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ASSOCIATION STUDIES ARTICLE

New insights into the genetics of primary open-angle glaucoma based on meta-analyses of intraocular pressure and optic disc characteristics

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Abstract

Primary open-angle glaucoma (POAG), the most common optic neuropathy, is a heritable disease. Siblings of POAG cases have a ten-fold increased risk of developing the disease. Intraocular pressure (IOP) and optic nerve head characteristics are used clinically to predict POAG risk. We conducted a genome-wide association meta-analysis of IOP and optic disc parameters and validated our findings in multiple sets of POAG cases and controls. Using imputation to the 1000 genomes (1000G) reference set, we identified 9 new genomic regions associated with vertical cup-disc ratio (VCDR) and 1 new region associated with IOP. Additionally, we found 5 novel loci for optic nerve cup area and 6 for disc area. Previously it was assumed that genetic variation influenced POAG either through IOP or via changes to the optic nerve head; here we present evidence that some genomic regions affect both IOP and the disc parameters. We characterized the effect of the novel loci through pathway analysis and found that pathways involved are not entirely distinct as assumed so far. Further, we identified a novel association between *CDKN1A* and POAG. Using a zebrafish model we show that *six6b* (associated with POAG and optic nerve head variation) alters the expression of *cdkn1a*. In summary, we have identified several novel genes influencing the major clinical risk predictors of POAG and showed that genetic variation in *CDKN1A* is important in POAG risk.

Introduction

In primary open-angle glaucoma (POAG), loss of retinal ganglion cells and nerve fibres manifests itself clinically as optic nerve damage, which leads to visual field loss and, eventually in 15% of these to visual impairment and blindness (1–3). The optic nerve damage is characterized by an increase in cup area, the central portion of the optic nerve head (or optic disc), and/or a decrease in the rim area (the area of the disc occupied by the retinal nerve fibre axons). This damage can be quantified by the vertical cup-disc ratio (VCDR), comparing the vertical diameter of the cup with the vertical diameter of the total optic disc. The VCDR ranges from 0 to 1. In the clinical setting, optic nerve heads with a VCDR above 0.7 or an asymmetry between eyes above 0.2 are considered to be suspect for glaucoma. The interpretation of VCDR depends on the disc area, i.e. discs with larger area have on average a higher VCDR and the cut-off point between a normal and abnormal VCDR might be higher (4).

Elevated intraocular pressure (IOP) is a well-recognized risk factor for POAG and current therapies lower IOP by various mechanisms; eye pressure is considered to be normal between 8 and 21 mmHg, although many eyes with eye pressure in this range can exhibit glaucomatous optic nerve features. Analyses of first-degree relatives of POAG patients have shown that the sib relative risk is 10.4 ± 2.5 (5). Classic twin studies in POAG are lacking and there is no consensus on heritability for POAG, but work by Cuellar-Partida *et al.* estimated the heritability of POAG based on genome-wide array data at 0.42 ± 0.09 (6). Several genome-wide association studies (GWAS) have identified new POAG genes by examining POAG directly or studying endophenotypes like VCDR and IOP (7–16). Several genes associated with VCDR and IOP - *CDKN2B-AS1*, *SIX6* (VCDR); and *CAV1/CAV2*, *TMCO1*, *ABCA1* and *ARHGEF12* (IOP) - are associated with POAG. Notably, no genes have been genome-wide associated with both VCDR and IOP. Charlesworth *et al.* previously found a genetic correlation between VCDR and IOP (RhoG = 0.45, $P = 0.0012$), however, genes underlying this relationship have not yet been identified (17).

The aims of this study were to (1) identify new genes associated with the POAG endophenotypes IOP, VCDR, cup area, and disc area, and ultimately POAG, using the 1000 Genomes imputations reference panel, and (2) investigate the genetic overlap between the different endophenotypes. To accomplish these aims we performed a meta-analysis of GWAS of these four traits within the International Glaucoma Genetics Consortium (IGGC).

Results

Intraocular pressure

We first conducted a meta-analysis of GWAS in the European cohorts. Top hit variants were replicated in the meta-analysis of the Asian cohorts. Next, we performed a meta-analyses of the European and Asians cohorts. After removal of single nucleotide polymorphisms (SNPs) with minor allele frequency (MAF) < 0.01 and low imputation quality, approximately 8 million SNPs were included. Whilst the meta-analysis of individuals of European descent yielded no novel associations, combined meta-analysis of individuals of European and Asian descent ($n = 37{,}930$, $\lambda = 1.05$; Supplementary Materials, Figs S1A and B, S2B) yielded nine genomic regions reaching genome-wide significance, of which eight genomic regions were already known (Supplementary Materials, Figs S1A and B, S2B, and Table S3) (9,11,13). The peak SNP in the new genomic region was rs55796939 on chromosome 11q25 near *ADAMTS8* (Supplementary Materials, Figs S3 and S4).The estimated heritability (h^2) of IOP using the LD score regression method (18) and the summary statistics of European-only meta-analysis was 0.13.

Vertical cup-disc ratio

In the meta-analysis of individuals of European descent ($n = 23{,}899$, $\lambda = 1.08$), 21 genomic regions were genome-wide significant (Supplementary Materials, Figs S5A, S6A and Table S4). Five genomic regions were novel (near to the genes *RPE65* on chr. 1p31, *F5* on chr. 1q23, *PDZD2* on chr. 5p13.3, *RREB1* on chr. 6p25, and *DGKB* on chr. 7p21.2) (Supplementary Materials, Figs S7 and S8); the other genomic regions have been previously associated with VCDR or cup area, two highly correlated traits (19–21). Of the five novel genomic regions, *RREB1* (*P*-value $= 4.13 \times 10^{-3}$) was nominally significant in the analysis of individuals of Asian descent ($n = 8{,}373$, $\lambda = 1.01$). In the combined analysis ($n = 32{,}272$, $\lambda = 1.06$), another four novel genomic regions, near to the genes *VCAN* on chr. 5q14.3, *PSCA* on chr. 8q24.2, *ENO4* on chr. 10q25.3, and *RBM23* on chr. 14q11.2 (Supplementary Materials, Figs S5B and S6B), were genome-wide significant leading to a total of nine (5 + 4) novel genomic regions associated with VCDR. Of these novel genomic regions, *F5* has been associated with the disc area previously (21). Disc area influences the VCDR (22), and therefore we corrected VCDR for disc area in a secondary analysis. After correction for disc area, the β (*P*-value) decreased from −0.007 (2.15×10^{-9}) to −0.002 (5.60×10^{-2}) in the subset with disc area available, suggesting that *F5* acts primarily on disc area and secondary to VCDR through its relation to disc area. The calculated h^2 of VCDR using the European -only meta-analysis was 0.31.

Cup area

The meta-analysis of individuals of European descent ($n = 22{,}489$, $\lambda = 1.06$) yielded 17 genome-wide significant regions of which 14 regions were already implicated for cup area or VCDR (Supplementary Materials, Figs S9A, S10A and Table S5) (20,21). There were three novel associations on chr. 1q42.11 near *CDC42BPA*, chr. 8q21.11 near *CRISPLD1*, and on chr. 15q26.3 near *FAM169B* (Supplementary Materials, Figs S11 and S12). *CDC42BPA* has previously been associated with disc area and the fact that the association with cup area adjusting for disc area is genome-wide significant suggests an independent effect on cup area. In the combined analysis of European and Asian individuals ($n = 29{,}828$, $\lambda = 1.06$, Supplementary Materials, Figs S9B and S10B) all loci except *FAM169B* and *CRISPLD1* remained genome-wide significant, and there was one additional genome-wide significant SNPs at chr. 6p21.2 (*CDKN1A*) and one highly suggestive significant SNP at chr. 9q34.2 (*ABO*; previously associated with IOP). For cup area the estimated h^2 was 0.27.

Disc area

The meta-analysis of individuals of European descent ($n = 22{,}504$, $\lambda = 1.06$) resulted in 13 genome-wide significant regions, of which two were not previously associated with disc area: *UGT8* on chr. 4q26 and *CTNNA3* on chr. 10q22.2 (Supplementary Materials, Figs S13A, S14A, S15, S16, and Table S6). These SNPs were not significant in the meta-analysis of individuals of Asian descent ($n = 7{,}307$, $\lambda = 1.02$). An additional four SNPs reached genome-wide significance in the combined meta-analysis ($n = 29{,}811$, $\lambda = 1.07$): *PRDM16* on chr. 1p36.23-p33, *GADD45A* on chr. 1p31.2, *VGLL4* on chr. 3p25.3, and *ASB7* on chr. 15q26.3 (Supplementary Materials, Figs S13B and S14B). The estimated h^2 for disc area was 0.27.

Characterization of the lead association signals

In total, 82 SNPs were associated with one or more of the above endophenotypes. Functional characterization of the 82 SNPs was performed using a range of bioinformatics tools (see Methods). In total, 650 variants in linkage disequilibrium (LD) with the 82 lead SNPs ($R^2 > 0.8$) were examined for functional annotation. Overall, 61% (50/82) of the associated loci are in LD with variants located in regulatory regions according to the ENCODE data (*e.g.* DNase I hypersensitive sites, transcription factor binding sites and motifs; see Supplementary Material, Table S7). We investigated the expression levels of the identified candidate genes using the UniGene database (23). Of all reviewed genes, *CDKN1A, PAX6* and *DUSP1* showed the highest number of transcripts per million in the eye (Supplementary Material, Table S8). According to the Ocular Tissue database (24), *CDKN1A* is highly expressed in the optic nerve head, as well as *DUSP1*, which also shows high expression in the trabecular meshwork. Both genes were associated with optic nerve head parameters. *PAX6* is highly expressed in the ciliary body and retina; in this study we found it associated with disc area. Other highly expressed genes in the optic nerve include *EFEMP1* and *ABI3BP*, which are associated with cup area and disc area, respectively (Supplementary Material, Table S9). To evaluate whether associated genes are highly expressed in a particular tissue, we performed tissue enrichment analyses for each trait using suggestive SNPs (*P*-value $< 1.00 \times 10^{-05}$) in DEPICT (25). No FDR significant tissue enrichment was found for IOP, VCDR or disc area. However, for cup area we found an enrichment for membranes, joints, stem cells and other related connective tissues. Similar results were found when suggestive SNPs associated with VCDR, cup and disc area (the optic nerve parameters) were analyzed together (Table S10).

