

Hypothesis-independent pathway analysis implicates GABA and Acetyl-CoA metabolism in primary open-angle glaucoma and normal-pressure glaucoma

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Abstract Primary open-angle glaucoma (POAG) is a leading cause of blindness worldwide. Using genome-wide association single-nucleotide polymorphism data from the Glaucoma Genes and Environment study and National Eye Institute Glaucoma Human Genetics Collaboration comprising 3,108 cases and 3,430 controls, we assessed biologic pathways as annotated in the KEGG database

for association with risk of POAG. After correction for genic overlap among pathways, we found 4 pathways, butanoate metabolism (hsa00650), hematopoietic cell lineage (hsa04640), lysine degradation (hsa00310) and basal transcription factors (hsa03022) related to POAG with permuted $p < 0.001$. In addition, the human leukocyte antigen (HLA) gene family was significantly associated with POAG ($p < 0.001$). In the POAG subset with normal-pressure glaucoma (NPG), the butanoate metabolism pathway was also significantly associated ($p < 0.001$) as well as the MAPK and Hedgehog signaling pathways (hsa04010 and hsa04340), glycosaminoglycan biosynthesis-heparan sulfate pathway (hsa00534) and the phenylalanine, tyrosine

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and tryptophan biosynthesis pathway (hsa0400). The butanoate metabolism pathway overall, and specifically the aspects of the pathway that contribute to GABA and acetyl-CoA metabolism, was the only pathway significantly associated with both POAG and NPG. Collectively these results implicate GABA and acetyl-CoA metabolism in glaucoma pathogenesis, and suggest new potential therapeutic targets.

Introduction

Glaucoma is the second leading cause of blindness worldwide, with an estimated 60 million people affected (Quigley 2011; Quigley and Broman 2006). In the United States, primary open-angle glaucoma (POAG) is the most common type of glaucoma (Friedman et al. 2004). POAG is an age-related progressive optic nerve degeneration caused by irreversible destruction of retinal ganglion cells eventually leading to blindness. Risk factors for POAG, aside from age, include elevated intraocular pressure, family history and African-American race (Caprioli and Varma 2011; Racette et al. 2003; Shin et al. 1977). A subgroup of POAG patients develops optic nerve degeneration without elevation of intraocular pressure (IOP) and this POAG subset is called normal-pressure glaucoma (NPG).

POAG is genetically and phenotypically complex (Fan and Wiggs 2010). Recent genome-wide association studies (GWAS) of large case-control and population-based

cohorts have identified genes and genomic regions associated with POAG risk including an intergenic region between caveolins 1 and 2 (*CAV1/CAV2*) (Thorleifsson et al. 2010; Wiggs et al. 2011), *TMCO1* and *CDKN2BAS* (Burdon et al. 2011; Wiggs et al. 2012), *SIX1/SIX6* (Wiggs et al. 2012) and 8q22 in the NPG subgroup (Wiggs et al. 2012).

While these GWAS have identified multiple genes potentially involved in glaucoma pathogenesis, the stringent correction for multiple testing and type I error identifies only variants with the largest main effects as statistically significant (Manolio et al. 2008). Many biologically meaningful associations of smaller effect size may go undetected, including variants with smaller effect size that in aggregate could reveal novel biological pathways or systems underlying disease susceptibility. Pathway-based analysis groups gene variants into biologically meaningful entities to distinguish the actual from the false positive associations. In short, this type of analysis can be used to uncover additional genotype-phenotype associations that the single-allele GWAS analysis may have missed.

Pathway analysis by randomization incorporating structure (PARIS) is a pathway analysis algorithm that uses single-allele GWAS data and assigns significance to a pathway through permutation of the genome rather than permutation of affection status (Yaspan et al. 2011). Through genomic randomization, PARIS accounts for multiple testing, gene

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size/SNP coverage and linkage disequilibrium (LD) biases present in the original GWAS dataset. Here, we report the use of PARIS in a hypothesis-independent approach to identify novel pathways and genes associated with POAG and the NPG subgroup with optic nerve degeneration independent of IOP.

Results

The demographics and ocular characteristics for GLAUGEN and NEIGHBOR study participants have been reported previously (Wiggs et al. 2011, 2012, 2013). We used the GWAS p values from the case–control meta-analysis in the NEIGHBOR and GLAUGEN datasets and executed the PARIS algorithm (Yaspan et al. 2011) using the KEGG pathways ($n = 209$) as a framework. We found significant association with POAG in 14 of the 209 pathways in the KEGG database (Table 1). These pathways were loosely grouped into three KEGG categories: metabolism, cellular adhesion and signaling, and autoimmune disorders. For a complete list of all 209 pathways and their permuted p values, see Supplemental Table 1.

