Testosterone Pathway Genetic Polymorphisms in Relation to Primary Open-Angle Glaucoma: An Analysis in Two Large Datasets


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Primary open-angle glaucoma (POAG), a leading cause of chronic progressive optic nerve degeneration worldwide, is a strongly age-related disease. Testosterone and estradiol production decline with age, and accumulating evidence suggests that the retina and optic nerve are sex hormone–sensitive tissues; thus, these sex hormones may be implicated in the glaucomatous process. Postmenopausal therapy with estrogen alone was associated with modest reductions in IOP in a post hoc analysis from a randomized clinical trial, and with lower POAG risk in a large observational study; yet, there is a gap in our understanding of the role testosterone plays, if any, in the glaucomatous process.

Nongonadal sources of sex hormone precursors from the adrenal gland provide dehydroepiandrosterone (DHEA), which is converted to sex hormones in peripheral tissues (Fig.). Interestingly, Coca-Prados and colleagues documented that the neuroendocrine secretory ciliary epithelium expresses 17β HSD isoforms 2, 5, and 7, and these cells actively metabolize androgen and other sex hormones. However, genetic variants of 17β HSD have been little studied specifically in relation to POAG.

Using a pathway analysis, we previously showed that, collectively, genetic variants in estrogen metabolism enzymes were associated with POAG in women and not men. Here, we formed a custom testosterone metabolism genetic variant panel in case-control datasets from the United States and Australia/New Zealand to further evaluate if there is an association overall, or stratified by sex, with POAG or POAG subtypes defined by IOP. This panel focused on genes related to the intracrine generation of testosterone, as local production of sex steroid may be most relevant to the glaucomatous process.

METHODS

Description of the Study Populations

The US data are derived from the National Eye Institute Glaucoma Human Genetics Collaboration Heritable Overall Operational Database (NEIGHBORHOOD), a genetic consortium that includes the following eight independent datasets: Massachusetts Eye and Ear Infirmary; National Eye Institute Glaucoma Human Genetics Collaboration; Iowa; Marshfield; the Ocular Hypertension Treatment Study; the Women’s Health Initiative Eyes; the Toronto Eye Study; the Early-Onset Glaucoma Study; and the National Health and Nutrition Examination Survey (NHANES). The Australia/New Zealand datasets are derived from the Australian-Glaucoma-Prediction-Study (AGPS) and the Auckland Eye Study (AES) datasets, respectively. Details of the datasets are provided in Table S1. We constructed a combined US data set and an Australian/New Zealand data set.

**Keywords:** primary open-angle glaucoma, testosterone metabolism pathway

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Genome Health Study; and two datasets from the Glaucoma Genes and Environment Study: one genotyped on the Affymetrix platform and the other genotyped on the Illumina HapMap Series. The NEIGHBORHOOD dataset has a total of 3853 POAG cases and 33,480 controls. The Australian and New Zealand data are derived from the Australian and New Zealand Registry of Advanced Glaucoma (ANZRAG) and consist of 1115 advanced POAG cases and 1992 controls genotyped on the Illumina Omni 1M or the OmniExpress array. Cases and controls were drawn from Southern Adelaide Health Service/Flinders University, University of Tasmania, Queensland Institute of Medical Research, and the Royal Victorian Eye and Ear Hospital. All participants in both datasets were of European ancestry. The institutional review boards of all participating institutions approved this study.

### Ophthalmic Characteristics of Cases and Controls

Across the eight datasets in NEIGHBORHOOD, cases and controls lacked evidence of secondary IOP elevation on slit lamp biomicroscopy, such as exfoliation syndrome, pigment dispersion syndrome, or trauma. For cases and controls, slit lamp examination or gonioscopy did not reveal evidence of significant irido-trabecular meshwork apposition suggestive of angle closure in either eye. For cases, fundus examination revealed a cup-disc ratio (CDR) of 0.07 or an intereye difference in CDR of at least 0.2. Each case had at least one eye with visual field (VF) loss consistent with nerve fiber layer dropout on a reliable test. In the absence of VF loss, the CDR was 0.8 or higher in both eyes. Elevated IOP was not a criterion for inclusion as a case or a control. Controls had a CDR of 0.6 or less in both eyes and a CDR intereye difference of 0.1 or less. IOP at diagnosis was collected and used to categorize POAG cases into high-tension glaucoma (HTG with IOP $\geq 22$ mm Hg) and normal tension glaucoma (NTG with IOP $< 22$ mm Hg) subtypes when available. The exact definition of POAG across the eight NEIGHBORHOOD sites can be found in Supplementary Table 2 of Cooke Bailey et al.

