temporal/inferior	0.756	26.4
GPS nasal	0.755	24.5
GPS nasal/superior	0.750	24.5
GPS nasal/inferior	0.753	26.4

FSM discriminant function* [31]	0.748	6.5
RB discriminant function* [32]	0.729	3.2
Bathija, et al. discriminant function* [15]	0.762	7.0

* Glaucomatous visual field loss is read as "normal" and no glaucomatous visual field loss as "outside normal lir
No sensitivities calculated because MRA has a categorical outcome; AUC = Area under the receiver c
characteristic curve; RNFL = retinal nerve fiber layer; CLM = contour line modulation; MRA = Moorfield reg
analysis; GPS = Glaucoma probability score; s.d. = standard deviation.

Figure 2. Receiver Operator Characteristic Curves of Heidelberg Retina Tomograph linear cup-disc ratio (LCDR) and the three linear discriminant functions as published by Mikelberg et al.,[31] Burk et al.,[32] and Bathija et al..[15]



The sensitivities of the disc-area corrected variables were 35% for HRT3 LCDR and 19% for ImageNet VCDR (p=0.021; McNemar's test) at a specificity of 97.5%, for those participants with glaucomatous visual field loss and available data for both systems (N=48). Table 5 gives the positive predictive values and sensitivities for a range of cut-off values for disc-area corrected HRT3 LCDR. If we redefined glaucoma using the ISGEO criteria, only 55 participants met the criteria for being classified as having glaucoma. The corresponding sensitivity for the 97.5th percentile of the disc-area corrected HRT3 LCDR was 38.2% at a specificity of 97.5%.

Figure 3. Mean values (bars) and 97.5th percentiles (lines) for Heidelberg Retina Tomograph linear cup-disc ratio (left) and ImageNet vertical cup-disc ratio (right), stratified for disc area (small, medium sized, large; see Figure 4), based on the 1680 healthy subjects.

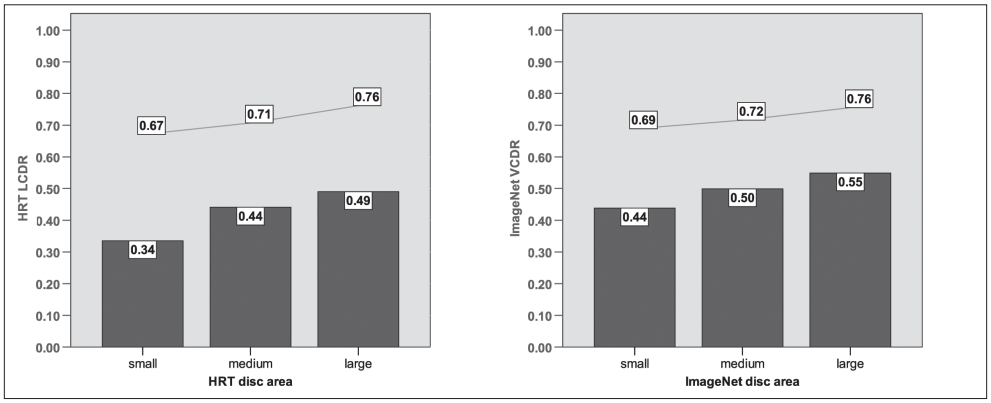


Figure 4. Distributions of the square root of the disc area for the Heidelberg Retina Tomograph and ImageNet, based on the 1680 healthy subjects.

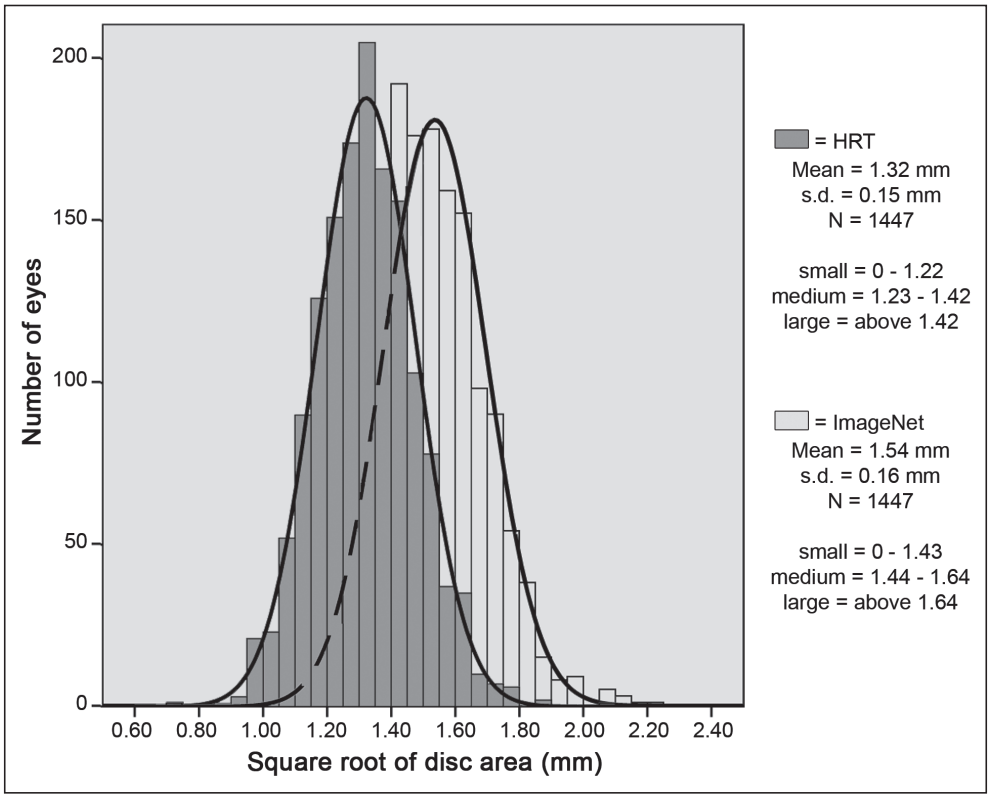


Table 5. Positive predictive values and sensitivities for a range of cut-off percentiles (specificities) for the disc-area corrected Heidelberg Retina Tomograph parameter linear cup-disc ratio

Percentile	Positive Predictive Value (%)	Sensitivity (%)
50th	5.0	75.0
80th	10.6	64.6
90th	18.5	56.3
95th	24.7	41.7
97.5th	32.1	35.4
99th	44.4	16.7

DISCUSSION

In this article, we presented population-based normative values for a large number of HRT3 variables. Of these variables, the LCDR turned out to be the most suitable variable for defining glaucomatous optic neuropathy for epidemiological purposes in this white population.

Some sectorial GPS variables had a similar sensitivity as LCDR at the required specificity of 97.5%. An advantage of GPS is that it does not require manual outlining of the optic disc margin and this advantage makes GPS, at first sight, the better parameter in population-based epidemiology.[39] However, adjusting for disc area appeared to improve the sensitivity of LCDR. A similar adjustment in case of GPS would still require manual outlining. Moreover, although the mean value of GPS seems to depend on disc area,[23,39-42] its 97.5th percentile did not (data not shown) and as a consequence the diagnostic performance of this parameter cannot be improved by adjusting for disc area. Another drawback of GPS turned out to be the fact that the software failed to calculate this variable in a significant number of eyes. Ideally, in a research setting, disc-margin delineation should be performed by a glaucoma-fellowship trained specialist. We found that manual outlining of the disc margin by different technicians yielded very similar LCDR values. This has also been found in other studies.[43-49] Although identical LCDR values do not guarantee perfect disc-margin delineation, the agreement shows that any imperfections in the delineation do not compromise the diagnostic performance for this variable. Hence, disc-area adjusted LCDR appears to be the most suitable variable for an HRT3-based epidemiological definition of glaucomatous optic neuropathy. An earlier study also showed that, among the conventional stereometric parameters, the LCDR had the highest discriminating ability between glaucoma and healthy subjects.[11]

Obviously, a sensitivity of 35% seems not very high. It is difficult to compare this sensitivity with clinical studies, since sensitivity depends on the cut-off point (specificity) applied and on the characteristics of the study population, including the age distribution of the participants (a higher age could contribute to false-positive perimetric test results) and the distribution of the glaucoma severity amongst the glaucoma cases. Due to the inclusion criterion of a minimum age of 55 years (see Methods section), the mean age of our study

population was relatively high compared to other population-based studies.[22,50] However, other population-based studies reported a similar mean age.[51-53] Regarding glaucoma severity, the median mean deviation of the glaucoma cases in our study was -9.3 dB. Some population-based studies reported a somewhat better mean deviation compared to ours;[22,50,54] whereas other studies reported a similar or even worse value.[51,53] In the Early Manifest Glaucoma Trial study, the mean mean deviation at inclusion was -5 dB. Their study population, however, was on average 10 years younger than our glaucoma cases.[55] Apart from possible differences in glaucoma severity, glaucoma cases in clinical studies presumably have more pronounced disc abnormalities. The reason for this difference is that - unless the IOP is obviously elevated - the appearance of the optic disc triggers doing perimetry in clinical settings and this might result in higher HRT sensitivities in clinical studies. If we applied the ISGEO criteria, which include optic disc properties in its glaucoma definition, the sensitivity increased to 38.2% at the aforementioned specificity of 97.5%. Area under the ROC curve values comparable to the values found in the current study have been reported in several clinical studies.[17,23,40,41] Interestingly, the population-based Tajimi Study evaluated the HRT3 in a population-based setting and found similar low sensitivities for the mentioned algorithms at an even lower specificity of around 86.7% (to be compared to 97.5% in our study). This suggests a modest performance of the HRT in population-based settings. However, the sensitivity we found was significantly higher (p=0.021; McNemar test) than the sensitivity found with ImageNet at the same specificity. Hence, a rather low sensitivity in a population-based setting is not uniquely for the HRT3. Despite this low sensitivity, the prevalence of glaucomatous optic neuropathy is about 14 times higher in cases with glaucomatous visual field loss (35%) than in controls (2.5%, by definition). The Tajimi Study found positive predictive values ranging from 10% to 29% at specificities ranging from 58% to 96%.[22] These findings are in good agreement with our results (Table 5). It should be emphasized that it is not our aim to employ or advocate the HRT for population-based screening programs as part of the health care system – the low sensitivity precludes this application and there are better devices for this purpose.[56] Rather, glaucomatous optic neuropathy is one of the features that contribute to the classification of glaucoma cases and controls in population-based studies. In an accompanying study, we showed that the 97.0th or 97.5th percentile is an appropriate cut-off point for risk-factor analyses conducted with population-based HRT data.[Ramdas, et al.; Chapter 2.2]. To some extend, the finding that HRT abnormalities are not one-to-one related to visual field abnormalities is not a weakness of our study or the HRT, but a property of glaucoma. It should be noted that many cases with so-called pre-perimetric glaucoma are picked-up correctly by the HRT: after the exclusion of cases with glaucomatous visual field loss, IOP remained a highly significant risk factor for glaucomatous optic neuropathy according to the HRT.[27]

We found no significant differences for gender in HRT3 measurements except for a slightly higher mean retinal nerve fiber layer thickness in females. This has also been found in other studies.[21,54,57] Refractive error was significantly related to rim area, rim volume and topographic standard deviation. In addition, myopic discs tended to be larger, but this was –

possibly related to the application of the conservative Bonferroni correction – not significant. These findings are in agreement with other studies that presented HRT data of healthy adults.[57-61] We found no differences between left and right eyes, which has also been found in another population-based study,[21] except for a higher mean retinal nerve fiber layer thickness in left eyes. This is presumably a chance finding, since another study found a higher thickness in right eyes.[59] Previous studies have found relationships between age and several HRT parameters.[60,61] This was not addressed in our study, as the age range of participants in our study was small (Table 1). The difference of the maximum cup depth between normal subjects and participants with glaucomatous visual field loss did not reach statistical significance due to the application of Bonferroni correction (p=0.019; Table 2); a significant difference was found, however, for the mean cup depth (p<0.001). It should be noted that, unlike our study, none of the studies, except the Tajimi study, cited above were based on HRT3, and that two of them were based on Japanese persons.[60,61] Our study was based on an almost completely (96%) white population.

Earlier definitions of glaucomatous optic neuropathy were also based on asymmetry in cup-disc ratio between left and right eyes,[62-69] and on the minimal neural rim width.[2] Minimal neural rim width had to be skipped, because HRT3 cannot calculate this variable. We decided to skip asymmetry too, for two reasons. First, asymmetry causes ambiguity in the definition of incident glaucomatous optic neuropathy: a subject may have a decreasing asymmetry caused by an increasing VCDR in the eye with the lower VCDR at baseline. Second, the additional yield of adding asymmetry to the HRT3-based glaucomatous optic neuropathy definition appeared to be very small (see Results section).

At first sight, it seems strange that we ended up with a basic variable such as (disc-area adjusted) LCDR whereas several more sophisticated compound variables have been developed.[15,18,31,32] These compound variables tended to have higher area under the ROC curve values than the basic HRT3 variables (Table 4). However, at a specificity of 97.5%, the sensitivities of these compound variables tended to be lower than those of the basic variables. This paradox is illustrated in Figure 2: the higher area under the ROC values of the compound variables are related to a high sensitivity at a moderate specificity; for higher specificities, the curve of the LCDR surpasses that of the compound variables.

As mentioned in the Methods section, all measurements were performed with HRT2 and were thereafter converted to HRT3. The results of HRT2 and HRT3 may differ slightly because of a change in horizontal scaling implemented in HRT3. However, this difference is not expected to occur in stereometric parameters (e.g. LCDR) that represent ratios.[70] Therefore the HRT parameters remain backwards compatible, which is especially useful in longitudinal studies.

According to the HRT manual, using a cylinder correction in cases with astigmatism values of 1.00 D and above is recommended. These cylinders were introduced by Heidelberg Engineering at the beginning of 2002. As mentioned in the Methods, we did not use the cylindrical HRT lenses to adjust for astigmatism. We explored the effect of this omission in

an independent population. All subjects who visited the research center between November 2007 and March 2008, and who had an astigmatism of at least 1.00 D, were included for this analysis (N=141). One random eye per subject was scanned with and without the appropriate cylinder attached to the HRT camera. We found no significant difference between scanning with or without the cylinder when stratified for astigmatism (using LCDR and GPS as the outcome measures; data not shown). These findings are consistent with other studies.[71,72]

HRT scans of participants with glaucomatous visual field loss had higher topographic standard deviations than participants without glaucomatous visual field loss (Table 1). In this comparison, all scans with a topographic standard deviation of over 50 µm (9.7% of all scans; Figure 1) were excluded. In a setting where data are used for research, the exclusion of unreliable test results is an appropriate approach. If the HRT would be used for screening (not an obvious choice, as mentioned above), persons with unreliable test results cannot be excluded: they are either sent home after reassurance or are referred for further examination. In such a setting, unreliable test results can either be interpreted as if they were reliable or can be considered abnormal without interpretation.[73] If we would include the unreliable HRT scans, the sensitivity would not change, but the specificity would decrease from 97.5 to 95.7%; if we would consider all unreliable HRT scans abnormal, the sensitivity would increase from 35 to 46% but the specificity would decrease from 97.5 to 87.8%. An earlier study found that unreliable tests were associated with disc size.[42] Also in our study failure of the GPS algorithm happened more often in small optic discs compared to medium-sized ($p<0.001$) and large ($p<0.001$) optic discs. We did not observe a difference between medium-sized and large optic discs ($p=0.427$).

In conclusion, we presented normative data of a general healthy non-glaucomatous population for the HRT3 and found that the built-in algorithms of the HRT did not perform well in a population-based setting. The disc-area adjusted HRT3 variable LCDR showed the best diagnostic performance in detecting glaucomatous visual field loss in this population-based study. Hence, this variable can be used to define glaucomatous optic neuropathy, which can subsequently be used as part of a definition of glaucoma. Detailed knowledge of the performance of the HRT in population-based epidemiology is a prerequisite for the interpretation of the results of earlier studies and the optimal conduct of new studies.

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Defining glaucomatous optic neuropathy from a continuous measure of optic nerve damage - the optimal cut-off point for risk-factor analysis in population-based epidemiology

ABSTRACT

Purpose: Diseases characterized by a continuous trait can be defined by setting a cut-off point for the disease measure in question, accepting some misclassification. The 97.5th percentile is commonly used as a cut-off point. However, it is unclear whether this percentile is the optimal cut-off point from the point of view of risk-factor analysis. The optimal cut-off point for risk-factor analysis can be found with a statistical method that minimizes the effect of misclassification. We applied this method to glaucomatous optic neuropathy. Here, the continuous trait is the cup-disc ratio. Aim of this study was to determine the optimal cup-disc ratio cut-off point for risk-factor analysis in population-based epidemiology.

Methods: All participants in the population-based Rotterdam Study underwent intraocular pressure (IOP) measurements, assessment of the cup-disc ratio with the Heidelberg Retina Tomograph and visual field testing. In the statistical method, the cup-disc ratio (the continuous trait) and the IOP (a major risk factor) were independent variables, and glaucomatous visual field loss (the true glaucoma endpoint) the dependent variable in a logistic regression model. The optimal cup-disc ratio cut-off point was found by minimizing the influence of IOP in this model. Variability of the approach was assessed by using a bootstrap resampling technique.

Results: Of 2444 included participants, 93 had glaucomatous visual field loss. The median optimal cup-disc ratio cut-off point was the 97.0th percentile with a 95% central range from 95.5 to 98.5.

Conclusion: The optimal cup-disc ratio cut-off point for risk-factor analysis is close to the commonly used 97.5th percentile.

INTRODUCTION

Open-angle glaucoma, from here on called glaucoma, is a neurodegenerative disease that causes a progressive damage to the optic nerve (glaucomatous optic neuropathy; GON), resulting in loss of sight (glaucomatous visual field loss; GVFL). Several risk factors have been identified; a major risk factor is an elevated intraocular pressure (IOP).

In diseases characterized by a continuous trait, subjects can be divided – arbitrarily – in abnormal and normal by setting a cut-off point for the disease measure in question. In risk-factor analysis, abnormal subjects are considered to be cases; the normal subjects serve as controls. In glaucoma, the damage to the optic nerve is visible as an increased excavation of the optic disc and is commonly quantified by a continuous measure of this excavation. In most population-based studies, GON is considered to be present if the excavation exceeds the 97.5th percentile of the healthy population.[1-3]

Although the 97.5th percentile is a commonly used cut-off point in population-based glaucoma research, it is unknown if this cut-off point is optimal from the point of view of risk-factor analysis. A cut-off point corresponding to a high specificity precludes false-positive classification, which is presumed to be more harmful in risk-factor analysis than false-negative classification in diseases with a low prevalence.[4,5] However, a too high specificity has its drawbacks as well, as the corresponding low sensitivity will decrease the number of cases and thus reduce the power of risk-factor analysis.[6,7] Hence, the intriguing question is if the power of population-based glaucoma risk-factor analysis can be improved by making a better choice with regard to the cut-off point applied.

The aim of the present study was to find the optimal cut-off percentile for the excavation of the optic disc, which maximizes the power of glaucoma risk-factor analysis in population-based epidemiology in a general population, aged 55 years and older. To achieve this goal, we employed an approach described by Prentice[8] and chose the cut-off percentile that validated his criteria for surrogacy. In our study, GON is the “surrogate” endpoint and GVFL the true end-point. Obviously, GVFL can be measured itself and can be used as dependent variable in risk-factor analysis as well. However, the optic disc can be assessed much easier and more reliably than the visual field, especially in the elderly. Since the prevalence of glaucoma increases strongly with age, the use of a surrogate is an interesting option. To estimate the variability of the approach in finding the optimal cut-off point for defining GON, we used a bootstrap resampling technique.

MATERIALS AND METHODS

Participants

The present study was performed within the Rotterdam Study, a prospective population-based

cohort study of residents aged 55 years and older living in Ommoord, a suburb of Rotterdam, the Netherlands. The rationale and study design have been described elsewhere.[9] All measurements were conducted after the Medical Ethics Committee of the Erasmus University had approved the study protocol and all participants had given a written informed consent in accordance with the Declaration of Helsinki. Baseline examinations took place between 1991 and 1993; follow-up examinations for glaucoma were performed from 1997 to 1999 and from 2002 to 2006. All participants from whom reliable data regarding the optic disc, the visual field and the IOP were available were included in this study.

Ophthalmic examination

The ophthalmic assessment in the Rotterdam Study included a medical history, Goldmann applanation tonometry (Haag-Streit, Bern, Switzerland), autorefraction (Topcon RM-A2000; Tokyo Optical Co, Tokyo, Japan), keratometry (Topcon OM-4 Ophthalmometer; Tokyo Optical Co, Tokyo, Japan), visual field testing (see below), fundus photography, ophthalmoscopy, and imaging of the optic nerve head with the Heidelberg Retina Tomograph (HRT; Heidelberg Engineering, Dossenheim, Germany) in both eyes after applying tropicamide 0.5% and phenylephrine 2.5% mydriatic drops.

The IOP was measured at baseline and at every follow-up round. For this study, the baseline value was used. Three measurements were taken in both eyes after applying oxybuprocaine 0.4% eye drops and fluorescein. The Goldmann applanation tonometer was calibrated once a year.

All participants who attended the second follow-up round were examined with the HRT2 device. After an upgrade, all previously collected data were converted to HRT3 format using the HRT3 software (Heidelberg Eye Explorer [HEyEx] version 1.5.10.0). The HRT3 variable “linear cup-disc ratio” (LCDR) was used to quantify the excavation of the optic disc. The rationale of this choice was explained in an accompanying article.[Ramdas, et al.; Chapter 2.1] The LCDR is the square root of the ratio of the area of the cup and the area of the optic disc. Adjustment for optic disc area was done by stratification in three equally-sized groups:[Ramdas, et al.; Chapter 2.1] small (up to 1.5 mm^2), medium, and large (above 2.0 mm^2). 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 within a single HRT scan. Scans with a topographic standard deviation of more than 50 µm were excluded as recommended in the HRT manual.

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.[1,10] Visual field loss 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 visual field loss 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 visual field loss, 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[10] and the full-threshold HFA 24-2 test results[11] have been described before. In short, visual field loss was considered to be GVFL only if reproducible and after excluding all other possible causes. For the present study, eyes were considered to have GVFL if they had GVFL during either follow-up round (some patients that could still be imaged with HRT at the second follow-up round were not able to perform [reliable] perimetry anymore).

Statistical analysis

One eye per participant was included in the analysis. For the IOP, we used the median of the three measurements taken in the included eye (see above).[1,12] In cases with unilateral GVFL, the eye with GVFL was selected; in all other cases, a random eye was chosen. Hence, fellow eyes of unilateral GVFL cases were not allowed to serve as controls. Differences between participants with and without GVFL were analysed with independent t-tests and chi-square statistics.

The aim of this study was to determine which cut-off percentile of disc-area corrected HRT LCDR maximized the surrogacy of GON for the outcome of main interest, GVFL. For this purpose, the three criteria modified from Prentice were used:[8,13]

1. a risk factor is selected that is associated with the outcome measure
2. the range of cut-off percentiles of the surrogate is determined where the risk factor is statistically significantly associated with the surrogate
3. for the range of cut-off percentiles selected in step 2, the cut-off percentile that minimizes the effect of the risk factor when surrogate and risk factor together predict the outcome measure, is determined

In this study, IOP was used as the risk factor, GON as the surrogate, and GVFL as the outcome measure or true endpoint. To assess the variability of the above-described procedure to obtain the optimal cut-off point, we used the bootstrap resampling technique.[14,15]

For the first step, we performed a logistic regression analysis with IOP at baseline as independent variable and GVFL as dependent variable, resulting in an odds ratio (OR) with corresponding 95% confidence interval (CI). For the second step, we ran logistic regression analyses with IOP at baseline as independent variable and GON (defined by a disc-area corrected LCDR percentile) as dependent variable. This was done for every bootstrap sample for every possible disc-area corrected LCDR cut-off percentile, in percentile steps of 0.5. A total of 3000 bootstrap samples were performed. For the third step, again logistic regression analyses were performed for every bootstrap sample with IOP at baseline and GON as independent variables and GVFL as dependent variable, for the range of LCDR cut-off percentiles wherein IOP was significantly associated with GON during the second step. In

this way, the LCDR cut-off percentile yielding the smallest contribution of IOP to the model was determined for every bootstrap sample. Finally, the mean, median and 95% central range (as the data showed a skewed distribution) of these 3000 LCDR cut-off percentiles were determined. All logistic regression analyses were adjusted for IOP treatment at baseline, age, and gender.

A p-value of 0.05 or less was considered statistically significant. All statistical analyses were performed using SPSS version 15.0.0 for Windows (SPSS inc., Chicago, IL, USA; 2006) and R statistical package version 2.7.2 for Mac (www.r-project.org).

RESULTS

Of 2996 subjects who attended at least the baseline and the second follow-up examinations of the Rotterdam Study, 2444 (81.6%) were included in this study. Of the 552 excluded participants, 485 were excluded because of unreliable or missing HRT and/or perimetry data and another 67 because of missing IOP data at baseline. Of these 2444 participants, 93 (3.8%) had GVFL.

Table 1 summarizes the characteristics of the study population according to GVFL status. Participants with GVFL were significantly older, had a significantly higher baseline IOP, more often received IOP-lowering treatment and had a higher LCDR. All this indicates that GVFL is a useful measure of the presence of glaucoma.

First step

IOP at baseline was significantly associated with GVFL as demonstrated by an OR of 1.17 (95% CI 1.10-1.25; p<0.001) per mmHg increase in IOP. Hence, IOP may serve as the risk factor in the analyses.

Table 1. Characteristics of the study population with and without glaucomatous visual field loss presented as mean ± standard deviation (range) unless stated otherwise

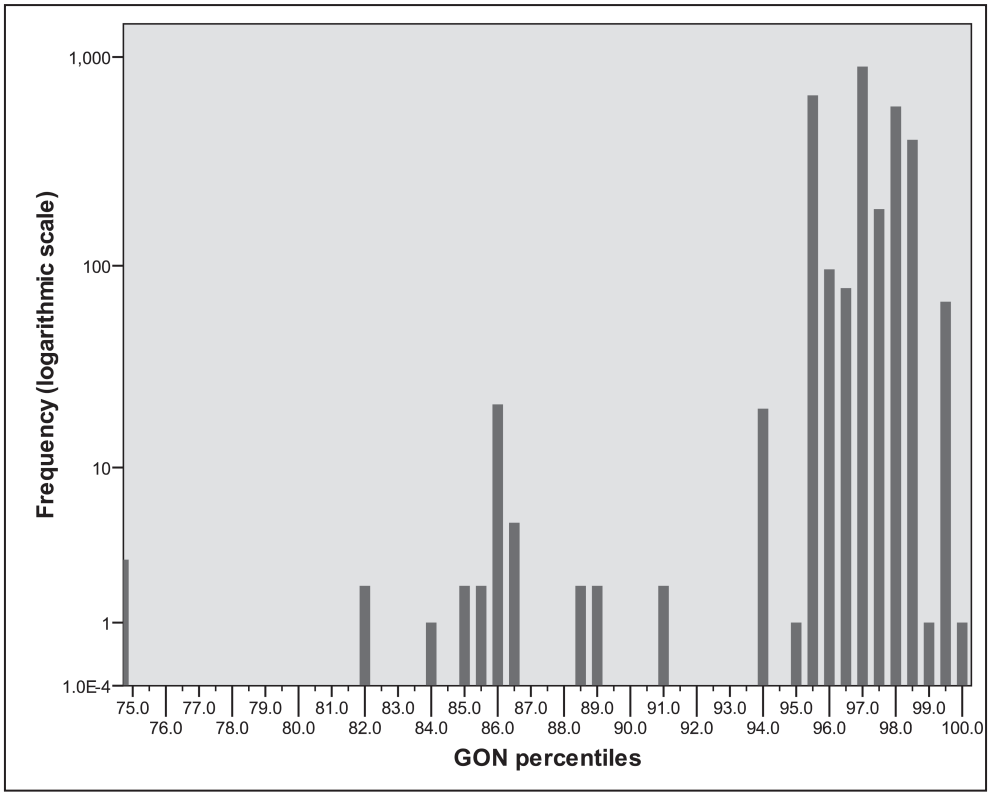
	No GVFL (N=2351)	GVFL (N=93)	p-value
Age at baseline (year)	65.3 ± 5.8 (56 to 86)	68.2 ± 5.8 (57 to 82)	<0.001*
Gender, N (%) female	1371 (58.3)	47 (50.5)	0.136
Eye, N (%) OD	1226 (52.1)	52 (55.9)	0.476
IOP at baseline (mmHg)	15.0 ± 3.0 (6 to 29)	16.8 ± 3.7 (8 to 26)	<0.001*
IOP-lowering treatment, N (%)	46 (2.0)	19 (20.4)	<0.001*
HRT3 disc area (mm^2)	1.8 ± 0.4 (0.7 to 4.3)	1.8 ± 0.4 (0.8 to 3.3)	0.176
HRT3 linear cup-disc ratio	0.4 ± 0.2 (0.0 to 1.0)	0.6 ± 0.2 (0.0 to 0.9)	<0.001*

* = significant at a p-value of 0.05; GVFL = glaucomatous visual field loss; IOP = intraocular pressure.

Second and third step

Figure 1 shows the results of the 3000 bootstrap cycles: the distribution of the LCDR percentiles for which IOP had the highest p-value (smallest contribution to the model) in step 3 and for which IOP was significantly associated with GON in step 2. For the original dataset, the 97.0th percentile was the most optimal GON percentile. For the bootstrap samples, the mean optimal GON percentile was the 96.8th percentile with a 95% range from 95.5 to 98.5. The median was the 97.0th percentile. The corresponding disc-area corrected LCDR cut-off values for the 97.0th percentile were 0.65, 0.69 and 0.74 for respectively small, medium and large sized optic nerve heads. At this specificity (97%), the sensitivity was 40%.

Figure 1. Results from the 3000 bootstrap samples: distribution of the percentiles of HRT LCDR for which IOP had the highest p-value in step 3 and for which IOP was significantly associated with GON in step 2 (see Materials and Methods for a description of steps 2 and 3; GON = glaucomatous optic neuropathy).



DISCUSSION

The 97.0th percentile of the disc-area corrected LCDR turned out to be the most suitable cut-off

percentile to define GON for studying glaucoma risk factors in population-based epidemiology. Interestingly, the commonly used but never validated 97.5th cut-off percentile is quite close to this value, and within the 95% central range as found in our study. This suggests that the power of risk-factor analyses cannot be improved substantially by replacing the commonly used 97.5th percentile.

As mentioned in the Materials and methods section, only participants with valid data on HRT, visual field, and IOP were included. This resulted in a significant proportion of excluded participants. The 552 participants with invalid data were significantly older than the included participants ($p<0.001$), but no difference in gender or IOP was observed ($p=0.155$ and $p=0.328$, respectively). Since we adjusted our analyses for age, the inevitable exclusion of participants with invalid data will presumably not have affected our findings significantly (see also below).

Generally speaking, to minimize the effect of misclassification it is necessary to take into consideration the distribution of the continuous variable in the healthy population, in the diseased population, and the relative sizes of the two populations, that is, the disease prevalence.[16] For example, if the prevalence of a disease is high, it would be more appropriate to take a lower cut-off percentile to reach an optimal balance between false-positive and false-negative classification (that is, to minimize misclassification).[1-3,17,18] The HRT LCDR cut-off percentile as determined in this study is appropriate for studying glaucoma risk factors in a population-based setting with GON as surrogate. It cannot automatically be applied to other diseases, the same disease in other settings, other surrogates, or other purposes. In a clinical setting, for example, the prevalence of a disease is higher and a good sensitivity is the principal concern to avoid overlooking disease. If – in a population-based setting – the purpose is screening, then a high specificity is mandatory in order to avoid false-positive labeling. In risk-factor research in the same population-based setting, the most efficient discrimination between cases and controls is the primary goal. We optimized our cut-off percentile for the latter purpose and setting. Any glaucoma risk factor analysis may be performed with the determined cut-off percentile, as long as GON is used as the surrogate and the study is conducted in a population-based setting. As an example, we analyzed myopia, a presumed risk factor of glaucoma, in three ways. With GVFL as the outcome measure, the odds ratio (95% confidence interval) was 1.38 (0.90 to 2.11); with GON (using the 97th percentile of the disc-area corrected LCDR), this analysis yielded an odds ratio of 1.32 (0.96 to 1.82). Combining both outcome measures resulted in an odds ratio of 1.31 (1.01 to 1.71). All these analyses were adjusted for age and gender; myopia was defined as a spherical equivalent of less than 0.00 D in the included eye.

In glaucoma, GON may precede GVFL several years and, the other way round, GVFL may be present without an LCDR value beyond the chosen percentile. The latter discrepancy is reflected in the low sensitivity (40% at a specificity of 97%; see Results). Hence, assessment of the optic disc (GON) cannot replace perimetry (GVFL) in health care settings; we aimed for applications in epidemiological research.

Surrogate endpoints are frequently used in other medical research fields, like in oncology

and cardiology. In these fields, the true endpoint often is death, which may be reached only after a very long follow-up period.[19,20] A validated surrogate that can be measured easily and in an earlier stage is obviously of great interest in such studies. For a convincing surrogate a statistical relationship alone is not sufficient; a surrogate is usually proposed on the basis of a biological rationale. The method described by Prentice[8] for the use of surrogates was originally proposed in randomized clinical trials and not in observational studies. For that reason, his criteria should not be used in the latter case without further consideration.[21] Especially, the results are only valid under the assumption of no other unmeasured confounding factors. Therefore, as mentioned in the Material and methods, we adjusted for the most obvious confounding factors: age, gender and IOP-lowering treatment.

If we focus on longitudinal glaucoma studies, there are at least two good reasons for preferring a surrogate endpoint like GON rather than a true endpoint like GVFL. First, sample sizes are large in these studies, for power reasons, and perimetry is a time consuming enterprise. Second, especially in the elderly, perimetry is awkward because the results tend to be increasingly unreliable and increasingly difficult to interpret with increasing age due to cognitive impairment, fatigue and concurrent eye diseases like cataract and macular degeneration, and neurological diseases like stroke. Obviously, excluding elderly people from glaucoma studies is not a logical solution since glaucoma is most common in the elderly. Imaging with HRT is much less hampered by aging, which justifies its use as a surrogate.

In conclusion, the 97.0th percentile of HRT LCDR appears to be the optimal cut-off percentile for defining GON in population-based glaucoma risk-factor analysis.

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Chapter 3

Incidence and risk factors for open-angle glaucoma

Incidence of glaucomatous visual field loss: a ten-year follow-up from the Rotterdam Study

ABSTRACT

Purpose: To determine the 10-year incidence of glaucomatous visual field loss (GVFL) and to investigate the influence of risk factors for open-angle glaucoma on this incidence.

Design: Population-based cohort study.

Participants: Participants aged 55 years and older from the Rotterdam Study.

Methods: Of the 7983 participants in the Rotterdam Study, 6806 underwent ophthalmic examinations at baseline (1990-1993). In 6723 of these 6806 participants (99%) both visual field screening and an assessment of the optic disc were performed. After exclusion of 93 participants with GVFL at baseline, 6630 participants at risk of developing GVFL remained. These participants underwent similar ophthalmic examinations during two follow-up visits (1997-1999 and 2002-2006). The incidence of GVFL was determined as an incidence rate and recalculated to a 10-year risk. Risk factors for open-angle glaucoma (age, gender, positive family history of glaucoma, baseline IOP, myopia and baseline vertical cup-disc ratio [VCDR]) were assessed using Cox regression. Dependent variable was the development of GVFL.

Main Outcome measures: 10-year risk and incidence rates of GVFL. Hazard Ratios of the abovementioned risk factors.

Results: Of 6630 participants, 3939 (59%) completed at least one follow-up examination and 2571 (39%) completed both; 108 developed GVFL. The overall incidence rate and 10-year risk of GVFL were 2.9/1000 person-years (95% confidence interval 2.4-3.5) and 2.8% (2.3-3.4%) respectively. The 10-year risk increased from 1.9% at age 55 to 59 years to 6.4% at age 80 years and older ($p<0.001$). The incidence increased by 11% per mmHg increase in IOP (Hazard Ratio 1.11; 95% confidence interval 1.06-1.15). Male gender (1.62; 1.10-2.38), high myopia (spherical equivalent -4 D and more myopic; 2.31; 1.19-4.49) and a baseline VCDR above the 97.5th percentile (4.64; 2.72-7.91) were associated with the development of GVFL. A positive family history was only significantly associated with the development of GVFL if IOP was removed from the model (2.0; 1.2-3.3; $p=0.012$).

Conclusions: These data provide an estimate of the incidence of GVFL in a white population. The development of GVFL was related to higher IOP, older age, high myopia, male gender, a positive family history of glaucoma and a larger baseline VCDR.

INTRODUCTION

Open-angle glaucoma (OAG) is a chronic progressive optic neuropathy with elevated intraocular pressure (IOP) as its main risk factor. Loss of retinal ganglion cells leads to a structural change in the optic disc (excavation), called glaucomatous optic neuropathy (GON). This loss results in a gradual decline of the visual field: glaucomatous visual field loss (GVFL).

Epidemiological research is one of the ways to uncover information needed for health care planning and to increase the understanding of the pathophysiology of the disease. Although many studies have reported on the prevalence of OAG,[1-11] only a limited number of studies have addressed its incidence.[12-17] Furthermore, risk-factor analyses based on current knowledge suffer from wide confidence intervals, and therefore, more well-described incidence data from population-based studies are indispensable.[18] Finally, recent studies that aimed to address the feasibility of OAG screening were not able to settle this issue definitively because sufficiently reliable data on the incidence of GVFL and OAG were lacking.[19-21]

The aims of the present study were: (1) to determine the 10-year incidence of GVFL as a function of age and gender in a general elderly white population, and (2) to investigate the influence of previously reported risk factors for OAG (IOP, myopia and a positive family history of glaucoma) and the presence of GON without GVFL at baseline on this incidence.

METHODS

Study population

The present study is part of the Rotterdam Study, a prospective population-based cohort study aimed at studying the occurrence of and risk factors for chronic diseases in the elderly. Its objectives, methods and major findings have been described elsewhere.[22] The Medical Ethics Committee of the Erasmus University had approved the study protocol and all participants had provided written informed consent in accordance with the Declaration of Helsinki. After the baseline examinations between 1990 and 1993, follow-up examinations for open-angle glaucoma (OAG) were performed from 1997 to 1999 and from 2002 to 2006.

Of the original eligible cohort of 10275 individuals, all residents aged 55 years and older living in one suburb of Rotterdam, 7983 persons (78%) participated in the study. As the ophthalmic part of the Rotterdam Study became operational after the screening of the randomly invited participants had started, 6806 of these 7983 participants underwent the ophthalmic examinations. Of these, 6723 (99%) had both visual field (VF) screening and an assessment of the optic disc at baseline. The baseline examination identified 93 participants with GVFL in at least one eye, leaving a cohort of 6630 (6723-93) participants at risk of incident GVFL (iGVFL).

Data collection

The ophthalmic examinations have been described in detail previously.[11] To summarize, the standardized protocol included refraction, measurement of the best corrected visual acuity, Goldmann applanation tonometry (Haag-Streit, Bern, Switzerland), fundoscopy, color fundus photography of the optic disc and macula region, stereoscopic fundus photography of the optic disc (see below), and VF testing (see below). Examinations were identical at baseline and follow-up except for the fact that optic disc assessment during the second follow-up examination was performed with the Heidelberg Retina Tomograph (HRT; Heidelberg Engineering, Dossenheim, Germany), because the simultaneous stereoscopic imaging system was no longer available.

Perimetry

The VF of each eye was screened using a 52-point supra-threshold test that covered the central VF with a radius of 24° (Humphrey Field Analyzer [HFA]; Carl Zeiss, Oberkochen, Germany). The test was modified from a standard 76-point screening test and tests the same locations as used in the Glaucoma Hemifield Test.[23] When the participant did not respond to the light stimulus (6 dB above a threshold-related estimate of the hill of vision) in at least three contiguous test points (or four including the blind spot), VF loss (VFL) was considered as present. If the first VF 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. In participants in which VFL 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 the Goldmann perimetry test results has been described before.[24] During the second follow-up, two ophthalmologists (RCWW and NMJ) independently classified the full-threshold HFA test results. They were masked to clinical data including optic disc data. The full-threshold test was considered unreliable if either false-positive catch trials exceeded 33% or both false-negative catch trails exceeded 33% and fixation losses exceeded 20%. To be classified as abnormal, a test had to be reliable and at least one of three criteria had to be met: a Glaucoma Hemifield Test ‘outside normal limits’, a minimum of three contiguous points in the pattern deviation probability plot with a sensitivity decreased to $p<0.05$ of which at least one point to $p<0.01$ (modified LTG-P criterion),[25] or a Pattern Standard Deviation $p<5\%$. VFL was considered to be present if it was reproducible, that is, the abnormalities on the full-threshold test and the supra-threshold tests had to be at least partially overlapping. Output of this process was a qualitative description (no abnormalities, unreliable test result, defect compatible with glaucoma, reduction in sensitivity presumably caused by cataract, homonymous defect suggesting a central nervous system lesion, central defect that might be related to e.g. macula degeneration). Photographs of the disc and

macula of participants with VFL were classified independently by two other ophthalmologists (PTVMdeJ and JRV) who looked for the presence and localization of fundus signs that could cause VFL. They were masked for the outcome of the VF test results. Results of VF scoring and photograph scoring were combined and compared. In case no cause compatible with the VFL was seen, we checked the results from the ophthalmologic and neurologic histories and examinations. Additional information was obtained from medical records (ophthalmologist, general practitioner, neurologist). When all this information could not provide a cause, the VFL, after excluding hemianopia, quadrantanopia and isolated central scotomas, was considered to be GVFL. Discrepancies were resolved by consensus.

Optic disc assessment

At baseline and during the first follow up, simultaneous stereo color transparencies were taken at a fixed angle of 20^{O} and analyzed with a computerized image analyzer (Topcon ImageNet System; ImageNet, Topcon Corporation, Tokyo, Japan), yielding the vertical cup-disc ratio (VCDR) as a primary measure of the excavation.[11,13] During the second follow-up, optic discs were assessed with the HRT. Here, the linear cup-disc ratio (LCDR) was used as a measure of the excavation. Cut-off values for ImageNet VCDR were based on the 97.5^{th} percentile as determined in participants without GVFL, an IOP of 21 mmHg or less at all visits, no IOP lowering treatment, and a negative family history of glaucoma. GON was considered to be present for ImageNet VCDR values exceeding 0.69 for small discs (up to 2.1 mm^2), 0.72 for medium size discs and 0.76 for large discs (above 2.7 mm^2). For HRT LCDR, the corresponding cut-off values were 0.67 for small discs (up to 1.6 mm^2), 0.71 for medium size discs and 0.76 for large discs (above 2.0 mm^2 [Ramdas, et al. Chapter 2.1]). If neither ImageNet nor HRT data were available, fundoscopy data were used and GON was considered to be present if the VCDR was larger than 0.7 (no stratification for disc area was performed in case of fundoscopy-based GON).[11]

Definitions of incident glaucomatous visual field loss and open-angle glaucoma

In this study, only GVFL due to OAG was investigated, that is, cases had to have an open anterior chamber angle and no history or signs of angle closure or secondary glaucoma was allowed.[11]

We defined iGVFL as no GVFL in both eyes at baseline and GVFL in at least one eye during at least one of the two follow-up visits. GVFL, once diagnosed, was considered to stay present throughout the total follow-up period. Hence, a participant without GVFL at baseline and with GVFL during the first follow-up visit (1997-1999) but either unreliable perimetry, perimetry classified as no GVFL, additional visual field damage due to concurrent disease inhibiting classification or no perimetry at all during the second follow-up visit (2002-2006) was considered an iGVFL case (see Discussion). According to our previously published

definitions,[13] participants with both GVFL and GON were considered to be definite OAG cases, participants with GVFL but no GON as probable OAG cases based on GVFL and participants with GON but no GVFL as possible OAG cases (the previously applied subdivision in possible GON [97.5th percentile] and probable GON [99th percentile] was abandoned for statistical reasons [Ramdas, et al.; Chapter 2.2]). GON was evaluated at the last follow-up visit.

IOP, myopia and family history

For IOP, the median of three measurements was recorded at baseline for each eye, and the highest median of both eyes was used. For refraction, the spherical equivalent refractive error was calculated at baseline as (sphere + [cylinder/2]) measured in diopters (D). Refraction was stratified into three categories: -4.00 D and more myopic (high myopia), -3.99 to -0.01 D (low myopia), and 0 D and a positive refractive error. Those eyes with a cataract extraction before baseline were excluded from this analysis. In cases with one eye with iGVFL, the refraction of that eye was used. In participants without iGVFL or with iGVFL in both eyes, the refraction of a random eye was used. The family history of glaucoma was determined by interviews at baseline and was considered positive if the participant reported a history of glaucoma in parents, siblings or offspring.

Statistical analysis

We used univariate analyses of covariance to compare baseline characteristics of participants (those who completed at least one follow-up examination) and nonparticipants (those who refused or were unable to participate in any follow-up round; the characteristics of those who did not participate at the baseline examination were described before).[13] Incidence was calculated using incidence rates, because we had non-negligible differences in follow-up durations between participants. Incidence rates were calculated by counting the number of iGVFL cases and the number of person-years for the cohort as a whole and for various subgroups. For participants without GVFL, person-years were counted from the baseline visit to the last visit with reliable perimetry. For iGVFL cases, GVFL was assumed to have developed halfway between the last visit without GVFL and the first visit with GVFL. The 10-year risk (cumulative incidence) of GVFL was subsequently calculated from the incidence rate using: 10-year risk = $1-e^{-10xIR}$, where IR is the incidence rate and *e* the base of the natural logarithm.[26] Gender-specific incidence rates were calculated by stratification to gender; age-specific incidence rates by stratification to age, in five-year age categories. The 95% confidence intervals (CIs) of the incidence rates were calculated using Poisson standard errors.

Subsequently, several previously reported OAG risk factors were analyzed in this cohort.[18] These risk-factors were baseline IOP, myopia and a positive family history of glaucoma. We also investigated the influence of the presence of baseline GON on iGVFL. First, univariate comparisons between participants with and without iGVFL were performed

with the chi-square test for dichotomous variables and the t-test for continuous variables. Subsequently, the influence of these factors, and age and gender, was analysed using a Cox proportional hazards model, expressed as hazard ratios (HRs) and corresponding 95% CIs. Follow-up duration was used as time-axis of the model.

All statistical analyses were performed using SPSS version 15.0.1 for Windows (SPSS Inc., Chicago, IL, USA). A p-value of 0.05 or less was considered statistically significant.

RESULTS

The mean time between baseline and the first follow-up was 6.5 years (range 5.0–9.4 years) and between baseline and the second follow-up 11.1 years (range 7.9-13.9 years). Figure 1 shows the course over time of the 6630 participants at risk of iGVFL; 3939 of 6630 participants (59%) completed at least one follow-up examination; 2571 (39%) completed both follow-up examinations. The mean follow-up time was 9.8 years (range 5.0-13.9 years).

Figure 1. Flow-chart specifying the follow-up of the 6630 participants at risk of incident glaucomatous visual field loss at baseline.

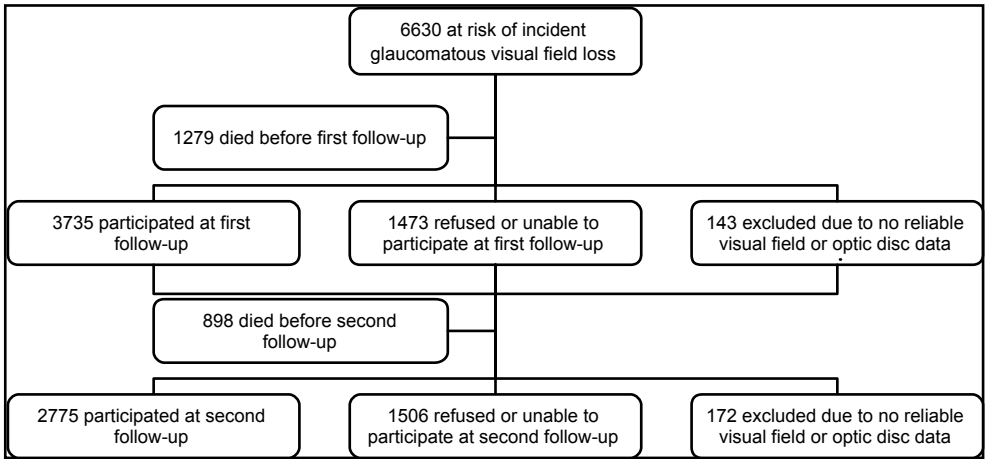


Table 1 shows the baseline characteristics of participants who completed at least one follow-up examination and non-participants, with univariate comparisons. Compared with participants, non-participants were older, more often female, more often had a history of stroke and less frequently reported a positive family history of glaucoma.

Table 2 gives an overview of iGVFL at first and second follow-up visit. During 37775 person-years of follow-up, 108 participants developed GVFL. The overall incidence rate and 10-year risk of GVFL were 2.9 per 1000 person-years (95% CI 2.4-3.5) and 2.8% (2.3-3.4%) respectively.

Table 1. Baseline characteristics of 6630 persons at risk of developing glaucomatous visual field loss (mean with standard deviation between brackets unless stated otherwise)

Baseline characteristics	Participated in at least one follow-up visit (N=3939)	Participated at baseline only (N=1291) or without reliable data at follow-up (N=121)	Died before first follow-up (N=1279)
Age, years	65.8 (6.9)	71.4 (8.9)*	77.5 (9.1)*
Female, %	58	68*	54*
IOP, mmHg	15.1 (3.2)	15.3 (3.4)	15.0 (3.8)
IOP treatment, %	1.8	2.9	2.9
Positive family history of glaucoma, %	8.4	6.1	4.0#
GON, %	3.7	3.2	5.1
History of stroke, %	1.3	3.8#	7.3*
Demented, %	0.2	3.7	15.6*
Institutionalized, %	0.7	6.0	28.7*

IOP = intraocular pressure; GON = glaucomatous optic neuropathy; *p < 0.001; #p ≤ 0.05.

Table 2. Overview of incident glaucomatous visual field loss at first and second follow-up in 6630 participants without glaucomatous visual field loss at baseline

First follow-up	Second follow-up	
No GVFL (N=3684)	No GVFL	2496
	Incident GVFL	48
	No reliable visual field and/or optic disc data	127
	Declined or unable	613
	Died	400
Incident GVFL (N=51)	Normal screening	11
	Abnormal screening unconfirmed with full-threshold HFA	5
	Confirmed GVFL	11
	No reliable visual field and/or optic disc data	1
	Declined or unable	15
	Died	8
Participated at baseline only (N=1291)	Declined or unable	828
	Died	463
Participated at baseline and second follow-up only (N=182)	No GVFL	155
	Incident GVFL	6
	No reliable visual field and/or optic disc data	21
No reliable visual field and/or optic disc data (N=143)	No GVFL	40
	Incident GVFL	3
	No reliable visual field and/or optic disc data	23
	Declined or unable	50
	Died	27
Died after baseline (N=1279)	-	-

GVFL = glaucomatous visual field loss; HFA = Humphrey field analyzer.

Table 3 presents age- and gender-specific incidence rates and 10-year risks of GVFL. The 10-year risk increased from 1.9% at age 55 to 59 years to 6.4% at age 80 years and older (p<0.001).

Of the 108 iGVFL cases, 20 (18.5%) had GON at baseline and another 24 developed GON during follow-up. Hence, of the 108 iGVFL cases, 44 cases could be classified as definite OAG cases and 64 as probable OAG cases,[13] resulting in incidence rates of 1.2 per 1000 person-years for definite OAG and of 1.7 per 1000 person-years for probable OAG based on GVFL. Of the 3939 participants that completed at least one follow-up examination, 147 (3.7%) had GON at baseline and another 177 developed GON without GVFL during follow-up. From these data, the incidence rate of possible OAG could tentatively be estimated to be 4.7 per 1000 person-years.