We next investigated possible relationships among the 14 pathways associated with POAG. We found that nine of these pathways contain a substantial amount of genic overlap due to the HLA gene family (*HLA-A*, *HLA-B*, *HLA-C*, *HLA-E*, *HLA-F*, *HLA-G*, *HLA-DMA*, *HLA-DMB*, *HLA-DOA*, *HLA-DOB*, *HLA-DPA1*, *HLA-DPB1*, *HLA-DQA1*,

HLA-DQA2, *HLA-DQB1*, *HLA-DRA*, *HLA-DRB1*, *HLA-DRB5*, *HLA-E*, *HLA-F*, and *HLA-G*). All nine of the pathways did not have all of these genes, but most of these pathways have several and all nine pathways include *HLA-DOB*, *HLA-DQA2* and *HLA-C*. A gene-based permutation test using PARIS (each gene within the pathway is considered as a separate entity), identified significant association ($p < 0.001$) for these three HLA gene family members (Fig. 1). To test the impact of all 21 HLA genes, we removed them from the nine pathways containing HLA gene family members and re-ran PARIS and found that all nine of these pathways were no longer statistically significant. Although the overall pathways were not significant, several genes within the 9 HLA-containing pathways continued to demonstrate interesting association using the gene-based test (Fig. 1). The viral myocarditis pathway (hsa05416) contains *CAVI* (Caveolin 1) previously shown to be associated with POAG (Thorleifsson et al. 2010; Wiggs et al. 2011). In the cell adhesion pathway, *JAM2* is a component of the neural retina and retinal pigment epithelium (Daniele et al. 2007) and *PVRL3* has previously been shown to contribute to ocular developmental defects (Lachke et al. 2012).

Next we used the gene-based permutation test to identify the genes within each of the remaining five pathways with significant association ($p < 0.001$). Three of the remaining five pathways—butanoate metabolism, lysine degradation, and limonene and pinene degradation—contained the significantly associated genes *ALDH2* and *ALDH3A2* (Fig. 1).

Table 1 Pathways associated with POAG overall

Pathway name	Total SNP count	Description	Gene count (nominally significant)	Complex feature count (nominally significant)	Simple feature count (nominally significant)
hsa00650	992	Butanoate metabolism	35 (19)	142 (33)	137 (14)
hsa00310	1,146	Lysine degradation	46 (24)	174 (37)	176 (18)
hsa00903	264	Limonene and pinene degradation	8 (7)	38 (12)	38 (7)
hsa04514	6,086	Cell adhesion molecules	130 (79)	922 (152)	1,538 (119)
hsa04612	1,443	Antigen processing and presentation	69 (32)	160 (31)	605 (56)
hsa04640	2,385	Hematopoietic cell lineage	82 (47)	364 (75)	521 (39)
hsa04672	1,300	Intestinal immune network for IgA production	46 (22)	175 (31)	373 (35)
hsa04940	1,592	Type I diabetes mellitus	41 (23)	191 (35)	629 (58)
hsa05140	1,835	Leishmaniasis	70 (38)	248 (49)	491 (43)
hsa05310	720	Asthma	28 (14)	87 (20)	282 (30)
hsa05320	1,359	Autoimmune thyroid disease	50 (22)	163 (27)	516 (50)
hsa05330	1,093	Allograft rejection	35 (19)	109 (22)	505 (51)
hsa05332	1,091	Graft-versus-host disease	37 (19)	110 (22)	509 (49)
hsa05416	2,226	Viral myocarditis	69 (37)	306 (49)	709 (58)

All pathways are human (*Homo sapiens*) and all pathways listed in this table have p values < 0.001 after permutation testing. The number of features (simple, complex or whole gene) that include at least one SNP with nominal significance ($p < 0.05$) is shown in parentheses. Complex features are the number of LD (linkage disequilibrium blocks). Simple features are SNPs in linkage disequilibrium

