Evaluation of the myocilin mutation Gln368Stop (rs74315329) demonstrates reduced penetrance in European populations

Abhishek Nag¹, Han Lu², Matthew Arno², Adriana I. Iglesias³,⁴, Pieter Bonnemaijer³,⁴, Linda Broer⁵, Andre G. Uitterlinden³,⁵,⁶, Caroline C.W. Klaver⁴, Cornelia van Duijn³, Pirro G. Hysi¹,⁷, Christopher J. Hammond¹,⁷

¹Department of Twin Research and Genetic Epidemiology, King's College London, St. Thomas' Hospital, London, UK
²King's Genomics Facilities, King's College London, London, UK
³Department of Epidemiology, Erasmus Medical Center, Rotterdam, the Netherlands
⁴Department of Ophthalmology, Erasmus Medical Center, Rotterdam, the Netherlands
⁵Department of Internal Medicine, Erasmus Medical Center, Rotterdam, the Netherlands
⁶Netherlands Consortium for Healthy Ageing, Netherlands Genomic Initiative, the Hague, the Netherlands
⁷Department of Ophthalmology, King’s College London, London, UK

Corresponding author

Prof. Christopher J. Hammond
Department of Twin Research and Genetic Epidemiology,
King’s College London,
St. Thomas’ Hospital,
London
United Kingdom
Email: chris.hammond@kcl.ac.uk
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No conflicting relationship exists for any author

Running Title

Gln368Stop myocilin mutation and glaucoma

Abbreviations and Acronyms

MYOC (myocilin), POAG (primary open-angle glaucoma), OPTN (optineurin), GWAS (genome-wide association study), JOAG (juvenile open-angle glaucoma), OR (odds ratio), IOP (intraocular pressure), ORA
(Ocular Response Analyser), RS (Rotterdam Study), HRC (Haplotype Reference Consortium), GVFL (glaucomatous visual field loss), MAF (minor allele frequency), CI (confidence intervals), OMIM (Online Mendelian Inheritance in Man)
Abstract

OBJECTIVE: Sequence variations in the myocilin (MYOC) gene account for ~2–4% of the glaucoma cases. One particular MYOC mutation, Gln368Stop (dbSNP accession number: rs74315329), is the most common genetic mutation causing glaucoma, by raising the intraocular pressure. The objective of this study was to evaluate the effect of this MYOC mutation on intraocular pressure using data from large-scale European population panels (directly sequenced and imputation-based).

DESIGN: Cross-sectional, cohort study

PARTICIPANTS: For this study (combined sample size of over 17,000), the discovery and the replication phases were conducted in population-based cohorts, the TwinsUK and the Rotterdam Study, respectively.

METHODS: Carriers of the risk allele for rs74315329 were identified using whole-genome sequencing and imputation data (based on 1000 Genomes and Haplotype Reference Consortium panels). The effect of this variant was evaluated using intraocular pressure measurements and data on visual field testing / a diagnosis of glaucoma (if available).

MAIN OUTCOME MEASURES: The penetrance of the variant rs74315329 was estimated from the percentage of the carriers of the risk
allele of the variant that had high intraocular pressure (ocular hypertension) and/or glaucoma.

**RESULTS:** In our study, the observed penetrance of the variant rs74315329 in relation to raised intraocular pressure was 12.5% and 19.4% in the TwinsUK and the Rotterdam Study respectively, suggesting a much lower penetrance for high intraocular pressure (and hence, glaucoma) in comparison to that reported previously.

**CONCLUSIONS:** The significance of this finding is that higher numbers of healthy individuals in the population are expected to be carriers of this mutation, which in turn reduces the utility of identifying carriers of this mutation as a screening tool for glaucoma.
Introduction

Glaucoma is the leading cause of irreversible blindness in the world\(^1\).

Primary open-angle glaucoma (POAG) is the commonest subtype of glaucoma occurring in Caucasian populations, accounting for about 50% of all the glaucoma cases.

Several studies have reported positive family history as a risk factor for glaucoma\(^2-4\), thus suggesting a role of genetic factors in the development of glaucoma.

Previously, linkage studies have implicated several genetic loci in the Mendelian forms of glaucoma that segregated in families (www.omim.org), with the causal gene identified at three of these loci – myocilin (MYOC) at the GLC1A locus\(^5\), optineurin (OPTN) at the GLC1E locus\(^6\) and TBK1 at the GLC1P locus\(^7\). More recently, the advent of genome-wide association studies (GWAS) has led to the identification of many genetic loci in association with POAG\(^8-10\) and its intermediate phenotypes\(^11-15\).