Gene-based test

To identify new loci not found through per-SNP tests, we performed gene-based testing using VEGAS2. Reflecting the smaller number of tests, our gene-based significance threshold is $P_{gene\text{-}based} < 0.05/24{,}769 = 2.02 \times 10^{-6}$ (24,769 genes tested). Using the gene-based test we found several novel loci (Supplementary Material, Table S11). *C9* was significantly associated with IOP (*P*-value 1.61×10^{-6}); *RARB* (*P*-value 1.86×10^{-6}) and *HORMAD2-AS1* (*P*-value 1.04×10^{-6}) were associated with VCDR. These genes were previously associated with the disc area, so the novel associations with VCDR could possibly be driven by the influence of the disc area on VCDR (21). In the cup area analysis, the genes *LRP10* (*P*-value 1.20×10^{-6}) and *REM2* (*P*-value 1.55×10^{-6}), and *THSD4* (*P*-value 5.44×10^{8}) were significantly associated. The first two genes are located near to *RBM23*, which was significant in the per-SNP test. *THSD4* is located near to *KPNB1*, which was associated with VCDR in our previous meta-analysis (20). In the disc area analysis, we found two genes that were significantly associated with disc area: *ANKRA2* (*P*-value 8.42×10^{-7}) and *LOC149950* (*P*-value 3.87×10^{-7}).

Characterizing the overlap in biological pathways involved in glaucoma endophenotypes

In total, 86 SNPs were associated with one or more of the above endophenotypes. The effect estimates and *P*-values of these SNPs for all four endophenotypes are shown in Table 1–3. *ADAMTS8* (IOP and VCDR, Tables 1 and 2B) and *ABO* (IOP and cup area, Table 1) were genome-wide significantly associated with two traits. Of note

Table 1. Single nucleotide polymorphisms (SNPs) that are genome-wide significantly associated with IOP and show and association with vertical cup-disc ratio.

			IOP			VCDR			Cup area			Disc area		
SNP	Nearest gene	A1/A2	β	SE	P	β	SE	P	β	SE	P	B	SE	P
rs10918274	*TMCO1*	t/c	0.26	0.04	**5.64E-12**	0.005	0.002	8.38E-03	0.010	0.003	2.47E-03	0.000	0.006	9.49E-01
rs7635832	*FNDC3B*	g/t	−0.22	0.03	**6.61E-13**	−0.001	0.001	3.35E-01	−0.004	0.003	1.27E-01	0.002	0.005	7.08E-01
rs10281637	*CAV1/CAV2*	c/t	0.20	0.03	**3.96E-13**	0.004	0.001	5.28E-03	0.006	0.003	1.23E-02	−0.002	0.005	6.01E-01
8:78380944*	*PKIA*	i/r	1.00	0.17	**7.54E-09**	0.000	0.010	9.74E-01	−0.018	0.017	3.00E-01	0.018	0.031	5.61E-01
rs7815043	*PKIA*	c/t	−0.10	0.03	**4.41E-05**	−0.001	0.001	3.13E-01	−0.001	0.002	8.32E-01	−0.002	0.004	5.66E-01
rs7944735	*Many genes*	c/g	0.19	0.03	**6.00E-11**	0.001	0.001	4.37E-01	0.006	0.003	3.33E-02	0.000	0.005	9.68E-01
11:120357425	*ARHGEF12*	d/r	0.18	0.03	**2.02E-09**	0.001	0.001	6.12E-01	0.001	0.003	6.45E-01	0.001	0.005	8.38E-01
rs12794618	*ARHGEF12*	c/t	0.17	0.03	**7.86E-09**	0.001	0.001	4.14E-01	0.002	0.003	4.84E-01	0.004	0.005	4.53E-01
rs55796939	*ADAMTS8*	t/c	0.36	0.06	**2.31E-08**	0.003	0.003	3.61E-01	0.006	0.006	3.19E-01	−0.003	0.010	7.95E-01
rs2472496	*ABCA1*	g/a	0.17	0.02	**1.93E-13**	0.004	0.001	**6.83E-05**	0.010	0.002	**9.63E-07**	0.003	0.004	4.75E-01
rs8176741	*ABO*	a/g	0.24	0.04	**3.47E-10**	0.007	0.002	**4.51E-05**	0.019	0.003	**7.12E-08**	0.004	0.006	5.42E-01
rs9913911	*GAS7*	g/a	−0.17	0.02	**7.01E-12**	−0.006	0.001	**1.84E-07**	−0.008	0.002	**2.48E-04**	−0.001	0.004	8.41E-01

For these SNPs, the associations with the other traits are also included. SNPs that are Bonferroni significantly associated with other traits are shown in bold (*P*-value $< 5.37 \times 10^{-4}$; 0.05/93). In the first rows, the SNPs genome-wide significantly associated with intraocular pressure (IOP) are shown. Next, the SNPs associated with IOP, vertical cup-disc ratio (VCDR), and cup area are shown. Nearest gene, reference NCBI build37; A1, reference allele; A2, other allele; β, effect size on the endophenotype (IOP, VCDR, cup area or disc area) based on allele A1; SE, standard error of the effect size; i, insertion; d, deletion; r, reference. *This locus was not considered as new because the signal came from only one INDEL (8:78380944).

Table 2A. Single nucleotide polymorphisms (SNPs) that are genome-wide significantly associated with at least one of the optic disc parameters.

			IOP			VCDR			Cup area			Disc area		
SNP	Nearest gene	A1/A2	β	SE	P	β	SE	P	β	SE	P	B	SE	P
rs6804624	*COL8A1*	c/t	−0.01	0.03	6.54E-01	0.008	0.001	**8.63E-12**	0.013	0.002	**1.99E-08**	0.020	0.004	**9.67E-07**
rs7916697	*ATOH7*	a/g	0.01	0.03	7.43E-01	−0.018	0.001	**2.46E-45**	−0.017	0.002	**1.32E-12**	−0.094	0.004	**1.34E-102**
10:96008348	*PLCE1*	d/r	0.01	0.03	5.73E-01	0.007	0.001	**4.57E-08**	0.013	0.002	**1.72E-08**	0.015	0.004	**2.22E-04**
rs324780	*TMTC2*	g/a	0.03	0.02	2.79E-01	−0.011	0.001	**7.16E-23**	−0.016	0.002	**1.57E-13**	−0.029	0.004	**8.58E-13**
rs4299136	*ASB7*	c/g	−0.03	0.03	4.22E-01	0.010	0.002	**2.68E-12**	0.018	0.003	**4.09E-10**	0.024	0.005	**4.02E-06**
16:51461915	*SALL1*	r/i	0.02	0.03	4.34E-01	0.010	0.001	**2.62E-13**	0.013	0.003	**6.78E-07**	0.032	0.005	**2.38E-12**
rs4784295	*SALL1*	c/g	0.02	0.03	5.63E-01	0.009	0.001	**3.93E-13**	0.013	0.003	**1.63E-07**	0.031	0.005	**1.12E-11**
rs5752773	*CHEK2*	g/c	0.01	0.03	6.91E-01	−0.012	0.001	**1.49E-20**	−0.024	0.003	**4.12E-21**	−0.024	0.005	**1.48E-07**
rs2092172	*CARD10*	a/g	0.00	0.03	8.86E-01	0.009	0.001	**3.08E-12**	0.011	0.003	**3.34E-05**	0.032	0.005	**1.44E-11**
rs7717697	*VCAN*	c/t	0.01	0.02	7.21E-01	−0.007	0.001	**6.66E-09**	−0.009	0.002	**1.19E-05**	−0.018	0.004	**4.84E-06**
rs1681739	*ENO4*	t/c	0.03	0.02	2.23E-01	0.006	0.001	**2.44E-08**	0.011	0.002	**3.70E-07**	0.019	0.004	**1.85E-06**
rs60779155	*ASB7*	a/g	−0.02	0.04	6.61E-01	0.010	0.002	**3.76E-10**	0.019	0.003	**3.75E-09**	0.030	0.006	**8.26E-08**
rs1830890	*PLCE1*	g/a	0.01	0.02	8.14E-01	0.006	0.001	**3.02E-08**	0.012	0.002	**1.06E-07**	0.013	0.004	5.51E-04
rs482507	*TMTC2*	c/t	0.02	0.02	3.48E-01	−0.011	0.001	**2.19E-19**	−0.017	0.002	**2.56E-14**	−0.030	0.004	**4.49E-13**
rs4436712	*SIX6*	t/g	−0.04	0.02	1.47E-01	0.009	0.001	**5.48E-14**	0.025	0.002	**1.50E-29**	−0.018	0.004	**6.59E-06**
rs738722	*CHEK2*	t/c	0.02	0.03	3.57E-01	−0.012	0.001	**4.94E-20**	−0.024	0.003	**7.81E-22**	−0.021	0.005	**2.63E-06**
rs2684249	*HSF2*	c/t	0.03	0.02	2.08E-01	−0.006	0.001	**1.64E-07**	−0.012	0.002	**3.04E-08**	−0.015	0.004	**1.49E-04**
rs34222435	*ASB7*	t/c	−0.03	0.03	3.86E-01	0.010	0.002	**3.07E-12**	0.019	0.003	**1.07E-10**	0.025	0.005	**2.98E-06**
rs7916410	*ATOH7*	t/c	0.00	0.03	9.76E-01	−0.018	0.001	**1.14E-45**	−0.017	0.002	**6.11E-12**	−0.097	0.004	**7.06E-109**
rs442376	*TMTC2*	c/t	−0.03	0.03	3.09E-01	0.011	0.001	**1.50E-17**	0.017	0.002	**3.18E-12**	0.032	0.004	**4.92E-14**
rs1345467	*SALL1*	g/a	0.01	0.03	6.53E-01	0.009	0.001	**4.96E-12**	0.012	0.003	**1.07E-06**	0.032	0.005	**6.41E-13**
rs5762752	*CHEK2*	c/g	0.01	0.03	6.61E-01	−0.011	0.001	**4.83E-18**	−0.021	0.002	**6.72E-19**	−0.023	0.004	**2.26E-08**
rs11129176	*RARB*	a/g	0.02	0.03	4.17E-01	0.005	0.001	**3.17E-05**	0.010	0.002	**1.01E-05**	0.023	0.004	**3.40E-08**
rs1997404	*COL8A1*	g/t	−0.03	0.03	3.24E-01	0.008	0.001	**2.39E-11**	0.013	0.002	**7.71E-08**	0.024	0.004	**1.90E-08**
rs34935520	*SIX6*	g/a	−0.04	0.02	1.13E-01	0.009	0.001	**7.95E-14**	0.025	0.002	**6.96E-29**	−0.023	0.004	**7.61E-08**

For these SNPs, the associations with the other traits are also included. Here the SNPs genome-wide significantly associated with at least one of the optic disc parameters and that are Bonferroni significantly associated with the other disc parameters are shown in bold (*P*-value $< 5.37 \times 10^{-4}$; 0.05/93).Nearest gene, reference NCBI build37; A1, reference allele; A2, other allele; β, effect size on the effect size on the endophenotype (IOP, VCDR, cup area or disc area) based on allele A1; SE, standard error of the effect size; i, insertion; d, deletion; r, reference.

