ARHGEF12 influences the risk of glaucoma by increasing intraocular pressure

Henriët Springelkamp[1,2,‡], Adriana I. Iglesias[2,‡], Gabriel Cuellar-Partida[3], Najaf Amin[2], Kathryn P. Burdon[4], Elisabeth M. van Leeuwen[2], Puya Gharahkhani[3], Aniket Mishra[3], Sven van der Lee[2], Alex W. Hewitt[4,5], Fernando Rivadeneira[2,6,7], Ananth C. Viswanathan[8], Roger C.W. Wolfs[1], Nicholas G. Martin[9], Wishal D. Ramdas[1], Leonieke M. van Koolwijk[2], Craig E. Pennell[10], Johannes R. Vingerling[1,2], Jenny E. Mountain[11], André G. Uitterlinden[2,6,7], Albert Hofman[2,7], Paul Mitchell[12], Hans G. Lemij[13], Jie Jin Wang[12], Caroline C.W. Klaver[1,2], David A. Mackey[4,14], Jamie E. Craig[15], Cornelia M. van Duijn[2,‡], Stuart MacGregor[3,‡,*]

[1]Department of Ophthalmology, Erasmus Medical Center, Rotterdam, 3000 CA, the Netherlands

[2]Department of Epidemiology, Erasmus Medical Center, Rotterdam, 3000 CA, the Netherlands

[3]Statistical Genetics, QIMR Berghofer Medical Research Institute, Royal Brisbane Hospital, Brisbane, Queensland 4006, Australia

[4]School of Medicine, Menzies Research Institute Tasmania, University of Tasmania, Hobart, Tasmania 7000, Australia

[5]Centre for Eye Research Australia (CERA), University of Melbourne, Royal Victorian Eye and Ear Hospital, Melbourne, Victoria 3002, Australia

[6]Department of Internal Medicine, Erasmus Medical Center, Rotterdam, 3000 CA, the Netherlands

[7]Netherlands Consortium for Healthy Ageing, Netherlands Genomics Initiative, the Hague, 2593 CE, the Netherlands

[8]NIHR Biomedical Research Centre, Moorfields Eye Hospital NHS Foundation Trust and UCL Institute of Ophthalmology, London EC1V 2PD, UK

[9]Genetic Epidemiology, QIMR Berghofer Medical Research Institute, Royal Brisbane Hospital, Brisbane, Queensland 4006, Australia

[10]School of Women's and Infants' Health, University of Western Australia, Crawley 6009, Western Australia, Australia

[11]Telethon Kids Institute, Subiaco 6008, Western Australia, Australia

[12]Centre for Vision Research, Department of Ophthalmology and Westmead Millennium Institute, University of Sydney, Sydney, New South Wales 2006, Australia

[13]Glaucoma Service, The Rotterdam Eye Hospital, Rotterdam, 3011 BH, the Netherlands

[14]Centre for Ophthalmology and Visual Science, Lions Eye Institute, University of Western Australia, Perth 6009, Western Australia, Australia

[15]Department of Ophthalmology, Flinders University, Adelaide, South Australia 5042, Australia

[*]Corresponding Author: Stuart MacGregor (300 Herston Road, Herston, Brisbane, Queensland 4006, Australia, fax: +61 7 3362 0101, phone: +61 7 3845 3563, Stuart.MacGregor@qimrberghofer.edu.au)

[‡]Henriët Springelkamp and Adriana I. Iglesias contributed equally to this work. Cornelia M. van Duijn and Stuart MacGregor jointly supervised this work.

Abstract

Primary open-angle glaucoma (POAG) is a blinding disease. Two important risk factors for this disease are a positive family history and elevated intraocular pressure (IOP), which is also highly heritable. Genes found to date associated with IOP and POAG are *ABCA1*, *CAV1/CAV2, GAS7*, and *TMCO1*. However, these genes explain only a small part of the heritability of IOP and POAG. We performed a genome-wide association study of IOP in the population-based Rotterdam Study I and Rotterdam Study II using single nucleotide polymorphisms (SNPs) imputed to 1000 Genomes. In this discovery cohort (n = 8,105) we identified a new locus associated with IOP. The most significantly associated SNP was rs58073046 (β = 0.44, p-value = 1.87×10^{-8}, minor allele frequency = 0.12), within the gene *ARHGEF12*. Independent replication in five population-based studies (n = 7,471) resulted in an effect size in the same direction that was significantly associated (β = 0.16, p-value = 0.04). The SNP was also significantly associated with POAG in two independent case-control studies (n = 1,225 cases and n = 4,117 controls; OR = 1.53, p-value = 1.99×10^{-8}), especially with high-tension glaucoma (OR = 1.66, p-value = 2.81×10^{-9}; for normal-tension glaucoma OR = 1.29, p-value = 4.23×10^{-2}). *ARHGEF12* plays an important role in the RhoA/RhoA kinase pathway, which has been implicated in IOP regulation. Furthermore, it binds to *ABCA1* and links the *ABCA1*, *CAV1/CAV2*, and *GAS7* pathway to Mendelian POAG genes (*MYOC*, *OPTN*, *WDR36*). In conclusion, this study identified a novel association between IOP and *ARHGEF12*.