POAG Primary open-angle glaucoma

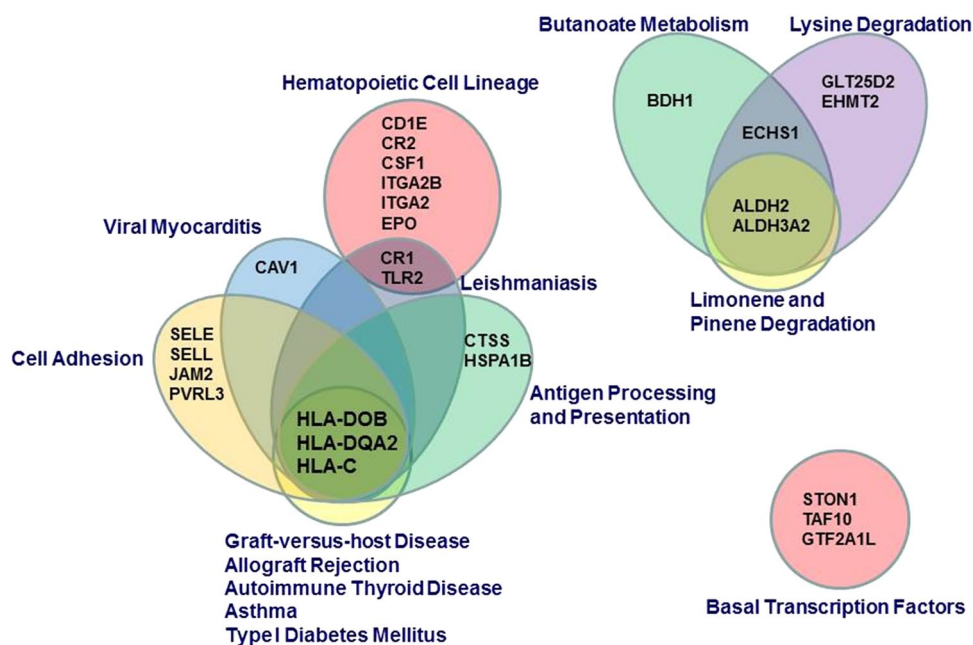


Fig. 1 KEGG pathways significantly associated with POAG overall. Pathways with a permuted p of <0.001 are presented (*blue font*). The individual genes within the associated pathways with gene-based permuted p values <0.001 are listed in *black font* and are contained in the ellipse corresponding to the pathway. Genes that are present in more than one pathway are indicated by *overlapping ellipses*. Nine pathways contained the 3 associated HLA genes *HLA-DOB*, *HLA-DQA2* and *HLA-C*. *ALDH2* and *ALDH3A2* were found in three pathways: (butanoate metabolism, lysine degradation, and limonene and pinene degradation). Two genes in the Leishmaniasis pathway overlap with the hematopoietic cell lineage pathway. The significantly associated genes in the basal transcription factor pathway were independent of all other pathways. *POAG* Primary open-angle glaucoma; *SELE* selectin E; *SELL* selectin L; *JAM2* junctional adhesion mole-

cule 2; *PVRL3* poliovirus receptor-related 3; *CAV1* caveolin 1; *HLA-DOB*, *HLA-DQA2*, *HLA-C* human leukocyte antigen- DOB, DQA2, C; *CDE1* T cell surface glycoprotein CD1e; *CR2* complement receptor 2; *CR1* complement receptor 1; *CSF1* colony-stimulating factor 1; *ITGA2B* integrin alpha chain 2b; *ITGA2* integrin alpha chain 2; *EPO* erythropoietin; *TLR2* toll-like receptor 2; *CTSS* cathepsin S; *HSPA1B* heat shock 70 kDa protein 1B; *BDH1* 3-hydroxybutyrate dehydrogenase, type 1; *ECHS1* enoyl-Coenzyme A hydratase, short chain, 1; *ALDH2* aldehyde dehydrogenase 2; *ALDH3A2* aldehyde dehydrogenase 3A2; *GLT25D2* glycosyltransferase 25 domain containing 2; *EHMT2* euchromatic histone-lysine *N*-methyltransferase 2; *STON1* stonin 1; *TAF10* TATA box binding protein (TBP)-associated factor; *GTF2A1L* general transcription factor IIA, 1-like

Two other genes in the lysine degradation pathway are also associated with POAG (*GLT25D2*, *EHMTA*) and one other gene in the butanoate metabolism pathway (*BDH1*) was significantly associated with POAG. The only significant genes in the limonene and pinene degradation pathway are *ALDH2* and *ALDH3A2*. Two genes in the Leishmaniasis pathway overlap with the hematopoietic cell lineage pathway. The significantly associated genes in the basal transcription factor pathway were independent of all other pathways (Fig. 1). Overall, after correcting for genic overlap, we identified four pathways (butanoate metabolism, lysine degradation, hematopoietic cell lineage and basal transcription factor) and HLA genes *HLA-DOB*, *HLA-DQA2* and *HLA-C* associated with POAG.