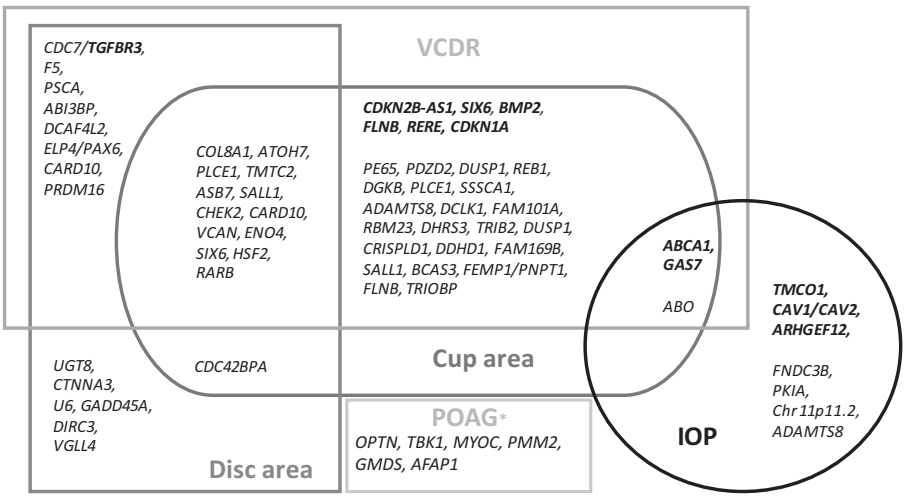

Figure 1. Overlap between the genes associated with one or more endophenotypes. Genes with a genome-wide significant association for at least one trait are shown. These genes are counted as overlapping genes if they are Bonferroni significantly associated with the other trait(s). Chr 11p11.2 (see intraocular pressure circle) means a region on chromosome 11p11.2 that is associated with IOP and has many genes in it; the likely causative gene in this region is not identified yet. Genes in bold are genes associated with primary open-angle glaucoma (POAG) in our meta-analysis of four case-control studies.*Genes associated with familial forms of POAG (*e.g.* *MYOC* and *OPTN*) or found in case-control association studies which did not show an association with the endophenotypes explored in this study.

is that there were different variants involved in *ADAMTS8*: rs55796939 for IOP and rs4936099 for VCDR ($r^2 = 0.03$ between these SNPs in 1000G European samples). Figure 1 shows the overlap in associations across endophenotypes — we depict annotated genes for which at least one SNP was genome-wide significant in at least one trait — Overlap is defined as nominal significance or stronger for the second trait. The figure shows as expected a strong overlap in variants associated with disc area, cup area and VCDR. Further, overlap is noted in genes associated with IOP, cup area and VCDR. We next explored the genetic overlap between optic disc parameters and IOP using our GWAS results from the European-only meta-analyses, which comprised a larger sample size ($n = 22{,}489$–$29{,}578$). As expected genetic correlation between optic disc parameters was high; VCDR and cup area showed the highest correlation (RhoG = 0.83, *P*-value $< 1.0 \times 10^{-308}$), followed by VCDR and disc area (RhoG = 0.62, *P*-value $= 7.36 \times 10^{-23}$). The genetic correlation between cup and disc area was 0.31 (*P*-value $= 1.00 \times 10^{-04}$). No significant genetic correlation was found between IOP and optic disc parameters; the highest correlation was between IOP and VCDR (RhoG = 0.06, *P*-value 0.3) (Table S12).

To further characterize the overlap in biological functions, gene set enrichment of loci associated with IOP and optic disc parameters was performed using DEPICT (25). We first investigated enriched pathways or gene sets using only genome-wide associated SNPs. No significant pathways were found after FDR correction. However, the pathways involved in metabolic processes such as "increased circulating leptin level", "abnormal fat cell morphology" and "increased insulin sensitivity" were suggestive when we analyzed the list of SNPs associated with VCDR, cup area and disc area (FDR < 0.2, see Supplementary Material, Table S13). We next searched for enriched pathways using suggestive SNPs (*P*-value $< 1.0 \times 10^{-5}$). We further investigated the potential overlap in pathways across the endophenotypes, and found 57 significant pathways when using VCDR, cup area and IOP variants, and 100 pathways when analysing suggestive VCDR, cup area and disc area variants. Note that in the first analysis we investigated pathways enriched when IOP genes are taken into account, while in the second one we analysed genes influencing the optic nerve head characteristics. Due to a high degree of redundancy between pathways, we clustered the significant pathways into meta-pathways, resulting in 11 meta-pathways for VCDR, cup area and IOP (Fig. 2A, Supplementary Material, Table S14); and 17 for VCDR, cup area, and disc area (Fig. 2B, Supplementary Material, Table S15). Most of the gene sets found in both analyses highlighted pathways involved in cell differentiation, notch signalling, regulatory DNA binding and embryonic development, which reflects the pathways found when VCDR and CA variants are analyzed (Supplementary Material, Fig. S17). Furthermore, we found "abnormal fat cell morphology" and "abnormal liver morphology" significantly enriched; a key gene in these pathways is *ABCA1*. When IOP genes are included the elongation factor, RNA Polymerase II (ELL2) protein complex" shows an enrichment. When disc area genes are included, pathways such as "blood vessel development", "protein import into nucleus", "Thrombospondin 1 (THBS1) and SMAD3 protein complex", and "abnormal eye morphology" were significant. Key genes in the latter include: *CDKN2B*, *FAT4*, *LRIG3*, *SIX6*, *COL8A1*, *SOX11*, *RND3*, *BOC*, *WNT2B* and *CYP26A1*.

From endophenotypes to primary open-angle glaucoma

To evaluate the implications of our findings in the context of glaucoma, we examined the association between the genome-wide significant SNPs found in this study and 4 independent POAG studies ($n = 6{,}429$ cases and 41,404 controls). In total, 75 independent (*i.e.* $R^2 < 0.8$) SNPs associated with one or more of the endophenotypes were assessed in the case/control studies. Of these, 32 were nominal significantly associated with POAG (*P*-value < 0.05; the chance that 32 SNPs of 75 SNPs have a *P*-value < 0.05 is $< 2.2 \times 10^{-16}$), and 11 independent SNPs were Bonferroni significantly associated with POAG (*P*-value $0.05/75 = 6.67 \times 10^{-4}$) (Table 4). Two out of the 11 Bonferroni significant SNPs, the rs2487048 in the *ABCA1* gene and the 11:120357425 in the *ARHGEF12* showed high heterogeneity (I^2). To estimate the common effect size we performed a random effect meta-analysis. The odds ratio (OR) remained almost the same for both variants, although *P*-values were not significant after adjusting for multiple testing, which is in line with the heterogeneity observed. All other nine SNPs surpassed the Bonferroni threshold for significance in both fixed and

Table 2B. Single nucleotide polymorphisms (SNPs) that are genome-wide significantly associated with vertical cup-disc ratio or with cup area