Introduction

Glaucoma is a heritable eye disease affecting the optic nerve, which leads to irreversible visual field loss and eventually to blindness. Primary open-angle glaucoma (POAG) is the most common form of glaucoma. Individuals with a first-degree family member affected with POAG have a ten-fold increased risk of developing the disease (1). Variants in *MYOC*, *OPTN*, and *WDR36* explain some familial forms of POAG (2-7). However, disease-causing mutations in these genes are rare in POAG patients and therefore explain only a small part of the overall heritability. Genome-wide association studies (GWAS) have identified *CAV1/CAV2*, *TMCO1*, *SIX6* and *CDKN2B-AS1* as POAG genes, and recently *ABCA1*, *AFAP1*, and *GMDS* were added to the list (8-12).

Elevated intraocular pressure (IOP) is an important risk factor for glaucoma and the target of glaucoma therapy is lowering the IOP. IOP is highly heritable with heritability estimates ranging between 0.29-0.67(13, 14). *TMCO1, GAS7, FAM125B* were implicated in IOP, as well as the *CAV1/CAV2* region (12, 15). The International Glaucoma Genetics Consortium (IGGC) recently published a meta-analysis of IOP, reporting four new genes for IOP (*FNDC3B*, *ABCA1*, *ABO*, and a region on chromosome 11.p11.2 with many genes in it), and showed that one of the new genes (*ABCA1*) also influences the risk of developing POAG (16). This has shown that investigating the genetics of IOP is a fruitful approach to discover genes related to POAG.

The IGGC meta-analysis utilised data imputed to the HapMap 2 reference panel. In this study we aimed to identify new genetic variants associated with IOP using 1000 Genomes reference panel to increase the number of variants analysed in the population-based Rotterdam Study.

Results

After exclusion of 95 subjects with a history of IOP-lowering laser or surgery, 8,105 subjects were included in the meta-analysis of the discovery cohorts (Rotterdam Study I [RS-I] and Rotterdam Study II [RS-II]). The demographics of all individual studies are shown in Table 1. The inflation

factor (λ) was 1.03 for RS-I and 1.01 for RS-II, indicating good control of population substructures. The λ of the meta-analysis was 1.04 (Supplementary Figure 1). In the meta-analysis, 3 single nucleotide polymorphisms (SNPs) reached genome-wide significance (Figure 1). These 3 SNPs were located on chromosome 11q23.3 in the *ARHGEF12* gene. The most significantly associated SNP was the intronic variant rs58073046 (β = 0.44, p-value = 1.87 x 10^{-8}, minor allele frequency [MAF] = 0.12; Figure 2 and Table 2). Since IOP can be influenced by the central corneal thickness (CCT) we adjusted for CCT. Adjustment for CCT was possible in only 25% of the dataset. In this small subset, by chance the effect of rs58073046 on IOP without adjustment for CCT was smaller (β = 0.34, p-value = 4.44 x 10^{-2}, n = 2,036). After adjustment for CCT, the effect estimate was 0.36 and remained marginally significant despite the small sample size (p-value for effect of rs58073046 on IOP corrected for CCT = 3.35 x 10^{-2}, n = 2,036).

When combining the results of the validation cohort, rs58073046 was replicated (β = 0.16, p-value = 4.13 x 10^{-2}, n = 7,471; Table 2). The effect estimates of each individual study are shown in Figure 3. Figure 2 shows that 93 SNPs in the chromosome 11q23.3 region reached a p-value below 5.0 x 10^{-5} in the discovery cohort. All 93 SNPs were included in the combined meta-analysis of the discovery and replication cohorts (see Supplementary Table 1). The two most significant associations are two SNPs in linkage disequilibrium (pairwise correlation r2 is 1 in 1000 Genomes Pilot 1 in Northern Europeans) which has thus similar effect sizes and p-values (rs58073046: β = 0.30, p-value = 6.12 x 10^{-8}; rs11217863: β = 0.30 for the minor allele, p-value = 6.22 x 10^{-8}; for all studies together). Twenty-four of the 93 SNPs included in the combined meta-analysis are located in regulatory elements, particularly at enhancers (16/24) and promoter flanking regions (7/24), and one at a CTCF-binding site, suggesting an effect on IOP by altering regulation of *ARHGEF12* or other genes.

Expression profile of human *ARHGEF12* was investigated using UniGene, an expressed sequence tag (EST) database from NCBI. Positive expression was found in various tissues, being particularly high in the eye, vascular tissue, ear, adipose tissue, mouth, uterus, and skin (Supplementary Table 2). Eye specific expression of *ARHGEF12* was examined through the eye-centric genome browser, EyeBrowse, which showed that *ARHGEF12* is expressed in the cornea, lens, iris, trabecular

meshwork, retina, optic nerve, and human fetal eye (Supplementary Table 3a). Compared to other

genes in the neighbourhood (*POU2F3* and *TMEM136*) *ARHGEF12* presents the highest EST counts

in the eye (Supplementary Table 3b). This finding is consistent with microarray data from the Ocular

Tissue Database in which the highest expression of *ARHGEF12* occurred in the trabecular meshwork

(Supplementary Table 4).