In the NEIGHBOR and GLAUGEN single-allele meta-analysis, we discovered that the NPG subgroup of cases was driving the genome-wide signals despite a sample size ($n = 720$) of less than half of the high-pressure glaucoma (HPG) subset ($n = 1,669$) (Wiggs et al. 2012). As POAG is phenotypically heterogeneous we used PARIS to test the

209 KEGG pathways in the NPG subset (Supplemental Table 1). Seven pathways were statistically significant in the NPG subgroup (Fig. 2; Table 2). Using the gene-based permutation test to identify the genes with significant association ($p < 0.001$) with NPG we found that there was substantial genic overlap between the MAPK signaling pathway and the Fc Epsilon RI signaling pathway, and between the butanoate pathway and the synthesis and degradation of ketone bodies pathway, leaving five pathways significantly associated with NPG: the MAPK signaling pathway, butanoate pathway, Hedgehog signaling, phenylalanine, tyrosine and tryptophan biosynthesis, and the glycosaminoglycan–heparan sulfate biosynthesis pathway. Of these the butanoate metabolism pathway is the only pathway that was significant in both the overall POAG analysis and the NPG analysis.

While the entire butanoate metabolism pathway may affect POAG/NPG etiology, not all of the genes within it may be of interest. Using the gene-based permutation test we evaluated each gene within the pathway to identify

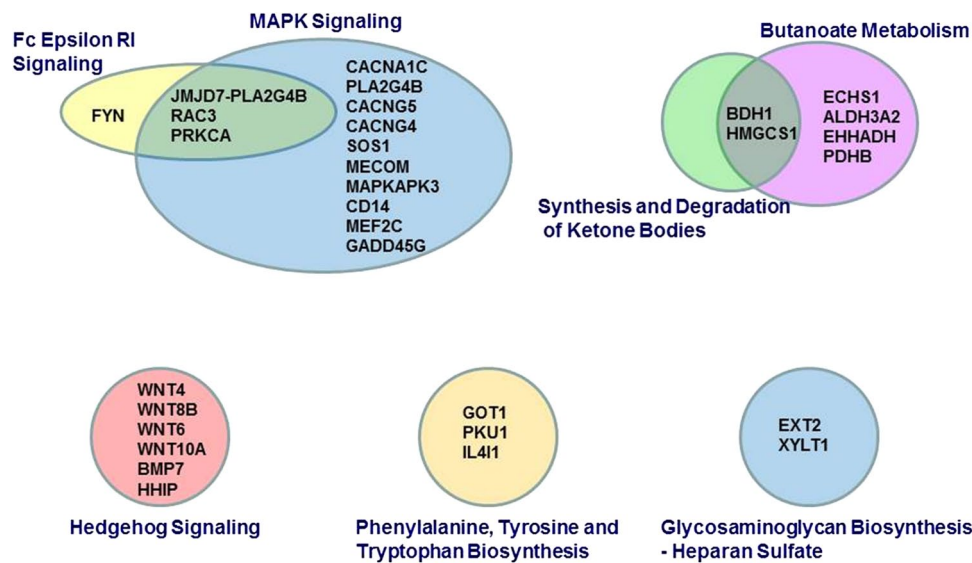


Fig. 2 KEGG pathways significantly associated with NPG. Pathways with a permuted p of <0.001 are presented (*blue font*). The individual genes within the associated pathways with gene-based permuted p values <0.001 are listed in *black font* and are contained in the ellipse corresponding to the pathway. Genes that are present in more than one pathway are indicated by *overlapping ellipses*. With the exception of *FYN*, the genes with significant association in the Fc epsilon RI signaling pathway are also included in the MAPK signaling pathway. Similarly, both of the significant genes in the synthesis and degradation of ketone bodies pathway (*BDH1*, *HMGCS1*) are also found in the butanoate pathway. The significantly associated genes in the Hedgehog signaling pathway, the phenylalanine, tyrosine and tryptophan biosynthesis pathway and the glycosaminoglycan biosynthesis-heparan sulfate pathway did not overlap with any other pathway. *NPG* normal-pressure glaucoma; *FYN* tyrosine-protein kinase Fyn; *JMJD7-PLA2G4B* jumonji domain containing 7-phospholipase A2, group IVB (cytosolic) read-through; *RAC3* ras-related C3 botulinum toxin substrate 3; *PRKCA* protein kinase C, alpha; *CACNA1C* calcium channel, voltage-dependent, L type, alpha 1C subunit; *PLA2G4B* phospholipase A2, group IVB