Bilateral iGVFL occurred in 23 of 108 (21%) iGVFL cases. The 10-year risk of bilateral GVFL in persons aged 55 to 80 years was 0.6% (95% CI 0.4-0.9) and 2.1% (0.5-8.2) at age

Table 3. Incidence rates and 10-year risks of glaucomatous visual field loss as a function of age and gender

Age Group (years)	Number of cases	Person-Years at risk	Incidence Rate*	95% CI of Incidence Rate*	10-year risk (%)	95% CI of 10-year risk
Men						
55-59	1	901	1.1	0.2-7.9	1.1	0.2-7.6
60-64	4	3030	1.3	0.5-3.5	1.3	0.5-3.5
65-69	11	4474	2.5	1.4-4.4	2.4	1.4-4.3
70-74	16	3739	4.3	2.6-7.0	4.2	2.6-6.7
75-79	14	2206	6.3	3.8-10.7	6.2	3.7-10.2
80+	9	1246	7.2	3.8-13.9	7.0	3.7-13.0
Overall	55	15597	3.5	2.7-4.6	3.5	2.7-4.5
Women						
55-59	3	1143	2.6	0.8-8.1	2.6	0.8-7.8
60-64	4	4058	1.0	0.4-2.6	1.0	0.4-2.6
65-69	8	5760	1.4	0.7-2.8	1.4	0.7-2.7
70-74	10	5134	1.9	1.0-3.6	1.9	1.0-3.6
75-79	12	3528	3.4	1.9-6.0	3.3	1.9-5.8
80+	16	2555	6.3	3.8-10.2	6.1	3.8-9.7
Overall	53	22178	2.4	1.8-3.1	2.4	1.8-3.1
Total						
55-59	4	2044	2.0	0.7-5.2	1.9	0.7-5.1
60-64	8	7088	1.1	0.6-2.3	1.1	0.6-2.2
65-69	19	10234	1.9	1.2-2.9	1.8	1.2-2.9
70-74	26	8873	2.9	2.0-4.3	2.9	2.0-4.2
75-79	26	5734	4.5	3.1-6.7	4.4	3.0-6.4
80+	25	3802	6.6	4.4-9.7	6.4	4.3-9.3
Overall	108	37775	2.9	2.4-3.5	2.8	2.3-3.4

CI = confidence interval; *per 1000 person-years.

80 years and older (p=0.04). Of those with GVFL on full-threshold HFA (N=57), the average mean deviation was -9.7 dB (standard deviation 5.0 dB).

Table 4 shows univariate comparisons for age, gender, and the previously mentioned OAG risk factors between participants with and without iGVFL. Table 5 presents the corresponding multivariate analysis. This analysis revealed a significant difference in iGVFL between men and women. The risk of iGVFL significantly increased by 11% per mmHg increase in baseline IOP. High myopia and GON at baseline were significantly associated with iGVFL, but low myopia was not. No significant association between a positive family history of glaucoma and iGVFL was found when adjusted for age, gender, IOP, treatment for IOP, myopia and GON (HR 1.57; 95% CI 0.92-2.67; p=0.1). If IOP was removed from the model, however, a significant association between iGVFL and a positive family history of glaucoma was found (2.0; 1.2-3.3; p=0.012).

Table 4. Univariate comparisons of age, gender and presumed risk-factors for open-angle glaucoma, all measured at baseline, between participants with and without incident glaucomatous visual field loss

	iGVFL (N=108)	No iGVFL (N=3831)	p-value
Female	53 (49.1%)	2248 (58.7%)	0.046
Age, yrs	68.4 (7.2)	65.7 (6.8)	≤ 0.001
IOP, mmHg*	17.3 (4.7)	15.0 (3.1)*	≤ 0.001
IOP treatment	13 (12%)	58 (1.5%)	≤ 0.001
Positive family history of glaucoma**	18 (16.7%)	311 (8.1%)	0.002
Low myopia***	22 (23.2%)	770 (21.4%)	0.674
High myopia***	10 (12.0%)	186 (6.2%)	0.030
Glaucomatous optic neuropathy	20 (18.5%)	127 (3.3%)	≤ 0.001

IOP = intraocular pressure; iGVFL = incident glaucomatous visual field loss; *15 persons had missing IOP measurements at baseline; **8 persons had missing data on their family history of glaucoma status at baseline; ***47 persons had excluded refraction measurements at baseline due to prior cataract surgery.

Table 5. Multivariate model with age, gender and presumed risk-factors for open-angle glaucoma as independent variables and incident glaucomatous visual field loss as the dependent variable

	Hazard Ratio	95% confidence interval	p-value
Low myopia	1.16	0.72-1.88	0.54
High myopia	2.31	1.19-4.49	0.01
IOP	1.11	1.06-1.15	≤ 0.001
Treatment for high IOP	2.91	1.42-5.96	0.004
Age, years	1.07	1.04-1.10	≤ 0.001
Gender, male	1.62	1.10-2.38	0.015
Positive family history of glaucoma	1.57	0.92-2.67	0.1
Glaucomatous optic neuropathy	4.64	2.72-7.91	≤ 0.001

IOP = intraocular pressure.

DISCUSSION

Our longitudinal study with long-term follow-up shows that the 10-year risk of GVFL in a general elderly white population is highly dependent on age. The 10-year risk increases from about 2% in the sixth decade to approximately 6% in the highest age category. The incidence is significantly associated with higher baseline IOP, high myopia, male gender, a positive family history of glaucoma and GON at baseline.

OAG has been defined in the Rotterdam Study – and in most other epidemiological studies as well - as a composite of GON and GVFL, independent of IOP.[11,13] Following our definitions of definite, probable and possible OAG (see Methods), the 108 iGVFL cases in the current study can be classified as either probable OAG cases (GVFL without GON) or definite OAG cases (both GON and GVFL). The reason that we presented iGVFL here separately is that defining incident GON reliably was somewhat difficult, because we had to switch our optic disc assessment technique (see Methods). However, if we would apply our GON definitions to the 108 iGVFL cases, 44 (41%) could be classified as definite OAG cases (see Results). Although 41% GON in GVFL cases seems rather low, it should be realised that a high cut-off point was used for defining GON (97.5th percentile). With this cut-off point, GON is 16 times more frequent in the iGVFL cases as would be expected in a normal population. After exclusion of the 44 iGVFL cases with GON (definite OAG), IOP remained a highly significant risk factor (HR 1.10; 1.03-1.19; p=0.008). This result supports the finding, although counter-intuitive, that agreement between disc appearance and VF test results is - at least in the early stages of OAG - very much the exception rather than the rule.[27-31] Hence, depending on the definition applied, the 10-year risk of OAG could be estimated from our data to range from 1.2% (definite OAG only) to 2.8% (definite and probable OAG based on GVFL) or to about 7%, if possible OAG is also considered (definite, probable and possible OAG together). As argued above, however, the latter percentage only reflects a rough estimate.

During the follow-up, we changed our confirmatory perimetric tool from Goldmann perimetry (baseline and first follow-up visit) to HFA (second follow-up visit; see Methods). However, in all visits the same screening perimetric tool was used (supra-threshold static perimetry). Due to a presumed higher sensitivity of static perimetry, some iGVFL cases detected during the second follow-up visit could actually have been prevalent at baseline. However, the majority of the incident cases (86 of 108) had a normal VF screening test at baseline, and of the remaining 22 cases 16 were confirmed at the first follow-up visit with Goldmann perimetry. This strongly suggests that the vast majority of the incident cases are incident cases rather than cases already prevalent at baseline. Some participants with an abnormal screening VF test at the first follow-up visit that was not confirmed with Goldmann perimetry could have been detected if HFA confirmation would have been used during that visit. Actually, 48 participants had an abnormal screening VF test at the first follow-up visit that was not confirmed with Goldmann perimetry. Of these 48 participants, 29 also participated in the second follow-up visit and could have been picked-up by HFA at that time. At that

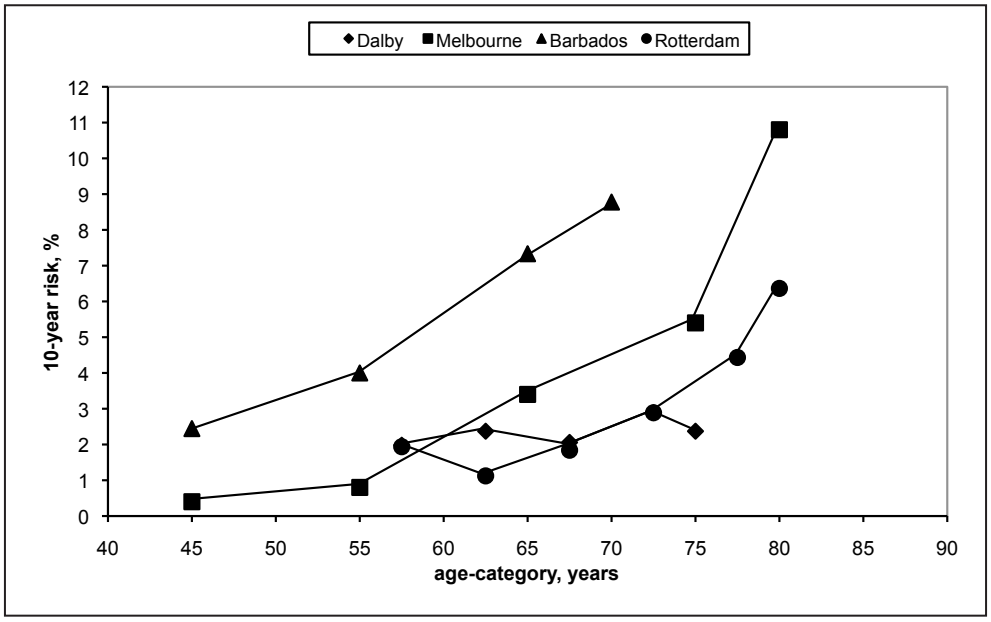
time, 21 of 29 were deemed to have a normal VF. If we assume that all remaining 19 (48-29) participants that did not participate in the second follow-up visit were iGVFL cases that were missed by not using HFA confirmation at the first follow-up visit, the overall 10-year risk of GVFL would have been 3.3% instead of 2.8%.

Performing perimetry and interpreting its results becomes increasingly difficult with ageing. For that reason, we considered GVFL, once diagnosed, to stay present throughout the total follow-up period (see Methods). It should be emphasized that, even with this approach, at least three abnormal fields had to be recorded before GVFL was diagnosed (two supra-threshold fields and a full-threshold or Goldmann confirmation). Nevertheless, this approach might have resulted in some overestimation of the incidence. As can be seen in Table 2, 11 participants with GVFL diagnosed at the first follow-up visit reversed to normal screening, and 5 participants with GVFL at the first follow-up visit had an abnormal screening unconfirmed with full-threshold testing. Of these 5 participants, 2 refused full-threshold testing. If we would exclude the remaining 3 participants and the 11 participants with normal screening, the overall 10-year risk of GVFL would be 2.5%. One possible explanation of the reversal to normal could be the development of cataract. The applied supra-threshold strategy and the full-threshold strategy are both threshold-related, and thus early glaucomatous defects might become invisible with decreasing overall sensitivity.

Several other population-based studies measured the incidence of OAG. In Dalby, Sweden, an annual incidence of OAG of 0.24% (10-year risk approximately 2.4%) was found.[12] In the Dalby study, both GVFL and GON were required to be present. This could be interpreted as definite glaucoma (to be compared with our 10-year risk of definite OAG of 1.2%, but no quantitative GON cut-off point was applied). Hence, some probable OAG cases might have been classified as incident cases as well. From Melbourne, Australia, a 5-year risk of OAG of 1.1% (95% CI 0.8-1.4%) was reported. In that study, OAG classification was based on a consensus panel and it was not stated whether GVFL was a mandatory feature of OAG.[17] Hence, their incident cases presumably were a mixture of definite, probable and possible OAG. In the Barbados Eye Study, the 9-year risk of OAG with both GON and GVFL was 4.4% (3.7-5.2%).[16] Given all these differences and uncertainties, our 10-year risk values seem to be in good agreement with the values found in Dalby and Melbourne. As to be expected, the Barbados Eye Study, mostly a black population, found a higher incidence. Comparing our results with that from Tierp, Sweden, and Olmsted, Minnesota, is somewhat difficult because of the high prevalence of pseudoexfoliation in Tierp and the study design in Olmsted.[14,32] Figure 2 shows the age-specific incidence data as found in four population-based studies.

We found a relationship between iGVFL and high myopia but not between iGVFL and low myopia. In the Barbados Eye Study, an association between myopia (≤ -0.5 D) and OAG was found (Odds Ratio [OR] 1.4; 95%CI 1.1–2.0); in the Blue Mountains Eye Study, both low myopia (-1.0 to -3.0 D; OR 2.3; 1.3-4.1) and high myopia (≤ -3.0 D; OR 3.3; 1.7-6.4) were associated with OAG.[33,34] Also in the Beaver Dam Eye Study an association between myopia (≤ -1.0 D) and OAG (OR 1.6; 1.1–2.3) was found, as was in the Malmo study.[35,36] Compared to

Figure 2. Age-specific incidence data of glaucomatous visual field loss as found in four population-based studies.



these cross-sectional studies, our incident data are less prone to misclassification of myopic VFL, and support the cross-sectional findings that myopia is a risk factor for OAG.

Male gender proved to be a risk factor for iGVFL in the present study. Previously, we found a tendency towards a higher incidence of OAG in men after 5 years of follow-up (2.0% versus 1.6%).[13] Similar non-significant findings indicating an increased risk in men were found in other incidence studies except for the Dalby study.[12,15-17]

In this study, no statistically significant association between a positive family history of glaucoma and iGVFL was found if adjusted for age, gender, baseline IOP, baseline IOP treatment, myopia and baseline VCDR. However, a positive family history was significantly associated with iGVFL if IOP was removed from the model (2.0; 1.2-3.3; p=0.012). This indicates that at least part of the heritability of OAG is IOP mediated. The Melbourne Visual Impairment Project reported an approximately doubling of the risk of developing OAG in individuals who reported a positive family history of glaucoma (Relative Risk 2.1; 1.0-4.2) and the Barbados Eye Study showed a similar increased risk (2.4, 1.3-4.6).[37,38] Furthermore, several cross-sectional studies have supported this association, except for the Tajimi Study that did not find any association.[39-42]

In addition to the population-based design, a major strength of our study was the long term follow-up. However, a long follow-up in elderly participants has its limitations as well. Only 59% of the participants at baseline completed at least one follow-up examination and only 39% completed two follow-up examinations. Non-participants were on average older

than participants. Hence, some underestimation of the overall incidence of GVFL will be the result because the incidence of GVFL increases with age. One third of the participants died during follow-up, but this is presumably unrelated to the disease studied.[43,44] Therefore, the age-specific incidence values we calculated should be essentially unbiased.

In conclusion, these new data provide an estimate of the long-term risk of GVFL in a white population. Our findings demonstrate an increase in the incidence of GVFL with higher baseline IOP, older age, high myopia, male gender, a positive family history of glaucoma and a larger baseline VCDR.

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Ocular perfusion pressure and the incidence of glaucoma: real effect or artifact? - The Rotterdam Study

ABSTRACT

Purpose: To determine the association between the ocular perfusion pressure (OPP; essentially the difference between the blood pressure and the intraocular pressure [IOP]) and incident open-angle glaucoma (OAG).

Methods: A subset of 3882 participants of the population-based Rotterdam Study for whom data from ophthalmic examinations at baseline and follow-up and blood pressure measurements at baseline were available, and who did not have OAG at baseline, were included. Associations between the mean, systolic and diastolic OPP and incident OAG were assessed using Cox regression models adjusted for age and sex, with and without adjustment for IOP.

Results: During a mean follow-up of 9.8 years, 103 participants (2.7%) developed OAG. The association between the mean OPP and incident OAG was not significant (hazard ratio 0.995 per mmHg increase in mean OPP; 95% confidence interval 0.971-1.019) when adjusted for IOP, but became significant if not adjusted for IOP (0.968; 0.945-0.992). The systolic and diastolic OPP showed a pattern similar to that of the mean OPP, though less significant.

Conclusions: The OPP appears to be associated with incident OAG but this association seems to be due to the fact that the IOP, a strong risk factor for OAG, is part of the OPP, rather than that OPP is an independent OAG risk factor itself.

INTRODUCTION

Open-angle glaucoma (OAG) is a neurodegenerative disease that causes progressive damage to the optic nerve head and leads to visual field loss. Some of the risk factors for OAG have been identified; the most obvious risk factor is an increased intraocular pressure (IOP).[1-3] It has been proposed that vascular components are also involved in the pathophysiology of OAG.[4-10] One of the first studies showing a possible link between (systemic) blood pressure and OAG was done in 1911 in Germany.[11] Thereafter, many researchers studied the relationships between blood pressure and IOP and blood pressure and OAG. Blood pressure appears to be associated with the IOP; no unambiguous effect of blood pressure on OAG has been found thus far.[12-21]

Blood pressure may affect the perfusion of the optic nerve head by influencing the perfusion pressure and also, especially in longstanding hypertension, by influencing the vessel diameter.[22] The actual perfusion of the optic nerve head can be measured but these measurements are difficult to perform reliably, especially on a large scale.[23] For that reason, in epidemiological studies, a proxy of the perfusion pressure is used rather than a measurement of the actual perfusion of the optic nerve head. In most tissues, the perfusion pressure equals essentially the difference between the arterial and venous blood pressure. In the eye, the actual mean ocular perfusion pressure (mean OPP [MOPP]) is commonly estimated by the difference between the arterial blood pressure and the IOP (defined more precisely in the Methods section).[5,24]

The relationship between the arterial blood pressure and OAG is complicated. As explained above, blood pressure is part of the MOPP, and a higher MOPP is presumed to decrease the risk of OAG. On the contrary, an increased blood pressure may reduce the vessel diameter and this might increase the risk of OAG. It becomes even more complicated when the IOP is taken into account as well. Because blood pressure is positively associated with IOP, systemic hypertension indirectly increases the risk of OAG. IOP is presumed to have a direct mechanical effect on the axons of the ganglion cells, but is also part of the MOPP - a higher IOP implies a lower MOPP. This implies that MOPP could pop-up as a risk factor for OAG solely because IOP is part of it, and this might explain the contradicting results regarding the relationship between MOPP and OAG[25-28]: some studies adjusted their analyses for IOP,[16,19,29] whereas others did not.[13,17,18,30]

The aim of this study was to clarify the intertwined relationships between blood pressure, MOPP and IOP, and OAG. Simply incorporating blood pressure, MOPP and IOP as three independent variables in a single model with OAG as the dependent variable is not informative. This because, apart from being mutual confounding factors, the variables may be correlated too strongly (blood pressure and MOPP; IOP and MOPP) and may form a causal pathway (for example, IOP constitutes a causal pathway between blood pressure and OAG). For that reason, we first analyzed the association between MOPP and OAG with adjustment for IOP. Next, we repeated the analysis without adjustment for IOP. We further explored the

role of the IOP in the MOPP by replacing the blood pressure value in the MOPP of each participant by a randomly allocated blood pressure value of another participant and comparing the associations between MOPP and OAG before and after this replacement. Other factors that might be related to perfusion were also explored. Earlier studies reported a decreasing prevalence of OAG with an increasing diastolic OPP (DOPP).[13,18] Therefore, we also assessed the relationships between systolic OPP (SOPP) and OAG and DOPP and OAG. Finally, we analyzed the association between blood pressure and IOP.

METHODS

Participants

The present study was performed within the Rotterdam Study, a prospective population-based cohort study of residents aged 55 years and older living in Ommoord, a district of Rotterdam, the Netherlands. The rationale and study design have been described elsewhere.[31,32] All measurements were conducted after the Medical Ethics Committee of the Erasmus University had approved the study protocol and all participants had given a written informed consent in accordance with the Declaration of Helsinki. Baseline examination took place between 1991 and 1993; follow-up examinations for OAG were performed from 1997 to 1999 and from 2002 to 2006. The present study included only participants who completed at least one follow-up examination, had no OAG at baseline, and who had valid data on OAG, IOP and blood pressure.

Ophthalmic examination and incident open-angle glaucoma

The ophthalmic examinations at baseline and follow-up included a medical history, autorefraction (Topcon RM-A2000; Tokyo Optical Co., Tokyo, Japan), keratometry (Topcon OM-4 Ophthalmometer; Tokyo Optical Co., Tokyo, Japan), measurement of the best corrected visual acuity with ETDRS optotypes, Goldmann applanation tonometry (Haag-Streit, Bern, Switzerland; see below), fundus photography of the posterior pole (Topcon TRC-50VT, Tokyo Optical Co., Tokyo, Japan), simultaneous stereoscopic fundus photography of the optic nerve head (Topcon ImageNet System, Topcon TRC-SS2, Tokyo Optical Co., Tokyo, Japan), imaging of the optic nerve head with the Heidelberg Retina Tomograph (Heidelberg Engineering, Dossenheim, Germany) and visual field testing (Humphrey Field Analyzer II 740 [HFA]; Carl Zeiss, Oberkochen, Germany).

The IOP was measured at baseline and at every follow-up round with Goldmann applanation tonometry after applying oxybuprocaine 0.4% eye drops and fluorescein from a paper strip. Three measurements were taken on each eye and the median value of these three measurements was recorded.[33] In the analysis we used the highest median IOP of both eyes.

The visual field of each eye was screened using a 52-point threshold-related supra-threshold test that covered the central field with a radius of 24°.[34,35] Visual field loss was defined as non-response to a light stimulus of 6 dB above a threshold-related estimate of the hill of vision in at least three contiguous test points, or four including the blind spot. In participants with reproducible abnormalities on supra-threshold testing, Goldmann perimetry (Haag-Streit; baseline and first follow-up)[34] or full-threshold HFA 24-2 testing (second follow-up)[36] was performed on both eyes. The classification process of the perimetry test results have been described before.[34,36] In short, visual field loss was considered to be glaucomatous visual field loss only if reproducible and after excluding all other possible causes.

Participants were considered to have incident OAG if neither eye had glaucomatous visual field loss at baseline and at least one eye showed glaucomatous visual field loss at follow-up.[36] Cases with a history or signs of angle closure (gonioscopy was performed in all identified cases) or secondary glaucoma were excluded.

Blood pressure and ocular perfusion pressure

Blood pressure was measured at baseline after the participant had been seated for at least 5 minutes. Systolic blood pressure (SBP; first Korotkoff phase) and diastolic blood pressure (DBP; fifth Korotkoff phase) were measured twice on the right arm using a random-zero sphygmomanometer with a 14x38 cm cuff. Afterwards, we calculated the mean of the two SBP values and of the two DBP values.[37] The mean arterial blood pressure (MABP) was calculated according to MABP = DBP+(SBP-DBP)/3, where SBP-DBP is the pulse pressure.[38] The MOPP was calculated according to MOPP = (2/3)MABP-IOP.[38] The SOPP and DOPP were calculated by subtracting the IOP from the SBP and DBP, respectively.

Potential confounders

Other factors that might be related to perfusion are, following the Hagen-Poiseuille law, factors that influence the blood rheology (viscosity) or the vessel diameter (the diameter has a much a larger influence than vessel length and is more subject to change). The hematocrit is the major determinant of viscosity in the general population.[7] Apart from blood pressure and hypertension, the vessel diameter may be influenced by smoking, diabetes mellitus and serum cholesterol. For smoking and diabetes, trained research assistants asked participants about their smoking habits and if they had diabetes. Smoking was analyzed using nominal categories: never, former and current smokers. Hematocrit and cholesterol levels were derived from blood samples taken at the research center. Serum cholesterol was quantified as the ratio of the high-density lipoprotein-bound cholesterol (HDL-C) and total cholesterol levels (HDL-C/Cholesterol ratio). Another potential confounder is the body mass index.[39,40] Body mass index was calculated as body mass in kilograms divided by the square of the height in meters. All potential confounders were determined at baseline.

Statistical analysis

Differences in baseline characteristics were analyzed with independent t-tests and chi-square statistics. We used Cox proportional hazard regression to calculate hazard ratios (HR) with corresponding 95% confidence intervals (CI) to analyze whether subjects with a high MOPP had a lower risk of developing OAG. The model fits were evaluated with C-statistics. Follow-up duration was used as the time variable. For participants without incident OAG, the follow-up duration was counted from the baseline visit to the last visit with reliable perimetry. For incident OAG cases, the follow-up was counted till the first visit in which glaucomatous visual field loss was detected. The multivariate model was created by first entering all covariates in the model. In the final multivariate model we included MOPP, age, sex, and those covariates reaching a significance of p=0.05 or less in the initial multivariate model - except for IOP-lowering treatment (see Discussion). This final model was built with and without adjustment for IOP.

MOPP and IOP together in a multivariate model might lead to multicollinearity issues, because the IOP is part of the MOPP. To assess whether multicollinearity played a role in our analysis of MOPP adjusted for IOP we calculated the Pearson correlation coefficient and the variance inflation factor (VIF).

As mentioned in the Introduction section, MOPP could pop-up as a risk factor for OAG solely because IOP is part of it. To assess whether the blood pressure has an additional contribution to the significance of MOPP as a risk factor for OAG, we further explored the role of the MOPP by recalculating the MOPP after replacing the blood pressure value of each participant by a randomly allocated blood pressure value of another participant (sampling without replacement). Next, we recalculated the HR of the association between MOPP - without adjustment for IOP - and OAG. This was repeated 30 times. The resulting 30 HRs were compared with that of the original model. The same approach was applied for SOPP and OAG and DOPP and OAG.

The relationships between SOPP and OAG and DOPP and OAG were further explored by stratifying both SBP and DBP into five categories each containing approximately 19 OAG cases. For each of the SBP pentiles, a Cox proportional hazard regression was performed with SOPP as independent variable, and for each of the DBP pentiles, this analysis was performed with DOPP as independent variable. This was done with and without adjustment for IOP.

The relationship between blood pressure and IOP at baseline was analyzed by performing multiple linear regression analyses with IOP as the dependent variable and blood pressure, age, sex, treatment for IOP and treatment for hypertension as the independent variables. This model was run for three different blood pressure variables: MABP, SBP and DBP.

A p-value of 0.05 or less was considered statistically significant. All statistical analyses

were performed using SPSS version 15.0.0 for Windows (SPSS inc., Chicago, IL, USA; 2006) and R statistical package version 2.9.1 for Mac (http://www.r-project.org).

RESULTS

The ophthalmic part of the Rotterdam Study comprised 6806 participants, of which 3939 had no OAG at baseline and participated at least in one follow-up round. Of these, 57 participants were excluded because of missing data on blood pressure or IOP. From the remaining participants, 103 out of 3882 (2.7%) developed OAG during follow-up.

Table 1 summarizes the baseline characteristics of the study population according to incident OAG status. Compared to participants without OAG, participants who developed OAG during the study were significantly older (p<0.001), had higher baseline IOP (p<0.001), and were more often treated for IOP at baseline (p<0.001). None of the potential confounders (hematocrit, blood pressure, usage of anti-hypertensive drugs, smoking, diabetes mellitus, HDL-C/Cholesterol ratio, and body mass index) showed significant differences between participants without OAG and those who developed OAG during follow-up. Body mass index was the only potential confounder that was significant in the initial multivariate analysis. We did not incorporate this variable in the final models because its presence did not change

Table 1. Baseline characteristics of the study population with and without incident open-angle glaucoma presented as mean ± standard deviation unless stated otherwise with univariate comparisons*

		Incident open-angle glaucoma (N=103)	No open-angle glaucoma (N=3779)	p-value
Age (year)		67.8 ± 7.0	65.2 ± 6.8	<0.001
Sex, N(%) female		51 (49.5)	2221 (58.8)	0.060
IOP (mmHg)		18.2 ± 4.7	16.0 ± 3.1	<0.001
Treatment for IOP, N(%)		16 (15.5)	85 (2.2)	<0.001
Hematocrit level (x100)		42.1 ± 3.0	41.5 ± 3.2	0.089
Systolic blood pressure (mmHg)		136.9 ± 21.0	135.7 ± 20.7	0.587
Diastolic blood pressure (mmHg)		73.2 ± 12.7	73.6 ± 10.8	0.721
Anti-hypertensives, N(%)		28 (27.5)	976 (25.8)	0.715
Smoking,N (%)	Never	33 (32.0)	1256 (33.5)	0.526
	Former	52 (50.5)	1698 (45.3)	
	Current	18 (17.5)	791 (21.1)	
Diabetes mellitus, N(%)		7 (6.8)	142 (3.8)	0.120
HDL-C/Cholesterol ratio		0.21 ± 0.06	0.21 ± 0.06	0.695
Body mass index (kg/m^2)		25.7 ± 2.9	26.3 ± 3.5	0.075

* = 324 participants had missing data on one of more covariates; IOP = intraocular pressure; HDL-C = High-density lipoprotein-bound cholesterol.

either the effect estimates or the significances of the relevant variables (IOP, MOPP, SOPP and DOPP).

Table 2 presents the final multivariate models for MOPP with and without adjustment for IOP. We could not find an association between MOPP and incident OAG if adjusted for IOP (HR: 0.995 per mmHg increase in MOPP; 95% CI: 0.971-1.019), but the association became significant after we removed IOP from the model (HR: 0.968; 95% CI: 0.945-0.992). The C-statistics of the models with and without adjustment for IOP were 0.70 and 0.67, respectively. The Pearson correlation coefficient of MOPP and IOP was -0.227 ($p<0.001$). The resulting VIF of 1.054 suggests that multicollinearity had no significant effect.

The role of adjusting for IOP or not, and thus the contribution of IOP to MOPP, was

Table 2. Multivariate Cox proportional hazard model for mean ocular perfusion pressure and the risk of developing open-angle glaucoma with (A) and without (B) adjustment for IOP

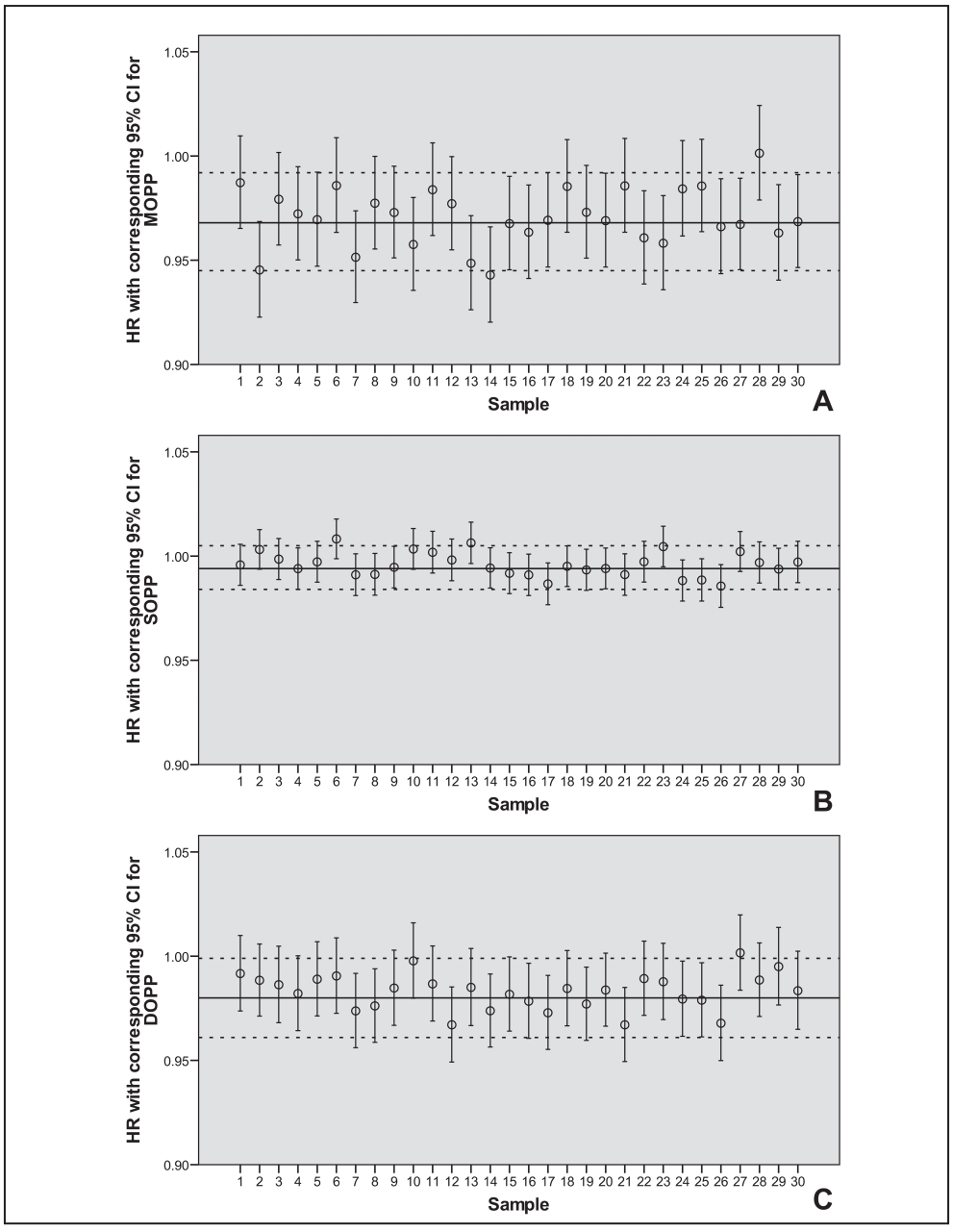
	Hazard Ratio	95% confidence interval	p-value
A			
Age (year)	1.074	1.043 - 1.105	<0.001
Sex (female)	0.594	0.393 - 0.898	0.014
IOP (mmHg)	1.168	1.114 - 1.224	<0.001
MOPP (mmHg)	0.995	0.971 - 1.019	0.675
B			
Age (year)	1.080	1.050 - 1.112	<0.001
Sex (female)	0.554	0.368 - 0.834	0.005
MOPP (mmHg)	0.968	0.945 - 0.992	0.010

IOP = intraocular pressure; MOPP = mean ocular perfusion pressure.

further assessed with the resampling technique as described in the Methods section. Figure 1A shows the results. The HRs of the resampled MOPP data scattered around the HR of the original dataset (solid line in Figure 1A), indicating that the significance of the MOPP in a model without adjustment for IOP is essentially due to the fact that the IOP is part of the MOPP. Next, we investigated whether this finding could be explained by the fact that both the SBP and the DBP were taken together in the formula for computing the MOPP variable. If only one of these variables would contribute significantly, the statistical noise added by the other variable could have masked the effect. Models with SOPP and DOPP instead of MOPP revealed, with adjustment for IOP, a HR of 0.998 (95% CI 0.987-1.008) for SOPP and a HR of 0.997 (95% CI 0.978-1.016) for DOPP. The same models without adjustment for IOP yielded a HR of 0.994 (95% CI 0.984-1.005) for SOPP and a HR of 0.980 (95% CI 0.961-0.999) for DOPP. Figures 1B and 1C show the corresponding results of the resampling technique applied to the SOPP (B) and DOPP (C) data, respectively. Again, there was no clear difference between the original HRs (solid lines in Figure 1B and C) and that of the resampled datasets, neither for SOPP nor for DOPP.

Figure 2 presents the relationships between SOPP and incident OAG and DOPP and

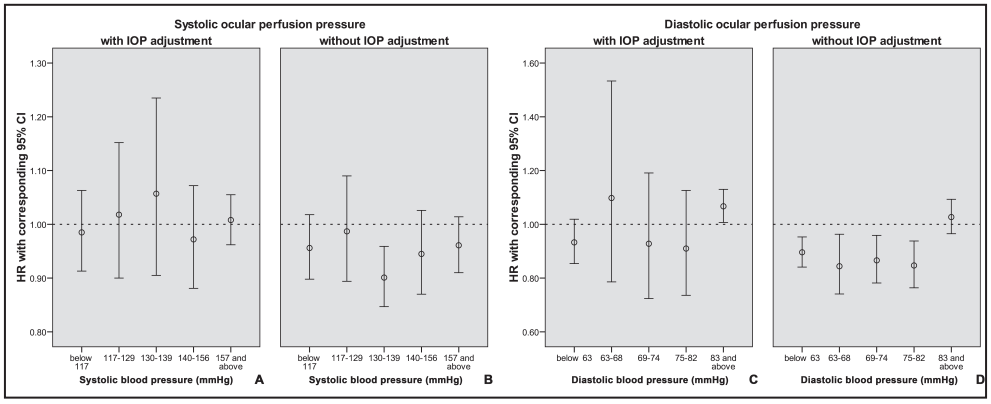
Figure 1. Hazard ratios (HR) and corresponding 95% confidence intervals (CI) for the resampled datasets of the associations between mean ocular perfusion pressure (MOPP) and open-angle glaucoma (A), systolic ocular perfusion pressure (SOPP) and open-angle glaucoma (B), and diastolic ocular perfusion pressure (DOPP) and open-angle glaucoma (C). In the resampled datasets, the blood pressure value of each participant was replaced by a randomly allocated blood pressure value of another participant. Solid line represents the HR of the original dataset; dotted lines the corresponding 95% CI.



incident OAG stratified for SBP and DBP, for models with and without adjustment for IOP. There were no clear associations with incident OAG, except for DOPP without adjustment for IOP, especially in subjects with lower DBP values (Figure 2D).

Finally, we assessed the relationships between IOP and the blood pressure variables MABP, SBP and DBP. IOP was strongly associated with all three variables ($p<0.001$). The corresponding regression coefficients were 0.035, 0.025 and 0.029 mmHg increase in IOP per mmHg increase in MABP, SBP and DBP, respectively. The percentages of variance in IOP explained by the MABP, SBP and DBP (R^2) were 2.0%, 2.5% and 1.0%, respectively.

Figure 2. Hazard ratios (HR) and corresponding 95% confidence intervals (CI) of the associations between systolic ocular perfusion pressure and open-angle glaucoma, as a function of the systolic blood pressure (A,B), and between diastolic ocular perfusion pressure and open-angle glaucoma, as a function of the diastolic blood pressure. Analyses adjusted for age and sex with (A,C) and without (B,D) adjustment for the intraocular pressure (IOP).



DISCUSSION

The MOPP appeared not to be an independent risk factor for OAG. Models without adjustment for IOP suggested a protective effect of a higher MOPP. This finding, however, could be explained by the fact that the IOP is part of the MOPP. A similar - albeit less obvious - protective effect was found for DOPP, but not for SOPP. This difference is most likely caused by the smaller standard deviation of the DBP compared to that of the SBP (Table 1). A smaller standard deviation in a blood pressure variable results in a larger contribution to the variability of the corresponding OPP variable explained by the IOP. This hypothesis is further supported by the finding that the highest DBP pentile had the highest standard deviation and the least significant DOPP (Figure 2D). The blood pressure variables were significantly associated with the IOP, but the variance of IOP explained by these variables was low, suggesting a minor importance in the pathophysiology of OAG (a 40 mmHg change in SBP, for example, corresponded to a 1 mmHg change in IOP).

Studies describing the possible association between MOPP and OAG used models with different covariates, making it difficult to compare results between these (epidemiological) studies. Obviously, the most important covariate in this association is the IOP. Studies in which OPP was adjusted for IOP, The Barbados Eye Study,[15] The Early Manifest Glaucoma Trial[27] and The Blue Mountain Eye Study,[19] could not find a significant association between MOPP and OAG. The Blue Mountains Eye Study found a modest increase in risk of OAG in participants with hypertension, especially in those who were poorly controlled. They also evaluated the potential relationship of OPP with OAG and OPP with ocular hypertension (the latter not adjusted for IOP). The relationship of OPP with OAG was significant for neither MOPP nor DOPP, but a marginal significance was found for SOPP with OAG (p=0.05). For the relationship between OPP and ocular hypertension they found that only DOPP had a protective effect (p=0.0008).[19] Studies in which OPP was not adjusted for IOP, The Baltimore Eye Survey,[18] The Egna-Neumarkt Study,[17] Proyecto VER[30] and previously our Rotterdam Study (but limited to persons receiving anti-hypertensives),[13] found a reduced DOPP to be a risk factor for OAG. Hence, most studies reporting a low OPP as a risk factor for OAG were not adjusted for IOP - as was our approach in Table 2B, and most studies that failed to find an association between OPP and OAG were adjusted for IOP - as was our approach in Table 2A.[41]

Apart from possible limitations in the way MOPP is calculated from brachial artery measurements,[42] the most obvious limitation of epidemiological studies is the fact that measurements are performed only once and only during daytime. In this way, any circadian influence on blood pressure or IOP will be overlooked, as will be the influence of any other fluctuation. It has been suggested that fluctuations in IOP are more damaging to the optic disc than an increase in IOP.[43-45] In addition, another study suggested that patients with progression of OAG despite a normalized IOP suffer from insufficient autoregulation due to vascular dysregulation.[46] One study, evaluating the diurnal fluctuations (between 7 AM and 10 PM) of IOP and MOPP in participants with and without OAG, reported that patients with OAG do not have significant diurnal changes in IOP, but they observed significant fluctuations in the MOPP.[38] The range of diurnal fluctuations in IOP may be narrowed by IOP-lowering treatment and might be captured by analyzing large numbers of participants examined during the day. However, less is known about what happens during the night.[47] Patients suffering from unstable blood flow, due to vascular dysregulation, may be unable to compensate for physiologic fluctuations in IOP and blood pressure in order to maintain MOPP.[48] Besides, nocturnal dipping of blood pressure and circadian fluctuations in OPP are associated with the development and progression of OAG,[49] which is probably due to vascular dysregulation resulting in ischemia.[28,50] Related to this, serum concentrations of endothelin-1 (vasoconstrictor) have been found to be slightly increased in patients with progressing glaucomatous visual field loss despite normal IOP.[28]

Almost all participants in the Rotterdam Study are from European descent. Differences in properties of vascular factors (such as hypertension) across populations of different

ethnicities have been described,[51] and as a consequence findings on OPP might differ between populations.

The effect of IOP-lowering treatment was not taken into account in the main analyses of the present study - albeit significant in the univariate comparison (Table 1). Unlike the other covariates in Table 1, the IOP-lowering treatment is not in a potential physiological pathway affecting the risk of developing OAG. Moreover, this covariate is highly correlated with the IOP and the risk of developing OAG. It reflects the clinician's concern about the risk of glaucoma that may take into consideration for example the appearance of the optic disc and the family history. Nonetheless, if we would add this variable to the analyses, the results did not alter significantly. Some anti-hypertensive drugs (e.g., calcium channel blockers) have been implicated in OAG.[52,53] The current analyses evaluating the relationship between OPP and OAG were not adjusted for the usage of anti-hypertensive drugs (because the corresponding p-value was above 0.05 in the initial multivariate analysis). Reentering either the usage of any anti-hypertensive drugs in the model or adding the usage of calcium channel blockers to the model did not change either the effects estimates or the significances of the relevant variables (IOP, MOPP, SOPP and DOPP; data not shown). The results on the relationship between MABP, SBP and DBP and IOP were adjusted for the usage of anti-hypertensive drugs. The removal of this covariate did not change the findings. The body mass index was the only possible confounder that was significant in the initial multivariate model, but its presence in the model did not have any influence on either the IOP or the OPP variables (see results section). Other factors such as, smoking, diabetes mellitus and cholesterol levels, are known to affect the vascular wall thickness through atherosclerosis. However, none of these factors was significant in the initial multivariate model. In an earlier study, we did not find an association between atherosclerosis and OAG.[54] In agreement with this, it has been suggested that it is not atherosclerosis but vascular dysregulation and insufficient autoregulation that leads to a low OPP (see also above).[6,55]

In conclusion, we found no independent significant effect of OPP on the development of OAG. The current findings suggest that, in epidemiological studies, the observed association between OPP and OAG is essentially due to the fact that the IOP is part of the OPP.

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Lifestyle and risk of developing open-angle glaucoma - The Rotterdam Study

ABSTRACT

Objective: To determine whether lifestyle-related risk factors, such as socioeconomic status, smoking, alcohol consumption, and obesity, are associated with open-angle glaucoma (OAG).

Methods: Participants from the Rotterdam Study, a prospective population based cohort study, were considered eligible if they participated at both baseline and follow-up and if they had no OAG at baseline. All participants underwent an identical ophthalmologic examination at all visits, including intraocular pressure measurements, optic nerve head assessment, and perimetry. Lifestyle-related factors were assessed by questionnaires by trained research assistants or measured during the examinations (body mass index and waist-to-hip ratio). Cox proportional hazard regression analysis was applied to calculate hazard ratios.

Results: Of 3939 eligible participants, 108 (2.7%) developed OAG during 9.7 years' mean follow-up. No statistically significant effect of socioeconomic status, smoking or alcohol intake was found. In women each unit increase in body mass index resulted in a 7% decrease in the risk of developing OAG ($p=0.04$). There was a significant increasing effect of body mass index on IOP ($p<0.001$) in women.

Conclusions: Obesity appears to be associated with a higher intraocular pressure and a lower risk of developing OAG. These associations were only present in women. Other lifestyle-related factors, such as socioeconomic status, smoking and alcohol consumption were not associated with OAG.

INTRODUCTION

Open-angle glaucoma (OAG) is a chronic eye disease characterized by glaucomatous optic neuropathy and corresponding glaucomatous visual field loss. Scientific research has identified several risk factors for OAG. Some of them are modifiable (e.g. intraocular pressure [IOP]) whereas others are not (e.g. age, sex, myopia, and ethnicity).[1,2]

A lower socioeconomic status (SES) (income and education) might be a risk indicator for OAG.[3,4] While SES cannot be changed easily by a patient, some other lifestyle-related risk factors can. Other lifestyle-related risk factors potentially involved in OAG are smoking, alcohol intake, and obesity.[5,6] Studies on smoking could neither find a clear association with OAG nor with IOP.[7-10] The same seems to be true for alcohol consumption and OAG,[7] but interestingly, there is evidence that a higher alcohol intake is associated with a higher IOP.[10,11] Similar but apparently conflicting results have been reported on obesity. Obesity has been reported to be inversely related to OAG,[12,13] but positively related to IOP.[14] Especially because the lifestyle-related risk factors smoking, alcohol intake, and obesity are modifiable, these contradicting findings need further evaluation.

The aim of the present study was to determine whether lifestyle-related risk factors are associated with OAG. For this purpose, we used data from the longitudinal population-based Rotterdam Study. Since earlier studies reported conflicting results regarding the effects on OAG and IOP, we investigated the effects on both the incidence of OAG and the IOP.

METHODS

Participants

The Rotterdam Study is a prospective population-based cohort study of residents aged 55 years and older living in a suburb of Rotterdam, the Netherlands. The rationale and study design have been described elsewhere.[15] All measurements were conducted after the medical ethics committee of the Erasmus University had approved the study protocol and all participants had given written informed consent in accordance with the Declaration of Helsinki. The baseline examination took place between 1991 and 1993; follow-up examinations for OAG were performed from 1997 to 1999 and from 2002 to 2006. For this study, we used data from a subset of participants who did not have OAG (see later) at baseline and who completed at least 1 follow-up examination.

Ophthalmic examination

The examinations at baseline and follow-up included autorefraction (Topcon RM-A2000; Tokyo Optical Co, Tokyo, Japan), keratometry (Topcon OM-4 Ophthalmometer; Tokyo Optical Co),

measurement of the best corrected visual acuity with Early Treatment Diabetic Retinopathy Study optotypes, Goldmann applanation tonometry (Haag-Streit, Bern, Switzerland; see later), fundus photography of the posterior pole (Topcon TRC-50VT; Tokyo Optical Co), simultaneous stereoscopic fundus photography of the optic nerve head (Topcon ImageNet System, Topcon TRC-SS2; Tokyo Optical Co), imaging of the optic nerve head with the Heidelberg Retina Tomograph (Heidelberg Engineering, Dossenheim, Germany) and visual field testing (see later).

The IOP was measured at baseline and at every follow-up round. At each visit, 3 measurements were taken on each eye and the median value of these 3 measurements was recorded.[16] In the analysis, we used the higher median of the IOP of both eyes.

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.[17,18] Visual field loss was defined as non-response in at least 3 contiguous test points (or 4 including the blind spot). If the first test results were unreliable (>33% false-positive or false-negative responses) or a reliable test result showed visual field loss in at least 1 eye, a second supra-threshold test was performed on that eye. If the second supra-threshold test result was unreliable or showed visual field loss, Goldmann perimetry (baseline and first follow-up)[17] or a full-threshold HFA 24-2 test (second follow-up)[19] was performed on both eyes. The classification process of the Goldmann perimetry test results[17] and the full-threshold HFA 24-2 test results[19] have been described before. In short, visual field loss was considered to be glaucomatous visual field loss only if reproducible and after excluding all other possible causes. Participants were considered to have incident OAG (iOAG) if neither eye had glaucomatous visual field loss at baseline and at least 1 eye showed glaucomatous visual field loss at follow-up.[19]

Assessment of lifestyle

For SES we assessed income (salary) and education level separately. Participants were interviewed at baseline by using a questionnaire including questions about their net income (salary minus tax) and education level. For net household income, we used the equivalent household income in Dutch guilders. The value of a Dutch guilder at the baseline of the study roughly equaled that of a dollar in 2010 (using the percentage of change in consumer price index, a measure for economic inflation).[20] The equivalent household income was calculated as follows: each participants household income was classified into 1 of 13 precoded categories. Because in a household more than 1 person would be dependent on a single income, the midpoint of each income category was divided by the number of persons who were living on that income raised to the power 0.36.[21] This transformation provided the so-called “equivalent household income” and was analyzed as a continuous variable.[22] Education level was categorized in 4 groups according to highest completed education: primary (elementary),

lower (vocational/secondary), intermediate (vocational/secondary) and higher (vocational/university).

For smoking, trained research assistants asked participants at baseline about their current and past smoking habits, including type of smoking: cigarette, cigar, or pipe. Smoking was analyzed using nominal categories: never, former, and current smokers.

For alcohol intake, all participants were interviewed at baseline at the study center by a trained dietician using an extensive semiquantitative food frequency questionnaire.[23] Participants reported the number of alcoholic beverages they consumed on a weekly basis in each of the following 4 groups: beer, wine, moderately strong alcoholic beverages such as port wine or sherry, and liquor. For each of these 4 groups, the number of drinks was multiplied by the average amount of ethanol in 1 drink of the alcoholic beverage. A “drink” was defined as 200 mL of beer that contained 8.0 g of ethanol, 100 mL of wine that contained 10.0 g of ethanol, 75 mL of moderately strong alcoholic beverages that contained 10.5 g of ethanol, or 50 mL of liquor that contained 14.0 g of ethanol.[24] Earlier studies reported protective effects for persons with a low intake of alcohol on several diseases (e.g. cardiovascular diseases) compared with participants with no alcohol intake at all or a high intake. This suggests a U-shaped relation for alcohol consumption.[25] Therefore, we analyzed alcohol consumption using nominal categories: no intake, intake up less than 10 g, 10 to 20 g, and more than 20 g, after summing the 4 alcohol groups.[26]

Finally, we studied 2 anthropometric measures related to obesity: body mass index (BMI) and waist-to-hip ratio.[27] The BMI was calculated as weight in kilograms divided height in meters squared. The waist-to-hip ratio is a measure of body shape, that is, of relative abdominal obesity. Waist-to-hip ratio was calculated by dividing waist circumference by hip circumference and expressed as percentages (that is, multiplied by 100). Weight, height, and waist and hip circumference were measured at the research center.

Statistical analysis

Differences in baseline characteristics between participants with and without iOAG were analyzed with independent t-tests and chi-square statistics. Differences in education level were assessed twice, with and without adjustment for myopia. This was done because myopia has been reported to be associated with both education level (as a surrogate measure for near work) and OAG.[19,28-32] Myopia was stratified into 3 categories by using the spherical equivalent at baseline: high (≤-4.00 diopters [D]), low (-3.99 to -0.01 D), and no myopia (≥0 D). Those eyes with a cataract extraction before baseline were excluded from this analysis. In persons with 1 eye with iOAG, the refraction of that eye was used. In participants without OAG or with OAG in both eyes, the refraction of a random eye was used.

For the multivariate analysis, we used Cox proportional hazard regression analysis to calculate hazard ratios (HRs) with corresponding 95% confidence intervals (CIs) to analyze whether participants had a lower or higher risk of developing OAG. Follow-up duration was

used as the time variable. Lifestyle risk factors with p<0.20 in the univariate comparisons were included in the multivariate analysis. The model was further adjusted for age, IOP at baseline, and IOP-lowering treatment, and stratified by sex. For the analyses on IOP, we conducted multiple linear regression analyses adjusted for these variables, except for baseline IOP, and stratified by sex.