The most significantly associated SNP (rs58073046) explained 0.4% (RS-I) and 0.3% (RS-II) of the

variance in IOP (Table 3). The explained phenotypic variance increased to 1.0% (RS-I) and 0.6%

(RS-II) by adding the 24 regulatory variants at 11q23.3, and to 2.2% (RS-I) and 2.6% (RS-II) by

adding all the other 11q23.3 variants which reached a p-value below 5.0×10^{-5} in the discovery

cohort, but the differences in explained variance are not statistically significant between the models.

The SNP (rs58073046) was also genome-wide significantly associated with POAG in 1,225 cases and

4,117 controls (Odds Ratio [OR] = 1.53, p-value = 1.99×10^{-8}; see Table 4). The association of

rs58073046 was stronger for high-tension glaucoma (HTG) (OR = 1.66, p-value = 2.81×10^{-9}) than

for normal-tension glaucoma (NTG) (OR = 1.29, p-value = 4.23×10^{-2}).

Figure 4 shows a network map of protein interactions created using the Ingenuity Pathway Analysis

(IPA) software. *ARHGEF12* binds directly to *ABCA1* and RhoA proteins, and interacts through other

proteins with genes implicated in POAG by GWAS (*CAV1/CAV2*, *GAS7*) or linkage analysis (*MYOC*,

OPTN and *WDR36*). No evidence was found for interactions with protein products of other known

IOP genes such as *ABO*, *TMCO1* or *FNDC3B*.

Discussion

The aim of this study was to identify new genetic variants that influence IOP using GWAS datasets

imputed to the 1000 Genomes reference panel. We have identified a new region, chromosome

11q23.3, associated with IOP. The SNP rs58073046 is located in *ARHGEF12*. This gene was

previously associated with POAG but the findings did not replicate (8). The association of the region with IOP is new.

Gharahkhani et al. previously reported an association between POAG and rs11827818 (OR 1.52, p-value = 9.2 x 10^{-9}), an intronic SNP located within the *TMEM136* gene near to *ARHGEF12*, in 1,155 cases and 1,992 controls from the ANZRAG study (8). We checked the association between the variant found by Gharahkhani et al. and POAG in the Genetic Research in Isolated Populations (GRIP)/Erasmus Rucphen Family (ERF) study consisting of 110 POAG cases. The magnitude for rs11827818 was smaller (OR 1.15 for overall glaucoma and OR 1.36 for HTG) than the magnitude of the most associated SNP rs58073046 observed in our study (OR 1.46 for overall POAG and OR 1.79 for HTG). These two SNPs (rs11827818 and rs58073046) are in partial linkage disequilibrium (pairwise correlation r2 is 0.51 in 1000 Genomes Pilot 1 in Northern Europeans). Gharahkhani et al. used the genotyped SNP rs2276035 within *ARHGEF12* for replication in other POAG case-control studies, however, this SNP did not clearly replicate. In our GRIP/ERF study, rs2276035 was not associated with POAG (OR 0.99 for overall POAG and OR 1.22 for those with HTG).

In our analysis of IOP, the SNP found by Gharahkhani et al. (rs11827818) was associated with an increased mean IOP level in our discovery cohorts but did not reach genome-wide significance (β = 0.30, p-value = 6.41 x 10^{-6}). In our replication cohorts the effect of rs58073046 on IOP was heterogeneous between studies, particularly in one small study (BATS) in which the effect was in the opposite direction. The I^2 for heterogeneity was 41.5 in the combined analysis of all studies. However, after removal of BATS the heterogeneity I^2 was 0.0 and the p-value became 5.04 x 10^{-9}. BATS is a relatively small and younger sample, which might explain the failure to replicate the findings. The effect estimates from all other replication cohorts were in the same direction as that from our discovery cohorts, though smaller in magnitude.

ARHGEF12 (Rho guanine nucleotide exchange factor (GEF) 12; previously known as Leukemia-Associated Rho Guanine Nucleotide Exchange Factor or *LARG*) may regulate RhoA GTPases (17). Rho proteins are important for numerous cellular processes. Activation of RhoA protein will lead to

the activation of ROCK, a RhoA kinase. It has been shown that RhoA/RhoA kinase signalling plays a role in regulation of trabecular meshwork plasticity, fibrogen activity, and myofibroblast activation (18). Activation of RhoA proteins can also decrease the permeability of Schlemm's canal cells (19). This links *ARHGEF12* to POAG as the regulation of IOP is a balance between the production of aqueous humour by the ciliary body and the outflow through the trabecular meshwork and Schlemm's canal cells. The changes in trabecular meshwork and Schlemm's canal cells lead to an increased resistance for the aqueous humour outflow and subsequently an elevated IOP. ROCK-inhibitors can decrease IOP by inducing relaxation of trabecular meshwork and ciliary body muscles and seems to be a good new target for IOP-lowering therapy (20).

Interestingly, *ARHGEF12* links the *ABCA1, CAV1/CAV2,* and *GAS7* genes, which has been previously associated with IOP as well as with POAG, to Mendelian POAG genes (*MYOC*, *OPTN*, and *WDR36*). The *ARHGEF12* gene interacts with *ABCA1*. *ARHGEF12* can extend the half-life of the ABCA1 protein, by binding to its C terminus and subsequently activating RhoA, which in turn prevents ABCA1 degradation (21). *ABCA1* plays a role in the transport of different molecules across extra- and intra-cellular membranes and the interference of *ARHGEF12* in the degradation of ABCA1 protein might extend the transportation of molecules. *ABCA1* is not the only glaucoma gene that has a role in the transport of vesicles. *CAV1*, *CAV2*, and *FAM125B* have been also implicated in vesicle transport (15).