(cytosolic); *CACNG5* calcium channel, voltage-dependent, gamma subunit 5; *CACNG4* calcium channel, voltage-dependent, gamma subunit 4; *SOS1* son of sevenless homolog 1; *MECOM* MDS1 and EVI1 complex locus; *MAPKAPK3* mitogen-activated protein kinase-activated protein kinase 3; *CD14* cluster of differentiation 14; *MEF2C* myocyte enhancer factor 2C; *GADD45G* growth arrest and DNA-damage-inducible, gamma; *BDH1* 3-hydroxybutyrate dehydrogenase, type 1; *HMGCS1* 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1; *ECHS1* enoyl-Coenzyme A hydratase, short chain, 1; *ALDH3A2* aldehyde dehydrogenase 3A2; *EHHADH* enoyl-Coenzyme A, hydratase/3-hydroxyacyl-Coenzyme A dehydrogenase; *PDHB* pyruvate dehydrogenase (lipoamide) beta; *WNT4* wingless-type MMTV integration site family, member 4; *WNT8B* wingless-type MMTV integration site family, member 8b; *WNT6* wingless-type MMTV integration site family, member 6; *WNT10A* wingless-type MMTV integration site family, member 10a; *BMP7* bone morphogenetic protein 7; *HHIP* hedgehog interacting protein; *GOT1* glutamic-oxaloacetic transaminase 1; *PKU1* phenylalanine hydroxylase; *IL4I1* interleukin 4 induced 1; *EXT2* exostosin glycosyltransferase 2; *XYLT1* xylosyltransferase I

those genes important to the signal of the pathway as a whole for both POAG overall and for the NPG subgroup. Of the 35 genes in the butanoate pathway, 9 were nominally significant for POAG overall and 8 were nominally significant in the NPG subgroup (Table 3). Among these 17 are three genes encoding enzymes that regulate GABA synthesis (*GAD1*, *ABAT*, *ALDH5A1*), an important inhibitory neurotransmitter in the mammalian central nervous system and retina (Bringmann et al. 2013). Two genes had permuted p values of <0.001 for both POAG and NPG: *ALDH3A2*, an aldehyde dehydrogenase involved in Sjogren Larsson syndrome, a neurodevelopmental disorder that includes ocular phenotypes (van der Veen et al. 2010) and *BDH1*, (3-hydroxybutyrate dehydrogenase), a mitochondrial protein that oxidizes 3-hydroxybutyrate to form acetoacetate and NADH+ (Maurer et al. 2011). Of the 8 genes with at least nominal association with NPG, 7 of them influence

acetyl-CoA production in mitochondria (Fig. 3). Using an alternative and different analytic algorithm for gene-based analysis (VEGAS) that sums the Chi square statistic for all variants assigned to each gene and accounts for LD using a Monte Carlo simulation (Liu et al. 2010), we also found at least nominal evidence of association for *ALDH1B1* and *BDH1* with POAG and *ALDH3A2*, *ALDH5A1*, *BDH1* and *ECHS1* with NPG providing additional support for these results (Table 3).

Discussion

In this study, we performed a hypothesis-independent pathway analysis using the KEGG pathways and GWAS data from a large POAG/NPG dataset (Wiggs et al. 2012). Pathway analyses make it possible to use biologically

Table 2 Pathways associated with NPG

Pathway name	Total SNP count	Description	Gene count (nominally significant)	Complex feature count (nominally significant)	Simple feature count (nominally significant)
hsa00650	992	Butanoate metabolism	35 (18)	142 (37)	137 (13)
hsa00072	237	Synthesis and degradation of ketone bodies	9 (6)	32 (12)	18 (4)
hsa04010	9,196	MAPK signaling	267 (139)	1,482 (244)	1,956 (116)
hsa04340	1,622	Hedgehog signaling	56 (25)	265 (49)	332 (25)
hsa04664	2,851	Fc epsilon RI signaling	79 (39)	453 (80)	638 (39)
hsa00534	1,136	Glycosaminoglycan biosynthesis-heparan sulfate	26 (16)	188 (35)	220 (22)
hsa00400	165	Phenylalanine, tyrosine and tryptophan biosynthesis	5 (4)	21 (6)	20 (6)

All pathways are human (*Homo sapiens*) and all pathways listed in this table have p values <0.001 after permutation testing. The number of features (simple, complex or whole gene) that include at least one SNP with nominal significance ($p < 0.05$) is shown in parentheses. Complex features are the number of LD (linkage disequilibrium blocks). Simple features are SNPs in linkage disequilibrium