All statistical analyses were performed using SPSS version 15.0.0 for Windows (SPSS, Chicago, Illinois, USA) and R statistical package version 2.9.1 for Mac (http://www.r-project.org). A p-value of 0.05 or less was considered statistically significant.

RESULTS

During a mean follow-up of 9.7 years (range, 5.0-13.9 years), 108 of 3939 participants (2.7%) developed OAG. Table 1 summarizes the baseline characteristics of the study population according to iOAG status with corresponding univariate comparisons. Participants who developed iOAG during follow-up were significantly older, had more often high myopia, and were more often male compared to those without iOAG.

Table 1. Characteristics and univariate analyses of the study population with and without incident OAG

		Incident OAG (N=108)	No OAG (N=3831)	p-value
Age (year), mean ± SD		67.9 ± 7.1	65.2 ± 6.8	<0.001
Gender, N (%) female		53 (49.1)	2248 (58.7)	0.05
IOP at baseline (mmHg), mean ± SD		17.3 ± 4.7	15.0 ± 3.1	<0.001
IOP > 21 mmHg at baseline, N (%)		13 (12.0)	89 (2.3)	<0.001
IOP treatment at baseline, N (%)		17 (15.7)	88 (2.3)	<0.001
IOP at follow-up (mmHg), mean ± SD		16.5 ± 4.6	14.2 ± 3.2	<0.001
IOP > 21 mmHg at follow-up, N (%)		14 (13.0)	51 (1.4)	<0.001
IOP treatment at follow-up, N (%)		41 (38.0)	226 (5.9)	<0.001
Myopia N (%)*	Low	22 (21.0)	770 (20.3)	0.67
	High	10 (9.5)	186 (4.9)	0.03

* = Forty-seven participants were excluded because of cataract surgery before baseline; IOP = intraocular pressure; OAG = open-angle glaucoma; SD = standard deviation.

Table 2 shows the assessed lifestyle risk factors according to iOAG status with corresponding univariate comparisons. At the p<0.20 level, participants with iOAG had a lower BMI than those without iOAG (p=0.12). No other differences between participants with and without iOAG were found. The differences in education level between participants with and without iOAG remained insignificant after adjustment for myopia (p=0.49).

Table 2. Univariate analyses of assessed lifestyle-related risk factors for participants with and without incident OAG

		Incident OAG (N=108)	No OAG (N=3831)	p-value
Net household income (in /1000,-), mean ± SD		2.2 ± 0.9	2.3 ± 1.0	0.54
Education N(%)	Primary	35 (32.4)	1144 (30.1)	0.55
	Lower	26 (24.1)	1132 (29.8)	
	Intermediate	37 (34.3)	1136 (29.9)	
	Higher	10 (9.3)	388 (10.2)	
Smoking	Never	19 (17.6)	808 (21.3)	0.60
	Former	53 (49.1)	1719 (45.3)	
	Current	36 (33.3)	1269 (33.4)	
Total alcohol intake (g/d), median (IQR)		5.0 (0.4-14.8)	4.3 (0.3-15.4)	0.72
Alcohol intake N (%)	No intake	14 (15.4)	588 (17.2)	0.40
	<10 g/d	50 (54.9)	1574 (46.1)	
	10-20 g/d	11 (12.1)	557 (16.3)	
	>20 g/d	16 (17.6)	692 (20.3)	
Alcohol intake (g/d), median (IQR)	Beer	0.3 (0.0-4.7)	0.2 (0.0-3.5)	0.27
	Wine	0.4 (0.0-1.4)	0.3 (0.0-1.8)	0.57
	Liquor	5.0 (0.0-14.0)	2.0 (0.0-14.3)	0.93
	Sherry	1.0 (0.0-6.8)	0.7 (0.0-4.8)	0.58
Body mass index (kg/m^2), mean ± SD*		25.8 ± 2.9	26.3 ± 3.5	0.12
Waist-to-hip ratio (x100), mean (SD)		89.6 ± 8.6	90.0 ± 9.2	0.69

SD = standard deviation; IQR =interquartile range; * = Calculated as weight in kilograms divided by height in meters squared.

In the multivariate analysis, BMI was associated with a reduced risk of developing iOAG (HR, 0.94 per unit increase in BMI; 95% CI, 0.89-1.00; p=0.03). After stratification by sex, this association appeared to be only present in women. A higher BMI in women showed a protective effect on OAG (women: HR, 0.93; 95% CI, 0.86-1.00; p=0.04; men: HR, 0.96; 95% CI, 0.87-1.06; p=0.38).

Table 3 shows the results of the multiple linear regression analyses with IOP as the outcome measure for all assessed lifestyle risk factors, stratified by sex. None of the assessed variables, except BMI, showed a significant association. Body mass index turned out to have an increasing effect on IOP in women, but not in men.

DISCUSSION

We did not find any evidence for an association between income, education level, smoking or alcohol intake and iOAG. We showed in a multivariate analysis that BMI has a protective

Table 3. Multiple linear regression analysis of lifestyle-related risk factors and intraocular pressure, stratified by sex*

		Men		Women	
		Beta ± SE	p-value	Beta ± SE	p-value
Net household income (in /1000,-)		0.02 ± 0.10	0.82	-0.02 ± 0.08	0.77
Education	Primary	1 [Reference]		1 [Reference]	
	Lower	-0.13 ± 0.31	0.67	-0.30 ± 0.18	0.09
	Intermediate	0.20 ± 0.32	0.55	-0.13 ± 0.20	0.52
	Higher	-0.31 ± 0.30	0.31	-0.65 ± 0.36	0.07
Smoking	Never	1 [Reference]		1 [Reference]	
	Former	-0.82 ± 0.38	0.03	0.06 ± 0.16	0.70
	Current	-0.40 ± 0.49	0.41	-0.22 ± 0.21	0.29
Body mass index (kg/m^2)		0.06 ± 0.04	0.09	0.08 ± 0.02	<0.001
Waist-to-hip ratio (x100)		0.01 ± 0.02	0.58	0.01 ± 0.01	0.46
Alcohol intake (g/d)	Beer	-0.01 ± 0.02	0.58	0.01 ± 0.06	0.88
	Wine	-0.02 ± 0.05	0.67	-0.001 ± 0.04	0.98
	Liquor	0.01 ± 0.01	0.18	-0.01 ± 0.01	0.36
	Sherry	0.03 ± 0.03	0.25	-0.02 ± 0.01	0.22

* = all variables adjusted for age and intraocular pressure-lowering treatment; SE = standard error.

effect on OAG in women, but not in men. In addition, BMI was associated with a higher IOP in women.

The present findings on SES (income and education level) are in line with those of the Los Angeles Latino Eye Study, which also did not find an association for income or education level with OAG.[33] In contrast, a case-control study of 220 OAG cases found that persons with OAG more often had a lower SES, in terms of having vehicles and owning their houses.[3] The current study was based on persons living in a single and rather homogeneous suburb, and as a consequence, the variability in income was limited, making it difficult to find significant associations. Furthermore, mortality rates in persons with lower SES are higher than those with higher SES.[34] This effect might have caused an underrepresentation of participants with lower SES in the higher age categories in our population-based cohort. Since OAG is most common in the higher age categories, this might have biased the results.

Studies on the involvement of smoking in IOP or OAG showed conflicting results.[7,35] A case-control study exploring the relationship between smoking and OAG found a positive association of smoking with OAG.[35] In contrast, the Beaver Dam Eye Study did not confirm this association.[7] Regarding the relationship between smoking and IOP, the Blue Mountains Eye Study reported a modest cross-sectional positive association for current smokers and IOP.[9,10] A meta-analysis of several epidemiological studies on smoking found a higher risk of developing OAG for current smokers, but not for former smokers.[36] However, a large prospective study among more than 100000 health professionals throughout the United States, which assessed smoking status for more than a decade, found that neither former smokers nor current smokers had an increased risk of developing OAG.[37] Nonetheless, the

same study reported a borderline inversed association with smoking pack-years.[37] A problem in analyzing smoking as a risk factor might be that environmental tobacco smoke cannot be included reliably. Such an exposure misclassification is usually similar in cases and controls and might contribute to a conservative risk estimate. There are scarce data on the possible effect of environmental tobacco smoke on OAG.[38]

The results of alcohol intake from case-control and prevalence studies are also mixed regarding OAG. Although some studies did not find a significant relationship between alcohol intake and OAG,[7,33,39,40] others found an association between alcohol intake and IOP, but only in men.[10,11,41] This was not found in the current study. Also, the insignificant findings when dividing alcohol into groups (beer, wine, liquor, and sherry) are in line with another prospective study.[39]

Finally, the relationships between BMI and OAG and BMI and IOP seem to be contradictory. In agreement with our findings, earlier studies found a positive association between BMI and IOP,[14,42] and other studies suggested an inverse relation of BMI to OAG.[11-13,43] Of these studies, only the Barbados Eye Study[43] and Pasquale, et al.[13] mentioned sex effects. The former found a significant association with OAG in men as well as women, and the latter study found for each unit increase in BMI a significant reduction of 6% in the risk of OAG for women, but not for men. This is in line with the present study, which presents a 7% reduction in risk of developing OAG for each unit increase in BMI in women, but no significant effect in men. The Singapore Malay Eye Study, which investigated optic disc parameters, reported a significantly higher BMI in persons with small cup-disc ratios, which also suggests a protective effect of BMI on OAG.[44,45] We could not find a significant association for BMI with cup-disc ratio, but independent of BMI, a higher waist-to-hip ratio was significantly associated with a smaller cup-disc ratio (data not shown).

A possible mechanism explaining the inverse association of BMI with IOP regardless of sex could be an increased orbital pressure because of excess fat tissue, with a rise in episcleral venous pressure and a consequent increase in IOP. With obesity, there may also be an increase in the viscosity of blood, with a consequent increase in outflow resistance of the episcleral veins.[46,47] As mentioned in the “Methods” section, we measured IOP using Goldmann applanation tonometry (mounted on a slitlamp). Interestingly, a study on obese patients (consisting mostly of women) reported a significantly higher IOP when measured with Goldmann applanation tonometry compared with Perkins hand-held tonometry, both with patients in a sitting position. This difference was much smaller in the control group.[48] An explanation for this finding is that, with Goldmann applanation tonometry, the thorax and abdomen are pushed against the slit lamp table while breath-holding works like a Valsalva maneuver. This is especially relevant for obese women. Therefore, measurement of IOP with Goldmann applanation tonometry in women with a high BMI might lead to an overestimation of the actual IOP and as a consequence might contribute to the remarkable relationships between BMI and IOP and BMI and OAG in women. The higher IOP in women with a high BMI should have resulted in an expected higher incidence of OAG. However, this effect was

not observed and thus the multivariate analysis yielded a protective effect of BMI on OAG incidence in women. Another explanation might be that high estrogen levels and hormone therapy might be protective to OAG,[49,50] and obesity seems to be positively related with postmenopausal plasma estrogen levels.[51]

Some studies on lifestyle-related risk factors adjusted for cardiovascular-related variables, assuming that these variables may be related to lifestyle and may also have an effect on OAG. Previously, we investigated the relationships between OAG and atherosclerosis and diabetes mellitus but could not find any association.[52,53] Moreover, adjustment for blood pressure, diabetes mellitus or cholesterol level did not alter the current results (data not shown).

In conclusion, we could not find an association between SES, smoking, or alcohol consumption and OAG. Although these findings are in line with those from earlier studies, our findings are based on a relatively low number of iOAG cases, and as a consequence, small effects of these lifestyle-related risk factors cannot be ruled-out because of power limitations. We found a protective effect of a high BMI on the development of OAG in women. This effect seems to be IOP independent, but an overestimation of IOP as assessed with Goldmann applanation tonometry in obese women may also have contributed to this inverse association.

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Nutrition and risk of open-angle glaucoma - The Rotterdam Study

ABSTRACT

Objective: To investigate whether dietary intake of nutrients (omega-3 and omega-6 fatty acids, carotenoids, minerals, vitamins, and flavonoids) is associated with incident open-angle glaucoma (OAG).

Design: Prospective population-based study.

Participants: Participants aged 55 years and older of the Rotterdam Study (N=3502) for whom dietary data at baseline and data from ophthalmic examinations at baseline and follow-up were available and who did not have glaucoma at baseline.

Methods: Ophthalmic examinations including measurements of the intraocular pressure and perimetry were performed at baseline and after an average follow-up duration of 9.7 years. Dietary intake of nutrients was assessed by validated questionnaires and adjusted for energy intake. Incident OAG was defined as the development of glaucomatous visual field loss during follow-up.

Main outcome measures: Hazard ratios of associations between the baseline intake of nutrients and incident OAG were calculated using the Cox regression model adjusted for age, gender, IOP, IOP-lowering treatment, and body mass index.

Results: During follow-up, 91 of 3502 participants (2.6%) developed OAG. The hazard ratio for magnesium (highest versus lowest tertile) was 2.25 (95% confidence interval 1.16-4.38), for retinol equivalents 0.45 (0.23-0.90), and for vitamin B1 0.50 (0.25-0.98). Similar hazard ratios were found after the exclusion of participants taking supplements. No clear associations between the intake of nutrients and the IOP were found.

Conclusions: A high intake of magnesium and a low intake of retinol equivalents and vitamin B1 appear to be associated with an - IOP independent - increased risk of OAG.

INTRODUCTION

One of the most important sight-threatening eye disorders in ophthalmology is open-angle glaucoma (OAG). This progressive neurodegenerative disease leads to glaucomatous optic neuropathy and eventually, through glaucomatous visual field loss, to loss of sight. Together with age-related macular degeneration it is the most common cause of irreversible blindness.

Earlier we found, in a population-based epidemiological setting, an inverse association of high dietary intake of anti-oxidants on age-related macular degeneration.[1] This is in line with the results reported by a multicenter case-control study on dietary intake,[2] and by clinical trials with antioxidant supplementation.[3-5] It has been suggested that oxidative stress also may play a role in the pathogenesis of OAG.[6,7] Oxidative stress was reported to induce and maintain degeneration of the optic nerve.[8,9] Furthermore, it has been suggested that oxidative stress damages the trabecular meshwork, resulting in an increase in intraocular pressure (IOP).[8] The IOP is an important risk factor of OAG. For these reasons, the effects of nutrients with anti-oxidant activity are of great interest,[10] especially because the intake of nutrients is modifiable. Common nutrients with anti-oxidant activity are carotenoids (present in most fruits and vegetables), vitamins B, C and E, and polyphenolic flavonoids (present in tea, especially green tea, and coffee). Other nutrients of interest are the omega-3 and omega-6 fatty acids, which are well known in the cardiovascular literature because of their effect on blood flow regulation by serving as direct precursors of prostaglandins. There is increasing evidence that ocular blood flow plays a role in the pathogenesis of OAG and it has been suggested that omega-3 and vitamin E may be of therapeutic value in OAG.[11,12] Previous studies suggested that calcium and magnesium may play a role in the pathogenesis of OAG by affecting the peripheral circulation.[13] Since magnesium interacts with several minerals that may compete with its absorption, we also included phosphorus and calcium in our analyses. Although several studies hypothesized a role for various nutrients in OAG, large (population-based) studies on the risk of OAG appear to be lacking.[14-16]

The aim of the present study was to explore the influence of nutrition on the development of OAG. For this purpose, we studied the associations between the intakes of omega-3 and omega-6 fatty acids, carotenoids, minerals, vitamins, and flavonoids and incident OAG in a prospective population-based setting. As there may be effects both directly on the optic nerve and through the IOP, we also studied the associations between these intakes and the IOP.

METHODS

Participants

The present study was performed within the Rotterdam Study, a prospective population-based cohort study comprising 7983 residents aged 55 years and older living in Ommoord,

a district of Rotterdam, the Netherlands.[17] The baseline examination of the ophthalmic part of the Rotterdam Study included 6806 participants and took place between 1991 and 1993; follow-up examinations for OAG were performed from 1997 to 1999 and from 2002 to 2006. Participants were included in this study if they (1) participated both at baseline and at least at one follow-up examination, (2) had complete data on OAG and dietary intake, and (3) were not classified as having OAG at baseline. All measurements were conducted after the Medical Ethics Committee of the Erasmus University had approved the study protocol and all participants had given a written informed consent in accordance with the Declaration of Helsinki.

Ophthalmic examination

The examinations at baseline and follow-up included autorefraction (Topcon RM-A2000; Tokyo Optical Co., Tokyo, Japan), keratometry (Topcon OM-4 Ophthalmometer; Tokyo Optical Co., Tokyo, Japan), measurement of the best corrected visual acuity with ETDRS optotypes, measurement of the IOP (see below), fundus photography of the posterior pole (Topcon TRC-50VT, Tokyo Optical Co., Tokyo, Japan), simultaneous stereoscopic fundus photography of the optic nerve head (Topcon ImageNet System, Topcon TRC-SS2, Tokyo Optical Co., Tokyo, Japan), imaging of the optic nerve head with the Heidelberg Retina Tomograph (Heidelberg Engineering, Dossenheim, Germany) and visual field testing (HFA II 740; Zeiss, Oberkochen, Germany; see below).

The IOP was measured at baseline and at every follow-up round with Goldmann applanation tonometry (Haag-Streit, Bern, Switzerland) after applying oxybuprocaine 0.4% eye drops and fluorescein from a paper strip. Three measurements were taken on each eye and the median value of these three measurements was recorded.[18] In the analysis we used the highest IOP of both eyes.

The visual field of each eye was screened using a 52-point supra-threshold test that covered the central visual field with a radius of 24°.[19,20] Visual field loss was defined as non-response in at least three contiguous test points (or four including the blind spot). In participants with reproducible abnormalities on supra-threshold testing, Goldmann perimetry (Haag-Streit, Bern, Switzerland; baseline and first follow-up) or a full-threshold HFA 24-2 test (second follow-up) was performed on both eyes. Details about the classification process were described before.[19,21] Cases had to have an open anterior chamber angle; a history or signs of secondary glaucoma were not allowed.

Incident OAG was defined as no glaucomatous visual field loss in both eyes at baseline and glaucomatous visual field loss in at least one eye at follow-up.[21]

Assessment of nutrition

All participants were interviewed at baseline for food assessment using an extensive

semiquantitative food frequency questionnaire at the study center by a trained dietician. The adaptation of the semiquantitative food frequency questionnaire has been shown to be a valid and time-efficient dietary assessment instrument.[22] The dietary intake of nutrients was calculated using The Dutch Food Composition Database (NEVO) from 1993 and 2006. "Energy-adjusted" nutrient intakes were computed as the unstandardized residuals from a (linear) regression model in which total caloric intake served as the independent variable and the absolute nutrient intake as the dependent variable. Because residuals have a mean of zero and thus include negative values, the expected mean nutrient intakes of the study population was added to the residuals as derived from the regression analysis.[23] Next, we divided the energy adjusted intake into tertiles (low, medium, and high intake). This process was repeated for all nutrients.

Included nutrients were omega-3 fatty acids (α-linolenic acid [ALA; C18:3 (n-3) cis], eicosapentaenoic acid [EPA; C20:5 (n-3) cis], docosahexaenoic acid [DHA; C22:6 (n-3) cis]), omega-6 fatty acids (linoleic acid [LA; C18:2 (n−6) cis]), carotenoids (α-carotene, ß-carotene, lutein, zeaxanthin, ß-cryptoxanthin and lycopene), minerals (calcium, phosphorus and magnesium), vitamins (retinol equivalents, B1, B6, B12, E and C), and flavonoids.

Body mass index was calculated as weight in kilograms divided height in meters squared and measured at the research center.

Statistical analysis

Differences in baseline characteristics were analyzed with independent t-tests and chi-square statistics. Nutrients with $p<0.10$ in the univariate comparisons (between participants with and without incident OAG) were included in a multivariate analysis. For the latter, we used a Cox proportional hazard model to calculate hazard ratios (HR) with corresponding 95% confidence intervals (CI) to analyze whether participants had a lower or higher risk of incident OAG. Follow-up duration was used as the time variable. Follow-up duration was counted from the baseline visit to the last visit with reliable perimetry. Incident OAG cases were censored at the first visit in which glaucomatous visual field loss was detected. The model was adjusted for age, gender, IOP, IOP-lowering treatment and body mass index, all measured at baseline. Adjustment for IOP was done to analyse whether the effects of the nutrients were independent of IOP. For each nutrient, the lowest tertile (i.e., the participants with the lowest intake) served as the reference group. Tertiles were analyzed as nominal categories, that is, we did not assume beforehand monotonic relationships between nutrient intakes and outcome measure. We also explored whether there was interaction between age or gender and each of the nutrients from the final model. As a secondary analysis, we repeated the above-mentioned analysis after exclusion of participants who reported to have used any supplements (vitamins or other health pills) at baseline.

To assess the associations between nutrients and IOP, we performed for each nutrient a linear regression analysis (IOP at follow up versus intake). These analyses were adjusted

for IOP-lowering treatment at follow-up. Nutrients were analyzed as nominal categories (see above). Nutrients with p<0.10 were included in a multiple linear regression analysis with IOP at follow-up as the dependent variable. This model was adjusted for age, gender, body mass index, and IOP-lowering treatment during follow-up.

We used complete case analysis and considered a p-value of 0.05 or less statistically significant. All statistical analyses were performed using SPSS version 15.0.0 for Windows (SPSS inc., Chicago, IL, USA; 2006) and R statistical package version 2.11.1 for Mac (http://www.r-project.org).

RESULTS

During a mean follow-up of 9.7 years (range 5.3-13.9 years), 91 (2.6%) of 3502 eligible participants developed OAG. Table 1 summarizes the general characteristics of the study population at baseline according to incident OAG status. Participants who developed OAG were significantly older, more often males, and had a lower body mass index than those without incident OAG. Their use of supplements did not differ from that of participants without incident OAG (p=0.86).

Table 2 shows the descriptive data of the mean daily dietary intake of nutrients for all participants adjusted for total energy intake, with corresponding univariate comparisons between participants with and without incident OAG. At the p<0.10 level, participants with incident OAG had a higher intake of magnesium and vitamin E, and a lower intake of ß-carotene, retinol equivalents, vitamin B1 and vitamin B12 compared to participants without incident OAG. No other differences in nutrition were found between participants with and without incident OAG. Table 3 shows the results of the multivariate analysis, both for all participants and after exclusion of participants using supplements. Participants with a high intake of magnesium had a strongly increased risk of OAG, whereas a high intake of retinol

Table 1. Baseline characteristics and univariate analyses of the study population with and without incident open-angle glaucoma presented as mean ± standard deviation (range) unless stated otherwise

	Incident open-angle glaucoma (N=91)	No open-angle glaucoma (N=3411)	p-value
Age (year)	67.6 ± 7.0	65.1 ± 6.6	<0.001
Gender (N[%] female)	47 (51.6)	2031 (59.5)	0.13
IOP (mmHg)	17.5 ± 4.8	15.0 ± 3.1	<0.001
IOP-lowering treatment (N[%])	14 (15.4)	73 (2.1)	<0.001
Body mass index (kg/m^2)	25.5 ± 2.9	26.3 ± 3.5	0.03
Supplement users (N[%])	33 (36.3)	1269 (37.2)	0.86

IOP = intraocular pressure.

Table 2. Mean dietary intake of the assessed nutrients by tertile (low, medium and high intake), adjusted for total energy intake for each tertile, with corresponding univariate comparisons between participants with and without incident open-angle glaucoma

	Low intake		Medium intake		High intake		p-value*
	Mean±s.d.	range	Mean±s.d.	range	Mean±s.d.	range	
OMEGA FATTY ACIDS							
ALA (C18:3 (n-3) cis) (g/day)	0.62±0.14	(<0.81)	0.95±0.09	(0.81-1.12)	1.56±0.44	(>1.12)	0.470
EPA (C20:5 (n-3) cis) (g/day)	0.00±0.01	(<0.01)	0.03±0.01	(0.01-0.05)	0.11±0.09	(>0.05)	0.729
DHA (C22:6 (n-3) cis) (g/day)	0.02±0.01	(<0.04)	0.07±0.02	(0.04-0.10)	0.21±0.14	(>0.11)	0.704
LA (C18:2 (n−6) cis) (g/day)	6.37±2.47	(<9.47)	12.04±1.56	(9.48-14.94)	19.87±4.54	(>14.94)	0.410
ANTI-OXIDANTS							
α-carotene (µgram/day)	590.72±205.44	(<852.18)	1059.18±119.34	(852.40-1278.48)	1851.95±996.30	(>1278.76)	0.438
ß-carotene (µgram/day)	2431.61±613.88	(<3216.03)	3775.53±334.72	(3219.18-4399.33)	5879.44±2609.44	(>4399.61)	0.075
Lutein (µgram/day)	1417.24±318.64	(<1842.51)	2164.79±188.46	(1842.72-2504.99)	3235.32±1156.42	(>2506.38)	0.238
Zeaxanthin (µgram/day)	72.54±17.16	(<96.48)	117.06±11.85	(96.49-137.88)	175.99±46.14	(>137.89)	0.865
ß-cryptoxanthin (µgram/day)	74.77±53.60	(<173.59)	265.80±61.07	(174.27-371.22)	530.05±191.52	(>371.31)	0.809
MINERALS							
Calcium (mg/day)	773.27±149.99	(<964.91)	1095.41±76.58	(965.19-1238.44)	1527.75±285.00	(>1238.61)	0.358
Phosphorus (mg/day)	1117.50±144.93	(<1319.64)	1515.03±130.32	(1320.59-1786.33)	2498.47±777.77	(>1787.00)	0.865
Magnesium (mg/day)	252.94±28.63	(<287.87)	309.41±12.38	(287.88-331.51)	369.48±34.67	(>331.52)	0.001
VITAMINS							
Retinol equivalents (mg/day)	0.57±0.10	(<0.68)	0.76±0.04	(0.68-0.84)	1.11±0.46	(>0.84)	0.003
Vitamin B1 (mg/day)	0.87±0.11	(<1.01)	1.11±0.06	(1.01-1.22)	1.39±0.15	(>1.22)	0.017
Vitamin B6 (mg/day)	1.31±0.14	(<1.47)	1.59±0.07	(1.47-1.70)	1.92±0.21	(>1.70)	0.988
Vitamin B12 (mg/day)	2.78±0.75	(<3.69)	4.33±0.39	(3.70-5.07)	8.48±6.92	(>5.08)	0.066
Vitamin E (mg/day)	8.83±1.99	(<11.38)	13.29±1.13	(11.38-15.33)	19.42±4.04	(>15.35)	0.086
Vitamin C (mg/day)	72.07±17.17	(<96.49)	114.99±10.87	(96.50-134.74)	177.97±45.30	(>134.79)	0.956
FLAVONOIDS							
Flavonoids	16.52±4.77	(<23.09)	27.94±2.89	(23.10-33.23)	42.18±8.70	(>33.23)	0.923

* = p-values of the univariate analyses between participants with and without incident open-angle glaucoma; s.d. = standard deviation; ALA = α-linolenic acid; EPA = eicosapentaenoic acid; DHA = docosahexaenoic acid; LA = linoleic acid.

Table 3. Multivariate analysis for nutrition and open-angle glaucoma, for all participants and after exclusion of participants taking supplements

		Hazard Ratio (95% CI; p-value)	Hazard Ratio (95% CI; p-value)*
Age (year)		1.07 (1.04-1.11; <0.001)	1.09 (1.05-1.14; <0.001)
Gender		0.74 (0.48-1.15; 0.178)	0.72 (0.41-1.25; 0.243)
IOP (mmHg)		1.16 (1.10-1.22; <0.001)	1.17 (1.10-1.24; <0.001)
IOP-lowering treatment		3.87 (1.95-7.69; <0.001)	6.21 (2.68-14.41; <0.001)
Body mass index (kg/m^2)		0.93 (0.87-0.99; 0.026)	0.96 (0.89-1.04; 0.372)
ß-carotene	1st	1.00 (reference)	1.00 (reference)
	2nd	1.62 (0.97-2.71; 0.063)	0.99 (0.52-1.86; 0.966)
	3rd	1.08 (0.59-2.00; 0.795)	0.97 (0.47-2.00; 0.930)
Magnesium	1st	1.00 (reference)	1.00 (reference)
	2nd	0.54 (0.27-1.07; 0.076)	0.53 (0.21-1.35; 0.185)
	3rd	2.25 (1.16-4.38; 0.016)	3.19 (1.42-7.20; 0.005)
Retinol equivalents	1st	1.00 (reference)	1.00 (reference)
	2nd	1.16 (0.72-1.87; 0.543)	1.04 (0.57-1.89; 0.901)
	3rd	0.45 (0.23-0.90; 0.023)	0.33 (0.14-0.80; 0.014)
Vitamin B1	1st	1.00 (reference)	1.00 (reference)
	2nd	0.40 (0.21-0.77; 0.006)	0.28 (0.11-0.68; 0.005)
	3rd	0.50 (0.25-0.98; 0.044)	0.39 (0.17-0.90; 0.027)
Vitamin B12	1st	1.00 (reference)	1.00 (reference)
	2nd	1.60 (0.97-2.64; 0.066)	1.09 (0.55-2.16; 0.800)
	3rd	0.95 (0.51-1.78; 0.882)	1.37 (0.65-2.89; 0.415)
Vitamin E	1st	1.00 (reference)	1.00 (reference)
	2nd	0.69 (0.39-1.22; 0.199)	0.87 (0.44-1.74; 0.696)
	3rd	1.34 (0.81-2.22; 0.250)	1.27 (0.66-2.48; 0.476)

* = participants using supplements were excluded (N=1302); CI = confidence interval; IOP = intraocular pressure.

equivalents and vitamin B1 had a protective effect. For these nutrients the crude hazard ratios (that is, only adjusted for age) were statistically significant as well (p=0.014, p=0.019, and p=0.047 for high intake of magnesium, retinol equivalents and vitamin B1, respectively). We found no significant interactions between age or gender and the nutrients. The effects were stronger if we excluded participants using supplements.

Regarding IOP, we found in the multivariate analysis significant associations for a medium intake of ß-cryptoxanthin (beta: -0.35 mmHg; p=0.049) and a medium intake of vitamin B12 (beta: -0.35 mmHg; p=0.039) with IOP. A high intake of these two nutrients, however, was not significantly associated with IOP, precluding the presence of a clear dose-response relationship.

DISCUSSION

Magnesium intake appears to be associated with an increased risk of OAG whereas retinol equivalents (vitamin A) and vitamin B1 seem to have an inverse association. Since the analyses were adjusted for IOP, these effects should be considered IOP-independent. This is in agreement with the fact that we did not find a significant effect of these nutrients on IOP. None of the other nutrients had an unequivocal effect on IOP as well. The effects of magnesium, retinol equivalents and vitamin B1 on OAG became stronger when excluding participants taking supplements. Interestingly, none of the nutrients that affect anti-oxidant defense were significantly associated with OAG.

Little is known about the possible associations between nutrition and OAG. The Nurses' Health Study and the Health Professionals Follow-up Study, two large prospective cohorts comprising over 100000 participants, studied the influence of anti-oxidants and vitamins (vitamin A, C, and E) on OAG. They could not find a significant association.[14] Our results on anti-oxidants, vitamin C and E (but not vitamin A) were in line with their results (no significant effect on OAG). A possible explanation for the non-significant finding of vitamin A in their study could be that their OAG cases were based on self-report. The resulting selection bias should not be present in our study in which the OAG cases were detected by systematic screening only. Of interest is that vitamin A and retinoic acid (a metabolite of vitamin A) were associated with Alzheimer's disease, like OAG a neurodegenerative disorder.[24]

Another study by the Nurses' Health Study and the Health Professionals Follow-up Study showed an increased risk of OAG in participants with a high ratio of omega-3 to omega-6 polyunsaturated fat (relative risk [95% CI]: 1.49 [1.11-2.01]).[15] However, the latter association was only present in participants with high-tension OAG (OAG with IOP > 21 mmHg; relative risk [95% CI]: 1.68 [1.18-2.40]) and not in participants with normal-tension OAG (OAG with IOP ≤ 21 mmHg; relative risk [95% CI]: 0.82 [0.20-3.47]). The ratio of omega-3 to omega-6, adjusted for total energy intake, was in our study higher in participants with OAG (mean 0.19) compared to participants without OAG (0.12), but this difference did not reach statistical significance (p=0.22). This lack of significance might be explained by power limitations or by the fact that the majority (79%) of our incident OAG cases had an IOP ≤ 21 mmHg and were untreated at baseline. The corresponding small number of cases with an IOP > 21 mmHg or on IOP-lowering treatment at baseline hampers a secondary subgroup analysis. In contrast, a case-control study conducted in Nigeria reported that patients with OAG have reduced blood levels of EPA and DHA (both omega-3), and a concomitant increase in omega-6 resulting in a lower ratio of omega-3 to omega-6 polyunsaturated fat in participants with OAG.[25] A limitation of the latter study is the fact that they included only ten patients with (severe) glaucoma and eight controls which were the healthy siblings of the patients.

A review of natural therapies (that is, by means of changing nutrition habits) for ocular disorders highlighted the role of magnesium supplementation in OAG therapy.[26] Magnesium administration improves the peripheral circulation[27] and has been reported to have a beneficial

effect on the visual field in patients with OAG with cold-induced vasospasm.[13] However, there are some concerns regarding the latter study. First, they based their analyzes on only ten glaucoma patients (of which six with OAG). Second, the follow-up was very short (one month) and the observed improvement in the mean deviation of the visual field (on average 1 dB) did not reach statistical significance (p=0.09). Third, they did not report baseline magnesium levels and changes after the onset of substitution.[28] Absorption and elimination of magnesium is affected by many variables, making it difficult to study the effects of magnesium supplementation in small groups.[29] As mentioned in the Introduction, magnesium may interact with phosphorus and calcium; however, we found no significant interaction (data not shown). Furthermore, magnesium is present in many fruits, vegetables and dairy products, and thus, the association of magnesium with OAG might be explained by other nutrients present in magnesium-rich products. Interestingly, the rationale of magnesium substitution is that magnesium has similar effects as calcium channel blockers.[13,29] A double-blind controlled trial with oral magnesium aspartate HCl supplements, presented an inverse association with systemic blood pressure.[30] The role of calcium channel blockers in OAG, however, is all but clear. Although a recent clinical study suggested a small protective effect,[31] large epidemiological studies including our study reported an apparently harmful effect.[32,33] Adjusting for the use of calcium channel blockers at baseline did not alter the harmful effect of magnesium in our study (data not shown).

Finally, patients with OAG are reported to have a lower thiamine (vitamin B1) level than controls.[34] This supports the current finding of a protective effect of vitamin B1. We showed the protective effect of vitamin B1 on OAG to be independent of IOP. This is in agreement with the association between vitamin B1 deficiency and degeneration of ganglion cells of the brain and spinal cord in animal experiments,[26] and between vitamin B1 deficiency and a reduced thickness of the retinal ganglion cell layer in rats.[35] Furthermore, a link between vitamin B1 deficiency and other optic neuropathies has also been suggested.[36] Obviously, most participants with a low intake of vitamin B1 in the current study may not have had a manifest vitamin B1 deficiency. Alcohol consumption could be a confounder here, but alcohol consumption was not associated with either IOP or OAG.[37] Recently, a synthetic derivative of vitamin B1, sulbutiamine, has been shown to have a neuroprotective effect on retinal ganglion cells, probably due to its anti-apoptotic properties.[38]

The strengths of the present study are its large sample size, its population-based setting, and the long follow-up. The study population presumably consumed a healthy diet as the median nutrient intake of the study population was at or above the recommended daily allowance.[1] A possible limitation of our study is that only one food frequency questionnaire was used at baseline. Although this could initiate misclassification (dietary changes may result, amongst others, as part of treatment for chronic illnesses like hypertension, diabetes and age-related macular degeneration), such misclassification would unlikely result in false-positive findings (non of the illnesses that have dietary measures as part of its treatment are clearly associated with OAG). Another possible limitation is the limited number of participants who

developed OAG during follow-up. Almost half of the cohort deceased or did not participate at follow-up. Although a selective non-response is possible, we found no significant differences in dietary intake of nutrients between participants and non-participants (data not shown). As we tested a large number of nutrients, this might have resulted in false-positive findings. To cope at least partially with this, we only included nutrients that were earlier addressed in the literature or for which a clear pathophysiological rationale could be found (see Introduction section). If we would apply the very strict Bonferroni correction for multiple comparisons to the univariate analyses as presented in Table 1, a p-value of 0.003 (0.05/19) would have been considered as statistically significant in the univariate analysis. According to Table 1 magnesium and the retinol equivalents are also significant at this p-value.

The percentage of participants taking supplements in our study may seem high (36.2-37.2%; Table 1), however, it was very similar to other studies conducted in the early nineteens (the baseline of this study). For example, the National Health and Nutrition Examination Survey reported that in the age range of 60-69 years, 39.2% of men and 51.6% of women used supplements in the United States.[39] A study from Sweden reported supplement use in 22.2% in men and 33.3% in women.[40] Finally, we did not adjust for variables like diabetes mellitus, blood pressure and cholesterol level, as was done in the Nurses' Health Study and the Health Professionals Follow-up Study.[14] However, in the present study none of these variables showed significant univariate differences at baseline (p=0.096, p=0.976 and p=0.682, respectively), and thus they would have been excluded from the final model - if they would have been included initially. Nevertheless, as magnesium has been associated with diabetes, we added it to the model. This did not change the findings (data not shown).

In conclusion, the current findings suggest that people with a high intake of magnesium have an about threefold increase in risk of OAG compared to those with a low intake, and people with a high intake of retinol equivalents or vitamin B1 have an about twofold lower risk of OAG compared to those with a low intake of these nutrients. These findings might be helpful in the unraveling of the largely unknown pathophysiology of OAG. After confirmation of these findings in other cohorts, modification of the intake of these nutrients should be further explored as a new option for the treatment of OAG, especially for those patients in whom disease progression continues despite an apparently sufficient IOP reduction – because the effects appeared to be IOP independent.

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Chapter 4

Genetics of glaucoma-related traits

4.1 *A genome-wide association study of optic disc parameters.*
Ramdas WD*, van Koolwijk LM*, Ikram MK*, Jansonius NM, de Jong PT, Bergen AA, Isaacs A, Amin N, Aulchenko YS, Wolfs RC, Hofman A, Rivadeneira F, Oostra BA, Uitterlinden AG, Hysi P, Hammond CJ, Lemij HG, Vingerling JR, Klaver CC*, van Duijn CM*.
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4.2 *Genome-wide association studies in Asians confirm the involvement of ATOH7 and TGFBR3, and further identify CARD10 as a novel locus influencing optic disc area.*
Khor CC*, Ramdas WD*, Vithana EN*, Cornes BK, Sim X, Tay WT, Saw SM, Zheng Y, Lavanya R, Wu R, Wang JJ, Mitchell P, Uitterlinden AG, Rivadeneira F, Teo YY, Chia KS, Seielstad M, Hibberd M, Vingerling JR, Klaver CC, Jansonius NM, Tai ES, Wong TY, van Duijn CM, Aung T.
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4.3 *Common genetic determinants of intraocular pressure and primary open- angle glaucoma.*
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[submitted]

4.4 *Genetic architecture of open angle glaucoma and related determinants.*
Ramdas WD, Amin N, van Koolwijk LM, Janssens AC, Demirkan A, de Jong PT, Aulchenko YS, Wolfs RC, Hofman A, Rivadeneira F, Uitterlinden AG, Oostra BA, Lemij HG, Klaver CC, Vingerling JR, Jansonius NM, van Duijn CM.
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A genome-wide association study of optic disc parameters

ABSTRACT

The optic nerve head is involved in many ophthalmic disorders, including common diseases such as myopia and open-angle glaucoma. One of the most important parameters is the size of the optic disc area, and the vertical cup-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 7360 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 117 kb of the *CDC7* gene, and rs1900004 on chromosome 10q21.3-q22.1 ($p=2.67x10^{-33}$) within 10 kb 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 kb of the *SIX1* gene. Findings were replicated in two independent Dutch cohorts (Rotterdam Study III and Erasmus Rucphen Family study; N=3612), 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 for VCDR. *ATOH7* was also associated with VCDR independent of optic disc area. Three 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.

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-) ophthalmologic 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-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 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 occurrence 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,[9-12] suggesting a major role for genetic factors. 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.

MATERIALS AND METHODS

Study Populations

The Rotterdam Study I (RS-I) is a prospective population-based cohort study of 7983 residents aged 55 years and older living in Ommoord, a suburb of Rotterdam, the Netherlands.[13] 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 3011 residents aged 55 years and older and 3392 residents aged 45 years and older respectively. The rationale and study design are similar to those of the RS-I.[13] 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 in a genetically isolated population in the southwest of the Netherlands with over 3000 participants aged between 18 and 86 years. Cross-sectional examination took place between 2002 and 2005. The rationale and study design of this study have been described elsewhere.[14,15] 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 10000 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 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.[16]

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 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.[17]

HRT 2, used in RS-III and ERF, uses a focused 670-nm diode laser light beam to acquire scans of the optic nerve head region, using the confocal principle. 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 µm below the peripapillary retinal surface in the region of the papillomacular bundle.

Imaging was performed after entering the participant’s keratometry data into the software and after adjusting the settings in accordance with the refractive error. In RS-III all HRT 2 data was 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 µm were excluded. The inter-observer variability and agreement for both systems have been described elsewhere.[18] Details of the optic disc measurements in TwinsUK are described elsewhere.[19]

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 using 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.[20,21] 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[20] and the full-threshold HFA 24-2 test results [Czudowska, et al.; Chapter 3.1] have been described before. 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.[21] 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 are described elsewhere.[22] After exclusion of participants for reasons of low-quality DNA, a total of 5974 participants were available with genotyping data from RS-I, 2157 participants from RS-II and 2082 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, 2385 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 regression 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 using SPSS version 15.0.0 for Windows (SPSS inc., Chicago, IL, USA; 2006), MACH2 QTL as implemented in GRIMP[23] 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.[24]

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.[25] 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.[26] 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 if they 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 optic nerve head appearance and glaucomatous visual field loss. This was done by using logistic regression analyses adjusted for age and gender.

RESULTS

Study samples

The discovery cohorts included 5312 (RS-I) and 2048 (RS-II) participants who were genotyped and had reliable optic disc data, resulting in a total of 7360 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, 1966 participants were included, and from ERF 1646, resulting in a total of 10972 when combining the discovery and replication cohorts from the Netherlands, and 11815 when also including the 843 participants of TwinsUK. 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 only 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 and 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)	7360	1966	1646	843
Age (years)	67.0 ± 8.4	55.6 ± 5.5	46.8 ± 14.1	56.1 ± 12.7
	(55 - 99)	(45 - 89)	(18 – 84)	(16-83)
Gender, N(%) female	4208 (57.2)	1102 (56.1)	942 (57.2)	818 (97.0)
Disc area (mm^2)*	2.40 ± 0.48	1.92 ± 0.45	1.92 ± 0.37	2.59 ± 0.65
	(0.58 – 6.20)	(0.70 – 7.20)	(1.07 – 4.33)	(0.75 – 6.96)
Vertical cup-disc ratio*	0.50 ± 0.14	0.42 ± 0.17	0.46 ± 0.15	0.32 ± 0.10
	(0.00 – 0.89)	(0.00 – 1.00)	(0.00 – 0.84)	(0.07 – 0.70)

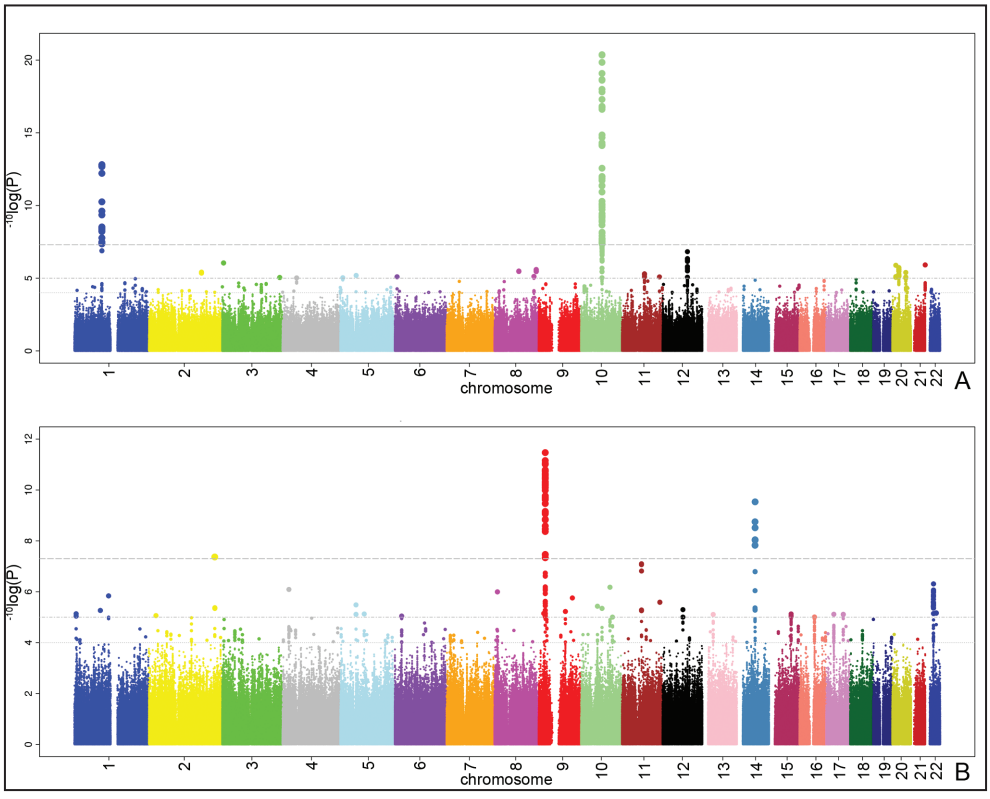
* = In RS-I, RS-II and TwinsUK measured with stereoscopic images, in RS-III and ERF with confocal scanning laser ophthalmoscopy.

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 (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 comparing those heterozygous with homozygous persons for the reference allele), and p-value $2.05x10^{-32}$ for rs1900004 (optic disc area decreased by 0.068 ± 0.006 mm^2). Table 2 shows the results of 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 joining the Dutch data with the TwinsUK series (Table 3), this region became genome-wide significant ($p=5.07x10^{-9}$). Table 3 shows that also 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*) gene, but the SNPs in the intergenic region were most significant. The genome-wide significant region on chromosome 10q21.3-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, solute carrier family 25 (mitochondrial carrier; Graves disease autoantigen), member 16 (*SLC25A16*) gene. However, the most significant evidence was found in the region between

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



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-disc ratio

All analyses for 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 reaching genome-wide significance at a threshold of $5x10^{-8}$. Adjustment for the intraocular

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

SNP	Chromosome location	Position	MA	RS-I/RS-II				RS-III				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	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	$1.85x10^{-6}$	0.21	-0.033	0.008	$5.28x10^{-5}$	0.22	-0.068	0.006	$2.05x10^{-32}$*	ATOH7/ PBLD	9021	175
rs1192415	1p22	91849685	G	0.18	0.091	0.010	$6.72x10^{-19}$*	0.18	0.059	0.019	$1.69x10^{-3}$	0.25	0.049	0.008	$2.39x10^{-10}$*	0.22	0.064	0.006	$1.82x10^{-27}$*	CDC7/ TGFBR3	116719	61
rs1362756	16q12.1	50015791	C	0.29	0.036	0.009	$4.85x10^{-5}$	0.28	0.032	0.016	$4.92x10^{-2}$	0.27	0.023	0.007	$1.56x10^{-3}$	0.28	0.028	0.005	$6.48x10^{-8}$	SALL1	1154095	10

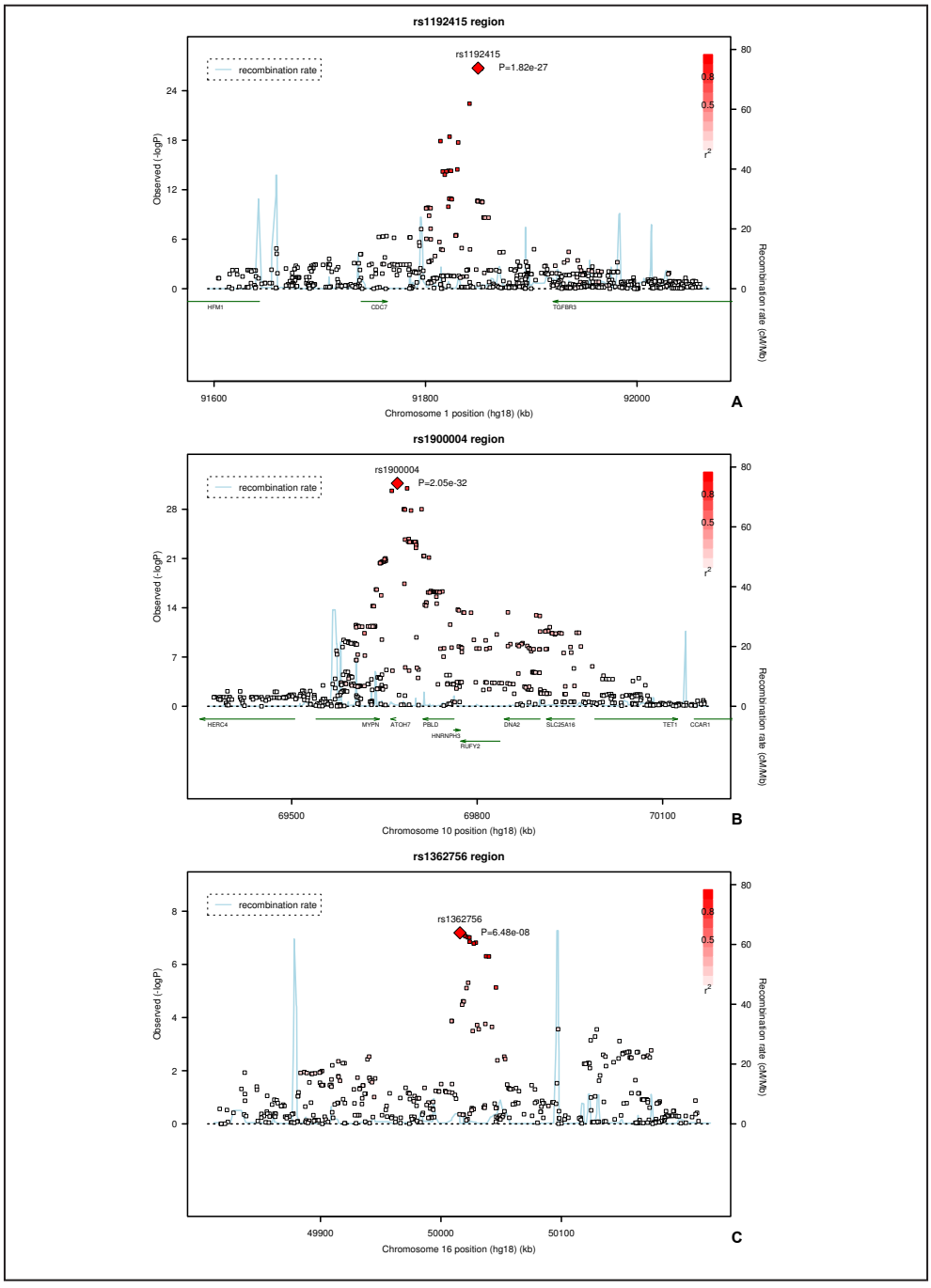
* = significant at a p-value of $5x10^{-8}$; SNP = single nucleotide polymorphism; MA(F) = minor allele (frequency); SE = standard error

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

Most significant SNP	Minor allele	Minor allele frequency	Chromosome location	Position	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	
rs1900004	T	0.24	10q21.3-q22.1	69670887	-0.133	0.038	$4.64x10^{-4}$*	-0.070	0.006	$2.71x10^{-35}$**
rs1192415	G	0.18	1p22	91849685	0.091	0.041	$2.60x10^{-2}$*	0.065	0.006	$2.77x10^{-28}$**
rs1362756	C	0.30	16q12.1	50015791	0.097	0.037	$8.29x10^{-3}$*	0.030	0.005	$5.07x10^{-9}$**

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

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



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 for association and reached genome-wide significance in the combined analysis of all Dutch cohorts (Table 4). This 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 of these three regions 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 combining 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 six loci associated with VCDR explained 2.2% of the variation in the VCDR. Also for the VCDR none of the loci were associated to myopia at $p<0.05$. When we evaluated the association to 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 six genetic loci (9q21, 14q22-23, 10q21.3-q22.1, 11q13, 13q13, 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 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

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

SNP	Chromosome location	Position	MA	RS-I/RS-II				RS-III				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	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	$1.38x10^{-2}$	0.47	-0.015	0.005	$2.54x10^{-3}$	0.46	-0.014	0.002	$1.96x10^{-14}$*	CDKN2B	0	88
rs10483727	14q22-23	60142628	T	0.40	0.014	0.002	$2.93x10^{-10}$*	0.39	0.001	0.005	$7.81x10^{-1}$	0.45	0.014	0.005	$4.95x10^{-3}$	0.41	0.012	0.002	$9.30x10^{-11}$*	SIX1	39878	10
rs17146964	11q13	65005721	G	0.21	-0.014	0.003	$7.94x10^{-8}$	0.21	-0.013	0.007	$5.65x10^{-2}$	0.21	-0.010	0.006	$1.05x10^{-1}$	0.21	-0.014	0.002	$4.43x10^{-9}$*	SCYL1	43403	8
rs1547014	22q12.1	27430711	T	0.29	-0.011	0.002	$7.20x10^{-6}$	0.30	-0.019	0.006	$1.02x10^{-3}$	0.32	-0.010	0.005	$7.34x10^{-2}$	0.29	-0.011	0.002	$1.96x10^{-8}$*	CHEK2	0	29
rs1900004	10q21.3-q22.1	69670887	T	0.22	-0.012	0.003	$4.49x10^{-6}$	0.23	-0.021	0.006	$8.90x10^{-4}$	0.21	-0.007	0.006	$2.98x10^{-1}$	0.22	-0.013	0.002	$2.06x10^{-8}$*	ATOH7/ PBLD	9021	10
rs1926320	13q13	35550617	C	0.24	0.011	0.003	$1.45x10^{-5}$	0.25	0.020	0.006	$1.29x10^{-3}$	0.27	0.008	0.006	$1.41x10^{-1}$	0.24	0.012	0.002	$4.85x10^{-8}$*	DCLK1	0	15
rs8068952	17q23	56641426	G	0.24	-0.012	0.003	$7.85x10^{-6}$	0.24	-0.014	0.006	$2.54x10^{-2}$	0.20	-0.007	0.006	$2.47x10^{-1}$	0.23	-0.012	0.002	$3.11x10^{-7}$	BCAS3	0	2
rs12025126	1p36.2-p36.1	8682141	C	0.28	-0.009	0.003	$3.93x10^{-4}$	0.27	-0.011	0.006	$6.62x10^{-2}$	0.32	-0.019	0.005	$3.82x10^{-4}$	0.29	-0.011	0.002	$4.14x10^{-7}$	RERE	0	5
rs2159128	19p13.3	901380	G	0.13	-0.016	0.005	$3.16x10^{-4}$	0.14	-0.021	0.010	$3.67x10^{-2}$	0.11	-0.032	0.011	$2.45x10^{-3}$	0.13	-0.019	0.004	$7.05x10^{-7}$	ARID3A	0	1

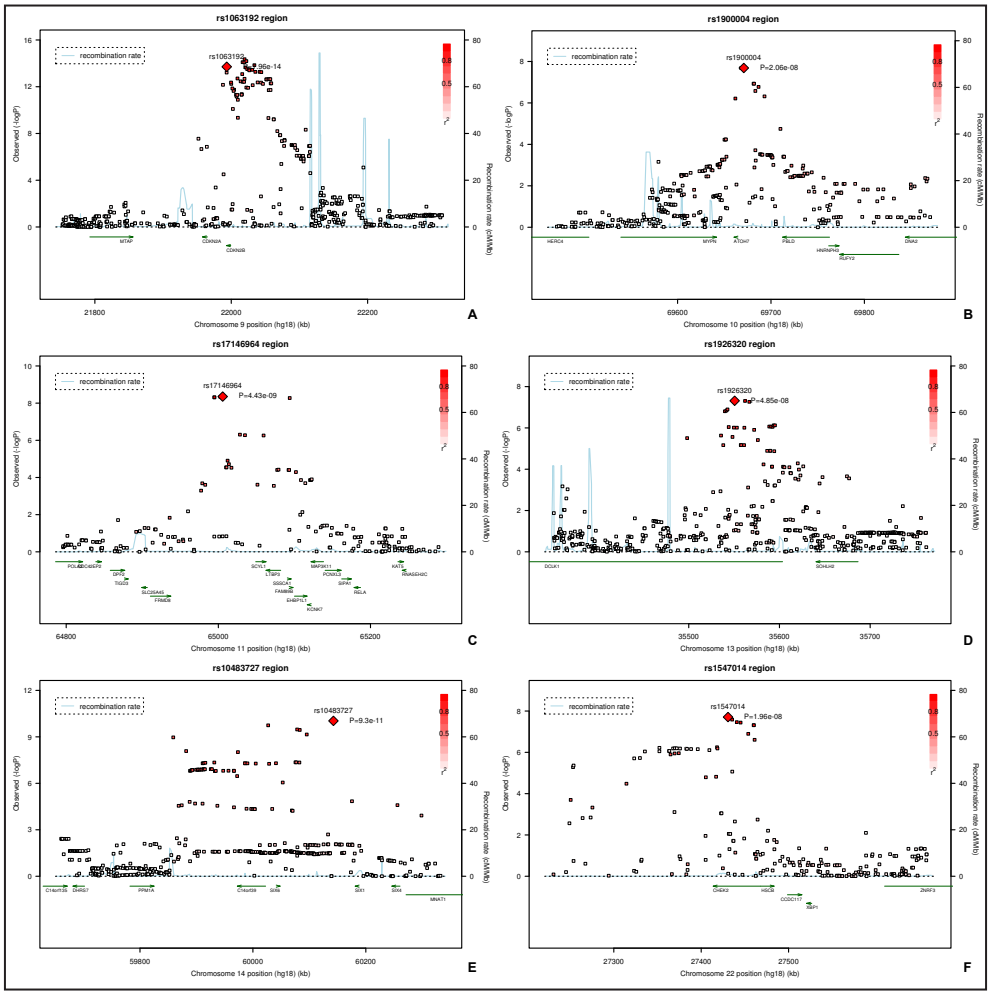
* = significant at a p-value of $5x10^{-8}$; SNP = single nucleotide polymorphism; MA(F) = minor allele (frequency); SE = standard error.