In flies, *RhoGEF2* is the single homologue of mammalian *ARHGEF1*, *ARHGEF11* and *ARHGEF12*, and has been extensively studied in the context of tumorigenesis (22). Flies lacking *RhoGEF2* showed an early embryonic lethality (23, 24), while overexpression of this gene in eye resulted in small eyes, ablation of eye tissue, aberrant proliferation patterns, tissue morphology, and partially blocked differentiation (22). Overexpression of *Rho1* GTPase results in a rough eye phenotype with reduced retinal thickness (25), but in the presence of *RhoGEF2* the retina thickness is recovered (23), supporting the role of *RhoGEF2* as upstream activator of *Rho1* in the developing eye. No data about eye morphology or histology has been described in either knockout flies or mice. Absence of *arhgef12* in mice leads to embryonic lethality with incomplete penetrance, which might be explained

by redundancy of *arhgef11* and *arhgef12* (26). These findings suggest that *arhgef12* expression is required during eye and general development and that its absence may impact animal viability.

POU2F3 is another gene in the region on chromosome 11. It is a member of the POU domain family of transcription factors, which regulate cell type-specific differentiation pathways. *POU2F3* specifically regulates differentiation of keratinocytes (27). *POU2AF1* is a POU class-associating factor and is associated with CCT (28). Because IOP is related to CCT, we performed an additional analysis with extra adjustment for CCT in the discovery cohorts. Only a small subset of the discovery cohorts had CCT data available, therefore the association did not reach genome-wide significance after this additional adjustment. However, the beta was similar, suggesting that the signal of association on chromosome 11 is independent of CCT.

TMEM136 (transmembrane protein 136) is the gene between *ARHGEF12* and *POU2F3*. Compared to *TMEM136* and *POU2F3*, *ARHGEF12* showed the highest expression in the eye and particularly in the trabecular meshwork and ciliary body (Supplementary Table 3b and 4).These findings, besides its interaction with known POAG and IOP genes, are compatible with the view that *ARHGEF12* is most likely the gene causing the association signal. Nonetheless, further functional studies focusing on eye phenotypes are needed to clarify the role of chromosome 11q23.3 in the regulation of IOP and its influence on the risk of glaucoma.

In summary, our meta-analysis of GWAS has identified a new locus that may be important for the regulation of IOP and the risk of glaucoma. *ARHGEF12* is the most likely gene causing the association signal. It plays a role in the RhoA/RhoA kinase signalling which has been proven to be an important new target for glaucoma therapy. Our study shows that investigating the genetics of IOP is a fruitful way to elucidate the genetics of glaucoma.

Methods

We performed a meta-analysis of GWAS in two discovery cohorts -- RS-I and RS-II -- which are identical in population structure. Our replication cohorts include the Brisbane Adolescent Twins Study (BATS), Blue Mountains Eye Study (BMES), the Western Australian Pregnancy Cohort (Raine) Study, the Rotterdam Study III (RS-III), and Twins Eye Study in Tasmania (TEST). Next, we validated our findings in the Australian & New Zealand Registry of Advanced Glaucoma (ANZRAG) and GRIP/ERF POAG case-control studies. All studies adhered to the tenets of the Declaration of Helsinki and written, informed consent was obtained from all participants.

The Rotterdam Study

The Rotterdam Study is a population-based study established in Rotterdam, the Netherlands (29). It consists of three cohorts. The original cohort, RS-I, started in 1990 and includes 7,983 subjects aged 55 years and older. The second cohort, RS-II, was added in 2000 and includes 3,011 subjects aged 55 years and older. The last cohort, RS-III, includes 3,932 subjects of 45 years of age and older and started in 2006. In all three cohorts, IOP was measured for both eyes with Goldmann applanation tonometry (Haag-Streit, Bern, Switzerland). The measurement was done twice. If the second measurement was different from the first measurement, a third measurement was performed and the median of all three values was taken. A subset of participants from RS-I underwent CCT measurements at baseline using ultrasound pachymetry (Allergan Humphrey 850, Carl Zeiss Meditec, Dublin, CA, USA). Another subset of participants from RS-I, RS-II and RS-III underwent CCT measurements at follow-up using a non-contact biometer (Lenstar LS900, Haag-Streit, Köniz, Switzerland). Other ophthalmic baseline and follow-up examinations, which are still ongoing, were described previously (30). DNA was isolated from whole blood according to standard procedures. Genotyping of SNPs was performed using the Illumina Infinium II HumanHap550 array (RS-I), the Illumina Infinium HumanHap 550-Duo array (RS-I, RS-II), and the Illumina Infinium Human 610-Quad array (RS-I, RS-III). Samples with low call rate (<97.5%), with excess autosomal heterozygosity (>0.336), or with sex-mismatch were excluded, as were outliers identified by the

identity-by-state clustering analysis (outliers were defined as being >3 standard deviation (s.d.) from population mean or having identity-by-state probabilities >97%). A set of genotyped input SNPs with call rate >98%, MAF >0.001 and Hardy-Weinberg Equilibrium (HWE) p-value >10^{-6} was used for imputation. The Markov Chain Haplotyping (MACH) package version 1.0 software (Rotterdam, The Netherlands; imputed to plus strand of NCBI build 37, 1000 Genomes phase I version 3) and minimac version 2012.8.6 were used for the analysis. GWAS analyses were performed using the ProbABEL package (31). The analyses were adjusted for age, sex, and the first five principal components. The Rotterdam Study has been approved by the institutional review board (Medical Ethics Committee) of the Erasmus Medical Center and by the review board of The Netherlands Ministry of Health, Welfare and Sports.