			IOP			VCDR			Cup area			Disc area		
SNP	Nearest gene	A1/A2	β	SE	P	β	SE	P	β	SE	P	B	SE	P
rs1925953	*RPE65**	t/a	−0.02	0.02	3.26E-01	0.006	0.001	**1.55E-07**	0.010	0.002	**1.50E-05**	0.006	0.004	1.08E-01
rs72759609	*PDZD2*	c/t	−0.04	0.05	3.50E-01	−0.012	0.002	**7.10E-09**	−0.020	0.004	**1.98E-06**	−0.021	0.008	5.62E-03
rs114503346	*DUSP1*	t/c	−0.12	0.08	1.27E-01	−0.021	0.004	**1.31E-08**	−0.035	0.007	**2.90E-07**	−0.035	0.013	5.83E-03
rs4960295	*RREB1*	a/g	0.02	0.02	4.75E-01	0.007	0.001	**2.49E-10**	0.009	0.002	**3.73E-05**	0.012	0.004	3.29E-03
rs10274998	*DGKB*	t/c	0.02	0.03	4.38E-01	0.008	0.001	**4.68E-08**	0.012	0.003	**8.08E-06**	0.011	0.005	2.65E-02
rs2157719	*CDKN2B-AS1*	c/t	−0.04	0.02	9.81E-02	−0.013	0.001	**3.75E-35**	−0.024	0.002	**3.31E-28**	−0.008	0.004	3.03E-02
rs3891783	*PLCE1*	g/c	0.04	0.02	1.01E-01	0.007	0.001	**1.06E-10**	0.011	0.002	**3.28E-07**	0.012	0.004	1.52E-03
rs1346	*SSSCA1*	t/a	−0.05	0.03	1.20E-01	−0.013	0.002	**7.51E-18**	−0.019	0.003	**9.31E-11**	−0.016	0.005	2.10E-03
rs4936099	*ADAMTS8*	c/a	−0.03	0.03	2.38E-01	−0.007	0.001	**6.70E-09**	−0.013	0.002	**4.96E-08**	−0.006	0.004	1.72E-01
13:36629905	*DCLK1*	d/r	−0.02	0.03	5.70E-01	0.007	0.001	**2.98E-08**	0.018	0.002	**2.20E-14**	−0.005	0.004	2.36E-01
rs7323428	*DCLK1*	t/g	−0.02	0.03	4.13E-01	0.007	0.001	**1.86E-08**	0.019	0.002	**1.67E-15**	−0.005	0.004	2.23E-01
rs8015152	*SIX6*	t/c	−0.06	0.02	2.27E-02	0.010	0.001	**2.86E-18**	0.024	0.002	**8.15E-26**	−0.011	0.004	6.18E-03
rs6107845	*BMP2*	a/g	0.03	0.02	2.80E-01	−0.009	0.001	**3.44E-17**	−0.017	0.002	**2.90E-15**	−0.004	0.004	3.27E-01
rs6764184	*FLNB*	t/g	0.05	0.03	5.03E-02	0.007	0.001	**1.89E-08**	0.015	0.002	**1.30E-10**	0.010	0.004	1.92E-02
rs7311936	*FAM101A*	c/g	−0.03	0.02	1.69E-01	−0.006	0.001	**2.48E-09**	−0.013	0.002	**4.52E-09**	0.003	0.004	5.14E-01
14:23388793	*RBM23*	r/d	0.02	0.03	3.99E-01	0.007	0.001	**2.56E-08**	0.013	0.003	**2.01E-07**	0.009	0.005	4.29E-02
rs3794453	*RBM23*	a/t	0.01	0.02	7.22E-01	0.007	0.001	**7.25E-08**	0.011	0.002	**2.88E-07**	0.009	0.004	3.11E-02
rs2252865	*RERE*	t/c	0.05	0.03	4.11E-02	0.005	0.001	**2.66E-05**	0.014	0.002	**1.33E-09**	0.003	0.004	5.08E-01
			IOP			**VCDR**			**Cup area**			**Disc area**		
SNP	**Nearest gene**	**A1/A2**	**β**	**SE**	**P**	**β**	**SE**	**P**	**β**	**SE**	**P**	**B**	**SE**	**P**
rs13016883	*TRIB2*	c/g	0.01	0.03	5.64E-01	0.006	0.001	**3.44E-06**	0.016	0.002	**1.83E-11**	0.001	0.004	8.30E-01
rs35084382	*DUSP1*	c/t	−0.10	0.07	1.32E-01	−0.018	0.003	**2.05E-08**	−0.033	0.006	**2.17E-08**	−0.031	0.011	5.51E-03
rs117598310	*CRISPLD1**	t/g	−0.05	0.05	3.10E-01	0.009	0.002	**1.07E-04**	0.021	0.004	**1.66E-06**	0.022	0.008	5.47E-03
rs1360589	*CDKN2B-AS1*	c/t	−0.04	0.02	8.42E-02	−0.013	0.001	**1.43E-34**	−0.024	0.002	**2.90E-28**	−0.008	0.004	4.45E-02
rs11613189	*FAM101A*	t/c	−0.03	0.03	2.27E-01	−0.005	0.001	**6.04E-06**	−0.016	0.002	**2.01E-12**	0.002	0.004	6.42E-01
rs2251069	*DDHD1*	c/t	0.01	0.02	7.29E-01	−0.006	0.001	**7.41E-08**	−0.013	0.002	**1.20E-09**	0.001	0.004	7.11E-01
rs6598351	*FAM169B**	t/c	−0.02	0.03	5.26E-01	0.006	0.001	**2.80E-05**	0.012	0.003	**1.77E-05**	−0.004	0.005	3.90E-01
rs11646917	*SALL1*	t/g	−0.01	0.03	6.65E-01	−0.009	0.001	**4.83E-10**	−0.015	0.003	**4.76E-09**	−0.015	0.005	1.30E-03
rs11867840	*BCAS3*	g/a	0.04	0.03	1.04E-01	−0.006	0.001	**4.86E-06**	−0.018	0.002	**2.35E-13**	0.011	0.004	1.00E-02
rs6054375	*BMP2*	t/g	0.03	0.03	2.45E-01	−0.010	0.001	**6.92E-15**	−0.018	0.002	**1.83E-15**	−0.003	0.004	4.74E-01
rs3791679	*EFEMP1/PNPT1*	g/a	0.04	0.03	1.72E-01	−0.005	0.001	**1.17E-04**	−0.013	0.002	**4.92E-08**	0.003	0.004	5.14E-01
rs12494328	*FLNB*	a/g	0.04	0.03	1.52E-01	0.006	0.001	**1.56E-06**	0.016	0.002	**6.03E-11**	0.009	0.004	4.50E-02
6:36592986	*CDKN1A*	d/r	−0.02	0.03	5.32E-01	0.006	0.001	**1.92E-05**	0.015	0.003	**1.12E-08**	−0.006	0.005	2.09E-01
rs72852338	*CDKN1A*	c/a	−0.02	0.03	5.46E-01	0.006	0.001	**3.29E-05**	0.014	0.003	**3.17E-08**	−0.005	0.005	2.97E-01
rs1074407	*TRIOBP*	t/a	0.11	0.02	**4.00E-06**	0.006	0.001	**3.32E-07**	0.012	0.002	**1.90E-08**	0.008	0.004	3.92E-02

For these SNPs, the associations with the other traits are also included. Here the SNPs genome-wide significantly associated with vertical cup-disc ratio or cup area and that are Bonferroni significantly associated with VCDR or cup area are shown in bold (*P*-value $< 5.37 \times 10^{-4}$; 0.05/93).Nearest gene, reference NCBI build37; A1, reference allele; A2, other allele; β, effect size on effect size on the endophenotype (IOP, VCDR, cup area or disc area) based on allele A1; SE, standard error of the effect size; i, insertion; d, deletion; r, reference.

*Genome-wide associated in the European-only meta-analysis.

random-effect models. The association between *CDKN1A* and POAG is novel (OR = 1.14, *P*-value = 7.4×10^{-7}). In our previous paper, the SNP rs6054374 near to *BMP2* was already associated with POAG (OR = 0.92, *P*-value 3.74×10^{-3}), but the most significantly associated SNP in the current meta-analysis rs6107845 near to *BMP2* shows a slightly larger effect on POAG (OR = 0.89, *P*-value = 8.52×10^{-6}). *CDKN1A*, the novel associated POAG candidate gene belongs to the cyclin dependent kinase inhibitor (CDKN) gene family as well as *CDKN2A*, *CDKN2B/CDKN2B-AS1*, which all lie in a well-known glaucoma associated locus on chr.9p21.

Expression of cdkn1a after knockdown of six6b in zebrafish

Previous studies have shown that the transcription factor *SIX6* alters the expression of *CDKN2A* and *CDKN2B* (26,27). All three genes (*SIX6*, *CDKN2A* and *CDKN2B*) are in loci associated with POAG, suggesting a possible functional link between these POAG-loci. Given that *CDKN1A* is part of the same gene family as *CDKN2A* and *CDKN2B* we tested whether SIX6 also regulates *CDKN1A*. To assess the potential for functional regulation of *CDKN1A* through *SIX6*, we first performed *in silico* analyses and observed that *SIX6* binds to *CDKN1A* (core score = 0.812). Then we used a previously studied zebrafish model (27), in which we tested whether knockdown of *six6b* alters the expression of *cdkn1a in vivo*. Knockdown of *six6b* was achieved using morpholino technology (27). 85% of the knockdown embryos showed a small eye phenotype, reduced optic nerve thickness and an upregulation of the expression levels of *cdkn2a/cdkn2b*, as observed in previous studies ($n = 220$) (27,28). In zebrafish, there is only one gene which is analogous to the human *CDKN2A* and *CDKN2B* and it is referred to in this paper as *cdkn2a/cdkn2b*. We evaluated the expression levels of *cdkn1a* in eyes of *six6b* deficient embryos by RT-qPCR. A 41-fold overexpression of *cdkn1a*

Table 2C. Single nucleotide polymorphisms (SNPs) that are genome-wide significantly associated with vertical cup-disc ratio or with disc area

			IOP			VCDR			Cup area			Disc area		
SNP	Nearest gene	A1/A2	β	SE	P	β	SE	P	β	SE	P	B	SE	P
rs1192414	*CDC7/TGFBR3*	a/g	0.06	0.03	5.66E-02	0.014	0.001	**1.78E-23**	0.007	0.003	1.12E-02	0.087	0.005	**7.44E-71**
rs10753787	*F5*	t/c	−0.03	0.02	1.69E-01	−0.007	0.001	**2.48E-09**	−0.005	0.002	2.14E-02	−0.019	0.004	**1.60E-06**
rs2920293	*PSCA*	g/c	0.00	0.02	8.57E-01	−0.006	0.001	**5.04E-09**	−0.007	0.002	9.17E-04	−0.015	0.004	**9.94E-05**
rs4658101	*CDC7/TGFBR3*	a/g	0.06	0.03	4.46E-02	0.013	0.001	**5.19E-23**	0.007	0.003	1.13E-02	0.089	0.005	**8.01E-77**
1:169530520	*F5/SELP*	i/r	0.02	0.03	4.22E-01	0.007	0.001	**7.20E-07**	0.005	0.003	5.44E-02	0.033	0.005	**1.49E-12**
rs2239854	*F5/SELP*	a/g	0.03	0.03	2.64E-01	0.006	0.001	**8.37E-07**	0.005	0.002	5.04E-02	0.030	0.004	**7.60E-13**
rs9843102	*ABI3BP*	a/g	0.00	0.03	9.84E-01	−0.006	0.002	**2.18E-04**	−0.002	0.003	5.88E-01	−0.036	0.005	**1.35E-11**
8:88744441	*DCAF4L2*	d/r	−0.01	0.02	6.98E-01	0.006	0.001	**6.66E-07**	0.006	0.002	4.53E-03	0.026	0.004	**2.04E-11**
rs6468996	*DCAF4L2*	t/c	0.00	0.02	9.12E-01	0.005	0.001	**2.52E-07**	0.006	0.002	2.14E-03	0.025	0.004	**5.16E-11**
rs61101201	*ELP4/PAX6*	g/t	0.02	0.03	5.51E-01	0.006	0.001	**2.27E-06**	0.005	0.002	4.51E-02	0.028	0.004	**1.53E-10**
rs56385951	*CARD10*	a/g	−0.06	0.04	9.08E-02	0.011	0.002	**1.87E-11**	0.008	0.003	8.83E-03	0.047	0.006	**1.49E-16**
1:3046430	*PRDM16*	i/r	−0.04	0.04	4.14E-01	0.007	0.002	5.35E-04	−0.002	0.004	7.15E-01	0.044	0.007	**1.79E-09**
rs12028027	*PRDM16*	c/t	−0.03	0.04	4.97E-01	0.007	0.002	**2.15E-04**	−0.001	0.004	8.58E-01	0.043	0.007	**1.46E-09**

For these SNPs, the associations with the other traits are also included. Here the SNPs genome-wide significantly associated with vertical cup-disc ratio or with disc area that are Bonferroni significantly associated with the VCDR or disc area are shown in bold (*P*-value $< 5.37 \times 10^{-4}$; 0.05/93).Nearest gene, reference NCBI build37; β, effect size on effect size on the endophenotype (IOP, VCDR, cup area or disc area) based on allele A1; SE, standard error of the effect size; i, insertion; d, deletion; r, reference.