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

Most significant SNP	Minor allele	Minor allele frequency	Chromosome location	Position	delta vertical cup-disc ratio per allele		P-value	delta vertical cup-disc ratio per allele in meta-analysis of all five cohorts		P-value in meta-analysis of all five cohorts
					Beta	SE		Beta	SE	
rs1063192	G	0.44	9p21	21993367	-0.007	0.005	$1.33x10^{-1}$	-0.013	0.002	$4.35x10^{-15}$**
rs10483727	T	0.44	14q22-23	60142628	0.012	0.005	$1.36x10^{-2}$*	0.012	0.002	$1.01x10^{-11}$**
rs17146964	G	0.20	11q13	65005721	-0.004	0.006	$5.25x10^{-1}$	-0.012	0.002	$3.72x10^{-9}$**
rs1547014	T	0.26	22q12.1	27430711	-0.005	0.005	$3.15x10^{-1}$	-0.011	0.002	$1.48x10^{-8}$**
rs1900004	T	0.24	10q21.3-q22.1	69670887	-0.005	0.006	$3.83x10^{-1}$	-0.012	0.002	$1.72x10^{-8}$**
rs1926320	C	0.25	13q13	35550617	0.010	0.006	$5.92x10^{-2}$	0.012	0.002	$1.23x10^{-8}$**
rs8068952	G	0.19	17q23	56641426	-0.018	0.007	$6.69x10^{-3}$*	-0.012	0.002	$2.81x10^{-8}$**
rs12025126	C	0.28	1p36.2-p36.1	8682141	-0.010	0.005	$6.64x10^{-2}$	-0.011	0.002	$5.69x10^{-8}$
rs2159128	G	0.08	19p13.3	901380	-0.012	0.010	$2.41x10^{-1}$	-0.018	0.004	$2.98x10^{-7}$

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

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



SNPs involved in the optic disc area explained 2.7%, while the six 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 included multiple genes. Although the genome-wide significant region is very large for the optic disc area analysis, the *ATOH7* gene (also known as *Math5*) showed the most significant evidence for association with VCDR. This gene is expressed in the retina where it controls photoreceptor development.[27] In animal studies with mice, *ATOH7* expression has been found in the developing optic nerve during embryogenesis.[28] During retinogenesis, seven different

major classes of cells develop out of the progenitor cells in the eye: photoreceptors (rods and cones), bipolar cells, horizontal cells, amacrine cells, retinal ganglion cells (RGC; these are the cells involved in OAG) and Müller cells. Degeneration of these cells may lead to blindness.[29] In mutant mice and zebrafish without *ATOH7*, optic nerves and RGC are not further developed, while amacrine cells and cones are formed in excess.[30,31] Overexpression of *ATOH7* and interaction with the *neuroD* gene in chickens increases the amount of RGC and photoreceptors.[32] The duration of expression of *ATOH7* is regulated by several proteins, including Growth and Differentiation Factor 11 (*GDF11*).[33] 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).[34] Thus the *SHH* and *GDF11* regulate *ATOH7*, which in turn regulates *Brn3b*. This gene may play a role in further differentiation of RGC and is expressed in post-mitotic RGC precursors. First, RGC differentiate into the lower retinal epithelium (later becoming the RGC 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.[34] Although *ATOH7* has been implicated in retinal development in animals, this gene has not been linked to the development of the optic nerve pathology in humans. The analysis of VCDR showed that the *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 as in 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 for 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 the *TGFBR3* is of interest because of the interaction of *ATOH7* with *GDF11*, a member of the bone morphogenetic protein (BMP) and the TGFbeta superfamily. The genes therefore point to the same signaling pathway. *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 a relation of *LTBP3* to VCDR (see below). While *CDKN2A* is not known to be involved in TGFbeta signaling, *CDKN2B* has been implicated in this pathway. As 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*).[35] The *p15Ink4b* protein phosphorylates and inactivates the retinoblastoma tumor suppressor (*pRb*) protein.[36] Deletions of this gene and of the retinoblastoma 1 gene are often found in malignant gliomas and melanomas.[37] 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.[38] 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 signaling has been implicated in a wide variety of diseases including inflammation, autoimmune disorders, fibrosis, cancer and cataracts. The region has recently also been associated with myocardial infarction and type 2 diabetes mellitus.[39] The *CDKN2B/CDKN2A* and *CDC7*/*TGFBR3* loci influence the VCDR independently of optic disc area as these genes were not significantly associated with the optic disc area (p>0.05). However, *TGFBR3* appears to be involved in VCDR through its role in optic disc area, as the effect of this gene on VCDR increased two fold when we did not adjust for optic disc area (RS-I: unadjusted beta=0.015, standard error=0.004, $p=2.45x10^{-5}$ compared to beta=0.007, standard error=0.003 in the adjusted analysis).

Regarding the optic disc area, we found one additional region genome-wide significantly associated when pooling the data of the Dutch and TwinUK. 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 and the bronchio-oto-renal syndrome, two autosomal dominant disorders.[40] Only rare variants have been implicated in Townes-Brocks syndrome and bronchio-oto-renal syndrome, while the association we report here is with common variants. One of the traits involved in the latter syndrome is myopia.[41] However, in our analyses we could not find evidence for an association of the common SNPs in the *SALL1* region to myopia (rs1362756; p=0.802). *SALL1* encodes a zinc finger transcriptional repressor. When considering the protein pathway, *SALL1* interacts with *SIX1*.[42] Rare variants in *SIX1* are involved in the bronchio-oto-renal syndrome.[43] 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). This gene is involved in eye development and has been related to congenital glaucoma. Defects in this gene have been associated with anophtalmia in mice[44] and in humans.[45,46] Embryological studies have shown expression in the ventral optic stalk, which later becomes the optic nerve.[47] In the adult mouse retina, *Optx2* mRNA has been found in cells within the ganglion cell layer and inner nuclear layer.[48] This gene is expressed in the developing retina, optic nerve and other brain structures.[45]

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.[49] However, also the presence *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.[50,51] The *DCLK1* gene on 13q13 is expressed in the optic tectum.[52] 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 has been associated with several types of cancer, including breast cancer.[53] 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[54] 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.[55] 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 pinpoint the two endophenotypes 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 for OAG. However, the findings were far from genome-wide significance 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.[18,56,57] 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 (Macgregor, et al. unpublished data). 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 associated 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 *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 *CDKN2B* on chromosome 9p21, *SIX1* on chromosome 14q22-23, *SCYL1* 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 signaling pathway that appears to play a key role. Further research is needed to implicate these finding to pathology of the eye.

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SUPPLEMENTARY APPENDIX

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 and C) and for the meta-analysis (D).

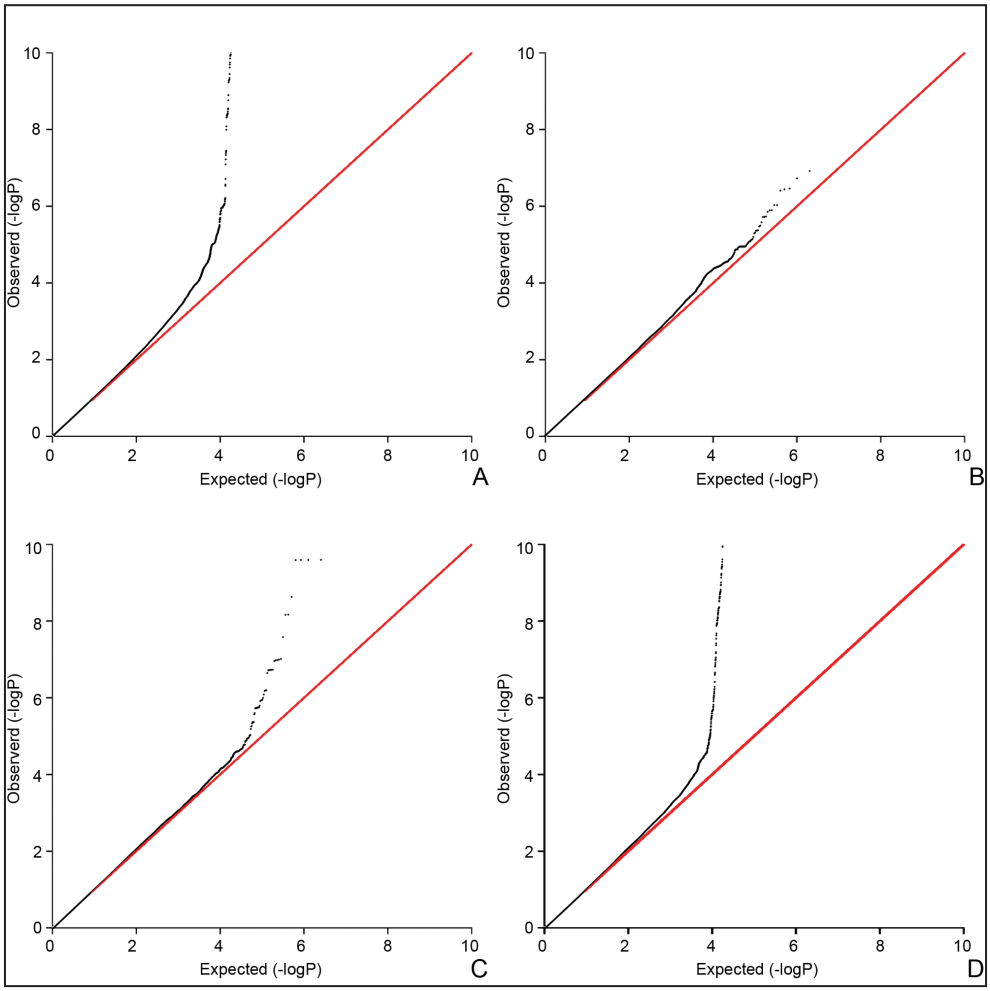


Figure S2. Vertical cup-disc ratio Q-Q plots for the observed versus expected p-values for the discovery cohorts (A), the individual replication cohorts (B and C) and for the meta-analysis (D).

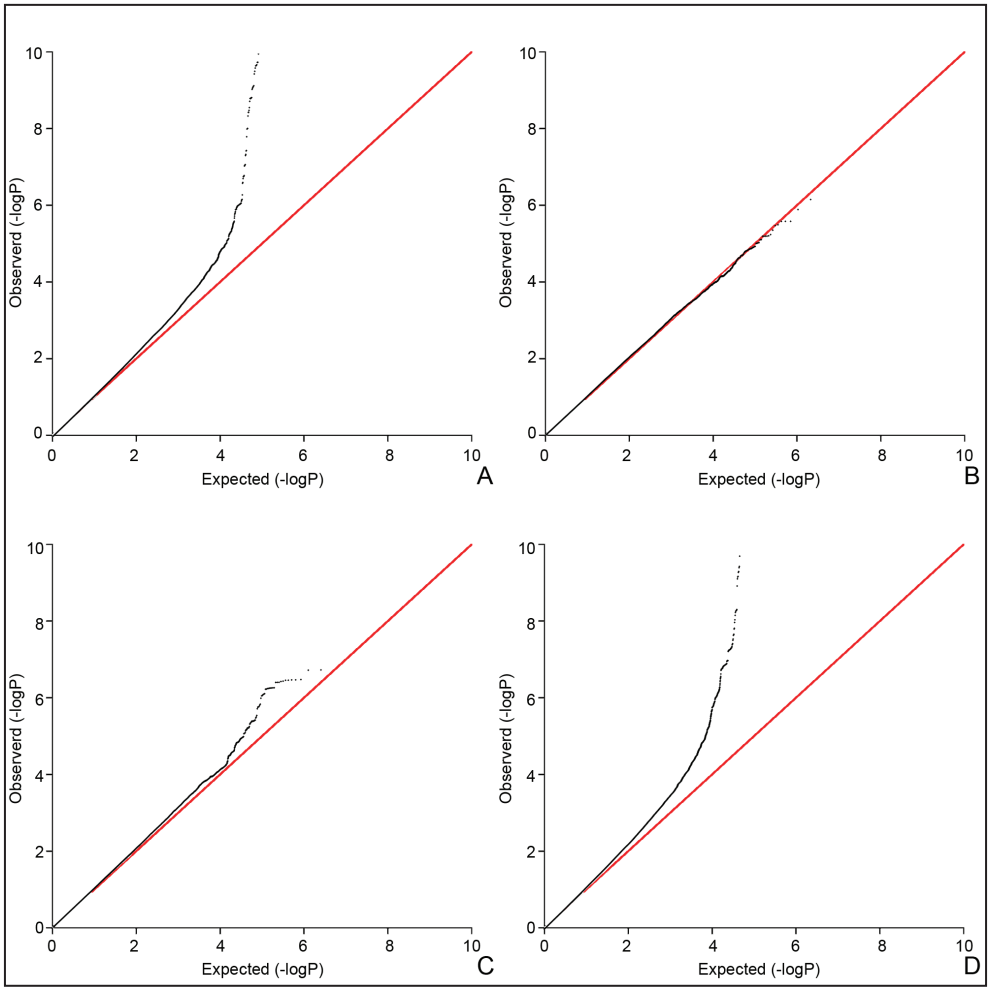


Table S1. Characteristics of the open-angle glaucoma patients presented as mean ± standard deviation (range) unless stated otherwise

	RS-I	
	Cases (N=188)	Controls (N=5548)
Age (years)	75.5 ± 7.4	74.5 ± 7.8
	(56 - 94)	(55 - 105)
Gender, N (%) female	85 (45.2)	3289 (59.3)
Intraocular pressure (mmHg)	18.2 ± 6.2	15.2 ± 3.5
	(6.0 – 54.6)	(5.0 – 58.5)
Intraocular pressure treatment, N (%)	37 (19.7)	93 (1.7)

RS = Rotterdam Study.

Genome-wide association studies in Asians confirm the involvement of ATOH7 and TGFBR3, and further identify CARD10 as a novel locus influencing optic disc area

ABSTRACT

Damage to the optic nerve (e.g., from glaucoma) has an adverse and often irreversible impact on vision. Earlier studies have suggested that the size of the optic nerve head could be governed by hereditary factors. We conducted a genome-wide association study on 4445 Singaporean individuals (N = 2132 of Indian, and N = 2313 of Malay ancestry, respectively), with replication in Rotterdam, the Netherlands (N = 9326 individuals of Caucasian ancestry) using the most widely reported parameter for optic disc traits, the optic disc area. We identified a novel locus on chromosome 22q13.1, *CARD10*, which strongly associates with optic disc area in both Singaporean cohorts as well as in the Rotterdam Study (rs9607469, per-allele change in optic disc area = 0.051 mm^2; *P*meta = $2.73x10^{-12}$) and confirmed the association between *CDC7* / *TGFBR3* (lead SNP rs1192415, *P*meta = $7.57x10^{-17}$) and *ATOH7* (lead SNP rs7916697, *P*meta = $2.00x10^{-15}$) and optic disc area in Asians. This is the first Asian-based genome-wide association study on optic disc area, identifying a novel locus for optic disc area, but also confirming results found in Caucasian persons suggesting that there are general genetic determinants applicable to the size of optic disc across different ethnicities.

INTRODUCTION

The optic disc or nerve head is the location where ganglion cell axons exit the eye to form the optic nerve. The axon-less central depression within the disc is called the optic cup. Measurement of optic disc parameters is important in the evaluation of glaucoma, the leading cause of irreversible blindness worldwide.[1] Population-based eye studies such as the Blue Mountains Eye Study and the Reykjavik Eye Study have indicated that larger optic disc area is associated with glaucoma, suggesting that optic disc area may be related to susceptibility to glaucoma.[2,3] Studies in both Caucasian and Asian populations have also found larger optic disc area measures associated with myopic eyes and smaller areas with hyperopic eyes.[4,5]

The heritability of the optic disc area and another correlated measurement, vertical cup-disc ratio (VCDR) has been estimated to be around 52-59% and 48-80%, respectively.[6-9] A recent genome-wide association study (GWAS) using data from over 11000 individuals of European descent identified three loci for optic disc area and another six for VCDR.[10] The genes identified for optic disc area were *CDC7*/*TGFBR3* on chromosome 1p22, *ATOH7* on chromosome 10q21.3-22.1, and *SALL1* on chromosome 16q12. The genes for VCDR were *CDKN2B* on chromosome 9p21, *SIX1* on chromosome 14q22-23, *SCYL1* on chromosome 11q13, *CHEK2* on chromosome 22q12.1, *ATOH7* on chromosome 10q21.3-22.1, *DCLK1* on chromosome 13q13, and the borderline significant gene *BCAS3* on chromosome 17q23.[10] Of these genes, *ATOH7* appears to be involved in both optic disc area and VCDR. This locus was confirmed in two independent Australian twin cohorts for optic disc area.[11]

Optic disc area has been reported to vary between races,[12-16] and is likely to differ between Caucasians and Asians, due to differences in prevalence of glaucoma and myopia,[17] and other ethnic specific factors. The aim of this study is to identify genes for optic disc area that may be unique in Asian populations as well as genes shared with Caucasians. For this purpose, we conducted GWAS including data from two population-based cohorts from Singapore, comprising individuals of Indian (SINDI) and Malay ethnicity (SiMES), respectively. To increase the likelihood of identifying true genetic determinants of optic disc area, we combined genome-wide genotyping data from these two cohorts in a formal meta-analysis. We then sought direct replication of the top single nucleotide polymorphism (SNP) markers exceeding the formal threshold of genome-wide significance ($P < 5.0 \times 10^{-8}$) by looking up directly genotyped SNPs in three independent cohorts of Caucasian ancestry from the Rotterdam Study (RS), the Netherlands.

MATERIALS AND METHODS

Study population

Asian

Both the Singapore Malay Eye Study (SiMES) and Singapore Indian Chinese Cohort (SICC) Eye Study adhered to the Declaration of Helsinki. Ethics approval was obtained from the Singapore Eye Research Institute (SERI) Institutional Review Board (IRB). All participants were given a choice to provide written, informed consent in either Tamil, Mandarin, Malay or English using bilingual interviewers. Both versions of study information sheet and informed consent form were approved by the SERI IRB before the study commenced.

SiMES is a population-based, cross-sectional study of 3280 Malay adults aged from 40 to 79 years. Details of the SiMES design, sampling plan, and methods have been reported elsewhere.[18] In brief, an age-stratified random sampling of all Malay adults, aged 40 to 80 years, residing in 15 residential districts in the southwestern part of Singapore was drawn from the computer-generated random list of 16069 Malay names provided by the Ministry of Home Affairs. A total of 1400 names from each decade of age (40-49, 50-59, 60-69, and 70-79 years), thus 5600 names, were selected. Of these, 4168 individuals (74.4%) were determined to be eligible to participate. A person was considered ineligible if he or she had moved from the residential address, had not lived there in the past 6 months, was deceased, or was terminally ill. Of the 4168 eligible individuals, 3280 participants (78.7%) took part in the study. The study was conducted from August 2004 to June 2006.

The SICC is designed to complement the SiMES in ethnic Indians and ethnic Chinese residents of Singapore. This study was further divided into the Singapore Indian Eye Study (SINDI) and Singapore Chinese Eye Study (SCES). Further information about the SICC has been published elsewhere.[19] Similar to SiMES, the SINDI study is a population-based, cross-sectional epidemiological study, but of ethnic Indian adults aged between 40 and 80+ years residing in Singapore. As with SiMES, the Ministry of Home Affairs provided an initial computer-generated list of 12000 ethnic Indian names derived from a simple random sampling of all ethnic Indian adults aged 40-80+ years of age residing in 15 residential districts in South-Western Singapore. From this list, a final sampling frame of 6350 ethnic Indian residents were derived using an age-stratified random sampling strategy similar to SiMES. SINDI was conducted from March 2007 to May 2007 and recruited 3400 (75% response rate) participants.

Caucasian

The Rotterdam Study I (RS-I) is a prospective population-based cohort study of 7983 residents aged 55 years and older living in Ommoord, a suburb of Rotterdam, the Netherlands.[20] 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 3011 residents aged 55 years and older and 3392 residents aged 45 years and older respectively. The rationale and study design are similar to those of the RS-I.[20] 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.

Measurements of Optic disc area

Asian

Measurement of optic disc area in SINDI and SiMES was performed using Heidelberg Retina Tomography 2 (HRT 2), as previously described.[21,22]

Caucasian

For the Rotterdam cohorts, ImageNet, which was used in RS-I and RS-II, takes simultaneous stereoscopic images of the optic disc at a fixed angle of 20 degree, using a simultaneous stereoscopic fundus camera (Topcon TRC-SS2; Tokyo Optical Co., Tokyo, Japan). Images were analyzed 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.[23]

HRT 2, used in SiMES, SINDI and RS-III uses a focused 670-nm diode laser light beam to acquire scans of the optic nerve head region, using the confocal principle. 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 µm below the peripapillary retinal surface in the region of the papillomacular bundle.

Imaging was performed after entering the participant’s keratometry data into the software and after adjusting the settings in accordance with the refractive error. In RS-III all HRT 2 data was 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 µm were excluded.[10]

Genotyping and data quality control

Asian

Study participants were genotyped using the Illumina Human610-Quad BeadChips, which assays 620901 SNPs across the genome, according to manufacturer’s protocols. Quality control (QC) criteria included the following: SNPs that had missingness > 5%, gross departure from Hardy-Weinberg equilibrium (HWE; $P < 10^{-6}$) or were monomorphic were excluded from subsequent analysis. Per-sample QC was then performed. Samples with an overall call

rate <95% were excluded from analysis. Samples were subjected to biological relationship verification by using the principle of variability in allele sharing according to the degree of relationship. Identity-by-state (IBS) information was derived using PLINK.[24] Those individuals who showed evidence of cryptic relatedness (possible either due to duplicated or biologically related samples) were removed before principal component (PC) analysis was performed. PC analysis was undertaken using EIGENSTRAT[25] to account for spurious associations resulting from ancestral differences of individual SNPs. PCs showing significant effect on univariate analysis (PC1, PC2, and PC3 for SINDI and PCs 1 and 2 for SiMES) were used to correct for any underlying population substructure within a linear regression framework. Lastly, samples showing gender discrepancies between the clinical gender and genetically inferred gender were removed.

In SiMES, 170 individuals showed evidence of admixture and were consequently excluded. This confirmed that participants were drawn from the same, single population. Biological relationship verification revealed a total of 279 samples with cryptic relatedness and 37 samples with impossible biological sharing or heterogeneity, probably because of contamination or high missingness. In addition, 44 individuals were removed due to gender discrepancies. Overall, 530 samples were excluded leaving a total of 2542 individuals for statistical analysis, of which 2313 had complete data for optic disc measurements, age, gender, and PCs 1 and 2. Likewise in SINDI, a total of 415 samples were removed from future analysis: 39 for population structure, 16 for gender discrepancies, 326 samples for evidence of cryptic relatedness, and 34 samples with impossible biological sharing or heterogeneity. This left a total of 2538 individuals for statistical analysis, of which a total of 2132 individuals had complete data for optic disc measurements, age, gender, and PCs 1 to 3.

After sample removal, SNP checks were performed only on the autosomes, yielding 600450 SNPs, of which 20 were first removed in both studies, as they could not be synchronized to the forward strand. In SiMES, SNPs were excluded from the analysis when (i) they were not called in at least 95% of the individuals (*N*=26343); (ii) they were monomorphic or had a MAF<5% (*N*=13230); or (iii) their genotype frequencies deviated from HWE ($P < 10^{-6}$) (*N*=3053). This left a total of 557823 SNPs for association analysis. In SINDI, 26602 SNPs were removed due to high rates of missingness (>5%) (i); as well as 11771 SNPs that were either monomorphic or had MAF<5% (ii); and 2956 SNPs for gross departures from HWE ($P < 10^{-6}$) (iii). Hence we were left with a total of 559118 SNPs for association analysis in SINDI. For the meta-analysis of SiMES and SINDI, we analyzed 551808 SNPs found in common in both cohorts after the application of stringent QC criteria.

In both cohorts, genotyping clusters were directly visualized for SNPs showing suggestive evidence of association with optic disc area at $P < 1.00 \times 10^{-5}$, and confirmed to be good before inclusion for statistical analysis.

Caucasian

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 are described elsewhere.[10] A total of 9326 individuals passed QC and ancestry checks and were available for 'look-up' replication of significant results from SINDI and SiMES.

Statistical Analysis

The PLINK software (version 1.06)[24] was used for primary association testing, as well as modeling within a linear regression framework. Individual genotypes were coded according to the number of copies of the variant allele present: 0 for the wild-type genotype, 1 for heterozygotes, and 2 for homozygote variants. A trend test incorporated within a linear regression model was used for primary association testing between genotypes and optic disc area as a quantitative trait, adjusting for age, gender, and genetic ancestry (reflected by principal components).

Manhattan (-10log p-plots) and linkage disequilibrium (LD) plots were created with the use of Haploview (version 3.2).[26] Quantile-quantile and regional association plots were created using the software R (www.r-project.org).[27]

Meta-analysis of results across cohorts was performed using the inverse variance method, as previously described for quantitative traits.[28] This method weighs each study according to effective sample size and cohort-specific MAF of the associated variants. To avoid an otherwise unacceptable number of false positive signals as an artifact of multiple testing, the formal threshold for genome-wide significance, $P < 5.00 \times 10^{-8}$, was considered to be statistically significant.

RESULTS

Many of the optic disc parameters (e.g., VCDR, optic cup area and neuroretinal rim area) are inter-correlated traits that are derived from the size of the optic disc. In this study we therefore focused on the key anatomical parameter most widely reported: overall optic disc area. Summary tables for SINDI, SiMES, and the Rotterdam Study cohorts are presented in Table 1. The Singapore GWAS cohorts - SINDI and SiMES - included 2132 and 2313 participants passing quality control (QC) checks and having complete optic disc area and demographic data (age and gender), respectively. The SINDI GWAS included a total of 559118 autosomal SNPs which passed QC which comprises per-SNP call rate of ≥ 95%, per-sample call-rate of ≥ 95%, minor allele frequency (MAF) ≥ 1%, and Hardy-Weinberg $P \geq 10^{-6}$. Similarly, the SiMES GWAS had a total of 557823 SNPs passing QC (see Materials and Methods section for detailed QC exclusions).

Quantile-quantile (Q-Q) plots of *P*-value distributions specific to SINDI and SiMES are shown in Figure S1A and B. Using inverse variance weights, we combined the cohort-

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

	SINDI	SiMES	RS-I	RS-II	RS-III
Total sample size	2132	2313	5312	2048	1966
Age (mean±SD),	57.5±9.7	58.3±10.8	68.0±8.4	64.3±7.8	55.6±5.5
range	(43–84)	(40–80)	(55–99)	(55–98)	(45–89)
Gender (% female)	48.9	50.6	58.3	54.2	56.1
Disc area	1.97±0.45	2.15±0.49	2.42±0.48	2.32±0.48	1.92±0.45
(mean±SD), range	(0.64–4.71)	(1.00–5.00)	(0.58–5.44)	(1.06–6.20)	(0.70–7.20)

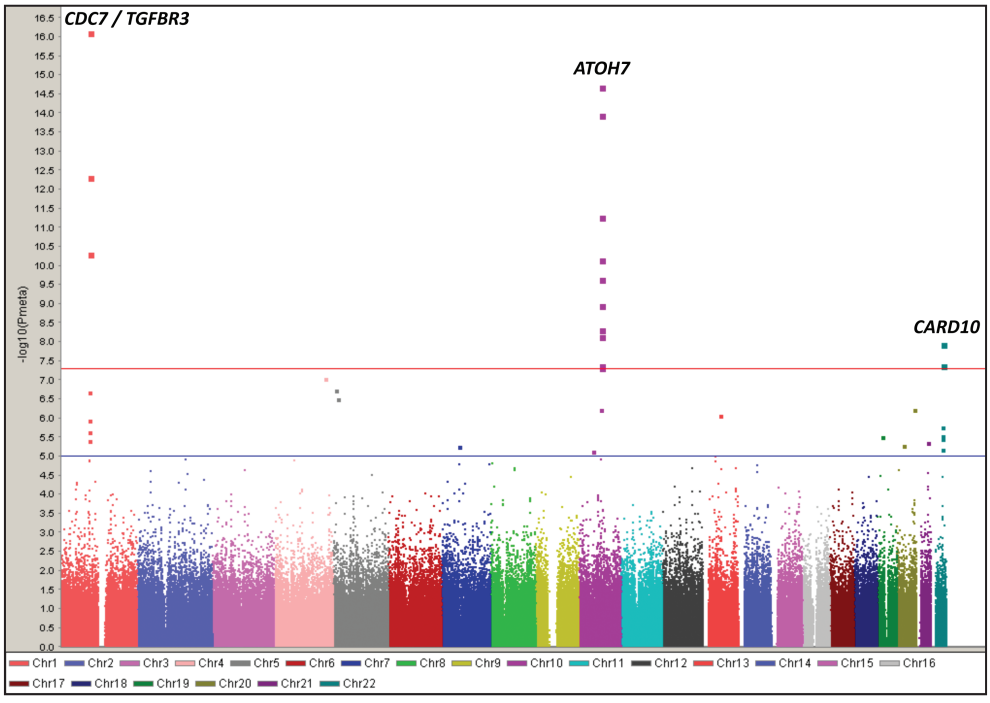
Age is in years; Disc area is in mm^2; SINDI: Singapore Indian Eye Study; SiMES: Singapore Malay Eye Study; RS-I: Rotterdam Study I; RS-II: Rotterdam Study II; RS-III: Rotterdam Study III.

specific results under an additive genetic model (assuming a trend per-copy of the variant allele) from both cohorts in fixed-effects meta-analysis. The Q-Q plots for the meta-analysis showed no deviation of the observed *P*-values against their expected distribution, except at the extreme tail (Figure S1C). This was observed against minimal overall inflation (Genomic inflation factor; λgc = 1.008), suggesting that these deviations at the tail end of the distribution could reflect true associations with optic disc area. Removal of SNPs at *CDC7* / *TGFBR3* and *ATOH7*, two loci previously described to be strongly associated with optic disc area in Caucasians,[10] still resulted in a significant excess of small *P*-values at the extreme tail of the distribution (Figure S1C).

Single-locus analysis revealed three genomic regions to be independently associated with optic disc area at $P < 5.00 \times 10^{-8}$ (Figure 1). Two of them (Chromosome 1p22 near *TGFBR3* and Chromosome 10q21.3-22.1 near *ATOH7*) unequivocally confirmed previously reported findings in Caucasian populations with multiple SNPs showing $P < 1.00 \times 10^{-10}$ in the combined SINDI and SiMES meta-analysis (Figure 1, Table S1). The third locus is novel and maps to the gene encoding for Caspase recruitment domain-containing protein 10 (*CARD10*) on Chromosome 22q13.1 where the association signals of two SNPs (rs6000762 and rs9607469) were observed to exceed genome-wide significance (Figure 1 and Table 2). Cohort-specific signals in SINDI and SiMES were consistent with a per-allele change of ~0.06 mm^2 for the lead SNPs on optic disc area (Table 2).

We then proceeded to seek confirmation for the two lead SNPs, rs6000762 and rs9607469, together with three other corroborating SNPs from the *CARD10* locus in three independent population-based studies comprising a total of 9326 Caucasian individuals recruited in Rotterdam, the Netherlands.[10] All five SNPs showed a significant association with optic disc area ($P < 0.0005$) as well as a consistent direction of effect in Caucasians. The per-allele effect size was somewhat smaller in the overall Rotterdam cohort (~0.034 and 0.045 mm^2 for the lead SNPs respectively) compared to SINDI and SiMES. Meta-analysis of all samples (N = 13771) for the five SNPs each revealed strong association with optic disc area (Table 2), where the most significantly associated SNP (rs9607469) accounted for ~0.8 percent of the phenotypic variance (a change of 0.051 mm^2 per copy of the minor allele, $P = 2.73 \times 10^{-12}$). A regional association plot revealed that all five SNPs were bound within a tight

Figure 1. Manhattan plot for the meta-analysis of both SINDI and SiMES cohorts.



The upper line denotes P = 5.00×10^{-8}, the formal threshold for genome-wide significance. The lower line shows P = 1.00×10^{-5}, an indication of suggestive evidence of association.

linkage disequilibrium block (D' > 0.8, Figure S2) within *CARD10*, and that no significant evidence of association was observed outside of this block. Conditional regression analysis revealed no further independent signal of association above and beyond that of rs9607469 (data not shown). On further analysis, we did not observe evidence of association with optic disc area in either SINDI or SiMES at the previously reported chromosome 16q12.1 locus near *SALL1* (Table S2). We did not observe significant evidence for gene-gene effects between *ATOH7* and *CARD10*, or between *TGFBR3* and *CARD10* which influenced optic nerve size in the Singaporean cohorts (*P* > 0.5 for each comparison).

No association was observed between all five *CARD10* SNPs and VCDR after adjusting for optic disc area (Table S3). We also did not observe association between *ATOH7* and *TGFBR3* SNPs and VCDR in both Singaporean cohorts (*P* > 0.1 for all SNPs, Table S4), despite being empowered to confirm strong evidence of association at chromosome 14p22-23 (near *SIX1*; Table S5).[10]

Table 2. Association results between the top 5 *CARD10* SNPs and optic disc area in each individual cohort and meta-analysis results of all cohorts studied

SNP	BP	Effect allele	Cohort	EAF	β (mm^2)	SE (mm^2)	*P*	*P*het
rs6000762	36248418	C	SINDI (N = 2132)	0.29	0.057	0.014	$4.06x10^{-5}$	
			SiMES (N = 2313)	0.3	0.063	0.016	$7.47x10^{-5}$	
			All Singapore (N = 4445)		**0.059**	**0.01**	**$1.36x10^{-8}$**	0.778
			RS-I (N = 5312)		0.013	0.012	0.27	
			RS-II (N = 2048)		0.081	0.019	$2.32x10^{-5}$	
			RS-III (N = 1966)		0.042	0.018	0.021	
			All Rotterdam (N = 9326)	**0.2**	**0.034**	**0.009**	**$9.09x10^{-5}$**	0.009
			All data (N = 13771)		**0.045**	**0.0067**	**$2.50x10^{-11}$**	0.013
rs9607469	36249213	A	SINDI (N = 2132)	0.26	0.06	0.014	$3.60x10^{-5}$	
			SiMES (N = 2313)	0.26	0.06	0.017	$3.20x10^{-4}$	
			All Singapore (N = 4445)		**0.06**	**0.011**	**$4.06x10^{-8}$**	1.000
			RS-I (N = 5312)		0.033	0.013	0.012	
			RS-II (N = 2048)		0.087	0.022	$6.81x10^{-5}$	
			RS-III (N = 1966)		0.033	0.021	0.11	
			All Rotterdam (N = 9326)	**0.14**	**0.045**	**0.01**	**$9.30x10^{-6}$**	0.090
			All data (N = 13771)		**0.051**	**0.0074**	**$2.73x10^{-12}$**	0.197
rs6000764	36250043	C	SINDI (N = 2132)	0.29	0.055	0.014	$6.88x10^{-5}$	
			SiMES (N = 2313)	0.29	0.048	0.016	0.003	
			All Singapore (N = 4445)		**0.052**	**0.011**	**$7.15x10^{-7}$**	0.742
			RS-I (N = 5312)		0.012	0.011	0.276	
			RS-II (N = 2048)		0.063	0.018	$5.96x10^{-4}$	
			RS-III (N = 1966)		0.051	0.018	0.0045	
			All Rotterdam (N = 9326)	**0.22**	**0.032**	**0.009**	**$1.40x10^{-4}$**	0.025
			All data (N = 13771)		**0.04**	**0.0066**	**$1.20x10^{-9}$**	0.043
rs6000766	36251975	G	SINDI (N = 2132)	0.31	0.053	0.014	0.00011	
			SiMES (N = 2313)	0.29	0.051	0.016	0.0016	
			All Singapore (N = 4445)		**0.052**	**0.01**	**$5.79x10^{-7}$**	0.925
			RS-I (N = 5312)		0.012	0.011	0.29	
			RS-II (N = 2048)		0.063	0.018	$6.27x10^{-4}$	
			RS-III (N = 1966)		0.051	0.018	0.0045	
			All Rotterdam (N = 9326)	**0.22**	**0.032**	**0.009**	**$1.60x10^{-4}$**	0.025
			All data (N = 13771)		**0.04**	**0.0066**	**$1.10x10^{-9}$**	0.044
rs8139526	36252360	C	SINDI (N = 2132)	0.31	0.051	0.014	$2.30x10^{-4}$	
			SiMES (N = 2313)	0.34	0.047	0.015	0.0018	
			All Singapore (N = 4445)		**0.049**	**0.01**	**$1.38x10^{-6}$**	0.845
			RS-I (N = 5312)		0.008	0.011	0.468	
			RS-II (N = 2048)		0.063	0.018	$5.2x10^{-4}$	
			RS-III (N = 1966)		0.049	0.017	0.0051	
			All Rotterdam (N = 9326)	**0.23**	**0.029**	**0.008**	**$3.90x10^{-4}$**	0.014
			All data (N = 13771)		**0.037**	**0.0064**	**$7.13x10^{-9}$**	0.027

SNP: Single nucleotide polymorphism; BP: basepair position; EAF: Effect allele frequency; β: per-allele change in optic disc area; SE: Standard error for ascertainment of β; SINDI: Singapore Indian Eye Study; SiMES: Singapore Malay Eye Study; RS-I: Rotterdam Study I; RS-II: Rotterdam Study II; RS-III: Rotterdam Study III; Phet: P-value for heterogeneity between studies.

DISCUSSION

In this GWAS of two cohorts of Asian ethnicity from Singapore (comprising 2132 Indians and

2313 Malays, respectively), we report strong evidence of association at *CARD10*, a locus not previously implicated in optic disc area. We replicated this finding in the combined Rotterdam Study cohorts which included Caucasian persons. We also confirmed a strong association with earlier reported genetic variations at two loci (*CDC* / *TGFBR3* on chromosome 1p22, and *ATOH7* on chromosome 10q21.3-22.1).[10,11]

Detailed assessment of the association results at *CARD10* revealed some degree of heterogeneity between RS-I, RS-II, and RS-III with four of the five associated SNPs (rs6000762, rs6000764, rs6000766, and rs8139526; Table 2). However, the lead SNP rs9607469 did not show significant heterogeneity either within the three Rotterdam cohorts ($P = 0.09$), or when all five cohorts (3 from Rotterdam and 2 from Singapore) were meta-analysed ($P = 0.197$). As rs9607469 showed more consistent, non-heterogenous association across all 5 cohorts, and the strongest evidence of association overall (Pmeta = 2.73×10^{-12}), it is likely to be a closer correlate of the yet undetected functional variant(s) compared to the other four SNPs. The weaker and more inconsistent results with the other four SNPs corroborating rs9607469 (observed particularly within the Rotterdam cohorts) probably reflect the incomplete linkage disequilibrium between them and rs9607469 (Table S6). In light of this, genotyping of all five of the *CARD10*-associated SNPs would be necessary in further replication attempts in order to best capture the information of optic disc area association at this locus.

As a carrier of the CARD domain, *CARD10* is intimately involved in the regulation of caspase activation and apoptosis.[29] In addition to that, *CARD10* also belongs to the family of membrane-associated guanylate kinase proteins whose role is to assemble membrane-associated signaling complexes. *CARD10* signals the activation of NF-kappaB, a well-characterized transcription factor with multiple physiological and pathological functions via B-cell lymphoma/leukemia 10 (BCL10), another member in the apoptosis and NF-kappaB signaling pathway.[30-35] This pathway has been implicated in other major neurodegenerative disorders such as Alzheimer's disease. Studies in bcl10-deficient mice have shown that, while bcl10 is dispensable for the execution of apoptosis, it is important for neural tube closure and specifically required for lymphocyte proliferation dependent on antigen receptor-mediated activation of NF-kappaB.[36] It has been postulated that bcl10 - inhibitor of NF-kappaB kinase a and b (IKKa/IKKb) - NF-kappaB pathway plays a role in normal central nervous system development, possibly via positive regulation of neuronal survival.[36] Given the interaction with BCL10 it is thus possible for genetic polymorphisms within *CARD10* to influence optic disc area via a role in the cell survival process.

Regulation of cell death via apoptosis is critical for neuronal cells. Mutations in another apoptosis regulating gene (Optic atrophy 1; *OPA1)* are associated with autosomal dominant optic atrophy (DOA), the most common inherited optic neuropathy, in which retinal ganglion cells are lost and visual acuity is impaired from an early age.[37,38] *OPA1* is involved in mitochondrial homeostasis, associated with regulation of mitochondrial fusion and sequestration of cytochrome c in the mitochondria.[39-42] *OPA1* and Mitofusin 1 are proposed to have a protective role within the cell,[43] acting as anti-apoptotic GTPases, that is, protecting

the cell from spontaneous apoptosis and the detrimental consequences of apoptotic stimuli. Interestingly, a small study on 28 DOA patients with *OPA1* mutations showed a significantly smaller optic disc area compared with controls.[44] This suggests that mutations in the apoptosis regulating gene *OPA1* may also determine the previously unrecognized feature of a smaller optic disc area. Our findings on *CARD10* therefore add to this body of evidence that indicate a relationship between apoptosis regulating genes, ganglion cell death/survival and optic disc area.

The fact that *CARD10* controls optic disc area possibly through ganglion axonal survival via the activation of the NF-kappaB signaling pathway may have implications for neuro-protection research in glaucoma and other neurodegenerative disease. Much attention has now been focused on neuroprotection as a strategy in therapies for glaucomatous optic neuropathy as a means of preserving retinal ganglion cells and their axonal projections. Although studies in glaucoma have shown reduction of intraocular pressure to be an effective modality in the treatment of glaucomatous optic neuropathy, not all patients respond to or can achieve meaningful intraocular pressure reductions, and such patients may benefit from neuroprotective therapies. The influence of NF-kappaB on cell survival could be protective or destructive, depending on types, developmental stages of cells, and pathological conditions.[45-47] Many compounds with neuroprotective actions are strongly associated with the inhibition of NF-kappaB, leading to speculation that blocking the pathological activation of NF-kappaB could offer neuroprotective effects in certain neurodegenerative conditions.[45] With the linkage of optic nerve area to NF-kappaB pathway through *CARD10*, we speculate that compounds that can modulate the activity of *CARD10* could potentially offer neuroprotection in glaumatous optic neuropathy.

Primary open-angle glaucoma (POAG) represents a heterogeneous group of eye disorders for which several endophenotypes have been identified including optic disc area.[48-50] A GWAS in 1263 POAG cases and 34877 controls has recently been performed in an Icelandic cohort, identifying polymorphisms near *CAV1* and *CAV2* as putative POAG susceptibility genes.[51] Surprisingly, *ATOH7*, which has been the strongest and most robustly associated gene with optic disc area, was neither reported to be significantly associated ($P < 10^{-7}$) with POAG in the GWAS, nor within the top 32 SNPs exceeding $P < 10^{-4}$ for POAG. These findings, as well as the statistically heterogeneous observations between the Icelandic discovery GWAS and the other European replication samples with regards to the most strongly associated SNPs at *CAV1-CAV2* further testify to the heterogeneity of POAG as a broad diagnosis group. It has been proposed that dissecting the genetic architecture of such a heterogeneous disorder could best be achieved by considering individual endophenotypes underlying the disease.[50,52] Quantitative endophenotypes allow individuals to be ranked along the continuum of risk, thus providing substantially more information than dichotomous measures of affection status. It is thus unsurprising that there has been very limited success to date in the identification of discrete POAG genes by using disease diagnosis as a dichotomous measure.[51,53,54]

In light of this, marker-sets compiled from univariate analysis of endophenotypes

could be more informative. Thus, future work should entail consolidation of most consistently associated SNPs in each of the quantitative traits to be tested in large glaucoma cohorts. An in depth analysis of genes such as deep sequencing, may also be required if POAG involves functional variants with a wide spectrum of allele frequencies, ranging from rare to common.

It is remarkable that despite the major anatomical difference between the eye of Caucasian and Asian populations and the differences in genetic make-up we have identified three key genes that play a role in individuals from these different ethnicities. This does not only support the conserved biological relevance of these genes but also opens the opportunities for joint discovery studies across these populations. However, one potential limitation in this study is that identical methods for disc assessment were not used in Rotterdam (ImageNet for RS-I and RS-II; Heidelberg Retina Tomography for RS-III) and Singapore (Heidelberg Retina Tomography for both SiMES and SINDI), which is a potential limitation. However, as both methods of disc assessment were objective and used standardized protocols for all subjects in each study, the potential bias caused by this limitation is likely to be minimal. Furthermore, the similar results through the cohorts (Table 2) suggest that the potential difference between both assessment techniques is more likely to be a systematic difference,[Ramdas, et al.; Chapter 2.1] and thus may not have harmed the current findings.

In summary, in this GWAS on optic disc area conducted in Asians, we report a novel locus, *CARD10* on chromosome 22q13.1, and have added to the list of genes that control a key glaucoma endophenotype. We suggest it is opportune to investigate whether any of the recently identified quantitative trait loci relevant to glaucoma do indeed contribute to the development of glaucoma and whether they explain a significant proportion of individual differences in disease predisposition. If they do, these genes have the potential to contribute significantly to the development of a diagnostic and prognostic model for POAG, which can facilitate the early detection and treatment of at risk individuals to prevent irreversible optic nerve degeneration and eventually blindness.

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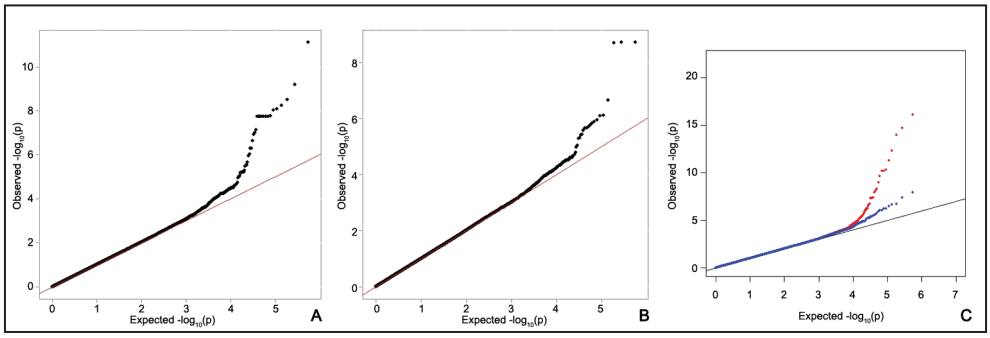
SUPPLEMENTARY APPENDIX

Figure S1. Quantile-quantile plot of SINDI (A), SiMES (B), and the meta-analysis with SiMES and SINDI for optic disc area.