NPG Normal-pressure glaucoma

meaningful entities to identify variants that individually may not reach genome-wide statistical significance, but in aggregate can be statistically associated with a disease trait. We found that the butanoate metabolism pathway (hsa00650) is significantly associated with both POAG overall and the NPG subgroup, a result that includes significant association of genes contributing to the formation of 4-aminobutanoate (GABA) (Fig. 3). GABA is a neurotransmitter that has a critical role in visual responsiveness (Zhang and McCall 2012). GABA activity has been implicated in retinal ganglion cell degeneration in a rat hypertensive glaucoma model, and in a mouse model of glaucoma (Moreno et al. 2008; Okumichi et al. 2008). In addition, glutamate, a precursor to GABA, is excitotoxic in retinal ganglion cells (Nguyen et al. 2011). Interestingly, visual field loss is a side effect of the antiepileptic, Vigabatrin, an inhibitor of GABA transaminase (encoded by *ABAT*) (Lawden et al. 1999). Vigabatrin-related visual field loss resembles glaucomatous loss and is likely due to biochemical disruption of the photoreceptor–Mueller cell–retinal ganglion cell complex (Clayton et al. 2012). In one study 44 % of cases (those with seizures taking vigabatrin) had evidence of visual field loss compared to 7 % of controls (Lux et al. 2004).

In addition to GABA metabolism, the butanoate pathway includes enzymes responsible for acetyl-CoA biosynthesis and required for ATP production in mitochondria. Interestingly, of the 8 butanoate pathway genes associated with NPG, 7 of them influence acetyl-CoA production (Fig. 3). Retinal ganglion cells have high-energy requirements and are dependent on mitochondrial ATP production for survival (Yu et al. 2013), and previous studies have suggested that mitochondrial dysfunction is a risk factor for ganglion cell death in glaucoma (Chrysostomou et al. 2013;

Lee et al. 2012). Our results support these hypotheses and suggest that further research on mitochondrial genes, proteins and function in glaucoma would be of interest.

The butanoate pathway also impacts thiamine synthesis, and thiamine deficiency is known to cause optic atrophy and other neurologic problems (Sedel et al. 2008). Pyruvate dehydrogenase (lipoamide) beta (*PBHB*), responsible for converting pyruvate to a thiamine precursor, was significantly associated ($p < 0.001$) with NPG providing support for a role of thiamine biosynthesis in optic nerve degeneration in glaucoma. Vitamin B1 (Thiamine) intake has also been shown to be associated with a reduced risk of POAG (Ramdas et al. 2012).

We also found a significant association with the HLA family of genes potentially indicating a contribution of immune responsiveness to glaucoma pathogenesis. A number of studies have suggested a role for the immune system in glaucoma (Wax 2011); in particular, genetic associations between POAG and HLA DRB1 and DQB1 have been evaluated in various populations, although with inconsistent results (Gil-Carrasco et al. 1999; Suzuki et al. 2010). It is possible that the differential North to South distribution of HLA alleles in Europeans may create spurious associations between HLA SNPs and POAG that are not fully corrected by including the selected eigenvectors in the logistic regression models. Nevertheless, our findings suggest that further investigation into the role of HLA genes and glaucoma may be of interest.

Two other pathways were associated with POAG overall: the Basal transcription factor pathway and the hematopoietic cell lineage pathway that includes *EPO*, encoding erythropoietin which has been shown to be neuroprotective in an animal model of glaucoma (Sullivan et al. 2012). While mutations in genes coding for several transcription

Table 3 Butanoate metabolism pathway (hsa00650) genes associated with POAG and NPG