Table 3. Single nucleotide polymorphisms (SNPs) that are genome-wide significantly associated with optic nerve head parameters (cup area and disc area)

			IOP			VCDR			Cup area			Disc area		
SNP	Nearest gene	A1/A2	β	SE	P	β	SE	P	β	SE	P	B	SE	P
1:227562773	*CDC42BPA*	d/r	−0.10	0.05	3.01E-02	0.003	0.002	2.37E-01	0.024	0.004	**8.05E-09**	−0.055	0.008	**3.65E-13**
rs73102394	*CDC42BPA*	t/c	−0.09	0.05	4.34E-02	0.003	0.002	1.62E-01	0.022	0.004	**4.16E-08**	−0.053	0.007	**5.01E-13**
rs11811982	*CDC42BPA*	a/c	−0.12	0.05	1.35E-02	0.004	0.002	5.54E-02	0.027	0.004	**2.31E-10**	−0.062	0.008	**2.02E-15**
rs10021731	*UGT8*	c/t	0.01	0.02	8.23E-01	−0.002	0.001	5.56E-02	−0.002	0.002	2.68E-01	−0.020	0.004	**7.48E-07**
rs12220165	*CTNNA3**	g/c	0.02	0.03	5.88E-01	−0.004	0.002	1.47E-02	−0.004	0.003	1.92E-01	−0.023	0.005	**2.51E-05**
rs787541	*U6, GADD45A*	c/g	0.07	0.03	7.08E-03	0.002	0.001	7.47E-02	0.002	0.002	4.82E-01	0.023	0.004	**6.66E-08**
rs1367187	*DIRC3*	c/t	−0.07	0.03	9.74E-03	0.002	0.001	2.46E-01	−0.002	0.003	4.87E-01	0.026	0.005	**1.03E-08**
rs2443724	*VGLL4*	c/g	0.00	0.02	8.62E-01	−0.003	0.001	1.53E-02	0.000	0.002	9.15E-01	−0.022	0.004	**4.72E-08**
rs1013830	*CTNNA3*	t/c	0.00	0.05	9.49E-01	−0.007	0.002	4.80E-03	−0.004	0.005	4.10E-01	−0.046	0.008	**5.45E-08**

For these SNPs, the associations with the other traits are also included. SNPs that are Bonferroni significantly associated with other traits are shown in bold (*P*-value $< 5.37 \times 10^{-4}$; 0.05/93). In the first rows, the SNPs genome-wide significantly associated with cup area are shown. Next, SNPs associated with only disc area, are shown. Nearest gene, reference NCBI build37; A1, reference allele; A2, other allele; β, effect size on the endophenotype (IOP, VCDR, cup area or disc area) based on allele A1; SE, standard error of the effect size; i, insertion; d, deletion; r, reference.

*Genome-wide associated with the European-only meta-analysis.

in the eye of *six6b* knockdown embryos was found (*P*-value = 0.001) (Fig. 3), showing that *in vivo* downregulation of *six6b* affects the expression levels not only of *cdkn2a/cdkn2b* but also of *cdkn1a*, likely by binding to their sequence, repressing their expression.

Discussion

This meta-analysis within the IGGC identified a novel genomic region associated with IOP, nine genomic regions associated with VCDR, five with cup area, and six with disc area. Eleven genomic regions were associated with POAG. Of these regions, the association between *CDKN1A* and POAG is novel.

We identified some specific loci that underlie the genetic correlation between IOP and VCDR described earlier (17). *ADAMTS8* and *ABO* were genome-wide significant for both IOP and VCDR or cup area. Variants found close to *ABO* (rs8176672 for cup area and rs8176741 for IOP) are in LD ($r^2 > 0.85$) with rs12216891, which lies in an enhancer and promoter histone mark, suggesting a potential regulatory mechanism in that region. Furthermore, *TRIOBP* is genome-wide significant for cup area, and reached a *P*-value of 3.42×10^{-6} for IOP. Interestingly, *TRIOBP* is approximately 180 kb away from *CARD10* which is associated with disc area. There is a large overlap between VCDR/cup area and disc area. Since VCDR is related to disc area, it might be that the effect found for VCDR is due to the effect of disc area. Most of these overlapping genes are still Bonferroni significant in the cup area analysis in which we corrected for disc area. Only *CDC7/TGFBR3* and *F5* are genome-wide significant for VCDR as well as for disc area, but the effect is negligible after correction for disc area, suggesting that these two genes play primarily a role in disc area.

When suggestive SNPs (*P*-value $< 1.0 \times 10^{-5}$) for VCDR and cup area were analyzed together using DEPICT, we found an enrichment of pathways involved in cell differentiation, development, regulatory DNA binding and Notch signalling. Including disc area SNPs to the VCDR and cup area analysis reveals additional joint pathways: 1) eye and blood vessel development, 2)

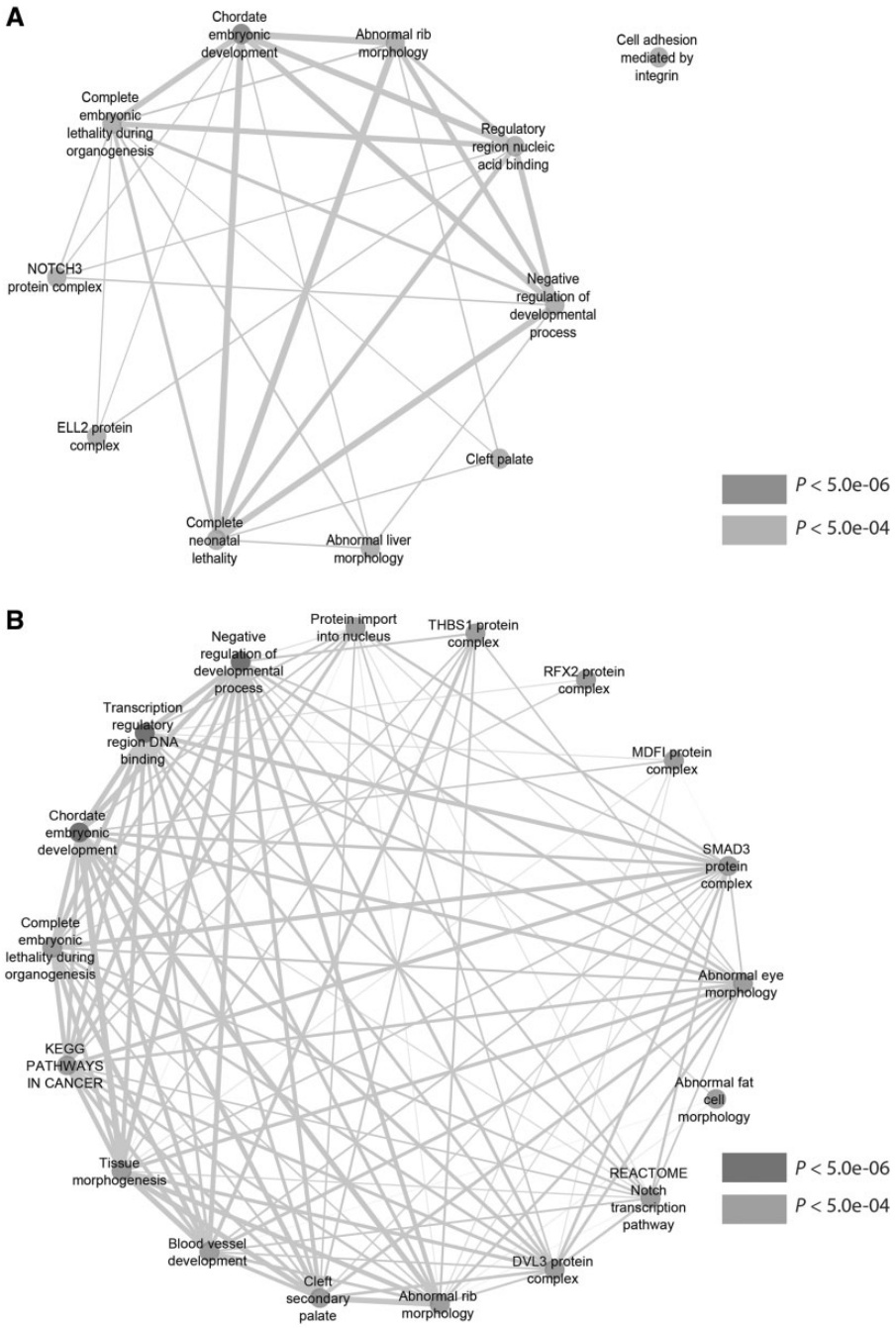

Figure 2. Pathways significantly enriched for: (**A**) Loci associated with the vertical cup-disc ratio, cup area and intraocular pressure (*P*-value $<7.0 \times 10\text{-}6$ in the GWAS). In total 11 meta-pathways were identified after clustering the 57 pathways identified by DEPICT. B) Loci associated with vertical cup-disc ratio, cup area and disc area (*P*-value $<1.0 \times 10\text{-}5$). In total 17 meta-pathways were identified after clustering the 100 pathways identified by DEPICT. In both figures, meta-pathways are represented by nodes coloured according to statistical significance, and edges are scaled according to the correlation between meta-pathways. *The pathway Abnormal eye morphology clustered with the meta-pathway Chordate embryonic development. ELL2 = Elongation Factor, RNA Polymerase II, DVL3= Dishevelled Segment Polarity Protein 3, THBS1 = Thrombospondin 1, RFX2= Regulatory Factor X, 2. MDFI = MyoD Family Inhibitor.

cancer, 3) protein import into nucleus, and 4) thrombospondin 1 and SMAD3 complexes, related to the extracellular matrix. The extracellular matrix pathway has been previously implicated in optic nerve degeneration (20). *ADAMTS8*, *COL8A1* and the novel identified gene *VCAN* (versican) are involved in the metabolism and composition of the extracellular matrix. Interestingly, mutations in *VCAN* have been implicated in several ophthalmologic disorders (29). Of interest, known POAG genes also fit in the pathways identified in this study, for example *GAS7* and *SIX6* play a role during development (27,30), *TGFBR3* has been implicated in extracellular matrix regulation (31) and in cancer as well as *GMDS* (32).