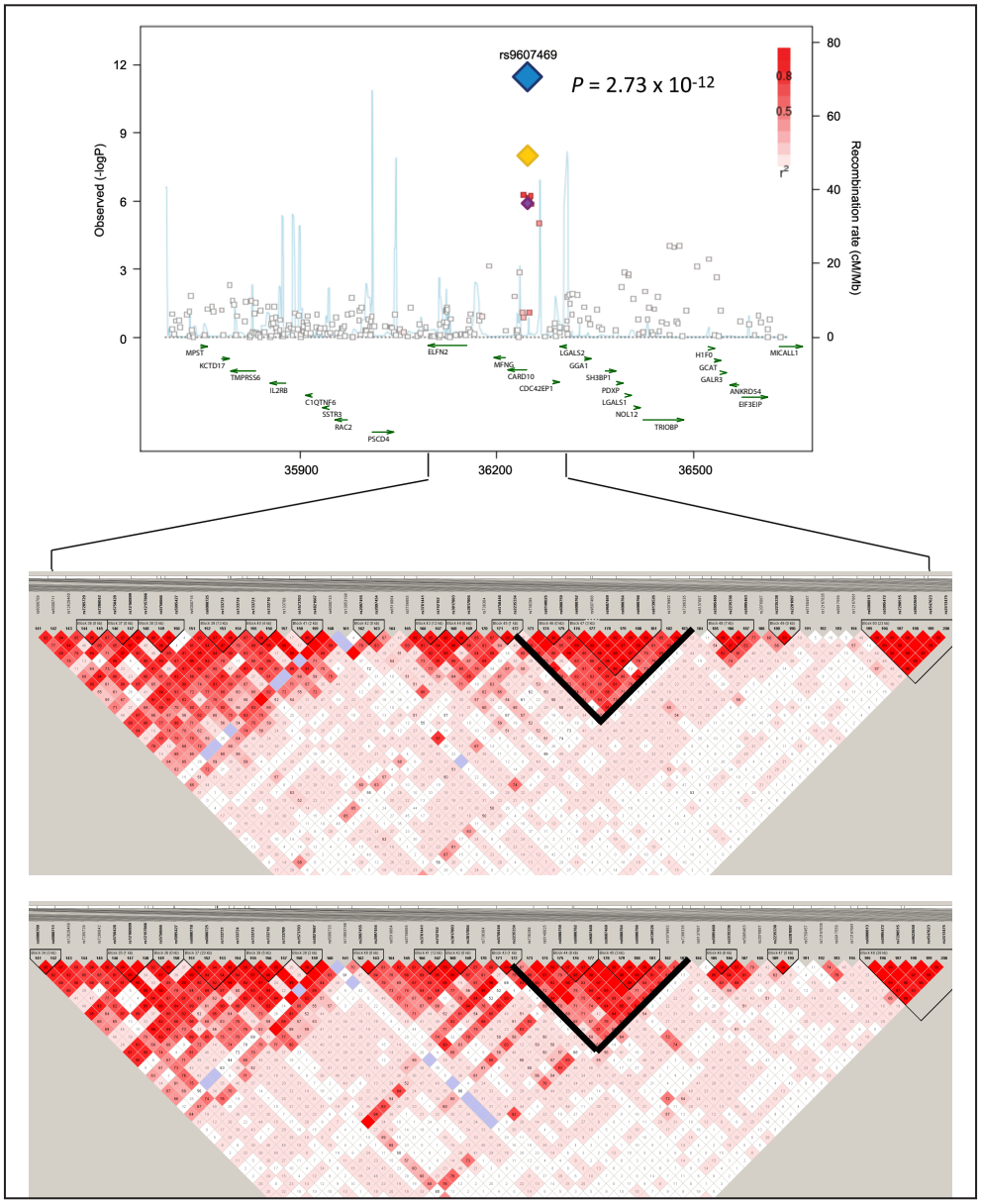
The upper dots represent the plot with all analyzed SNPs and the lower dots represent the plot after removal of genome-wide significant SNPs from *CDC7*/*TGFBR3* and *ATOH7* (C). The diagonal black line represents the null-hypothesis, and the formal threshold for genome-wide significance is reflected at P = 5.0×10^{-8}.

Table S1. Evidence of association at Chromosome 1p22 and Chromosome 10q21.3-22.1 with optic disc area in SINDI and SiMES

				SINDI (N = 2132)		SiMES (N = 2313)		Meta-analysis (N = 4445)	
SNP	Chromosome	Position	Effect allele	β (mm^2)	*P*	β (mm^2)	*P*	β (mm^2)	*P*
rs1192415*	1p22	91849685	G	0.097	7.69×10^{-12}	0.093	2.61×10^{-6}	0.0959	7.57×10^{-17}
rs1041159	1p22	91849111	C	0.08	5.73×10^{-9}	0.083	2.12×10^{-5}	0.0811	4.71×10^{-13}
rs1192404	1p22	91841555	G	0.077	1.16×10^{-7}	0.08	1.10×10^{-4}	0.0780	4.74×10^{-11}
rs3858145	10q21.3-q22.1	69681844	G	-0.078	8.95×10^{-9}	-0.096	2.25×10^{-7}	-0.0843	1.08×10^{-14}
rs7916697	10q21.3-q22.1	69661859	A	-0.092	6.21×10^{-10}	-0.097	8.04×10^{-7}	-0.0938	2.00×10^{-15}
rs12571093	10q21.3-q22.1	69689377	A	-0.068	6.30×10^{-6}	-0.13	1.15×10^{-6}	-0.0835	2.21×10^{-10}
rs2241970	10q21.3-q22.1	69701073	G	-0.072	2.17×10^{-6}	-0.13	1.47×10^{-6}	-0.0863	6.62×10^{-11}
rs17231602	10q21.3-q22.1	69682295	A	-0.061	8.18×10^{-5}	-0.13	1.76×10^{-6}	-0.0778	7.15×10^{-9}
rs4746752	10q21.3-q22.1	69717883	C	-0.069	5.19×10^{-7}	-0.082	2.02×10^{-6}	-0.0742	5.1×10^{-12}

β : per-allele change in optic disc area; * β = 0.064 mm^2 per-allele; *P* = 1.82×10^{-27} in Ramdas et al..[10]

Figure S2. Regional association and linkage disequilibrium plots of the region of interest on chromosome 22.



Top panel refers to the regional association plot at the chromosome 22 region surrounding *CARD10*, focusing on rs9607469. The large diamond refers to combined analysis of SINDI, SiMES, and Rotterdam cohorts. The middle diamond refers to the combined analysis of SINDI and SiMES only. The small diamond refers to the original hit in SINDI. The middle panel is an LD plot of the highlighted region in SINDI. The lower panel is an LD plot of the highlighted region in SiMES. The region of association is bounded in thick black lines in both LD plots. For both LD plots, the LD coefficient used here is D'. Dark squares indicate pairs in strong LD (D' > 0.8), Gray squares in moderate LD (D' between 0.4 - 0.8), and white squares in weak LD (D' < 0.4).

Table S2. Association analysis at the chromosome 16q12.1 locus near *SALL1* with optic disc area

				SINDI (N = 2132)		SiMES (N = 2313)	
SNP	Chromosome	Position	Effect allele	β (mm^2)	*P*	β (mm^2)	*P*
rs6499196	16q12.1	50008820	T	0.01035	0.4727	0.01387	0.3949
rs4784274	16q12.1	50011624	C	0.01085	0.513	0.005213	0.7612
rs17616757	16q12.1	50017614	A	0.06278	0.4522	0.2161	0.3749
rs11645416	16q12.1	50018168	C	-0.02369	0.3909	-0.0763	0.4365
rs2063099	16q12.1	50018372	A	0.004946	0.7479	0.007377	0.6905
rs2017684	16q12.1	50026393	C	0.001967	0.8892	-0.00194	0.9021
rs17532886	16q12.1	50026847	G	-0.04995	0.07155	-0.00824	0.9373
rs1345467	16q12.1	50039822	G	0.009489	0.5778	0.02071	0.3116

SNP = single nucleotide polymorphism; β : per-allele change in optic disc area.

Table S3. Association analysis at *CARD10* with vertical cup-disc ratio in SINDI and SiMES

			SINDI (N = 2132)		SiMES (N = 2313)	
SNP	Position	Effect allele	β	*P*	β	*P*
rs6000762	36248418	C	0.00496	0.25	-0.0012	0.76
rs9607469	36249213	A	0.00226	0.61	-0.0007	0.87
rs6000764	36250043	C	0.00528	0.22	-0.0012	0.76
rs6000766	36251975	G	0.00450	0.29	-0.0015	0.72
rs8139526	36252360	C	0.00296	0.48	-0.0019	0.61

SNP = single nucleotide polymorphism; β : per-allele change in vertical cup-disc ratio.

Table S4. Evidence of association at Chromosome 1p22 and Chromosome 10q with vertical cup-disc ratio in SINDI and SiMES

				SINDI (N = 2132)		SiMES (N = 2313)	
SNP	Chromosome	Position	Effect allele	β	*P*	β	*P*
rs1192415	1p22	91849685	G	0.0005705	0.90	-0.005801	0.25
rs1041159	1p22	91849111	C	-0.0002702	0.95	-0.004982	0.32
rs1192404	1p22	91841555	G	0.001524	0.73	-0.005173	0.33
rs3858145	10q21.3 - q22.1	69681844	G	-0.0001101	0.98	-0.001061	0.82
rs7916697	10q21.3 - q22.1	69661859	A	-0.006653	0.15	-0.004469	0.37
rs12571093	10q21.3 - q22.1	69689377	A	-0.00157	0.74	-0.001337	0.84
rs2241970	10q21.3 - q22.1	69701073	G	-0.002738	0.56	-0.001274	0.85
rs17231602	10q21.3 - q22.1	69682295	A	-0.002985	0.53	-0.003127	0.66
rs4746752	10q21.3 - q22.1	69717883	C	-0.001009	0.81	-0.004365	0.32

SNP = single nucleotide polymorphism; β : per-allele change in vertical cup-disc ratio.

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

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 11972 participants from 4 independent population-based studies in The Netherlands. We replicated our findings in 7482 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=1432 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.

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] This 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 and onset of 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 11972 participants from 4 independent population-based studies in The Netherlands, and we replicated our findings in 7482 participants from 4 additional independent cohorts of Caucasian ancestry. We investigated whether the IOP associated SNPs were also related to glaucoma in 1432 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.

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 11972 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),[24] 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 7983 residents 55 years of age and older living in Ommoord, a suburb of Rotterdam, The Netherlands.[25] 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 3011 new respondents in the same age range as RS-I.[25] 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 3932 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 3000 participants 18 years of age and older.[16,26] 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.[16,27] 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 strongest association in the discovery phase were carried forward and assessed for association with IOP in 2235 participants from the TwinsUK Study, 1807 from the Australian Twin Study, 1304 from the DCCT/EDIC Study, and 2136 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.[28] 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/).[29] 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).[29-31]

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.[32] The genome-wide threshold for statistical significance was set at a p-value of $5x10^{-8}$ to adjust for multiple testing.[33] 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.[34] 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 90^{th}, 50^{th} and 10^{th} percentile of the mean expression intensity to categorize our data into groups with high (>90^{th}), moderate (50^{th}-90^{th}), low (10^{th}-50^{th}) and very low (<10^{th}) 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.

RESULTS

Discovery studies

Genotypic and IOP data were available for 11972 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).[35] 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),[35] which is located 7.6 MB from *MYOC*.

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).[35] 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

Table 1. Characteristics of the discovery cohorts

Characteristic	RS-I	RS-II	RS-III	ERF
Participants with valid data (N)	5794	2102	2041	2035
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-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)

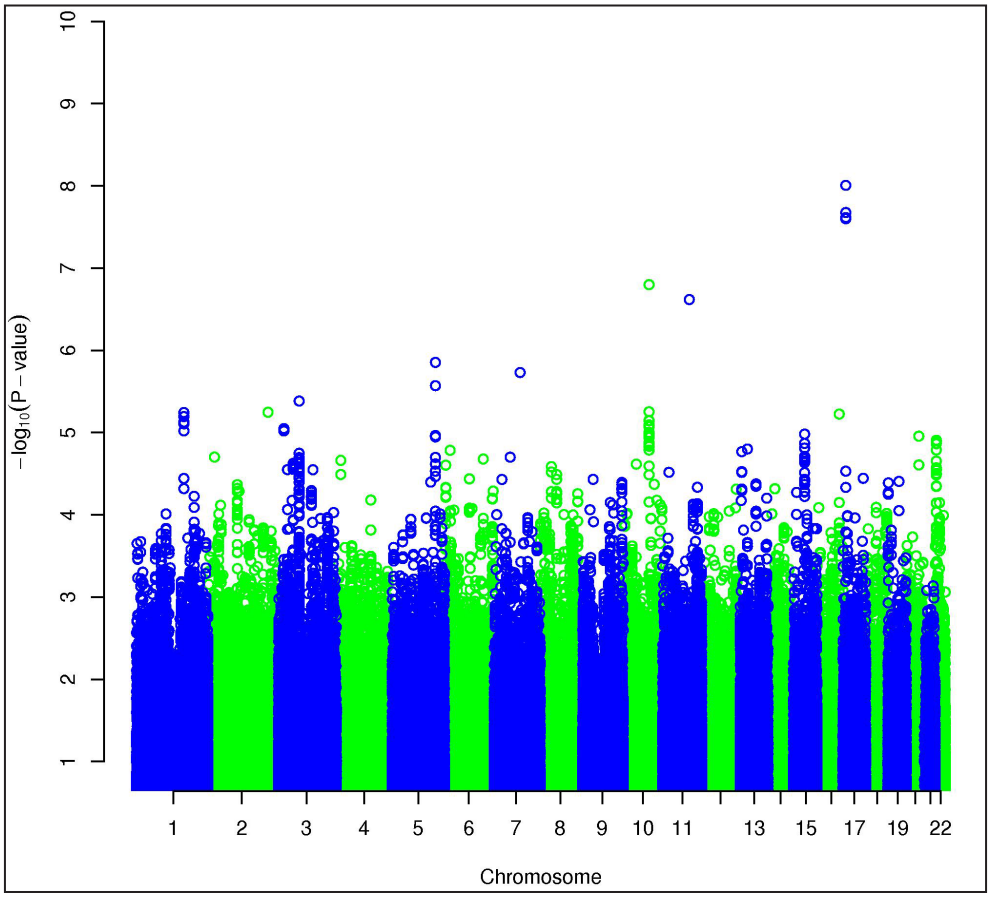
IOP = intraocular pressure; SD = standard deviation; RS= Rotterdam Study; ERF = Erasmus Rucphen Family study.

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	$9.8x10^{-9}$
rs7894966	10q23.2	88608604	G	0.04	*BMPR1A*	8	0.67	0.13	$1.6x10^{-7}$
rs216146	5q32	149426114	T	0.39	*CSF1R*	2	0.22	0.05	$1.4x10^{-6}$
rs2117760	3p13	70933151	A	0.32	*FOXP1*	1	0.22	0.05	$4.1x10^{-6}$
rs7555523	1q24.1	163985603	C	0.13	*TMCO1*	11	0.30	0.07	$5.7x10^{-6}$
rs1826598	16q23.1	76130456	A	0.11	*ADAMTS18, NUDT7*	1	0.32	0.07	$6.0x10^{-6}$
rs9841621	3p24.3	18384081	G	0.01	*SATB1*	5	-0.81	0.18	$8.9x10^{-6}$

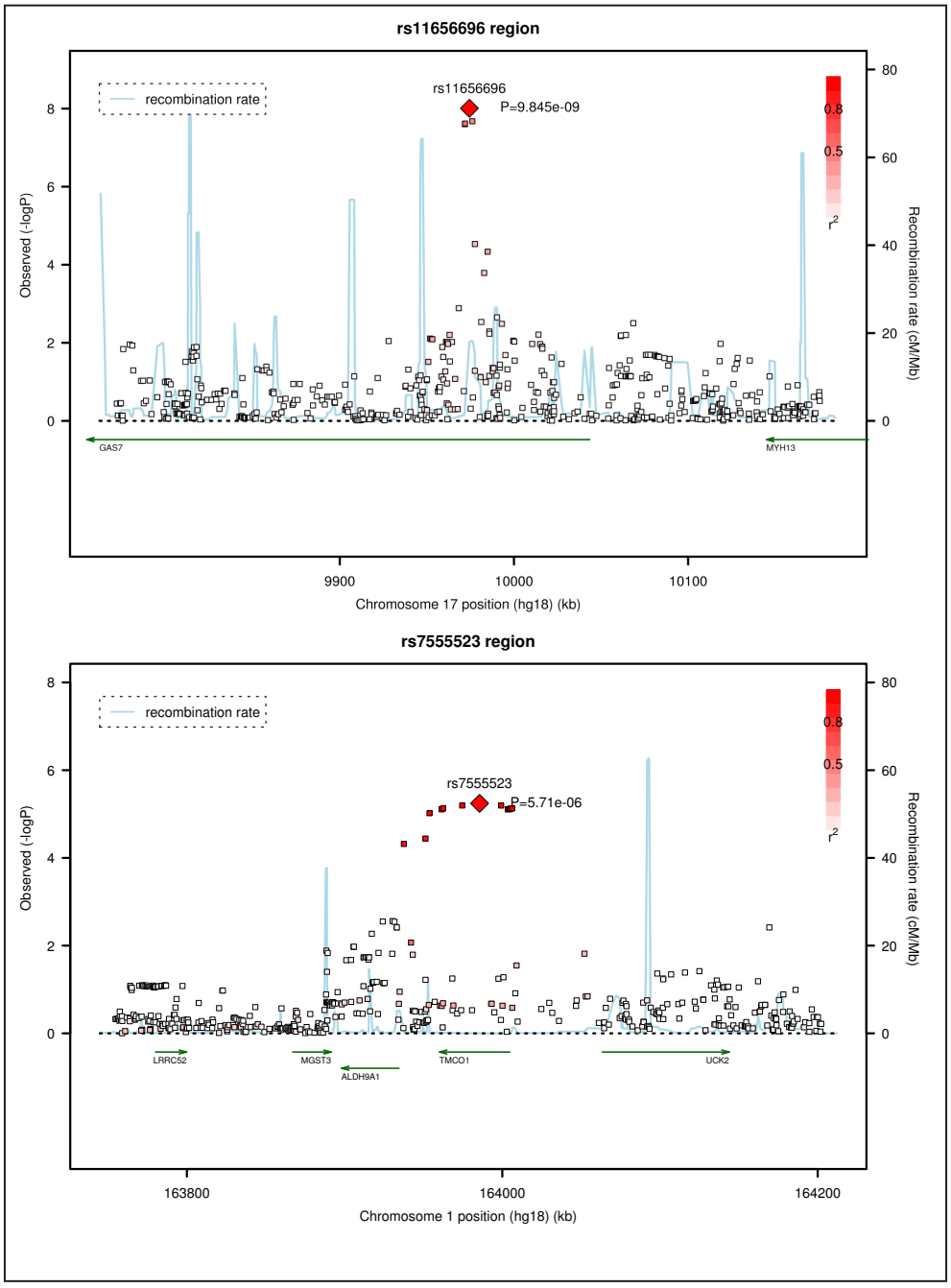
SNP = single nucleotide polymorphism; Chrom = chromosome; MA(F) = minor allele (frequency); SE = standard error; * = number of SNPs with $p<10^{-5}$ in the region; According NCBI build 37.1, rs11656696 is located at position 10033679 in the growth-arrest-specific gene *GAS7* while an earlier build allocated the SNP at 9974404 (http://www.ncbi.nlm.nih.gov).

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



Japan, only rs7081455 on chromosome 10 showed nominal evidence for association 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 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*.

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



Replication studies

Replication of the IOP association was done in 4 additional cohorts from the TwinsUK study (N=2235), the Australian Twin study (N=1807), the Diabetes Control and Complications Trial/ Epidemiology of Diabetes Interventions and Complications study (DCCT/EDIC; N=1304), and the Wellcome Trust Case-Control Consortium 2 / Blue Mountains Eye Study (WTCCC2/BMES; N=2136) (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												**Joint analysis of discovery and replication cohorts**		
		Twins-UK			Australian Twins			DCCT/EDIC			WTCCC2/BMES					
SNP	Chrom	Beta	SE	P-value	Beta	SE	P-value	Beta	SE	P-value	Beta	SE	P-value	**Beta**	**SE**	**P-value**
rs11656696	17p13.1	-0.32	0.11	$3.9x10^{-3}$	-0.11	0.10	$2.9x10^{-1}$	0.04	0.11	$6.8x10^{-1}$	-0.06	0.09	$4.9x10^{-1}$	**-0.19**	**0.03**	**$1.4x10^{-8}$**
rs7894966	10q23.2	-1.15	0.37	$1.7x10^{-3}$	-0.32	0.29	$2.6x10^{-1}$	-0.11	0.29	$6.9x10^{-1}$				**0.30**	**0.10**	**$3.6x10^{-3}$**
rs216146	5q32	0.00	0.11	$9.8x10^{-1}$	-0.08	0.11	$4.8x10^{-1}$	-0.08	0.11	$4.7x10^{-1}$	-0.08	0.09	$3.4x10^{-1}$	**0.09**	**0.03**	**$4.9x10^{-3}$**
rs2117760	3p13	0.12	0.11	$2.9x10^{-1}$	-0.03	0.11	$7.7x10^{-1}$	0.05	0.11	$6.5x10^{-1}$				**0.16**	**0.04**	**$2.8x10^{-5}$**
rs7555523	1q24.1	0.24	0.15	$9.6x10^{-2}$	0.23	0.16	$1.4x10^{-1}$	0.18	0.17	$2.9x10^{-1}$	0.30	0.13	$1.8x10^{-2}$	**0.28**	**0.05**	**$1.6x10^{-8}$**
rs1826598	16q23.1	0.15	0.15	$3.4x10^{-1}$	0.10	0.17	$5.6x10^{-1}$	0.20	0.18	$2.7x10^{-1}$	0.01	0.13	$9.3x10^{-1}$	**0.22**	**0.05**	**$2.0x10^{-5}$**
rs9841621	3p24.3	-0.42	0.36	$2.4x10^{-1}$	-0.34	0.33	$3.0x10^{-1}$	-0.31	0.40	$4.3x10^{-1}$	-0.15	0.31	$6.3x10^{-1}$	**-0.54**	**0.12**	**$1.4x10^{-5}$**

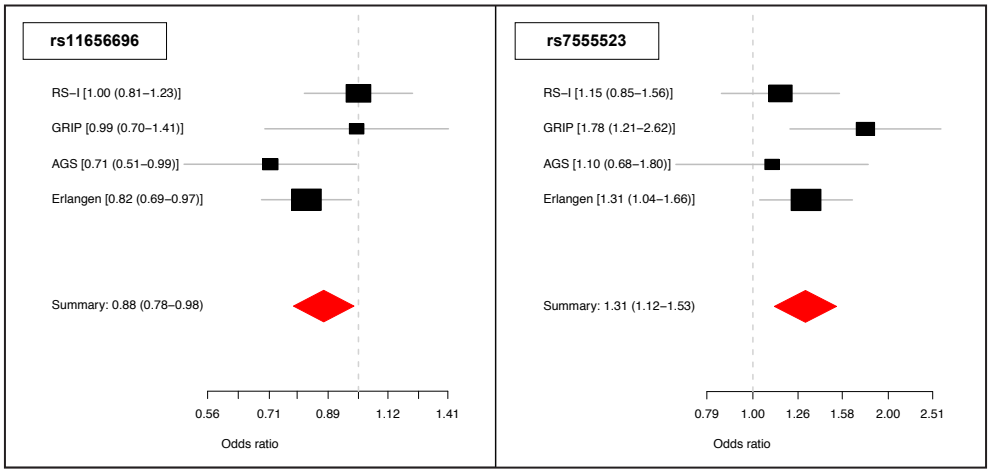
SNP = single nucleotide polymorphism; Chrom = Chromosome; SE = standard error; DCCT/EDIC = Diabetes Control and Complications Trial / Epidemiology of Diabetes Interventions and Complications study; WTCCC2/BMES = Wellcome Trust Case-Control Consortium 2 / Blue Mountains Eye Study

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.

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 (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.

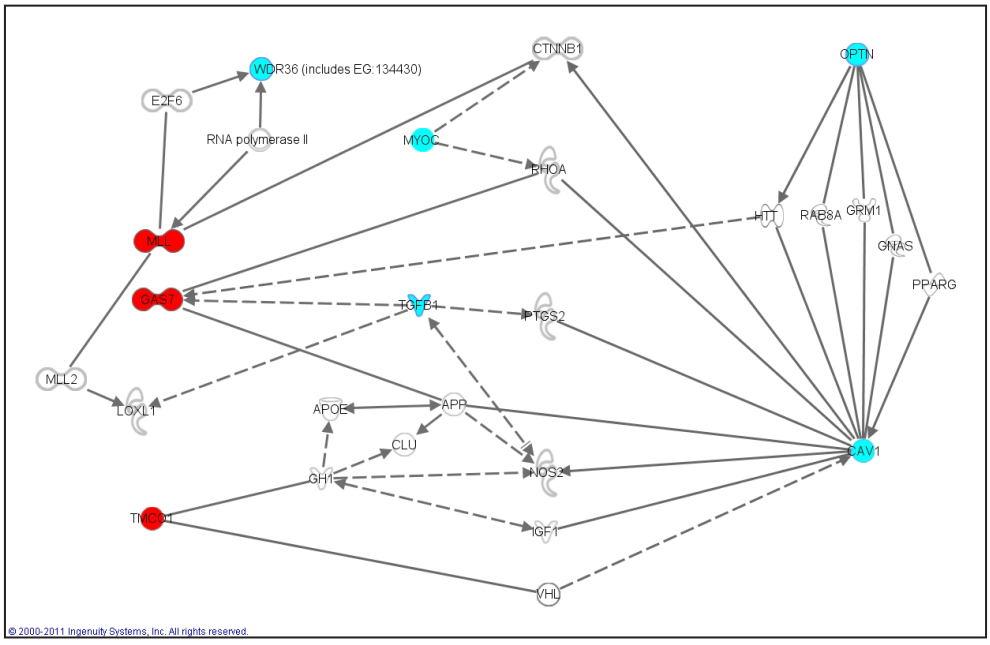
GAS7 is located in a chromosomal region previously identified by a linkage study of glaucoma.[36] We observed moderate to high expression of *GAS7* in the ciliary body (CB), the secretory neuroepithelium that produces the aqueous humor. Previously, Liton and colleagues already reported expression of *GAS7* in human trabecular meshwork (TM), which is the main tissue involved in aqueous humor outflow.[37] Together, the CB and TM largely control IOP. Significant downregulation of *GAS7* expression was observed in TM of glaucomatous eyes.[37] In absence of the (in vivo) typical mechanical forces on the TM, a similar effect was also observed in cultured TM cells.[37] 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.[38] 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 associate with actin and mediate the reorganization of microfilaments.[39,40] In neuronal cells, GAS7 expression is critical for neurite formation.[39,41] *MYOC*, the major glaucoma gene previously associated with elevated IOP cases, also affects the actin cytoskeletal structure and neurite outgrowth.[42] 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.[43] *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.[44] *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.[45] *GAS7* interacts with both *MYOC* and *CAV1* through β-catenin (*CTNNB1*) and RhoA (*RHOA*). β-catenin anchors the actin cytoskeleton and is part of the Wnt signalling pathway, which has previously been implicated in trabecular outflow regulation.[46,47] RhoA signalling regulates the intracellular levels of phosphorylated myosin light chain, which directly influence trabecular meshwork cellular contraction and thus aqueous humor outflow.[48] 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).[49-51] 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.[52,53] Rs7555523 is located in a region which previously showed suggestive evidence for linkage with blood pressure.[54] IOP and blood pressure have already been shown to correlate.[55] *TMCO1* is highly expressed in the human TM and CB, and to a lesser extent in the retina (Table 4).[37] *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.[52] 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 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 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]

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. Known glaucoma disease genes are given in blue. 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.

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 glaucoma patients 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.[56,57] CCT has previously been

reported to account for 1-6% of the variance in IOP measured with Goldmann applanation tonometry.[58-61] 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 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.

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SUPPLEMENTARY APPENDIX

Additional Methodology

Genotyping and imputation methods 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 5974 participants from RS-I, 2157 (RS-II), 2082 (RS-III), and 2385 (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 2235 participants, from 1417 sibships/families (of which 209 monozygotic), were included in the analyses. For a subset of 2093 participants from 1331 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-value ≥ 10^{-6}, and a SNP call rate >95% or Illumina Beadstudio Gencall Score ≥0.7, resulting in 543862 SNPs. Imputation was done with reference to HapMap release 22 CEU using MACH (http://www.sph.umich.edu/csg/abecasis/MACH/).[2] Data from 1807 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 in 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 1441 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 841342 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 participant 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 1304 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 using 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-value > $1x10^{-3}$, 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 2136 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 radius 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.

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

SNP	Chrom	RS-I			RS-II			RS-III			ERF		
		Beta	SE	P-value	Beta	SE	P-value	Beta	SE	P-value	Beta	SE	P-value
rs11656696	17p13.1	-0.351	0.064	$3.4x10^{-8}$	-0.107	0.107	$3.1x10^{-1}$	-0.241	0.101	$1.7x10^{-2}$	-0.156	0.120	$1.9x10^{-1}$
rs7894966	10q23.2	0.909	0.195	$3.0x10^{-6}$	0.226	0.308	$4.6x10^{-1}$	0.561	0.267	$3.6x10^{-2}$	0.697	0.300	$2.0x10^{-2}$
rs216146	5q32	0.238	0.067	$3.7x10^{-4}$	0.115	0.110	$3.0x10^{-1}$	0.247	0.098	$1.2x10^{-2}$	0.252	0.113	$2.6x10^{-2}$
rs2117760	3p13	0.242	0.070	$5.5x10^{-4}$	0.336	0.114	$3.2x10^{-3}$	0.130	0.104	$2.1x10^{-1}$	0.150	0.112	$1.8x10^{-1}$
rs7555523	1q24.1	0.331	0.097	$6.2x10^{-4}$	0.515	0.162	$1.5x10^{-3}$	0.223	0.142	$1.2x10^{-1}$	0.100	0.166	$5.5x10^{-1}$
rs1826598	16q23.1	0.517	0.103	$5.4x10^{-7}$	0.185	0.171	$2.8x10^{-1}$	0.127	0.157	$4.2x10^{-1}$	0.164	0.177	$3.5x10^{-1}$
rs9841621	3p24.3	-1.079	0.277	$1.0x10^{-4}$	-0.434	0.466	$3.5x10^{-1}$	-0.777	0.422	$6.5x10^{-2}$	-0.620	0.368	$9.2x10^{-2}$

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			RS-II			RS-III			ERF		
			Beta	SE	P-value	Beta	SE	P-value	Beta	SE	P-value	Beta	SE	P-value	Beta	SE	P-value
Nakano, et al.																	
rs547984 [C]	1q43	0.55	0.03	0.04	$4.9X10^{-1}$	0.03	0.06	$6.8X10^{-1}$	-0.17	0.11	$9.8X10^{-2}$	0.06	0.10	$5.1X10^{-1}$	0.21	0.11	$4.8X10^{-2}$
rs540782 [G]	1q43	0.57	0.04	0.04	$3.6X10^{-1}$	0.04	0.06	$5.0X10^{-1}$	-0.17	0.11	$1.1X10^{-1}$	0.08	0.10	$3.8X10^{-1}$	0.19	0.11	$7.2X10^{-2}$
rs693421 [G]	1q43	0.57	0.04	0.04	$3.5X10^{-1}$	0.04	0.06	$4.9X10^{-1}$	-0.17	0.11	$1.2X10^{-1}$	0.09	0.10	$3.6X10^{-1}$	0.19	0.11	$7.3X10^{-2}$
rs2499601 [T]	1q43	0.46	0.06	0.04	$1.9X10^{-1}$	0.08	0.06	$2.3X10^{-1}$	-0.13	0.10	$2.0X10^{-1}$	0.17	0.09	$7.5X10^{-2}$	0.06	0.11	$5.5X10^{-1}$
rs7081455 [G]	10p12.31	0.45	0.12	0.04	$4.6X10^{-3}$	0.16	0.06	$1.4X10^{-2}$	0.02	0.11	$8.4X10^{-1}$	0.15	0.10	$1.3X10^{-1}$	0.11	0.11	$2.9X10^{-1}$
rs7961953 [G]	12q21.31	0.88	0.01	0.07	$8.5X10^{-1}$	0.07	0.10	$4.7X10^{-1}$	-0.25	0.16	$1.2X10^{-1}$	-0.06	0.15	$6.9X10^{-1}$	0.18	0.15	$2.2X10^{-1}$
Jiao, et al.																	
rs1533428 [T]	2p16	0.30	0.02	0.05	$7.0X10^{-1}$	0.02	0.07	$7.6X10^{-1}$	0.09	0.12	$4.5X10^{-1}$	0.10	0.10	$3.4X10^{-1}$	-0.16	0.12	$1.6X10^{-1}$
rs12994401 [T]	2p16	0.19	-0.04	0.05	$4.8X10^{-1}$	0.04	0.08	$6.2X10^{-1}$	-0.14	0.13	$2.8X10^{-1}$	-0.02	0.12	$8.8X10^{-1}$	-0.16	0.13	$2.1X10^{-1}$
Thorleifsson, et al.																	
rs4236601 [A]	7q31	0.29	0.19	0.05	$1.1X10^{-4}$	0.24	0.07	$7.6X10^{-4}$	0.08	0.11	$4.7X10^{-1}$	0.27	0.11	$1.1X10^{-2}$	0.05	0.12	$6.9X10^{-1}$
rs1052990 [G]	7q31	0.36	0.17	0.04	$1.6X10^{-4}$	0.22	0.07	$8.7X10^{-4}$	0.00	0.11	$9.7X10^{-1}$	0.26	0.10	$8.9X10^{-3}$	0.11	0.11	$3.2X10^{-1}$

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

Table S3. Characteristics of the replication cohorts

Characteristic	TwinsUK	Australian Twins	DCCT/EDIC	WTCCC2/BMES
Participants with valid data (N)	2235	1807	1304	2136
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 (6.5 – 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	5548	104	2035	152	141	988	378
Age (y), mean ± SD	75.5 ± 7.4	74.5 ± 7.8	73.3 ± 9.2	48.8 ± 14.4	73.1 ± 11.1	72.2 ± 8.2	66.5 ± 14.1	73.9 ± 6.4
(range)	(56 - 94)	(55 - 105)	(51 – 91)	(18 – 86)	(27 – 98)	(55 – 92)	(12 – 104)	(34 – 97)
Male gender (%)	54.8	40.7	47.1	43.3	54.4	43.7	39.0	40.2
IOP (mmHg), mean ± SD	18.2 ± 6.2	15.2 ± 3.5	25.9 ± 8.6	15.3 ± 3.1	27.0 ± 8.3	19.6 ± 7.7	27.8 ± 9.3	<21.0
(range)	(6 – 55)	(5 – 59)	(12 – 62)	(6 – 33)	(13 – 54)	(13 – 48)	(11 – 65)	
IOP ≥ 22 mm Hg (%)	20.7	3.3	60.6	1.2	73.3	*	50.8	0.0
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

Figure S1. Regional association plots of loci associated with IOP ($5x10^{-8}$ < P-value < $1x10^{-5}$) in meta-analysis

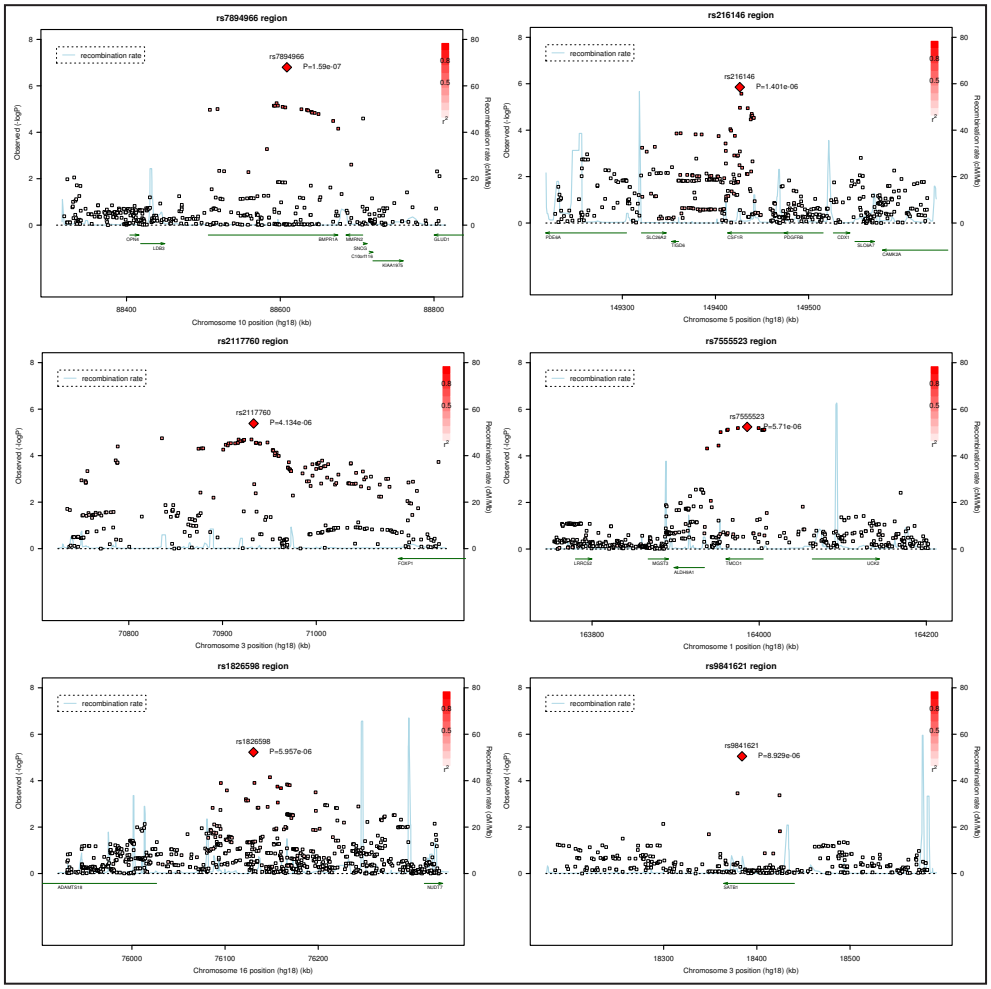
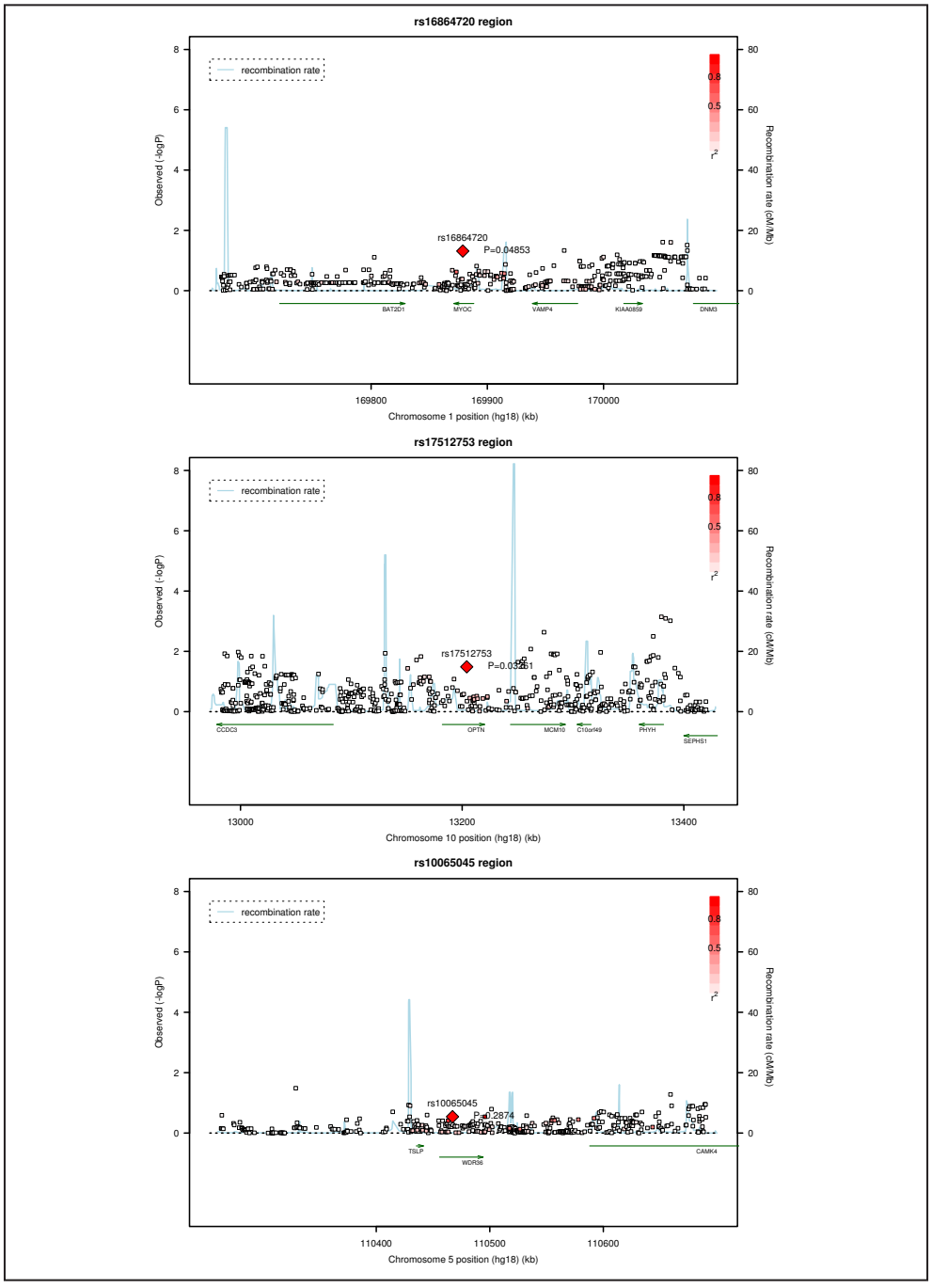


Figure S2. Regional association plots of *MYOC*, *OPTN*, and *WDR36* regions in meta-analysis



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Genetic architecture of open-angle glaucoma and related determinants

ABSTRACT

Background: Although the vertical cup-disc ratio (VCDR) and intraocular pressure (IOP) are important determinants of open-angle glaucoma (OAG), it is unclear to what extent the genetic origin of these traits overlap with those of OAG. We evaluated whether the same genes that determine VCDR and IOP also predict OAG.

Methods: Genetic risk scores were constructed from single nucleotide polymorphisms (SNPs) using genome-wide association data of 9326 participants from the Rotterdam Study cohorts (mean±standard deviation age: 64.6±9.1). These risk scores were used to calculate the explained variance of VCDR and IOP in an independent cohort (Erasmus Rucphen Family study) consisting of 1646 participants (mean±standard deviation age: 46.8±14.1) and the OAG risk in a subset of the Rotterdam Study cohorts. To evaluate false positive findings, we generated two new variables containing randomly sampled values to serve as a negative control.

Results: The explained variance of VCDR increased when increasing the number of SNPs included in the risk score, suggesting a polygenic model. We found no clear evidence for a similar model for IOP, suggesting that a small number of SNPs determine the susceptibility to IOP. The SNPs related to IOP in terms of p-values contributed little to VCDR. The risk scores associated with VCDR were also associated significantly with OAG. This suggests a common polygenic background for VCDR and OAG

Conclusions: We found evidence for a polygenic model underlying one of the major traits of OAG, VCDR, and OAG itself. The IOP did not show any evidence for such a model.

INTRODUCTION

Open-angle glaucoma (OAG) is a neurodegenerative disease that leads to progressive damage to the retinal ganglion cells and nerve fibers, resulting in glaucomatous visual field loss. It is one of the leading causes of irreversible blindness in the world, affecting over 60 million persons worldwide.[1] Although the etiology of OAG is still obscure, it has been recognized for long that genetic factors play a role in its pathogenesis. Relatives of patients with OAG have an increased risk of OAG.[2] Several rare genetic variants associated with a high risk of disease have been implicated in familial forms of OAG, but these variants only explain a small percentage of OAG patients.[3] This raises the question whether the genetic etiology of OAG is determined by a large number of rare variants with major effects on the disease risk (rare variant, common disease hypothesis) or whether there are multiple common variants underlying the disease (common variant-common disease hypothesis).

Genome-wide association studies (GWAS) have identified a large number of common variants (single nucleotide polymorphisms [SNPs]) with a low risk for a large number of common diseases (e.g. type 2 diabetes, Alzheimer's disease, and various forms of cancer), but also for diseases that are less common such as multiple sclerosis and Crohn's disease.[4] GWAS have also been powerful in studies on quantitative risk factors of diseases such as blood pressure, glucose levels and height.[4] More recently, GWAS were used to identify polygenic forms of inheritance for schizophrenia, implicating ten thousands of genetic variants covering the whole genome rather than a distinct number of variants.[5] The essence of the latter approach is that a gene discovery sample is used to define risk scores, and the risk is subsequently predicted in an independent sample. In this approach, not only the variants that are genome-wide significant are used but also those who do not reach this threshold. This method can also be applied to test for overlapping of genes between diseases and their underlying liability.

If there is a polygenic model that explains part of the susceptibility to a disease, the classical theory of Fisher predicts that the number of risk alleles is distributed according to the Gaussian distribution in the population.[6] It is easy to show that if there are >30 genetic variants, no person in the population carries zero risk alleles but everyone carries a gradient number of risk alleles.[7] Thus, a polygenic model implies that individuals in a population always carry risk alleles for a disease and that the liability to disease increases with the number risk alleles one carries. It also follows from this model that underlying a disease that is defined as present or absent, there is probably a quantitative trait. The persons in the tail of the distribution of this trait are considered to be "diseased". If the quantitative trait (or risk factor) is related to the genotypic distribution and increases with the number of risk alleles, the risk of disease will behave accordingly.

If we translate this to OAG, there are two clinical measures consistently associated with this disease and may thus determine its (polygenic) liability to OAG. First, OAG is characterized by damage to the optic nerve head, which is visible as an (increased) excavation (cupping)

upon ophthalmological examination. The extent of the excavation is commonly quantified as the vertical cup-to-disc ratio (VCDR), ranging from 0 to 1.[8] Both an increase in VCDR and an unusually large VCDR at a single observation may indicate glaucomatous changes of the optic nerve head.[9] The second clinical measure that may determine the (polygenic) liability to OAG is the intraocular pressure (IOP). Current treatment for OAG is based on lowering the IOP. VCDR as well as IOP are highly heritable, making these two variables potentially important endophenotypes for OAG.[10] Until now it is unknown to which extent the genes involved in VCDR overlap with those involved in IOP, and to which extent they actually predict OAG.

The aim of this study was to investigate whether there is a polygenic model in which many variants apply to VCDR and IOP and whether these genes also predict the risk of OAG. For this study, we used the Rotterdam Study I, Rotterdam Study II and Rotterdam Study III (RS-I, RS-II and RS-III, respectively) as discovery cohorts to derive genetic risk scores that capture the genes involved in VCDR and IOP. Next, we assessed whether these risk scores predicted VCDR and IOP in an independent target cohort (the Erasmus Rucphen Family [ERF] study). For the prediction of OAG, RS-I served as the target cohort, because no OAG cases were included in ERF.

METHODS

Study Populations

The RS-I is a prospective population-based cohort study of 7983 residents aged 55 years and older living in Ommoord, a suburb of Rotterdam, the Netherlands. Baseline examination for the ophthalmic part took place between 1991 and 1993; follow-up examinations were performed from 1997 to 1999 and from 2002 to 2006.[11,12] RS-I included 188 OAG cases that either already had OAG at baseline or developed OAG during follow-up.

The RS-II and RS-III are two other prospective population-based cohort studies of respectively 3011 residents aged 55 years and older and 3392 residents aged 45 years and older. The rationale and study design are similar to that of the RS-I.[11] The baseline examination of RS-II took place between 2000 and 2002; the follow-up examination was performed from 2004 to 2005. Baseline examination of RS-III took place between 2006 and 2009.[13]

The ERF Study is a family-based cohort in a genetically isolated population in the southwest of the Netherlands with over 3000 participants aged between 18 and 86 years. Cross-sectional examinations took place between 2002 and 2005. The rationale and study design of this study have been described elsewhere.[14,15] 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.

Ophthalmic examination

The ophthalmic assessment in RS-I and RS-II, both for baseline and follow-up, included a medical history, autorefraction, keratometry, IOP measurement, visual field testing and optic nerve head imaging with simultaneous stereoscopic photography (ImageNet; Topcon Corporation, Tokyo, Japan) of both eyes after mydriasis with tropicamide 0.5% and phenylephrine 2.5% eye drops.

RS-III was similar to RS-I except for optic nerve head imaging with Heidelberg Retina Tomograph 2 (HRT; Heidelberg Engineering, Dossenheim, Germany). The ophthalmic assessment in ERF included a medical history, autorefraction, keratometry, IOP measurement, and optic nerve head imaging with HRT of both eyes after pharmacologic mydriasis.

In all cohorts we measured IOP at baseline and at every follow-up round with Goldmann applanation tonometry (Haag-Streit, Bern, Switzerland) after applying oxybuprocaine 0.4% eye drops. The median value of three successive measurements was recorded.[16]

The OAG definition was based on the presence of glaucomatous visual field loss. Details have been described elsewhere. Neither VCDR nor IOP were part of the diagnosic criteria of OAG.[17,18]

ImageNet, which was used for optic nerve head imaging 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 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.[19]

HRT 2, used for optic nerve head imaging in RS-III and ERF, uses a focused 670-nm diode laser light beam to acquire scans of the optic nerve head region, using the confocal principle. 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 µm below the peripapillary retinal surface in the region of the papillomacular bundle. Imaging was performed after entering the participant’s keratometry data into the software and after adjusting the settings 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 µm were excluded. The inter-observer variability and agreement for both systems have been described elsewhere.[20][Ramdas, et al.; Chapter 2.1]

Genotyping

In the RS-I, RS-II and RS-III cohorts, DNA was genotyped using the Illumina Infinium II HumanHap arrays according to the manufacturer's protocols. After exclusion of participants for reasons of low-quality DNA, a total of 5974 participants were available with genotyping data from RS-I (HH550 v3.0®), 2157 participants were from RS-II and 2082 from RS-III (HH550Duo and HH610Quad®). Details are described elsewhere.[21]

In ERF, DNA was genotyped on four different platforms (Illumina 6k, Illumina 318K, Illumina 370K and Affymetrix 250K), which were then merged and imputed to 2.5 million SNPs hapmap using build 36 HapMap (release 22) CEU population as a reference cohort. After exclusion of participants for whom genotyping data were unavailable, 2385 had genotyping data. Extensive quality control analyses have been performed in each cohort.

Statistical analysis

Statistical analysis within the discovery cohorts

If data of both eyes were available, we randomly chose one. In case of missing or unreliable baseline data of both eyes, we used follow-up data whenever possible. For each of the discovery cohorts (comprising RS-I, RS-II and RS-III) linear regression models were used to examine the associations between the risk scores and VCDR and IOP, adjusted for age and gender. For VCDR we also adjusted for optic disc area. For IOP the mean value of both eyes was included and adjusted for IOP lowering treatment. This adjustment was done by adding 30% upon the measured IOP for medical treatment and by fixing the IOP at 30 mmHg for surgical/laser treatment in the past.

To summarize the results through the three discovery cohorts, we performed a meta-analysis with Metal for Linux (www.sph.umich.edu/csg/abecasis/metal) using the inverse variance method of each effect size estimate.[22]

All statistical analyses were performed using SPSS version 15.0.0 for Windows (SPSS inc., Chicago, IL, USA; 2006), PLINK[23] and R statistical package version 2.10.1 for Mac (www.r-project.org).