Brisbane Adolescent Twins Study and Twins Eye Study in Tasmania

The Australian Twin Eye Study comprises participants examined as part of TEST or BATS. In most participants, the IOP was measured with the TONO-PEN XL (Reichert, Inc. New York, USA) (32). The Australian twin cohorts were genotyped on the Illumina Human Hap610W Quad array. The inclusion criteria for the SNPs were a MAF >0.01, HWE p-value ≥10^{-6}, and a SNP call rate >95% or Illumina Beadstudio Gencall Score ≥0.7, resulting in 543,862 SNPs. Imputation was done with reference to the August 4, 2010 version of the publicly released 1000 Genomes Project European genotyping using MACH. For BATS data, 1,152 people from 517 families were included in the analyses. For TEST data, 663 individuals from 350 families were included. Association analyses were performed in Merlin (http://www.sph.umich.edu/csg/abecasis/merlin/) by using the –fastassoc option. Ancestry, initially determined through self-reporting, was verified through Principal Component decomposition. The analyses were adjusted for age, sex, the technique of IOP measurement, and the first five principal components. The studies were approved by the human ethics committees of the University of Tasmania, Royal Victorian Eye and Ear Hospital, and Queensland Institute of Medical Research.

Blue Mountains Eye Study

The Blue Mountains Eye Study is a population-based cohort study of common eye diseases in older Australians living in the Blue Mountains region, west of Sydney, Australia. IOP was measured using Goldmann applanation tonometry (Haag-Streit, Bern, Switzerland) (33). DNA was extracted from whole blood and quality was validated by Sequenom iPLEX assay. Genotyping was performed on the Illumina Infinium platform using the Human660W-Quad, a Wellcome Trust Case Control Consortium 2 designed custom chip containing Human550 probes with 60,000 additional probes to capture common copy-number variations from the Structural Variation Consortium(34). Genotyped data were filtered to include SNPs with genotyping rate $\geq$0.97, MAF$\geq$1 %, HWE p-value $\geq 10^{-6}$. Samples with call rates less than 95% were excluded from analysis. Relatedness filtering based on estimated identity by descent was performed so that no pairs of individuals shared more than 20% of their genome. Ancestry outliers with >6 s. d. from 1000 Genomes northern European ancestry samples were removed. The IMPUTE2 software was used for imputation of data on 1000 Genomes phase 1 release version 3 (35, 36). The association test was performed using SNPTEST _v2.5-beta4 (37, 38). The analyses were adjusted for age, sex, and the first five principal components. The study was approved by the Human Research Ethics Committees of the University of Sydney and Sydney West Area Health Service.

Raine

The Western Australian Pregnancy Cohort (Raine) Study is an ongoing prospective cohort study of pregnancy, childhood, adolescence and young adulthood in Perth, Western Australia (39). At the initiation of the study, 2,900 pregnant women were recruited at 16-18 weeks' gestation from the state's largest public women's hospital and surrounding private practices for a randomized clinical trial investigating effects of intensive ultrasound and Doppler studies in pregnancy outcomes. Following this study, the offspring of the recruited individuals have been evaluated in detail during childhood and adolescence. At the 20-year review of the cohort, Raine participants underwent a comprehensive ocular examination for the first time (40). As part of this examination, IOP was

measured using an Icare TAO1i Tonometer (Icare Finland Oy, Helsinki, Finland). DNA samples and

consents for GWAS studies were available from the previous assessments. Genotype data were

generated using the genome-wide Illumina 660 Quad Array at the Centre for Applied Genomics

(Toronto, Ontario, Canada). Relatedness filtering based on estimated identity by descent was

performed so that no pairs of individuals shared more than 20% of their genome. We also excluded

people who had a high degree of missing genotyping data (> 3%). The data were filtered for a HWE

p-value > $1x10^{-6}$, SNP call rate >95%, and a MAF >0.01. GWAS imputation was performed in the

MACH v1.0.16 software using the November 23, 2010 version of the 1000 Genome Project European

genotyping. The association analyses were adjusted for age, sex, and the first two principal

components. This study was approved by the Human Research Ethics Committee of the University of

Western Australia.