Advanced POAG cases in ANZRAG had best-corrected visual acuity worse than 6/60 due to POAG or a reliable 24-2 VF with a mean deviation worse than $-22$ dB or at least two of four central fixation squares affected with a pattern SD of $<0.5$. The less severely affected eye was also required to have signs of glaucomatous disc damage with care taken to exclude secondary glaucomas of all types. Unscreened participants from the Australian Cancer Study (225 esophageal cancer cases, 317 Barrett’s esophagus cases, and 552 controls) and from a study of inflammatory bowel diseases (303 cases and 595 controls) were chosen as controls.

### Genotyping Data and Imputation

Details regarding the genotyping of the US and Australian datasets, including information about the genotyping platforms and quality control measures, can be found in the Supplemental Note of Cooke Bailey et al. Estimated genotypic probabilities for the loci in the US and Australian dataset imputed to the 1000 Genomes Project reference panel (March 2012) were analyzed.

### Generation of Genetic Data for the Testosterone Pathway Analysis

The genome-wide associations between single nucleotide polymorphism (SNP) allele dosage in relation to POAG were analyzed with ProBABEL (GenABEL project developers; http://www.genabel.org/) for NEIGHBORHOOD and SNPTEST (University of Oxford; http://mathgen.stats.ox.ac.uk) in ANZRAG. Logistic regression models adjusting for age, study-specific eigenvectors, and study-specific covariates for each dataset were evaluated. Using METAL (Center for Statistical Genetics, University of Michigan; http://csg.sph.umich.edu/abecasis/metal/index.html) we performed a meta-analysis to assess SNP dosages in relation to POAG across the US datasets. SNPs with imputation quality score $>0.7$ and minor allele frequency $>0.05$ were carried forward for the US and Australian datasets.

For this work, we used the $P$ values for association with POAG from the 2974 gene variants in NEIGHBORHOOD and the 2617 gene variants in ANZRAG (with 2609 consensus SNPs between datasets) that were attributable to testosterone metabolism for pathway analyses (see Fig. for genes). The number of gene variants differed slightly for each analysis.

### Pathway Analysis by Randomization Incorporating Structure (PARIS) Analysis

As part of the testosterone pathway, we chose to include genes involved in the formation of androstenediol and testosterone, because although they are made in the testes, they are also formed from DHEA produced by the adrenal glands and then undergo intracrine conversion to both androgens and estrogens in local tissues. We generated a custom SNP panel derived from 16 genes across 12 chromosomes comprising the testosterone metabolic pathway, as depicted in the Figure. We submitted the $P$ values from SNPs within 50 kB of the start and end sites of these genes to PARIS (v2.4). We have previously described PARIS and used a prior version of this software to assess the estrogen metabolism pathway gene variants in relation to POAG. PARIS derives a $P$ value for association between a given gene variant set and outcome of interest using a permutation procedure. Specifically, it first creates a random collection of SNPs with genomic features that mimic features of the user-defined pathway (in this case, testosterone metabolism), then compares the number of statistically significant ($P < 0.05$) features within the user-defined pathway to the random pathway. We chose to permute 10,000 times to determine an overall likelihood of the random pathway containing more significant features than the user-defined one. For example, for the testosterone pathway SNP set association with POAG among men in NEIGHBORHOOD, PARIS reported 44 significant features; specifically, 27 of 238 “simple features” (SNPs not in any linkage disequilibrium block [LD block]) and 17 of 45 “complex features” (an LD block with two or more SNPs) had $P$ value less than 0.05 for association with POAG. PARIS calculated a permuted $P = 0.0001$, indicating that only 1 of 10,000 random pathways with genetic architectures similar to the testosterone pathway had a higher significant feature count ($>44$ significant features with $P < 0.05$). Initially, these analyses were carried out in men and women together for the outcomes of overall POAG as well as of the HTG and NTG subtypes. Subsequently, associations between testosterone metabolism SNPs and POAG were repeated in men and women separately. We also used the “-paris-details” flag to investigate specifically which of the genes in the testosterone metabolism pathway were contributing to the significant signal in the overall pathway. Analyses in NEIGHBORHOOD were repeated in ANZRAG using a dataset-specific testosterone SNP set, that is, SNPs in the ANZRAG dataset located within 50 kB of the start and end sites of the 16 genes comprising the testosterone metabolism pathway, because various platforms were used across studies, and different sets of SNPs passed the quality control filters. However, in secondary analysis, we did use the 2609 consensus SNPs between both datasets as the exposure of interest in relation to the various glaucoma phenotypes. Finally,
**Table 2. The Mean Age and Distribution of POAG Cases and Controls in the ANZRAG, Stratified by Sex and HTG or NTG**