Risk scores within the independent cohort

The risk score profiling method tests the association of a genetic score variable that reflects a combined effect of a number of selected SNPs with a trait. Details of this method have been described elsewhere.[5] The results of the SNPs of the meta-analyzed discovery cohorts were categorized according to p-value and examined in an independent cohort (ERF). The p-value thresholds were as follow: $p<10^{-10}$, $p<10^{-9}$, $p<10^{-8}$, $p<10^{-7}$, $p<10^{-6}$, $p<10^{-5}$, $p<10^{-4}$, $p<10^{-3}$, $p<10^{-2}$, $p<0.1$, $p<0.2$, $p<0.3$, $p<0.4$, $p<0.5$, $p<0.6$, $p<0.7$, $p<0.8$, $p<0.9$, $p<1.0$. For each category the effect (beta) of each SNP was multiplied by the corresponding number of alleles of each

participant to calculate a score for that particular SNP. The mean of all these scores for the respective participant was the risk score. In case of missing SNP data from a participant of the replication cohort the allele frequencies were used and multiplied by the betas. Next, we ran linear regression analyses with the VCDR as the outcome measure and the risk scores adjusted for age and gender as the determinant in order to calculate the proportion of explained variance (PEV) of each group of SNPs (i.e. p-value threshold) as determined in the discovery cohort. A p-value of 0.05 or smaller was considered as statistically significantly associated with the trait.

The same analysis was also done with IOP as the outcome. To examine whether a “non-ophthalmic” variable reveals the same results we performed the same analysis for a negative control. For this variable we used a sampling method without replacement. We sampled the VCDR respectively IOP in the target cohort, and thus, the distribution remained the same. In addition, we created a second normal distributed variable by assigning a random number (with mean: 10; standard deviation: 1) to all participants.

For the analysis of the family-based data (ERF) we used SOLAR version 4.1.5 for Linux to adjust for the pedigree structure.[24]

Finally, we applied the same approach for the OAG cases of RS-I by calculating the Nagelkerke R-square of OAG for the VCDR and IOP risk scores. This was done using logistic regression analyses adjusted for age and gender. Since, as a matter of course, individuals with OAG may have an increased VCDR and/or IOP, we performed a secondary analysis in which we excluded RS-I from the meta-analysis of the discovery cohorts to avoid possible biased results. Differences in general characteristics between cases and controls were analyzed with independent t-tests and chi-square statistics.

Only autosomal SNPs that were available in all four cohorts (~520.000 SNPs) were included. To have less overlap of linkage disequilibrium blocks we did not use imputed data. SNPs that deviated significantly from Hardy-Weinberg equilibrium ($p<0.0001$) or that had a minor allele frequency less than 0.05 were excluded from the present study.

RESULTS

Study samples

Of the 5974 participants that were genotyped in the RS-I cohort 5312 had valid VCDR data. The remaining 662 were excluded because of missing or unreliable data. From the RS-II cohort 2048 from 2157 genotyped participants were included in this study. For 109 persons we did not have (reliable) data. From the RS-III cohort a total of 1966 participants were included. The target cohort for the quantitative analysis (ERF) included 1646 participants. Table 1 summarizes the general characteristics for the discovery cohorts and the replication cohort. The participants of the target set were significantly younger ($p<0.001$), and had lower VCDR

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

	Discovery cohorts			Target cohort
	RS-I	RS-II	RS-III	ERF
Total sample size (N)	5312	2048	1966	1646
Age (years)	68.0 ± 8.4	64.3 ± 7.8	55.6 ± 5.5	46.8 ± 14.1
	(55 - 99)	(55 - 98)	(45 - 89)	(18 – 84)
Gender, N(%) women	3099 (58.3)	1109 (54.2)	1102 (56.1)	942 (57.2)
Vertical cup-disc ratio*	0.50 ± 0.14	0.50 ± 0.14	0.42 ± 0.17	0.46 ± 0.15
	(0.00 – 0.89)	(0.05 – 0.87)	(0.00 – 1.00)	(0.00 – 0.84)
Intraocular pressure (mmHg)**	14.8 ± 3.4	14.4 ± 3.4	13.6 ± 3.0	14.8 ± 3.0
	(5.0 – 58.5)	(7.0 – 31.5)	(5.0 – 30.0)	(6.0 – 30.0)

* = In RS-I and RS-II measured with ImageNet and in RS-III and ERF with Heidelberg Retina Tomograph; ** = Sample sizes for intraocular pressure analyses were 5794 for RS-I, 2102 for RS-II, 2041 for RS-III, and 2035 for ERF.

values ($p<0.001$). The latter difference can be explained by the use of different measurement techniques for optic disc imaging (since we analyzed the cohorts separately and subsequently meta-analyzed the findings, this difference will not influence our findings). The distributions of VCDR and IOP for the four cohorts are shown in Supplementary Figure S1. Genetic outliers of non-European ancestry were excluded. No institutional heterogeneity between the cohorts or residual population sub-stratification was noticed after inspecting the genotype data.[25] Inflation factors for the included cohorts for VCDR and IOP ranged from 1.024 to 1.061 and from 1.006 to 1.037, respectively.

Risk score analysis

For VCDR, age and gender explained 0.3% of the total variance. Figure 1A presents the PEV attributable to the risk scores when risk scores from the VCDR analysis in the discovery sample were used to predict VCDR in the target sample ERF. Figure 1B shows the PEV when the same risk scores were used to predict a random variable (negative control). For VCDR, the risk score based on the p-value threshold of $p<10^{-10}$ (first bar) consisted of 9 SNPs (Table 2) and explained only 0.1% (in addition to age and gender; region above the dotted line) of the variance in VCDR (Figure 1A) in the target population. Increasing the number of SNPs included in the risk scores (i.e. increasing the p-value thresholds for the SNPs to be included) resulted in a gradual increase in the PEV until the $p<10^{-2}$ threshold was used (consisting of 7260 SNPs; $p=8.68\times10^{-4}$). The PEV increased up to 1.0% ($p=1.91\times10^{-4}$) at the threshold of $p<0.2$ for the SNPs to be included in the risk score and then stabilized. This suggests that there is a polygenic model underlying the genetics of VCDR. No similar pattern was observed for the negative controls, i.e., the random variable used to evaluate false positive associations other than due to confounding by admixture (lowest observed $p=0.204$). For the second negative control, based on sampling without replacement, increasing the p-value thresholds

Table 2. Number of single nucleotide polymorphisms (SNPs) included in each p-value category presented as N(%)

p-value threshold	Vertical cup-disc ratio	Intraocular pressure
$<10^{-10}$	9 (0.0)	-
$<10^{-9}$	10 (0.0)	-
$<10^{-8}$	12 (0.0)	1 (0.0)
$<10^{-7}$	18 (0.0)	2 (0.0)
$<10^{-6}$	34 (0.0)	4 (0.0)
$<10^{-5}$	61 (0.0)	13 (0.0)
$<10^{-4}$	179 (0.0)	88 (0.0)
$<10^{-3}$	966 (0.2)	755 (0.1)
$<10^{-2}$	7260 (1.4)	6429 (1.2)
<0.1	60029 (11.5)	56798 (10.9)
<0.2	114122 (21.8)	110338 (21.1)
<0.3	166995 (31.9)	163261 (31.2)
<0.4	219096 (41.9)	215470 (41.2)
<0.5	270510 (51.7)	267418 (51.2)
<0.6	321288 (61.5)	318625 (60.9)
<0.7	371823 (71.1)	369764 (70.7)
<0.8	422175 (80.8)	420547 (80.4)
<0.9	472496 (90.4)	471532 (90.2)
<1.0	522762 (100.0)	522782 (100.0)

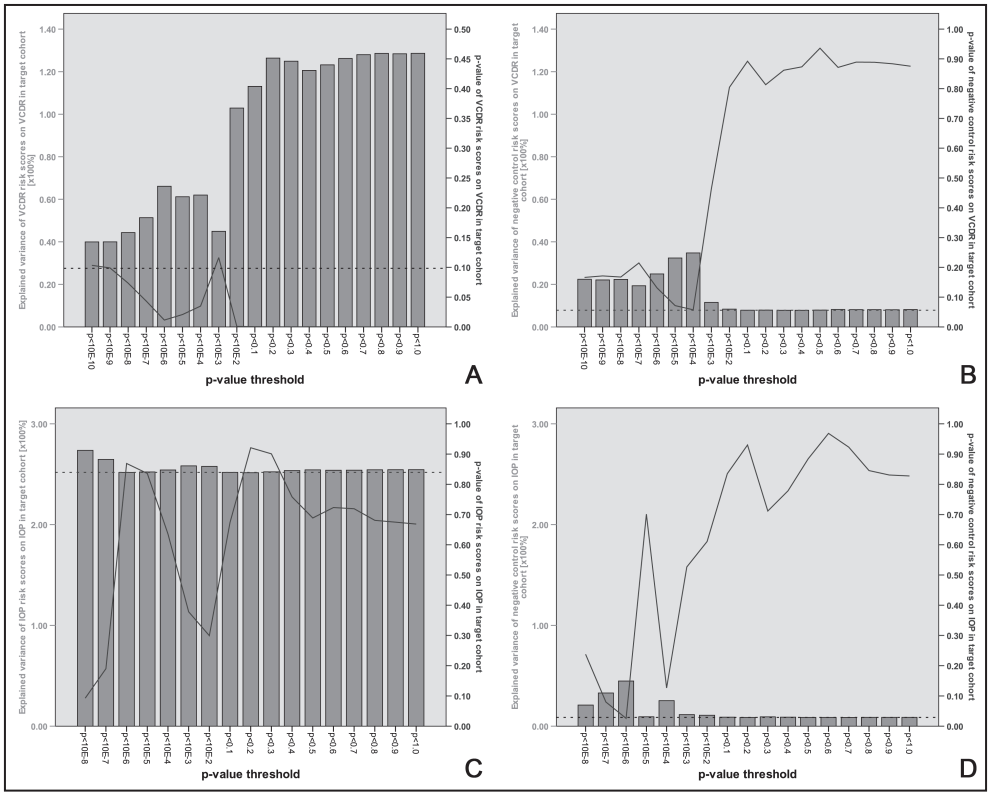
for the SNPs did not have any effect on the PEV, neither for the negative control based on random generated numbers (Figure 1B; Supplementary Figure S2).

Figures 1C and 1D present the corresponding graphs for IOP. For IOP, age and gender explained 2.5% of the total variance, while the first risk score based on a threshold of $p<10^{-8}$ explained an additional 0.2% (region above the dotted line) of the variation in IOP in the target sample ERF. This score included only one SNP and did not reach nominal significance (p=0.093). However, the addition of more SNPs did not yield any increase in PEV. None of the more extended risk scores were even marginally significant.

To evaluate whether there is a shared genetic component between VCDR and OAG and IOP and OAG, we tested the association of risk scores based on VCDR and IOP in the discovery sample with OAG cases and controls in RS-I. Table 3 shows the descriptive data of the OAG cases and controls in RS-I. A total of 5304 participants of RS-I had complete data for optic nerve head measurements, IOP and reliable visual fields. Of these, 171 were classified as having OAG. This classification was based on the presence or absence of glaucomatous visual field loss. OAG cases were significantly older and more often men compared to controls. Not surprisingly, OAG cases had a larger VCDR and an increased IOP compared to controls, although these measures were not part of the classification process.

Figure 2A displays the PEV (Nagelkerke R-square) of OAG in RS-I attributable to VCDR genetic risk scores. Age and gender explained 4.0% of the variance of OAG and the

Figure 1. The explained variance of risk scores of the vertical cup-disc ratio (VCDR) and intraocular pressure (IOP) for each p-value threshold for their respective trait in the target cohort ERF (A and C, respectively); and the explained variance of the risk scores of VCDR and IOP for each p-value threshold for the negative control (B and D, respectively).



The y1-axis (left; histogram) depicts the explained variance (in %) against different p-value thresholds on the x-axis that were used to construct the risk scores. The y2-axis (right; line) depicts the p-values from the association analysis of the risk scores with the trait. Dotted line represents the attribution of age and gender to the explained variance.

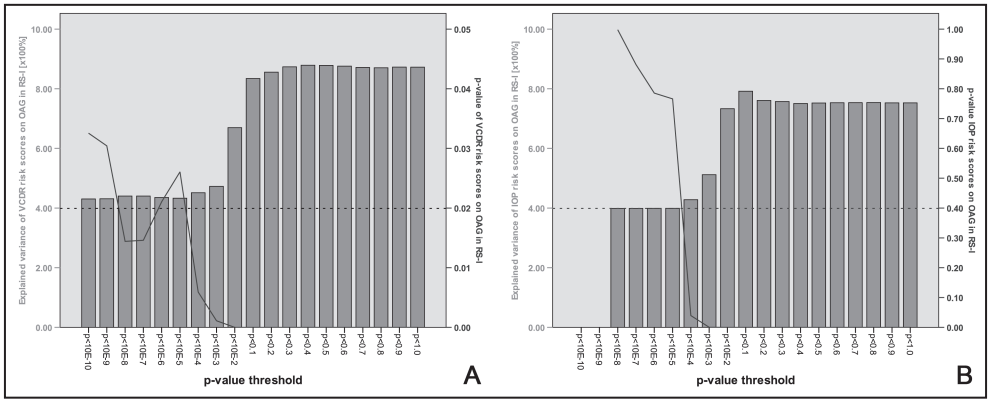
Table 3. Descriptive data of open-angle glaucoma cases and controls in RS-I, presented as mean ± standard deviation (range) unless stated otherwise

	Cases (N=171)	Controls (N=5133)	p-value	RS-I (Total N=5304)
Age (years)	77.4 ± 7.2 (56 – 94)	74.0 ± 7.5 (55 – 99)	< 0.001*	74.1 ± 7.5 (55 – 99)
Gender, N(%) women	81 (47.4)	3013 (58.7)	< 0.001*	3094 (58.3)
Vertical cup-disc ratio	0.59 ± 0.15 (0.15 – 0.89)	0.50 ± 0.14 (0.00 – 0.83)	< 0.001*	0.50 ± 0.14 (0.00 – 0.89)
Intraocular pressure (mmHg)	16.8 ± 4.7 (5.5 – 39.7)	14.7 ± 3.2 (5.0 – 35.8)	<0.001*	14.7 ± 3.3 (5.0 – 39.7)

* = significant at a p-value of 0.05.

first risk score based on the p-value threshold of $p<10^{-10}$ (first bar) explained only an additional 0.3% of the OAG variance. However, when more SNPs with higher p-values were added to the score, the PEV increased sharply from $p<10^{-2}$ and kept on increasing up to the inclusion of SNPs in the risk score with p<0.3 in the discovery set. The latter score explained up to 4.7% of the variance of OAG above the attribution of age and gender, which is more than what is explained by age and gender together. The pattern of Figure 2A suggests a polygenic model for OAG as well and also a large genetic component shared by both VCDR and OAG. In a secondary analysis we excluded RS-I from the meta-analysis of the discovery cohorts and found a similar trend (Figure 3A). Nevertheless, as a consequence the PEV was much lower due to a major reduction in power. Results for IOP were similar to VCDR if we included RS-I, in that the risk scores were significantly associated with OAG (Figure 2B). However, in contrast to VCDR the evidence for a polygenic model underlying IOP disappeared in the secondary analyses (i.e. after excluding RS-I from the meta-analysis of the discovery cohorts; Figure 3B).

Figure 2. The Nagelkerke R-square of risk scores of the vertical cup-disc ratio (VCDR) and intraocular pressure (IOP), adjusted for age and gender, for each p-value threshold on open-angle glaucoma (OAG) in RS-I (A and B, respectively).

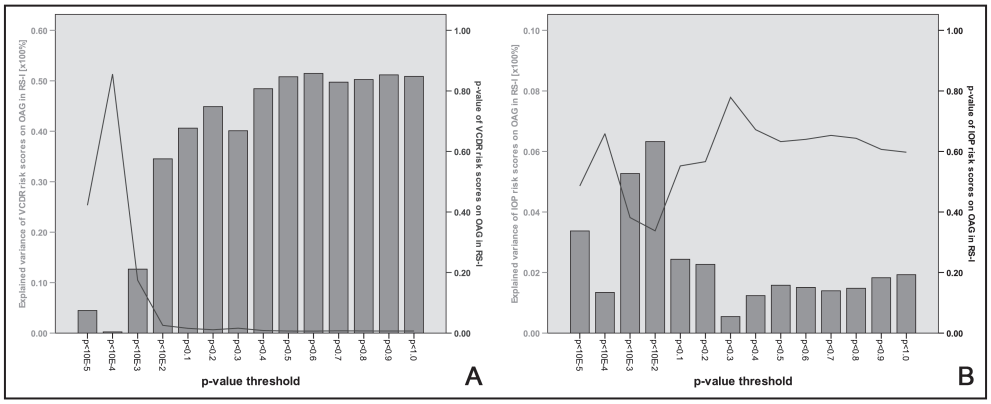


The y1-axis (left; histogram) depicts the explained variance (in %) against different p-value thresholds on the x-axis, which were used to construct risk scores. The y2-axis (right; line) depicts the p-values from the association analysis of the risk scores with the trait.

DISCUSSION

In the present study of GWAS data from Caucasian participants, we found evidence for a polygenic model underlying VCDR, one of the major known measures for OAG. In contrast, IOP did not show significant evidence for a polygenic model in the current study. Of these two traits, VCDR is the most relevant trait related to the diagnosis of OAG, while IOP is the most relevant trait for OAG treatment in patients with elevated IOP. Of interest is the finding that the risk scores based on the VCDR also predicted OAG. Although both VCDR and elevated IOP are putative endophenotypes for OAG, only VCDR showed evidence for a joint polygenic origin with OAG.

Figure 3. The Nagelkerke R-square of risk scores of the vertical cup-disc ratio (VCDR) and intraocular pressure (IOP), adjusted for age and gender, for each p-value threshold on open-angle glaucoma (OAG) in RS-I (A and B, respectively) after excluding RS-I from the discovery cohort. To display the pattern more clear the attribution of age and gender has already been subtracted.



The y1-axis (left; histogram) depicts the explained variance (in %) against different p-value thresholds on the x-axis, which were used to construct risk scores. The y2-axis (right; line) depicts the p-values from the association analysis of the risk scores with the trait.

In the approach of the present study a large discovery cohort is crucial, while the size of the target cohort is less important. Therefore, we combined data of three independent cohorts from the Rotterdam Study, and used a single target cohort (ERF). We also repeated our analysis in which we used RS-III as a second replication cohort, rather than as one of the discovery cohorts. The results were similar, further supporting the evidence for a polygenic model of VCDR (data not shown).

At first sight, it seems unexpected that we did not find a similar model for IOP as for VCDR, because an elevated IOP has been implicated as the most important determinants of both glaucomatous optic neuropathy (often referred to as an increased VCDR) and glaucomatous visual field loss. In some patients with OAG the IOP is not elevated. This form of disease is referred to as normal tension glaucoma, which represents about 15-40% of all patients with OAG.[26] In these patients, however, IOP lowering also causes a decrease in the rate of disease progression, suggesting that IOP matters in normal tension glaucoma as well. Due to the relatively low number of cases in our study, we did not separately analyze patients with normal tension glaucoma. For both traits, IOP and VCDR, strong evidence of genetic correlation with OAG susceptibility has been found. These two traits also correlate with each other in our population (Pearson's $p<0.001$) and in other populations.[27] Nonetheless, when we tested whether the risk scores based on IOP predicted VCDR in the target cohort we found 0.54% as the highest significant PEV for VCDR of IOP-related SNPs (at $p<10^{-8}$; data not shown). This finding suggests there is little overlap in the genes determining IOP and VCDR. Combined with the current findings this suggests that there are other, possibly rare variants with strong effects or yet unidentified environmental risk factors that determine IOP. However, a drawback of our analysis of IOP is that we did not adjust for the central corneal thickness,

while the measurement of IOP may also be affected by central corneal thickness.[28]

All OAG cases were only derived from RS-I. A potential problem in the interpretation of the findings on OAG might be that the OAG cases derived from RS-I were also included in the discovery cohorts for the VCDR genetic risk scores. Although this may create an autocorrelation between the VCDR risk scores defined in the discovery set and OAG prediction conducted in the same set, this problem also occurred for the IOP scores. As the IOP score did not associate with the risk of OAG, it appears unlikely that any autocorrelation bias explained the overlap in genes involved in VCDR and OAG. Furthermore, it is important to realize that the diagnosis for OAG in RS-I was primarily based on glaucomatous visual field loss, thus not on VCDR or IOP. Moreover, the results did not alter when we excluded RS-I from the discovery cohorts.

As far as we know, the current study is the first to describe the phenomenon of a polygenic model for optic disc cupping (expressed in the VCDR). We further showed that the same risk scores predicted OAG. This finding sheds new light on the etiology of OAG. The amount of variance explained by the VCDR risk scores exceeded that of age and gender. Nevertheless the PEV of both was still too low to allow the prediction of OAG in persons at risk.

Rare variants with large effects have also been implicated in the etiology of OAG. However, the current data suggest that many SNPs (probably with very small effects) may collectively account for a substantial proportion of variation in VCDR. This implies that many common variants have not been identified yet, because of low effect estimates of risks associated with single SNPs. Larger GWAS are needed to detect those variants and to further replicate our findings.

In conclusion, the present study has three major implications. First, in our large epidemiological study there is little overlap between the genes involved in VCDR and IOP. Second, the current study provides evidence for a polygenic model in VCDR involving many common SNPs that are not shared by a random variable, but specific to VCDR itself. This polygenic basis is not shared by the IOP, which may suggest that either the trait IOP has underlying rare variants, or the variants involved in IOP have such small effects that they were not well detected in the discovery set because of lack of power. Third, the genetics of VCDR overlap convincingly with that of OAG.

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SUPPLEMENTARY APPENDIX

Figure S1. The distributions of VCDR and IOP for RS-I, RS-II, RS-III, and ERF (A and B, respectively)

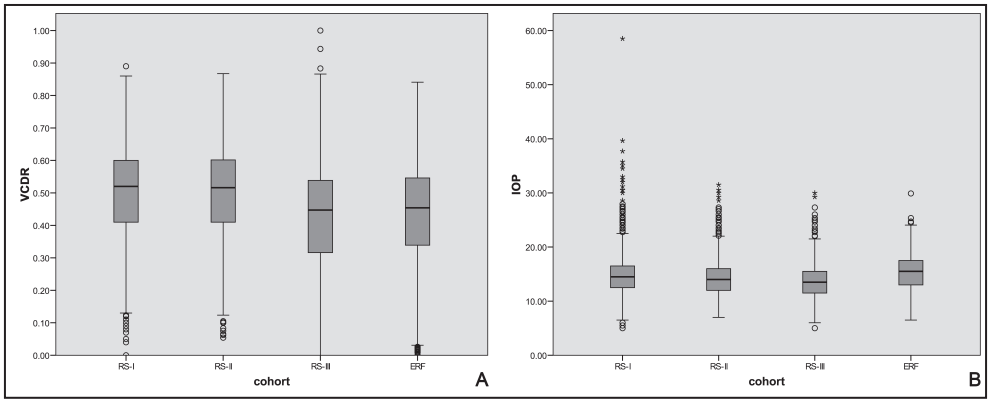
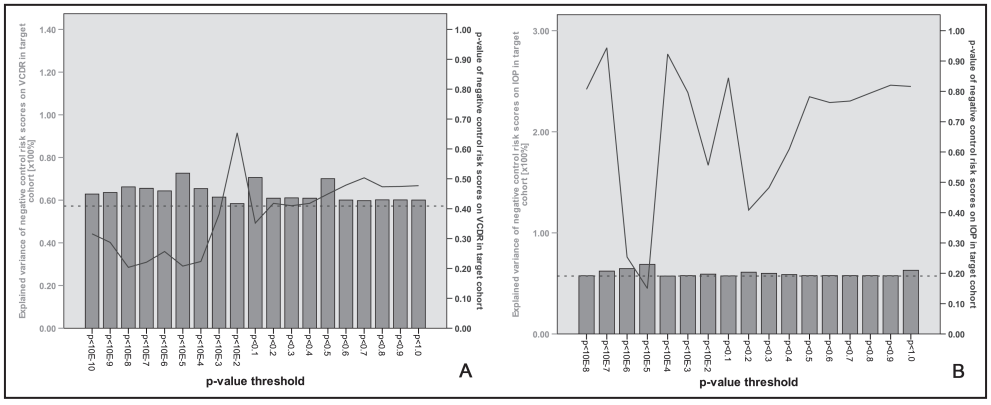


Figure S2. The explained variance of the risk scores of VCDR and IOP for each p-value threshold for the second negative control (A and B, respectively).



The y1-axis (left; histogram) depicts the explained variance (in %) against different p-value thresholds on the x-axis that were used to construct the risk scores. The y2-axis (right; line) depicts the p-values from the association analysis of the risk scores with the trait. Dotted line represents the attribution of age and gender to the explained variance.

Chapter 5

Implications of genetics in open-angle glaucoma

5.1 *Common genetic variants associated with open-angle glaucoma.*
Ramdas WD, van Koolwijk LM, Lemij HG, Pasutto F, Cree AJ, Thorleifsson G, Janssen SF, Ten Brink J, Amin N, Rivadeneira F, Wolfs RC, Walters GB, Jonasson F, Weisschuh N, Mardin CY, Gibson J, Zegers RH, Hofman A, de Jong PT, Uitterlinden AG, Oostra BA, Thorsteinsdottir U, Gramer E, Welgen-L ƒlen UC, Kirwan JF, Bergen AA, Reis A, Stefansson K, Lotery AJ, Vingerling JR, Jansonius NM, Klaver CC, van Duijn CM.
Hum Mol Genet. 2011 Jun 15;20(12):2464-2471.

5.2 *Clinical implications of old and new genes for open-angle glaucoma.*
Ramdas WD, van Koolwijk LM, Cree AJ, Janssens AC, Amin N, de Jong PT, Wolfs RC, Gibson J, Kirwan JF, Hofman A, Rivadeneira F, Oostra BA, Uitterlinden AG, Ennis S, Lotery AJ, Lemij HG, Klaver CC, Vingerling JR, Jansonius NM, van Duijn CM.
Ophthalmology. 2011 Aug 26.

Common genetic variants associated with open-angle glaucoma

ABSTRACT

Open-angle glaucoma (glaucoma) is a major eye disorder characterized by optic disc pathology. Recent genome-wide association studies identified new loci associated with clinically relevant optic disc parameters, such as the optic disc area and vertical cup-disc ratio (VCDR). We examined to what extent these loci are involved in glaucoma. The loci studied include *ATOH7, CDC7*/*TGFBR3* and *SALL1*, for optic disc area, and *CDKN2B*, *SIX1*, *SCYL1*/*LTBP3*, *CHEK2*, *ATOH7* and *DCLK1*, for VCDR. We performed a meta-analysis using data from six independent studies including: the Rotterdam Study (N= 5736), Genetic Research in Isolated Populations combined with Erasmus Rucphen Family study (N=1750), Amsterdam Glaucoma Study (N=296), and cohorts from Erlangen and Tübingen (N=1363), Southampton (N=702), and deCODE (N=36151) resulting in a total of 3161 glaucoma cases and 42837 controls. Of the eight loci, we found significant evidence ($p=1.41x10^{-8}$) for the association of *CDKN2B* with glaucoma (odds ratio [OR] for those homozygous for the risk allele: 0.76; 95% confidence interval [CI]: 0.70-0.84), for the role of *ATOH7* (OR: 1.28; 95% CI: 1.12-1.47), and for *SIX1* (OR: 1.20; 95% CI: 1.10-1.31) when adjusting for the number of tested loci. Furthermore, there was a borderline significant association of *CDC7*/*TGFBR3* and *SALL1* (both p=0.04) with glaucoma. In conclusion, we found consistent evidence for three common variants (*CDKN2B*, *ATOH7* and *SIX1*) significantly associated with glaucoma. These findings may shed new light on the pathophysiological protein pathways leading to glaucoma, and point to pathways involved in the growth and development of the optic nerve.

INTRODUCTION

Open-angle glaucoma (from here on called glaucoma) is a chronic neurodegenerative disease that leads to progressive damage to retinal ganglion cells and nerve fibers, resulting in visual field loss.[1] Glaucoma is recognized as the commonest cause of irreversible blindness worldwide. However, the etiology of glaucoma remains obscure. Risk factors for glaucoma include old age, elevated intraocular pressure, myopia, African descent and positive family history.[2,3] Only three causative genes have been established (*MYOC*, *OPTN* and *WDR36*) for late-onset glaucoma.[4] High-risk variants in these genes are predominantly observed in familial cases of glaucoma, but their frequency in sporadic patients from the general population is low (3-5%).[4]

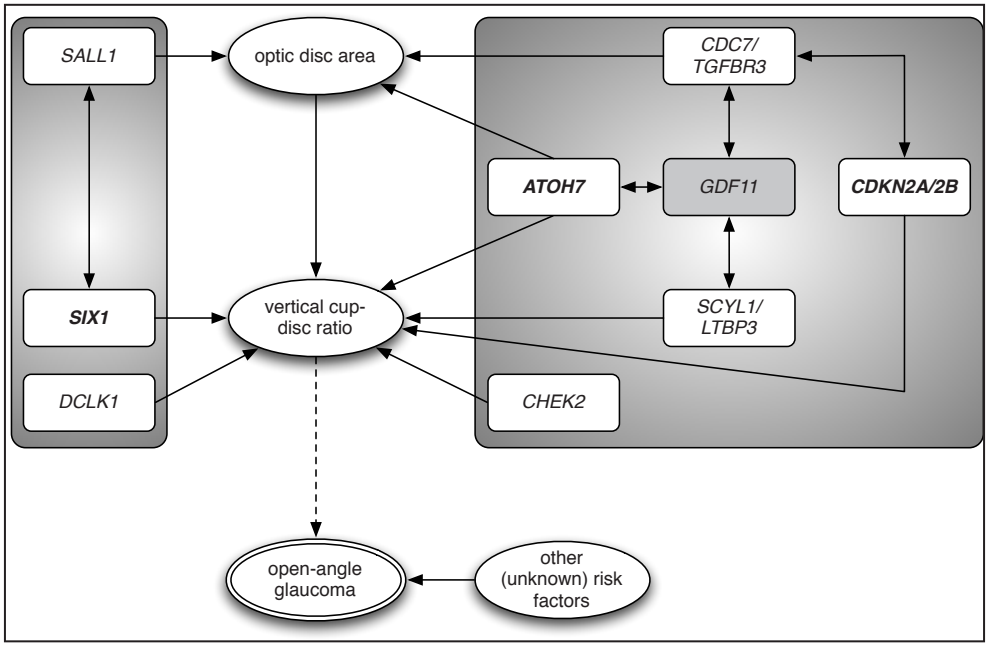
One of the first signs of glaucoma is damage to the optic disc (optic nerve head), visible as an increased excavation (cupping). The optic disc cupping occurs more in the vertical direction, which is commonly quantified as the vertical cup-to-disc ratio (VCDR).[1,5] An increased VCDR is a significant determinant of the risk of developing glaucoma.[6-8] Recently, we identified new loci involved in the optic disc area as well as VCDR: *ATOH7, CDC7*/*TGFBR3* and *SALL1* for optic disc area, and *CDKN2B*, *SIX1*, *SCYL1*/*LTBP3*, *CHEK2*, and *DCLK1*, in addition to *ATOH7*, for VCDR (Table 1).[9] When considering the function of the proteins these genes encode for, two protein pathways emerge (Figure 1).[9] To further elucidate the relation of genes implicated in the optic disc area and VCDR to glaucoma, we examined to what extent these loci are involved in glaucoma. For this purpose we performed a meta-analysis using data from six independent studies comprising of Caucasian persons: the Rotterdam Study (N= 5736), Genetic Research in Isolated Populations combined with Erasmus Rucphen Family study (N=1750), Amsterdam Glaucoma Study (N=296), and cohorts from Erlangen and Tübingen (N=1363), Southampton (N=702) and deCODE (N=36151) resulting in a total of 3161 glaucoma cases and 42837 controls.

MATERIALS AND METHODS

Study Populations

The first cohort, the Rotterdam Study (RS-I) is a prospective population-based cohort study of 7983 residents aged 55 years and older living in Rotterdam, the Netherlands.[10] RS-I waspreviously included in the gene discovery study.[9] In this paper, we included cases and controls from RS-I. The second study included glaucoma cases from the Genetic Research in Isolated Populations (GRIP; N=104) and controls from the Erasmus Rucphen Family study (ERF). For GRIP, medical records in three local hospitals were assessed to identify patients with glaucoma. ERF is a family-based study in a genetically isolated population in the southwest of the Netherlands with over 3000 participants aged 18 to 86 years.[11,12] Participants

Figure 1. Overview of the biological interaction of the investigated genes in relation to open-angle glaucoma (left part: developmental pathway; right part *TGFB*-signaling/growth pathway; genes associated with open-angle glaucoma in the present study are in bold).



of GRIP were ascertained independently of ERF, but lived in the same region. The third study, the Amsterdam Glaucoma Study (AGS), included 148 cases and 148 controls collected from eye clinics, meetings of the glaucoma patients' association, nursing homes and fairs for the elderly from all over the Netherlands. The fourth study included participants from Erlangen and Tübingen, Germany, comprising 986 glaucoma cases and 377 controls. Cases and controls were recruited from the same geographic regions. For the fifth study, from Southampton, glaucoma cases and controls (N=470 and 232, respectively) were collected from specialist glaucoma and general clinics at the Southampton Eye Unit, UK. Finally, in deCODE, the sixth study, 1265 glaucoma cases were recruited from the Reykjavik Eye Study[13] and Icelandic glaucoma clinics. Controls (N=34886) were selected among individuals who had participated in the various genetic programs at deCODE. The present study included a total of 3161 glaucoma cases and 42837 controls, all of Caucasian ethnicity. All measurements in these studies were conducted after the respective relevant medical ethics committees had approved the study protocols and all participants had given a written informed consent in accordance with the Declaration of Helsinki.

Ophthalmic examination

The ophthalmic assessment in RS-I included a medical history, autorefraction, keratometry,

Goldmann applanation tonometry, visual field testing (Humphrey Field Analyzer II 740 [HFA; Carl Zeiss, Oberkochen, Germany] or Goldmann kinetic perimetry [Haag-Streit, Bern, Switzerland]) and optic nerve head imaging with Topcon ImageNet System (Topcon Corporation, Tokyo, Japan) of both eyes after pharmacologic mydriasis. In GRIP 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. The ophthalmic assessment in ERF was similar to RS-I, but no visual field testing was included, medical records were checked for any glaucoma pathology (LMEvK and HGL) and optic nerve head imaging was done with Heidelberg Retina Tomograph 2 (HRT; Heidelberg Engineering, Dossenheim, Germany). In AGS all persons underwent ophthalmoscopy and biomicroscopy with a 90 diopter lens, and digital stereo images of the optic nerve head were taken after mydriatic drops. Participants from Erlangen and Tübingen 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 examination included optic nerve head imaging (HRT 1 and 2; or biomicroscopy with Goldmann lens and a Haag-Streit slit lamp), and 24-hour Goldmann applanation tonometry profile with five measurements.[14,15] Patients from Southampton were examined by an experienced glaucoma specialist at the Southampton University Hospital Eye Unit. Biomicroscopy was performed and visual fields were measured using HFA 24-2 and HFA 30-2. Examination of participants from deCODE included biomicroscopy and visual field testing using the Octopus 123 perimeter (Haag-Streit, Köniz, Switzerland). Details have been described elsewhere.[13]

Criteria for glaucoma

In RS-I, glaucoma diagnosis was primarily based on the presence of glaucomatous visual field loss, and not on the VCDR. The visual field of each eye was screened using a 52-point supra-threshold test that covered the central visual field with a radius of 24°,[16,17] and tested the same locations as used in the Glaucoma Hemifield Test. In participants in whom visual field loss was reproducible on a second supra-threshold test, Goldmann kinetic perimetry or full-threshold HFA testing with 24-2 grid was performed on both eyes by a skilled perimetrist. Details about the classification process have been described before.[8,16] Cases had to have an open anterior chamber angle and no history or signs of secondary glaucoma or manifest exfoliation were allowed.

In GRIP, 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, combined with a matching glaucomatous visual field defect and open angles upon gonioscopy. Visual field test results had to be reliable and reproducible. Patients with any other disease that could cause visual field defects were excluded.

In AGS, glaucoma cases had to have glaucomatous optic neuropathy with corresponding glaucomatous visual field loss in at least one eye or a VCDR≥0.8 when no

visual field was available. In 84.5% of all cases we had visual fields. In order to be eligible as a control person, one had to be aged 55 years or older, have a VCDR≤0.6 on ophthalmoscopy and fundus photography.

In Erlangen and Tübingen glaucoma was defined as the presence of glaucomatous optic disc damage (in at least one eye), visual field defects in at least one eye and intraocular pressure higher than 21 mmHg in one eye without therapy. Optic disc damage was classified according to Jonas, et al..[18,19] A pathologic visual field was defined by a pathologic Bebié curve, three adjacent test points with more than 5 dB sensitivity loss, or at least one point with more than 15 dB sensitivity loss. In addition, controls were age and gender matched to the patients.[14]

Details of the glaucoma cases from Southampton have been reported previously.[20] In brief, diagnosis was made on the basis of characteristic glaucomatous visual field loss/ glaucomatous optic disc damage/increased intraocular pressure. Patients presenting with narrow-angle, developmental or secondary glaucoma or any other known abnormalities of the anterior segment were excluded. Controls had no history of glaucoma and were not on any treatment to lower intraocular pressure.

Finally, in deCODE glaucoma was based on glaucomatous optic neuropathy and glaucomatous visual field loss.[21] Cases had to have an open anterior chamber angle on gonioscopy. Exfoliation syndrome was specifically looked for and if detected the participant was excluded. Controls with a reported history of glaucoma were excluded from the control group.

Laboratory analysis

In the RS-I, DNA was genotyped using the Illumina Infinium II HumanHap550chip v3.0® array according to the manufacturer's protocols.[22,23] After exclusion of participants for reasons of low-quality DNA, a total of 5974 participants were available with genotyping data from RS-I, of whom 5736 had reliable optic disc measurements and visual fields. In ERF and GRIP, DNA was genotyped on four different platforms (Illumina 6k, Illumina 318K, Illumina 370K and Affymetrix 250K), which were then merged. A total of 2385 had genotyping data, of which 1646 from ERF had reliable optic disc data. For the AGS study, the SNPs were characterized by using TaqMan®. The Erlangen and Tübingen cohorts were genotyped using selected pre-developed TaqMan® Genotyping Assays (Applied Biosystems, Foster City, CA, USA), following the manufacturer's instructions. Genotyping of Southampton cases and controls was carried out using KASPar chemistry (KBioscience, Hoddesdon, UK). Finally, in deCODE samples were assayed with the Illumina HumanHap300 or HumanHapCNV370 bead chips (Illumina, SanDiego, CA, USA).

Statistical analyses

Within each study logistic regression analyses were used to examine the associations

between the top single nucleotide polymorphisms (SNPs; Table 1) and glaucoma adjusted for age and gender. With these logistic regression models, we calculated odds ratios (ORs) with corresponding 95% confidence intervals (CIs). The minor allele of the SNPs was considered as the risk allele. Next, we performed meta-analyses using fixed-effects models to calculate the joint effect through the six independent cohorts for the heterozygous and homozygous effect of the SNPs. To adjust for multiple testing, we used Bonferroni's correction; a p-value of 0.003 (0.05/8 SNPs/2 [for hetero- and homozygous effect]) or smaller was considered statistically significant. Heterogeneity of the meta-analyses was measured by calculating I^2.[24] Finally, as a secondary analysis, we ran an allelic analysis assuming the risk associated with the genotype is multiplicative to the number of risk alleles. All statistical analyses were performed using SPSS version 15.0.0 for Windows (SPSS inc., Chicago, IL, USA; 2006), and R statistical package version 2.11.1 for Mac (www.r-project.org).

RESULTS

Table 2 summarizes the general characteristics of the cases and controls for all cohorts. Cases of GRIP and Southampton were significantly older ($p<0.001$) than their controls. As expected, in all studies, the intraocular pressure and intraocular pressure-lowering treatment were increased in glaucoma cases.

All studies showed marginal evidence for association ($p<0.003$; adjusted for multiple testing) of rs1900004 (close to *ATOH7*), rs1063192 (*CDKN2B*) and rs10483727 (close to *SIX1*) with glaucoma for the homozygous effect (Figure 2). For rs1900004 (*ATOH7*) as well as rs10483727 (*SIX1*), we found significant ORs for glaucoma of 1.28 (95% CI: 1.12-1.47; $p=2.49x10^{-4}$), and 1.20 (95% CI: 1.10-1.31; $p=7.65x10^{-5}$), respectively, for those homozygous for the T-allele. For rs1063192 (*CDKN2B*), we found evidence for association with glaucoma in persons heterozygous and homozygous for the G-allele. The OR for the heterozygous ones was 0.85 (95% CI: 0.77-0.94; p=0.002) and for the homozygous 0.76 (95% CI: 0.70-0.84; $p=1.41x10^{-8}$). The latter translates into a 1.32 increase in risk for the C-allele. Testing for heterogeneity showed no significant differences across the studies ($I^2<22.5\%$).

We could not find evidence for a significant association for the other loci with glaucoma when adjusting for multiple testing. Nonetheless, the associations of the homozygous effect for rs1192415 (*CDC7*/*TGFBR3*) and rs1362756 (close to *SALL1*) with glaucoma were borderline significant (p=0.044 and p=0.040, respectively). However, rs1192415 (*CDC7*/*TGFBR3*) is a rare SNP, which appeared to be monomorphic in the Southampton cohort. For this SNP, findings were inconsistent through the other studies. Finally, we evaluated whether the findings were robust when ignoring specific recessive effects for those hetero- and homozygous. The allelic effect showed significant evidence for rs1063192 (*CDKN2B*) and rs10483727 (*SIX1*; see Table S1) with glaucoma.

Table 1. Loci investigated in the current study

Most significant SNP	MA	MAF	Chromosome location	Position	Nearest gene (*symbol*; name)	Distance (b)	Quantitative trait associated with by GWAS	Direction of effect size
rs1900004	T	0.22	10q21.3-q22.1	69670887	*ATOH7*; atonal homolog 7	9021	Optic disc area/VCDR	-/-
rs1192415	G	0.22	1p22	91849685	*CDC7*/*TGFBR3*; cell division cycle 7 homolog/transforming growth factor, beta receptor III	116719	Optic disc area	+
rs1362756	C	0.28	16q12.1	50015791	*SALL1*; sal-like 1	1154095	Optic disc area	+
rs1063192	G	0.44	9p21	21993367	*CDKN2B*; cyclin-dependent kinase inhibitor 2B	0	VCDR	-
rs10483727	T	0.44	14q22-23	60142628	*SIX1*; sine oculis-related homeobox 1 homolog	39878	VCDR	+
rs17146964	G	0.20	11q13	65005721	*SCYL1*/*LTBP3*; SCY1-like 1/latent transforming growth factor beta binding protein 3	43403	VCDR	-
rs1547014	T	0.26	22q12.1	27430711	*CHEK2*; CHK2 checkpoint homolog	0	VCDR	-
rs1926320	C	0.25	13q13	35550617	*DCLK1*; doublecortin-like kinase 1	0	VCDR	+

SNP = Single nucleotide polymorphism; MA(F) = minor allele (frequency); GWAS = genome-wide association study; VCDR = vertical cup-disc ratio.

Table 2. Characteristics of the open-angle glaucoma cases presented as mean ± standard deviation (range) unless stated otherwise

	RS-I		ERF/GRIP		AGS		Erlangen and Tübingen		Southampton		deCODE	
	Cases (N=188)	Controls (N=5548)	Cases (N=104)	Controls (N=1646)	Cases (N=148)	Controls (N=148)	Cases (N=986)	Controls (N=377)	Cases (N=470)	Controls (N=232)	Cases (N=1265)	Controls (N=34886)
Age (years)	75.5±7.4 (56–94)	74.5±7.8 (55–105)	73.3±9.2 (51–91)	46.8±14.1 (18–84)	72.9±11.1 (27–98)	72.3±8.0 (55–92)	66.5±14.1 (12–104)	73.9±6.4 (34–97)	74.3±10.5 (38–96)	69.1±10.3 (50–91)	84.1±11.8 (30–103)	61.6±21.3 (5–106)
Gender, N (%) women	85 (45.2)	3289 (59.3)	55 (52.9)	942 (57.2)	68 (45.9)	84 (56.8)	600 (61.0)	101 (59.8)	232 (49.7)	119 (51.3)	682 (53.9)	19818 (56.8)
Intraocular pressure (mmHg)	18.2±6.2 (6.0–54.6)	15.2±3.5 (5.0–58.5)	25.9±8.6 (12.0–62.0)	15.4±3.0 (6.0–30.0)	26.9±8.5 (13.0–54.0)	16.7±3.4 (13.0–28.0)	27.8±9.3 (11.0–65.0)	<21.0	26.0±5.5 (14.0–54.0)	NA	NA	NA
Intraocular pressure treatment N (%)	37 (19.7)	93 (1.7)	104 (100.0)	13 (0.8)	148 (100.0)	0 (0.0)	almost 99%	0 (0.0)	470 (100.0)	0 (0.0)	NA	NA

RS = Rotterdam Study; ERF = Erasmus Rucphen Family; GRIP = Genetic Research in Isolated Populations; AGS = Amsterdam Glaucoma Study.

DISCUSSION

The present study yielded one significant gene (*CDKN2B*) involved in glaucoma in those heterozygous as well as those homozygous. The minor allele of the corresponding SNP (rs1063192) was genome-wide significantly associated with a lower VCDR and a reduced risk of glaucoma.[9] In addition, there was also significant evidence for a role of *ATOH7* and *SIX1* in glaucoma when adjusting for multiple testing. For these genes the effect appeared to be recessive, although the association remained significant when testing a multiplicative model for *SIX1*. Those homozygous for the minor allele had an increased risk of glaucoma. The three genes showed consistent evidence for a recessive effect through all cohorts. The other five regions (*CDC7*/*TGFBR3*, *SALL1*, *SCYL1*/*LTBP3*, *CHEK2* and *DCLK1*) that were previously reported to be associated with either the optic disc area or VCDR could not be significantly related to glaucoma. None of the genes was identified before in genome-wide association studies (GWAS) on glaucoma.[21,25]

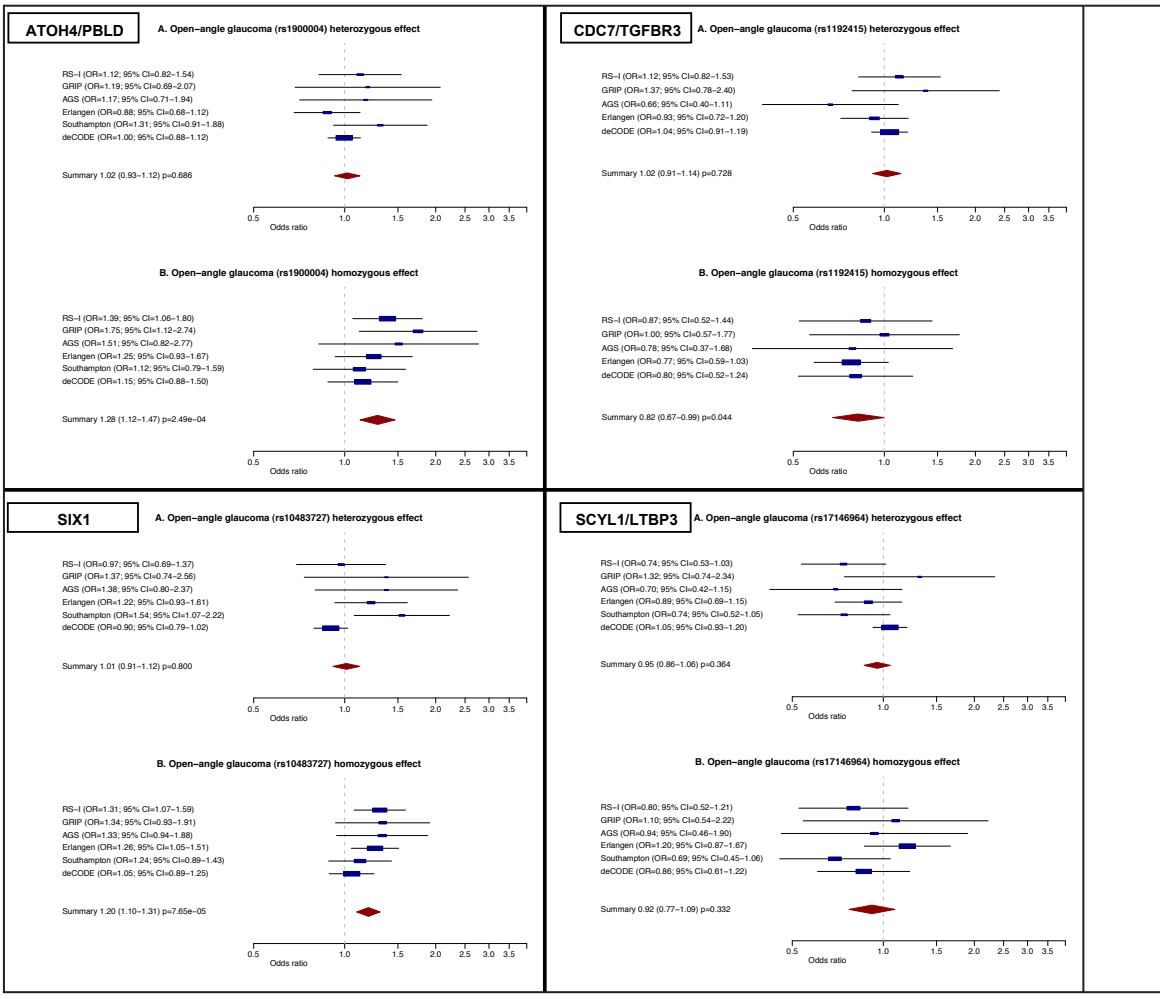
The region of *CDKN2B* has been implicated in other diseases (e.g. diabetes, myocardial infarction and gliomas).[26-28] Different variants have been associated with different disorders. The variant associated with glaucoma in our study was earlier implicated in glioma.[28] Glioma and glaucoma appear to share the same risk allele. Most of the risk variants at this locus are in non-coding regions. The consistent association with several diseases suggests these variants act by influencing the expression of nearby genes. The SNP associated with glaucoma in the current study is highly correlated with increased CDKN2B antisense RNA (*ANRIL*) expression. Thus the SNP is involved in regulating *CDKN2B* levels in blood and other tissues, suggesting that modulation of *ANRIL* expression may mediate disease susceptibility.[29] At present, little is known about the function of *ANRIL* in general and in neuronal tissue specifically.

ATOH7 has been implicated in eye development before and point to a role of early development of the optic nerve (see further). Recently, *ATOH7* has also been associated with optic nerve hypoplasia in humans.[30] Deficiency of *ATOH7* in mice may result in a critical reduction in retinal ganglion cells.[31]

SIX1 acts within a network of genes that trigger eye organogenesis.[32] These findings combined with the current findings are of interest and may shed new light on the etiology of glaucoma.

Increased intraocular pressure is the predominant risk factor for glaucoma. About half of the glaucoma patients have a statistically normal intraocular pressure. Earlier, we showed that adjustment for intraocular pressure did not alter the findings for the investigated SNPs (Table 1), in that the association with the VCDR remained significant.[9] This suggests that other mechanisms independent of intraocular pressure explain these associations. When we combine current findings with previous ones, two pathways emerge that may be relevant in addition to intraocular pressure (Figure 1). One pathway related to growth, including *CDKN2B* and *ATOH7* involved through the *TGFB* pathway, which has been implicated before in the pathogenesis of glaucoma,[33-35] and the other pathway related to development, including *SIX1*.