Australian & New Zealand Registry of Advanced Glaucoma

ANZRAG recruits cases of advanced glaucoma Australia-wide through ophthalmologist referral. The

cohort also included participants enrolled in the Glaucoma Inheritance Study in Tasmania (GIST) who

met the criteria for ANZRAG. This cohort has been described previously (9). Advanced POAG was

defined as best-corrected visual acuity worse than 6/60 due to POAG, or a reliable 24-2 Visual Field

with a mean deviation of worse than -22db or at least 2 out of 4 central fixation squares affected with

a Pattern Standard Deviation of < 0.5%. The less severely affected eye was also required to have signs

of glaucomatous disc damage. Clinical exclusion criteria for this advanced POAG study were: i)

pseudoexfoliation or pigmentary glaucoma, ii) angle closure or mixed mechanism glaucoma; iii)

secondary glaucoma due to aphakia, rubella, rubeosis or inflammation; iv) infantile glaucoma, v)

glaucoma in the presence of a known associated syndrome. The ANZRAG cohort included 1,155

ANZRAG glaucoma cases and 1,992 controls genotyped on Illumina Omni1M or OmniExpress arrays

and imputed against 1000 Genomes Phase 1 Europeans. The case set included all samples from the

previously published GWAS (9). Controls were drawn from the Australian Cancer Study (225

oesophageal cancer cases, 317 Barrett's oesophagus cases and 552 controls) or from a study of

inflammatory bowel diseases (303 cases and 595 controls). The quality control methods were

performed in PLINK by removing individuals with more than 3% missing genotypes, SNPs with call rate <97%, MAF < 0.01 and HWE p-value < 0.0001 in controls and HWE p-value $< 5 \times 10^{-10}$ in cases (41). The same quality control protocol was used before merging the cases and controls to avoid mismatches between the merged data sets. After merging, the genotypes for 569,249 SNPs common to the arrays were taken forward for analysis. Relatedness filtering based on estimated identity by descent was performed so that no pairs of individuals shared more than 20% of their genome. Principal components were computed for all participants and reference samples of known northern European ancestry (1000G British, CEU and Finland participants) using the smartpca package from EIGENSOFT software (42, 43). Participants with principal component 1 or 2 values >6 s.d. from the known northern European ancestry group were excluded. Imputation was conducted using IMPUTE2 in 1-Mb sections, with the 1000 Genomes phase 1 Europeans (March 2012 release) used as the reference panel (35, 36). SNPs with imputation quality score >0.8 and MAF > 0.01 were carried forward for analysis. Association testing on the imputed data was performed in SNPTEST _v2.5-beta3 using an additive model (-frequentist 1) and full dosage scores (-method expected) with sex and the first six principal components fitted as covariates (37, 38). All were Australians of European ancestry. Approval was obtained from the Human Research Ethics Committees of Southern Adelaide Health Service/Flinders University, University of Tasmania, QIMR Berghofer Institute of Medical Research (Queensland Institute of Medical Research) and the Royal Victorian Eye and Ear Hospital.

Peak IOP measures were available for 1,039 of the 1,155 cases in the ANZRAG cohort. Of these cases, 330 (31.8%) had NTG (IOP ≤ 21 mm Hg) and 709 (68.2%) had HTG (IOP >21 mm Hg). Association testing for NTG and HTG was performed in SNPTEST _v2.5-beta3 as explained above, using 1,992 shared population controls.

Erasmus Rucphen Family study and Genetic Research in Isolated Populations program

The ERF study is a family-based cohort in a genetically isolated population in the southwest of the Netherlands with over 3,000 participants aged between 18 and 86 years (44, 45). In the region of the ERF population, a total of 110 patients with glaucoma who did not participate in the ERF study were

recruited in three local hospitals. Their visual fields were tested with standard automated perimetry (Humphrey Field Analyzer c24-2 SITA Standard test program) or the Octopus 101 (G2 program with TOP strategy) (Haag-Streit, Bern, Switzerland). The diagnosis of glaucoma was made by the patient's ophthalmologist and confirmed by a glaucoma specialist (HGL). It 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 seen by gonioscopy. Classification of HTG (IOP > 21 mmHG) and NTG (IOP ≤ 21 mmHG) was based on IOP at the time of diagnosis. Participants from the ERF study were used as control group (n = 2,125). Genotyping was performed with the 318K array of the Illumina Infinium II whole-genome genotyping assay (HumanHap300-2). Samples with low call rate (<97.5%), with excess autosomal heterozygosity (>0.336), or with sex-mismatch were excluded. A set of genotyped input SNPs with call rate >98%, with MAF >0.01, and with HWE p-value $>10^{-6}$ was used for imputation. We used the MACH package version 1.0.18.c software (Rotterdam, The Netherlands; imputed to plus strand of NCBI build 37, 1000 Genomes Phase I version 3) and minimac version 2012.8.15 for the analyses. Association tests were performed using the ProbABEL package (31). The analyses were adjusted for age and sex. All measurements in these studies were conducted after the Medical Ethics Committee of the Erasmus University had approved the study protocols.

Expression data

We investigated the expression profile of several genes using NCBI's UniGene(46), which is an organized view of the transcriptome that evaluates semi quantitatively the EST calculated as number of transcripts per million (online available at http://www.ncbi.nlm.nih.gov/unigene/). The EST data for "Breakdown by body site" that shows the approximate gene expression pattern in different tissues was chosen.

Expression of genes in eye tissues was evaluated using two databases: the EyeBrowse and the Ocular Tissue Database. The EyeBrowse is a customized eye-centric version of the UCSC Genome Browser, which includes A) eye-derived ESTs from the National Eye Institute (47) and B) the EyeSage project

(48, 49). The EyeBrowse is available at http://eyebrowse.cit.nih.gov/. We only selected human data. In the Ocular Tissue Database, the gene expression is indicated as Affymetrix Probe Logarithmic Intensity Error (PLIER) normalized value. The PLIER normalization method was described by Wagner et al (50). The Ocular Tissue Database is available at https://genome.uiowa.edu/otdb/.