Common variants in *CDKN2B* and its antisense *CDKN2B-AS1* have been robustly found associated with POAG. The gene

Table 4. Association with primary open-angle glaucoma in a meta-analysis of four independent glaucoma case-control studies (ANZRAG, NEIGHBORHOOD, Singapore, and Southampton).

	Nearest gene	A1/A2	OR (95% CI)	OR (R)	*P*-value	*P*-value (R)	Direction	I^2	*P*-value of heterogeneity
IOP SNPs									
rs10918274	*TMCO1*	t/c	1.39 (1.30-1.50)	1.39	**2.75E-19**	**1.37E-09**	++++	38.4	1.82E-01
rs7635832	*FNDC3B*	g/t	0.89 (0.83-0.95)	0.91	1.41E-03	3.65E-02	—?	33.9	2.20E-01
rs10281637	*CAV1/CAV2*	c/t	1.13 (1.07-1.20)	1.13	**2.32E-05**	**2.32E-05**	++++	0	4.89E-01
rs2487048	*ABCA1*	a/g	1.26 (1.19-1.33)	1.26	**2.65E-15**	3.82E-03	++++	82.9	5.53E-04
rs8176741	*ABO*	a/g	1.07 (0.99-1.17)	1.04	7.36E-02	5.25E-01	−+-+	58.5	6.51E-02
rs7944735	Many genes (*NUP160, PTPRJ*)	c/g	1.06 (1.01-1.13)	1.07	2.99E-02	2.99E-02	++++	0	8.99E-01
11:120357425	*ARHGEF12*	d/r	1.16 (1.09-1.23)	1.19	**1.52E-06**	3.02E-02	++++	83.2	4.65E-04
rs55796939	*ADAMTS8*	t/c	1.07 (0.94-1.24)	1.17	2.72E-01	4.46E-01	+?−	78.6	9.35E-03
rs9913911	*GAS7*	g/a	0.80 (0.76-0.84)	0.80	**1.08E-17**	**1.08E-17**	—-	0	7.50E-01
VCDR SNPs									
rs1925953	*RPE65*	t/a	1.07 (1.02-1.13)	1.10	4.21E-03	2.01E-02	++++	46.7	1.31E-01
rs1192414	*CDC7/TGFBR3*	a/g	1.08 (1.02-1.16)	1.08	9.26E-03	9.26E-03	++++	0	7.27E-01
rs10753787	*F5*	t/c	0.97 (0.93-1.03)	0.97	3.67E-01	3.67E-01	—-	0	9.92E-01
rs6804624	*COL8A1*	c/t	0.99 (0.94-1.05)	0.99	8.14E-01	8.14E-01	—+	0	8.42E-01
rs72759609	*PDZD2*	c/t	0.90 (0.83-0.99)	0.91	3.20E-02	3.20E-02	—-	0	9.53E-01
rs114503346	*DUSP1*	t/c	1.00 (0.80-1.25)	1.00	9.99E-01	8.80E-01	+?-+	42	1.78E-01
rs4960295	*RREB1*	a/g	0.99 (0.95-1.05)	1.00	9.50E-01	9.09E-01	−+-+	4.6	3.70E-01
rs10274998	*DGKB*	t/c	1.03 (0.98-1.10)	1.04	2.16E-01	2.16E-01	++-+	0	5.38E-01
rs2157719	*CDKN2B-AS1*	c/t	0.69 (0.66-0.74)	0.69	**1.29E-40**	**1.29E-40**	—-	0	5.67E-01
rs1900005	*ATOH7*	a/c	1.01 (0.96-1.07)	1.01	6.98E-01	6.77E-01	+-++	5.1	3.67E-01
10:96008348	*PLCE1*	d/r	1.02 (0.97-1.09)	1.04	3.38E-01	3.15E-01	+-+?	35.3	2.13E-01
rs1346	*SSSCA1*	t/a	0.90 (0.85-0.97)	0.91	2.41E-03	2.41E-03	—-	0	9.04E-01
rs4936099	*ADAMTS8*	c/a	0.94 (0.9-1.00)	0.94	5.75E-02	5.75E-02	—-	0	9.63E-01
rs324780	*TMTC2*	g/a	0.93 (0.89-0.99)	0.93	1.35E-02	1.35E-02	—-	0	7.69E-01
13:36629905	*DCLK1*	d/r	0.99 (0.94-1.05)	0.99	7.53E-01	8.00E-01	−+-	6.2	3.62E-01
rs8015152	*SIX6*	t/c	1.21 (1.16-1.28)	1.19	**3.90E-15**	**7.08E-05**	++++	62.4	4.62E-02
rs4299136	*ASB7*	c/g	1.03 (0.97-1.10)	1.03	3.55E-01	3.55E-01	++-+	0	8.29E-01
16:51461915	*SALL1*	i/r	0.94 (0.89-1.00)	0.94	3.85E-02	3.85E-02	—-	0	7.82E-01
rs6107845	*BMP2*	a/g	0.89 (0.85-0.94)	0.91	1.02E-05	6.94E-03	—-	43.1	1.53E-01
rs5752773	*CHEK2*	g/c	0.92 (0.88-0.98)	0.92	4.63E-03	4.63E-03	—-	0	9.12E-01
rs2092172	*CARD10*	a/g	0.97 (0.92-1.04)	0.98	4.35E-01	4.35E-01	−+-	0	7.76E-01
rs6764184	*FLNB*	t/g	1.07 (1.02-1.13)	1.02	5.73E-03	7.66E-01	++-+	86.1	8.14E-05
rs7717697	*VCAN*	c/t	0.98 (0.93-1.04)	0.98	5.26E-01	5.26E-01	—?	0	7.30E-01
rs2920293	*PSCA*	g/c	1.03 (0.98-1.09)	1.03	2.25E-01	2.25E-01	++-?	0	3.79E-01
rs1681739	*ENO4*	t/c	1.02 (0.97-1.08)	1.03	3.92E-01	3.99E-01	+−+	49.2	1.16E-01
rs7311936	*FAM101A*	c/g	0.99 (0.95-1.04)	1.00	8.12E-01	8.59E-01	+—	11	3.38E-01
14:23388793	*RBM23*	r/d	1.03 (0.98-1.10)	1.03	1.83E-01	1.83E-01	+++?	0	4.61E-01
Cup area SNPs									
rs2252865	*RERE*	t/c	1.11 (1.06-1.18)	1.11	5.76E-05	2.87E-02	++-+	59.3	6.10E-02
rs4846112	*DHRS3*	a/g	0.95 (0.91-1.01)	0.96	1.18E-01	1.18E-01	—-	0	5.53E-01
1:227562773	*CDC42BPA*	d/r	0.87 (0.79-0.97)	0.90	1.14E-02	2.11E-01	−+?	48.6	1.43E-01
rs13016883	*TRIB2*	c/g	1.08 (1.03-1.14)	1.08	4.25E-03	4.25E-03	+++?	0	8.63E-01
rs35084382	*DUSP1*	c/t	1.04 (0.85-1.29)	1.05	6.72E-01	6.72E-01	+?-+	0	3.91E-01
rs117598310	*CRISPLD1*	t/g	1.08 (1.00-1.19)	1.09	5.39E-02	5.39E-02	+++	0	8.01E-01
rs1360589	*CDKN2B-AS1*	c/t	0.69 (0.66-0.73)	0.69	**1.90E-42**	**1.90E-42**	—-	0	6.47E-01
rs10998036	*ATOH7*	c/g	1.01 (0.96-1.08)	1.02	5.42E-01	5.72E-01	+—	26	2.55E-01
10:96008348	*PLCE1*	d/r	1.02 (0.97-1.09)	1.04	3.38E-01	3.15E-01	+-+?	35.3	2.13E-01
rs1346	*SSSCA1*	t/a	0.90 (0.85-0.97)	0.91	2.41E-03	2.41E-03	—-	0	9.04E-01
rs482507	*TMTC2*	c/t	0.94 (0.89-0.99)	0.94	2.03E-02	2.03E-02	—-	0	7.46E-01
rs11613189	*FAM101A*	t/c	0.99 (0.95-1.05)	0.99	8.25E-01	7.77E-01	++−	18.5	2.98E-01
rs7323428	*DCLK1*	t/g	0.99 (0.94-1.05)	1.00	7.83E-01	8.87E-01	+-+-	13.6	3.25E-01
rs2251069	*DDHD1*	c/t	0.95 (0.91-1.00)	0.96	7.62E-02	7.62E-02	−+-	0	4.08E-01
rs4436712	*SIX6*	t/g	1.24 (1.19-1.31)	1.23	**5.77E-18**	**1.52E-07**	++++	48.8	1.19E-01
rs6598351	*FAM169B*	t/c	0.99 (0.93-1.06)	0.99	8.06E-01	8.06E-01	−+−	0	7.11E-01
rs11646917	*SALL1*	t/3g	0.98 (0.93-1.04)	0.98	5.49E-01	5.49E-01	−++	0	5.97E-01
rs11867840	*BCAS3*	g/a	1.06 (1.01-1.13)	1.06	1.83E-02	2.12E-02	++++	8.3	3.51E-01
rs6054375	*BMP2*	t/g	0.89 (0.85-0.94)	0.91	8.52E-06	9.93E-03	—-	47.1	1.29E-01
rs738722	*CHEK2*	t/c	0.93 (0.89-0.99)	0.93	1.26E-02	1.26E-02	—-	0	9.05E-01
rs3791679	*EFEMP1/PNPT1*	a/g	0.96 (0.92-1.02)	0.96	2.23E-01	2.23E-01	—-	0	5.51E-01