Figure 2. The heterozygous (A) and homozygous effect (B) of the top SNPs on open-angle glaucoma in RS-I, GRIP, AGS, Erlangen

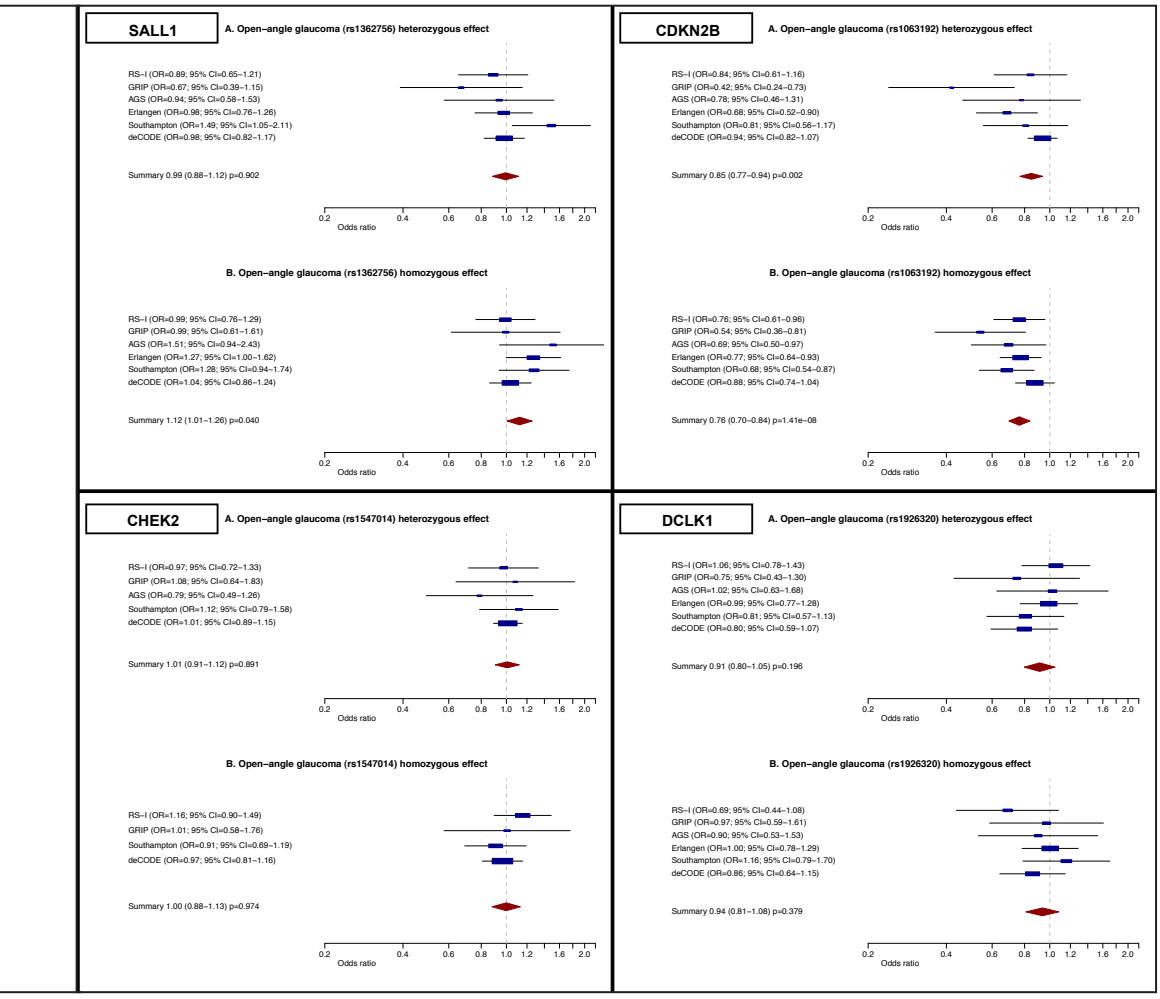


The diamonds indicate the overall OR. The size of the gray box is inversely proportional to the variance. Horizontal lines indicate and Tübingen; no participant of AGS was homozygous for this SNP. The SNP rs1192415 was monomorphic in the Southampton

Only three out of the eight loci that were previously associated with the optic disc area and VCDR were significantly associated with glaucoma in the current study. It remains to be determined whether other genes associated with the VCDR will be relevant when studying larger samples.

So far, only one genome-wide significant gene has been identified and consistently replicated for glaucoma using GWAS.[21] It is of interest that this gene (*CAV1*) interacts with *CDKN2A* through *CDKN1A* (www.ingenuity.com). Nevertheless, in the present study the gene showing the strongest association in terms of p-values is *CDKN2B*. This gene reaches

and Tübingen, Southampton, deCODE, and the joint effect. (OR=odds ratio; CI=confidence interval).



95% CI. The dashed vertical line in each panel shows the value for no effect (OR=1.0). Genotyping of rs1547014 failed in Erlangen cohort.

genome-wide significance ($p<5x10^{-8}$) in our study. At present, there is no interaction known of this gene with *CAV1*. Identification of the causal variant in the region is needed to increase our knowledge of the causal pathways.

Although this is one of the largest studies on the genetics of glaucoma, the power to detect genes with small effects is still limited. Furthermore, one of the major problems in glaucoma in general is the lack of standardized clinical criteria, which will remain a problem in future research. Despite this hampering our findings, the consistency in ORs suggests that this problem may have primarily affected the statistical power rather than heterogeneity of

glaucoma cases.

In conclusion, the present study reveals three common variants implicated in glaucoma and supports the hypothesis of the involvement of the *TGFB* pathway in glaucoma. Further exploration of our findings may include expression and translational studies. The role of these genes in non-white populations (such as some African populations with a markedly higher prevalence of glaucoma) remains to be established. Nonetheless, we could relate three of the eight loci to glaucoma, opening new avenues to improve our understanding for this common form of sight-threatening disease.

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SUPPLEMENTARY APPENDIX

Table S1. Allelic effect of the top SNPs on open-angle glaucoma in RS-I, GRIP, AGS, Erlangen and Tübingen, Southampton, deCODE, and the joint effect presented as odds ratio (95% confidence interval)

SNP	Gene	RS-I	GRIP/ERF	AGS	Erlangen and Tübingen	Southampton	deCODE	Joint effect
rs1900004	*ATOH7*	1.269 (1.007-1.600)	1.512 (1.016-2.252)	1.300 (0.863-1.958)	1.023 (0.839-1.248)	1.207 (0.918-1.585)	1.029 (0.931-1.137)	1.090 (1.010-1.177) p=0.027
rs1192415	*CDC7/TGFBR3*	1.040 (0.795-1.360)	1.180 (0.773-1.801)	0.696 (0.443-1.093)	0.859 (0.699-1.057)	NA	0.996 (0.885-1.121)	0.996 (0.984-1.007) p=0.451
rs1362756	*SALL1*	0.948 (0.755-1.191)	0.826 (0.546-1.249)	1.214 (0.839-1.756)	1.133 (0.937-1.370)	1.369 (1.055-1.776)	1.027 (0.943-1.118)	1.054 (0.984-1.130) p=0.136
rs1063192	*CDKN2B*	0.774 (0.626-0.956)	0.499 (0.338-0.738)	0.705 (0.510-0.974)	0.754 (0.634-0.898)	0.690 (0.542-0.878)	0.936 (0.861-1.018)	0.845 (0.791-0.903) p=5.90x10^{-7}*
rs10483727	*SIX1*	1.277 (1.037-1.572)	1.344 (0.936-1.931)	1.335 (0.947-1.883)	1.254 (1.052-1.495)	1.197 (0.946-1.513)	1.003 (0.924-1.089)	1.097 (1.027-1.172) p=0.006*
rs17146964	*SCYL1/LTBP3*	0.760 (0.577-0.999)	1.228 (0.773-1.949)	0.763 (0.493-1.181)	1.001 (0.813-1.233)	0.722 (0.541-0.963)	1.007 (0.902-1.124)	0.950 (0.874-1.032) p=0.227
rs1547014	*CHEK2*	1.082 (0.862-1.357)	1.032 (0.683-1.559)	0.788 (0.491-1.264)	NA	0.973 (0.761-1.243)	0.990 (0.910-1.077)	0.993 (0.923-1.069) p=0.858
rs1926320	*DCLK1*	0.906 (0.706-1.162)	0.861 (0.570-1.300)	0.966 (0.653-1.430)	0.998 (0.821-1.212)	0.949 (0.724-1.244)	1.007 (0.912-1.111)	0.999 (0.923-1.081) p=0.682

*= Joint effect significant at a Bonferroni adjusted p-value of 0.006 (0.05/8 SNPs); SNP = single nucleotide polymorphism; RS = Rotterdam Study; ERF = Erasmus Rucphen Family; GRIP = Genetic Research in Isolated Populations; AGS = Amsterdam Glaucoma Study; Genotyping of rs1547014 failed in Erlangen and Tübingen; no participant of AGS was homozygous for this SNP. The SNP rs1192415 was monomorphic in the Southampton cohort.

Clinical implications of old and new genes for open-angle glaucoma

ABSTRACT

Objective: Genome-wide association studies have revealed new insights into the genetic determinants of open-angle glaucoma (OAG). We performed a study to determine to what extent variants within established genes (*MYOC*, *OPTN* and *WDR36*) and newly identified common genetic variants (*ATOH7*, *CDKN2B* and *SIX1*) contribute to the risk of OAG.

Design: a population-based setting, family-based setting and a case-control study.

Participants: The Rotterdam Study I cohort (N=5312; mean age±standard deviation [SD]: 68.0±8.4 years). Findings were replicated in the Genetic Research in Isolated Populations combined with the Erasmus Rucphen Family study (N=1750; mean age±SD: 48.3±15.2 years), and a cohort from Southampton (N=702; mean age±SD: 72.5±10.7 years).

Methods: After identifying common variants associated with OAG within the established genes, risk of OAG was analyzed using logistic regression. Discriminative accuracy was assessed by comparing the area under the receiver operator characteristic (ROC) curves (AUC) for models including the number of risk alleles, intraocular pressure (IOP), age and gender, with the AUC for the same model but without the risk alleles.

Main outcome measures: Odds ratios and AUCs of individual and combined risk alleles.

Results: No consistent significant associations for the established genes (*MYOC*, *OPTN* and *WDR36*) with OAG were found. However, when comparing the load of risk variants between cases and controls, 2 of 3 studies showed a significant increased risk of OAG for participants carrying more risk alleles of the three established genes. When combining all 6 genes, participants carrying a high number of risk alleles (highest tertile) had a 2.29-fold to 3.19-fold increase in risk of OAG compared with those carrying only a few risk alleles. The addition of the newly identified genes to IOP, age and gender resulted in a higher AUC compared with the AUC without the newly identified genes (p=0.027).

Conclusions: We found a significant contribution to the risk of OAG for the new common variants identified by recent genome-wide association studies, but not for variants within the established genes. Participants carrying a high number of risk alleles had an about 3-fold increase in the risk of OAG compared with those with a low number of risk alleles.

INTRODUCTION

Open-angle glaucoma (OAG) is a neurodegenerative disease that leads to progressive damage to the retinal ganglion cells and nerve fibers, resulting in visual field loss. It is one of the leading causes of irreversible blindness in the world.[1] Despite decades of research, the etiology of OAG remains obscure although it has been recognized for a long time that a positive family history of OAG is a risk factor and that genes may be involved in the pathogenesis of OAG.[2-4] To date 3 genes have been established (*MYOC*, *OPTN* and *WDR36*) for late-onset OAG.[5] Various mutations in these genes have a large impact on the risk of OAG, although this explains the disease only in a limited number of families. Recently, a common variant involved in OAG related pathology was discovered,[6] and it was shown that common genetic variants associated with the clinically important vertical cup-disc ratio (VCDR) may overlap with those of OAG.[7] Three genes (*ATOH7*, *CDKN2B* and *SIX1*) that are implicated in VCDR are associated consistently with OAG across populations.[8-10] Compared with the effect of mutations in the established genes, the variants in the newly identified genes are more frequent but individually they seem to have small effects, emphasizing the existence of polygenic and complex forms of OAG.

The aim of the present study was to determine the clinical implications of the joint effect of variants within the established genes and of the newly identified common variants or single nucleotide polymorphisms (SNPs) in OAG. For this purpose, the regions of the established genes (*MYOC*, *OPTN* and *WDR36*) were explored to find the most significant SNP for OAG in the population-based Rotterdam Study I (RS-I). Next, the risk of each gene was analyzed separately and combined, and whether having more risk alleles of established and newly identified genes increased the risk of OAG was determined. Results were replicated in 2 independent populations: 1) a series of OAG cases from the Genetic Research in Isolated Populations (GRIP) program using the family-based Erasmus Rucphen Family (ERF) study as controls and 2) a case-control study from Southampton. Finally, the contribution of the established and recently discovered genes to the discriminative accuracy was determined by comparing the area under the receiver operator characteristic (ROC) curves (AUC) for models including the number of risk alleles in the established and newly identified genes, age, gender, and intraocular pressure (IOP), with the AUC for a model including only age, gender, and IOP.

METHODS

Study Populations

The RS-I is a prospective, population-based cohort study of 7983 residents aged 55 years and older living in Ommoord, a suburb of Rotterdam, the Netherlands. Baseline examination for the ophthalmic part took place between 1991 and 1993; follow-up examinations were

performed from 1997 to 1999 and from 2002 to 2006.[11] A total of 5974 participants were available with genotyping data (see below), of whom 5736, including 188 OAG cases, had reliable optic disc measurements and visual fields.

For cases of GRIP (N=104) medical records in 3 local hospitals were assessed to identify patients with OAG. The ERF study, arising from the same population base served as a control group (N=1646) for GRIP. The ERF study is a family-based cohort in a genetically isolated population in the southwest of the Netherlands with more than 3000 participants between 18 and 86 years of age. Cross-sectional examination took place between 2002 and 2005.[12,13] OAG cases of GRIP were ascertained independently of ERF, but lived in the same region. After exclusion of participants for whom genotyping data were unavailable, 2385 had genotyping data, of which 1646 had reliable optic disc data.

From Southampton, glaucoma cases and controls (N=470 and 232) were recruited in 2005 and collected from glaucoma specialist and general clinics at the Southampton Eye Unit, United Kingdom.[14] Controls were selected from a previously collected control cohort.[15]

All cohorts consisted of Caucasian participants. All measurements in these studies were conducted after the Medical Ethics Committee of the Erasmus University (the Netherlands), and the Southampton and South West Hampshire Local Research Ethics Committee (United Kingdom) approved the study protocols and all participants had given a written informed consent in accordance with the Declaration of Helsinki.

Ophthalmic examination

The ophthalmic assessment in RS-I included a medical history, autorefraction, keratometry, visual field testing (Humphrey Field Analyzer II 740 [HFA]; Carl Zeiss, Oberkochen, Germany) and optic nerve head imaging with Topcon ImageNet System (Topcon Corporation, Tokyo, Japan) and Heidelberg Retina Tomograph (Heidelberg Engineering, Dossenheim, Germany) of both eyes after mydriasis with tropicamide 0.5% and phenylephrine 2.5%.

In GRIP visual fields were tested with standard automated perimetry by means of the HFA 24-2 Swedish interactive threshold algorithm standard test program or the Octopus 101 (Haag-Streit, Bern, Switzerland) G2 program with TOP strategy. The ophthalmic assessment in the ERF study was similar to that of RS-I, but no visual field testing was included. Glaucoma was excluded in these controls by two of the authors (LMEvK and HGL), who checked the medical records for any glaucoma pathologic features.

Patients from Southampton were examined by an experienced glaucoma specialist at the Southampton University Hospital Eye Unit. The examination included medical history, biomicroscopy, and visual field testing using the HFA 24-2 and HFA 30-2.

In all 3 cohorts, the IOP was measured with Goldmann applanation tonometry (Haag-Streit, Bern, Switzerland) after applying oxybuprocaine 0.4% eye drops. In the RS-I and the ERF study the median value of 3 successive measurements was recorded.[16]

Open-angle glaucoma diagnosis

In the RS-I, OAG diagnosis was primarily based on glaucomatous visual field loss, and not on VCDR.[17] The visual field of each eye was screened using a 52-point supra-threshold test that covered the central visual field with a radius of 24°.[18,19] In participants with reproducible visual field loss on repeat second supra-threshold testing or unreliable tests, Goldmann kinetic perimetry (Haag-Streit, Bern, Switzerland) or full-threshold HFA testing with 24-2 grid was performed on both eyes by a skilled perimetrist. Details about the classification process have been described before.[17,18] In short, visual field loss was considered to be glaucomatous visual field loss only if reproducible and after excluding all other possible causes using all available data. Cases had to have an open anterior chamber angle and no history or signs of secondary OAG or manifest exfoliation were allowed.

In GRIP, the diagnosis of OAG was made by the ophthalmologist in attendance and was 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 upon gonioscopy. Visual field test results had to be reliable and reproducible. Patients with any other disease that could cause visual field defects were excluded.

Details of the OAG cases from Southampton have been reported previously.[14] In brief, diagnosis was made on the basis of characteristic glaucomatous visual field loss or glaucomatous optic disc damage or increased IOP. Patients presenting with narrow-angle, developmental, or secondary glaucoma or any other known abnormalities of the anterior segment were excluded. Controls had no history of glaucoma and were not receiving any treatment to lower IOP. As in the other studies, participants with any other disease that could cause visual field loss were excluded.

Genotyping

In the RS-I cohort, participants were genotyped using the Illumina Infinium II HumanHap 550 chip v3.0 array according to the manufacturer's protocols. Details are described elsewhere.[20] In the ERF study and GRIP, participants were genotyped using four different platforms (Illumina 6k, Illumina 318K, Illumina 370K and Affymetrix 250K), which were then merged. Because the same microarray was not used for the different study populations, the genotype data was imputed using HapMap CEU as reference population, resulting in over 2.5 million SNPs. Investigated SNPs were extracted from this database. Genotyping of Southampton cases and controls was carried out using KASPar chemistry (KBioscience, Hoddesdon, United Kingdom).

Statistical analysis

Statistical analysis within the cohorts

In cases with unilateral OAG, the eye with OAG was selected; in all other cases, a random eye was chosen. For IOP the highest value of both eyes was included and adjusted for IOP or glaucoma treatment. This adjustment was done by adding 30% upon the measured IOP for medical treatment and by fixing the IOP at 30 mmHg for surgical or laser treatment.[21][Van Koolwijk, et al.; Chapter 4.3] A p-value of 0.05 or less was considered statistically significant. All statistical analyses were performed using SPSS version 15.0.0 for Windows (SPSS inc., Chicago, IL, USA; 2006) and R statistical package version 2.9.1 for Mac (www.r-project.org).

Cumulative risk of common variants for open-angle glaucoma

Because no in-depth assessment of rare variants in *MYOC*, *OPTN* and *WDR36* was available, first, all genotyped and imputed SNPs in the regions of the established genes (within 200 kb distance from *MYOC*, *OPTN* and *WDR36*) were analyzed and their association with OAG were evaluated. SNPs that deviated significantly from Hardy-Weinberg equilibrium ($p<0.0001$) or that had a minor allele frequency <0.05 were excluded. The SNP with the strongest association with OAG in terms of p-values was selected for the corresponding gene. For each SNP we applied logistic regression to calculate risk of OAG as odds ratio (OR) with corresponding 95% confidence interval (CI), and the explained variance (Nagelkerke R^2). All analyses were adjusted for age and gender. To calculate the cumulative genetic risk, the risk alleles of the respective SNPs in the OAG cases was summed, they were divided into tertiles (low-risk, medium-risk and high-risk group) and subsequently the ORs of OAG were calculated, with the first tertile (i.e., the group with lowest amount of risk alleles) serving as the reference. The trend of the risk alleles was assessed by computing the predictive probabilities with 95% CI for OAG. Next, the same approach was applied to analyze the 3 novel loci (*ATOH7*, *CDKN2B* and *SIX1*) recently identified by genome-wide association (GWA) studies on VCDR after inspecting the distribution of the SNPs in cases and controls. For this purpose, all SNPs were analyzed in separate models to determine which allele resulted in an OR of more than 1 for OAG. The genotyped data were imputed, and therefore the allele dosages of the SNPs were recoded as follow: <0.5=0, 0.5 to 1.5=1 and >1.5=2. All regression analyses were adjusted for age and gender.

Finally, the discriminating ability for the established genes and for all genes combined were compared by comparing the AUC using a paired test, as described by Hanley and McNeil.[22,23] This was carried out by plotting the predictive probabilities from logistic regression models, including the number of risk alleles of the established genes, and all assessed SNPs. The AUCs of these models were compared with the AUC of the model including only 1 of the strongest known risk factors for glaucoma, IOP adjusted for age and gender. The IOP measurements were not performed in controls in the Southampton cohort; therefore this analysis was carried out only in RS-I. A secondary analysis was performed to evaluate the difference in risk between the studies. For this purpose, a genetic risk score prediction model was created using logistic regression.[24] In this model, we included only the number of risk alleles of the 6 genes and did not include IOP. The risk score was calculated as a weighted

sum of the number of risk alleles multiplied by the logarithm of the OR (see above) for each of the individual genes for the specific cohorts. The percentage of the total variance in OAG explained by the risk score was estimated by Nagelkerke R^2 from the logistic regression model, with the risk score as a quantitative predictor and OAG as the outcome. The C-statistic was estimated as an AUC provided by the above-mentioned model.

RESULTS

A total of 762 OAG cases and 7426 controls spread over 3 cohorts were included. Table 1 summarizes the general characteristics of the 3 cohorts. There was no significant difference in age between cases and controls in the RS-I (p=0.083), but cases were more often men (p<0.001). Controls from the ERF study were significantly younger (p<0.001) compared with cases from GRIP as well as to the other cohorts. In both the GRIP and the ERF cohort and Southampton cohort, no difference in gender was found (p=0.688 and p=0.381, respectively). Cases from the Southampton cohort were significantly older than their controls (p<0.001).

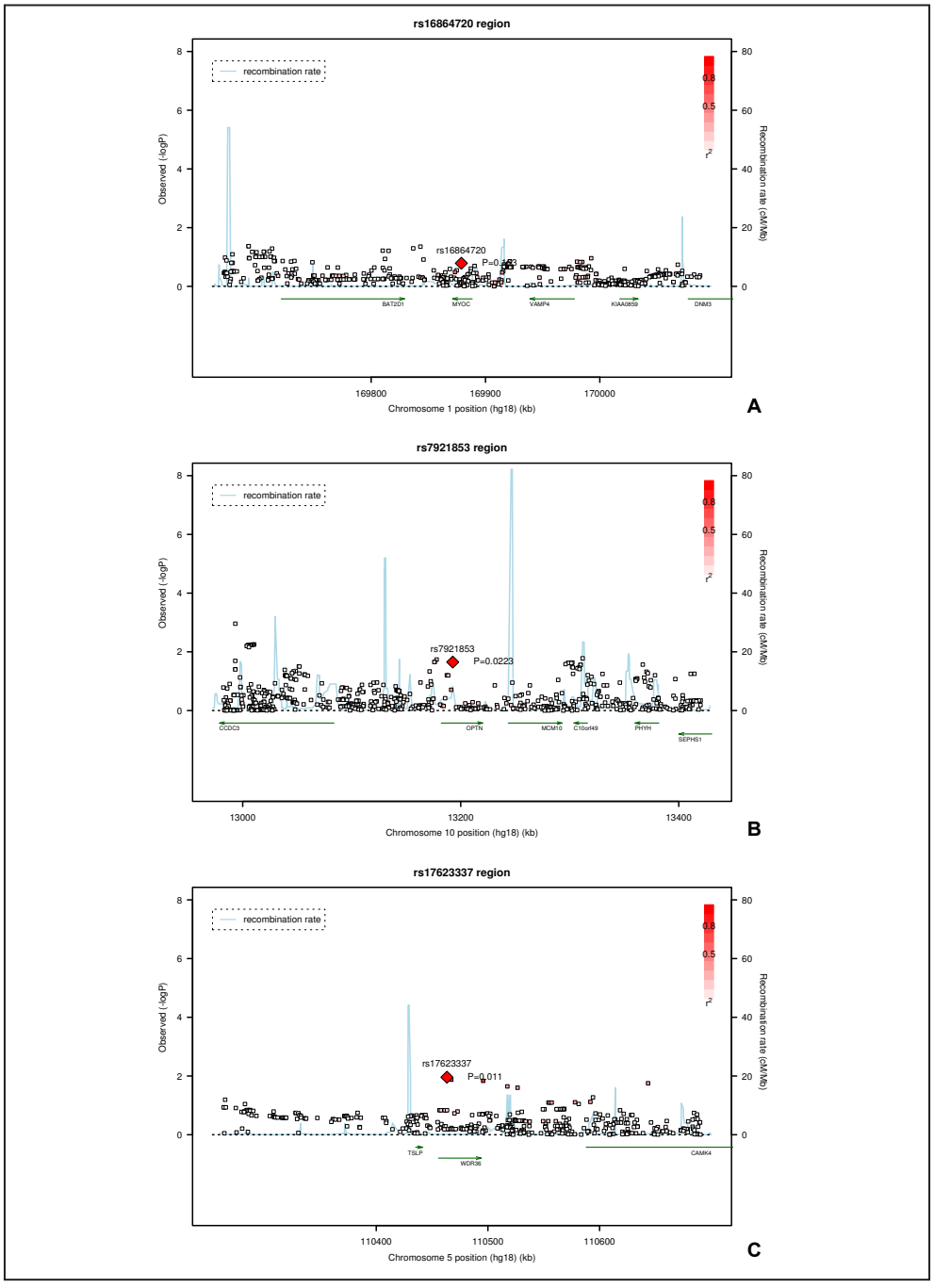
First, the association of *MYOC*, *OPTN* and *WDR36* were evaluated with OAG in RS-I. The SNPs in regions including the genes *MYOC*, *OPTN* and *WDR36* showed marginal evidence for an association with OAG when adjusting for the number of SNPs tested in these

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

	RS-I		GRIP/ERF		Southampton	
	Cases (N=188)	Controls (N=5548)	Cases (N=104)	Controls (N=1646)	Cases (N=470)	Controls (N=232)
Age (years)	75.5 ± 7.4 (56 - 94)	74.5 ± 7.8 (55 - 105)	73.3 ± 9.2 (51 – 91)	46.8 ± 14.1 (18 – 84)	74.3 ± 10.5 (38 – 96)	69.1 ± 10.3 (50 – 91)
Gender, N(%) women	85 (45.2)	3289 (59.3)	55 (52.9)	942 (57.2)	232 (49.7)	119 (51.3)
Vertical cup-disc ratio*	0.59 ± 0.15 (0.15 – 0.89)	0.50 ± 0.14 (0.00 – 0.83)	NA	0.46 ± 0.15 (0.00 – 0.84)	0.71 ± 0.14 (0.08 – 1.00)	NA
Disc area (mm^2)*	2.46 ± 0.57 (1.18 – 4.76)	2.42 ± 0.48 (0.58 – 5.44)	NA	1.92 ± 0.37 (1.07 – 4.33)	NA	NA
Intraocular pressure (mmHg)	18.2 ± 6.2 (6.0 – 54.6)	15.2 ± 3.5 (5.0 – 58.5)	25.9 ± 8.6 (12.0 – 62.0)	15.4 ± 3.0 (7.0 – 30.0)	26.0 ± 5.5 (14.0 – 54.0)	NA, but <21.0
Treatment for intraocular pressure, N(%)	37 (19.7)	93 (1.7)	104 (100.0)	13 (0.8)	almost 99%	0 (0.0)

RS = Rotterdam Study; ERF = Erasmus Rucphen Family; GRIP = Genetic Research in Isolated Populations; * = In RS-I measured with Topcon ImageNet System, in ERF with Heidelberg Retina Tomograph, and in Southampton with slit lamp clinical examination.

Figure 1. Regional plots of the three genes *MYOC*, *OPTN* and *WDR36* (respectively A to C) with their association with open-angle glaucoma in the Rotterdam Study-I.



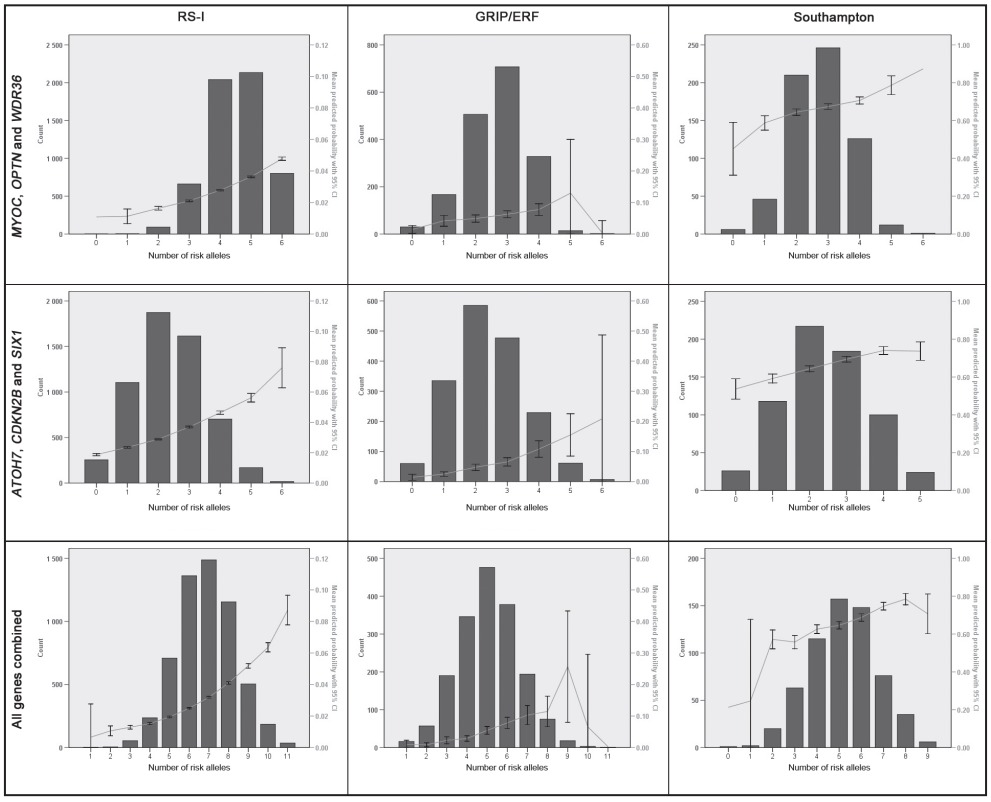
regions (Figure 1). The SNPs within the genes *MYOC*, *OPTN* and *WDR36* with the strongest association with OAG in terms of p-values were rs16864720 (p=0.164), rs7921853 (p=0.025) and rs17623337 (p=0.012), respectively.

Table S2 shows the ORs for OAG for the established genes (*MYOC*, *OPTN* and *WDR36*) and the 3 newly identified genes (*ATOH7*, *CDKN2B* and *SIX1*) in all 3 cohorts. The associations were not significant through the 3 cohorts for *MYOC*, *OPTN* and *WDR36*, but the ORs pointed to the same direction. Although the p-value for *MYOC* was only 0.164 in the RS-I, the selected SNPs were significant at a p-value of 0.05 in the GRIP and ERF cohorts and Southampton cohort. When combining the effects of these 3 genes, a significant increased frequency of the SNPs was seen in the RS-I (p-trend=0.008) and Southampton cohort (p-trend=0.041), and borderline significant in the GRIP and ERF cohort (p-trend=0.062; Table S3). The joint effect of the risk alleles of *MYOC*, *OPTN* and *WDR36* explained a small proportion of OAG (0.9%, 0.4% and 2.1% in RS-I, GRIP/ERF and Southampton, respectively). The joint effect of the risk alleles of the three newly identified genes (*ATOH7*, *CDKN2B* and *SIX1*) explained together 1.1%, 2.2% and 2.2% of OAG in the RS-I, the GRIP and ERF cohort and Southampton cohort, respectively. In all studies, the explained variance of the newly identified genes exceeded that of *MYOC*, *OPTN* and *WDR36*. Participants carrying more risk alleles had a significantly higher risk of OAG (in each study p<0.002; Table S3).

As expected, when combining the number of risk alleles of all SNPs (i.e., *MYOC*, *OPTN*, *WDR36*, *ATOH7*, *CDKN2B* and *SIX1*) each allele resulted in a significant increase in risk of OAG (RS-I: OR, 1.29; 95%CI, 1.17-1.43; $p=2.96x10^{-7}$, GRIP and ERF: OR, 1.48; 95%CI 1.23-1.78; $p=2.35x10^{-5}$; Southampton: OR, 1.22; 95%CI, 1.09-1.37; p=0.001). The association was independent of IOP, in that the significance did not diminish when adding IOP to the model (RS-I: OR, 1.32; 95%CI 1.20-1.46; $p=5.14x10^{-8}$; GRIP and ERF: OR, 1.64; 95%CI, 1.66-2.25; p=0.001). Participants carrying a high number of risk alleles (i.e., those in the high-risk tertile) had a 2.29 to 3.19-fold increase in risk of OAG compared with those in the low-risk tertile (Table S3). Figure 2 shows the distributions and predicted probabilities of the established genes, the newly identified genes, and all genes combined for OAG in all cohorts. In the population-based, family-based and case-control study almost all participants carried at least one risk allele.

Discriminating ability of the genes for OAG was analyzed by comparing the ROC curves of four models (Figure 3). In the RS-I the AUC for the model including only age and gender was 0.59 (95% CI, 0.58-0.61), and increased significantly when adding IOP to the model (AUC: 0.68; 95% CI, 0.67-0.70; p<0.001). In the third model, that is, when adding the number of risk alleles of *MYOC*, *OPTN* and *WDR36* to the model, the AUC increased slightly to 0.70 (95% CI, 0.68-0.71), though the ROC curve was not significantly different from that of the previous model, that is, without the established genes (p=0.28). The fourth model included *ATOH7*, *CDKN2B* and *SIX1*. The addition of these genes showed a significant increase in discriminative accuracy (AUC: 0.72; 95% CI, 0.71-0.73; p=0.024 compared with the model without the risk alleles) for OAG. To compare the cumulative risk conferred by these variants

Figure 2. Predicted probabilities (lines) and distribution (bars) of the number of risk alleles (for *MYOC*, *OPTN* and *WDR36* [upper row], for *ATOH7*, *CDKN2B*, and *SIX1* [middle row], and for all genes combined [lower row], respectively) and open-angle glaucoma for all cohorts.



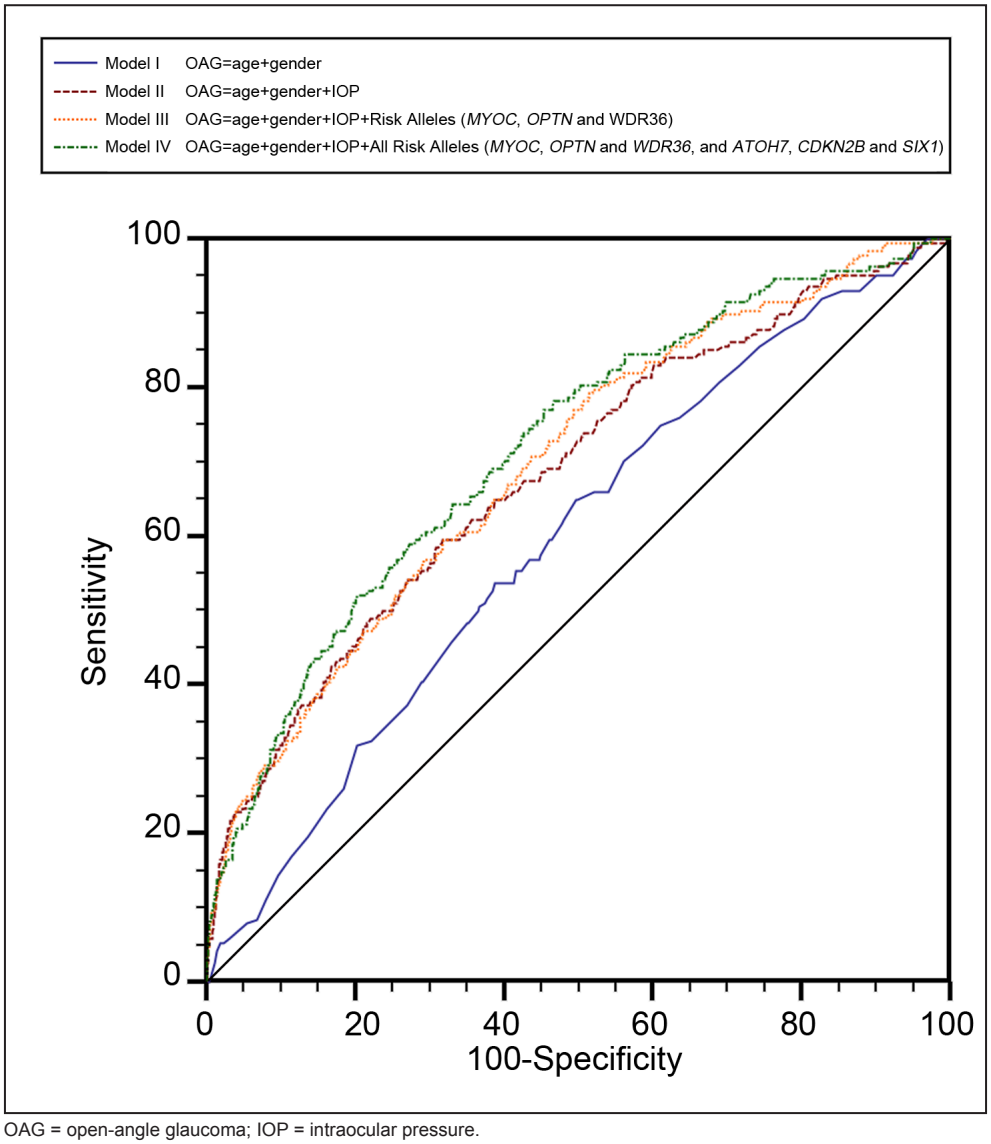
Vertical black lines represent 95% confidence interval.

in RS-I with the 2 other cohorts (GRIP and ERF cohort and Southampton cohort), a genetic risk score was computed (Table S4). The risk score model was similar in all cohorts and collectively explained 4–6% of the variation in risk of OAG for all 6 genes combined.

DISCUSSION

In the present study, *MYOC* was the only established gene that was consistently associated with OAG. However, when combining *MYOC* with the other 2 established genes (*OPTN* and *WDR36*), participants carrying a high number of risk alleles of common variants within these genes had a significantly increased risk of OAG in 2 of 3 studies. The addition of the newly identified genes (*ATOH7*, *CDKN2B* and *SIX1*) contributed significantly to the prediction of

Figure 3. Area under the Receiver Operator Characteristic (ROC) curves for four models with open-angle glaucoma in the Rotterdam Study-I.



OAG = open-angle glaucoma; IOP = intraocular pressure.

OAG. When combining the number of risk alleles, each risk allele resulted in an OR varying from 1.22 to 1.48 for OAG. When comparing participants in the high-risk tertile with those in the low-risk tertile, a 3-fold increase in the risk of OAG was seen for those in the high-risk tertile.

When combining all risk alleles of established genes and newly identified genes

together it becomes clear that these risk alleles are widely spread through the populations (Figure 2; Table S4). Regarding the number of risk alleles, Janssens and van Duijn[25] showed that if there are more than 30 genetic risk variants associated with a disease, no person in a population carries 0 risk alleles but everyone carries a gradient number of risk alleles. When considering the risk alleles from the (only) 6 SNPs investigated in the current study, only 1 participant from the 3 cohorts carried zero risk alleles. This participant was one of the controls from the Southampton case-control study.

The common variants used in the current study for the genes *MYOC*, *OPTN* and *WDR36*, were identified in RS-I by analyzing all SNPs within a window of 200 kb around these genes. However, none of the common variants within the genes *MYOC*, *OPTN* and *WDR36* were even close to genome-wide significance ($p<5x10^{-8}$). This does not exclude that these rare variants may be involved in the etiology of, in particular, familial OAG cases, as GWA studies do not cover these variants which are difficult to assess, nor was there sufficient power to detect the effects of such rare variants in the present study.[26,27] Frequencies of mutations in all 3 established genes were reported to be moderate and were associated with OAG in only a small fraction of patients.[28] Thus, extremely large sample sizes are needed to detect and investigate these variants. In the general population, in which also the bulk of patients with sporadic forms of the disease are included, these rare variants are found in no more than 3% to 5% of the patients.[5] Earlier, the RS-I revealed that only 2.2% of participants with OAG carried a high-risk mutation in *MYOC*.[29] Another study reported a similar overall frequency (approximately 2%-4%) of *MYOC* mutations in 5 populations (of Caucasian, Australian, Canadian, African American and Asian origin).[30] The frequency of polymorphisms within the *OPTN* gene in patient populations varies with regard to ethnic background and is very low.[31] The same also holds for *WDR36*.[32] Variants within these genes may modify disease severity of individuals with OAG, including patients with *MYOC* mutations.[33] Therefore the effects of common variants were targeted and the effect of the genes was pooled. Although the causative variants are not included on GWA study arrays, there is debate on the question whether these variants can be captured by common variants and create synthetic association.[34,35] The RS-I was used to detect these variants and the findings were replicated in the GRIP and ERF cohort and Southampton cohort. When combining these genes with the newly identified genes, a consistent increase in risk of OAG with the number of risk alleles in all 3 studies is clear. The newly identified genes were primarily found to be associated with optic disc parameters, for example, VCDR. This might induce some confounding due to the relationship between VCDR and OAG; however, of note is that the effect of the genes remained significant ($p<0.001$ in RS-I) when additionally adjusting for IOP, VCDR and optic disc area. This suggests that other – yet unknown – mechanisms (independent of IOP) may be involved in the pathogenesis of OAG. Furthermore, neither IOP nor VCDR nor optic disc area were included in the definition of OAG in the RS-I.

Overall the results in the RS-I were slightly better than the GRIP and ERF cohort and the Southampton cohort. This may be because the variants within the established genes were

identified in the RS-I. Further, among the 3 cohorts the RS-I was by far the largest cohort. Previously the newly identified variants were identified in the RS-I,[9] which might be a third reason explaining the slightly better results of the RS-I.

Regarding the assessed SNPs of the newly identified genes used in this study, are all based on genetic studies of OAG. GWA studies on glaucoma have revealed other SNPs to be associated with OAG.[6,36] However, these SNPs could not be replicated consistently in the present study (Table S5). A possible explanation for the non-significant findings in the RS-I may be the number of OAG cases compared with the studies that revealed these SNPs. The significance found in the GRIP and ERF cohort may be explained by that the OAG cases of GRIP consisted of more severe OAG cases in terms of IOP-levels and IOP-lowering treatment compared with the RS-I (Table 1). Further research is needed to validate these and the investigated SNPs by replication studies.

In addition to age and gender, the IOP is in clinical practice used as the main predictor of OAG. The AUC based on these determinants was 0.68 in the RS-I. A similar AUC for IOP, age and gender has also been found in the Blue Mountain Eye Study (AUC, 0.67). In the Blue Mountain Eye Study the discriminative accuracy of several visual tests did not differ (AUC range, 0.67 [for IOP] – 0.87 [for visual field loss]).[37] Although the increase in AUC was marginal, the addition of the risk alleles of all genes combined resulted in a significant improvement of the AUC (AUC, 0.72) of the model including only age, gender and IOP without the risk alleles.

In conclusion, the clinical implications of the current knowledge of the genetics of OAG were explored. None of the newly discovered or established genes explained a significant proportion of OAG in the general population on its own. However, by combining all available genetic information in a single risk profile, where the total number of risk alleles of all genes together is counted, ORs of about 3 were reached, which is similar to that of, for example, myopia with OAG.[17,38-41] This suggests that, albeit not yet routinely, genetic counseling may become helpful in challenging diagnostic cases, in particular when in the near future new OAG genes will be found by new generation genome studies.

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Table S2. Association of single nucleotide polymorphisms of *MYOC*, *OPTN* and *WDR36*, and three new indentified loci with open-angle glaucoma

								RS-I		GRIP/ERF		Southampton	
	Chromosome (base pair) location	Closest gene	Coded allele (Al1)	Non-coded Allele (Al2)	Coded Allele Frequency	Allele with OR>1	Allele with OR>1 Frequency	OR (95% CI)	p-value	OR (95% CI)	p-value	OR (95% CI)	p-value
Single nucleotide polymorphisms of established genes													
rs16864720	1q23-q24 (169878890)	*MYOC*	G	A	0.86	G	0.86	1.25 (0.91-1.72)	0.164	1.72 (1.06-2.82)	0.030*	1.58 (1.14-2.19)	0.006*
rs7921853	10p13 (13192672)	*OPTN*	T	G	0.45	T	0.45	1.26 (1.03-1.54)	0.025*	1.50 (0.52-4.29)	0.455	1.06 (0.84-1.32)	0.646
rs17623337	5q22.1 (110463317)	*WDR36*	G	A	0.94	G	0.94	2.17 (1.19-3.97)	0.012*	1.02 (0.70-1.48)	0.923	1.29 (0.73-2.26)	0.382
New identified single nucleotide polymorphisms													
rs1900004	10q21.3-q22.1 (69670887)	*ATOH7*	C	T	0.78	T	0.22	1.27 (1.01-1.60)	0.043*	1.51 (1.02-2.25)	0.042*	1.21 (0.92-1.59)	0.177
rs1063192	9q21 (21993367)	*CDKN2 B*	A	G	0.55	A	0.55	1.29 (1.05-1.60)	0.017*	2.00 (1.36-2.96)	<0.001*	1.45 (1.14-1.84)	0.003*
rs10483727	14q22-23 (60142628)	*SIX1*	C	T	0.59	T	0.41	1.28 (1.04-1.57)	0.021*	1.34 (0.94-1.93)	0.110	1.20 (0.95-1.51)	0.134

* = significant at a p-value of 0.05; OR = odds ratio; CI = confidence interval; RS = Rotterdam Study; GRIP = Genetic Research in Isolated Populations; ERF = Erasmus Rucphen Family.

Table S3. Risk of combined risk alleles for *MYOC*, *OPTN*, *WDR36* and three new genes for open-angle glaucoma

	RS-I	GRIP/ERF	Southampton
MYOC, *OPTN* and *WDR36*			
Number of risk alleles. Mean ± SD (range)	4.5 ± 0.9 (0 – 6)	2.7 ± 1.0 (0 – 6)	2.7 ± 0.9 (0 – 6)
Medium-risk tertile, OR (95% CI)	1.08 (0.77-1.50)	1.16 (0.64-2.10)	1.61 (1.10-2.37)*
High-risk tertile, OR (95% CI)	1.81 (1.23-2.67)*	1.97 (0.99-3.90)	1.48 (0.94-2.32)
p-trend	0.008*	0.062	0.041*
ATOH7, *CDKN2B* and *SIX1*			
Number of risk alleles. Mean ± SD (range)	2.3 ± 1.2 (0 – 6)	2.4 ± 1.2 (0 – 6)	2.4 ± 1.2 (0 – 5)
Medium-risk tertile, OR (95% CI)	1.33 (0.95-1.87)	1.94 (1.05-3.60)*	1.62 (1.07-2.44)*
High-risk tertile, OR (95% CI)	1.92 (1.32-2.78)*	3.20 (1.70-6.02)*	2.24 (1.30-3.84)*
p-trend	0.001*	<0.001*	0.002*
All genes combined			
Number of risk alleles. Mean ± SD (range)	6.8 ± 1.5 (1 – 11)	5.1 ± 1.5 (1 – 11)	5.2 ± 1.5 (0 – 9)
Medium-risk tertile, OR (95% CI)	1.43 (1.03-2.01)*	2.09 (1.13-3.85)*	1.26 (0.86-1.85)
High-risk tertile, OR (95% CI)	2.32 (1.53-3.51)*	3.19 (1.65-6.16)*	2.29 (1.34-3.93)*
p-trend	<0.001*	<0.001*	0.004*

* = significant at a p-value of 0.05; SD = standard deviation; OR = odds ratio; CI = confidence interval; RS = Rotterdam Study; GRIP = Genetic Research in Isolated Populations; ERF = Erasmus Rucphen Family.

Table S4. Distribution and risk scores for the cumulative risk alleles for *MYOC*, *OPTN*, *WDR36* and three new genes for open-angle glaucoma

	RS-I		GRIP/ERF		Southampton	
Number of risk alleles	Frequency, cases/controls	Risk score**, Mean±s.d.	Frequency, cases/controls	Risk score**, Mean±s.d.	Frequency, cases/controls	Risk score**, Mean±s.d.
0	-	-	-	-	0.00 / 0.01	0.00 ± 0.00
1	0.00 / 0.00	0.04 ± 0.00	0.00 / 0.01	0.08 ± 0.02	0.00 / 0.01	0.06 ± 0.00
2	0.00 / 0.00	0.19 ± 0.08	0.00 / 0.04	0.17 ± 0.03	0.02 / 0.05	0.13 ± 0.03
3	0.01 / 0.01	0.25 ± 0.05	0.05 / 0.11	0.25 ± 0.03	0.09 / 0.14	0.17 ± 0.03
4	0.01 / 0.04	0.31 ± 0.05	0.09 / 0.20	0.33 ± 0.04	0.19 / 0.17	0.22 ± 0.04
5	0.05 / 0.13	0.36 ± 0.04	0.26 / 0.27	0.41 ± 0.04	0.23 / 0.29	0.25 ± 0.03
6	0.25 / 0.24	0.40 ± 0.03	0.30 / 0.21	0.48 ± 0.04	0.25 / 0.22	0.29 ± 0.03
7	0.25 / 0.26	0.45 ± 0.03	0.18 / 0.11	0.56 ± 0.04	0.16 / 0.06	0.32 ± 0.03
8	0.24 / 0.20	0.49 ± 0.02	0.08 / 0.04	0.63 ± 0.04	0.06 / 0.05	0.35 ± 0.03
9	0.12 / 0.09	0.53 ± 0.02	0.05 / 0.01	0.69 ± 0.03	0.01 / 0.02	0.38 ± 0.02
10	0.05 / 0.03	0.58 ± 0.01	0.00 / 0.00	0.78 ± 0.00	-	-
11	0.04 / 0.01	0.61 ± 0.01	0.00 / 0.00	-	-	-
12	-	-	-	-	-	-
P-value*	2.01×10^{-7}		1.88×10^{-9}		2.75×10^{-5}	
C-statistic (95% CI)*	0.60 (0.56-0.64)		0.68 (0.63-0.73)		0.60 (0.55-0.65)	
Nagelkerke R^2*	0.02		0.06		0.04	

s.d. = standard deviation; CI = confidence interval; RS = Rotterdam Study; GRIP = Genetic Research in Isolated Populations; ERF = Erasmus Rucphen Family; * = for the risk score prediction model; ** = risk scores were calculated on the basis of the effect estimates for each specific cohort.

Table S5. Results of SNPs identified by genome-wide association studies on open-angle glaucoma

Reference	Nakano, et al. 2009						Thorleifsson, et al. 2010
SNP	rs547984	rs540782	rs693421	rs2499601	rs7081455	rs7961953	rs4236601
Chromosome	1	1	1	1	10	12	7
Minor Allele	A	C	T	T	G	A	A
MAF	0.45	0.43	0.43	0.46	0.45	0.12	0.29
Rotterdam Study-I (RS-I)							
Odds Ratio	0.92	0.94	0.95	1.14	1.01	0.93	1.12
95% CI	0.75-1.13	0.76-1.16	0.77-1.17	0.93-1.40	0.82-1.24	0.67-1.28	0.89-1.40
p-value	0.427	0.583	0.602	0.215	0.951	0.649	0.333
Genetic Research in Isolated Populations (GRIP) program combined with the Erasmus Rucphen Family (ERF) study							
Odds Ratio	1.10	1.03	1.03	0.84	1.01	0.98	1.74
95% CI	0.76-1.60	0.81-1.45	0.71-1.50	0.58-1.21	0.71-1.44	0.58-1.64	1.16-2.61
p-value	0.615	0.871	0.871	0.345	0.971	0.924	0.008*

* = significant at a p-value of 0.05; SNP = single nucleotide polymorphism; MAF = minor allele frequency; OR = odds ratio; CI = confidence interval; Southampton was already part of Thorleifsson, et al. 2010.

Chapter 6

General discussion

General discussion

Although numerous advances in diagnostic and medical treatment of open-angle glaucoma (OAG) have become available, there is still no cure for OAG. A strong increase in the prevalence of OAG is expected for the coming years.[1] After decades of research only a few risk factors for OAG have been discovered, and when concerning the (genetic) etiology of OAG we know even less. Research presented in this thesis was aimed twofold. Its first aim was to view epidemiological aspects of OAG. The second aim was to shed light on the genetics of endophenotypes and OAG itself.