Gene	Description	E.C. number	POAG Gene <i>p</i> value <i>n</i> /1,000	NPG Gene <i>p</i> value <i>n</i> /1,000	VEGAS POAG	VEGAS NPG	General metabolic function
AACS	Acetoacetyl-CoA synthetase	6.2.1.16	0.097	0.1	0.139	0.407	Acetyl-CoA
ABAT	4-Aminobutyrate aminotransferase	2.6.1.19	0.031	0.441	0.469	0.476	GABA
ACADS	Acyl-Coenzyme A dehydrogenase, C-2 to C-3 short chain	1.3.8.1	0.121	1	0.675	0.768	Acetyl-CoA
ACAT1	Acetyl-Coenzyme A acetyltransferase 1	2.3.1.9	0.089	0.099	0.305	0.252	Acetyl-CoA
ACAT2	Acetyl-Coenzyme A acetyltransferase 2	2.3.1.9	1	0.225	0.951	0.636	Acetyl-CoA
ACSM1	Acyl-CoA synthetase medium-chain 1	6.2.1.2	1	1	0.486	0.807	Acetyl-CoA
ACSM2A	Acyl-CoA synthetase medium-chain 2A	6.2.1.2	0.18	1	0.503	0.708	Acetyl-CoA
ACSM2B	Acyl-CoA synthetase medium-chain 2B	6.2.1.2	1	1	0.843	0.507	Acetyl-CoA
ACSM3	Acyl-CoA synthetase medium-chain 3	6.2.1.2	1	1	0.278	0.955	Acetyl-CoA
ACSM4	Acyl-CoA synthetase medium-chain 4	6.2.1.2	0.217	1	0.875	0.825	Acetyl-CoA
ACSM5	Acyl-CoA synthetase medium-chain 5	6.2.1.2	0.23	1	0.621	0.805	Acetyl-CoA
AKR1B10	Aldo-keto reductase 1, B10	1.1.1.21	1	0.01	0.881	0.073	Acetyl-CoA
ALDH1B1	Aldehyde dehydrogenase 1B1	1.2.1.3	0.003	0.238	0.014	0.354	Acetyl-CoA
ALDH2	Aldehyde dehydrogenase 2	1.2.1.3	<0.001	1	0.542	0.796	Acetyl-CoA
ALDH3A2	Aldehyde dehydrogenase 3A2	1.2.1.3	<0.001	<0.001	0.167	0.059	Acetyl-CoA
ALDH5A1	Aldehyde dehydrogenase 5A1	1.2.1.24	0.301	<0.001	0.361	0.043	GABA Acetyl-CoA
ALDH7A1	Aldehyde dehydrogenase 7A1	1.2.1.3	0.426	1	0.933	0.672	Acetyl-CoA
ALDH9A1	Aldehyde dehydrogenase 9A1	1.2.1.3	0.094	0.235	0.114	0.674	Acetyl-CoA
BDH1	3-Hydroxybutyrate dehydrogenase, type 1	1.1.1.30	<0.001	<0.001	0.0,082	0.0005	Acetyl-CoA
BDH2	3-Hydroxybutyrate dehydrogenase, type 2	1.1.1.30	1	1	0.541	0.479	Acetyl-CoA
ECHS1	Enoyl-Coenzyme A hydratase, short chain, 1	4.2.1.17	0.003	<0.001	0.117	0.0342	Acetyl-CoA Fatty acid oxidation
EHHADH	Enoyl-Coenzyme A, hydratase/3-hydroxyacyl-Coenzyme A dehydrogenase	4.2.1.17, 1.1.1.35	0.005	<0.001	0.289	0.0773	Acetyl-CoA Peroxisome beta-oxidation
GAD1	Glutamate decarboxylase 1	4.1.1.15	0.002	0.277	0.0887	0.27	GABA
GAD2	Glutamate decarboxylase 2	4.1.1.15	0.107	0.345	0.409	0.543	GABA

Table 3 continued

Gene	Description	E.C. number	POAG Gene <i>p</i> value <i>n</i> /1,000	NPG Gene <i>p</i> value <i>n</i> /1,000	VEGAS POAG	VEGAS NPG	General metabolic function
HADH	Hydroxyacyl-Coenzyme A dehydrogenase	1.1.1.35	0.118	1	0.436	0.816	Acetyl-CoA
HADHA	Hydroxyacyl-Coenzyme A dehydrogenase, alpha subunit	4.2.1.17	1	1	0.58	0.459	Acetyl-CoA
HMGCL	3-Hydroxymethyl-3-methylglutaryl-coenzyme A lyase	4.1.3.4	1	1	0.722	0.328	Acetyl-CoA
HMGCS1	3-Hydroxy-3-methylglutaryl-coenzyme A synthase 1	2.3.3.10	0.039	<0.001	0.636	0.19	Acetyl-CoA
HMGCS2	3-Hydroxy-3-methylglutaryl-coenzyme A synthase 2	2.3.3.10	1	0.415	0.568	0.545	Acetyl-CoA
L2HGDH	L-2-Hydroxyglutarate dehydrogenase	1.1.99.2	1	1	0.824	0.841	GABA
OXCT1	3-Oxoacid CoA transferase 1	2.8.3.5	1	1	N/A	N/A	Acetyl-CoA
OXCT2	3-Oxoacid CoA transferase 2	2.8.3.5	1	0.099	0.981	0.0507	Acetyl-CoA
PDHB	Pyruvate dehydrogenase (lipoamide) beta	1.2.4.1	1	<0.001	0.725	0.339	Acetyl-CoA Pyruvate Thiamine
PDHA1	Pyruvate dehydrogenase (lipoamide) alpha 1	1.2.4.1	1	1	N/A	N/A	Acetyl-CoA Pyruvate Thiamine
PDHA2	Pyruvate dehydrogenase (lipoamide) alpha 2	1.2.4.1	1	1	0.989	0.234	Acetyl-CoA Pyruvate Thiamine