(continued)

Table . (Continued)

	Nearest gene	A1/A2	OR (95% CI)	OR (R)	P-value	P-value (R)	Direction	I^2	P-value of heterogeneity
rs12494328	*FLNB*	a/g	1.13 (1.07-1.20)	1.13	**1.28E-05**	**5.89E-04**	++-+	26.9	2.50E-01
rs6804624	*COL8A1*	c/t	0.99 (0.94-1.05)	0.99	8.14E-01	8.14E-01	—+	0	8.42E-01
6:36592986	*CDKN1A*	d/r	1.14 (1.09-1.21)	1.15	**7.74E-07**	**1.04E-04**	++++	36.6	1.93E-01
rs2684249	*HSF2*	c/t	0.92 (0.88-0.97)	0.94	1.08E-03	1.66E-01	—+	63.3	4.25E-02
rs8176672	*ABO*	t/c	1.00 (0.91-1.11)	1.00	9.49E-01	9.49E-01	−+-?	0	3.69E-01
rs4936099	*ADAMTS8*	c/a	0.94 (0.90-1.00)	0.94	5.75E-02	5.75E-02	—-	0	9.63E-01
rs34222435	*ASB7*	t/c	1.03 (0.97-1.10)	1.03	3.66E-01	3.66E-01	++-+	0	8.74E-01
rs1074407	*TRIOBP*	t/a	1.04 (1.00-1.10)	1.04	4.92E-02	8.66E-02	++++	32.9	2.15E-01
Disc area SNPs									
rs4658101	*CDC7/TGFBR3*	a/g	1.08 (1.02-1.16)	1.08	7.81E-03	7.81E-03	++++	0	7.22E-01
1:169530520	*F5/SELP*	i/r	1.01 (0.96-1.08)	1.02	5.40E-01	5.40E-01	++-?	0	7.14E-01
rs11811982	*CDC42BPA*	a/c	0.87 (0.80-0.97)	0.90	1.19E-02	8.28E-02	−++	20.5	2.87E-01
rs9843102	*ABI3BP*	a/g	0.92 (0.86-0.98)	0.92	1.37E-02	1.37E-02	—-	0	6.24E-01
rs10021731	*UGT8*	c/t	1.01 (0.96-1.06)	1.01	6.82E-01	6.82E-01	−++	0	6.50E-01
8:88744441	*DCAF4L2*	d/r	1.03 (0.99-1.09)	1.04	0.1225	1.39E-01	++-+	4.9	3.68E-01
rs12220165	*CTNNA3*	g/c	1.08 (1.01-1.16)	1.09	1.14E-02	1.14E-02	++++	0	9.04E-01
rs7916410	*ATOH7*	t/c	1.00 (0.96-1.06)	1.00	7.63E-01	7.45E-01	+-++	3.9	3.73E-01
rs61101201	*ELP4/PAX6*	g/t	1.00 (0.94-1.06)	1.00	9.77E-01	9.77E-01	−+-?	0	9.63E-01
rs442376	*TMTC2*	c/t	1.04 (0.99-1.10)	1.05	7.94E-02	7.94E-02	−+++	0	6.82E-01
rs1345467	*SALL1*	g/a	1.07 (1.01-1.14)	1.07	1.86E-02	1.86E-02	++++	0	8.73E-01
rs5762752	*CHEK2*	c/g	0.92 (0.88-0.98)	0.92	4.90E-03	4.90E-03	—-	0	8.29E-01
rs56385951	*CARD10*	a/g	0.99 (0.92-1.07)	1.00	9.15E-01	9.15E-01	+-+-	0	9.88E-01
1:3046430	*PRDM16*	i/r	0.97 (0.87-1.10)	0.98	7.13E-01	8.72E-01	+−?	63.9	6.28E-02
rs787541	*U6, GADD45A*	c/g	0.98 (0.94-1.04)	0.98	6.10E-01	9.06E-01	−++	50.7	1.08E-01
rs1367187	*DIRC3*	c/t	0.95 (0.90-1.01)	0.96	1.11E-01	4.12E-01	+-+-	46.1	1.35E-01
rs2443724	*VGLL4*	c/g	0.91 (0.87-0.97)	0.91	1.04E-03	2.61E-02	−+-	38	1.84E-01
rs11129176	*RARB*	a/g	0.99 (0.94-1.05)	1.00	8.85E-01	9.93E-01	+—	40.4	1.69E-01
rs1997404	*COL8A1*	g/t	1.00 (0.95-1.06)	1.00	9.60E-01	9.60E-01	−+++	0	6.18E-01
rs34935520	*SIX6*	g/a	1.26 (1.20-1.33)	1.26	**2.82E-20**	**6.73E-14**	++++	21.5	2.81E-01
rs60779155	*ASB7*	a/g	1.02 (0.96-1.10)	1.03	4.52E-01	4.52E-01	+−+	0	5.02E-01

Results are shown for the most significantly associated single nucleotide polymorphisms from the endophenotype analyses. Nearest gene, reference NCBI build37; A1, reference allele; A2, other allele; OR, estimated odds ratio for allele A1; 95% CI, confidence interval; OR (R), estimated odds ratio for allele A1 in random effect meta-analysis; P-value (R), P-value in random effect meta-analysis; I^2 statistic measuring heterogeneity on a scale of 0–100%; i, insertion; d, deletion; r, reference.

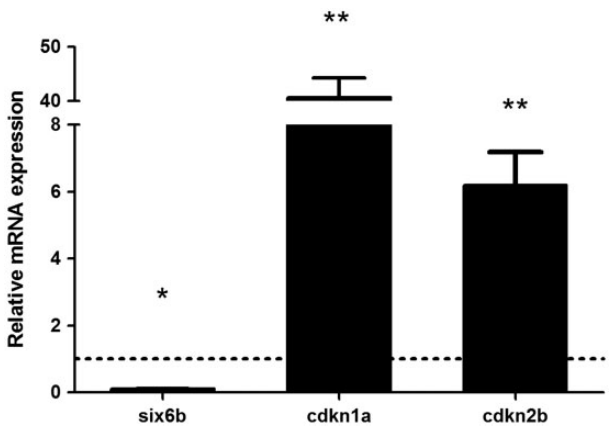

Figure 3. *cdkn1a* mRNA expression change Overexpression of *cdkn1a* and *cdkn2a/cdkn2b* in response to six6b depletion is shown. All samples expression were normalized to the control gene sdha. Relative expression was calculated by setting the wild-type expression level at 1. Values represent mean ± standard error of the mean. *P < 0.05; **P < 0.005.

CDKN1A, also known as *p21*, *CIP-1* or *WAF-1*, belongs to the same family as *CDKN2B* and also encodes a cyclin-dependent kinase inhibitor. Upregulation of *CDKN1A* causes G1 arrest and inhibits proliferation of the cell. Herein, for the first time, we provide genome-wide significant evidence for association of *CDKN1A* variants with cup area. Two prior small cohort studies suggested a possible role of *CDKN1A* in POAG. Tsai *et al.* (33) found an association between a codon 31 polymorphism in *CDKN1A* and POAG in 58 patients and 59 controls from China (OR = 2.39 [1.14–5.01]). Saglar *et al.* found no statistically significant association between the codon 31 polymorphism and POAG in 75 patients and 119 controls from Turkey (OR = 1.70, P-value = 0.25) (34). Our study provides strong evidence for the role of *CDKN1A* in POAG risk in a large sample consisting of 6,429 cases and 41,404 controls and shows the first convincing evidence for an association of *CDKN1A* and POAG in individuals of European descent. A potential functional interaction between POAG-loci (*i.e. SIX6* and CDKN genes) has been shown previously (26,27). We performed *in vivo* studies in embryonic zebrafish eye and found that knockdown of *six6b* upregulates both *cdkn2a/cdkn2b* and *cdkn1a*. The mechanism behind the genetic variation in *SIX6*, and the CDKN genes remains obscure. However, in a recent study, Skowronska-Krawczyk *et al.* showed that *SIX6* regulates the expression of *CDKN2A* in the context of IOP elevation (26). More comprehensive studies at the individual tissue level *e.g.* retinal ganglion cell layer or optic nerve should be performed to evaluate the consequences of the genetic variation associated with POAG; for example, if associated variants in *SIX6* lead to up-regulation of CDKN genes.

Genetic variation in CDK inhibitor genes has been found associated with various diseases and traits. SNPs close to *CDKN1A*, for example, have been associated with electrocardiographic measures (35) and colorectal cancer (36). While the *CDKN2A/CDKN2B* and *CDKN2B-AS1* locus has been found in GWAS of Type 2 Diabetes (37), fasting glucose (38), intracranial aneurysm (39), myocardial infraction (40), coronary heart disease (41) and various cancers, including basal cell carcinoma (42), breast (43) and prostate (44) cancer, and glioma (45). Interestingly, the same allele in *CDKN2B* that decrease risk of POAG, was found associated with increased risk of glioma. The functional significance of the genetic variation in the CDK genes remains to be determined, possible mechanism in POAG might be related with the disruption of a delicate balance between proliferation and apoptosis.

It has been suggested that p53 plays a role in POAG, especially in POAG with paracentral visual field loss (46). Intriguingly, p53 has been also related to the CDK-inhibitors and to four of the new genes pointed out by this study (*GADD45A*, *PDZD2*, *RREB1* and *PSCA*). *GADD45A* is involved in growth arrest through p53-dependent and independent mechanisms (47,48) and can interact via *CDKN1A* (49). *PDZD2* is a tumour suppressor gene, reported to activate p53 by transcriptional regulation (50). *RREB1* has an effect on p53 by binding to its promotor and transactivates its expression (51). While *PSCA* expression correlates with the expression of p53 in choriocarcinoma, suggesting a role of *PSCA* in cell growth through p53-related pathways (52). Other genes play a role in apoptosis or cell growth via other mechanisms than p53: *VGLL4* inhibits Bax- and TNFa-induced apoptosis (53) and *DGKB* is a regulator of diacylglycerol, which is important for cell growth and differentiation. *UGT8* plays a role in the biosynthesis of the sphingolipids of myelin membranes of the central and peripheral nervous system; sphingolipids are also implicated in apoptosis (54).