<table>
<thead>
<tr>
<th>Sex</th>
<th>n</th>
<th>Age, y (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>POAG Males</td>
<td>563</td>
<td>59.9 (14.9)*</td>
</tr>
<tr>
<td>POAG Females</td>
<td>592</td>
<td>61.1 (13.8)*</td>
</tr>
<tr>
<td>Controls Males</td>
<td>1270</td>
<td>58.4 (13.2)</td>
</tr>
<tr>
<td>Controls Females</td>
<td>722</td>
<td>56.6 (15.1)</td>
</tr>
<tr>
<td>HTG Males</td>
<td>370</td>
<td>59.1 (15.3)*</td>
</tr>
<tr>
<td>HTG Females</td>
<td>339</td>
<td>59.0 (13.9)*</td>
</tr>
<tr>
<td>NTG Males</td>
<td>143</td>
<td>62.2 (14.5)*</td>
</tr>
<tr>
<td>NTG Females</td>
<td>187</td>
<td>64.1 (14.0)*</td>
</tr>
</tbody>
</table>

*Age and/or maximum known IOP were missing on 116 POAG cases.

we explored whether our outcomes differed if we excluded SNPs from five genes that coded for overlapping estrogen-metabolizing enzymes (CYP19A1, SULT1E1, HSD17B1, HSD3B1, and SRD5A1). Although unlikely, we also excluded the four small genes with only one feature (HSD17B8, SULT1A1, HSD3B1, and HSD3B2) to minimize any bias they might introduce, as we were primarily interested in collections of genes that worked in biochemical pathways in relation to glaucoma outcomes.

**RESULTS**

The mean ages of cases and controls stratified by sex and POAG subtype (HTG versus NTG) are provided in Tables 1 and 2 for the US and Australian datasets, respectively. There is a preponderance of female controls in the US dataset due to the large size of the Women’s Genome Health Study, which has a case-cohort design.

In NEIGHBORHOOD, the testosterone pathway was not associated with POAG overall (permutated $P = 0.77$; Table 3) but a significant association was noted in ANZRAG (permutated $P = 0.018$; Table 3). In both datasets, the testosterone pathway was associated with POAG overall among men (permutated $P \leq 0.033$) but not among women (permutated $P \geq 0.42$). In both datasets, the testosterone pathway was significantly associated with HTG (permutated $P \leq 0.011$), but there were inconsistent results with respect to NTG (Table 3). Although the testosterone pathway was associated with NTG in ANZRAG (permutated $P < 0.0001$), it was not associated with NTG in NEIGHBORHOOD (permutated $P = 1.00$). These results were essentially identical when evaluating only the overlapping SNPs (as opposed to the dataset-specific SNP sets) between the US and Australian datasets (see Supplementary Material). Further stratification by sex for the POAG subtypes of HTG and NTG as outcomes was not performed due to the smaller sample sizes in both datasets. In both datasets, the relationship between the testosterone pathway and POAG stratified by sex was similar if the five genes that overlap between the estrogen metabolism pathway and the testosterone pathway (CYP19A1, SULT1E1, HSD17B1, HSD3B1, and SRD5A1) were excluded from analysis (data not shown). Furthermore, we also performed an analysis deleting four small genes with only one feature (HSD3B1, HSD3B2, HSD17B8, and SULT1A1), as such genes will yield a $P < 0.0001$ if there is only one SNP in the feature block with $P < 0.05$; the results of this analysis were the same as the main results (see Supplementary Material).