Genes encoding proteins that are included in the KEGG butanoate metabolism pathway (*Homo sapiens*), hsa00650 are listed alphabetically with their corresponding protein name and enzyme commission (E.C.) number (based on the chemical reactions catalyzed). The *p* value (based on 1,000 permutations) for primary open-angle glaucoma (POAG) and normal-pressure glaucoma (NPG) is given. The *p* values for the VEGAS test and the contribution of the protein to the general metabolic processes related to butanoate metabolism are listed

factors cause Mendelian early-onset glaucoma (Fan and Wiggs 2010), basal transcription factors have not been associated with common complex forms of glaucoma such as POAG or NPG. The robust association of *CDKN2BAS* with POAG and NPG (Burdon et al. 2011; Wiggs et al. 2012), a long noncoding RNA that regulates expression of *CDKN2B* (p16) an inhibitor of cell cycle regulatory protein, CDK4 (cyclin-dependent kinase 4) (Wan et al. 2013), suggests that cell cycle progression may be an important regulator of glaucoma pathogenesis and the basal transcription factors also contribute to cell cycle regulation overall.

In addition to the butanoate pathway, three other pathways associated with NPG are of interest. The MAPK signaling pathway can affect neuronal development and degeneration (Dapper et al. 2013). Associated genes in this pathway, *RAC3* (Albertinazzi et al. 2003) and *PRKCA* (Guo

et al. 2012), influence ocular neurite outgrowth and axon guidance, respectively. The Hedgehog signaling pathway was associated with NPG overall, and several associated genes within this pathway have important roles in ocular development (*WNT4*, *WNT8b*, *WNT6*, *BMP7*) (Maurus et al. 2005; Fokina and Frolova 2006; Martinez et al. 2009; Wyatt et al. 2010). Finally *EXT2*, a member of the glycosaminoglycan biosynthesis-heparan sulfate pathway, also contributes to WNT signaling and to axon sorting in the optic tract (Lee et al. 2004).

There are several limitations to our study. First, our approach used the collection of pathways annotated in the KEGG database, which, while comprehensive, is not exhaustive and is limited to current knowledge published in the literature. Second, although we have employed a rigorous permutation test to reduce false positives, no other current POAG/NPG data set of comparable size is currently

of the University of Pittsburgh, Johns Hopkins University, Duke University (Duke clinic and CATHGEN), University of West Virginia, University of Miami, University of Michigan (Michigan clinic and CIGTS and AGIS), Stanford University, Marshfield Clinic, and the University of California, San Diego.

Single allele and meta-analysis

We performed logistic regression to assess the association between individual SNPs and POAG using PLINK v1.07 (Purcell et al. 2007). For GLAUGEN, the logistic regression model included age, gender, study site, DNA source, DNA extraction method and three eigenvectors (EV 1, 2 and 6). For NEIGHBOR, the logistic regression model included age, gender, study site and two eigenvectors (EV1 and 2). Principal component analyses (using EIGENSOFT <http://www.hsph.harvard.edu/alkes-price/software/>) for both the POAG group vs controls and the NPG subgroup vs. controls are shown in Supplemental Fig. 1A and 1B. Quantile–quantile plots were used to estimate genomic inflation factors that were 1.009 for GLAUGEN and 1.034 for NEIGHBOR. Combined meta-analysis of the GLAUGEN and NEIGHBOR datasets was done using the METAL software package (Willer et al. 2010). We analyzed each study using logistic regression as described previously (Wiggs et al. 2011, 2012). Then, we combined the results using the inverse-weighted variance method based on the regression coefficients and standard errors estimated from each study as implemented in the program METAL. The GENOMIC CONTROL option was set to ON to adjust for genomic inflation differences between the studies (Wiggs et al. 2012).

PARIS

Results from the meta-analysis of the GLAUGEN and NEIGHBOR studies were used for the pathway analysis which was done using the PARIS pathway analysis software package (Yaspan et al. 2011). Biologic pathways were identified using the KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway database for homo sapiens (hsa), version 54.1 (Kanehisa and Goto 2000). SNPs from the GWAS were considered to reside in a pathway gene if the SNP fell within the ENSEMBL genomic interval ± 50 kb to either side of the gene. If the overlap included another gene in the pathway, the overlapping SNP(s) were counted once. We set a threshold of a single-allele p value of < 0.05 as nominally significant for use within the PARIS analysis. PARIS utilizes a permutation test to determine the significance of its pathway analyses. We used 1,000 permutations for this analysis. A KEGG pathway was considered to be significant at the $p < 0.001$ level if none of the 1,000

randomized pathways had more significant SNP signals than the actual pathway. To determine which of the genes in each pathway were contributing to the significant signal in the pathway, we used the “-I” (Investigate) option within PARIS. Gene-based results were also investigated using VEGAS (Liu et al. 2010), using the web-based interface (<http://gump.qimr.edu.au/VEGAS/>) and the default parameters, which includes the HapMap CEU for assessing LD.

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