Another interesting novel gene is *RPE65* (retinal pigment epithelium -specific protein 65kDa). This gene has been associated with retinitis pigmentosa (RP) (55,56) and Leber congenital amaurosis type 2 (LCA2) (57). As the name implies, the encoded protein is located in the retinal pigment epithelium (58). Both diseases (RP and LCA2) are not characterized by an excavation of the optic nerve head. However, we have checked several online databases for expression in different tissues. In the eye, it is also highly expressed in the optic nerve head (Tables S8 and S9) suggesting that this gene could be involved in other ocular processes. Little expression is found in the brain, with no expression in other tissues or organs in the body. Future studies are necessary to confirm our finding.

Of the genes identified by gene-based testing, *C9* (complement component 9) is especially interesting. Its protein is part of the membrane attack complex (MAC), together with the proteins C5b, C6, C7, and C8. This complex activates several steps that lead to cell death, and cells protect themselves by removing the complex through endocytosis. Caveolin is one of the proteins involved in endocytosis and the *CAV1/CAV2* genes are associated with IOP and POAG. It has been shown that inhibition of caveolin-1 inhibits the endocytosis of MAC (59).

To our best knowledge, this meta-analysis is the largest study of IOP and optic nerve head parameters to date, using well-characterized datasets from populations world-wide. A limitation of our study is the lack of an available dataset for replication of the novel associations detected by combined European and Asian ancestry samples. However, the heterogeneity of these novel genomic regions is generally low in the meta-analysis. For VCDR, cup area, and disc area we have identified novel SNPs in the analysis of individuals with European ancestry. Of the nine novel associations found in these populations (*RPE65*, *PDZD2*, *RREB1*, *DGKB* for VCDR; *CDC42BPA, CRISPLD1* and *FAM169B* for cup area; and *CTNNA3* and *UGT8* for disc area), only *RREB1* was nominally significant in the individuals with Asian ancestry. Five of the seven non-significant SNPs in the individuals with Asian ancestry had an effect estimate in the same direction. As the analysis in individuals with Asian ancestry contains a smaller number of individuals, this could be due to lack of power.

We have identified 21 genetic variants associated with POAG endophenotypes: IOP, VCDR, cup area, and disc area. The effect estimates of single SNP associations are small. We expected small effects of many SNPs, since glaucoma endophenotypes are highly polygenic traits (60). Although individual SNP effects are small, the joint effect of the various genetic variants could yield a clinical relevant parameter. These association results do not imply that the variants described here have a causal effect. Fine-mapping and functional studies are required to identify the causal variants tagged by our findings and the exact molecular mechanisms involved in POAG. Although the overlap between IOP-loci and the optic disc parameters-loci is not large, this is the first study showing a genome-wide significant evidence of the genetic correlation between IOP and VCDR; we expect that larger sample sizes and improved imputation accuracy may help to find more of the loci underlying the genetic correlation between these two endophenotypes. Of the novel associations, *CDKN1A* is strongly associated with POAG, This finding is in line with other studies (26), pointing to the CDK-inhibitor genes as key players in the development of POAG. The p53 pathway has been implicated in POAG and interact in different cellular contexts with four of the new genes pointed out by this study (*GADD45A*, *PDZD2*, *RREB1* and *PSCA*). Functional studies need to be performed to assess the role of *p53* and CDK-inhibitors in the pathophysiology of POAG. A more comprehensive study of these mechanisms may inform the development of new therapies for POAG.

Materials and Methods

Study design

We performed a meta-analysis on directly genotyped and imputed SNPs to the 1000 Genomes reference panel. We analyzed four outcomes: IOP, VCDR, cup area, and disc area; the same approach was used for all four outcomes. In the first stage, we conducted a meta-analysis of GWAS including 22,489–29,578 individuals of European ancestry. Subsequently, we evaluated the genome-wide significant SNPs from the first stage in a meta-analysis of GWAS including 7,307–8,373 individuals of Asian ancestry. Finally, we performed a mega/meta-analysis of GWAS from all individual studies including individuals of European and Asian ancestry. We subsequently tested the effect of all genome-wide significant SNPs on POAG in four independent case-control studies ($n = 6{,}429/41{,}404$). These cases are not part of our primary meta-analyses which focused on quantitative traits in the general population (IOP, VCDR, cup area and disc area). Three of the case-control studies were of European ancestry (7,61,62) and one of Asian ancestry (63).

Subjects, phenotyping and genotyping

All 19 studies included in this meta-analysis are part of the IGGC (Table S1A). Details for each individual study can be found

in Supplementary Material and Tables S1B and C and S2. The ophthalmological examinations included measurements of IOP and optic nerve head assessment. All 19 studies contributed to the IOP mega/meta-analysis, 18 to the VCDR and 16 to the cup area and disc area mega/meta-analysis. Validation of mega/meta-analysis results was performed in four independent POAG case-control studies. Studies performed genomic imputation using 1000 Genomes phase 1 reference samples. Study-specific quality control can be found in the Supplementary Material. All studies were performed with the approval of their local medical ethics committee, and written informed consent was obtained from all participants in accordance with the Declaration of Helsinki.

Statistical analysis

In the IOP analysis, individuals who underwent IOP-lowering laser or surgery were removed from the analysis; in individuals receiving IOP-lowering medication, the IOP value was multiplied by 1.3 to estimate a pre-medication IOP value (64). The mean IOP, VCDR, cup area, and disc area of both eyes was used for the analyses. SNPs with MAF < 0.01 and imputation quality scores <0.3 (proper-info of IMPUTE) or $R^2 < 0.3$ (MACH) were removed from the analyses. Each individual study performed a linear regression between each endophenotype (IOP, VCDR, cup area, and disc area) and the SNPs, under the assumption of an additive model for the effect of the risk allele. Analyses were adjusted for age, sex and the first five principal components (for population-based studies) or family structure (for family-based studies).

We performed an inverse variance weighted fixed-effect meta-analysis with METAL software (65). P values for heterogeneity were calculated by using the Cochran's Q-test for heterogeneity. SNPs with a *P*-value for heterogeneity <0.001 were removed from the results, as well as SNPs only present in three studies. We used the 'genomic control' option in METAL to correct the standard error of each individual study for estimated genomic inflation. To evaluate if our results exhibit signs of population stratification we estimated the intercept using the LD score regression method (18) in the European-only meta-analyses. The intercept, which estimates inflation after removing polygenic signals, was: 1.0142 for IOP, 1.0199 for VCDR, 1.0074 for cup area and 1.0167 for the disc area (Table S16).

In the meta-analyses of individuals with European ancestry, a *P*-value $<5.0 \times 10^{-8}$ (the genome-wide threshold of association) was considered significant. In the second stage, these genome-wide significant SNPs were validated in individuals with Asian ancestry, and in this look-up a p value <0.05 was considered significant. Finally, in the mega/meta-analysis of individuals with European and Asian ancestry a *P*-value of $<5.0 \times 10^{-8}$ was considered significant. In total, we identified 75 independent SNPs across different genomic regions for all the traits together. Therefore, the significance level after Bonferroni correction in the meta-analysis of POAG cohorts was $= 6.67 \times 10^{-4}$ (0.05/75 independent SNPs). To estimate the common effect size of the top SNPs associated with IOP, optic disc parameters and their effect in the look-up in the POAG cohorts a random-effect meta-analysis was performed using plink (66) http://pngu.mgh.harvard.edu/purcell/plink/ parameter *–meta-analysis*. Manhattan, regional and forest plots were made using R (67) and LocusZoom (68).

The genetic correlation between optic disc parameters (VCDR, cup and disc area) and IOP was estimated using the LD score regression method (18,69). GWAS summary statistics from the Europeans-only meta-analyses were used to calculate the genetic overlap. To restrict the analyses to well imputed SNPs, we included only SNPs with MAF > 0.01 that were present in HapMap3. GWAS data were harmonized using the munge_sumstats.py function. For analyses, we used the pre-computed LD scores for Europeans eur_w_ld_ch available at https://github.com/bulik/ldsc/wiki. The ldsc.py function (with all default settings) was used to calculate the genetic correlation between traits. In addition, we calculated the heritability (h^2) estimates from the GWAS meta-analysis using the same function.

Gene-based test using VEGAS

A gene-based test was performed using the VEGAS2 software (70), with a 50kb gene boundary. We used the parameter '-top 100' (default) to perform gene-based tests. This parameter considers association test statistics of all variants mapped to a gene to compute gene-based test statistics. The 1000 Genomes European and Asian populations were used as a reference to calculate LD for European and Asian ancestry data respectively. After calculation of gene-based test statistics for Asian and European ancestry populations separately, meta-analyses were conducted using Fisher's method for combining *P*-values.

Functional characterization, expression data, zebrafish and gene-set enrichment

We investigated for evidence of regulatory functions of associated loci HaploReg version 2 (71) and Regulomedb version 1.1 (72). We investigated the expression of the associated genes using NCBI's UniGene (23) and The Ocular Tissue Database (24). We also investigated the expression of *cdkn1a* in a *six6b* knockdown zebrafish and used DEPICT to investigate gene-set enrichment. More information about these analyses can be found in the Supplementary Material.

Data access

Access to the summary association statistics for IOP and optic disc parameters is available at https://www.dropbox.com/sh/3j2h9qdbzjwvaj1/AABFD1eyNetiF63I5bQooYura?dl=0.

Supplementary Material

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. Dr. Pasquale has been a paid speaker for Allergan. He also served as a nonpaid consultant to Novartis and a paid consultant to Bausch + Lomb. He has received support to travel to the Exfoliation Glaucoma Think Tank Meeting in NYC by the Glaucoma Foundation.

Dr. Jonas: Consultant for MundiPharma Co.; Allergan Inc.; Merck Sharp & Dohme Co., Inc.; Alimera Co.; Boehringer Ingelheim Co., Sanofi Co., Pfizer Co.; Patent holder with CellMed AG, Alzenau, Germany and with University of Heidelberg/Germany.

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