Similar to the pathway approach, we tested the association between the 16 testosterone pathway genes and our various outcomes to determine the genes that were driving observed associations. When comparing the US and Australian datasets, there was no overlap in the significant driver genes with $>|
TABLE 3. Relation Between the Testosterone Pathway Genetic Variants and POAG HTG and NTG With Sex-Stratified Results

<table>
<thead>
<tr>
<th>Gene</th>
<th>Chr</th>
<th>No. of Simple Features Based on US Data†</th>
<th>No. of Complex Features Based on US Data†</th>
<th>Gene P Value, POAG HTG in NEIGHBORHOOD</th>
<th>Gene P Value, POAG HTG in ANZRAG</th>
<th>Gene P Value, POAG NTG in NEIGHBORHOOD</th>
<th>Gene P Value, POAG NTG in ANZRAG</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSD3B1</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0.0001</td>
</tr>
<tr>
<td>HSD3B2</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>HSD17B7</td>
<td>1</td>
<td>25</td>
<td>1</td>
<td>0.44</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>SBD5A</td>
<td>2</td>
<td>18</td>
<td>4</td>
<td>0.081</td>
<td>0.36</td>
<td>0.0001</td>
<td>1</td>
</tr>
<tr>
<td>SBD5A</td>
<td>2</td>
<td>21</td>
<td>2</td>
<td>0.077</td>
<td>0.18</td>
<td>0.22</td>
<td>0.0021</td>
</tr>
<tr>
<td>SULT1E1</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0.070</td>
<td>0.058</td>
<td>0.056</td>
</tr>
<tr>
<td>SBD5A</td>
<td>5</td>
<td>22</td>
<td>2</td>
<td>1</td>
<td>0.0035</td>
<td>1</td>
<td>0.68</td>
</tr>
<tr>
<td>HSD17B8</td>
<td>6</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0.0001</td>
</tr>
<tr>
<td>HSD17B3</td>
<td>9</td>
<td>16</td>
<td>4</td>
<td>0.60</td>
<td>0.0088</td>
<td>1</td>
<td>0.021</td>
</tr>
<tr>
<td>AKR1C3</td>
<td>10</td>
<td>57</td>
<td>6</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0.47</td>
</tr>
<tr>
<td>HSD17B12</td>
<td>12</td>
<td>13</td>
<td>3</td>
<td>0.0024</td>
<td>0.42</td>
<td>0.0001</td>
<td>1</td>
</tr>
<tr>
<td>CYP19A1</td>
<td>15</td>
<td>38</td>
<td>10</td>
<td>1</td>
<td>0.0002</td>
<td>0.42</td>
<td>0.092</td>
</tr>
<tr>
<td>HSD17B2</td>
<td>16</td>
<td>11</td>
<td>5</td>
<td>0.012</td>
<td>0.0037</td>
<td>0.014</td>
<td>0.051</td>
</tr>
<tr>
<td>SULT1A1</td>
<td>16</td>
<td>0</td>
<td>1</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.0001</td>
<td>1</td>
</tr>
<tr>
<td>HSD17B1</td>
<td>17</td>
<td>15</td>
<td>1</td>
<td>0.40</td>
<td>0.45</td>
<td>0.11</td>
<td>0.0008</td>
</tr>
<tr>
<td>HSD17B14</td>
<td>19</td>
<td>24</td>
<td>2</td>
<td>0.032</td>
<td>0.41</td>
<td>0.0013</td>
<td>0.35</td>
</tr>
</tbody>
</table>

P values < 0.05 are shown in bold. Simple features refer to SNPs not in any LD block. Complex features refer to LD blocks with two or more types of SNPs. Gene names can be found in the Figure legend. Chr, chromosome.