In this chapter, I will present a summary and the main findings of my work as described in this thesis. Next, I will discuss methodological considerations, potential clinical implications, and finally, suggestions for further research.

Summary and main findings

Epidemiological research is one of the ways to increase our understanding in the etiologic, diagnostic and therapeutic mechanisms of a disease, for example, by analyzing possible environmental or genetic risk factors. An accurate and consistent case selection is the first step in this type of research by establishing a definition that can resemble clinical aspects. There are large differences between a clinical setting and epidemiological studies. First, to distinguish healthy from non-healthy persons we aim for a test with a high sensitivity in clinical settings, whereas in epidemiological studies we require a high specificity accepting the corresponding lower sensitivity. Second, in a clinical setting a patient with a certain condition is examined according to the possible differential diagnosis set by the ophthalmologist resulting in disease-specific examinations, and disease is often in a more severe stage. In epidemiological studies most participants are in general healthy and are examined for several diseases. Third, due to large sample sizes in epidemiological studies examinations that are very time-consuming, in particular perimetry for OAG screening, may increase the costs and therefore not preferable to other examinations.

Most (epidemiological) definitions of OAG include morphologic changes of the optic nerve head, depicted as glaucomatous optic neuropathy (GON), in combination with glaucomatous visual field loss (GVFL).[2-4] Although intraocular pressure (IOP) is nowadays considered a risk factor for OAG, some also include IOP in their definition.[5]

For GON several instruments have been developed to analyze the optic nerve head in an objective and standardized fashion. For example, scanning laser polarimetry (GDx Nerve Fiber Analyzer; Laser Diagnostic Technologies, San Diego, CA),[6,7] optical coherence tomography

(OCT; Humphrey-Zeiss, Dublin, CA; Heidelberg Engineering, Dossenheim, Germany; Topcon Corporation, Tokyo, Japan)[8], and confocal scanning laser ophthalmoscopy (Heidelberg Retina Tomograph [HRT]; Heidelberg Engineering, Dossenheim, Germany).[9] Several studies have compared these instruments; an about similar performance in their ability to distinguish OAG from "normal" was found.[10] Of these imaging devices only HRT has shown long-term stability and backwards compatibility.[11,12]

We presented morphologic characteristics of the optic nerve head in a "healthy" almost completely (96%) Caucasian population, and proposed a definition for GON.[Chapter 2.1] The latter was done by calculating the 97.5th percentile of the variable that showed the highest sensitivity at this level. This turned out to be the disc-area corrected linear cup-disc ratio. The linear cup-disc ratio is comparable with the vertical cup-disc ratio (VCDR) and therefore here further referred to as VCDR. A requirement for calculating most of the stereometric parameters with HRT is outlining of the optic disc margin. We found that manual outlining by two technicians yielded similar values. This suggests that manual outlining is not a limitation of the HRT, though it is time-consuming. Interestingly the disc-area corrected linear cup-disc ratio variable showed a better discriminating ability, than built-in compound variables of the HRT. This is probably due to that these so called linear discriminant functions were established in clinical studies and thus may have been optimized at other sensitivities/specificities.[13-16] The same holds for the Glaucoma Probability Scale (GPS), which is supposed to be more objective than other HRT variables in that there is no need to outline the optic disc margin manually. Nevertheless, it has been shown that the performance of GPS depends on the disc area.[17-21] Adjusting GPS for disc area would reenter the need of manual outlining of the disc margin. In our study, this adjustment did not improve its diagnostic performance. Other epidemiological studies showed similar performances of these functions in population-based studies. The results of the normative values of optic nerve head parameters were in line with those from other population-based studies.[22-24]

As described in this thesis the method of using the 97.5th percentile of a continuous trait to distinguish between "normal" and "abnormal" has also been applied in other studies.[2,25,26] Although it is a commonly used cut-off point in population-based research, it is unknown whether this cut-off point is optimal from the point of view of risk-factor analysis. On the basic principles of surrogates used in clinical trials[27] we presented a rather sophisticated method to find the optimal percentile to define GON, in which the effect of misclassification is minimal, the power maximal, and the variability estimated.[Chapter 2.2] This percentile turned to be the 97.0th percentile, which appears to be close to the commonly used 97.5th percentile.

For GVFL we defined strict criteria to cover the limitations of perimetry: reliability and reproducibility. We found that the 10-year risk of GVFL in participants from the Rotterdam Study was highly dependent on age, showing an increase from 2% in participants aged 60-70 years to over 6% in participants aged 80+ years. The incidence of GVFL reported in this thesis seemed to be in agreement with those reported by other population-based studies.[28,29] Incident

GVFL, in the Rotterdam Study referred to as incident OAG, was significantly associated with male gender, elevated IOP, high myopia, a positive family history, and GON at baseline. [Chapter 3.1] The association of male gender with OAG had been reported previously for the 5-year risk of OAG in the Rotterdam Study.[30] Similar but non-significant findings have also been found by other research groups.[29,31] On the other hand, when including only women of the Rotterdam Study women with early menopause showed an increased risk of OAG.[32] This has also been found in other studies.[33] Furthermore, female sex hormones may be protective against OAG.[34] These inconclusive results show that the effect of gender on OAG is still debatable. Recently a review highlighting the role of gender in OAG reported that current evidence suggests that older women are at risk for OAG.[35] The relationship between myopia and OAG has been established by several other studies.[36-38]

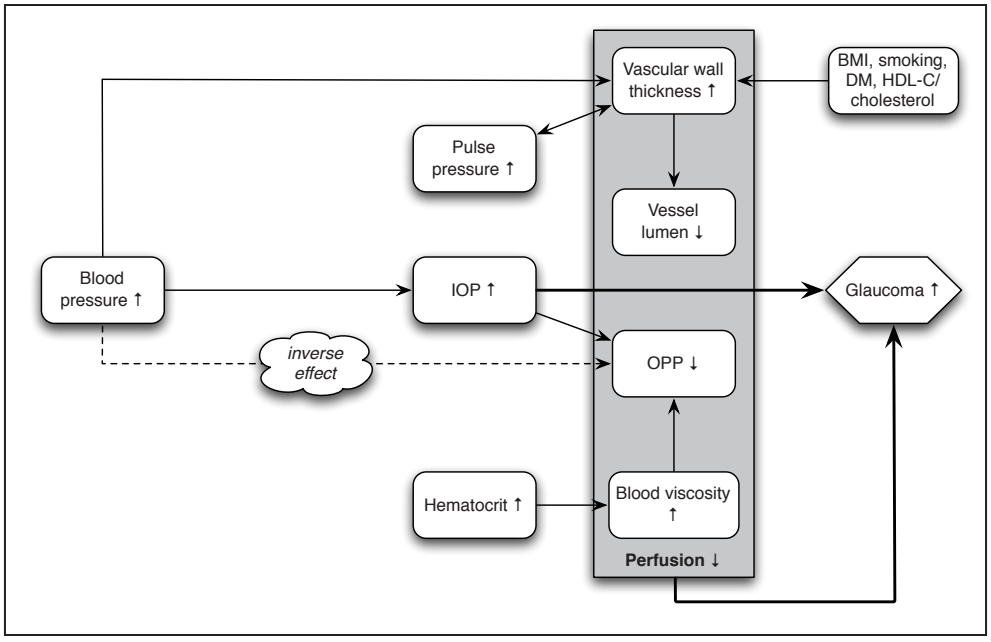
For IOP several theories have been proposed for its role in the pathophysiology of OAG. The two main mechanisms are mechanical by compression of the axons of the ganglion cells, and vascular through affecting the ocular perfusion pressure. The latter is dependent on the blood pressure and IOP. Many studies demonstrated an association of blood pressure with IOP, but the association of blood pressure with OAG is debatable.[39-48] The same holds for the mean ocular perfusion pressure.[40,43-46,49,50] The association of mean ocular perfusion pressure with OAG may depend on the adjustment of IOP.[51] Following the Hagen-Poiseuille law, various factors are involved in the perfusion. Figure 1 summarizes the complex relationships between IOP, blood pressure and mean ocular perfusion pressure, and OAG.

We assessed the relationship between the mean ocular perfusion pressure and OAG by using a sampling technique for the blood pressure and accordingly recalculating the mean ocular perfusion pressure. When interpreting our data we found no IOP-independent effect of mean ocular perfusion pressure on OAG and sampling of the blood pressure did not change the respective effect estimates. The former has also been found by other population-based studies (most studies reporting an association were not adjusted for IOP).[40,42,44-46,50,52] Based on these findings we concluded that the association of the mean ocular perfusion pressure with OAG was primarily driven by the IOP.[Chapter 3.2]

A drawback of large epidemiological studies is the fact that as well as IOP as blood pressure are measured only once and only during daytime. This precludes the possibility to assess circadian variations of either IOP or blood pressure. Patients suffering from unstable blood flow, due to vascular dysregulation, may be unable to compensate for physiologic fluctuations in IOP and blood pressure in order to maintain ocular perfusion pressure.[53] Besides, nocturnal dipping of blood pressure and circadian fluctuations in ocular perfusion pressure seems to be associated with the development and progression of OAG.[54]

Currently IOP is the major modifiable risk factor of OAG making it the most important clinical target for OAG treatment.[55,56] Next to lowering IOP there is considerable interest in (other) measures to prevent progression of OAG. Particularly for patients who are at risk for OAG it is important to know which precautionary measures can be taken, except of eye drops

Figure 1. Schematic overview of the possible pathways from mean ocular perfusion pressure to open-angle glaucoma. Factors from the Hagen-Poiseuille law are shown in the shaded area.



OPP = ocular perfusion pressure; BMI = body mass index; DM = diabetes mellitus; HDL-C = High-density lipoprotein-bound cholesterol.

or eye surgery. To assess whether environmental factors are related to OAG, we analyzed the risk of OAG for lifestyle- and nutrition-related factors.

Differences in lifestyle including socioeconomic status, smoking, alcohol consumption, and obesity, have been implicated before but failed to reach a consensus.[57] We related these lifestyle-related factors to IOP and OAG. Socioeconomic status (income and education level), smoking and alcohol consumption could not be related to OAG, but obesity, quantified using body mass index on a continuous scale, turned to have on the one hand a protective effect on OAG and on the other hand an increasing effect on IOP.[Chapter 3.3]

Starting with socioeconomic status, the relationship of socioeconomic status to OAG has also been assessed by a another population-based study showing insignificant results as well.[58] Smoking is an important risk factor for various systemic and ocular diseases; however, evidence for the involvement of smoking in OAG is controversial. A meta-analysis of several epidemiological studies on smoking showed a higher risk of developing OAG for current smokers, but not for former smokers.[59] We could not confirm this association. Also the findings regarding alcohol consumption and OAG are mixed.[60] Like several prospective studies, who could not find a relationship between alcohol and OAG,[61,62] we also failed to demonstrate a significant association of alcohol with OAG.

The only lifestyle-related risk factor that was found to be significant was body mass index.

Although our findings seem to be contradictory, they are in agreement with those reported by another follow-up study.[63] Especially the gender effect is of interest, in that the effect seemed to be driven by women. Possible mechanisms explaining the inverse association of body mass index with IOP regardless of gender could be an increased orbital pressure because of an excess fat tissue, with rise in episcleral venous pressure and consequent increase in IOP. Also the method of measuring IOP may play a crucial role in the association of body mass index with IOP in women.[64] With Goldmann applanation tonometry (mounted on slitlamp), the thorax and abdomen are pushed against the slit lamp table while breath-holding works like a Valsalva maneuver. This is especially relevant for obese women. Therefore, measurement of IOP with Goldmann applanation tonometry in female persons with a high body mass index might lead to an overestimation of the actual IOP, and as a consequence might contribute to the remarkable relationships between body mass index and IOP and body mass index and OAG in women. Another explanation might be the influence of female hormones on body mass index and OAG (see above).

The implications of several nutrients in OAG have been reported before, though little evidence exists. We related nutrition-related factors to IOP and OAG in the longitudinal Rotterdam Study. Nutrients included were omega-3 and omega-6 fatty acids, anti-oxidants, minerals, vitamins, and polyphenolic flavonoids. Of the assessed nutrients significant IOP-independent associations with OAG were found for the dietary intake of magnesium (positive association), retinol equivalents (inverse association), and vitamin B1 (inverse association). [Chapter 3.4] The effect of magnesium might be similar to that of calcium channel blockers.[65,66] The latter were found to have a harmful effect on OAG in two epidemiologic studies,[67,68] but not in clinical settings.[69] Retinol equivalents (vitamin A) have been implicated in some ocular pathologies but little is known regarding OAG. Vitamin B1 (thiamine) is involved in several optic neuropathies. One study showed reduced levels of vitamin B1 in patients with OAG compared to controls.[70] Like our study another large study on anti-oxidants and the risk of OAG did not find a significant association.[71] A literature search yielded no other large studies relating dietary intake of nutrients to OAG.

As mentioned earlier, one of the most important aspects of the glaucomatous eye but also of other ocular manifestations is the morphology of the optic nerve head. Its size, the disc area, and VCDR are both highly heritable[72] and together with IOP indispensable in clinical management and epidemiological research on OAG. Although the high heritability's suggest a major role for genetic factors, the underlying genetic determinants remain largely undetermined. This prompted us to study the disc area, VCDR, and IOP as endophenotypes for OAG. To identify the possible underlying genetic determinants, we conducted the first large genome-wide association (GWA) study on all three variables, and replicated our findings in other cohorts.

For disc area, we identified three genetic loci. The first locus was located between the cell division cycle 7 (*CDC7*) and the transforming growth factor beta receptor 3 (*TGFBR3*)

gene on chromosome 1p22, the second locus was close to the atonal homolog 7 (*ATOH7*) gene on chromosome 10q21.3-q22.1, and the third locus Sall-like 1 (*SALL1*) gene on chromosome 16q12.1.[Chapter 4.1] The gene *ATOH7* has been confirmed by a study from Australia and Singapore.[73] The latter study also confirmed *CDC7/TGFBR3* and revealed a new locus: caspase recruitment domain-containing protein 10 (*CARD10*) on chromosome 22q13.1, which was replicated in the Rotterdam Study cohorts.[Chapter 4.2]

For VCDR we identified in addition to *ATOH7* five other loci: cyclin-dependent kinase inhibitor 2A and B (*CDKN2A/2B*) gene, sin oculis homeobox homolog 1 (*SIX1*) gene, SCY1-like 1 (*SCYL1*) gene/latent transforming growth factor beta binding protein 3 (*LTBP3*) gene, CHK2 checkpoint homolog (*CHEK2*) gene, and doublecortin-like kinase 1 (*DCLK1*) gene. [Chapter 4.1]

When considering the function of the proteins these genes encode for, two protein pathways emerge.[Figure 1 Chapter 5.1] *CDKN2A*/*2B* is known to interact with *CDC7* and *TGFbeta*. Overexpression of *CDC7* has been found in neoplastic transformations in some tumors. *ATOH7* is expressed in the retina where it controls photoreceptor development.[74] The duration of expression of *ATOH7* is regulated by several proteins, including *GDF11*, a member of the bone morphogenetic protein (*BMP*) and the *TGFbeta* superfamily.[75] Both *ATOH7* and *GDF11* interact with *TGFBR3*. These genes therefore point to the same signaling pathway. *GDF11* interacts with *LTBP3*, which is located in the same region as *SCYL1*. *SALL1* is involved in the bronchio-oto-renal syndrome, which includes myopia,[76] and interacts with *SIX1*, which belongs to the SIX family involved in eye development.[77] Rare variants in *SIX1* are involved in the bronchio-oto-renal syndrome.[78] Finally, *CHEK2* has been associated with cancer,[79] and *DCLK1* is expressed in the optic tectum.[80] Of interest is that *LTBP1*, *LTBP2* (also known as *LTBP3*) and *TGFB1* (all members of the *TGFbeta* latent complex) have been implicated in the exfoliation syndrome,[81,82] which plays an etiologic role in OAG and is one of the most important single identifiable risk factor for glaucoma worldwide.[82,83] Furthermore, null mutations in *LTBP2* may cause primary congenital glaucoma.[84]

For IOP we performed another GWA study, which revealed two loci: growth-arrest-specific 7 gene (*GAS7*) and trans-membrane and coiled-coil domains 1 gene (*TMCO1*). The former gene is involved in the formation of neuritis and is regulated by transforming growth factor *TGFbeta*, while the function of the latter gene, *TMCO1*, is largely unknown.

The implication of genetic determinants in OAG follows from the finding that a positive family history of OAG was suggested to be a risk factor for OAG dated more than a century ago.[85] It has been proposed that dissecting the genetic architecture of such a heterogeneous disorder like OAG may best be achieved by considering individual endophenotypes underlying the disease. Quantitative endophenotypes allow individuals to be ranked along the continuum of risk, thus providing substantially more information than dichotomous measures of affection status.[86] It is thus unsurprising that there has been very limited success to date in the identification of discrete OAG genes by using the disease itself as a dichotomous measure.

To date over 20 genetic loci have been reported for OAG, but with conflicting results. This is probably due to small sample sizes[87] and the fact that OAG is multifactorial with multiple interacting loci each with a small effect size.[88] The latter raises the question whether there is a polygenic model in which many variants scattered over the genome apply to the disease of interest. It has been shown that if there are >30 genetic risk variants associated with a disease, no person in a population carries zero risk alleles but everyone carries a gradient number of risk alleles.[89] We investigated whether there is a polygenic model underlying VCDR and IOP, and whether these genes overlap with the genetic profile of OAG. The respective data described in this thesis showed two main findings. First, many SNPs (probably with very small effects) may collectively account for a substantial proportion of the variation in VCDR. Second, the genetics of VCDR overlap with that from OAG.[Chapter 4.4] To address the first, larger GWA studies are needed to detect these common variants for VCDR.[Chapter 4.1] The second can be addressed by relating the genes found to be associated with VCDR to OAG. [Chapter 5.1]

Of the six loci identified for VCDR three were shown to be associated with OAG. Using data from six independent studies, we found *CDKN2B*, *SIX1*, and *ATOH7*, to be consistently associated with OAG.[Chapter 5.1] These genes had not been implicated before in OAG. Possible pathways concerning these genes in OAG, suggest two pathways that may be relevant in addition to intraocular pressure.[Figure 1 Chapter 5.1] One pathway related to growth, including *CDKN2B* and *ATOH7* involved through the *TGFbeta*-pathway, which has been implicated before in the pathogenesis of glaucoma,[90-92] and the other pathway related to development, including *SIX1*.

The addition of the newly identified genes (*CDKN2B*, *SIX1* and *ATOH7*) to the established genes (*MYOC*, *OPTN* and *WDR36*) showed a significant contribution to the risk of OAG for the newly identified genes. It has been reported that the established genes account for <5% of all OAG in the general population.[93] Also the newly identified genes seemed to have a very low explained variance in OAG. Nevertheless, together the risk alleles resulted in an about three-fold increase in the risk of OAG.[Chapter 5.2] These findings suggest that a risk profile based on these genes may give useful information on the risk of OAG. For comparison, the reported odds ratios are higher than those of myopia or an IOP of 21 mmHg, two risk factors for OAG.[36,37,94-97]

Methodological considerations

All studies described in this thesis were conducted in the Rotterdam Study. The strengths of this study are its population-based setting, its large size, the long follow-up duration, and the collection of data on various clinical characteristics of all participants gathered at every follow-up round. Besides ophthalmic examinations, participants also underwent cardiovascular, neurologic, and many other examinations (including laboratory tests of blood samples,

magnetic resonance imaging, X-rays, testing of cognition, etc.). One of the weaknesses of using a population-based study is that participants who can not visit the research center are rather ignored in this study. Reasons not to participate are for example a poor health, but also poor vision. The latter may include participants with reduced sight due to OAG. Poor health is related to age, which is among the most important risk factors of OAG. Furthermore, as the prevalence of OAG is relatively low compared to diseases like myocardial infarction or cancer, only a small number of participants develop OAG. The long follow-up has limitations as well. Only 59% of the participants who attended the baseline completed at least one follow-up round. As a consequence the power to detect a statistical significant difference between a certain determinant and OAG is low.

Another concern is the definition of OAG used in the Rotterdam Study. The definition of OAG changed during follow-up of the participants. Dielemans, et al. described the first definition for the Rotterdam Study.[98] A problem with this definition was that it included the IOP. A few years later Wolfs, et al. described another definition in which the IOP was not included.[2] OAG was based on the presence of GON and GVFL divided into possible/probable/definite GON and possible/probable/definite OAG. This seemed to be a rather sophisticated definition with several limitations. Therefore, we simplified the definition of OAG by defining 4 stages: no/possible/probable/definite OAG (Table 1). OAG still included GON and GVFL.

Table 1. Four stages to define open-angle glaucoma in the Rotterdam Study

Stage	Presence of		Remarks
	GON	GVFL	
No	No	No	Those with a VCDR close to the cut-off may switch to ”Possible” and back, due to variation/errors in measurement technique
Possible	Yes	No	Those with a VCDR close to the cut-off may switch to ”No” and back, due to variation/errors in measurement technique
Probable	No	Yes	Once GVFL detected, a person will remain a case at follow-up
Definite	Yes	Yes	Once GVFL detected, a person will remain a case at follow-up but may switch to ”Probable” if VCDR is close to the cut-off

GON = glaucomatous optic neuropathy; GVFL = glaucomatous visual field loss; VCDR = vertical cup-disc ratio.

The definition of GON had been changed, because of several reasons. At baseline, first and second follow-up VCDR was measured using stereoscopic slideviewing imaging (Topcon ImageNet System, Tokyo, Japan). In addition to ImageNet, ophthalmic examination at second follow-up included HRT. Following the previous ImageNet-based definition, the presence of GON depended on the VCDR (the 97.5th and 99.5th percentile), the minimal neural rim width, and the asymmetry between left and right eyes.[2] However, we found that the 99.5th percentile did not have a better discriminative ability compared to the 97.5th percentile.[Chapter 2.2] The minimal neural rim width was skipped, because HRT cannot calculate this variable. Besides, using the previous definition most cases with GON were based on the VCDR rather than on the minimal neural rim width. We decided to skip asymmetry too, because of three

reasons. First, asymmetry may cause misclassification in the definition of incident GON: a subject may have a decreasing asymmetry caused by an increasing VCDR in the eye with the lower VCDR at baseline. Second, there appeared to be poor agreement between HRT and ImageNet for smaller VCDR, the range crucial for defining asymmetry. Better agreement was found for higher VCDR, the range needed for defining the 97.5th percentile of VCDR. Third, the additional yield of adding asymmetry to the HRT-based GON definition appeared to be negligible.[Chapter 2.1] Therefore the definition of GON had been changed to only the 97.5th percentile of the VCDR adjusted for the size of the optic disc when measured with a standardized device, or in case of missing or unreliable data based on the VCDR estimated by ophthalmoscopy.

The definition of GVFL was not altered, but there was a small change in the method of perimetry at second follow-up. At baseline and first follow-up visual fields were assessed using Goldmann perimetry, and at second follow-up using a Humphrey Field Analyzer (HFA). However, during the whole study all participants were screened using the same device: a HFA. If and only if participants showed a reproducible visual field defect a Goldmann perimetry or HFA was performed. As mentioned previously participants who were once classified as having GVFL were considered as having GVFL irrespective of passing visual field screening at a later follow-up. We applied this rule because of several reasons. First, as the age increases during follow-up perimetry results may become less reliable and more difficult to interpret. Second, GVFL cannot improve regardless of treatment. Third, small visual field defects due to OAG can be missed when only performing the visual field screening test, which incorrectly may suggest no GVFL at all. Possible explanations for the reversal to normal visual field could be the development of cataract, cognitive impairment, fatigue, concurrent eye diseases like anterior ischemic optic neuropathy and age-related macular degeneration, and neurological diseases like stroke.[Chapter 2.2]

Even though the methods for defining OAG changed during follow-up, the prevalence and incidence data did not change significantly.[Chapter 3.1] Furthermore, other research groups have verified many findings presented in this thesis, suggesting that it might not have hampered our findings. Unfortunately there is no uniform definition of OAG to date. Different diagnoses of OAG are used across different studies, making it difficult to compare results. The Rotterdam Study is worldwide one of the largest population-based studies in ophthalmology. Nevertheless, as reported in chapter 5.1, the power to detect genes for OAG with small effects is still limited. Despite this hampering our findings, the consistency in our findings suggests that this problem may affect primarily the statistical power due to misclassification.

Clinical implications

Although the findings of the current thesis may not have a direct effect on clinical practice, there are some considerations to be made.

Findings reported in Chapter 2 may support epidemiological research that could improve clinical research in the future. The confirmation of the increase in incidence of OAG with age is especially of importance in health care planning, because the life expectancy continues to increase. Our finding on perfusion pressure may provide new insights in the understanding of the pathophysiology of OAG. As the effect of blood pressure is almost negligible in the average patient with OAG, the clinician should primarily focus on IOP rather than the blood pressure in management of OAG.

The relationship between body mass index and IOP and OAG is of clinical interest because it shows that caution should be taken when measuring IOP in obese women using Goldmann applanation tonometry mounted on a slit lamp. A false-positive high IOP might influence the decision of an ophthalmologist to treat the patient against high IOP, which in turn may lead to unnecessary complications and health care costs.

Regarding the relationship of dietary intake of nutrients with OAG, persons at risk for OAG and patients in whom disease progression continues despite an apparently sufficient reduction in IOP, may benefit from a modification of the intake of magnesium, retinol equivalents, and vitamin B1.

Effective inexpensive lifestyle or nutrition measures that would favorably alter the risk of developing OAG would certainly be welcome as the healthcare costs attributable to OAG escalate as the disease progresses to later stages.

In the last few years several large commercial companies started to offer personal risk prediction of diseases based on genetic profiles. This risk prediction is speculated to lead personal medicine. The risk of a number of diseases is estimated from the genetic profile of the client. Personal genome medicine in OAG remains still close to science fiction, with the exception of families in which the disease is caused by a dominant mutation. Genetic research presented in this thesis started together with a new era in genetic epidemiology: the GWA studies, in which many genes are being implicated in several diseases. The clinical diagnostic and therapeutic value in “real-life” is to be determined. In general, ophthalmologists are not inclined to determine genetic risk profile of a glaucoma patient with a negative family history. Nonetheless, the joint effect of the genes identified for OAG described in this thesis appear to have a significant contribution on the risk of OAG and serve as a starting point where gaps are to be filled in by future findings. This suggests that a risk profile based on these genes may provide useful clinical information on the risk of OAG.

Future research

One of the main findings of this thesis is the identification of several genes for OAG and related parameters, which were not implicated before. The methodological design presented

in this thesis may be essential for future research. First, we searched for quantitative clinical important parameters related to the disease of interest. Next, we performed a GWA study to identify common variants associated with these quantitative traits. Thereafter, we analyzed whether these traits follow a polygenic model and underlie the genetic profile of the disease of interest. This supports further research on the respective quantitative trait. Finally, the common variants associated with the "best" quantitative traits could be related to the disease of interest to reveal associated genes.

This thesis presents important results for future research regarding the genetics of OAG. Especially, the finding of two genetic pathways involved in OAG is promising and provides new opportunities for genetic research. In GWA studies millions of single nucleotide polymorphisms (SNPs) are genotyped and analyzed. The large number of tests results in statistical problems referred to as multiple testing. To adjust for multiple testing we often adjust the certainty level of our analyses by considering a lower p-value threshold as significant. However, as a consequence we need more statistical power by analyzing larger samples sizes to detect small differences. SNPs associated with a disease often have small effects on the disease, making it even more difficult to assess the genetics of a disease by GWA studies, especially if genetic variants have low effect estimates.[100] Furthermore, in population-based studies statistical power can also be affected if the disease of interest has a low prevalence. One way to handle these problems is by setting up large collaborations including many studies and/or by analyzing quantitative traits.

Considering the successes in genetics by GWA studies, especially those on quantitative traits (e.g. blood pressure) have the potential to gain more success than those on binary traits (e.g. hypertension). As described in this thesis, a GWA on quantitative traits of the optic disc revealed genes that were associated with VCDR, one of the quantitative traits that overlap with the genetic architecture of OAG, and genes that were implicated in OAG.

One of the major problems in OAG research in general is the lack of standardized clinical criteria. A framework for a definition of OAG for population-based research has been described and closely resembles our definition.[4] Large collaborations may unify their definition of OAG and adapt it to clinical definitions to reduce noise and yield more power to find statistical significant associations.

Recently, the application of 1000-genomes instead of HapMap has been suggested as a reference population. HapMap from the International HapMap Project aimed to develop a haplotype map of the human genome, the HapMap, which describes the common patterns of human DNA sequence variation.[101,102] The Caucasian sample of HapMap has been based on <100 United States residents with northern and western European ancestry. The 1000-genomes project aims to sequence the genomes of a larger number of people (>2500 of which >500 Caucasians), to provide a more comprehensive resource on human genetic variation.

Most GWA studies are performed on Caucasian populations like the Rotterdam Study. Improvements in sequencing technology have sharply reduced the cost of sequencing, while

it is still very expensive for research groups in underdeveloped countries. These countries, especially on the African continent, have a higher prevalence of OAG than European countries. GWA studies on OAG in participants with other ancestry than Caucasian might provide valuable information.

Another important approach will be large-scale generation sequencing. This approach may be extremely powerful to identify causal variants with relatively large effects. The approach can be used to screen specific regions identified by GWA linkage studies on a genome-wide scale.

Several risk factors for OAG have been described in the literature, though only a few are consistent. In this thesis we also found some non-genetic risk factors, which still require validation. The dietary intake of magnesium, retinol equivalents, and vitamin B1 should be further explored as a new option for the treatment of OAG. Large (population-based) studies and clinical trials on dietary intake of nutrients and the risk of OAG are scarce.[99] Because of the multifactorial behavior of OAG research on gene-environment interaction may be of interest as well.

In summary, we need (1) large consortia to perform GWA studies on OAG or, in case of a limited number of cases, quantitative traits related to OAG; (2) a uniform definition of OAG; (3) to combine and verify results from other studies; (4) to evaluate the expression and functional role of the discovered genes and the proteins they encode for in OAG; (5) to compare genetic differences and similarities across different study populations with OAG; (6) to assess more environmental risk factors; (7) to test for gene-environment interaction. This will increase our knowledge of OAG.

In conclusion, we need to work!

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Chapter 7

Samenvatting
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Samenvatting

SAMENVATTING

Dit proefschrift bestaat uit een verzameling van artikelen die epidemiologische en genetische aspecten van de aandoening open-kamerhoek glaucoom beschrijven.

Hoofdstuk 1 geeft een korte inleiding weer over glaucoom en de opzet van dit proefschrift. Een samenvatting van de beschouwende wetenschap uit dit proefschrift volgt hieronder.

Aangezien bij glaucoom primair de oogzenuw (waarvan het bij oogspiegelen zichtbare deel papil is genaamd) is aangedaan, worden in Hoofdstuk 2.1 de anatomische kenmerken van de papil beschreven. Immers, om een onderscheid te kunnen maken tussen een afwijkende en een normale papil moeten we eerst definiëren wanneer iets normaal is. Allereerst hebben we de kenmerken van de papil, zoals de grootte en de mate van excavatie (cup-disc ratio), geanalyseerd in een grote groep mensen uit de algemene populatie (deelnemers van de ERGO-studie). Tevens hebben we criteria voor glaucomateuze papilafwijkingen (glaucomateuze opticoneuropathie) opgesteld. Binnen de ERGO-studie zijn de papillen van de deelnemer met behulp van verschillende technieken geanalyseerd. Om de objectiviteit en continuïteit (zo min mogelijk verandering over tijd) te waarborgen werd gekozen voor de Heidelberg Retina Tomograph (HRT). Vervolgens werd bepaald welke maat van de papil de grootste verschillen vertoont tussen mensen met en zonder glaucomateus gezichtsveldverlies. Dit bleek de lineaire cup-disc ratio (LCDR) te zijn. Een verdere verbetering werd bereikt door te corrigeren voor de grootte van de papil (disc area). Het resultaat werd vergeleken met een andere veel gebruikte techniek, namelijk het Topcon ImageNet System. Daar bleek de verticale cup-disc ratio (VCDR) het grootst onderscheidend vermogen te hebben voor mensen met en zonder glaucomateus gezichtsveldverlies, hoewel de HRT LCDR beter presteerde.

In de epidemiologie is het gebruikelijk om waardes die bij slechts 2,5% of minder van de populatie voorkomen als afwijkend te beschouwen. Dit zijn dus alle waardes boven het 97.5^{e} percentiel in de algemene populatie. Echter, het is de vraag of dit percentiel wel het meest optimale is voor het doen van risicofactoranalyses. Een statistische methode om dit te bepalen voor glaucoom, maar ook voor andere aandoeningen, wordt in Hoofdstuk 2.2 beschreven. In onze studie vinden we dat het optimale percentiel voor de LCDR de 97,0^{e} is. Met dit percentiel is de ruis die ontstaat door misclassificatie van deelnemers geminimaliseerd en haal je het maximaal mogelijke uit risicofactoranalyses.

In Hoofdstuk 3 worden de incidentie van glaucomateus gezichtsveldverlies en mogelijke risicofactoren daarvoor beschreven. Hoofdstuk 3.1 geeft het beloop van de deelnemers van het ERGO-onderzoek weer en hun risico op glaucomateus gezichtsveldverlies. De incidentie van

glaucomateus gezichtsveldverlies toont een sterke toename met de leeftijd. Een vergelijkbare trend is ook waargenomen in andere studies. We vonden een sterke associatie tussen de bekende risicofactoren voor glaucoom en incident glaucomateus gezichtsveldverlies. Onder deze risicofactoren vallen myopie (bijziendheid), verhoogde oogdruk, mannelijk geslacht, en glaucomateuze opticoneuropathie zoals beschreven in Hoofdstuk 2.1. In de navolgende hoofdstukken worden andere mogelijke risicofactoren belicht.

Beginnende met fysiologische aspecten van het oog, wordt eerst de oculaire perfusiedruk in Hoofdstuk 3.2 beschreven. Een schatting van de oculaire perfusiedruk in epidemiologische studies is mogelijk o.b.v. een formule beschreven door Bill in 1975.[1] In essentie wordt de perfusiedruk van het oog bepaald door een verschil tussen de systemische bloeddruk en de oogdruk. De associatie tussen de oculaire perfusiedruk en glaucoom is door de tegenstrijdige bevindingen van verschillende epidemiologische studies nog onduidelijk. Een belangrijk conclusie in Hoofdstuk 3.2 is dat de associatie tussen oculaire perfusiedruk en glaucoom veroorzaakt wordt door de sterke associatie tussen de perfusiedruk en de oogdruk en dat de bloeddruk slechts een zeer kleine rol speelt.

Momenteel is de behandeling van glaucoom gebaseerd op het controleren van de oogdruk. Dit, omdat de oogdruk de enige tot dusver bekende modificeerbare risicofactor is. Aangezien glaucoom soms progressie toont ondanks een goed gereguleerde oogdruk, is het van belang om te onderzoeken wat andere modificeerbare factoren doen zoals bijv. leefstijl- en voedinggewoontes. In Hoofdstuk 3.3 worden leefstijl-gerelateerde factoren onderzocht, waaronder: socio-economische status, roken, alcoholgebruik en obesitas. De bevindingen suggereren dat bij vrouwen met overgewicht vaak een fout-positieve te hoge oogdruk wordt gemeten en dat zij een lager risico op glaucoom lijken te hebben. Bij mannen is dit niet het geval. De overige genoemde leefstijl-gerelateerde factoren blijken niet geassocieerd te zijn met glaucoom.

Voedingsgewoonten, zoals de inname van omega-3 en omega-6 vetzuren, anti-oxidanten, spoorelementen, vitamines en flavenoïden, worden in Hoofdstuk 3.4 beschreven. De bevindingen suggereren dat mensen die veel magnesium innemen een verhoogd risico op glaucoom hebben, terwijl een hoge inname van de vitamines A en B1 juist een beschermend effect op glaucoom lijkt te hebben.

Hoofdstuk 4 en 5 hebben betrekking op de genetica omtrent glaucoom. Zoals in Hoofdstuk 2 beschreven, zijn van de papilkenmerken de VCDR en de disc area één van de belangrijkste maten voor glaucoom. Daarnaast is een te hoge oogdruk de belangrijkste risicofactor voor het ontstaan van glaucoom. Voor het bepalen van genetische varianten die geassocieerd zijn met deze uitkomsten hebben we een zogenaamde genome-wide association (GWA) studie gedaan. Hiervoor wordt het DNA van duizenden deelnemers bepaald, waaruit vervolgens vaak voorkomende varianten op nucleotide niveau worden bepaald, die ofwel gegenotypeerd ofwel geïmputeerd worden. Dit betreffen vaak miljoenen varianten en worden ook wel single nucleotide polymorphisms (SNPs) genoemd. Hoofdstuk 4.1 beschrijft een GWA studie voor

twee belangrijk maten voor glaucoom: de VCDR en de disc area. Met behulp van data van vijf verschillende populaties worden in totaal acht genen (cell division cycle 7 [*CDC7*]/transforming growth factor beta receptor 3 [*TGFBR3*], atonal homolog 7 [*ATOH7*], sall-like 1 [*SALL1*], cyclin-dependent kinase inhibitor 2A en B [*CDKN2A/2B*], sin oculis homeobox homolog 1 [*SIX1*], SCY1-like 1 [*SCYL1*]/latent transforming growth factor beta binding protein 3 [*LTBP3*], CHK2 checkpoint homolog [*CHEK2*], en doublecortin-like kinase 1 [*DCLK1*]) geïdentificeerd, waarvan één (*ATOH7*) met beide uitkomstmaten geassocieerd is.

Hoofdstuk 4.2 geeft een GWA studie weer voor de disc area gemeten in een Aziatische populatie. Deze studie repliceert de genen beschreven in Hoofdstuk 4.1 en onthult nog een gen: caspase recruitment domain-containing protein 10 (*CARD10*) op chromosoom 22q13.1.

Hoofdstuk 4.3 is een uiteenzetting van wederom een GWA studie, maar nu gericht op de oogdruk. Na samenvoegen van verschillende studies vinden we twee genen die significant geassocieerd blijken te zijn met de oogdruk: growth-arrest-specific 7 (*GAS7*) en trans-membrane and coiled-coil domains 1 (*TMCO1*). Deze twee genen blijken tevens geassocieerd te zijn met glaucoom.

Uit onderzoek blijkt dat indien een ziekte verklaard wordt door >30 genen verspreid over het genoom, er zeer waarschijnlijk niemand is die geen enkel risicovariant draagt.[2] Dit betekent dus dat iedereen genetisch een substantieel risico op de ziekte heeft. Hoofdstuk 4.4 laat zien dat in de algemene bevolking de VCDR en glaucoom niet door een beperkt aantal genen met grote effecten worden verklaard, maar door heel veel genen met kleine effecten verspreid over het hele genoom (ook wel polygeen model genoemd). Zoals verwacht blijkt verder dat genen die geassocieerd zijn met de VCDR overlappen met de genetica van glaucoom.

Aangezien de VCDR makkelijker te bepalen is dan de diagnose glaucoom vast te stellen is, is het interessant om de VCDR te analyseren en die genen uit te zetten tegen glaucoom. Dit laatste is gedaan in Hoofdstuk 5.1. In een studie, bestaande uit zes populaties, waarbij verschillende landen over de wereld betrokken zijn geweest, blijkt dat drie van de acht genen geassocieerd met de VCDR en/of de disc area ook gerelateerd kunnen worden aan glaucoom. De implicaties van deze genen en de al reeds bekende genen worden beschreven in Hoofdstuk 5.2.

Er zijn slechts drie genen bekend die een significante rol kunnen spelen bij het ontstaan van glaucoom op late leeftijd. Onder deze genen vallen: het Myociline-gen (*MYOC*), Optineurine-gen (*OPTN*) en WD40-repeat 36-gen (*WDR36*).[3] Echter, de prevalentie van de risicovarianten van deze genen is zeer laag in de algemene bevolking. Van belang is dus wat de (klinische) implicaties zijn van deze genen, maar ook van de nieuw gevonden genen. Deze laatste zijn de SNPs die we ontdekt hebben in Hoofdstuk 4. Daarnaast is het ook van belang het gecombineerde risico, dat wil zeggen het risico op glaucoom in dragers met meerdere risicovarianten te beschrijven. Drie verschillende studies met elk een verschillende opzet: population-based, family-based en case-control studie tonen consistente resultaten. Er blijkt

een klinisch significante toegevoegde waarde te zijn van de nieuw gevonden genen boven de al bekende (*MYOC*, *OPTN* en *WDR36*) genen voor glaucoom. Deelnemers die met een groot aantal risicovarianten worden geboren hebben een 2-3 keer zo hoge kans op het ontwikkelen van glaucoom ten opzichte van deelnemers met weinig risicovarianten. Tenslotte, zijn er recentelijk ook genen gevonden in andere studies te weten uit Japan en IJsland. Deze genen hebben wij ook onderzocht, echter, deze SNPs repliceerden niet in onze studie.[4,5]

In dit proefschrift hebben we verschillende genen ontdekt die geassocieerd zijn met determinanten en risicofactoren van glaucoom. De genen duiden op een rol van groei/ ontwikkeling en *TGFbeta* in het ontstaan van glaucoom.

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Dankwoord

Dankwoord

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Wishal

December 2010

About the author

About the author

Wishal Ramdas was born on July 10th, 1982 in Dorsten, Germany. In 2000, he completed his secondary school in Emmen, the Netherlands, followed by his medical education at the Erasmus University Rotterdam. In 2004, he participated in research on central corneal thickness measurements at the department of Ophthalmology of the Erasmus Medical Center Rotterdam (supervisor: Dr. R.C.W. Wolfs). Before obtaining his medical degree in January 2007, he conducted another study on the association between falls and topical beta-blocker use in the elderly at the departments of Internal Medicine section of Geriatric Medicine and Ophthalmology of the Erasmus Medical Center Rotterdam (supervisors: Dr. T.J.M. van der Cammen and Dr. R.C.W. Wolfs). This resulted in his first publication.

In April 2007, he started the work described in this thesis at the ophthalmic epidemiology unit (head: Prof.dr. P.T.V.M. de Jong, later followed by Prof.dr. J.R. Vingerling) of the department of Epidemiology (head: Prof.dr. A. Hofman) of the Erasmus Medical Center Rotterdam (supervised by: Dr. N.M. Jansonius). As from the middle of 2008 he collaborated with the genetic epidemiology unit (head: Prof.dr.ir. C.M. van Duijn). In August 2009, he obtained a Master of Science degree in Clinical Epidemiology at the Netherlands Institute for Health Sciences (NIHES).

In February 2011, he started his specialist training in Ophthalmology at the Erasmus Medical Center Rotterdam.

PhD Portfolio

Erasmus MC
Universitair Medisch Centrum Rotterdam

Summary of PhD training and teaching

Name PhD student: Wishal Djainath Ramdas		
Erasmus MC Departments: Epidemiology and Ophthalmology		
Research School: Netherlands Institute for Health Sciences (NIHES)		
PhD period: April 2007 – December 2010		
Promotors: Prof.dr. J.R. Vingerling and Prof.dr.ir. C.M. van Duijn		
Supervisor: Dr. N.M. Jansonius		
1. PhD training		
	Year	**Workload (ECTS)**
General research skills		
- Master in Clinical Epidemiology (NIHES)	2007-2009	30
Specific courses (e.g. Research school, Medical Training)		
- "BC SNPMax course", Rotterdam, the Netherlands	2008	1
- "R-course", Rotterdam, the Netherlands	2008	1
- "Humphrey cursus dag", Leiden, the Netherlands	2009	0.5
- "Cursus: 1st Research Course in Ophthalmology and Visual Science met als thema Ocular-Immumunology", Amsterdam, the Netherlands	2010	0.5
- "Minicursus Methodologie van Patientgebonden Onderzoek en Voorbereiding van Subsidieaanvragen", Rotterdam, the Netherlands	2010	0.5
- "LVAO-dag Refractie en Optica", Utrecht, the Netherlands	2010	0.5
Seminars and workshops		
- Weekly scientific seminars Dept. of Epidemiology	2007-2010	2
Presentations		
- Dept. of Ophthalmology: "*Definition of glaucomatous optic neuropathy for epidemiological studies based on the Heidelberg Retina Tomograph (HRT3) – The Rotterdam Study*"	2008	1
- Rotterdam Eye Hospital: "*Definition of glaucomatous optic neuropathy for epidemiological studies based on the Heidelberg Retina Tomograph (HRT3) – The Rotterdam Study*"	2008	1
- Dept. of Ophthalmology: "*SNPs associated with endophenotypes of OAG*"	2011	1
- Dept. of Ophthalmology: "*Lifestyle, Nutrition, and the risk of open-angle glaucoma*"	2011	1
(Inter)national conferences and presentations		
- Nederlands Oogheelkundig Gezelschap (NOG) jaarvergadering 2008, Maastricht, the Netherlands	2008	1
- Association for Research in Vision and Ophthalmology – Nederland 2008	2008	1

Annual Meeting, Leiden, the Netherlands; paper presentation "*Heidelberg Retina Tomograph (HRT3) in population-based epidemiology: normative values and a definition of glaucomatous optic neuropathy*"		
- Association for Research in Vision and Ophthalmology (ARVO) 2009 Annual Meeting, Fort Lauderdale, USA; poster presentation "*Heidelberg Retina Tomograph (HRT3) in population-based epidemiology: normative values and a definition of glaucomatous optic neuropathy*"	2009	1
- Congress of the European Society of Ophthalmology (SOE) 2009, Amsterdam, the Netherlands, paper presentation "*Definition of glaucomatous optic neuropathy for epidemiological studies based on the Heidelberg Retina Tomograph (HRT3) - The Rotterdam study*"	2009	1
- Association for Research in Vision and Ophthalmology – Nederland 2009 Annual Meeting, Rotterdam, the Netherlands; paper presentation "*The optimal cup-disc ratio cut-off point for an HRT3-based definition of glaucomatous optic neuropathy in population-based epidemiology*"	2009	1
- Association for Research in Vision and Ophthalmology (ARVO) 2010 Annual Meeting, Fort Lauderdale, USA; poster presentation "*A Genome-Wide Association Study on Glaucomatous Optic Neuropathy*"	2010	1
- XIX Biennial Meeting of the International Society for Eye Research (ISER) 2010 Montreal, Canada; oral presentation "*Genome-wide association studies: a roadmoap to unraveling the genetics of open-angle glaucoma*"	2010	1
- Nederlands Oogheelkundig Gezelschap (NOG) jaarvergadering 2011, Maastricht, the Netherlands	2011	1
- World Glaucoma Congress (WGC) 2011, Paris, France; oral presentation "*The road from GWA discovery to the clinical implications for open-angle glaucoma*"	2011	1
2. Teaching		
	Year	**Workload (ECTS)**
Supervising Master's theses		
- Supervised Andrea Gasten: "*Three genes interacting with SIX1 and SALL1 are associated with optic disc area*"	2010-2011	5
Other		
- Reviewer for: Investigative Ophthalmology & Visual Science (IOVS)	2011	0.5
- Reviewer for: Archives of Ophthalmology	2011	0.5

Articles included in this thesis

ARTICLES INCLUDED IN THIS THESIS

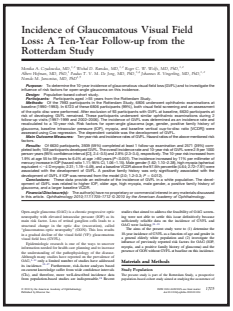
Chapter 2.1

Heidelberg Retina Tomograph (HRT3) in Population-based Epidemiology: Normative Values and Criteria for Glaucomatous Optic Neuropathy

Chapter 2.2

Defining Glaucomatous Optic Neuropathy from a Continuous Measure of Optic Nerve Damage- The Optimal Cut-off Point for Risk-factor Analysis in Population-based Epidemiology

Chapter 3.1



Incidence of Glaucomatous Visual Field Loss: A Ten-Year Follow-up from the Rotterdam Study

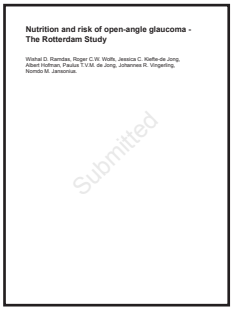
Chapter 3.2

Ocular Perfusion Pressure and the Incidence of Glaucoma: Real Effect or Artifact: The Rotterdam Study

Chapter 3.3

Lifestyle and Risk of Developing Open-Angle Glaucoma

Chapter 3.4



Nutrition and risk of open-angle glaucoma - The Rotterdam Study

Chapter 4.1

A Genome-Wide Association Study of Optic Disc Parameters

Chapter 4.2

Genome-wide association studies in Asians confirm the involvement of *ATOH7* and *TGFBR3*, and further identify *CARD10* as a novel locus influencing optic disc area

Chapter 4.3

Common Genetic Determinants of Intraocular Pressure and Primary Open-Angle Glaucoma

Chapter 4.4

Genetic architecture of open angle glaucoma and related determinants

Chapter 5.1

Common genetic variants associated with open-angle glaucoma

Chapter 5.2

Clinical Implications of Old and New Genes for Open-Angle Glaucoma

List of publications

List of publications

Antithrombotic medication and incident open-angle glaucoma.
Marcus MW, Müskens RP, **Ramdas WD**, Wolfs RC, de Jong PT, Vingerling JR, Hofman A, Stricker BH, Jansonius NM.
[in preparation]

Medical Characteristics of Patients with Macular Telangiectasia Type 2 (MacTel Type 2) - MacTel Project Report No. 3.
Clemons TE, Gillies MC, Chew EY, Bird AC, Peto T, Wang JJ, Mitchell P, **Ramdas WD**, Vingerling JR, and the Macular Telangiectasia Project Research Group
[submitted]

Linkage and association analysis of glaucoma related traits in a large pedigree from a Dutch genetically isolated population.
Axenovich TI, Zorkoltseva IV, Belonogova NM, Van Koolwijk LM, Borodin PM, Kirichenko AV, Babenko VN, **Ramdas WD**, Amin N, Despriet DD, Vingerling JR, Lemij HG, Oostra BA, Klaver CC, Aulchenko YS, van Duijn CM
[submitted]

Nutrition and risk of open-angle glaucoma - The Rotterdam Study.
Ramdas WD, Wolfs RC, Kiefte-de Jong JC, Hofman A, de Jong PT, Vingerling JR, Jansonius NM.
[submitted]

Common genetic determinants of intraocular pressure and primary open-angle glaucoma.
van Koolwijk LM*, **Ramdas WD***, Ikram MK, Jansonius NM, Pasutto F, Hysi PG, Macgregor S, Janssen SF, Hewitt AW, Viswanathan AC, ten Brink JB, Hosseini SM, Amin N, Despriet DD, Willemse-Assink JJ, Kramer R, Rivadeneira F, Struchalin, 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, Wong TY, Czudowska MA, Hofman A, Uitterlinden AG, Wolfs RC, de Jong PT, Oostra BA, Paterson AD, Wellcome Trust Case Control Consortium 2, Mackey DA, Bergen AA, Reis A, Hammond CJ, Vingerling JR, Lemij HG, Klaver CC, van Duijn CM.
[submitted]

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Marcus MW, Müskens RP, **Ramdas WD**, Wolfs RC, de Jong PT, Vingerling JR, Hofman A, Stricker BH, Jansonius NM.
[submitted]

Corticosteroids and open-angle glaucoma: a population-based cohort study and a systematic review of published cases
Marcus MW, Müskens RP, **Ramdas WD**, Wolfs RC, de Jong PT, Vingerling JR, Hofman A, Stricker BH, Jansonius NM.
[submitted]

Three genes interacting with SIX1 and SALL1 are associated with optic disc area.
Gasten AC*, **Ramdas WD***, Broer L*, Ikram MK, de Jong PT, Aulchenko YS, Wolfs RC, Hofman A, Rivadeneira F, Uitterlinden AG, Klaver CC, Jansonius NM, Vingerling JR, van Duijn CM.
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An automated 3-D method for the correction of axial artifacts in spectral-domain optical coherence tomography images.
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Ramdas WD, Rizopoulos D, Wolfs RC, Hofman A, de Jong PT, Vingerling JR, Jansonius NM. Ophthalmic Epidemiol. [accepted]

Common genetic variants associated with open-angle glaucoma.
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