MYOC, the causal gene at the GLC1A locus (OMIM 601652), was identified in pedigrees with juvenile open-angle glaucoma (JOAG), a term used to refer to POAG with an earlier age of onset and an autosomal dominant mode of inheritance\(^16\). Sheffield et al. (1993)\(^17\) first reported the GLC1A linkage locus (mapping to chromosome 1q21-q31) in a family with 22 members
affected with JOAG. Subsequently, Stone et al. (1997)\(^5\), using a combination of fine-mapping and candidate gene approach at the 1q21-q31 locus, identified missense mutations within the *MYOC* gene [also known as trabecular meshwork-induced glucocorticoid response protein (*TIGR*) gene] that segregated with the disease. Since then, several other investigators have reported *MYOC* mutations segregating within families of JOAG as well as adult-onset POAG\(^{18-21}\). Moreover, analysis of *MYOC* mutations in sporadic cases of POAG from various ancestries (Caucasian, Asian and African-American) has demonstrated that a range of probable disease-causing *MYOC* mutations account for ~2–4% cases\(^{22,23}\).

To date over 100 *MYOC* gene mutations (~85% of which are missense) have been reported in association with JOAG and POAG (http://www.myocilin.com/variants.php, last accessed 25 March 2016). Of these, Gln368Stop (dbSNP accession number: rs74315329) is the commonest glaucoma-causing *MYOC* mutation (accounting for >40% of the POAG cases due to *MYOC* mutations) in the population\(^5,22,23\). The higher frequency of Gln368Stop among all the known *MYOC* mutations, coupled with the fact that it has been reported in majority of the populations investigated so far, suggested that there might be a possible founder effect for this mutation\(^{23}\). Subsequently, studies have demonstrated that the same
disease haplotype for the Gln368Stop mutation was present in 15 unrelated affected Caucasian families settled in Australia\textsuperscript{24} and a large affected French-Canadian family\textsuperscript{25}, supporting a common ancestral origin for this mutation.

So far, the studies that have evaluated the Gln368Stop MYOC mutation in sporadic POAG cases, have demonstrated a high odds of developing the disease with this mutation – Fingert et al. (1999)\textsuperscript{23} found that 27/1,693 POAG cases carried the mutation compared to 1/793 controls (OR = 12.84). More recently, Gharahkhani et al. (2015)\textsuperscript{26} used an imputation-based method with the 1000 Genomes reference panel in advanced POAG cases from the ANZRAG study, and further affirmed the high effect of the Gln368Stop mutation (OR = 15.53).

The Gln368Stop MYOC mutation appears to increase POAG risk by raising the intraocular pressure (IOP): sporadic as well as familial POAG cases harbouring the Gln368Stop mutation have a higher mean IOP compared to the general population, with the mean IOP for the mutation carriers ranging between 27.7 and 30 mm Hg\textsuperscript{20,22,27–29}.

The recent availability of large-scale population-based sequencing data has made it possible to evaluate the effect of Gln368Stop on POAG risk in the population at large. Here we aim to test the effect of Gln368Stop (hereafter
also referred to as rs74315329) on IOP, and by extension on POAG, in European population panels using directly sequenced and imputation-based data.
Materials and Methods

The primary study was conducted in the TwinsUK cohort, a population-based study of healthy twin volunteers\textsuperscript{30}. Volunteering twin siblings were unaware of the eye studies interests at the time of enrolment and gave fully informed consent under a protocol reviewed by the St. Thomas’ Hospital Local Research Ethics Committee.

As a part of the UK10K project\textsuperscript{31}, 1,854 unrelated subjects from the TwinsUK had their whole genome sequenced at a low coverage (average depth of coverage was 6x) at the Wellcome Trust Sanger Institute’s core sequencing facility with Illumina’s GAII sequencing machines. In addition, another subset of 1,190 TwinsUK subjects (which included some twin pairs) was sequenced at a much higher average depth of coverage (30x-40x). Discrete genotype calls were available for all the variable sites that were identified in the sequencing dataset, which was used to identify the TwinsUK subjects that were carriers of the allele A (hereafter referred to as the ‘risk allele’) for the variant rs74315329 in the \textit{MYOC} gene.