† Genetic architecture is based on the US dataset considering POAG as the outcome. Genetic architecture for the Australian dataset and or different outcomes varied only slightly, and these differences can be seen in the Supplementary Material.

DISCUSSION

Very little is known about the role of testosterone metabolism in POAG. This work assessed the relationship between gene variants related to the intracrine testosterone metabolism and POAG using two large datasets. In both datasets, we observed that the assembled testosterone pathway SNP set was consistently associated with HTG in two datasets. Furthermore, the pathway was consistently associated with POAG in men but not in women.

Some of the testosterone pathways genetic associations across the US and Australian datasets were not consistent. Specifically, although the testosterone SNPs were not associated with POAG overall in the US dataset (permuted P = 0.77), a significant association was found in the Australian dataset (permuted P = 0.018). Also, the relationship between the testosterone SNP set and NTG was null in the US dataset, whereas it was significant in the Australian dataset. Various sensitivity analyses using only overlapping SNPs or excluding genes predominately involved in estrogen metabolism did not change the results that were consistent between the US and Australian datasets. We suspect that the inconsistencies between the datasets are due to differing sample size, as the individual genes have very modest effects and no common gene sets emerged as driving the pathway results replicating.
Table 5. Gene Significance Within the Testosterone Pathway for POAG Patients Stratified by IOP in NEIGHBORHOOD (United States) and ANZRAG (Australia)

<table>
<thead>
<tr>
<th>Gene</th>
<th>US</th>
<th>Australia</th>
<th>US</th>
<th>Australia</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSD3B1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>HSD3B2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>HSD17B7</td>
<td></td>
<td></td>
<td>0.0002</td>
<td>0.34</td>
</tr>
<tr>
<td>SRD5A2</td>
<td>&lt;0.0001</td>
<td>1</td>
<td>&lt;0.18</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>SRD5A3</td>
<td>&lt;0.0001</td>
<td>0.44</td>
<td>0.49</td>
<td>0.55</td>
</tr>
<tr>
<td>SULT1E1</td>
<td>1</td>
<td>0.026</td>
<td>0.054</td>
<td>0.036</td>
</tr>
<tr>
<td>SRD5A1</td>
<td>1</td>
<td>0.18</td>
<td>0.18</td>
<td>1</td>
</tr>
<tr>
<td>HSD17B8</td>
<td></td>
<td>&lt;0.0001</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>HSD17B3</td>
<td>1</td>
<td>0.098</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>AKR1C3</td>
<td>1</td>
<td>0.66</td>
<td>0.92</td>
<td>0.0001</td>
</tr>
<tr>
<td>HSD17B12</td>
<td>&lt;0.0001</td>
<td>0.17</td>
<td>0.014</td>
<td>0.24</td>
</tr>
<tr>
<td>CYP19A1</td>
<td>0.62</td>
<td>&lt;0.0001</td>
<td>0.95</td>
<td>0.65</td>
</tr>
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<td>0.10</td>
<td>1</td>
<td>&lt;0.0001</td>
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<tr>
<td>SULT1A1</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>1</td>
</tr>
<tr>
<td>HSD17B1</td>
<td>1</td>
<td>0.21</td>
<td>1</td>
<td>0.085</td>
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<tr>
<td>HSD17B14</td>
<td>0.0077</td>
<td>1</td>
<td>0.57</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

*P values <0.05 are shown in bold. Gene names can be found in the Figure legend.

*All P values are permuted P values as discussed in the Methods section.

across the datasets; however, we cannot rule out different disease definitions and environmental influences as the source of the differences we report.