Ensembl Genome Browser

The Ensembl Genome Browser release version 77 was used to investigate regulatory variants in genome-wide significant regions (51).

Ingenuity Pathway Analysis

Network map was created using the IPA software (Ingenuity Systems, http://www.ingenuity.com, Redwood City, CA, USA), where a) *ARHGEF12*, b) known IOP associated genes (*ABO* and *FNDC3B*), c) known genes associated with both IOP and POAG (*ABCA1, CAV1/CAV2, GAS7* and *TMCO1*), as well as d) known genes involved in familial forms of glaucoma (*OPTN, TMCO1, WDR36*) were selected. The "Path explorer" function (shortest +1) was used to map protein-protein interactions between *ARHGEF12* and the rest of included genes. All direct and indirect interactions are supported by at least one reference from the literature, a textbook, or canonical information stored in the Ingenuity Pathways Knowledge Base.

Statistical analysis

We used the mean IOP of right and left eye for the analysis. If IOP was missing for one eye, the IOP of the other eye was used. For participants receiving IOP-lowering medication, we added 30% to the IOP measurement to estimate a pre-medication IOP value (52). Participants who underwent IOP-lowering laser or surgery were excluded from the analysis. GWAS was performed on each individual study as described above under the assumption of an additive model for the effect of the risk allele. In a secondary analysis in the discovery phase CCT was included as an extra covariate. We used METAL software to carry-out an inverse variance weighted fixed-effect meta-analysis between RS-I and RS-II (53). SNPs with MAF <0.01 or with imputation quality score R^2 <0.5 were excluded. For

the meta-analysis of RS-I and RS-II a p-value of $<5.0 \times 10^{-8}$ (threshold of genome-wide significance) was considered statistically significant. Next, we validated the association results of the SNPs that reached genome-wide significance in five other studies (BMES, BATS, Raine, RS-III, and TEST). In the validation phase, a p-value <0.05 was considered statistically significant. Furthermore, in the discovery and validation cohorts we meta-analysed all the SNPs with p-value $<5.0 \times 10^{-5}$ in the region that reached genome-wide significance in the discovery cohort. We calculated the explained variance (R^2) of IOP by the new SNPs in RS-I and RS-II. In the first model, we calculated the explained variance for the most significantly associated SNP. Next, we added SNPs located within a regulatory element or all SNPs with p-value $<5.0 \times 10^{-5}$ to the model. The nested models were compared using an F test. Finally, we investigated the effect of the genome-wide significant SNPs on POAG in ANZRAG and ERF. A Manhattan plot, regional plots and forest plots were made using R (54) and LocusZoom (55).

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Conflict of interest

The authors declare no competing financial interests.

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Legends to Figures

Figure 1. Manhattan plot of the meta-analysis of genome-wide association studies for intraocular pressure in the discovery phase (n = 8,105). Each dot represents a single nucleotide polymorphism (SNP). The plot shows -log10 –transformed p-values for all SNPs. The upper black-dotted horizontal line represents the threshold of genome-wide significance (p-value < 5.0 x 10^{-8}); the lower black-dotted horizontal line represents a p-value of 1 x 10^{-5}.

Figure 2. Regional association and recombination plot of the 11q23.3 region in the meta-analysis of the discovery cohorts. Plots are centered on rs58073046 (purple diamond), the most significantly associated single nucleotide polymorphism (SNP) in this region, and flanked by the meta-analysis results for SNPs in the 400-kb region surrounding it. SNPs are shaded according to their pairwise correlation (r^2) with rs58073046. The blue line represents the estimated recombination rates; the gene annotations are shown below the figure.

Figure 3. Forest plot for rs58073046 (chromosome 11q23.3). For each study, the square shows the beta linear regression coefficient or the average difference in intraocular pressure for each additional copy of the minor allele (G) and the lines represent the standard error of the estimate.

Abbreviations: BATS = Brisbane Adolescent Twins Study; BMES = Blue Mountains Eye Study; RS = Rotterdam Study; TEST = Twins Eye Study in Tasmania

Figure 4. Network map of protein-protein interactions between *ARHGEF12* with a) previously known genes associated with IOP and glaucoma (*ABCA1*, *CAV1/CAV2*, *GAS7*), and b) known genes involved in familial forms of glaucoma (*MYOC*, *OPTN*, *WDR36*). Map was built using Ingenuity Pathway Analysis. Solid lines imply direct relationships between proteins (e.g. physical protein-protein interaction or enzyme-substrate); dotted lines imply indirect functional relationships, such as co-expression, phosphorylation/dephosphorylation, activation/deactivation, transcription or inhibition. Proteins in **bold** correspond to known glaucoma genes. Meaning of symbols is shown on the right side of the figure.

TABLES

Table 1. Characteristics of the discovery and replication studies.