An additional subset of 3,048 TwinsUK subjects (which included some twin pairs), that was not sequenced but for which Chip genotype data were available (genotyped using two different Illumina genotyping platforms:
317K Duo and HumanHap610K-Quad arrays), imputed genotypes based on the 1000 Genomes Phase 3 reference panel ([http://csg.sph.umich.edu//abecasis/MACH/download/1000G.Phase3.v5.htm](http://csg.sph.umich.edu//abecasis/MACH/download/1000G.Phase3.v5.htm)) were used to identify additional carriers of the risk allele for rs74315329. Phasing of the genotypes was done using the software MaCH ([http://csg.sph.umich.edu//abecasis/MACH/tour/imputation.html](http://csg.sph.umich.edu//abecasis/MACH/tour/imputation.html)) and genotype imputation for markers in the 1000 Genomes reference panel was done using the software Minimac ([http://genome.sph.umich.edu/wiki/Minimac](http://genome.sph.umich.edu/wiki/Minimac)). Imputed data contains the probabilities of the possible genotypes at each marker. An arbitrary threshold of 80% for the probability of a heterozygous genotype was used to identify carriers of the risk allele for rs74315329.

In the TwinsUK cohort, IOP was measured using the Ocular Response Analyser (ORA), a non-contact air-puff tonometer. The mean IOP of the two eyes was used for the analysis. Throughout this study, an IOP reading greater than 21 mm Hg will be considered as ‘high IOP’ i.e. ocular hypertension. For the individuals that were identified as carriers of the risk allele for rs74315329, the most recent IOP readings, visual field testing information and POAG status were obtained through follow-up with their local optician (which was on an average five years after their initial
recruitment). In all the cases, follow-up IOP at the local optician was measured using a non-contact tonometer. These individuals were also enquired regarding any history of taking IOP-lowering medication and POAG diagnosis / surgery.

For the TwinsUK individuals that were carriers of the risk allele for rs74315329 (identified using the whole-genome sequencing and the 1000 Genomes imputation datasets, as described above), validation of the genotype for this variant was performed using Sanger sequencing. Further details of the Sanger sequencing methodology that was used are provided in the Supplementary Information (available at www.aaojournal.org).

The Rotterdam Study, a population-based study based in Rotterdam (Netherlands) was used for the replication of the findings observed in the TwinsUK. The Rotterdam study comprises of three cohorts – RSI, RSII and RSIII (combined N = 11,189). In the Rotterdam Study cohorts, imputation data was used to identify the carriers of the risk allele for rs74315329. Genotyping in these cohorts was performed using a combination of genotyping platforms - 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). Imputation was performed on the Michigan Imputation Server.
using the reference panel released by the Haplotype Reference Consortium (HRC). An arbitrary threshold of 80% for the probability of a heterozygous genotype for rs74315329 was used to identify the risk allele carriers. Exome sequencing data that was available in a subset of the RS-I subjects\textsuperscript{32} was used to confirm the genotype for rs74315329 for the individuals that were identified as carriers of the risk allele for this variant using the imputation data. For all three Rotterdam cohorts, IOP from the most recent assessment, measured using Goldmann applanation tonometry, was used. The subjects in all three Rotterdam cohorts also had their optic discs and visual fields assessed in order to detect the presence of glaucomatous optic neuropathy or glaucomatous visual field loss (GVFL)\textsuperscript{33}. In addition, any history of taking IOP-lowering medication and POAG diagnosis / surgery was also available. Further details on the Rotterdam Study are available in Hofman et al. (2016)\textsuperscript{34}. 
Results

In the TwinsUK, the average read depth for rs74315329 was ~7.5x and ~33x in the low-coverage and the high-coverage sequencing subsets, respectively. Seven individuals (out of a total 3,044) from the sequencing dataset were identified as heterozygous (carriers) for the risk allele (allele A) of the variant rs74315329. No individual was homozygous for the risk allele of this variant.

In the 1000 Genomes–based imputation dataset for the TwinsUK, the variant rs74315329 was well imputed, with an imputation quality score (r-squared) of 0.56. The imputation dataset identified an additional two TwinsUK individuals with greater than 80% probability of a heterozygous genotype for rs74315329. In the imputation dataset, a high probability (>90%) of a heterozygous state for rs74315329 was observed in six of the seven TwinsUK individuals that were initially identified as carriers of the risk allele for this variant in the sequencing dataset (Table 1).