For the associations between the testosterone SNP set and HTG in the US and Australian datasets, there was no common genetic driver of the relationship between testosterone metabolism SNPs and HTG. Different 17β HSD isoforms play critical roles in the testosterone pathway and are involved in the intracellular generation of markers that bind both androgen and estrogen receptors (Fig.) via the interconversion of androstenedione and testosterone as well as the interconversion of estrone and estradiol. The literature would suggest that the trabecular meshwork cells26 and retinal cells, including retinal ganglion cells,9,27 are under estrogenic control, whereas ocular adnexal tissues are predominately under androgenic control.28 However, little is known of the relationship between the various 17β HSD isoforms and glaucoma, particularly the product of HSD17B14, which was first discovered in the human retina29 and predicts a favorable response to tamoxifen in estrogen receptor-positive breast cancer tissue.30 As for 17β HSD isoforms 7 and 12, which were associated with HTG only in the US dataset, they are involved in local production of estradiol and feature prominently in the endoplasmic reticulum,31 whose subcellular organelles are abundant in the trabecular meshwork.32 More work is needed to understand if and how testosterone metabolism and functional polymorphisms in the various 17β HSD enzymes contribute to endoplasmic reticulum stress found in POAG.33

The association between the testosterone gene variant set and POAG in men but not women in two datasets was a notable finding. There is some evidence that declining estrogen levels are linked to higher IOP in postmenopausal women34 and that in women, postmenopausal hormones might lower IOP35,36 and be associated with lower glaucoma risk.11,36 In contrast, there are scarce data of the impact of sex hormones on health outcomes in aging males.37 There are, however, specific case reports of syndromic male hypogonadism associated with elevated IOP.38,39 Although specific genes in the testosterone pathway that accounted for this apparent sexual dimorphism were identified in both datasets (for example, HSD17B7 in the US dataset and HSD17B1 in the Australian dataset), no common gene drivers of this relation were found. There are a myriad of reasons why gene drivers common to the US and Australian datasets could not be found, including the dissimilar sample sizes, the slight variation in case-control ascertainment, and differences in genotyping platforms that were used. Furthermore, differences in the quality control and the imputation process generated different SNP sets that conformed to our definition of testosterone gene variants in the US and Australian datasets. Nonetheless, the lack of consistent cross-study driver genes raises questions about whether the relationship between testosterone metabolism SNPs and POAG is truly sex specific. This is in contrast to the relationship between the estrogen pathway SNPs and POAG, where we found COMT to be a driver gene for the sexual dimorphic association with glaucoma (associated with HTG in women but not men) in two separate datasets.15

Study limitations include the fact that none of the individual testosterone pathway SNPs were significantly associated with POAG stratified by sex or with POAG subtypes of HTG/NTG after correcting for multiple comparisons. Nonetheless, it is well known that existing genome-wide association studies of POAG are underpowered to find statistically meaningful gene variants of modest effects due to the need to minimize false discovery rates. In addition, the testosterone metabolic pathway could be construed to extend beyond biochemical pathways focused on intracellular production of sex steroids, such as cholesterol biosynthesis.40 Finally, there is a lack of evidence that a genetic signature associated with sex steroid metabolism is related to varying concentrations of estradiol or testosterone in cells relevant to POAG. Nonetheless, there is accumulating evidence that exposures altering estrogen levels modify the risk of POAG.11,36,41,42

Our study does have strengths, including the use of two large datasets, the use of common imputed SNPs across the genome, and the use of updated PARIS software with its enhanced ability to refine gene margins. By including a second dataset to compare findings, we were able to provide a more careful interpretation of the relationship between the testosterone metabolic pathway and POAG and POAG subtypes stratified by sex. By jointly analyzing association signals across a large number of genetic variants, pathway analysis allowed for identification of modest cumulative effects, which could have been missed in standard analyses of individual variants.

In conclusion, in this study involving 40,440 participants from two continents, we observed a significant relationship between the testosterone metabolism SNPs collectively and POAG among men but not among women. We also found that these SNPs were associated with the high-tension subtype of POAG in both the US and Australian dataset, although there was no consensus on driver genes for these pathway associations across the two datasets.

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References


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APPENDIX

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