	Discovery cohorts (n = 8,105)		Replication cohorts (n = 7,471)				
	RS-I	RS-II	RS-III	BATS	BMES	Raine	TEST
n included in analysis	6010	2095	2992	1152	1769	895	663
mean age (SD)	69.2 (9.0)	64.8 (7.9)	57.2 (6.8)	20.1 (4.0)	64.0 (8.3)	20.0 (0.4)	25.6 (18.8)
% male	40	46	44	53	43	49	60
mean IOP (SD)	14.7 (3.2)	14.2 (3.1)	13.6 (2.9)	15.8 (2.9)	16.1 (2.7)	14.9 (4.7)	15.8 (3.1)
n of participants with IOP lowering medication	112	40	35	-	38	-	-
n of participants with IOP lowering laser/surgery	59	36	12	-	18	-	-

Abbreviations: BATS = Brisbane Adolescent Twins Study; BMES = Blue Mountains Eye Study; IOP

= intraocular pressure; n = number of samples; RS = Rotterdam Study; SD = standard deviation;

TEST = Twins Eye Study in Tasmania

Table 2. Summary of the discovery and replication findings of the genome-wide search for intraocular pressure related genes using data imputed to the 1000 Genomes reference.

				Discovery stage (n = 8,105)			Replication stage (n = 7,471)			Meta-analysis (n = 15,576)			
SNP	Chr/pos	A1/A2	MAF	β	SE	P-value	β	SE	P-value	β	SE	P-value	I^2
rs58073046	11/120248493	g/a	0.12	0.44	0.08	1.87×10^{-8}	0.16	0.08	4.13×10^{-2}	0.30	0.06	6.22×10^{-8}	41.6

Abbreviations: A1 = allele 1, the effect allele; A2 = allele 2; β = effect size on intraocular pressure based on allele 1; Chr = chromosome; MAF = minor allele frequenty (=A1); $I^2 = I^2$ for heterogeneity between all samples; pos = position; SE = standard error; SNP = single nucleotide polymorphism

Table 3. The explained variance of intraocular pressure in the Rotterdam Study I (RS-I) and

Rotterdam Study II (RS-II). Models with different predictors were tested and the p-value shows the p-

value of the difference in explained variance for model 2, model 3, and model 4 compared to model 1.

	RS-I		RS-II	
	Explained variance (%)	P-value	Explained variance (%)	P-value
Model 1 = rs58073046	0.4%		0.3%	
Model 2 = model 1 + promotor flanking region SNPs	0.6%	0.28	0.4%	0.89
Model 3 = model 2 + enhancers + CTCF binding site	1.0%	0.19	0.6%	0.82
Model 4 = model 3 + all other SNPs (93 in total)	2.2%	0.06	2.6%	0.16

Table 4. Result of the association of rs58073046 with primary open-angle glaucoma (POAG). The table shows the association result for all POAG, as well as for the subtypes high-tension glaucoma (intraocular pressure >21 mmHg) and normal-tension glaucoma (intraocular pressure ≤21 mmHg).

		All POAG				HTG				NTG			
	controls (n)	cases (n)	OR	95% CI	P-value	cases (n)	OR	95% CI	P-value	cases (n)	OR	95% CI	P-value
ANZRAG	1,992	1,115	1.54	1.32-1.80	3.14×10^{-7}	709	1.65	1.38-1.97	2.12×10^{-7}	330	1.33	1.03-1.72	3.01×10^{-2}
ERF/GRIP	2,125	110	1.46	0.91-2.35	1.27×10^{-1}	68	1.79	1.04-3.09	4.10×10^{-2}	42	0.92	0.41-2.06	8.36×10^{-1}
Meta-analysis	4,117	1,225	1.53	1.32-1.78	1.99×10^{-8}	777	1.66	1.41-1.97	2.81×10^{-9}	372	1.29	1.01-1.64	4.23×10^{-2}

Abbreviations: ANZRAG = Australian & New Zealand Registry of Advanced Glaucoma; CI = confidence interval; ERF/GRIP = Erasmus Rucphen Family study and Genetic Research in Isolated Populations; HTG = high-tension glaucoma; NTG = normal-tension glaucoma; OR = Odds Ratio; POAG = primary open-angle glaucoma

Please note that the sum of HTG and NTG is not equal to the total number of cases in the ANZRAG cohort, since peak IOP measures were only available for 1,039 of the 1,155 cases.

List of abbreviations

A1 = allele1, the effect allele

A2 = allele2, the other allele

ANZRAG = Australian & New Zealand Registry of Advanced Glaucoma

β = effect size on intraocular pressure based on allele 1

BATS = Brisbane Adolescent Twins Study

BMES = Blue Mountains Eye Study

CCT = central corneal thickness

Chr = chromosome

CI = confidence interval

ERF = Erasmus Rucphen Family

EST = expression sequence tag

GIST = Glaucoma Inheritance Study in Tasmania

GRIP = Genetic Research in Isolated Populations

GWAS = genome-wide association study

HTG = high-tension glaucoma

HWE = Hardy-Weinberg Equilibrium

IGGC = International Glaucoma Genetics Consortium

IOP = intraocular pressure

IPA = Ingenuity Pathway Analysis

MACH = Markov Chain Haplotyping

MAF = minor allele frequency

NTG = normal-tension glaucoma

OR = Odds Ratio

PLIER = Probe Logarithmic Intensity Error

POAG = primary open-angle glaucoma

Pos = position

RS = Rotterdam Study

SD = standard deviation

SE = standard error

SNP = single nucleotide polymorphism

TEST = Twins Eye Study in Tasmania