Consequently, a total of nine unrelated individuals in the TwinsUK (out of a combined panel of 6,092 individuals) were identified as carriers of the risk allele for rs74315329, using a combination of whole genome sequencing and imputation-based data (Table 1). Seven of the nine risk allele carriers did not have data on their co-twin (in their respective datasets). In the case of the
remaining two risk allele carriers that had data available on their co-twin (both were dizygotic twin pairs), the co-twin was not a carrier of the risk allele i.e. they were homozygous for the non-risk allele of rs74315329.

Sanger sequencing confirmed the heterozygous genotype in eight of the nine carriers of the risk allele for rs74315329 in the TwinsUK (Supplementary Figure 1, available at www.aaojounal.org). The one risk allele carrier that failed to validate on Sanger sequencing exhibited a homozygous genotype for the non-risk allele (allele G) of rs74315329. This individual had been identified as a risk allele carrier in the sequencing dataset, and the imputation data suggested that this individual had greater than 99% probability for a heterozygous state for rs74315329 (Table 1). Since this individual belonged to the sequencing dataset, in which only one co-twin per pair was sequenced, data on the co-twin of this individual was not available.

The minor allele frequency (MAF) of rs74315329 in the combined TwinsUK panel was ~0.07% (for either scenario – eight or nine risk allele carriers), which is similar to that observed in the 1000 Genomes project (0.06%). The exome sequencing databases such as EVS (http://evs.gs.washington.edu/EVS/) and ExAC (http://exac.broadinstitute.org/) report a comparatively higher MAF for rs74315329 (0.14% and 0.15%, respectively). Exome sequencing projects
typically sequence at a much higher depth of coverage as compared to whole-genome sequencing projects (such as the 1000 Genomes project) – accordingly, a possible under-calling of heterozygous genotypes in the latter could explain the comparatively lower MAF (but in compliance with the 1000 Genomes project) for rs74315329 observed in the TwinsUK.

Table 1 provides the list of the TwinsUK samples that were identified as carriers of the risk allele for rs74315329 and their findings in the different datasets that were used.

IOP information (either on initial recruitment or on follow-up) was available for eight of the nine TwinsUK individuals that were carriers of the risk allele for rs74315329. Only one of these eight mutation carriers recorded a high IOP (>21 mm Hg), either on initial measurement or on follow-up (Table 2). The mutation carrier with high IOP (Sample No. 7), on follow-up, had also developed visual field defects and had undergone trabeculectomy in both the eyes. For this individual, the IOP prior to trabeculectomy was available only for the left eye (27.3 mm Hg), while the same for the right eye was not available. Post-trabeculectomy, the most recent IOP of this individual had reduced to 12.3 mm Hg (13.3 mm Hg and 11.3 mm Hg, for the right and left eye, respectively).
None of the remaining seven mutation carriers recorded a higher than normal IOP, either on initial measurement or on follow-up (a mean of five years later). In addition, none of these seven individuals had any history of taking IOP-lowering medication; and for five of these seven individuals, visual field data available from the most recent assessment at their local optician showed no GVFL.

In the Rotterdam cohorts, HRC-based imputation data was used to identify individuals with a heterozygous genotype (carriers) for rs74315329. Twelve, seven and twelve individuals were identified as carriers of the risk allele for rs74315329 in the RS-I, RS-II and RS-III, respectively. The heterozygous genotype for rs74315329 was confirmed in all six carriers (out of twelve) in the RS-I that also had exome sequencing data. In the RS-I, three of the twelve carriers of the risk allele for rs74315329 who had normal IOP on assessment were on IOP-lowering medication; it is therefore assumed that these three individuals had ocular hypertension before the initiation of treatment. None of the 12 mutation carriers showed any GVFL. In the RS-II, two of the seven mutation carriers were previously diagnosed with glaucoma (with high IOP) and have had laser surgery for the same (only one of these two individuals showed GVFL on assessment). Another RS-II mutation carrier, who initially had normal IOP on IOP-lowering medication, provided
history of glaucoma laser surgery on follow-up visit. In the RS-III, none of
the 12 mutation carriers had high IOP, were on IOP-lowering medication,
showed GVFL on assessment, or reported any history of glaucoma. Details
such as the heterozygous genotype probability, IOP and age of the mutation
carriers in the RS-I, RS-II and RS-III are summarised in Supplementary
Table 1 (available at www.aaojournal.org).
Overall, in the TwinsUK, one of the eight carriers of the risk allele for the
MYOC variant rs74315329 had high IOP; while in the Rotterdam Study (RS
I-III), 6 of the 31 risk allele carriers had high IOP, and three of those six
individuals have been diagnosed with POAG. For rs74315329, this
corresponds to a penetrance of 12.5% (95% C.I. = 0.7% - 53.3%) and 19.4%
(95% C.I. = 8.1% - 38.1%) with respect to high IOP or ocular hypertension,
in the TwinsUK and the Rotterdam Study, respectively. Likewise, the
penetrance of rs74315329 with respect to POAG is 9.7% (95% C.I. = 2.5% -
26.9%) in the Rotterdam Study, in which a complete assessment for POAG
was available. An age-dependent penetrance (with respect to high IOP and
POAG) of the variant rs74315329 in the TwinsUK and the Rotterdam Study
is summarised in Table 3.
Only one other MYOC variant (rs202176570), of the remaining 17 variants
that have been catalogued in the OMIM database
(http://www.omim.org/entry/601652), was non-monomorphic in the TwinsUK sequencing dataset. This variant, however, was too rare (just one heterozygous individual) to enable any meaningful assessment of its effect on IOP.
Discussion

In this study, we analysed the effect of the known glaucoma-causing MYOC mutation Gln368Stop (variant rs74315329) using whole genome sequence data and imputed data based on large scale population-based sequencing panels. POAG cases with this variant often have very high IOPs, on average 30 mm Hg\textsuperscript{35}. The protein product of the MYOC gene, which has a cytoskeletal function, is widely expressed in ocular tissues, in particular the trabecular meshwork\textsuperscript{18,36}. MYOC mutations (including the Gln368Stop mutation) lead to a buildup of abnormal protein in the trabecular meshwork, which impairs the trabecular outflow of aqueous humour, thus raising the IOP\textsuperscript{37}. This suggests that the MYOC mutations possibly exert a toxic gain-of-function effect, a finding that has been verified by MYOC-knockout studies in mice\textsuperscript{38}. For some of the subjects in our study, comprehensive glaucoma assessment (optic disc imaging and visual field testing) was unavailable or was obtained via their local optician. Given that the disease-causing MYOC mutations (including rs74315329) cause POAG by raising the IOP, the complete availability of IOP measurements in the risk allele carriers for rs74315329 allowed us to evaluate its penetrance with relation to POAG, using IOP as a proxy. In our study, seven of the eight risk allele carriers for rs74315329 in the TwinsUK, and 25 of the 31 risk allele carriers
for rs74315329 in the Rotterdam Study, had IOP less than or equal to 21 mm Hg.

So far, majority of the studies that have evaluated the association of the MYOC variant rs74315329 (the Gln368Stop mutation) with ocular hypertension and/or POAG have implicated a much higher penetrance compared to what was observed in our study\textsuperscript{22,27,28,35}. For instance, Allingham et al. (1998)\textsuperscript{27} observed that the penetrance of the Gln368Stop mutation was 100\% and 78\% with respect to ocular hypertension and POAG respectively by age 70; similarly, for the same mutation, Fingert et al. (2002)\textsuperscript{35} reported a penetrance with respect to POAG as high as 90\%. In our study, the observed penetrance of the MYOC variant rs74315329 for ocular hypertension was 12.5\% and 19.4\% in the TwinsUK and the Rotterdam Study, respectively. Part of the reason for the reduced penetrance of rs74315329 observed in our study is that previous studies might have overestimated its penetrance, they being either family-based studies or population-based case-control studies. Mutations can show increased penetrance in family-based studies due to more ready ascertainment of families with multiple affected individuals as well as due to the existence of additional genetic or environmental modifiers within families\textsuperscript{39}. On the other hand, population-based case-control studies, by design, tend to oversample
for cases, which can overestimate the penetrance of mutations if the population prevalence of the disease is not accounted for\textsuperscript{40}.

Accounting for the known population prevalence of POAG, we estimated the expected penetrance of rs74315329, based on the previously reported odds ratios (12.8 to 15.5) for the risk allele of rs74315329\textsuperscript{23,26}. Given a population prevalence rate for POAG in Caucasians aged between 40 and 80 years (age group of the risk allele carriers for rs74315329 observed in the TwinsUK and the Rotterdam Study) of \textasciitilde2.5\%\textsuperscript{41}, the expected POAG prevalence rate among the risk allele carriers for rs74315329 (i.e. the expected penetrance of rs74315329) can be estimated to range between 24.2\% and 27.9\% (the calculation has been approximated based on the fact that the population prevalence of POAG and the MAF of rs74315329 are both small). The observed penetrance of rs74315329 for ocular hypertension in the TwinsUK and the Rotterdam Study (12.5\% and 19.4\% respectively) may be even lower for POAG, since not all individuals with ocular hypertension progress to POAG\textsuperscript{42}. In fact, POAG assessment in the Rotterdam Study has shown that thus far only three of the six mutation carriers with high IOP show any evidence of GVFL. As is evident, even after accounting for the known population prevalence of POAG, the penetrance of rs74315329 for ocular hypertension, and by extension for
POAG, observed in our study, is lower than what would be expected based on the odds ratio estimates reported by previous studies.

Since the penetrance of the variant rs74315329 in relation to POAG is known to increase with age\textsuperscript{27,28}, a finding that was also corroborated by our study (Table 3), the age of our cohorts in comparison to the previous studies is an important consideration. Alward et al. (1998)\textsuperscript{22}, one of the studies that we used for the estimation of the expected penetrance of rs74315329 (as described above), reported that the average age of POAG diagnosis among the Gln368Stop mutation carriers in their study was 59 years. In our study, the mean age of the 32 mutation carriers that did not have raised IOP or a diagnosis of POAG was 65.2 years. In the case of our youngest cohort (RS-III), though all the twelve mutation carriers (without raised IOP or a diagnosis of POAG), barring one, were older than 50 years, only three of them were older than 59 years. For the remaining three cohorts (TwinsUK, RS-I and RS-II), 19 of the 20 mutation carriers without raised IOP or a diagnosis of POAG were older than 59 years. Thus, barring the RS-III to an extent, we do not expect the age of our cohorts to significantly impact the estimation of the penetrance of the MYOC variant.

To date, in the studies that evaluated the penetrance of mutations, the number of control subjects that were analysed was limited in number. The
recent availability of large-scale population-based sequencing panels has made it possible to ascertain the penetrance of a number of known disease-causing mutations using sufficiently powered population-based studies. We believe that our estimation of the penetrance of the Gln368Stop \textit{MYOC} mutation i.e. rs74315329 using a population-based panel of over 17,000 subjects might represent a more “realistic” measure of its penetrance compared to its previous estimates. This observation of a lower than expected penetrance of the \textit{MYOC} variant rs74315329 is in accordance with recent findings for mutations that have previously been implicated in diseases\textsuperscript{31,43,44}. Narasimhan et al. (2016)\textsuperscript{44} sequenced the exomes of \textasciitilde3,000 Pakistanis with high levels of consanguinity and observed that there were as many as 29 instances of rare homozygous loss-of-function mutations in genes catalogued in the OMIM database in individuals that showed no manifestations consistent with the expected OMIM phenotype. The implication of the reduced penetrance of the Gln368Stop \textit{MYOC} mutation (which results in premature termination of the myocilin protein) is that higher numbers of healthy individuals in the population are expected to be carriers of this mutation than estimated previously. Hence, the finding of this mutation in an individual would require cautious interpretation. The
reduced penetrance also implies that the genotype-phenotype correlation between this mutation and POAG is likely to be much weaker than estimated previously, thus potentially reducing the utility of knowing the genotype at rs74315329 as a predictive tool in identifying those at a high-risk of developing POAG. Nonetheless, given the difficulties and poor efficacy of screening for glaucoma with current paradigms\(^{45}\), using genetic variants that offer even modest predictive value might still be useful to target subjects for screening.

A limitation of the TwinsUK cohort is that it is a volunteer-based cohort with a potential “healthy volunteer” bias. If such a bias were true, then twins suffering from sight-impairing severe glaucoma might be less likely to volunteer. However, the prevalence of common diseases and lifestyle characteristics in the TwinsUK is comparable to that of age-matched population-based studies\(^{46}\). Moreover, the estimated prevalence of the variant rs74315329 in the TwinsUK is similar to the expected population prevalence. Another potential limitation of our study is that in the Rotterdam cohorts the variant rs74315329 was imputed, and it was possible to confirm the imputation calls in only a subset of them that were also sequenced. But given that Sanger sequencing validated the imputation calls for this variant in the TwinsUK, and that previous studies have confirmed the validity of
imputation for this variant\textsuperscript{26}, this is unlikely to have a significant impact on our results.

In conclusion, we reported the findings of the largest study to date evaluating the penetrance of the most common genetic mutation causing POAG. Given the rarity of this mutation and its much lower penetrance (than known previously) for ocular hypertension (and hence, POAG), our study suggests that screening for this mutation would not be useful on its own. But known carriers of this mutation would require careful monitoring, although they might be reassured that it is not always disease-causing.
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References


